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Polysaccharide microarrays for high-throughput screening of transglycosylase activities in plant extracts

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Abstract Polysaccharide transglycosylases catalyze disproportionation of polysaccharide molecules by cleaving glycosidic linkages in polysaccharide chains and transferring their cleaved portions to hydroxyl groups at the non-reducing ends of other polysaccharide or oligosaccharide molecules. In plant cell walls, transglycosylases have a potential to catalyze both cross-linking of polysaccharide molecules and grafting of newly arriving polysaccharide molecules into the cell wall structure during cell growth. Here we describe a polysaccharide microarray in form of a glycochip permitting simultaneous high-throughput monitoring of multiple transglycosylase activities in plant extracts. The glycochip, containing donor polysaccharides printed onto nitrocellulose-coated glass slides, was incubated with crude plant extracts, along with a series of fluorophore-labelled acceptor oligosaccharides. After removing unused labelled oligosaccharides by washing, fluorescence retained on the glycochip as a result of transglycosylase reaction was detected with a standard microarray scanner. The glycochip assay was used to detect transglycosylase activities in crude extracts from nasturtium (*Tropaeolum majus*) and mouse-ear cress *(Arabidopsis thaliana*). A number of previously unknown saccharide donor-acceptor pairs active in transglycosylation reactions that lead to the formation of homo- and hetero-glycosidic conjugates, were detected. Our data provide experimental support for the existence of diverse transglycosylase activities in crude plant extracts.

Keywords Glycochip · Microarray · Oligosaccharides · Plant cell wall · Transglycosylation · XET

Abbreviations

SR	sulforhodamine
HEC	hydroxyethyl cellulose
XET	xyloglucan endotransglycosylase, EC 2.4.1.207
XTH	xyloglucan endotransglycosylase/hydrolase
XG	xyloglucan
XGOs	xyloglucan-derived oligosaccharides
CEOs	cellooligosaccharides
LAOs	laminarioligosaccharides
XYLOs	(1-4)-β-D-xylan derived oligosaccharides
MLGOs	mixed-linkage (1-3;1-4)-β-D-glucan oligosaccharides
XLLG	nonasaccharide $Glc_4Xyl_3Gal_2$ derived from xyloglucan
TLC	thin-layer chromatography
GPC	gel-permeation chromatography
MALDI-TOF	matrix-assisted laser-desorption/ionization time-of-flight
HPLC	high-performance liquid chromatography

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PMSF phenylmethylsulfonyl fluoride
DP degree of polymerization

Introduction

The microarrays are powerful analytical tools enabling sensitive, high-throughput miniaturized detection of molecular interactions. Although their main applications were initially in analyses of gene expression, the microarrays are being increasingly applied to almost every area of experimental biology and medicine [reviewed in 7]. Microarrays that contain immobilized carbohydrates, also known as glycochips, have been employed as high-throughput analytical tools for studying carbohydrate-protein interactions [8, 13, 33] including carbohydrate-active enzymes, [26, 34], antibodies [23, 32, 40], lectins and microbes [3, 22, 39]. For example, microarrays containing defined polysaccharides and plant extracts have been probed with panels of monoclonal antibodies to characterize the polysaccharide composition of plant cells [40]. Also, the carbohydrate microarrays proved to be a very promising tool for rapid screening and diagnosis of microbial infections [39].

