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Roswitha Schröder · Teresa F. Wegrzyn
Karen M. Bolitho · Robert J. Redgwell

Mannan transglycosylase: a novel enzyme activity in cell walls of higher plants

Abstract Mannan transglycosylase is a novel cell wall enzyme activity acting on mannan-based plant polysaccharides in primary cell walls of monocotyledons and dicotyledons. The enzyme activity was detected by its ability to transfer galactoglucomannan (GGM) polysaccharides to tritium-labelled GGM-derived oligosaccharides generating tritium-labelled GGM polysaccharides. Mannan transglycosylase was found in a range of plant species and tissues. High levels of the enzyme activity were present in flowers of some kiwifruit (*Actinidia*) species and in ripe tomato (*Solanum lycopersicum* L.) fruit. Low levels were detected in mature green tomato fruit and activity increased during tomato fruit ripening up to the red ripe stage. Essentially all activity was found in the tomato skin and outermost 2 mm of tissue. Mannan transglycosylase activity in tomato skin and outer pericarp is specific for mannan-based plant polysaccharides, including GGM, galactomannan, glucomannan and mannan. The exact structural requirements for valid acceptors remain to be defined. Nevertheless, a mannose residue at the second position of the sugar chain and the absence of a galactose substituent on the fourth residue (counting from the non-reducing end) appear to be minimal requirements. Mannan-based polysaccharides in the plant cell wall may have a role analogous to that of xyloglucans, introducing flexibility and forming growth-restraining networks with cellulose. Thus mannan transglycosylase and xyloglucan endotransglycosylase, the only other known transglycosylase activity in plant cell walls, may both be involved in remodelling and refining the cellulose framework in developmental processes throughout the life of a plant.

R. Schröder (✉) · T. F. Wegrzyn · K. M. Bolitho
Mt. Albert Research Centre, The Horticulture and Food Research Institute of New Zealand, Private Bag 92 169,
Auckland, New Zealand
E-mail: rschroeder@hortresearch.co.nz
Fax: +64-9-8154202

R. J. Redgwell
Nestlé Research Centre, Nestec Ltd., Vers-chez-les-Blanc,
PO Box 44, 1000 Lausanne 26, Switzerland

Introduction

The hemicelluloses xyloglucan, glucuronoarabinoxylan, glucomannan and galactoglucomannan are widespread components of primary cell walls of mono- and dicotyledonous plants. They are closely associated with cellulose microfibrils, creating a stable framework in which a matrix of complex pectin polymers is embedded (Carpita and Gibeaut 1993). This hemicellulose–cellulose framework is regarded as the major load-bearing structure in the primary plant cell wall.

Of the hemicelluloses, the structure and role of xyloglucan (XG) in this framework has been best characterised. XG coats cellulose microfibrils via hydrogen bonding, and also cross-links cellulose microfibrils, creating a xyloglucan–cellulose network that restricts turgor-driven cell expansion (McCann et al. 1990). It has been postulated that a transient loosening of this growth-constraining network can only be accomplished by an enzyme acting in endotransglycosylase mode, cutting an XG chain and attaching the newly created chain end to another chain, thereby restoring the original strength of the cell wall (Albersheim 1976). The existence of such an endotransglycosylase mechanism for XG was verified by Smith and Fry (1991) and

Nishitani and Tominaga (1991). The enzyme activity was subsequently identified and purified as xyloglucan endotransglycosylase (XET) (Farkas et al. 1992; Fry et al. 1992; Nishitani and Tominaga 1992).

XET expression and enzyme activity are usually positively correlated with processes of cell wall loosening during elongation (Fry et al. 1992; Xu et al. 1996; Vissenberg et al. 2000) or fruit ripening (Redgwell and Fry 1993). However, McQueen-Mason et al. (1993) showed that XET is not the causal agent for cell expansion. Instead, the enzyme seems to continuously modify the XG–cellulose network during different stages of plant development by breaking and rejoining existing or newly synthesised XG molecules to existing XG (Thompson and Fry 2001). In this manner, it probably restores and refines the XG–cellulose network during developmental processes.

There is a strong likelihood that there are transglycosylases other than XET that play a role in modulating polysaccharides of the hemicellulose–cellulose framework. Two important polysaccharides in this category, glucomannan and galactoglucomannan (GGM), are found throughout the plant kingdom as hemicellulose components of primary and secondary cell walls. They are characterised by a β-$(1 \rightarrow 4)$-mannose backbone that may be interspersed with glucose residues. Also included in this family are the pure mannans and galactomannans (Matheson 1990).

Like XG, glucomannan and GGM drastically reduce the crystallinity of cellulose microfibrils in composite materials (Hackney et al. 1994; Whitney et al. 1995, 1998), thereby introducing properties of flexibility and toughness (Newman et al. 1994). This effect may also occur in planta, as glucomannan coats cellulose microfibrils in differentiating tracheids only at night when water pressure is high, but not during the day when transpiration is low (Hosoo et al. 2002).

Whitney et al. (1998) showed that glucomannan has the ability to form cross-links with cellulose in composite materials, and concluded that XG may not be the only polysaccharide that can cross-link cellulose. This result has been confirmed in elongating maize coleoptiles, where glucomannan is found coating the cellulose microfibrils and also as interstitial material between them (Carpita et al. 2001). Also, GGM is likely to contribute to a network with cellulose despite its lower molecular weight ($\approx$30 kDa; Schröder et al. 2001) compared to glucomannan ($\approx$900 kDa; Matheson 1990) or XG ($\approx$500 kDa for kiwifruit XG; Schröder et al. 1998). Whitney et al. (2000) showed that XG approximately the size of GGM was still able to create a network, albeit having fewer cross-links compared to high-molecular-weight XG–cellulose networks.

The similarity in the relationships between glucomannan/GGM and cellulose, and XG and cellulose, supports the idea that a transglycosylase is active on mannan-based hemicelluloses in the same way as XET acts on XG. In this paper, we report the occurrence of a new cell wall enzyme activity, mannan transglycosylase, which is able to perform transglycosylase reactions on glucomannan and GGM, as well as on other plant-derived mannan-polysaccharides. By applying an approach analogous to that used to identify XET activity, mannan transglycosylase was detected in a range of plant and tissue types using GGM and tritium-labelled GGM-derived oligosaccharides as substrates. Some preliminary data on the enzyme characteristics of mannan transglycosylase are presented and possible roles for this new enzyme activity discussed.

