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Screening for hetero-transglycosylating activities in extracts from nasturtium (*Tropaeolum majus*)

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Abstract—Using combinations of different polysaccharides as glycosyl donors and of oligosaccharides fluorescently labeled by sulforhodamine (SR) as glycosyl acceptors, we screened for the presence of transglycosylating activities in extracts from nasturtium (*Tropaeolum majus*). Besides xyloglucan endotransglycosylase/hydrolase (XTH/XET, EC 2.4.1.207) activity, which transfers xyloglucanosyl residues from xyloglucan (XG) to XG-derived oligosaccharides (XGOs), a glycosyl transfer from XG to SR-labeled cellooligosaccharides and laminarioligosaccharides has been detected. The XGOs also served as acceptors for the glycosyl transfer from soluble cellulose derivatives carboxymethyl cellulose and hydroxyethylcellulose. The effectivity of these polysaccharides as glycosyl donors for transfer to XG-derived octasaccharide [1-^{3}H]XXLGol decreased in the order XG > HEC > CMC. Isoelectric focusing in polyacrylamide gels showed that bands corresponding to hetero-transglycosylase activities coincided with zones corresponding to XTH/XET. These results can be explained as due either to substrate non-specificity of certain isoenzymes of XTH/XET or to existence of enzymes catalyzing a hetero-transfer, that is the formation of covalent linkages between different types of carbohydrate polymers.

Keywords: Fluorescent labeling; Oligosaccharides; Transglycosylation; XTH; XET; Xyloglucan; Nasturtium; *Tropaeolum majus*

1. Introduction

Current models describe the primary cell wall of higher plants as a composite of insoluble cellulose microfibrils embedded in an alkali-soluble matrix composed predominantly of neutral hemicelluloses and acidic pectins.[1–3] The hemicelluloses are attached to cellulose groundwork via hydrogen bonds, while the linkages between pectins and hemicelluloses may be mediated by Ca^{2+} bridges or are of covalent nature. Recent studies confirm the widespread occurrence of covalent polysaccharide-to-polysaccharide links, for example, between pectin and xyloglucan in angiosperms,[4] in xylan–xyloglucan–pectin complexes in cauliflower stems,[5] xylan–xyloglucan cross-linking via feruloyl acid esters in maize suspension cultures[6] or the linkage between galactan and rhamnogalacturonan I (RG-I) in suspension-cultured potato cells.[7] Golgi-enriched preparations from etiolated pea epicotyls were reported to catalyze the transfer of [U-^{14}C]galactose from UDP[U-^{14}C]galactose into β-(1→4)-galactan side chains on a pectin–xyloglucan complex.[8]

The mechanism of cell wall formation involves biosynthesis of individual matrix components in the Golgi and their secretion to the cell wall where they become anchored by hydrogen bonds to cellulose or they are mutually linked by different types of ionic and/or covalent bonds. It seems probable that the glycosidic interpolymeric linkages are realized in situ, by the action of transglycosylases located in the cell wall.[1] The existence of transglycosylases catalyzing the formation of interpolymeric links in the plant cell wall has been envisaged

Abbreviations: CEOs, cellooligosaccharides; LAOs, laminarioligosaccharides; CMC, Na-carboxymethyl cellulose; HEC, hydroxyethyl cellulose; SR, sulforhodamine; XG, xyloglucan; XGOs, xyloglucan-derived oligosaccharides; XXLGol, reduced xyloglucan-derived octasaccharide $Glc_3Xyl_3GalGlucitol$.

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by several authors in the past[9,11] but the direct proof of their existence has been lacking.

In the present work, we used combinations of different polysaccharides and their fluorescently and/or radioactively labeled oligosaccharides as substrates for screening of transglycosylase activities other than XET. We found that the extracts from nasturtium cotyledons catalyzed transglycosylation with the following donor: acceptor combinations: XG:XGOs-SR, XG:CEOs-SR, HEC:XGOs-SR, CMC:XGOs-SR, and XG:LAOs-SR.

