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Cellular approach to understand bitter pit development in apple fruit

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ABSTRACT

Bitter pit (BP), a Ca^{2+} deficiency disorder of apple fruit (*Malus domestica*), is a complex process that involves not only the total input of Ca^{2+} into the fruit, but also a proper Ca^{2+} homeostasis at the cellular level. The objective of this study was to test the hypothesis that Ca^{2+} accumulation into storage organelles and binding to the cell wall is associated with BP development in apple fruit. The experiment was carried out on 'Granny Smith' apples stored at 0 °C for 60 d. After storage, fruit were segregated into two lots for analysis, apples with the water-soaked initial visual symptoms of BP and those not showing this symptom. Cytochemical and ultrastructural observations showed an accumulation of Ca^{2+} in the vacuole of individual outer cortical cells of pitted fruit. We also observed an increase in the expression of genes encoding four pectin methylesterases, a greater degree of pectin deesterification and therefore more Ca^{2+} binding sites in the cell wall, and a higher fraction of the total cortical tissue Ca^{2+} content that was bound to the cell wall in pitted fruit compared with non-pitted fruit. Cells of the outer cortical tissue of pitted fruit consistently had higher membrane permeability than outer cortical cells of non-pitted fruit. The results provide evidence that Ca^{2+} accumulation into storage organelles and Ca^{2+} binding to the cell wall represent important contributors to BP development in apple fruit.

1. Introduction

Bitter pit (BP) is an important Ca^{2+} deficiency disorder in apple. It begins with water-soaked symptoms caused by plasma membrane breakdown, followed by tissue disintegration and dehydration, eventually resulting in corky, dark and depressed spots on the fruit surface (Fuller, 1980). Despite many years of research, the mechanisms involved in BP development are still not well understood and a better understanding of these mechanisms is needed to devise more effective control strategies.

Calcium contributes to cellular membrane structure and function by binding to phospholipids and proteins at the membrane surface (Hanson, 1960; Clarkson and Hanson, 1980; Hirschi, 2004). Calcium binding to the membrane also delays phospholipid and monogalactosyldiacylglycerol catabolism, which preserves membrane integrity by reducing senescence-related membrane lipid changes, and by increasing membrane restructuring processes (Picchioni et al., 1996, 1998). The maintenance of proper membrane structure and function has been reported to be dependent on >0.1 mM free Ca^{2+} in the apoplastic pool (Hanson, 1960), whereas cytosolic Ca^{2+} levels are maintained at extremely low concentrations (0.1–0.2 μM) and may not play the same roles (Plieth, 2001).

Bitter pit development in apple fruit is mainly the result of low total Ca^{2+} concentrations in fruit tissue (Fuller, 1980). However, the incidence of BP is not always correlated with total Ca^{2+} content and new hypotheses have pointed to other possible factors involved in BP development (Saure, 1996, 2005). The disorder may also involve an abnormal Ca^{2+} homeostasis in the cells, where a depletion of the free apoplastic Ca^{2+} affects plasma membrane structure and function, eventually leading to BP development. It has been reported that about 40% of the Ca^{2+} in fruit tissue is located in the vacuole, with most of the remaining 60% located in the cell wall (Bangerth, 1979; Harker and Venis, 1991; White and Broadley, 2003). The high vacuolar and cell wall Ca^{2+} contents make them important regulators of Ca^{2+} homeostasis in the cell. Thus, metabolic changes in Ca^{2+} content in these compartments might be related to Ca^{2+} depletion in the apoplastic solution, which can weaken plasma membrane structures, leading to cell death and BP symptoms. These ideas are in agreement with other studies, suggesting that normal cellular metabolism requires an apoplastic pool of free Ca^{2+} directly accessible to the plasma membrane for proper membrane stabilization and function (Hanson, 1960; White and Broadley, 2003). An increase in Ca^{2+} storage in the vacuole and/or binding to the cell wall could explain the fact that, frequently, fruit showing Ca^{2+} deficiency have the same or even higher total Ca^{2+} content as sound fruit grown under the same agronomic conditions (Ferguson and Watkins, 1989; Saure, 2005). In addition, chemical and X-ray analysis have shown that damaged tissues actually have

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higher concentrations of Ca^{2+} than the surrounding healthy tissues (Chamel and Bossy, 1981).

Calcium movement to storage organelles such as the vacuole is accomplished by the activity of Ca-ATPases and Ca^{2+}/proton antiporter (CAX) proteins. Calcium-ATPases use ATP as a source of energy whereas CAXs use the membrane's electrochemical gradient energy to pump Ca^{2+} into storage organelles. Since, the electrochemical gradient across the tonoplast membrane is generated by the vacuolar-ATPase (V-ATPase) and H^+-pyrophosphatase (PPase), these proteins indirectly affect Ca^{2+} movement into the vacuolar compartment. The expression and activity of these proteins represent an important mechanism for Ca^{2+} homeostasis regulation in the cell, since most of the Ca^{2+} in the vacuole forms complexes with phenolics, oxalates, carbonates, and phosphates, which makes Ca^{2+} unavailable for other cellular functions (White and Broadley, 2003). High expression of an *Arabidopsis thaliana* *Ca^{2+}/H^+ exchanger* in tomato plants not only increased the total amount of Ca^{2+} in the fruit, but also increased the incidence of the Ca^{2+} deficiency disorder, blossom-end rot (BER), by 90% (Park et al., 2005). The sCAX1 tomato plant phenotypes, in conjunction with the biochemical properties of sCAX1 in yeast (Hirschi et al., 1996; Hirschi, 1999), suggest that expression of this transporter potentially altered Ca^{2+} homeostasis by increasing organellar and decreasing apoplastic Ca^{2+} pools which may have resulted in higher membrane permeability and Ca^{2+} deficiency development.

