Fruit softening: evidence for pectate lyase action in vivo in date (Phoenix dactylifera) and rosaceous fruit cell walls

Thurayya Z. S. Al Hinai¹, Robert A. M. Vreeburg¹, C. Logan Mackay², Lorna Murray², Ian H. Sadler² and Stephen C. Fry¹,*

¹The Edinburgh Cell Wall Group, Institute of Molecular Plant Sciences, The University of Edinburgh, Daniel Rutherford Building, The King’s Buildings, Max Born Crescent, Edinburgh EH9 3BF, UK
²EastCHEM School of Chemistry, The University of Edinburgh, The King’s Buildings, Edinburgh EH9 3FJ, UK

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INTRODUCTION

Fruit softening in general

The programmed softening that occurs during the ripening of many fruit species requires cell wall loosening and a reduction in cell–cell adhesion as a result of dissolution of the pectin-rich middle lamella (Jarvis et al., 2003; Brummell, 2006). Characteristic modifications include solubilization and depolymerization of pectin, loss of neutral sugars from pectic side chains, cell wall swelling and disassembly of the xyloglucan–cellulose network (Paniagua et al., 2017). These modifications are partly due to non-enzymic reactions with reactive oxygen species (especially the hydroxyl radical, •OH; Dumville and Fry, 2000; Airianah et al., 2016) or expansins (Brummell et al., 1999), and partly the result of wall-modifying enzymes secreted into the apoplast during ripening. These enzymes act by cleaving polysaccharides, resulting in mechanical weakening. There are three such types of enzyme activity: hydrolases, transglycosylases and lyases, requiring specific substrates (Moya-León et al., 2019). Endo-acting wall-modifying enzymes studied in relation to fruit softening include xyloglucan endotransglycosylase/hydrolases (XTHs) (Saladié et al., 2006; Miedes and Lorences, 2009), cellulases (Dong et al., 2018), endo-polygalacturonases (EPGs) (Wu et al., 1993; Asif and Nath, 2005; Quesada et al., 2009), pectate lyases (PLs) (Marín-Rodríguez et al., 2003; Dong et al., 2018) and rhamnogalacturonan lyases (Ochoa-Jiménez et al., 2018; Méndez-Yañez et al., 2020). In addition, pectin methylesterases (Tieman et al., 1992; Phan et al., 2007) and exo-polygalacturonases (Bartley, 1978; Yang et al., 2018) attack pectin but not by mid-chain cleavage. However, the link...
between enzyme activities (measured in vitro after extraction of the enzymes) and fruit softening was often contradictory.

Pectins

In tomato, the most extensively studied model fruit, and in many other fleshy fruits, pectin modification is the most pronounced cell wall change during ripening. Pectin has three major domains (reviewed by Fry, 2010): homogalacturonan (HG; ‘pectate’), which consists of a mainly unbranched chain of anionic (1→4)-α-d-galacturonic acid (GalA) residues plus neutral blocks of methyl-esterified (1→4)-α-GalA residues; rhamnogalacturonan-I, which has a backbone of repeating disaccharide units of (1→4)-α-d-GalA-(1→2)-α-l-Rha (where Rha = rhamnose), with neutral side chains of β-galactose and α-arabinose usually attached to approx. 50 % of the rhamnose residues at their O-4 position; and rhamnogalacturonan-II, which consists of eight or more (1→4)-α-d-GalA residues as a backbone to which five different side chains are attached, making a highly complicated structure. Another, often minor, domain of pectin is xylogalacturonan which has an α-d-GalA backbone (with or without methyl esters) with β-d-xylene and α-l-fucose side chains. The present study focuses on HG, which is usually the most abundant pectic domain.

HG-acting enzymes

Plants possess two enzyme activities capable of cleaving the backbone of anionic HG domains in mid-chain: EPG and PL. Both of these act only on anionic HG domains, and therefore prior de-methylesterification by pectin methylesterase may be necessary (Tieman et al., 1992; Dong et al., 2018). In addition, plants have exo-PG (α-d-galacturonidase), which removes GalA residues one at a time from the non-reducing end of HG, presumably having relatively little effect on the cell wall’s mechanical properties. (In this paper, we use ‘EPG’ specifically for endo-polygalacturonase and ‘PG’ for polygalacturonase where we feel the data do not distinguish endo- from exo-.)

EPG, which catalyses endo-hydrolysis (Fig. 1A, reaction i), is the most studied pectin-cleaving enzyme, yet its effect on fruit softening may be low (Wang et al., 2018). Genes encoding EPGs are often upregulated during fruit ripening (Tucker and Grierson, 1982), suggesting that this enzyme may be produced during softening. This is supported by reports of PG activity extractable from fruit (Wu et al., 1993; Orr and Brady, 1993; Villarreal et al., 2008; Zhang et al., 2020). However, many such reports have not satisfactorily distinguished between EPG and PL, and even exo-PG, activities. For example, ‘EPG’ activity in strawberry extracts was often assayed as in-vitro production of new reducing termini (i.e. as total reducing groups) from a substrate of pure HG (Villarreal et al., 2009; Figueroa et al., 2010; Zhou et al., 2015, based on an influential study by Gross, 1982); however, reducing groups are generated from HG by endo-PG, exo-PG and PL, and also by OH reactions, so these three enzyme activities and the reactive oxygen species would not have been distinguished in such studies.

Transformation experiments with antisense PG genes in tomato and strawberry produced discrepant data. In tomato, PG expression (measured as mRNA levels by northern blotting) could be reduced to 1 % of that of the wild type without affecting softening (Smith et al., 1990; Brummell and Harpster, 2001), whereas in strawberry and apple, firmer fruits were produced when PG expression was reduced to 5–25 % of that of the wild type (Quesada et al., 2009; Atkinson et al., 2012; Posé et al., 2015).

