A Barley Xyloglucan Xyloglucosyl Transferase Covalently Links Xyloglucan, Cellulosic Substrates, and (1,3;1,4)-β-D-Glucans*

Molecular interactions between wall polysaccharides, which include cellulose and a range of noncellulosic polysaccharides such as xyloglucans and (1,3;1,4)-β-D-glucans, are fundamental to cell wall properties. These interactions have been assumed to be noncovalent in nature in most cases. Here we show that a highly purified barley xyloglucan xyloglucosyl transferase HvXET5 (EC 2.4.1.207), a member of the GH16 group of glycoside hydrolases, catalyzes the in vitro formation of covalent linkages between xyloglucans and cellulosic substrates and between xyloglucans and (1,3;1,4)-β-D-glucans. The rate of covalent bond formation catalyzed by HvXET5 with hydroxyethylcellulose (HEC) is comparable with that on tamarind xyloglucan, whereas that with (1,3;1,4)-β-D-glucan is significant but slower. Matrix-assisted laser desorption ionization time-of-flight mass spectrometric analyses showed that oligosaccharides released from the fluorescent HEC:xyloglucan conjugate by a specific (1,4)-β-D-glucan endohydrolase consisted of xyloglucan substrate with one, two, or three glucosyl residues attached. Ancillary peaks contained hydroxethyl substituents (m/z 45) and confirmed that the parent material consisted of HEC covalently linked with xyloglucan. Similarly, partial hydrolysis of the (1,3;1,4)-β-D-glucan:xyloglucan conjugate by a specific (1,3;1,4)-β-D-glucan endohydrolase revealed the presence of a series of fluorescent oligosaccharides that consisted of the fluorescent xyloglucan acceptor substrate linked covalently with 2–6 glucosyl residues. These findings raise the possibility that xyloglucan endo-transglucosylases could link different polysaccharides in vivo and hence influence cell wall strength, flexibility, and porosity.

The structural integrity of land plants is dependent, in large part, on the collective strength and flexibility of the walls that surround individual cells, whereas key elements of cellular function, such as water and nutrient exchange, depend upon the porosity of the walls. Plant cell walls are dynamic structures that are altered during cell division, growth, and differentiation to enable cells to adapt to changing functional requirements and to environmental and pathogen-induced stresses. In addition, walls are important for intercellular cohesion and cell-cell communication and must be selectively permeable to water, nutrients, and growth regulators.

The primary cell walls of vascular plants consist of cellulosic microfibrils that are embedded in a chemically complex matrix consisting mostly of polysaccharides but also containing structural proteins, enzymes, and phenolic acids (1, 2). Xyloglucans and pectic polysaccharides are the major noncellulosic polysaccharides of primary walls from dicotyledonous plants, whereas in the Poales and related commelinoid monocots, including commercially important cereals and grasses, glucuronarabinoxylans and (1,3;1,4)-β-D-glucans are the predominant noncellulosic wall polysaccharides, and levels of pectic polysaccharides, glucomannans, and xyloglucans are relatively low (3). In addition, wall composition and the fine structures of component polysaccharides vary depending upon the growth phase, cell type, cell position, and local region within the wall (4, 5). In some cell types, lignin is deposited throughout the wall during secondary thickening (6, 7), and in response to pathogen attack, the rapid formation of a cross-linked protein network, together with the deposition of lignin and callose, can strengthen walls and create new physical barriers to invading microorganisms (8).

Although the types and abundance of polysaccharides in plant cell walls have been defined in detail, little information is available on the molecular interactions between constituent polysaccharides in the wall. In most wall models it is assumed that different polysaccharides are held in place through extensive intermolecular hydrogen bonding rather than through covalent interactions (1, 9). However, there is recent circumstantial evidence (10) to support earlier suggestions (11–13) that xyloglucans might be covalently linked to pectic polysaccharides.

Here we have shown that a highly purified xyloglucan xyloglucosyl transferase (EC 2.4.1.207), also known as xyloglucan
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endo-transglucosylase (XET),3 from barley seedlings can catalyze the formation of covalent linkages between xyloglucans and cellulose substrates and between xyloglucans and (1,3,1,4)-β-d-glucans. The polysaccharides are linked “head-to-tail” through their respective nonreducing and reducing termini to form heteropolysaccharides.

