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Overwintering Survival of Strawberry (Fragaria x ananassa): Proteins Associated with Low Temperature Stress Tolerance during Cold Acclimation in Cultivars

For the degree of Doctor of Philosophy

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OVERWINTERING SURVIVAL OF STRAWBERRY (FRAGARIA X ANANASSA): PROTEINS ASSOCIATED WITH LOW TEMPERATURE STRESS TOLERANCE DURING COLD ACCLIMATION IN CULTIVARS

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LIST OF ABBREVIATIONS

ºC	Degree Celsius
2DE	Two-dimensional electrophoresis
2-HPCL	2-hydroxyacyl-CoA lyase
6PGL	6-phosphogluconolactonase
ABA	Abscisic acid
ACN	Acetonitrile
ADH	Alcohol dehydrogenase
ADH-3	Alcohol dehydrogenase class-3
AdoMet synthase	S-adenosylmethionine synthetase
AFP	Antifreeze protein
AGI	Arabidopsis genome initiative
AHC	Agglomerative Hierarchical Clustering
AK	Adenylate kinase
AKR	Aldo-keto reductase
ANOVA	Analysis of variance
ANR	Anthocyanidin reductase
ANS	Anthocyanidin synthase
apgm	2,3-biphosphoglycerate-independent phosphoglycerate mutase
β-1, 3-glucanase	Beta-1,3-glucanase
β-galactosidase	Beta-galactosidase
CAD	Cinnamyl-alcohol dehydrogenase
cAPX	Cytosolic ascorbate peroxidase
CAT	Catalase
CBB	Coomassie brilliant blue
CBF	C-repeat/drought-responsive element binding factor

CBS domain	Cystathionine beta-synthase domain
CCoAOMT	Caffeoyl-CoA 3-O-methyltransferase
CDPK	Calcium-dependent protein kinase
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
CHI	Chalcone isomerase 1
chl a/b p26	Chlorophyll a/b-binding protein
chl a/b, II	Chlorophyll a/b-binding, photosystem II type I
CHS	Chalcone synthase
ClpC	Chloroplast import chaperone
COMT	Caffeic acid 3-O-methyltransferase
COR	Cold-regulated genes
CSD	Cold shock domain
CSDP	Cold shock domain proteins
CSP	Cold shock proteins
Cu/Zn SOD	Cu/Zn superoxide dismutase
Cyt b6-f FeS	Cytochrome b6-f complex iron-sulfur subunit
DFR	Dihydroflavonol 4-reductase
DHN	Dehydrin
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	Escherichia coli
eEF1A	Elongation factor 1-alpha
eIF4A	Eukaryotic initiation factor 4A
ERD	Early Response to dehydration
EST	Expressed sequence tags
F3H	Flavonoid 3'-hydroxylase
FA	Formic acid
FBPase	Fructose-1,6-bisphosphate aldolase
Fra	Fragaria allergen
FtsH1	ATP-dependent zinc metalloprotease FTSH
GADPDH	Glyceraldehyde-3-phosphate dehydrogenase

gb code	Genbank code
GEM-like	Forming homology-interacting protein 1
gi	Gene identifier
GLDH	L-galactono-1,4-lactone dehydrogenase
Gly Clvg	Glycine cleavage system H protein
Gly I	Glyoxalase I (lactoylglutathione lyase)
Gly II	Glyoxalase II (hydroxyacylglutathione hydrolase)
GO	Gene Ontology
GPI	Glucose-6-phosphate isomerase
GR-RBP	Glycine-rich-RNA-binding proteins
GST	Glutathione S-transferase
HPLC	High-pressure liquid chromatography
Hsc	Heat shock cognate
HSD	Honestly Significant Difference
HSP	Heat shock protein
ICE1	Inducer of CBF expression 1
IDH1	Isocitrate dehydrogenase 1 (NADP+), soluble
IDH2	Isocitrate dehydrogenase 2 (NADP+), mitochondrial
IEF	Isoelectric focusing
IFR	Isoflavone reductase related protein
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LEA	Late embryogenesis abundant protein
LFQP	Label-free quantitative proteomics
LOS2	Low expression of osmotically responsive gene 2
LTI	Low-temperature induced
LTQ	Linear trap quadrupole
MDH1	Malate dehydrogenase, cytosolic
MDH2	Malate dehydrogenase, mitochondrial
MLP	Major latex protein
mM	Millimolar
mRNA	Messenger RNA