PL cleaves anionic HG domains by a β-elimination reaction (i.e. non-hydrolytically) to give a product with a 4-deoxy-β-l-threo-hex-4-eno-pyronuronosyl residue (abbreviated as ΔUA, for ‘unsaturated uronic acid’) at the newly formed non-reducing end (Fig. 1B, reaction i) (Fuchs, 1965; Shaligram and Singhal, 2010; Nasuno and Starr, 1967; Iqbal et al., 2016; Zhou et al., 2016). (Note: rules of carbohydrate nomenclature dictate that a β-l-ΔUA residue is the product expected when a lyase catalyses an elimination reaction starting with a pectic α-d-GalA residue; this does not imply any change in the configuration at carbon-1.) Earlier work had reported a microbial pectin lyase (not PL) that acts on methylsterified HG (Albersheim et al., 1960). PL gene expression (monitored as mRNA accumulation) has been reported in ripening fruits including strawberry (Benitez-Burraco et al., 2003; Figueroa et al., 2008), banana (Dominguez-Puigjana et al., 1997; Pua et al., 2001), mango (Chourasia et al., 2006; Deshpande et al., 2017) and grapes (Nunan et al., 2020). Despite early negative reports (e.g. Besford and Hobson, 1972), recent studies have suggested a central role for PL genes in tomato fruit softening: tomato fruits with silenced PL genes had reduced PL mRNA expression, reduced extractable PL enzyme activity and increased fruit firmness (Uluisk et al., 2016; Yang et al., 2017). A putative PL gene was ascribed a possible role in softening (Jiménez-Bermúdez et al., 2002; Marín-Rodríguez et al., 2002). PL activity (assayed in vitro) is extractable from ripening strawberry (Zhou et al., 2016), banana (Marín-Rodríguez et al., 2003) and several other fruits (Wang et al., 2018).

Expression, activity and action

Fruit species clearly differ in the reactions modifying HG during ripening, and in no species can the reaction(s) which contribute the ‘key’ role in softening be precisely defined. Often, mRNA accumulation has been taken as evidence of ‘contribution’. Fewer studies have assayed extractable enzyme activities, and very few have tested whether the enzymes exhibit action in the fruit in vivo. Activity is measured in katal among optimized conditions in vitro; action is what can be observed in vivo, in living fruit tissue. Direct evidence for enzyme action can potentially be provided by analysis of changes in polysaccharide chemistry during ripening.

There are several plausible reasons why an enzyme that exhibits in vitro activity when extracted from the plant might not exhibit action within the living plant. For example (Fry, 2004), the enzyme and its substrate may be spatially separated, specific inhibitors may be present, the apoplastic redox potential, pH or ionic strength may not be optimal, or the prior action of
Fig. 1. EPG and PL action on homogalacturonan followed by Driselase digestion. (A) EPG attacking the (1→4) glycosidic bond between de-esterified GalA residues of HG, producing a new reducing terminus and a new saturated non-reducing terminus by hydrolysis. Digestion of EPG products with Driselase cleaves the remaining methyl ester groups and the whole chain of HG to monomeric GalA by its combination of hydrolysing enzymes including PME, EPG and galacturonidase. (B) PL attacking the same substrate, producing a new reducing terminus and a new unsaturated non-reducing (ΔUA) terminus by β-elimination. Digestion of PL products with Driselase cleaves the remaining methyl ester groups and the whole chain of HG to GalA monomers plus the dimer, ΔUA–GalA, the unique PL action fingerprint.
a necessary helper enzyme (pectin methylesterase in the case of EPG and PL) may not have occurred.

Dates

The present work focuses on fruit of the date (Phoenix dactylifera), a dioecious monocot in the commelinid family Arecaceae. It is widely cultivated in the Middle East and North Africa. Date ripening in many varieties is marked by a decrease in water content and an increase in soluble sugar (Ahmed et al., 1995; El Arem et al., 2011). A remarkable decrease in the cell wall content of the fruit pulp has also been reported in ripe date as well as other fleshy fruits (Vicente et al., 2007; Griba et al., 2013).

In date fruits, pectin is the major non-cellulosic cell wall component, rather than hemicelluloses as in commelinid grasses. During date ripening, a decreased degree of HG methylesterification was reported (Griba et al., 2013), making it a potential substrate for hydrolysis by PG and β-elimination by PL. Moreover, an increase in extractable cellulase, β-galactosidase (Rastegar et al., 2012) and PG (Serrano et al., 2001) activities was reported in date. β-Galactosidase and PG activities peaked at the full ripe stage, after which the PG activity was reduced while β-galactosidase activity remained high. The increase in the extractable activities of these two enzymes was correlated with fruit softness during ripening (Serrano et al., 2001). No data are available about PL in dates – either PL activity in extractable proteins or PL action in vivo. We aimed to supply the first evidence for PL action in fruits.

Strategy for detecting PL action

Each of the proposed mechanisms of HG endo-cleavage leaves a fingerprint on the fruit’s pectin which may be used as a tool to examine the in-vivo contribution of each mechanism to ripening. Oxidation by -OH leaves mid-chain oxo groups (Arianah et al., 2016), hydrolysis by EPG leaves a new non-reducing terminal GalA residue, and β-elimination by PL leaves a new non-reducing terminal ΔUA residue. It had not been tested whether PL exhibits action in vivo – in the fruit of any species, or indeed in any other plant organs. Here, we provide the first evidence of PL’s in-vivo action by detecting its unique fingerprint (containing ΔUA) in ripe fruits of several species.

MATERIALS AND METHODS

Materials

Ripe date (Phoenix dactylifera ‘Khalas’) fruits were collected from three randomly selected trees from a date palm field in Oman in June 2018. The samples were stored at ~80 °C. Pear (Pyrus communis ‘Conference’), rowan (Sorbus aucuparia) and apple (Malus pumila ‘Bramley’) fruits were collected from a private garden in Edinburgh, UK.

Cellvibrio japonicus PL, purchased as an ammonium sulfate suspension (from Megazyme; https://www.megazyme.com), was centrifuged at 14 500 g for 3 min and the pellet was re-dissolved in water at 10 U mL⁻¹. Aspergillus aculeatus EPG, purchased as an ammonium sulfate suspension (Megazyme; https://www.megazyme.com), was not pelletable and was therefore dialysed against pyridine/acetic acid/0.5 % chlorobutanol buffer (1:1:98), then diluted to 10 U mL⁻¹. Driselase (from Basidiomycetes sp. 067K1305, Sigma) was purified by ammonium sulfate precipitation and gel permeation chromatography (Fry, 2000). Homogalacturonan (= ‘polygalacturonic acid’ or ‘sodium polypectate’), CAPS [3-(cyclohexylamino)-1-propanesulfonic acid] and CaCl₂ were from Sigma–Aldrich (https://www.sigmaaldrich.com/united-kingdom.html). Aluminium-backed F254 silica-gel thin-layer chromatography (TLC) plates (1.0554.0001) were from Merck (https://www.merckgroup.com/uk-en). We found that for oligogalacturonide analysis, aluminium-backed plates gave much better chromatography than the corresponding plastic-backed plates.

PL in-vitro activity products

A reaction mixture of 6.6 mg mL⁻¹ HG, 50 mM CAPS (Na⁺, pH 10), 1 mM CaCl₂ and 3.3 U mL⁻¹ PL was incubated at 20 °C. The reaction was stopped at the desired time points by addition of 0.2 volumes of formic acid. Products were used as (unsaturated) ΔUA–GalAₙ markers.

