Final project report **Year 2/2**

WTFRC Project#: PH-04-0444 **Organization project:** AFHRC-53086

Project Title: Proteomic approach to study scald disorder of apples

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Objectives:

To improve our fundamental understanding of scald disorder, the objectives of this study were:

1) Using proteomic tools, characterize proteins associated with scald development in scald susceptible apple cultivars.

2) Map protein changes associated with factors that impart scald resistance including ripeness and treatment with DPA or 1-MCP.

3) Identify proteins that are responsible for scald development and resistance and propose metabolic pathways involved.

Significant findings (2004):

1) Improved the methodologies of 2-dimensional electrophoresis (2-DE) analysis and characterization of proteins in apples.
2) Significant development of scald was found in untreated "Red Delicious" apples during storage. Treatment with 1-MCP and DPA inhibited scald development in all tested apples.
3) Using proteomic tools, characterized proteins associated with scald development in the scald susceptible apple cultivar "Red Delicious".

Significant findings (2005):

1) Data analysis from experiments conducted in 2004-2005 was completed. The analysis of 2-DE gels, mapped apple proteins and identified up- and down-regulated proteins associated with control and treatments with DAP and 1-MCP were conducted. At day 0, a total of 16, 28 and 29 proteins were found to be in specially associated with the scald, DPA and 1-MCP treatment, respectively. Among them, 5, 17 and 17 proteins from the control, DPA and 1-MCP treatments, respectively were identified. Four proteins were found to be present in fruit treated with DPA or 1-MCP. At day7, 134 proteins were found to be specific to the DPA treatment, while 71 proteins were common to both the DPA and 1-MCP treatments.
2) In 2005-2006, two additional proteomic studies were conducted to analyze 2-DE gels, mapped apple proteins and identify up- and down-regulated proteins associated with control, DAP and 1-MCP treatments.
First, we characterized proteins profiles in scald and non-scald tissues. Gel analysis identified 211 proteins in non-scald tissues, while 90 proteins in scalded tissues. Among them, 69 proteins in non-scald tissues and 54 in scalded tissues were present with a normalized intensities great enough to be identified. In total, 114 protein spots were excised from gel and sent for protein identification.
3) A chemiluminescence detection based technology was developed to measure the H_2O_2 concentration in peel and flesh tissues of apples.
4) Established collaborations with Drs. Beaudry at MSU and Zhu at USDA to conduct gene expressions studies in association with scald development using micro-array and substractive hybridization expression techniques.

Materials and Methods:

Apple fruit

2004: "Cortland", "Law Rome" and "Red Delicious" were included in this year's study. Fruit were harvested before the climacteric stage, with internal ethylene concentration below 0.1-0.2 ppm (one week before commercial harvest). After harvest, fruit were divided into three groups, control, DPA treatment and 1-MCP treatment. DPA (2000 µl/L) and 1-MCP (1.0 µl/L) were applied. Fruit were stored under cold air and CA (3.0 kPa +1.0 kPa CO_2) at 0-1°C for 4 and 6 months. Scald development was evaluated immediately after removal from storage, or after an additional 7 days at

22 °C. Scald was rated using a scale of 1 to 5: 1=no scald; 2=1-10%; 3=11-33%; 4=34-66 %; and 5=67-100% (Whitaker, et al., 2000). Two sub-samples of peel from eight apples were taken from each treatment group. Peels were quick frozen in liquid N_2, and stored at -86 °C for further analysis. 2005: Further research in 2005-2006 was concentrated on the "Red Delicious" cultivar with a new experimental design. Fruit were obtained from two orchards and two harvest maturities (early and late, one week before commercial harvest, before the climacteric stage, with internal ethylene concentration below 0.1-0.2 ppm and post-climacteric stage). After harvest, fruit were divided into three groups, control, DPA treatment and 1-MCP treatment. DPA (2000 µl/L) and 1-MCP (1.0 µl/L) were applied. Fruit were stored under cold air at 0-1°C for 5 months. Scald development was evaluated immediately after removal from storage, or after an additional 7 days at 22 °C as described above. Three sub-samples of peel from twenty apples were taken from each treatment group. Peels were quick frozen in liquid N_2, and stored at -86 °C for further analysis.

