Ethylene regulation of fruit softening and cell wall disassembly in Charentais melon

Kiyomi Nishiyama[1,*], Monique Guis[2,*], Jocelyn K. C. Rose[3], Yasutaka Kubo[1], Kristen A. Bennett[4], Lu Wangjin[1], Kenji Kato[1], Koichiro Ushijima[1], Ryohei Nakano[1], Akitsugu Inaba[1], Mondher Bouzayen[2], Alain Latche[2], Jean-Claude Pech[2] and Alan B. Bennett[4,†]

[1] *Graduate School of Natural Science and Technology, Okayama University, Okayama, 700-8530 Japan*

[2] *UMR990 INRA/INP-ENSA Toulouse, Avenue de l'Agrobiopole, BP 32607, F-31326 Castanet-Tolosan, France*

[3] *Department of Plant Biology, Cornell University, Ithaca, NY 14853, USA*

[4] *Department of Plant Sciences, University of California, Davis, CA 95616, USA*

Abstract

Cell wall disassembly in ripening fruit is highly complex, involving the dismantling of multiple polysaccharide networks by diverse families of wall-modifying proteins. While it has been reported in several species that multiple members of each such family are expressed in the same fruit tissue, it is not clear whether this reflects functional redundancy, with protein isozymes from a single enzyme class performing similar roles and contributing equally to wall degradation, or whether they have discrete functions, with some isoforms playing a predominant role. Experiments reported here sought to distinguish between cell wall-related processes in ripening melon that were softening-associated and softening-independent. Cell wall polysaccharide depolymerization and the expression of wall metabolism-related genes were examined in transgenic melon (*Cucumis melo* var. cantalupensis Naud.) fruit with suppressed expression of the 1-aminocyclopropane-1-carboxylate oxidase (ACO) gene and fruits treated with ethylene and 1-methylcyclopropene (1-MCP). Softening was completely inhibited in the transgenic fruit but was restored by treatment with exogenous ethylene. Moreover, post-harvest application of 1-MCP after the onset of ripening completely halted subsequent softening, suggesting that melon fruit softening is ethylene-dependent. Size exclusion chromatography of cell wall polysaccharides, from the transgenic fruits, with or without exogenous ethylene, indicated that the depolymerization of both pectins and xyloglucans was also ethylene dependent. However, northern analyses of a diverse range of cell wall-related genes, including those for polygalacturonases, xyloglucan endotransglucosylase/hydrolases, expansin, and β-galactosidases, identified specific genes within single families that could be categorized as ethylene-dependent, ethylene-independent, or partially ethylene-dependent. These results support the hypothesis that while individual cell wall-modifying proteins from each family contribute to cell wall disassembly that accompanies fruit softening, other closely related family members are regulated in an ethylene-independent manner and apparently do not directly participate in fruit softening.

Key words: Cell wall modification, enzyme, fruit softening, gene expression, 1-MCP, melon fruit, transgenic plant.

Introduction

Fruit ripening is a genetically programmed event that is characterized by a number of biochemical and physiological processes that alter fruit firmness, fruit colour, flavour,

* These authors contributed equally to the work presented here.

† To whom correspondence should be addressed. E-mail: abbennett@ucdavis.edu

Abbreviations: ACO, 1-aminocyclopropane-1-carboxylate oxidase; AIS, alcohol-insoluble solid; CDTA, 1,2-cyclohexanediaminetetraacetic acid; DAP, days after pollination; EGase, endo-1,4-β-glucanase; β-Gals, β-galactanase/β-galactosidase; 1-MCP, 1-methylcyclopropene; PCR, polymerase chain reaction; PG, polygalacturonase; XTH, endotransglycosylase/hydrolase.

aroma, and texture (Brady, 1987; Brummell and Harpster, 2001). Fruit softening is a major factor that determines fruit quality and post-harvest life. In melon (*Cucumis melo* L.), a typical climacteric fruit, some cultivars show an extremely rapid decrease in flesh firmness during ripening, limiting their transport, storage, and shelf-life.

The plant primary cell wall consists of three co-extensive polymer networks: cellulose microfibrils coated with and cross-linked with hemicellulose and embedded in a co-extensive pectin network (Carpita and Gibeaut, 1993). Disassembly of the primary cell wall and reduction in cell–cell adhesion following dissolution of the pectinaceous middle lamella are believed to contribute to fruit softening (Crookes and Grierson, 1983; Wakabayashi, 2000). Xyloglucan, the predominant hemicellulose in the cell walls of most dicotyledons, also typically undergoes degradation during fruit ripening (Rose and Bennett, 1999), and the depolymerization of a specific subset of xyloglucan polymers that are tightly bound to cellulose microfibrils has been proposed as one of the earliest ripening-associated wall changes in some fruit (Rose *et al*., 1998). These modifications in cell wall polymers during ripening involve the co-ordinated and interdependent action of numerous cell wall-modifying enzymes and proteins.

A range of enzymes that contribute to pectin modification and disassembly, including polygalacturonase (PG), pectate lyase, pectin methylesterase, β-galactanase/β-galactosidase (β-Gal), and α-arabinase/α-arabinofuranosidase (Rose *et al*., 2003) have the capacity to reduce the apparent molecular size of pectic polymers by cleaving the backbone or side chain residues (Ranwala *et al*., 1992; De Veau, 1993; Hadfield *et al*., 1998). Similarly, several classes of proteins have been proposed to participate in ripening-related disassembly and reorganization of the cellulose–xyloglucan network (Rose and Bennett, 1999). For example, endo-1,4-β-glucanase (EGase) mRNA and enzyme activity levels have been correlated with the onset of ripening and softening of many fruits (Rose and Bennett, 1999) as has the expression of expansins, which have been hypothesized to participate in hemicellulose depolymerization by exposing previously inaccessible xyloglucan polymers to the action of wall hydrolases (Rose *et al*., 1997). However, the *in vivo* substrates of plant EGases and expansins have still not been determined, and suppression or overexpression of specific members of these gene families in transgenic tomato has not been sufficient to prevent fruit softening (Brummell and Harpster, 2001). Alternatively, xyloglucan endotransglycosylase/hydrolases (XTHs), which specifically use xyloglucan as a substrate, might contribute to xyloglucan depolymerization in ripening fruit by acting in a hydrolytic mode, or by acting as a transglycosylase and using xyloglucan oligosaccharides as acceptor substrates (Rose *et al*., 2002; Saladié *et al*., 2006), although this has yet to be directly tested.