EPG in-vitro activity products

Commercial EPG at 10 U mL⁻¹ was used to digest 20 mg mL⁻¹ HG in pyridine/acetic acid/0.5 % chlorobutanol buffer (1:1:98), pH 4.7. The reaction mixture was incubated on a wheel at 20 °C overnight and products were used as (saturated) GalAₙ markers.

Paper chromatography

PL and EPG in-vitro digestion products were loaded on Whatman No. 1 paper and run in ethyl acetate/acetate acid/water (EAW) 10:5:6 for 30 h. The paper was then dried and stained with AgNO₃ (Fry, 2000).

Preparation of alcohol-insoluble residue (AIR)

We prepared AIR as the source of cell walls by homogenizing (using a pestle and mortar) 9 g of fresh fruit in 36 mL of 75 % ethanol containing 5 % formic acid. The homogenate was incubated on a wheel at 20 °C overnight and then centrifuged at 3220 g for 5 min. The pellet was washed twice in 75 % ethanol and then saponified in 10 mL of aqueous 0.2 M Na₂CO₃ at 4 °C for 16 h. The Na₂CO₃ was neutralized by acetic acid, then ethanol was added to a final concentration of 75 % and the suspension was kept overnight at 4 °C (thus any polysaccharides solubilized in Na₂CO₃ would be reunited with the insoluble wall fraction). The mixture was centrifuged at 3220 g for 5 min. The pellet was washed three times in 75 % ethanol...
and twice in acetone for 1 h each on a wheel. The final pellet of (de-esterified) AIR was dried and stored at room temperature for analysis.

Driselase digestion

De-esterified date AIR (25 mg d. wt) was digested in 3 mL of 0.05 % Driselase in pyridine/acetic acid/water (1:1:98 v/v/v, containing 0.5 % chlorobutanol) at 37 °C for 3 d. Digestion was stopped by addition of 0.2 volumes of formic acid and the products were stored at −20 °C.

High-pressure liquid chromatography (HPLC)

HPLC was performed on a column of CarboPac PA1 (250 × 4 mm; Dionex UK Ltd, https://www.thermofisher.com/uk/en/home/industrial/chromatography/dionex.html) eluted at 1 mL min⁻¹ with a linear gradient of 100 % solution A (500 mm NaOH)→100 % solution B (500 mm NaOH in 500 mm sodium acetate) in 30 min followed by isocratic B for 10 min (García-Romera and Fry, 1995) Carbohydrates in the eluate were monitored by use of a pulsed amperometric detector with a gold electrode (Dionex).

High-voltage paper electrophoresis

Samples of the AIR/Driselase digestion products were loaded as a 20 cm streak (200 µL cm⁻¹) on Whatman No. 3 paper. Electrophoresis was conducted at pH 2.0 in a volatile buffer [formic acid/acetic acid/water (1:3.5:35.5 v/v/v)] at 3 kV for 4 h. The apparatus and methods are described by Fry (2020). Papers were dried and viewed under a 254 nm ultraviolet (UV) lamp. A small part of the paper (the fringe of the sample streak plus the whole neighbouring marker mixture) was stained with AgNO₃ (Fry, 2000).

Unsaturated oligogalacturonides were eluted from specific zones of the unstained part of the paper electrophoretogram in 75 % ethanol, dried and re-dissolved in 50 µL of H₂O.

Thin-layer chromatography

Samples eluted from paper electrophoretograms were loaded on TLC plates as 0.8 cm streaks (2.5 µL of each sample). The plate was run in butanol-ol/acetic acid/water (2:1:1) for 7 h, then dried and stained by dipping in thymol solution (0.5 % w/v thymol and 5 % H₂SO₄ v/v in ethanol) followed by re-drying and then heating in an oven at 105 °C for 5 min.

Nuclear magnetic resonance (NMR) spectroscopy

A sample of putative ΔUA–GalA was prepared by complete digestion of 6.6 mg mL⁻¹ HG in 3.3 U mL⁻¹ PL in 50 mM CAPS (Na⁺, pH 10) and 1 mM CaCl₂. The resulting ΔUA–GalA was purified by a preparative high-voltage paper electrophoresis, eluted in 75 % ethanol and then dried. The 1-D and 2-D proton and ¹³C-NMR spectra were recorded on a Bruker AVANCE NEO instrument (18.8 T; 800 MHz for protons) using d₇-methanol as solvent. Proton spectra were referenced to the residual CD₃OD signal at 3.33 ppm and ¹³C spectra were referenced to CD₂OD at 49.0 ppm. Chemical shifts are given in ppm (δ) relative to tetramethylsilane, and scalar coupling constants (J) are given in Hz.

Mass spectrometry

A sample of putative ΔUA obtained by Driselase digestion of de-esterified date fruit cell walls and preparative paper electrophoresis was prepared for electrospray analysis at a concentration of approx. 10 µg in acetonitrile/water (1:1). Analysis was performed on a 12-tesla SolarixX 2XR Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bruker Daltonics) operating in negative mode. Each spectrum was the sum of 20 scans, with a dataset size of 2 million words. Fragmentation was performed by collision-induced dissociation (CID) with argon as a neutral gas. The collision voltage was 10 V. Data interpretation was achieved with DataAnalysis 5.0 (Bruker Daltonics).

RESULTS

Products formed by action of commercial PL or EPG on commercial HG in vitro

A time-course for the digestion of commercial HG by commercial PL in vitro revealed a range of unsaturated oligogalacturonides even after 2 min at 20 °C, as visualized by TLC (Fig. 2A). The concentration of the smallest product (confirmed below to be a dimer; ΔUA–GalA), indicated by thymol stain intensity, continuously increased with time up to 128 min, by which time the dimer was almost the sole product. The concentration of each of the bigger oligosaccharides transiently peaked and then diminished. A pentasaccharide (ΔUA–GalA₄), visible at 2 and 4 min, appeared to be the largest product capable of migrating from the origin.

A priori, it could be suggested that Driselase or commercial EPG themselves possess PL activity which would generate ΔUA–GalA₄ even from unmodified HG. However, this was shown not to be the case, as Driselase and EPG digestion of commercial HG generated only saturated products. Driselase produced a spot of GalA as the only final product visualized on TLC, and EPG digestion produced GalA, GalA₂ and GalA₃ (Fig. 2B).

