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Biosynthetic origin and longevity in vivo of α-D-mannopyranosyl-(1 → 4)-α-D-glucuronopyranosyl-(1 → 2)-*myo*-inositol, an unusual extracellular oligosaccharide produced by cultured rose cells

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Abstract. A non-reducing trisaccharide, α-D-mannopyranosyl-(1 → 4)-α-D-glucuronopyranosyl-(1 → 2)-*myo*-inositol (MGI) accumulated in the spent medium of cell-suspension cultures of 'Paul's Scarlet' rose (*Rosa* sp.) predominantly during the period of rapid cell growth. This trisaccharide was also produced by cultures of sycamore (*Acer pseudoplatanus* L.) but not by those of the graminaceous monocots maize (*Zea mays* L.) and tall fescue grass (*Festuca arundinacea* Schreb.). When added to cultured *Rosa* cells, [^{14}C]MGI was neither taken up by the cells nor bound to the cell surface and was not metabolised extracellularly. When D-[6-^{14}C]glucuronic acid was fed to cultured *Rosa* cells, extracellular [^{14}C]MGI started to appear only after a 5-h lag period, compared with a 0.5-h lag period for labelling of extracelluar polysaccharides. Furthermore, [^{14}C]MGI continued to accumulate in the medium for at least 20 h after the accumulation of ^{14}C-polymers had ceased. These observations indicate that extracellular MGI was produced from a slowly turning-over pool of a preformed intermediate. Structural considerations indicate that the intermediate could be a glucuronomannan or a phytoglycolipid (glycophosphosphingolipid). No *Rosa* polysaccharides could be found that generated MGI in the presence of living *Rosa* cells. We therefore favour phytoglycolipids as the probable biosynthetic origin of MGI.

Key words: Glucuronomannan – *myo*-Inositol – Oligosaccharide – Oligosaccharin biosynthesis – Phytoglycolipid – *Rosa* (extacellular oligosaccharide)

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Abbreviations: CDTA = *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; GalA = D-galacturonic acid; GI = α-D-glucuronopyranosyl-(1 → 2)-*myo*-inositol; GlcA = D-glucuronic acid; Man = D-mannose; MGI = α-D-mannopyranosyl-(1 → 4)-α-D-glucuronopyranosyl-(1 → 2)-*myo*-inositol; TFA = trifluoroacetic acid

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Introduction

Oligosaccharins are those oligosaccharides that evoke biological responses when applied at low concentrations to living plant cells. They have been proposed to play important intercellular signalling roles in higher plants (York et al. 1984; Darvill et al. 1992; Aldington and Fry 1993, 1994; Auxtová et al. 1995; Warneck et al. 1998). In studies of their physiological roles, it would be of great interest to investigate changes in the extraprotoplasmic concentrations of endogenous oligosaccharins, e.g. in response to environmental stimuli. However, such analyses are hindered by the low concentrations likely to be present in vivo. In addition, extraprotoplasmic fluid is not easily prepared in large volumes from plant organs. As an alternative, the spent medium of plant cell-suspension cultures can be collected in large volumes, facilitating the analysis of extraprotoplasmic oligosaccharides present at low concentration.

Plant cell cultures have been shown to accumulate in the culture medium biologically relevant concentrations of certain oligosaccharins, including those derived from xyloglucans (Fry 1986), pectins (Tani et al. 1992), *N*-linked glycoproteins (Priem et al. 1990; Priem and Gross 1992) and unidentified sources (Schröder and Knoop 1995; Roberts et al. 1997). We are investigating the secretion of oligosaccharins by cultured *Rosa* cells (García-Romera and Fry 1995, 1997), and recently discovered in this source a novel trisaccharide, α-D-mannopyranosyl-(1 → 4)-α-D-glucuronopyranosyl-(1 → 2)-*myo*-inositol (MGI) (Smith et al. 1999). In some experiments, MGI at 10^{-8}–10^{-3} g/l significantly inhibited protein biosynthesis in *Rosa* cell cultures (Smith 1998), indicating that MGI is an oligosaccharin.

The trisaccharide MGI is highly unusual because it is a non-reducing compound with *myo*-inositol at the terminus. The biosynthetic origin of MGI is unclear. Extraprotoplasmic oligosaccharides can potentially arise by de-novo synthesis and secretion, or they can be generated secondarily by partial hydrolysis of a preformed precursor, e.g. a wall polysaccharide. The latter biosynthetic origin has been demonstrated for extrapro-

toplasmic XXFG, an oligosaccharin derived from xyloglucan (McDougall and Fry 1991). In the present work, we have investigated aspects of MGI metabolism: how the production of MGI changes during the culture growth cycle, the longevity of MGI in the presence of living plant cells, and whether MGI is synthesised de novo or via a pre-formed polymer.