Tomato fruit ripening and softening have been intensively studied, but the underlying molecular mechanisms may not be entirely representative of fruit softening in other species. While it is likely that fruits of most species have similar complements of cell wall enzymes, the corresponding patterns of transcript and protein abundance and enzyme activities vary substantially between fruit species (Brummell and Harpster, 2001). Thus, a comprehensive understanding of fruit softening requires investigation of several species. Charentais melon (*Cucumis melo* var. cantalupensis Naud.) undergoes remarkably rapid softening in the transition from the onset of ripening to over-ripe stages (Rose *et al*., 1998), thereby providing an excellent model plant to study fruit ripening and softening (Ayub *et al*., 1996; Rose *et al*., 1998; Hadfield *et al*., 2000; Perin *et al*., 2002). During ripening, Charentais fruit exhibit modification of both pectic and hemicellulosic polymers (Rose *et al*., 1998), as has been observed in tomato (Huber, 1983), avocado (Huber and O'Donoghue, 1993; Sakurai and Nevins, 1997), European pears (Hiwasa *et al*., 2004), and other species. Rose *et al*. (1998) demonstrated that the initial softening process of Charentais fruit coincides with a loss of galactose from polyuronides and xyloglucan disassembly, with the depolymerization of polyuronides occurring predominantly in the late (over-ripe) stages. However, relatively little is known about the expression of genes encoding cell wall-modifying enzymes in melons, other than those encoding PGs (Hadfield *et al*., 1998).

Ethylene plays a major role in regulating ripening and softening of climacteric fruit, including Charentais melon (Ayub *et al*., 1996; Hadfield *et al*., 2000), and accordingly, the expression of some ripening-related cell wall-associated genes and activities, including those of PGs (Sitrit and Bennett, 1998; Hiwasa *et al*., 2003, 2004), expansins (Rose *et al*., 1997), and EGases (Lashbrook *et al*., 1994). Two approaches have been used to determine the precise role of ethylene in fruit ripening and softening: transgenic suppression of ethylene production and the use of volatile inhibitors of ethylene action. Genetically engineered tomatoes and melons with suppressed ethylene biosynthesis exhibit delayed and reduced fruit softening (Murray *et al*., 1993; Picton *et al*., 1993; Ayub *et al*., 1996; Guis *et al*., 1997; Flores *et al*., 2001). However, whether melon fruit softening is totally, or partially, dependent on ethylene has not been clearly established, since transgenic melon with reduced expression of the 1-aminocyclopropane-1-carboxylate oxidase (ACO) gene showed a delayed but significant decrease in flesh firmness during ripening (Ayub *et al*., 1996; Guis *et al*., 1997; Flores *et al*., 2001). The application of a highly potent inhibitor of ethylene perception, 1-methylcyclopropene (1-MCP; Sisler and Sereck, 1997) provides an alternative approach to assess the role of ethylene in fruit softening. It was previously demonstrated that treatment of pear fruit with 1-MCP after

the onset of ripening restricted softening and prevented the accumulation of *PG* mRNA and endo-PG activity (Hiwasa *et al*., 2003).

In this report, the role of ethylene in Charentais melon fruit softening was assessed using transgenic lines with suppressed *ACO* expression and by treating fruits with 1-MCP after the onset of ripening. The depolymerization of pectic and hemicellulosic cell wall polymers was then analysed and the expression of genes that may contribute to their degradation was monitored in order to evaluate the potential contribution of each gene to softening and the degree to which they exhibit ethylene regulation.

Materials and methods

Plant materials

For experiments with transgenic fruits exhibiting suppressed ethylene synthesis, a stable homozygous line of Charentais melon (*C. melo* var. cantalupensis Naud. 'Vedrantais') harbouring an antisense *ACO* gene (Balague *et al*., 1993; Ayub *et al*., 1996) and its untransformed wild-type control were used. Flowers were tagged on the date of anthesis and only two fruit were kept on each plant. Maximal ethylene production and abscission of wild-type fruit were observed at 43 d after pollination (DAP) and 48 DAP, respectively. Transgenic fruit exhibited no ethylene peak, and basal ethylene levels remained <0.5% of those of the wild type at the climacteric peak, and the development of the abscission zone was also completely inhibited. Wild-type fruit were harvested at 32, 36, 40, 43, and 46 DAP, and transgenic fruit were harvested at the same stages with an additional harvest at 50 DAP. Some transgenic fruit were treated on the vine at 46 DAP with 50 µl l^{-1} ethylene for 4 d in 32.0 l vessels containing KOH to absorb respiratory CO_2. The vessels were ventilated every 12 h to prevent oxygen deprivation and to adjust ethylene concentrations.

For experiments involving 1-MCP treatments of wild-type fruits, Charentais melon plants were cultivated in a greenhouse in the Kochi Prefecture, Japan. Preclimacteric fruit (25–27 DAP) were harvested and treated with 5000 ppm propylene at 25 °C for 48 h to induce uniform ripening. After the propylene treatment, fruit were either stored at 25 °C in ambient conditions (control) or exposed to 10 ppm of 1-MCP (Rohm and Haas, Philadelphia, PA, USA) for 24 h and then stored in the same conditions as the control fruit. Rates of ethylene production and flesh firmness were determined at 0, 2, 4, 7, 10, and 14 d after the start of the experiment. Ethylene production was determined by using a gas chromatograph (model GC-4CMPE, Shimadzu, Kyoto, Japan) and flesh firmness was measured at four equatorial regions of the peeled flesh using a penetrometer (model SMT-T-50, Toyo Baldwin, Tokyo, Japan) fitted with an 8 mm plunger. Fruit mesocarp tissues were flash frozen in liquid nitrogen and stored at −80 °C prior to analysis.

