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Effect of Pre-harvest Application of Hexanal and Growth Regulators in Enhancing Shelf Life and Regulation of Membrane Associated Genes in Strawberry

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Abstract

The role of hormones during fruit development and ripening in strawberry (*Fragaria ananassa*) is poorly understood. In this study, two strawberry cultivars ('Jewel' and 'Wendy') were chosen based on their shelf life quality, intermediate and excellent, respectively. Hexanal and growth regulators were applied to the fruit as two pre-harvest sprays. Fruits treated with 'Enhanced Freshness Formulation' (a formulation with hexanal as key ingredient) showed improvement in shelf life of both cultivars. Auxin application reduced loss of fruit firmness while ABA was able to accelerate the ripening process without resulting a significant effect on the relevant fruit quality parameters. The regulation of gene expression during ripening in relation to hexanal and hormones was examined in 21 genes potentially involved in cell wall degradation. Gene expression profiles showed similar pattern in the two cultivars with more prominent amplitude in 'Wendy'. The expression of hormone responsive genes responded in an antagonistic manner to the exogenous hormone applications supporting their role in ripening and fruit development. Hexanal application induced a clear reduction in the transcript level of two Phospholipase D genes and other key enzymes involved in cell wall degradation. These findings indicate that ripening in strawberry is associated with the expression of specific genes and the modulation of this gene expression by hexanal supports its role in increasing the fruit shelf-life.

Keywords

Hexanal,–Absciscic acid,–Auxin,– *Fragaria × ananassa*,– Gene expression, ,–Ripening.

Introduction

Strawberry is a soft fruit, characterized by rapid loss of texture during ripening. The significant postharvest loss in such fruits due to rapid and excessive softening have evoked considerable attention into the mechanism that govern fruit softening process (Perkins-Veazie 1995; Brummell and Harpster; 2001; Li et al. 2010). Strawberries are grown in all provinces of Canada. Agriculture and Agri-Food Canada reported in 2013 that Quebec and Ontario have the greatest acreages, 1,758 hectares (ha) or 48% of the national acreage and 933 ha (25% of the national acreage), respectively. Nevertheless, a recent study indicated that 56% of edible strawberries grown in Ontario, by volume, are wasted through the course of the supply chain. The percentage estimates of strawberries wasted at each stage are: 15% during agricultural production; 11% during post-harvest handling and distribution; 9% during retail; and 35% during consumption (Siu 2014). The cell-wall disassembly and the reduction of cell to cell adhesion, as result of middle lamella dissolution are the main factors that cause fruit softening (Brummell and Harpster 2001; Brummell 2006). The significant modifications in cell-wall structure leading to loss of firmness includes the depolymerization of matrix glycans, the

solubilization and/or depolymerization of pectins, and the loss of neutral sugars from sidechains of pectins (Brummell 2006; Goulão and Oliveira 2008).

Biochemical and genetic studies of fruit softening indicated that cell-wall biosynthesis and degradation is the result of coordinated expression of several families of genes encoding cell-wall metabolism proteins, such as expansins, pectin methylesterases, polygalacturonases, pectate lyases, β -galactosidases, α -L-arabinofuranosidases, endo-(1,4)- β -D-glucanases, β -xyloxidases, xyloglucanases, endotransglucosylases, endo-mannanases, and phospholipase D (PLD) (Benítez-Burraco et al. 2003; Redondo-Nevado et al. 2001; Salentijn et al. 2003; Trainotti et al. 2001; Tiwari and Paliyath 2011). For instance, N-glycan processing enzymes, including α -mannosidase and β -D-N-acetylhexosaminidase, act by breaking the glycosidic bonds between carbohydrates, or between carbohydrates and non-carbohydrate structural molecules (Brummell and Harpster 2001). Pectate lyases (PLs) operate by catalyzing the cleavage of glycosidic bounds of unsaturated regions of pectins by a β -elimination reaction (Marín-Rodríguez et al. 2002). Expansins facilitate cell-wall loosening through disruption of non-covalent bonds between matrix glycans and cellulose microfibrils (Rose et al. 1997; Rose and Bennett 1999; Brummell and Harpster 2001). Finally, phospholipase D causes membrane deterioration through catalyzing the hydrolysis of phospholipids, which maintain cell viability and homeostasis into phosphatidic acid (Dawidowicz 1987; Exton 1997). Suppressing such enzymes in fruit tissues considerably reduces softening rate, improve fruit quality, and extend shelf life (Brummell et al. 1999; Sesmero et al. 2007, 2009; Meli et al. 2010). It was found that a primary alcohol such as hexanol, and an aldehyde such as hexanal were potent inhibitors of PLD activity (Paliyath et al. 1999). Hexanal was proven to be a more potent inhibitor, apparently due

to the lack of hydroxyl group that may interrupt the complete hydrolysis of substrate-enzyme intermediate. Hexanal is a naturally occurring compound and is also considered as a GRAS (generally regarded as safe) compound for food use. PLD inhibition technologies for the postharvest preservation of fruits and vegetables (US Patent # 6,514,914; 7,198,811) were developed based on this property (Paliyath et al. 1999). Hexanal treatment showed promising results in enhancing shelf life of several fruits, such as apple, banana, cherry, peach, strawberry, vegetables such as broccoli, tomato, and several fresh-cut vegetables, and flowers such as carnation and rose (Paliyath and Subramanian 2008). Usually, hexanal is applied as a vapour, spray-, or dip treatment in specialized formulations containing antioxidants such as α -tocopherol and ascorbic acid (Paliyath and Subramanian 2008). Hexanal treatment offers several advantages over 1-MCP treatment as it does not impair color and flavour development while delaying senescence in apple and tomato fruits (Kondo et al. 2005; Cliff et al. 2009).