Materials and methods

Plant material

For the general survey of mannan transglycosylase activity, tomatoes and other fruit and vegetables were purchased ripe from a retail source in Auckland, New Zealand. Lettuce, onions and peas (see Table 1 for botanical names) were purchased as seeds, germinated in the greenhouse under ambient light conditions and harvested after 12 days. Flowers from *Actinidia* (kiwifruit) species were obtained from HortResearch's orchard in Te Puke, New Zealand. Pine seedlings were grown in the greenhouse under ambient light conditions and young, green, non-lignified side branches harvested from 10-month-old plantlets. Cherry tomato seedlings (*Solanum lycopersicum* L. var. *cerasiforme*) for assays of activity during fruit ripening were purchased commercially, grown outdoors in West Auckland and harvested after 12 weeks. Tomato fruit (*Solanum lycopersicum* L. cv. Daniela) for determination of enzyme characteristics, and tomato flowers, were obtained from Status Produce in Pukekohe, New Zealand.

Polysaccharide and oligosaccharide substrates

The preparation of 4 M KOH-soluble GGM and XG from outer pericarp of ripe kiwifruit was carried out after Schröder et al. (2001). Galactomannan (from carob; low viscosity, borohydride reduced), glucomannan (from konjac; native, low viscosity), and mannan (from ivory nut) were purchased from Megazyme, Ireland, and solubilised according to the manufacturer's instructions. Mannan from baker's yeast (*Saccharomyces cerevisiae*) was purchased from Sigma–Aldrich, Australia. All polysaccharides were made up as 1% solutions in water and stored at -20°C.

Mannanase-derived GGM oligosaccharides (GGMOs) for general assays were prepared from ripe kiwifruit GGM (18 mg) using endo-β-mannanase (50 U) from *Aspergillus niger* (Megazyme) after Schröder et al. (2001). The mannanase-derived GGMOs were converted to their radioactive alditols by reduction with NaB[^{3}H]$_4$ (925 MBq; Amersham, UK). The [^{3}H]GGMO mixture was dissolved in water (4 μg μl^{-1}; 1.6×10^6 cpm μl^{-1}), and stored at -20°C.

Table 1 Survey of mannan transglycosylase activity in tissues of ripe fruit, flowers, and seedlings using GGM and [³H]GGMOs as substrates. Tissue was extracted with MES buffer containing 1 M NaCl and Complete protease inhibitors (Roche, New Zealand) at pH 5.0. After centrifugation, the pH of all supernatants was adjusted to 4.6–4.8 using acetic acid or KOH. Extracts (15 µl) were assayed with 15 µl of MES (0.2 M) pH 5.0, 5 µl GGM (1%) and 1 µl [³H]GGMOs (approx. 1.6×10^6 cpm). Reactions were stopped after 2 h and label incorporation determined using the paper assay. Values are means of one or two experiments with two replicates each. Protein levels were between 1.4 (cucumber) and 15.7 mg ml extract^{-1} (closed *A. eriantha* flowers). Extractions were also carried out using MES buffer at pH 6.0 or Na-acetate buffer at pH 5.0, and similar results were obtained in either system (data not shown)

Plant	Common name	Tissue extracted	Enzyme activity [cpm (mg FW)$^{-1}$ h^{-1}]
Fruit			
Malus pumila 'Pacific Rose'	Apple	Skin + underlying flesh	1.44
Prunus domestica	Plum	Skin + underlying flesh	1.94
Prunus persica	Nectarine	Skin + underlying flesh	0.84
Cucurbita pepo var. *melopepo*	Courgette	Skin + underlying flesh	1.04
Vitis vinifera	Grape	Skin + underlying flesh	0.20
Musa×paradisiaca	Banana	Pericarp	0.14
Cucumis sativus	Cucumber	Outer pericarp	1.17
Actinidia deliciosa 'Hayward'	Kiwifruit	Outer pericarp	0.95
		Core	1.48
Solanum lycopersicum	Tomato	Outer pericarp	12.65
Flowers			
Brassica oleracea var. *italica*	Broccoli	Unopened	0.00
Brassica oleracea var. *botrytis*	Cauliflower	Unopened	1.57
Asparagus officinale	Asparagus	Unopened	0.00
Actinidia deliciosa 'Hayward'	Kiwifruit	Unopened	19.98
		Open	10.69
Actinidia rufa	–	Unopened	0.68
		Open	0.00
Actinidia eriantha	–	Open	88.38
Solanum lycopersicum	Tomato	Open	7.57
Malus pumila 'Granny Smith'	Apple	Open	4.21
Seedlings			
Pinus radiata	Pine	Whole, no root	1.37
Arabidopsis thaliana	Arabidopsis	Whole, no root	0.57
Lactuca sativa	Lettuce	Whole, no root	4.93
Pisum sativum	Pea	Hypocotyl	6.21
		Leaves	5.60
Allium cepa	Onion	Whole, no root	0.00

For assaying acceptor specificity, mannan-oligosaccharides (M_2–M_5) and galactomannan pentasaccharide (G_2M_5) were purchased from Megazyme, and 0.25 mg of each reduced to radioactive alditols with NaB[³H]$_4$. The freeze-dried oligosaccharides were taken up in water (4 µg µl^{-1}, approx. 1.4×10^6 cpm µl^{-1}) and stored at −20°C until use.

Cellulase-derived GGMOs were prepared according to Schröder et al. (2001) using purified cellulase from *Trichoderma viride* (Sigma–Aldrich). Gel filtration of an aliquot of the cellulase-derived GGMOs on Toyopearl HW40S (Tosohaas, Montgomery, PA, USA) showed the same elution profile as GGMOs derived from mannanase treatment (data not shown). The cellulase-derived GGMO mixture was radiolabelled with NaB[³H]$_4$ as described above, dissolved in water (4 µg µl^{-1}; 1.7×10^6 cpm µl^{-1}) and stored at −20°C until use.

