



Thaumatococcus-like proteins and their possible role in protection against chilling injury in peach fruit

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ABSTRACT

Peaches are highly perishable; they ripen and deteriorate quickly at ambient temperature, and cold storage is used to slow these processes. The cell wall protein composition of two peach cultivars, and total protein composition were examined at harvest and after cold storage (3 weeks, 5 °C) by two-dimensional polyacrylamide gel electrophoresis. The two peach cultivars used were 'Oded', a white-, melting-flesh, cling-stone, early season cultivar resistant to chilling injury, and 'Heremoza', a white-, melting-flesh, free-stone, mid-season cultivar susceptible to chilling injury. Following storage, peptides in the cell wall with molecular masses ranging from 18 kDa to 60 kDa were identified by amino acid sequence to be thaumatococcus-like protein 1 precursor and thaumatococcus-like protein 2 precursor. qRT-PCR analysis revealed that the thaumatococcus-like protein 1 precursor transcript accumulated significantly in both cultivars during storage. However, after 1 and 2 weeks of cold storage at 5 °C the thaumatococcus-like protein 1 precursor transcript levels were significantly higher in the chilling injury-resistant peach 'Oded' than the susceptible peach 'Heremoza'. This early accumulation of the thaumatococcus-like protein 1 precursor transcript in the resistant peach suggests that thaumatococcus-like protein 1 precursor (and perhaps thaumatococcus-like protein 2 precursor) might be involved in protecting against chilling injury. Although thaumatococcus-like proteins accumulated to high levels in cell walls of chilling injury-sensitive 'Heremoza', the kinetics of transcript accumulation suggest that the early appearance of the transcript for this protein family might be involved in shielding the fruit from the dramatic cell wall-structure changes that accompany the onset of chilling injury in stone fruit, and that result in woolliness development.

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1. Introduction

Peaches are highly perishable; they ripen and deteriorate quickly at ambient temperature (Lurie and Crisosto, 2005). Therefore, low temperature storage (0–5 °C) is a common strategy used to slow the ripening processes as well as decay development during storage and/or shipment (Crisosto et al., 1999; Lurie and Crisosto, 2005). However, if susceptible varieties of peach, nectarine, and other stonefruit such as plum and apricot are held too long at a low temperature, they will not ripen properly when rewarmed and will develop chilling injury (CI) (Crisosto et al., 1999; Zhou et al., 2000a, 2001; Crisosto and Labavitch, 2002; Brummell et al., 2004b; Manganaris et al., 2005, 2006).

In peach fruit that develop CI symptoms, cell wall modifications have been extensively studied (Zhou et al., 2000a,b,c; Brummell et al., 2004a,b). Long-term cold storage of suscep-

tible peaches is known to result in dramatic changes in the cell wall properties. The manifestation of CI in peaches includes defective cell wall disassembly and the development of a dry, woolly rather than soft, juicy texture (Lurie and Crisosto, 2005). Recent studies have shown considerable changes in transcription in cold-stored peaches compared to unstored fruit (Gonzalez-Aguero et al., 2008; Ogundiwin et al., 2008; Tittarelli et al., 2009; Vizoso et al., 2009). This leads to the hypothesis that changes in cell wall proteins may be associated with the cold-induced development of woolliness, the most ubiquitous symptom of CI in peach.

Proteomics is a powerful tool for studying and identifying global changes in structure and abundance of plant proteins in response to developmental and environmental signals (Rampitsch and Srinivasan, 2006; Shi et al., 2008). There has been an increasing trend in use of proteomic methods in the field of fruit and vegetable physiology over the last few years (Rocco et al., 2006; Hjerno et al., 2006; Pedreschi et al., 2008). However, little research has utilized proteomic approaches in the field of postharvest physiology (Pedreschi et al., 2008; Shi et al., 2008), although, proteomic approaches utilizing two-dimensional polyacrylamide gel elec-

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trophoresis (2D-PAGE) together with mass spectrometry have been used to study plant responses to low temperature stress (Cui et al., 2005; Amme et al., 2006).

Proteomic methods have been tested and employed successfully to study different plant organelles including cell walls (Feiz et al., 2006), chloroplasts, peroxisomes and mitochondria (Peltier et al., 2000; Fukao et al., 2002; Heazlewood et al., 2003). Proteomic examination of plant cell walls is extremely challenging because of contamination with polysaccharides, polyphenolics and cytosolic proteins (Feiz et al., 2006). To prevent contamination of cytosolic proteins stringent washes must be used as part of the extraction procedure.

Thus far, to our knowledge no one has employed a proteomic strategy to study low temperature stress in cell walls of peach, and no proteomic data of cell wall proteins are yet available. Therefore, the present study provides the first fundamental cell wall proteomic analyses of the cold-stored peach fruit tissue. In the present study, and as part of our ongoing efforts to understand physiological and molecular responses of peach fruit to cold storage, we used 2D-PAGE analysis joined with reverse HPLC microspray mass spectrometry (HPLC/MS/MS) to examine changes in the cell wall proteins and total proteins of peach fruit subjected to cold storage. The 2D-PAGE results were also confirmed with quantitative reverse transcriptase-PCR (qRT-PCR) analysis.

2. Materials and methods

2.1. Plant material and treatments

'Hermoda', a white fleshed, free-stone, melting-flesh, mid-season peach susceptible to CI, and 'Oded', a white fleshed, cling-stone, melting-flesh, early season peach resistant to CI, were sampled at harvest, harvest plus 3 d ripening at 20 °C, after 1, 2 and 3 weeks storage at 5 °C, and 3 weeks storage at 5 °C plus 3 d ripening at 20 °C. At each time of sampling, fruit were cubed, weighed and flash frozen in liquid nitrogen, and stored at –80 °C. Five fruit were sampled per treatment.

