Plant Physiol. (1994) 105: 965–974

Endo-1,4-β-Glucanase, Xyloglucanase, and Xyloglucan Endo-Transglycosylase Activities Versus Potential Substrates in Ripening Tomatoes[1]

Gordon Maclachlan* and Colin Brady

Department of Biology, McGill University, 1205 Avenue Dr. Penfield, Montréal, Québec H3A 1B1, Canada (G.M.); and Division of Horticulture, Commonwealth Scientific and Industrial Research Organization, Delhi Road, North Ryde, New South Wales 2113, Australia (C.B.)

In ripening fruits of tomato (*Lycopersicon esculentum* L. var 83-G-38), the amounts of cellulose and xyloglucan (XG) remained constant during tissue softening, but the relative molecular weight (M_r) of XG decreased markedly and the M_r of cellulose declined slightly. These changes could have been due to activities of nonspecific endo-1,4-β-glucanases and/or buffer-soluble XG endotransglycosylase, both of which increased when tissue firmness declined most rapidly. Tomato extracts also reduced the viscosity of XG solutions, especially in the presence of added XG oligosaccharides. This depolymerizing (XGase) capacity differed from β-glucanase and XG transglycosylase activity (a) by being almost entirely buffer insoluble, and (b) by declining precipitously during fruit softening. Although it disappeared from ripe fruit, XGase may have functioned in promoting wall loosening at earlier stages of fruit development when its activity was highest. By contrast, during aging of fruit in the ripening-inhibited mutant *rin* there was no change in M_r of XG or cellulose, and activities of β-glucanases and XG transglycosylase were lower than in wild-type tomato. Nevertheless, some softening of the fruit did take place over time and XG amounts declined, possibly because high XGase activity was maintained in the mutant, unlike in wild-type fruit.

The aim of the present study was to determine whether XG and/or cellulose are degraded during tissue softening and liquifying reactions that accompany tomato (*Lycopersicon esculentum*) fruit ripening, and, if so, to establish whether activities of any of the known hydrolases or transglycosylase(s) that can cleave these major wall components are correlated with the loss of tissue firmness. The possibility that 1,4-β-glucosyl linkages in wall materials are broken during ripening needs to be examined in view of questions raised recently about the adequacy of pectin catabolism, by itself, to explain the process. It has been demonstrated repeatedly that high levels of endopolygalacturonase activity and depolymerization of pectic materials both occur in ripening tomato and many other maturing fruits (reviewed by Brady, 1987). Nevertheless, when antisense RNA to this enzyme was expressed in tomato fruit in such a way that its activity was reduced to <1% of normal levels and pectin breakdown was prevented, tissue softening still took place (Sheehy et al., 1988; Smith et al., 1988, 1990). Likewise, when low polygalacturonase levels in *rin* mutants were transformed to near-normal levels by gene transfer, with the result that pectic materials were extensively degraded, fruit softening still failed to take place (Giovannoni et al., 1989; DellaPenna et al., 1990). These results have led to proposals (e.g. Campbell et al., 1990; Fischer and Bennett, 1991) that partial breakdown of other structural components of the wall, such as the cleavage of cellulose or XG, may be required along with the degradation of pectic materials to bring about sufficient relaxation for ripening fruits to soften as observed.

Levels of endo-1,4-β-glucanase activity, as assayed by measuring loss of viscosity of solutions of CMC (EC 3.2.1.4), often show increases during fruit ripening along with levels of polygalacturonase activity (Brady, 1987; Campbell et al., 1990; Fischer and Bennett, 1991). Tomato fruit contains at least three nonspecific CMCases that can also hydrolyze XG (Maclachlan and Brady, 1992). Two of these enzymes are currently being purified and sequenced (Lashbrook et al., 1993) with the aim of conducting experiments with antisense genes to better define their roles in fruit ripening. However, these are not the only enzymes that can cleave XG. Ripening tomato fruit also contains specific XG-depolymerizing activity (referred to here as XGase) that reduces the viscosity of solutions of XG but does not react with CMC. This XGase activity differs from the tomato CMCase activities in pH optimum, net charge, hydrophobicity, and solubility. The addition of XG oligosaccharide subunits to tomato extracts greatly increases its capacity to depolymerize XG (Maclachlan and Brady, 1992).

In the present study, these enzyme activities were assayed during fruit maturation in a tomato variety that ripens normally (wild-type 83-G-38) and in *rin*. Separate assays were conducted on locular and pericarp tissues since the former normally gels or liquifies before the latter softens. In addition, cellulose and cellulose-bound XG were isolated from these

[1] Experimental work was conducted partly in Commonwealth Scientific and Industrial Research Organization laboratories, where G.M. was a guest on sabbatical. Studies at McGill University were supported by the Natural Sciences and Engineering Research Council of Canada.

* Corresponding author; fax 1–514–398–5069.

Abbreviations: CMC, carboxymethylcellulose; DP, degree of polymerization; *rin*, ripening-inhibited mutant tomato; V_o, void volume; XET, xyloglucan-specific endo-transglycosylase; XG, xyloglucan.

tissues and measurements were made of their amounts and DP.

Yet another XG-cleaving enzyme has been described in extracts of pea epicotyls and other growing tissues (Fry et al., 1992), as well as in nasturtium cotyledons (Farkas et al., 1992; Fanutti et al., 1993), that degrades XG without hydrolysis via transglycosylation reactions. This enzyme has been detected at relatively high activity levels in ripening kiwi fruit (Redgwell and Fry, 1993). XET activity breaks chains of XG and transfers the new potentially reducing ends to XG acceptors without any net increase of reducing power in the system. If the donors are long XG chains and the acceptors are short XG oligosaccharide subunits, then transglycosylation should result in a decline in net XG mol wt and translate into a fall in viscosity of XG solutions. Whether an oligosaccharide-dependent viscosity loss of XG solutions, which was first observed in pea epicotyl extracts (Farkas and Maclachlan, 1988) and later in ripening tomato fruit (Maclachlan and Brady, 1992), is due entirely to XET activity as suggested by Lorences and Fry (1993) and Redgwell and Fry (1993), or to oligosaccharide-activated (allosteric) XGase as contemplated in earlier studies, or to a mixture of such enzymes, remains to be established.

The specific XGase that was purified from germinating nasturtium seeds during mobilization of XG reserves and assayed by generation of reducing ends from XG (Edwards et al., 1985, 1986) responds to added XG oligosaccharides by rapidly reducing the viscosity of XG solutions and, at the same time, preferentially using XG fragments instead of water as acceptors for cleaved XG chain transfer (Farkas et al., 1992; Fanutti et al., 1993). This is an example of a hydrolase (XGase) transformed into a transglycosylase (XET) in the presence of suitable acceptor concentrations. In contrast, the active XET that was purified from *Vigna* epicotyls (Nishitani and Tominaga, 1992) could not be considered a XGase because it had no detectable hydrolytic activity, nor did it have any capacity to generate XG fragments with molecular masses less than 10 to 20 kD. Thus, the possibility arises that tomato fruit contains a specific oligosaccharide-activated XGase, or a XET that can employ oligosaccharide as acceptor, or enzyme(s) with potential to catalyze both reactions.