Enzyme extractions

Tissues were snap-frozen in liquid N$_2$ and stored at −80°C until use. Frozen tissue and extraction buffer were ground to a fine powder in liquid N$_2$ using a mortar and pestle, and insoluble polyvinylpyrrolidine added (50–100 mg g^{-1} tissue). The ratio of tissue to buffer was 1:3 unless stated otherwise. Extraction buffers used were McIlvaine [0.2 M Na$_2$HPO$_4$·7H$_2$O, 0.1 M citric acid (pH 5.0), 0.5 M NaCl], Na-acetate [0.25 M Na-acetate (pH 5.0), 0.4 M NaCl, 1 mM DTT] or MES (0.2 M

MES (pH 5.0 or 6.0), 0.4 M NaCl]. After thawing, extracts were kept on ice for 30–60 min. The supernatants were then recovered by centrifugation for 10 min at 13,000 g at 4°C and assayed for mannan transglycosylase activity. Protein concentrations were measured using the BCA protein assay reagent (Pierce, Rockford, IL, USA).

Mannan transglycosylase assays

Reaction mixtures contained 5 µl of 1% polysaccharide solution, 1 µl of [³H]GGMOs, enzyme extract and buffer as indicated in the results. All reactions were carried out at 22°C and stopped by adding 40% formic acid (50 µl). For background reactions, 50 µl of formic acid (40%) was added before addition of enzyme extract, and these values were subtracted from values obtained using active enzyme extract. Activities are usually described in cpm, as quenching levels were similar in all assays.

Detection of mannan transglycosylase activity

The tritium-labelled polysaccharides produced in the assays were separated from [³H]oligosaccharides by either: (i) their ability to bind to paper during prolonged washing with water (paper assay), or (ii) their immobility during paper chromatography.

The paper assay was modified after Fry et al. (1992) and used with GGM or XG as polysaccharide substrates, as these polymers strongly bind to paper. Reaction mixtures were spotted onto paper, marked, and the paper washed for 2 h in running tap water. The use of [^{14}C]GGM confirmed that the polymer bound easily to paper and did not wash off in running tap water. The paper was dried, the spots cut out and assayed for ^{3}H by scintillation counting using organic counting scintillant (OCS; Amersham). The convenience of the paper assay with GGM as donor substrate made it the preferred method for general screening for mannan transglycosylase in a wide range of plants and tissue types.

The paper binding capacity of galactomannan and glucomannan was tested by applying a 1% solution (50 μl) to a Whatman 3MM paper strip and eluting it with water, using the descending method. The eluates tested positive for carbohydrate with phenol–sulfuric reagent. Therefore, for the assay of these polymers, and mannan, paper chromatography was used. The reaction products were applied to 46 cm × 27 cm sheets of Whatman 3MM paper and unreacted [^{3}H]oligosaccharides removed by chromatography in ethyl acetate–acetic acid–water (10:5:6, by vol.) for 24 h after Fry et al. (1992). The polysaccharide material, which is immobile in this solvent, was assayed for ^{3}H using scintillation counting as described above.

Results

Mannan transglycosylase activity—survey of plant tissues

Transglycosylase reactions involving polysaccharides are not easy to monitor as the start and end products do not vary in composition and not much in size. To overcome this obstacle for the assay of XET, Fry et al. (1992) developed a method that used tritiated xyloglucan oligosaccharides and polysaccharides as reaction partners. During this reaction, the polysaccharide chain is cleaved and the newly generated reducing end of the polysaccharide chain attached to the non-reducing end of the tritiated acceptor oligosaccharide. The occurrence of tritiated polysaccharides points to transglycosylation reactions. Using this modified XET assay, mannan transglycosylase activity was discovered by its ability to transfer GGM polysaccharides to GGM-derived, tritium-labelled oligosaccharides ([^{3}H]GGMOs), generating tritium-labelled GGM polysaccharides. The tritium-labelled GGM produced in the reaction could be separated from [^{3}H]GGMOs as GGM binds to paper in an aqueous environment (paper assay). Control reactions with only [^{3}H]GGMOs both as donor and acceptor substrates showed no radioactivity, indicating the reaction was dependent on the presence of polysaccharide donor.

Initially, mannan transglycosylase activity was assayed in rapidly growing tissues and fruit to determine whether the enzyme, like XET, may play a role in cell expansion or fruit ripening (Rose and Bennett 1999; Table 1). Of the fruit tissues assayed, only the outer pericarp from ripe tomato had significant mannan transglycosylase activity. All other fruit tissues had no (banana, grape) or very low activity (apple, plum, nectarine, courgette, cucumber). Ripe kiwifruit, although being the source of the substrates, showed only low levels of mannan transglycosylase activity.

Of the flowers assayed, mannan transglycosylase activity varied greatly among members of the genus *Actinidia*, despite having similar morphology. Flowers of *Actinidia eriantha* showed the highest activity of all tissues assayed, whereas in flowers of *A. rufa*, a distant relative to *A. eriantha*, no mannan transglycosylase activity was detected. Flowers of *A. deliciosa* (closely related to *A. eriantha*) showed activity between these two extremes, at about the level of activity found in tomato outer pericarp (Table 1). Tomato and apple flowers had moderate levels of activity, whereas cauliflower flower tissue showed low levels. In broccoli and asparagus flowers, no mannan transglycosylase activity was detected.