Proteomic analysis

Total protein extraction: In 2004, total protein was extracted from 2.0 g peel samples with 12 mL SDS extraction buffer containing 2% SDS, 20 % glycerol, 60 mM DTT and 40 mM Tris-base buffer (pH 8.5). In 2005, total protein was extracted from ground fruit peel tissue (2.5 g) with a protocol modified phenol-based protocol from Saravanan and Rose (1). The final extract corresponds to the total protein extract and was stored at B86 °C for further analysis.

Protein assay

Protein concentration was measured using the RC DC protein assay kit (Bio-Rad Laboratories), with BSA (bovine serum albumin) as a standard. The protein concentration was expressed as mg/g FWt.

2-D electrophoresis (2-DE)

For 2-DE analysis, proteins were first separated by iso-electrofocusing (IEF). IEF was carried out with the IPG Phor System II (Amersham Bioscience) using Immobiline DryStrip gels (18 cm) with non-linear pH gradients (pH 3-11 NL) according to the manufacturer=s instruction. 80.0 µg protein was resolublized in DeStreak Rehydration solution (Amersham Biosciences) loaded onto the gel and incubated overnight or for 10 h at room temperature using the in-gel rehydration procedure. After the first dimension separation, second dimension separation was conducted on large format 12.5% acrylamide SDS-PAGE gels (24 cm x 18 cm) on an Ettan Dalt*six* multi gel system (Amersham Biosciences) for 30,000VH at 20°C. After 2-D electrophoresis, gels were placed in a protein fixation solution in preparation for staining. Fluorescent stain (SYPRO Ruby, Bio-Rad Laboratories) was applied to visualize proteins.

Image analysis and protein identification

2-DE gel images were captured with a digital camera and analyzed using computer assisted image analysis software (PDQuest Version 7.4, Bio-Rad Laboratories). Spot detection, quantification and gel comparisons were conducted. Spots that changed in association with scald development were selected and cut from gels to send for MS identification.

Protein identification:

Excised spots were identified by NanoSpray LC/MS/MS (3200 Q Trap LC/MS/MS system. Mass spectrometry was performed on a hybrid quadrupole linear ion trap (Q-TRAP LC/MS/MS, Applied Biosystems, Foster City, CA, USA) equipped with a nanospray ion source at the DalGEN Proteomics Core Facility, Atlantic Research Centre, Dalhousie University, Nova Scotia. The raw MS/MS data were searched against NCBI viridiplantae entries, 278115 sequences, updated Nov 7th 2006 (NIH, Bethesda, MD, USA) using the MASCOT algorithm (Matrix Science, London, UK). The MS and MS/MS mass tolerance was 0.8 and 0.5 Da respectively and one missed cleavage was allowed.

Carboxamidomethyl cysteins and oxidized methionines were set as variable modifications. Proteins with significant peptide matches were selected for error tolerant searching. The data was also searched against the SwissProt database, 234112 sequences, updated Dec 11th 2006 (Sprot version 50.8) to ensure no peptides from trypsin or keratin were present. Peptide ion scores greater than 41 indicate identity or extensive homology ($p < 0.05$) and are referred to as significant hits. Peptides below the significance threshold were only reported where other significant hits to the same protein were present. The Pro ID algorithm (Applied Biosystems, Foster City, CA, USA) was used to search data against the EST database for apple, 195553 entries, updated Dec 19th 2005 and strawberry, 9213 entries, updated Dec 19th 2005 (Genome Database for Rosaceae, Washington State University, Pullman, WA, USA http://www.mainlab.clemson.edu/gdr). Search parameters for Pro ID were the same as for those used with MASCOT. Peptides with a Pro ID confidence value at least 95 were considered significant hits.

Statistical analysis.
Experimental design and data analysis were conducted using GenStat, Release 8.1, (VSN International Ltd., 2005. Lawes Agricultural Trust). ANOVA was conducted from a factorial design with three cultivars, three treatments, two storage conditions, two removals, two evaluations and two sub-samples for 2004 and with one cultivar, two locations, two harvests, three treatments, and two removals for 2005.

Results and Discussion
2004

Incidence of scald disorder. Scald development in three apple cultivars was evaluated for "Red Delicious", "Cortland" and Law Rome". Significant scald developed only on the "Red Delicious" with a scald index rating between 1-2 after 4 and 6 months storage. There was a slight increase in scald development from 4 to 6 months, while there was no significant increase of scald following 7 days at room temperature after storage. Treatment with DPA and 1-MCP inhibited scald development (Tab. 1). The low scald development on "Cortland" and "Law Rome" may be due to the relative cool season last year. Therefore, our further analyses have been focusing on "Red Delicious".