EXPERIMENTAL PROCEDURES

Materials—AmpholineTM isoelectric focusing (IEF) polyacrylamide gels (pH range 3.5–9.5), molecular mass marker proteins (20–94 kDa), and dextran 500 were from GE Healthcare; Bio-Gel P-60, phenyl-Sepharose, and pl marker proteins 4.45–9.6 were from Bio-Rad; Microcon microconcentrators were from Amicon (Beverly, MA); 3MM paper was from Whatman; Microlath (22–25-μm pore size) was from Calbiochem, and ampholines were from Serva (Heidelberg, Germany). Phenylmethylsulfonyl fluoride, 2-mercaptoethanol, glucose, EDTA, bovine serum albumin (fraction V), polyethylene glycols 8000 and 1450, bovine serum albumin, pectin (citrus fruit), esterified pectin (potassium salt; citrus fruit), polygalactouronic acid, and laminarin (from Laminaria digitata) were supplied by Sigma. Lissamine rhodamine B sulfonate chloride (sulforhodamine SR) was from Acros Organics (Morris Plains, NJ); the Coomasie protein assay reagent was from Pierce; EcoLume scintillation fluid was from MP Biomedicals (Irvine, CA); chromatography 3MM paper was from Whatman, and acetonitrile was from BDH (Poole, UK). barley (1,3,1,4)-β-d-glucans (average molecular masses of 450 and 40 kDa), β-d-galactans (from lupin and potato), lichenin, rhamnogalactouronan, barley arabinobioxyan, tamarind xyloglucan (TXG), haxanogalacturan (soybean pectin fiber), xyloglucan-derived heptasaccharide (XXXG), its reduced form XXXGol, and (1,3,1,4)-β-d-glucanase from Bacillus subtilis were from Megazyme (Bray, Ireland). Carboxymethylcellulose of degree of substitution 0.54 was from Imperial Chemical Industries (Dingley, Australia); arabinogalactan protein (from gum arabic) was kindly donated by the late Dr. Peter Biely (Institute of Chemistry, Slovak Republic); a low viscosity locust-bean gum galactomannan was from Seikagaku Corp. (Tokyo, Japan). Cello-oligosaccharides (CEO) of degree of polymerization (DP) of DP 2–6 and laminari-oligosaccharides (LAO) of DP 2–6, were from Seikagaku Corp. (Tokyo, Japan). Cello-β-heptose and cello-octaose were prepared by acid hydrolysis from Antigum CS6 (System Bio-Industries, Paris, France). Hydroxyethylcellulose (HEC) of medium viscosity (~1500 mPa s, 5% in H2O at 20 °C), of average molecular mass 450 kDa, and of a degree of substitution ~0.3 was from Fluka Biochemicals (Buchs, Switzerland). Beechwood 4-O-methyl-(1,4)-β-d-glucuronoxylan was from Institute of Chemistry (Slovak Republic); a low viscosity locust-bean gum galactomannan was donated by Dr. Peter Biely (Institute of Chemistry), sulfuric acid swollen cellulose of average molecular mass of 12–15 kDa and a degree of substitution −0.25 was provided by Professor Bruce Stone (La Trobe University, Australia), and 1,4-β-d-glucan endohydrolase EGI from Trichoderma reesei was kindly donated by the late Dr. Marianne Hayn (University of Graz, Austria).

Extraction of HvXET5—Barley (Hordeum vulgare L., cv. Clipper) (2 kg dry weight) was surface-sterilized for 10 min in 0.1% (w/v) NaOCl, washed successively with tap water, 0.5 M NaCl, and sterile water, and steeped for 24 h in sterile water containing chloramphenicol (100 μg/ml), neomycin (100 μg/ml), penicillin G (100 units/ml), and nystatin (100 units/ml). Germinating grains were maintained at ~40% (w/w) moisture content by regular application of fresh antibiotic solution for 7 days at 21 ± 2 °C in the dark. Bacterial or fungal contamination of the grains was not evident at any stage during this period. The germinated grain and young seedlings were homogenized at 4 °C in 2.0 volumes of homogenization buffer, pH 6, containing 0.1 M imidazole-HCl buffer, 1 M NaCl, 2 mM EDTA, 1 mM 2-mercaptoethanol, and 1 M phenylmethylsulfonyl fluoride (buffer A), in a Waring Blender six times with 1-min intervals with intermittent cooling (2 min) on ice. The homogenate was held for 1 h at 4 °C to extract proteins; insoluble material was removed by centrifugation (4000 × g, 60 min, 4 °C), and the extract filtered through Miracloth. The extract was precipitated to 90% with solid (NH4)2SO4; the precipitate was collected (8000 × g, 45 min, 4 °C) and resuspended in 4 liters of buffer A (without NaCl). The extract was stored in 0.5-liter aliquots at ~20 °C.

Purification of HvXET5—The HvXET5 enzyme was purified from extracts of 7-day-old barley seedlings using Sepharose Q, phenyl-Sepharose, chromatofocusing on PBE-94, and size-exclusion chromatography on Bio-Gel P-60 (Table 1). The activity of HvXET5 during enzyme purification was determined radiometrically at 30 °C in 100 mM succinate or ammonium acetate buffers, pH 6.0, containing 5 mM calcium chloride, 0.3% (w/v) TXG, and 3H-labeled xyloglucan-derived saccharide heptaisosaccharide (3HGGol, specific radioactivity 83 MBq/μmol−1) (14) with ~30,000 dpm per reaction mixture. The radioactivity incorporated in reaction products was counted on 2 × 1-cm Whatman chromatography 3MM paper strips in plastic vials and using LSC6500 scintillation counter (Beckman Instruments, Fullerton, CA) with ~48% efficiency for tritium, using EcoLume scintillation fluid, and with 70% quenching. Enzyme activity of HvXET5 during purification is expressed in katals, where 1 katal represents 1 mol of product formed per s; specific activity is expressed in picokatals·mg−1 protein.

