

GGL-family tissue specific activity

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Summary

Xylans are a major component of plant secondary cell walls and their understanding is vital to the optimisation of bio-fuel production. Despite this, the biosynthesis of these polysaccharides is still poorly understood. I have investigated the xylans of root and etiolated hypocotyl tissue and shown significant amounts to be present in both tissues. Previous studies have shown that two genes, GGL4 and GGL5, are responsible for adding glucuronic acid to the xylan backbone of glucuronoxytan in *Arabidopsis thaliana* stem cell wall (Mortimer, unpublished data). In this study, I investigated the functions of these and related genes, GGL1, GGL3 and GGL6, for addition of glucuronic acid to glucuronoxytan and glucuronoarabinoxylan in root and etiolated hypocotyl secondary cell wall. This was done by structurally characterising the xylan of mutants in these genes by polysaccharide analysis using carbohydrate gel electrophoresis in root and etiolated hypocotyl tissue and high-performance anion exchange chromatography with pulsed amperometric detection. This paper provides evidence that GGL4 and/or GGL5 are responsible for the addition of some glucuronic acid to glucuronoxytan in root and etiolated hypocotyl tissues but are not the sole glucuronic acid transferase in these tissues. In both root and etiolated hypocotyl GGL3 is also required for addition of glucuronic acid. High-performance anion exchange chromatography with pulsed amperometric detection, however, showed no detectable change in the relative monosaccharide composition of the hemicellulosic cell wall. In addition, I report that there is significantly poorer digestion of root xylan than in stem or etiolated hypocotyl which may indicate the presence of glucuronoarabinoxylan in the root.

Abbreviation	Full name
AIR	Alcohol insoluble residue
ANTS	8-amino-naphthalene-1,3,6-trisulfonic acid
RsAraf1	α -L-arabinofuranosidase/ β -xylosidase
Abf51A	α -L-arabinofuranosidase (EC3.2.1.55)
Arabidopsis	Arabidopsis thaliana
DMSO	Dimethyl sulphoxide
GAX	Glucuronoarabinoxylan
GGL	Glycogenin-Like
HPAEC-PAD	High-performance anion exchange chromatography with pulsed amperometric detection
[Me]GlcA	Glucuronic acid / Methylglucuronic acid
NaCNBH3	Sodium cyanoborohydride
PACE	Polysaccharide analysis using carbohydrate gel electrophoresis
Xylanase	1,4- β -xylan xylanoxylase (EC 3.2.1.8)
XGAT	Xylan glucuronic acid transferase
(Xyl) _n	1,4- β -linked xylose (n= degree of polymerisation)

Introduction

Secondary cell wall

Once elongation is complete, some plant cells (such as those in the xylem vessels) lay down a highly rigid secondary cell wall inside the expandable primary cell wall. This structure includes a diverse array of covalently and non-covalently cross-linked pectins, phenolics, proteins and hemicelluloses (Alberts *et al*, 2002).

Xylans are hemicelluloses and make up approximately 30% of the secondary cell wall (Ebringerova and Heinze, 2000). The backbone chain of 1,4- β -linked xylan is usually decorated on the O-2 position with branches of glucuronic acid (the mixture of methylated and unmethylated glucuronic is denoted by [Me]GlcA), arabinose (also on the O-3 in grasses) or a mixture of both branch types (Teleman *et al*, 2002). Glucuronoxylan (GX) is more common in dicots and glucuroarabinoxylan (GAX) is more common in the monocots (Darvill *et al*, 1980). There is some evidence that it may be present in *Arabidopsis thaliana* (Zablackis *et al*, 1995) though it is still uncertain. Xylans are thought to hydrogen bond to cellulose cross-linking microfibrils (Linder *et al*, 2003) to provide structural rigidity to the cell wall. [Me]GlcA has been shown to disrupt interactions between the xylan and cellulose but enhance those to lignin by forming covalent ester linkages (Imamura *et al*, 1994). The Ara of GAX has also been observed to bind to the ferulic acid of lignin (Grabber *et al*, 1998).

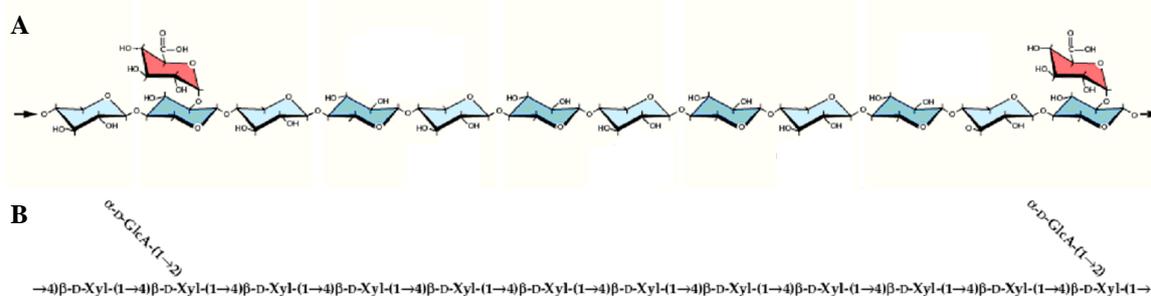


Figure 1 - Structure of dicot and noncommelinoid monocot GX. (A) Chemical structure of GX. (B) Simplified representation of GAX. (Adapted from Biochemistry & Molecular Biology of Plants (Buchanan, Gruissem and Jones, eds)

The cell wall has a complex structure and, considering its vital importance to the biology of the plant (including roles in development, defence and physiology), its biosynthesis is poorly characterised (York and O'Neill, 2008). Understanding the biosynthesis of xylans is not only useful for creating better models of the cell wall, but also commercially. The projection for an increased production of biofuels (Ragauskas *et al*, 2006) has led to greater interest in how to manipulate these pathways to produce tissue that is more easily used in industry whilst still retaining viable plants that are easily grown on a large scale. The high degree of side branching in the xylans makes them difficult to digest. This is a problem in biofuels as a large amount of the potential sugar is wasted as

oligosaccharides. If plants could be engineered to have less substitution of the xylan backbone a higher yield could be gained from biofuel plants however it is important to fully understand the effects of interfering with the biosynthetic pathways before discoveries can be applied to industry.

