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Do polyamines contribute to plant cell wall assembly by forming amide bonds with pectins?

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Abstract

Two new reducing glycoconjugates [*N*-D-galacturonoyl-putrescinamide (GalA–Put) and *N*,*N*′-di-D-galacturonoyl-putrescinamide (GalA–Put–GalA)] and homogalacturonan–putrescine ($GalA_n$–Put) conjugates were synthesised as model compounds representing possible amide (isopeptide) linkage points between a polyamine and either one or two pectic galacturonate residues. The amide bond(s) were stable to cold acid and alkali (2 M TFA and 0.1 M NaOH at 25 °C) but rapidly hydrolysed by these agents at 100 °C. The amide bond(s) were resistant to Driselase and to all proteinases tested, although Driselase digested $GalA_n$–Put, releasing fragments such as $GalA_3$–Put–$GalA_3$. To trace the possible formation of GalA–polyamine amide bonds in vivo, we fed *Arabidopsis* and rose cell-cultures and chickpea internodes with [^{14}C]Put. About 20% of the ^{14}C taken up was released as $^{14}CO_2$, indicating some catabolism. An additional ~73% of the ^{14}C taken up (in *Arabidopsis*), or ~21% (in rose), became ethanol-insoluble, superficially suggestive of polysaccharide–Put covalent bonding. However, much of the ethanol-inextractable ^{14}C was subsequently extractable by acidified phenol or by cold 1 M TFA. The small proportion of radioactive material that stayed insoluble in both phenol and TFA was hydrolysable by Driselase or hot 6 M HCl, yielding ^{14}C-oligopeptides and/or amino acids (including Asp, Glu, Gly, Ala and Val); no free ^{14}C-polyamines were released by hot HCl. We conclude that if pectin–polyamine amide bonds are present, they are a very minor component of the cell walls of cultured rose and *Arabidopsis* cells and chickpea internodes.

Keywords: *Arabidopsis thaliana*; *Rosa* sp.; *Cicer arietinum*; Cell wall; Cross-links; Amide bonds; Isopeptide bonds; D-galacturonic acid; Pectins; Polyamines; Putrescine

1. Introduction

The primary cell wall must possess a high mechanical stability that opposes the turgor pressure of the protoplast but, at the same time, it must be extensible enough to permit cell expansion (growth). In dicotyledons and non-gramineous monocotyledons, the primary wall consists mainly of a cellulose–xyloglucan framework embedded in a complex network of pectic polysaccharides. Pectins represent about 30% of wall's total dry mass. In addition, there are covalent linkages (whose precise structure remains to be elucidated) between xyloglucans and pectic polysaccharides (Thompson and Fry, 2000; Popper and Fry, 2005; Abdel-Massih et al., 2003).

Abbreviations: AIR, alcohol-insoluble residue; AHP, aniline hydrogenphthalate; APyAW, BAW, EAW, etc., PC solvents (see Experimental); DGP, *N*,*N*′-di-D-galacturonoyl-putrescinamide; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; GABA, γ-aminobutyrate; $GalA_n$, homogalacturonan; MGP, *N*-D-galacturonoyl-putrescinamide; NHS, *N*-hydroxysuccinimide; *Q*, net charge on an ion; TFA, trifluoroacetic acid; PAW, phenol/acetic acid/H_2O (2:1:1, w/v/v); PC, paper chromatography or chromatogram; PE, paper electrophoresis or electrophoretogram; Put, putrescine; Spd, spermidine; Spm, spermine.

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Three main domains of pectic polysaccharides exist: homogalacturonans (GalA$_n$s) and the rhamnogalacturonans (RG-I and RG-II) (O'Neill et al., 1990; Visser and Voragen, 1996). GalA$_n$s are linear α-(1 → 4)-linked chains of D-galacturonic acid (GalA) residues (Willats et al., 2001), some of which carry methyl and/or acetyl ester groups (Ishii, 1997; Needs et al., 1998; Perrone et al., 2002). GalA$_n$s are synthesised in the Golgi apparatus and secreted into the wall in a highly methyl-esterified form (O'Neill et al., 1990; Mohnen, 1999). Some of the methyl groups are then removed by pectin methylesterases, enabling GalA$_n$s to bind Ca^{2+} and form gels (Ridley et al., 2001). RG-I is based on a backbone of the repeat unit . . .α-D-Gal*p*A-(1 → 2)-α-L-Rha*p*-(1 → 4)-. . . with side-chains rich in Gal and Ara residues (McNeil et al., 1980; Lau et al., 1985). Its GalA residues are not methyl-esterified, but they can be *O*-acetylated at C-2 and/or C-3 (Komalavilas and Mort, 1989; Ishii, 1997; Perrone et al., 2002). RG-II has a backbone of α-(1 → 4)-linked GalA residues (O'Neill et al., 1990) and side-chains composed of 12 different monosaccharides linked by a wide variety of glycosidic bonds (Stevenson et al., 1988). RG-II can form intermolecular cross-links through a borate molecule, which forms a tetra-ester with apiose residues of two RG-II molecules (O'Neill et al., 1996). The backbone of RG-II (Whitcombe et al., 1995), and probably also of RG-I (because it is efficiently solubilised from the cell wall when only GalA$_n$ is enzymically cleaved; McNeil et al., 1980), appears to be glycosidically contiguous with GalA$_n$s.

Since pectins are polyanionic, they are partially neutralised in vivo by Ca^{2+} (Jarvis, 1984) and potentially by other cations such as polyamines (D'Orazi and Bagni, 1987; Messiaen et al., 1997; Messiaen and Van Cutsem, 1999). Mariani et al. (1989) have reported the presence of ionically bound polyamines in pectic fractions. The three main polyamines are putrescine (Put; 1,4-diaminobutane), spermidine (Spd) and spermine (Spm) – polycations occurring in almost every cell compartment, including the wall (Galston, 1983). At physiological pH, they can bind ionically to anionic macromolecules, including nucleic acids (Basu et al., 1990), proteins (Apelbaum et al., 1988), phospholipids (Tassoni et al., 1996) and pectins (Goldberg and Perdrizet, 1984; D'Orazi and Bagni, 1987; Messiaen et al., 1997). Polyamines are powerful modulators of the supramolecular conformation of pectins: they interfere with Ca^{2+}-bridging by displacing Ca^{2+} (Grant et al., 1973) with an efficiency depending on the polyamine's valency ($Spm^{4+} > Spd^{3+} > Put^{2+}$) (Messiaen et al., 1997).

Polyamines can also form covalent (amide; isopeptide) bonds with various low-M_r cellular carboxylic acids, for example forming *N*-feruloyl-Put and *N*-caffeoyl-Put (Martin-Tanguy et al., 1978; Balint et al., 1987). By comparable reactions, pectin–polyamine amide bonds could also theoretically form. In this context, model compounds representing pectin–lysine amide bonds have been synthesised (Perrone et al., 1998), but their natural occurrence in plant cell walls remains inconclusive (Qi et al., 1995; Perrone et al., 1998). Pectin–polyamine amide linkages would allow polyamines to act as covalent cross-linkers between pectins, contributing to cell wall assembly, maintaining wall coherency during cell expansion, and strengthening the wall during biotic stress responses. Moreover, increasing evidence indicates that, in both animals and plants, polyamines can form amide bonds with side-chain –COOH groups of polypeptides. This reaction, catalysed by transglutaminases, occurs between the γ-carboxyamide group (R-$CONH_2$) of a glutamine residue and an amino group of a polyamine (Aeschlimann and Paulsson, 1991; Serafini-Fracassini et al., 1995; Waffenschmidt et al., 1999), e.g.

$$\text{R-CO}\mathit{N}\text{H}_2 + \text{NH}_2\text{-(CH}_2)_4\text{-NH}_2 \rightarrow \text{R-CONH-(CH}_2)_4\text{-NH}_2 + \mathit{N}\text{H}_3$$

Since polyamines have *two* –NH_2 groups, such reactions could generate protein–Put–protein cross-links (Waffenschmidt et al., 1999). In theory, a related reaction could involve a methylesterified pectic GalA residue (R-$COOCH_3$), e.g.

$$\text{R-COO}\mathit{C}\text{H}_3 + \text{NH}_2\text{-(CH}_2)_4\text{-NH}_2 \rightarrow \text{R-CONH-(CH}_2)_4\text{-NH}_2 + \mathit{C}\text{H}_3\text{OH}$$

potentially catalysed by a pectin methylesterase acting as a transacylase rather than a hydrolase. Again, since Put is bifunctional, this raises the possibility of pectin–Put–pectin or pectin–Put–protein cross-links, which could be important structural features in cell wall architecture.

