Versatile high-resolution oligosaccharide microarrays for plant glycobiology and cell wall research

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Running title*: Oligosaccharide microarrays for plant glycobiology

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Background: Microarrays of plant-derived oligosaccharides are potentially powerful tools for the high-throughput discovery and screening of antibodies, enzymes and carbohydrate binding proteins.

Results: Oligosaccharide microarrays were produced and their utility demonstrated in several applications.

Conclusion: A new generation of oligosaccharide microarrays will make an important contribution to plant glycomic research.

Significance: High throughput screening technology enables the more effective production of carbohydrate active enzymes and molecular probes.

SUMMARY

Microarrays are powerful tools for high throughput analysis and hundreds or thousands of molecular interactions can be assessed simultaneously using very small amounts of analytes. Nucleotide microarrays are well established in plant research, but carbohydrate microarrays much less so and one reason for this a lack of suitable glycans with which to populate arrays. Polysaccharide microarrays are relatively easy to produce because of the ease of immobilising large polymers non-covalently onto a variety of microarray surfaces but they lack analytical resolution because polysaccharides often contain multiple distinct carbohydrate sub-structures. Microarrays of defined oligosaccharides potentially overcome this problem but harder to produce because oligosaccharides are usually require coupling prior to immobilisation. We have assembled a library of well characterised plant oligosaccharides produced either by partial hydrolysis from polysaccharides or by de novo chemical synthesis. Once coupled to protein these neoglycoconjugates are versatile reagents that can be printed as microarrays onto a variety of slide types and membranes. We show that these microarrays are suitable for the high
throughput characterization of the recognition capabilities of monoclonal antibodies, carbohydrate-binding modules and other oligosaccharide-binding proteins of biological significance and also that they have potential for the characterization of carbohydrate-active enzymes.

Glycans are crucial for plant life and are used for storage, defence and signalling and as structural cell wall components (1–6). Plant oligo- and polysaccharides are also important components of food and feed and have numerous industrial applications. Starch is the most common carbohydrate in the human diet whilst plant cell walls provide bulk materials including timber, paper and cloth, as well as fine chemicals, food ingredients and biofuel feedstocks (1, 6–8). The complexity and diversity of plant polysaccharides underpin their biological roles and many of their industrially important characteristics, but also produces challenges for research and optimal utilisation. A detailed knowledge of the structures, functions, interactions and occurrence of plant glycans is essential for understanding their complex contributions to plant life and to fully exploit their commercial potential. However, unlike proteins and nucleotides, complex carbohydrates are not readily amenable to sequencing or synthesis and existing biochemical techniques for glycan analysis although powerful are usually low throughput (3, 9).

The development of rapid genome sequencing methods and improvements in protein expression techniques enable the production of large numbers of carbohydrate-active enzymes and carbohydrate-binding proteins including carbohydrate-binding modules (CBMs). There has been an exponential increase in the number of entries in the carbohydrate active enzyme (CAZy) database (10) of these proteins but this has not been matched by structural analysis or determination of their biochemical activities (11). Similarly, monoclonal antibodies (mAb) are immensely valuable molecular probes for carbohydrate research but their usefulness is dependent on knowledge of the epitopes they recognise (12–15). The rate limiting step in CBM, mAb and enzyme production is often a lack of efficient methods for screening their specificities. There is therefore a clear need in plant biology for high throughput (HTP) and high resolution techniques for the analysis of carbohydrate-active proteins including enzymes.

Microarray technology has underpinned the development of multiplexed assays that have revolutionised the HTP analysis of nucleotides, proteins and increasingly, carbohydrates (16–18). Using microarrays, the abundance of, and interactions between hundreds or thousands of molecules, can be assessed simultaneously using very small amounts of analytes (16–18). Carbohydrate microarrays were first produced in 2002 and a variety of approaches have been developed for the printing and immobilisation of oligo- and polysaccharides (19–26). However the representation of glycomes on these arrays is generally far less comprehensive than is the coverage of transcriptomes/genomes and proteomes by nucleotide and protein arrays respectively. The primary reason for this is the lack of facile methods for the production of sets of homogeneous, sequence-defined plant oligosaccharide structures (27, 28). In contrast, partially defined polysaccharides are relatively easy to obtain and microarrays and enzyme linked immunosorbent assays (ELISAs) populated with such samples have shown potential for HTP screening (29–31). However, most plant polysaccharides and especially those from plant cell walls are complex heteropolymers and, even if pure will typically accommodate a range of smaller oligosaccharide sub-structures (or epitopes) and so polysaccharide-based assays, whether they be microarrays or ELISAs lack analytical resolution (30–32).

