An enzyme activity capable of endotransglycosylation of heteroxylan polysaccharides is present in plant primary cell walls

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Abstract

Heteroxylans in the plant cell wall have been proposed to have a role analogous to that of xyloglucans or heteromannans, forming growth-restraining networks by interlocking cellulose microfibrils. A xylan endotransglycosylase has been identified that can transglycosylate heteroxylan polysaccharides in the presence of xylan-derived oligosaccharides. High activity was detected in ripe fruit of papaya (*Carica papaya*), but activity was also found in a range of other fruits, imbibed seeds and rapidly-growing seedlings of cereals. Xylan endotransglycosylase from ripe papaya fruit used a range of heteroxylans, such as wheat arabinoxylan, birchwood glucuronoxylan and various heteroxylans from dicotyledonous primary cell walls purified from tomato and papaya fruit, as donor molecules. As acceptor molecules, the enzyme preferentially used xylopentaitol over xylohexaitol or shorter-length acceptors. Xylan endotransglycosylase was active over a broad pH range, and could perform transglycosylation reactions up to 55°C. Xylan endotransglycosylase activity was purified from ripe papaya fruit by ultrafiltration and cation exchange chromatography. Highest endotransglycosylase activity was identified in fractions that also contained high xylan hydrolase (xylanase) activity, and correlated with the presence of the endoxylanase CpaEXY1. Recombinant CpaEXY1 protein transiently over-expressed in *Nicotiana benthamiana* leaves showed both endoxylanase and xylan endotransglycosylase activities *in vitro*, suggesting that CpaEXY1 is a single enzyme with dual activity *in planta*. Purified native CpaEXY1 showed 2 to 4-fold higher endoxylanase activity than endotransglycosylase activity, suggesting that CpaEXY1 may act primarily as a hydrolase. We propose that xylan endotransglycosylase activity (like xyloglucan and mannan endotransglycosylase activities) could be involved in remodelling or re-arrangement of heteroxylans of the cellulose-non-cellulosic cell wall framework.

Keywords: Cell wall, Endoxylanase, Heteroxylan, Papaya, Xylan endotransglycosylase

Abbreviations

CTAB	Cetyltrimethylammonium bromide
WAX	Wheat arabinoxylan
PAHBAH	<i>p</i> -hydroxybenzoic acid hydrazide
XET	Xyloglucan endotransglycosylase
XTH	Xyloglucan endotransglycosylase/ hydrolase
Xyl ₂ -ol	Xylobiitol

Xyl ₃ -ol	Xylotriitol
Xyl ₄ -ol	Xylotetraitol
Xyl ₅ -ol	Xylopentaitol
Xyl ₆ -ol	Xylohexaitol

Introduction

The framework upon which the primary cell wall of land plants is based, and from which it derives its strength, is a structural network of cellulose microfibrils held together non-covalently by pectins and a group of polysaccharides known as the hemicelluloses (Park and Cosgrove 2012). The resulting network forms the major load-bearing structure in the primary cell wall and, apart from providing support, is also responsible for restricting growth. Plants have mechanisms for loosening and modifying this structure during growth and development without loss of wall strength, allowing turgor-driven cell expansion and the incorporation of new materials into the expanding wall.

The composition of hemicelluloses varies with species, tissue types and stages of development, but is diverse and usually consists of xyloglucans, heteroxylans (xylans) and heteromannans in varying proportions. In the primary walls of dicotyledonous plants and non-commelinoid monocotyledons, the most abundant hemicellulose is xyloglucan, a polysaccharide with a (1→4)-β-D-glucan backbone that is regularly substituted with xylose residues and, depending on species and tissue, can be further decorated with galactose, fucose and/or arabinose as well as non-glycosyl (o-acetyl) residues. The integration of new xyloglucan into the wall is accomplished by the endotransglycosylase activity of xyloglucan endotransglycosylase/hydrolases (XTH). Here, a xyloglucan chain is cleaved by xyloglucan endotransglycosylase (XET) activity and the newly-created reducing end attached to the non-reducing end of another xyloglucan molecule (Farkaš et al. 1992; Fry et al. 1992; Nishitani and Tominaga 1992). XTHs are members of a large gene family with most members possessing only XET activity and lacking xyloglucanase activity, although a few proteins are predominantly xyloglucanases and have the potential for XET activity under certain conditions (Eklöf and Brumer 2010; Baumann et al. 2007). Xyloglucanase activity is most commonly present in germinating seeds, where the activity hydrolyses storage xyloglucans to provide substrates for the growing embryo (Edwards et al. 1986), and in ripening fruit during fruit softening (Maclachlan and Brady 1994).

Heteromannans have a backbone consisting either of (1→4)-β-D-mannan (mannans, galactomannans) or of alternating regions of (1→4)-β-D-mannose and (1→4)-β-D-glucose

which can be either unsubstituted (glucomannan) or substituted with galactose (galactoglucomannan) (Melton et al. 2009). Glucomannans and galactoglucomannans also contribute to non-covalent microfibril cross-linking, although galactoglucomannans are generally shorter polymers than xyloglucans and bind less strongly to cellulose (Whitney et al. 1998). A mannan endotransglycosylase activity has been characterised in ripe tomato fruit and was identified as the endo- β -mannanase LeMAN4 (Schröder et al. 2004, 2006). Unlike most XTH proteins, LeMAN4 is a dual activity enzyme. Whereas endo- β -mannanase activity is associated with the breakdown of mannans either during seed germination or fruit softening (Bewley et al. 1997, 2000), mannan endotransglycosylase activity has been proposed to have an equivalent role to XET in the plant cell wall, remodelling it during all developmental stages (Schröder et al. 2009).

Heteroxylans are a divergent group of polymers consisting of a (1 $\rightarrow$ 4)- β -D-xylan backbone, which is substituted in all land plants with short side chains (usually monosaccharide) of arabinose and/or glucuronic acid residues. In the primary walls of the commelinoid monocots, that includes the Poales (grasses), glucuronoarabinoxylans constitute up to 40% of the wall, where, together with unbranched (1 $\rightarrow$ 3, 1 $\rightarrow$ 4)- β -D-glucan, they are the major structural non-cellulosic polysaccharide (Brummell and Schröder 2009). Heteroxylans are also commonly present as structural components in the primary cell walls of dicots, although they are less abundant than xyloglucan. The structure and properties of these dicot xylans are less well known, as are the potential modifications occurring during the cell wall loosening that allow growth and during the cell wall disassembly that cause fruit softening. It is likely that in both dicot and commelinoid monocots cell walls, heteroxylans interact non-covalently with cellulose and form inter-microfibril cross links, in a manner similar to that of either xyloglucans or mannans, although the interactions are less strong (Carpita 1983).

Endo-(1 $\rightarrow$ 4)- β -xylanase (endoxylanase) activity is well known in plants, including in germinating seeds of some monocots (notably cereals) and dicots, and in ripening fruit tissue of some dicot species (Brummell and Schröder 2009). Plant endoxylanases are typically found as small gene families, for example 12 genes in *Arabidopsis* and 10 in rice (Yokoyama and Nishitani 2004), and at least 4 and possibly 6 genes in papaya (Ming et al. 2008). *Arabidopsis* possesses four putative endo-1,4- -xylanases with a putative signal sequence for secretion to the apoplast, suggesting that heteroxylan metabolism is involved in some aspects of plant development. A large increase in endoxylanase activity accompanies fruit ripening in papaya (Paull and Chen 1983), indicating that in some species heteroxylan depolymerisation may be a component of the wall disassembly leading to fruit softening. All plant endoxylanases undergo extensive post-translational modifications where C- and N-terminal ends, possibly acting as

chaperones during protein folding, are removed before a catalytic domain of approximately 32 kDa is released, representing the active enzyme (Brummell and Schröder 2009).