Protein quantification and SDS-PAGE: With the standardized protein procedure developed previously, proteins were successfully extracted from all samples. More than one hundred sharp bands were seen on the SDS-PAGE gels with no evidence of protein breakdown or contamination (data not shown). This procedure ensured that our protein extraction procedure was successful and reliable.

Improvement of methodology for 2-DE protein analysis: Several improvements have been archieved during this study, including maximizing protein extraction capacity while maintaining good protein quality, analyzing proteins on large scale SDS-PAGE gels and analyzing proteins qualitatively and quantitatively after fluorescence staining (Sypro Ruby). Similar sensitivity to silver stain was achieved by the use of a modified fluorescence stain. This protocol allows us to quantitatively detect ~1-10 ng protein. More than 900 polypeptides (spots) from apple fruit are shown on a 2-DE SDS-PAGE gel (Fig.1), some of which have been identified.

Identification of proteins from 2004 study: Gel analysis of the 2004-2005 study indicated that protein populations and profiles changed significantly among control, DPA and 1-MCP treated apples during storage. To date, we have focused on "Red Delicious" apples under cold storage and have compared scalded fruit (control) with those treated with DPA or 1-MCP after 6 months storage. Apple peel proteins were separated by 2-DE and gels stained with SYPRO Ruby. Based on the protein extraction protocol used in 2004, protein profiles from control fruit (with scald), DPA and 1-

MCP treated fruit are shown in Fig 1. (1A, 1B, and 1C). A total of 900, 851, and 954 spots have been visualized for the control, DPA and 1-MCP treated fruits, respectively. Substantial quantitative and qualitative differences can be seen. At day 0, there were 16, 28 and 29 proteins associated with the scald, DPA and 1-MCP treatments, respectively. Among them, three proteins were consistently present in fruit treated with DPA or 1-MCP. At day7, 134 proteins were found to be specific to DPA treatment, while 71 proteins were common to both the DPA and 1-MCP treatments. LC/MS identification was conducted on day 0 samples, and 5, 17 and 17 proteins from control, DPA and 1-MCP respectively, were putatively identified and annotated in Table 3. For control fruit, the identified proteins are endoplamin homolog precursor, malic enzyme, plastid 5, 10-methylene-tetrahyrofolate dehydrogenase and non-symbiotic hemoglobin class 1. In DPA treated apples, NAD binding/ glyceraldehyde-3-phosphate dehydrogenase, pectin methylesterase, malate dehydrogenase, alcohol dehydrogenase, cholorphyll a/b binding enzyme, polyphenol oxidase, heat shock proteins and ATP synthase subunits were identified, indicating that multiple pathways involved in cell wall, chloroplast function, browning and electron transport system may act as protecting mechanism against scald. The occurance of NAD binding/ glyceraldehyde-3-phosphate dehydrogenase, malate dehydrogenase, and alcohol dehydrogenase in DPA treated fruit may be similar to a report that all at these proteins being inhibited or brokendown by H_2O_2 treatment of *Arabidopsis*, and are related to oxidative stress responses in plants (2, 3). 1-MCP treated fruit had four proteins the same as in DPA treated fruit (Fig.1D), which were phosphopyruvate hydratase, pectin methyl esterase, malate dehydrogenase, and alcohol dehydrogenase. In addition, calmodulin binding, sucrose synthase, isopropylmalate synthase, cinnamoyl CoA reductase, glutamate decarboxylase 2 and putative 26S proteasome beta subunit were also identified. Both cinnamoyl CoA reductase and glutamate decarboxylase 2 are also stress response proteins in plants. These findings indicated that additional mechanisms are involved in tissue protection against scald. Among the scald hypotheses, some evidence suggested that oxidation products of (*E.E*)-α-farnesene could be the causal agents of apple scald (4, 5). Other evidence suggests that a general oxidative stress triggered by disruption of mitochondrial electron transport at low temperature results in the production of superoxide, which may lead to scald (6). DPA inhibits oxidation of α-farnesene *in vivo* and *in vitro* and prevent scald development (7, 8). Application of 1-methylcyclopropene (1-MCP) can also prevent scald development in apples (9). The blocking of ethylene action seems to be critical to reduce the production of α-farnesene and the development of scald (10, 11), while the complete prevention of scald by 1-MCP was better correlated with antioxidant capacity (12). At this stage of this study, we speculate that DPA and 1-MCP might have their own unique function against scald, but may also share a common mechanism by protecting fruit tissue from oxidative stress damage caused by H_2O_2 and/or ROS. A much clearer picture will be drawn, when protein identifications from 2005-2006 are completed.