In contrast to climacteric fruits, considerably less is known about the hormonal control of ripening in non-climacteric fruits such as strawberry (Adams-Phillips et al. 2004). There is some evidence implicating auxin, gibberellins (GA) and abscisic acid (ABA), but much of this evidence relies on hormones quantification during fruit development or exogenous hormone application studies (Manning 1993; Fait et al. 2008; Symons et al. 2012).

Strawberry cultivars can vary in the deterioration rate of their fruit quality traits during shelf life storage in which loss-of-firmness was placed in the top of such characters. In this study, two strawberry cultivars 'Jewel' and 'Wendy' that vary in their shelf life longevity were characterized. Physiological traits such as fruit firmness, weight, acidity, fruit size and TSS were evaluated during storage at 4°C. To determine any potential hormonal regulation of pre-harvest

shelf life characteristics, fruit quality traits were also assessed in fruit pre-treated with auxin and ABA, the major two hormones contributing to strawberry fruit development and ripening (Chen et al. 2011; Jia et al. 2011). Moreover, the potential role of hexanal, as an inhibitor of phospholipase D, in increasing the shelf life of fruits was assessed. Further, relative transcript abundance levels of a set of 21 genes putatively encoded proteins associated with cell wall degradation enzymes were evaluated along with metabolite production in order to correlate gene expression and physiological responses of key pathways during developmental stages of the two distinguishable strawberry varieties.

Material and Methods

Plant material and postharvest treatments

Fruits were harvested from two strawberry (*Fragaria X ananassa*) cultivars, 'Jewel' and 'Wendy', grown under field conditions (Guelph, ON, Canada). These two varieties were chosen based on their shelf life characteristics, intermediate and excellent, respectively. For molecular studies, fruit were selected at six developmental stages (Fig. 1): small green; SG (green receptacle and green achenes), large green; LG (green receptacle with enlarged green achenes), small white; SW (small white receptacle and green achenes); large white; LW (white receptacle with some rosy spots and brown achenes); pink; P (colour starting to appear on the receptacle), and red; R (ripe red receptacle). The experiment was carried out in a completely randomized block design with three biological and six technical replicates per cultivar. Each block was divided in half, and plants in one half were sprayed with hexanal formulation or hormone while the other half was sprayed with water (control). Stock hexanal formulation consisted of hexanal (1% vol/vol), geraniol (1%), α -tocopherol (1% wt/vol), ascorbic acid (1% wt/vol), cinnamic acid (0.1% wt/vol) tween 20 (10% vol/vol) and ethanol (10% vol/vol).

To evaluate the physiological and molecular effects of hexanal and other hormones in strawberry fruit quality and shelf-life longevity, fruit from both cultivars were subjected to different pre-harvest treatments, as follows: two different hexanal formulations, EFF1 (0.01% v/v hexanal) and EFF2 (0.02% v/v hexanal), as described in Misran et al. (2015), 1-naphthalene acetic acid (NAA; 200mg/L), and abscisic acid (ABA; 200 μ M). All treatments were applied at 7 days and 3 days before commercial harvest.

Fruit shelf-life assessment

To determine shelf life characteristics, fruit of similar size and developmental stage from both cultivars were harvested at commercial maturity. After harvest, fruits were washed in tap water, and randomly divided into groups of six fruit that were placed into small boxes and stored at 4°C. Fruits were then evaluated at 0, 3, 6 and 9 days postharvest. Several physical and chemical strawberry fruit quality attributes, such as firmness, total soluble solids (TSS), titratable acidity (TA), fruit size and weight, and visual inspection of fruit decay were evaluated on samples that were retrieved at 0, 3, 6 and 9 days postharvest. After assessing physiological characteristics, all samples (20 fruit/replicate; three independent biological replicates) were frozen in liquid N₂ and stored at -80°C for further analysis. TSS (%) of the strawberry juice was measured with a digital refractometer (PR-32 α Palette; Atago, Japan). For titratable acidity evaluation, 2 mL of fruit juice in 50 mL dH₂O was titrated with 0.1 M NaOH to pH 8.2, expressed as percentage of citric acid (%). Firmness of intact fruit (N) was determined using a digital penetrometer equipped with a 2 mm diameter flat probe (FHT200, Exttech Instruments, USA). Loss of fruit weight was calculated as % of the initial fruit weight at harvest.

Nucleic acid extraction and qPCR assays

Total RNA extraction, DNase treatment and cDNA synthesis were performed as described previously (El-Sharkawy et al. 2014). Quantitative reverse transcription PCR (qRT-PCR) was conducted for 21 genes chosen to represent a variety of biological functions during the ripening and different developmental stages (Table 1). Gene-specific primers were designed

using Primer Express (v3.0, Applied Biosystems, Carlsbad, CA, USA), (Table 1). Two micrograms of total RNA were treated with DNaseI (Invitrogen, Burlington, ON, Canada) prior to cDNA synthesis using Superscript II reverse transcriptase (Invitrogen, Canada). PCR reactions were performed in 10 μ L, containing SYBR Green master mix [0.2 mM dNTPs, 0.3U Platinum Taq Polymerase (Invitrogen), 0.25 $\times$ SYBR Green, and 0.1 $\times$ ROX], 20 ng of cDNA and 300 nM of each primer. Three biological and three technical replicates for each reaction were analysed on a CFX96 Real-Time PCR Detection System (BioRad, Mississauga, ON, Canada) with a first step of 95°C for 2 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Melting curves were generated using the following program: 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s. Specific primers for β -actin (*FvAct*) were used as the internal control for the normalization of the RNA steady-state level, and the relative changes in gene expression were quantified using the 2 $^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). Strawberry β -actin (*FvAct*), GenBank XP_004309498) was used as a reliable internal reference gene for qPCR, since expression of this gene did not show significant differences between different treatments or developmental stages.