Protein Determination, SDS-PAGE, and Amino Acid Sequencing—Protein concentration determinations during purification and characterization of HvXET5 and SDS-PAGE were performed as described previously (15). During HvXET5 purification, protein was detected with colloidal Coomassie Brilliant Blue G-250 in methanol for 30 h at ambient temperature (16). This staining technique detects ~6–10 ng of protein per band or 0.7 ng per mm2 (16, 17). Automated amino acid sequence analysis of HvXET5 was performed by Edman degradative analysis as described previously (18). A single primary sequence was detected, with no secondary sequence. Clear signals for each phenylthiohydantoin amino acid residue derivative were detected.
Isoelectric Focusing—The crude protein extracts and purified preparations were separated on a flatbed IEF apparatus (GE Healthcare) in 1-mm polyacrylamide gels using a pH gradient of 3.5–9.5. Pre-focused gels were run at 600 V for 30 min, followed by 800 V for a further 20 min. Proteins were detected with a Coomassie Brilliant Blue dye after the gels were fixed in 20% (w/v) trichloroacetic acid. Apparent pI values were estimated by reference to marker proteins with pI values of 4.45–9.6. Enzyme activity in gels was detected by overlaying the separation gels with a 1.5-mm 1.3% (w/v) agarose detection gel containing 0.2% (w/v) TXG and 5–10 μM of SR-labeled xyloglucan-derived oligosaccharides XGO-SR (XXXG-SR, XXLG-SR, XLLG-SR molar ratios were 1:1.6:1.8) (19, 20) in 0.1M succinate buffer, pH 6, containing 5 mM calcium chloride. The separation gels contacted with detection gels were incubated for 1–5 h at 30 °C, depending on the activity of the preparation under investigation. The detection gels were immediately fixed and de-stained in 60% (v/v) ethanol containing 5% (v/v) formic acid. The detection gel with fluorescent zones was evaluated under a UV lamp at 366 nm.

pH Optimum and Enzyme Stability—The effect of pH on the activity of HvXET5 was determined by incubating 1 nM HvXET5 at 30 °C for 60 min in 50 mM citric acid, 100 mM sodium dihydrophosphate (McIlvaine) buffers, pH 4.0–8.5, in the presence of 0.02% (w/v) bovine serum albumin. A comparison of succinate, ammonium acetate, or sodium phosphate buffers, each at 50–200 mM, indicated that HvXET5 activity was unaffected by the ionic strength of these buffers. The thermal stability of HvXET5 was determined after 15 min of incubation at 0–70 °C. The freeze/thaw stability of HvXET5 at 1 nM concentration was determined after three cycles of freezing (−80 °C) and thawing (4 °C), each at duration of 3 min. Activity was subsequently measured at 30 °C in 100 mM ammonium acetate buffer, pH 6, containing 5 mM calcium chloride, without and with the addition of 10% (v/v) glycerol. Enzyme activity was determined radiometrically as specified above, and expressed as % activity relative to maximal activity. Assays were performed in triplicate and standard errors of 8–14% were observed.

Effects of Divalent Cations—The effect of Ca²⁺ (as calcium chloride) and Mg²⁺ (as magnesium sulfate), both in 0–15 mM

TABLE 1
Enzyme yields and purification factors of HvXET5 from 7-day-old seedlings

| Purification step                  | Yield          | Specific activity | Recovery | Purification factor |
|------------------------------------|----------------|------------------|----------|---------------------|
|                                    | Protein (mg)   | Activity (picokatal) |          |                     |
| Crude homogenate (1 M NaCl extract) | 6,397          | 7,777            | 1.2      | 100                 | 1.0 |
| Sepharose Q, pH 6.8                | 5,375          | 8,512            | 1.6      | 110                 | 1.3 |
| Phenyl-Sepharose, pH 6.0           | 1,254          | 7,771            | 6        | 100                 | 5   |
| Bio-Gel P-60, pH 7.0               | 450            | 6,909            | 15       | 89                  | 13  |
| PBE-94, pH 5.0–8.3                 | 50             | 2,996            | 60       | 39                  | 50  |
|                                    | 0.1            | 363              | 3,630    | 5                   | 3,025 |

a Total enzyme activity in selectively pooled fractions was assayed radiometrically.
b Recoveries are expressed as percentage of a total enzyme activity in a crude homogenate.
c Purification factors are based on specific activities.

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FIGURE 2. pH and temperature dependences of relative activities of HvXET5. 
A, pH values. B, temperature dependences were determined radiometrically after 15 min of incubation at the indicated pH or temperatures values.

Concentration ranges, and of EDTA in a 0–20 mM concentration range, were determined by incubating 1 nM HvXET5 in 100 mM succinate buffer, pH 6. Enzyme activities were determined radiometrically as specified above, in triplicate and with a standard error of 8–10%.