Biosynthetic genes

From over 800 putative glycosyltransferases and glycosyl hydrolases (Brown *et al.* 2007) that have been identified by bioinformatics in Arabidopsis, two genes have recently been identified that are required for the addition of [Me]GlcA to GX in the stem. Mutants in these genes, glycogenin-like genes GGL4 and GGL5, have a bare xylan backbone (Mortimer *et al.*, unpublished data) and are to be renamed xylan glucuronic acid transferase XGAT1 and XGAT2. This makes it unlikely that FRA8 is a GX-specific glucuronyltransferase as proposed by R. Zhong *et al.*, (2005) as the *ggl4/ggl5* mutant has not [Me]GlcA substitution of xylan in the stem despite the expression of FRA8 in vessel and fiber cells.

As GGL4 and GGL5 are expressed most strongly in the stem, it is possible that other genes perform the same function on other tissues. A small family of genes with high sequence homology to GGL4 and GGL5 are hypothesized to have similar activities. This study will investigate the functions of these GGL genes by analysis of the phenotypes of transposon mutants.

GGL1 and GGL6 are strongly expressed strongly in the roots based on expression data from the AtGenExpress project (Schmid *et al.*, 2005) and GUS promoter fusion for GGL1 (Richardson, 2003) therefore cell wall from root tissue will be investigated. The cell wall of etiolated hypocotyl will also be analysed to examine the role of the GGL genes in development. GGL3 is expressed throughout the plant. GGL4 and GGL5 are included in the study for comparison and because their activity in the stem may also extend to other secondary wall-producing vascular tissue such as that of the root. It is possible that the related genes are involved in addition of glucuronic acid to the xylan backbone in a different tissue, such as the root or the developing hypocotyl, or they might add glucuronic acid to GAX rather than GX in some or all tissue types. Due to the similar expression patterns of GGL1 and GGL6, a *ggl1/ggl6* double mutant is used to maximize the chance of an observable phenotype in case of redundancy. There are two different transposon knockouts of GGL3 to act as a control for the efficacy of the mutagenesis.

There are several endo- β -1-4-xylanase enzymes that can cut the xylan polysaccharide backbone (used both by the plant itself for recycling and remodeling the cell wall as well as necrophytes and parasites). Xylanase 11A (1,4- β -xylan xyloxydrolase), a glycosyl hydrolase, is used in these experiments because it is a small enzyme able to penetrate into the cell wall to digest it but is unable to cut linkages adjacent to a branch due to its deep active site (Katapodis *et al*, 2002). The reducing end of the resulting fragments can be coupled with a charged fluorophore, 8-amino-naphthalene-1,3,6-trisulfonic acid (ANTS) and resolved on a polyacrylamide gel (Figure 2). Carbohydrates are resolved according to their degree of polymerization (chain length) and type (monomer size, linkage and charge). The fragment pattern produced can be used to infer the composition of the original xylans before digestion.

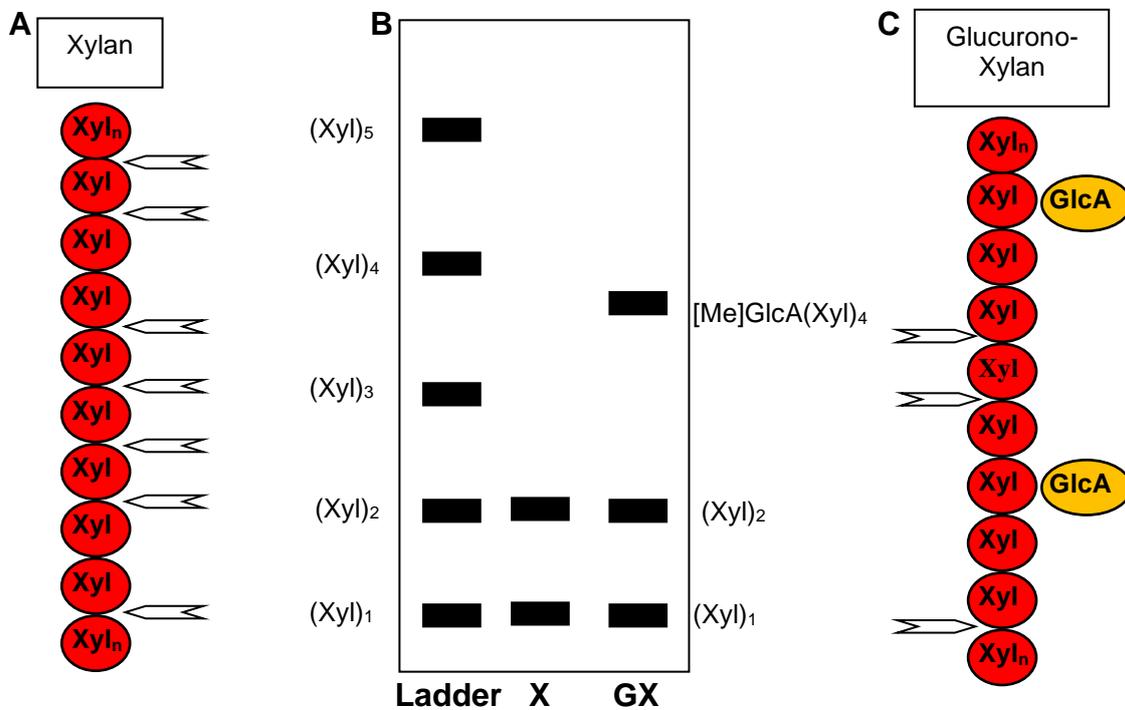


Figure 2 - Diagram and description of idealised X and GX PACE gels when digested with xylanase 11A. (A) Schematic of hypothetical unsubstituted xylan. White arrows represent hydrolysis sites for xylanase 11A. (B) A ladder (left) can be made by mixing known amounts of purified oligosaccharides from an incomplete digest. The digestion of unsubstituted xylan (centre) gives mostly $(Xyl)_1$ and $(Xyl)_2$. The digestion of glucuronoxylan (right) gives the same bands as seen for unsubstituted xylan but also a band of $[Me]GlcA(Xyl)_4$ which is shifted down relative to $(Xyl)_4$ by the charge of the GluA. (C) Schematic of hypothetical glucuronoxylan. White arrows represent cut sites for xylanase 11A.