Statistical analysis

Data was collected for analysis of fruit quality parameters and was analyzed using repeated measures ANOVA in SAS (SAS Version 9.4, SAS Institute, Raleigh, NC) using the PROC GLIMMIX (Generalized Linear Mixed Model) procedure. An F test was used to test the equality of the variance of the fixed effects and a likelihood ratio test to validate the significance of the random effects. An identity link function and restricted maximum likelihood estimation were used under the GLIMMIX model. Based on the data

obtained, a suitable distribution function was fit for the analysis. The distribution function was selected based on fit statistic values (lower AICC value). The spatial power (sp (pow)) covariance type was used for most of the analyses such as firmness, total soluble solids, titratable acidity, fruit weight and size, as was able to account for the unequal spacing of time measures, homogenous error variances and an exponential decline in correlation over time. Error assumptions of the variance analysis (random, homogenous, normal distribution of error) were tested using the studentized residual plots and the Shapiro-Wilk normality test. Means for the analyses was determined using the LSMEANS statement and means separation conducted using Tukey-Kramer adjusted multiple means comparison test. Outliers were analyzed using the studentized residuals to check for deviation by more than ± 3.4 . The type 1 error rate was set at 0.05 and 0.01 for all statistical comparisons. **Results**

Physicochemical characteristics

Several fruit quality and physiological variables were evaluated to determine the effect of the hexanal and the hormones on both cultivars. As a result of EFF treatment, 'Wendy' exhibited a higher firmness (1.65N-1.75 N) than 'Jewel' (1.15N-1.30 N) and their loss of firmness was gradual while control fruit exhibited a steady reduction in fruit firmness with the progression in storage time. In 'Wendy' and 'Jewel', control (untreated) fruit exhibited a constant reduction in fruit firmness in storage (~32% and 39% by the end of storage, respectively). ABA treated fruit showed similar reductions in firmness to the control fruit (~35% and 38% by the end of storage for 'Wendy' and 'Jewel', respectively). However, more stability in firmness throughout the storage period was observed in EFF1 (~6% and 9%), EFF2 (~5% and 11%), and auxin (~9% and 7%), respectively. No significant difference in the TSS content was observed due to the exogenous applications as both cultivars exhibited constant increase in TSS levels throughout

the storage period. Interestingly, hexanal application significantly slowed down the reduction in TA levels during storage (Table 2). At the end of storage period (day 9) mature fruits in both varieties had a significantly higher TA levels than control fruits (Table 2).

Strawberry fruit has a short postharvest life and the main firmness reduction occurred between the LG and R stages. During storage period, only control and ABA-treated fruit exhibited significant rotting resulting in high loss of fruit (Table 2). The ripening date of both cultivars in this study did not considerably change due to treatments, excluding NAA- and ABA-treated fruit that showed significant delay (~7 days) and acceleration (~5 days) in ripening process, respectively (Fig. 2).

The firmness values records started at the day of harvest and continued through the ninth day of storage. The two hexanal formulations and auxin applications significantly enhanced the fruit firmness in both cultivars (Table 2). Hexanal-treated fruit had significantly higher firmness values during the period from harvest to the ninth day of storage compared to the control fruit (Table 2). Over the nine days of storage, the firmness of 'Jewel' declined from 0.90 N to 0.17 N for control, compared to 1.30 N to 0.80 N for EFF1. Table 2 also shows that NAA treated fruit exhibited significantly higher fruit firmness than control fruit as storage time progressed. EFF1 and NAA treatments caused a significant increase in fruit size ('Jewel': 13% and 32%; 'Wendy': 15% and 27%, respectively) and weight ('Jewel': 32% and 92%; 'Wendy': 25% and 64%, respectively). In contrast, 'Jewel' and 'Wendy' ABA-treated fruit exhibited a reduction in fruit size (18% and 12%, respectively) and weight (37% and 23%, respectively).

Gene expression during developmental stages of strawberry fruit ripening

Quantitative reverse transcription PCR (qRT-PCR) was conducted for 21 genes encoding cell wall modifying enzymes. Seventeen of these genes represent four groups involved in the remodelling of plant cell wall: N-glycoprotein processing enzymes, phospholipase D (PLD), expansin and pectin esterases. In order to develop a comprehensive picture of the transcriptional response that occurs during fruit ripening, a gene representative to the following three groups was also included in the transcript profile analysis; growth regulated related genes, ascorbate oxidase, and aquaporin. Expansin, PLD1, PME1a, XTH1 and Cel2 showed a steady and dramatic increase in their transcript levels during fruit development, while alpha galactosidase (α Gal, Ascorbate Oxidase, PE6 and 4 members of α -Mannosidase gene family (α -MA1a, α -MA1b, α -MA3 and α -MA4) exhibited a significant decrease in their expression (Fig. 3). PLD2 and XTH2 genes had a very low and steady transcript level at the first four stages of fruit development then increased only in the pink and red stages. The transcript levels of α -MA2a and β -Hex2 were almost undetectable at any of the developmental stages. This expression pattern was similar in both cultivars with more prominent and higher amplitude in 'Wendy'.

Changes in gene expression during days post-harvest storage

To understand the regulation of fruit softening during shelf-life, the transcription levels of these genes were assessed during post-harvest storage. The heatmap cluster analysis (Fig. 4) shows distinct temporal patterns of expression, and functional classification of those genes implied molecular processes that coincide with the progression of the storage period. For instance, the

expression profile indicated a strong induction of the genes belonging to the α -mannosidase gene family (α -MA1a, α -MA1b, α -MA2a, α -MA2b, α -MA3 and α -MA4), two phospholipase D genes (PLD1 and PLD2), the two β -D-N-acetylhexosaminidase (β -Hex1 and β -Hex2) as well as the ASR and SHP genes with a maximum expression level at 6 and 9 days postharvest (Fig.4). This expression pattern was similar in both cultivars with significantly higher levels in Wendy. In contrast, the transcript level of ascorbate oxidase and PME1a genes declined as the days in storage increased (days 6 and 9). Some genes showed very low expression right after harvest (day 0) and then increased gradually to reach their maximum level at day 9 postharvest. This pattern was common in the four xyloglucan transglucosylase family (XTH1, XTH2, α Gal and Cel2) as well as PE6 and aquaporin (PIP2.7), (Fig.4). The expansin was the only gene in this study which showed a relative abundance peak at day 3 and day 6 then declined at day 9 postharvest. The expression patterns of all the transcripts had the same trend in both cultivars but the levels were much lower in 'Jewel'.