Some evidence supports the existence of covalently wall-bound polyamines. For example, Berta et al. (1997) showed that in tobacco thin cell layers, inhibitors of polyamine synthesis caused various developmental changes which were accompanied by changes in the cell wall and/or middle lamella: the walls became more amorphous, were partially lysed and became uneven in thickness, and cell–cell adhesion was lost. Exogenous polyamines reversed some of these changes. These results are compatible with the idea that polyamines contribute to wall architecture, but it was impossible to be certain whether they influence wall architecture directly (by acting as ionic or covalent links between wall components) or indirectly (by controlling development in a 'hormonal' way, as with auxins or cytokinins). In embryogenic cultures of spruce, up to half the total endogenous Spd (but almost none of the Put) was found to be bound to material that was inextractable in cold aqueous 0.2 M perchloric acid (Santanen and Simola, 1992). This could represent polysaccharide–Spd or protein–Spd covalent bonding. In addition, covalent linkages between polyamines and the cell wall could theoretically be achieved by oxidative coupling between the aromatic moiety of conjugates such as *N*-feruloyl-Put and the phenolic components of the cell wall such as lignin (Razem and Bernards, 2002; Facchini et al., 2002).

The aims of this work were therefore to synthesise and characterise pectin–Put amide bonds, to devise a method by which to investigate the natural occurrence of such bonds, and to apply it to various kinds of plant material.

2. Results and discussion

2.1. Preparation and properties of GalA–Put amides

Two new model glycoconjugates, *N*-D-galacturonoyl-putrescinamide (MGP) and *N*,*N*′-di-D-galacturonoyl-putrescinamide (DGP) (Fig. 1), were synthesised by condensation of D-galacturonic acid (GalA) with [^{14}C]Put. After cation-exchange chromatography of the products, fractions were tested for (a) radioactivity and (b) ninhydrin staining. The two major radioactive products were obtained: the more abundant one showed characteristics compatible with those expected for DGP in that it did not bind the cation-exchange resin, it stained yellow with aniline hydrogen-phthalate (AHP) for the presence of reducing GalA residues, and it did not stain with ninhydrin, indicating that both the amino groups of Put were protected. The second radioactive product showed the characteristics expected of MGP: it bound to the cation-exchange resin, stained yellow with AHP and stained pink with ninhydrin, indicating a free amino group.

On paper electrophoresis (PE) at pH 2.0 and 3.5, DGP had no net charge, indicating the absence of free –COOH and –NH_2 groups. MGP moved towards cathode owing to the presence of a free amino group. Both molecules were readily resolved from authentic Put, which migrated faster towards the cathode because it has two free amino groups and a lower M_r than MGP and DGP.

2.2. Susceptibility of GalA–Put bonds to hydrolysis

The susceptibility of MGP and DGP to various hydrolytic treatments is summarised in Table 1. The amide linkage(s) of both molecules were labile in hot acid and hot alkali, but stable to cold acid and relatively stable to cold alkali.

After 1 h in hot trifluoroacetic acid (TFA; 1 M at 120 °C), MGP had been partially hydrolysed to free Put and GalA, whereas DGP had given MGP as intermediary product together with some Put and GalA. By 6 h, all the MGP had been destroyed, yielding Put as the main ninhydrin-positive product and at least two minor unidentified ninhydrin-staining by-products (Fig. 2); the GalA had been largely degraded after 6 h in hot acid, as expected (free uronic acids being unstable during prolonged treatments with hot acid; Fry, 2000).

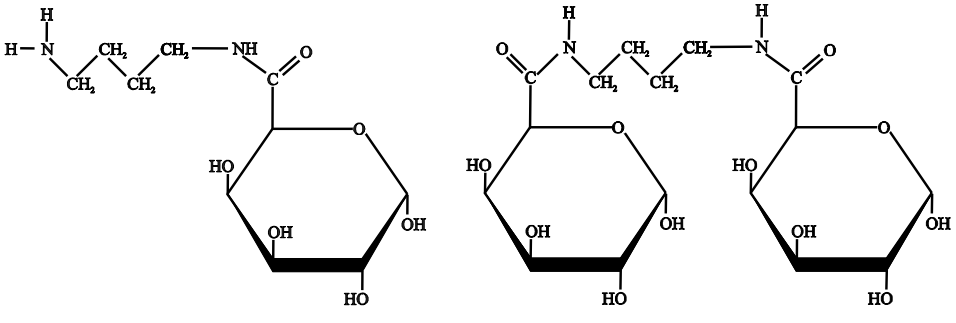
Table 1
Qualitative summary of the susceptibility to cleavage of GalA–Put amide bonds

Method of cleavage	Susceptibility to cleavage	
	MGP	DGP
2 M TFA, 25 °C	−	−
2 M TFA, 100 °C	++	++
2 M TFA, 120 °C	+++	+++
0.1 M NaOH, 25 °C	±	−
0.1 M NaOH, 100 °C	+++++	+++++
0.5% Pronase	−	−
0.5% Trypsin	−	−
0.5% Papain	−	−
0.5% Proteinase K	−	−
0.5% Driselase	−	−

−, Essentially resistant.
±, Very slow or partial degradation.
+, Slow degradation.
+++++, Very rapid degradation.

In cold alkali (0.1 M NaOH at 25 °C), both the amide bonds of DGP were stable for 16 h (no Put was liberated), although the reducing groups were gradually destroyed (probably oxidised) between 6 and 16 h, as indicated by loss of AHP staining. However, under the same conditions, MGP was slowly hydrolysed (data not shown): by 2 h some free Put had appeared and by 16 h all the MGP had been degraded to Put (main product) plus at least three minor ninhydrin-positive by-products. Released GalA was not expected to be observed since reducing sugars are short-lived in NaOH (Sowden, 1957). At 100 °C, alkaline hydrolysis of the amide bond(s) of both MGP and DGP was very rapid (complete in <30 min), yielding Put (main product) and minor ninhydrin-positive by-products.

MGP and DGP were resistant to enzymic digestion with all proteases tested (Table 1) and with Driselase (data not shown). Driselase is a mixture of hydrolytic enzymes able



MGP DGP

Fig. 1. Chemical structures of *N*-D-galacturonoyl-putrescinamide (MGP) and *N*,*N*′-di-D-galacturonoyl-putrescinamide (DGP).

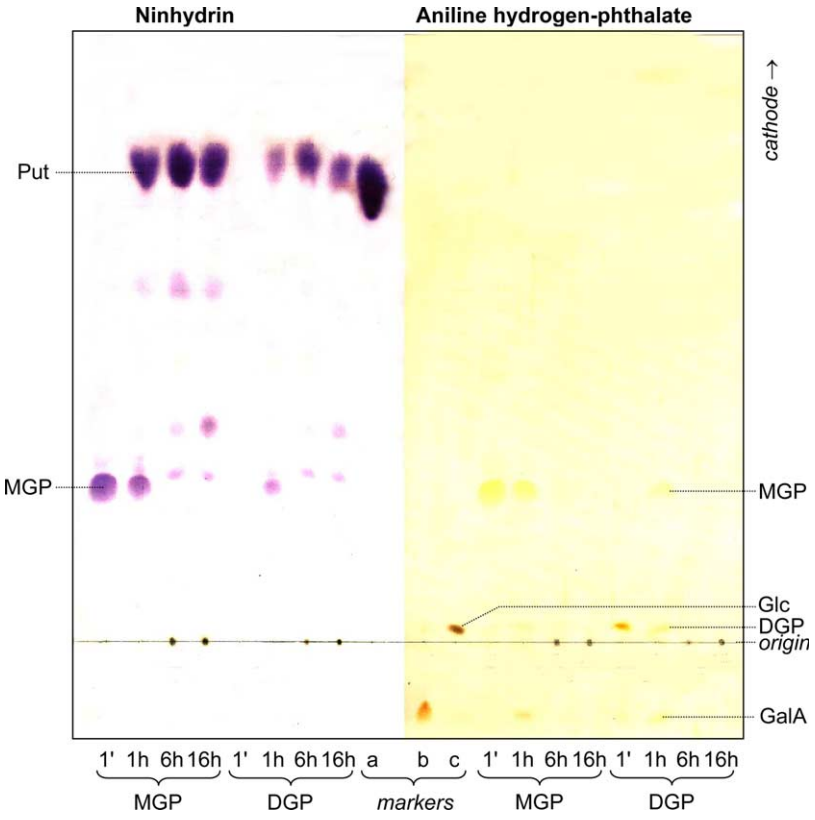


Fig. 2. Effect of hot acid on MGP and DGP. Samples were treated with 1 M TFA at 120 °C for various times and then subjected to PE at pH 3.5 (3 kV, 15 min). Duplicate electrophoretograms were stained for free amino groups with ninhydrin (left) or for reducing sugar groups with AHP (right). Markers: a, putrescine (Put); b, galacturonic acid (GalA); **c**, glucose (Glc).

to hydrolyse plant cell wall polysaccharides essentially to completion (Fry, 2000). In particular, the pectinolytic enzymes present in Driselase hydrolyse homogalacturonan and rhamnogalacturonan-I almost completely to galacturonic acid and associated neutral monosaccharides. Also, the pectin-esterases present in Driselase can remove the methyl ester groups from all pectic GalA residues except the few that are (or are adjacent to) *O*-acetylated GalA residues (Perrone et al., 2002). Driselase could thus potentially be used to hydrolyse pectins to low-M_r products without cleaving GalA–Put amide bond(s).

