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The pectic disaccharides lepidimoic acid and β-D-xylopyranosyl-(1→3)-D-galacturonic acid occur in cress-seed exudate but lack allelochemical activity

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Running title: Acidic oligosaccharides in cress-seed exudate

ABSTRACT

• **Background and aims** Cress-seed (*Lepidium sativum*) exudate exerts an allelochemical effect, promoting excessive hypocotyl elongation and inhibiting root growth in neighbouring *Amaranthus caudatus* seedlings. We investigated acidic disaccharides present in cress-seed exudate, testing the proposal that the allelochemical is an oligosaccharin — lepidimoic acid (LMA; 4-deoxy-β-L-*threo*-hex-4-enopyranuronosyl-(1→2)-L-rhamnose).

• **Methods** Cress-seed exudate was variously treated [heating, ethanolic precipitation, solvent partitioning, high-voltage paper electrophoresis and gel-permeation chromatography (GPC)], and the products were bioassayed for effects on dark-grown *Amaranthus* seedlings. Two acidic disaccharides, including LMA, were isolated and characterised by electrophoresis, thin-layer chromatography (TLC) and nuclear magnetic resonance (NMR) spectroscopy, and then bioassayed.

•**Key results** Cress-seed exudate contained low-M_r, hydrophilic, heat-stable material that strongly promoted *Amaranthus* hypocotyl elongation and inhibited root growth, but that separated from LMA on electrophoresis and GPC. Cress-seed exudate contained ~250 µM LMA, whose TLC and electrophoretic mobilities, susceptibility to mild acid hydrolysis, and NMR spectra are reported. A second acidic disaccharide, present at ~120 µM, was similarly characterised, and shown to be β-D-xylopyranosyl-(1→3)-D-galacturonic acid (Xyl→GalA), a repeat-unit of xylogalacturonan. Purified LMA and Xyl→GalA when applied at 360 and 740 µM, respectively, only slightly promoted *Amaranthus* hypocotyl growth, but equally promoted root growth and thus had no effect on the hypocotyl:root ratio, unlike total cress-seed exudate.

•**Conclusions** LMA is present in cress seeds, probably formed by rhamnogalacturonan lyase action on rhamnogalacturonan-I during seed development. Our results contradict the hypothesis that LMA is a cress allelochemical that appreciably perturbs the growth of potentially competing seedlings. Since LMA and Xyl→GalA slightly promoted both hypocotyl and root elongation, their effect could be nutritional. We conclude that rhamnogalacturonan-I and xylogalacturonan (pectin domains) are not sources of oligosaccharins with allelochemical activity, and the biological roles (if any) of the disaccharides derived from them are unknown. The main allelochemical principle in cress-seed exudate remains to be identified.

Abbreviations
ΔUA, 4-deoxy-L-*threo*-hex-4-enuronic acid (product of elimination of H_2O from either D-GalA or D-GlcA).
BAW, butan-1-ol/acetic acid/water (2:1:1)
COSY, proton–proton chemical shift correlation spectroscopy
EPAW, ethyl acetate/pyridine/acetic acid/water (6:3:1:1)
GalA, D-galacturonic acid
GlcA, D-glucuronic acid
HMBC, heteronuclear multiple-bond correlation
HSQC, heteronuclear single-quantum coherence (single-bond C–H correlation)
HVPE, high-voltage paper electrophoresis
LMA, lepidimoic acid [4-deoxy-β-L-*threo*-hex-4-enopyranuronosyl-(1→2)-L-rhamnose]
OG, orange G
RG, rhamnogalacturonan
Rha, L-rhamnose
TBA, thiobarbituric acid
TLC, thin-layer chromatography
Xyl, D-xylose

INTRODUCTION

Allelopathy — the injurious effect of a plant on the germination, growth or development of neighbouring plants of other species — has been known for millennia. For example, the allelopathic effects of chickpea (*Cicer arietinum*) and barley (*Hordeum vulgare*) on weeds were known before 300 BC (Rice, 1984). Allelochemicals, the substances responsible for allelopathy, are often secondary metabolites that are released by roots, rhizomes, leaves, stems or seeds. They may enter the environment via various routes e.g. leaching, volatilisation, root exudation, seed-coat exudation, or microbial decomposition (Rice, 1984; Higashinakasu *et al.,* 2004). When susceptible plant species are exposed to allelochemicals, germination may be inhibited or the seedlings may show abnormal growth and development. Typical effects include inhibition of root growth, lack of root hairs, abnormally long or short shoots, swollen seeds and low reproductive ability (Rice, 1979).

Natural plant growth regulators also include oligosaccharins: biologically active oligosaccharides, especially those that are generated by partial degradation of the plant cell wall (Darvill *et al.*, 1992; Aldington and Fry, 1993; Field 2009; Cabrera *et al.*, 2013). Numerous oligosaccharins have been artificially prepared from cell-wall hemicelluloses (York *et al.*, 1983; McDougall and Fry, 1988, 1989; Zablackis *et al.*, 1996; Kollarová *et al.*, 2005; Benová-Kakošová *et al.*, 2006; Kaida *et al.*, 2010; Zhao *et al.*, 2013) and pectins (Marfà *et al.*, 1991; Altamura *et al.*, 1998; Dumville and Fry, 2000; Balandran-Quintana *et al.*, 2002; Costales *et al.*, 2007). Although oligogalacturonides are not generated extracellularly by healthy *Rosa* cell cultures (García-Romera and Fry, 1997), some other living plant cells and tissues do generate oligosaccharins *in vivo* by the partial degradation of pre-formed wall polysaccharides (Fry, 1986; McDougall and Fry 1991; Zabotina *et al.*, 1995; Boudart *et al.*, 2003) or membrane glycolipids (Smith and Fry 1999; Smith *et al.*, 1999) or by transglycosylation of existing oligosaccharides (Dumville and Fry, 2003).

Hasegawa *et al.* (1992) proposed that 'lepidimoide', the sodium salt of an unsaturated acidic disaccharide which they discovered in cress (*Lepidium sativum*) seedlings, has allelochemical activity. This would be the first example of an allelochemical that is an oligosaccharin. Lepidimoide is the sodium salt of 4-deoxy-β-L-*threo*-hex-4-enopyranuronosyl-(1→2)-L-rhamnose (conveniently abbreviated as ΔUA→Rha, where ΔUA represents the 4,5-unsaturated 4-deoxyuronic acid residue). The free acid was called lepidimoic acid (LMA; Yokotani-Tomita *et al.*, 1998); however, since the acid and

its salts no doubt freely interconvert *in vivo*, and both were reported to have similar biological effects (Yamada *et al.*, 1996; Hirose *et al.*, 2003), we will use the term LMA consistently. LMA is not confined to cress but was also detected in germinating seeds of numerous other plant species including monocots (Yamada *et al.*, 1995, 1997).