2.2. Firmness and extractable juice measurements

Physiological parameters were measured following a protocol described by Zhou et al. (2000c). In brief, firmness was measured on two pared sides of each fruit using a penetrometer fitted with an 8-mm diameter plunger. After firmness was determined, the fruit was cut into halves and woolliness was estimated both by visual observation and organoleptically. Juicy fruit with no signs of woolliness were classed as healthy. The amount of expressible juice was determined by removing a tissue plug weighing about 2 g from each fruit with a cork borer, passing it through a 5 mL syringe into an Eppendorf tube, centrifuging and separately weighing the juice and solids (Lill and van der Mespel, 1988). Fifteen fruit were examined at each observation time for each cultivar.

2.3. Isolation of cell walls

The cell walls were isolated from 10 g of peach mesocarp stored at –80 °C following a protocol described by Feiz et al. (2006) with minor modifications. In short, the mesocarp was transferred into 50 mL of 5 mM acetate buffer, pH 4.6, 0.4 M sucrose and protease inhibitor cocktail (Roche Diagnostics GmbH, Germany) 1 tablet per 50 mL of the buffer. The mixture was ground in a blender (Osterizer, USA) for 2 min. After adding polyvinyl polypyrrolidone (PVPP; 0.5 g per 10 g of the tissue), the mixture was incubated in a cold room for 30 min while shaking. Cell walls were separated from soluble cytoplasmic fluid by centrifugation of the homogenate for

15 min at 1000 × g at 4 °C. The pellet was resuspended and recentrifuged in 15 mL of 5 mM acetate buffer, pH 4.6, containing first 0.6 M and then 1 M sucrose. The residue was washed with 200 mL of 5 mM acetate buffer, pH 4.6, on 30 µm pore size nylon net (Millipore, USA). The resulting cell wall fraction was ground in liquid nitrogen in a mortar and pestle prior to lyophilization. This process resulted in about 0.5 g of dry powder from 10 g of frozen tissue.

2.4. Cell wall protein extraction

The cell wall material from 10 g of frozen tissue was used for extracting the cell wall proteins using the method of Feiz et al. (2006) with slight adjustments. Proteins were extracted from the isolated cell walls by successive salt solutions in the following order: two extractions each time with 7.5 mL of CaCl₂ (5 mM acetate buffer, pH 4.6, 0.2 M CaCl₂ solution and 1 tablet of the protease inhibitor cocktail per 50 mL of the buffer), followed by two extractions with 7.5 mL of LiCl solution (5 mM acetate buffer, pH 4.6, 6 M LiCl and 1 tablet of the protease inhibitor cocktail per 50 mL of the buffer). After each incubation, the suspension was centrifuged for 15 min at 4000 × g and 4 °C. Supernatants were desalted and concentrated to a volume of 100–200 µL using 15-mL Amicon Ultra Centrifugal Filter Device (Millipore, USA) with a membrane cut-off of 3 kDa. This concentrate was mixed with two volumes of precooled absolute phenol, and precipitated overnight at –20 °C. The following day, after centrifugation, the pellet was air dried for 30–60 min and dissolved in immobilized pH gradient (IPG) re-swelling buffer containing 9 M urea, 3% CHAPS, 0.5% Triton X-100, 2% IPG buffer (pH 3–10). The whole extracted cell wall proteins from 10 g of the frozen fruit tissue was used to rehydrate 13-cm IPG strips (pH 3–10) (Amersham Biosciences, Sweden) for the 2D-PAGE analysis.

2.5. Total protein extraction

Total proteins were extracted from frozen fruit tissue with phenol–protein extraction protocol as described previously (Barent and Elthon, 1992). In brief, 5 g of tissue was frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. The frozen powder was suspended in 10 mL of protein extraction buffer [PEB; 700 mM sucrose, 50 mM Tris, 30 mM HCl, 2 mM dithiothreitol (DTT), 100 mM KCl and 5 mM Na₂EDTA] in a 50 mL centrifuge tube and then 10 mL of water-saturated phenol was added. The tube was sealed and shaken for 5 min at room temperature. The organic and aqueous phases were separated by centrifugation at 7000 × g for 10 min. The phenol phase was taken and 10 mL of the PEB and 2 mL of water-saturated phenol added, shaken and centrifuged as above. The phenol phase was re-extracted with an equal volume of the PEB and 1 mL of water-saturated phenol. Following centrifugation, the soluble proteins were precipitated from the phenol phase by adding five volumes of 0.1 M ammonium acetate in methanol (precooled at –20 °C prior to use). Protein precipitation occurred at –20 °C overnight. The following day, the solution was centrifuged for 10 min at 7000 × g. The pellets were washed three times with 0.1 M ammonium acetate in methanol and once with cold acetone. The pellets were air dried and solubilized in the IPG re-swelling buffer for (2D-PAGE). The protein content was determined with Quant-iT™ Protein Assay Kit (Invitrogen, USA) according to the manufacturer's instructions. Extracted total proteins (200 µg) was used to rehydrate 13-cm IPG strips (pH 3–10) (Amersham Biosciences, Sweden) for the 2D-PAGE analysis.

2.6. Two-dimensional polyacrylamide gel electrophoresis

The 2D-PAGE was performed according to the IPG principles and methods of Amersham Biosciences (Piscataway, USA) as described

earlier (Shi et al., 2008) with some modifications. For analytical and preparative gels, 13-cm IPG strips (pH 3–10) (Amersham Biosciences, Sweden) were rehydrated overnight with 250 μ L of IPG re-swelling buffer at room temperature. Isoelectric focusing (IEF) was conducted at 18 °C with a Pharmacia Multiphor II separation unit (Amersham Biosciences, Sweden). The running conditions were: 300 V for 2 h, followed by 800 V for 15 min, 1300 V for 15 min, 1800 V for 15 min, 2300 V for 15 min, 2800 V for 15 min, 3300 V for 15 min, and finally 3500 V for 4 h. The focused strips were equilibrated twice for 30 min each time, first in 2 mM tributylphosphine (TBP) and then in 2.5% (w/v) iodoacetamide (IAA) prepared in equilibration buffer containing 50 mM Tris–HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, and 2% (w/v) sodium dodecyl sulfate (SDS). The electrophoresis phase was in a 12.5% acrylamide gel with a SE600 Series instrument (Hoefer Scientific Instruments, USA). The protein spots in analytical gels were visualized by staining with silver nitrate (BDH, England) suitable for reverse HPLC/MS/MS analysis according to the manufacturer's recommendations.