2) Further research in 2005-2006 was concentrated on "Red Delicious" with a new design that included two blocks, two harvest maturities (early and late) and treatments with DAP and 1-MCP. 2005: *Incidence of scald disorder*. Significant scald developed only on the "Red Delicious" with a scald index rating between 4-5 after 5 months storage. There was a significant increase in scald development from 4 to 6 months, while there was no significant increase of scald following 7 days at room temperature after storage. Treatment with DPA and 1-MCP inhibited scald development (Tab.2).

Identification of proteins from 2005study:

In 2005-2006, two proteomic studies were conducted using an improved protein protocol (13). We analyzed 2-DE gels, mapped apple proteins and identified up- and down-regulated proteins associated with control and treatments with DAP and 1-MCP. On average, the number of spots in each proteins populations increased from 900 to 1400. This will allowed us to investigate even broader protein profiles.

First, we characterized proteins profiles in scald and non-scald tissues. Gel analysis was completed and 211 spots were identified to be present in non-scald tissues, while 90 spots were found to be in

scald tissues. Among them, 69 spots in non-scald tissues and 54 in scald tissues were found to be present with a normalized intensity great enough to be excised from gel (Fig.2). A total of 114 protein spots were excised from gels and sent to protein identification by LC/MS in Dec. 2006.

Second, we characterized protein populations and profiles from tissues associated from control and DAP and 1-MCP treatments. Gel analysis of this study is currently being conducted. Spot identifications will be conducted in summer 2007.

Potential outcome and benefits:

1) Application of proteomic technology is a feasible tool to investigate the biochemical mechanisms of apple scald. Hundreds of proteins and enzyme system can be monitored at one time as apples respond to the conditions which cause scald and to the compounds that prevent scald.
2) Investigation on the effects of DPA and 1-MCP treatments to alter protein profiles in apple fruit may help to identify proteins responsible for scald development and/or resistance.

Identification and quantitative analysis of the proteins profiled in scald tissue, combined with the knowledge of previous findings, may help to identify the proteins and possible metabolic pathway(s) involved in the development of the disorder. Understanding the mechanism of scald formation in stored apples and the physiology/biochemistry of resistance could lead to new, effective strategies to reduce or eliminate this disorder. Using this technology on apples may lead to new insights into the long-standing mystery of scald resistance and development.

Project title: Proteomic approach to study scald disorders of apple fruits
PI: Dr. Jun Song
Proposed project duration: Two years (2004-2005)
Current year request (2005): $30,000

Item	Year 1 (2004)		**Year 2 (2005)**	
	WTFRC	AAFC[1]	**WTFRC**	**AAFC**
Salaries[2]	$15,000	$14,000	**$14,000**	**$14,000**
Benefits	$3,000	$3,000	**$3,000**	**$3,000**
Equipment[3]	$5,000	$8,000	**$2,000**	**$7,000**
Supplies[4]	$3,000	$1,000	**$7,000**	**$1,500**
MS identification[5]	$3,000	$2,000	**$4,000**	**$2,000**
Statistics[6]	$0	$0	**$0**	**$500**
Travel[7]	$0	$500	**$0**	**$1,500**
Miscellaneous	$0	$500	**$0**	**$500**
Total	$29,000	$29,000	**$30,000**	**$30,000**

[1]Matching funds in Canadian dollars from Agriculture and Agri-Food Canada's Matching Investment Initiative (Contingent on approval of WTFRC funding). [2]Salary dollars (part) for a postdoctoral research associate for two years (Dr. Zheng, QiFa, start date June 06, 2005). [3]Additional image analysis equipment and upgrades. [4]Fruit, chemicals, gases, electrophoresis, staining supplies. [5]Identification of proteins at proteomic facility. [6]Support for statistical analysis.
[7]Travel to present results of project.

