A transcriptomics-based kinetic model for enzyme-induced pectin degradation in apple (*Malus × domestica*) fruit

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Abstract

Modifications of cell wall pectin during apple softening involve the concerted action of several enzymes. A mathematical model was developed to describe the action of cell wall hydrolases resulting in specific modifications in cell wall pectin polysaccharides during softening of ‘Jonagold’ apple. A conceptual model of enzyme-induced pectin breakdown was formulated based on current knowledge. Activities of different cell wall enzymes were considered to be directly correlated to the expression levels of the respective genes. The first pectin modification to occur was assumed to be cleavage of side chain neutral sugars from the polymer backbone, by the action of side chain debranching enzymes, beta-galactosidase and alpha-L-arabinofuranosidase. It was considered that this increases cell wall porosity, facilitating the action of pectin methyl esterase, which de-methylates pectin polysaccharides, providing a substrate for polygalacturonase. Polygalacturonase degrades the de-methylated pectin chain, producing smaller pectin fragments which are easily solubilised in water. Coupled differential equations

were written based on these reactions. The model was calibrated using data on expression of cell wall-related genes, activities of enzymes encoded by these genes, and the associated modifications in pectin polysaccharides during softening in ‘Jonagold’ apples stored at 1°C under regular atmosphere or controlled atmosphere. The model could explain 79 % of the variation in the data. Using the model, *in silico* investigations on the impact of downregulation of different key cell wall-related genes on softening were carried out, and the results were compared successfully with currently available literature data.

Keywords: modelling, firmness, cell wall, polygalacturonase, beta-galactosidase

1. Introduction

Loss in flesh firmness resulting from enzyme-induced modifications in cell wall pectin is a major cause of postharvest quality losses in apples. Consequently, several studies aimed at understanding the modifications that occur within cell wall pectin polysaccharides and how these modifications are regulated during softening (Wu et al. 1993; Atkinson et al. 2012; Ng et al. 2013; Gwanpua et al. 2016b). A number of cell wall hydrolases have been implicated in pectin modifications during fruit softening, such as polygalacturonase (PG), pectin methyl esterase (PME), beta-galactosidase (β -GAL), alpha-arabinofuranosidase (α -AF), and pectate lyase (Goulao et al. 2007; Gwanpua et al. 2014). Also, non-pectolytic proteins, in particular expansins, have been shown to be involved in reorganizing the cellulose–xyloglucan network (Rose et al. 1997).

Characterization of cell wall pectin changes provides information on its degradation (Hadfield 1998). Pectin characterization is particularly important when studying softening because *in vitro* activities measured for most enzymes may significantly deviate from *in vivo* activities (Christiaens et al. 2012). The relationship between expression of cell wall-related genes, activities of cell wall hydrolases, and pectin modifications is not straightforward. The current hypothesis assumes that PG action results in pectin depolymerisation and solubilisation, while β -GAL, α -AF, and softening-related expansins increase cell wall porosity (Brummell 2006). A useful way to ascertain this hypothesis is to quantify the relationships using mathematical models (Hertog et al. 2011). Several models have been developed

to understand and predict apple softening (Tijskens et al. 1999; Hertog et al. 2001; Johnston et al. 2001; Gwanpua et al. 2012; van der Sman and Sanders 2012; Gwanpua et al. 2013). However, these models so far were limited to describing the effect of temperature and composition of atmospheric gases on firmness, as a physical property, rather than explaining the underlying biochemical changes responsible for fruit softening. An exception is the model developed by Róth et al. (2008) for predicting enzymatic softening of apples, based on a simplified mechanism of sequential degradation of pectin backbone by β -GAL and PG. However, no data on pectin properties was used in that model, and the actual mechanism of modifications in pectin polysaccharides during apple softening is much more complex.

With the goal of developing a detailed kinetic model to mechanistically describe enzyme-induced cell wall breakdown during apple softening, we collected data on cell wall pectin characterization, activities of cell wall enzymes, and expression of cell wall-related genes during ripening in ‘Jonagold’ apples kept under different storage conditions (Gwanpua et al. 2014; Gwanpua et al. 2016a). The present study uses these data to develop a kinetic model describing the events from the expression of cell wall-related genes, over synthesis of cell wall hydrolases to modifications in pectin polysaccharides leading to softening of ‘Jonagold’ apples, similar to an approach earlier used in modelling ethylene biosynthesis in apples (Van de Poel et al. 2014). The model was used *in silico* to investigate the impact of downregulation of different cell wall-related genes on cell wall modifications, and the findings were compared to literature data.