ms	Methionine synthase
MW	Molecular weight
NAD-SDH	NAD-dependent sorbitol dehydrogenase
NaF	Sodium orthovanadate
NH4HCO3	Ammonium bicarbonate
nsHb-1	Non-symbiotic hemoglobin class 1
OMT	O-methyltransferase
PAL	Phenylalanine ammonia-lyase
PCA	Principal Component Analysis
PDC	Pyruvate decarboxylase
PGD	Phosphogluconate dehydrogenase
PGK	Phosphoglycerate kinase
pI	Protein isoelectric point
PK	Pyruvate kinase
PLD alpha	Phospholipase D alpha
PP2C	Protein phosphatase type 2C
PR	Pathogenesis-related
PVPP	Polyvinylpolypyrrolidone
PTM	Post-translationally modified
qPCR	Quantitative real time polymerase chain reaction
RAB18	Responsive to ABA
RBP	RNA-binding protein
REF	REF/SRPP-like protein (rubber elongation factor)
rgp	Reversibly glycosylated polypeptide (α 1,4-glucan protein synthase)
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RuBisCO LS	Ribulose-1,5-bisphosphate carboxylase oxygenase large subunit
RuBisCO SS	Ribulose-1,5-bisphosphate carboxylase oxygenase small subunit
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGT1	Suppressor of G2 allele of SKP1
smHSP	Small heat shock protein

sti1	Stress-inducible protein
SSR	Simple-sequence repeats
TBP	Tris buffered phenol
Thiolase 1	Acetyl-CoA C-acetyltransferase
TPP	Trans-Proteomic Pipeline
TPX	Thioredoxin-dependent peroxidase
UGGT	UDP-glucose glucosyltransferase
UGGT	UDP-glucose glucosyltransferase
UGPase	UTP-glucose-1-phosphate uridylyltransferase
USP	Universal stress protein
wt	weight
v	volume
µM	Micromolar

ABSTRACT

Koehler, Gage. Ph.D., Purdue University, December 2011. Overwintering Survival of Strawberry (*Fragaria* × *ananassa*): Proteins Associated with Low Temperature Stress Tolerance during Cold Acclimation in Cultivars. Major Professor: Stephen Randall.

Winter survival is variable among commercially grown strawberry (*Fragaria* × *ananassa*) cultivars. The main objectives of this study were to evaluate the molecular basis that contribute to this difference in strawberry cultivars and to identify potential biomarkers that can be used to facilitate the development of new strawberry cultivars with improved overwintering hardiness. With these goals in mind, the freezing tolerance was examined for four cultivars, 'Jonsok', 'Senga Sengana', 'Elsanta', and 'Frida' (listed from most to least freezing tolerant based on survival from physiological freezing experiments) and the protein expression was investigated in the overwintering relevant crown structure of strawberry. Biomarker selection was based on comparing the protein profiles from the most cold-tolerant cultivar, 'Jonsok' with the least cold-tolerant cultivar 'Frida' in a comprehensive investigation using two label-free global proteomic methods, shotgun and two dimensional electrophoresis, with support from univariate and multivariate analysis. A total of 143 proteins from shotgun and 64 proteins from 2DE analysis were identified as significantly differentially expressed between 'Jonsok' and 'Frida' at one or more time points during the cold treatment (0, 2, and 42 days at 2 °C). These proteins included molecular chaperones, antioxidants/detoxifying enzymes, metabolic enzymes, pathogenesis related proteins and flavonoid pathway proteins. The proteins that contributed to the greatest differences between 'Jonsok' and 'Frida' are candidates for biomarker development. The novel and significant aspects of this work include the first crown proteome 2DE map with general characteristics of the strawberry crown proteome, a list of potential biomarkers to facilitate the development of new strawberry cultivars with improved cold stress tolerance.