It was initially reported that >3 μM LMA promotes hypocotyl elongation (superficially resembling gibberellin action) whereas >100 μM LMA inhibits root growth (superficially resembling auxin action) in neighbouring — potentially competing — seedlings such as those of *Amaranthus caudatus*. At 3 μM and 1000 μM, LMA was reported to cause an approximately 1.5- and 5-fold promotion, respectively, of *Amaranthus* hypocotyl elongation (Hasegawa *et al.*, 1992). Subsequently, the same laboratory reported that the promoting effect of LMA on *Amaranthus* hypocotyls required at least 300 μM, at which concentration it caused only a ~1.2-fold promotion of hypocotyl elongation; and the root inhibiting effect was not mentioned (Yamada *et al.*, 1996). Likewise, 300 μM LMA was reported to promote hypocotyl elongation in dark-grown seedlings of *Celosia argentea* (cockscomb, another member of the Amaranthaceae) 1.2-fold (Yokotani-Tomita *et al.*, 1998) or 1.7-fold (Hirose *et al.*, 2004); again, effects on root growth were not reported.

The structural requirements for LMA's bioactivity are relatively lax (Yamada *et al.*, 1996). For example, the rhamnose moiety can be replaced by its 2-epimer (6-deoxy-L-glucose) (Hirose *et al.*, 2004). The ΔUA residue cannot be replaced by its methyl ester, but surprisingly the (water-insoluble) per-*O*-acetylated methyl ester of LMA was reported to be highly active. Furthermore the ΔUA residue can be replaced by an α-D-glucuronic acid residue, a 4-deoxy-α-D-glucuronic acid (synonymous with 4-deoxy-α-D-galacturonic acid) residue, or — particularly effectively — by a neutral α-D-glucose residue. Thus, the most effective disaccharide found was α-D-glucopyranosyl-(1→2)-L-rhamnose (Yamada *et al.*, 1996).

The Hasegawa laboratory also reported that LMA exerts several other effects on plants, including the promotion of adventitious root formation in excised cucumber cotyledons (Yamada *et al.*, 2010), the regulation of fructose 2,6-bisphosphate concentrations in *Amaranthus* seedlings (Kato-Noguchi *et al.*, 2001), the promotion of growth and development in *Arabidopsis* (Goto *et al.*, 1995), the promotion of chlorophyll accumulation in sunflower and cucumber cotyledons (Yamada *et al.*, 1998), the inhibition of chlorophyll loss in senescent oat leaves (Miyamoto *et al.*, 1997a) and the retardation of bean petiole abscission (Miyamoto *et al.*, 1997b). The only other laboratory to report a biological effect of LMA (Kong *et al.*, 2006) reported that, at 15 μg

per g soil, it mimicked the presence of *Echinochloa* seedlings in inducing momilactone B synthesis in some rice cultivars.

Aldington *et al.* (1993) pointed out that the structure of LMA suggests that it could arise by the action of a lyase on rhamnogalacturonan-I (RG-I), which is a cell-wall pectic domain. Polysaccharide lyases cleave sugar polymers via a β-elimination mechanism, introducing a double bond between C-4 and C-5, forming a 4-deoxy α,β-unsaturated carboxylate residue at the new non-reducing end. The presence of RG lyase activity has been reported in many micro-organisms and possibly, at low activity, in some plants (Stratilová *et al.*, 1998; Naran *et al.*, 2007). RG-I is a major component of the seed mucilage and/or root exudate in some plants such as okra (Sengkhamparn *et al.*, 2009), cress (Ray *et al.*, 1988; Deng *et al.*, 2009) and *Arabidopsis* (Haughn and Western, 2012). It has a backbone composed of the repeating disaccharide unit [→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→], some of the Rha residues bearing Ara/Gal-rich neutral side-chains (Albersheim *et al.* 2011; Fry, 2011a). The other widely occurring pectic domains are homogalacturonan, xylogalacturonan and RG-II. The generation of an allelochemical through the partial digestion of a pectic domain by a lyase would strengthen the concept that cell-wall polysaccharides are a source of biologically relevant oligosaccharins.

Since the bioactivity of LMA has only been reported by two laboratories, independent confirmation would be welcome. Iqbal and Fry (2012) confirmed that cress-seed exudate indeed has the reported effects on the growth of *Amaranthus* and lettuce seedlings. We showed that the cress-seed factor targets cell <u>expansion</u> in the *Amaranthus* hypocotyl. Any effect on cell division was too small, or in the wrong direction, to account for the growth response. The growth factor was already present in dry cress seeds, and progressively released even from heat-killed seeds (Iqbal and Fry, 2012). It is possible that the factor's effects on non-cress seedlings enhance the cress seedlings' chance of establishment by weakening potentially competing neighbours. Whether or not this is correct, it is of interest to characterise the bioactive substance(s) present in cress-seed exudate. We have now re-evaluated the hypothesis that LMA is the 'allelochemical' exuded by cress seeds.

MATERIALS AND METHODS

Materials

ΔUA-α-(1→3)-GalNAc was from Elicityl, France (described by the suppliers as β-, agreeing with the β-D-GlcA residue in the chondroitin from which the disaccharide was prepared; however, since ΔUA is an L-*threo*-derivative, the same linkage in the unsaturated disaccharide should be designated α-). Cress seeds (*Lepidium sativum*) were purchased from Sutton Seeds, UK. Sterile 5-cm plastic Petri dishes were obtained from Sterilin Ltd. (Caerphilly, United Kingdom). Filter paper discs (Whatman No. 1) and chromatography paper (Whatman No. 1 or 3) were from Sigma–Aldrich. Merck silica-gel TLC plates were ordered from Merck (Darmstadt, Germany). Organic solvents for chromatography solvent mixtures and volatile electrophoresis buffers were from Sigma-Aldrich (Poole, UK), BDH AnalaR Chemicals Limited (Poole, UK), Arcos Organics (Geel, Belgium) and Fisher Chemicals (Fisher Scientific, Loughborough, UK). Other general laboratory chemicals were from Sigma–Aldrich, Poole, Dorset, UK.

Thiobarbituric acid assay for ΔUA residues

ΔUA residues in isolated oligosaccharides were assayed by a modification of the method of Koseki *et al.* (1978). The sample (40 μl of aqueous solution) was mixed with 20 μl 0.2 M sodium periodate (= meta-periodate) and kept at 20°C for 40 min. Then 40 μl 7.5 M phosphoric acid was added followed immediately by 200 μl 10% (w/v) sodium arsenite (= *m*-arsenite), and the mixture was shaken until the brown colour disappeared. Next 600 μl 0.6% (w/v) 2-thiobarbituric acid (pre-adjusted to pH 2 by addition of a little NaOH) was added, and the solution was incubated in a boiling water-bath for 15 min. After cooling, 400 μl dimethylsulphoxide was added, and the mixture was centrifuged at 3000 g for 3 min. The A_{549} of the clear pink supernatant was read. A standard curve (Fig. S1) was produced with 0.1–4.0 mM malondialdehyde or α-ΔUA-(1→3)-GalNAc (from chondroitin) as the 'sample'.

Preparation of total and low-M_r cress-seed exudate

For Fig. 1, total cress-seed exudate (TCSE) was used: 2.7 g of dry seeds (≈ 1000 seeds) were imbibed at 4°C for 24 h, and the total released exudate was collected and freeze-dried (yield: 200 mg).