In the present study assays were conducted for both XGase and XET activities by measuring loss of viscosity of XG solutions and the incorporation of labeled XG oligosaccharide into high mol wt XG. The same reaction conditions were employed in both assays and the same mixture of XG oligosaccharides was used as the acceptors/activators. Extracts of pericarp and locular tissue were examined in wild-type (83-G-38) tomatoes during their maturation from mature green (unripe, firm) through "breaker" to red (ripened, soft) fruit, and in *rin* mutants during yellowing with age.

MATERIALS AND METHODS

Plant Materials

Tomato plants (*Lycopersicon esculentum* L.) were grown in recirculating Hoagland nutrient solution in a greenhouse at 25 ± 2°C. The varieties were a normally ripening wild-type line of 83-G-38 (De Ruiter Zonen BV, Bleiswijk, The Netherlands) that was resistant to tobacco mosaic virus, and *rin*, which is backcross 6 from *rin* from a Rutgers background into 83-G-38. Flowers were self-pollinated and a single fruit was allowed to develop from each flower cluster. Tomatoes were harvested at intervals between 30 and 65 d after anthesis at dates that were chosen to provide as full a range of tissue firmness as possible, i.e. from mature green (unripe but fully grown) fruit to red and softened wild-type tissue (83-G-38) or to yellow and only partially softened mutant tissue (*rin*). Firmness of the sampled fruits was measured by the pressure required to puncture the equatorial region of the pericarp (epidermis removed) using a calibrated Effigy "penetrometer" (circular shaft, 1 cm diameter). Fruits were washed in 1% Na hypochlorite, quartered, and divided into (a) pericarp tissue adjacent to but not including the epidermis and (b) locules (including seeds), which represented approximately 28 and 15% (w/w), respectively, of the fresh weight of the fruits. The tissues were frozen in liquid nitrogen, freeze dried, and stored at −90°C.

Polysaccharide Preparation and Assay

To prepare intact cell wall polysaccharides separated into standard wall fractions according to solubility, freeze-dried tomato tissue (2 g) was suspended in boiling 80% ethanol (150 mL), homogenized while hot in a blender, and boiled (30 min) in a reflux condenser to ensure the inactivation of any destructive enzymes and the extraction of low mol wt solutes. Ethanol-insoluble material was collected by centrifugation and extracted (30 min) with 0.1 M EDTA, 1.0 M NaCl, pH 6.8 (75 mL) in a boiling water bath. The mixtures were centrifuged, the extraction was repeated, and the supernatants were combined. The EDTA-insoluble residue was extracted twice with 4% KOH (w/v), 0.1% $NaBH_4$ (total volume 60 mL) at room temperature for 48 h in a shaking device. The combined supernatants were neutralized with glacial acetic acid and chilled, and the supernatant was clarified by centrifugation. Two volumes of ethanol were added, the mixtures were chilled, and the precipitate was dissolved in 0.01% NaN_3, 0.1 M Tris, pH 7.0 (10 mL). The 4% KOH-insoluble residue was shaken in 24% KOH (w/v), 0.1% $NaBH_4$ (25 mL) at room temperature for 48 h and centrifuged. This was repeated and the combined supernatants were neutralized and mixed with 2 volumes of ethanol. The suspension was chilled and the precipitate was dissolved in 0.01% NaN_3, 0.1 M Tris, pH 7.0 (20 mL). The 24% KOH-insoluble residue was neutralized, washed thoroughly with H_2O, and freeze dried.

XG was estimated in neutralized tissue extracts and in aliquots of fractions by adding I-KI solution and measuring the amount of iodine:XG complex at A_{640} (Kooiman, 1960). Tamarind XG was extracted in 2 N NaOH, 0.05% $NaBH_4$ from seed freshly collected in Barbados and used as enzyme substrate and as standard in the Kooiman assay after purification by precipitation with ethanol and copper (Edwards et al., 1985). EDTA and alkali extracts of tomato contained starch, particularly in unripe green tissues, and its reaction with iodine interfered with the XG assay. Accordingly, all neutralized extracts were treated with α-amylase (human saliva, type IX-A, Sigma, 25 units in 10 μL of 1 mM $CaCl_2$)

and incubated at room temperature for 24 h. This was repeated until the blue-purple color of starch-iodine disappeared.

Cellulose was estimated in 24% KOH-insoluble, washed residues after freeze drying by dissolving a weighed sample in hot anhydrous DMSO:paraformaldehyde (Gordon and Maclachlan, 1989) and assaying for anhydro-Glc with the phenol-sulfuric acid reagent (Dubois et al., 1956) using cellulose microcrystals (Avicel, PH-101, Fluka) as standard. XG that remained bound to cellulose after 24% KOH extraction was assayed in the DMSO extracts by precipitating cellulose with added I-KI reagent and measuring XG-iodine color development at 640 nm in the supernatant.

Polysaccharide Fractionation

The size distribution of XG was examined by fractionating approximately 2 mg on columns (1.0 × 90 cm) of Sepharose CL-6B (Pharmacia) and eluting with 0.1 N NaOH. Fractions were collected and aliquots neutralized with glacial acetic acid and assayed for XG with I-KI. Columns were calibrated with dextrans of known size (Sigma). The size distribution of cellulose was estimated by dissolving 10 mg of freeze-dried residues in anhydrous DMSO:paraformaldehyde (1.0 mL) at 110 to 135°C and fractionating approximately 2 mg of the methylol derivative on columns (1.0 × 90 cm) of de-aerated controlled pore glass beads (PG-1400, pore size 1273 Å, 80–120 mesh, Sigma) (Gordon and Maclachlan, 1989). Fractions were eluted with DMSO and cellulose was estimated with phenol-sulfuric acid.

Enzyme Preparation

Aliquots (10 g) of freeze-dried tomato tissue were suspended in 100 mL of cold 20 mM Na_2HPO_4, 5% glycerol, 1 mM DTT, 0.1% NaN_3, homogenized for 1 min in an omnimixer, and adjusted to pH 6.0 with 1 M NH_4OH. After stirring in the cold for 1 h, the suspension was centrifuged (10,000*g*, 20 min) and decanted through Miracloth. The volume of this clarified "buffer-soluble" extract was recorded, separated into 15-mL aliquots, and frozen. The buffer-insoluble residue was rehomogenized in 1 volume (= fresh weight of the tissue) of extraction buffer containing 1 M NaCl, adjusted to pH 6, and stirred in the cold overnight. It was centrifuged and filtered, and this "buffer-insoluble, salt-soluble" extract was frozen in aliquots. Protein was assayed using the Bio-Rad reagent with BSA as standard. The buffer-soluble and buffer-insoluble, salt-soluble extracts of 1 g fresh weight of pericarp or locule tissue contained approximately 0.4 and 0.15 mg of protein, respectively.