2.3. Preparation and Driselase-digestion of $GalA_n$–Put conjugates

We prepared pectin–Put conjugates as model substrates on which to develop a methodology for seeking GalA–polyamine amide bonds in vivo. $GalA_n$ was treated with a limited supply of [^{14}C]Put (1 μmol of Put per 2380 μmol of GalA residues) in the presence of various doses of the coupling reagent (EDAC/NHS). The proportion of the [^{14}C]Put that was thereby condensed to the polysaccharide was estimated by PC (Fig. 3). With 4 μl of coupling reagent, only ~25% of the ^{14}C became $GalA_n$-bound, and thus the mono-amide ($GalA_n$–Put) was expected to predominate; with 64 μl of coupling reagent, a plateau of polymer yield had been reached, and thus an appreciable

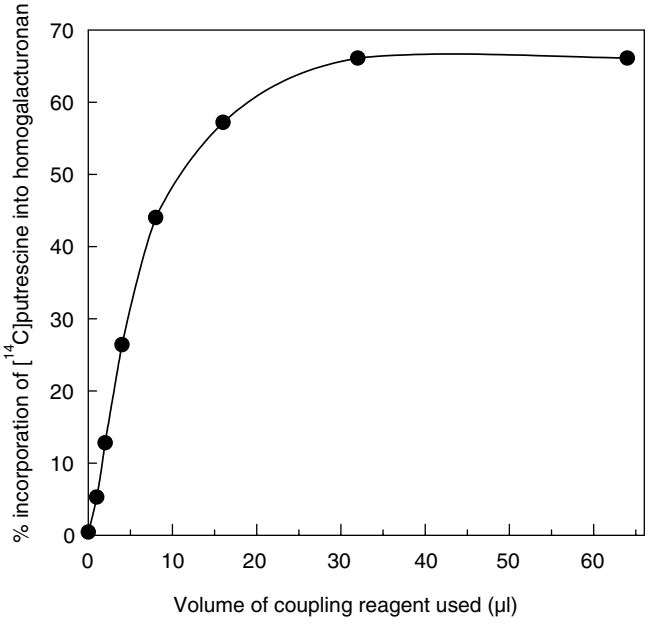


Fig. 3. Effect of dose of coupling reagent (EDAC/NHS) on the condensation of [^{14}C]Put with $GalA_n$. To 100 μl of a solution containing $GalA_n$ and [^{14}C]Put, we added 0–64 μl of EDAC/NHS reagent. After 1 h at 20 °C, the polysaccharide was assayed for radioactivity.

proportion of the di-amide ($GalA_n$–Put–$GalA_n$) was expected to be present. Owing to the low Put:$GalA_n$ ratio used, the vast majority of the GalA residues would have

remained unconjugated (and therefore not radioactive) even under the latter conditions. The two samples, produced by use of 4 and 64 μl of reagent, were selected as 'low-amide' and 'high-amide' $GalA_n$ preparations, respectively (Fig. 3).

After removal of unincorporated Put, the ''low-'' and ''high-amide'' $GalA_n$ samples were digested with Driselase and the products analysed by electrophoresis at pH 2.0 and 6.5 (Fig. 4). At pH 2.0 (i.e., when free amino groups are almost fully ionised but free carboxy groups are only slightly ionised), at least two radioactive digestion-products (**X**, **Y**) migrated towards the cathode (Fig. 4(a) and (b)). Their positive charge shows that one of the amino groups in the Put moiety was not amide-linked. These two cationic products are likely to be $GalA_2$ and $GalA_3$ amide-linked to one Put moiety (see structural representations in Fig. 4(b)); they would have similar net charge ($Q \approx +1.0$) but differ in mass and thus be separable, since PE mobility is approximately

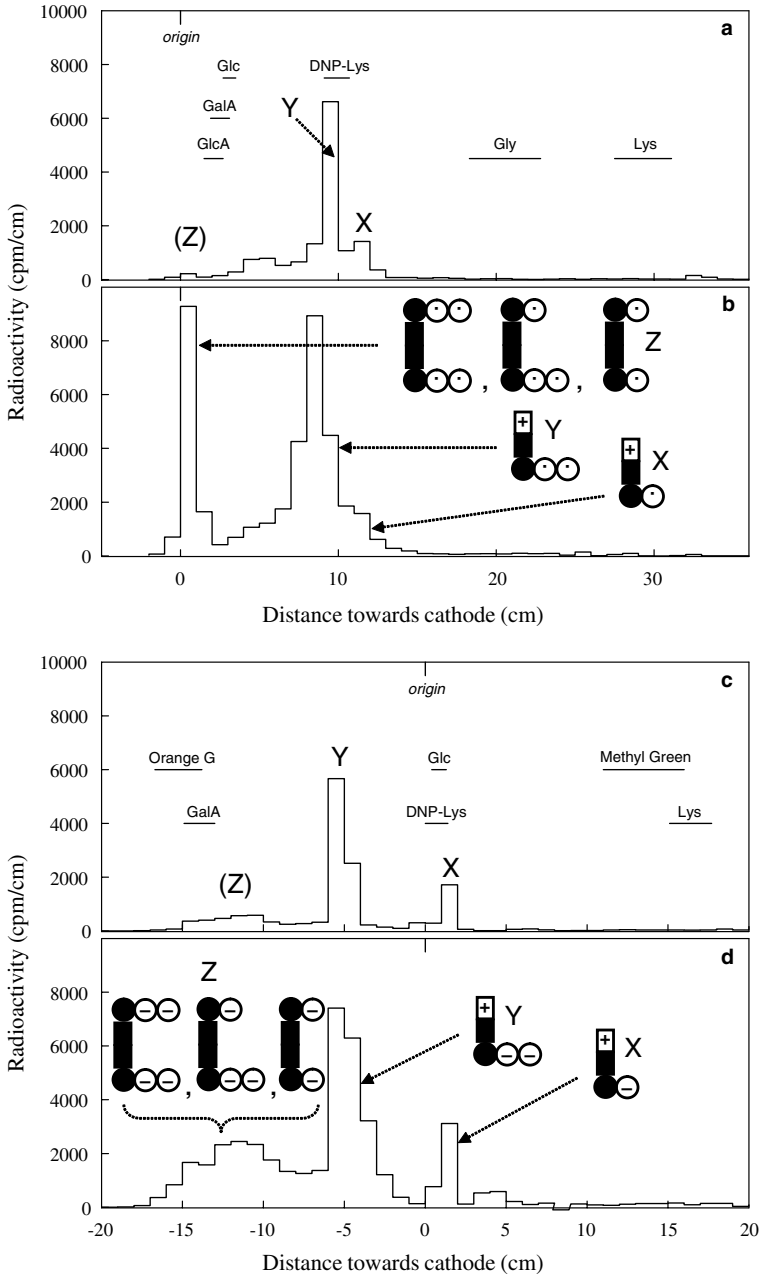


Fig. 4. High-voltage electrophoresis of products obtained by Driselase digestion of two preparations of $GalA_n$–[^{14}C]Put conjugate. (a,b) Electrophoresis at pH 2.0; (c,d) electrophoresis at pH 6.5. The products shown were obtained from $GalA_n$ to which a low (a,c) or high (b,d) proportion of the [^{14}C]Put had been coupled, by use of 4 or 64 μl of the coupling reagent, respectively (cf. Fig. 3). Proposed structures for the major radioactive products are indicated: rectangle = Put; circle = GalA residue; black-filled = involved in amide linkage (therefore uncharged); white = unsubstituted and therefore possessing an ionisable amino group (+) or carboxy group [(−) or (·), representing a full or slight negative charge, respectively, depending on the pH of the electrophoresis buffer].

proportional to $Q/M_r^{2/3}$ (Offord, 1966). No MGP was released, which migrates almost as fast as glycine (see Gly marker in Fig. 4(a)); thus, as expected, an amide-linked Put group protects at least one glycosidic linkage from Driselase digestion. Digestion of the "high-amide" $GalA_n$ gave an enhanced yield of an additional peak (**Z**) with little net electrophoretic mobility at pH 2.0 (Fig. 4(b)), proposed to contain several compounds having a Put moiety amide-linked via both its amino groups to $GalA_2$ and/or $GalA_3$.