**Substrate Specificities**—The incubation mixtures contained 1.2% (w/v) soluble polysaccharides as donor substrates and as acceptor substrates either 23–27 µM XGO-SR, SR-labeled cellulose-oligosaccharides (CEO-SR) (DP 2–8), or SR-labeled laminari-oligosaccharides (LAO-SR) (DP 2–6) (20) in 100 mM ammonium acetate buffer, pH 6, containing 5 mM calcium chloride and 0.5–1 mM purified HvXET5. The molar ratios of individual oligosaccharides in the two oligosaccharide-SR mixtures were 1:0.78:0.62:0.41:0.16:0.03:0.013 for C2-SR to C8-SR (SR-labeled cellobiose to celloctaose), and 1.0:1.7:0.87:0.41:0.2 for L2-SR to L6-SR (SR-labeled laminaribiose to laminari-hexaose). All incubations proceeded for 18 h at 30 °C. Enzymes inactivated by boiling for 3 min served as controls. The efficiency of transfer of selected polysaccharides onto fluorescent acceptors was determined by size-exclusion HPLC. The SR-labeled oligosaccharides and polysaccharides were detected following HPLC by fluorescence detection (excitation 568 nm and emission 584 nm) or by evaporative light scattering detection (ELS5D) at 568 nm, and by MALDI-TOF mass spectrometry analyses. Enzyme activities were determined by integrating peak areas, after subtracting background level obtained from boiled enzyme control reactions. Relative activities of HvXET5 are expressed as % of activity observed with TXG as a donor substrate and XGO-SR as an acceptor. In all instances assays were performed in duplicate with standard errors of 8–12%. Relative activities are expressed as percentage of the integrated peak area of the reaction with TXG and XGO-SR. The incubation mixtures were performed in duplicate with standard errors of 8–12%. Relative activities are expressed as percentage of the integrated peak area of the reaction with TXG and XGO-SR. The efficiency of transfer of selected polysaccharides onto [3H]XXXGol was further evaluated by ascending chromatography in 60% (v/v) ethanol on Whatman chromatography 3MM paper strips, and radioactivity in paper strips was determined by liquid scintillation counting as specified above.

**Preparation of HEC:XXXG-SR and (1,3;1,4)-β-d-Glucan: XXXG-SR Conjugates**—XXXG-SR was prepared as described (19, 20) and purified on a reversed phase column with a water/acetonitrile gradient. A relatively homogeneous HEC and (1,3,1,4)-β-d-glucan fractions (collected after separations by size-exclusion chromatography) at 0.8% (w/v) concentrations were dissolved in 100 mM ammonium acetate buffer, pH 6, containing 5 mM calcium chloride and incubated with 8 µM XXXG-SR, in the presence of 4 nM purified HvXET5 at 30 °C with shaking. Every 16 h (HEC) or 24 h ((1,3;1,4)-β-d-glucan), a fresh HvXET5 enzyme (¼ of the original amount) was added. The reactions were terminated by boiling after 48 and 144 h, for HEC and (1,3;1,4)-β-d-glucan, respectively.

The rates of synthesis of HEC:XXXG-SR conjugate by HvXET5 at 1 nM concentration were followed for 0, 16, and 24 h. The rates of transfer of 130 µM XXXGol onto 0.1% (w/v) HEC:XXXG-SR conjugate by HvXET5 (1 nM) were followed for 0, 6, and 16 h. Boiled enzymes served as controls in all instances. The products were analyzed by HPLC with fluorescent and ELSD detectors.

**Characterization of HEC:XXXG-SR and (1,3;1,4)-β-d-Glucan:XXXG-SR Conjugates**—Approximately 0.5 mg of HEC: XXXG-SR or (1,3;1,4)-β-d-glucan:XXXG-SR was dissolved in 50 mM ammonium acetate buffer, pH 5, and incubated with 10 nM of the purified 1,4-β-d-glucan endohydrolase from *T. reesei* (family GH5 glycoside hydrolase (21)) or 1 nM of the purified (on Bio-Gel P-60) (1,3;1,4)-β-d-glucanase (a family GH16 glycoside hydrolase (22)) from *B. subtilis* for 1 h at 30 °C. The reactions were stopped by boiling, and the hydrolysis products of HEC:XXXG-SR and (1,3;1,4)-β-d-glucan:XXXG-SR were separated by HPLC and analyzed by MALDI-TOF mass spectrometry.

**HPLC Analysis**—Native polysaccharides were fractionated by size-exclusion chromatography on either a P3000 or P4000 PolySep GFC columns (particle size not specified, 300 × 7.8 mm) (Phenomenex, Torrance, CA) with water or 100 mM ammonium acetate as eluant at a flow rate of 0.8 ml/min. Fractions of the mixture of XGO-SR (XXXG-SR, XXLG-SR, and XLLG-SR) and the hydrolysis products of HEC:XXXG-SR and (1,3;1,4)-β-d-glucan:XXXG-SR were performed on a Hyperil ODS column (5 µm, 250 × 2.1 mm) (Thermo Electron Corporation).
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Corporation, Waltham, MA) with a linear gradient of 23.45–26.65% aqueous acetonitrile at a flow rate of 0.2 ml/min. A model 1090 liquid chromatograph with diode-array detector, controlled by ChemStation software (Agilent Technologies, Palo Alto, CA), and fluorescence (model RF-10AXL, Shimadzu, Kyoto, Japan) and ELSD (model 800, Alltech Associates Inc., Deerfield, IL) connected in series to the 1090 DAD, were used for analyses of enzymic reactions. The eluant flow from the fluorescence detector to the ELSD was split in the ratio 5 (to collect) to 1 (ELSD). The ELSD was operated at 40 °C and a nitrogen pressure of 1.5 bar, and the column temperature was 21 °C. Size-exclusion HPLC of SR-labeled polysaccharides and oligosaccharides were carried out on a BioSep SEC S3000 column (5 μm, 300 × 7.8 mm); the eluant was 100 mM ammonium acetate in 20% (v/v) acetonitrile at a flow rate of 1.0 ml/min.