Enzyme Assays

Endo-cleavage of β-glucan chains was assayed viscometrically in Cannon-Manning semimicro viscometers in a final volume of 1.0 mL using 0.5% (w/v) CMC or tamarind XG as substrate and measuring the initial rate of loss of viscosity evoked by added tomato enzyme (approximately 400 μg of protein from buffer-soluble extracts, 150 μg from salt-soluble extracts), as described by Maclachlan (1988). The time it took to lose 10 to 20% of initial viscosity was determined and units of activity were calculated. One unit was defined as the amount of enzyme that reduced viscosity by 1%/h at 32°C. CMCase activities were assayed at pH 5 and 7 to distinguish the main isozymes that have been identified in tomato fruit. XG depolymerization was assayed at pH 6 since this was the pH at which the addition of 0.02% (w/v) tamarind XG oligosaccharides caused maximum enhancement of viscosity loss of XG solutions (Maclachlan and Brady, 1992). XET activity was also assayed with 0.5% tamarind XG as donor and 0.02% tamarind XG oligosaccharides as acceptor, but in a total volume of 250 μL with the octasaccharide components labeled with [^{14}C]Fuc. Activity was measured by the initial rate of incorporation of ^{14}C into material insoluble in cold 50% ethanol, as described by Farkas et al. (1992). It has been shown (Farkas and Maclachlan, 1988; Farkas et al., 1992; Lorences and Fry, 1993) that the common XG oligosaccharides, whether fucosylated or not, are all effective as acceptors in the XET assay or as promoters of the depolymerization of XG in the viscometric assay. XET assays were routinely conducted in quadruplicate; the average SE was ±6% of the mean values.

Oligosaccharides were prepared by hydrolysis of purified tamarind XG with *Trichoderma* cellulase and fractionation on Bio-Gel P_4 (Bio-Rad) columns as described by Maclachlan et al. (1992). They contained a mixture of XG subunits with seven, eight, or nine sugars (Glc_4Xyl_3 with zero, one, or two terminal Gal units) in a ratio (Farkas et al., 1992) that yielded an average mol wt of 1350. [^{14}C]Fuc-labeled oligosaccharides were prepared by *Trichoderma* cellulase hydrolysis of tamarind XG that had been fucosylated by solubilized pea XG fucosyltransferase (Hanna et al., 1991), using as donor GDP-[U-^{14}C]Fuc (8.32 GBq $mmol^{-1}$, New England Nuclear). The specific activity of the XG oligosaccharides labeled with [^{14}C]Fuc was 2 to 3 Bq μg^{-1}.

RESULTS

Tissue Softening

Green tomato fruit required 4 to 4.5 kg pressure/cm^2 of exposed pericarp for a penetrometer to break through to the underlying locules (Fig. 1). In wild-type tomatoes (83-G-38), during the rapid color change from green to red (breaker stage) between 38 and 41 d after anthesis, fruit firmness decreased dramatically to about one-fourth of the penetrometer value of mature green tomatoes. Thereafter it gradually declined further to about 0.5 kg/cm^2. The *rin* also lost firmness with time, but relatively slowly, reaching half of its original value by about 60 d.

XG and Cellulose during Fruit Softening

No trace of iodine-reactive XG (absorption at 640 nm) was found in amylase-treated hot EDTA or dilute (4%) NaOH extracts of tomato tissue. Since I-KI will not yield a color reaction with XG chains that have a molecular mass below about 20 kD (Hayashi, 1989; Farkas et al., 1992), the possible existence of XG oligosaccharides with DP up to about 60 Glc units cannot be ruled out. It was evident, however, that tomato fruits contain no significant amount of readily ex-

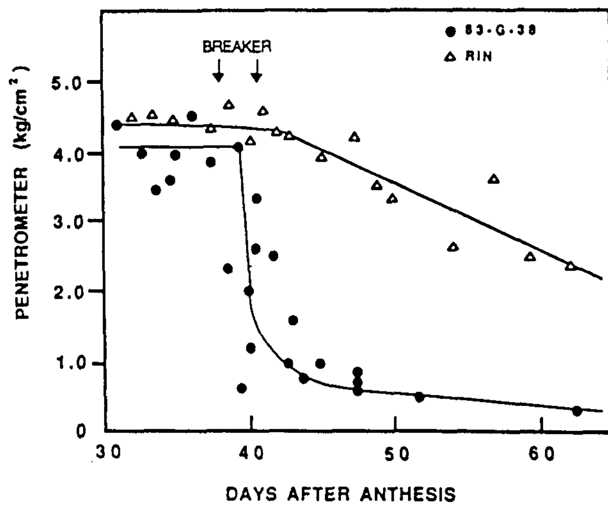

Figure 1. Pressure required to penetrate pericarp of wild-type (83-G-38) and *rin* mutants of tomato fruit harvested at intervals after anthesis. The color change from green to red occurred between 39 and 42 d in 83-G-38, but *rin* never turned red and only gradually faded to yellow-green.

tracted reserve XG, as do nasturtium and tamarind seeds. The XG that was recovered from both pericarp and locule tissue required 24% KOH to solubilize from the cellulosic residue, i.e. an alkali concentration sufficient to cause swelling of cellulose but not its dissolution. Very little XG remained associated with cellulose after this alkali treatment (the average I-KI estimate of XG in the residue insoluble in 24% KOH was equivalent to only 3% of the phenol-sulfuric acid test for total carbohydrate in pericarp tissue and 0.6% in locules). We conclude that tomato fruit XG was tightly bound to cellulose and possibly intercalated into the crystalline microfibrillar network, as depicted in the model by Hayashi (1989).

None of the XG samples taken from locules or pericarp at any stage of the ripening process reacted detectably with Fuc-binding lectin from *Ulex europaeus* in double-diffusion Ouchterlony tests (as did pea XG; Hayashi and Maclachlan, 1984). This confirms the observation of Seymour et al. (1990), who found only traces of Fuc in methylated tomato walls. However, all of our samples yielded clear precipitates with the β-galactoside-binding lectin from *Ricinus communis*. Thus, the composition of tomato fruit XG resembles that from seeds rather than that from growing dicots, because it lacks terminal Fuc, but it differs by being tightly bound to cellulose.

Neither bound XG nor cellulose declined in total amount during the softening process in ripening wild-type tomatoes (Table I). Cellulose levels in pericarp and locule plus seeds were low and comparable on a dry weight basis (approximately 3%). XG levels in the pericarp tissues (approximately 1% dry weight) were much greater than in locules (0.15–0.2% dry weight), but were still relatively low compared to those from growing dicot tissues (Hayashi, 1989).

The size distribution of XG from pericarp and locule, as visualized by fractionation on columns of Sepharose CL-6B, decreased significantly during wild-type tomato ripening, most markedly at the time when fruits became red (Fig. 2). In pericarp tissue, most of the XG in green fruit eluted between the peaks of dextran 264 kD and V_o. The DP began to decline toward the end of the breaker stage to reach sizes that were equivalent to dextran 100 to 200 kD in fully ripe fruit. Locular XG possessed a lower average size than pericarp XG, but this also declined to relatively very low values in ripe fruit.