Hexanal application modulates gene expression

In order to strengthen understanding of the independent and mutual contributions of hexanal in coordinating fruit ripening, fruit treated with EFF1 (data not shown) or EFF2 were analysed for the expression of selected genes. Gene expression was studied at various days post-harvest - at 3, 6 and 9 days after storage. The most striking and prominent effect of hexanal was observed in 'Wendy' especially with EFF2. Thus, we focused our results only on the effect of EFF2. Based on the gene expression patterns, we classified the genes into three main groups. Group-1 was comprised of genes that were downregulated by hexanal and this group has 10

genes as follows: 5 members of α -Mannosidase gene family (α -MA1a, α -MA2a, α -MA2b, α -MA3 and α -MA4), two members of the acetylhexosaminidase (β -Hex1 and β -Hex2), PLD1, α -Gal and Ascorbate oxidase. Group-2 included five genes that expressed in hexanal dependent manner (PIP2.7, PME1a, PLD2, Expansin, and XTH2). The detailed expression pattern of these five genes was shown in the supplemental information in Fig.S1. The third group contains all transcripts with no visible response to hexanal application, including the two growth hormone regulated genes (ASR and SHP) as well as α -MA1b, Cel2, XTH1 and PE6. The global gene expression pattern is presented in Fig. 5. In view of the natural variations between 'Jewel' and 'Wendy' in terms of firmness, ripening behavior and fruit shelf-life characteristics, the question was raised whether the prolonged shelf life in 'Wendy' is partially due to inadequate regulatory mechanism to coordinate gene expression involved in cell wall degradation. To determine this regulatory mechanism(s), the expression of the different cell wall associated genes (Group-1) was quantified in both cultivars (Fig. 6). Clear differences between the two cultivars in the downregulation of nine genes belonging to group-1 were detected. For instance, EFF2 reduced the expression by 71% and 76% in β -Hex1 and β -Hex2 respectively in 'Wendy' while both genes were expressed at basal constants level in 'Jewel' (Fig. 6). Among all studied transcripts in this group1, only α -MA2a showed an average transcription reduction by 58 % and 77 % in 'Jewel' and 'Wendy', respectively (Fig. 6), indicating that the effect of hexanal on gene expression may be cultivar-dependent.

Altered gene expression by either auxin or ABA

To understand the postharvest regulation of fruit softening and determine the role of exogenous hormone, gene expression was assessed during the shelf life phase after the application of either NAA or ABA. Cell wall metabolism genes such as α -mannosidase (α -MA1a, α -MA1b, α -MA2b, α -MA2b, α -MA3 and α -MA4) and β -D-N-acetylhexosaminidase (Hex1 and Hex2) were downregulated by ABA but their transcript levels did not response to NAA application (Fig. 7). The expression level of ASR gene did not alter by NAA application but increased by ABA. The contrary effect of the two hormones was evident for some genes such as PE6 and XTH1 (Fig. 7). The transcript level of PE6 gene was increased by NAA and reduced by ABA while the expression of XTH1 had an opposite pattern. However, expression of α Gal, PLD2 and XTH2 showed a continuous increase in their transcript levels by ABA and NAA. In contrast, both hormones caused a reduction in the expression levels of PME1a, AO, Expansin, PiP2.7 and Cel2 genes (Fig. 7). PLD1 and SHP appeared to be insensitive to both hormones as their expression was not altered in response to the exogenous application.

Discussion

Ripening-related cell wall metabolism and associated textural changes have been a major focus of ripening and it was mostly studied in the climacteric fruits (Almeida et al. 2007; Gapper et al. 2014). Strawberry is a non-climacteric fruit, with a short postharvest life. In the absence of a climacteric rise in respiration the development of the strawberry receptacle can be envisaged as a continuous process, encompassing overlapping phases of cell division, expansion, and senescence. As it turns red the receptacle becomes obviously senescent, easily damaged mechanically and as a result, shows little resistance to pathogen attack (Amil-Ruiz et al. 2011).

Yet the biochemical basis of cell wall degradation in strawberry fruit has not been clearly understood. Hexanal treatment showed promising results in enhancing shelf life of several fruits by reducing membrane degradation initiated by PLDs, (Paliyath and Subramanian 2008). In the present study, we used two strawberry cultivars with different levels of firmness. Despite these differences, the two cultivars responded similarly to hexanal or hormone application. Hexanal (EFF1 or EFF2) and auxin applications were able to reduce the loss of firmness and thus increase the shelf-life in both cultivars while ABA did not show any effect on firmness or shelf-life. None of our treatments (hexanal or hormones) had an effect on the sugar content and had only a minor effect on the acidity and that too only after 9 days post-harvest. These results suggest that the treatments are not having any adverse effects on the sugar/acid content as well as the general quality of the fruits.

Previous studies have reported that strawberry cultivars can vary in their firmness and softening due to their differences in timing of polysaccharide solubilisation and depolymerization, (Salentijn et al. 2003, Rosli et al. 2004). In the present study, the expression and regulation of 21 genes encoding divergent classes of cell wall proteins that contribute to fruit cell wall metabolism were analysed.