For all other Figures, low-M_r cress-seed exudate (LCSE) was used. Imbibed but ungerminated cress seeds (5 g dry weight, soaked for 24 h in 50 ml of water at 4°C) were placed inside a length of 12-kDa-cut-off dialysis tubing (diameter 2 cm), which was

inserted into 50 ml water in a 100-ml measuring cylinder such that the sac was just submerged, and incubated at 4°C for 48 h with occasional inversion. The external solution was filtered through filter-paper and labelled LCSE. The total solute content of LCSE after drying was typically 1.6 mg/ml.

Properties of total cress-seed exudate

To investigate heat-stability of the active principle(s), we incubated samples of TCSE (5 mg/ml) at 100 or 130°C for various periods, then bioassayed them. Ethanol solubility was tested by addition of ethanol to 70% (v/v) and incubation at 20°C for 15 h, then polymers were sedimented by centrifugation at 4000 g for 20 min. The supernatant was then adjusted to, sequentially, 80 then 90% ethanol, and the incubation/centrifugation were repeated. The 90% ethanolic supernatant and each pellet were dried *in vacuo* and redissolved at 2.5 mg/ml in water and bioassayed. Phase partitioning was tested in further samples of TCSE (after adjusting to pH 2, 6 or 12 with HCl or NaOH) by shaking with an equal volume of ethyl acetate; the two phases in each case were separated and dried, then the residues were re-dissolved in the original volume of water and bioassayed.

Bioassay

The solution to be tested was pipetted onto two 4.7-cm discs of Whatman No. 1 filter paper in a 5-cm plastic Petri dish, then 10 *Amaranthus* seeds were placed (well spaced) on the paper. The lids were sealed with Parafilm and the dishes incubated in the dark at 25°C for 4.5 days. The seedlings were then submerged for 10 min in 5 ml of stain solution (0.01% w/v aniline blue in 5% v/v acetic acid), rinsed in water, arranged on an acetate overhead-projector sheet on a background of graph paper, and scanned. The roots stain blue but the hypocotyls (which have a cuticle) remain white, facilitating their separate measurement (Long *et al.*, 2008). Hypocotyls were measured from the hook to the junction with the root; the whole tap-roots (there were no laterals) were also measured.

High-voltage paper electrophoresis (HVPE)

HVPE was conducted on 57-cm-long sheets of Whatman No. 1 or No. 3 paper in volatile buffers at pH 6.5 or 2.0 (voltages and times as specified in individual experiments), then dried to remove the buffers, all as described by Fry (2011b). When the separated zones were to be bioassayed, the paper was dried, dipped through acetone/methanol (2:1) and re-dried, and this cycle was repeated several times; strips of the paper were then eluted with water, and the eluate was dried *in vacuo* and re-dissolved in water.

Compounds on paper electrophoretograms were stained with aniline hydrogen-phthalate (for reducing sugars), $AgNO_3$ (total sugars) and ninhydrin (amino acids); these methods are summarised by Fry (2000). UV-absorbing sugars were documented under a 254-nm UV lamp against a fluorescent background created by dipping the paper through 0.001% fluorescein diacetate (in acetone), drying and then exposing to ammonia vapour for ~10 min in a glass tank (converting fluorescein diacetate to the fluorescent compound fluorescein). UV-absorbing sugars showed up as dark spots against the uniformly fluorescent background.

Thin-layer chromatography (TLC)

TLC was conducted on silica-gel plates in either of two solvents — butan-1-ol/acetic acid/water (2:1:1 by vol.; BAW) or ethyl acetate/pyridine/acetic acid/water (6:3:1:1; EPAW). Carbohydrates were stained with thymol/H_2SO_4 (Jork *et al.*, 1994) or molybdate reagent. For the latter, 50 ml of an aqueous solution containing 10% (w/v) ammonium molybdate and 10% (v/v) H_2SO_4 was added to 150 ml acetone, then the TLC plate was immediately dipped through this mixture, dried, and heated in an oven at 120°C until spots appeared.

Nuclear magnetic resonance (NMR) spectroscopy
Samples B3 and B4 were examined in D_2O on a Bruker AVANCE III 800-MHz spectrometer operating at 799.72 MHz for protons and at 201.10 MHz for ^{13}C nuclei. The structures of B3 and B4 were determined by a series of standard one-dimensional (1D) and two-dimensional (2D) NMR spectroscopy experiments as detailed in the Results section.

<center>RESULTS</center>

Cress seeds exude low-M_r, hydrophilic, heat-stable 'allelochemicals'

In preliminary tests of the properties of bioactive compound(s) exuded from cress seeds, we initially used a solution of the exudate from imbibed (but ungerminated) seeds as the growth medium for *Amaranthus* seedlings. This solution promoted hypocotyl elongation and inhibited root elongation, as reported before (Hasegawa *et al.*, 1992; Iqbal and Fry, 2012). The hypocotyl-promoting principle was highly heat-stable, retaining its activity after lengthy periods at 100°C (Fig. 1a) and even after 48 h at 130°C (Fig. 1b). The root-inhibiting principle lost a small proportion of its activity upon prolonged heating.

The majority of the active principle remained in solution in 70–90% (v/v) ethanol (Fig. 1c), which precipitates polysaccharides, suggesting that it has a low M_r. Furthermore, on partitioning between water and ethyl acetate, the active principle favoured the aqueous phase (Fig. 1d), indicating that it is hydrophilic. These data are compatible with the suggestion that the active principle is a disaccharide such as LPA.

Further characterisation of the active principle focused on low-M_r cress-seed exudate (LCSE), which we routinely separated from the viscous mucilage by dialysis. The solution harvested from outside the dialysis sac typically had a total solute concentration of ~1.6 mg/ml. LCSE had effects on *Amaranthus* similar to those of live cress seeds and whole cress-seed exudate (data not shown).

The major hypocotyl-stimulating component of LCSE does not co-electrophorese with lepidimoic acid

Lepidimoic acid (LMA) is a 322-dalton acidic disaccharide with 1 carboxy group (expected to be almost fully ionised in neutral solutions) and 2 sugar moieties, and thus a charge:mass (Q/M) ratio $\approx -1:321$ at pH 6.5. On electrophoresis in pH 6.5 buffer, the LMA anion will therefore migrate between glucose (Q/M = 0) and free glucuronate (Q/M $\approx -1:193$). Samples of LCSE were subjected to HVPE at pH 6.5, and strips of the electrophoretogram were bioassayed. The mobilities of common acidic, basic and neutral sugars (Fig. 2a) and amino acids (Fig. 2b) are shown. No significantly bioactive principle was detected migrating between the fastest-running anion tested (aspartate) and the fastest cation (lysine; Fig. 2c).

To confirm and extend these findings we fractionated the components of a new batch of LCSE by preparative HVPE and bioassayed these (Fig. 3). Authentic sugar markers and

a fringe from the preparative electrophoretogram were silver-stained, and a sugar band was indeed detected in the position expected of LMA (Fig. 3a). However, the 'LMA' region of the electrophoretogram only slightly promoted hypocotyl growth in comparison with crude cress-seed exudate (Fig. 3c; compare Fig. 1) and did not increase the hypocotyl:root length ratio.