We have developed a new generation of glycan microarrays for plant research based on defined oligosaccharide structures produced either by isolation from polysaccharides or by de novo chemical synthesis. Once coupled to bovine serum albumin (BSA) these neoglycoprotein sets are highly versatile and microarrays can be printed on a variety of slides and membranes. Most of the oligosaccharides we describe here are derived from, or based on, cell wall polysaccharides that are amongst the most complex in nature and present particular challenges for HTP
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analysis, but we have also included starch related oligosaccharides and novel synthesised structures.

EXPERIMENTAL PROCEDURES

Oligosaccharide samples - Oligosaccharides were produced either by partial enzymatic or chemical hydrolysis of source polysaccharides followed by fractionation and purification or were prepared by chemical synthesis. For detailed information on all oligosaccharide samples see Supplementary Table 1.

MALDI-TOF analysis of conjugation efficiency - Analysis was performed as described in (33).

Monoclonal antibodies and CBMs - Previously characterised cell wall-directed rat monoclonal antibodies used in this study included LM5 (34), LM6 (35), LM13 and LM16 (36), LM10 and LM11(37), LM15 (38) and LM21 and LM22 (39). Novel rat monoclonal antibodies were obtained as follows: LM23 was derived subsequent to immunization with a complex pectic immunogen from apple fruits and this antibody binds to xylosyl residue in a range of antigenic contexts including xylogalacturonan and xylan. LM24 and LM25 were derived subsequent to immunisation with a neoglycoprotein generated from a mixture of XXLG and XLLG xyloglucan oligosaccharides (Megazyme, Bray, Ireland). LM12 was derived subsequently to immunization with a neoglycoprotein generated with oligosaccharide structure 16. In all these cases immunization and hybridoma isolation protocols were carried out as described (35). CBMs were produced as described (40, 41).

BSA conjugation reaction - Oligosaccharides were conjugated to BSA essentially as described (42). Briefly, BSA was mixed with oligosaccharides and NaCNBH$_3$ (Sigma-Aldrich) in a borate buffer at pH 8. The reaction was allowed to progress at ambient temperature for 96 h. Where necessary, conjugates were purified from reaction mixtures using a spin column with a 10 KDa cut off (Pall, Lund, Sweden). The conjugates were stored in the reaction buffer at 20 °C before use and were stable for at least 6 months whereby CN’ from the reducing agent presumably acts as a preservative.

Microarray printing - Carbohydrate microarrays were printed using two types of microarrays robot, a pin based MicroGrid II (Digilab/Genomic solutions, Huntingdon UK) and a piezolectric Sprint (Arrayjet, Roslin, UK). For printing on the MicroGrid II: Microarrays were printed using 4 split pins or 4 solid pins (Digilab, Huntingdon, UK) and oligosaccharides were diluted to a 2 mg/ml and a 0.04 mg/ml concentration in de-ionised water immediately before use and transferred to a 96 well micro plate for printing. Microarrays were printed at 16 °C at 35% humidity with 1 deposit per spot. The same procedure was used to print onto nitrocellulose membrane with a pore size 0.45 μm (Whatman, Maidstone, UK), FastSlides (Whatman, Maidstone, UK) nitrocellulose coated glass slides (Schott, Mainz, Germany) and a range of other surface modified glass slides (Schott, Mainz, Germany). For printing on the Arrayjet Sprint: The Sprint microarrayer was equipped with a 12-sample high capacity jetspyder sample pick up device. Microarrays were printed at 19 °C at 55% humidity, using 6 drops per spots when printing on nitrocellulose membrane, and 2 drops per spot when printing on all glass slide types. Samples were printed in 55.2% glycerol, 44% water 0.8% Triton X 100 and the same slides and nitrocellulose as for the MicroGrid II were used.