MALDI-TOF Mass Spectrometry Analyses—MALDI TOF spectra were acquired using a Bruker Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) operating in a reflectron mode. Samples (1 μl) dissolved in water were mixed with 1 μl of a 5 g/liter solution of dihydroxybenzoic acid in 1% (v/v) phosphoric acid and spotted on a matt-steel target plate. External calibration was performed using peptide standards (Bruker Daltonik GmbH, Bremen, Germany), which were analyzed under the same conditions. Spectra were acquired using between 800–3,000 laser shots. The ionization voltages were IS1 = 25.0 kV, IS2 = 21.7 kV, and lens = 8.2 kV. The mass spectrometer was calibrated with XXXG-SR, XXLG-SR, and XLLG-SR that were purified by tandem normal phase and size-exclusion HPLC.

RESULTS

Purification of the Barley HvXETS—The barley HvXET5 enzyme was purified ~3,000-fold from extracts of 7-day-old barley seedlings, using ammonium sulfate precipitation, ion exchange and hydrophobic chromatography, chromatofocusing, and size-exclusion chromatography (Table 1). The numbering of the barley XET isoforms is based upon the gene nomenclature proposed by Strohmeier et al. (23). The specific activity of the purified HvXET5 was 3,630 picokatals·mg⁻¹, which is about six times higher than the specific activity of a recombinant Populus XET PttXET16A (24) and close to that of a cauliflower XET purified by affinity chromatography on xyloglucan (25).
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A

B

C

D
It was necessary to use 1 M NaCl in the imidazole buffer for efficient enzyme extraction, presumably because the XET enzymes are tightly associated with cell wall material. The key steps during the purification of HvXET5 were chromatofocusing on PBE-94 and chromatography on Bio-Gel P-60, where several HvXET isoenzymes were separated from each other and from major contaminating proteins (Table 1). The enzymes bound strongly to phenyl-Sepharose and were not eluted by a 3–0 M linear gradient of NaCl. A 30–70% linear gradient of ethylene glycol was required for elution, suggesting that the enzymes were highly hydrophobic. The HvXET5 isoenzyme also bound to Bio-Gel P-60 and 0.01% (v/v) Tween 20, and 0.2 M NaCl were required to elute the enzyme from the column.

The final purified enzyme preparation showed a single band of 34 kDa on SDS gels at high protein loadings (Fig. 1A), with no other protein species present. These data indicated that contaminating proteins accounted for less than 6 ng or 0.25% of the protein used for SDS-PAGE analysis. Furthermore, a single protein and activity band of pl 7.6 was detected on an isoelectric focusing gel and on a zymogram detection gel containing TXG- and SR-labeled xyloglucan oligosaccharides XGO-SR (Fig. 1B). A single sequence was detected during NH2-terminal amino and SR-labeled xyloglucan oligosaccharides XGO-SR (Fig. 1).

The pH optimum and enzyme stability—The pH optimum of the purified HvXET5 was 6.0, and the temperature optimum varied between 28 and 30 °C (Fig. 2). The enzyme also operated efficiently at 0 °C, where it showed ~40% of the activity observed at 30 °C. A similar tolerance to low temperature has also been observed with recombinant TCH4 XET enzyme from Arabidopsis (26). As for the freeze/thaw stability of HvXET5, the purified enzyme retained its activity for at least a year when stored at −20 °C and did not lose activity after several freeze-thaw cycles. The addition of 10% (v/v) glycerol had no affect on HvXET activity after several freeze-thaw cycles.

The Effects of Divalent Cations—Effects of the divalent cations Ca2+ and Mg2+ were tested on the activity of HvXET5 under optimal conditions. Although Ca2+ at concentrations between 5 and 15 mM stimulated HvXET5 activity by ~7–8%, Mg2+ inhibited the activity of HvXET5 by 3–4% in the same concentration range. The chelating agent EDTA inhibited the activity of HvXET5 by ~30% in 2–20 mM concentration ranges (data not shown).