The chief textural changes resulting in fruit softening are due to enzyme-mediated alterations in the structure and composition of cell walls that lead to partial or complete solubilisation, de-esterification, and depolymerization of cell-wall polysaccharides, accompanied by a loss of neutral sugars and galacturonic acid (Brummell and Harpster 2001; Rosli et al. 2004). Cell wall degradation is an inherent part of fruit ripening, and involves the degradation of cellulose, pectin and other carbohydrate polymers involved in the cell wall architecture. Excessive

degradation of cell wall can result in the loss of fruit quality due to loss of firmness. Phospholipase D is a key enzyme in the membrane deterioration pathway (Paliyath and Droillard 1992). PLD initiates the phospholipid breakdown by removing the head group of phospholipids, and other enzymes that act downstream such as phosphatidic acid (PA) and lipolytic acyl hydrolase act in tandem with PLD. None of the downstream enzymes can directly cause the catabolism of phospholipids. Thus, by inhibiting PLD, the entire catabolic pathway can be slowed down. The discovery of hexanal as a PLD inhibitor and its potential to block the hydrolysis at the active site of PLD may be a key mechanism that could help in reducing membrane deterioration. These effects suggest that hexanal is capable of influencing the gene expression. Thus, the present study was an attempt to understand the differences in the molecular mechanism of action between hexanal, and hormones with known molecular regulatory effects on expression of genes involved in membrane integrity. As expected, the transcript levels of the two PLD genes declined after the hexanal treatment supporting their role on the membrane deterioration process. Multiple members of N-glycan processing enzymes (eg. α -mannosidase and β -D-N-acetylhexosaminidase) showed a significant reduction in their transcript abundance after hexanal and auxin treatments suggesting they are negative regulators of membrane degradation. Although the role of individual N-glycan enzymes are unknown, Meli et al. (2010) have showed that the suppression of two ripening-specific N-glycoprotein modifying enzymes, α -mannosidase (α -Man) and β -D-N-acetylhexosaminidase (β -Hex) in tomato enhances fruit shelf life, owing to the reduced rate of softening. However, N-glycan processing enzymes remain an active area of investigation. Genes encoding enzymes expected to be involved in cell wall degradation such as PMEs, expansin, alpha-galactosidase

and xyloglucan endotransglucosylase were upregulated during ripening, however their regulation by hexanal or hormones (NAA and ABA) vary and cannot be generalized between gene family members. In strawberry, seven expansins have been identified, two of them are fruit specific and their expression increases during ripening (Dotto et al. 2006). Xyloglucan endotransglucosylases/hydrolases (XTHs) are cell wall modification enzymes that are thought to be involved in disassembly of the cellulose-xyloglucan matrix by cleaving the xyloglucan β -D-glucan backbones and then linking xyloglucan segments into them to loosen the cross-links between cellulose (Eklof and Brumer 2010). Some XTHs members family have been shown to act as transglucosylases (xyloglucan endotransglucosylase (XET)), catalyzing the transfer of a xyloglucan molecule fragment to another xyloglucan molecule. Some, other *XTHs* act preferentially as hydrolases (xyloglucan endohydrolase (XEH)), which hydrolyzes one xyloglucan molecule, while some of the *XTH* proteins present both activities (Nishitani, 1997; Rose et al. 2002; Fry 2004). Two ripening-related genes regulated by hormones ASR (Absciscic acid stress ripening) and SHP (Shatterproof-like) were also analysed in this study. ASR proteins have been reported to act as a downstream component involved in ABA signal transduction and Chen et al. (2011) indicated that *FaASR* may be involved in strawberry fruit ripening. It has been shown that C-type MADS-box genes like *SHATTERPROOF* may act as modulators of ripening in strawberry (Daminato et al. 2013). The expression of these two genes responded as expected, in an antagonistic manner to the hormones supporting their role in ripening and regulated by either auxin or ABA. For ripening to start, the auxin content of the strawberry fleshy receptacle must decrease (Given et al. 1988). Ripening-related processes that are sensitive to auxin comprise softening, change of colour, and the formation of aroma (Aharoni and O'Connell

2002). Concomitant with the start of ripening, ABA is synthesized and contributes to the control of the process (Chen et al. 2011; Jia et al. 2011). Therefore, the two hormones are not significantly overlapping in their control of ripening, which is negative for auxin and positive for ABA. On the contrary, their repressed expression in this study remains an active area of investigation. Although these data suggest that post-harvest technologies based on hexanal appear to offer great promise for enhancing the shelf life and quality in fruits, the molecular basis of its mode of action require further studies.

References

- Adams-Phillips, L., Barry, C., and Giovannoni, J. 2004. Signal transduction systems regulating fruit ripening. *Trends in Plant Sci.* **9**: 331-338.
- Aharoni, A., and O'Connell, A. 2002. Gene expression analysis of strawberry achene and receptacle maturation using DNA microarrays. *J. Exp. Bot.* **53**: 2073–2087.
- Almeida, J.R.M., D'Amico, E., Preuss, A., Carbone, F., de Vos, R.C.H, Deiml, B., Mourgues, F., Perrotta, G., Fischer, T.C., and Bovy, A.G. 2007. Characterization of major enzymes and genes involved in flavonoid and proanthocyanidin biosynthesis during fruit development in strawberry (*Fragaria ×ananassa*). *Arch Biochem. Biophys.* **465**: 61–71.
- Amil-Ruiz, F., Blanco-Portales, R., Muñoz-Blanco, J., and Caballero, J.L. 2011. Strawberry plant defense mechanism: a molecular review. *Plant Cell Physiol.* **52**:1873-1903.
- Benítez-Burraco, A., Blanco-Portales, R., Redondo-Nevado, J., Bellido, M.L., Moyano, E., Caballero, J.L., and Munoz-Blanco, J. 2003. Cloning and characterization of two ripening-related strawberry (*Fragaria×ananassa* cv. Chandler) pectate lyase genes. *J Exp. Bot.* **54**: 633–645.
- Brummell, D.A, Harpster, M.H., Civello, P.M., Palys, J.M., Bennett, A.B., and 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.
- Brummel, D.A., and Harpster, M.H. 2001. Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. *Plant Mol. Biol.* **47**: 311-340.