2. Results and discussion

Recently, we have described a sensitive method for visualizing the transglycosylating activity of XTH/XET (xyloglucan endotransglycosylase/hydrolase, EC 2.4.1.207) isoenzymes after their separation by flat-bed isoelectric focusing by using xyloglucan as the glycosyl donor and fluorescently labeled xyloglucan-derived oligosaccharides (XGO-SR) as the glycosyl acceptors. The transglycosylation catalyzed by XET caused incorporation of the fluorescent label into the high-M_r polysaccharide, which after removal of unreacted XGO-SRs by washing with organic solvents, could be observed under ultraviolet light.[10] In the present work we adopted this method for screening of putative hetero-transglycosylase activities in plant extracts using detection gels containing different polysaccharide/oligosaccharide combinations. The fixation of the fluorescently labeled oligosaccharides into the 60% ethanol-insoluble polysaccharide material was detected with the following donor:acceptor pairs: XG:XGOs-SR, XG:CEOs-SR, XG:LAOs-SR, HEC:XGOs-SR, and CMC:XGOs-SR (Fig. 1).

In some instances, it was possible to detect the hetero-transglycosylating activities also by the conventional radiometric assay. In these experiments, the ^{3}H-labeled XG-derived octasaccharide alditol [1-^{3}H]XXLGol and [1-^{3}H]cellooligosaccharides as the respective acceptors and polysaccharides xyloglucan, carboxymethylcellulose and/or hydroxyethylcellulose as the respective glycosyl donors were used. With [1-^{3}H]XXLGol as an acceptor, the incorporation of the label into polymeric materials was detected with all the three polysaccharides. The efficiency of individual polysaccharides as glycosyl donors decreased in the order XG > HEC > CMC (Fig. 2). It should be noted however that this comparison takes into account neither the size-polydispersity of used cellulose derivatives nor their different degree of substitution. Interestingly, we were unable to detect any glycosyl transfer from the tested polysaccharide donors when [1-^{3}H]cellooligosaccharides were used as the glycosyl acceptors in spite that the reaction gave positive signal with CEOs-SR in the gel assays (Fig. 1). At this stage of work, we are unable to explain the differences

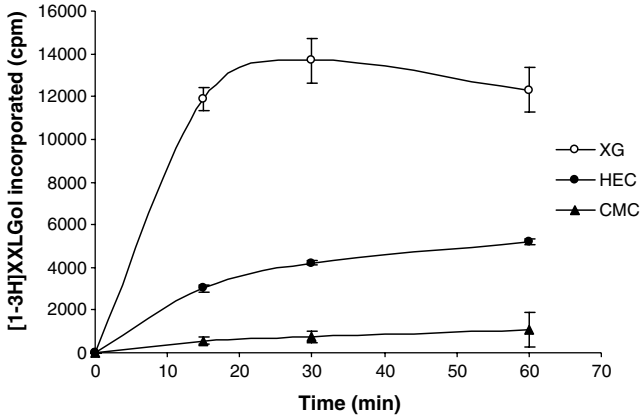

Figure 2. Incorporation of [1-^{3}H]XXLGol into polymeric fraction during incubation with: (○), XG; (●), HEC; (▲), CMC. The points are average from three parallel determinations ± SD. On the left are the isoelectric points of protein standards. Values obtained with boiled enzyme were subtracted. The conditions were as described in Experimental section.