Microarray probing - Arrays were blocked by incubation for 1 h in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.7 mM KH$_2$PO$_4$, pH 7.5) containing 5% w/v low fat milk powder (MPBS) for nitrocellulose probing, 0.05% Tween 20 for FastSlides and Schott nitrocellulose coated glass slides and 0.5 M borate buffer containing 50 mM ethanolamine, pH 8.5 for all other slide types. Arrays were probed for 2 h with antibodies diluted 1/10 in PBS containing 5% w/v low fat milk powder, for nitrocellulose membrane or PBS containing 0.05% Tween 20 for all slide types. After washing with PBS (all microarray types), nitrocellulose microarrays microarrays were incubated for 2 h in either anti-rat or anti-mouse secondary antibodies conjugated to alkaline phosphatase (Sigma, Poole, UK) diluted 1/5000 in 5% MPBS. After further
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Washing in PBS microarrays were developed using a substrate containing 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium (NBT) in BCIP/NBT buffer (100 mM NaCl, 5 mM MgCl₂, 100 mM diethanolamine, pH 9.5). All glass based slides were incubated for 2 h in either anti-rat or anti-mouse secondary antibodies conjugated to AlexaFluor 555 (Invitrogen, Life Technologies Nærum, Denmark) and subsequently washed in PBS and then de-ionised water.

Scanning and analysis - Microarrays on nitrocellulose membrane were scanned using a flatbed scanner (Cannon 8800, Søborg, Denmark) and converted to 16 bit grey-scale TIFFs. Slides were scanned using a slide scanner (GenePix 4100, Molecular Devices, Sunnyvale, USA). The output from all scanning was analysed using microarray analysis software (ImaGene 6.0, BioDiscovery, El Segundo, CA, USA). Output from the analysis was further processed as necessary using Excel (Microsoft Denmark, Hellerup, Denmark) and presented as heatmaps in which colour intensity is correlated to mean spot signals.

Oligosaccharide microarray analysis of phosphorylase activity - The array was blocked by incubation for 1 h in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.7 mM KH₂PO₄, pH 7.5) containing 5% w/v BSA powder. After blocking, the array was washed three times for 5 min with 100 mM MOPS pH 7.0. Following the washes, the array was probed with 1 mg/mL rabbit muscle phosphorylase a (P1261, Sigma, Poole, UK) and 50 kBq [C14]Glucose-1-phosphate (GE healthcare, UK) for 2 h at 25 °C under continuous shaking (25 rpm). After incubation, the array was washed with PBS for 15 min. This was followed by two washes with two times PBS with 0.05% v/v Tween 20 for each 5 min. After this a sample from the washing buffer was taken and the radioactivity was analysed. If radioactivity was reduced to baseline levels the membrane was left drying and subsequently analysed on a phosphorimager system (445 SI, Molecular Dynamics).

Immunofluorescent labeling of tobacco sections - The cell wall imaging of xyloglucan mAb binding to tobacco stem pith parenchyma cell walls pre-treated withpectate lyase, was performed as described (38).

RESULTS

A library of plant oligosaccharides - A set of oligosaccharides was assembled either by the enzymatic cleavage of polysaccharides followed by purification of constituent oligosaccharides, or by chemical synthesis (Figure 1A). Each structure shown in Figure 1A has been assigned a code number (italicised to distinguish from manuscript reference numbers) which is consistent throughout the text. Some of the oligosaccharides have been previously described whilst four chemically synthesised galactosyl oligosaccharides were newly produced (structures 19, 20, 21 and 22). Details of all oligosaccharides are provided in Supplementary Table 1. The purities of the oligosaccharides were determined by a variety of methods which have been described previously and referenced in Supplementary Table 1. The purity of oligosaccharides produced from polysaccharides was generally at least 90% and for chemically synthesised oligosaccharides approximately 99%. Oligosaccharides were coupled to BSA by reductive amination with sodium cyanoborohydride which produced a ring opened sugar residue between the oligosaccharide and the BSA (Figure 1B).