Brummell, D. A. 2006. Cell wall disassembly in ripening fruit. *Functional Plant Biology* **33**: 103-119.

Chen, F., Liu, H., and Yang, H. 2011. Quality attributes and cell wall properties of strawberries (*Fragaria ananassa* Duch.) under calcium chloride treatment. *Food Chemistry* **126**: 450–459.

Cliff, M., Lok, S., Lu, C., and Toivonen, P.M.A. 2009. Effect of 1-methylcyclopropene on the sensory, visual, and analytical quality of greenhouse tomatoes, *Postharvest Biol. Technol.* **53**: 11-15.

Daminato, M., Guzzo, F., and Casadoro, G. 2013. A SHATTERPROOF-like gene controls ripening in non-climacteric strawberries, and auxin and abscissic acid antagonistically affect its expression. *J. Exp. Bot.* **12**: 3775–3786.

Dawidowicz, E.A. 1987. Dynamics of membrane lipid metabolism and turnover. *Annu. Rev. Biochem.* **56**: 43–61.

Dotto, M.C., Martínez, G.A., and Civello, P.M. 2006. Expression of expansin genes in strawberry varieties with contrasting fruit firmness. *Plant Physiol. Biochem.* **44**:301–307.

El-Sharkawy, I., Sherif, S., Jones, B., Mila, I., Kumar, P.P., Bouzayen, M., and Jayasankar, S. 2014. TIR1-like auxin-receptors are involved in the regulation of plum fruit development. *J. Exp. Bot.* **65**: 5205–5215.

Eklof, J.M., and Brumer, H. 2010. The XTH gene family: an update on enzyme structure, function, and phylogeny in xyloglucan remodeling. *Plant Physiol.* **2**:456–66.

Exton, J.H. 1997. Phosphoipase D: enzymology mechanisms of regulation, and function. *Physiol. Rev.* **77**: 303–320.

Fait, A., Hanhineva, K., Beleggia, R., Dai, N., Rogachev, I., Nikiforova, V.J., Fernie, A.R. and Aharoni, A. 2008. Reconfiguration of the achene and receptacle metabolic networks during strawberry fruit development. *Plant physiol.* **2**: 730-750.

Fry, S.C. 2004. Primary cell wall metabolism: tracking the careers of wall polymers in living plant cells. *New Phytol.* **161**: 641–675.

Gapper, N. E., Giovannoni, J. J., and Watkins, C. B. 2014. Understanding development and ripening of fruit crops in an ‘omics’ era. *Hortic. Res.* **1** 14034 10.1038/hortres.2014.34.

Given, N.K., Venis, M.A., and Grierson, D. 1988. Hormonal regulation of ripening in the strawberry, a non-climacteric fruit. *Planta* **174**: 402–406.

Goulão, L.F., and Oliveira, C.M. 2008. Cell wall modification during fruit ripening: when a fruit is not the fruit. *Trends Food Sci. Tech.* **19**: 4-25.

Jia, H.F., Chai, Y.M., Li, C.L., Lu, D., Luo, J.J., Qin, L., and Shen, Y.Y. 2011. Absciscic acid plays an important role in the regulation of strawberry fruit ripening. *Plant Physiol.* **157**: 188–199.

Kondo, S., Setha S., Rudell, D.R., Buchanan, D.A., and Mattheis, J.P. 2005. Aroma volatile biosynthesis in apples affected by 1-MCP and methyl jasmonate, *Postharvest Biol. Technol.* **36**: 61-68.

Li, X., Xu, C., Korban, S.S., and Chen, K. 2010. Regulatory mechanisms of textural changes in ripening fruits. *Crit. Rev. Plant Sci.* **29**: 222–243.

Livak, K.J., and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* **25**:402-408.

Manning, K. 1993. Soft fruits In: Taylor JE, Tucker GA, eds. *Biochemistry of fruit ripening*. Cambridge, UK: Chapman and Hall, 347-373.

Marín-Rodríguez, M.C., Orchard, J., and Seymour, G.B. 2002. Pectate lyases, cell wall degradation and fruit softening. *J. of Exp. Bot.* **53**: 2115-2119.

Meli, V.S., Ghosh, S., Prabha, T.N., Chakraborty, N., Chakraborty, S. and Datta, A. 2010. Enhancement of fruit shelf life by suppressing *N*-glycan processing enzymes. *Proc. Nat. Acad. Sci. U.S.A.* **107**: 2413–2418.

Misran A, Padmanabhan P, Sullivan JA, Khanizadeh S, Paliyath G. 2015. Composition of phenolics and volatiles in strawberry cultivars and influence of preharvest hexanal treatment on their profiles. *Can J Plant Sci* **95**: 115-126.

Nishitani, K. 1997. The role of endoxyloglucan transferase in the organization of plant cell walls. *Int. Rev Cytol.* **173**:157–206.

Paliyath, G., and Droillard, M. J. 1992. The mechanism of membrane deterioration and disassembly during senescence. *Plant Physiol. Biochem.* **30**: 789-812.

Paliyath, G., Pinhero, R.G., Yada, R.Y., and Murr, D.P. 1999. Effect of processing conditions

on phospholipase D activity of corn kernel subcellular fractions, J. Agric. Food Chem. **47**: 2579-2588.