Electrophoresis thus did not support the hypothesis that the principal allelochemical present in cress-seed exudate is LMA. Nevertheless, as the LMA hypothesis has been reported many times (reviewed by Hasegawa *et al.*, 2007), we analysed additional aliquots of the same HVPE-eluates by TLC. This demonstrated the presence of numerous neutral sugars migrating 0–2 cm towards the cathode (Fig. 3d). [Neutral compounds migrate slightly during electrophoresis owing to electro-endo-osmosis.] Anionic sugars were also detected, including GalA and putative LMA centred at about 6 and 4 cm, respectively, towards the anode (Fig. 3d). Thus LMA appeared to be present, albeit almost inactive, in LCSE.

Size-range of active principle(s) present in LCSE

As an alternative means of testing the nature of the active principle(s) present in LCSE, we fractionated it by gel-permeation chromatography (GPC) on Bio-Gel P-2 and analysed the fractions. TLC revealed numerous carbohydrates of various apparent degrees of polymerisation, most of which eluted from the Bio-Gel P-2 column as narrow peaks; however, the putative LMA eluted as a broad peak (dotted yellow box in Fig. 4a).

The same Bio-Gel P-2 fractions were bioassayed for effects on *Amaranthus* seedling growth (Fig. 4b,c). No hypocotyl growth promotion coincided with the LMA zone (fractions 20–27, with a maximum in 26), corroborating the HVPE data indicating that LMA was not a major active principle. When the fractions were assayed at ½-strength (Fig. 4b), a peak of hypocotyl promotion was found in fractions 30 and 31, but this peak was set against a moderately inhibitory background effect (seen in the surrounding fractions, 27–29 and 32). To eliminate hypocotyl inhibition, we repeated the bioassays, in duplicate, on selected fractions at ¼-strength. Under these conditions, there was a peak of hypocotyl promotion spanning fractions 28–32 (Fig. 4c), which embraced the fractions containing GalA, sucrose, galactose, glucose and fructose. A broad zone of root inhibition was also seen in and near this region (fractions 27–32), possibly due to the high osmotic pressure of these sugar-rich fractions; little or no promotion of root growth was observed.

The stock solution of crude LCSE (20 mg/ml, as loaded on to the Bio-Gel column) had a pH of 7.85 and the Bio-Gel P-2 fractions (at full strength) had pH values of 6.2–7.3 (Fig. 4b). These pH values are higher than are usually recommended for seedling growth, and may themselves have had some growth effects; however, regardless of whether dilutions of crude LCSE were made in water or in HEPES buffer (pH 7.45), similar biological effects were obtained (Fig. 4b), suggesting that pH was not appreciably influencing our bioassay results.

The data support the other work in this paper in indicating that LMA is not a major growth regulatory component of cress-seed exudate.

Purification and characterisation LMA and a novel acidic disaccharide

The bioassays described above were conducted with LCSE fractions likely to contain LMA, but these fractions were not pure LMA. As an alternative means of evaluating the proposed bioactivity of LMA, we purified this compound from LCSE, characterised it physico-chemically, and bioassayed it in pure form. Unexpectedly, an additional, acidic disaccharide was also detected, characterised and bioassayed.

Six 500-µg aliquots of LCSE were subjected to HVPE at pH 6.5, the loadings alternating with a marker-mixture. A spot (B4) corresponding to LMA showed up in LCSE (without staining) as a faint, UV-absorbing zone under a 254-nm lamp (Fig. 5a), as expected because of the C=C double bond of LMA. The six lanes were documented in various ways:

- One lane was stained for reducing sugars with aniline hydrogen-phthalate, which revealed heavy spots of neutral monosaccharides and free GalA, a weaker spot at the B4 position (putative LMA), and an incompletely resolved spot (B3) running slightly slower than LMA. B4, B3, and authentic ΔUA-GalNAc all ran very close to each other, suggesting that these all shared the characteristic of being disaccharides with one acidic and one neutral sugar unit (thus Q/M ≈ −1:350).
- Another lane was stained with $AgNO_3$, which detects both reducing and non-reducing sugars: this again revealed the above-mentioned four sugar zones plus several non-reducing sugars, including those labelled A, B1 and B2. Compound B2 ran only slightly slower than B3.

- A fluorescent background was imparted to an additional lane with fluorescein, and UV-absorbing spots were photographed. This faintly revealed B4 and the unsaturated acidic disaccharide ΔUA-GalNAc.

- Two further lanes were cut into strips, which were eluted and assayed for ΔUA residues by the thiobarbituric acid (TBA) assay (pink histograms on Fig. 5a). A major peak of ΔUA was detected at the B4 position, consistent with LMA, and a minor peak (A) had a lower mobility, representing an oligosaccharide with a lower Q:M ratio (possibly related to LMA but with additional neutral sugar residues).

- A final lane was also cut into strips, eluates from some of which were analysed in two TLC systems (Fig. 5b,c). Spots were detected that were ascribed to each of the anionic cress-seed sugars (A, B1, B2, B3, B4) mentioned above.

The properties of A, B1, B2, B3, B4 are summarised in Table 1. The two reducing sugars B3 and B4 were selected for further purification, characterisation and bioassay. To obtain larger quantities, we subjected LCSE to preparative HVPE (Fig. 6a). Guided by the UV-absorbing band, we eluted and dried the LMA-rich region from three identical electrophoretograms (zone *i*; total yield 14.0 mg) and a slightly slower-migrating region (zone *ii*; 9.29 mg), although the compounds in these bands overlapped. Each eluate was redissolved in water at 1% (w/v), and analysed by TLC (Fig. 6b).

It had been noted that samples containing B4 (LMA), even when obtained as an anionic zone from an electrophoretogram, also contained a trace of free (neutral) rhamnose, detectable by TLC (e.g. Fig. 5b), indicating a tendency of the anionic disaccharide to hydrolyse. This tendency was confirmed when TLC plates were developed by one *versus* two ascents (Fig. 6b). The second ascent revealed a new spot of rhamnose, generated by hydrolysis of B4 while the plate was being dried between the two runs (follow the yellow arrows in Fig. 6b). The hydrolysis occurred not only in the acidic chromatography solvent (BAW) but also, more surprisingly, in the buffered solvent (EPAW). Preparative TLC was therefore not favoured for the isolation of LMA. In contrast, compounds B1, B2 and B3 were evidently stable between the two TLC ascents (Fig. 6b).

Zone *i* in Fig. 6a contained both B4 and B3. To separate these compounds, we further fractionated a 4.9-mg sample of zone *i* material by preparative HVPE at pH 2.0. At this pH, most carboxylic acids are only slightly ionised and therefore migrate slowly (Fry, 2011b). HVPE at pH 2.0 cleanly resolved B4 from B3 (Fig. 7a), both of which were separately eluted, producing purified B4 (LMA; yield 3.2 mg) and B3 (1.6 mg), which

were re-dissolved in water at 0.8% and 0.4% (w/v) respectively. Re-electrophoresis (pH 2) of a small portion of these solutions showed that each was essentially pure (Fig. 7b).

To determine the composition of compounds B3 and B4, we subjected aliquots to acid hydrolysis (Fig. 7c,d): 24 µg of B4 yielded ~9 µg of rhamnose (close to the theoretical yield of 11.64 µg Rha from 24 µg LMA) plus a small amount of an unidentified sugar (presumably a degradation product of ΔUA). Hydrolysis of B3 gave roughly equimolar Xyl + GalA; and since aniline hydrogen-phthalate (Fig. 5a) had stained the reducing end of B3 orange (characteristic of a uronic acid) rather than red (characteristic of a neutral pentose), B3 was probably a Xyl→GalA rather than a GalA→Xyl disaccharide.