Construction and reproducibly of oligosaccharide microarrays - Several types of substrate surface were tested including a range of surface-treated glass slides (Figures 2A-2C), glass slides coated with nitrocellulose (Figures 2D and 2E) and nitrocellulose membrane (Figures 2F-2H). Two types of spotting robot were used, one piezoelectric (Arrayjet Sprint, Figures 2A-2F) and one pin-based (Microgrid II, Figures 2G and 2H). The test prints shown were made using BSA conjugated with (1→4)-β-D-mannohexaose and (1→5)-α-L-arabinopentaose printed in sextuplet and at 10 concentrations from 2 mg/mL to 7.6 ng/mL (Figures 2A-2H). The arrays were probed with the anti-mannan or
anti-arabinan mAbs LM21 and LM6, respectively. The oligosaccharides were successfully presented for recognition on all the surfaces used but there were differences in detection limits and spot morphologies. Of the glass slides, the nitrocellulose-coated Nexterion E slide (Figure 2D) and the FastSlide (Figure 2E) yielded the most sensitive detection (2 μg/mL for (1→4)-β-D-mannohexaose) and the least sensitive detection was obtained using the Nexterion P slide (125 μg/mL for (1→4)-β-D-mannohexaose) (Figure 2C). Although similar in detection limits, arrays produced on the FastSlide (Figure 2E) were superior to those produced on the Nexterion E slide because they had a more consistent spot size across the concentration range and this is an important consideration when quantifying spot signals. Nitrocellulose membrane was also a suitable substrate for microarray production using both the pin-based and piezoelectric printers. With the piezoelectric Arrayjet printer the detection limit on nitrocellulose was 122.1 ng/mL (Figure 2F) and using the pin-based printer the detection limit was 2 μg/mL for split pins (Figure 2G) and 0.5 μg/mL for solid pins (Figure 2H). These data demonstrated that the neoglycoprotein oligosaccharides are a versatile resource for the manufacture of microarrays on diverse surfaces. The reproducibility of microarrays produced on the piezoelectric Arrayjet robot were tested by producing 12 separate copies of arrays both on nitrocellulose membrane (Figure 2I) and slides (Figure 2J), probing with selected antibodies, and then quantifying the spot signals from these replicate experiments (Figures 2I and 2J). For both nitrocellulose and slides, 6 arrays were probed with the anti-mannan mAbs LM21, and 6 with the anti-arabinan mAb LM6 and mean spot signals from 3 arrays were compared with the corresponding 3 replicate arrays. The data sets were plotted against each other and r^2 values calculated (Figures 2I and 2J). In all cases there was a low level of variability in the arrays, with r^2 values of greater than 0.9 in all cases. Similarly high levels of reproducibility have been previously been reported for arrays produced using the pin-based MicroGrid robot (32).

Most of the applications envisaged for oligosaccharide microarrays involve the interrogation of arrayed samples by a number of different glycan-binding proteins, ligands or enzymes which must be kept separate during probing. To achieve this we designed our array production to be compatible with multi-pad slides that fit in to a probing apparatus that forms a seal around each pad. An example of a typical array set up used is shown in Supplemental Figure 1A, where each of the 16 pads on each slide accommodates at least 324 spots in an area 6 mm x 6 mm and each oligosaccharide is usually represented by four spots (two replicates at two different concentrations). As shown in in Supplemental Figure 1B, the probing apparatus accommodates four such slides so that in a single probing experiment 64 distinct mAbs, CBMs et cetera can be individually screened simultaneously against at least 80 oligosaccharides printed in quadruplet. 50 μL of probing solution is required to probe one of the 16 pads. The composite image in in Supplemental Figure 1C shows the binding of five different mAbs to their respective epitopes on multiple copies of the array shown in Supplemental Figure 1A.