2.7. Image and data analysis

The 2D-PAGE gels were scanned with a ChemImager 4400 instrument (Alpha Inotech, San Leandro, CA). Gel matching for protein quantification was performed with Z3 software (Compugen Inc., Israel). The abundance of each protein spot was estimated according to the percentage volume (vol%). Spot-detecting parameters were set according to the manufacturer's instructions; local background regions: 17; average spot size: 5; weak spot sensitivity: 20%; and noise cut-off 40%. To verify the auto-detected results, all spots were also confirmed visually. The spots were sequenced twice with reverse HPLC/MS/MS.

2.8. In gel proteolysis and mass spectrometry analysis

The proteins in the 2D-PAGE gels were reduced with 10 mM DTT, modified with 40 mM IAA and trypsinized (modified trypsin; Promega) at a 1:100 enzyme-to-substrate ratio. The resulting tryptic peptides were resolved by reverse-phase chromatography on 0.075 mm $\times$ 200 mm fused silica capillaries (J&W) packed with Reprosil reversed phase material (Dr. Maisch GmbH, Germany). The peptides were eluted with linear 65 min gradients of 5–45% and 15 min at 95% acetonitrile with 0.1% formic acid in water at flow rates of 0.25 μ L/min. Mass spectrometry was performed by an ion-trap mass spectrometer (Orbitrap, Thermo) in a positive mode using repetitively full mass spectrometry scan followed by collision induces dissociation (CID) of the 7 most dominant ions selected from the first mass spectrometry scan.

The mass spectrometry data was clustered and analyzed using the Sequest software (Eng et al., 1994) or/and Pep-Miner (Beer et al., 2004) searching against the plant part of the non-redundant (nr) database. A peptide was considered as high quality if its Pep-Miner identification score was greater than 80 and the Sequest score of 1.5 for singly charged peptides, 2.5 for doubly charged peptides and 3 for triply charged peptides.

2.9. Total RNA extraction

Total RNA was isolated from pooled mesocarp tissue of five fruit in each treatment using the method described earlier (Lopez-Gomez and Gomez-Lim, 1992). Four grams of the frozen tissues was ground in a mortar and pestle. The frozen powder was added to 10 mL lysis buffer [2% SDS, 1% mercaptoethanol, 50 mM ethylenediaminetetraacetic acid (EDTA), and 150 mM tris base with pH adjusted to 7.5 with 1 M boric acid; 2–3 mL g⁻¹ fresh tissue] at room temperature. The homogenate was quickly vortexed for 1 min with 0.25 volumes of absolute ethanol and 0.11 volumes of 5 M

potassium acetate. An equal volume of chloroform:isoamyl (49:1) was added, followed by strong vortexing, and the homogenate was centrifuged at 15,000 $\times$ g for 20 min. The aqueous phase was extracted twice with phenol:chloroform (1:1) and once with chloroform:isoamyl alcohol (49:1). The RNA was precipitated overnight with 3 M lithium chloride (final conc.) at –20 °C and collected by centrifugation at 15,000 $\times$ g for 90 min at 4 °C. The RNA was then resuspended in 500 μ L of 0.1% diethylpyrocarbonate (DEPC)-treated sterile water and precipitated with 0.3 M potassium acetate (final concentration) and two volumes of absolute ethanol. After overnight incubation at –20 °C, the RNA was repelleted by centrifugation at 15,000 $\times$ g for 90 min at 4 °C, washed twice with 75% ethanol, and resuspended in the DEPC-treated water. Purity of the extracted RNA was assayed by ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA), and stored at –80 °C until further analysis.

2.10. Quantitative reverse transcriptase-PCR analysis

Expression levels for the *TLP1* gene was calculated relative to *Initiation Factor eIF-4-Gamma (eIF-G)* gene as described before (Ogundiwin et al., 2008). The sequence of the *TLP1* gene (ChillPeachDB ID PPN003H07; Arabidopsis ID AT1G200300) forward primer is 5'-CAGCTCCGCCAGCTACATTAG-3' and reverse primer is 5'-TTGACGCCTTACAGTCGCC-3' with amplicon size of 141 base pairs (bp). The sequence of the *eIF-G* gene (ChillPeachDB ID PPN052D02; Arabidopsis ID AT5G57870) forward primer is 5'-CTCTGGAAGATCCACCATG-3' and reverse primer is 5'-GCAAATCAATGCCGATATCATC-3' with amplicon size of 144 bp.

Total RNA (1 μ g) was used to synthesize full-length cDNA using the Versco™ cDNA kit (Applied Biosystems, USA). Primers and cDNA concentrations used for the reactions were predetermined as described to enable linear and high efficient response (<http://www.abgene.com/downloads/article-SYBRoptimise.pdf>). Reaction mixture contained forward and reverse primers and Power SYBR Green PCR Master mix (Applied Biosystems, USA) in a 10 μ L total sample volume. Reactions were analyzed on a Rotor-Gene 3000 PCR machine (Corbett Life Research, Australia) using 35 cycles of 95 °C for 10 s, 60 °C for 15 s, 72 °C for 20 s, and 80 °C for 10 s. Data obtained were analyzed with Rotor-Gene 6 software. The qBase quantification Software (<http://medgen.ugent.be/qbase/>) was used and data are expressed according to the delta-delta-Ct method. Each biological sample was examined in duplicate with two-three technical replicates. Gene-specific oligonucleotide primers were designed using Primer Express® version 2.0 software (Applied Biosystems, USA).