An important group of carbohydrate-active enzymes are glycosyl hydrolases belonging to the family GH16 [23] that degrade or modify plant cell wall polysaccharides [35, 38]. It has been well documented that retaining β-D-glycosyl hydrolases could in principle catalyse transglycosylation reactions, which result from the consequences of catalytic reaction mechanism itself, whereby the transglycosylation occurs usually as a reverse reaction at relatively high concentrations of substrates (millimolar). In contrast, 'true' transglycosylases catalyze glycosyl transfer from the donor molecule to a carbohydrate acceptor at significantly lower concentrations (micromolar) [*e.g.* 15]. The best characterized transglycosylases so far have been the xyloglucan endo-transglycosylases/hydrolases (abbreviated XTHs) from higher plants [12, 25, 28]. The enzyme can exhibit both hydrolase (abbreviated as XEH) and transglycosidase (abbreviated as XET) activities. In the XET mode, the enzyme cleaves glycosidic linkages in the main polyglucose chain of the xyloglucan molecule and transfers the cleaved portions containing the newly created reducing end to the hydroxyl group at the C-4 of the non-reducing end of the acceptor molecule. In these transfer reactions, xyloglucan molecules or oligosaccharides derived from xyloglucan typically serve as the glycosyl donor and the glycosyl acceptor, respectively [12, 25]. Some XTH/XET isoenzymes when assayed *in vitro*, have been shown to exhibit a certain substrate promiscuity by catalyzing transglycosylation reactions between structurally distinct donor and acceptor substrates (the hetero-transglycosylation) [2, 16, 17]. Several studies published to date document the existence of covalent linkages between diverse types of polysaccharides in the plant cell walls [9, 10, 27], but the mechanisms by which these linkages are formed are not known. It has been suggested long ago that they may originate from the action of transglycosylases present in the cell wall [18]. If hetero-transglycosylations are shown to occur in plant cell walls, our understanding of cell wall structure, function, formation, and re-modelling in higher plants will substantially be changed.

Since transglycosylation does not result in the net formation of reducing ends, conventional reductometric methods cannot be used to assay the activity of transglycosylases. Existing assay methods can be subdivided into viscometric [21], radiometric [4, 12, 35], fluorimetric [11] and colorimetric [37]. Separation of reaction products can be achieved by size-exclusion HPLC [24, 25], paper chromatography [30] or high-performance capillary electrophoresis [29]. Unfortunately, none of these approaches is conducive to simultaneous, monitoring of multiple transglycosylase reactions. The importance of transglycosylases to plant cell wall biogenesis and modification and the necessity for their in-depth characterization prompted us to develop a high-throughput approach. Here we describe a method designed for producing polysaccharide microarrays (or glycochips) that contain a series of polysaccharide substrate donors attached to a nitrocellulose support. The glycochips are then probed with crude plant extracts for transglycosylating activities using a series of fluorescently labelled oligosaccharide acceptor molecules.

Materials and methods

Polysaccharides

The polysaccharides used to manufacture microarrays were purchased from commercial sources, obtained from other researchers or they were prepared in-house. Tamarind seed xyloglucan, M_r $>10^6$ was obtained from Dr. Mayumi Shirakawa, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan. The polysaccharide was dialyzed against distilled water and lyophilized before use. Hydroxyethylcellulose, medium viscosity, was purchased from Fluka. Beech wood β-D-glucuronoxylan was prepared by Dr. Peter Biely, Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia. Citrus potassium pectate (45% esterified) was purified from the commercial preparation (Genu Pectin, Copenhagen, Denmark) by washing with acidified 60% ethanol (5 ml conc. HCl in 100 ml of 60% ethanol). Locust bean gum galactomannan and laminarin were purchased from Sigma. Mixed-linkage (1,3;1,4)-β-D-glucan from barley (~97%), arabinoxylan from oat (~94%),

arabinoxylan from wheat (~95%), konjac glucomannan, and Islandic moss lichenan were purchased from Megazyme. Barley arabinoxylan (>96%) was prepared in-house by M. H. Potato arabinogalactan, arabinans from sugar beet, and from oat, as well as rhamnogalacturonan I from okra, were kind gifts from Dr. Henk A. Schols (University of Wageningen, The Netherlands). The oligo- and polysaccharides used in this study are listed in Table 1.

