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Analyzing Xyloglucan Endotransglycosylases by Incorporation of Synthetic Oligosaccharides into Plant Cell Walls

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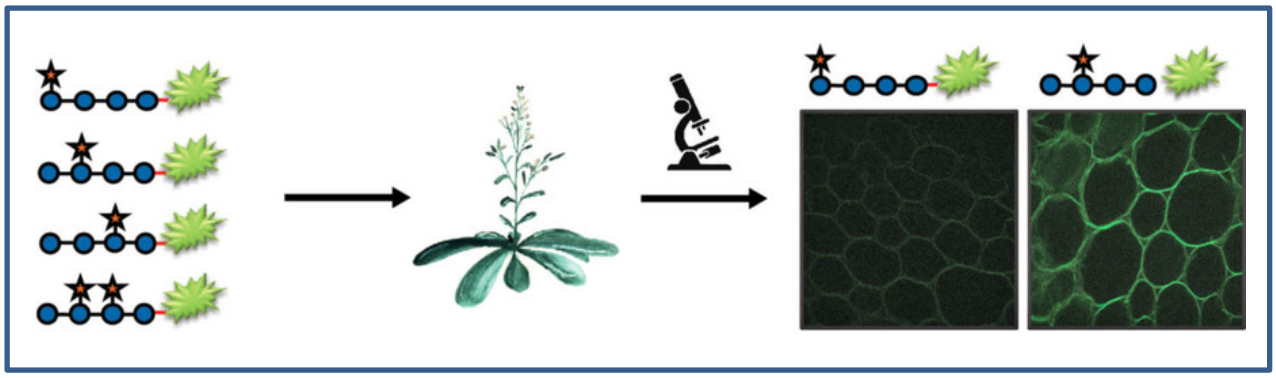

A different brick in the wall: Synthetic oligosaccharides obtained by automated glycan assembly were enzymatically incorporated into polysaccharides printed as microarrays as well as intact plant cell walls by the action of native xyloglucan endotransglycosylases. The differential incorporation of the oligosaccharides provided detailed information on the substrate specificities of these important plant cell wall remodeling enzymes.

Analyzing Xyloglucan Endotransglycosylases by Incorporation of Synthetic Oligosaccharides into Plant Cell Walls

Colin Ruprecht,[a] Pietro Dallabernardina,[a] Peter J. Smith,[b] Breeanna R. Urbanowicz,[b] and Fabian Pfrengle*[a]

Abstract: The plant cell wall is a cellular exoskeleton consisting predominantly of a complex polysaccharide network that defines the shape of cells. During growth, this network can be loosened through the action of Xyloglucan Endo-Transglycosylases (XETs), glycoside hydrolases that 'cut and paste' xyloglucan polysaccharides through a transglycosylation process. We have analyzed cohorts of XETs in different plant species to evaluate xyloglucan acceptor substrate specificities using a set of synthetic oligosaccharides obtained by automated glycan assembly. The ability of XETs to incorporate the oligosaccharides into polysaccharides printed as microarrays and into stem sections of *Arabidopsis thaliana*, beans, and peas was assessed. We found that single xylose substitutions are sufficient for transfer, and xylosylation of the terminal glucose residue is not required by XETs, independently of plant species. To obtain some information on the potential xylosylation pattern of the natural acceptor of XETs, i.e. the non-reducing end of xyloglucan, we further tested the activity of xyloglucan xylosyl transferase (XXT) 2 on the synthetic xyloglucan oligosaccharides. This data sheds light on inconsistencies between previous studies towards determining the acceptor substrate specificities of XETs and have important implications for further understanding plant cell wall polysaccharide synthesis and remodeling.