3. Results

3.1. Characterization of the phenotypes of 'Oded' and 'Heremoza' peaches

'Oded' peaches after 3 weeks 5 °C and SL had expressible juice similar to that of unstored fruit held for 3 d at 20 °C, while 'Heremoza' peach fruit had very low expressible juice (Table 1). After storage and SL, the 'Heremoza' fruit were woolly, while no wooliness developed in 'Oded' fruit when observed visually and determined organoleptically (data not shown). The 'Heremoza' fruit were firmer than the 'Oded' fruit at all the stages except after 3 weeks of storage (Table 1). Based on these results 'Oded' can be classified as 'resistant' to wooliness, and 'Heremoza' as a 'susceptible' cultivar.

Table 1

Fruit firmness and expressible juice (EJ) in the peaches 'Heremoza' and 'Oded' fruits at harvest, after harvest and shelf-life (SL; 3 d at 20 °C), after cold storage (CS; 5 °C, 3 weeks), and after CS plus shelf-life (SL; 3 d at 20 °C). Data are mean values $\pm$ SD of 10–12 individual fruit.

Sample	Harvest	Harvest and SL		3 weeks CS	3 weeks CS and SL	
	Firmness ^a (N)	Firmness (N)	EJ (%)	Firmness (N)	Firmness (N)	EJ (%)
Oded	51.8 $\pm$ 1.6	15.4 $\pm$ 0.9	51.6 $\pm$ 5.1	19.8 $\pm$ 1.1	6.7 $\pm$ 0.3	60.7 $\pm$ 8.0
Heremoza	61.2 $\pm$ 2.1	20.6 $\pm$ 5.9	56.4 $\pm$ 1.3	26.2 $\pm$ 9.7	8.1 $\pm$ 4.9	17.5 $\pm$ 9.8

^a Firmness measured in Newton (N).

3.2. Changes in cell wall proteins of 'Heremoza' and 'Oded' peaches after exposure to cold storage

In 'Heremoza' peaches we examined the changes in cell wall protein abundance at harvest, shelf-life after harvest (SL; 3 d at 20 °C), and in healthy fruit and fruit with internal breakdown after 3 weeks cold storage at 5 °C (Fig. 1). The same time points were examined in 'Oded' peach, with the addition of shelf-life after storage (Fig. 2). Silver stained 2D-PAGE gels showed spots at *pI* values ranging from 3.0 to 10.0, and molecular masses of 10–60 kDa (Figs. 1 and 2). The majority of the protein spots in the gels of the fruit tissue were below 30 kDa in molecular masses.

In the 2D-PAGE gels of cell wall proteins from 'Heremoza' fruit tissue (Fig. 1) at different stages, more than 100 spots were shown by silver nitrate staining. Compared to proteins seen at harvest (Fig. 1A), 9 spots either appeared or increased in abundance after cold storage in healthy fruit tissue (Fig. 1C), and among these only 4 spots were abundant in fruit tissue with internal breakdown (Fig. 1D). All the 9 spots were identified as either TLP1 (GI no. 25091405) or TLP2 (GI no. 25091406) from *Prunus persica* with *pI* values ranging from 3.0 to 9.8, and molecular masses of 18–60 kDa (Table 2).

Most of the proteins seen in the cell wall at harvest (Fig. 1A), were still present in cell walls after SL without storage and following cold storage, but disappeared in cell wall proteins of mealy fruit (Fig. 1). These proteins were for the most part unknown proteins

(Table 3). An unknown protein (GI no. 148807158) from *Prunus dulcis* represented major peptides with *pI* values ranging from 7.0 to 9.0, and molecular masses of 10–28 kDa (Table 3). In addition, a peroxidase (GI no. 82698813) from *Sesamum indicum* and a hypothetical protein (GI no. 147782384) from *Vitis vinifera* were also found, with low peptide coverage (%) and *pI*/molecular masses (kDa) values of 9.0/28 and 4.1/20, respectively (Table 3). A similar change in the composition of the cell wall proteins was found in 'Oded' fruit when comparing harvest to storage (Fig. 2).

In the case of 'Oded' cell walls, 50 spots were abundant at different stages as identified by silver staining (Fig. 2). Similar to the 'Heremoza' fruit tissue, after cold storage the majority of peptides present in abundance in the cell walls were from the thaumatin family (Fig. 2C). These peptides remained present when the fruit were held at SL following storage (Fig. 2D).

3.3. Change in total proteins of 'Oded' peach after exposure to cold storage

Using a phenol based protocol for extraction of total proteins from the peach fruit tissue, we obtained high protein yields and clean 2D-PAGE gels. In the gels of 'Oded' peach (Fig. 3) from total proteins at different stages, more than 300 spots were shown by silver nitrate staining. In the examined gels of the 'Oded' fruit tissue the majority of the protein spots were above 20 kDa in molecular masses.