Oligosaccharides

A library of oligosaccharides was prepared by partial acid or enzymatic hydrolysis from their polymeric precursors. Typically, the acid digestion of 100 mg polysaccharide was carried out in 10 ml of 2 M trifluoroacetic acid (Sigma) at 100°C for 20–90 min. Reaction progress was monitored by thin-layer chromatography. The hydrolysates were evaporated to dryness, dissolved in a small amount of water, separated by size-exclusion chromatography (SEC) on the Biogel P6 (BioRad) column (2.4 × 105 cm) eluted with milliQ water. Fractions with the desired oligosaccharide size ranges were pooled and lyophilized. Oligosaccharides from arabinogalactan of degree of polymerisation (DP) 2-3 and laminarioligosaccharides of DP 2–9 were prepared as described above. Cellooligosaccharides of DP 2–7 were prepared by acetolysis of cellulose [41] and separated by SEC on the Biogel P6 column. Barley (1,3;1,4)-β-D-glucan of medium viscosity was hydrolyzed with a *Bacillus* (1,3;1,4)-β-D-glucanase (Megazyme) that hydrolyzes (1,4)-β-linkages adjacent to a (1,3)-linked β-D-glucosyl residue, yielding a mixture of oligosaccharides G4G3G, G4G4G3G and G4G4G4G3G, where G represents a β-D-glucosyl residue, and 3 and 4 are (1,3)- and (1,4)-β-glycosidic linkages, respectively, and the reducing end is positioned to the right. Barley arabinoxylan was digested with a *Trichoderma* (1,4)-β-D-xylan endohydrolase (Megazyme). Locust bean gum galactomannan and konjac glucomannan were hydrolyzed with a *Bacillus* (1,4)-β-D-mannan endohydrolase (Megazyme). Xyloglucan oligosaccharides of DP 7–9 were prepared by a partial digestion of tamarind seed xyloglucan with *Trichoderma* cellulase as described [37]. Beechwood (4-O-methyl-β-D-glucurono)-xylan (glucuronoxylan) was hydrolyzed with a partially purified *Trichoderma* cellulase (Cellulysin, Calbiochem). The hydrolysis was monitored by TLC on the Silicagel 60 plates (Merck), developed twice in *1*-butanol-ethanol-water (5:3:2, by vol). The sugars were detected on plates by spraying with 1% (w/v) orcinol in a 10 % (v/v) sulfuric acid/ethanol solution and heated at 100ºC for 5 min. The hydrolysate was fractionated on the BioGel P6 column as described above. Fractions corresponding to xylooligosaccharides of DP 2–4 were pooled and lyophilized. Oligogalacturonides of DP 2–10 were prepared from citrus potassium pectate by enzymatic hydrolysis with endopolygalacturonase Rohament P (Rohm GmbH, Germany) [14]. They were separated on a Sephadex G-25 (Fine) column (1.5 × 105 cm) eluted with water, pooled and lyophilized. Molecular masses of oligosaccharides and other fractions were confirmed by MALDI-TOF analyses performed on a Kratos Kompact MALDI 3, using 2,5-hydroxybenzoic acid as a matrix.

Fluorescent labelling of oligosaccharides

Oligosaccharides were labelled with sulforhodamine using the previously described procedure [19]. When necessary, the labelled oligosaccharides were purified by chromatog-

Table 1 Oligo- and polysaccharides used as glycosyl acceptor and donor substrates, respectively, for detecting transglycosylation activities

Donor substrates (source)	Acceptor substrates (SR- labelled oligosaccharides derived from)
Mixed-linkage (1-3;1-4)-β-D-Glucan (Barley)	
Galactomannan (Locust Bean Gum)	
Xyloglucan (Tamarind)	Xyloglucan, (DP 7–9)
Arabinoxylan (Wheat)	Cellulose, (DP 2–7)
Hydroxyethylcellulose (HEC)	Laminarin, (DP 2–8)
Laminarin (*Laminaria digitata*)	Pectinate, (DP 2–10)
Potassium pectinate (Citrus)	Glucuronoxylan, (DP 2–4)
Glucuronoxylan (Beechwood)	Mixed-linkage (1-3;1-4)–β-D-glucan, (DP 3–5)
Arabinogalactan type 1 (Potato)	Glucomannan, (DP 2–8)
Arabinogalactan type 2 (Stractan)	Galactomannan, (DP 3–6)
Linear arabinan (Sugar Beet)	Arabinoxylan, (DP 3–9)
Branched arabinan (Sugar Beet)	Arabinogalactan, (DP 2–3)
Arabinoxylan (Oat)	
Rhamnogalacturonan I (Okra, *Abelmoschus esculentus*)	

raphy on Silicagel column (1.5 × 40 cm) eluted with *n*-propanol-methanol-water (2:1:1, by vol.).