2. Materials and methods

Apples (*Malus × domestica* Borkh.) cv ‘Jonagold’ were harvested in 2012 from an orchard located at Opvelpsestraat, Bierbeek, Belgium. Fruit were harvested at commercial maturity and stored at 1 °C, either under controlled atmosphere (CA) storage or regular atmosphere for 6 months. Before storage and at intervals during storage, firmness, cell wall pectin properties, and activity of various cell wall hydrolases (PG, PME, β -Gal, and α -AF) were quantified. This data was earlier reported in Gwanpua et al. (2014). Firmness was measured as the maximum force needed for a self-cutting cylindrical probe, with a surface area of 1 cm², to penetrate the fruit to a depth of 8mm. Cell wall material was isolated

from apple cortex tissue as alcohol insoluble residue (AIR). The AIR was fractionated by extracting sequentially with water, CDTA, and Na₂CO₃, to obtain water extractable pectin (WEP), chelator extractable pectin (CEP), and Na₂CO₃ extractable pectin (NEP) fractions, respectively. The uronic acid content of the AIR and the different fractions were quantified. Also, the degree of methylation of the AIR and the WEP fraction were quantified. Furthermore, the molar mass and the neutral sugar content of the WEP fraction were quantified using size exclusion chromatography and high-performance anion exchange chromatography, respectively. Optimized protocols were used for obtaining crude extracts of the different cell wall hydrolases. PG activity was quantified by measuring the amount of reducing groups released from the hydrolysis of polygalacturonic acid, using 2-cyanoacetamide. PME activity was measured by incubating the crude enzyme extract with 0.35% apple pectin solution and titrating the released carboxylic acid with 0.01 M NaOH to maintain pH at 7.5. β -Gal and α -AF activities were measured by quantifying the release of *p*-nitrophenol from *p*-nitro phenyl- β -D-galactopyranoside and *p*-nitrophenyl- α -L-arabinofuranoside, respectively. For details on these protocols, the reader is referred to Gwanpua et al. (2014).

Additionally, tissue samples were collected, frozen in liquid nitrogen, and stored at -80 °C, and the expression of candidate cell wall-related genes were later analysed by real-time quantitative PCR. Details on the materials and methods used for quantification of the cell wall-related gene expression, and the data are reported in Gwanpua et al. (2016a).

3. Model development

3.1.Description

A schematic diagram describing the enzyme-induced breakdown of cell wall pectin polymers is shown in Fig. 1. The Na₂CO₃ extractable pectin fraction, NEP, constitute mainly of highly branched Rhamnogalacturonan-I (RG-I) pectin molecules tightly bound within the middle lamella and primary cell wall (Renard 2005; Brummell 2006). NEP may also constitute of Homogalacturonan (HG) pectin molecules that are attached to RG-I either as side chains (Voragen et al. 2003; Voragen et al. 2009) or via covalent linkages through backbone residues (Nakamura et al. 2002; Mohnen 2008; Caffall and

Mohnen 2009). In the model, it was assumed that the NEP is made up of long chain, highly methylated
 HG molecules, HG_{NEP} , linked to highly branched RG-I molecules, $RG-I_{NEP}$. The first modifications
 to occur in NEP are losses of side chain neutral sugars from the RG-I domain by the action of side-
 chain debranching enzymes, β -GAL and α -AF (Brummell and Harpster 2001; Peña and Carpita 2004;
 Gwanpua et al. 2016b). The action of these debranching enzymes weakens the interaction between
 $RG-I_{NEP}$ and HG_{NEP} , releasing highly methylated HG molecules, $HG_{CEP,highDM}$, into the CEP
 fraction. It was assumed that interaction of PME and PG with HG is limited in HG_{NEP} due to steric
 hindrance by highly branched RG-I molecules (Brummell and Harpster 2001). PME must first act on
 $HG_{CEP,highDM}$ to produce de-methylated HG molecule, $HG_{CEP,lowDM}$, as PG will only act on pectin
 molecules with low degree of methylation (Steele et al. 1997). The action of PG on $HG_{CEP,lowDM}$
 produces shorter chain molecules that are solubilised into the water extractable pectin (WEP) fraction,
 HG_{WEP} . Further degradation of HG_{WEP} was assumed possible by the action of PG to produce WEP
 molecules with shorter chains, $HG_{WEP,short}$.

WEP fraction was assumed to also constitute of smaller chains RG-I molecules, $RG-I_{WEP}$, resulting
 from side chain debranching of $RG-I_{NEP}$. This is supported by the fact that *in vitro* treatments of pectin
 extracted from mature green tomato fruit with purified β -GAL resulted to decreased apparent average
 molecular size and increased solubilisation of pectin (De Veau et al. 1993). Moreover, WEP fraction in
 apple fruits is rich in side chain neutral sugars (Ng et al. 2015; Gwanpua et al. 2016b). As the extent of
 branching of the WEP fraction decreases during softening (Gwanpua et al. 2014; Ng et al. 2015), further
 degradation of side chain neutral sugars in $RG-I_{WEP}$ was considered. It was assumed that no further
 demethylation occurred in the WEP fractions, as the DM of this fraction was constant throughout
 ripening (Gwanpua et al. 2014). The active amount of each cell wall enzyme was assumed to be driven
 by translation from the respective genes, and degradation due to protein turnover. The reaction scheme
 in Fig.1 was used to formulate a mathematical model for enzyme induced pectin degradation during
 softening in apples. The different enzyme catalysed reactions were modelled by mass-action kinetics

rather than the Michaelis-Menten kinetics, based on the assumption that substrate concentrations are not saturating. The use of mass-action kinetics was motivated by the need to reduce the number of model parameters so as to avoid overfitting, while maintaining the descriptive power of the model.