We have previously proposed that a xylan endotransglycosylase activity analogous to XET and mannan endotransglycosylase is likely to exist in higher plants (Schröder et al. 2009, 2010). Endotransglycosylase reactions involving xylo-oligosaccharides have been reported for bacterial xylanases, where xylo-oligosaccharides acted both as donor and acceptor, and acceptor concentrations had to be in the mM range for the transglycosylation reaction to occur (Roberge et al. 1999; Moreau et al. 1994). A similar reaction has been described for fungal glucanases (Biely et al. 1991), as well as for plant endo- β -mannanases (e.g. Coulombel et al. 1981; Marraccini et al. 2001) and xylan endo- and exotransglycosylases from several plant species, including oat and broad bean (Franková and Fry 2011; here named trans- β -xylanases and trans- β -xylosidases, respectively).

In this paper, we report the presence of xylan endotransglycosylase activities in a wide range of tissues and species, including ripe fruit, imbibed seeds and rapidly growing seedlings, by using high molecular weight polymeric heteroxylan polysaccharide donor substrates and tritiated xylo-oligosaccharides as acceptor substrates, with acceptor substrate concentrations being in the μ M range. In papaya, we show that xylan endotransglycosylase activity increases as the fruit ripens, characterise its biochemical properties, and show that it co-purifies with the xylanase CpaEXY1. The potential roles of xylan endotransglycosylase activity during re-arrangement of cell walls are discussed.

Materials and methods

Plant material

Papaya fruit *Carica papaya* L., 'Rainbow' and 'Sunset' fruit were harvested from the University of Hawaii at Manoa's Poamoho Experiment station in Central Oahu, Hawaii, USA and ripened at 22°C. Papaya fruit 'Sunrise' from Fiji and a yellow-fleshed variety of 'Solo' parentage termed 'PPY' from the Philippines were purchased at a local fruit market in Auckland, New Zealand. Ripeness was determined by skin colour (green = unripe, 50% yellow = medium ripe, 100% yellow = ripe). Ripe pineapple (*Ananas comosus*), banana (*Musa domestica*), avocado (*Persea americana*), kiwifruit (*Actinidia deliciosa* 'Hayward'), apple (*Malus pumila* 'Royal Gala'), pear (*Pyrus communis* 'Packham's Triumph'), Ya pear (*Pyrus bretschneideri*), and cucumber (*Cucumis sativus*) and celery (*Apium graveolens* var. *dulce*) were purchased at a local fruit market in Auckland, New Zealand. Fungicide-free barley, maize, oat and wheat seeds were purchased from

Newton Seeds (Onehunga, Auckland, New Zealand). The seeds were sterilized for 30 min in 1% bleach with a few drops of Tween 20, rinsed with sterile water, placed onto moist cotton wool in sterile clear plastic dishes, and kept at 21°C under a 12 h light followed by 12 h dark cycle. Imbibed seeds were harvested when the radicle had just emerged (max. 1 mm long). Seedlings were harvested (without root) at 2 to 3 cm long. All tissues were snap-frozen in liquid N₂ and stored at -80°C.

Crude enzyme extractions

Tissue was finely ground in liquid N₂ and extracted with 5 volumes of 0.1 M Mes, pH 5.5, containing 1.3 M NaCl, 0.2 M EDTA and protease inhibitor (Complete™, Roche). After thawing on ice, the extract was mixed by vortexing, left on ice for 45 min, and centrifuged at 11,000 *g* for 10 min at 4°C. The supernatant was collected and assayed for xylan endotransglycosylase and endoxylanase activity.

Polysaccharide and oligosaccharide substrates for endotransglycosylase assays

Wheat arabinoxylan (WAX; low viscosity) and birchwood xylan were purchased from Megazyme (Bray, Ireland). Xyloglucan and galactoglucomannan from kiwifruit were purified as described in Schröder et al. (2001). Polygalacturonic acid from citrus was purchased from Sigma, St Louis, MO).

Heteroxylans (1 M and 4 M KOH-soluble) from tomato and papaya were prepared from cell walls of ripe papaya fruit 'PPY' and tomato (*Solanum lycopersicum* 'Daniela') by homogenising in methanol-chloroform-water-formic acid (16:5:1:1, by vol.), and then sequentially extracted using dimethylsulfoxide, trans-1,2-cyclohexane diaminetetraacetic acid, Na₂CO₃, 1 M KOH and 4 M KOH as described in Prakash et al. (2012). Heteroxylans were purified from 1 M KOH- and 4 M KOH-soluble fractions by precipitation with cetyltrimethylammonium bromide (CTAB) as described in Schröder et al. (2001). In brief, fractions (1 M and 4 M KOH-soluble) were dissolved in water, CTAB (1%, by vol.) added to a final concentration of 0.05% (by vol.), and heteroxylans precipitated overnight at room temperature. The pellet was recovered by centrifugation and suspended in 4 M NaCl. CTAB was removed by repeatedly adding ethanol saturated with Na-acetate crystals, and the precipitate allowed to sediment. The precipitate was dissolved in water and dialysed against water and freeze-dried. Sugar composition was analysed according to Albersheim et al. (1967), Blumenkrantz and Asboe-Hansen (1973) and Ahmed and Labavitch (1977).

Galactoglucomannan-glucuronoxylan I and II (GGM-GX I, II) were prepared from ripe tomato cell walls according to Prakash et al. (2012). GGM-GX I was purified from the DMSO extract and GGM-GX II from the CTAB supernatant of the 4 M KOH extract. Linkage analysis by methylation of GGM-GX I was carried out as described by Pettolino et al. (2012).

For analysis of rate of hydrolysis, reduced WAX was used to keep background reducing power low in reducing end assays. WAX (250 mg) was incubated with NaB(H₄) (10 mg mL⁻¹, total volume 25 mL) overnight at room temperature. The reaction was stopped using glacial acetic acid until H₂ production ceased. Salts were removed by dialysis for 5 d at 4°C against water (molecular weight cut-off 12 kDa; daily water change), and the dialysate freeze-dried to give reduced WAX.

Heteroxylan-derived oligosaccharides (xylo-oligosaccharides) were purchased from Megazyme. For general xylan endotransglycosylase assays, xylotriose (Xyl₃), xylotetraose (Xyl₄) and xylopentaose (Xyl₅) were mixed at a ratio of 1:1.4:2.4 (by weight), and tritiated in 0.2 M NaOH as described in Schröder et al. (2006) using 0.74 GBq NaB[³H]₄ (American Radiolabeled Chemicals, St Louis, MO) by conversion to alditols. The tritiated xylo-oligosaccharide alditols were desalted, freeze-dried and dissolved in water (4 µg µL⁻¹; specific activity 37.3 kBq µg⁻¹ *or, on average, approximately 22 MBq µmol⁻¹*).