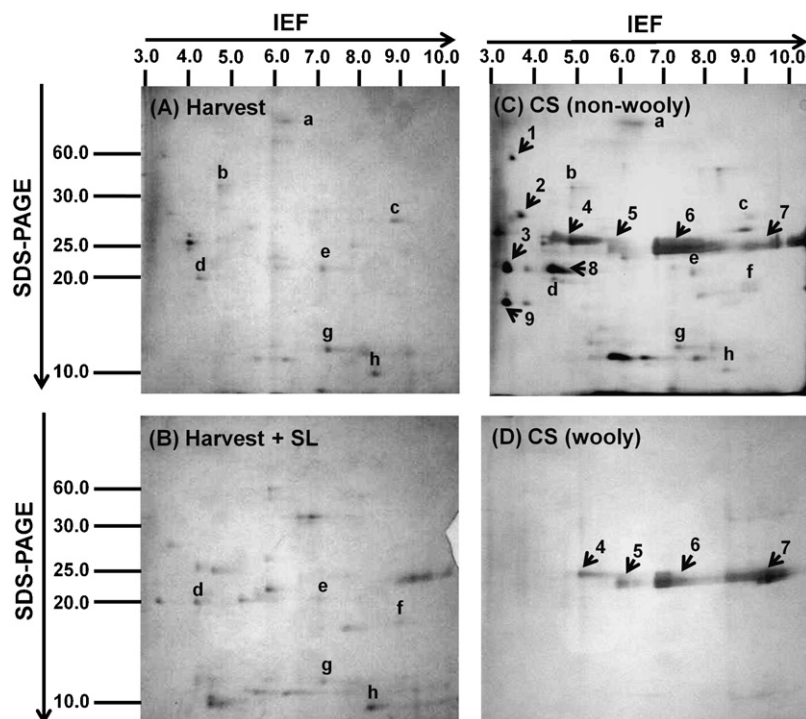


Fig. 1. Silver-stained 2D gels of cell wall proteins (CWP) extracted from 'Heremoza' peach tissues at harvest and after cold storage (CS). (A) At harvest; (B) at harvest + shelf-life (SL); (C) after CS, (non-woolly fruit tissue); (D) after CS, (woolly fruit tissue). CS was for 3 weeks at 5 °C and SL was for 3 d at 20 °C. Black arrows indicate the spots that increased in abundance compared to harvest. Alphabets indicate spots that were common in most panels.

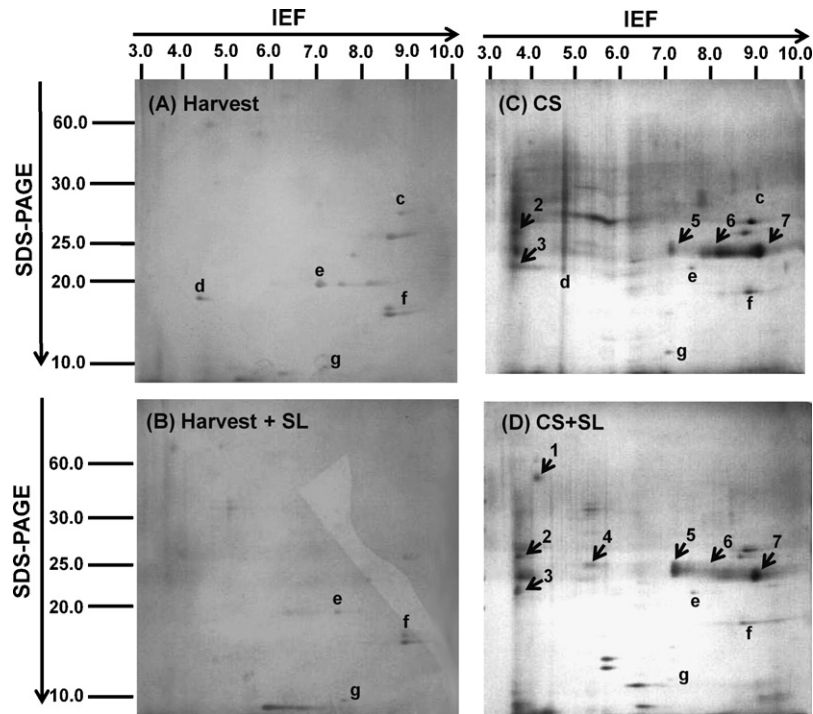


Fig. 2. Silver-stained 2D gels of cell wall proteins (CWP) extracted from the 'Oded' peach fruit tissue at harvest and after cold storage (CS). (A) At harvest; (B) at harvest + shelf-life (SL); (C) after CS; (D) after CS + SL. CS was for 3 weeks at 5 °C and SL was for 3 d at 20 °C. Black arrows indicate the spots that increased in abundance compared to harvest. Alphabets indicate spots that were common in most panels.

Table 2

Identification of cell wall proteins (CWP) in the peaches 'Heremoza' and 'Oded' fruit tissues (Figs. 1 and 2) accumulated at harvest, harvest and shelf-life (SL; 3 d at 20 °C) after cold storage (CS; 5 °C, 3 weeks), and after CS and SL.

Spot no. ^a	GI no. ^b	Protein identification	Organism	MP ^c	SC (%) ^d	M_r/pI (obs.) ^e
1	25091406	Thaumatococcus-like protein 2 precursor	<i>Prunus persica</i>	2	14	60/3.3
2	25091405	Thaumatococcus-like protein 1 precursor	<i>Prunus persica</i>	2	18	28/3.5
3	25091406	Thaumatococcus-like protein 2 precursor	<i>Prunus persica</i>	6	45	20/3.0
4	25091405	Thaumatococcus-like protein 1 precursor	<i>Prunus persica</i>	4	16	26/5.0
5	25091405	Thaumatococcus-like protein 1 precursor	<i>Prunus persica</i>	8	41	25/6.0
6	25091405	Thaumatococcus-like protein 1 precursor	<i>Prunus persica</i>	8	41	25/7.3
7	25091405	Thaumatococcus-like protein 1 precursor	<i>Prunus persica</i>	6	25	25/9.8
8	25091406	Thaumatococcus-like protein 2 precursor	<i>Prunus persica</i>	6	45	22/4.3
9	25091406	Thaumatococcus-like protein 2 precursor	<i>Prunus persica</i>	2	14	18/3.0

^a Spot numbers corresponds to the 2D-gels in Figs. 1 and 2.

^b Gene index number in NCBI non-redundant (nr) database and identified species name.

^c Matched peptides' numbers.

^d Percentage coverage of the matched sequence.

^e Observed molecular mass/ pI .

Table 3

Identification of cell wall proteins (CWP) in 'Heremoza' and 'Oded' peaches fruit tissues (Figs. 1 and 2) that were common at harvest, harvest and shelf-life (SL; 3 d at 20 °C), after cold storage (5 °C, 3 weeks) and after CS plus SL.