Specificity screening of monoclonal antibodies - We tested the microarrays for mAb screening by probing microarrays with a selection of 38 mAbs, some with previously well characterised specificities, and some new ones (Figure 3). Examples of representative arrays are shown in Figure 3A. The heatmap shown in Figure 3B is an overview of the whole data set and the expanded heatmaps (Figures 3C-3J) provide more detailed information about the binding of selected mAbs. Oligosaccharide structures are only shown where binding produced a mean spot signal of at least 15% of the highest mean signal in the entire data set. The microarray profiles obtained for mAb binding were consistent with data previously obtained by other techniques. For example, mAbs LM5 (34) and LM6 (35) bound predominantly to (1→4)-β-D-galactan (structure 19) and (1→5)-α-L-arabinans (Structures 9, 10, 11, 12, 13, 14) respectively (Figure 3C). As previously shown (39), mAbs LM21 and LM22 bound to mannan-containing oligosaccharides and LM22, but not LM21 bound strongly to galactomannan-derived
oligosaccharides (structures 32, 33 and 34) (Figure 3D). The inclusion of oligosaccharides with different degrees of polymerisation (DP) provided information about the epitope sizes recognised by some mAbs. For example, LM6 bound strongly to an arabinan dimer (structure 9) and similarly to arabianins with DPs up to 7 (Figure 3C). In contrast, LM13 binding was restricted to longer oligosaccharides and LM13 did not bind to an arabinan dimer (structure 9) and only very weakly to an arabinan trimer (structure 10), confirming and extending the previous analysis of the recognition of soluble oligosaccharides by competitive-inhibition ELISAs (36) (Figure 3C). Similarly, whilst LM22 (39) bound strongly to a mannan dimer (structure 27), mAb BS-400-4 (43) did not, and the minimum epitope size for this mAbs appears to be at least DP3 (Figure 3D). LM12, a novel rat mAb derived subsequent to immunization with a feruloylated-arabinosyl-BSA immunogen displayed recognition of feruloyl residues attached to a range of sugars (Figure 3F). In contrast to the previously described LM9, which is specific to feruloylated galactan (44), LM12 bound to oligosaccharides containing feruloylated arabinosyl residues (structures 15 and 16) as well as feruloylated galactosyl residues (structure 24) (Figure 3F). The anti-RGI mAb LM16 bound with greatest avidity to a new synthetic galactotriose (structure 21) (Figure 3F) and also showed unexpected binding to hexamino- (1→4)-β-D-glucopentaose (structure 78, Figure 3I) although this cross reactivity is unlikely to present problems for plant research since this polymer is unknown in the plant kingdom. A novel aldouronic acid epitope (glucoronyl- (1→2)- α-[(1→4)-β-D-xylotriose], structure 42) recognised by anti-arabinogalactan protein (AGP) mAbs, LM14 and JIM14 was also identified (Figure 3G). As expected the anti-(1→3),(1→4)-β-D-glucan mAb BS-400-3 bound to the (1→3),(1→4)-β-D-glucan structures 65 and 66 and did not cross react with the (1→3)-β-D-glucan oligosaccharides 57, 58 and 59 (45) and the anti-(1→3)-β-D-glucan mAb BS-400-2 also showed its expected binding to β-glucans containing 1,3-linkages (46) (Figure 3H). mAb JIM6 showed broad specificity for β-glucans containing 1,4-linkages but did not bind to β-glucans containing 1,3-linkages (Figure 3H).

Five antibodies directed to xyloglucan displayed subtle distinctions in recognition of xyloglucan-derived oligosaccharides with novel mAbs LM24 and LM25 displaying wider recognition of galactosylated xyloglucan oligomers (and in the case of LM25 weak binding to unsubstituted β-glucan) than the previously characterized mAb LM15 (38) (Figure 3J). The differences in the anti-xyloglucan mAb array binding profiles were reflected in differing binding profiles when applied to pectic lyase-treated transverse sections of tobacco stem pith parenchyma as shown in Figure 4. Previous work had demonstrated that after pectic HG removal the LM15 xyloglucan epitope is revealed abundantly at the corners of intercellular spaces (Marcus et al. 2008 and Figure 4B). In equivalent material the LM24 epitope was most abundant in adhered cell walls between intercellular spaces (Figure 4C) and the LM25 epitope was localised in cell walls lining intercellular spaces (Figure 4D).

Specificity screening of CBMs - We tested the oligosaccharide microarrays for CBM screening by probing arrays with a set of CBMs that were produced by mutation of CBM4-2 from Rhodothermus marinus (47–49) and with a variety of lectins (Figure 5). Wild type CBM4-2 is a xylan-binding CBM and as expected it bound to xylobiose (structure 38), xylotriose (structure 39), xylotetraose (structure 40) and xylopentaose (structure 41) (Figure 5A). CBM4-2 has also been reported to cross react with certain (1→3),(1→4)-β-D-glucans and semi-quantitative affinity electrophoresis indicated a similar level of binding of CBM4-2 to birch xylan as to barley (1→3),(1→4)-β-D-glucan (50). The array data were in agreement with this since in addition to the xylan oligosaccharides, CBM4-2 also bound to (1→3),(1→4)-β-D-glucotetraose and (1→3),(1→4)-β-D-glucopentaose (structure 65 and 66) but its much weaker binding to (1→3),(1→4)-β-D-glucotriose (structure 62) provides insight into the minimum DP required for optimal binding. X-6 showed a greater specificity for xylan oligosaccharides than CBM4-2 and bound with greatest avidity to xylopentaose (structure 41) and xylotetraose.
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(structure 40) and weakly to xylotriose (structure 39). X-6 appears to have a requirement for at least 3 xylose residues for binding since it did not bind to xylobiose (structure 38). A-12 showed a similar specificity to X-6 but also cross reacted with the linear (1→4)-β-linked glucopentose (structure 54). A-8 displayed an even more restricted binding profile to xylan oligomers than X-6 and bound strongly to xyloentaose (structure 41) but only weakly to xylotetraose (structure 40). The mutant CBMs M60-3 and MRT-5 also bound to the xyloentaose (structure 41) and MRT-5, but not M60-3, bound to the xylotetraose (structure 40). However, both CBMs had new cross-reactivities compared to wild type CBM4-2 and bound to xyloglucan oligosaccharides (structure 45) and (structure 46). The binding to MRT-5 appeared to be inhibited by the presence of galactose since this CBM bound only weakly to structure 46 but strongly to structure 45 which is identical apart from the galactose substitution of one xylose residue. Together, these data indicate that oligosaccharide microarrays are useful tools for the rapid screening of the binding profiles of CBMs. However, in contrast to most antibodies, CBMs often display moderate binding affinities and this may limit the application of these arrays for some CBM studies.