For determination of oligosaccharide specificity of crude papaya xylan endotransglycosylase, 3.1 mg Xyl₂ (xylobiose), and 7.0 - 7.5 mg Xyl₃, Xyl₄, Xyl₅ and Xyl₆ (xylohexaose) were tritiated individually, using 0.481 GBq NaB[³H]₄ for Xyl₃, Xyl₄ and Xyl₅ and 0.37 GBq NaB[³H]₄ for Xyl₂ and Xyl₆. The specific activity of the corresponding tritiated alditols was 96.4, 49.2, 42.5, 21.7 and 35 kBq µg⁻¹, respectively (*or, on a molar base, 27.7, 20.4, 23.2, 14.7, 28.4 MBq µmol⁻¹ respectively*).

For enzyme kinetics, Xyl₅ (10 mg) was tritiated with 0.74 GBq NaB[³H]₄, giving a specific activity of 50.3 kBq µg⁻¹ (*34 MBq µmol⁻¹*). Tritiated oligosaccharides were counted using Liquid Scintillation Cocktail (ACS-II, Amersham), with a counting efficiency of 28%.

Xylan endotransglycosylase assays

Reaction mixtures (20 µL) contained 0.25% (w/v) polysaccharide solution and 0.04% (w/v) [³H]Xyl₃-ol, [³H]Xyl₄-ol and [³H]Xyl₅-ol mixture (298 kBq) in 0.2 M Mes-NaOH buffer, pH 5.5. Reactions were carried out at 21°C. Volumes of extract and incubation time were adjusted as needed to provide linearity of assays. Reactions were stopped by adding 50 µL of 40% (v/v) formic acid. Background reactions were performed by adding formic acid before the enzyme extract, and values subtracted from those obtained using active enzyme extract. The [³H]-labelled polysaccharides produced in the assays were separated from un-reacted

[³H]oligosaccharides by their immobility during paper chromatography. Reaction products were applied to 46 x 27 cm sheets of 3MM paper (Whatman, Maidstone, UK) and mobile, unreacted [³H]oligosaccharides were removed by chromatography in ethyl acetate:acetic acid:water (10:5:6, by vol.) for 48 h after Fry et al. (1992). Initial trials using [³H]xylo-oligosaccharides and WAX without addition of active enzyme showed background radioactivity of approximately 5% with a standard deviation of 0.23% remaining at the site of original application. The addition of enzyme in background reactions mentioned above, or other donor substrates than WAX, did not significantly influence this value (data not shown). After chromatography, the paper was dried; the spots where the reaction mixture had been applied were cut out, and assayed for ³H by liquid scintillation counting using organic counting scintillant (OCS, Amersham) with a counting efficiency of 40%. Endotransglycosylase activities are given as Bq of radioactivity incorporated into high molecular weight products per kBq of radioactive oligosaccharides supplied per h, or as indicated.

Endoxylanase assays

Endoxylanase activity was determined by measuring the release of Remazol dye from water-soluble birch xylan after Chen and Paull (2003). The substrate [0.4 mL of 0.1% 4-*O*-methyl-D-glucurono-D-xylan-Remazol Brilliant Blue (RBB) (Sigma) and 10 µg bovine serum albumin in 50 mM citric acid, pH 5.5] was incubated with enzyme extract and buffer (final volume 50 µL) at 37°C. Incubation time was 4 h unless indicated otherwise. The reaction was terminated by the addition of 95% ethanol (0.9 mL) to precipitate non-degraded xylan-RBB. After 20 min, the mixture was centrifuged at 16000 *g* for 10 min and the absorbance of the supernatant measured at 595 nm.

Purification of xylan endotransglycosylase activity from papaya fruit

Ripe mesocarp of 'Rainbow' (100% yellow skin colour, flesh firmness of ca. 27 N) was blended in 25 mL of ice-cold buffer (50 mM NaH₂PO₄, pH 4.5, 1 mM dithiopyridine, 1 mM phenylmethylsulfonyl fluoride) until smooth, then centrifuged at 2°C at 14000 *g* for 15 min (Chen and Paull 2003). The supernatant was filtered through Miracloth (Calbiochem, San Diego, CA), and soluble pectin removed by ultrafiltration (MWCO 100 kDa). The flow-through containing xylan endotransglycosylase activity was concentrated by ultrafiltration (MWCO 10 kDa), and the retentate (1 mL) loaded onto S-Sepharose (10 x 10 mm; GE Healthcare, Piscataway, NJ) equilibrated in 50 mM phosphate buffer, pH 4.5, and eluted at a flow rate 1 mL min⁻¹ with 10 mL 200 mM NaCl in phosphate buffer, pH 4.5. After de-salting and concentrating

the eluate by ultrafiltration (MWCO 10 kDa), the retentate (1 mL) was applied to a column of MonoS HR 5/5 (GE Healthcare) equilibrated in phosphate buffer, pH 4.5, and eluted using a salt gradient from 0 to 500 mM NaCl, collecting 1 mL fractions. Fractions were assayed for xylan endotransglycosylase and xylanase activity.

For determining enzyme kinetics, activity was purified from ripe papaya 'PPY' fruit with the following modifications. After ultrafiltration (100 kDa followed by 30 kDa), the 30 kDa retentate (approximately 5 mL, equilibrated in 50 mM NaOAc, pH 5.0) was loaded onto a HiTrap SP HP column (5 mL bed volume) equilibrated in 50 mM NaOAc, pH 5.0, and eluted with a linear gradient of 50 mM NaOAc/0 mM NaCl to 50 mM NaOAc/500 mM NaCl over 50 mL, then to 50 mM NaOAc/1000 mM NaCl over 10 mL, and 2 mL fractions were collected. Fractions were assayed for xylan endotransglycosylase and xylanase activity. Fractions 12 and 13 were similar with respect to enzyme activities and purity as fraction 16 of the previous purification (data not shown).

Transient expression of recombinant CpaEXY1 protein in *Nicotiana benthamiana* Domin

The complete open reading frame of endoxylanase *CpaEXY1* (GenBank accession number AY138986, Chen and Paull 2003) was PCR-amplified using forward primer 5'-ACAGCATGCA GCTTGGAGAG AAGAACTTAC AG-3' and reverse primer 5'-AATCCTAGGT TAAACTTTAA TGAGAAGTAA TCTTGTTTG-3'. These primers added a unique *SphI* site at the translation initiation ATG (and changed the second amino acid residue from Lys to Gln), and added an *AvrII* site immediately downstream of the translation stop. The 1.7 kb PCR product was ligated into the viral vector TTOSA1 APE (Kumagai et al. 2000) that had been digested with *SphI* and *AvrII*, placing the *CpaEXY1* transgene under the control of a tobamovirus subgenomic promoter, to produce plasmid TTOSA1-XYL. Approximately 10 days after inoculation of lower leaves of young plants of *N. benthamiana*, leaf tissue from plants transfected with TTOSA1-XYL or with empty TTOSA1 vector, respectively, was finely ground in liquid N₂ and extracted with 50 mM sodium citrate, pH 4.5, containing 1 mM dithiopyridine, 1 mM phenylmethylsulfonyl fluoride. The supernatant was recovered after centrifugation and assayed for xylan endotransglycosylase and endoxylanase activity. Protein contents of extracts were 0.539 µg µL⁻¹ (TTOSA1-XYL) and 1.108 µg µL⁻¹ (empty TTOSA1 vector).