Cell wall enzymes can potentially affect the amount of free Ca^{2+} available for normal cell metabolism. Pectin methylesterase (PME) enzymes demethylate pectins, increasing the number of carboxyl groups that can form very strong electrovalent bonds with Ca^{2+} ions (Ralet et al., 2001). Solutions containing pectin and Ca^{2+} can have a ratio of total Ca^{2+} to pectin-bound Ca^{2+} of close to 1.0, depending on the amount of deesterified galacturonosyl carboxyl groups, pH and Ca^{2+} concentration (Tibbits et al., 1998). Increasing PME activity, possibly resulting from increased PME expression, can create new binding sites for Ca^{2+} in the cell wall during storage of apple fruit, reducing the levels of apoplastic free Ca^{2+}, increasing membrane permeability and, eventually, BP incidence. Pectin methylesterases are also involved in cell wall disassembly during fruit ripening. Pectin demethylation caused by PME activity normally precedes and favors the action of other enzymes involved in pectin solubilization/degradation such as endo-polygalacturonases (Endo-PG), exo-polygalacturonases (Exo-PG), β-galactosidases (β-Gal), and pectate lyases (PL) (Massiot et al., 1994; Goulao et al., 2007). In the case of BP, pectin methylesterase expression/activity early in storage may create binding sites for Ca^{2+} in the cell wall, reducing Ca^{2+} availability for normal cell function, eventually triggering local Ca^{2+} deficiency and cell death. Later in storage, when apoplastic free Ca^{2+} content is low, pectin degrading enzymes may release Ca^{2+} into the apoplast and reduce further development of BP. This is in agreement with the usual period of BP development; its appearance is greatest during the first 1–2 months of cold storage. Later, at the time when apple softening occurs, there is an increase in soluble pectins and loss of Ca^{2+} from the pectin-rich middle lamella (Stow, 1993; Goulao and Oliveira, 2008).

Considering that cellular storage organelles and the cell wall have great capacity to retain Ca^{2+}, the objective of this study was to test the hypothesis that Ca^{2+} accumulation into storage organelles and binding to the cell wall are associated with BP development in apple fruit.

2. Materials and methods

Apple fruit (*Malus domestica* cv. Granny Smith) were harvested in a commercial orchard in Stockton, California, divided into four replications of 100 fruit, and stored at 0 °C in the Postharvest Laboratory at the University of California, Davis. After 60 d of storage, each replication had 57 ± 10% BP incidence. The fruit in each replication were segregated into two lots, fruit with and fruit without the initial water-soaked visual symptoms of BP. The experimental design was completely randomized. The initial 100-fruit samples each yielded subsamples of 40 healthy fruit and 40 fruit showing BP symptoms. Thus, there were four replicate samples of 40 healthy fruit and 40 pitted fruit. Skin and outer cortical tissue samples were taken from the calyx-end region of the healthy and pitted fruits. Skin tissue with about 1 mm thickness was obtained by peeling the calyx-end region of each fruit. Cortical tissue with about 3 mm thickness was collected in the same region as the skin tissue. Skin and cortical samples from pitted fruit contained healthy and pitted tissue.

2.1. Gene expression

Total RNA was extracted separately from skin and outer cortical fruit tissue as described in the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The RNA concentration and purity were determined at 260 nm and 260 nm/280 nm, respectively, using a UV spectrophotometer (NanoDrop 2000, Thermo Scientific, Wilmington, DE, USA). For all samples, 3 μg of total RNA was reverse transcribed using SuperScript III (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Quantitative, real-time PCR was then performed with the addition of 1× SYBR green (Applied Biosystem, Foster City, CA, USA) to each sample containing about 100 ng of the synthesized cDNA. The data obtained were normalized based on the expression of the housekeeping apple 18S rRNA (Defilippi et al., 2005). All primers designed were 20 nucleotides long with a melting point temperature of 58 ± 3 °C. Nine putative Ca^{2+}/H^+ exchanger nucleotide sequences were obtained in the UCDavis apple (*Malus domestica*) Expressed Sequence Tags (EST) database (http://cgf.ucdavis.edu/home/). Among these ESTs, only three Ca^{2+}/H^+ exchangers were found to be expressed in the targeted fruit tissues. The expressed nucleotides were named *CAX1* (CTG1061171) 72% identity to *vacuolar Ca^{2+}/H^+ exchanger* (NCBI: NM_115045), *CAX2* (CTG1073422) 86% identity to *vacuolar Ca^{2+}/H^+ exchanger* (NCBI: NM_112177), and *CAX3* (CTG1076982) 81% identity to *vacuolar Ca^{2+}/H^+ exchanger* (NCBI: XM 002533684). Two putative *Ca-ATPases*, one *Vacuolar-ATPase* and one *H^+-pyrophosphatase* were also obtained in the UCDavis database and were found to be expressed in the targeted tissue. The *Ca-ATPases* were named *Ca-ATPase1* (CTG1060377) 78% identity to *Ca-ATPase* (NCBI: XM_002325215), and *Ca-ATPase2* (CTG1063686) 76% identity to *Ca-ATPase* (NCBI: AJ310848). The *Vacuolar-ATPase* was named *V-ATPase* (CTG1058311) 83% identity to *Vacuolar-ATPase* (NCBI: NM_001036222), and the *H^+-pyrophosphatase* was named *PPase* (EB143723) 82% identity to *H^+-pyrophosphatase* (NCBI: NM_101437). All nucleotide sequences for putative cell wall enzymes were obtained in the apple (*Malus domestica*) EST database in the NCBI gene bank (http://www.ncbi.nlm.nih.gov/Genbank/). The putative pectin methylesterases were named *PME1* (NCBI: CO168183) 98% identity to *pectin methylesterase* (NCBI: AB067684.1), *PME2* (NCBI: CO415488) 83% identity to *pectin methylesterase* (NCBI: XP 002278061.1), *PME3* (NCBI: CN994362) 84.2% identity to *pectin methylesterase* (NCBI: XP 002317777), and *PME4* (NCBI: CN994197) 88% identity to *pectin methylesterase* (NCBI Accession: X95991.1). The other cell wall enzymes studies were *Endo-PG* (NCBI: L27743) 96% identity to *endo-polygalacturonase* (NCBI: AJ504855), *Exo-PG* (CO417950) 66% identity to *exo-polygalacturonase* (NM_112258), *β-Gal* (L29451) 74% identity to *β-galactosidase* (NM_115144), and *PL* (AY376878) 77% identity to *pectate lyase* (NM_125713). All mentioned nucleotide sequences encoding cell wall enzymes are part of Unigene sequences.