**Competitive inhibition assays** - One potential concern of assays based on immobilised targets is that the binding of antibodies, CBMs or other probes might be either inhibited or promoted by the immobilisation itself, or by the coupling of the oligosaccharides to the carrier molecule, in this case BSA. To test for this we also used the arrays in a competitive inhibition format whereby arrays were probed in the presence of soluble unconjugated oligosaccharides used at a range of concentrations (Supplemental Figure 2). A set of mAbs and CBMs were tested and in all cases the results obtained were consistent with those obtained using immobilised neoglycoproteins. For example, the binding of the anti-AGP LM14 to glucoronyl- (1→2)-α-[ (1→4)-β-D-xylotriose-BSA was inhibited by the unconjugated version of this structure but not by β-(1→4)-D-xylotetraose (structure 39).

Similarly the binding of CBM4-2 to both (1→4)-β-D-xylotriose- BSA and 3’-β-D-glucosyl- (1→4)-β-D-glucobiose-BSA was inhibited by unconjugated (1→4)-β-D-xylohexaose and the binding of mAbs LM21 and LM22 was inhibited by appropriate haptens (Supplemental Figure 2).

**Oligosaccharide microarrays as multiplexed acceptors for enzyme assays** - Microarrays of immobilised sets of substrates or acceptors are potentially useful tools for HTP enzyme screening and characterisation that enable multiplexed analysis of activities. Arrays of polysaccharides (51) and limited numbers of oligosaccharides (52) have been used to explore glycosyltransferase activities and we were interested to test the applicability of our arrays for similar purposes. As proof of concept we tested rabbit muscle phosphorylase using [C14]glucose-1-phosphate as the glycosyl donor (Figure 6). The general mechanism of the method is shown in Figure 6A. Scanning the arrays with a phosphorimager after incubation with enzyme and glycosyl donor revealed specific [C14]-labelled spots representing the transfer of [C14]glucose onto α-linked glucan oligosaccharides that were present on this particular microarray (structures 67, 69 and 71) (Figure 6B). A control array that had been treated with enzyme inactivated by boiling did not show incorporation of [C14]glucose (Figure 6C).

**DISCUSSION**

It is generally recognised that there is a widening gap between our ability to discover genes and proteins and to understand their roles in plant glycobiology. For example, it is estimated that we can safely predict the activities of no more than 20% of the proteins within the carbohydrate active enzymes (CAZy) database (11). We show here that carbohydrate microarrays can make a valuable contribution to the HTP analysis of a variety of carbohydrate-protein interactions and whilst oligosaccharide microarrays have been described for use in medical animal and microbial research, equivalent technology has not previously been developed for plant research.
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The use of defined oligosaccharides rather than polysaccharides is important for obtaining detailed information about carbohydrate-interacting proteins and a library of cell wall derived oligosaccharides is a significant resource in itself and once coupled to protein can be used to produce microarrays on diverse surfaces. The versatility of being able to print microarrays on both nitrocellulose and slides is important. Whilst slide-based microarrays can only be analysed using specialised scanning equipment, membrane-based arrays can be used by non-experts and scanned using an ordinary office scanner and are thus ideal for wider distribution to researchers. Several linkers have been developed for the covalent and non-covalent attachment of oligosaccharides to substrates. For example coupling to lipids has also been shown to be a highly effective method for oligosaccharide microarray production (19, 20, 25, 26, 53). One reason for choosing BSA as a carrier molecule was because BSA-based neoglycoproteins are a multifunctional resource that can be used not just for microarray production but also as immunogens and as components of other assays for which immobilisation is required. Nevertheless, any coupling procedure that involves modification of reducing ends is likely to interfere with the activity of reducing end acting probes or enzymes, and this may be exacerbated by the large size of the BSA molecule. We found that BSA-coupled oligosaccharides arrayed as described were effective substrates for several exo-acting glycosyl hydrolases but not for endo-acting enzymes (data not shown). Presumably the exo-acting enzymes were non-reducing-end acting and the lack of activity of the endo-acting enzymes was a result of steric hindrance from the BSA.