At pH 6.5 (i.e., when free amino and carboxy groups are both almost fully ionised), some neutral material was detected (peak **X**; Fig. 4(c) and (d)), likely to be $GalA_2$ amide-linked to one Put moiety; and a product with a moderate net negative charge (peak **Y**), likely to be $GalA_3$ amide-linked to one Put moiety (net charge ≈ -1). In the high-amide $GalA_n$ preparation, there was in addition an enhanced yield of several partially resolved products with stronger net negative charges ('peak' **Z**), ascribed to a Put moiety amide-linked via both its amino groups to $GalA_2$ and/or $GalA_3$ (net charge -2, -3 or -4).

These observations support the conclusion that Driselase is capable of digesting Put-carrying pectic polysaccharides, including those in which the Put moiety may act as a cross-link between two pectic chains, to release electrophoretically amenable oligosaccharide products, while maintaining the amide bond(s) intact. We therefore undertook the following phase of the work to look for possible naturally occurring pectin–Put amide complexes.

2.4. Uptake and incorporation of [^{14}C]Put by cultured cells

In an attempt to radiolabel GalA–polyamine conjugates, we tested the ability of suspension-cultured cells of various plant species to take up and incorporate [1,4-^{14}C]Put. Since Put is the precursor of Spd and Spm (Pegg, 1986), this strategy was expected also to radiolabel any pectin-linked Spd and Spm as well as Put moieties.

After 5 h incubation, net uptake of ^{14}C from the medium was extensive (>80%) in 3- and 10-d-old cultures of rose, spinach and *Arabidopsis*, but uptake by 3- and 10-d-old maize cells was <6% (data not shown). Since Put is positively charged, it is unlikely to go through the plasma-membrane in absence of a specific carrier. The presence of a polyamine carrier mediating active transport was clearly shown by Antognoni et al. (1995) in carrot protoplasts. An adequate Put carrier was evidently not present in cultured maize cells. We therefore focused on two species (*Arabidopsis* and rose) that were highly active at Put uptake.

Radioactivity rapidly disappeared from the medium (>80% in 2 h) and accumulated in the cells of both rose and *Arabidopsis* (Fig. 5(a)). At the end of the first hour of incubation, only 39% of the cellular ^{14}C was still ethanol-soluble in *Arabidopsis* whereas in rose cells ~80% was ethanol-soluble. Thereafter there was a slight decrease in ethanol-soluble cellular ^{14}C, probably partly due to loss as $^{14}CO_2$. The ^{14}C in the alcohol-insoluble residue (AIR, which consists mainly of polysaccharides and proteins) had reached a plateau by 2 h, at about 73% of the cellular ^{14}C in *Arabidopsis* and 21% in rose.

To investigate in what manner the ^{14}C is associated with cellular polymers in the AIR, we sequentially extracted with acidified phenol (PAW; which would disrupt most ionic bonds and would solubilise most proteins), 70% ethanol (to remove PAW), and cold 1 M TFA (to disrupt any ionic bonds remaining after the PAW treatment). These extractants would not be expected to solubilise [^{14}C]Put residues that were amide-bonded to pectins. In both plant species, ~90% of the AIR-associated, ^{14}C-labelled material was PAW-soluble (Fig. 5(b)). Furthermore, 90–95% of the PAW-solubilised ^{14}C-material had a high mobility on PE at pH 2 (data not shown), indicating that it was of low M_r; the remaining 5–10% could have been incorporated into proteins. Subsequent treatment with 70% ethanol did not solubilise any additional ^{14}C, but cold aqueous TFA did release a small proportion (Fig. 5(b)), all of which was of low M_r. Since cold TFA is unable to hydrolyse GalA–Put amide linkages, we conclude that the TFA-soluble material had been associated with AIR polymers by strong ionic bonds, resistant to PAW extraction.

The residue that remained insoluble after PAW-, ethanol- and TFA-extraction still contained some ^{14}C, the proportion of which increased progressively with the time for which the cells had been incubated in the presence of [^{14}C]Put (Fig. 5(c)). After 6 h incubation, inextractable ^{14}C represented about 7% and 9% of the total ^{14}C-AIR in *Arabidopsis* and rose, respectively.

2.5. Assays for natural occurrence of pectin–Put amide linkages

To prepare a larger amount of ^{14}C-AIR, which could potentially contain GalA–Put bonds, we incubated cells of *Arabidopsis* and rose and differentiated chickpea internodes with [1,4-^{14}C]Put for 6 h. We confirmed that some of the ^{14}C was lost from the system (28%, 27% and 16% in *Arabidopsis*, rose and chickpea, respectively; data not shown), presumably as $^{14}CO_2$ (Singh et al., 1993). Isolated ^{14}C-labelled cell walls were washed with homogenisation buffer and 70% ethanol to remove weakly bound radiochemicals, then extracted in PAW, 70% ethanol and cold 1 M TFA as before (Fig. 6). About 3–6% of the cell-associated ^{14}C was recovered in the final TFA-insoluble residue.

The radioactive wall-residue (after extensive Pronase-digestion in the case of chickpea) was hydrolysed with Driselase or with hot 6 M HCl. If any pectin–Put amide linkages were present, Driselase would be expected to release compounds similar to those shown in Fig. 4. On the contrary, hot HCl would break the amide bonds, liberating Put or related polyamines (e.g., Spm and Spd).

Driselase-generated compounds were separated by PE at pH 2.0 (Fig. 7(a)). In both rose and *Arabidopsis*, three main peaks were detected (**A**–**C**). **A** had no net charge at pH 2.0, suggesting the presence of material derived from