Materials and methods

Plant cell-suspension cultures. Cell-suspension cultures of 'Paul's Scarlet' rose (*Rosa* sp.) were maintained in a medium (Fry and Street 1980), initially at pH 6.1, that contained inorganic salts, D-glucose (20 g/l), 2,4-dichlorophenoxyacetic acid (1 mg/l), kinetin (0.5 mg/l) and $Na_2EDTA \cdot 2H_2O$ (7.4 mg/l). No inositol was added. The cells were sub-cultured fortnightly by 4-fold dilution. Cell-suspension cultures of sycamore (*Acer pseudoplatanus* L.), maize (*Zea mays* L.) and tall fescue grass (*Festuca arundinacea* Schreb.) were maintained as described by Stuart and Street (1969), Lorences and Fry (1991) and McDougall and Fry (1994), respectively.

Total oligosaccharides in spent medium of Rosa *cells*. Spent medium from a 14-d-old 800-ml *Rosa* culture was filtered through 53-μm nylon gauze. Some of the soluble extracellular polymers were precipitated by addition of an equal volume of ethanol and storage at 4 °C overnight. The precipitate was filtered off on nylon gauze and the ethanol removed from the filtrate in vacuo. The concentrated aqueous solution was analysed by gel-permeation chromatography.

Uronate-containing oligosaccharides in spent medium of Rosa *cells*. An inoculum (2.0 ml) of 1-week-old *Rosa* cell culture was added to 5 ml of fresh medium containing 3.7 MBq of D-[6-^{14}C]glucuronic acid ([^{14}C]GlcA; 2.0 MBq/μmol; prepared as described before by Brown and Fry 1993) and incubated under standard conditions for a further 7 d. The culture was then filtered through nylon gauze, and the filtrate was analysed by gel-permeation chromatography.

Accumulation of extracellular MGI during the growth cycle of Rosa *cultures*. Duplicate 30-ml cultures of *Rosa* were harvested at intervals after sub-culture. Packed cell volume was determined after centrifugation at 1500 *g* for 5 min. The supernatant was dried in vacuo and the solutes were resuspended in 1.0 ml water and analysed by Dionex HPLC (Dionex, Camberley, UK) using eluent programme 1.

Survey of other cultures for MGI. Cell-suspension cultures of *Acer*, *Zea* and *Festuca* were harvested at 1 and 2 weeks after sub-culturing and the cells filtered off on nylon gauze. Some soluble extracellular polymers were precipitated by addition of an equal volume of ethanol and storage at 4 °C overnight. The precipitate was filtered off on nylon gauze and the ethanol removed from the filtrate in vacuo. The concentrated aqueous solution was analysed by Dionex HPLC using eluent programme 1.

Determination of the stability of [^{14}C]MGI in vivo. Purified [*GlcA*-^{14}C]MGI was filter-sterilised and added to a 7-d-old *Rosa* culture (or fresh culture medium as a control) at about 7 Bq/ml. Aliquots (1 ml) were transferred to sterile 5-ml Petri dishes and incubated under standard growth conditions. At intervals, duplicate dishes were harvested and the contents subjected to low-speed centrifugation; the cells were washed twice with 0.4 ml water. The medium and the washings were pooled and analysed by paper chromatography.

Kinetics of radiolabelling of MGI and extracellular polymers. Cells of *Rosa* (9.7 g) were collected on sterile muslin 5 d after subculturing, and added to 800 ml of fresh culture medium. D-[6-^{14}C]Glucuronic acid (filter-sterilised) was added at 3.5 kBq/ml, and aliquots of the culture (2.0 ml) were incubated in 5-cm Petri dishes under standard conditions. At intervals, duplicate dishes were harvested: cells were filtered off on nylon gauze and washed with 2 × 1.0 ml of water. The total filtrate was analysed by paper chromatography. Material of R_f 0.00 was taken to be ^{14}C-polymers. The [^{14}C]MGI was recognised by co-migration with an α-(1 → 4)-trigalacturonide ($GalA_3$) marker; the [^{14}C]MGI was not accompanied by detectable [^{14}C]$GalA_3$, which can be resolved from MGI by paper electrophoresis (García-Romera and Fry 1997; Smith et al. 1999).

Preparation of Rosa *cells walls and soluble extracellular polysaccharides*. Cells of *Rosa* (5 d after sub-culturing) were collected on nylon gauze, freeze-dried and repeatedly stirred overnight in fresh phenol/acetic acid/water (2:1:1, w/v/v; PAW) until the PAW contained no detectable protein (Fry 1988). The residue was then washed in 70% ethanol and repeatedly stirred overnight in fresh 90% dimethylsulphoxide until the extract contained no detectable starch. The final cell wall preparation was washed with 70% ethanol, then 100% acetone, and air-dried. Extracellular polysaccharides were collected from the initial culture filtrate by addition of 4 volumes of ethanol, incubation at 4 °C overnight and centrifugation at 1500 *g* for 10 min.