2.2. Cytochemical and ultrastructural observations of Ca^{2+} localization

Fruit outer cortical tissue with skin was fixed in 4% glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.2) containing 2% potassium antimonate. After washing in a rinse buffer (0.1 M phosphate buffer containing 2% potassium antimonate, pH 7.2), the tissue was post fixed in 1% osmium tetroxide in 0.1 M potassium phosphate buffer containing 2% potassium antimonate for 2 h at 4 °C. The tissue was dehydrated in a graded alcohol series and embedded in epoxy resin. For observation with the transmission electron microscope, ultrathin sections were prepared. Control grids mounted with tissue sections were immersed in a solution of 100 mM ethylene glycol tetraacetic acid (pH 8.0), a chelator with high affinity for calcium ions, and incubated at 60 °C for 1 h. After treatment, the grids were rinsed in distilled water. Electron micrographs were taken with a Philips CM120 Biotwin Lens electron microscope at 75 kV (F.E.I. Company, Hillsboro, OR) (Suzuki et al., 2003).

2.3. Mineral analysis

Outer cortical fruit tissue without skin (collected as described above) was frozen in liquid nitrogen and freeze-dried for later mineral analysis. Nitrogen was analyzed by a combustion method (AOAC, 2006). Potassium was extracted with 2% acetic acid and quantitatively assessed by atomic emission spectrometry (Johnson and Ulrich, 1959). Samples analyzed for Ca^{2+} and Mg^{2+} were subjected to microwave acid digestion/dissolution and then analyzed by inductively coupled plasma atomic emission spectrometry (Meyer and Keliher, 1992).

2.4. Quality evaluations

Fruit firmness was measured with a penetrometer (Güss Fruit Texture Analyzer, South Africa) fitted with an 11 mm probe. Skin was removed on two sides of each apple and firmness recorded on each side. Juice samples were extracted by squeezing two cortical wedges cut from both sides of the fruit in two layers of cheese cloth. Percentage of malic acid equivalents was determined with an automatic titrator (Radiometer, Copenhagen, Denmark) by titrating 4 mL of juice with 0.1 N NaOH to pH 8.2. Dry matter (%) was determined by freeze-drying the samples.

Membrane leakage was determined in 3 fruit discs of 1 cm diameter and 0.5 cm thickness (about 3 g fresh weight) cut from the calyx-end region of the fruit with a stainless steel cork borer, and sectioned with a double bladed knife beginning 1 mm under the skin. Each sample of 3 discs from 3 fruit represented one replication, and placed into a 50 mL conical tube with 20 mL of 0.35 mol L^{-1} isotonic mannitol, and the conductivity was recorded periodically for 6 h. Samples were then frozen and thawed twice to determine the total conductivity (Saltveit, 2002). Ion leakage values were expressed as percentage of total conductivity. The isotonic mannitol solution was determined by incubating pericarp discs in solutions containing different concentrations of mannitol. The isotonic solution was the one that did not change tissue weight after incubation.

2.5. Cell wall measurements

Fruit outer cortex tissue was stored at −80 °C for cell wall material (CWM) extraction according to Campbell et al. (1990). The samples were boiled in 95% ethanol (4 L kg^{-1}, fresh weight) for 20 min, homogenized at top speed in a Polytron (Brinkman Instruments, New York, NY) for 60 s, then centrifuged at 1500 × *g* for 10 min, and the supernatant was decanted. The pellet was resuspended in 80% ethanol by homogenization in the Polytron, recentrifuged to a pellet, and the supernatant again decanted; this washing cycle was repeated until the resulting supernatant was colorless. The crude cell wall pellet was dried under an air stream, then suspended in DMSO:water (9:1, v/v; 20 L kg^{-1}, dry weight) and stirred at room temperature for 24 h to remove starch. The slurry was centrifuged at 1500 × *g* for 10 min, DMSO was decanted, and the pellet was washed repeatedly in 95% ethanol to remove all traces of DMSO. The pellet was dried under air, then resuspended and washed once in acetone. The acetone-washed, starch-free material (alcohol-insoluble substances, AIS) was air-dried and analyzed as the CWM. Samples of the CWM were suspended in distilled H_2O for 1 h and centrifuged (10,500 × *g*, 25 min). The supernatant was collected and the previous step was repeated once. The two supernatants were combined and designated as the water-soluble pectin fraction. The remaining pellet was considered to contain the water-insoluble pectin fraction for the purposes of determining Ca^{2+} binding. The Ca^{2+} content was determined in the CWM, water-insoluble and water-soluble pectin fractions and presented as a percentage of the total cortical tissue dry weight. The relative Ca^{2+} content was calculated considering the fraction of Ca^{2+} content in the CWM, water-insoluble and water-soluble pectin fractions as representing the total cortical tissue Ca^{2+} content. The total uronic acid content of each pectin fraction was measured based on the method described by Ahmed and Labavitch (1977). The degree of deesterification was measured by the reductive method in which the esterified galacturonosyl carboxyl groups are reduced (i.e., converted from glacturonic acid to galactose) and then the deesterified uronic acids are quantified and represented as a percentage of the total uronic acid content present in the original unreduced samples (Klein et al., 1995). Pectin samples were incubated overnight at room temperature in 1 mL of 10 mg mL^{-1} $NaBH_4$ in 50% ethanol plus 50% 1 N NH_4OH. The samples were neutralized with acetic acid, dried, and washed several times with acetic acid:methanol (1:9) and then methanol. Later, reduced and unreduced samples were dissolved in 67% H_2SO_4 and the deesterified uronic acid content determined spectrophotometrically (Blumenkrantz and Asboe-Hansen, 1973; Ahmed and Labavitch, 1977).