Plant extracts

Extracts from 12-day old germinated seeds [2] of *Tropaeolum majus* and *Arabidopsis thaliana* were prepared by homogenization in 0.1 M imidazole-HCl buffer, pH 7.0 containing 1 M NaCl, 2 mM EDTA, 1 mM β-mercaptoethanol and 1 mM PMSF (buffer : biomass ratio= 1:1). All operations were carried out at 0–4°C. An equal volume of buffer was then added and the whole mixture was stirred overnight in the cold. To remove the cell debris, the mixture was filtered though Miracloth and centrifuged at 20,000 × g for 20 min in cold. The extracts were precipitated with ammonium sulfate to 90 % saturation in the cold, overnight. Afterwards, the mixtures were centrifuged at 20,000 × g for 20 min, the sediments were dissolved in small amounts of the homogenization buffer and dialyzed against several changes of 20 mM citrate phosphate buffer, pH 5.5 containing 1 mM β-mercaptoethanol and lyophilized. The dried "crude extracts" were stored at -20°C until further use. XET activity in the extracts was determined using a standard radiometric assay [12].

Microarray printing

Polysaccharides were deposited on FAST Slides® (Whatman, cat. no. 10485323) with a MicroGridII 610 (Digilab Genomic Solutions) robotic spotter and MicroSpot solid pins (Digilab Genomic Solutions) at 50 % relative humidity and 25°C. Pin loading and spot deposition calibration settings were optimized according to the manufacturer's standard operating procedure. To prevent cross-contamination, the pins were cleaned with 1 % (v/v) domestic bleach and then rinsed with milliQ water. Fourteen polysaccharides were printed in two different concentrations and in duplicate (Fig. 1). All nitrocellulose pads on the FAST Slides® contained the same assortment of polysaccharides, arranged in 7 rows and 8 columns of spots with a pitch of 0.5 mm. After printing, the FAST Slides® were heated in an oven with circulating air at 50°C for 15 min. Areas not occupied by polysaccharide samples on the nitrocellulose pads were blocked with 5 % (w/v) aqueous skimmed milk; the nitrocellulose pads were incubated under shaking at 45 rpm for 1 h. Non-adsorbed milk protein on the nitrocellulose pads was removed by washing with milliQ water under shaking at 45 rpm for 5 min. FAST Slides® were dried by centrifugation at 450×*g* for 2 min. Residual milliQ water droplets were removed with a Kenair air duster (Kenro).

Assay conditions

The printed and pre-treated polysaccharide microarrays were capped with a sixteen-well incubation chamber (Whatman, cat. no. 10486046) and inserted into a slide holder (Whatman, cat. no. 10486081). Eighty microliters of