From the dilutions and loadings used in the isolation of these two disaccharides, we estimate that crude LCSE, prepared by the dialysis method described, contained ~250 µM B4 and ~120 µM of B3.

Compound B4 [putative LMA, i.e. β-L-ΔUA-(1→2)-L-Rha] was stable in water at 120°C (Fig. 7e), but in 0.1 M acetic acid (pH ≈ 2.8) at 120°C it was completely hydrolysed to rhamnose plus a by-product tentatively identified as ΔUA (or a degradation product thereof). The latter was lost during hydrolysis in more concentrated or in stronger acids (data not shown). This behaviour is characteristic of ΔUA→sugar disaccharides, as shown by acid hydrolysis experiments on a comparable commercial standard, α-L-ΔUA-(1→3)-D-GalNAc (labelled 'Std' on Fig. 7f,g). In the case of the standard, replicate TLCs were stained with thymol (mainly revealing the putative free ΔUA product; Fig. 7g) and with molybdate (mainly revealing the slightly faster-migrating free GalNAc product; Fig. 7f). The ΔUA→GalNAc was stable in hot water but readily hydrolysed in hot dilute acid (and it was partially decomposed to unidentified products when heated in pyridine–acetate buffer at pH 6.5). Thus LMA (ΔUA→Rha) and its 'model', ΔUA→GalNAc, are highly labile in hot dilute acid but relatively stable in hot water.

Characterisation of compounds B3 and B4 by NMR spectroscopy

Purified B3 was characterised in detail by NMR spectroscopy. Analysis of the 1D proton NMR spectrum and the 2D proton COSY spectrum of B3 confirmed the presence of Xyl and GalA moieties. These spectra showed that two anomers were present in approximately equal quantities and gave the chemical shifts (δ_H) of all the protons in both anomers (Table 2). Proton–proton coupling constants (J_{HH}) were measured directly from the 1D spectrum. The marked difference in the chemical shifts and the coupling

constants for the GalA-1 protons in the two anomers showed that GalA was the reducing moiety. This was supported by the observation that, apart from the signals for Xyl-H1, corresponding proton signals for the other Xyl protons were superimposed. The large (11 Hz) coupling for the Xyl-1 protons showed the glycosidic link to be β. Carbon-13 chemical shifts (δ_C) for all carbons except that for GalA-C6 were determined from the 2D HSQC carbon–proton correlation spectrum. The carbon-13 chemical shift of GalA-C6 was determined from the 2D HMBC carbon–proton correlation spectrum by correlation with Gal-H5. Other expected multi-bond proton–carbon correlations within each moiety were also present in the HMBC spectrum. That the residues are linked via oxygen from Xyl-C1 to GalA-C3 is clear from the HMBC spectrum which shows 3-bond correlations of Xyl-H1 with GalA-C3 and of GalA-H3 with Xyl-C1. This is supported by the chemical shifts of GalA-C3 which are 9 ppm higher than in unsubstituted GalA (Ramos *et al.*, 1996). These data show that B3 is the disaccharide β-D-xylopyranosyl-(1→3)-D-galacturonic acid.

Purified B4 was also investigated by NMR. Analysis of the 1D proton NMR spectrum and the 2D proton COSY spectrum of B4 (LMA) confirmed the presence of Rha and ΔUA moieties. These spectra showed that two anomers were present and gave the chemical shifts (δ_H) of all the protons in both anomers (Table 3). Proton–proton coupling constants (J_{HH}) were measured directly from the 1D spectrum which gave the α-anomer : β-anomer ratio as 2:5. Carbon-13 chemical shifts (δ_C) for all carbons except those for C5 and C6 of the ΔUA residue were determined from the 2D HSQC carbon–proton correlation spectrum. The chemical shifts of C5 and C6 on the ΔUA acid residue were determined from the 2D HMBC carbon–proton correlation spectrum. C5 shows the expected correlations with H1, H3 and H4. C6 shows a correlation with H4. That the residues are linked via oxygen from C1 of ΔUA to C2 of Rha is confirmed by the HMBC spectrum which shows 3-bond correlations of ΔUA-H1 with Rha-C2 and of Rha-H2 with ΔUA-C1. This is supported by the chemical shifts of Rha-C2 which are 5–7 ppm higher than in unsubstituted Rha (Stothers 1973). The data for the β-anomer agree well with published data on LMA (Hasegawa *et al.* 1992; Hirose *et al.* 2004), but there are no published reports of data for the α-anomer.

The NMR spectral data thus supported the identification of B4 as LMA, an unsaturated disaccharide (ΔUA→Rha) likely to be formed during the elimination-degradation of the pectic domain, rhamnogalacturonan-I, by a lyase activity (Stratilová *et al.*, 1998; Naran *et al.*, 2007). LMA has previously been reported to occur naturally in cress-seed and

other seed exudates (Hasegawa *et al.* 1992; Yamada *et al.*, 1995, 1997). Compound B3 on the other hand is the disaccharide β-D-xylopyranosyl-(1→3)-D-galacturonic acid (Xyl-GalA), which does not appear to have been reported occurring in plants *in vivo*. Xyl-GalA is a known product of the *in-vitro* action of xylogalacturonan hydrolase on another pectic domain, xylogalacturonan (Zandleven *et al.*, 2005). However, polymeric xylogalacturonan was reported to be undetectable in *Arabidopsis* seeds (Zandleven *et al.*, 2007; although it has apparently not been tested for in cress seeds), so the occurrence of free Xyl→GalA in seed exudate was unexpected.

Bioassay of LMA and Xyl→GalA

Samples of the purified LMA (B4) and Xyl→GalA (B3) were bioassayed over a range of concentrations (Fig. 8a,b). We tested these disaccharides both in the presence and absence of 0.2 mM KCl, as a potential osmoticum. Effects of LMA and Xyl→GalA were very slight, but there appeared to be a slight promotion of hypocotyl growth with 360 μM LMA and 740 μM Xyl→GalA (Fig. 8a,b). Root growth was also slightly promoted by these disaccharide concentrations, so the hypocotyl:root length ratio was not increased by either disaccharide, contradicting the findings on LMA reported by Hasegawa *et al.* (1992). The disaccharide effects tended to be more pronounced without added KCl than with it, although the difference was not significant. Thus, there was no evidence for disaccharide/K^+ synergy (Fig. 8a,b).

To confirm these findings, we repeated bioassays of B4 and B3 in octuplicate, at the specific concentrations mentioned above (Fig. 8c,d). There was a slight but significant promotion of elongation of both hypocotyls and roots by both LMA and Xyl→GalA. There was no effect of these disaccharides on the hypocotyl:root ratio.