The size distribution of cellulose from pericarp and locules, after fractionation on columns of glass beads, peaked well above a dextran marker of 500 kD at all stages of ripening (Fig. 3). In pericarp cellulose, the ratio of amounts with a size greater than dextran 500 to amounts less than dextran 500 averaged 1.72 (SE 0.08) and showed no trend toward decreasing values as ripening progressed. The same ratio for locule cellulose showed a gradual decline from a value of 1.8 for green tomato to 1.5 for red, ripe tomato, suggesting that some modest depolymerization did take place during liquifaction of this tissue.

Table I. *Levels of cellulose and cellulose-bound XG in wild-type tomato during the ripening process*

Tomatoes (83-G-38) ripening on the vine were selected at times after anthesis (see Fig. 1) when they were still green and firm (penetrometer values 4.5–4.0 kg/cm²), or when color appeared and firmness rapidly declined (breaker), or later when fully red, ripe, and soft (1.0–0.5 kg/cm²). XG (cellulose-bound) was assayed by reaction with I-KI in neutralized fractions insoluble in 4% KOH but soluble in 24% KOH, and cellulose was estimated with phenol-H_2SO_4 in fractions insoluble in 24% KOH but soluble in paraformaldehyde:DMSO, as described in "Materials and Methods." Average SE of quadruplicate estimates were 4 to 5% of the values given.

Age	Color	Penetrometer	XG		Cellulose	
			Pericarp	Locule	Pericarp	Locule
d		*kg/cm²*	*mg/g dry wt*			
31	Green	4.5	9.3	1.7	32.9	30.1
35	Green	4.0	12.1	1.9	32.0	28.2
41	Yellow	3.2	10.1	1.5	32.6	30.3
41	Orange	2.3	10.0	1.6	33.4	33.1
45	Red	1.0	9.5	1.4	33.5	28.5
47	Red	0.5	10.2	1.5	34.9	28.8

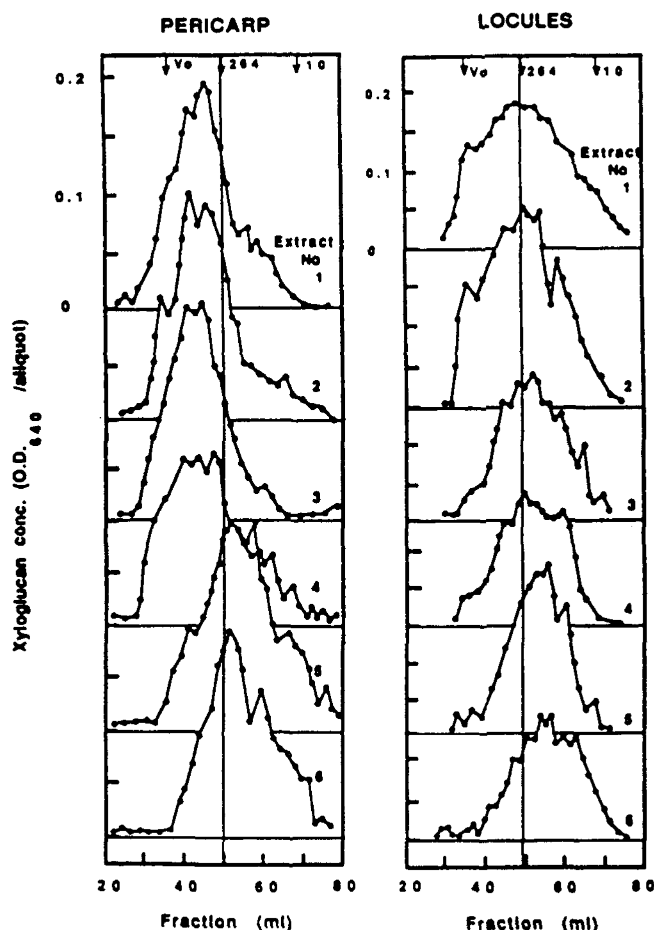

Figure 2. Size distribution of XG samples taken from ripening tomatoes selected for decreasing firmness. Extracts 1 to 6 were from 83-G-38 tomatoes with penetrometer readings between 4.5 and 0.5 kg/cm^2 as recorded between 31 and 47 d in Table I. XG was fractionated on columns of Sepharose CL-6B and estimated in aliquots of fractions with the I-KI reagent as described in "Materials and Methods." Numbers at the top of the figures refer to the peaks of dextran standards (kD). V_0 was the peak of dextran 2000.

The Range of XG-Cleaving Enzyme Activities in Tomato Fruit

Figure 4 shows the capacity of high salt extracts of wild-type tomato fruit at breaker stage to depolymerize XG, as assayed viscometrically with tamarind XG as substrate, and to carry out XET reactions, as assayed by measuring incorporation of [^{14}C]Fuc-labeled oligosaccharides into high mol wt XG. The extracts hydrolyzed XG slowly in the absence of added oligosaccharides (10% loss of viscosity in 90 min), which could have been due to nonspecific 1,4-β-glucanase (CMCase) or to specific XGase activity. Added oligosaccharides greatly enhanced the rate of XG depolymerization (10% loss of viscosity in 2 min). Under the same reaction conditions but with oligosaccharides [^{14}C]fucosylated, label was incorporated into XG by transglycosylation, even as the average chain length of XG was drastically reduced. By the time (20 min) that the viscosity of the tamarind XG solution had fallen to half of its original value, about 20% of the supplied oligosaccharide had been incorporated into XG insoluble in 50% ethanol (Fig. 4). Thus, it is possible to attribute the oligosaccharide-stimulated XG degradation to activated XGase or to XET or to a combination of such enzymes.

Changes in CMCase and XG-Cleaving Enzyme Activities during Fruit Ripening

Figure 5 compares the levels of these enzyme activities on a fresh weight basis in buffer-soluble and buffer-insoluble, salt-soluble extracts of wild-type tomato fruit pericarp as tissue firmness fell by about 90% from values in firm, green, mature fruit to those in red, ripe fruit. CMCase activities at pH 5 and 7 were distributed between buffer- and salt-soluble extracts, and their levels all increased as the tissue softened. XET activity levels in the buffer-soluble fraction increased sharply during ripening, but in the salt-soluble fraction it declined a little, so that total activity showed only a gradual increase.

In contrast, the capacity to depolymerize XG as assayed viscometrically in the presence of added oligosaccharide (Fig. 5) failed to follow the patterns of development or solubility profiles of any of the above enzymes. It was confined almost entirely to the salt-soluble extract, and its relatively very high level in green fruit fell markedly during the period of most rapid softening. The capacity of these extracts to reduce viscosity of XG solutions in the absence of added oligosaccharide, shown in Table II, was relatively much lower than in the presence of oligosaccharides, but it too was primarily extracted in the salt-soluble fraction and it declined in level during ripening.