This study highlighted some important technical aspects of oligosaccharide microarray production including the relative merits of different microarray robot printers. The pin-based MicroGrid II printer was suited for the production of microarrays on nitrocellulose membrane with larger spot sizes. However, we found that array quality often decreased with longer print runs such that some spots were missing or not properly printed and this is likely to resulted from the inevitable wear of the pins that occurs with contact printing. This drawback is avoided with non-contact printers such as the Arrayjet Sprint that dispel samples by a highly reproducible piezo-actuation process. Another major advantage of the Arrayjet Sprint was its much greater speed which is important not just to increase throughput but because the evaporation of sample buffer with a concomitant concentration of samples, can be highly problematic during long microarray print runs. Importantly, by printing arrays on multi-pad slides (as shown in Supplemental Figure S1) such that each pad is isolated by a gasket during probing, it is possible to simultaneously assess the binding of large numbers of mAbs, CBMs et cetera against many immobilised samples. For example, using ten 16-pad slides it is possible to simultaneously screen 160 antibodies, each against 400 immobilised glycans.

The primary goal of this work was to develop plant oligosaccharide microarray technology per se but we also obtained new epitope-level information about mAb specificities. For example, mAbs LM14 and JIM14 have previously been described as binding to unknown epitopes occurring on AGPs (32, 54). The oligosaccharide microarrays demonstrated that both mAbs bind with high specificity to glucoronyl-(1→2)-α-[1→4]-β-D-xylotriose (structure 42) (Figure 4g) which is a constituent of glucuronoxylan and glucuronarabinoxylans and this finding is therefore interesting because it implies that these two mAbs may bind to an epitope not usually associated with AGPs in addition to binding to glucuronyl residues decorating arabinogalactan structures. The synthetic galactosyl structures 20, 21 and 22 are not known to occur on any plant cell wall polysaccharide and it was therefore surprising that mAb LM16 bound strongly to 6′-β-D-galactotriose- (1→4)-β-D-galactotriose (structure 2f). LM16 has been described previously as binding to an epitope occurring on sugar beet RGI that is generated by arabinofuranosidase treatment and is galactosidase labile (36). Sugar beet arabinan side chains can in some cases be attached to RGI backbones via short galactosyl motifs and it is possible that LM16 recognises this structure once exposed by arabinofuranosidase (36). It has been shown...
that such short galactan stubs can be substituted with ferulic acid at the C6 position but substitution with another sugar has not been reported (55). LM16 does not bind to galactosyl residues per se since it does not bind to linear galactan, galactomannan or galactoxyloglucans. The strong binding of LM16 to both structure 2I and native sugar beet pectin therefore raises the intriguing possibility of a novel RGI epitope.

The importance of obtaining detailed information about epitope structures was clearly illustrated by the anti-xyloglucan mAbs LM15, LM24 and LM25. Oligosaccharide array analysis revealed subtle differences in the binding profiles of these mAbs which most likely would not have discriminated using previous polysaccharide-based ELISA or microarrays. Immunolabelling of tobacco sections with these mAbs showed that despite their relatively small differences in structure, the epitopes recognised had distinct cellular locations. Whilst the biological significance of these findings is unclear at present they show that a detailed evaluation of epitope structures that oligosaccharide arrays can provide is important for the subsequent interpretation of data produced in antibody studies.

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**FOOTNOTES**

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**FIGURE LEGENDS**

**FIGURE 1.** A library of plant oligosaccharides. A. Structures 1–6, 20, 21, 22 and 72 were chemically synthesized whilst all other oligosaccharides were produced by fractionation of polysaccharides followed by separation and purification. The oligosaccharides are shown after coupling to BSA by reductive amination as shown in B. α glycosidic linkages are indicated by the small α symbols on the structures and all other linkages are β. The structures shown are the predominant structures in that sample and the number codes of the structures are consistent throughout the manuscript. Further details of the oligosaccharides are provided in Supplementary Table S1. Note that the structures illustrated in Figure 1 correspond to those listed in Supplementary Table S1 but after conjugation and opening of the reducing end sugar.