Preparation of ^{14}*C-polysaccharides*. A 1-week-old *Rosa* cell culture (50 ml) was supplied with 150 kBq of D-[6-^{14}C]glucuronic acid and incubated under standard conditions for a further 7 d. Cell walls were freed of protein and starch as described above. ^{14}C-Pectic polysaccharides were repeatedly extracted in 50 mM *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (CDTA) (Na^+, pH 7.5), at room temperature overnight, until the extracts contained no more radioactivity. Further polysaccharide fractions were then extracted with 8 M urea [in 50 mM Hepes (Na^+), pH 7.5], 6 M NaOH containing 1% $NaBH_4$, and 6 M NaOH containing 4% H_3BO_3. In each case the extraction was repeated until no more radioactivity was detected in the supernatant. Each extract was de-salted on a Sephadex CL-4B column (150 ml bed volume; Sigma, Poole, UK) and freeze-dried.

Incubation of ^{14}*C-polysaccharides with* Rosa *cells*. The dried ^{14}C-polysaccharides were resuspended in 0.25 ml of water, added to 6-d-old *Rosa* culture or fresh culture medium (10 ml in a 9-cm Petri dish) and incubated under standard conditions for 24 h. The cells were filtered off on nylon gauze and washed with 2 × 1.0 ml of water. The total filtrate was analysed by paper chromatography.

Search for GI units in soluble extracellular ^{14}*C-polymers and* ^{14}*C-cells*. From *Rosa* cultures that had been incubated with D-[6-^{14}C]glucuronic acid for 25 h (see Fig. 4), the whole cells and the soluble extracellular polymers (= the origin of the paper chromatogram) were subjected to acid hydrolysis in 2 M trifluoroacetic acid (TFA). The products were analysed by paper chromatography. Radioactive material that co-migrated with authentic α-D-[^{14}C]glucuronopyranosyl-(1 → 2)-*myo*-inositol ([^{14}C]GI) was then eluted and subjected to electrophoresis.

Acid hydrolysis to release GI. Acid hydrolysis was conducted in 2 M TFA at 120 °C for 1 h. After removal of the TFA in vacuo, the hydrolysate was analysed by paper chromatography or HPLC (eluent programme 2).

Reference compounds for chromatography. The marker $GalA_3$ was isolated from a pectinase digest of commercial polygalacturonic acid. Pure MGI and [^{14}C]MGI were isolated as described by Smith et al. (1999). The [^{14}C]GI was prepared from [^{14}C]MGI by acid hydrolysis. Malto-oligosaccharides, D-glucuronic acid and D-glucuronolactone were from Sigma Chemical Co., Poole, UK.

Paper chromatography and electrophoresis. Chromatography was conducted on Whatman 3MM paper by the descending method for

16 h in ethyl acetate/acetic acid/water (1:1:1, by vol.). High-voltage electrophoresis was performed on Whatman No. 1 paper in acetic acid/pyridine/water (10:1:189, by vol., pH 3.5) at 3 kV for 90 min with white spirit as coolant (20–30 °C). Reducing sugars were stained on paper by the aniline hydrogen-phthalate method (Partridge 1949; modified by Fry 1988).

Gel-permeation chromatography. A 780- or 270-ml (bed volume) column of Bio-Gel P-2, eluted with acetic acid/pyridine/water (1:1:25, by vol., pH ≈ 4.7), was used for GPC. The column was calibrated using blue dextran (K_{av} = 0) and $CoCl_2$ (K_{av} = 1). Eluted sugars were assayed with phenol/sulphuric acid (Dubois et al. 1956). The chromatographic behaviour of compounds on GPC is reported as K_{av}, defined as [(elution volume of compound *minus* elution volume of blue dextran)/(elution volume of $CoCl_2$ *minus* elution volume of blue dextran)].

High-pressure liquid chromatography (HPLC). Oligosaccharides were analysed by HPLC on a CarboPac PA1 column (250 mm long, 4 mm i.d.; Dionex). The sample (20 µl) was injected and eluted according to one of the following eluent programmes. *Programme 1*: 10 min isocratic 20 mM NaOH/10 mM CH_3COO-Na, 10 min isocratic 50 mM CH_3COONa/100 mM NaOH, a 30-min gradient to 100 mM CH_3COONa/100 mM NaOH, 5 min isocratic 100 mM CH_3COONa/100 mM NaOH, a 25-min gradient to 500 mM CH_3COONa/500 mM NaOH, and 15 min isocratic 500 mM CH_3COONa/500 mM NaOH; flow rate 1 ml/min (reduced to 0.8 ml/min for 500 mM CH_3COONa/500 mM NaOH); post-column base (500 mM NaOH) was added at 0.5 ml/min. *Programme 2*: 5 min 10 mM NaOH, 25 min H_2O, a 40-min gradient to 800 mM NaOH, and 5-min isocratic 800 mM NaOH; flow rate 1 ml/min; post-column addition of base as above. Separated carbohydrates were detected by a pulsed amperometric detector fitted with a gold electrode. The detector response-factor was determined with pure MGI dispensed gravimetrically.