Assays for enzyme activities in locule extracts from wild-type tomatoes are shown in Table III. Total CMCase activity levels were higher in locules than in pericarp tissue (cf. Fig. 5) and they increased further with ripening. However, the capacity to depolymerize XG in the absence of added oligosaccharides was lower in extracts of locules than in pericarp (cf. Table II), and it fell to even lower levels in ripe fruit. The enhanced capacity to depolymerize XG in the presence of XG oligosaccharides also fell to very low levels in ripe fruit. XET activity levels were similar to those in pericarp and were maintained during ripening. In general, the levels of these enzyme activities in locules changed up or down during ripening in a manner that was similar to the direction of their changes in pericarp.

Changes in *rin* Mutant Fruit Pericarp

Figure 6 shows the size distribution of XG and cellulose from the pericarp of *rin* fruits after fractionation on columns of Sepharose CL-6B or glass beads, respectively. Profiles are compared at 41 and 62 d, i.e. during a period when the fruit gradually lost about half of its firmness and slowly turned yellow but not red (Fig. 1). These times were chosen to be equivalent to the times when wild-type tomato fruit entered the breaker phase and softened rapidly (39–42 d) and eventually became over-ripe (>50 d). In *rin*, XG levels fell from an average of 14.0 to 9.7 mg/g fresh weight (SE ± 0.5 mg) between 41 and 62 d, but the size distribution of the residual

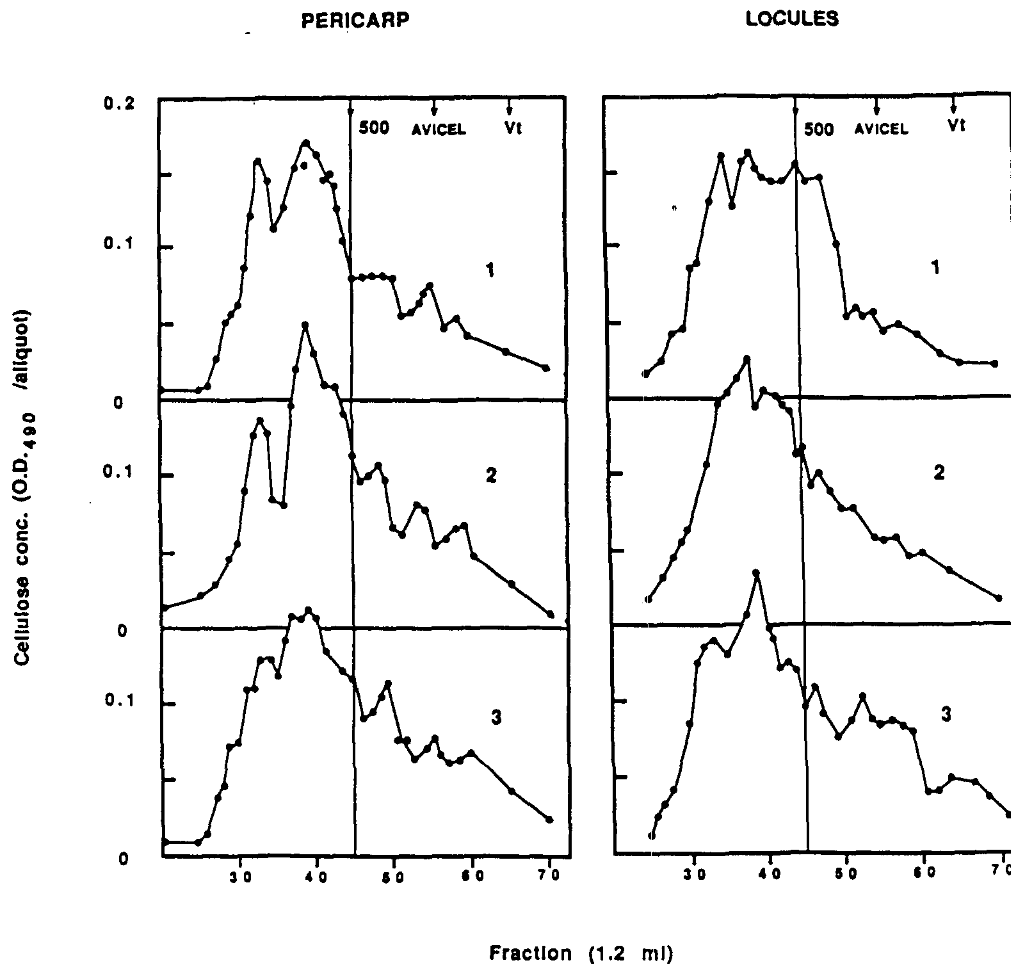

Figure 3. Size distribution of cellulose samples taken from ripening tomatoes. Samples 1, 2, and 3 derived, respectively, from tissue that was unripe (green), turning color (breaker), and ripe (red), with penetrometer readings of 4.5, 2.3, and 0.5 kg/cm^2 (see Fig. 1). Cellulose was dissolved in DMSO-paraformaldehyde and fractionated on a controlled-pore, glass-bead column eluted with DMSO as described in "Materials and Methods." Carbohydrate was determined in aliquots of eluted fractions with phenol-sulfuric acid. The elution volume of dextran 500 is indicated with a vertical line, and the peak elution volumes of microcrystalline cellulose (Avicel, DP approximately 200) and Glc (Vt) are indicated by arrows at the top of the graphs.

XG (Fig. 6) stayed constant and relatively high (DP was mainly between dextran 264 and V_o). Even by 62 d, the amounts and size of *rin* XG were comparable to those in unripe wild-type tomato (cf. Table I and Fig. 2). Cellulose showed no indication of any depolymerization in *rin* (Fig. 6) or in wild-type (Fig. 3) pericarp.

With respect to XG-cleaving enzyme activities in *rin* pericarp extracts, the levels of CMCases and XET were lower than those found in wild-type tomato (Table III, cf. Fig. 5), although they all increased with aging as in normal fruit. XGase activity, assayed viscometrically in the absence of added oligosaccharides, was relatively very low at 41 d in the mutant compared with XGase at the breaker stage in wild-type tomato (Table III, cf. Table II). By 62 to 63 d, both mutant and normal XGase activities were barely detectable. The main difference observed in the mutant extracts was in the capacity to depolymerize XG in the presence of oligosaccharides, which increased during aging rather than abruptly declining, as during normal ripening (Table III, cf. Fig. 5). This may account for the observation that XG levels declined over time in *rin* but not in ripening fruit.