Techniques such as mass spectrometry or NMR do not allow easy quantification and comparison between samples. In addition, NMR requires relatively large samples and as such is only suitable for analyzing total cell wall monosaccharide ratios or linkage in short oligosaccharides. PACE, however, is used because it can achieve picomolar sensitivity and so is able to detect the products of a digestion. This allows a more detailed idea of the original structure of the polysaccharide. Moreover, PACE can run samples in parallel allowing straightforward quantification and high throughput.

PACE can be used quantitatively because each oligosaccharide only has one free reducing end and as such only one fluorophore couples per molecule. The relative ratios of bands can be measured or, if standards are used, absolute quantities can be determined (Goubet *et al*, 2002).

This study focuses on the GGL family of genes to investigate whether they add GlcA sidechains to GX in other tissues or are they involved in GAX synthesis. Through this investigation of *ggl* mutants I discovered that the roles of known glucuronic acid transferases GGL4 and GGL5 extend to include activity in the roots and etiolated hypocotyl as well as their previously known function in the stem. GGL3 was also shown to have glucuronic acid transferase activity in the root and hypocotyl. The role of the genes in GAX synthesis, however, will require further investigation.

Results

Presence of xylan in root and etiolated hypocotyl

To determine whether GX could be detected in root or hypocotyl cell walls I harvested Arabidopsis root and etiolated hypocotyl tissue and prepared cell wall material. The wall was digested with xylanase and released oligosaccharides derivatised with ANTS and separated by gel electrophoresis. GX could be detected in both samples (data not shown). The amount of cell wall was optimised for complete xylanase digestion (data not shown). The visualisation of stem cell wall requires digestion of 50 µg of sample resuspended in 100 µl of urea to give clear bands when using 1 µl for analysis. To give bands of comparable intensity as stem samples, 500 µg of root cell wall was required and the equivalent of 250 µg of etiolated hypocotyl cell wall. Due to the small mass of sample extracted from the etiolated hypocotyls, only 100 µg was used but was resuspended in 40 µl of urea instead of 100 µl. This demonstrates that there is significant GX present in the roots and etiolated hypocotyl but that it is a much lower percentage (an order of magnitude in root) of the overall Alcohol Insoluble Residue (AIR) than in the stem. This is in agreement with previous studies which have shown the xylan level in the stem to be 2.62% of cell wall material

		[Me]GlcA substitution (%)	
Genotype		Biological replicate 1 (n=3)	Biological replicate 2 (n=3)
Root	WT	9.80 ± 0.29	12.18 ± 2.34
	<i>ggl1/6</i>	11.43 ± 0.87	11.62 ± 2.31
	<i>ggl4/5</i>	4.47 ± 1.34	6.37 ± 1.83
	<i>ggl3-2</i>	7.67 ± 1.54	6.84 ± 0.81
	<i>ggl3-3</i>	10.19 ± 0.25	11.88 ± 1.08
Genotype		Biological replicate 1 (n=2)	Biological replicate 2 (n=1)
Etiolated hypocotyl	WT	11.36 ± 0.33	9.58
	<i>ggl1/6</i>	12.16 ± 0.33	9.67
	<i>ggl4/5</i>	8.69 ± 0.56	4.65
	<i>ggl3-2</i>	5.25 ± 0.39	5.10
	<i>ggl3-3</i>	11.14 ± 1.54	

Table 1 – Degree of [Me]GlcA substitution in wild type and *ggl* mutants for root and etiolated hypocotyl tissue. Values are means ± SD for the indicated number of technical replicates.

and in the roots 0.286% of cell wall material (Wong, 2005). The level in the etiolated hypocotyl can therefore be estimated to be roughly 0.6% of cell wall material.

PACE gels of xylanase 11 digested cell wall from the roots and hypocotyls of wild type plants gave a similar level of glucuronic acid [Me]GlcA substitution of xylan to that previously observed in stem (11%-13%).