The plant cell wall represents an important renewable resource and a sustainable feedstock for numerous industrial applications. The plant cell wall is both rigid and flexible, and these vital biomechanical properties confer structural stability while enabling growth and expansion. The load-bearing structures in the plant cell wall are cellulose microfibrils that are embedded into a matrix of hemicelluloses and pectins.[1] One of the most important and abundant hemicellulosic polysaccharides in primary walls is xyloglucan, which has been proposed to be involved in coating and tethering the cellulose microfibrils and may be participating in the formation of 'biomechanical hotspots', and thus plays an important structural role in the wall.[1-2] Xyloglucan, which has been identified in all land plants studied to date, consists of a β-1,4-linked glucan backbone that is highly substituted with α-1,6-linked xylopyranose residues. These xylose residues are often successively elongated with β-1,2-linked galactose, and α-1,2-linked fucose (see Figure 1).[3] Further substituents include arabinofuranoses, arabino-pyranoses, galacturonic acids, and acetyl groups.[3-4] The degree of substitution varies between cell types and plant species, resulting in a diversity of xyloglucan polysaccharides within a single plant and even more so across organisms. Xyloglucans are remodeled in the cell wall by the action of Xyloglucan Endo-Transglycosylases (XETs).[1, 5] XETs cleave existing xyloglucan chains and then transfer the enzyme-bound xyloglucan fragment to the non-reducing end of another xyloglucan polysaccharide, thereby loosening and /or rearranging the proposed xyloglucan-cellulose network (Figure 1). The importance of XETs for plant growth has been illustrated by various experiments connecting XET activity with cell elongation.[6] Recent reports have not only shown that transglycosylation occurs between two xyloglucan polymers but also between different types of plant cell wall polysaccharides.[7] For example, a specific horsetail (*Equisetum*) enzyme from the XET gene family is able to use cellulose, mixed-linkage glucan, and xyloglucan as donors, transferring each of them onto xyloglucan oligosaccharide acceptors.[7b, 7e]

The activities of XETs against different donor and acceptor substrates have previously been analyzed using xyloglucan oligosaccharides.[5a, 8] Experiments using pea and bean extracts suggested that xylosylation of the terminal non-reducing glucose of the oligosaccharide acceptor is essential for XET activity,[5a, 8a] whereas other studies, using recombinantly expressed enzyme from *Populus* or purified enzyme from nasturtium, suggested that xylosylation at this position is not required.[8b, 8c] It remained elusive if the conflicting results originated in the use of XETs from different plant species. Unequivocal evidence requires the use of xyloglucan oligosaccharides with single xylose substitutions at different positions on the glucan backbone. However, the traditional method of isolating xyloglucan oligosaccharides from natural sources, by sequential enzyme digestion and purification steps, does not yield the structurally defined substrates necessary to probe this specificity.

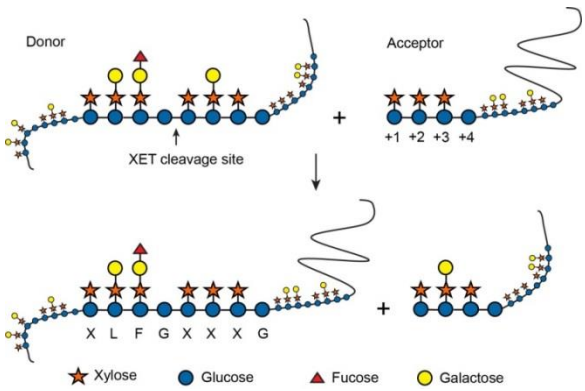

Figure 1. Schematic representation of XET transglycosylation activity. The enzyme cuts at the indicated site and forms a glycosyl-enzyme intermediate. The acceptor molecule then attacks the intermediate leading to formation of the polysaccharide product of the transglycosylation reaction. The one-letter nomenclature used to describe xyloglucan structure is given below the product.[9]

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On the other hand, chemical synthesis provides access to homogenous oligosaccharides of principally any composition, which are powerful tools that can be utilized to probe the large complement of carbohydrate active enzymes required for assembly and remodeling of the plant cell wall. Synthetic oligosaccharides have already proven very useful for identifying the epitopes of monoclonal antibodies directed at different classes of cell wall polysaccharides.[10] In the aforementioned example, oligosaccharides were produced by automated glycan assembly, in which protected monosaccharide building blocks are sequentially added to a solid-phase resin, with deprotection steps in between the individual glycosylation reactions, facilitating fine-tuned control of the final structures. After assembly, the oligosaccharides can be cleaved from the resin and globally deprotected, providing milligram quantities for biological and biochemical experiments. Herein, we report the use of synthetic xyloglucan oligosaccharides (XGOs) obtained by automated glycan assembly for investigating the acceptor substrate specificities of XETs present in both plant extracts and *in muro* from several diverse species.