DISCUSSION

For XG to play a critical role in regulating the firmness of fruit tissues, it must be tightly bound to cellulose, as it is in growing tissues (Hayashi, 1989), rather than simply secreted into periplasmic spaces, as it is in seeds that employ it as a reserve polysaccharide (Reis et al., 1987). We have confirmed earlier observations (Huber and Lee, 1986) that tomato fruit contains a polysaccharide that is not starch but that reacts with the Kooiman (1960) I-KI reagent to yield a distinctive blue-green color, and that requires concentrated alkali to solubilize it from the wall. This fraction has terminal and 2-

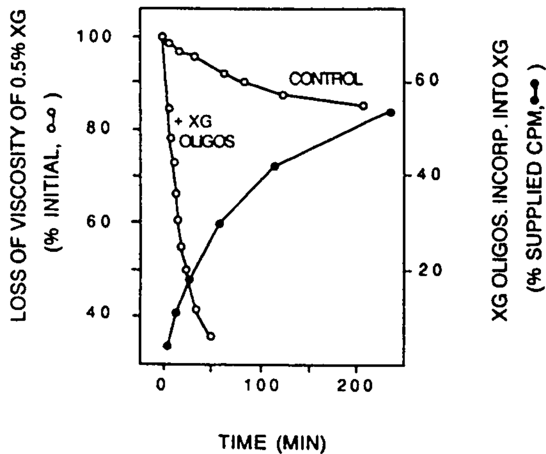

Figure 4. Progress curves for the decline in viscosity of tamarind XG solutions catalyzed by tomato fruit extract plus or minus (control) XG oligosaccharides (XG OLIGOS) (O) and for XET activity (●). Assays were conducted as described in "Materials and Methods" using enzyme extracted in buffer (pH 6) containing 1 M NaCl from whole tomato fruit harvested at breaker. Final concentrations of substrate XG (0.5%, w/v) were the same in the three assays, and XG oligosaccharides (0.02%, w/v) were derived from partial hydrolysates of tamarind XG that had been either [^{14}C]fucosylated (XET assay) or left unlabeled (viscometric assays).

linked xylosyl and 1,4,6-linked glucosyl units (methylation analysis) that are characteristic of all XGs (Tong and Gross, 1988; Seymour et al., 1990). Although Huber and Lee produced results suggesting that ripening in tomato was accompanied by a loss of hemicellulosic Xyl and a decline in amount and DP of I-KI-positive material, the latter studies were unable to duplicate these observations. It is possible that turnover or transglycosylation rates are variable under different nutritional regimes and may mask any net effects of "loosening" reactions on XG size and DP. Certainly Xyl and Glc continue to be incorporated into hemicellulosic and cellulosic fractions of the tomato wall until well into late stages of fruit ripening (Mitcham et al., 1989; Greve and Labavitch, 1991).

In the present study neither bound XG nor cellulose levels in wild-type tomato fruit tissues showed any significant decline in total amount during the ripening process (Table I). However, cellulose levels were low (approximately 3% dry weight) and the relative weight of XG:cellulose (30% in pericarp and 5% in locules) was much less than that found in this macromolecular complex from growing tissues (e.g. 70% in pea epicotyl; Hayashi and Maclachlan, 1984). It may be that these polysaccharides had already been partially degraded in developing tomato fruits during cell expansion (0–30 d postanthesis) before the present measurements began.

Clear evidence for a major reduction in the size distribution of XG chains was found in later stages of ripening in both pericarp and locular tissues (Fig. 2). The ratio of XG with a size greater than the equivalent of dextran 264 to XG with a size less than dextran 264 fell in pericarp tissue from about 3:1 (green) to less than 0.5:1 (red). In locules this ratio declined from about 0.9 to 0.4. There was no such evidence for any decline in cellulose DP in pericarp tissue during ripening (Fig. 3), perhaps because there was sufficient XG coating on the surface of microfibrils to impede access of XG-cleaving enzymes to the cellulose chains. In locules, where the low XG:cellulose ratio would barely suffice to provide a monolayer of XG to coat microfibril surfaces (Hayashi and Maclachlan, 1984), some cellulose depolymerization did occur during ripening (Fig. 3).

With respect to CMCase activities in ripening tomatoes, the buffer-insoluble, salt-soluble extracts of pericarp reduced the viscosity of CMC solutions at initial rates that increased sharply as ripening progressed (Fig. 5). Buffer-soluble CMCase levels also increased, but gradually. Total CMCase activities of locular tissue were especially high and increased even further with ripening (Table III). These are nonspecific 1,4-β-glucanases (Brady and Maclachlan, 1992) and could have been responsible for the partial depolymerization observed in XG and cellulose in ripe fruits (Figs. 2 and 3).

The level of XET activity in buffer-soluble extracts of

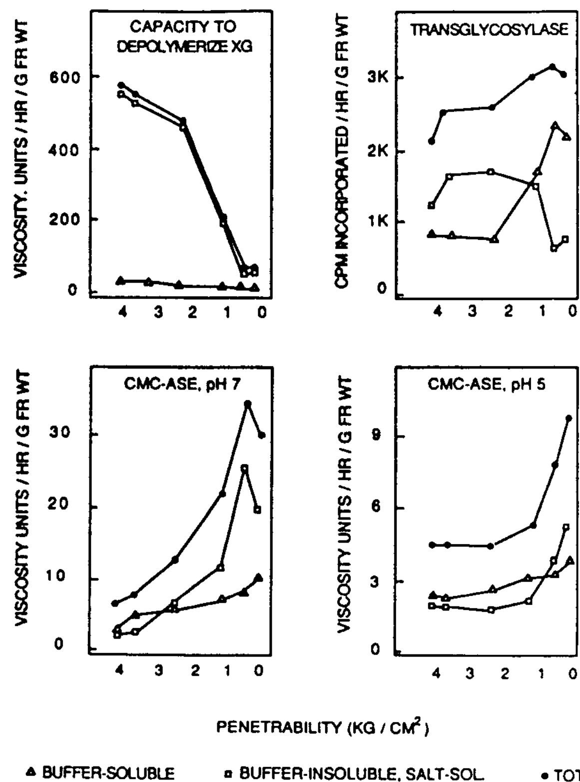

Figure 5. Changes in activity levels during tomato fruit ripening of enzymes from pericarp tissue that cleave XG or CMC. Fruits were harvested at intervals as they changed from green and firm (penetrability 4.1 kg/cm^2) to red and soft (0.4 kg/cm^2). Pericarp tissue was excised and extracted first with buffer at low ionic strength, then with buffer containing 1.0 M NaCl. CMCases, XET, and the capacity to depolymerize XG in the presence of XG oligosaccharides was assayed in these extracts as described in "Materials and Methods." △, Buffer-soluble; □, buffer-insoluble, salt-soluble; ●, total activity. The capacity to depolymerize XG in the absence of added oligosaccharide (XGase activity) is shown in Table II.

Table II. *The capacity of extracts from wild-type tomato fruit pericarp to depolymerize XG in the absence of added XG oligosaccharides (XGase activity)*

Assays were conducted viscometrically using 0.5% (w/v) tamarind XG as substrate (see "Materials and Methods") and enzyme from fruits (83-G-38) at various stages of ripening (see Fig. 1). Change in levels of other XG-cleaving enzyme activities and in the capacity to depolymerize XG in the presence of XG oligosaccharides are shown in Figure 5.