Paper electrophoresis separates PL products from EPG products

Paper electrophoresis in pH 2.0 buffer showed a good discrimination between PL and EPG products, providing an efficient method to distinguish the products of these two enzymes. PL products run faster than EPG products owing to the low pKᵢ of the ΔUA residue (Fig. 3A). Regardless of the number of GalA residues (ΔUA–GalAₙ₋₃), PL products...
ran to a specific region of the electrophoretogram, giving
a UV-absorbing spot (characteristic of the ΔUA residue),
while EPG products ran slower, with monomeric GalA being
the slowest migrating acidic product (Fig. 3A). Electrophoresis
at pH 2.0 thus effectively gave a group separation of satu-
rated from unsaturated oligogalacturonides. In contrast, during
electrophoresis in pH 6.5 buffer (at which pH all –COOH
groups are almost fully ionized; Fry, 2020), GalA₃ and ΔUA–
GalA (which both possess two –COOH groups and are of
similar molecular weight) were not well separated (Fig. 3B).
The PL and EPG products also overlapped when paper chro-
matography (Fig. 3C) was used instead of electrophoresis. We
Fig. 3. Paper electrophoresis and chromatography for separating PL products from EPG products. (A) Expected and observed products formed from HG by EPG digestion and PL digestion. Left: EPG (10 U mL⁻¹) was incubated at 20°C for 16 h with HG (20 mg mL⁻¹) in pyridine/acetic acid/water (1:1:98 v/v/v, containing 0.5 % chlorobutanol), pH 4.7, yielding GalA₃, GalA₂ and GalA. Right: PL (3.3 U mL⁻¹) was incubated at 20°C for 10 min with HG (6.6 mg mL⁻¹) in 50 mM CAPS buffer (Na⁺, pH 10) containing 1 mM CaCl₂, yielding ΔUA–GalA₃, ΔUA–GalA₂ and ΔUA–GalA. Centre: products were electrophoresed at pH 2.0 (3 kV, 4 h), alongside markers, and stained with AgNO₃. (Two independent preparations of ΔUA–GalA were run, differing in purity and concentration.) (B) Electrophoresis at pH 6.5 of comparable markers. Markers [left to right: ΔUA–GalA₂ (PL product); ΔUA–GalA (PL product); GalA, GalA, and GalA (EPG products); galacturonic acid; glucose] were fractionated by high-voltage paper electrophoresis at pH 6.5 (4 kV, 50 min). Each sample contained an internal marker (Orange G), which was marked in pencil prior to staining. (C) Paper chromatography of comparable markers in ethyl acetate/acetic acid/water (10:5:6) for 30 h.
therefore recommend electrophoresis at pH 2.0 as the preferred method for isolating PL ‘fingerprints’.

Driselase trims large PL products to the disaccharide whereas EPG trims them to a mixture of products

The PL products from a brief digestion (2 min) of commercial HG with commercial PL (as in Fig. 2A) followed by either Driselase or EPG digestion showed the smallest product of each. Driselase digestion for up to 1 week at 37 °C produced spots of monomer (GalA) and the unsaturated dimer (ΔUA–GalA), as visualized on TLC (Fig. 2C). On the other hand, EPG digestion for the same period at 20 °C produced a spot of the unsaturated trimer (ΔUA–GalA₂) in addition to the unsaturated dimer (ΔUA–GalA) plus saturated GalA, GalA₁, and GalA₂ (Fig. 2D). Driselase, producing a single unsaturated product, is therefore the preferred agent for isolating a specific PL ‘fingerprint’ (ΔUA–GalA).

Confirmation of conclusions by HPLC

Performing HPLC of the products formed by brief in-vitro PL action on HG confirmed the presence of a series of unsaturated oligogalacturonides (Fig. 4B) which did not co-elute with saturated oligogalacturonides (Fig. 4A). The ΔUA–GalA₁ (Fig. 4C), purified by preparative paper electrophoresis, was digested by Driselase to yield ΔUA–GalA plus free GalA (Fig. 4D).

NMR evidence for the structure of the proposed ΔUA–GalA

The identity of the proposed ΔUA–GalA, obtained from complete digestion of commercial HG with commercial PL and isolated by preparative high-voltage paper electrophoresis, was tested by NMR spectroscopic analysis.

The proton spectrum (Fig. 5) showed that the sample of ΔUA–GalA was a mixture of α- and β-anomers (60:40) at GalA. The proton COSY spectrum (Fig. 5) allowed the identification of the separate proton signals. The 13C spectrum showed 24 signals as expected. These were assigned from the HSQC 1-bond CH correlation spectrum. Spectral data are given in Table 1. The proton–proton coupling constants confirm the stereochemistry of the GalA residue. The position of the linkage between the two rings is clear from the HMBC spectrum, which showed three-bond correlations between H-1 of ΔUA and C-4 of GalA and between H-4 of GalA and C-1 of ΔUA. All the other signals show correlations between protons and carbons in the same ring. In addition to the expected responses from di-axial protons (close in space), the proton NOESY spectrum also confirmed the presence of the GalA fragment as there are responses between H-3 and H-4, and H-4 and H5, confirming that H-4 is equatorial (H-4 axial would be too far away to give these responses). The response between the ΔUA protons H1 and H2 demonstrates that the linkage there is β-L-. If this were α-L-, these protons would be too far apart to give a response. There are also responses between the H1 of ΔUA and H4 of GalA, supporting the position of linkage on GalA.
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Fig. 5. NMR evidence for the structure of the proposed ΔUA–GalA. (A) Proton 1-D and proton COSY NMR spectra (region approximately 3.3–5.4 ppm) of ΔUA–GalA produced by *in-vitro* action of commercial PL on commercial HG. Signals labelled U arise from ΔUA; other labelled signals arise from GalA. The signal at 3.33 ppm arises from CD$_2$HOD. (B) Proposed structure of ΔUA–GalA.

|   | α-anomer (60 %) | β-anomer (40 %) |
|---|----------------|----------------|
|   | $\delta_H$  | $J_{HH}$ (Hz) | $\delta_C$ | $\delta_H$  | $J_{HH}$ (Hz) | $\delta_C$ |
| GalA-1α | 5.279 | 3.6 | 94.4 | GalA-1β | 4.576 | 7.8 | 98.6 |
| GalA-2α | 3.750 | 3.6, 10.2 | 70.2 | GalA-2β | 3.454 | 7.8, 10.0 | 73.3 |
| GalA-3α | 3.969 | 3.2, 10.2 | 70.0 | GalA-3β | 3.658 | Obscured | 73.7 |
| GalA-4α | 4.596 | 3.2, u | 81.4 | GalA-4β | 4.541 | 3.1, u | 80.2 |
| GalA-5α | 4.740 | bs | 70.7 | GalA-5β | 4.371 | bs | 74.3 |
| GalA-6α | 172.4 |   |   | GalA-6β |   |   | 171.4 |
| ΔUA-1 | 5.093 | 1.9 | 101.5 | ΔUA-1 | 5.116 | 2.0 | 101.5 |
| ΔUA-2 | 3.984 | 1.9, 5.6 | 71.8 | ΔUA-2 | 3.658 | Obscured | 71.9 |
| ΔUA-3 | 4.216 | 3.8, 5.6 | 67.2 | ΔUA-3 | 4.262 | 3.6, 6.2 | 67.2 |
| ΔUA-4 | 6.071 | 3.6 | 112.6 | ΔUA-4 | 6.066 | 3.6 | 112.9 |
| ΔUA-5 | 142.7 |   |   | ΔUA-5 |   |   | 142.5 |
| ΔUA-6 | 165.3 |   |   | ΔUA-6 |   |   | 165.4 |

bs = broad singlet, u = unresolved.
Detection of PL products in date fruit cell walls