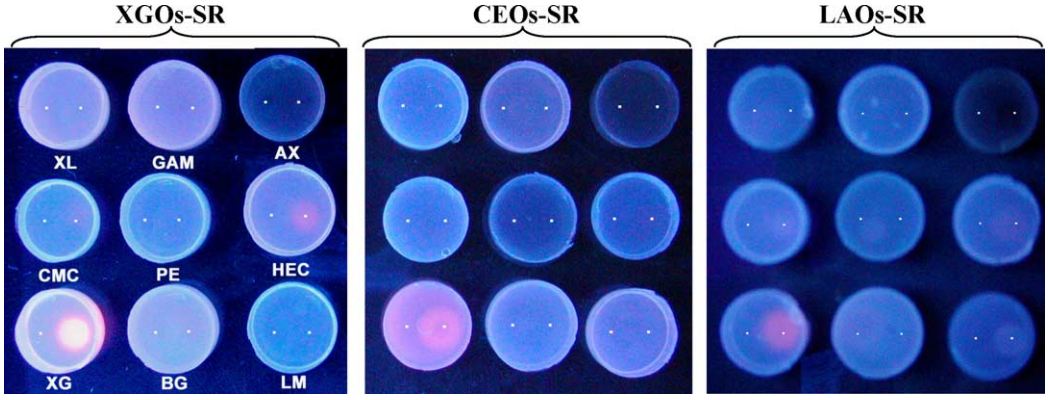

Figure 1. Spot-tests with crude extract from nasturtium seeds on agarose gels containing different combinations of polysaccharides and sulforhodamine-labeled oligosaccharide acceptors. Abbreviations: AX, arabinoxylan; BG, mixed-linkage β-(1→3)/β-(1→4)-glucan; CMC, Na-carboxymethylcellulose; GAM, galactomanan; HEC, hydroxyethylcellulose; LM, laminarin; PE, pectin; XG, xyloglucan; XL, xylan. The same arrangement of substrates is in all the three sets. Boiled extract was applied as control on the left part of each gel.

between the activity of the enzyme(s) toward the fluorescently labeled cellooligosaccharides and the inability to detect any activity with [1-^{3}H]cellooligosaccharides. A possible explanation could be that the bulky non-polar sulforhodamine group attached to the reducing end of the oligosaccharide interacts with some non-polar region on the protein thereby facilitating its accommodation in the active center of the enzyme. This would however implicate that the transfer between xyloglucan and the cellooligosaccharides is of artificial nature, not occurring under native conditions.

A common feature of all the polymers shown to be capable of serving as glycosyl donors is that they contain linear β-(1→4)- or β-(1→3)-linked polyglucosyl backbone. The carboxymethyl- and/or hydroxyethyl substituents on the β-(1→4)-polyglucosyl backbone of cellulose derivatives apparently do not represent any significant sterical hindrance for the enzyme(s). Moreover, the chains of CMC and HEC contain stretches of unsubstituted glucosyl units where the attack by the XTH/XET could take place.

Until now, the existence of covalent links between cellulose and xyloglucan has not been proved in plant cell walls. Studies from different laboratories showed that a portion of xyloglucan in primary plant cell walls is inaccessible to xyloglucan-degrading enzymes and therefore is considered to be embedded or 'woven' into the cellulose microfibrils.[11,12] The nature of the association of the interfibrillar xyloglucan with cellulose molecules, as well as the way how it is formed is not known. It may be mediated by hydrogen bonds but in view of the present results, covalent coupling cannot be excluded. Arguments presented recently by Thompson[13] support the notion that hydrogen bonds between cellulose and hemicelluloses alone are not strong enough to limit the cell wall expansion. If covalent linkages between xyloglucan and cellulose were to exist, they must be formed on nascent cellulose molecules, before their aggregation into microfibrils takes place.

IEF resolution of nasturtium-seed extracts showed that at least five zones exhibited XG:XGOs-SR transglycosylating activity. The major band with pI ~ 6.3 gave positive reaction also with substrate combinations XG:CEOs-SR, XG:LAOs-SR, and HEC:XGOs-SR (Fig. 3). Interestingly, of the four isoenzymes of XET present in the extracts from nasturtium epicotyls, only the minor isoform of pI ~ 6.3 proved positive also with XG:CEOs-SR as substrates (not shown). It is therefore possible that the pI ~ 6.3 isoforms of XET are identical but differently expressed both in cotyledons and epicotyls. The absence of hetero-transglycosylating activity of other epicotyl XET isoforms shows that there are variations in the substrate specificity between various forms of XET expressed in the same plant.

Our results indicate the existence of hetero-transglycosylases or of non-specific XTH/XETs capable to catalyze the formation of interpolymeric glycosidic linkages between different types of polysaccharides in