Paliyath, G., and Subramanian, J. 2008. Phospholipase D inhibition technology for enhancing shelf life and quality. G. Paliyath, D.P. Murr, A.K. Handa, S. Lurie (Eds.), pages 240-245. Postharvest Biology and Technology of Fruits, Vegetables, and Flowers (1st ed.), Wiley-Blackwell, USA.

Perkins-Veazie, P. 1995. Growth and ripening of strawberry fruit. Horticultural Reviews **17**: 267–297.

Redondo-Nevado, J., Moyano, E., Medina-Escobar, N., Caballero, J.L., and Muñoz-Blanco, J. 2001. A fruit-specific and developmentally regulated endopolygalacturonase gene from strawberry (*Fragaria × ananassa* cv. Chandler). J. Exp. Bot. **52**: 1941–1945.

Rose, J.K.C., Lee, H.H., and Bennett, A.B. 1997. Expression of a divergent expansin gene is fruit specific and ripening-regulated. Proc. Natl. Acad. Sci. U. S. A. **94**: 5955– 5960.

Rose, J.K.C., and Bennett, A.B. 1999. Cooperative disassembly of the cellulose– xyloglucan network of plant cell walls: parallels between cell expansion and fruit ripening. Trends Plant Sci. **4**: 176–183.

Rose, J.K.C., Braam, J., Fry, S.C., and Nishitani, K. 2002. The XTH family of enzymes involved in xyloglucan endotransglucosylation and endohydrolysis: current perspectives and a new unifying nomenclature. Plant and Cell Physiol. **43**:1421–1435.

Rosli, H.G., Civello, P.M., and Martinez, G.A. 2004. Changes in cell wall composition of three *Fragaria* × *ananassa* cultivars with different softening rate during ripening. *Plant Physiol. and Biochem.* **42**: 823–831.

Salentijn, E.M.J., Aharoni A., Schaart J.G., Boone, M.J., and Krens F.A. 2003. Differential gene expression analysis of strawberry cultivars that differ in fruit-firmness. *Physiol. Planta.* **118**: 571–578.

Sesmero, R., Quesada, M.A., and Mercado, J.A. 2007. Antisense inhibition of pectate lyase gene expression in strawberry fruit: Characteristics of fruits processed into jam. *J. Food Eng.* **79**: 194–199.

Sesmero, R., Mitchell, J.R., Mercado, J.A., and Quesada, M.A. 2009. Rheological characterisation of juices obtained from transgenic pectate lyase-silenced strawberry fruits. *Food Chem.* **116**: 426–432.

Siu A. (2014). An analysis of food waste in Ontario's domestic fresh strawberry supply chain (Master's thesis). Retrieved from <https://yorkspace.library.yorku.ca/xmlui/handle/10315/30195>

Symons, G.M., Chua, Y.J., Ross, J.J., Quittenden, L.J., Davies, N.W., and Reid, J.B. 2012. Hormonal changes during non-climacteric ripening in strawberry. *J Exp. Bot.* **63**: 4741–4750.

Tiwari, K., and Paliyath, G. 2011. Microarray analysis of ripening-regulated gene expression and its modulation by 1-MCP and hexanal. *Plant Physiol. Biochem.* **49**: 329–340.

Trainotti, L., Spinello, R., Piovan, A., Spolaore, S., and Casadoro, G. 2001. beta-Galactosidases with a lectin-like domain are expressed in strawberry. *J Exp. Bot.* **52**: 1635–1645.

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Figure 1: Strawberry fruit developmental stages.

Figure 2: Effect of different treatments in fruit size.

Figure 3: Cluster heatmap for gene expression during developmental stages.

Figure 4: Cluster heatmap for gene expression during days post-harvest.

Figure 5: Cluster heatmap for gene expression in response to EFF2 application.

Figure 6: Relative expression of genes that exhibited highly significant changes in their expression pattern after EFF2 application.

Figure 7: Cluster heatmap for gene expression after ABA or NAA application.