Several methods have been developed to measure transglycosylation activity in plants that rely on incorporation of labeled oligosaccharide acceptors into oligosaccharide donor molecules[8b, 8c] or, more commonly, into polysaccharide donors.[7b, 7c, 11] In the latter case, polysaccharides are usually immobilized by adsorption onto solid surfaces, including filter paper, silica-gel, glass fiber,[7a, 11] or nitrocellulose pads.[7c] After the transglycosylation reaction, the labeled oligosaccharide acceptors are washed away and the remaining signal is quantified. To systematically map the acceptor specificity of XETs, we chemically synthesized a set of xyloglucan oligosaccharides (XGOs) by automated glycan assembly according to previously published procedures.[12] This set contained oligosaccharides comprised of β-1,4-linked glucotetraosyl backbones appended with single α-1,6-linked xylose substitutions in the +1 position (counting from the non-reducing end of the acceptor, see Figure 1, XGGG), +2 position (GXGG), +3 position (GGXG)[12b], and with two xylose substitutions in both the +2 and +3 positions (GXXG)[12a]. The oligosaccharides are denoted according to the common nomenclature for xyloglucans[9]. Since the XGOs were obtained with an aminopentyl linker at the reducing end, the amino group could be directly exploited for attachment of fluorescein (FC) using *N*-hydroxyl succinimide (NHS) chemistry.

Initially, we tested XET-mediated incorporation of the synthetic XGOs into xyloglucan polysaccharides immobilized on filter paper. However, we found that the low-substituted acceptors adsorbed so tightly onto the cellulose surface that our attempts to wash away any non-covalently bound compounds failed. To enable effective and simple removal of unreacted acceptor oligosaccharides, we therefore decided to covalently link polysaccharides to *N*-hydroxysuccinimide (NHS)-activated glass slides.[13] Different plant polysaccharides, including xyloglucan, hydroxyethylcellulose (HEC), mixed-linkage glucan (MLG), arabinoxylan (AX), and glucuronoxylan (GX) were printed in four different concentrations using a microarray printer (Figure 2A). Next, extracts from *Arabidopsis thaliana*, pea, bean, and nasturtium were used to analyze the cohorts of different XETs within these extracts for activity against our XGO probe library. After overnight incubation on the polysaccharide array, we found incorporation of several of the XGOs into xyloglucan. Further, the amount of oligosaccharide incorporation was correlated with the concentrations printed onto the slide, and was absent when plant extracts were boiled to inactivate any proteins prior to the reactions (Figure 2B), indicating that we had measured enzymatic activity rather than passive adsorption. Extracts from all four species evaluated were able to incorporate Fluorescein (FC)-labeled oligosaccharides GXGG-FC, GGXG-FC and GXXG-FC, but were not able to use XGGG-FC as a substrate. Our results indicate that single xylose substitutions at the glucan oligosaccharide backbone either in the +2 or in the +3 position facilitate productive contacts with XET enzymes present in plant extracts from these four species. Conversely, the observed lack of XGGG-FC incorporation into xyloglucan clearly indicates that xylosylation at the terminal glucose is not sufficient for XET activity and is likely not required for transfer of a XGO. It is important to note that xylose substitution in the +1 position does not inhibit XET function, as previous work has established that XXXG can function as an acceptor for XETs.[6b, 8c, 8d] Further, we found that GXGG tended to be a better substrate than GGXG for XETs of all four species. This observation is consistent with previous studies using a heterologously expressed XET from *Populus*, in which only slightly increased XET activity on XXXG compared to XXG substrates was observed. This observation led to the conclusion that the +3 position has only a minor effect on the enzymatic reaction.[5f, 8c] Structural data on a poplar and a nasturtium XET previously suggested that xylosyl substitutions in the +1 and +2 positions form interactions with the enzyme, whereas the relevance of the substitution in the +3 position remained elusive.[14] Here, we show with the incorporation of GGXG-FC into immobilized xyloglucan that single xylose substitutions in the +3 position of XGOs are sufficient for enzymatic transfer by XETs.

Contrary to a previous study that used microarrays with polysaccharides immobilized on nitrocellulose to analyze transglycosylation activity in nasturtium and *Arabidopsis thaliana*,[7c] we found no incorporation of fluorescently labeled XGO probes into hydroxyethylcellulose (HEC) or xylan by extracted XETs from any species evaluated in this report (Figure 2B).