Polyacrylamide gel electrophoreses and protein gel blot analyses

Proteins were separated by one-dimensional SDS-PAGE using 10% polyacrylamide separating gels, which were stained with colloidal Coomassie stain. Protein molecular mass markers used

were Precision Plus Protein™ Dual Color (Bio-Rad, Hercules, CA). Protein concentrations were determined using a Protein Assay Kit (Bio-Rad) with bovine serum albumin as a standard. Protein gels were electroblotted to polyvinylidene fluoride membrane and immunodetection carried out as described in Nieuwenhuizen et al. (2007) using antibodies developed against the xylanase catalytic module from papaya (Manenoi and Paull 2007) at a concentration of 1:5000 (by vol.) and gel blots developed using goat anti-rabbit IgG–alkaline phosphatase secondary antibody (Sigma) and 1-Step NBT/BCIP as substrate (Pierce Chemicals, Dallas, TX). Protein gel images were scanned using a Typhoon 9400 Variable Mode Imager (GE Healthcare).

Peptide sequencing

Proteolytic fragments of the protein gel band corresponding to the major xylanase band after protein gel blot analysis, and using an aliquot of fraction 16 eluted from the MonoS column, were generated by digestion with trypsin. Gel fragments and 100 µg of protein from fraction 16 from the MonoS column were reduced with 10 mM DTT then alkylated with 10 mM iodoacetamide to prepare for tryptic digestion, which was performed following Jimenez et al. (2003). The peptide fragments were analyzed by LC-MS at Plant & Food Research, Ruakura, New Zealand against a database of all proteins in the papaya genome (<http://www.phytozome.net/papaya.php>).

Enzyme kinetics of xylan endotransglycosylase and endoxylanase activities of native CpaEXY1 protein

Assays for hydrolysis contained the following: WAX (reduced; 0.2% or 0.4% final concentration w/v), NaOAc pH 5.5 (32 mM), and purified native CpaEXY1 enzyme from ripe papaya 'PPY' (0.007 µg µL⁻¹ protein) in a total volume of 250 µL. Reactions were stopped by adding 750 µL PAHBAH reagent (1% p-hydroxybenzoic acid hydrazide in 0.5 M NaOH). To determine the number of reducing ends created through hydrolysis, samples were boiled for 5 min, and after cooling, absorbance was measured at 410 nm (Lever, 1972). For t=0, PAHBAH reagent was added before addition of enzyme. Total A_{410} values obtained with inactive enzyme (t=0) were subtracted from total A_{410} values obtained with active enzyme, and for determination of the hydrolysis reaction rate the nmol of reducing ends generated per minute per µg protein were calculated. Xylose in 50 mM NaOAc (pH 5.5) was used to create a standard curve.

Assays for transglycosylation were the same as the assays for hydrolysis except for the presence of [³H]Xyl₅-ol, in a total volume of 25 µL. [³H]Xyl₅-ol acceptors were tested at 467, 234, and 117 µM over a time period of 60 min. At 234 µM acceptor concentration and 30 min

reaction time, three different donor concentrations were tested. The reactions were terminated by adding 50 μ L of formic acid (40%), un-reacted [3 H]Xyl₅-ol acceptors were removed by paper chromatography, and enzyme activity quantified by scintillation counting using non-aqueous scintillant (counting efficiency 40%) as described above. The radioactivity per nmol acceptor [3 H]Xyl₅-ol offered in the reactions was estimated by counting appropriate concentrations on paper using non-aqueous scintillant, as the cpm values of tritiated oligosaccharides on paper were lower than in solution, presumably because they penetrate into the paper matrix (Fry et al. 1992). Results were calculated as nmol [3 H]Xyl₅-ol incorporated per minute per μ g protein, assuming each molecule of acceptor incorporated into WAX equalled an endotransglycosylase reaction. All reactions were carried out at 21°C.

Results

Characterisation of xylan endotransglycosylase activity in papaya extracts

Xylan endotransglycosylase activity was detected in crude extracts of ripe papaya fruit mesocarp by measuring the incorporation of tritiated ([3 H]) xylo-oligosaccharide substrates into a high molecular weight polymeric wheat arabinoxylan (WAX) substrate. The assay required removal of un-reacted [3 H]-xylo-oligosaccharides by their differential mobility during paper chromatography. This procedure was used, rather than the 'paper-binding assay' for assay of XET and mannan endotransglycosylase (Fry et al. 1992; Schröder et al. 2004), since neither WAX nor the tomato heteroxylans tested bound well to paper under aqueous conditions (data not shown). As acceptors, a mixture of [3 H]-xylo-oligosaccharides ([3 H]-Xyl₃-ol, [3 H]-Xyl₄-ol and [3 H]-Xyl₅-ol) was chosen to maximize the chance of discovering the activity. Using crude papaya extract (10 μ L) and a donor substrate concentration of 0.25% (w/v) with 0.04% (w/v) [3 H]xylo-oligosaccharides as acceptor, the assay was approximately linear for up to 180 min (data not shown). Therefore, xylan endotransglycosylase assays were routinely carried out under these substrate conditions and at a shortened assay time of 60 min.

To prove that the radioactive oligosaccharides were incorporated into high molecular weight WAX, the xylan endotransglycosylase product was treated with either trypsin or with a mixture of fungal xylanases (Prakash et al. 2012), respectively, and paper chromatography carried out to test whether tritiated polysaccharides had been degraded. The xylan endotransglycosylase reaction product was insensitive to protease treatment as [3 H] incorporation into WAX was identical before and after treatment (data not shown). The xylan endotransglycosylase reaction product was sensitive to fungal xylanase treatment, as

radioactivity at the origin after paper chromatography was reduced to background levels, showing that the radioactive oligosaccharides were incorporated, presumably by transglycosylation, into high molecular weight WAX.

Fig. 1 shows the incorporation of [^3H]-xylo-oligosaccharides into high-molecular weight WAX by separating the products using size exclusion chromatography. The eluate was monitored for total carbohydrate and [^3H] in reactions with and without active enzyme. The hashed area indicates incorporation of [^3H] into high-molecular weight polysaccharide, and had a similar shape to [^3H] incorporated into high-molecular weight xyloglucan by ripe kiwifruit XET (Schröder et al. 1998). Elution profiles at $t=0$ show that WAX and [^3H]-xylo-oligosaccharides were well separated under these conditions and that without addition of active enzyme, no [^3H] label was present in high molecular weight WAX.

Papaya xylan endotransglycosylase did not appear to bind tightly to the cell wall. A NaCl concentration of 50 mM in the extraction buffer was sufficient to release the enzyme activity, and a subsequent extraction of the residue with buffer containing 1.3 M NaCl did not release further activity (data not shown). However, since this may not be the case for xylan endotransglycosylase activity from other tissues or other species, protein was extracted using Mes-NaOH buffer containing 1.3 M NaCl for papaya and all other tissues used subsequently. Xylan endotransglycosylase in papaya extracts was stable at 4 C for at least ten days, and could be frozen at -80 C and thawed repeatedly with minimal loss of activity (data not shown).

McIlvaine buffer and a Universal Tribuffer system (Kuo et al. 1985) were used to determine the pH optimum of xylan endotransglycosylase from papaya fruit. With $\sim 50\%$ maximal activity at pH 4.0 or 7.0, the enzyme exhibited activity over a relatively broad pH range (Fig. 2a), with two possible optima, the first between pH 4.5 and 5.0 and the second around pH 6.2 (with slightly less activity). Interestingly, endoxylanase activity from papaya, which was also present in the crude extracts, followed a similar pH pattern (Chen and Paull 2003).