Cell wall extraction and size-exclusion chromatography of cell wall fractions

Isolation and extraction of cell wall fractions was performed as previously described (Rose *et al*., 1998), with slight modifications. Briefly, 50 g of frozen melon mesocarp was boiled in 95% ethanol, homogenized, and filtered through Miracloth (Calbiochem). After an ethanol, chloroform:methanol (1:1, v/v), and acetone wash, the remaining alcohol-insoluble solid (AIS) residue was oven-dried at 30 °C. The AIS residue from each sample was homogenized for 4 h in water containing 0.02% NaN_3 and centrifuged at 6000 *g* for 20 min. The supernatant was lyophilized and the water-insoluble pellet sequentially extracted with 50 mM 1,2-cyclohexanediaminetetra-acetic acid (CDTA), 50 mM Na_2CO_3, 4% KOH, and 24% KOH, as previously described (Rose *et al*., 1998). After centrifugation at 8500 *g* for 20 min, the solubilized fractions were collected, dialysed against distilled water, and lyophilized. Polymers from the lyophilized water-soluble and CDTA-soluble extracts were separated as previously described (Rose *et al*., 1998) and each column fraction assayed for uronic acid content (Blumenkrantz and Ashoe-Hansen, 1973). Polymers from 24% KOH-soluble extracts were fractionated and the xyloglucan content of the column fractions measured as before (Rose *et al*., 1998) and, in all fractions, total sugars were also assayed using a modified phenol–sulphuric acid assay (Rose *et al*., 1998).

Cloning of gene fragments encoding cell wall-modifying enzymes and probe preparation

Total RNA was extracted from mesocarp tissues using a modified hot borate method (Wan and Wilkins, 1994), and poly(A)$^+$ RNA was isolated from the total RNA using Oligotex dT30 (Takara, Otsu, Japan) for cDNA synthesis. First-strand cDNA was synthesized using reverse transcriptase according to the manufacturer's instructions and used as a template to amplify fragments of genes encoding cell wall-modifying enzymes. Polymerase chain reaction (PCR) primers were designed based on the amino acid sequences conserved among the coding sequences of the corresponding genes in the databases [XTHs, 5′-GA(AG)CA(CT)GA(CT)GA(AG)A-T(ACT)GA(CT)TT(CT)G-3′ and 5′-TCNGT(GA)CA(GA)TA (GA)TT(GA)TA(TGA)ATNG-3′; expansin, as in Rose *et al*. (1997); EGase, 5′-GGITA(CT)TA(CT)GCIGGIGA-3′ and 5′-NCCNA(AG)(AGT)AT(AG)TA(AG)TCNAC(CT) TG-3′; β-Gal, 5′-GAYTAYYTITGGTAYATGAC-3′ and 5′-CCATAYYTG ICCYTTICCCAT-3′]. After PCR amplification under adequate conditions, the amplified fragments of the XTH and expansin genes were cloned into pCR-Script (Stratagene Inc. La Jolla, CA, USA) and the fragments of EGase and β-Gal genes into pGEM-T Easy (Promega, Madison, WI, USA) according to the manufacturer's instructions, and sequenced. Based on sequence homology, a total of seven unique clones (two for XTH, one each for expansin and EGase, three for β-Gal) were selected and designated *CmXTH1* (accession no. DQ914794), *CmXTH3* (accession no. DQ914796), *CmExp1* (accession no. DQ914793), *CmEGase1* (accession no. AB271851), *CmGal1* (accession no. AB270923), *CmGal2* (accession no. AB270925), and *CmGal3* (accession no. AB270925), respectively.

In the experiment using transgenic fruit, radiolabelled probes were prepared using cloned fragments, random hexamer priming using [α-^{32}P]dATP (3000 Ci $mmol^{-1}$), and Klenow DNA polymerase (UBS, Cleveland, OH, USA). For the *PG* gene family (*MPG1*, *-2*, and *-3*), full-length cDNA clones were used as probes (Hadfield *et al*., 1998). In the experiment using 1-MCP, digoxigenin-labelled probes were prepared using cloned fragments and the PCR DIG probe synthesis kit (Roche Diagnostics, Mannheim, Germany).

RNA gel blot analysis

An aliquot of total RNA from each sample was subjected to electrophoresis on a 1.2% agarose denaturing gel containing 0.66 M formaldehyde and transferred to Hybond-N$^+$ membrane (Amersham, Piscataway, NJ, USA). In the experiment using transgenic fruit, hybridization was carried out in 10% (v/v) dextran sulphate, 2% (w/v) SDS, 1 M NaCl, and 100 µg ml^{-1} sonicated salmon sperm DNA at 65 °C for 16 h. The membranes were washed three times in 5× SSC, 0.1% SDS (w/v), 0.05% NaPPi at 42 °C, and three times

in 0.5× SSC, 0.1% SDS (w/v), 0.05% NaPPi at 65 °C, and exposed to film with an intensifying screen (Du Pont, Boston, MA, USA) at −80 °C. In the experiment using 1-MCP, membranes were hybridized overnight in high SDS buffer [50% deionized formamide (v/v), 5× SSC, 7% SDS, 2% Blocking Reagent (Roche Diagnostics), 50 mM sodium phosphate (pH 7.0), and 0.1% *N*-lauroylsarcosine sodium salt (w/v)] at 42 °C. Membranes were washed at a high stringency condition (twice in 2× SSC with 0.1% SDS for 15 min at 42 °C, and then in 0.1× SSC with 0.1% SDS for 30 min at 60 °C). Signals were detected by a chemiluminescent method using CDP-star (Roche Diagnostics).

Results

Fruit softening and cell wall disassembly in wild-type and transgenic melon with suppressed ACO expression

Maximal ethylene production and abscission of wild-type fruit was observed at 43 DAP and 48 DAP, respectively (data not shown). In wild-type fruit, flesh firmness decreased rapidly following the onset of ethylene production and reached the oversoft stage at 50 DAP (Fig. 1). By contrast, transgenic fruit with suppressed *ACO* expression exhibited no burst of ethylene synthesis and basal ethylene levels remained at <0.5% of the wild-type climacteric peak level. The development of the abscission zone and softening were also completely inhibited. Treatment of the transgenic fruit with ethylene induced a rapid decrease in flesh firmness within 4 d (Fig. 1).