The analysis of variance (ANOVA) for a completely randomized design was performed for each variable. The analyses were accomplished using SAS statistical package (SAS Institute, 2002). The mean values of four replicate samples were compared using Duncan's test ($P = 0.05$).

3. Results

The expression analysis after 60 d of storage revealed that three *CAXs*, two *Ca-ATPases*, one *V-ATPase*, and one *PPase* gene were expressed in the outer cortical tissue of apple fruit (Fig. 1A). All these genes were also expressed, although at much lower levels, in the skin tissue of healthy fruit, with the exception of the *Ca^{2+}/H^+ exchanger1* (*CAX1*) (Fig. 1B). Expression of *CAX1*, *Ca-ATPases 1* and *2*, and *V-ATPase* in the outer cortical tissue was the same whether or not fruit showed BP symptoms. *CAX2* had slightly higher expression and *CAX3* had slightly lower expression in pitted fruit. The *PPase* gene expression was approximately 4.5 times higher in fruit with BP, compared to fruit without BP (Fig. 1A). In the skin tissue, all of these genes had significantly lower expression in fruit with BP than in healthy fruit, although the BP-related reduction in *CAX2* expression was much less than the reductions in the expression of the other genes (Fig. 1B). The expression analysis of genes involved in pectin metabolism showed that four putative fruit *PMEs* were expressed in the outer cortical tissue and the skin of fruit with and without BP after 60 d of storage (Fig. 2A and B). Fruit with BP had higher expression of all four *PMEs* in the outer cortical tissue

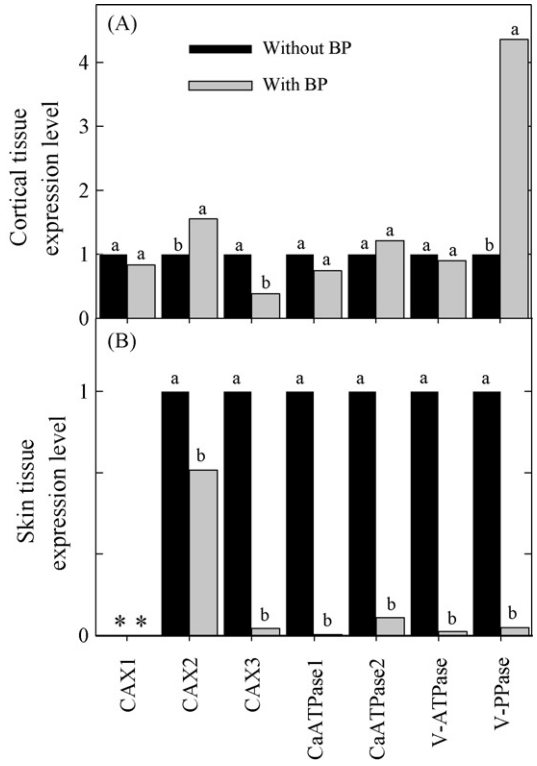

Fig. 1. Expression of genes involved in Ca^{2+} transport in the outer cortical tissue (A) and in the skin (B) of 'Granny Smith' apple fruit with and without bitter pit (BP) after 60 d in cold storage. Not detected (*). *CAX1*, *CAX2*, *CAX3* (putative Ca^{2+}/H^{+} *exchangers*), *Ca-ATPase1*, *Ca-ATPase2* (putative Ca^{2+} *pumps*), *V-ATPase* (vacuolar-ATPase) and *PPase* (pyrophosphatase) (putatives H^{+} *pumps*). Bars with similar letters are not significantly different ($P = 0.05$) according to Duncan's test.

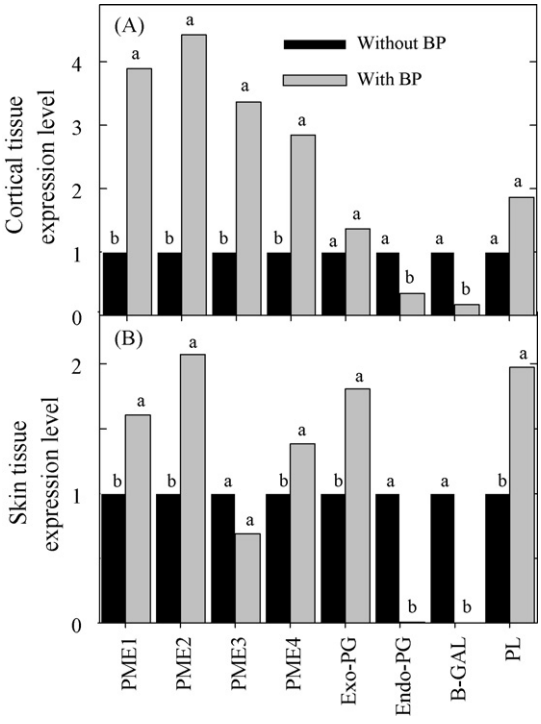

Fig. 2. Expression of genes involved in cell wall metabolism in the outer cortical tissue (A) and in the skin (B) of 'Granny Smith' apple fruit with and without bitter pit (BP) after 60 d in cold storage. *PME1*, *PME2*, *PME3*, *PME4* (putative *pectin methylesterases*) *Exo-PG* (putative *exo-polygalacturonase*), *Endo-PG* (putative *endo-polygalacturonase*), *β-Gal* (putative *β-galactosidase*), *PL* (putative *pectate lyase*). Bars with similar letters are not significantly different ($P = 0.05$) according to Duncan's test.