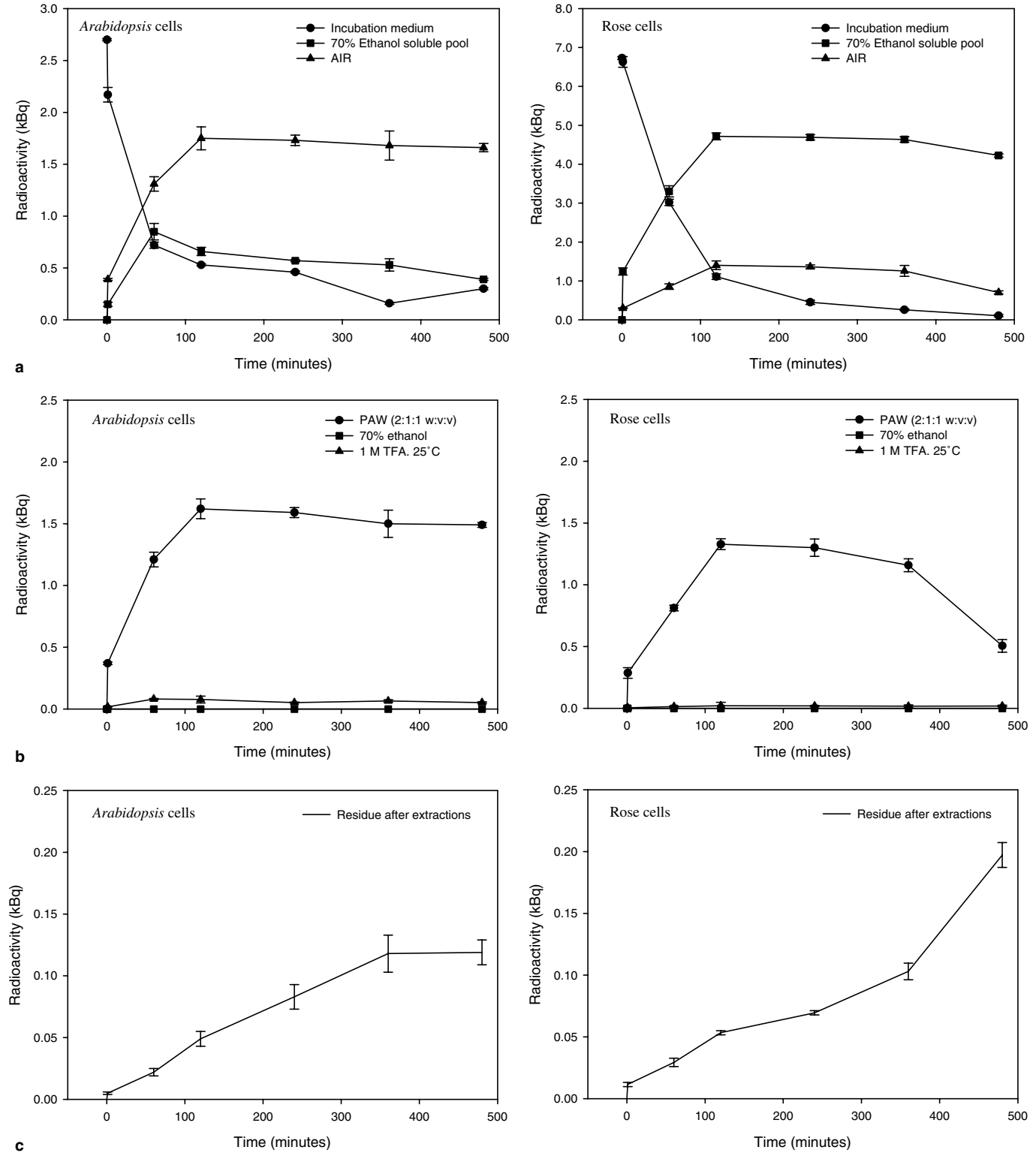


Fig. 5. Uptake of [^{14}C]Put by 7-d-old *Arabidopsis* and rose cultures from their medium and subsequent distribution of radioactive compounds. (a) Partitioning between ethanol-soluble and -insoluble pools. (b,c) Distribution of ^{14}C between four sub-fractions of the AIR defined by their extractability into (b) PAW, 70% EtOH and 1 M TFA (applied sequentially), and (c) the residue that resisted these three extractants. Left graphs, *Arabidopsis* cell-cultures; right graphs, rose cell-cultures.

incomplete polymer-digestion or possibly of relatively large neutral compounds similar to peak **Z** of Fig. 4(b) and thus lacking a free positively charged group. **B** was a rather wide peak, migrating a little slower than MGP (Fig. 7(a)) but mainly faster than peak **Y** of Fig. 4(b). Peak **B** of Fig. 7(a) could have included peak **X** of Fig. 4(b), but

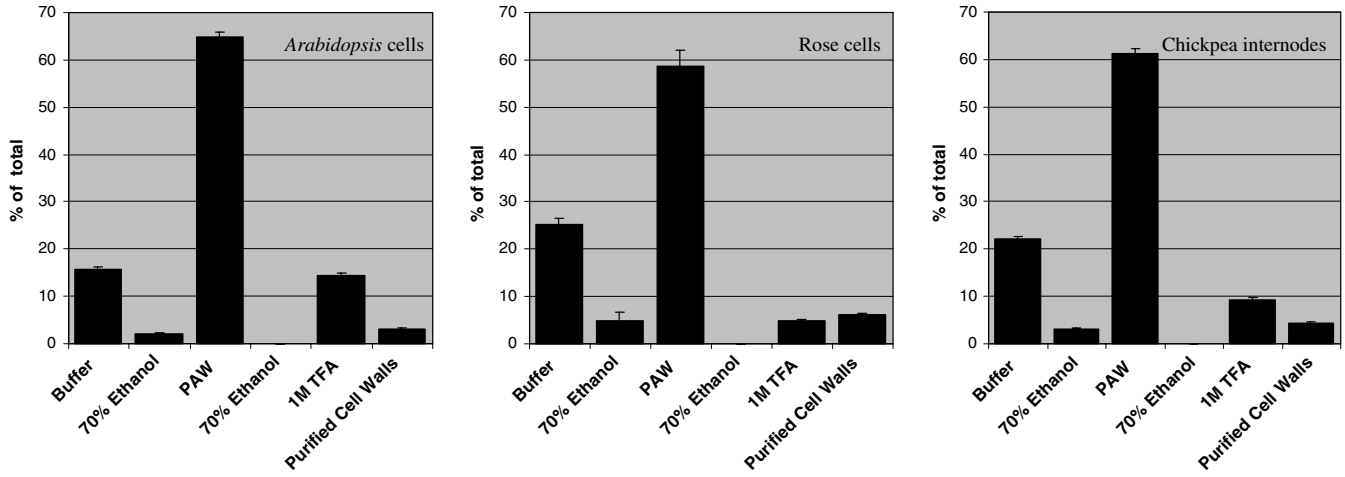


Fig. 6. Distribution of radioactivity taken up by *Arabidopsis* and rose cell-cultures and by chickpeas internodes, after 6 h incubation in presence of [1,4-^{14}C]Put. The *y*-axis reports the % distribution of total cellular ^{14}C between the six pools shown on the *x*-axis (i.e., excluding ^{14}C remaining in the medium or lost as $^{14}CO_2$). Data are the mean of three experiments ±SD.

accompanied by other compounds, causing peak **B** to broaden. **C**, probably two partially resolved peaks, approximately co-migrated with lysine and γ-aminobutyrate (GABA). Little or no ^{14}C co-migrated with free Put, Spd or Spm. In chickpea internodes, only one main peak was detected, near the origin; a very small peak approximately co-migrated with the free polyamine(s). The near-absence of free ^{14}C-polyamines, after Driselase digestion, indicated that the sequential extractions used had removed from the AIR all polyamines that had been ionically bound to pectins.

At pH 6.5, electrophoresis of the Driselase-digestion-products of rose cell walls (Fig. 8(a)) gave a large peak of ^{14}C-labelled material with no net charge, at least four negatively charged products (two co-migrating with Asp and Glu, and two approximately co-migrating with compound **Y** of Fig. 4(c) and (d)), and several minor products with a net positive charge (one approximately co-migrating with Lys). On PC in BAW, a wide range of ^{14}C-products were detected (Fig. 8(b)): major ones approximately co-migrated with Asp and Glu, minor ones with Lys, Val and Leu. PC in EAW broadly agreed with these conclusions (Fig. 8(c)); in particular, little ^{14}C migrated in the oligogalacturonide zone [where compounds such as **X**, **Y** and **Z** (Fig. 4) would have been expected].

Erwinia endopolygalacturonase solubilised very little of the ^{14}C from the walls of [^{14}C]Put-fed rose cells (data not shown).

Compounds generated by the HCl-hydrolysis of ^{14}C-labelled cell walls were also analysed by PE at pH 2.0 (Fig. 7(b)). In both rose and *Arabidopsis*, peaks similar to **A**, **B** and **C** were present. The yield of **A** was lower than after Driselase digestion, whereas ^{14}C in zone **B** was elevated, indicating that 6 M HCl can hydrolyse **A** to yield faster-migrating monomers; nevertheless, the absence of free polyamines clearly indicated that the AIR lacked pectin–Put amide bonds. Re-electrophoresis of **B** at pH 3.5 resolved four peaks (**a**–**d**) (Fig. 9(a)) which approximately co-migrated with external markers of Asp, Glu, Glc and Lys, respectively. Each peak was eluted and further characterised. Peaks **a** and **b** were submitted to more prolonged electrophoresis at pH 3.5 after addition of Asp and Glu, respectively, as internal markers. The exact co-migration of the ^{14}C with the relevant internal marker strongly supported the identity of **a** and **b** as Asp and Glu, respectively (Fig. 9(b)). When peak **c** was re-run by PE at pH 6.5 (Fig. 9(c)), it again showed no net charge. PC in BAW resolved three radioactive peaks co-migrating with Gly (major), Ala and Val, suggesting that **c** is a mixture of such amino acids. On PC in EAW, peak **d** was separated in two main compounds, likely to be Lys (∼80%) and GABA (∼10%) as indicated by use of internal markers (Fig. 9(d)).