DISCUSSION

Plant tissues exude a vast array of secondary metabolites, some of which are biologically active, including hormones and oligosaccharins. To separate any classical plant hormones (most of which are lipophilic weak acids) from other bioactive compounds in cress-seed exudate, partitioning between polar and non-polar phases at various pH values is an effective method (Nakayama *et al.*, 1996). In the present study, the bioactive compound(s) in cress-seed exudate were hydrophilic at all tested pH values (2, 6, 12), as judged by solvent partitioning between water and ethyl acetate. Thus,

although the exudate exhibited activities that are superficially auxin- and gibberellin-like, the major active principle(s) present were not any of these because at pH 2 both indole auxins and gibberellins would be expected to partition in to the organic phase rather than the water phase (Kelen and Sanli, 2009; Nakayama *et al.*, 1996). In addition, at pH 12, any auxins or gibberellins with hydrophilic ester-linked groups attached would have been hydrolysed to (hydrophobic) free auxins or gibberellins. Heat treatment of cress-seed exudate showed that the bioactive compound(s) are stable for prolonged periods at high temperatures, and their ethanol solubility is compatible with their being small and hydrophilic, like LMA. Size-fractionation by GPC confirmed that the active principle(s) were of low M_r. All these observations appeared to agree with the report by Hasegawa *et al.* (1992) that LMA is the major active principle exuded by cress seed(ling)s and capable of interfering allelochemically with the growth of neighbouring *Amaranthus* seedlings.

However, in our attempts to explore further the production and roles of the bioactive principle(s), we encountered results that clearly showed LMA not to be the major agent causing excessive hypocotyl elongation and inhibiting root growth in *Amaranthus*. In particular, the active principle was not found to co-migrate with LMA during electrophoresis, even though LMA was readily detectable on the electrophoretograms. Furthermore, purified LMA exerted only moderate effects on *Amaranthus* seedlings (Fig. 8a,c): it caused a slight increase in hypocotyl elongation (16% promotion), and it did not inhibit root growth. On the contrary, it caused a 10% promotion of root growth, so that the hypocotyl:root length ratio was almost unaffected (Fig. 8d), contradicting the findings of Hasegawa *et al.* (1992), who reported up to a 400% increase (5-fold promotion) in hypocotyl elongation and a strong inhibition of root growth.

Strictly speaking, we did not test lepidimoide (which is the sodium salt of LMA). However, we did test LMA in the presence of 0.2 mM KCl (thus the potassium salt of LMA). Furthermore, exogenous LMA and lepidimoide will freely interconvert *in vivo* since in aqueous solution any organic acid instantly forms salts of all cations present; indeed, it was previously reported that lepidimoide and LMA are functionally equivalent (Yamada *et al.*, 1996; Hirose *et al.*, 2003). Therefore, our conclusions about the bioactivity of LMA (and its K+ salt) can be equally applied to lepidimoide.

In the course of this work we discovered that cress seeds exude not only LMA but also, in comparable quantities, an additional acidic disaccharide, identified as β-D-xylopyranosyl-(1→3)-D-galacturonic acid (Xyl-GalA). Xyl-GalA had minor effects on

Amaranthus seedling growth similar to those of LMA. Thus, the moderate growth effects of LMA are not highly specific to the structure of this disaccharide. A similar lack of specificity for the moderate promotion of hypocotyl growth by LMA has also been reported by Hasegawa's group (Yamada *et al.*, 1996; Hirose *et al.*, 2004), so the effects may be nutritional rather than 'oligosaccharin' signalling effects. The small enhancement of both hypocotyl and root growth in *Amaranthus* seedlings cannot be accepted as a significant allelochemical effect — most allelochemicals having <u>adverse</u> effects on neighbouring plants.

Hasegawa's group reported that <u>pure</u> lepidimoide and LMA exert biological effects. We therefore did not systematically test LMA supplemented with additional components. However, our early preparations of LMA tested by bioassay (Figs. 2 and 3) would have been mixtures of LMA + Xyl-GalA (since these two disaccharides have very similar mobilities when electrophoresed at pH 6.5, as shown in Figs. 5 and 6), and our results show that this particular mixture did not exert the characteristic biological effects — hypocotyl promotion, root inhibition — reported by Hasegawa *et al.* (1992).

Both LMA and Xyl-GalA have structures compatible with their being generated by the action of enzymes on pectic polysaccharide domains: lyase action on RG-I and hydrolase action on xylogalacturonan. The disaccharides were present in the collected cress-seed exudate at ~250 µM LMA and ~125 µM Xyl-GalA after incubation of 5 g dry seeds in a total of 100 ml water. This means that these two disaccharides were generated to the extent of ~0.16% and 0.08% of the seed dry weight respectively. Most of the LMA and Xyl-GalA was probably already present in the dry seeds since the seeds were only imbibed with water at 4°C, making it unlikely that extensive enzymic fragmentation of pectic polysaccharide occurred during the harvesting of the exudate. Thus, any RG lyase and xylogalacturonan hydrolase action on seed polysaccharides probably occurred before seed maturation rather than during imbibition. It remains theoretically possible that the two disaccharides were manufactured as by-products during pectic polysaccharide synthesis in developing seeds. However, there is no known pathway for the *de-novo* biosynthesis of ΔUA residues; therefore the presence of LMA strongly suggests the *in-vivo* action of a rhamnogalacturonan lyase in developing cress seeds. This conclusion is novel because, as already mentioned, only very low RG lyase activity has previously been suggested in plants (Stratilová *et al.*, 1998; Naran *et al.*, 2007).

The true biological roles, if any, of LMA and Xyl-GalA in cress-seed exudate remain unknown. Speculatively, such roles could include interactions with soil-dwelling micro-

organisms, with the acidic disaccharides either signalling the presence of a cress root to initiate a symbiotic relationship such as endophytism or mycorrhizal association. Alternatively, the acidic disaccharides could serve as anti-microbial agents deterring colonisation by pathogens.

Despite the inability of LMA and Xyl-GalA to adversely affect *Amaranthus* (and lettuce) seedling development, it remains clear that whole cress-seed exudate does exert adverse 'allelochemical' effects on neighbouring competing seedlings (Fig. 1 and Hasegawa *et al.*, 1992). These effects, however, must be ascribed to different, unidentified, substances rather than to the disaccharides investigated to date.

CONCLUSIONS

Our results consistently dispute the reports (reviewed by Yamada *et al.*, 2007) that LMA strongly promotes hypocotyl elongation and inhibits root growth and thus greatly increases the hypocotyl:root ratio of target seedlings such as *Amaranthus*. Since LMA ($\Delta UA \rightarrow Rha$) and $Xyl \rightarrow GalA$ slightly promoted the growth of both organs in our work, though only at concentrations well above 3 µM, their effects could be nutritional (e.g. sugar supply) rather than being based on oligosaccharin-type signalling. The existence in cress-seed exudate of factor(s) that exert the reported effects (promoting hypocotyl elongation and inhibiting root growth) was confirmed, as was the occurrence of LMA in cress-seed exudate. However, we conclude that the nature of the major factor(s) exerting the growth effects on *Amaranthus* seedlings remains unknown and an interesting open question for further investigation.

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<u>LEGENDS TO FIGS</u>

Fig. 1. The main active principle of cress-seed exudate is heat-stable, ethanol-soluble and hydrophilic

(a) An aqueous solution of total cress-seed mucilage (TCSE; 5 mg/ml) was treated at 100°C for various times and then bioassayed on *Amaranthus* seedlings. Seedlings cultured in water alone were used as a control. Data represent the mean of 10 seedlings from a single Petri dish; error bar represents intra-plate SD (n = 9 or 10).