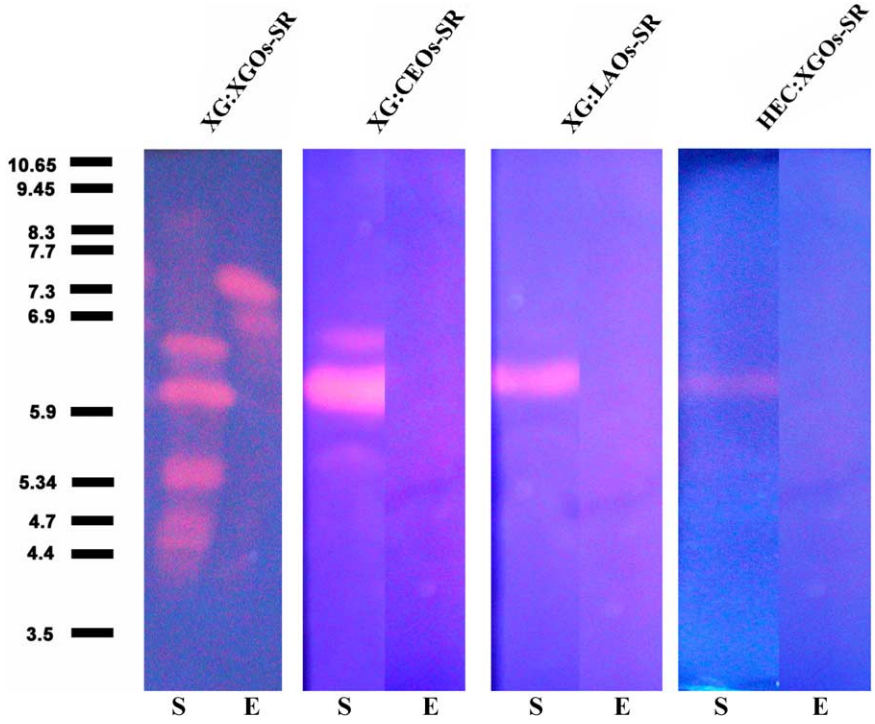

Figure 3. Visualization of transglycosylating activities after IEF separation of crude extract from nasturtium seeds (S) and epicotyls (E) using detection gels containing various donor:acceptor combinations.

plants. Further work is in progress to confirm this idea using assays exploiting diverse combinations of suitably labeled polymeric glycosyl donors and oligomeric glycosyl acceptors.

3. Experimental

3.1. Materials

Polysaccharides used in this study were of the following origin: tamarind seed xyloglucan from Dainippon Pharmaceutical Co., Ltd, Osaka, Japan, mixed-linkage barley β-(1→3)/β-(1→4)-glucan from Megazyme, Na-carboxymethylcellulose, medium viscosity from Serva, hydroxyethylcellulose from Fluka, arabinogalactan and laminarin from Sigma, birch glucuronoxylan from the Production Department of our Institute. Xyloglucan-derived oligosaccharides DP 7–9 were prepared by digestion of xyloglucan with *Trichoderma* cellulase,[14] cellooligosaccharides DP 2–7 by acetolysis of cellulose,[15] laminarioligosaccharides DP 2–8 by partial hydrolysis of laminarin in 2 M trifluoroacetic acid for 30 min at 100 °C. After hydrolysis, the oligosaccharides were purified by passing through a column of Sephadex G-15 (2.5 × 110 cm) eluted with water. Oligomer composition of the oligosaccharides used was assessed by MALDI spectrometry. The oligosaccharides were converted to the corresponding 1-deoxy-1-aminoalditols (glycamines) by reductive amination[16] or by electroreduction of their oximes[17] and subsequently coupled with sulforhodamine as described by Fry.[16] The oligosaccharides labeled with sulforhodamine (OS-SR) were further purified by flash-chromatography on silica gel column (1.6 × 30 cm), first using the system *n*-propanol–MeOH–water (3:2:1, v/v/v), which eluted the unreacted dye and then *n*-propanol–acetic acid–water (3:2:1, v/v/v), which chased the OS-SRs out of the column. Radioactive alditols from oligosaccharides derived from xyloglucan and cellulose were prepared by their reduction with Na-borotritide [^{3}H]$NaBH_4$ (ICN Radiochemicals, San Diego, CA) as described earlier.[18]

3.2. Plant extracts

Nasturtium (*Tropaeolum majus*) seeds were germinated in wet perlite at 20–22 °C under natural day/night periods for 12 days. The cotyledons were separated from epicotyls and homogenized separately. Plant tissues, 0.5–5 g, were homogenized with two volumes of extracting buffer by grinding with pestle in a mortar. The buffer contained 0.1 M imidazole-HCl, pH 6.0 and 1 M NaCl. All operations were carried out at 4 °C. After the extraction, the slurry was centrifuged at 20,000*g* for 15 min and the supernatants were precipitated with $(NH_4)_2SO_4$ to 90% saturation. The precipitates were collected by centrifugation and dissolved in a small volume of 1 M NaCl. The solutions were then dialyzed against 3 mM β-ME, lyophilized, and stored at −20 °C. Before analysis, the lyophilized extracts were dissolved in small amounts of distilled water (usually at a concentration of 10 mg/mL).