Xylan endotransglycosylase activity was equally efficient within a temperature range of 21°C to 55°C (Fig. 2b), above which the activity declined. In contrast, the competing endoxylanase activity showed increased efficiency between 21°C and 45°C , above which it declined sharply, confirming observations of Chen and Paull (2003). At temperatures above 60°C , essentially no endoxylanase activity was detected (Chen and Paull 2003). Endoxylanase activity was only about 30% of the maximum xylan endotransglycosylase activity at a temperature of 21°C .

To examine the donor substrate specificity of a crude papaya fruit xylan endotransglycosylase preparation, commercial preparations of WAX and birchwood glucuronoxylan together with dicot fruit cell wall heteroxylans from papaya and tomato were tested (Fig. 3a). Fractions derived from the CTAB precipitate of 1 M or 4 M KOH-soluble extracts

of papaya and ripe tomato cell walls were both enriched in xylose and uronic acid, indicating the presence of heteroxylans (Table 1). The galactoglucomannan-glucuronoxylan I and II complexes were derived, respectively, from the DMSO-soluble extract of the cell wall (GGM-GX I) and from the CTAB supernatant of the 4 M KOH-soluble extract of tomato (GGM-GX-II) (Prakash et al. 2012). The linkage composition of these fractions is shown in Table 2. Of the heteroxylan donor substrates tested, WAX, the 1 M KOH-soluble preparations from tomato and papaya cell walls, and tomato GGM-GX I were the most efficient donors. Glucuronoxylan from secondary birchwood cell walls was less active, as were the tightly-bound heteroxylans from tomato and papaya cell walls. Partially purified xyloglucan from kiwifruit and highly purified galactoglucomannan from kiwifruit were negligible donor substrates for xylan endotransglycosylase, whereas polygalacturonic acid from citrus showed some donor substrate activity.

To determine the preferences of the papaya enzyme activity for a certain length of acceptor substrate, xylo-oligosaccharides ($[^3\text{H}]\text{Xyl}_2\text{-ol}$ up to $[^3\text{H}]\text{Xyl}_6\text{-ol}$) were assayed for acceptor activity between approximately 100 and 500 μM , with WAX as the donor substrate. Fig. 3b shows that when assayed individually, $[^3\text{H}]\text{Xyl}_2\text{-ol}$ and $[^3\text{H}]\text{Xyl}_3\text{-ol}$ were very weak acceptors, whereas $[^3\text{H}]\text{Xyl}_4\text{-ol}$ and $[^3\text{H}]\text{Xyl}_6\text{-ol}$ were moderate acceptors. $[^3\text{H}]\text{Xyl}_5\text{-ol}$ was the preferred acceptor for xylan endotransglycosylase, being three-fold more effective than $[^3\text{H}]\text{Xyl}_4\text{-ol}$, and 2.5 fold more effective than $[^3\text{H}]\text{Xyl}_6\text{-ol}$. Why the longer $[^3\text{H}]\text{Xyl}_6\text{-ol}$ is a weaker acceptor than $[^3\text{H}]\text{Xyl}_5\text{-ol}$ is unclear, but worthy of further investigation.

Purification of xylan endotransglycosylase from ripe papaya fruit

Xylan endotransglycosylase was purified from papaya fruit 'Rainbow' through a combination of ultrafiltration and cation exchange chromatography on S-Sepharose and MonoS, followed by elution using a salt gradient. The MonoS eluate fractions had xylan endotransglycosylase activity in fractions 15-17, with a maximum in fraction 16 (Fig. 4a). A protein gel of these column fractions showed that most of the protein eluted before fraction 15 (Fig. 4b). From fraction 15 onwards, a few faint protein bands were visible using the colloidal Coomassie stain, indicating that the protein preparation was not pure. A protein band at approximately 32.5 kDa that cross-reacted with antibodies against papaya endoxylanase, an enzyme highly active in ripe papaya, indicated the presence of this protein in the preparation (Fig. 4c). Xylanase assays confirmed co-purification of endoxylanase, with the highest activity occurring in fraction 16 and corresponding to the xylan endotransglycosylase activity elution pattern (Fig. 4a).

The proteins present in fraction 16 were examined using LC-MS analysis to identify tryptic peptide fragments. The analysis yielded 45 peptide fragments that were 100% identical

to CpaEXY1 (Table S1), the major xylanase isoform in ripe papaya fruit (Chen and Paull 2003, GenBank accession number AY138986) and a member of glycoside hydrolase family 10 (GH10, http://www.cazy.org/GH10_eukaryota.html). Peptide fragments covering 54% of the catalytic domain of CpaEXY1 were identified (Fig. 5). No unique fragments corresponding to five other putative xylanase proteins in the papaya genome were detected (Table S2). Multiple peptide fragments corresponding to a thaumatin/osmotin protein (23 fragments), a serine protease (11), and a (1→3)- β -glucanase (14) were also observed (Table S1). LC-MS analysis of tryptic digests of the gel-purified immuno-detectable xylanase band at approximately 32.5 kDa also confirmed the presence of the CpaEXY1 protein (data not shown).

From these data we hypothesised that in papaya, xylan endotransglycosylase and endoxylanase activity could be dual activities of CpaEXY1. However, it was not possible to exclude that an additional protein that possessed xylan endotransglycosylase activity was also present at a very low concentration. To investigate this further, the full-length open reading frame of CpaEXY1 was transiently over-expressed in *N. benthamiana* leaves using a hybrid tobacco mosaic virus / tomato mosaic virus vector (TTOSA1-XYL). SDS-PAGE and western blot analyses using an antibody raised against the CpaEXY1 catalytic domain revealed a doublet of ~28-30 kDa (Fig. S1). This was smaller in size than the native processed immuno-detectable endo-xylanase protein from ripe papaya fruit, and showed that the CpaEXY1 protein was processed in a slightly different way in *N. benthamiana* than it was in papaya. No immuno-detectable protein bands were visible in extracts from leaves transfected with the empty vector.

Protein extracts from transfected *N. benthamiana* leaves with the viral vector TTOSA1-XYL were tested for xylanase and xylan endotransglycosylase activities and compared with those from leaves transfected with the empty TTOSA1 vector. The extract containing recombinant CpaEXY1 protein had both activities (Table 3), demonstrating that papaya CpaEXY1 is a single enzyme with both xylan endotransglycosylase and xylanase activities. After either extended assay times or in assays that contained protein concentrations above those stated in Table 3, the hydrolysis activity of endoxylanase caused decreasing transglycosylation activity by hydrolysing both donor and acceptor substrates (data not shown).

Kinetics of transglycosylase and hydrolase reactions of papaya endoxylanase

Due to the incorrect processing of the recombinant protein in *N. benthamiana*, it was inappropriate to use this for kinetic analysis of these activities. Instead we focussed on kinetic analysis of purified native protein from papaya with the assumption that a single protein possessed two activities. Endotransglycosylase and hydrolase reactions were carried out under

identical conditions, with the only difference being the presence of [³H]Xyl₅-ol as acceptor in the endotransglycosylase reaction.

The hydrolysis reaction was linear up to 125 min using 0.2 and 0.4 % (w/v) WAX, respectively (Fig. 6a). The doubling of the polysaccharide (donor) concentration approximately doubled the hydrolysis rate. Transglycosylation reactions were carried out using increasing acceptor concentrations at a constant donor concentration of 0.2% (w/v) WAX. Doubling the acceptor concentration from 234 to 467 μM [³H]Xyl₅-ol resulted in an almost doubling of the transglycosylation rate (Fig. 6b). An acceptor concentration of 117 μM resulted in a very low reaction rate, indicating that optimal acceptor concentrations were above this concentration. Increasing the donor concentration from 0.1% to 0.4% (w/v) at a constant acceptor concentration of 234 μM increased the endotransglycosylase reaction (Fig. 6c). In Fig. 6a and b, the transglycosylation and hydrolysis rates are directly comparable. At a donor concentration of 0.2% (w/v) WAX in both assays, the rate of hydrolysis was between 2 times (using acceptor concentration of 467 μM for transglycosylation) to 4 times (using acceptor concentration of 234 μM for transglycosylation) higher than the transglycosylation rate under identical conditions.