Assay of radioactivity. Radioactive compounds on chromatography paper were assayed by scintillation counting after the addition of about 2 ml of 'OptiScint HiSafe' scintillant (Wallac Chemicals). If the sample was needed for further analysis after scintillation counting, the scintillant was washed off with toluene and the paper allowed to dry; the sugar was then eluted with water by the method of Eshdat and Mirelman (1972). Radioactive sugars in aqueous solution were assayed by scintillation counting after addition of 10 volumes of 'OptiPhase HiSafe'.

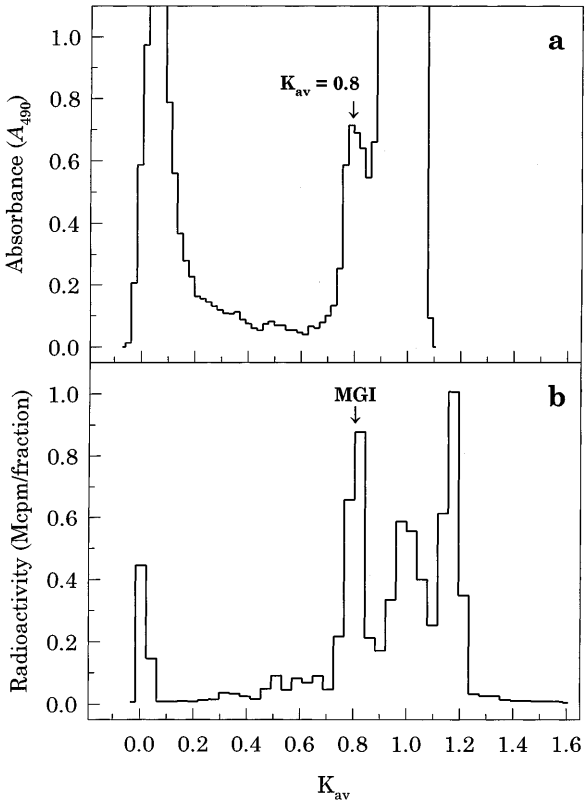


Fig. 1a,b. Size distribution of carbohydrates secreted by *Rosa* cell-suspension cultures. **a** Total carbohydrates. Concentrated spent medium from a 2-week-old, 800-ml cell-suspension culture of *Rosa*, after partial removal of soluble polysaccharides by ethanol precipitation, was fractionated by GPC on a 780-ml column of Bio-Gel P-2. Carbohydrate was assayed by the phenol–sulphuric acid method. **b** Uronic acid residues, biosynthetically ^{14}C-labelled. Cultured *Rosa* cells were incubated with D-[6-^{14}C]glucuronic acid for 7 d, and the spent medium was then analysed on a 270-ml column of Bio-Gel P-2. The peak at $K_{av} \approx 1.00$ contained mainly glucuronolactone; that at $K_{av} \approx 1.18$ contained mainly unmetabolised glucuronic acid. (1 Mcpm = 10^6 cpm)

Results

Expanding Rosa *and* Acer *cells accumulate extracellular MGI*. Gel-permeation chromatography of the spent medium from a non-radioactive, 14-d-old culture of *Rosa* cells (Fig. 1a) revealed the presence of oligosaccharides (K_{av} 0.1–0.8) in addition to some soluble extracellular polysaccharides (K_{av} = 0.0) that had not been removed by the ethanol treatment and a large amount of unmetabolised glucose (K_{av} = 1.0). The major component of the largest oligomer peak (K_{av} about 0.8) has been characterised as the novel non-reducing oligosaccharide, MGI (Smith et al. 1999). Gel-permeation chromatography of the spent medium from a similar 14-d-old culture that had been fed D-[6-^{14}C]glucuronic acid for the final 7 d (Fig. 1b) showed that the trisaccharide was the major uronic acid-containing oligomer present.

Dionex HPLC showed that MGI was not detectable in the medium during the 4-d lag period after subculture, but that thereafter it accumulated in the medium in parallel with cell growth (Fig. 2). Representative HPLC traces for 4- and 14-d-old *Rosa* cultures (Fig. 3a,b) illustrate this. An oligosaccharide co-eluting with MGI was also present in the spent medium of 7- and 14-d-old cell cultures of the dicot *Acer* (Fig. 3c–e), but not of the graminaceous monocots *Zea* (data not shown) or *Festuca* (Fig. 3f,g). The partially purified MGI from the K_{av}-0.8 peak of Fig. 1a is shown in Fig. 3h, and the elution pattern of malto-oligosaccharides (Fig. 3i) serves to illustrate the relative chromatographic properties of MGI.