Using the knowledge gained from the in-vitro PL activity experiments, we developed a protocol to detect PL action products in vivo. Driselase digestion of de-esterified date fruit cell walls (AIR) would cleave any PL action products, even large products such as ΔUA–GalAΔ to release the smallest unsaturated product (ΔUA–GalA) plus free GalA. Paper electrophoresis was then used to separate the highly acidic ΔUA–GalA from all other Driselase-generated sugars; TLC then helped to resolve and visualize the ΔUA–GalA, providing the proof for PL action in vivo. (Fig. 6A).

Paper electrophoresis (pH 2.0) of the products obtained by Driselase digestion of cell walls from ripe dates produced a heavy spot of neutral sugars, a heavy GaLA spot and a faster migrating, UV-absorbing spot indicating the presence of highly acidic, unsaturated products (Fig. 6A, left image). The electrophoretogram was cut into transverse strips, eluates of which were analysed by TLC. The neutral fractions (strips 4–6) gave a range of neutral sugars (probably including isoprimeverose, galactose, glucose and rhamnose) (Fig. 6A, right image). Fractions 7–10, which had co-electrophoresed with GalA, were confirmed by TLC to contain predominantly the monosaccharide GalA. TLC of the highly anionic, UV-absorbing fractions (14–16), which had co-electrophoresed with the ΔUA–GalAΔ species, revealed predominantly the dimer, ΔUA–GalAΔ (Fig. 6A), previously shown (Fig. 2C) to be the only unsaturated end-product of Driselase re-digestion of partial PL products.

Driselase digestion of HG (even if pre-digested by EPG) is expected to give only GaLA (Fig. 1A, reaction ii), whereas Driselase digestion of PL-pre-treated HG yields in addition one unsaturated dimer, ΔUA–GalAΔ for every PL event, from the non-reducing terminus (Fig. 1B, reaction ii). Thus the ΔUA–GalAΔ:GalAΔ ratio approximately indicates the number of PL-catalysed cuts per unit chain length of HG. In dates (Fig. 6A), the ΔUA–GalAΔ:GalAΔ ratio was estimated by pixel counting in Photoshop (Vreeburg et al., 2014) to be approx. 1:20, mol mol–1, suggesting that roughly one glycosidic bond in 20 of the endogenous HG domains had been cleaved by in-vivo PL action in dates. This approximation neglects the GaLA generated by Driselase digestion of fruit rhamnogalacturonan-I domains, but remains a reasonable approximation.

Further evidence that the ΔUA residue had been generated by the fruit in vivo (rather than artefactually by Driselase) came from a back-up study with commercial EPG, which lacks detectable PL activity (Fig. 2B). When Na4CO3-de-esterified fruit AIR was exhaustively digested with exogenous EPG, and the products were electrophoresed and fractions analysed by TLC, the major products were, as expected, three (saturated) hydrolysis products: GaLA, GaLAΔ and GaLAΔ (Fig. 6B). In addition, a substantial spot of ΔUA–GalAΔ and a trace of ΔUA–GalAΔ were detected: these electrophoresed with high mobility and ran on TLC in the expected positions. These observations confirm that endogenous PL had been acting in vivo on the pectin of live fruit.

Mass spectrometric confirmation of the identity of the in-vivo PL action product

Driselase digestion products of de-esterified date AIR were resolved by high-voltage paper electrophoresis as in Fig. 6A. The ΔUA–GaLA fraction was then analysed by negative-mode electrospray-ionization FT-ICR mass spectrometry (FT-ICR-MS). The simulated m/z of the ΔUA–GaLA anion is 351.05690 based on its formula of C12H15O12. Experimentally, molecular-ion negative-mode MS measured the m/z at 351.05677, i.e. the value expected with 0.37 ppm error (Fig. 7A).

The CID fragmentation of the ion observed at m/z 351.05677 resulted in several fragments that further supported the proposed structure (Fig. 7B).

DISCUSSION

Wall polysaccharide degradation in fruit: enzyme action contrasted with enzyme activity, gene transcription and protein synthesis

During fruit softening in many species, cell wall composition changes have been reported, especially in pectin domains, mostly presumed to be due to the actions of polysaccharide-modifying enzymes, although additional non-enzymatic wall degradation mechanisms can occur (Brummell et al., 1999; Dumville and Fry, 2003; Vreeburg et al., 2014; Arianah et al., 2016). Attention has focused on endo-enzymes, since these cleave polysaccharide molecules in mid-chain, potentially having a greater effect on wall mechanics than exo-enzymes, which only remove single monosaccharide residues. The two endo-enzyme activities that can cleave HG are EPG and PL. While initially reported to be absent (Besford and Hobson, 1972), and later somewhat side-lined, PL is becoming a focus of renewed interest (Marin-Rodriguez et al., 2002; Santiago-Doménech et al., 2008; Wang et al., 2018; Moya-León et al., 2019; Uluisik and Seymour, 2020). PL activity may be difficult to measure in conventional plant extracts in vitro as PLs are often deactivated during normal extraction protocols (Payasi et al., 2006); we therefore devised a method for detecting PL action in vivo.