Figure 1. A conceptual model of enzyme-induced cell wall pectin modifications during apple fruit softening. The Na_2CO_3 extractable pectin (NEP) constitutes Rhamnogalacturonan –I (RG-I) molecules to which are attached Homogalacturonan (HG) with high degree of methylation (DM). Beta-galactosidase (β -GAL) and alpha-arabinofuranosidase (α -AF) catalyse the removal of galactose (Gal) and arabinose (Ara), respectively, from RG-I, with rate constants $k_{\beta\text{-GAL}}$ and $k_{\alpha\text{-AF}}$, respectively. This results to the release of HG molecules with high DM, $\text{HG}_{\text{CEP,highDM}}$, and RG-I molecules that are extractable with water, RG-I_{WEP} . Further debranching occurs in RG-I_{WEP} , while pectin methyl esterase (PME) acts on $\text{HG}_{\text{CEP,highDM}}$, removing methyl groups (CH_3) from the galacturonic acid backbone to form HG molecules with low DM, $\text{HG}_{\text{CEP,lowDM}}$, with rate constant k_{PME} . Polygalacturonase (PG) catalyses the breakdown of $\text{HG}_{\text{CEP,lowDM}}$ to form HG molecules that are solubilisable

in water, HG_{WEP} , with a rate constant $k_{\text{PG,CEP}}$. Further breakdown of HG_{WEP} by PG to form shorter chains HG molecules, $\text{HG}_{\text{WEP,short}}$ is possible, with a rate constant $k_{\text{PG,WEP}}$. The activities of the different enzymes are controlled by translation and protein degradation, with rate constants $k_{t,p}$ and $k_{d,p}$, respectively (p represents respective enzyme).

3.2. Model equations

3.2.1. Synthesis of pectin degrading enzymes

The rate of accumulation of enzymes was modelled as a net balance between translation from the respective transcript, mRNA_p , with rate constant $k_{t,p}$, and degradation of the enzymes, with the protein-specific degradation rate $k_{d,p}$ (Eqs. (1) – (4), p refers to the specific enzyme involved).

$$\frac{d[p]}{dt} = k_{t,p} [\text{mRNA}_p] - k_{d,p} [p] \quad (1)$$

3.2.2. Enzyme-induced pectin modifications

The first reaction occurring within the pectin polysaccharide was assumed to be loss of side chains from NEP, according to Eq. (2).

$$\frac{d[\text{NEP}]}{dt} = -k_{\beta\text{-GAL}} [\beta\text{-GAL}] [\text{NEP}] - k_{\alpha\text{-AF}} [\alpha\text{-AF}] [\text{NEP}] \quad (2)$$

where $k_{\beta\text{-GAL}}$ and $k_{\alpha\text{-AF}}$ are the rate constants of $\beta\text{-GAL}$ -catalysed loss of galactose and $\alpha\text{-AF}$ -catalysed loss of arabinose, respectively, from RG-I pectin. As de-branching of NEP releases $\text{HG}_{\text{CEP,highDM}}$ that are converted to low degree of methylation (DM) HG molecules, the rate of accumulation of $\text{HG}_{\text{CEP,highDM}}$ was modelled according to Eq. (3).

$$\frac{d[\text{HG}_{\text{CEP,highDM}}]}{dt} = k_{\beta\text{-GAL}} [\beta\text{-GAL}] [\text{NEP}] + k_{\alpha\text{-AF}} [\alpha\text{-AF}] [\text{NEP}] - k_{\text{DM}} [\text{PME}] [\text{HG}_{\text{CEP,highDM}}] \quad (3)$$

where k_{DM} is the rate constant for pectin de-methylation. De-methylated HG pectin, $HG_{CEP,lowDM}$, is degraded by PG to shorter chains HG molecules, HG_{WEP} , that are extractable by water. The amount of $HG_{CEP,lowDM}$ was therefore modelled according to Eq. (4).

$$\frac{d[HG_{CEP,lowDM}]}{dt} = k_{DM} [PME] [HG_{CEP,highDM}] - k_{PG,CEP} [PG] [HG_{CEP,lowDM}] \quad (4)$$

where $k_{PG,CEP}$ is the rate constant for degradation of low DM CEP molecules by PG. Further degradation of HG_{WEP} by PG to form shorter chains molecules, $HG_{WEP,short}$, was modelled according to Eq. (5).

$$\frac{d[HG_{WEP}]}{dt} = k_{PG,CEP} [PG] [HG_{CEP,lowDM}] - k_{PG,WEP} [PG] [HG_{WEP}] \quad (5)$$

where $k_{PG,WEP}$ is the rate constant of depolymerisation of WEP. Different rate constants were used for depolymerisation of CEP and WEP, as the number of regions with long stretch of de-methylated pectin will be different in both molecules.

Further degradation of side chains in $RG-I_{WEP}$ was modelled according to Eq. (6) – (7).

$$\begin{aligned} \frac{d[RG-I_{WEP}]}{dt} = & k_{\beta-GAL} [\beta-GAL] [NEP] + k_{\alpha-AF} [\alpha-AF] [NEP] \\ & - k_{\beta-GAL} [\beta-GAL] [RG-I_{WEP}] - k_{\alpha-AF} [\alpha-AF] [RG-I_{WEP}] \end{aligned} \quad (6)$$

$$\frac{d[RG-I_{WEP,debr}]}{dt} = k_{\beta-GAL} [\beta-GAL] [RG-I_{WEP}] + k_{\alpha-AF} [\alpha-AF] [RG-I_{WEP}] \quad (7)$$

As both β -GAL and α -AF are exo-acting enzymes, their activity is affected by the number of branch points (free galactosyl and arabinosyl residues) within the pectin backbone. Since it can be assumed that it is primarily the length of the side chain branches that reduces during pectin debranching, rather than the number of branch points, common rate constants were used for all reactions catalysed by these enzymes.