Extracellular MGI is not metabolically labile. Exogenous [^{14}C]MGI was supplied to a 7-d-old culture of *Rosa* cells. Paper chromatography showed that in 72 h the [^{14}C]MGI was not metabolised to products with different R_f values. Exogenous [^{14}C]MGI was not appreciably taken up by the *Rosa* cells nor adsorbed to the cell surface; 96.6% of the added [^{14}C]MGI was recovered in the medium after 72 h (data not shown).

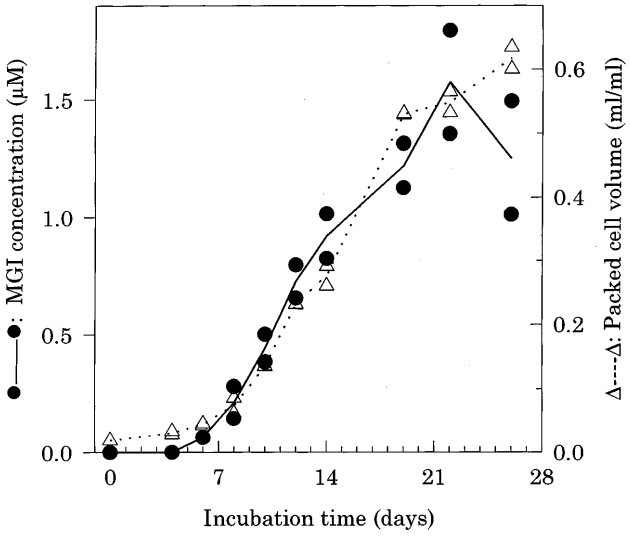
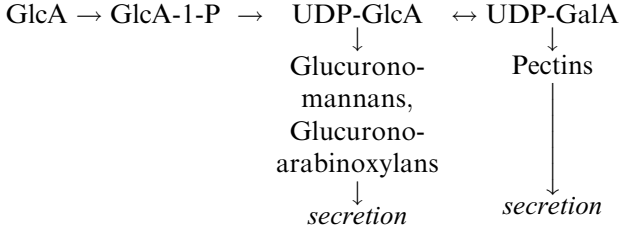


Fig. 2. Changes in MGI concentration in the *Rosa* medium as a function of culture age. Duplicate samples were harvested at intervals during a culture cycle. Δ · · · · · Δ, packed cell volume (ml packed cells per ml suspension culture); ●—●, concentration of trisaccharide MGI in culture filtrate

Biosynthesis of MGI by Rosa *cells*. Theoretically, the cells could synthesise MGI de novo as a trisaccharide (i.e., directly from sugar nucleotides) or they could release it by partial hydrolysis of a pre-formed polymer. To distinguish between these two modes of biosynthesis, 5-d-old *Rosa* cultures were supplied with D-[6-^{14}C]glucuronic acid and the kinetics of accumulation of extracellular ^{14}C-polymers and [^{14}C]MGI monitored. After a lag period of about 0.5 h, the rate of accumulation of extracellular ^{14}C-polymers quickly reached a high value (Fig. 4), which was maintained for about 7 h. Thereafter the rate decreased sharply owing to depletion of the exogenous [^{14}C]glucuronic acid. This labelling pattern is as expected for short, de-novo pathways:



in which all the intermediate pools are small, turn over rapidly and reach a constant specific radioactivity very soon after administration of the [^{14}C]GlcA. The major contributor to the 0.5-h lag period is likely to be the Golgi-vesicular transit time.

Extracellular [^{14}C]MGI, in contrast, had a much longer lag period (about 5 h), after which rapid accumulation began. Accumulation of [^{14}C]MGI was still rapid at 25–31 h. This pattern of ^{14}C-trisaccharide accumulation is not compatible with a de-novo pathway because the pool of UDP-GlcA would have been essentially non-radioactive by 25–30 h owing to depletion of the exogenous [^{14}C]GlcA. Rather, the data support the hypothesis that extracellular [^{14}C]MGI is formed from