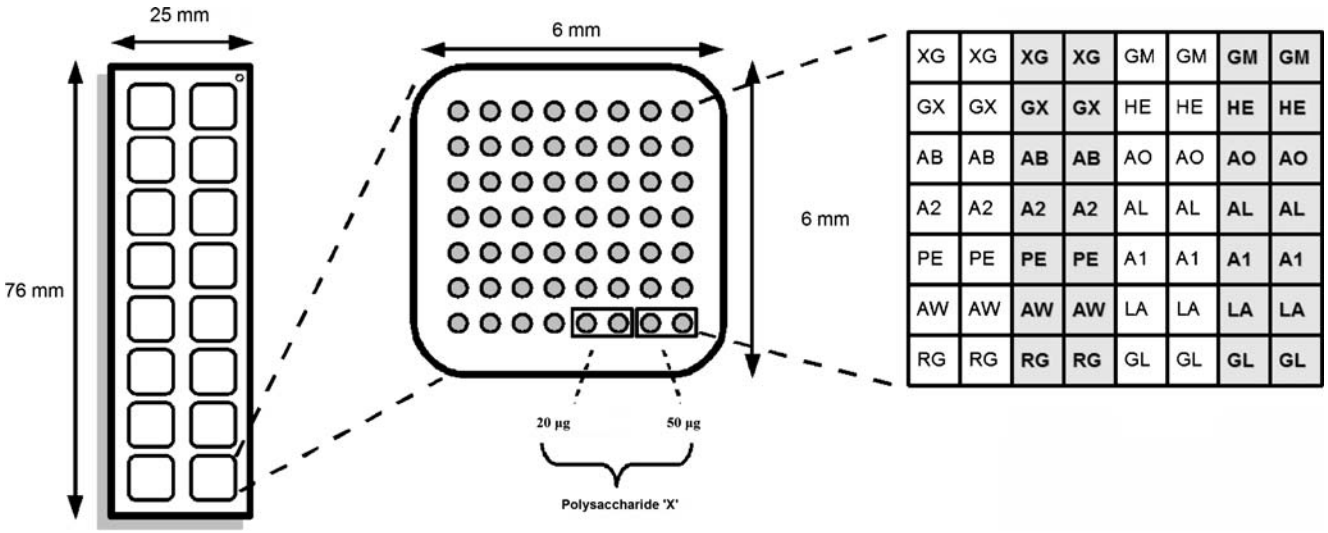

Fig. 1 Layout of the glycochip for high-throughput screening of transglycosylation activities. The FAST Slides® contained 2 × 8 nitrocellulose-coated pads, to which polysaccharides were printed at 20 and 50 µg quantities in juxtaposed duplicate spots. For Rhamnogalacturonan I, (RG), the corresponding amounts were 2 and 5 µg respectively. Abbreviations: *XG* Xyloglucan (Tamarind), *GM* Galactomannan (Locust Bean Gum), *GX* Glucuronoxylan (Beechwood), *HE* Hydroxyethylcellulose, *AB* Branched arabinan (Sugar Beet), *AO* Arabinoxylan (Oat), *A2* Arabinogalactan 2 (Stractan), *AL* Linear arabinan (Sugar Beet), *PE* Potassium pectinate (Citrus), *A1* Arabinogalactan 1 (Potato), *AW* Arabinoxylan (Wheat), *LA* Laminarin (*Laminaria digitata*), *RG* Rhamnogalacturonan (Okra, *Abelmoschus esculentus*), *GL* (1,3;1,4)-β-D-Glucan (Barley)

the reaction mixture consisting of 50 mM succinate buffer, pH 5.5, crude extract from *T. majus* (37 μg protein) or *A. thaliana* (40 μg protein) and 5 μM concentrations of the respective fluorescently labelled acceptor oligosaccharide were added to each well. The microarray assembly was covered with a microscope glass slide to prevent evaporation, placed in a plastic box and incubated at room temperature for 4–12 h. For all assays, the right-hand column in the nitrocellulose pads served as the negative control with enzyme inactivated by boiling for 5 min. The reaction was terminated and the unreacted labelled oligosaccharides were removed by washing with 60 % (v/v) ethanol with gentle shaking for 5 h, followed by washing with 60 % (v/v) ethanol containing 5 % (v/v) formic acid for 16 h. The washed microarrays were dried and scanned in the Cy3 channel using a GenePix 4000B scanner (Axon Instruments, UK) at a resolution of 5 μm per pixel.

Inhibitory studies

To monitor the interference of different non-labelled oligosaccharides in the reaction of XG with XLLG-SR, a fluorescent modification of the radiometric assay was employed. Briefly, 11 μL assay mixture contained 0.15% tamarind seed xyloglucan in 50 mM succinate buffer pH 5.5, 12 μM XLLG-SR, 0.14 μg of protein from crude extract and a range of concentrations of competing non-labelled oligosaccharides. The reactions were allowed to proceed at 25°C for different time intervals and were stopped by addition of 10 μL of 40% (v/v) formic acid. Four microliters from the stopped assay were spotted in quadruplicates onto a template made of Whatman 3MM chromatographic paper to fit exactly the size of 96-well ELISA microtitration plate and dried at 50°C for 5 min. The paper was then washed 3-times for 30 min in 66% (v/v) ethanol and subsequently in running tap water for 1 h and dried. The build-in fluorescence retained on the paper was quantified with a micro-plate reader equipped with fluorescent detector and appropriate filters with λ_{exc}=530±25 nm, λ_{em}=575±15 nm (Synergy HT-I, Biotek). The absolute amounts of products of the individual reactions were estimated from the calibration curve made with lissamine sulforhodamine chloride. The oligosaccharide concentrations causing 50% inhibition IC_{50} were estimated using the Origin 6.0 (Origin Lab Corporation) program.

Results and discussion

Transglycosylases have been predicted to catalyze the formation of covalent glycosidic links between individual polysaccharide molecules in plant cell walls during growth and in final phases of their formation [18]. Since these reactions proceed outside the cytoplasm, the free energy needed for the formation of new bonds must come from the cleavage of the preexisting glycosidic linkages. The best-characterized transglycosylase to date is xyloglucan endotransglycosylase (XET, EC 2.4.1.207), a member of xyloglucan endotransglycosylases/hydrolases (XTHs) subfamily of enzymes, which can act both as xyloglucan hydrolases (XEH activity, the glycosyl acceptor is water) and transglycosylases (XET activity, the glycosyl acceptor is xyloglucan) [12, 28]. Another known transglycosylating enzyme is the mannan endotransglycosylase/hydrolase (MTH, EC 3.2.1.78) acting on (1-4)-β-D-mannan-based plant polysaccharides from primary cell walls of kiwifruit and tomato [30, 31] and belonging to GH5 family of hydrolases (http://www.cazy.org/).