3.2.3. Output relations

It was not possible to quantify separately the different HG molecules. CEP was considered as the sum of $\text{HG}_{\text{CEP,highDM}}$ and $\text{HG}_{\text{CEP,lowDM}}$ (Eq. (8)).

$$[\text{CEP}] = \text{HG}_{\text{CEP,highDM}} + \text{HG}_{\text{CEP,lowDM}} \quad (8)$$

Similarly, only the total WEP fraction, $\text{WEP}_{\text{total}}$, was measured.

$$[\text{WEP}_{\text{total}}] = [\text{HG}_{\text{WEP}}] + [\text{HG}_{\text{WEP,short}}] + [\text{RG-I}_{\text{WEP}}] + [\text{RG-I}_{\text{WEP,debr}}] \quad (9)$$

Since $k_{\beta\text{-GAL}}$ and $k_{\alpha\text{-AF}}$ are both responsible for debranching and always appear together in the model equations, it is not possible to estimate specific values for each without directly linking them to the rate of galactose and arabinose loss respectively. Consequently, two additional odes for the loss of galactose and arabinose from RG-I_{WEP} were written (Eqs. (10) and (11)).

$$\frac{d[\text{Gal}_{\text{RG-I}_{\text{WEP}}}]}{dt} = -k_{\beta\text{-GAL}} [\beta\text{-GAL}] [\text{Gal}_{\text{RG-I}_{\text{WEP}}}] \quad (10)$$

$$\frac{d[\text{Ara}_{\text{RG-I}_{\text{WEP}}}]}{dt} = -k_{\alpha\text{-AF}} [\alpha\text{-AF}] [\text{Ara}_{\text{RG-I}_{\text{WEP}}}] \quad (11)$$

where $\text{Gal}_{\text{RG-I}_{\text{WEP}}}$ and $\text{Ara}_{\text{RG-I}_{\text{WEP}}}$ are the galactose and arabinose content of RG-I_{WEP} , respectively.

The measured galactose and arabinose content of the WEP fraction were related to the $\text{Gal}_{\text{RG-I}_{\text{WEP}}}$ and

$\text{Ara}_{\text{RG-I}_{\text{WEP}}}$ by the following relations:

$$\text{Gal}_{\text{WEP}_{\text{total}}} = \frac{[\text{Gal}_{\text{RG-I}_{\text{WEP}}}] [\text{RG-I}_{\text{WEP}}]}{\text{WEP}_{\text{total}}} \quad (12)$$

$$\text{Ara}_{\text{WEP}_{\text{total}}} = \frac{[\text{Ara}_{\text{RG-I}_{\text{WEP}}}] [\text{RG-I}_{\text{WEP}}]}{\text{WEP}_{\text{total}}} \quad (13)$$

where $\text{Gal}_{\text{WEP}_{\text{total}}}$ and $\text{Ara}_{\text{WEP}_{\text{total}}}$ are the galactose and the arabinose content of the WEP fraction, respectively. The relation in Eqs. (12) and (13) assumes the only source of galactose and arabinose in the WEP is RG-I_{WEP} .

An expression was used to correlate the measured molar mass of the WEP fraction, $\text{MM}_{\text{WEP},\text{total}}$, with the amount of the different WEP intermediates (Eq. (14)). The non-depolymerised WEP molecules were assumed to have the same average molecular mass, MM_{WEP} , while the short chain WEP molecules were assumed to have an average molar mass denoted by $\text{MM}_{\text{WEP},\text{short}}$.

$$\text{MM}_{\text{WEP},\text{total}} = \frac{\text{MM}_{\text{WEP}} \left([\text{HG}_{\text{WEP}}] + [\text{RG-I}_{\text{WEP}}] \right) + \text{MM}_{\text{WEP},\text{short}} \left([\text{HG}_{\text{WEP},\text{short}}] + [\text{RG-I}_{\text{WEP},\text{debr}}] \right)}{[\text{WEP}_{\text{total}}]} \quad (14)$$

The link between cell wall modifications and loss in tissue firmness is not straightforward. Some authors have investigated the relationship between specific cell wall modifications (mainly those induced by expansin) and tissue biomechanics (Wang et al. 2008; Park and Cosgrove 2012). However, there is no model linking different aspects of pectin modifications and loss in tissue firmness, with only simple linear regression used to correlated firmness and different pectin properties (Billy et al. 2008; Gwanpua et al. 2014). Extensive arabinan and galactan side chains of RG-I (in the NEP fraction) are important in maintaining cell wall integrity through covalent linkages with cellulose (Zykwinska et al. 2005) or by physical entanglement with other cell wall polymers (Brummell 2006). CEP is also involved in strengthening cell wall by forming Ca^{2+} crosslinks between HG molecules (Zykwinska et al. 2005). Flesh firmness, F , was therefore assumed to be proportional to the amount of NEP and CEP fractions present within the cell wall. After some preliminary data analysis, this was modelled by the following empirical relation:

$$F = \beta \left([\text{NEP}] + [\text{CEP}] \right)^\gamma \quad (15)$$

where β and γ are model parameters.