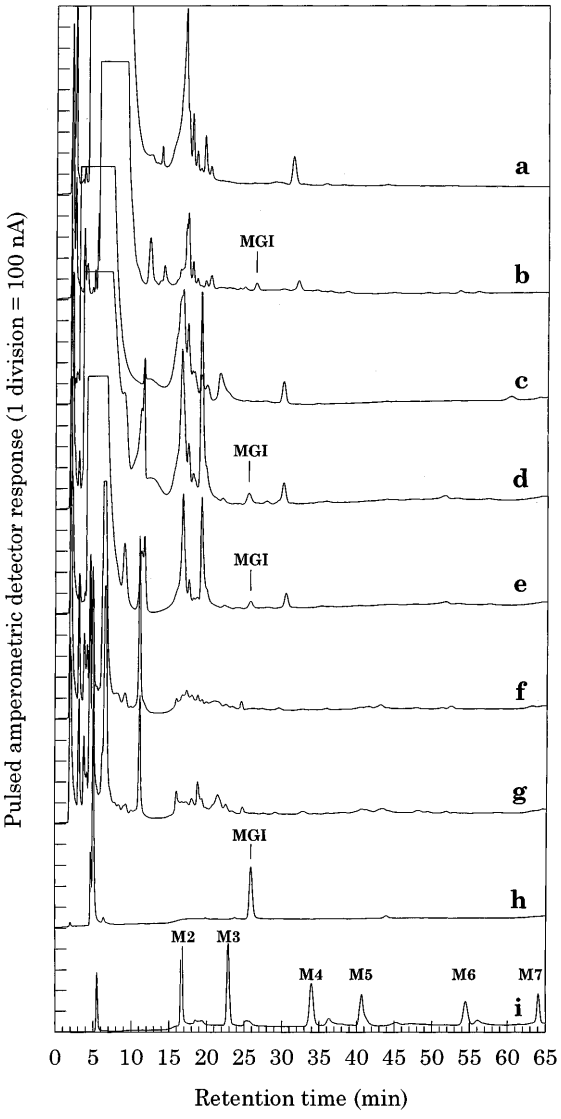


Fig. 3. Detection of MGI in certain culture filtrates by Dionex HPLC. **a**, 4-d-old *Rosa* culture; **b**, 14-d-old *Rosa* culture; **c**, fresh culture medium (*Acer*); **d**, 7-d-old *Acer* culture; **e**, 14-d-old *Acer* culture; **f**, 7-d-old *Festuca* culture; **g**, 14-d-old *Festuca* culture; **h**, partially purified MGI; **i**, malto-oligosaccharide markers for run *h*. Note: the oligogalacturonides $GalA_2$ to $GalA_{11}$ had retention times of 73.5, 76.3, 78.4, 80.2, 81.8, 83.2, 84.7, 85.8, 86.7, and 87.8 min in this system. HPLC was on CarboPac PA1 using eluent programme 1. M2–M7, maltose to maltoheptaose

a relatively stable, slowly turning-over precursor pool. Such a precursor pool could be a pre-formed [^{14}C]polymer: this hypothesis correctly predicts that the accumulation of [^{14}C]MGI would accelerate over approximately the first 10 h as the specific radioactivity of the precursor polymer increased (McDougall and Fry 1991).

Parent polymer of MGI. If MGI were produced from a precursor polysaccharide (with the inositol moiety at the 'reducing' end), acid hydrolysis of this polysaccharide would release α-D-glucuronosyl-(1 → 2)-*myo*-inositol (GI), which is relatively acid-stable (Smith et al. 1999).

The extracellular ^{14}C-polymers and the ^{14}C-labelled cells from the 25-h time-point in the experiment reported

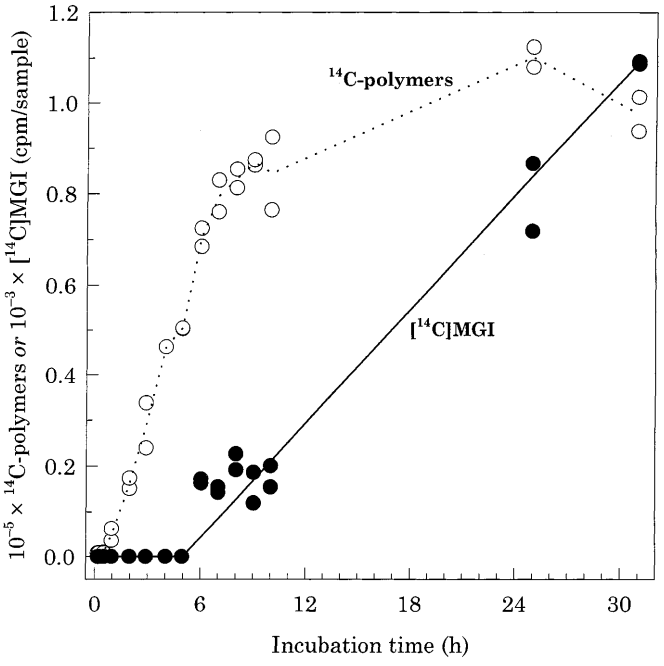


Fig. 4. Kinetics of accumulation of soluble extracellular ^{14}C-polymers and [^{14}C]MGI in the medium of *Rosa* cell-suspension cultures after presentation of D-[6-^{14}C]glucuronic acid

in Fig. 4 were subjected to acid hydrolysis and the products separated by paper chromatography (Fig. 5a, b). Both samples yielded radioactive material that co-migrated with GI. However, on electrophoresis at pH 3.5, this material failed to co-migrate with authentic GI (Fig. 5c,d). Such material would include aldobiouronic acids such as GalA-Rha, GlcA-Xyl and GlcA-Man, derived from rhamnogalacturonan-I, glucuronoarabinoxylans and glucuronomannans, respectively.