Size fractionation of water-soluble polymers indicated a clear decrease in the molecular mass of polyuronides between the immature and over-ripe stages in wild-type fruit, with a decrease in the average molecular mass from 260 kDa to ~36 kDa (Fig. 2A, B). The profile of polyuronide size distribution from 46 DAP transgenic *ACO*-suppressed fruit was similar to that from the immature wild type at 36 DAP (Fig. 2A, C), indicating that pectin degradation was substantially suppressed in the transgenic fruit. However, following ethylene treatment of *ACO*-suppressed transgenic fruit, a complete loss of high and intermediate molecular mass pectins was observed (Fig. 2D). Fractionation of CDTA-soluble polymers also illustrated a clear shift in the polyuronide profile between the immature and over-ripe stages in wild-type fruit, with a decrease in the average molecular mass and with a greatly reduced portion of polyuronides eluting in the void volume (Fig. 3A, B). High molecular mass pectins were predominant at 46 DAP in *ACO*-suppressed transgenic fruit, and the distribution appeared essentially identical to that from immature wild-type fruit (Fig. 3C). However, the molecular weight profile of pectins from ethylene-treated transgenic *ACO*-suppressed fruit was similar to that of the 46 DAP ripe wild-type fruit.

A molecular weight downshift in the 24% KOH-soluble cell wall polymer fraction, resulting from xyloglucan depolymerization, was observed during the ripening of wild-type melons (Fig. 4A, B), as was previously described (Rose *et al*., 1998). However, the profile of xyloglucan polymers from mature (46 DAP) *ACO*-suppressed transgenic fruit (Fig. 4C) was similar to that from immature wild-type fruit (Fig. 4B), and xyloglucan depolymerization was observed only after treatment with ethylene (Fig. 4D).

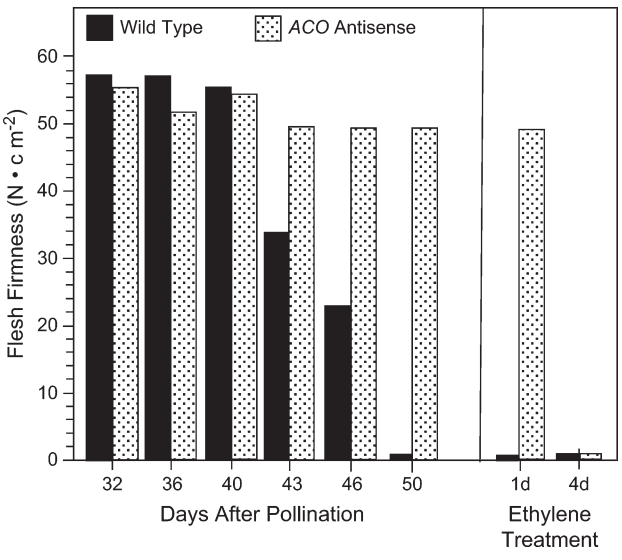

Fig. 1. Flesh firmness of wild-type and *ACO* antisense Charentais melons during ripening on the vine (left panel) and *ACO* antisense fruit after 1 d and 4 d of ethylene treatment (50 ppm) beginning at 46 DAP (right panel).

Expression of cell wall-associated genes in wild-type and transgenic melon with reduced ACO expression

Fruit softening and both pectin and xyloglucan depolymerization were strongly ethylene-dependent, as revealed by evaluation of the *ACO*-suppressed transgenic melon fruit, and so the abundance of mRNAs encoding a range of proteins potentially involved in melon wall disassembly was evaluated to assess which gene products might be responsible for the observed polymer depolymerization. The expression of three genes encoding PGs (*MPG1*, *MPG2*, and *MPG3*) was evaluated; *MPG1* mRNA accumulated to a high level at the peak of ethylene production in wild-type melon fruit at 43 DAP and then declined, while expression was undetectable at any stage in *ACO*-suppressed transgenic fruit (Fig. 5), but was markedly induced after 1 d and 4 d of exposure to exogenous ethylene (Fig. 5). The accumulation of *MPG2* mRNA in wild-type fruit started slightly before the onset of ethylene production (36 DAP) and reached a peak coincident with maximal ethylene levels. In the *ACO*-suppressed transgenic fruit, *MPG2* mRNA accumulation was slightly delayed (40 DAP) but reached a similar level to that of the wild type at later stages of ripening (46–50 DAP) and was only slightly affected by exogenous ethylene (Fig. 5). The expression of *MPG3* mRNA in

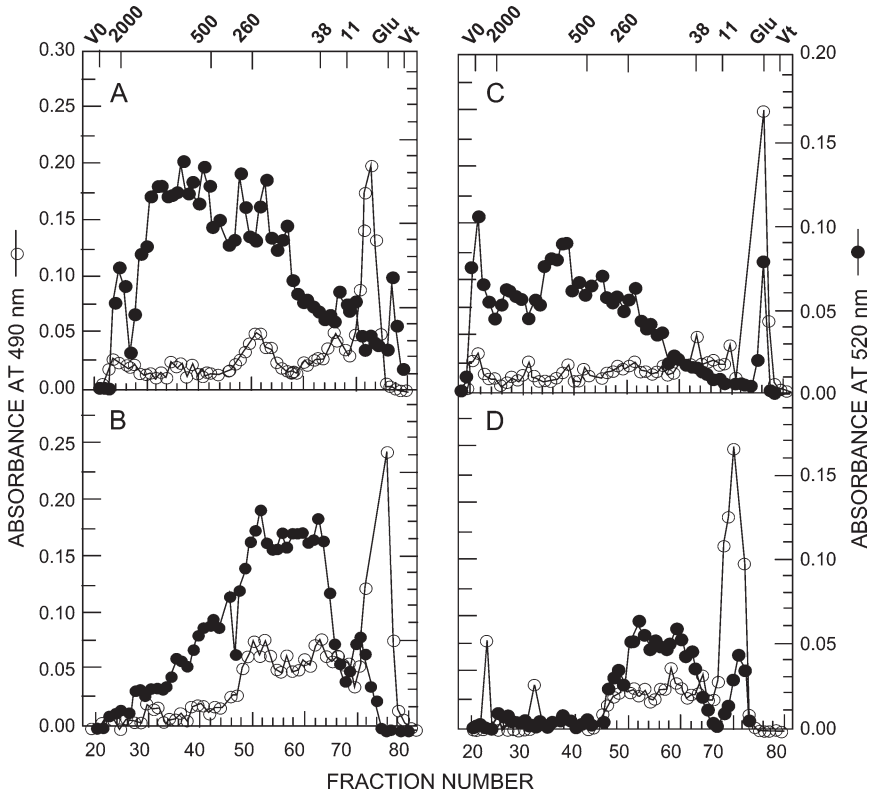

Fig. 2. Gel filtration profiles of water-soluble polysaccharides from wild-type fruit at 36 DAP (A) and 46 DAP (B), untreated *ACO* antisense fruit at 46 DAP (C), and transgenic fruit treated on the vine at 46 DAP with 50 ppm ethylene for 4 d (D). Water-soluble extracts were fractionated on a Sepharose CL-4B column and fractions assayed for uronic acid (filled circles) or total sugar (open circles) content. Dextran molecular mass markers (kDa) used as calibration standards are shown at the top. Vo, void volume; Vt, total volume.