3.3. Transglycosylase assays

Spot-tests in agarose gels were carried out in 2-mL assay gels containing 1% agarose, 50 mM succinate buffer, pH 5.5, 0.3% of respective polysaccharide, and 7–10 μM of individual sulforhodamine-labeled oligosaccharides (OS-SR) poured into small Petri dishes (28 mm in diameter). Plant extract solutions (10 μL) containing 30–100 μg protein were spotted on small discs of glass-fiber filter paper (Whatman GF/A, 5 mm diameter) layered on the surface of the gel. As controls, plant extracts that had been inactivated by boiling were used. The dishes were then covered and incubated at 30 °C for 5–16 h. After the incubation, the gels were washed with several changes of 60% ethanol containing 5% formic acid and observed under the ultraviolet light (312 nm). Isoelectric focusing of plant extracts and the detection of transglycosylating activities on the IEF gels was done as previously described.[10]

Radiometric assays were performed in 20 μL assay mixtures essentially as described by Fry et al.[19] but using different combinations of polysaccharides as glycosyl donors and tritiated oligosaccharide alditols as glycosyl acceptors. Boiled enzymes were used in controls. After different time intervals, the reactions were terminated by adding 20 μL of 40% (v/v) formic acid, the mixtures were applied by streaking onto chromatographic paper Whatman 3MM and chromatographed ascendingly in 60% (v/v) ethanol. During this procedure, the unreacted [1-^{3}H]-oligosaccharides moved with the front of the solvent whereby polymer molecules remained on the start. After drying, the areas at ±1 cm from the start were cut out and their radioactivity was determined by liquid scintillation counting.

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References

1. Fry, S. C. *New Phytol.* **2004**, *161*, 641–675.
2. Carpita, N. C.; Gibeaut, D. M. *Plant J.* **1993**, *3*, 1–30.

3. Cosgrove, D. J. *Plant Physiol. Biochem.* **2000**, *38*, 109–124.
4. Popper, Z.; Fry, S. C. *Ann. Bot.* **2005**, *96*, 91–99.
5. Femenia, A.; Rigby, N. M.; Selvendran, R. R.; Waldron, K. W. *Carbohydr. Polym.* **1999**, *39*, 51–164.
6. Kerr, E. M.; Fry, S. C. *Planta* **2004**, *219*, 73–83.
7. Geshi, N.; Jorgensen, B.; Scheller, H. V.; Ulvskov, P. *Planta* **2000**, *210*, 622–629.
8. Cumming, C.; Rizkallah, H.; McKendrick, K.; Abdel-Massih, R.; Baydoun, E.; Brett, C. *Planta* **2005**, *222*, 546–555.
9. Keegstra, K.; Talmadge, K. W.; Bauer, W. D.; Albersheim, P. *Plant Physiol.* **1973**, *51*, 188–196.
10. Farkaš, V.; Ait Mohand, F.; Stratilová, E. *Plant Physiol. Biochem.* **2005**, *43*, 431–435.
11. Hayashi, T. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1989**, *40*, 139–168.
12. Pauly, M.; Albersheim, P.; Darvill, A.; York, W. S. *Plant J.* **1999**, *20*, 629–639.
13. Thompson, D. S. *J. Exp. Bot.* **2005**, *56*, 2275–2285.
14. Sulová, Z.; Lednická, M.; Farkaš, V. *Anal. Biochem.* **1995**, *229*, 80–85.
15. Wolfrom, M. L.; Thompson, A. In *Methods in Carbohydrate Chemistry*; Whistler, R. L., Green, J. W., BeMiller, J. N., Wolfrom, M. L., Eds.; Academic Press: New York and London, 1963; Vol. 3, pp 143–150.
16. Fry, S. C. *Plant J.* **1997**, *11*, 1141–1150.
17. Fedoronko, M.; Stach, T.; Capek, P.; Farkaš, V. *Carbohydr. Res.* **1998**, *306*, 457–461.
18. Sulová, Z.; Takáčová, M.; Steele, N. M.; Fry, S. C.; Farkaš, V. *Biochem. J.* **1998**, *330*, 1475–1480.
19. Fry, S. C.; Smith, R. C.; Renwick, K. I.; Martin, D. J.; Hodge, S. K.; Mathews, K. J. *Biochem. J.* **1992**, *282*, 821–828.