(b) As (a) but heating was at 130°C for 48 h. Data represent means from triplicate Petri dishes; error bars represent inter-plate SE (n = 3).

(c) TCSE (10 ml of 5 mg/ml) was adjusted sequentially to 70%, then 80%, then 90% ethanol and centrifuged after each increase in ethanol; the three precipitates and the 90% ethanolic supernatant were dried and re-dissolved at 2.5 mg/ml in water and bioassayed. Control: *Amaranthus* seeds incubated in water alone. Data are means from triplicate Petri dishes; error bars show inter-plate SE (n = 3).

(d) Further samples of TCSE (10 mg/ml) were adjusted to different pH values by addition of HCl or NaOH and then shaken with an equal volume of ethyl acetate. The two phases were separated and dried, and the non-volatile material in each phase was re-dissolved in the original volume of water and bioassayed. Data are means from triplicate Petri dishes; error bars are inter-plate SE (n = 3).

Fig. 2. The active principle of cress-seed exudate is lost during electrophoresis

Eleven replicate preparations of low-M_r cress-seed exudate (LCSE) were applied to 4-cm-wide lanes on Whatman No. 3 paper (each lane loaded with 1.5 ml LCSE) alongside authentic sugar and amino acid markers, and electrophoresed at pH 6.5 and 3.0 kV for 22 min. The marker lanes were stained (a,b), and eluates of 3-cm-long strips of the eleven LCSE lanes were bioassayed on *Amaranthus* seed/lings (c). In (c), the green, pink and red arrows indicate the expected positions of glucose, LMA and GalA respectively (GalA runs very slightly slower than the marker GlcA in this electrophoresis system). Error bars indicate inter-plate SE (n = 11).

Fig. 3. Presence of acidic oligosaccharides in biologically inactive electrophoresis fractions

A 36-cm-wide lane of Whatman No. 3 paper was loaded with LCSE (36 ml containing a trace of Orange G), and electrophoresed at pH 6.5 and 3.0 kV for 22 min. External markers and a ~4-cm-wide fringe of the LCSE loading were stained with $AgNO_3$ (a), then the remaining unstained 32-cm-width of the LCSE loading was cut into 2-cm strips (b; only part shown), which were eluted, dried and re-dissolved in 1.3 ml water. Portions (0.1 ml) of each of these solutions were diluted to 1 ml and bioassayed (c), and in selected cases 4-µl portions of the same solutions were analysed for sugars by TLC in solvents BAW and EPAW (d). Non-standard abbreviations used: Fru, fructose; LMA, lepidimoic acid; M, marker mixture; M2, maltose; M3, maltotriose; M4, maltotetraose; Sucr, sucrose.

Fig. 4. The active principle of cress-seed exudate does not co-elute with LMA on gel-permeation chromatography

A 5.5-ml sample of 2% (w/v) LCSE (110 mg dry weight) was applied to a 185-ml bed-volume column of Bio-Gel P-2 and eluted in deionised water. Sixty fractions (F1–F60; each 3.7 ml) were collected and 3-µl portions of F16–F37 analysed by TLC in solvents BAW or EPAW (a). Further portions of each fraction were tested for pH (b, c; ●) and bioassayed at ½-strength (b) and ¼-strength (c), for effects on the growth of hypocotyls (green histograms) and roots (red) of *Amaranthus* seedlings. At the right-hand end of (b) and (c) are shown the bioassay results of pure water (H2O), a dilution series ($^1/_{128}$ or $^1/_{64}$- to full-strength) of crude 2% (w/v) LCSE in water (C/128 or C/64 to C); Fig. (b) also shows bioassays of 25 mM HEPES (Na^+) buffer pH 7.45 (H), and a dilution series of crude 2% LCSE in this buffer (CH/64 to CH). The seedlings showed only very stunted growth in full-strength 2% LCSE (indicated ×). The pH values are shown (●) only for the <u>full</u>-strength LCSE and Bio-Gel fractions. Dotted yellow box: position of putative LMA. Non-standard abbreviations used: Api, apiose; GlcAL, glucuronolactone; V_0, void volume.

Fig. 5. High-resolution electrophoresis of acidic components of cress-seed exudate

(a) Paper electrophoretogram showing six replicate lanes of a marker-mixture ('MM', containing 10 μg Glc, 10 μg GalA, a trace of ΔUA-GalNAc and a trace of orange G) and of LCSE (500 μg dry weight of crude low-M_r cress-seed exudate plus a trace of orange G). Electrophoresis was performed on Whatman No. 1 paper, at pH 6.5 and 3 kV, for 105 min. The various tracks were stained or otherwise documented as indicate at the top. Spots circled in pencil were visible under a hand-held 254-nm UV lamp before staining. The results of duplicate TBA assays (A_{549}, revealing ΔUA residues) are indicated by the pink histograms.

(b), (c) TLC of sugars eluted from selected strips of the electrophoretogram [marked as ✓ in (a), and labelled as cm migrated towards anode along the bottom edge of the TLCs]. TLC was performed by 1 ascent in the solvent mixtures BAW (b) or EPAW (c). Bands A and B1–B4 are LCSE components discussed in the text; B4 is putative LPA.

Fig. 6. Lepidimoic acid partially hydrolyses to rhamnose after TLC

(a) Two zones (*i*, *ii*) of acidic disaccharides from LCSE were isolated by preparative HVPE (3 identical electrophoretograms like the one shown were run on Whatman No 3 paper with a loading of LCSE at 2 mg/cm; electrophoresis was at pH 6.5 and 3 kV for 105 min). UV-absorbing bands were marked in pencil, guide strips were stained with AgNO₃ or aniline hydrogen-phthalate (AHP) as indicated at the top, and then the unstained parts of zones *i* and *ii* were eluted and dried, and the solutes redissolved at 10 mg/ml.

(b) Samples of *i* and *ii* were subjected to TLC in BAW or EPAW, with 1 or 2 ascents, as indicated. The images show that the LMA (B4) was partially hydrolysed to free rhamnose while the plate was being dried between the 1st and 2nd ascent. This rhamnose (labelled Rha*) does not co-migrate with marker rhamnose (follow the black arrows) because it sets off, for the 2nd run, from the 1st-run LMA position (yellow arrows). LBA, lactobionic acid. Compounds B1 to B4 are named as in Fig. 5. Prep *i* is richer in LMA than prep *ii*, but only prep *ii* contains B1.

Fig. 7. Purification of B3 (Xyl→GalA) and B4 (LMA), and their hydrolysis to monosaccharides

(a) Preparative HVPE (pH 2.0, 4 kV, 3 h) of zone *i* material (see Fig. 6a) to separate B4 and B3; the left edge of the electrophoretogram was silver-stained, and the remainder

cut for elution of B3 and B4 (red rectangles); B4 was recognised by its UV absorption (pencilled).

(b) Analytical HVPE (pH 2.0, 4 kV, 3 h) of small portions of purified B3 and B4.