Analysis by HPLC (eluent programme 2) of acid hydrolysis products of non-radioactive polysaccharide fractions from *Rosa* cell walls and soluble extracellular polymers also yielded no detectable GI (data not shown). Thus, neither the cell walls nor the soluble extracellular polymers could be shown to contain a polysaccharide that included MGI as part of its structure.

*(*uronate-^{14}*C)-Polysaccharides do not yield [*^{14}*C]MGI in the presence of* Rosa *cells*. As an alternative approach to the detection of the hypothetical polymer from which MGI is formed in vivo, we supplied replicate 6-d-old 10-ml *Rosa* cell cultures with various radioactive pectic and hemicellulosic fractions that had been extracted from (*uronate*-^{14}C)-labelled *Rosa* cells. It was thought that an apoplastic enzyme system might be capable of effecting the partial hydrolysis or transglycosylation of such polymers to yield [^{14}C]MGI. The cultures were supplied with CDTA-solubilised material (17 kcpm), urea-solubilised material (6.8 kcpm), NaOH/$NaBH_4$-solubilised material (7.0 kcpm) or a suspension of the NaOH-insoluble residue (3.4 kcpm). After 18 h incubation, the spent medium was analysed by paper chromatography. None of the ^{14}C-polymers yielded any chromatographically mobile radioactive material

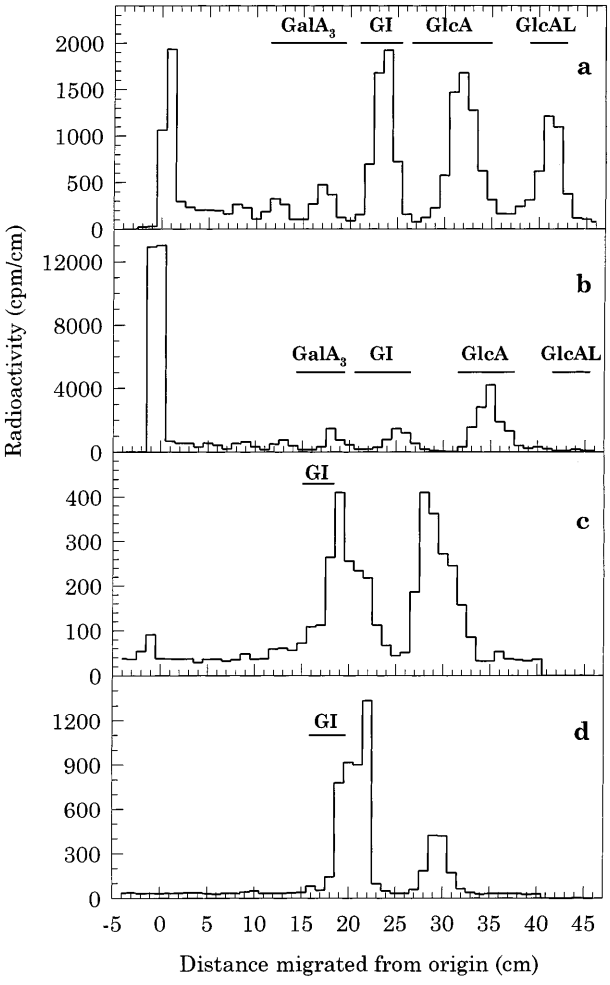


Fig. 5a–d. Analysis of acid-hydrolysis products of (*uronate*-^{14}C)-labelled soluble extracellular polymers and cell walls. **a,b** A *Rosa* culture was pre-labelled by incubation in the presence of D-[6-^{14}C]glucuronic acid for 25 h. Labelled polymers from the culture medium and the cells were then hydrolysed in 2 M TFA at 120 °C for 1 h and the products analysed by paper chromatography: **a** products from extracellular polymers; **b** products from cells. **c,d** The material that approximately co-migrated with authentic GI was then eluted and re-analysed by paper electrophoresis at pH 3.5: **c** putative GI from extracellular polymers; **d** putative GI from cells. External markers used were: *GalA*$_3$, α-(1 → 4)-trigalacturonide; *GI*, α-D-glucuronosyl-(1 → 2)-*myo*-inositol; *GlcA*, D-glucuronic acid; *GlcAL*, D-glucuronolactone

after incubation with the cells. Thus, no pectic or hemicellulosic precursor of MGI could be demonstrated by this method.

Discussion

This work shows that MGI, a recently discovered and unusual trisaccharide which may possess oligosaccharin activity, accumulates in the media of growing cultures of two dicots (*Rosa* and *Acer*), but not in the media of two graminaceous monocots (*Festuca* and *Zea*). The trisaccharide MGI is one of the major extracellular oligosaccharides secreted by the *Rosa* cells; it accumulated in the medium to a concentration of about 1.5 μM

by the stationary phase of the culture cycle. The work also shows that extracellular MGI is relatively stable in the presence of 7- to 10-d-old *Rosa* cells: it is not appreciably adsorbed to the cell surface, taken up, or metabolised.