Lettuce seedlings, and hypocotyls and leaves from pea seedlings showed moderate levels of mannan transglycosylase activity. Young pine shoots were low in activity, and onion and *Arabidopsis* seedlings had no activity.
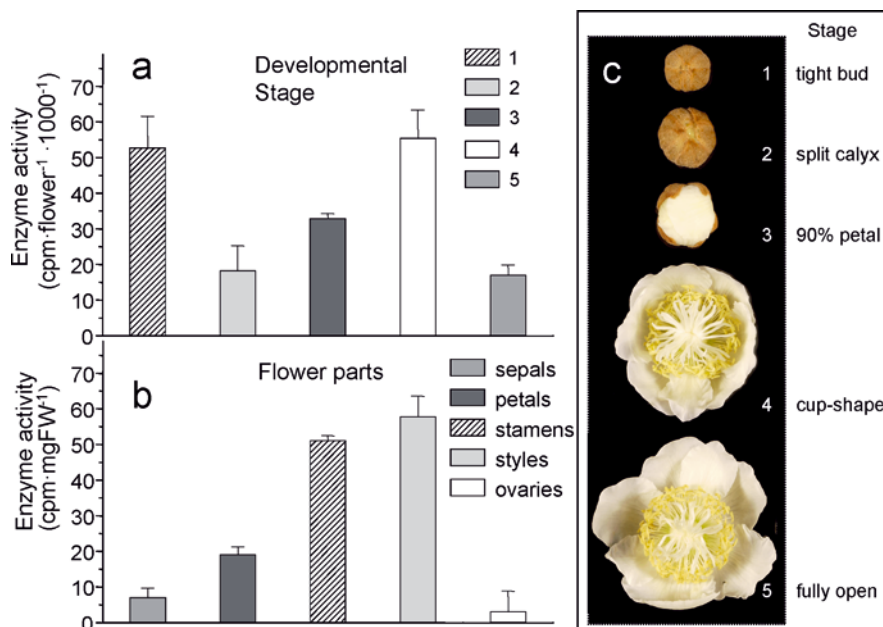
Mannan transglycosylase activity in *A. deliciosa* flowers

In the initial survey of plant tissues, extracts from *A. deliciosa* flower buds and open flowers had high levels of mannan transglycosylase activity with kiwifruit GGM and [^{3}H]GGMOs as substrates (Table 1). Subsequently the activity pattern of mannan transglycosylase was evaluated in a developmental series of *A. deliciosa* flowers (Fig. 1a,c), and in flower parts of 'cup-shape' flowers (Fig. 1b). Of all developmental stages, the tightly closed flower buds (stage 1) and 'cup-shape' flowers (stage 4) had the highest levels of activity (Fig. 1a). From closed buds to stage-2 flowers, a considerable drop in the levels of mannan transglycosylase activity occurred. After the calyx had split, the level of activity increased again once the flower began to open (stages 3 and 4), reaching a maximum in the 'cup-shape' stage.

Different parts of 'cup-shape' flowers were assayed using kiwifruit GGM and [^{3}H]GGMOs as substrates. High levels of activity were found in petals, stamens, and styles. No activity, however, was detected in sepals and ovaries (Fig. 1b).

Mannan transglycosylase activity in tomato fruit

Of all fruit tested in the initial survey of plant tissues, only outer pericarp from red ripe tomato had high levels of mannan transglycosylase activity using kiwifruit

Fig. 1 a, b Mannan transglycosylase activity during flower development of *Actinidia deliciosa* (**a**), and in flower parts of cup-shape flowers (**b**), with kiwifruit GGM and GGMOs as substrates. **c** Developmental stages of *A. deliciosa* flowers corresponding to stages assayed in **a**. Whole flowers or flower parts of *A. deliciosa* were sampled at orchard full-bloom and immediately frozen in liquid N$_2$. Tissues were extracted (1:6 tissue:buffer) in 0.2 M MES (pH 5.0), containing 0.4 M NaCl, 10 mM DTT and Complete protease inhibitors (Roche). The supernatants (45 µl for whole flowers, 30 µl for flower parts) were assayed using GGM and [^{3}H]GGMOs as substrates. Reactions were stopped after 2 h and label incorporation determined using paper assays. Values are means ± SD of two experiments with two replicates each

GGM and [^{3}H]GGMOs as substrates (Table 1). Therefore, the distribution of enzyme activity in different tissue types of ripe tomato was monitored. At the red ripe stage, most of the activity in tomato 'Daniela' was located in the skin and in the outermost 2 mm of the outer pericarp tissue beneath it (SK + OP) (Fig. 2a). In this cultivar, SK + OP comprises about 25% of total fruit weight, with the outer pericarp being the highly coloured parenchymous tissue between the smaller skin cells and the vascular network. A further experiment in which mannan transglycosylase activity in skin or outer pericarp only were compared, showed that these tissues gave similar values when expressed on a per milligram protein basis (data not shown). The higher activity per gram fresh weight seen for skin alone was therefore due to higher protein content in the smaller cells at the fruit surface, rather than a significant increase in the mannan transglycosylase specific activity of this tissue. Some activity was detected in the inner pericarp region. This may be due to difficulties in dissecting inner and outer pericarp, as the demarcation line between the two tissues was not always clearly visible. No activity was found in the locules and seeds of the fruit.

Subsequently, the activity pattern of mannan transglycosylase in SK + OP was assayed during tomato fruit development and ripening, using kiwifruit GGM and [^{3}H]GGMOs as substrates. Extracts of SK + OP in developing tomatoes 'Daniela' from about 5 mm diameter to mature green fruit showed no mannan transglycosylase activity (data not shown). From the mature green stage, mannan transglycosylase in SK + OP of cherry tomatoes increased during ripening, with the highest activity at the red ripe stage (Fig. 2b). Turning fruit showed only low levels of activity, while overripe tomato showed a significant drop in activity to levels below that of the mature green stage.
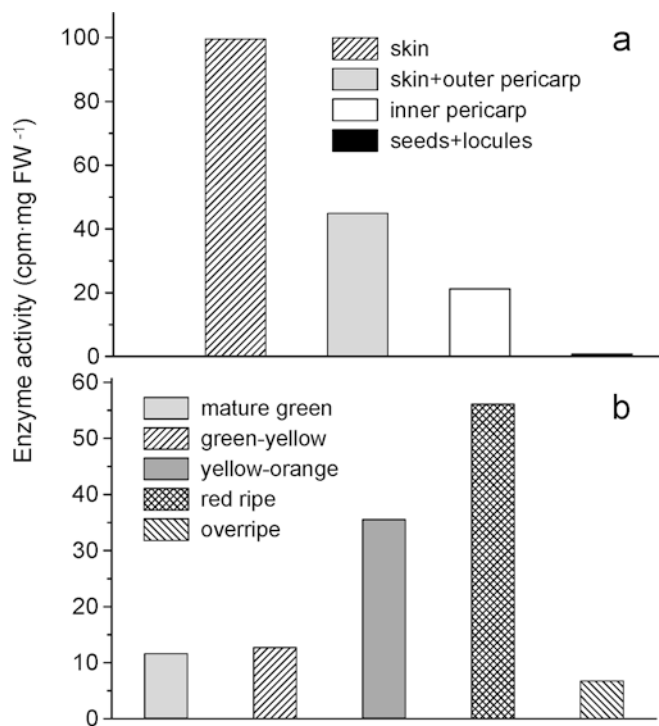
Characteristics of tomato mannan transglycosylase