A ripening-related increase in extractable PL activity, assayed in vitro, was reported in many fruits including tomato (Ulu̇isik et al., 2016), banana (Marin-Rodriguez et al., 2003) and strawberry (Zhou et al., 2016). A suggestion that endogenous PL may exhibit action in vivo comes from the observations that, in PL-silenced tomato fruits, less pectin became soluble (Yang et al., 2017) and its molecular weight remained relatively high (Ulu̇isik et al., 2016). However, an unambiguous demonstration of in-vivo PL action was lacking. In-vitro enzyme activity does not confirm in vivo action as there could be restrictions on substrate accessibility, presence of certain inhibitors and/or non-optimum action conditions in vivo.
Fig. 6. Detecting PL fingerprints in digests of date fruit cell walls by paper electrophoresis and TLC. (A) Driselase digestion. Date AIR (25 mg) was digested in 3 mL of Driselase (0.05 %) in PyAw, containing 0.5 % chlorobutanol at 37 °C. Left: the products were loaded as a 20-cm streak on Whatman No. 3 paper and electrophoresed at pH 2 (3 kV for 4 h). The left-hand fringe of the paper plus the markers were stained with AgNO₃, visualizing the products. The major portion, only part of which is shown (in grey), was not stained; green/white shading indicates a UV-absorbing band. The whole unstained portion was cut into seventeen 1-cm strips and products were eluted. Right: eluates from strips 2–17 were run by TLC in butan-1-ol/acetic acid/water (2:1:1) alongside marker mixtures, and stained with thymol. Marker mixtures were: S, saturated oligogalacturonides; U, unsaturated oligogalacturonides. (B) EPG digestion. As in (A), but digestion was with EPG (10 U mL⁻¹) instead of Driselase.
Fig. 7. Mass spectrometry of putative ΔUA–GalA obtained by Driselase digestion of de-esterified date fruit cell walls. (A) Negative-mode ESI FT–ICR mass spectrum. The in-silico simulated isotope distribution is highlighted (red dots). The mass error is 370 ppb. (B) Negative-mode ESI FT–ICR CID fragmentation mass spectrum of the species identified in (A). Observed m/z values are labelled in black; proposed identities and their calculated m/z values are in blue.
Although gene expression and extractable enzyme activity can suggest that a given enzyme-catalysed reaction could possibly be involved in a physiological process such as ripening, the demonstration of in-vivo action of the enzyme remains a gold standard that is difficult to achieve. By quantifying the in-vivo action of an enzyme, all transcriptional, post-transcriptional and post-translational modifications are taken into account, together with the regulation of enzyme activity by local cellular environments. In addition to providing a more biologically relevant proof of the in-vivo occurrence of polysaccharide modifications, determination of in-vivo enzyme action also circumvents problems associated with enzyme denaturation during extraction.

**A strategy for detecting products of PL action**

The unique fingerprint of PL action ($\Delta\text{UA–GalA}$), described by Fuchs (1965) and Nasuno and Starr (1967), is confirmed in this study. *In-vitro* digestion of HG chains with commercial PL produces oligogalacturonides with an unsaturated non-reducing terminus and a simple galacturonic acid at the reducing terminus (Fig. 1B, reaction i), with the unsaturated dimer ($\Delta\text{UA–GalA}$) being the smallest product detected (Fig. 6A). This highly acidic (low $pK_a$) dimer was separated by electrophoresis at pH 2.0 from all other products (Fig. 3A), to give a sample pure enough for us to prove its identity using TLC (Figs 6A and 8, Supplementary Fig. S1), MS (Fig. 7) and NMR spectroscopy (Fig. 5).

We recommend Driselase rather than EPG for routine analysis of in-vivo PL action products because (1) Driselase gave a single unsaturated product ($\Delta\text{UA–GalA}$) whereas EPG gave a mixture of $\Delta\text{UA–GalA}_2$ and $\Delta\text{UA–GalA}_3$; (2) EPG gives three saturated oligogalacturonides in addition to the unsaturated ones, whereas the only saturated acidic product of Driselase is the monomer, GalA; and (3) EPG requires the AIR to be pre-saponified, e.g. with Na$_2$CO$_3$, removing methylester groups, whereas Driselase contains esterases which can remove the methylester groups of HG.

The action of PL in fruit in vivo would be unlikely to digest the HG to products as small as $\Delta\text{UA–GalA}$ or $\Delta\text{UA–GalA}_2$. On the contrary, products of (partial) PL action in fruit would mainly be present in polymeric form (alcohol-insoluble polysaccharides in AIR), making them difficult to isolate and characterize. Therefore, further in-vitro hydrolysis of the AIR was performed with Driselase to release a small and well-defined PL action fingerprint, $\Delta\text{UA–GalA}$. Driselase was checked to show it has no pectate lyase activity of its own (Fig. 2B) and to be unable to cleave the unsaturated dimer to its monomers (Fig. 2C).

The PL action fingerprint ($\Delta\text{UA–GalA}$) was obtained by Driselase digestion of date fruit AIR and documented by electrophoresis and TLC. The mass of the putative $\Delta\text{UA–GalA}$ isolated from date fruits was confirmed by MS and found to be identical to that of the product obtained in vitro by digestion of commercial HG by commercial PL (Fig. 7). The identity of the PL ‘fingerprint’ compound was further confirmed chromatographically and electrophoretically (Figs 6 and 8; Supplementary Fig. S1) and by NMR spectroscopy (Fig. 5).

The action products of PL were also successfully detected by the same strategy in apples, pears and rowan berries (dicots; Rosaceae), confirming that fruit PL action is taxonomically widespread. It is interesting that this contributor to fruit
softening was observed both in true fruits (the monocot date and dicot rowan) and in the fleshy parts of false fruits (apple and pear). Another proposed in-vivo contributor to fruit softening – apoplastic hydroxyl radicals – was found in true fruits but not in false fruits (Al Hinai et al., 2016).

Another possible contributor to fruit softening could be rhamnogalacturonan lyase, which non-hydrolytically cleaves rhamnogalacturonan-I in mid-chain. Our finding of pectic polymers possessing ΔUA–GalA termini (the PL finger-print) concurs with the discovery in cress seed mucilage of the unsaturated disaccharide, ΔUA–rhamnose (lepidomic acid; Hasegawa et al., 1992), a probable indicator of in-vivo rhamnogalacturonan lyase action (Iqbal et al., 2016). It will be interesting to discover whether pectic polymers with ΔUA–rhamnose termini can be detected in fruits, indicating in-vivo rhamnogalacturonan lyase action.

Conclusions

This study deals with wall re-modelling in the living plant cell. Plants express numerous ‘wall-related’ genes, generating mRNAs which, if translated, would encode proteins whose in silico predicted enzymic activities suggest that they may be able to re-model the cell wall. In some cases, plant cell walls have been shown to contain the corresponding encoded proteins which, when extracted, exhibit in-vitro activity on wall-related polysaccharides. However, in many cases, it remains to be proven that these enzymes exert in-vivo action, actually re-modelling the walls of living plant cells. This important question has often been neglected. In the present work, we have developed methods to demonstrate that PL exhibits in-vivo action in several fruits. Such action, cleaving the backbone of the pectic HG domain, occurs at the right time and in the right place to play a role in fruit softening. The methods presented open the way to wider documentation of PL action, e.g. in fruits of other species and in non-fruit tissues that also express PL genes, complementing the evidence for in-vivo non-enzymic cleavage of polysaccharides by hydroxyl radicals (Al Hinai et al., 2016).