Also in chickpea internode cell walls, 6 M HCl did not generate free ^{14}C-polyamines (Fig. 7(b)), confirming the absence of pectin–Put amide bonds in differentiated tissues. Most of the radioactivity was incorporated into neutral compounds, possibly neutral sugars derived from Put catabolism.

The catabolism of Put, Spd and Spm is thought to begin with the action of diamine and polyamine oxidases, in reactions that generate H_2O_2 as well as pyrroline, diaminopropane and related products. For example, Put is oxidised to 4-aminobutanal, which non-enzymically cyclises to Δ^1-pyrroline, the latter being further oxidised by NAD^+ to yield GABA (Flores and Filner, 1985; Balint et al., 1987; Kumar and Thorpe, 1989; Rea et al., 2004). Besides catabolism of free Put, another proposed route of catabolism is after conjugation to caffeic acid: Put → hydroxycinnamoyl-Put → hydroxycinnamoyl-4-aminobutanal → hydroxycinnamoyl-GABA → GABA (Balint et al., 1987). Spd can probably be catabolised to GABA plus β-alanine, the latter formed via diaminopropane (Awal et al., 1997).

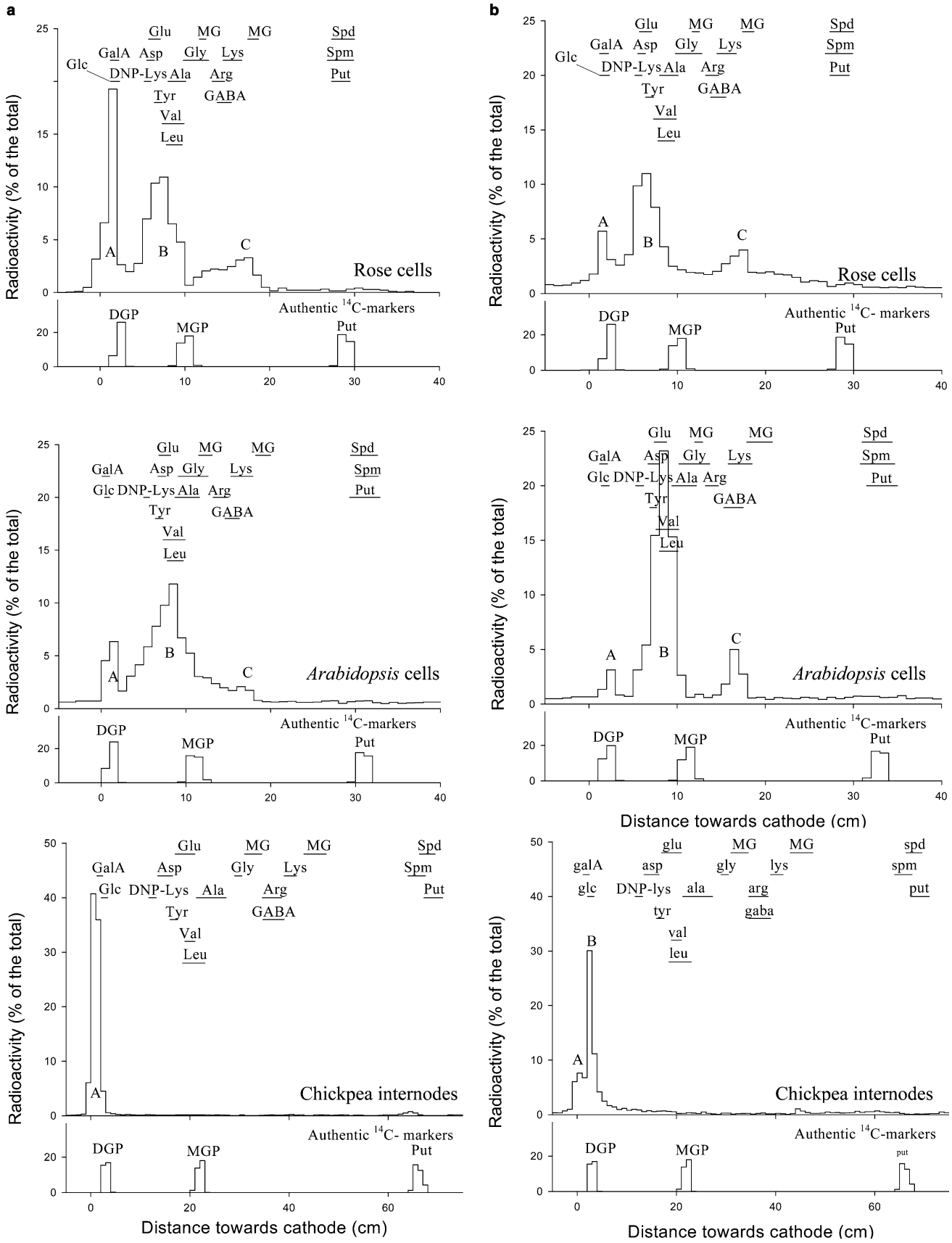


Fig. 7. Radioactive products obtained after Driselase digestion (a) or HCl hydrolysis (b) of cell wall polymeric material purified from [1,4-^{14}C]Put-fed rose cell-cultures (top), *Arabidopsis* cell-cultures (centre) and chickpea internodes (bottom). Digestion products were analysed by PE at pH 2.0 (3.0 kV for 30 min). MG = methyl green (marker; partially resolved into two spots).

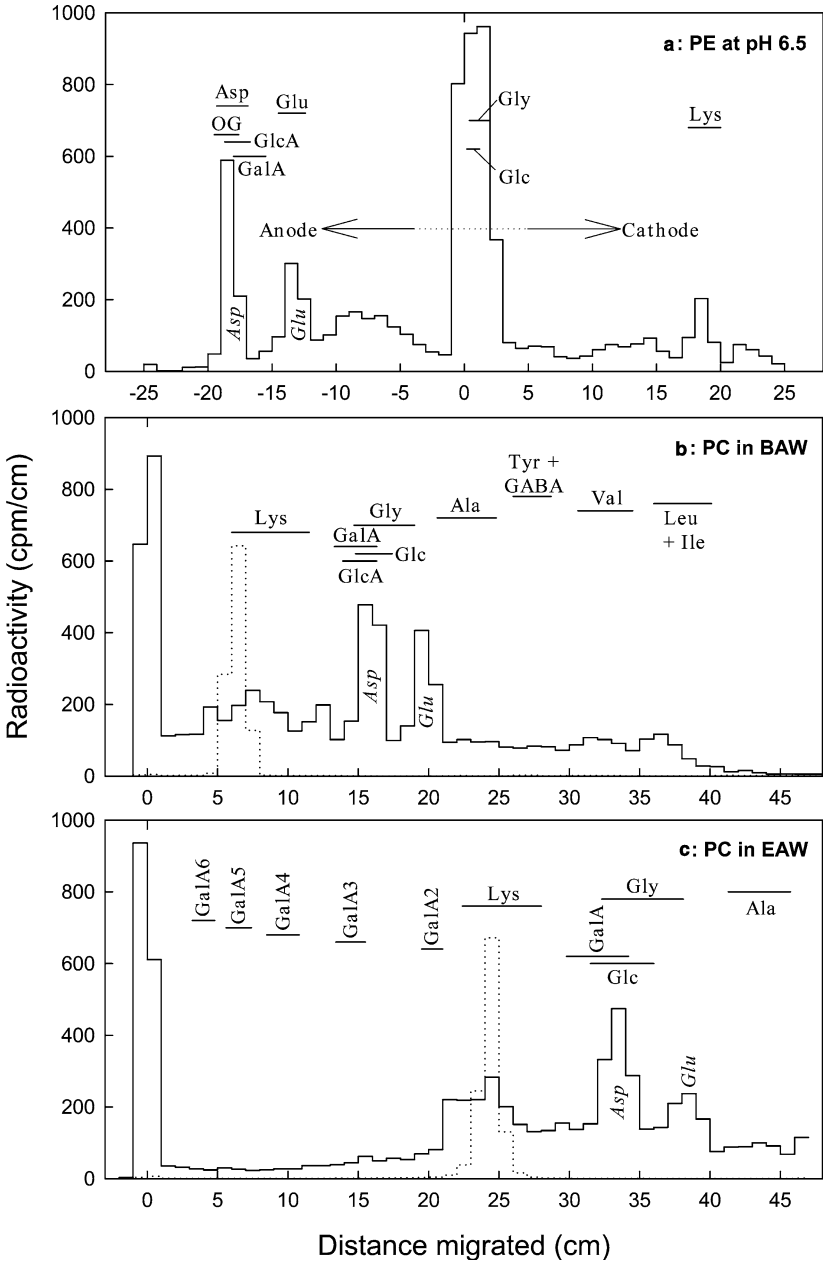


Fig. 8. Further radiochemical characterisation of products obtained by Driselase digestion of the walls of [^{14}C]Put-fed rose cells. Products were analysed by PE at pH 6.5 (a), or PC in BAW (b) or EAW (c). Horizontal lines show the positions of external markers (OG = Orange G; GalA2–6 = oligogalacturonides of degree of polymerisation 2–6). Italic labels indicate the radioactive peaks ascribed to aspartate and glutamate (see text). The dotted histograms (· · · · · ·) in (b) and (c) show the chromatographic behaviour of authentic [^{14}C]Put run simultaneously. In (a), free [^{14}C]Put migrated >25 cm towards the cathode (not shown). In (b), the solvent front was at 48 cm; in (c) it was run off the end of the paper.