wild-type fruit, although slightly lower, followed a similar pattern to that of *MPG1*, with maximum abundance at 43 DAP. In *ACO*-suppressed transgenic fruit, a basal low level of *MPG3* transcripts was detected between 32 DAP and 40 DAP, and a substantial increase occurred at later stages (43–50 DAP) that was enhanced by exposure to exogenous ethylene.

XTHs have been implicated in xyloglucan metabolism and so the expression of several XTH genes was also examined. In wild-type fruit, mRNA corresponding to the *CmXTH1* gene exhibited strong but transient accumulation before the onset of ethylene production (36 DAP), then declined during the increase in ethylene production (40 DAP) and was present at barely detectable levels during the later stages of ripening (Fig. 5). In the *ACO*-suppressed transgenic fruit, a slight and transient accumulation of *CmXTH1* mRNA occurred at 36 DAP and then a clear accumulation started at 46 DAP and continued to increase to 50 DAP. Application of exogenous ethylene further induced *CmXTH1* mRNA accumulation after 24 h; however, the *CmXTH1* transcripts were barely detectable after 4 d of ethylene treatment. The accumulation of *CmXTH3* mRNA in wild-type fruit was detected at 43 DAP, with expression levels declining at 46 DAP (Fig. 5). In the transgenic fruit, *CmXTH3* mRNA levels were undetectable until 46 DAP and then accumulation further increased at 50 DAP. Application of exogenous ethylene induced a substantial increase in both *CmXTH1* and *CmXTH3* mRNA, but their levels declined between 1 d and 4 d of ethylene exposure.

Expansins have also been proposed to act co-operatively with cell wall hydrolases to disassemble cell wall polymers (Rose and Bennett, 1999) and, accordingly, in wild-type fruit, *CmExp1* mRNA was first detected at the onset of ethylene production, concomitant with the initiation of the softening, and mRNA levels remained high throughout ripening (Fig. 5). In the *ACO*-suppressed transgenic fruit, a similar pattern of *CmExp1* mRNA accumulation was observed, although at somewhat reduced levels relative to wild-type fruit. Treatment of the transgenic fruit with exogenous ethylene further increased *CmExp1* mRNA abundance.

Fruit softening and ethylene production in 1-MCP-treated melon fruit

To test further the role of ethylene and specific gene products in melon fruit softening, wild-type melon fruit were treated with 1-MCP to inhibit ethylene perception. The treatment dramatically inhibited fruit senescence, resulting in minimal evidence of senescence or

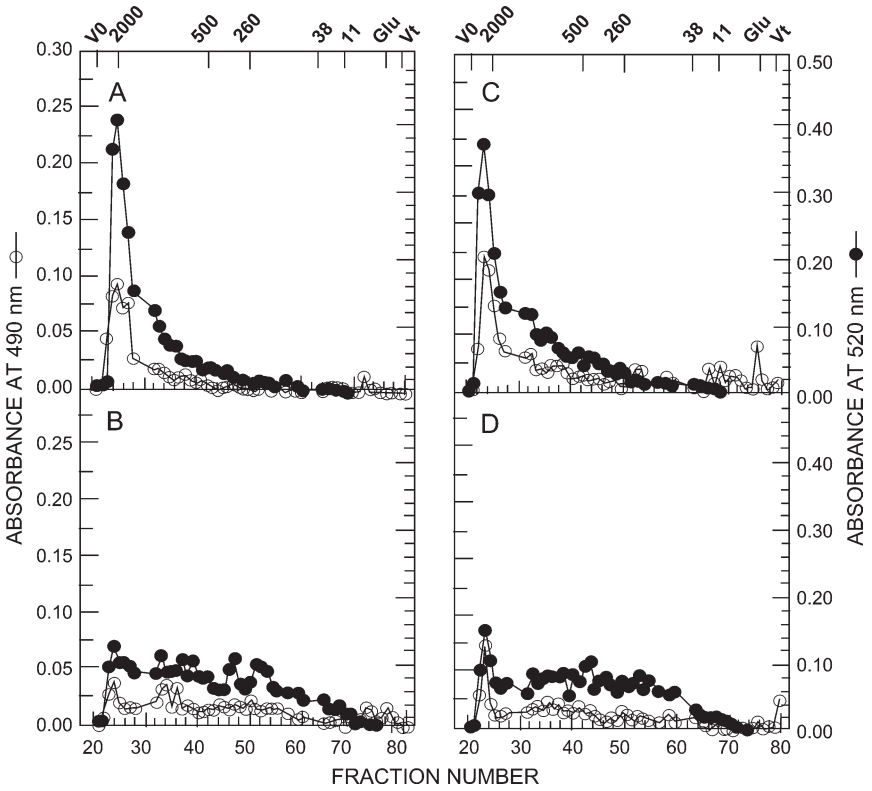

Fig. 3. Gel filtration profiles of CDTA-soluble polysaccharides from wild-type fruit at 36 DAP (A) and 46 DAP (B), untreated *ACO* antisense fruit at 46 DAP (C), and transgenic fruit treated on the vine at 46 DAP with 50 ppm ethylene for 4 d (D). Details are as described for Fig. 2.

post-harvest infection, and no development of the abscission zone, even 15 d after harvest (Fig. 7). Untreated control fruit showed a typical climacteric pattern of ethylene production, reaching a peak of 18.2 nl g^{-1} h^{-1} at 4 d after harvest, and then decreasing to 20% of its peak 10 d after harvest (Fig. 6). Ethylene production was significantly suppressed by 1-MCP treatment after the onset of ripening, declining to trace levels at 2 d after 1-MCP treatment, and gradually restoring production 5 d after the 1-MCP treatment.