**FIGURE 2.** Microarray printing surfaces and reproducibility. Coupling oligosaccharides to protein enabled printing on a wide variety of slides and membranes commonly used for microarray production. (1→4)-β-D-mannohexaose and (1→5)-α-L-arabinopentaose printed in sextuplet and at 10 concentrations from 2 mg/mL to 30.5 ng/mL. The arrays were probed with the anti-mannan or anti-arabinan monoclonal antibodies (mAbs) LM21 and LM6 respectively. Microarrays were printed using a non-contact piezoelectric robot (A–F) or a pin-based robot (G and H). Arrays were printed on
surface-modified glass slides (A-C), nitrocellulose coated glass slides (D and E) and nitrocellulose membrane (F-H). Reproducibility of the microarrays was tested by printing 12 copies of arrays on both nitrocellulose membrane (I) and nitrocellulose coated glass FastSlides (J). Arrays were probed with the anti-mannan mAbs LM21, or the anti-arabinan mAb LM6. Representative probed replicate arrays are shown and also graphs of mean spot signals from 3 arrays plotted against each other. Axes on the graphs are relative mean spot signals.

**FIGURE 3.** Specificity screening of monoclonal antibodies (mAbs). Oligosaccharide microarrays were probed with a set of 38 monoclonal antibodies and selected examples of probed arrays are shown in (A). The mean spot signals obtained from 3 experiments are presented in heatmaps in which colour intensity is correlated to signal (B-I). Expanded heatmaps (C-I) provide more detailed information about the binding of selected mAbs. The highest signal in the entire data set was set to 100 and all other values normalised accordingly. Oligosaccharide structures are only shown where binding produced a mean spot signal of at least 15% of the highest mean signal in the entire data set. Further details about the oligosaccharides are provided in **Supplementary Table S1.**

**FIGURE 4.** Localization of xyloglucan epitopes in Tobacco stems. Immunofluorescence imaging of transverse sections of tobacco stem pith parenchyma cell walls with anti-xyloglucan monoclonal antibodies (mAb) after a pectate lyase pretreatment. (A) Calcofluor fluorescence showing all cell walls. (B) The same section as (A) with immunofluorescence labelling with mAb LM15 showing abundant binding to cell walls at the corners of intercellular spaces (*). (C) Equivalent section showing immunofluorescence labelling with mAb LM24 indicating most abundant labelling in regions of adhered cell walls between intercellular spaces. (D) Equivalent section showing immunofluorescence labelling with mAb LM25 which binds abundantly to cell walls lining intercellular spaces. Scale bar = 10 μm.

**FIGURE 5.** Specificity screening of carbohydrate binding modules and lectins. A. Oligosaccharide microarrays were probed with the xylan-binding carbohydrate binding module (CBM) CBM4-2 and also mutant variants of CBM4-2: XG-34/1-X; XG-34/2-VI; X-6; X-13; M60-3; MRT-5; A-8; A-12. The highest signal in the entire data set was set to 100 and all other values normalised accordingly. Further details about the oligosaccharides are provided in **Supplementary Table S1.**

**FIGURE 6.** Oligosaccharide microarrays as multiplexed acceptors for enzyme assays. (A) Schematic showing the experimental setup used to assay the activity of rabbit muscle phosphorylase using a [C14]glucose-1-phosphate donor and an array immobilised oligosaccharide acceptor. (B) An oligosaccharide microarray was used to assay the activity of rabbit muscle phosphorylase a with [C14]Glucose-1-phosphate as a glycosyl donor. The incorporation of [C14] was detected using a phosphoimager system. The control array in (c) was probed with boiled phosphorylase a under the same conditions. The structures of the oligosaccharides that were the most effective acceptors are shown. Further details about the oligosaccharides are provided in **Supplementary Table S1.**
Figure 1

(1→4)-β-mannopentose

(1→4)-β-mannotetraose

Sodium borohydride

BSA

D-GlcA

D-Glc

D-Man

D-Gal

D-GalA

Me-D-GalA

L-Ara

2-D-GlcNAc

Feruloyl
Figure 2

Representative replicate arrays

Nitrocellulose membrane

Probed with mAb LM6

Probed with mAb LM21

Probed with mAb LM6

Probed with mAb LM21

Mean (N=3)

r²=0.9973

r²=0.9982

r²=0.9973

r²=0.9966
Figure 3
Figure 5
Figure 6

Phosphorylase
Donor: C14-glucose-1-phosphate

Phosphorimager

Microarrayed oligosaccharide acceptors

A

B

C