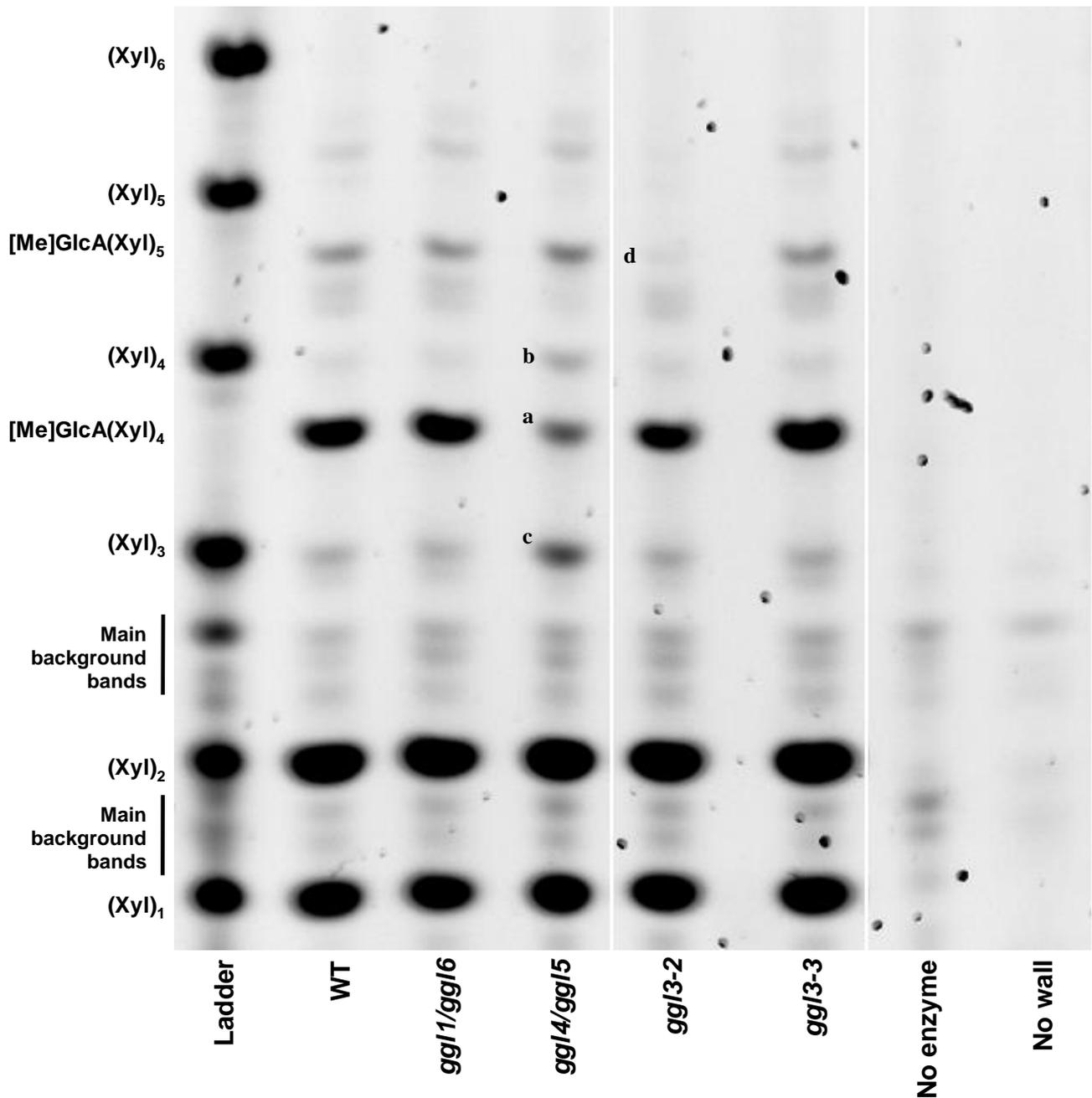


Figure 3 – Xylan analysis of *ggl* mutant root cell wall by PACE. Alcohol-insoluble cell wall was solubilised with NaOH and digested twice with xylanase 11A. The digestion products were then derivatised with ANTS and resolved by electrophoresis. Controls of undigested wall (no enzyme) and enzyme only (no wall) have been included to identify the main background bands. Mutant dependent bands are labelled (a-d). The two *ggl3* mutant lanes have been swapped for clarity.

Structural analysis of root xylan

To determine the requirement for any GGL genes for [Me]GlcA addition and the frequency of this branching, PACE gels of xylanase-11 digested cell wall from the roots of the mutants were prepared. They revealed differences in relative band intensities (Figure 3). A reduction in the intensity can be seen in the [Me]GlcA(Xyl)₄ band (a) of the *ggl4/ggl5* mutant as well as a compensatory increase in the (Xyl)₃ and (Xyl)₄ bands (b and c). A reduction can also be seen in the [Me]GlcA(Xyl)₅ band (d) of the *ggl3-2* mutant.

I quantified the degree of [Me]GlcA substitution in order to compare the *ggl* transposon mutants in root and etiolated hypocotyl (Table 1). Analysis by ANOVA of [Me]GlcA substitution in root tissue showed that there was a significant variation between the different plant lines ($p < 0.05$). Pair wise comparison by T-test demonstrated significant difference between wild type and *ggl4/ggl5* ($p < 0.05$) and between wild type and *ggl3-2* ($p < 0.05$).

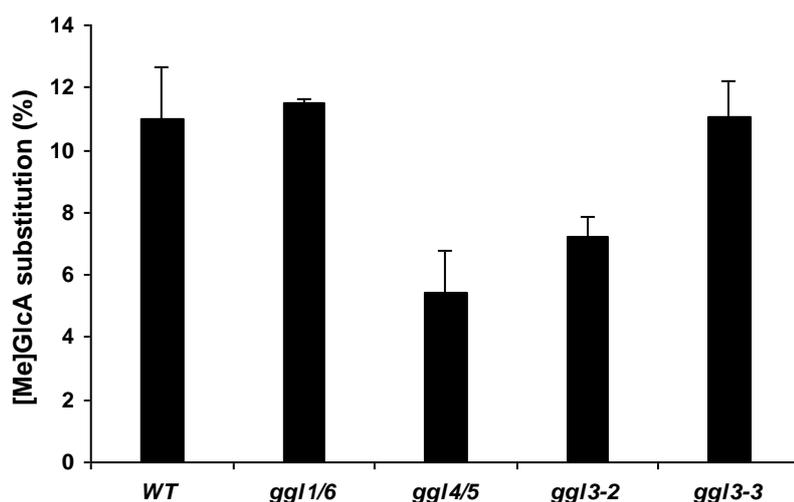


Figure 4 – Degree of [Me]GlcA substitution in root cell wall. Relative quantification of the amount of [Me]GlcA compared to Xyl by PACE analysis of root cell wall from *ggl* mutants digested twice with xylanase 11A. Error bars show standard deviation of the two biological replicates.

Structural analysis of etiolated hypocotyl xylan

The same procedure was applied to the data from etiolated hypocotyls and though significant variation existed between the plant lines ($p < 0.05$), ANOVA demonstrated that there was also significant difference between the biological repeats ($p < 0.05$) due to the degree of [Me]GlcA substitution being lower in all plant lines in one of the repeats. This difference could have been caused

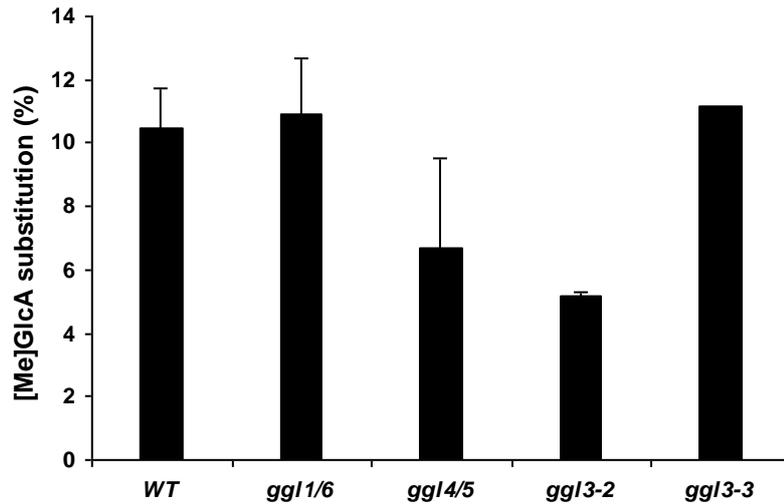


Figure 5 – Degree of [Me]GlcA substitution in etiolated hypocotyl cell wall. Relative quantification of the amount of [Me]GlcA compared to Xyl by PACE analysis of etiolated hypocotyl cell wall from *ggl* mutants digested twice with xylanase 11A. Error bars show standard deviation of the two biological replicates. There is only one biological replicate of *ggl3-3*

by harvesting the tissue at different stages of development as the cell wall might be rapidly remodeled during this period.