[^{14}C]GABA is further metabolised to a Krebs cycle intermediate, succinate (Flores and Filner, 1985; Bouche et al., 2003), from which ^{14}C can be incorporated into numerous diverse metabolites.

3. Conclusions

We report the synthesis and characterisation of model *N*-D-galacturonoyl-putrescinamides and homogalacturonan–putrescine conjugates, and a strategy by which to search for such novel compounds in plant cell walls. Our data clearly demonstrate the absence of newly synthesised secondary amide bonds linking Put (or its metabolic derivatives, Spd and Spm) to GalA, showing that if such amides are present, they represent a very minor component of plant cell wall in cultured rose and *Arabidopsis* cells and chickpea internodes. The ^{14}C incorporated into cell wall polymers was due mainly to radiolabelled amino acids and possibly neutral sugars produced by Put catabolism.

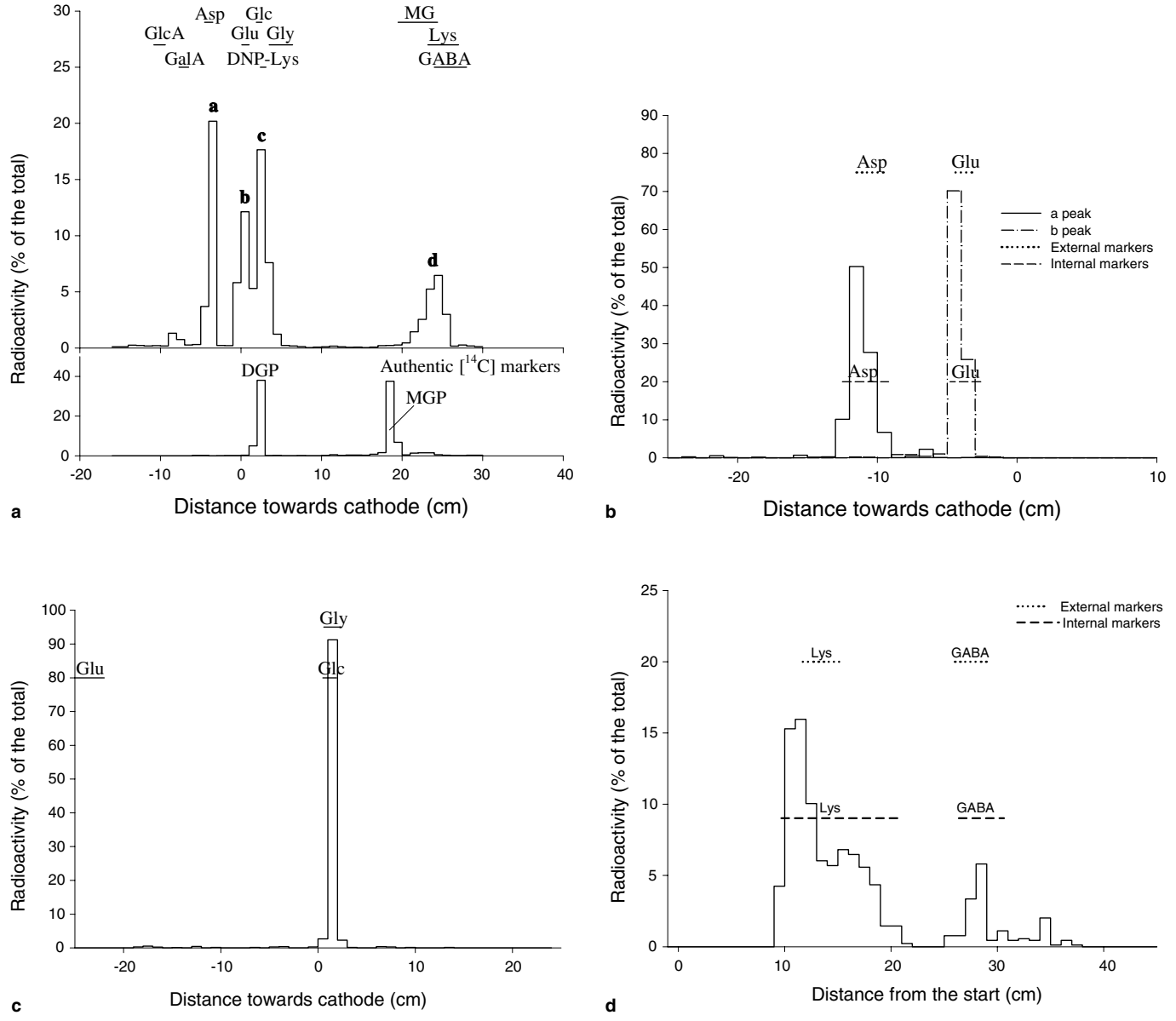


Fig. 9. Radioactive products of HCl hydrolysis of cell wall polymeric material obtained from [1,4-^{14}C]Put-fed *Arabidopsis* cells. (a) Hydrolysis products analysed by PE at pH 3.5 (3.0 kV for 50 min). Peaks **a**–**d** were eluted from the electrophoretogram and further analysed: (b) PE (pH 3.5; 3 kV; 90 min) of peaks **a** and **b**; (c) PE (pH 6.5; 3 kV; 60 min) of peak **c**; (d) PC in EAW of peak **d**.

4. Experimental

4.1. Chemicals and radiochemicals

Driselase (from a basidiomycete) was from Fluka (Milan, Italy). Pronase (protease type XIV from *Streptomyces griseus*), trypsin (protease type I from bovine pancreas), papain (from *Papaia* latex), proteinase K (from *Tritirachium album*) and all other chemicals were purchased from Sigma Chemical Co. [1,4-^{14}C]Putrescine dihydrochloride (3.96 GBq mmol^{-1}) was from Amersham International (Bucks, UK). Driselase was partially purified [precipitated with $(NH_4)_2SO_4$ and de-salted] as before (Fry, 2000).

4.2. Culture conditions

Cell-suspension cultures of rose (*Rosa* sp., "Paul's Scarlet") were routinely sub-cultured once per fortnight by dilution into 10 vol of a medium (Fry and Street, 1980) containing 2% D-glucose as sole carbon source. Cultures were incubated under constant dim illumination on an orbital shaker at 25 °C. *Arabidopsis thaliana* cell-suspension cultures were grown at 25 °C as described by May and Leaver (1993) and sub-cultured by regular transfer of 30 ml of a 7-d-old culture into 170 ml of fresh medium containing 3% sucrose as sole carbon source.

Chickpea (*Cicer arietinum* L.) seeds were soaked overnight in aerated water and grown in a greenhouse under

natural light condition for 10 d. Half-elongated fourth internodes were harvested and used for incubation.

4.3. Paper chromatography (PC) and paper electrophoresis (PE)

PC was performed on Whatman 3MM paper in Me_2CO/pyridine/HOAc/H_2O (4:1:3:2 by vol.; APyAW) for 16–18 h, in EtOAc/HOAc/H_2O (10:5:6 v/v/v; EAW) for 8–9 h, or in BuOH/HOAc/H_2O (12:3:5 v/v/v; BAW). PE was at 3 kV on Whatman 3MM at pH 2.0 [H_2O/HCOOH/HOAc (45:1:4 v/v/v)], pH 3.5 [H_2O/HOAc/pyridine (189:10:1 v/v/v)] or pH 6.5 [H_2O/HOAc/pyridine (300:1:33 v/v/v)]. Detection of markers was with 0.5% ninhydrin in Me_2CO for amino compounds and with AHP or $AgNO_3$ for reducing sugars (Fry, 2000).