References

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Table.1 Scald disorder in Red Delicious apple fruit at day 0 and 7, after 6 month storage under air and CA in 2004.

	Red Delicious					
	Control		DPA		1-MCP	
4 m	D0	D7	D0	D7	D	D7
	2.0	3.0	1.0	1.3	1.3	1.3
6 m	D0	D7	D0	D7	D0	D7
	2.5	3.0	1.0	1.0	1.0	1.0

Table.2 Scald disorder in Red Delicious apple fruit at day 0 and 7, after 5 month storage under air in 2005.

	Red Delicious					
Early	Control		DPA		1-MCP	
	D0	D7	D0	D7	D0	D7
	1.9	2.0	1.0	1.1	1.8	1.9
Late	D0	D7	D0	D7	D0	D7
	2.1	3.3	1.0	1.0	1.0	1.0

Table 3. Putative identification of protein extracted from "Red Delicious" apple in control and after 1-MCP and DPA treatments. Protein spots excised from gels stained with Sypro Ruby were subjected to digestion with trypsin and identified following mass spectrometry analysis (LC/MS/MS)*. Spot numbers are also shown in the figure 2**.**

Control		DPA		1-MCP	
Spot	Protein name	Spot	Protein name	Spot	Protein name
1	Endoplasmin homolog precursor (GRP94 homolog)	17	High molecular weight heat shock protein (*Malus x domestica*)	18	Phosphopyruvate hydratase (*Arabidopsis thaliana*)
		18	Phosphopyruvate hydratase (*Arabidopsis thaliana*)	35	NAD-dependent malate dehydrogenase (*Prunus persica*)
8	Malic enzyme/ oxidoreductase, acting on NADH or NADPH, NAD or NADP as acceptor (*Arabidopsis thaliana)*	19	Polyphenol oxidase precursor (*Prunus armeniaca*)	37	Allyl alcohol dehydrogenase (*Nicotiana tabacum*)
		20	Os04g0118400 [*Oryza sativa* (japonica cultivar-group)]	46	Pectin methylesterase (*Nicotiana tabacum*)
		21	Putative protein (Arabidopsis thaliana)	49	Phosphopyruvate hydratase (*Arabidopsis thaliana*)
		22	Eukaryotic initiation factor 4A-2 (ATP-dependent RNA helicase eIF4A-2) (eIF-4A-2)	50	Calmodulin binding (*Arabidopsis thaliana*)
		23	glyceraldehyde-3-phosphate dehydrogenase (*Pinus sylvestris*)	51	Sucrose synthase 1 (*Pyrus pyrifolia*)
11	Plastid 5,10-methylene-tetrahydrofolate dehydrogenase (*Prototheca wickerhamii*)			55	Putative 20S proteasome beta subunit 5 (*Prunus persica*)
		25	ATP synthase subunit alpha, mitochondrial	56	Actin (*Pisum sativum*)
		29	NAD binding / glyceraldehyde-3-phosphate dehydrogenase (phosphorylating)/ Glyceraldehyde-3-phosphate dehydrogenase (*Arabidopsis thaliana*)	57	Aminotransferases class-I pyridoxal-phosphate-binding site(*Medicago truncatula*)
				58	Isopropylmalate synthase (*Brassica oleracea*)
12	Prohibitin (ISS) (*Ostreococcus tauri*)	30	Glyceraldehyde-3-phosphate dehydrogenase (*Pinus sylvestris*)	63	Elongation factor 1-alpha (*Hordeum vulgare* subsp. Vulgare)
16	Non-symbiotic hemoglobin class 1 (*Malus x domestica*)	33	Light harvesting chlorophyll a /b binding protein (*Hedera helix*)	64	Unnamed protein product [*Oryza sativa* (japonica cultivar-group)]
		35	NAD-dependent malate dehydrogenase (*Prunus persica*)	67	Acidic chitinase III (*Nicotiana tabacum*)
			Allyl alcohol dehydrogenase (*Nicotiana tabacum*)	68	Cinnamoyl CoA reductase CCR2 (*Arabidopsis thaliana*)
		37	Trypsin inhibitor subtype A (Glycine max)		
		40	(Segment 5 of 10) Putative oxygen-evolving enhancer	70	Putative 20S proteasome beta subunit 5 (*Prunus persica*)
		43	protein 1 (OEE1) (33 kDa subunit of oxygen evol		
			ATP synthase F0 subunit b (*Pseudendoclonium akinetum*)	72	Glutamate decarboxylase 2 (*Brassica juncea*)
		44			
		46	Pectin methylesterase (*Nicotiana tabacum*)		

*Please note that *all* the MS/MS data can be made available as supplementary material in whatever format the WTFRC prefers.