3.3. Model calibration

Gene expression data were used as input in Eqs. (1) – (4). Isozymes within a gene family may encode different isoforms of a protein. Since we did not characterise different isoforms of the enzymes, the different isozymes were pooled and a constant average apparent translation rate was used. Therefore, the input used for the synthesis of each enzyme was simply the sum of the expression of the different isozymes within that gene family. Separate parameters for the rate constants were estimated for the two storage conditions, under CA and under regular atmosphere.

The model ordinary differential equations (odes) were solved numerically in MATLAB 2015b (The Mathworks Inc., Natick, USA), using the ode15s solver. Model implementation and parameter estimations were done using OptiPa, a MATLAB user interface for calibrating ode-based models (Hertog et al. 2007).

4. Results and discussion

4.1. Model simulations

The expression of two *PG* genes (*MdPG1* and *MdPG2*), two β -*GAL* genes (*Md β -GAL1* and *Md β -GAL2*), two α -*AF* genes (*Md α -AF1* and *Md α -AF2*), and three *PME* genes (*MdPME1*, *MdPME2* and *MdPME3*) were used as input for the synthesis of the respective enzymes (Fig. 2).

Figure 2. Expression of candidate cell wall-related genes during storage of 'Jonagold' apples at 1°C under controlled atmosphere (CA-stored) and regular atmosphere (air stored). Each data point is the mean of four measurements, with the error bar representing the standard error.

Although only a few of these genes were identified as key markers for softening (Gwanpua et al. 2016a), the expression data of all isogenes were used to account for any contribution made by these genes towards total cell wall enzyme activities. In (Gwanpua et al. 2016a, Table 1) only the expression levels after 6 months storage both under CA and regular atmosphere were provided, while a time series expression during storage is now shown in Fig. 2. The modelled enzyme activities of these four cell wall hydrolases, together with the individual measurements are shown in Fig. 3, with the estimated values of the model parameters reported in Table 1. The rate constants for the synthesis of the different cell wall enzymes were generally lower for the CA stored fruit. This suggests that the CA effect was not limited to down regulation of the cell wall-related genes, but influenced the rate of translation, and

possibly post-translational modifications. This is expected as CA regulates respiration, and most cell metabolism requires ATP, preferably coming from respiration.

Figure 3. The evolution of (A) PG activity, (B) PME activity, (C) β -GAL activity, and α -AF activity during storage of 'Jonagold' apples at 1°C under controlled atmosphere (CA-stored) and regular atmosphere (air stored). Both the modelled values and the individual measurements are shown.

The assay used for measuring the PG activity, which was based on measuring the amount of reducing groups following incubation with the crude enzyme extract, predominantly quantifies exo-PG activity, while the expression data are based on the expression of endo-PG genes, which has been shown to be more relevant in apple fruit softening (Costa et al. 2010; Atkinson et al. 2012; Longhi et al. 2013). Though it is well established that *Malus Endo-polygalacturonase 1* plays a key role in apple fruit softening, several studies have failed to measured endo-PG activity in ripe apples (Abeles and Biles 1991; Yoshioka et al. 1992; Goulao et al. 2007). While some of these authors have concluded that endo-PG activity is absent in apples, the very strong correlation between endo-PG genes expression and cell wall solubilisation and softening in apples (Gwanpua et al. 2016a), suggests that it is more likely that

the extraction protocols and assays used to quantify endo-PG action were not suitable, rather than complete absence of the enzyme. More relevance was therefore given to the expression data of the endo-PG than to the PG activity data, by assigning fixed values to the rate constants related to translation of *PG* genes and degradation of the PG enzymes. This explains why the model fit for PG activity was particularly poor, as the model attempts to relate the expression data with modifications in the pectin polysaccharide (which are much closer reflection of *in vivo* activity than the PG activity data), and loss in firmness.

The model simulations, together with the measured data, for different properties of cell wall pectin polysaccharides during storage of 'Jonagold' apples at 1°C under CA and regular atmosphere are shown in Fig. 4. As with the enzymes, the model generally followed the trend of the data during storage under both conditions. Again the estimated values of the rate constants were much higher for the fruit stored under regular atmosphere compared to CA storage. This indicates that in addition to regulating the amount of enzyme synthesized, respiration may influence the interaction between the enzyme and the substrate, by supplying energy for various active transports processes (Sondergaard et al. 2004). The simulated increase in WEP fraction was concurrent with a decrease in NEP and CEP fractions, as the model assumes that breakdown of NEP and CEP were responsible for increase in the WEP fraction. Initially, the CEP fraction increased as a result of degradation of side chain sugars from RG-I in the NEP fraction. During the rapid softening in air stored fruit, CEP was converted to WEP through PG catalysed depolymerisation of HG molecules, forming shorter chains pectin molecules that are solubilised in water. As this happened, the CEP fraction decreased in air stored fruit. This resulted to a rapid decline in the CEP fraction. After 50 d, there was a slower rate of firmness decline in the air stored fruit. This decline in softening coincided with an increase in the CEP fraction, suggesting more pectin molecules were released from the NEP fraction into the CEP fraction, than were degraded by PG into WEP molecules.