For characterisation of properties, acceptor and donor specificity of mannan transglycosylase activity, ripe tomato SK + OP was used as the tissue source.

Enzyme properties

Tomato mannan transglycosylase appeared to be ionically bound to the cell wall, as at least 0.4 M NaCl was needed to release activity in a number of extraction buffers. Nevertheless, salt-free McIlvaine buffer readily solubilised tomato mannan transglycosylase. A subsequent extraction of the residue with McIlvaine buffer containing 1 M NaCl did not release further activity. Therefore, the phosphate or citrate ions in the McIlvaine buffer seem to be enough to disrupt the enzyme–cell wall interaction. An increase in the pH of the extraction buffers did not release activity (data not shown). This differs from kiwifruit XET, which can be extracted from the cell wall by an increase in either the pH or salt concentration of the extraction buffer (Redgwell and Fry 1993).

Tissue samples snap-frozen in liquid N$_2$ and stored at −80°C, retained mannan transglycosylase activity for at
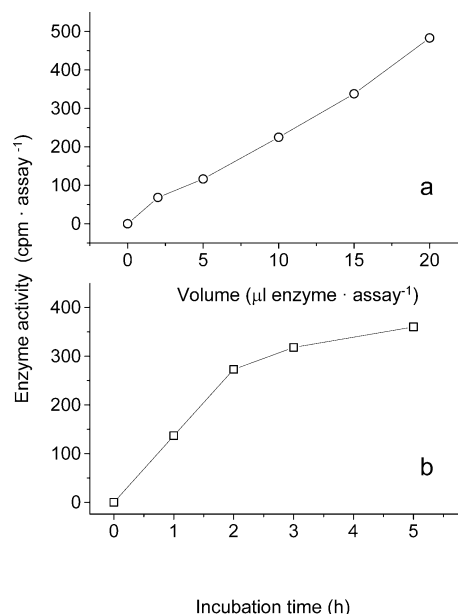
Fig. 2a, b Mannan transglycosylase activity of crude tomato (*Solanum lycopersicum*) extracts in different zones of red ripe tomato (**a**), and during tomato ripening (**b**), with kiwifruit GGM and GGMOs as substrates. Fruits of tomato 'Daniela' at the red ripe stage were dissected into skin, skin plus outer pericarp (skin plus 2 mm of non-vascular tissue beneath it), inner pericarp (solid tissue from extensive vascular zone to the locular gel), and locule contents. Extractions of these zones were made using MES buffer (pH 5.0). Cherry tomatoes for the ripening series were grouped into mature green, green–yellow, yellow–orange, red ripe and overripe fruit. These tissues were extracted using McIlvaine buffer. Extracts (15 μl for fruit zones, 50 μl for ripening series) were assayed with the addition of 5 μl of 1% GGM and 1 μl [^{3}H]GGMOs for 1 h, reactions stopped and label incorporation determined using paper assays. Values represent results from one typical extraction, with 2 replicate assays each

least 1 year. Enzyme from ripe tomato SK + OP extract was stable at 4°C for 24 h, and could also be frozen at −20°C (but only once) for about 1 month without loss of activity. After 5 min at 70°C mannan transglycosylase activity of the extract was reduced by approx. 65%, and after 20 min no enzyme activity was detectable (data not shown).

Figure 3 shows the dependence of mannan transglycosylase activity on extract volume and time, using GGM and [^{3}H]GGMOs as substrates. Up to 100 min, the assay was linear using up to 20 μl of enzyme extract (Fig. 3a). For 7.5 μl of extract, the reaction rate was linear for up to 2 h, with a doubling of the incorporated label between 1 and 2 h. After this time the quantity of reaction products levelled off. This could be due to either death of enzyme or the presence of substrate-competing hydrolase activity in the crude extract (Fig. 3b).

The pH optimum of tomato mannan transglycosylase was 5.0 in McIlvaine buffer (Fig. 4a). With about 50% maximal activity at pH 3.7 or 5.8, the enzyme exhibited
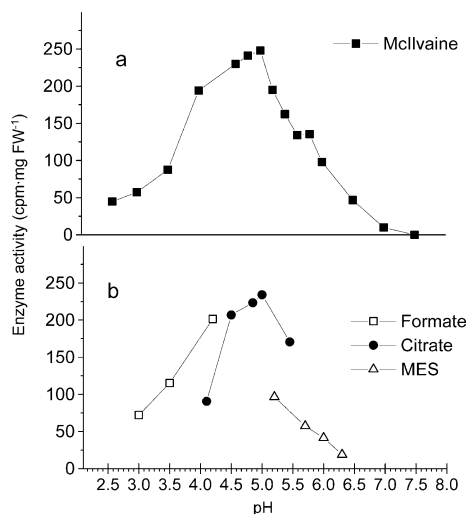

Fig. 3a, b Volume (**a**) and time (**b**) dependence of the reaction of tomato mannan transglycosylase using kiwifruit GGM and [^{3}H]GGMOs. Mannan transglycosylase activity was extracted as described in Table 1. For volume dependence of enzyme activity (**a**), 0, 2, 5, 10, 15, 20 μl of enzyme extract (5.7 ± 0.03 mg ml^{-1} protein) were made up to a final volume of 20 μl with MES buffer (0.2 M, pH 5.0) and substrates added. The reactions were stopped after 100 min and paper assays carried out. For time dependence of enzyme activity (**b**), enzyme extract (7.5 μl) was added to substrates. At the time points indicated the reactions were stopped and label incorporation determined using paper assays. Values represent results from a typical extraction, with 2 duplicate assays each

a relatively broad pH range of action. Activity measured with other buffers, including Na-formate, Na-citrate and MES, covering a pH range of 3.0–6.5 confirmed the pH optimum of mannan transglycosylase to be 5.0 (Fig. 4b).