4.4. Assay of radioactivity

Samples on dried strips of chromatography paper were placed in 2 ml of OptiScint HiSafe Scintillation fluid (Wallac) and assayed in a liquid scintillation counter at ~90% efficiency.

4.5. Synthesis of MGP and DGP

Two new model glycoconjugates (Fig. 1) were synthesised. To 20 ml of 50 mM MES (Na^+) buffer, pH 6.5, were simultaneously added: 0.1 g Put, 0.3 g galacturonic acid, 1.32 g 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) (Brown and Fry, 1993), 0.04 g *N*-hydroxysuccinimide (NHS) (Sehgal and Vijay, 1994) and 92.5 kBq [1,4-^{14}C]Put. The mixture was incubated for 3 h at ~25 °C with constant stirring. The reaction was stopped by addition of 1 ml 20% (w/v) NH_4OAc and the solution was applied to a 1.5 × 15 cm column of Dowex 50WX4 resin (pyridinium form). Solutes were eluted with a discontinuous gradient of 0–2 M pyridine/HOAc buffer, pH 4.7. Replicate aliquots (10 μl) from each fraction were spotted on to filter paper and stained with ninhydrin or AHP or assayed for radioactivity. Radioactive fractions were pooled, freeze-dried, dissolved in H_2O and subjected to preparative PE (pH 3.5, 3 kV, 45 min). Radioactive compounds with electrophoretic (im)mobility and susceptibility to staining compatible with those expected for DGP and MGP were eluted from the PE with H_2O as described by Eshdat and Mirelman (1972) and stored as aqueous solutions at −20 °C.

4.6. Synthesis of $GalA_n$–[^{14}C]Put amide bonds

To 100 μl of a solution containing 0.9% (w/v) $GalA_n$ ('polygalacturonic acid') and 85 kBq/ml carrier-free [1,4-^{14}C]Put in 45 mM MES (Na^+), pH 6.5, we added 0–64 μl of coupling reagent [EDAC/NHS; a freshly prepared solution containing 4.5% (w/v) EDAC and 0.3% (w/v) NHS]. After 1 h at 20 °C, a small proportion of the mixture was analysed by PC in EAW. Material with R_F 0.00 (polysaccharide) was assayed for radioactivity. The remainder of each preparation was de-salted on Bio-Gel P-2 and re-dissolved in PyAW (1:1:98).

4.7. Evaluation of susceptibility of isopeptide bonds to cleavage by acid, alkali and enzymes

Susceptibility of DGP and MGP to acid hydrolysis was tested in 1 M TFA at 120, 100 and 25 °C. Alkaline degradation was monitored in 0.1 M NaOH at 25 and 100 °C. Enzymic hydrolysis of DGP and MGP was tested for 48 h at 25 °C in 0.5% Driselase [in pyridine/HOAc/H_2O (1:1:98 v/v/v; PyAW), pH 4.7]; 0.5% Pronase [in 30 mM NH_4HCO_3, pH 7.9, containing 1 mM $CaCl_2$]; 0.5% trypsin [in 100 mM $(NH_4)_2CO_3$, pH 8.0, containing 1 mM $CaCl_2$]; 0.5% papain [in 100 mM NH_4OAc, pH 5.5]; 0.5% proteinase K [in 10 mM Tris–HCl, pH 7.5, containing 1 mM $CaCl_2$]. The $GalA_n$–[^{14}C]Put preparations were also digested in Driselase. All enzyme solutions contained 0.5% 1,1,1-trichloro-2-methylpropan-2-ol (chlorobutanol) to prevent microbial growth (Fry, 2000). Degradation- and hydrolysis-products were analysed by PE at pH 2.0 (20 min) or 3.5 (15 min) and by PC in APyAW.

4.8. Time-course of put uptake and cell wall radiolabelling

Aliquots (5 ml) of 7-d-old *Arabidopsis* or rose cell cultures were transferred into sterile 5.5-cm Petri dishes. Cells were pre-incubated for 30 min on a rotary shaker (100 rpm) at 25 °C in daylight. [1,4-^{14}C]Put was then added (37 kBq per dish). At intervals (1 min, then 2–8 h), 500 μl of cell-suspension was taken and centrifuged at 500*g* for 2 min at 4 °C and the supernatant was collected. The pellet (intact cells) was rapidly washed with 2 × 500 μl of 40 mM Hepes (Na^+), pH 7.5, containing 3% sucrose. The three pooled supernatants were assayed for soluble extracellular ^{14}C. The cells were resuspended in 1 ml cold 70% ethanol and shaken overnight at 4 °C. This step was repeated twice more. The pooled ethanolic extracts were assayed for ^{14}C. AIRs were shaken overnight at 25 °C in 1 ml PhOH/HOAc/H_2O (2:1:1 w/v/v; PAW) for extraction of proteins and ionically bound substances. PAW-insoluble cell walls were pelleted at 8800*g* for 10 min, and washed with 200 μl PAW. PAW-soluble supernatants were assayed for ^{14}C. In the same way, the pellets were sequentially washed with 1 ml + 200 μl 70% ethanol and 1 ml + 200 μl 1 M TFA (25 °C). Aliquots of the extracts and TFA-insoluble residues were assayed for ^{14}C.

4.9. In-vivo search for GalA–put amide bonds

Arabidopsis and rose cells were pre-incubated as above; excised 4th internodes of 10-d-old chickpea seedlings were pre-incubated in 5 ml sterile water. [1,4-^{14}C]Put was then added (~130 kBq per dish). After 6 h further incubation, cells were collected by centrifugation at 500*g* for 2 min at 4 °C and washed as above, whereas chickpea internodes

were removed from the medium and washed with 2×5 ml water. The pooled incubation media and washings were assayed for ^{14}C. *Arabidopsis* and rose cells were suspended in 5 ml homogenisation buffer [40 mM Hepes (Na^+), pH 7.5, containing 10 mM imidazole (as glucosidase inhibitor), 1 mM benzamidine, 5 mM 6-amino-*n*-hexanoic acid and 1 mM phenylmethylsulphonyl fluoride (as proteinase inhibitors), and 10 mM dithiothreitol] and sonicated at output 3 with pulsed ultrasound application (90% burst and 10% resting) for 4 min by a Branson (Model B-15) sonicator. Chickpea internodes were ground in liquid N_2, then suspended in 5 ml of homogenisation buffer and homogenised in a glass Potter–Elvehjem homogeniser. Aliquots of the homogenate were assayed for ^{14}C. Homogenates were centrifuged at 800*g* for 10 min. The pellet (cell walls) was washed with 3×1 ml of homogenisation buffer and 3×1 ml 70% ethanol. Washed walls were sequentially extracted and washed with 1 ml + 200 μl PAW, 1 ml + 200 μl 70% ethanol, and 1 ml + 200 μl 1 M TFA (25 °C) as previously described.

For chickpea samples, the TFA-insoluble wall residue was digested in 1 ml 0.5% Pronase in 30 mM NH_4HCO_3, pH 7.7, containing 1 mM $CaCl_2$, at 37 °C under constant stirring for 48 h. The sample was centrifuged at 8800*g* for 10 min. Pronase digestion was repeated twice more, and the final pellet was washed with 2×300 μl of 30 mM NH_4HCO_3. The *Arabidopsis* and rose cell walls and the Pronase-treated chickpea walls were then treated with either Driselase or HCl. For Driselase, samples were shaken for 48 h at 37 °C in 1 ml of 0.5% Driselase in PyAW (1:1:98 v/v/v, pH 4.7, containing 0.5% chlorobutanol) (Fry, 2000). For acid hydrolysis, samples were treated with 6 M HCl at 120 °C for 16 h in flame-sealed bottles: the hydrolysate was dried and repeatedly re-dried from H_2O for removal of excess HCl. Driselase- and HCl-products were analysed by PE at pH 2.0 (30 min).

Radioactive products of HCl hydrolysis of *Arabidopsis* walls were eluted from the pH 2.0 PE and further analysed by PE at pH 3.5 (50 min). Radioactive products were again eluted and subjected to further PE or PC (see Section 2 for specific solvent systems) with internal markers. After quantification of the ^{14}C profile, the strips of chromatography paper were taken out of the scintillation fluid, washed with toluene and dried; the internal marker was then stained. In this way, exact co-migration of ^{14}C with the internal marker was tested.

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