Softening of untreated control fruit began at the onset of ripening, and fruit firmness decreased to half of the initial level within the first 2 d (Fig. 6) and continued to an oversoft level within the next 2 d. By contrast, loss of fruit firmness in 1-MCP-treated fruits was markedly suppressed, with fruits remaining firm until day 10, at which time softening continued, but at a much slower rate than that observed from day 2 to day 4 in control fruit.

Expression pattern of genes encoding cell wall-modifying enzymes in non-treated and 1-MCP-treated melon

As with the *ACO*-suppressed transgenic fruit, the abundance of mRNAs encoding several proteins potentially involved in melon cell wall disassembly was examined. *MPG1* and *MPG2* mRNAs were not detected during the preclimacteric stage but started to accumulate with the increase in ethylene levels and the decrease in flesh firmness (Fig. 8). During ripening, mRNA levels reached a peak at day 2 and then decreased gradually, reaching only trace levels at day 10. *MPG1* mRNA abundance was strongly reduced following 1-MCP treatment at day 4 and day 7 and then returned to normal levels at day 10. By contrast, *MPG2* mRNA accumulation was largely unaffected by 1-MCP treatment. The mRNA encoding β-Gal (*CmGal1*) accumulated to high levels in young expanding fruit, peaked at 15 DAP, and decreased to trace levels towards the mature stage (data not shown). *CmGal1* mRNA accumulation was detected during ripening after day 4 in control fruit, while expression was completely suppressed by 1-MCP treatment. The mRNA accumulation of *CmGal2* showed a similar pattern to that of *MPG2*, except for the transient accumulation at the very early growing stage (data not shown). *CmGal3* and *CmEGase1* showed low levels of transcript abundance throughout growing and ripening stages in both control and 1-MCP-treated fruit.

Discussion

Ethylene and softening in Charentais melon fruit

Softening of climacteric fruit is regulated by ethylene, although it is still not clear whether this ethylene

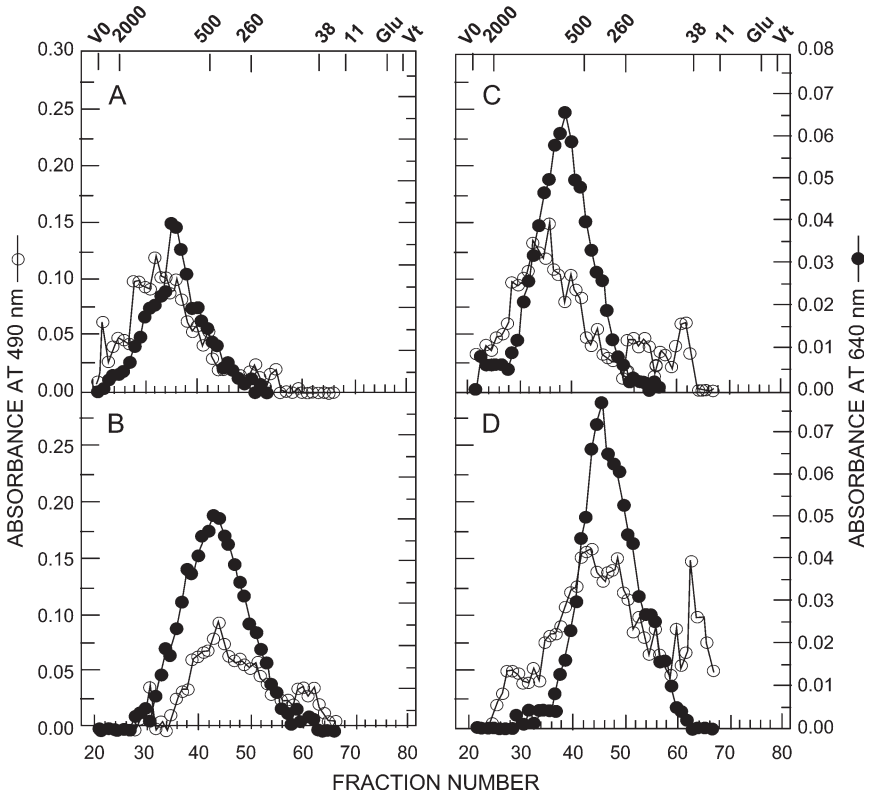

Fig. 4. Gel filtration profiles of 24% KOH-soluble polysaccharides from wild-type fruit at 36 DAP (A) and 46 DAP (B), untreated *ACO* antisense fruit at 46 DAP (C), and transgenic fruit treated on the vine at 46 DAP with 50 ppm ethylene for 4 d (D). KOH-soluble extracts were fractionated on a Sepharose CL-6B column and fractions assayed for xyloglucan (filled circles) or total sugar (open circles) content. Dextran molecular mass markers (kDa) used as calibration standards are shown at the top. Vo, void volume; Vt, total volume.

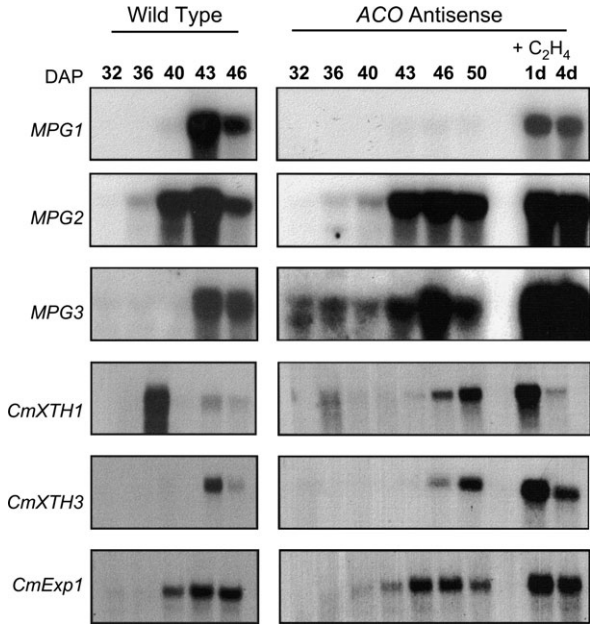

Fig. 5. RNA gel blot analysis of the expression of three PG genes (*MPG1*, *-2*, and *-3*), two XTH genes (*CmXTH1* and *-3*), and an expansin gene (*CmExp1*). RNA was extracted at various DAP in wild-type Charentais melon fruit, untreated *ACO* antisense fruit, and transgenic fruit treated at 46 DAP with 50 ppm ethylene for 1 d and 4 d.