Xylan endotransglycosylase activity is found in a range of fruit, vegetable and cereal tissues

Xylan endotransglycosylase and xylanase activities were assayed in ripening papaya cultivars, other fruit and vegetable tissues, and in imbibed seeds and growing seedlings of monocots, to determine whether the enzyme played a role in either fruit ripening, mobilisation of seed storage xylans or cell expansion during growth. Tissues were chosen on the basis that they either had high heteroxylan content in the cell walls or were rich in xylanase activity (Brummell and Schröder 2009).

Fruit from a range of papaya cultivars was examined at different ripening stages to investigate whether xylan endotransglycosylase and xylanase activities showed a similar pattern during softening or whether either of the activities was predominant (Fig. 7a). Overall, the papaya cultivars showed different amounts of xylan endotransglycosylase and xylanase activity. In 'Sunset', 'PPY' and 'Sunrise', where different ripening stages were examined, both activities increased during ripening, except for 'Sunrise', where xylan endotransglycosylase activity did not increase between the 50% and 100% ripe stages. In 'PPY' and 'Sunrise', xylan endotransglycosylase activity was lower than in 'Sunset' and 'Rainbow'; here, xylanase activity seemed the predominant reaction.

Xylan endotransglycosylase activity was detected in protein extracts prepared from a range of other fruit and vegetable tissues (Fig. 7b), although in all cases the activity was much less than in ripe papaya fruit. Of these, xylan endotransglycosylase activity was found in celery

stalks, in flesh of ripe fruit of avocado and pineapple, as well as in apple peel protein extracts. Negligible activity was found in ripe tomato skin and outer pericarp, kiwifruit outer pericarp, flesh of European pear and Ya pear, and lettuce leaves (data not shown). Except for avocado, none of the tissues assayed showed detectable xylanase activity.

Xylan endotransglycosylase and xylanase activities were determined in extracts of imbibed germinating seeds and in rapidly growing seedlings of barley, oat, maize and wheat. Xylan endotransglycosylase activity was present in rapidly growing seedlings of all four species tested, whilst xylanase activity was barely detectable (Fig. 7c). In imbibed seeds, xylan endotransglycosylase and xylanase activities were found in oat and barley, and at much lower amounts in maize and wheat. The low xylanase and xylan endotransglycosylase activities could be due to either unsuitable pH or substrate conditions in the assay or the presence of endogenous xylanase inhibitors (Biely et al. 2008; Simpson et al. 2003). Although these inhibitors have so far been shown to act only as a defence mechanism against microbial pathogens, suggestions have been made that they may also have a potential role in the regulation of plant xylanases (Simpson et al. 2003).

Discussion

The present study has established that xylan endotransglycosylase activity is present in primary cell walls and is able to carry out transglycosylation reactions on a range of heteroxylan polysaccharides. Assaying the enzyme activity by its ability to attach the reducing ends of xylan donors to the non-reducing ends of tritiated xylan oligosaccharide acceptors, we found the widespread existence of xylan endotransglycosylase activity in both monocotyledonous and dicotyledonous plants, at different developmental stages. The highest activity was present in ripening papaya fruit. Here, the activity could be attributed to papaya endoxylanase CpaEXY1. Together with XET and mannan endotransglycosylase, xylan endotransglycosylase completes the suite of enzymes necessary for the incorporation of hemicelluloses into the developing plant cell wall.

Native papaya xylan endotransglycosylase used a wide range of heteroxylans from different sources as donor, although highest activity was found with WAX, suggesting that the enzyme preferentially used a more highly substituted xylan backbone rather than a less-substituted one such as birchwood 4-*O*-methyl-D-glucuronoxylan. Differences in donor molecular weight could also conceivably play a role in substrate preference. The donor polysaccharide concentrations used in the enzyme assays were similar to those used in assays for mannan endotransglycosylase or XET activity (Fry et al. 1992; Schröder et al. 2004). A

variation in donor preference was also found amongst the highly substituted substrates purified from tomato and papaya fruit primary cell walls. The loosely bound heteroxylans purified from 1 M KOH-soluble cell wall polysaccharides were better donor substrates than the tightly bound 4 M KOH-derived polysaccharides, and the DMSO-soluble very loosely bound GGM-GX I from tomato was much better than the firmly bound GGM-GX II purified from a 4 M KOH-soluble extract. This may be because of the relatively higher xylose contents of these preparations.

Papaya xylan endotransglycosylase and endoxylanase activities had similar pH optima, but the temperature sensitivity of the reactions was markedly different. This may indicate that papaya endoxylanase and xylan endotransglycosylase activity are due to either different but related proteins (possibly of the -xylanase family) or to different isoforms of CpaEXY1. The papaya genome contains a xylanase gene family of up to six members (Ming et al. 2008; Table S2), three of which possess a signal sequence targeting the mature protein to the apoplast. However, tryptic digests of the highly purified protein fraction from MonoS chromatography yielded only xylanase fragments homologous to CpaEXY1 and not other putative xylanases, showing that isoforms of CpaEXY1 were the most abundant protein in the preparation.

Papaya fruit endoxylanase, like all other plant xylanases, undergoes extensive post-translational modifications (Chen and Paull 2003). The *CpaEXY1* gene encodes a 65 kDa primary translation product, and after cleavage of a signal peptide directing the protein to the apoplast, and further cleavage of C-terminal and N-terminal domains, an active catalytic domain of 32.5 kDa remains. Although xylanases are typically found as small gene families, xylanase isoforms generated by differential proteolytic processing seem to be common. In the aleurone layer of cereals, processing produced many xylanase intermediates (Caspers et al. 2001), and active enzymes of molecular masses of 55 and 30 kDa in wheat (Cleemput et al. 1997), and 29 kDa (Dashek and Chrispeels 1977) and 41 kDa (Banik et al. 1996) in barley have been reported. A similar situation seems to exist in papaya, with different isozymes being created by differential processing of CpaEXY1. Two-dimensional protein gel electrophoresis of proteins isolated from ripe mesocarp of 'PPY' papaya fruit followed by immuno-staining of the protein gel blot using an antibody against papaya xylanase showed multiple cross-reacting spots in the 25 to 33 kDa size range and with pI from 6.0 to 7.0 (Fig. S2). Of these spots, three were close to the predicted size of the catalytic subunit of 32.5 kDa. The lack of correlation between the relative xylan endotransglycosylase and endoxylanase activities in germinating cereal grains and seedlings also points to the possibility that the two activities are either due to separate proteins or to additional isoforms resulting from differential processing of the same protein. The detection of xylan endotransglycosylase activity in a range of species and tissues in which endoxylanase activity was undetectable also suggests that multiple proteins may be involved.