and higher expression of three *PMEs* in the skin tissue compared to fruit tissues without BP (Fig. 2A and B). Both *Exo-PG* and *PL* had equal expression levels in pitted and healthy cortical tissue, but they were more highly expressed in the skin of pitted fruit than in the skin of healthy fruit (Fig. 2A and B). *Endo-PG* and *β-Gal* were expressed at lower levels in pitted cortical and skin tissues (Fig. 2A and B).

Cytochemical and ultrastructural studies of Ca^{2+} localization showed an unusually high accumulation of Ca^{2+} inside the vacuoles of randomly distributed cells in the outer cortical tissue of pitted fruit (Fig. 3A). This elevated vacuolar Ca^{2+} accumulation was not observed in healthy fruit tissues (Fig. 3B). Plasmolysis followed by plasma membrane breakdown was observed in fruit tissue with early BP symptoms (Fig. 3C and D). The complexity of BP development is shown by the presence of healthy (Fig. 3C-a), plasmolyzed (Fig. 3C-b), and senescing (Fig. 3C-c) cells close to each other in pitted fruit tissue during the early stages of pit development.

In BP compared with healthy fruit, the total Ca^{2+} concentration was lower in outer cortical tissue cell wall, and in both soluble and insoluble pectin fractions (Fig. 4A). The degrees of pectin deesterification (Fig. 4B) and the concentrations of deesterified uronic acids (Fig. 4C) were higher in the total cell wall, and in insoluble and soluble pectin fractions, of cortical tissue from fruit with BP compared with those without BP symptoms. The relative percentages of total cortical tissue Ca^{2+} were higher in the cell wall and insoluble pectin fractions of pitted fruit compared with non-pitted fruit, but were equal in the soluble pectin fractions from the healthy and pitted tissues (Fig. 4D).

Cortical tissue of fruit with BP had higher membrane leakage (Table 1) and lower Ca^{2+} concentration (Fig. 5) than tissues of fruit without BP. The nutrients Mg^{2+} and K^{+} were higher in fruit with BP than in fruit without BP and the N concentration was similar (Fig. 5). The N/Ca^{2+} and $[K^{+} + Mg^{2+}]/Ca^{2+}$ ratios were higher in pitted fruit, reaching values as high as 118.1 and 109.1, respectively, while fruit without BP had N/Ca^{2+} and $[K^{+} + Mg^{2+}]/Ca^{2+}$ ratios of 75.6 and 60.4, respectively (Fig. 6). The dry matter content and malic acid content were higher in fruit with BP (Table 1). Pitted fruit had lower flesh firmness than healthy fruit, but total soluble solids content was statistically equal in pitted and non-pitted fruit (Table 1).

4. Discussion

4.1. Role of cellular storage organelles as sinks for Ca^{2+} and relationship to BP development

Storage compartments, such as the vacuole and endoplasmic reticulum, are strong sinks for Ca^{2+} in the cell, representing about 40% of the total Ca^{2+} in the tissue (Harker and Venis, 1991). A slight increase in the transport of Ca^{2+} into these storage organelles can potentially deplete other pools of Ca^{2+} available for cellular functions, resulting in a cellular localized Ca^{2+} deficiency, particularly if these other pools normally have relatively low Ca^{2+} contents (White and Broadley, 2003). Bitter pit is characterized by dark brown spots that originate in the outer cortical fruit tissue as a result of the death of localized clusters of cells (Fuller, 1980). Calcium accumulation inside the vacuoles of localized cells in the outer cortical tissue may represent a withdrawal of free apoplastic Ca^{2+} that could otherwise contribute to plasma membrane structure and stability. If so, the localized accumulation of Ca^{2+} inside the vacuole may trigger a localized depletion of apoplastic Ca^{2+}, resulting in an increase in membrane leakiness, plasmolysis, and eventually localized cell death (i.e., pit formation). These results may explain previous reports of higher concentrations of Ca^{2+} in pitted tissue than in healthy tissue surrounding the pitted regions (Chamel and Bossy, 1981).