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of Figure S1: detecting ΔUA–GalA in Driselase digest of date AIR from three different date samples.

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LITERATURE CITED

Ahmed 1A, Ahmed AWK, Robinson RK. 1995. Chemical composition of date varieties as influenced by the stage of ripening. Food Chemistry 54: 305–309.

Al Hinai OB, Vreeburg RA, Fry SC. 2016. Pectic polysaccharides are attacked by hydroxyl radicals in ripening fruit: evidence from a fluorescent fingerprinting method. Annals of Botany 117: 441–455.

Albersheim P, Neukom H, Deuel H. 1960. Über die Bildung von ungesättigten Abbauprodukten durch ein pektinabhägebauendes Enzym. Helvetica Chimica Acta 43: 1422–1426.

Asif MH, Nath P. 2005. Expression of multiple forms of polygalacturonase gene during ripening in banana fruit. Plant Physiology and Biochemistry 43: 177–184.

Atkinson RG, Sutherland PW, Johnston SL, et al. 2012. Down-regulation of POLYGALACTURONASE1 alters firmness, tensile strength and water loss in apple (Malus × domestica) fruit. BMC Plant Biology 12: 129.

Barley IM. 1978. Exo-polygalacturonase of apple. Phytochemistry 17: 213–216.

Benitez-Burcaco A, Blanco-Portales R, Redondo-Navedo J, et al. 2003. Cloning and characterization of two ripening-related strawberry (Fragaria × ananassa cv. Chandler) pectate lyase genes. Journal of Experimental Botany 54: 633–645.

Besford RT, Holson GE. 1972. Pectic enzymes associated with the softening of tomato fruit. Phytochemistry 11: 2201–2205.

Brumme LL, Harpster MH. 2001. Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. Plant Molecula Bi 47: 311–340.

Brumme LL, Harpster MH, Civello PM, Palys JM, Bennett AB, Dunsmuir P. 1999. Modification of expansin protein abundance in tomato fruit alters softening and cell wall polymer metabolism during ripening. The Plant Cell 11: 2203–2216.

Chourasia A, Sane VA, Nath P. 2006. Differential expression of pectate lyase during ethylene-induced postharvest softening of mango (Mangifera indica var. Dashehari). Physiologia Plantarum 128: 586–555.

Deshpande AB, Anamika K, Jha V, et al. 2017. Transcriptional transitions in Alphonso mango (Mangifera indica L.) during fruit development and ripening explain its distinct aroma and shelf life characteristics. Scientific Reports 7: 8711.

Dominguez-Puigjaner E, Llop I, Vendrell M, Prat S. 1997. A cDNA clone highly expressed in ripe banana fruit shows homology to pectate lyases. Plant Physiology 114: 1071–1076.

Dong Y, Zhang S, Wang Y. 2018. Compositional changes in cell wall polyuronides and enzyme activities associated with melting/mealy textural property during ripening following long-term storage of 'Comice' and 'd’Anjou' pears. Postharvest Biology and Technology 135: 131–140.

Dumville JC, Fry SC. 2000. Uronic acid-containing oligosaccharins: their bio-synthesis, degradation and signalling roles in non-diseased plant tissues. Plant Physiology and Biochemistry 38: 125–140.

Dumville JC, Fry SC. 2003. Solubilisation of tomato fruit pectins by ascorbate: a possible non-enzymic mechanism of fruit softening. Planta 217: 951–961.

El Arem A, Flamini G, Behija SE, et al. 2011. Chemical and aroma compositions of date palm (Phoenix dactylfera L.) fruits at three maturation stages. Food Chemistry 127: 1744–1754.

Figueroa CR, Pimentel P, Gaete-Eastman C, et al. 2008. Softening rate of the Chilean strawberry (Fragaria chiloensis) fruit reflects the expression of polygalacturonases and pectate lyase genes. Postharvest Biology and Technology 49: 210–220.

Figueroa CR, Rosli HG, Civello PM, Martinez GA, Herrera R, Moya-León MA. 2010. Changes in cell wall polysaccharides and cell wall degrading enzymes during ripening of Fragaria chiloensis and Fragaria × ananassa fruits. Scientia Horticulturae 124: 454–462.

Fry SC. 2000. The growing plant cell wall: chemical and metabolic analysis. Caldwell, NJ: Blackburn Press.

Fry SC. 2004. Primary cell wall metabolism: tracking the careers of wall poly- mers in living plant cells. New Phytologist 161: 641–675.

Fry SC. 2010. Cell wall polysaccharide composition and covalent crosslinking. Annual Plant Reviews 41: 1–42.

Fry SC. 2020. High-voltage paper electrophoresis (HVPE). Methods in Molecular Biology 2149: 1–31.

Fuchs A. 1965. The trans-eliminative breakdown of Na-polygalacturonate by Pseudomonas fluorescens. Antonie Van Leeuwenhoek 31: 323–340.

García-Romera I, Fry SC. 1995. The longevity of biologically-active oligogalacturonides in rose cell cultures: degradation by exo-polygalacturonase. Journal of Experimental Botany 46: 1853–1857.
Griba A, Dardelle F, Lehner A, et al. 2013. Effect of water deficit on the cell wall of the date palm (Phoenix dactylifera ‘Deglet nour’, Arecales) fruit during development. Plant, Cell & Environment 36: 1056–1070.

Gross KC. 1982. A rapid and sensitive spectrophotometric method for assaying polygalacturonase using 2-cyanoacetamide. HortScience 17: 933–934.

Hasegawa K, Mizutani J, Kosemura S, Yamamura S. 1992. Isolation and identification of lepidimoide, a new allelopathic substance from mucilage of germinated cress seeds. Plant Physiology 100: 1059–1061.

Iqbal A, Miller JG, Murray L, Sadler IH, Fry SC. 2016. The pectic saccharides lepidimoic acid and [l-p-xylopyranosyl-(1→3)-d-galacturonic acid occur in cross-seed exudate but lack allelochemical activity. Annals of Botany 117: 607–623.

Jarvis MC, Briggs SPI, Knox JP. 2003. Intercellular adhesion and cell separation in plants. Plant, Cell & Environment 26: 977–989.

Jiménez-Bermúdez S, Redondo-Nevado J, Muñoz-Blanco J, et al. 2002. Manipulation of strawberry fruit softening by antisense expression of a pectate lyase gene. Plant Physiology 128: 751–759.

Martin-Rodríguez MC, Orchard J, Seymour GB. 2002. Pectate lyases, cell wall degradation and fruit softening. Journal of Experimental Botany 53: 2115–2119.

Martin-Rodríguez MC, Smith DL, Manning K, Orchard J, Seymour GB. 2003. Pectate lyase gene expression and enzyme activity in ripening banana fruit. Plant Molecular Biology 51: 851–857.