XTH and endo- β -mannanase families possess multiple isoforms with a range of endotransglycosylase and hydrolase activities. Most *Arabidopsis* XTHs are strict XETs, and only two out of the >30 members of this gene family are predicted to show xyloglucanase activity (Baumann et al. 2007). Molecular modelling and mutagenesis have shown that proteins with XET and xyloglucanase activities are very similar, and that xyloglucanase activity is at least in part due to the presence of a small loop in the protein that is absent in endotransglycosylases (Baumann et al. 2007). However, XTHs belong to GH16 and adopt a β -jellyroll conformation (Baumann et al. 2007), whereas plant endo- β -xylanases including CpaEXY1 from papaya belong to GH10 and fold into a $(\alpha/\beta)_8$ -barrel (Pell et al. 2004), and so are structurally more related to the endo- β -mannanases of GH5 (Bourgault et al. 2005). All of these GH families use a double displacement/retaining mechanism of glycosyl transfer, which potentially allows for endotransglycosylase reactions (Roberge et al. 1999; Moreau et al. 1994; Biely et al. 1991; Sinnott 1990).

Determining the relative rates of transglycosylation and hydrolysis of native CpaEXY1 *in vitro* showed that the hydrolysis rate was 2 to 4 times higher than the transglycosylation rate. However, lower endotransglycosylase activity does not necessarily mean that the endotransglycosylase reaction is insignificant in biological terms. It is also important to recognise that *in vitro* quantitative and qualitative studies of enzyme activity may not necessarily reflect what is happening *in vivo*. The activity of cell wall enzymes can differ in free solution compared to in the apoplast where, due to either the pore size of the cell wall or binding to cell wall polysaccharides, protein mobility or action may be restricted. Cell wall conditions of water concentration, ionic milieu, and local pH are also difficult to replicate exactly *in vitro*. For example, endo- β -mannanase in ripe tomato fruit exhibits both mannanase and mannan endotransglycosylase activities *in vitro*; however, *in muro* it is possible that if the enzyme acts at all, it acts as an endotransglycosylase since mannans do not change in size during tomato ripening (Prakash et al. 2012). To gain a better insight into xylanase and endo- β -mannanase enzymes, assays aimed at investigating their endotransglycosylase activity *in muro* must be carried out, as outlined for XTH by Fry (2004). Those plant species which exhibited comparatively high endotransglycosylase and low hydrolase activity would make ideal sources in which true transglycosylases might be found.

Endoxylanase activity has been identified in several plant species and tissues. In cereal grains, xylanase hydrolyses primary cell wall heteroxylans for the mobilisation of endosperm nutrients during germination (Caspers et al. 2001). In maize, xylanase activity is involved in the lysis of the tapetum-containing anther wall at pollen maturation (Bih et al. 1999) and the hydrolysis of the stigma surface for pollen tube penetration (Wu et al. 2002), and in *Arabidopsis*

is associated with secondary cell wall metabolism during vascular bundle development (Suzuki et al. 2002). In ripe papaya fruit, the activities of both endoxylanase (Paull and Chen 1983) and xylan endotransglycosylase (Fig. 6a) increased as fruit softened during ripening. It has been shown that the molecular weight of hemicelluloses declines during softening (Paull et al. 1999), although a specific examination of xylans has not been made. Xylan endotransglycosylase activity was also found in ripe fruit of avocado, banana, pineapple, and apple. We observed considerable endoxylanase activity in extracts of imbibed seeds of oat and barley, tissues that are rich in storage heteroxylans. Endoxylanase activity was much lower in growing seedlings, although both tissues also exhibited xylan endotransglycosylase activity, at least *in vitro*. These activities could of course be due to the presence of different proteins with specific either xylan endotransglycosylase or endoxylanase activity. In growing seedlings, xylan endotransglycosylase activity may be acting to reorganise xylans in the cell wall to support wall modifications. Evidence in support of a xylan endotransglycosylase reaction during rapid growth is reported in maize suspension cultures, where the molecular mass of xylans increased dramatically in the first hours after deposition into the cell wall (Kerr and Fry 2003). The incorporation of new xylan polymers into the expanding wall via transxylosylation may therefore be a major function for xylan endotransglycosylase enzymes in young tissues. Overall, the discovery of a xylan endotransglycosylase activity is consistent with recent reports (Franková and Fry 2011, 2012) that transglycosylation may be a much more common feature of primary cell wall metabolism than previously appreciated.

Electronic Supplementary Material

The following materials are available in the online version of this article:

Table S1 Peptide fragments in fraction 16 eluted from the MonoS column

Table S2 Predicted xylanase proteins and open reading frames in the papaya genome

Fig. S1 Analysis of the recombinant protein products produced in *Nicotiana benthamiana* leaves using viral vector TTOSA1-XYL

Fig. S2 2D-gel electrophoresis and gel blot of papaya fruit proteins

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Figure legends

Fig. 1 Incorporation of [^3H]xylo-oligosaccharides (OS) into high-molecular weight wheat arabinoxylan (WAX) at $t=0$ without addition of papaya extract (WAX, $t=0$) and after 60 min with extract (Product). Crude papaya extract (100 μL) was incubated with [^3H]xylo-oligosaccharide mixture (0.04% w/v), WAX (0.25% w/v), and 0.2 M Mes buffer, pH 5.5, for 1 h at 21°C. For the $t=0$ reactions, WAX and the [^3H]xylo-oligosaccharide mixture were incubated for 1 h without the addition of enzyme. Products were separated by size exclusion chromatography on Sepharose S300 (75 x 1.5 cm; eluent 0.05 M Na-acetate pH 6.0, 0.125 M NaCl, 0.05% chlorobutanol; fraction size 20 min, flow rate $\sim 6 \text{ mL h}^{-1}$). Elution profiles were monitored as total carbohydrates (TC) using the phenol sulphuric assay. [^3H] label was measured using scintillation counting with aqueous scintillant at $t=0$ (^3H Xylan OS) and after 60 min with papaya extract (Product ^3H label). The hashed area indicates [^3H] label incorporation into high-molecular weight polymeric product. Maximum radioactivity (728 kBq) was eluted at approximately 115 mL elution volume, consisting of unincorporated [^3H]xylo-oligosaccharides.

Fig. 2 pH and temperature optima of xylan endotransglycosylase from crude papaya fruit protein extract. **a** pH optimum was assessed using McIlvaine buffer (0.1 M Na citrate and 0.2 M NaH_2PO_4) and Universal Tribuffer (0.05 M diethanolamine, 0.1 M Mes and 0.05 M 4-ethylmorpholine), with 0.25% wheat arabinoxylan and 0.04% [^3H]xylo-oligosaccharide mixture as substrates. Reactions were carried out at 21 °C for 1 h at the pH indicated. **b** Temperature optimum was determined using the same substrate concentrations as described above, with 10 μL of extract in a reaction volume of 20 μL containing 30 mM Mes buffer, pH 5.5. For comparison, the temperature optimum of papaya xylanase is also shown (determined using xylan-RBB in the appropriate buffer as described in Materials and Methods). Endotransglycosylase and hydrolase reactions were incubated at the temperature indicated for 1 h. Data are expressed as % maximum activity, and were derived from the means of two triplicated experiments SD.