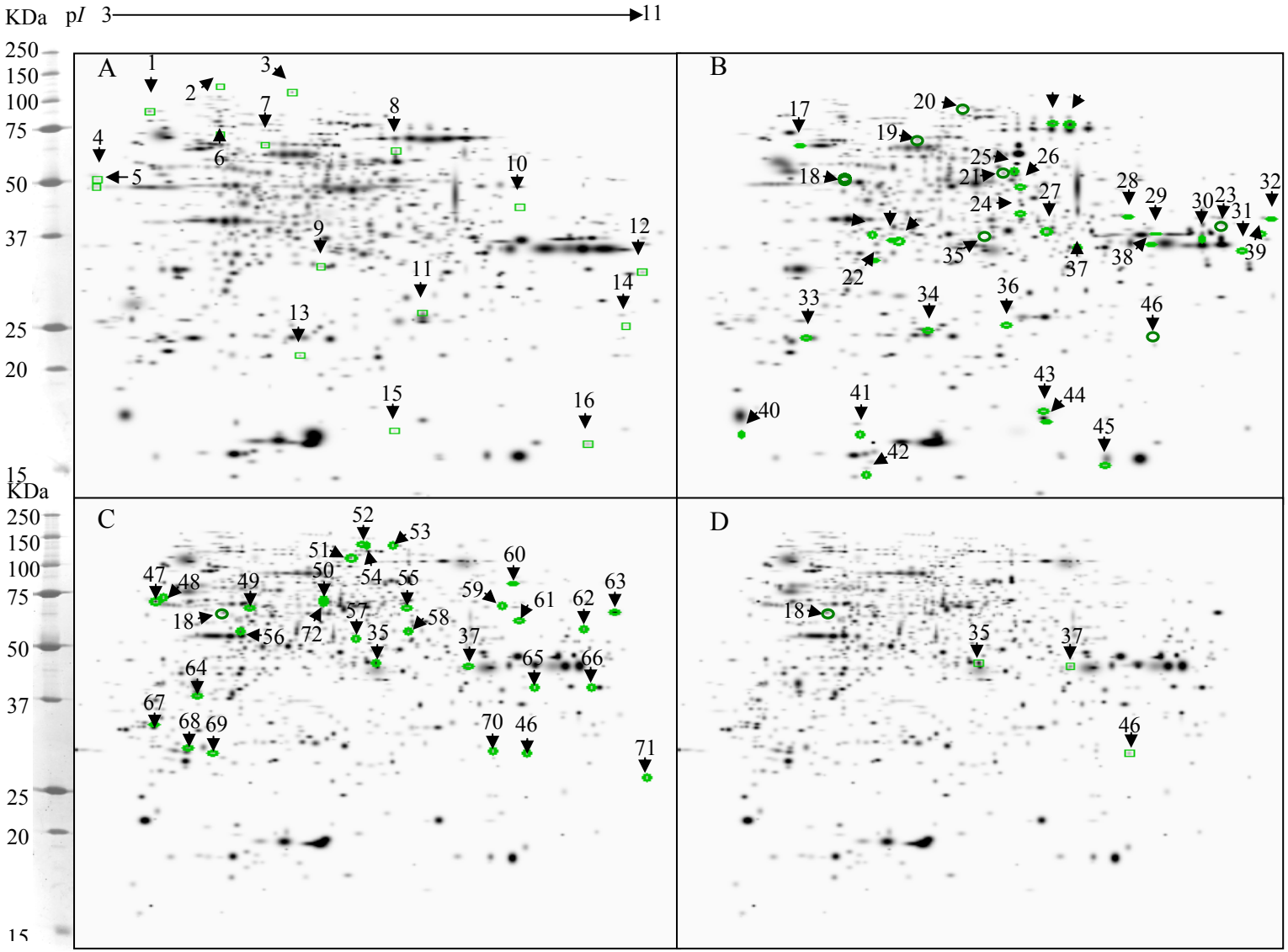

Fig. 1. 2-DE analysis of proteins from apples stored for 6 months under air or CA storage. 80.0 µg protein was loaded onto a 12.5 % polyacylamide large format gel and visualized with SYPRO Ruby stain. The molecular weight of protein standards are indicated on the left. The image is inverted from a dark image with red spots. Arrows indicate proteins identified by LC/MS/MS with corresponding numbers listed in Table 3. A: Control; B: DPA; C: MCP; and D: Proteins present in both DPA and 1-MCP.