Figure 4. The evolution of (A) Na_2CO_3 extractable pectin (NEP) fraction, (B) chelator extractable pectin (CEP) fraction, (C) water extractable pectin (WEP) fraction, (D) galactose content of WEP, (E) arabinose content of WEP, (F) degree of branching (Dbr), (G) molar mass of WEP, and (H) firmness during storage of 'Jonagold' apples at 1°C under controlled atmosphere (CA-stored) and regular atmosphere (air stored). Both the modelled values and the individual measurements are shown. AIR denotes alcohol insoluble residues and GalA denotes galacturonic acid.

While both the expression of the α -AF genes and the measure enzyme activity were much lower than those of β -GAL, the loss in side chain arabinose was comparable to the loss in side chain galactose (Fig. 4D and 4E). This explains why the estimated rate constant for the action of α -AF, $k_{\alpha\text{-AF}}$, was much higher than the estimated rate constant for β -GAL action, $k_{\beta\text{-GAL}}$ (Table 1). This means compare to β -GAL, a relatively smaller amount of active α -AF is needed to increase cell wall porosity. It could also mean that the measured *in vitro* activity is not a true reflection of the *in vivo* activity.

Table 1: Parameter estimates and the 95% confidence interval (CI) for pectin degradation model during softening in ‘Jonagold’ apples stored at 1 °C under controlled atmosphere (CA) or in air. CI were obtained using the model based error resampling bootstrap techniques.

*Parameter		Estimates†			Units§
		Mean	95% CI		
			Lower bound	Upper bound	
$k_{t,\text{PG}}$		0.001			U g ⁻¹ fruit d ⁻¹ relative expression ⁻¹
$k_{t,\text{PME}}$	CA	0.0025	0.0007	0.0044	U g ⁻¹ fruit d ⁻¹ relative expression ⁻¹
	air	0.0043	0.0030	0.0056	U g ⁻¹ fruit d ⁻¹ relative expression ⁻¹
$k_{t,\alpha\text{-AF}}$	CA	1.6×10 ⁻⁶	1.9×10 ⁻⁸	3.5×10 ⁻⁶	U g ⁻¹ fruit d ⁻¹ relative expression ⁻¹
	air	4.6 ×10 ⁻⁶	2.9×10 ⁻⁶	6.2×10 ⁻⁶	U g ⁻¹ fruit d ⁻¹ relative expression ⁻¹
$k_{t,\beta\text{-GAL}}$	CA	2.3×10 ⁻⁶	6.6×10 ⁻⁹	6.3×10 ⁻⁶	U g ⁻¹ fruit d ⁻¹ relative expression ⁻¹
	air	1.4×10 ⁻⁶	8.2×10 ⁻⁷	2.1×10 ⁻⁶	U g ⁻¹ fruit d ⁻¹ relative expression ⁻¹
$k_{d,\text{PG}}$		0.0001			d ⁻¹
$k_{d,\text{PME}}$		2.5×10 ⁻⁵	1.4×10 ⁻⁶	7.5×10 ⁻⁵	d ⁻¹
$k_{d,\alpha\text{-AF}}$		1.6×10 ⁻⁵	2.2×10 ⁻⁶	5.9×10 ⁻⁵	d ⁻¹
$k_{d,\beta\text{-GAL}}$		0.0047	0.0014	0.0092	d ⁻¹
$k_{\alpha\text{-AF}}$	CA	0.35	0.009	0.79	U ⁻¹ g fruit d ⁻¹
	air	0.42	0.09	0.78	U ⁻¹ g fruit d ⁻¹
$k_{\beta\text{-GAL}}$	CA	0.0013	0.00001	0.008	U ⁻¹ g fruit d ⁻¹
	air	0.017	0.002	0.032	U ⁻¹ g fruit d ⁻¹
k_{PME}	CA	0.00081	0.0001	0.0015	U ⁻¹ g fruit d ⁻¹
	air	0.00081	0.0001	0.0015	U ⁻¹ g fruit d ⁻¹
$k_{\text{PG,CEP}}$	CA	0.0014	0.0001	0.0029	U ⁻¹ g fruit d ⁻¹
	air	0.035	0.013	0.10	U ⁻¹ g fruit d ⁻¹
$k_{\text{PG,WEP}}$	CA	0.039	0.007	0.110	U ⁻¹ g fruit d ⁻¹
	air	0.047	0.002	0.224	U ⁻¹ g fruit d ⁻¹
γ		1.3	1.1	1.6	/
β		1.0	0.3	2.2	N (Mol % GalA in AIR) ⁻¹
$[\text{PG}]_0$		0.0029	0.0001	0.0128	U g ⁻¹ fruit

$[PME]_0$	2.3	1.8	3.0	U g ⁻¹ fruit
$[\alpha\text{-AF}]_0$	0.00082	0.000001	0.00260	U g ⁻¹ fruit
$[\beta\text{-GAL}]_0$	0.097	0.087	0.108	U g ⁻¹ fruit
$[NEP_n]_0$	21	19	24	Mol % GalA in AIR
$[RG\text{-I}_{WEP}]_0$	8.3	5.5	11.6	Mol % GalA in AIR
$[RG\text{-I}_{WEP,debr}]_0$	0.019	0.002	0.095	Mol % GalA in AIR
$[HG_{CEP,highDM}]_0$	0.095	0.0003	0.682	Mol % GalA in AIR
$[HG_{CEP,lowDM}]_0$	8.4	7.2	9.5	Mol % GalA in AIR
$[HG_{WEP}]_0$	6.9	3.9	9.6	Mol % GalA in AIR
$[HG_{WEP,short}]_0$	1			Mol % GalA in AIR
$[Gal_{RG\text{-I}_{WEP}}]_0$	9.7	7.1	12.8	Mol % GalA
$[Ara_{RG\text{-I}_{WEP}}]_0$	11.5	8.4	15.4	Mol % GalA
MM_{WEP}	0.1			MDa
$MM_{WEP,short}$	3.0	2.6	3.4	MDa

*Separate values of the rate constants were estimated for apples stored in CA and air.