	Age (d after anthesis)					
	39	41	43	45	52	63
Penetrometer (kg/cm^2)	4.1	3.6	2.3	1.1	0.6	0.4
XGase Activity[a]						
Buffer-soluble	1.6	1.0	0.5	0.7	0.5	0.3
Buffer-insoluble, salt-soluble	4.9	4.2	3.6	1.3	0.8	0.2
Total	6.5	5.2	4.1	2.0	1.3	0.5

[a] Viscosity units h^{-1} g^{-1} fresh weight.

pericarp also increased markedly during ripening (Fig. 5). The timing was such that transglycosylation reactions could have helped maintain the net level of XG in this tissue even as its DP declined (Table I; Fig. 2). However, XET activity in buffer-insoluble, salt-soluble extracts drifted downwards in pericarp tissue during ripening (Fig. 5), as did total XET activity in locules (Table III). This may reflect the presence of more than one XET isozyme or changes in intracellular localization of the enzyme in this fruit over time.

Changes in the rate at which wild-type tomato extracts depolymerized XG, as assayed viscometrically in the presence of XG oligosaccharide, differed fundamentally from the patterns of development of CMCase and XET activities in two respects: first, the capacity for depolymerization was almost entirely confined to buffer-insoluble extracts, and second, its level declined precipitously rather than rising or drifting when the tissue softened (Fig. 5). Evidently the increases observed in CMCase and XET activities at this time could not have contributed significantly to the total capacity of fruit extracts to depolymerize XG in vitro, whatever role they may have had in metabolizing XG in vivo. The capacity of tomato extracts to reduce the viscosity of XG solutions in the absence of added oligosaccharides (XGase activity, Tables II and III) was relatively weak, but it too was found predominantly in the salt-soluble enzyme fraction, and it declined in level as tissue softened. The similarity in these profiles suggests that it represents a latent form of XGase that was activatable by oligosaccharide. It seems less likely to have been a weak hydrolase that changes to an active transglycosylase in the presence of oligosaccharide acceptors, since XET activity was substantial and mainly buffer soluble in ripe tomatoes (Fig. 5; Table III). Clearly, it will be necessary to purify these activities and compare physical properties in detail before the number of enzymes in tomato that are responsible for XGase and XET activities is resolved.

At this point it can be concluded that tomatoes contain a variety of enzymes that cleave 1,4-β-glucosyl linkages in XG or cellulose, none of which can be dismissed as irrelevant to fruit development or the fruit-ripening process. The capacity to depolymerize XG, being largely buffer-insoluble but extractable by salt (wall bound?) and much more active in green wild-type or *rin* mutants than in ripe fruit, appears to be due

Table III. *Activities of enzymes that cleave XG or CMC in extracts of wild-type (83-G-38) tomato locules and in rin mutant tomato pericarp*

Enzyme activities in 83-G-38 are the sum of those in buffer- and salt-soluble extracts (see "Materials and Methods"), and in *rin* are those solubilized by one extended extraction in buffer, 1 M NaCl. XG-depolym. refers to the capacity of extracts to reduce viscosity of 1% XG solutions. oligos, Oligosaccharide subunits of XG.

	Tissue			
	83-G-38 locules		*rin* pericarp	
	35 d[a]	52 d	39 d	62 d
Color	Green	Red	Green	Yellow
Penetrometer (kg/cm^2)	3.6	0.6	4.4	2.4
1,4-β-Glucan-cleaving activity[b]				
CMCase, pH 5	94	117	3	6
CMCase, pH 7	22	104	12	32
XG-depolym., minus oligos	1.3	0.3	0.8	0.6
XG-depolym., plus oligos	50	5	175	338
XET activity[c]	1981	1828	795	1130

[a] d after anthesis. [b] Viscosity units h^{-1} g^{-1} fresh weight. [c] cpm incorporated h^{-1} g^{-1} fresh weight.

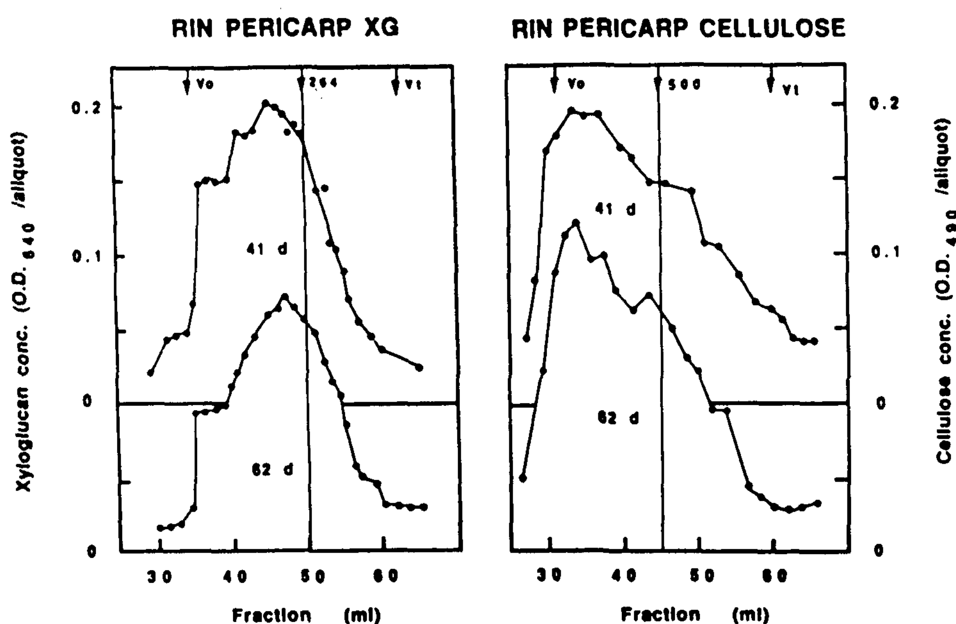

Figure 6. Size distribution of XG and cellulose samples taken from the pericarp of *rin* mutant tomatoes at 41 and 62 d after anthesis. The mutant fruit changed from green and firm at 41 d to yellow and partly softened at 62 d (see Table III). XG was extracted and fractionated on a column of Sepharose CL-6B with dextran 264 kD as standard, and cellulose was fractionated on glass beads with dextran 500 kD as standard, as described in "Materials and Methods." XG was assayed with I-KI and cellulose with phenol-sulfuric acid.

to a specific XGase activity that may be particularly useful as a wall-loosening device during turgor-driven fruit expansion. In contrast, the capacity for XG-transglycosylation was mainly buffer soluble and concentrated in softening red fruit, where it and nonspecific β-glucanases are the best candidates for catalyzing the decline in degree of polymerization of XG that is observed in vivo at that time. It is evident that transgenic experiments to selectively manipulate the levels of these enzymes are necessary before their potential roles in influencing the plasticity and firmness of tomato fruit can be clearly established.

ACKNOWLEDGMENTS

We wish to thank Dr. Anne Camirand, who prepared the [^{14}C]-Fuc-labeled XG from tamarind XG that we used as source of labeled oligosaccharide. We are grateful to Ms. Glenda Orr and Dr. David Brummell for technical assistance and many useful discussions during the course of this work.