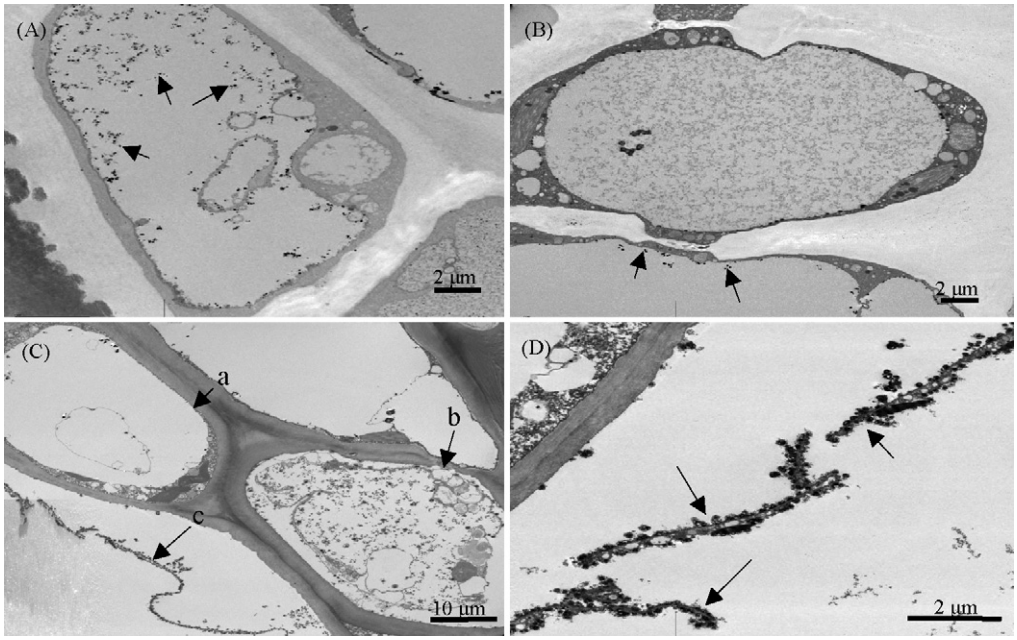

Fig. 3. Cytochemical and ultrastructural observations of Ca^{2+} localization in apple fruit tissue after 60 d in cold storage. Comparison of Ca^{2+} localization inside cellular storage organelles (vacuole) in bitter pitted fruit (A) and healthy fruit (B) tissue. Arrows pointing to black spots resulting from the reaction between potassium antimonate and Ca^{2+} in the cells (A and B). Bitter pit symptom development in pitted fruit tissue (C and D). Arrows indicate healthy (a), plasmolyzed (b), and senescing (c) cells. Arrows indicate membrane disintegration during bitter pit (BP) development (D).

Our analysis of the expression of genes putatively encoding apple Ca^{2+} transporters and pumps is based on the presumption that apple ESTs with high homology to identified genes encoding Ca^{2+} transporters and pumps in other plants do, in fact, encode apple fruit Ca transporters and pumps. This analysis showed that several genes involved in Ca^{2+} movement into the vacuole had similar expression in the outer cortical tissue of pitted and healthy fruit, despite the observed localized accumulation of Ca^{2+} inside the vacuole. Analyses of gene expression in the entire outer cortical tissue at the calyx end may have diluted a localized increase in the expression of genes for these proteins. Alternatively, there may not have been changes in gene expression for these Ca^{2+} transport proteins, but only a localized increase in their activity that led to higher accumulation of Ca^{2+} in the vacuole of localized cells. The high expression of *PPase* observed in the outer cortical tissue at the calyx end of pitted fruit is in agreement with Casado-Vela et al. (2005), who used a proteomic approach to study the development of Ca^{2+} deficiency in tomato fruit. PPase activity contributes to the electrochemical gradient across the tonoplast membrane that is required by CAX proteins to move Ca^{2+} into the vacuole. Thus, if our data for *PPase* expression reflect substantially elevated transcription in localized cell clusters that will eventually form pits, PPase activity could be associated with higher localized activity of CAX proteins, increasing Ca^{2+} movement into the vacuole.

The pattern of gene expression for proteins putatively involved in Ca^{2+} transport into the vacuole in skin tissue was different from the pattern observed in the cortical tissue, with decreased expression of almost all genes in pitted fruit tissue. The only exception was CAX3 that showed a reduction in expression in both skin and cortical tissues of pitted fruit. A possible explanation could be that skin tissue has a different response to lower Ca^{2+} levels in the tissue, down regulating the expression of these genes to avoid Ca^{2+} deficiency development. This mechanism could also explain the fact that BP always start in the outer cortical tissue, but not in the skin tissue that eventually collapses in response to the cell death underneath in the outer cortical tissue.

Although V-ATPase and PPase proteins are only located in the membranes of Ca^{2+} storage organelles, CAX and Ca-ATPase proteins can also be located on the plasma membrane, transporting Ca^{2+} from the cytosol to the apoplast (White and Broadley, 2003). In this case, the final balance between the activity of CAX and Ca-ATPase located in organellar membranes and plasma membrane could determine the concentration of apoplastic Ca^{2+} and BP development. Therefore, further work is required to determine the function and the cellular location of these putative CAXs and Ca-ATPases.

4.2. The cell wall as a potential binding site for Ca^{2+} and a mechanism to trigger BP development

The cell wall represents the biggest pool of Ca^{2+} in fruit tissue and small changes in the ability of the cell wall to bind Ca^{2+} may represent a large variation in the amount of Ca^{2+} available for other cellular functions. Our results show that the outer cortical tissues in the calyx-end region of pitted fruit have higher expression of

Table 1
Dry matter, total soluble solids, malic acid equivalents, flesh firmness, and membrane leakage of 'Granny Smith' apple fruit at harvest and after 60 d in cold storage with and without bitter pit (BP).

Fruit	Dry matter (%)	Total soluble solids (%)	% Malic acid equivalents	Flesh firmness (*N*)	Ion leakage (%)[a]
At harvest	14.8 c[b]	11.0 b	0.706 b	73.0 a	0.52 b
No BP	16.3 b	12.4 a	0.784 b	71.8 b	0.61 b
BP	16.9 a	13.1 a	0.894 a	70.3 c	1.46 a
CV	0.56	4.18	1.21	2.08	8.12

[a] Calculated based on the change in conductivity per hour as a percentage of the total conductivity.
[b] Mean values with similar letters are not significantly different ($P = 0.05$) according to Duncan's test.