(c,d) TLC of purified B4 and B3 and their acid hydrolysis products in BAW (c) or EPAW (d). \Hydrolysis was by heating in 1.6 M TFA at 100°C for 1 h. In (d) a dilution series of rhamnose is included, from which the yield of B4-generated rhamnose was estimated.

(e) Graded acid hydrolysis of B4 (LMA). Replicate samples of zone *i* material (see Fig. 6a) were incubated in water or 0.1 M acetic acid at 20, 70 or 120°C, then the products were resolved by TLC in BAW and stained with thymol.

(f,g) Hydrolysis of the LMA 'model compound', ΔUA-(1$\rightarrow$3)-GalNAc (labelled Std on the image) in various solutions at 120°C. Replicate TLC plates (run in BAW) were stained with molybdate reagent (f) or thymol (g). M, MM, marker (mixture).

Fig. 8. Effect of purified LMA and Xyl$\rightarrow$GalA on *Amaranthus* seedlings

(a,b) Dose–response curve for effect of purified LMA and Xyl$\rightarrow$GalA on *Amaranthus* seedlings in the presence (filled symbols) and absence (open symbols) of 0.2 mM KCl. The dotted green and red lines show the mean hypocotyl and root length in the absence of KCl. Any statistical significance of an oligosaccharide effect is indicated relative to the corresponding zero-oligosaccharide control (*, $p<0.05$; **, $p<0.005$). Black arrows: treatments selected for replication in (c) and (d). Error bars show intra-plate SE (n = 7–10 seedlings for the oligosaccharide-treated samples, or 15–19 seedlings for the zero-oligosaccharide controls).

(c,d) Highly replicated bioassays using treatments selected from (a) and (b) (360 µM LMA and 740 µM Xyl$\rightarrow$GalA, in water). Standard errors are inter-plate (n = 8 plates); each plate being scored from the mean of 7–10 measureable seedlings. Labels on bars refer to a comparison with the water treatment (*ns*, no significant difference; *, $p<0.05$; **, $p<0.005$).

TABLES

Table 1. Characteristics* of selected sugars present in low-M_r cress-seed exudate (LCSE)

Compound (see Fig. 5)	m_{OG} at pH 6.5	UV abs	TBA +ve	AgNO$_3$ +ve	AHP	R_{Glc} in BAW	R_{Glc} in EPAW
A	0.473	?	+	+	–	0.69	0.15
B1	0.623	–	–	+	–	1.26	1.10
B2	0.672	–	–	+	–	0.73	0.14
B3 (Xyl-GalA)	0.693	–	–	+	orange	0.69	0.08
B4 (LMA)	0.716	+	+	+	brown	0.94	0.50
Glc	**0**	–	–	+	brown	**1**	**1**
Rha	0.000	–	–	+	brown	1.30	2.40
GalA	0.924	–	–	+	orange	0.74	0.07

*<u>Abbreviations used</u>:

m_{OG} = electrophoretic mobility (at pH 6.5, towards the anode) of the compound relative to that of Orange G (m_{OG} = 1) and glucose (m_{OG} = 0).

UV abs: producing a dark spot, absorbing 254-nm UV radiation and thus diminishing the background fluorescence of the electrophoresis paper.

TBA: pink colour in thiobarbituric acid assay for ΔUA residues.

AgNO$_3$: brown spot with silver nitrate (reaction given by reducing and non-reducing sugars).

AHP: coloured spot with aniline hydrogen-phthalate, indicating a reducing sugar (neutral hexoses brown, neutral pentoses red, uronic acids orange).

R_{Glc}: mobility on TLC in the solvent mixture indicated, relative to that of glucose.

Table 2. NMR data for the acidic disaccharide B3

	β-anomer				α-anomer		
	δ_H	J_{HH} (Hz)	δ_C		δ_H	J_{HH} (Hz)	δ_C
GalA-1	4.59 (d)	J_{12} = 8.0	92.2		5.29 (d)	J_{12} = 3.9	95.8
GalA-2	3.63 (dd)	J_{23} = 8.9	70.7		3.96 (dd)	J_{23} = 10.2	67.1
GalA-3	3.80 (dd)	J_{34} = 3.5	82.3		4.01 (dd)	J_{34} = 3.2	79.2
GalA-4	4.40 (dd)	J_{45} = 0.9	69.8		4.47 (dd)	J_{45} = 1.0	70.4
GalA-5	4.09 (d)		74.9		4.61 (d)		70.6
GalA-6			174.3				175.0
Xyl-1	4.61 (d)	J_{12} = 7.9	104.5		4.62 (d)	J_{12} = 7.9	104.5
Xyl-2	3.35 (dd)	J_{23} = 9.3	73.2		3.35 (dd)	J_{23} = 9.3	73.2
Xyl-3	3.44 (t)	J_{34} = 9.3	75.7		3.44 (t)	J_{34} = 9.3	75.7
Xyl-4	3.61 (ddd)	J_{45a} = 10.1	69.3		3.61 (ddd)	J_{45a} = 10.1	69.3
Xyl-5a	3.31 (dd)	J_{45e} = 5.5	65.1		3.31 (dd)	J_{45e} = 5.5	65.1
Xyl-5e	3.95 (dd)	J_{55} = 11.8			3.95 (dd)	J_{55} = 11.8	

a = axial, d = doublet, dd = doublet of doublets, ddd = doublet of doublet of doublets, e = equatorial, t = triplet

Table 3. NMR data for the acidic disaccharide B4 (LMA)

	β-anomer				α-anomer		
	δ_H	J_{HH} (Hz)	δ_C		δ_H	J_{HH} (Hz)	δ_C
Rha-1	5.25 (d)	J_{12} = 1.6	91.7		4.96 (s)	J_{12} = 0.0	93.8
Rha-2	4.13 (dd)	J_{23} = 3.5	77.5		4.21 (d)	J_{23} = 3.3	79.1
Rha-3	3.84 (dd)	J_{34} = 9.8	69.2		3.62 (dd)	J_{34} = 9.8	71.9
Rha-4	3.38 (t)	J_{45} = 9.8	72.2		3.29 (t)	J_{45} = 9.8	71.9
Rha-5	3.87 (dq)	J_{56} = 6.3	68.5		3.41 (dq)	J_{56} = 6.3	72.3
Rha-6	1.26 (d)		16.7		1.28 (d)		16.7
ΔUA-1	5.19 (d)	J_{12} = 2.4	98.2		5.32 (d)	J_{12} = 2.4	100.5
ΔUA-2	3.82 (dd)	J_{23} = 7.3	70.0		3.87 (dd)	J_{23} = 7.1	70.2
ΔUA-3	4.37 (dd)	J_{34} = 3.2	65.8		4.40 (dd)	J_{34} = 3.1	65.8
ΔUA-4	5.97 (d)		110.4		5.97 (d)		110.4
ΔUA-5			142.8				142.8
ΔUA-6			167.3				167.3

d = doublet, dd = doublet of doublets, dq = doublet of quartets, s = singlet

Fig. S1. Standard curve for the assay of ΔUA residues and related compounds by a modification of the method of Koseki *et al.* (1978).

The *x*-axis plots the quantity of compound assayed in a 40-μl aqueous sample, and the *y*-axis shows the absorbance of the final 1.3-ml reaction mixture.