Received September 8, 1993; accepted March 23, 1994.
Copyright Clearance Center: 0032-0889/94/105/0965/10.

LITERATURE CITED

Brady C (1987) Fruit ripening. Annu Rev Plant Physiol **38:** 155–178

Campbell AD, Hysamer M, Stotz HV, Greve LC, Labavitch JM (1990) Comparison of ripening processes in intact tomato fruit and excised pericarp discs. Plant Physiol **94:** 1582–1589

DellaPenna D, Lashbrook CC, Toenjes K, Giovannoni JJ, Fischer RL, Bennett AB (1990) Polygalacturonase isozymes and pectin depolymerization in transgenic *rin* tomato fruit. Plant Physiol **94:** 1882–1886

Dubois M, Gills KA, Hamilton JK, Rebers RA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem **28:** 350–356

Edwards M, Dea ICM, Bulpin PV, Reid JSG (1985) Xyloglucan (amyloid) mobilization in the cotyledons of *Tropaeolum majus* L. seeds following germination. Planta **163:** 133–140

Edwards M, Dea ICM, Bulpin PV, Reid JSG (1986) Purification and properties of a novel xyloglucan-specific endo-1,4-β-D-glucanase from germinated nasturtium seeds. J Biol Chem **261:** 9489–9494

Fanutti C, Gidley MJ, Reid JSG (1993) Action of a pure xyloglucan endo-transglycosylase (formerly called xyloglucan-specific endo-(1,4)-β-D-glucanase) from the cotyledons of germinated nasturtium seed. Plant J **3:** 691–700

Farkas V, Maclachlan G (1988) Stimulation of pea 1,4-β-glucanase activity by oligosaccharides derived from xyloglucan. Carbohydr Res **184:** 213–219

Farkas V, Sulova Z, Stratilova E, Hanna R, Maclachlan G (1992) Cleavage of xyloglucan by nasturtium seed xyloglucanase and transglycosylation to xyloglucan subunit oligosaccharides. Arch Biochem Biophys **298:** 365–370

Fischer RL, Bennett AB (1991) Role of cell wall hydrolases in fruit ripening. Annu Rev Plant Physiol Plant Mol Biol **42:** 675–703

Fry SC, Smith RC, Renwick KF, Martin DJ, Hodge SK, Matthews KJ (1992) Xyloglucan transglycosylase, a new wall-loosening enzyme activity from plants. Biochem J **282:** 821–828

Giovannoni JJ, DellaPenna D, Bennett AB, Fischer RL (1989) Expression of a chimeric polygalacturonase gene in transgenic rin (ripening inhibited) tomato fruit results in polyuronide degradation but not fruit softening. Plant Cell **1:** 53–63

Gordon R, Maclachlan G (1989) Incorporation of UDP-[^{14}C]glucose into xyloglucan by pea membranes. Plant Physiol **91:** 373–378

Greve LC, Labavitch JM (1991) Cell wall metabolism in ripening fruit. Plant Physiol **97:** 1456–1461

Hanna R, Brummell DA, Camirand A, Hensel A, Russell EF, Maclachlan G (1991) Solubilization and properties of GDP-fucose:xyloglucan 1,2-α-L-fucosyltransferase from pea epicotyl membranes. Arch Biochem Biophys **290:** 7–13

Hayashi T (1989) Xyloglucans in the primary wall. Annu Rev Plant Physiol Plant Mol Biol **40:** 139–168

Hayashi T, Maclachlan G (1984) Pea xyloglucan and cellulose. I. Macro-molecular organization. Plant Physiol **75:** 596–604

Huber DJ, Lee JH (1986) Comparative analysis of pectins from pericarp and locular gel in developing tomato fruit. *In* ML Fishman, JJ Jew, Chemistry and Function of Pectins. American Chemical Society, Washington, DC, pp 141–156

Kooiman P (1960) A method for the determination of amyloid in plant seeds. Recl Trav Chim Pays-Bas **79:** 675–678

Lashbrook CC, Brummell DA, Gonzalez-Bosch C, del Campillo E, Bennett A (1993) Molecular characterization of differentially expressed endo-β-1,4-glucanase (cellulase) genes in tomato flower abscission and fruit ripening (abstract No. 555). Plant Physiol **102:** S-99

Lorences EP, Fry SC (1993) Xyloglucan oligosaccharides with at least two α-D-xylose residues act as acceptor substrates for xyloglucan endotransglycosylase and promote the depolymerization of xyloglucan. Physiol Plant **88:** 105–112

Maclachlan G (1988) β-Glucanases from *Pisum sativum*. Methods Enzymol **160:** 382–391

Maclachlan G, Brady C (1992) Multiple forms of 1,4-β-glucanase in ripening tomato fruits include a xyloglucanase activatable by xyloglucan oligosaccharides. Aust J Plant Physiol **19:** 137–146

Maclachlan G, Levy B, Farkas V (1992) Acceptor requirements for GDP-fucose: xyloglucan 1,2-α-L-fucosyltransferase activity solubilized from pea epicotyl membranes. Arch Biochem Biophys **294:** 200–205

Mitcham E, Gross K, Ng T (1989) Tomato fruit cell wall synthesis during development and senescence. Plant Physiol **89:** 477–481

Nishitani K, Tominaga R (1992) Endo-xyloglucan transferase, a novel class of glycosyltransferase that catalyses transfer of a segment of xyloglucan molecule to another xyloglucan molecule. J Biol Chem **267:** 21058–21064

Redgwell RJ, Fry SC (1993) Xyloglucan endo-transglycosylase activity increases during kiwi fruit (*Actinidia deliciosa*) ripening. Plant Physiol **103:** 1399–1406

Reis D, Vian B, Darzens D, Roland J-C (1987) Sequential patterns of intramural digestion of galactoxyloglucan in tamarind seedlings. Planta **170:** 60–73

Seymour GB, Colquhoun IJ, DuPont MS, Parsley KR, Selvendran RR (1990) Changes in the cell wall polysaccharides of tomato fruit during ripening. Phytochemistry **29:** 725–731

Sheehy RE, Kramer MK, Hiatt WR (1988) Reduction of polygalacturonase activity in tomato fruit by antisense RNA. Proc Natl Acad Sci USA **85:** 8805–8809

Smith CJS, Watson CF, Morris PC, Bird CR, Seymour GB, Gray JE, Arnold C, Tucker GA, Schuch W, Harding S, Grierson D (1990) Inheritance and effect on ripening of antisense polygalacturonase genes in transgenic tomatoes. Plant Mol Biol **14:** 369–379

Smith CJS, Watson CF, Ray J, Bird CR, Morris PC, Schuch W, Grierson D (1988) Antisense RNA inhibition of polygalacturonase gene expression in transgenic tomatoes. Nature **334:** 724–726

Tong CBS, Gross KC (1988) Glycosyl-linkage composition of tomato fruit cell wall hemicellulosic fractions during ripening. Physiol Plant **74:** 365–370