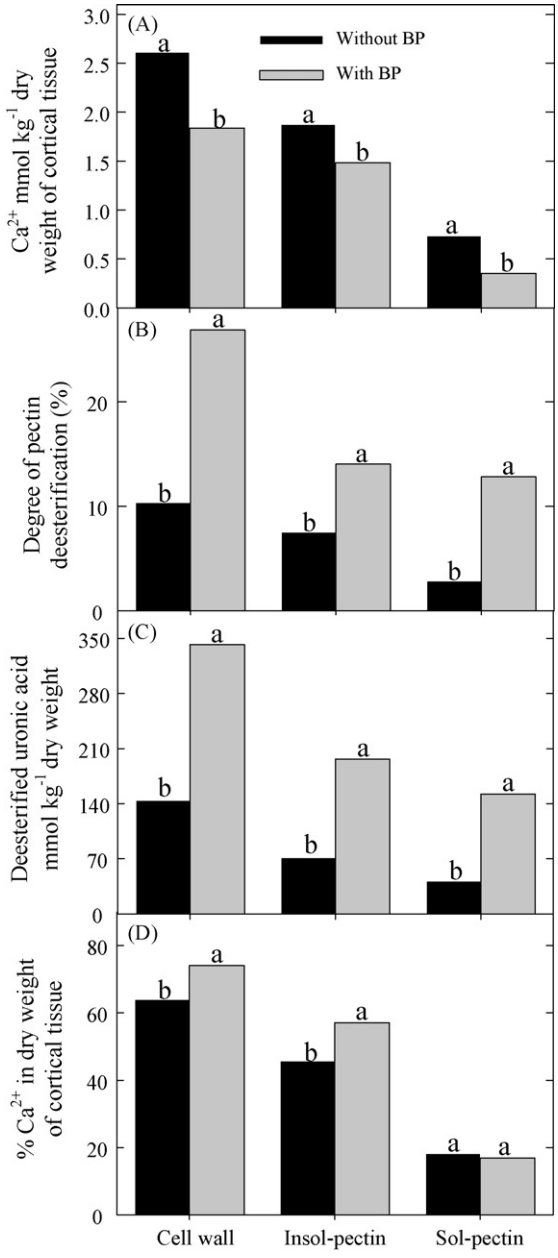

Fig. 4. Calcium concentration (A), degree of pectin deesterification (B), deesterified uronic acid concentration (C), and relative Ca^{2+} content (D) in 'Granny Smith' apple fruit with and without bitter pit (BP) after 60 d in cold storage. Cell wall = total cell wall; Insol-pectin = water-insoluble pectin; Sol-pectin = water-soluble pectin. Bars with similar letters are not significantly different ($P = 0.05$) according to Duncan's test.

PMEs and a higher degree of pectin deesterification, likely reflecting the greater PME activity. Potentially, the higher *PME* expression and the resulting pectin deesterification are involved in development of Ca^{2+} deficiency. Pitted fruit had lower total cortical tissue Ca^{2+} concentration and a higher fraction of this Ca^{2+} was bound to the cell wall than in healthy fruits. A greater proportion of the Ca^{2+} bound to the cell wall of pitted fruit was in the insoluble pectin fraction, where Ca^{2+} bridges parallel deesterified pectin backbones, forming an "egg-box" structure that tightly retains Ca^{2+} (Ridley et al., 2001; Prasanna et al., 2007). Pectins can bind almost 100% of the Ca^{2+} in solution when the ratio of deesterified carboxyl groups in the pectin backbone to total Ca^{2+} is close to 2, depending also on the apoplastic pH (Tibbits et al., 1998). This ratio represents the "egg-box" structure and shows that Ca^{2+} can be tightly bound inside the

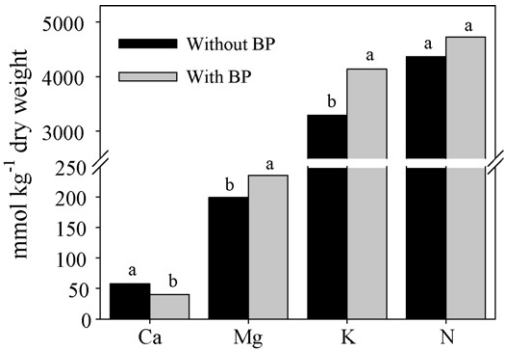

Fig. 5. Calcium, Mg^{2+}, K, and N concentration in the outer cortical tissue of 'Granny Smith' apple fruit with and without bitter pit (BP) after 60 d in cold storage. Bars with similar letters are not significantly different ($P = 0.05$) according to Duncan's test.

pectin network, potentially depleting the apoplastic pool of free Ca^{2+}, affecting membrane structure and integrity, and eventually leading to Ca^{2+} deficiency symptoms in the tissue. The increase in *PME* expression that we observed was substantial enough to be measured in the skin and outer cortical tissue of pitted fruit, but we do not know if this reflects a general increase in PME across these tissues or more localized increases in PME expression, perhaps paralleling the highly localized accumulation of Ca^{2+} inside the vacuole and possibly localized expression and/or activity of proteins involved in Ca^{2+} movement to the vacuole. These uncertainties could be addressed using *in situ* hybridization to identify where in the tissues *PME*, CAX, *Ca-ATPase*, *V-ATPase*, or *PPase* mRNAs are found. The deesterification of pectins in the outer cortical apple tissue may lead to a general reduction in the fruit's apoplastic pool of free Ca^{2+}; leading to a further Ca^{2+} depletion that could contribute to localized membrane breakdown and cell death. The use of monoclonal antibodies that distinguish between pectins which have low *vs.* high levels of methyl-esterification or localized patches of Ca-associated pectins (Guillemin et al., 2005) may prove useful. If the scenario for triggering BP development that we have proposed herein is accurate, there will be a positive correlation between the levels of gene expression and/or cell wall pectin changes with BP development.