Spot no. ^a	GI no. ^b	Protein identification	Organism	MP ^c	SC (%) ^d	M_r/pI (obs.) ^e
c	82698813	Peroxidase and	<i>Sesamum indicum</i>	3	13	28/9.0
	148807158	Unknown protein	<i>Prunus dulcis</i>	2	16	28/9.0
d	147782384	Hypothetical protein	<i>Vitis vinifera</i>	2	2	20/4.1
e	148807158	Unknown protein	<i>Prunus dulcis</i>	5	40	21/7.0
f	148807158	Unknown protein	<i>Prunus dulcis</i>	3	24	18/9.0
g	148807158	Unknown protein	<i>Prunus dulcis</i>	5	40	12/7.2
h	148807158	Unknown protein	<i>Prunus dulcis</i>	2	14	10/8.5

^a Spot numbers corresponds to the 2D-gels in Fig. 1.

^b Gene index number in NCBI non-redundant (nr) database and identified species name.

^c Matched peptides' numbers.

^d Percentage coverage of the matched sequence.

^e Observed molecular mass/ pI .

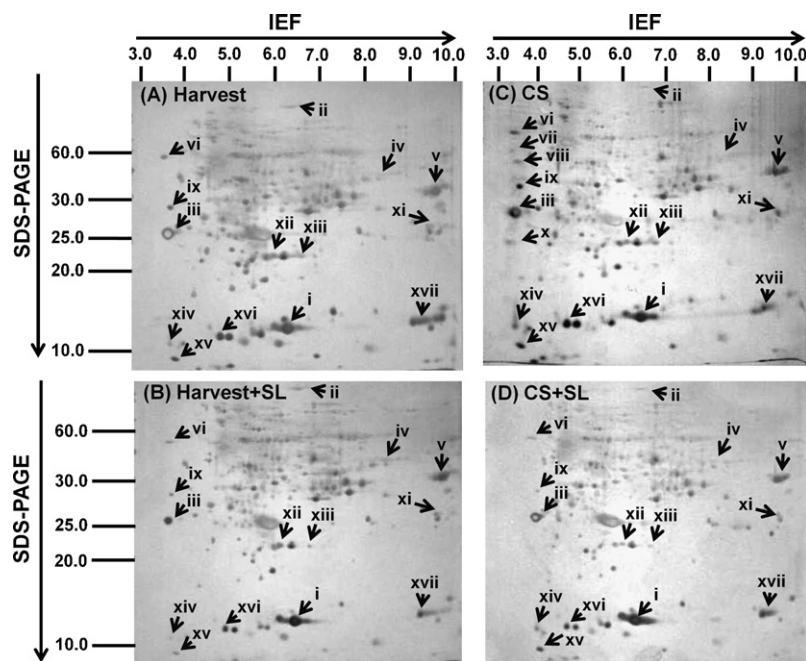


Fig. 3. Silver-stained 2D gels of total proteins (TP) extracted from the 'Oded' peach fruit tissue at harvest and after cold storage (CS). (A) At harvest; (B) at harvest + shelf-life (SL); (C) after CS; (D) after CS + SL. CS was for 3 weeks at 5 °C and SL was for 3 d at 20 °C. Roman numerals are spots that were sequenced and described in Tables 4A and 4B.

The different classes of proteins that were common at all four stages examined include stress-induced proteins (PR proteins: GI no. 159794689 and 159794693 from *Prunus domestica*), a ripening-related protein (endo-polygalacturonase: GI no. 85680278 from *P. persica*), photosynthesis (ATP synthase: GI no. 57013987 from *Nicotiana tabacum*), gluconeogenesis (malate dehydrogenase: GI no. 5123836 and 114479586 from *N. tabacum* and *Citrus junos*, respectively), citric acid cycle (aconitase: GI no. 34851120 from *Prunus avium* and isocitrate dehydrogenase: GI no. 15982950 from *P. per-*

sica), glycolysis (triosephosphate isomerase: GI no. 136057 from *Coptis japonica*), allergen proteins (GI no. 82492265, 4887129 and 44409474 from *P. persica*, *Prunus armeniaca* and *P. avium*, respectively), and unknown and hypothetical proteins (GI no. 148807158, 147797489 and 118482100 from *P. dulcis*, *P. armeniaca* and *Populus trichocarpa*, respectively) with *pI* values ranging from 3.4 to 9.6, and molecular masses of 12–80 kDa (Fig. 3 and Table 4A). Compared to proteins seen at harvest (Fig. 3A), only a few spots either appeared or increased in abundance after cold storage (Fig. 3C and D and

Table 4A

Identification of proteins that were common at harvest, harvest and shelf-life (SL; 3 d at 20 °C), after cold storage (CS; 5 °C, 3 weeks), and after CS and SL. Data was obtained from total proteins (TP) in 'Oded' peach fruit tissues (Fig. 3).

Spot no. ^a	GI no. ^b	Protein identification	Organism	MP ^c	SC (%) ^d	<i>M_r</i> / <i>pI</i> (obs.) ^e
i	82492265	Major allergen and	<i>Prunus persica</i>	11	71	12/6.2
	159794689	PR ^f	<i>Prunus domestica</i>	11	71	12/6.2
ii	34851120	Putative aconitase	<i>Prunus avium</i>	18	19	80/6.5
iii	4887129	Putative allergen protein	<i>Prunus armeniaca</i>	5	29	26/3.8
iv	222855635	Predicted protein	<i>Populus trichocarpa</i>	11	22	40/8.5
	222846542	Predicted protein and	<i>Populus trichocarpa</i>	6	10	40/8.5
	85680278	Endo-polygalacturonase	<i>Prunus persica</i>	3	7	40/8.5
v	148807158	Unknown protein and	<i>Prunus dulcis</i>	7	43	30/9.6
	5123836	Malate dehydrogenase and	<i>Nicotiana tabacum</i>	2	6	30/9.6
	114479586	Malate dehydrogenase	<i>Citrus junos</i>	2	6	30/9.6
vi	57013987	ATP synthase	<i>Nicotiana tabacum</i>	5	11	70/3.4
xii	147797489	Hypothetical protein	<i>Prunus armeniaca</i>	10	39	22/6.0
xiii	136057	Triosephosphate isomerase	<i>Coptis japonica</i>	3	16	22/6.8
xvi	44409474	Major cherry allergen	<i>Prunus avium</i>	7	42	12/4.8
xvii	118482100	Unknown protein and	<i>Populus trichocarpa</i>	4	20	15/9.1
	159794693	PR10 ^f	<i>Prunus domestica</i>	4	32	15/9.1

^a Spot numbers corresponds to the 2D-gels in Fig. 3.