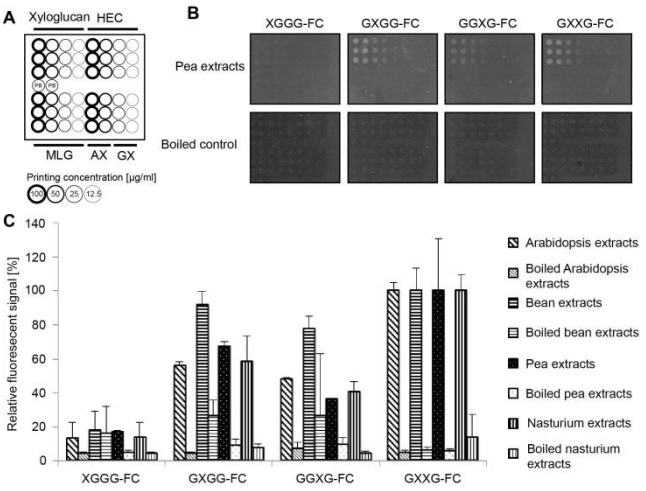

Figure 2. A glycan microarray platform enables characterization of xyloglucan endotransglycosylase activity.
(A) Printing pattern of polysaccharides on the microarray. HEC: hydroxyethylcellulose; MLG: mixed-linkage glucan; AX: arabinoxylan from wheat; GX: glucuronoxylan from beech. (B) Example of microarray scan for pea extracts. (C) Incorporation of fluorescently labeled xyloglucan oligosaccharides by xyloglucan endotransglycosylase activity in extracts isolated from different plant species. The average of the four concentrations was normalized to the corresponding value obtained for the GXXG-FC oligosaccharide of each species. The representative result of three biological replicates is shown. Error bars indicate standard deviation of technical replicates.

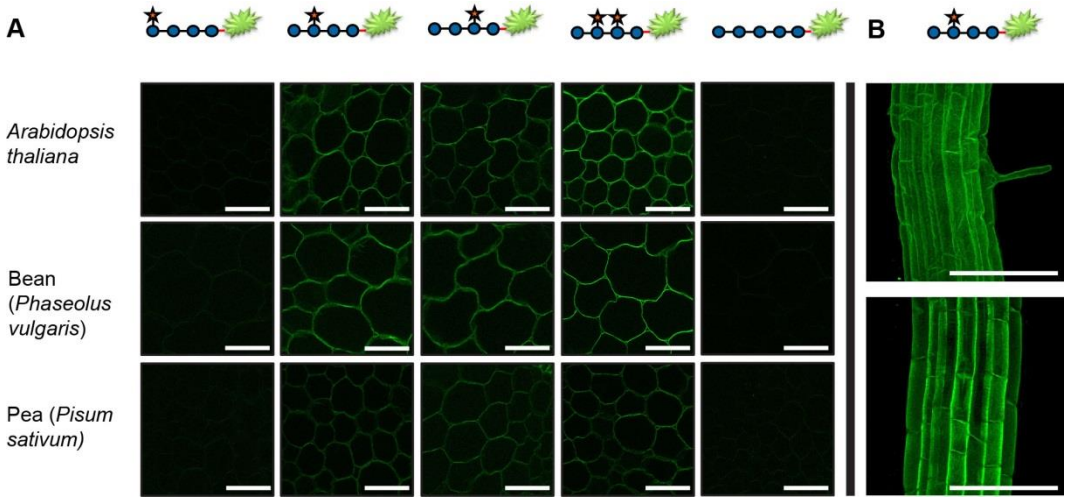

Figure 3. Incorporation of synthetic xyloglucan oligosaccharides into plant cell walls.

(A) Freshly cut stem sections were incubated for 16h with the indicated fluorescein labeled oligosaccharides. Displayed are pith cells from stems of the three species. (B) Maximum intensity projection of *Arabidopsis thaliana* cells in the differentiation (upper) and elongation zone (lower) of a 4 d old root showing labeling after incubation with GXGG-FC. Scale bars indicate 100µm. Stem sections from heat-inactivated controls are shown in Supplementary Figure 1.