Substrate Specificity of the Purified HvXET5—Xyloglucan oligosaccharides (XGO-SR) and cello-oligosaccharides (CEO-SR) fluorescently labeled with sulforhodamine (SR) were used as acceptor substrates for the purified HvXET5. Transferase activity was observed when tamarind xyloglucan (TXG), hydroxyethylcellulose (HEC), sulfuric acid-swollen cellulose, and barley (1,3;1,4)-β-D-glucan were used as donor polysaccharides (Table 2). No hydrolytic activity was detected with any of the donor substrates. The transfer of nonfluorescent donor polysaccharides onto fluorescent acceptors was determined by size-exclusion HPLC, where dramatic increases in molecular size of fluorescent material showed that the transfer reaction had occurred. In Fig. 3A the progressive formation over 24 h of high molecular mass, fluorescent HEC from unlabeled HEC donor substrate, and the fluorescent XGO-SR acceptor molecule can be seen. In Fig. 3B, the transfer reaction by HvXET5 is shown with the high molecular mass product of the reaction presented in Fig. 3A, whereby the fluorescent component XGO-SR of the high molecular mass HEC:XXXG-SR material from Fig. 3A (shaded fractions) was removed from the reducing end of the polysaccharide and replaced with the nonfluorescent oligosaccharide XXXGol. As this occurred, low molecular mass fluorescent oligosaccharides XGO-SR were progressively released. A schematic representation of the transfer reaction shown in Fig. 3, A and B, is summarized in Fig. 3C.

The HvXET5 enzyme also catalyzes the transfer of TXG, celluloses, and (1,3;1,4)-β-D-glucan onto fluorescently labeled CEO-SR, albeit at low levels (Table 2). The fluorescence assay technique used in this study was sufficiently sensitive to confidently measure activities that were better than 0.1 pmol of XGO-SR or 1 × 10−5 % of the amount of XGO-SR acceptor used in standard enzyme reactions.

The capacity of HvXET5 to form covalent linkages between xyloglucan fragments and either cellulose or (1,3;1,4)-β-D-glucan was first shown with the oligosaccharide mixture XGO-SR and later confirmed with the single xyloglucan-derived heptasaccharide XXXG-SR (Figs. 4 and 5). Similarly, formation of covalent linkages by HvXET5 between xyloglucan fragments and cellulose or (1,3;1,4)-β-D-glucan was confirmed independently using radiometric analysis with 3H-labeled xyloglucan-derived heptaisotol and paper chromatography. The slower transfer of the (1,3;1,4)-β-D-glucan donor onto the XXXG-SR acceptor substrate, compared with donors with (1,4)-β-D-glucan backbones, probably results from the molecular kinks introduced into this substrate by the (1,3)-β-linkages (27) and hence from less favorable binding to the active site of the enzyme.

In control experiments, either the acceptor or donor substrates were omitted or the enzyme was inactivated by boiling. In no instance was any change in molecular size of fluorescent material observed. The absence of transferase activity, when either the donor or acceptor substrate was omitted, was particularly important, because it indicated that transglycosylation
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A

Graph showing fluorescence and ELSD over time with peaks at 0h and 144h.

B

Graph showing fluorescence with peaks labeled 1 to 5.

C

Graph showing intensity over m/z with peaks labeled 1 to 5.
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Xyloglucans consist of a backbone of (1,4)-β-D-glucan substituted with xylosyl, galactosyl, and fucosyl residues (28). The molecular sizes of xyloglucans can be altered after their deposition into the cell wall (29), and this process is likely to be mediated by a class of enzymes broadly known as xyloglucan endotransglycosylases/hydrolases (XTHs). However, enzymes within this group can have XET activity or both xyloglucan endotransglycosylase and xyloglucan endohydrolase activities (14, 30, 31). The XETs are abundant in the apoplastic space; they cleave the (1,4)-β-D-glucan backbone of xyloglucans and, in the case of XETs, transfer the nonreducing fragment of the original substrate that remains bound to the enzyme directly onto the nonreducing terminus of another xyloglucan chain (14, 31). The xyloglucan molecule that is cleaved by the enzyme initially is referred to as the donor substrate, whereas the xyloglucan chain to which the product of hydrolysis is transferred is known as the acceptor substrate. The transglycosylation activity of XETs can theoretically result in the disproportionation of xyloglucan molecules, such that some will increase in molecular mass, and others will decrease in molecular mass (14, 31).

Sequences encoding XTHs are surprisingly abundant in barley EST databases, given the relatively low levels of xyloglucans in walls of most barley tissues (23, 32). There are at least 22 XTH genes in barley (23), about 30 in rice (33), about 40 in Populus trichocarpa (34), and about 33 in Arabidopsis (35). In an attempt to reconcile the relatively low abundance of xyloglucans in cell walls of barley against the large number of XTH genes and their high expression levels in many tissues of barley, Stroehmeier et al. (23) suggested that some of the XTHs might be active on the more abundant matrix phase polysaccharides of cell walls in barley, namely the arabinoxylans and the (1,3; 1,4)-β-D-glucans. A role for XTHs in the modification of highly abundant (1,3,1,4)-β-D-glucans and arabinobiose in walls of the commelinoid monocots would be consistent with the abrupt increase in molecular size of heteroxylans that has been observed in suspension-cultured maize cells following the deposition of the polysaccharide into the walls (29).