dependence is complete or partial. Flores *et al*. (2001) observed that in *ACO*-suppressed transgenic melon fruit, softening was accelerated by exogenous ethylene, but continued, albeit at a reduced rate, after the removal of exogenous ethylene, suggesting that softening was partially ethylene-regulated. Here, no decline in flesh firmness of untreated *ACO*-suppressed transgenic fruit was observed (Fig. 1) and 1-MCP treatment at 2 d after the onset of ripening inhibited the subsequent softening dramatically (Fig. 6). The results of these two different approaches indicate that softening of Charentais fruit is completely dependent on ethylene, even after the initiation of ripening. Similarly, 1-MCP treatment after the onset of ripening resulted in transient but complete cessation of fruit softening in 'Galia' melons (Ergun *et al*., 2005) and pears (Hiwasa *et al*., 2003).

The loss of ethylene production in *ACO*-suppressed transgenic fruit resulted in minimal pectin and xyloglucan depolymerization, unlike the substantial degradation that was observed in normal ripening fruit (Figs 2, 3). Moreover, the application of exogenous ethylene stimulated disassembly of both polymer networks (Fig. 4), indicating that these processes are ethylene-regulated, and therefore that the corresponding wall-modifying genes might be similarly regulated.

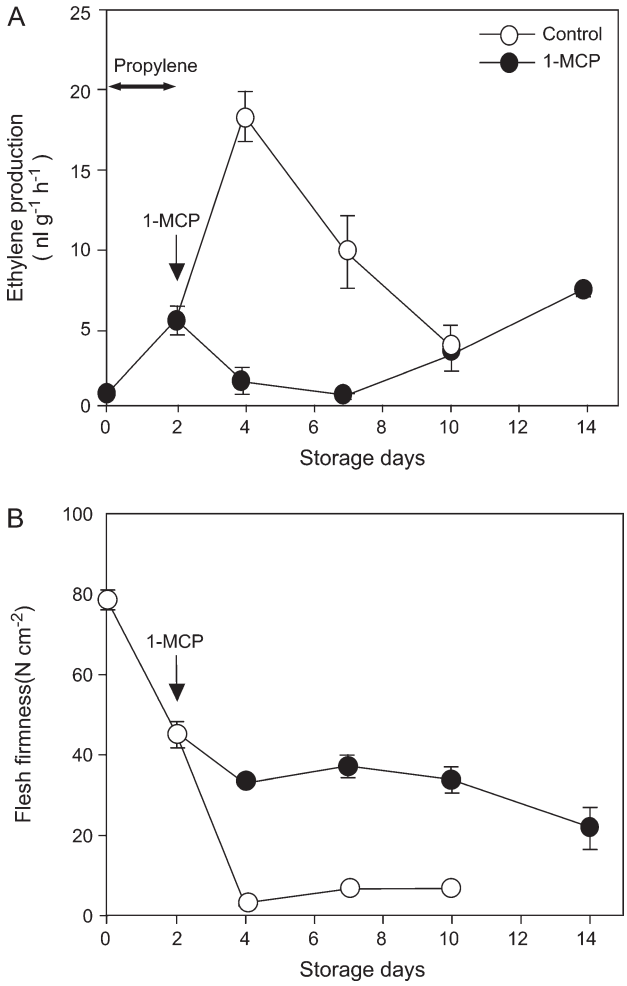

Fig. 6. Ethylene production and flesh firmness in Charentais melon fruit treated with or without 1-MCP. All fruit were treated with 5000 ppm propylene for the first 2 d to ripen uniformly and stored at 25 °C. Some fruit were treated with 5 ppm 1-MCP overnight of day 2. Error bars represent standard errors (n=4).

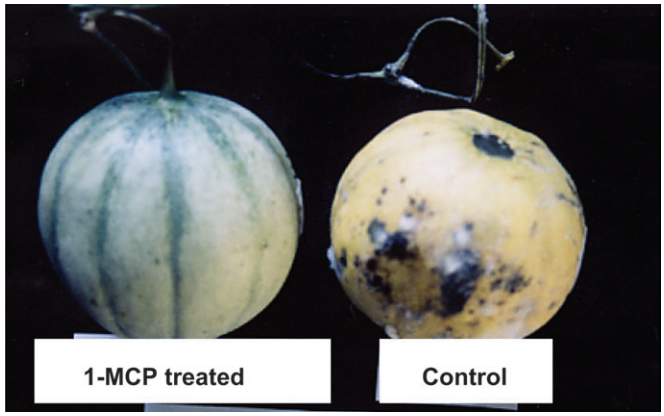

Fig. 7. Untreated wild-type Charentais melon (control) or a fruit treated with 1-MCP after 15 d storage at 25 °C.

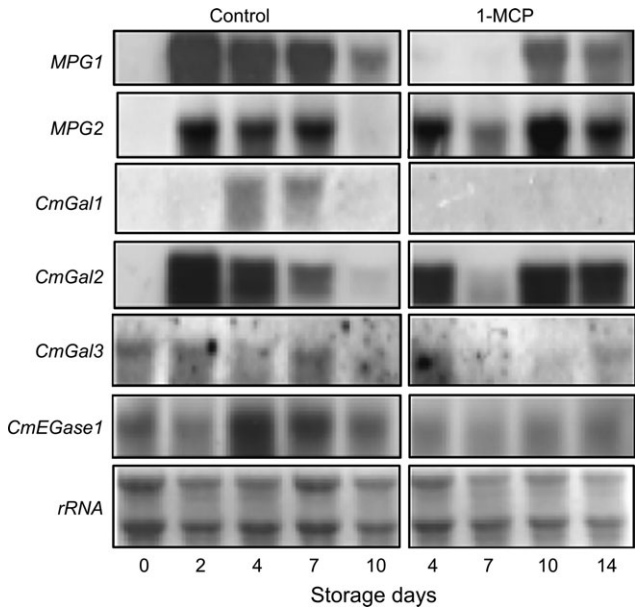

Fig. 8. RNA gel blot analysis of genes encoding cell wall-modifying enzymes. Fruit were exposed to 5000 ppm propylene for the first 2 d after harvest and then stored at 25 °C. A subset of fruit were treated with 5 ppm 1-MCP overnight of day 2. Ethidium bromide-stained rRNA is shown as a loading control.