Donor substrate specificity

Other mannan-based polymers such as galactomannan, glucomannan, mannans from ivory nut and yeast, and XG were assayed for their ability to act as donor substrates for mannan transglycosylase (Table 2). Unlike GGM or XG, glucomannan, galactomannan and mannan do not bind well to paper in an aqueous environment and were therefore assayed using paper chromatography to separate unreacted [^{3}H]GGMOs from [^{3}H]polymer. With GGM and [^{3}H]GGMOs as substrates, both assay methods showed similar activity with 179.5 ± 9.8 cpm (mg FW)$^{-1}$ h^{-1} for the paper assay and 176.4 ± 46.5 cpm (mg FW)$^{-1}$ h^{-1} for paper chromatography using mannan transglycosylase activity from ripe tomato SK + OP (Table 2).

Mannan transglycosylase also acted on galactomannan, glucomannan and mannan from ivory nut, producing activity levels only slightly lower than those obtained with kiwifruit GGM. Hence, the absence of a

Fig. 4a, b pH-dependence of mannan transglycosylase activity in crude tomato extracts in McIlvaine buffer or an array of buffers including formate, citrate or MES (all in Na^+-form), with kiwifruit GGM and GGMOs as substrates. Tomato extracts were prepared as described in Table 1, and 35 µl added to 400 µl of **a** McIlvaine (0.1 M citrate, 0.2 M NaH_2PO_4), **b** Na-acetate (0.2 M), Na-formate (0.2 M), or MES (0.2 M) buffer at the pH indicated. Of these solutions, 35 µl was added to substrates, reactions stopped after 275 min and label incorporation determined using paper assays. Values reported are results of a typical extraction, with 2 replicate assays each

Table 2 Donor substrate specificity of mannan transglycosylase activity from red ripe tomato skin and outer pericarp. Tomato SK + OP was extracted in MES buffer (see Table 1) and the extract (7 µl) assayed with 5 µl of each polymer (1%) and 1 µl [³H]GGMOs (approx. 1.6×10^6 cpm). After 3 h, reactions were stopped, and label incorporation determined using paper assays or paper chromatography. Values are means ± SD of three experiments with duplicate assays each. *n.a.* Not applicable, *n.d.* not determined

	Enzyme activity [cpm (mg FW)$^{-1}$ h^{-1}]	
	Paper assay	Paper chromatography
GGM	179.5 ± 9.8	176.4 ± 46.5
Galactomannan	n.a.	162.2 ± 9.3
Glucomannan	n.a.	150.5 ± 17.3
Ivory nut mannan	n.a.	144.1 ± 4.9
Yeast mannan	n.a.	38.1 ± 4.6
Xyloglucan	0.7 ± 0.7	n.d.

mannose–glucose backbone (mannan, galactomannan) or galactose side stubs (mannan, glucomannan) did not compromise the activity of the enzyme.

Mannan from baker's yeast [β-(1 → 6)-linked mannose backbone] was a poor substrate for mannan transglycosylase from ripe tomato SK + OP, indicating a clear preference for β-(1 → 4)-linked mannose backbone in the substrate molecule. A β-(1 → 4) glucan backbone (present in kiwifruit XG) was not a donor substrate, indicating a requirement for β-linked mannan backbones.

Acceptor substrate specificity

Hydrolysis of GGM with endo-mannanase from *Aspergillus niger* resulted in a mixture of four main GGM-derived oligosaccharides (GGMOs II to V) and traces of mannobiose (Schröder et al. 2001). GGMOs II to V vary in the length of their glucose–mannose backbone, and in the presence and length of the galactose side chains (Table 3). After separation of the tritiated GGMO mixture by TLC and assay of individual [³H]GGMOs, only GGMO IV was highly active as an acceptor for GGM in the transglycosylation reaction (Table 3). GGMOs II and III showed only weak acceptor activity, whereas GGMO V showed no activity. This indicates a certain optimum chain length for the enzyme acceptor binding-site, as GGMOs II and III are shorter than GGMO IV. Another acceptor requirement seems to be an unsubstituted mannose (or mannitol) residue at the fourth position (if there is one) from the non-reducing end of the sugar chain, the only difference between GGMO IV (active) and GGMO V (inactive).

As the reducing ends of the acceptors do not participate in the creation of new glycosidic bonds, borohydride reduction of the terminal mannose residues to mannitol did not interfere with the mannan transglycosylase assay. This has also been found for xyloglucan oligosaccharide acceptors for XET (Fry et al. 1992).
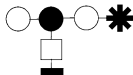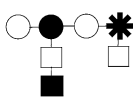
Mannan- and galactomannan-derived oligosaccharides were used to determine which structural features were pre-requisite for the acceptors to be valid substrates for tomato mannan transglycosylase (Table 4). Using the mannan-derived oligosaccharides, the enzyme was about twice as active with M_4 compared to M_3 confirming a certain optimum chain length for acceptors. Mannobiitol (M_2) was not active under the conditions used. The results also show that an alternating glucose–mannose backbone or the presence of galactose side stubs were not structural requirements for acceptors.

Pure M_5 was only slightly active in the enzyme assay despite fulfilling structural requirements of minimum size and possessing an unsubstituted mannose residue at the fourth position from the non-reducing end of the sugar chain. In contrast, a heptaose G_2M_5 derived from galactomannan, structurally identical to M_5 except for the presence of two galactose side stubs was used by tomato mannan transglycosylase as acceptor substrate. It is possible that the galactose side stubs protected the G_2M_5 acceptor from being degraded by a hydrolytic activity in the crude tomato extract. G_2M_5 was also more active than M_4 or M_3, indicating a preference of the enzyme activity for longer acceptor chains.