Radioactivity from exogenous D-[6-^{14}C]glucuronic acid is readily incorporated into the pools of UDP-uronic acids [UDP-GlcA and UDP-galacturonic acid (UDP-GalA)], which are the precursors for uronic acid residues of polysaccharides. Decarboxylation of UDP-GlcA to UDP-xylose (UDP-Xyl) results in loss of the ^{14}C as $^{14}CO_2$. Thus, exogenous D-[6-^{14}C]glucuronic acid gives specific radiolabelling of the uronic acid residues of polymers, while pentose and neutral hexose residues remain non-radioactive (Brown and Fry 1993). The kinetics of radiolabelling of polymers (Fig. 4) indicate that, after administration of D-[6-^{14}C]glucuronic acid, the UDP-uronic acid pools quickly reached a high specific radioactivity, which was then maintained approximately constant for at least 7 h. The contrasting kinetics of [^{14}C]MGI accumulation show that this compound was not rapidly secreted as a finished trisaccharide. Rather, the long initial lag and subsequent prolonged accumulation of [^{14}C]MGI show that MGI was derived from a slowly turning-over precursor pool, assumed to be a pre-formed polymer.

It is of interest to consider the nature of the proposed polymeric precursor of MGI. One hypothesis is that it is a polysaccharide with an inositol moiety already attached at the 'reducing' end, from which MGI could be released by enzymic hydrolysis of the third glycosidic bond from that end:

$$\ldots\text{X–X–X–X–M–G–I} \rightarrow \ldots\text{X–X–X–X} + \text{M–G–I}$$

(where X = unspecified sugar residue). However, no evidence could be obtained for the existence in *Rosa* cells or spent medium of a polysaccharide that contains the acid-stable G–I unit.

A second hypothesis is that the polymer does not itself possess an inositol moiety but acts as the donor substrate of a transglycosylation reaction in which a disaccharide moiety is transferred to free inositol as the acceptor substrate:

$$\text{M–G–X–X–X–X}\ldots + \text{I} \rightarrow \text{M–G–I} + \text{X–X–X–X}\ldots$$

The repeating backbone unit of the minor cell wall polysaccharide, glucuronomannan [α-D-Man*p*-(1 → 4)-β-D-Glc*p*A-(1 → 2)-]$_n$ (Mori and Kato 1981; Redgwell 1983; Honda et al. 1996) is structurally compatible with this hypothesis if the anomeric configuration of the glucuronosyl residue is reversed by the transglycosylation reaction. However, no polysaccharide could be detected that yields MGI when co-incubated with cultured *Rosa* cells.

A third hypothesis is that the precursor of MGI is a phytoglycolipid. Phytoglycolipids (also known as glycophosphosphingolipids) are a group of minor glycolipids found in plants (leaves, seeds and possibly cell cultures) and fungi but not in animals or bacteria (Laine and Hsieh 1987; Perotto et al. 1995). They possess the core structure, [GlcA–*myo*-inositol–phosphate–ceramide] (Carter et al. 1969; Hsieh et al. 1981; Laine and Hsieh 1987), to which additional sugar residues may be attached, forming structures such as

α-D-Man*p*-(1 → 2)
|
α-D-Glc*p*N-(1 → 4)-α-D-Glc*p*A-(1 → 6)-*myo*-inositol-1–phosphate–ceramide

(Carter et al. 1969) and

α-D-Glc*p*N-(1 → 4)-α-D-Glc*p*A-(1 → 2)-*myo*-inositol-1–phosphate–ceramide

(Hsieh et al. 1981), where GlcN = 2-amino-2-deoxy-glucose. The structural similarity of MGI,

α-D-Man*p*-(1 → 2)
|
α-D-Glc*p*A-(1 → 2)-*myo*-inositol

to the above phytoglycolipids is compatible with the hypothesis that MGI is formed by partial hydrolysis of a phytoglycolipid.

Inositol–phosphate bonds are very resistant to hot acid, and therefore a phytoglycolipid would not be expected to yield GI during acid hydrolysis. In pea root nodules, a putative phytoglycolipid has been shown by immunocytochemical techniques to be present in the plasma membrane (Perotto et al. 1995). In our experiments it is unlikely that an exogenous, water-insoluble [^{14}C]phytoglycolipid would have reached the appropriate (plasma membrane?) site for enzymic hydrolysis to MGI.

The available evidence is thus compatible with a phytoglycolipid, i.e. a membrane component, being the precursor of MGI. However, the possibility of MGI being formed by a glucuronomannan-to-inositol transglycosylation reaction cannot at present be excluded.

In animals, 'inositolphosphoglycans' (structurally related to MGI) are released from membrane-bound glycosylphosphatidylinositols, and function as second messengers in the action of insulin (Caro et al. 1997; Rademacher 1998). The possibility that MGI serves a signal-transduction role in plants is under investigation.

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