Regulation of genes encoding cell wall-modifying enzymes in Charentais melon fruit

Ripening of Charentais melons is characterized by a substantial solubilization and depolymerization of pectins in the later stages of ripening (Rose *et al*., 1998) that is largely ethylene-regulated, as observed in molecular size analysis of water- and CDTA-soluble pectins from wild-type and *ACO*-suppressed transgenic melon fruit (Figs 2, 3). An increase in PG activity and mRNA levels has been reported in several fruit species concomitant with the degradation of pectin polysaccharides and softening (Huber, 1983; Fischer and Bennett, 1991). Hadfield *et al.* (1998) showed that the expression of *MPG1*, *-2*, and *-3* increased during Charentais fruit ripening and correlated temporally with an increase in pectin-degrading activity. A similar ripening-associated expression pattern of *MPG* was also observed in non-treated wild-type fruit (Figs 5, 8); however, each *MPG* gene showed a distinct pattern of ethylene regulation. *MPG1* expression was barely detectable in the *ACO*-suppressed transgenic fruit, but was induced to high levels by exogenous ethylene. In addition, 1-MCP application completely suppressed *MPG1* expression for several days. These observations indicate that *MPG1* expression is totally dependent on ethylene and suggest that *MPG1* is one of the primary candidates responsible for ethylene-dependent pectin disassembly. By contrast, *MPG2* expression was only slightly affected by either transgenic *ACO* suppression or 1-MCP treatment, indicating that regulation of *MPG2* expression is ethylene-independent. Expression of *MPG3* appeared to be

regulated by both ethylene-dependent and -independent factors, as *MPG3* mRNA was detected in *ACO*-suppressed transgenic fruit and expression levels were enhanced by exogenous ethylene. Since substantial pectin depolymerization and fruit softening did not occur in the *ACO*-suppressed transgenic fruit in the absence of ethylene, *MPG2* and *-3* are not likely to be major factors in inhibition of ethylene-mediated softening and pectin degradation, or their action in the cell wall may require the activities of other key wall-modifying proteins that were inactive in the absence of ethylene. It is also likely that *MPG3* encodes an exo-PG, and this activity would not have been detected in the size exclusion chromatography separations (Hadfield *et al*., 1998).

The loss of galactose has also been proposed to contribute to pectin mobilization during ripening, and genes encoding β-Gals have been characterized in several fruit species, such as tomato (Smith and Gross, 2000), Japanese pear (Tateishi *et al*., 2001), and pear (Mwaniki *et al*., 2005). Smith *et al*. (2002) demonstrated that down-regulating the expression of a tomato β-Gal gene (*TBG4*) in transgenic plants resulted in fruit that were, on average, 40% firmer than controls at the red-ripe stage. In Charentais melon fruit, the galactose content in the pectin and hemicellulose wall extracts decreased during ripening, especially at late softening stages (Rose *et al*., 1998), and purified β-Gals have been shown to have the capacity to catalyse an apparent decrease in the molecular size of pectins *in vitro* (Ranwala *et al*., 1992). In this study, three β-Gal genes were cloned and characterized. While the accumulation of *CmGal1* and *-2* mRNAs was not detectable at the preclimacteric stage, *CmGal2* mRNA accumulated abruptly with the onset of ripening and *CmGal1* mRNA levels increased following a 2 d lag after the onset of ripening (Fig. 8). Similarly, 1-MCP treatment completely inhibited mRNA accumulation of *CmGal1* but had less effect on *CmGal2*. These observations suggest that ripening-associated expression of *CmGal1* is likely to be dependent on ethylene, but that *CmGal2* expression could be ethylene-independent. *CmGal1* mRNA was not detected in 1-MCP-treated fruit even at later stages (day 10 and day 4), when *MPG1* mRNA levels had returned to normal. This suggests a role for *CmGal1* in the gradual reinitiation of softening in 1-MCP-treated fruit, and a potential co-operative action between *CmGal1* and *MPG1*. In addition, *CmGal1* could be involved less in the early rapid softening process but more in the later softening process.

Rose *et al*. (1998) suggested that depolymerization of a xyloglucan fraction that is tightly bound to cellulose microfibrils represents one of the early events in ripening-related wall disassembly. The results described here support the correlation between depolymerization of this xyloglucan fraction and fruit softening, and further suggest that this aspect of wall disassembly is ethylene-regulated (Fig. 4). While the enzymatic basis of this process has not been established, cell wall-modifying proteins, including EGases, XTHs, and expansins, have been suggested to play a synergistic role in the restructuring of the cellulose–xyloglucan network (Rose and Bennett, 1999). Two melon XTH genes (*CmXTH1* and *CmXTH3*) and an expansin gene (*CmExp1*) showed ripening-related patterns of expression (Fig. 5). No major differences were observed in overall mRNA levels of XTH genes and *CmExp1* between wild-type and non-softening *ACO*-suppressed transgenic fruit, except a temporal delay in the transgenic fruit (Fig. 4), although exogenous ethylene enhanced the mRNA levels of these genes. Therefore, expression of *CmXTH1* and *-3* and *CmExp1* is likely to be ripening-associated but only partially ethylene-dependent, suggesting that they do not play a significant role in ethylene-dependent melon fruit softening. While a potential function for these genes in the softening process is not as apparent for *MPG1* and *CmGal1*, it is still possible that they represent important members of the battery enzymes employed in cell wall disassembly, and it should further be noted that the detection of wild-type levels of mRNAs in the transgenic fruit does not preclude post-transcriptional gene regulation as an important part of the overall control of cell wall modification.

This study indicates that while the overall process of cell wall disassembly in ripening Charentais melons is clearly ethylene-regulated, considerable variation exists between the patterns of regulation of divergent families of wall-modifying proteins and between individual members within those families. This work lays the foundation for further investigation into the role of specific members of these gene families, with the goal of discerning their contribution to the wall disassembly during fruit softening and the mechanisms by which their expression is co-regulated as part of the ripening programme.

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