Galactomannan in combination with mannose oligosaccharides or G_2M_5 acceptors gave the same activity pattern as with GGM as donor substrate (data not shown).
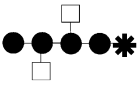
As the backbone of GGM from kiwifruit is composed of alternating glucose–mannose residues (Schröder et al. 2001), we deduced that digestion of GGM with purified cellulase leads to GGMOs that have the same basic

Table 3 Acceptor substrate activity of individual [³H]GGMOs. Tritiated GGMOs (120 µg) were separated by thin-layer chromatography and eluted after Schröder et al. (2001). Purified GGMOs II, III, IV and V were taken up in 30 µl of H₂O and 5 µl of each was assayed with 1% kiwifruit GGM (5 µl) and 20 µl tomato extract (prepared as described in Table 1). The reactions were stopped after 2 h and the degree of radiolabel incorporation determined using paper assays. The assays were carried out twice with duplicate assays each (average cpm ± SD)

Name		Compound	Enzyme activity (cpm·assay⁻¹)
GGMO II	○✹	β-D-Glc-(1→4)- D-Man-ol	75 ± 19
GGMO III	○✹ □	β-D-Glc-(1→4)[α-D-Gal-1→ 6)]- D-Man-ol	53 ± 20
GGMO IV	○●○✹ □ ■	β-D-Glc-(1→4)[β-D-Gal-(1→ 2)-α-D-Gal(1→6)]- β-D-Man-(1→4)- β-D-Glc-(1→4)- D-Man-ol	709 ± 35
GGMO V	○●○✹ □ □ ■	β-D-Glc-(1→4)[β-D-Gal-(1→ 2)-α-D-Gal(1→6)]- β-D-Man-(1→4)- β-D-Glc-(1→4)-[α-D-Gal(1→6)]- D-Man-ol	16 ± 16

Symbols: ● β-linked mannose ○ β-linked glucose □ α-linked galactose ■ β-linked galactose

✹ Mannitol [³H]

Table 4 Acceptor substrate specificity of mannan transglycosylase from red ripe tomato SK + OP using GGM as donor. Reaction mixtures with mannan- or galactomannan-derived oligosaccharides contained 1 µl of [³H]oligosaccharide (4 µg, approx. 1.4×10⁶ cpm µl⁻¹), 5 µl kiwifruit GGM (1%) and 20 µl of crude tomato extract. The reactions were stopped after 3 h and radioactive incorporation determined using paper chromatography. Values are means ± SD of two experiments with duplicate assays each. *OS* Oligosaccharide

Source	Name		Compound	Enzyme activity (cpm·nmol OS in assay⁻¹)
Mannan-derived oligosaccharides	M₂	●✹	β-D-Man-(1→4)- D-Man-ol	4.7 ± 4.7
	M₃	●●✹	β-D-Man-(1→4)- β-D-Man-(1→4)- D-Man-ol	187.0 ± 36.5
	M₄	●●●✹	β-D-Man-(1→4)- β-D-Man-(1→4)- β-D-Man-(1→4)- D-Man-ol	465.6 ± 58.3
	M₅	●●●●✹	β-D-Man-(1→4)- β-D-Man-(1→4)- β-D-Man-(1→4)- β-D-Man-(1→4)- D-Man-ol	60.8 ± 24.8
Galactomannan-derived oligosaccharide	G₂M₅	●●●●✹ □ □	β-D-Man-(1→4)-[α-D-Gal(1→6)]- β-D-Man-(1→4)-[α-D-Gal(1→6)]- β-D-Man-(1→4)- β-D-Man-(1→4)- D-Man-ol	568.9 ± 41.3

Symbols: ● β-linked mannose □ α-linked galactose ✹ Mannitol [³H]

structure as the mannanase-derived GGMOs. Due to the substrate specificity of cellulase, however, glucose forms the reducing end of the oligosaccharides instead of mannose, and mannose (mostly substituted with galactose side stubs) the non-reducing end instead of glucose. Cellulase-derived [³H]GGMOs had no acceptor activity, indicating that either the glucose (glucitol) residue at the reducing end or substituted mannose residue at the non-reducing end of the oligosaccharide chains prevents them from being active acceptors for mannan transglycosylase activity from crude tomato extract (data not shown).

Discussion

Mannan transglycosylase is a new transglycosylase activity that acts on a range of mannan-based plant polysaccharides including glucomannan and galactoglucomannan, hemicelluloses that are present in primary walls of monocotyledonous or dicotyledonous plants. Mannan transglycosylase activity was assayed using pure GGM polysaccharide and tritium-labelled GGM-

derived oligosaccharides in a modified paper assay originally developed for XET (Fry et al. 1992). The activity was detected in crude extracts prepared from a range of plant species, and from tissues such as ripening fruit, expanding flower parts and growing seedlings.

Activity was measured using oligosaccharide acceptor concentrations that ranged from 130 µM (G₂M₅) to 300 µM (M₃), using mannan transglycosylase in crude tomato extracts. These numbers are within the same order of magnitude as the reported K_m for oligosaccharide substrates of pea stem XET (50 µM; Fry et al. 1992) or ripe kiwifruit XET (100 µM; Schröder et al. 1998), indicating that the mannan transglycosylase reaction operates in a physiological range.

Although it was present in a wide range of tissues, mannan transglycosylase activity was neither ubiquitous nor as widespread as XET, the only other known transglycosylase activity in plant cell walls. However, assays of enzyme activity in crude extracts are only indicative, as they can be affected by the presence of inhibitory compounds, enzymes that degrade the product, and failure to solubilise enzyme activity during extraction. It is unlikely that substrate requirements of

some mannan transglycosylases have not been met by the GGM components derived from kiwifruit, as mannan transglycosylase activity from ripe tomato skin and outer pericarp was promiscuous in its use of donor and acceptor substrates.