Fig. 3 Donor and acceptor substrate preferences of xylan endotransglycosylase from crude papaya fruit protein extract. **a** Donor substrate specificity was determined using a mixture of [^3H]xylo-oligosaccharides as acceptors and a range of polysaccharide substrates as donors: WAX (wheat arabinoxylan), birchwood xylan, heteroxylan-enriched cell wall fractions obtained by

CTAB precipitation of 1 M and 4 M KOH-soluble extracts of tomato or papaya fruit cell walls, galactoglucomannan-glucuronoxylan I and II (GGM-GX I, II) from ripe tomato cell walls, kiwifruit xyloglucan, citrus polygalacturonic acid (PGA), kiwifruit galactoglucomannan (GGM). **b** Acceptor substrate specificity was determined using WAX as donor and the [³H]xylo-oligosaccharide indicated as acceptor. The acceptors were assayed between approximately 100 and 500 μ M for 1 h. Experiments were carried out 2-3 times, and values are means of a typical, triplicated experiment $\pm$ SD.

Fig. 4 Purification of xylan transglycosylase and xylanase activities from 'Rainbow' papaya fruit using ion exchange chromatography. **a** xylan endotransglycosylase and xylanase activity of MonoS column fractions. Transglycosylase assays were carried out using WAX as donor and the tritiated xylo-oligosaccharide mixture as acceptors for 1 h at 21°C, and hydrolase assays using RBB-xylan, for 1 h at 37°C. Xylan endotransglycosylase activities are given as Bq incorporated per kBq provided per μ g protein per h, and xylanase as A_{595} per μ g protein per h. **b** Protein gel of the column fractions, stained with colloidal Coomassie Blue. **c** Protein gel blot reacted with anti-xylanase antiserum. The arrows indicate the predicted size of the mature protein (~32.5 kDa).

Fig. 5 The amino acid sequence of papaya endoxylanase CpaEXY1 (GenBank accession number AY138986) with the predicted signal sequence (1-28; double underlined) and predicted carbohydrate binding module (51-182; underlined). The two short regions boxed in grey (232-235 and 518-520) were the predicted sites of proteolysis to produce the mature protein described in Chen and Paull (2003). Bolded residues were part of fragments obtained by LC-MS analysis of fraction 16 eluted from the MonoS column (Table S1).

Fig. 6 Kinetics of the xylan endotransglycosylase and xylanase reaction. **a** Xylanase activity at two different WAX donor concentrations. Xylanase assays were performed by assaying reducing termini using the PAHBAH assay. **b** Xylan transglycosylase activity at two acceptor concentrations, 234 μ M and 467 μ M [³H]Xyl₅-ol, using 0.2% WAX as donor. **c** Xylan transglycosylase activity at donor concentrations from 0.1 to 0.4% (w/v) WAX using 234 μ M [³H]Xyl₅-ol as acceptor, assayed for 30 min. Experiments were carried out three times using highly purified CpaEXY1 from ripe papaya fruit; shown are the results of a typical triplicated experiment $\pm$ SD. WAX donor substrate concentrations are given in % as the molecular weight of

the polysaccharide is too disperse to be calculated in molarities (see elution profile WAX at $t=0$ in Fig. 1).

Fig. 7 Xylan endotransglycosylase and xylanase activities in crude protein extracts of various tissues and species. **a** Comparison of activities between four cultivars of ripening papaya fruit at different ripening stages. **b** Activities in a range of fruit and vegetable tissues. **c** Activities in imbibed cereal grains and in the corresponding young growing seedlings. Values are means of two triplicated experiments $\pm$ SD.

Figure 4

Figure 5

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1  MKLGEKNLQF YFLLVLPYAL LFPGLETNAL SYDYTASIQC LENPQKAQYG
51  GGIITNPELN QGLKGWSTFG DAKIQHRVAG SNSFIVAHR S QPHDSVSQT
101 LYLQSNKLYT FSAWIRVSEG KTPVKAIFKT KSGYKYAGAV VAESNCWSML
151 KGGLTVDASG PAELYFETDN TSVEIWIDSI SLQPFTQQEW KSHQDQSIKK
201 IRKKNVRIQA VDKLGNPLPN TTVSISPKKI GFPFGCAINR NILNNNAYQS
251 WFSSRFTVTT FENEMKWAST EPSQGHEDYS TADAMVQFAK KNGIAIRGHN

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301 **VFWD**DPKYQS **GWVSS**LSPND **LNAA**ATKRIN **SVMN**RYKGQV **IGWD**VVNENL
 351 **HFSF**FESKLG ANASAVFYGE AHK**TDP**STTL **FMNE**YNTVED **SRDG**QATPAK
 401 YLEKLRSIQS LPGNGNMGIG LESHFSSSP NIPYMRSID TLAATGLPVW
 451 LTEVDVQSGG NQAQSLEQIL REAHSHPKVR **GIVI**SAWSP **NGCY**RMCLTD
 501 **NNFH**NLPTGD **VVDK**LLREWG GGATVKGKTD QNGFFQSSLF HGDYEIKVQV
 551 NHPSKLPSSS SHHTFKLNST DDESKQTRLL LIKV

Table 1 Sugar composition of CTAB-insoluble pellets from papaya and tomato fruit KOH-soluble extracts used as donor substrates for testing xylan endotransglycosylase activity. Values are means of two analyses. The standard deviation was < 2 mol%.

Sugar	Sugar composition (mol%)			
	Papaya		Tomato	
	1 M KOH	4 M KOH	1 M KOH	4 M KOH
Rhamnose	2.0	1.8	4.6	4.5
Fucose	1.2	0.5	0.1	0.1
Arabinose	1.4	1.1	8.4	8.3
Xylose	56.9	54.5	53.5	35.0
Mannose	3.5	1.3	0.3	4.5
Galactose	2.9	3.6	5.5	7.1
Glucose	10.8	3.3	2.1	11.6
Uronic acid	21.3	34.0	25.7	28.7

Table 2 Sugar linkages of galactoglucomannan-glucuronoxylan (GGM-GX) complexes derived from cell walls of ripe tomato. Both GGM-GX I and II contained about 5 mol% of glucuronic acid (Prakash et al. 2012).

Glycosyl linkage	GGM-GX I	GGM-GX II
	(mol%)	
4-Xyl	23.0	10.0
2-Xyl	3.0	n.d.
2,4-Xyl	4.0	n.d.
T-Xyl	*	n.d.
4-Man	11.0	37.0

4,6-Man	7.0	9.0
4-Glc	23.0	25.0
4,6-Glc	3.5	3.0
T-Glc	8.0	n.d.
2-Gal	4.0	*
4-Gal	n.d.	*
T-Gal	10.0	15.0
5-Ara	*	n.d.
T-Ara	*	n.d.

*) present in trace amounts (<0.5 mol%); n.d. = not detected

Table 3 Activities of recombinant CpaEXY1 protein produced by transient over expression in *Nicotiana benthamiana* leaves using viral vector TTOSA1-XYL. Endoxylanase activity was assayed versus methyl-glucuronoxylan-RBB. Xylan endotransglycosylase activity was assayed using wheat arabinoxylan (WAX) and [³H]xylo-oligosaccharides.

	Protein in extract (μg)	Xylan endotransglycosylase activity (Bq inc kBq prov ⁻¹ assay ⁻¹)	Endoxylanase activity (A ₅₉₅ assay ⁻¹)
Extract containing	0.013	0.269 ± 0.200	0.005 ± 0.007
recombinant	0.027	0.415 ± 0.378	0.007 ± 0.004
CpaEXY1 protein	0.067	0.357 ± 0.189	0.010 ± 0.010
	0.135	0.715 ± 0.271	0.031 ± 0.010
	0.270	1.178 ± 0.213	0.069 ± 0.003
Tobacco leaf control	0.270	0.150 ± 0.060	0.000 ± 0.000

Figure 2

Figure 3

Figure 6

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