†Rate constants for translation of PG genes and degradation of PG enzymes were kept fixed as measured activity was ignored during model fitting. The initial values of all the intermediates could not be estimated simultaneously (coupled parameters), some had to be kept fixed. Average molar mass (MM) for small size water extractable pectin (WEP) was kept fixed at 0.1 MDa.

§1 U of PG activity was defined as the amount of enzyme producing 1 µmol of reducing groups per minute under the assay conditions (Gwanpua et al. 2014).

1 U of PME activity was defined as the amount of enzyme required to release 1 µmol of carboxyl group per min, under the assay conditions (Gwanpua et al. 2014).

1 U of β-GAL activity was defined as the amount of enzyme that hydrolyses the release of 1 µmol of p-nitrophenol from the substrate per min, under the assay conditions (Gwanpua et al. 2014).

1 U of α-AF activity was defined as the amount of enzyme that hydrolyses the release of 1 µmol of p-nitrophenol from the substrate per min, under the assay conditions (Gwanpua et al. 2014).

4.2.Relationship between mRNA abundance and enzyme activity

Many studies have shown that there may be little or no correlation between mRNA abundance and enzyme activity. For example, PME activity and protein abundance increased throughout ripening in tomato fruit, from the early stage to the red ripe stage, while PME mRNA abundance increased during fruit development and declined at the onset of ripening (Harriman et al. 1991). Also, a number of studies have shown that the endo-PG gene, *PGI*, is involved in regulating cell wall modifications during softening in apples (Tacken et al. 2010; Costa et al. 2010; Atkinson et al. 2012; Gwanpua et al. 2016a), while several studies have failed to measure endo-PG activity in ripe apples (Yoshioka et al. 1992; Goulao et al. 2007). Discrepancies between gene expression and enzyme activity is not unusual, as

enzyme activity is not only determined by translation, but by post-translation modifications of the proteins or regulation of the activity of the protein. An example is PME, whose activity is strongly regulated by PME inhibitors, that are often co-expressed with the PME genes (Giovane et al. 2004; Reca et al. 2012). Additionally, the activity of enzymes within the fruit tissue is very much dependent on the pH of the apoplast and accessibility of the enzyme to relevant substrates. The *in vitro* enzyme activity can be seen as a measure of the protein abundance, as the assay for measuring enzyme activity is often based on ideal conditions for the enzyme of interest, which may be very different from the *in vivo* environment where the enzyme may be compartmentalized, or the pH of the apoplast may not be optimal.

By adopting a complete systems approach, starting from gene expression to protein synthesis, and modifications in cell wall pectin, any discrepancy between mRNA abundance and enzyme activity resulting from possible post-translational modifications was accounted for. However, specific aspects of post-translational modifications are not incorporated into the model. Consequently, the rate constants for translation reported in Table 1 should not be seen as absolute values representing only translation, but also encompass the specific activity of the respective enzymes.

4.3. Relationship between cell wall pectin characteristics and firmness

The modelled firmness, together with the individual firmness data, is shown in Fig. 4H. The simple relationship in Eq. (15) was sufficient to describe the relationship between firmness and cell wall pectin modifications. The model assumed that decrease in the amount of NEP and CEP was responsible for firmness losses. However, the further degradation that occurred within the solubilised pectin may further reduce the “gluing” properties of pectin, thereby causing further cell wall weakening and softening. There is also the role played by loss in cell turgor resulting from moisture losses (Jackman et al. 1992). Atkinson et al. (2012) reported that suppression of *PGI* gene resulted in a reduction of water loss in ripe apple fruit, and changes in the structure of the hypodermal cell layers. The observed retention in firmness in *PGI* suppressed fruit in the same study was most likely a combination of stronger intercellular adhesion and higher cell turgor. To better understand and model the relationship between pectin modification and texture, there is need for dedicated studies investigating the link

between cell wall properties, turgor, and cell wall biomechanics (Wang et al. 2008; Park and Cosgrove 2012).