The tomato enzyme showed no marked preference for any of the β-(1 → 4)-linked plant mannan-type donor polymers tested, which included GGM, galactomannan, glucomannan and mannan. Active acceptors so far comprise a mannose residue at the second position of the sugar chain counting from the non-reducing end that can be substituted with galactose side stubs. Most of the data suggest that the enzyme is not strongly influenced by the nature of the fourth residue counting from the non-reducing terminus of the acceptor since this residue can be reduced to a sugar alcohol (mannitol) or even absent. Galactose side stubs on the fourth residue, however, result in inactive acceptors. This relative indifference of mannan transglycosylase towards the reducing end terminus of the acceptor chain makes it theoretically possible that two polysaccharides participate in the reaction. Also, acceptor molecules with a longer backbone chain were more active as substrates than smaller ones, therefore indicating a certain optimum acceptor chain length in the enzyme–substrate binding site.

Despite theoretically fulfilling all acceptor requirements, mannopentaose (M_5) was not used as an acceptor substrate. This oligomer may have been hydrolysed during the assay, possibly by endo-β-mannanase (EBM), a mannan-specific glycosyl hydrolase present in ripe tomato (Pressey 1989). The oligosaccharide G_2M_5 however functioned as an acceptor, indicating it may have been resistant to hydrolysis by EBM because of the galactose substituents. Thus, the unambiguous establishment of the structural requirements for valid oligosaccharide acceptors for mannan transglycosylase will require a more detailed investigation than the present study, using pure enzyme to minimise reactions on substrates by competing hydrolases.

In tomato, EBM has been immunologically detected in the same tissue zones as mannan transglycosylase (Bewley et al. 2000). It follows the same pattern of increasing activity during fruit ripening to a maximum at full ripeness (Bewley et al. 2000; Bourgault et al. 2001), although its role in terms of changes to cell wall composition is unclear. EBMs found in seeds with galactomannan as storage polysaccharide (Dulson and Bewley 1989; Nonogaki et al. 1995; Marraccini et al. 2001) play a part in their hydrolysis during germination. In common with other plant glycosyl hydrolases whose main role in vivo is polysaccharide hydrolysis, some of these seed EBMs have been reported as catalysing transglycosylation in vitro when mannan-derived oligosaccharides were present as sole substrates. These reactions occurred, however, at oligosaccharide levels approximately 10 to 50 times higher than used in combination with mannan polysaccharides (Coulombel et al. 1981; Marracini

et al. 2001). In tomato, mannan transglycosylase and EBM activities may therefore be two different enzymes or isoforms of the same enzyme exhibiting different modes of action.

A comparison of enzymatic features of mannan transglycosylase and EBM activities does not resolve this question. In comparison with mannan transglycosylase, the pH optimum reported by Pressey (1989) for tomato EBM (pH 4.5–5.0) is lower, and tomato EBM needed salt for extraction with McIlvaine buffer (Bewley et al. 2000). In ripening tomato, mannose-containing polymers extracted in the hemicellulosic fraction do not change in size or composition during ripening (Huber 1983; Tong and Gross 1988; Seymour et al. 1990). One possibility is that transglycosylation rather than hydrolysis of these polymers is occurring. Therefore, if EBM and mannan transglycosylase are the same enzyme, hydrolysis may not be the preferred mode of action in vivo during tomato fruit ripening. Interestingly, in Nasturtium seeds having XG as a storage polymer, XET was originally characterised as a hydrolase (XEH; Edwards et al. 1986). The ability of this enzyme to take part in transglycosylation reactions between polysaccharide and oligosaccharide substrates was only proven after XET activity had been described in other tissues (Fanutti et al. 1993), giving rise to the re-naming of the enzyme to xyloglucan transglucosylase/hydrolase (XTH) to acknowledge its hydrolytic and transglycolytic functions (Rose et al. 2002).

The patterns of mannan transglycosylase activity in developing kiwifruit flowers and ripening tomato support an involvement in the structural reorganisation of tissues during growth and ripening. The optimal pH range of the tomato activity, together with its association with the cell wall, are consistent with a cell wall enzyme acting at the lower pH found in the tomato apoplast later in ripening (Almeida and Huber 1999) or during acid-induced cell wall growth (Rayle and Cleland 1992). Tomato extracts with the highest activity were measured in fruit where the skin was just able to be pulled away from the outer pericarp and the pericarp had begun to give way to finger pressure, so that the enzyme may be acting to increase cell wall flexibility. This flexibility may be a feature specific to ripening fruit, as there was no measurable activity in developing or mature green fruit. In flower development of A. deliciosa the pattern of mannan transglycosylase activity coincided with the time of maximum elongation of petals, stamens, and styles during flower opening (Brundell 1975). In fully open flowers, when elongation of flower parts has ceased and flower senescence has begun, no activity was detected. There was also a period of increased enzyme activity in tightly closed Actinidia flower buds, when ovule formation begins and petals first begin to expand inside the flower bud. Ovule formation requires some elaboration of new cell wall structures, as a canal has to be established (and maintained during growth) from ovules through to stigmata, to allow fertilisation to occur (Polito and Grant 1984).

Characterisation of the purified enzyme, its mannan substrates, and their behaviour in vivo are needed to identify the role of mannan transglycosylase in the modification of cell wall properties during growth and fruit ripening. In most tissues the types of mannan-based polymer present in cell walls have not yet been identified, let alone their detailed structures and how these affect cell wall properties during developmental changes. There are increasing numbers of reports on interactions between glucomannans or galactoglucomannans with cellulose that influence the structural properties of the cell wall (Hackney et al. 1994; Whitney et al. 1998, 2000; Carpita et al. 2001; Hosoo et al. 2002). Multiple roles have been assigned to XET during plant development, and the transglycosylation of xyloglucans seems to be important—though not the causal agent—in processes as divergent as elongation and fruit ripening. The discovery of another hemicellulose transglycosylase suggests that polysaccharide modification by transglycosylation may be a more common event in plant cell walls than was previously thought, particularly for structural components associated with cellulose. This raises the possibility that some of the many changes to the primary structure of cell wall polysaccharides during growth and development that have been attributed to hydrolytic or to biosynthetic processes, may be a consequence of transglycosylation.

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