To confirm our results obtained using glycan microarrays and plant protein extracts, we aimed to further analyze XET activity *in muro* with the fluorescently labeled XGOs using previously published procedures.[6c, 15] We prepared freshly cut stem sections of *Arabidopsis thaliana*, peas, and beans, and incubated them with the XGO donor substrates overnight. Consistent with the microarray results, we found incorporation of GXGG-FC, GGXG-FC, and GXXG-FC into the cell walls of all three species, relative to boiled controls. This data corroborates our *in vitro* data and confirm that the presence of a single xylose substituent, either in the +2 or the +3 position of the acceptor, is sufficient for recognition and utilization by diverse XETs (Figure 3). We did not detect fluorescent labeling of the cell walls when fresh plant sections were supplied with XGGG-FC or GGGGG-FC as acceptor substrates (Figure 3A, Supplementary Figure 1). It is important to note that XET enzymes are encoded by large *Xyloglucan Endo-Transglycosylase/Hydrolase (XTH)* gene families in land plants. For example, 33 and 41 members of the *XTH* family are present in *Arabidopsis thaliana* and *Populus trichocarpa*,[16] respectively, and thus several different *XET* genes were likely expressed in the tissues and cells that we analyzed. While the XET activity that we measured in plant cell walls and *in vitro* might therefore stem from several putative isoforms of XETs, the lack of XGGG-FC incorporation by all species using both experimental formats clearly indicates that a xylose substitution in the +1 position is not sufficient for any of the XET isoforms present in our samples.

Next, we tested whether we would be able to see incorporation of XGOs into roots of *Arabidopsis thaliana* seedlings. Similar experiments have been previously performed with purified XGO fluorophore conjugates using root cells of *Arabidopsis thaliana* and tobacco.[6c, 17] After incubating 4-day-old seedlings in GXGG-FC-containing buffer overnight, we found strong fluorescent labeling of cell walls in both the differentiation and elongation zones of the root (Figure 3B), indicating the incorporation of the chemically synthesized oligosaccharides into living plant tissue.

Xyloglucan is synthesized in the Golgi apparatus and then transported via vesicles to the cell wall.[3] After secretion, the nascent xyloglucan chains are incorporated into the existing xyloglucan network by the action of XETs. In the plant cell wall, important natural acceptor substrates of XETs are therefore the non-reducing ends of xyloglucan. To analyze the putative xylosylation pattern of these natural XET acceptor substrates, we tested a xyloglucan xylosyltransferase (XXT) from *Arabidopsis thaliana* [18] on our synthetic XGO structures. We were particularly interested in whether the terminal glucose of these xyloglucan substrates is likely to be xylosylated, which was initially suggested to be essential for XET activity[8a] and which corresponds to the usual illustrations of XET activity. The catalytic domain of *At*XXT2 was transiently expressed in Human embryonic kidney cells 293 (HEK293) cells[19] and the purified enzyme was incubated with the XGOs and UDP-xylose. We found that *At*XXT2 only slowly transferred a xylose residue from UDP-xylose to GXGG to form XXGG, but quickly transferred xylose to GGXG to produce GXXG (Figure 4). These two products have very similar retention times when separated and analyzed by high-performance liquid chromatography (HPLC), and were therefore distinguished by treating the reaction products with a β-glucosidase, which cleaves off unsubstituted glucose units at the non-reducing end of the oligosaccharides, prior to analysis (Supplementary Figure 2). Moreover, we found that *At*XXT2 was unable to xylosylate the terminal backbone glucosyl residue at the non-reducing end of GXXG (underlined). It is important to note that *At*XXT2 is part of a five member gene family in *Arabidopsis thaliana;* therefore, it remains a possibility that one of the other XXTs may be capable of catalyzing the addition of xylose at the terminal glucose. Previous reports, however, suggested that XXT1, XXT2, and XXT5 have similar substrate specificities.[18a, 20] We therefore hypothesize that the terminal glucose residue at the non-reducing end of xyloglucan polysaccharides is not xylosylated during biosynthesis, but may be trimmed later by β-glucosidases.[21] Since we found that XETs do not require xylosylation of the terminal glucose residue for transfer, we further hypothesize that XETs are able to graft both trimmed and untrimmed non-reducing ends of freshly synthesized xyloglucan onto existing cell wall polymers.