Pectins are a major class of cell wall polysaccharides, and are degraded by both solubilization and depolymerization (Ridley et al., 2001). In apple fruit, it has been demonstrated that there is an elevated degree of solubilization, but little depolymerization of pectins after harvest (Prasanna et al., 2007). The action of pectin metabolism-related enzymes such as β-Gal can facilitate the action of pectin depolymerization enzymes, which can degrade pectin backbones and release Ca^{2+} to the free soluble pool in the apoplast

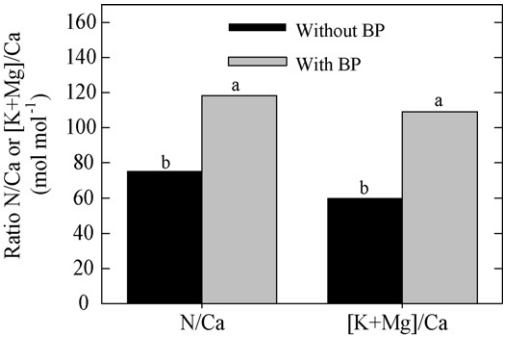

Fig. 6. Ratio of N/Ca^{2+} and $[K^+ + Mg^{2+}]/Ca^{2+}$ in the outer cortical tissue of 'Granny Smith' apple fruit with and without bitter pit (BP) after 60 d in cold storage. Bars with similar letters are not significantly different ($P = 0.05$) according to Duncan's test.

solution (Prasanna et al., 2007; Goulao and Oliveira, 2008). The lower expression of *β-Gal* and *Endo-PG* observed in the cortical and skin tissue of pitted fruit at the time of BP development may have delayed pectin degradation and Ca^{2+} release into the apoplastic solution; this, in turn, could enhance the probability of BP development in the fruit. On the other hand, the higher expression of *Exo-PG* and *PL* observed in the skin tissue of pitted fruit might have increased Ca^{2+} release in the apoplast solution and reduced the chances of BP development in the skin tissue. This would be consistent with previous reports showing that BP symptom development is limited first to the outer cortical tissue, only later triggering epidermal cell death (Ferguson and Watkins, 1989). It is likely that Ca^{2+} in the cell wall is not static, but rather it is in a constant state of flux moving in and out of the cell wall. An imbalance favoring the cell wall-bound Ca^{2+} in localized cells may lead to BP development.

The higher membrane permeability in apples with BP possibly resulted in higher water loss, concentrating dry matter, total soluble solids, and malic acid content in pitted fruit. Flesh firmness is influenced by cell wall strength and turgor pressure (Mignani et al., 1995). Fruit with BP had a lower total Ca^{2+} concentration in the cell wall, which could explain, in part, the lower flesh firmness in pitted fruit. However, it is also likely that leakier plasma membranes and higher water loss, reduced flesh firmness of pitted fruit by reducing cellular turgor pressure.

4.3. Better understanding BP development in apple

The visual symptoms of BP development observed in this study started with plasmolysis followed by plasma membrane breakdown and cell death. Membrane structure and integrity are believed to be dependent on a free pool of Ca^{2+} in the apoplast (Hanson, 1960; Plieth, 2001). When the concentration of Ca^{2+} in the apoplast is below the threshold required to maintain membrane structure and function, membrane leakiness results and pits develop in the tissue. However, Ca^{2+} concentration in the apoplast alone may not be informative in relation to BP development. Other nutrients, such as Mg^{2+}, K^+, and N, may also be involved in BP development in apples and the ratios $[K^+ + Mg^{2+}]/Ca^{2+}$ and N/Ca^{2+} have been used to predict fruit susceptibility (Dris et al., 1998; Lanauskas and Kvikliene, 2006). The effects of Mg^{2+} and K^+ may result from their competition with Ca^{2+} for binding sites at the plasma membrane surface, but these elements cannot replace Ca^{2+}'s role in membrane structure and stability (Schonherr and Bukovac, 1973; Yermiyahu et al., 1994).

Our results show a higher concentration of Mg^{2+} and K^+ and a higher ratio $[K^+ + Mg^{2+}]/Ca^{2+}$ in pitted fruit tissue. This may indicate a competition between $[K^+ + Mg^{2+}]$ and Ca^{2+} for binding sites at the plasma membrane surface and more Ca^{2+} being released to the apoplast solution. In this context, the higher affinity of the deesterified pectins for Ca^{2+} in relation to other nutrients (Demarty et al., 1978), may favor a selective movement of the Ca^{2+} displaced from plasma membranes to the cell wall-bound form of Ca^{2+}. Consequently, less Ca^{2+} will be bound to the plasma membrane, which will become leakier, eventually leading to plasmolysis, membrane breakdown, and cell death. Nitrogen has also been reported to be involved in BP development, but the mechanism of action is still not well understood (Ferguson and Watkins, 1989; Saure, 2005). Our results show a similar concentration of N in both pitted and healthy fruit and the higher N/Ca^{2+} ratio observed in pitted fruit was solely the result of lower tissue Ca^{2+}. High $[K^+ + Mg^{2+}]/Ca^{2+} + N/Ca^{2+}$ ratios have also been observed in apple fruit with lenticel breakdown (LB) symptoms (Kupferman, 2009), suggesting that high ratios of these nutrients may be part of a common mechanism involved in BP and LB development.

This study supports the potential involvement of Ca^{2+} storage in organelles and in the cell wall in BP development in apple fruit. As discussed, at the tissue level, PME activity could increase Ca^{2+} binding to the cell wall and, thus, reduce the apoplastic pool of free Ca^{2+}. Localized increases in the expression and/or activity of CAXs and/or Ca-ATPases present in the membrane of Ca^{2+} storage organelles could increase Ca^{2+} accumulation inside these organelles, further depleting the apoplastic pool of free Ca^{2+} and resulting in localized membrane breakdown and, subsequently, BP development. Future work will focus on factors that trigger changes in proper Ca^{2+} homeostasis, leading to increased Ca^{2+} movement into storage organelles and binding to the cell wall, and more precisely localize changes in cell walls and gene expression that have thus far been described only at the tissue level. A better understanding of the mechanisms involved in BP development and the factors that trigger this disorder will allow the development of more effective control strategies.

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