4.4. *In silico* investigation of downregulation of cell wall-related genes

In addition to providing plausible explanations on the mechanism of pectin degradation during softening, another application of the model developed in this study is its use for simulations on the impact of downregulation of one or more cell wall-related genes on pectin modifications and softening. To this end, the expression level of *MdPG1* was set to 8 % of its normal expression, similar to reduction in *PG1* mRNA abundance in transgenic PG1as-41 (Atkinson et al. 2012). The effect of this on cell wall modifications and softening was simulated (Fig. 5). Atkinson et al. (2012) reported a 30 N and 40 N loss in firmness for the PG1as-41 and the control (Royal Gala), respectively. As the ripening condition used in that study (16-week storage at 5 °C in regular atmosphere) was different from those used in developing the model, it was not possible to compare both results on a time basis. However, as it took about 175 d for the apples stored at 1 °C in regular atmosphere to soften by 40 N, it was assumed that ripening Royal Gala apples at 5 °C for 16 weeks equates to ripening Jonagold apples at 1 °C for 175 d. The loss in firmness reported by Atkinson et al. (2012) for PG1as-41 and the control was subtracted from the initial firmness (82 N), and plotted together with the firmness predicted by the model (Fig. 5G). The firmness predicted by the model was comparable to those obtained for PG1as-41. Generally, the difference in firmness seems to be larger earlier on, but reduces with time. This is related to the fact that initially there is only partial pectin degradation by the limited PG in PG1as-41, but the enzyme appears to build up with time to levels sufficient to induce depolymerisation of HG molecules (Fig. 5F). The observed softening in PG1as-41 could also have resulted from the action of side chain debranching enzymes, since β -GAL have been shown to result in decreased pectin molar mass and increased pectin solubilisation (Ranwala et al. 1992). The simulated galactose and arabinose content of the WEP for PG1as-41 was higher than in the control between day 0 and 100 (Fig 5D and 5E). This is because the WEP in PG1as-41 was predominantly RG-I resulting from side chain debranching, with little solubilisation resulting from depolymerisation of HG molecules. It was not possible to quantitatively compare the results with predicted changes in WEP and CEP fractions, as Atkinson et al. (2012)

reported values in absolute amount, while we expressed as percentage uronic acid in AIR. However, PG1as-41 had a lower amount of WEP and a higher amount of CEP than the control, similar to those predicted by the model (Fig. 5B and 5C). A shift in molar mass towards higher molecular weight was measured for the WEP fraction in PG1as-41, similar higher molar mass predicted for the WEP fraction when expression level of *MdPGI* was reduced by 92 % (Fig 5F).

Figure 5. Simulations of the effect of downregulation of cell wall-related genes on the modifications in cell wall pectin properties and softening. (A) Na_2CO_3 extractable pectin (NEP) fraction, (B) chelator extractable pectin (CEP) fraction, (C) water extractable pectin (WEP) fraction, (D) Galactose (Gal) content of the WEP, (E) Arabinose (Ara) content of the WEP, (F) molar mass (MM) of WEP, and (G) firmness during 6month storage at 1°C under regular air. AIR denotes alcohol insoluble residues and GalA denotes galacturonic acid.

We also investigated the effect of downregulation of the main β -GAL gene, *Md β -GAL2*, that was identified in Gwanpua et al. (2016a) to be key in regulating softening. The expression of *Md β -GAL2* was set to 5% of its normal expression, and the resulting changes in pectin properties and firmness during storage at 1°C under regular atmosphere were predicted (Fig. 5). The simulated effect of downregulation of this single β -GAL gene had an impact on pectin solubilisation and softening. This is because our model assumes that pectin debranching is crucial for any other cell wall enzyme to act on the native pectin chain. There are no transgenic studies in literature on the effect of downregulation of β -GAL genes on apple fruit softening. However, a study on tomato showed that downregulation of a beta-galactosidase gene (TBG4) resulted in a 40 % decrease in softening (Smith et al. 2002). As pectin debranching was a result of both losses in galactose and arabinose neutral sugars from the pectin backbone, it is expected that even when *Md β -GAL2* is downregulated, the action of α -AF can still lead to some pectin debranching. To investigate the effect of almost complete absence of side-chain debranching on softening, both *Md β -GAL2* and *Md α -AF2* were set to 5% of their normal expression, and again the resulting changes in pectin properties and firmness during storage at 1°C under regular atmosphere were predicted (Fig. 5). A much larger reduction in softening was obtained, as the action of the other pectin modifying enzymes were strongly limited by the low porosity of the pectin chain. This highlights the importance of considering multiple-gene silencing in generation of transgenic lines with a desired trait. Based on the model simulations, the main difference between the effect of downregulation of *MdPG1* gene and downregulation of *Md β -GAL2* gene is that the former affects the rate of initial softening, while the latter affects firmness loss after the fruit had already undergone substantial softening.

The current model considers enzyme-induced cell wall modifications during postharvest softening, but the effect of downregulation of cell wall-related genes may also influence the evolution of cell wall properties and firmness during fruit development (Ng et al. 2013). The current model could therefore be further developed to consider changes in cell wall properties and firmness during apple fruit development.

5. Conclusion

Enzyme regulated cell wall pectin degradation during softening is a complex process. By using a mathematical modelling approach, the kinetics of pectin degradation during ‘Jonagold’ apple fruit softening was described. Using data on the expression of cell wall-related genes as input, the evolution of cell wall hydrolases, the associated modifications in the cell wall pectin polysaccharide, and loss in firmness during ripening in controlled atmosphere or regular atmosphere can be simulated. The model provides a useful tool for interpreting data on pectin biochemistry, and can be used to assist in selection of genes for breeding programs that have retention of firmness as a desirable trait. The current model was aimed at explaining only those changes that occur in pectin held in walls by ester bonds, bound by ionic interactions, and solubilised in water, as the main cell wall modifications during softening in apple fruit occurred in these fractions. For fruit species undergoing more extensive cell wall modifications during softening (such as in tomato, kiwifruit and avocado), the model will need to be extended to include modifications in other pectin fractions, and also non-pectolytic modifications, such as those occurring in the cellulose–xyloglucan network.

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