A T-test between the [Me]GlcA substitution of *ggl3-2* and *ggl3-3* in root cell wall reveals a significant difference between the lines ($p < 0.05$) which confirms the repeated discrepancy between *ggl3-2* and *ggl3-3* (which should be equivalent as they are two different transposon mutants of the same gene).

Presence of glucuronoarabinonxylan in root

To investigate whether the GGL genes were involved in the synthesis of other xylans, the degree of digestion quantified. PACE gels of roots showed significantly ($p < 0.05$) greater proportion of high-weight bands ([Me]GlcA(Xyl)₅ or above compared to (Xyl)₄ or below) than stem tissue or etiolated hypocotyl (Figure 6, Figure 7A). This could indicate incomplete digestion due to insufficient enzyme used (the same amount of enzyme was used for 50 µg of stem cell wall but 500 µg of root cell wall due to lower xylan content in the roots). Another possibility is that the digestion was being hampered by arabinose side chains which could indicate the presence of GAX in Arabidopsis. No significant decrease in high weight bands was observed when the number of xylanase 11 digests was increased (Figure 7B) implying that the high-weight bands are not the result of insufficient enzyme.

If the larger oligosaccharides were indigestible due to arabinose side-chains then it might be possible hydrolyse the arabinose by using an arabinosidase enzyme. I therefore tested whether the ratio of higher weight to lower weight bands in stem and root could be affected by the bifunctional enzyme

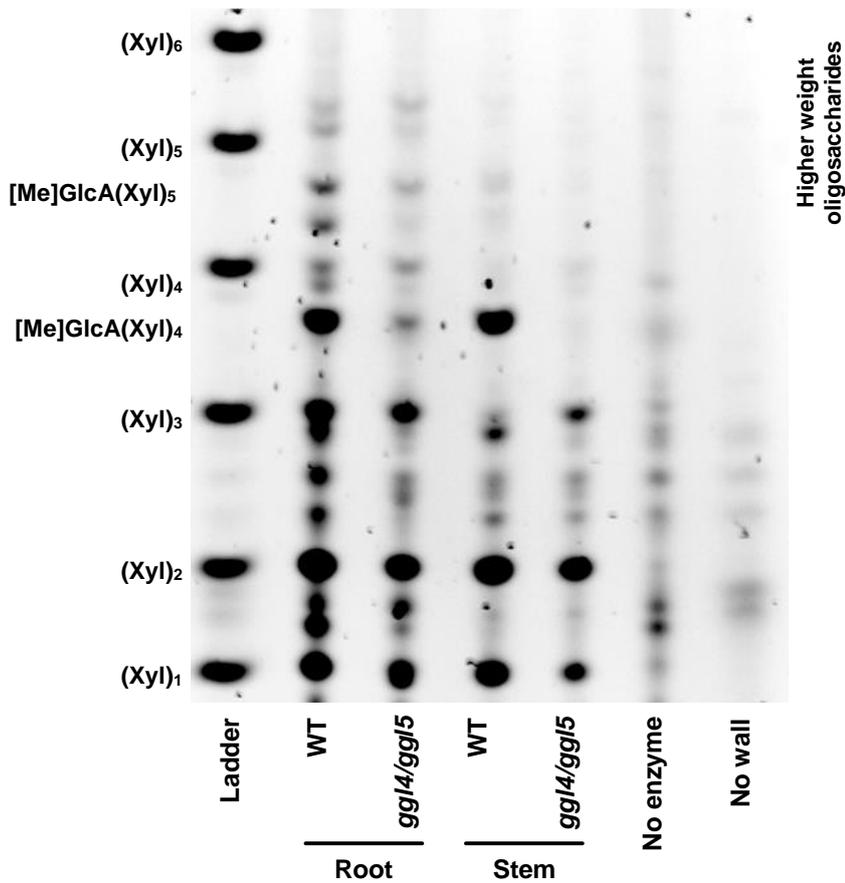


Figure 6 – Xylan analysis of xylanase-digested stem and root cell wall of wild type and *ggl4/ggl5* by PACE. The digestion shows the difference in banding patterns from stem and from root. Root tissue gives a greater number of high molecular weight bands. The [Me]GlcA(Xyl)₄ band is also more noticeably reduced in stem tissue (b) from *ggl4/ggl5* than root tissue (a).

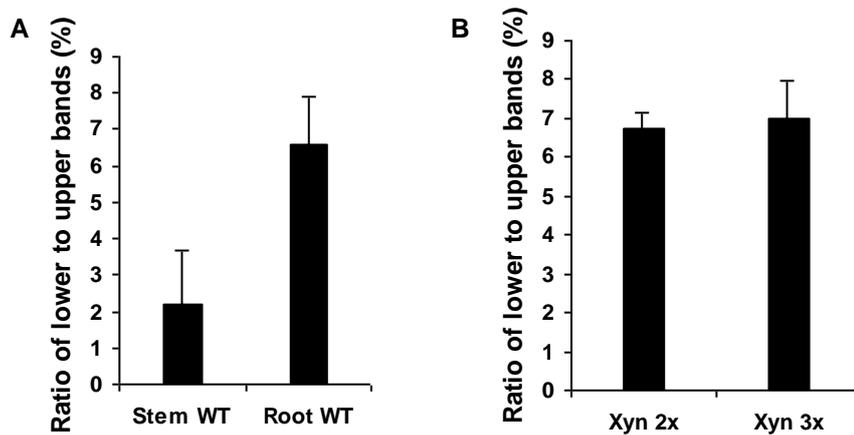


Figure 7 – Ratio of large to small fragments in WT cell wall. Relative quantification of the amount of (Xyl)₅ and [Me]GlcA(Xyl)₅ to all smaller oligosaccharides by PACE analysis of twice xylanase 11A digested root and stem cell wall from WT Arabidopsis. (A) Ratios in root and stem tissue. Error bars show standard deviation of the three technical replicates. (B) Ratio in root tissue when digested twice or three times by xylanase 11A. Error bars show standard deviation of the two technical replicates.