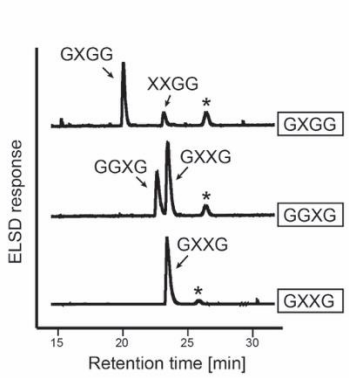

Figure 4. Probing acceptor substrate specificity of xyloglucan xylosyltransferase 2 (XXT2) using synthetic oligosaccharides.
Chemically synthesized xyloglucan oligosaccharides were incubated with *At*XXT2 for 1h, and the reaction products were analyzed using HPLC coupled with an ELSD. Acceptor substrates are indicated with boxes, and the peaks marked with asterisks correspond to UDP. Results for the 24h incubation and enzymatic digests of the *At*XXT2 reaction products with β-glucosidase are shown in Supplementary Figure 2.

To conclude, we employed a set of chemically synthesized xyloglucan oligosaccharides (XGOs) to analyze the acceptor substrate specificities of Xyloglucan Endo-Transglycosylases (XETs) in four different plant species. We developed a glycan microarray that can be used for simple and rapid analysis of transglycosylation activity in plant extracts and found that β-1,4-linked glucan oligosaccharides with single xylose substituents either in the +2 or the +3 position, but not in the +1 position, are suitable acceptor substrates for XETs. These results were confirmed *in muro* by incorporating fluorescently labeled XGOs into cell walls of plant stem sections, consistent with the observation that xylosylation of the non-reducing end of xyloglucan is not required for transfer by XETs. Further, analysis of the substrate specificity of *At*XXT2 using the synthetic XGOs suggested that the terminal glucose of xyloglucan is probably not xylosylated during biosynthesis. Future studies will aim at using other synthetic cell wall oligosaccharides for studying plant cell wall-remodeling enzymes such as the recently reported xylan and mannan transglycosylases.[7a, 22]

Experimental Section

Plant material
Seeds for bean (*Phaseolus vulgaris* L. cv. *Canadian Wonder*), pea (*Pisum sativum* L. cv. *Kelvedon Wonder*), and nasturtium (*Tropaeolum majus*) were obtained from a commercial source (Chrestensen, Erfurt, Germany). Plants were grown in the greenhouse under a 16h light-8h dark regime. Stem material for the enzyme extracts and for the sections was harvested from 8 week-old *Arabidopsis thaliana* (Col-0) plants, and from 2 week-old bean, pea, and nasturtium plants. *Arabidopsis thaliana* (Col-0) seedlings were sterilized with 70 % ethanol, vernalized for two days at 4°C, and then grown vertically on agar plates including Murashige and Skoog (MS) medium under a 16h light-8h dark regime.

Polysaccharides and oligosaccharides
Polysaccharides were purchased from different sources. Xyloglucan from tamarind, arabinoxylan from rye, and glucuronoxylan from beech wood were procured from Megazyme (Bray, Ireland). Hydroxyethylcellulose was obtained from Merck (Darmstadt, Germany) and barley mixed-linkage glucan was purchased from Sigma (USA). Xyloglucan oligosaccharides were synthesized using automated glycan assembly according to previously described procedures.[12] The synthesis of XGGG, GXGG, and GGXG is described in detail in[12b], and the synthesis of GXXG is described in[12a]. In brief, using an automated oligosaccharide synthesizer, suitably protected monosaccharide building blocks were added in iterative glycosylation and deprotection cycles to a linker-functionalized Merrifield resin. After complete assembly, the linker was cleaved and the oligosaccharides were globally deprotected to yield the desired glycans directly equipped with an aminopentyl linker.

Coupling fluorescein to synthetic oligosaccharides
The synthetic xyloglucan oligosaccharides were fluorescently labelled with *N*-hydroxyl succinimide (NHS)-activated fluorescein (Thermo Fisher) by reaction with the aminopentyl linker according to following reaction setup: 0.5-1 µg oligosaccharide, 1.5 eq NHS-fluorescein, and 20 eq of diisopropylethylamine (DIPEA) in 500 µl DMSO. After incubation for 2h at room temperature, the sample was lyophilized, redissolved in water:acetonitrile (90:10), and purified using a water:acetonitrile gradient on a Synergi Hydro-RP column (4.6 mm diameter, 4 µm particle size, Phenomenex, Torrence, CA, USA) using an Agilent 1200 Series HPLC.