The principle of the fluorimetric activity assay for XET can be written schematically as follows:

$$\begin{aligned} &XG - XG' + XGO^* \rightarrow XG - XGO^* + XG' \\ &(\text{donor}) + (\text{acceptor})^* \rightarrow (\text{product1})^* + (\text{product2}), \end{aligned}$$

where, XG-XG' is xyloglucan polymer, XGO* is xyloglucan-based fluorescently labelled oligosaccharide at its reducing terminus and the label is marked with an asterisk. The product of the reaction carrying the fluorescent tag, XG-XGO*, is separated and its fluorescence built-in by the transglycosylation reaction is determined [11, 24, 25]. In case of the so-called hetero-transglycosylation reactions, the donor and the acceptor molecules have distinct chemical structure [2].

Construction of a glycochip for the transglycosylation assay requires an efficient immobilization of polysaccharide donors on solid supports, where the immobilized polymers should withstand the conditions of aqueous environment. Both non-covalent adsorption and covalent immobilization of polysaccharides have been described [6, 11, 20, 33, 34, 40]. In theory, transglycosylases could cleave polysaccharide molecules immobilized on a solid support randomly at several linkage positions. As shown in Fig. 2, only enzymatic attacks at sites B and C produce detectable fluorescent products that remain attached to the solid support, whereas the labelled products formed at site A are lost due to diffusion. Under these conditions, transglycosylation reactions will always produce a detectable signal because the glycochips contain excess of polysaccharides randomly attached on the solid support, thereby permitting experimentalists to perform fluorimetric screens for transglycosylation reactions using glycochips containing a panel of polysaccharide glycosyl donors.

In this work, we opted to use as the support commercially available glass slides coated with a nitrocellulose polymer (FAST Slides®, Whatman). Nitrocellulose surfaces have favourable adsorption capacity for polysaccharides,

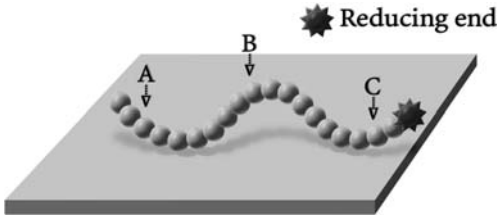

Fig. 2 Scheme of a polysaccharide molecule adsorbed in a random fashion on the surface of a glycochip. The fluorescent products are formed as a result of the transglycosylase action attacking substrates at sites B and C, where the fluorescent products remain attached to the nitrocellulose support, whilst those formed at a site A are lost through diffusion and therefore undetectable

which eliminates the necessity for chemical derivatization prior to fixation [40]. The FAST Slides® nitrocellulose coating is also compartmentalized into discrete pads (Fig. 1), permitting simultaneous assays with reaction mixtures containing a range of different substrates or under different conditions.

The prepared glycochips together with the expanded library of fluorescently labelled oligosaccharides were used