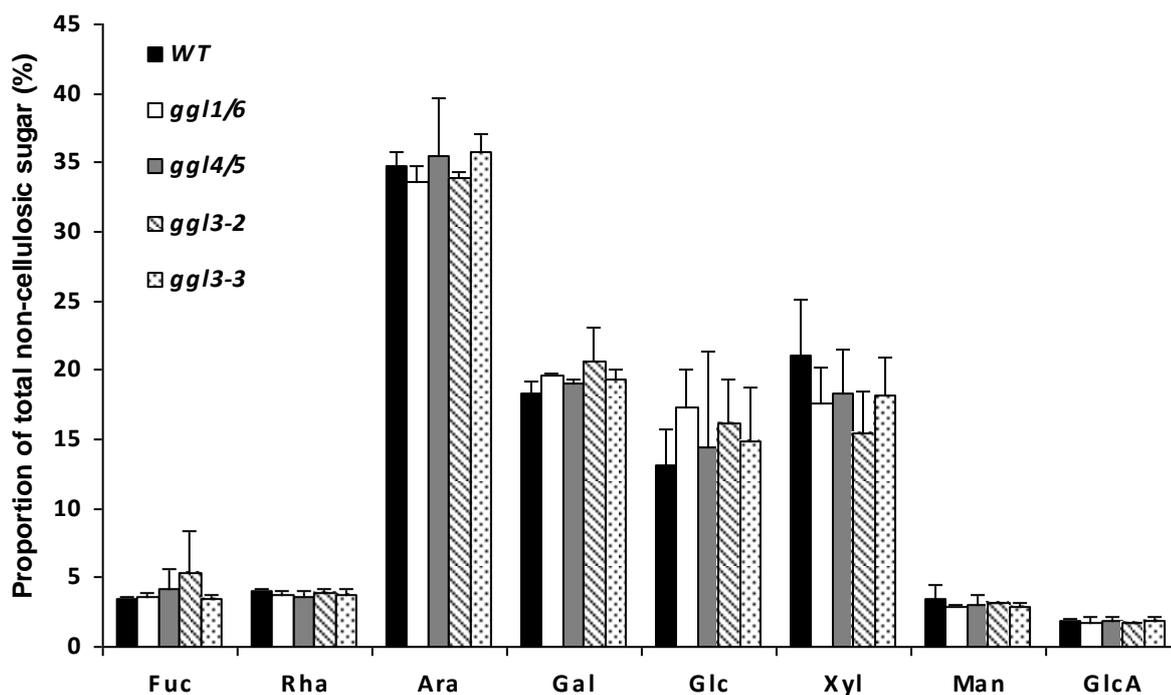


Figure 8 – Relative levels of different monosaccharides in the root hemicellulose of *ggl* mutants. Relative quantification of the amount of monosaccharides by HPAEC-PAD of trifluoroacetic acid hydrolysed root cell wall from WT and *ggl* mutant Arabidopsis. The individual sugars fucose (Fuc), rhamnose (Rha), arabinose (Ara), galactose (Gal), glucose (Glc), xylose (Xyl), mannose (Man) and glucuronic acid (GlcA) are expressed as a percentage of the total recoverable sugar. Error bars show standard deviation of the two biological replicates.

α -L-arabinofuranosidase/ β -xylosidase (RsAraf1) which had previously been shown to cleave arabinose residues from arabinogalactan proteins (Kotake *et al*, 2006).

Two sequential digestions with RsAraf after three xylanase digestions failed to give a difference to cell wall digested with the xylanase only in all samples and a positive control of pure wheat arabinoxylan. This demonstrates that RsAraf it is unable to cleave arabinose from arabinoxylan and hence is probably also ineffective on GAX. The same assay was performed with the enzyme α -L-arabinofuranosidase (Abf51A) which is known to release arabinose from arabinoxylan (Saha, 2000) but data was inconclusive due to an experimental error as Abf51A was added to the negative control and unfortunately there was no time to repeat the experiment.

To analyze the monosaccharide composition of the GGL mutants I treated samples with trifluoroacetic acid which hydrolysed the hemicellulosic polysaccharides whilst leaving the cellulose intact (Morrison, 1988). High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) showed that the levels of free monosaccharides released by this procedure do not differ significantly between wild type and any of the *ggl* mutants (Figure 6). The ratio of GlcA to Xyl is also constant across the mutants (data not shown).

Discussion

The aim of this study was to determine whether the GGL family of genes had any function in adding [Me]GlcA to GX and GAX in the stem and etiolated hypocotyl. It was found that the *ggl4/5* mutant had lower levels of glucuronic acid substitution of xylans in both of these tissues as did the *ggl3-2* mutant. Overall monosacchride levels were not noticeably different and experiments to investigate GAX were inconclusive.

The level of [Me]GlcA substitution is similar in stem, root and hypocotyls. The amount of root cell wall required for PACE are consistent with the known xylan levels in different tissues (Wong, 2005) and the xylan content of hypocotyls, which was previously unknown, can be said to be roughly double the level in roots (roughly 0.6% of cell wall material). Absolute quantisation could be achieved by running PACE gels with standards of known amount. To be accurate the mass of etiolated hypocotyl collected would need to be an order of magnitude greater than that collected in this study. The amount of xylan in etiolated hypocotyls seems to be more than in young stem material implying that development under normal light conditions and in the dark may put different requirements on secondary cell wall formation.