Thus, molecular modeling established a potential structural connection between XTHs and (1,3; 1,4)-β-D-glucan endohydrolases and with certain (1,4)-β-D-xylan endohydrolases (23). The models were subsequently supported by the published three-dimensional structure of the Populus tremula × tremuloides XET (36). The plant XTHs and microbial (1,3; 1,4)-β-D-glucan endohydrolases are all classified in the family GH16 group of glycoside hydrolases, although a small number of microbial xyloglucan endohydrolases are also classified within families GH5, GH12, GH44, and GH74 (37).

In an attempt to test suggestions that some barley XET enzymes could catalyze transfer of xyloglucan onto acceptors

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DISCUSSION

Analysis of the products of HvXET5 action—To confirm the results obtained from the fluorescence assays, the high molecular mass HEC:XXXG-SR conjugate generated by incubation of the HvXET5 with XXXG-SR and nonfluorescent HEC (Fig. 4A) was partially hydrolyzed with a highly purified (1,4)-β-D-glucan endohydrolase from T. reesei that contained no β-D-glucosidase or other contaminating activities. Three major fluorescent oligosaccharide fractions were released from the HEC:XXXG-SR (Fig. 4, A and B) during the partial hydrolysis with the (1,4)-β-D-glucan endohydrolase. MALDI-TOF mass spectrometry analyses showed that these oligosaccharides had molecular masses corresponding to XXXG-SR with one, two, or three additional glucosyl residues attached (Fig. 4B). Furthermore, ancillary m/z peaks, which corresponded to Glc:XXXG-SR, Glc-Glc:XXXG-SR, and Glc-Glc-Glc:XXXG-SR containing hydroxyl substituents (m/z 45) from the donor substrate, were also detected in the spectra (Fig. 4, C and D) and confirmed that the parent material consisted of HEC covalently linked with the XXXG-SR.

Similarly, a (1,3;1,4)-β-D-glucan endohydrolase from B. subtilis was used for digestion of the (1,3;1,4)-β-D-glucan: XXXG-SR conjugate (Fig. 5, A and B). The enzyme was purified from the commercially available preparation by size-exclusion chromatography on Bio-Gel P-60, to remove contaminating endo-β-D-glucans. Partial hydrolysis of the (1,3;1,4)-β-D-glucan:XXXG-SR conjugate (Fig. 5A) released a series of fluorescent oligosaccharides (Fig. 5B) that were shown by MALDI-TOF mass spectrometry to consist of the XXXG-SR acceptor substrate with 2–6 covalently attached glucosyl residues (Fig. SC). These data indicated that the high molecular mass fluorescent material generated by the HvXET5 contained polymeric barley (1,3;1,4)-β-D-glucan covalently linked to the XXXG-SR acceptor substrate.

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FIGURE 5. Characterization of (1,3;1,4)-β-D-glucan:XXXG-SR conjugate synthesized by HvXET5. A, HPLC of high molecular mass, fluorescent (1,3;1,4)-β-D-glucan:XXXG-SR synthesized after 144 h from unlabeled (1,3;1,4)-β-D-glucan and XXXG-SR by HvXET5. The material eluting between 8 and 12 min was pooled for hydrolysis by B. subtilis (1,3;1,4)-β-D-glucan endohydrolase. Fluorescence and ELSD profiles have nonlinear detection responses. B, purification by HPLC of five major oligosaccharides released from (1,3;1,4)-β-D-glucan:XXXG-SR by (1,3;1,4)-β-D-glucan endohydrolase. C, MALDI-TOF mass spectra of (1,3;1,4)-β-D-glucan-derived oligosaccharides conjugates 1–5 from B showed the presence of XXXG-SR with 2–6 covalently attached glucosyl residues at its nonreducing termini. The position of XXXG-SR is marked by an arrow. The positions of molecular mass standards of (1,3;1,4)-β-D-glucan (40 kDa), polyethylene glycol 1450, and glucose (180) are indicated. a.u., arbitrary units.
Barley XET Covalently Links Different Polysaccharides

other than xyloglucans, an XET isoenzyme was purified from extracts of barley seedlings to a near monodisperse form. The difficulties encountered during the purification procedure of HvXET5 were largely attributable to the presence of numerous hydrophobic patches on the surface of the enzyme, as predicted from the three-dimensional structure of the Populus XET (36). However, after the HvXET5 enzyme was purified, its activity remained more or less constant for at least a year at −20 °C. The difficulties with enzyme purification might also explain why so few XTHs have been purified from plant tissue extracts (24, 38).

Most of the known enzymic properties of XTHs have been determined following heterologous expression of the corresponding cDNAs (25, 26, 39–41).