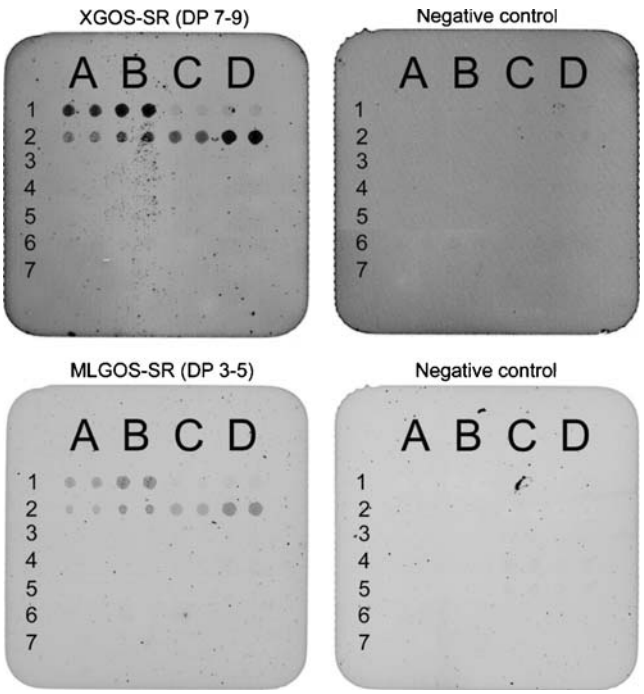

Fig. 3 Examples of the detection of transglycosylation activities in crude extract from nasturtium. FAST Slides® containing printed polysaccharide microarrays were incubated with crude extracts from nasturtium seedlings and SR-labelled XGOs-SR (top panels) and oligosaccharides derived from mixed-linkage (1-3;1-4)-β-D-glucan (MLGOs-SR) (bottom panels), respectively. Dark spots are indicative of the formation of fluorescent transglycosylation products that remained attached to the pads. The pads on the right represent negative controls incubated with boiled crude extracts. The polysaccharide layouts are depicted in Fig. 1

Table 2 Donor-acceptor combinations exhibiting transglycosylation activities with crude extracts from nasturtium (*Tropaeolum majus*) seedlings. New donor-acceptor combinations identified in this work are marked with asterisks

Donor polysaccharide (Position on glycochip)	Acceptors: SR-oligosaccharides derived from
Xyloglucan (A1, B1)	Xyloglucan
	Cellulose
	Laminarin
	Glucomannan*
	Mixed-linkage (1-3;1-4)-β-D-glucan*
	Galactomannan*
Galactomannan (C1, D1)	Xyloglucan*
	Cellulose*
	Mixed-linkage (1-3;1-4)-β-glucan*
Glucuronoxylan (A2, B2)	Xyloglucan*
	Cellulose*
	Mixed-linkage (1-3;1-4)-β-glucan*
	Galactomannan*
Hydroxyethyl cellulose (C2, D2)	Xyloglucan
	Cellulose*
	Mixed-linkage (1,3;1,4)-β-D-glucan*

to examine 140 potential donor-acceptor combinations applied in picomolar-ranges. Eighteen donor-acceptor pairs gave positive signals, indicating that the transglycosylation reactions took place (an example is shown in Fig. 3). The donor-acceptor combinations producing positive reactions with crude extracts from nasturtium and *Arabidopsis* seedlings are summarized in Tables 2 and 3. Most of the found reactions had not previously been detected in crude extracts from nasturtium [2]. With *Arabidopsis thaliana* extract, we only observed two positive reactions occurring between the XGO-SR as acceptors and tamarind XG and beechwood xylan as the respective donors (Table 3).

The identification of a relatively large number of homo- and hetero-transglycosylation reactions *in vitro* raises a question, whether the observed cross-linking reactions between different types of polysaccharides could exist in cell wall environment? It can be expected that identification of linkages between different types of polysaccharides in

Table 3 Donor-acceptor combinations exhibiting transglycosylation activities with crude extracts from *Arabidopsis thaliana* seedlings

Donor polysaccharide (Position on glycochip)	Acceptors: SR-labelled oligosaccharides derived from
Xyloglucan (Tamarind) (A1, B1)	Xyloglucan
Glucuronoxylan (Beechwood) (A2, B2)	Xyloglucan

plant cell walls represents due to their relative scarcity a rather difficult task. Recent studies do provide experimental support to the existence of interpolymeric links in plant cell walls and thus of hetero-transglycosylation reactions. As examples could serve the presence of pectic-xylan-xyloglucan covalent complexes in cauliflower [9]; covalent linkage between xyloglucan and acidic polysaccharides in angiosperms [27]; and, linkages between (1-4)-β-D-galactan and pectin-xyloglucan complexes [1]. Although some of these linkages could be formed by glycosyl transferases from activated sugars in the Golgi apparatus prior to secretion of polysaccharides into the cell walls [1], some could equally well be formed *in muro*, by transglycosylases localized in the cell wall. Recently, an apparently authentic hetero-transglycosylase activity, the (1-3;1-4)-β-D-glucan: xyloglucan endotransglycosylase (MXE), grafting predominantly mixed-linkage glucan (MLG) to xyloglucan oligosaccharides, was detected in horsetail (*Equisetum*) species. However, the amino acid sequence of this enzyme has not been reported [10]. It is believed that transglycosylases acting on β-D-xylans or other cell wall polysaccharides could also occur in plants [5, 36].