It is most likely that the genes are required because they encode for transferases but the transferase activity has not been specifically assayed in this study of mutant phenotypes. It is therefore possible that they could be necessary accessory proteins for the transferases. There is no evidence, however, that any related genes from the superfamily perform such an accessory function (arabidopsis.org)

These data indicate a role for GGL4 and/or GGL5 in glucuronic acid transfer in tissues other than the stem. Further experiments will have to be performed with the single mutants in order to discern whether they act combinatorially in these tissues or whether only one of them is active. They are, however, not the only genes required for glucuronic acid transfer in the root and etiolated hypocotyl as indicated by the ability of *ggl4/ggl5* mutant plants to substitute the xylan backbone. In the root, the *ggl4/ggl5* mutant has only a ~50% reduction in [Me]GlcA substitution compared to wild type and ~30% reduction in cell wall from etiolated hypocotyl. This is consistent with redundancy to other genes in these tissues, the most likely candidate being GGL3 as the *ggl3-2* mutant also shows a reduction in [Me]GlcA substitution in both root and etiolated hypocotyl. The *ggl3-3* mutant seems likely to be an incomplete knockout as it still has wild type activity.

Aside from trivial errors (e.g. seed mislabeling) *ggl3-3* may not have a reduction in gene function if the transposon is spliced out or the protein can fold and function even with the transposon sequence translated. These alternatives could be distinguished by the use of RT-PCR. It is also possible (though unlikely) that GGL3 does not have a role in addition of [Me]GlcA to xylan but there is a mutation in a gene that does in the *ggl3-2* plant line.

There is no significant difference between the wild type substitution level and the sum of the *ggl4/ggl5* and *ggl3-2* mutants' substitution indicating that it is possible that between them, these genes contribute all of the required [Me]GlcA transferase activity. If GGL3, GGL4 and GGL5 are the only genes to add [Me]GlcA to GX, a triple *ggl3/ggl4/ggl5* mutant would show a complete lack of branched bands. If there was still some substituted xylan in the triple mutant either one of the other GGL genes also adds [Me]GlcA to GX. When knocked out on its own the other genes mask the effect by increasing their activity to compensate, the assay may not be sensitive enough to measure 10% change from WT, or a non-GGL gene may serve the function.

The banding pattern from the xylanase digest showed that the reduction in [Me]GlcA substitution of the residual xylan structure in the cell wall of *ggl3* roots is not equivalent to that in *ggl4/ggl5* roots (Figure 3). This may imply that there exist two pools of GX, each created by either GGL3 or GGL4/GGL5 and that the pool created by GGL3 gives [Me]GlcA(Xyl)₅ as the main branched digestion product whereas GX synthesized by GGL4/GGL5 gives mostly [Me]GlcA(Xyl)₄. GGL3, therefore, may not only act in a different tissue to GGL4/GGL5, but also add the [Me]GlcA branch in a different pattern or at a different density.

The glucuronic acid side branches may not be distributed regularly or randomly along the xylan backbone and may be clustered in regions and leave bare xylan in others. GGL4 and GGL5 would then be hypothesized to add [Me]GlcA more densely along the backbone than GGL3 is able to. It is also possible that there are three pools, one of bare xylan, another of xylan substituted every 4th residue by a branch (by GGL4 and GGL5) and a third pool of xylan substituted every 5th residue (by GGL3). These pools could be different organs in the same tissue or different types of xylan in the same wall. The [Me]GlcA substitution levels in the wild type are similar in stem, hypocotyl and root (i.e. about 1 in 8) so if the different GGL genes give a different branch spacing, there must be a compensating effect to give the same overall ratio of glucuronic acid to xylose. This may involve a varied pool of unsubstituted xylan.

As the degree of [Me]GlcA substitution affects how strongly the GX interacts with other components of the cell wall, controlling the details of the spacing between [Me]GlcA branches may be a method of modulating rigidity in the secondary cell wall.

There is a trend in etiolated hypocotyl that both *ggl4/ggl5* and *ggl3-2* have lower [Me]GlcA substitution but it is not statistically significant because the biological repeats differ too greatly from each other. The difference could be improved by growing more repeats and keeping a stricter growth time before harvesting. The trend appears to be that, again, both GGL4/GGL5 and GGL3 play a role. It might have been expected that the genes responsible to adding the [Me]GlcA to GX in etiolated hypocotyl would be the same as in stem because the tissues are separated only by the cotyledons but the preliminary data do not support this. This might imply that regulation of GX biosynthesis plays a

role in growth and development. A time-course could be done to investigate whether the cause of the discrepancy is due to developmental changes in gene activity.

GAX has been shown to comprise roughly 5% of the cell wall of the dicot, *Acer pseudoplatanus* (Darvill *et al.*, 1980) and has been suggested to exist in *Arabidopsis* cell (Zablackis *et al.*, 1995). I have provided evidence that the xylan from the root digests to larger oligosaccharides than xylan from the stem. It is possible that the arabinose branches of GAX cause the greater amount of high molecular weight oligosaccharides seen on PACE gels. It is also possible that full digestion is being interfered with by other factors that are specific to the root tissue or a combination of any of these causes. There does not appear to be any difference between the mutants and wild type in the high to low weight ratio of oligosaccharides. The ratio of high to low weight bands may not be a good indicator of GAX. This could be tested by analyzing the ratios of these bands in the digestion products of xylnans from plants with known levels of GAX. It may be the case that none of the GGL genes are involved in GAX synthesis. It is also possible that the method is not sensitive enough to detect the changes involved (for example if the bands caused by GAX are masked by bands from the more abundant GX).

To further investigate whether GAX is present and the cause of the difference in banding pattern between stem and root digests, an assay could be optimised to show whether the larger products of xylanase digestion are sensitive to different arabinosidase enzymes. If the oligosaccharides from the xylanase digestion were then digested with arabinosidase it should release $(Ara)_1$. $(Ara)_1$ has the same mass and charge as $(Xyl)_1$ and so should co-migrate with it and might be detectable as an increase in the intensity of this PACE band. Alternatively it could be tested if the addition of arabinosidase renders larger fragments digestible to a second round of xylanase digests which would be seen as a reduction in upper bands on a PACE gel when subsequent arabinosidase and xylanase digests are performed. Each mutant could then be systematically tested for alterations for GAX.