Glycan microarray printing
Polysaccharides were diluted in four concentrations (100, 50, 25 and 12.5 µg/ml) in printing buffer (80% 50 mM sodium phosphate, pH 8.5, 0.005% CHAPS, 20% PEG400) and printed on CodeLink NHS ester-activated glass slides (SurModics Inc., Eden Prairie, MN, USA) using a non-contact piezoelectric spotting device (S3; Scienion, Berlin, Germany). The printing was performed at room temperature and 40% humidity. After printing, the slides were quenched for 1 h at room temperature in 100 mM ethanolamine, 50 mM sodium phosphate, pH 9, and washed twice with deionized water.

Measurement of XET activity using glycan microarrays
Plant material was harvested, frozen in liquid nitrogen, and ground into a fine powder using a mortar and pestle. After adding extraction buffer (0.1 M MES pH 5.5, 0.5 M NaCl) including a proteinase inhibitor (cOmplete™ tablets, Roche), the suspension was vortexed and centrifuged for 10 min at 15,000 g, at 4°C. The supernatant was collected, and ammonium sulfate was added to obtain an 80% saturated solution. The precipitated proteins were pelleted by centrifugation for 10 min at 15,000 g, 4°C, and re-dissolved in 0.1 vol of extraction buffer. After another round of centrifugation for 10 min at 15,000 g, 4°C, the supernatant was used for the assays. For use as controls, aliquots of the respective plant extracts were boiled for 10 min at 90°C. To incubate the microarrays with different combinations of plant extracts and fluorescently labelled xyloglucan oligosaccharides (30µM), we applied a FlexWell 64 grid (Grace Bio-Labs, USA) to the slide. The slides were blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at room temperature, and then washed twice with extraction buffer. The extracts were incubated for 16 h on the glycan array and then the unreacted fluorescently labelled xyloglucan oligosaccharides were removed by washing twice for 10 min with extraction buffer and subsequently with deionized water. After drying the slides by centrifugation (300 x g, 2 min), the fluorescent signal on the slides was scanned with a GenePix 4300A microarray scanner (Molecular Devices, USA). Quantification of the fluorescent signal was carried out with GenePix Pro 7 software (Molecular Devices).

Incorporation of synthetic xyloglucan oligosaccharides into plant sections
Hand-cut plant stem sections and *Arabidopsis thaliana* seedlings were incubated with fluorescently labelled xyloglucan oligosaccharides (30µM) for 16 h in extraction buffer (0.1 M MES pH 5.5, 0.5 M NaCl). Control sections and seedlings were boiled in extraction buffer for 10 min at 90°C before adding the fluorescently labelled xyloglucan oligosaccharides. The sections were washed twice with extraction buffer and then imaged using a Leica SP8 confocal microscope using a 40x magnification for the stem samples and a 63x magnification for the root cells.

Analyzing *At*XXT2 activity on synthetic xyloglucan oligosaccharides
Expression of the catalytic domain of *At*XXT2 was performed in HEK293 cell suspension culture as previously described[19]. For the enzyme assays (10 µl), 9.5 µg *At*XXT2 was incubated with 2 mM UDP-Xylose and 1 mM XGO in 50 mM HEPES pH 7.4, 1 mM $MgCl_2$, and 1 mM $MnCl_2$ for either 1 h or 24 h at room temperature. Reactions were terminated by heating for 5 min at 90°C, and the products were analyzed on an Agilent 1200 Series HPLC equipped with an Agilent 6130 quadrupole mass spectrometer (MS) and an Agilent 1200 Evaporative Light Scattering Detector (ELSD). The oligosaccharides were separated on a Hypercarb

column (150 x 4.6 mm, Thermo Scientific) using a water (including 0.1% formic acid)-acetonitrile solvent system as previously described.[23] The peaks in the ELSD traces were assigned based on their retention times and corresponding masses in the MS. To further analyze the reaction products, 4 mU β-glucosidase from *Aspergillus niger* (BGLUC, Megazyme) was added to the terminated *At*XXT2 reactions, and another incubation for 16 h was performed. The digested oligosaccharides were analyzed on the HPLC in parallel with undigested controls.

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Keywords: carbohydrates • xyloglucan endotransglycosylase (XET) • plant cell wall • polysaccharide glycan array • fluorescent labelling · xyloglucan xylosyltransferase 2 (XXT2)

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