^b Gene index number in NCBI non-redundant (nr) database and identified species name.

^c Matched peptides' numbers.

^d Percentage coverage of the matched sequence.

^e Observed molecular mass/*pI*.

^f Pathogenesis-related.

Table 4B

Identification of total proteins (TP) in 'Oded' peach fruit tissues (Fig. 3): proteins seen in greater abundance after cold storage (CS; 5 °C, 3 weeks) compared to other times of examination.

Spot no. ^a	GI no. ^b	Protein identification	Organism	MP ^c	SC (%) ^d	M _r /pI (obs.) ^e
vii	15982950	NADP-dependent isocitrate dehydrogenase	<i>Prunus persica</i>	12	31	60/3.3
viii	10180029	Putative protein disulfide-isomerase	<i>Prunus avium</i>	8	27	50/3.4
ix	82568691	2-Oxoacid-dependent dioxygenase	<i>Prunus mume</i>	5	20	40/3.5
x	108864390	Putative 40S ribosomal protein S5	<i>Oryza sativa</i>	2	17	25/3.3
xi	25091405	Thaumatococcus-like protein 1 precursor	<i>Prunus persica</i>	3	13	25/9.8
xiv	30421435	Calmodulin	<i>Pyrus communis</i>	9	49	12/3.5
xv	24473796	60 s acidic ribosomal protein	<i>Prunus dulcis</i>	5	58	10/3.6

^a Spot numbers corresponds to the 2D-gels in Fig. 3.

^b Gene index number in NCBI non-redundant (nr) database and identified species name.

^c Matched peptides' numbers.

^d Percentage coverage of the matched sequence.

^e Observed molecular mass/pI.

Table 4B), representing protein classes that include intracellular signaling (calmodulin: GI no. 30421435 from *Pyrus communis*), protein folding (disulfide-isomerase: GI no. 10180029 from *P. avium*), ethylene biosynthesis (2-oxoacid-dependent dioxygenase: GI no. 82568691 from *Prunus mume*). Moreover, a selective analysis of some of the major peptides from the gels also showed the increase in appearance of a TLP1 (GI no. 25091405) from *P. persica* after cold storage (Fig. 3C, spot no. xi).

3.4. Expression of TLP1 transcript during cold storage and shelf-life

To examine if high levels of TLP1 proteins in the cell wall coincided with higher expression levels, qRT-PCR analysis of *TLP1* gene was performed. *TLP1* gene was up-regulated in fruit tissues of both peaches during cold storage (Fig. 4), while the transcript levels decreased in SL following storage (data not shown). The rate of increase during storage was quite different in the resistant cultivar Oded in comparison to the sensitive cultivar 'Heremoza'. In 'Oded' the TLP1 transcript increased greatly after 1 week and 2 weeks of cold storage at 5 °C while the transcript levels in 'Heremoza' increased only following 3 weeks of the storage when the tissue were already injured (Fig. 4).

4. Discussion

Low temperature stress affects the function and survival of all organisms to various extents. In several plants, changes in gene expression, and assembly and degradation of proteins have been reported in response to variations in environmental conditions, including cold stress (Guy et al., 1985; Mohapatra et al., 1987; Durham et al., 1991; Cregoe et al., 1993). Levitt (1980) reported increase in freezing tolerance of many plant species when exposed to low but above the freezing point temperatures. A similar adaptation strategy has been found for plants sensitive to low temperature (Cui et al., 2005; Shi et al., 2008). Recently, such changes have been reported in peach fruit, where transcriptional profiling analysis of cold-responsive genes in peach fruit using DNA microarrays discovered differentially expressed genes in the cold-treated fruit mesocarp tissues (Ogundiwin et al., 2008; Vizoso et al., 2009). However, it has not been elucidated how change in genes expression, transcription of new genes and assembly of specific proteins cause improved cold tolerance, even though much research has been conducted to identify and analyze cold-responsive genes and to understand their function and regulation (Mohapatra et al., 1989; Cattivelli and Bartels, 1990; Hajela et al., 1990; Cregoe et al., 1993; Ogundiwin et al., 2008).

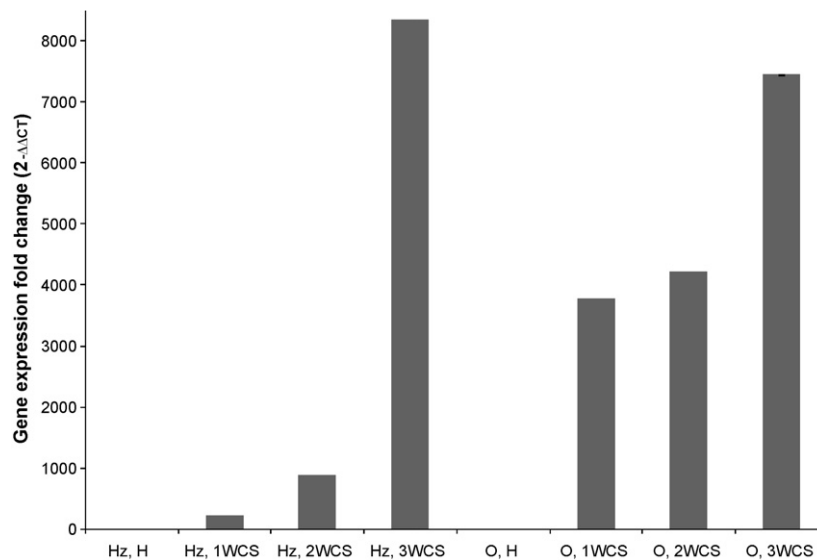


Fig. 4. Response to cold treatment and subsequent shelf-life of *Thaumatococcus-like 1 protein precursor* (*TLP1*) transcript. Relative transcript levels of the *TLP1* were determined in Hz, 'Heremoza' peach; O, 'Oded' peach at harvest (H) and weeks after cold storage at 5 °C (WCS). The data represent the mean value ± SD. The gene expression level was normalized against peach *Initiation Factor eIF-4-Gamma*. Small SD bars are hidden by the vertical bars.