It was hoped that HPAEC-PAD would give extra information on the changes in the cell wall occurring in different mutants however the lack of difference between the mutants and the wild type can have several interpretations. It may be that there is no change in monosaccharide levels because the *ggl4/ggl5* and *ggl3-2* mutants compensate for the reduced GlcA in GX by increasing the amount of GlcA in other hemicellulosic polysaccharides but this redistribution cannot be seen because HPAEC-PAD records the overall composition of the hemicellulose such as AGP without distinguishing between the different sources of the monosaccharides. It could also be that the changes in GlcA in GX are not compensated elsewhere but the reduction in relative GlcA is swamped by more abundant sources from other hemicelluloses which stay constant. With enough repeats it may be sensitive enough to detect a small change in GlcA even if the change in the total amount of GlcA in the hemicellulose is very small. HPAEC-PAD is a time consuming process, however, and it may not be practical to do the number of repeats needed to get small enough errors to draw solid conclusions for these mutants.

In this study I have extended the model of the biosynthesis of GX, showing that though only GGL4 and GGL5 are required for addition of [Me]GlcA to the xylan backbone in stem tissue, GGL 3 is required in both roots and etiolated hypocotyl demonstrating both tissue and development specific differences in the genes required. It is possible that the genes act in different tissues in order to synthesise different forms of GX, perhaps required for differences in cell wall properties. I have also provided evidence that the lower digestibility of root xylans is not due to insufficient enzyme. These findings open up areas of further study both in refining our models of cell wall biosynthesis and in improving assays to investigate xylan structure.

Materials and Methods

Plant material

Homozygous transposon mutants were used. The T-DNA insertion lines and wild type plants were grown in identical conditions.

	Genes knocked out	Transposon insert lines
Wt	None	None
<i>ggl4/5</i>	At4g33330/ At3g18660	GABI_722F09 x Salk_063763
<i>ggl1/6</i>	At1g08990/ At1g54940	Unknown x Salk_123905
<i>ggl3-2</i>	At1g77130	Salk_009971
<i>ggl3-3</i>	At1g77130	GK-166E01.01

Seeds from these lines were planted out on agar (0.8% w/v) plates containing MS nutrients and B5 vitamins and plants were grown as in (Murashige and Skoog, 1962). To collect etiolated hypocotyls the plants were grown for 6 days at 22°C in the dark before being harvested. To collect root material the plants were grown for two weeks at 22°C under a 16 h day light regime of 150–180 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ before being transferred to floats for four weeks and then being harvested.

Cell wall preparation

Harvested material was boiled in ethanol (96% v/v, 30 min, 70°C) and homogenized in a Mixer Mill MM200 (Glen Creston, UK) and the homogenate centrifuged (14000 rpm, 15 min) The pellet was washed with ethanol (96% v/v), ethanol (100% v/v), methanol/chloroform (2:3 v/v, overnight), methanol/chloroform (2:3 v/v, 1 h), ethanol (100% v/v), ethanol (65% v/v), ethanol (80% v/v) and ethanol (100% v/v). The remaining pellet of alcohol insoluble residue (AIR) was dried overnight at 60°C.

Enzyme hydrolysis

AIR samples were treated with sodium hydroxide to solublise and de-acetylate the cell wall components to allow for better enzyme access (4M, 1 hr) and neutralized to pH 3-9 with hydrochloric acid (1M). Ammonium acetate (adjusted to pH 6 with acetic acid) was added to a final concentration of 1M for 500 μl . The specified enzyme was added (indicated amount, >5 hrs, 21°C) and the reaction quenched by boiling (20 min, 100°C). Several digests were performed sequentially as indicated. After all desired digests were carried out the sample was dried by centrifugal vacuum evaporation.

Polysaccharide analysis using carbohydrate gel electrophoresis

The dry sample was derivatised to ANTS overnight (37°C) as in Goubet et al. (2002) in acetic acid:water:DMSO (3:17:20 v/v, 10 μl). The samples were then resuspended in urea (3M, 100 μl for root, 40 μl for etiolated hypocotyl). Ladders of xylose oligosaccharides (Xyl 1-6, 1 μl of each) were derivatised in the same manner but re-suspended in urea (6M, 100 μl) and stored at -20°C. PACE gels

were loaded with samples (2 µl) and ladder (1 µl) and run, scanned with a G:BOX UV transilluminator (Syngene, UK) and quantified as in Goubet et al. (2002).

Calculation of substitution

For root, each biological repeat is based on the average of three technical repeats (full repeats of digestion, fluorophore derivatisation and electrophoresis). Due to insufficient etiolated hypocotyl material, the first biological repeat is the average of two technical repeats and the second biological repeat had only technical repeat. All statistics were performed on the two biological repeats.

When bands were quantified, the intensity from the no-enzyme control lane was subtracted to compensate for background. The ratio of [Me]GlcA to Xyl was calculated by the formula: $1x([\text{Me}]\text{GlcA}(\text{Xyn})_4) + 1x([\text{Me}]\text{GlcA}(\text{Xyn})_5) / 1x(\text{Xyl})_1 + 2x(\text{Xyl})_2 + 3x(\text{Xyl})_3 + 4x[\text{Me}]\text{GlcA}(\text{Xyn})_4 + 4x(\text{Xyl})_4 + 5x[\text{Me}]\text{GlcA}(\text{Xyn})_5 + 5x(\text{Xyl})_5$. The ratio of upper to lower bands was calculated as $[\text{Me}]\text{GlcA}(\text{Xyn})_5 + (\text{Xyl})_5 / (\text{Xyl})_1 + (\text{Xyl})_2 + (\text{Xyl})_3 + [\text{Me}]\text{GlcA}(\text{Xyn})_4 + (\text{Xyl})_4$.

High-performance anion exchange chromatography with pulsed amperometric detection

50 µg AIR samples were treated with trifluoroacetic acid (2M, 1 hour, 120°C) to hydrolyze the non-cellulosic cell wall. The samples were then dried by centrifugal vacuum evaporation and resuspended in acetonitrile:water (1:19 v/v, 20 µl). Chromatography was performed on samples as in Currie and Perry (2006).

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