The high number of the positive reaction pairs and the high sensitivity of the assay (Tables 2 and 3) call for an extreme caution when interpreting the results. A part of the false positives can be explained by substrate non-specificity of the present XETs, the other part could be due to insufficient purity of acceptors and/or donors used in the assays. The false positives can be ruled out by performing separate additional assays. In an attempt to distinguish between non-specific transfer catalyzed by XET and the reactions catalyzed by 'authentic' hetero-transglycosylases, we performed inhibition assays with non-labelled oligosaccharides, both on the microchip and *in solutio*. The rationale behind these experiments was that the oligosaccharides acting as the natural acceptors would exert the strongest inhibitory effect. As inhibitors, we selected oligosaccharides whose labelled counterparts proved as acceptors in the reaction with xyloglucan (Table 2). Thus, in the reaction of the substrate pair XG:XGOs-SR performed on the glycochip, the strongest inhibition was found with nonlabelled XGOs while the oligosaccharides derived from cellulose (CEOs), (1-4)-β-D-xylan (XYLOs) and/or laminarin (LAOs) exhibited lesser effect (Supplemental Fig. S1). This observation was confirmed by the finding that the half-maximal inhibitory concentration IC_{50} in the same reaction was the lowest with XG-derived nonasaccharide (Table 4) thereby leading to a conclusion that XG and XG- derived oligosaccharides were the natural substrates and that the heterotransglycosylation can be ascribed to substrate non-specificity of XET(s) rather than to action of specific hetero-transglycosylases.

Table 4 Half-maximal inhibitory concentrations IC_{50} of the selected oligosaccharides in the transglycosylation reaction XG: XLLG-SR catalyzed by crude extract from nasturtium. The reaction conditions were as described in Materials and methods section

Inhibitor oligosaccharide (linkage type)	IC_{50} (μM)
Xyloglucan nonasaccharide (XLLG)	58.7
Cellotetraose, (β1-4)	2,516
Xylotetraose, (β1-4)	3,050
Laminaritetraose, (β1-3)	8,315

On the other hand, the false negative results may also occur and could be caused by the presence of polysaccharide hydrolases in plant crude extracts. The hydrolases could decompose substrates and products of the transglycosylation reactions, thereby increasing the likelihood of the false negative results. Decomposition by hydrolases could be minimized by using different dilutions of crude extracts, by varying reaction conditions or shortening the incubation times.

The effect of pH should also be taken into consideration. As the most characterized transglycosylases have their pH optima between 4 and 8 [10, 12, 16, 17, 25, 29, 30], we used pH 5.5 as a compromise in our incubations. In more detailed screens, the transglycosylation reactions could be performed at various pH ranges, to avoid overlooking unknown transglycosylase activities. As an example, we have shown that during incubation of the glycochip with XGO-SR, the signals for reactions with xyloglucan and hydroxyethyl cellulose as the donors were strongest at pH 5.5 while the other reactions were much less influenced by pH change (Supplemental Fig. S2).

The described examples show that polysaccharide microarrays in combination with a range of fluorescently labelled oligosaccharides can offer a new approach to screen for transglycosylase reactions *per se*. This approach permits high-throughput screenings that only require small amounts of substrates and proteins and offers substantial savings in time and cost, because once the polysaccharide microarrays had been printed, they can be stored and used at any time. The method presented here could be useful *e.g.* for monitoring the expression patterns of transglycosylases during plant growth and development under normal conditions and under conditions of biotic and abiotic stress, for detection of transglycosylase enzymes in various plant tissues and organs, for mapping differences between plant varieties and for the high-throughput screening of inhibitors of transglycosylase reactions. Moreover, the results presented in this paper confirm the existence of the so-called hetero-transglycosylations taking place *in vitro* between structurally different polysaccharides [1]. In order to verify their existence in plant cell walls, more detailed studies will

be needed. It will be of equal importance to discern between the possibility that the hetero-transglycosylations are due to substrate promiscuity of some XET isoenzymes or are the result of action of as yet undiscovered 'authentic' hetero-transglycosylases.

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