Proteins with amino acid sequences similar to thaumatin have been reported in most studied plant species and termed thaumatin-like proteins (TLP). TLP are high-molecular-weight proteins that are classified as class 5 pathogenesis-related proteins (PR-5) (Hiroyuki and Terauchi, 2008). TLP are divided in two subcategories; one basic form found in vacuole and another acidic form that is apoplasmic (Stintzi et al., 1993; Fils-Lycaon et al., 1996). TLP are encoded by a multigene family; in the *Arabidopsis thaliana* genome there are 26 different annotated TLP. Members of the TLP family have also been discovered in animals, for example, in the desert locust *Schistocerca gregaria* (Brandazza et al., 2004). However, the majority of TLP lack the characteristic sweet taste and high *pI* value of thaumatin.

TLP are reported to have diverse functions; among them antifungal activity, antifreeze activity, and involvement in fruit ripening. In a few cases their action against pathogenic microorganisms has been associated with endo- α -1,3-glucanase activity (Grenier et al., 1999; Menu-Bouaouiche et al., 2003) and α -amylase inhibiting properties (Franco et al., 2002). However, membrane-permeabilizing activity has also been reported (Koiwa et al., 1999). A TLP from ripe fruit of emperor banana (*Musa basjoo* cv. Emperor Banana) inhibited mycelial growth of *Fusarium oxysporum* and *Mycosphaerella arachidicola* (Ho et al., 2007). A potential role of TLP as antifreeze substances has been demonstrated in plants (Hoshino et al., 1999). In another study, antifreeze proteins from cold-acclimated winter rye (*Secale cereale* L.) leaves were found to be similar to members of PR proteins including endochitinases, endo- α -1,3-glucanases, and TLP (Hon et al., 1995). TLP are known to be expressed in fruit and seeds (Neale et al., 1990; Jayasankar et al., 2003) and accumulate in a ripening specific manner in banana, cherry, pepper, and grape (Vu and Huynh, 1994; Fils-Lycaon et al., 1996; Tattershall et al., 1997; Barre et al., 2000; Kim et al., 2002).

Until now, to our knowledge, no one has employed a proteomic approach to study chilling stress in stone fruits at the cell wall protein level, and, therefore, the present study provides the first fundamental cell wall proteomic analyses of peach fruit tissue to low temperature. We have optimized a protocol for isolating cell wall bound proteins from frozen peach fruit tissue with minimum contaminating cytosolic proteins, and indeed no cytosolic protein has been identified. Most of the proteins present in the cell wall of stored fruit either belong to the TLP family or are similar to unknown proteins reported mainly from *Prunus* sp., but also from *V. vinifera* and *P. trichocarpa*. The protocol involved cell disruption, a series of stringent washes to remove contaminating proteins, and extraction of cell wall proteins with CaCl_2 and LiCl . The difficulties associated with polysaccharides and polyphenolics removal, which impeded the focusing in the first dimension and caused streaking and background staining in the second dimension have been minimized with a series of rigorous washes, resulting in protein preparations that are convincingly free of contaminating materials and contain a considerable number of proteins. Most of the protein spots were easily identified by the reverse HPLC-MS/MS following silver staining.

Although most of the proteins seen in cell walls of fruit at harvest were still present if they were ripened without storage, following storage a different group of cell wall proteins appeared. The majority of the new peptides present in cell walls after storage were from the thaumatin family, and identified as either TLP1 or TLP2 (Figs. 1 and 2C and D, and Table 2).

In comparing total cellular proteins in 'Oded' fruit seen at harvest (Fig. 3A) and after cold storage (Fig. 3C), not many differences and changes were reported, only a few spots either appeared or increased in abundance, and TLP1 was found to increase in cold storage. While TLP1 in total protein extract constitute a small fraction of the total proteins, it comprises the majority of proteins in cell walls.

Expression analysis using qRT-PCR showed differing kinetics of transcript accumulation of the *TLP1* gene during cold storage of the resistant and sensitive cultivars (Fig. 4). Similar results of transcript abundance were reported in another peach study where the relative expression of the *TLP1* gene increased following 1 week of cold storage at 5 °C (Ogundiwin et al., 2008). In peaches stored at 5 °C for 21 d, four thaumatin-like genes were among 164 cold-induced genes (Tittarelli et al., 2009). The early accumulation of the *TLP1* transcript in the CI-resistant peach in comparison to sensitive cultivar, suggests that *TLP1* (and perhaps *TLP2*) might be involved in protecting against CI.

Overall, the data presented in this study reveal similar responses of TLP accumulation in two different peach cultivars (late-season and early-season variety) in response to 3 weeks cold storage/low temperature stress. Although TLP accumulate to high levels in cell walls of CI-sensitive 'Herzozeta', the kinetics of transcript accumulation suggest late appearance of this protein family in storage might be part of the reason that these fruit develop woolliness. In contrast, the early appearance and high level of TLP transcript in the resistant cultivar, 'Oded', might be involved in shielding the fruit from the dramatic cell wall-structure changes that accompany the onset of CI in stonefruit. Further studies are needed to investigate the precise role and mode of action of the TLP and other reported unknown cell wall proteins for their involvement in cell wall changes of cold-stored peaches showing CI symptoms.

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