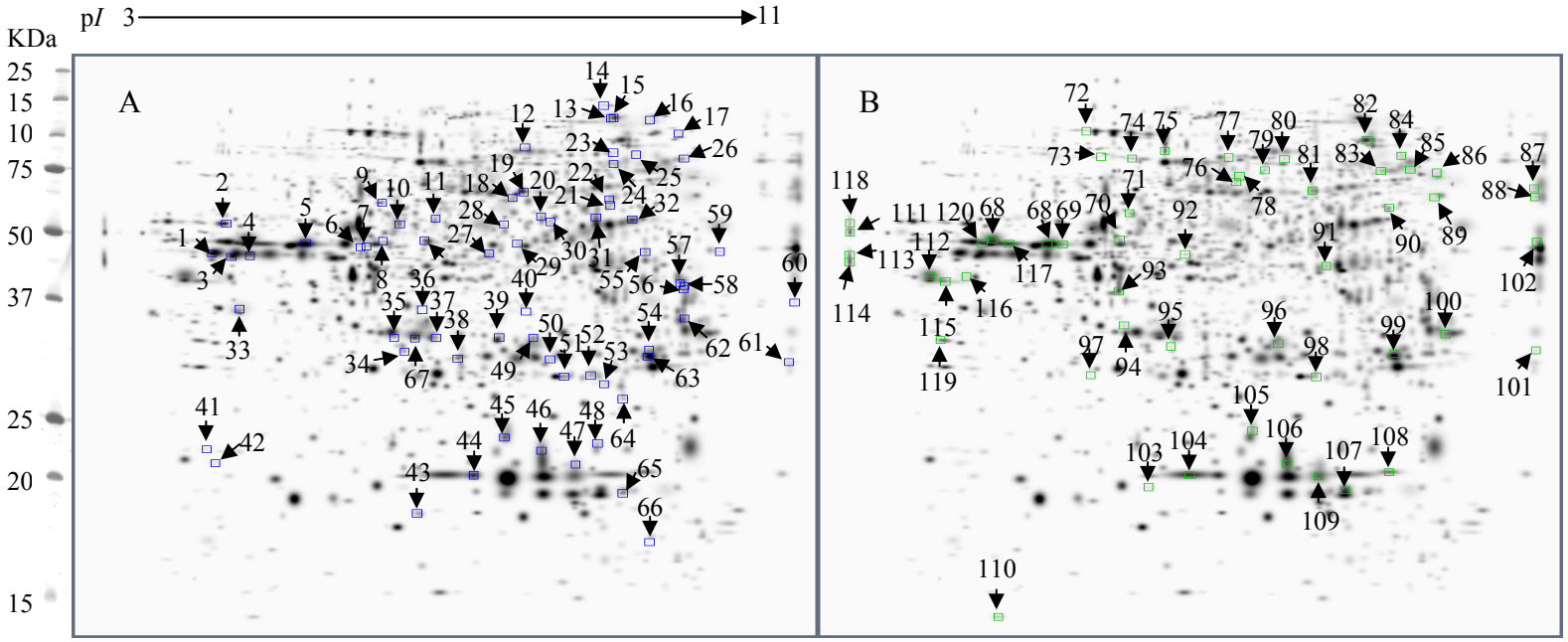

Fig. 2. 2-DE analysis of proteins from apples stored for 6 months under air or CA storage. 100.0 µg protein was loaded onto a 12.5 % polyacylamide large format gel and visualized with SYPRO Ruby stain. The molecular weight of protein standards are indicated on the left. Arrows indicate proteins which are only present in the group and will be identified by LC/MS/MS. A: Only in non-scald tissues; B: Only in scald tissues.