Méndez-Yañéz A, González M, Carrasco-Orellana C, Herrera R, Moya-León MA. 2020. Isolation of a rhamnogalacturonase lyase expressed during ripening of the Chilean strawberry fruit and its biochemical characterization. Plant Physiologu and Biochemistry 146: 411–419.

Miedes E, Lorences EP. 2009. Xyloglucan endotranogalactosylase/endoxyloglucan endotransglucosylase (XTHs) during tomato fruit growth and ripening. Journal of Plant Physiology 166: 489–498.

Moya-León MA, Mattas-Araya E, Herrera R. 2010. Molecular events occurring during softening of strawberry fruit. Frontiers in Plant Science 10: 615.

Nasuno S, Starr MP. 1967. Polygalacturonic acid trans-eliminase of Xanthomonas campestris. The Biochemical Journal 104: 178–185.

Nunan DJ, Davies C, Robinson SP, et al. 2020. Expression patterns of cell wall-modifying enzymes during grape berry development. Planta, 214: 257–264.

Ochoa-Jiménez VA, Berumen-Varela G, Burgara-Estrella A, et al. 2018. Functional analysis of tomato rhamnogalacturonase lyase gene Solyc11g011300 during fruit development and ripening. Journal of Plant Physiology 231: 31–40.

Orr G, Brady C. 1993. Relationship of endopolygalacturonase activity to softening in a freestone peach. Postharvest Biology and Technology 3: 121–130.

Paniagua C, Santiago-Doménech N, Kirby AR, et al. 2017. Structural changes in cell wall pectins during strawberry fruit development. Plant Physiology and Biochemistry 118: 55–63.

Payasi A, Misra PC, Sanwal GG. 2006. Purification and characterization of pectate lyase from banana (Musa acuminata) fruits. Phytochemistry 67: 861–869.

Phan TD, Bo W, West G, Lycett GW, Tucker GA. 2007. Silencing of the major salt-dependent isomerase of pectinesterase in tomato alters fruit softening. Plant Physiology 144: 1960–1967.

Posé S, Kirby AR, Paniagua C, et al. 2015. The nanostructural characterization of strawberry pectins in pectate lyase or polygalacturonase silenced fruits elucidates their role in softening. Carbohydrate Polymers 132: 121–130.

Pua E, Ong C, Liu P, Liu J. 2001. Isolation and expression of two pectate lyase genes during fruit ripening of banana (Musa acuminata). Physiologia Plantarum 113: 92–99.

Quesada MA, Blanco-Portales R, Posé S, et al. 2009. Antisense down-regulation of the FaPG1 gene reveals an unexpected central role for polygalacturonase in strawberry fruit softening. Plant Physiology 150: 1022–1032.

Rastegar S, Rahemi M, Baghizadeh A, Gholami M. 2012. Enzyme activity and biochemical changes of three date palm cultivars with different softening pattern during ripening. Food Chemistry 134: 1279–1286.

Saladí M, Rose JK, Cosgrove DJ, Catalá C. 2006. Characterization of a new xyloglucan endotransglucosylase/hydrolase (XTH) from ripening tomato fruit and implications for the diverse modes of enzymatic action. The Plant Journal Biology 47: 282–295.

Santiago-Domenech N, Jiménez-Bemúdez S, Matas AJ, et al. 2008. Antisense inhibition of a pectate lyase gene supports a role for pectin depolymerization in strawberry fruit softening. Journal of Experimental Botany 59: 2769–2779.

Serrano M, Pretel MT, Botella MA, Amorós A. 2001. Physicochemical changes during date ripening related to ethylene production. Food Science and Technology International 7: 31–36.

Shaligram NS, Singhal RS. 2010. Surfactin – a review on biosynthesis, fermentation, purification and applications. Food Technology and Biotechnology 48: 119–134.

Smith CJ, Watson CF, Morris PC, et al. 1990. Inheritance and effect on ripening of antisense polygalacturonase genes in transgenic tomatoes. Plant Molecular Biology 14: 369–379.

Tieman DM, Harriman RW, Ramamohan G, Handa AK. 1992. An anti-sense pectin methylesterase gene alters pectin chemistry and soluble solids in tomato fruit. The Plant Cell 4: 667–679.

Tucker GA, Grierson D. 1982. Synthesis of polygalacturonase during tomato fruit ripening. Planta 155: 64–67.

Ulusik S, Seymour GB. 2020. Pectate lyases: their role in plants and importance in fruit ripening. Food Chemistry 309: 125559.

Ulusik S, Chapman NH, Smith R, et al. 2016. Genetic improvement of tomato by targeted control of fruit softening. Nature Biotechnology 34: 950–952.

Vicente AR, Saladie M, Rose JK, Labavitch JM. 2007. The linkage between cell wall metabolism and fruit softening: looking to the future. Journal of the Science of Food and Agriculture 1243: 1237–1243.

Villarreal NM, Rosli HG, Martínez GA, Civello PM. 2008. Polygalacturonase activity and expression of related genes during ripening of strawberry cultivars with contrasting fruit firmness. Postharvest Biology and Technology 47: 141–150.

Villarreal NM, Martínez GA, Civello PM. 2009. Influence of plant growth regulators on polygalacturonase expression in strawberry fruit. Plant Science 176: 749–757.

Vreeburg RA, Airiahnaz OB, Fry SC. 2014. Fingerprinting of hydroxyl radical-attacked polysaccharides by N-isopropyl-2-aminocridone labeling. The Biochemical Journal 463: 225–237.

Wang D, Yeats TH, Ulusik S, Rose JK, Seymour GB. 2018. Fruit softening: revisiting the role of pectin. Trends in Plant Science 23: 302–310.

Wu Q, Szakacas-Dobozi M, Hemmat M, Hrazdina G. 1993. Endopolygalacturonase in apples (Malus domestica) and its expression during fruit ripening. Plant Physiology 102: 219–225.

Yang L, Huang W, Xiong F, et al. 2017. Silencing of SiPG1, which encodes a pectate lyase in tomato, confers enhanced fruit firmness, prolonged shelf-life and reduced susceptibility to grey mould. Plant Biotechnology Journal 15: 1544–1555.

Yang Y, Yu Y, Liang Y, Anderson CT, Cao J. 2006. Comparative analysis of polygalacturonase in the fruit of strawberry cultivars. Genetics and Molecular Research: GMR 14: 12776–12787.

Zhou HC, Li G, Zhao X, Li IJ. 2015. Comparative analysis of polygalacturonase in relation to softening in strawberry fruits. Canadian Journal of Plant Science 96: 604–612.