The HvXET5 isoenzyme that was purified in the present work catalyzed, in vitro, the formation of covalent linkages between celluloses such as chemically modified or paracrystalline HEC and sulfuric acid swollen cellulose or (1,3;1,4)-β-D-glucans and xyloglucans (Figs. 3–6). The polysaccharides are linked from reducing to nonreducing ends of donor and acceptor substrates, respectively, rather than by cross-linking of the type observed between arabinoxylan chains through esterified hydroxycinnamic acids (7) or between pectic polysaccharides through borate (1, 42). The results are consistent with recent data from Ait Mohand and Farkas (43), who showed hetero-transglycosylating activity in unpurified extracts from nasturtium (Tropaeolum majus), but they were not able to unambiguously assign the enzyme responsible. The HvXET5 activity represents a non-Leloir type of biosynthetic reaction, insofar as the energy required for the formation of the new glycosidic linkage is provided from an existing glycosidic linkage rather than from a sugar nucleotide-activated donor. The data shown in Fig. 3A is particularly important with respect to the action pattern of the barley XET. The presence of fluorescent material of intermediate molecular mass, which is with a molecular mass between that of the starting HEC and the fluorescent acceptor substrate XGO-SR, indicates that the enzyme acts in an essentially stochastic manner. Conversely, when the HEC is tagged at its reducing terminus with the fluorescent XGO-SR, the absence of fluorescent products of intermediate sizes (Fig. 3B) indicates that the enzyme has a preference for binding and cleaving at the xylosylated XGO-SR tag, which is positioned at the reducing end of the HEC:XGO-SR conjugate. It should also be noted that during chemical modification of cellulose with hydroxyethyl groups, the HEC product is likely to be substituted primarily on the more reactive C-6 hydroxyethyl groups, perhaps in a block-wise fashion (44). If this were the case, the HEC substrate might represent a structural analog of xyloglucan. However, the barley (1,3;1,4)-β-D-glucan clearly acts as a donor substrate, and cello-oligosaccharides act as acceptor substrates. We therefore believe that the potential for these hetero-transglycosylation reactions to occur in vivo warrants further investigation.

The substrate specificity of XET enzymes, which involves cleaving a (1,4)-β-D-glucosyl linkage in the donor substrate before transfer to the nonreducing end of the acceptor substrate, would suggest that the HvXET5 re-forms a (1,4)-β-linkage between the reducing end glucosyl residue of the donor polysaccharide, whether that be the HEC or the barley (1,3;1,4)-β-D-glucan, and the nonreducing end of the XXXG-SR acceptor substrate. It is considered unlikely that the polymeric donor molecules would be attached to the xylosyl residues of the XXXG-SR acceptor substrate.

The rate of the reaction catalyzed by the HvXET5 enzyme described here with HEC is comparable with that on TXG (Table 2). Values for the $K_m$ and $k_{cat}$ constants with TXG were 3 mg·ml$^{-1}$ and 1 × 10$^{-7}$ s$^{-1}$, respectively, and for the acceptor substrate XXXGol the values of $K_m$ and $k_{cat}$ were 69 × 10$^{-6}$ M and 1.5 × 10$^{-7}$ s$^{-1}$, respectively.4 The rate of the reaction with (1,3;1,4)-β-D-glucan is relatively slow (Table 2), but it would be anticipated that contact between a large molecular mass donor-enzyme complex and the nonreducing terminus of the acceptor substrate might not occur quickly. If the HvXET5 enzyme were

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to catalyze these reactions in vivo, cellulose, (1,3;1,4)-β-D-glucans and xyloglucans in barley walls could potentially be linked to create a very large, continuous molecular network within the wall and would significantly alter the strength of walls, their porosity, and flexibility (Fig. 6). Potential accessibility and diffusion limitations in the cell wall environment could greatly reduce the catalytic rates in muro. The cell might compensate for this through the synthesis of relatively large amounts of stable enzyme, and this would be consistent not only with the high levels of XET mRNA transcripts that are found in plant cells (23 and data not shown) but also with the long term stability of the HvXET5 observed here. In any case, it has not yet been demonstrated that the ability of XETs to covalently link different polysaccharides has in muro significance. To do so would require the isolation of a short fragment of 10 or fewer glycosyl residues that can be clearly shown to originate from two distinct polysaccharide types (such as cellulose and xyloglucan). These “linkage regions” of two different polysaccharide types will only constitute a tiny proportion of total wall polysaccharides and have not been detectable using current technologies.

Emerging information on the remodeling of fungal cell walls during spore formation and under stress indicates that glycosylphosphatidylinositol-anchored transferase enzymes, some of which are members of family GH16, might also be involved in linking different polysaccharides such as β-D-glucans and chitin in the wall (45). There are also indications that pectic polysaccharides might be covalently linked with xyloglucans in plant cell walls (10–13). However, the purified HvXET5 enzyme did not link polygalacturonan or β-D-galactans to xyloglucan, nor did the HvXET5 enzyme link arabinoxylan to xyloglucans, despite suggestions based on molecular modeling (23) that this was a possibility. However, there are multiple isoforms of XETs in plant cells (23, 33–35, 46), and it remains possible that other isoforms might prefer different donor and acceptor substrate specificities. Preliminary data show that the partially purified HvXET6 enzyme has lower catalytic rates with celluloses and (1,3;1,4)-β-D-glucan (not shown).

If covalent linkages between different polysaccharides do occur in plant cell walls, they will have important implications for wall rigidity, strength, and porosity. A thorough understanding of covalent linkages between wall polysaccharides would also provide opportunities to genetically manipulate agro-industrial processes such as paper production, food quality and texture, malting and brewing, bioethanol production, dietary fiber, and ruminant digestibility. We are now generating transgenic barley lines, in which selected HvXET genes have been up- and down-regulated, to further investigate this potential.

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