Table 1. Genes and gene specific primers used for qRT-PCR in this study

Primer name	Sequence	Accession number	Gene
FaMa.1a(F)	CAATGTGCCTGATCGTGTT	XP_004297489.1	α -mannosidase
FaMa.1a(R)	CTGTTCCCATGGTCCACAT		
FaMa.1b(F)	ACGAACAGCAGAAGCTTGGT	XP_011463212.1	
FaMa.1b(R)	ACCGTAGGAATCCACCACAC		
FaMa.2a(F)	CGGAGCACATTTAGGCAAT	XP_004306319.1	
FaMa.2a(R)	GCTGAAACCAAGTGGTGGT		
FaMa.2b(F)	GCCAATGATTACGCAAAAAG	XP_011468215.1	
FaMa.2b(R)	CCAGAAGCAGACTCAACCAA		
FaMa.3(F)	AGCACAGCGAAAGGGAGATA	XP_011462234.1	
FaMa.3(R)	AAGGCATTGGCAAAAATCTG		
FaMa.4(F)	GCACAGCGAAAGGGAGATA	XP_011462239.1	
FaMa.4(R)	GTGACGAGGAAAGGCATTG		
FaHex1(F)	AGTACTCTCCCGCGGATGT	XP_004307561.1	beta-hexosaminidase 1
FaHex1(R)	TATGGCCAGGTGAGTCGAT	XP_004301810.1	beta-hexosaminidase 2
FaHex2(F)	CTTTTCCACTTGGGTGGTG		
FaHex2(R)	GCCTTTGGCAGTCATGTTT		
FaASR-F	CAGTTGCTGCAGTTGGATCT	JN006160.1	ABSCISIC ACID STRESS RIPENING
FaASR-R	GCCATGAGCCTCTTTTTCTT		
FaSHP-F	CCGTCAAGGACCTCAAGAAT	KC676787.1	SHATTERPROOF-like protein
FaSHP-R	CGATTTTCAGCAAACAGCATT		
Fa-AO-(F)	AACCCACAGGGATCATACCA	XM_004291264.2	L-ascorbate oxidase
Fa-AO-(R)	CCAACACGGAAAACTCCACT		
FaPE6-(F)	GGTTGATTGAGCCACAAGGT	XP_004297462.2	Pectinesterase
FaPE6-(R)	AACCGTAAACTTCCCTGCCT		
FaPMEIa-(F)	CAAATCTGTGGACGAGCTGA	XP_004307821.1	Pectin methylesterase inhibitor
FaPMEIa-(R)	GAACCCATCAAGGCAAGTGT		
FaExpA1-(F)	ACTCTGACCCATTGTTTGC	XM_004304241.2	Expansin
FaExpA1-(R)	CCACAAGCTCCACCCATAGT		
FaPIP2.7-(F)	GCTGTTCTTGGCTCGTAAGG	XP_004290727.1	Aquaporin
FaPIP2.7-(R)	ACACAGCAAACCCAATAGGC		
PLD1-Fw	GGAGTTACCAAGTGAAGGAGAA	AAW83125.1	PhospholipaseD-1
PID1-Rev	GGAGGAAGGTAGTCGGATTTG	XP_004287315.1	PhospholipaseD-2
PLD2-Fw	TGACTCTGGGAGAGCTTCTTA		
PLD2-Rev	CCCATCAAGCCATCTCTCTTT		
FaXTH1-(F)	GGTGACCGTGAACAGAGGAT	GQ367550.1-F	Xyloglucan endotransglucosylase/hydrol
FaXTH1-(R)	TGCTGTTCTTGAACACTCGG		
FaXTH2-(F)	TTGACGTGACGTTTGGTGAT	XM_004303811.2	xyloglucan endotransglucosylase/hydrol
FaXTH2-(R)	ATCTGCATGTCGATCCTTCC		
Fa- α Gal(F)	TTTCCATCTGGAATCAAGGC	XM_004299700.2-F	alpha-galactosidase
Fa- α Gal(R)	TCCTCATGGCCTAGTGATCC		
FaCel2-(F)	CTGCCTTCTTGGCTACCTTG	XM_004301543.2-F	endoglucanase 25
FaCel2-(R)	CTATGGTGACACGTTTTGG		
FaAct(F)	TGACCTTGCTGGACGAGAT	XP_004309498.1	Actin
FaAct(R)	TCACGTACAATTTCCCGTTC		

Table 2 Quality parameter of Jewel and Wendy cultivars fruit after different pre-harvest treatments

Parameter	Treatment	Jewel				Wendy			
		Storage time (d)				Storage time (d)			
		0	3	6	9	0	3	6	9
Firmness (N)	Ctrl.	0.90	0.85	0.18	0.17	1.70	0.95	0.85	0.60
	EFF1	1.30	1.15*	1.00**	0.80**	1.75	1.45**	1.30**	1.18**
	EFF2	1.15	1.10*	0.90**	0.70**	1.65	1.35*	1.30**	1.25**
	NAA	1.10	0.90	0.80**	0.60**	1.60	1.30*	1.25*	1.15**
	ABA	0.88	0.70	0.24	0.15	1.27	0.95	0.85	0.70
TSS (°Brix)	Ctrl.	7.3	7.3	8.0	8.05	6.6	6.85	7.65	8.67
	EFF1	6.7	7.5	7.7	8.15	7.1	7.6	7.85	8.35
	EFF2	6.9	7.2	7.2	7.65	7.1	7.2	7.25	8.0
	NAA	6.7	7.4	8.0	8.3	7.0	7.05	7.25	8.25
	ABA	6.5	6.8	7.9	8.2	6.55	6.60	7.20	8.35
TA (g/100ml citric acid)	Ctrl.	2.13	1.92	1.74	1.49	1.18	1.18	1.12	0.89
	EFF1	2.00	1.73	1.55	1.56*	1.28	1.28	1.28	1.25*
	EFF2	1.94	1.86	1.70	1.63*	1.21	1.20	1.15	1.15
	NAA	2.34	2.13*	1.93*	1.82*	1.58	1.16	1.16	1.03
	ABA	1.82	1.73	1.49*	1.45	1.06	1.06	1.06	1.03
Weight (g)	Ctrl.	25.8	23.9	21.5	20.6	20.2	19.9	18.0	16.1
	EFF1	31.2**	30.8**	28.6**	25.8**	28.8**	26.6**	25.4**	23.2**
	EFF2	30.6*	25.9	24.9*	22.9	24.5*	23.4*	20.1	18.2
	NAA	40.5**	39.1**	38.2**	37.3**	40.7**	39.8**	35.5**	34.7**
	ABA	19.9**	17.8**	15.2**	14.0**	13.7**	12.7**	10.2**	9.4**
Size (mm)	Ctrl.	41.8	39.9	38.7	36.4	37.8	36.7	35.8	35.3
	EFF1	46.8**	44.9**	43.2**	41.4**	43.9**	41.1**	40.2**	39.9**
	EFF2	42.7	41.2	40.9	40.5*	40.4	37.8	36.2	35.8
	NAA	53.4**	52.2**	50.3**	48.4**	52.2**	50.9**	48.0**	47.4**
	ABA	37.6**	35.4**	33.1**	29.5**	30.2**	29.4**	28.9**	28.0**
Decay (%)	Ctrl.	0	13	25	33	0	6	9	12
	EFF1	0	0	0	0	0	0	0	0
	EFF2	0	0	0	0	0	0	0	0
	NAA	0	0	0	7	0	0	0	4
	ABA	0	20*	29*	38**	0	15*	17*	14

Values represent the mean of six replicates. Means followed by asterisks indicates significant differences between control and treatments at the same storage time ($P \leq 0.05$ or 0.01).