CHAPTER 1. INTRODUCTION

1.1. Processes Associated with Reliable Overwintering Survival

Perennial plants that are evolutionarily adapted to temperate regions have seasonal acclimation processes that contribute to increasing tolerance levels associated with freezing, desiccation, anoxia, ice-encasement and pathogen attack. Overwintering survival depends heavily on the capacity for freezing tolerance. The biophysical, and biochemical changes that occur in plants during cold acclimation and in response to low and freezing temperatures have been extensively studied especially in the model system *Arabidopsis thaliana* (Ruelland et al., 2009; Zhu et al., 2007). Adaptive strategies that have evolved to surmount the physical and biochemical challenges imposed by freezing temperatures such as modifying membrane composition, activating reactive oxygen scavenging systems, protecting proteins from misfolding, and neutralizing toxic by-products, are represented in species that have reliable overwintering success. Even though these and other general mechanisms are fundamental to our understanding about low temperature tolerance, more meaningful practical applications can be gained when implementing this knowledge towards improving specific crop(s) freezing tolerance.

The analyses of large scale data sets generated from global genomic and proteomic experiments have potential to expand our understanding about the molecular basis for overwintering and freezing tolerance. The introduction that follows highlights evidence supporting specific changes in metabolic machinery leading to an increased cold stress tolerance.

1.1.1. Membrane Modifications and Lipid Biosynthesis

Cellular life would not be possible without membranes. Cellular processes such as energy production, signaling and transport are linked to the integrity of the membrane. Irreversible membrane damage is associated with the formation of expansion-induced lysis during freezing and/or thawing cycles and hexagonal II phase formations caused by freezing induced dehydration (Uemura et al., 1995; Uemura et al., 2006). It is a long held view that the plasma membrane is the primary site for freezing damages (Steponkus, 1984). The ability to regulate the cell membrane fluidity by altering lipid composition is a fundamental adaptation in organisms that do not have internal temperature regulation mechanisms.

Maintaining membrane fluidity at low temperatures is achieved through altering the properties of amphipathic lipids that compose cellular membranes, namely by the chain length, level of saturation, and presence or absence of phytosterol(s). The alteration of membrane composition, increasing level of fatty acid desaturation is induced by low temperature and is positively correlated with cold stress tolerance (Horiguchi et al., 2000).

In the model plant Arabidopsis, the isolation of fatty acid desaturase mutants with altered lipid compositions has facilitated biochemical and molecular approaches to understanding the importance of the level of unsaturated fatty acids in the lipid components of temperature stress. Generally, plants with more unsaturated fatty acids in the lipid components have greater cold tolerance and plants with higher tolerance for heat have more saturated fatty acids. Freezing sensitivity is conferred by mutants; *fad*2 (Miquel, 1993) *fad*3 (Zhou et al., 2010) and *fad*8 (Kodama et al., 1994). Levels of unsaturated fatty acids have also been correlated with freezing tolerance levels in potatoes, *Solanum. commersonii* and *Solanum.tuberosum* (Palta et al., 1993).

1.1.2. Cytoskeleton in Response to Cold Exposure

Microtubules, composed of α- and β-tubulin heterodimers and actin filaments, interact closely with cellular membranes. Cold-induced membrane rigidification is a direct and early consequence to cold exposure (Örvar et al., 2000). Subsequent events to the increase of

membrane rigidity include calcium influx into the cytosol, reorganization of the actin cytoskeleton, and activation of cold induced genes associated with low temperature tolerance (Huang et al., 2007; Örvar et al., 2000; Sangwan et al., 2001; Wasteneys and Yang, 2004). This positions the cytoskeleton reorganization as an early response to cold exposure. In addition, the cytoskeletal reorganization is necessary and important for supporting cellular processes during long term low temperature exposure.

The establishment of a cold stable cytoskeleton is likely achieved in part through the cytoskeleton-associated proteins that are involved in nucleation, membrane anchoring, polymerization and depolymerization dynamics (e.g., growing and shrinking of polymers), severing, and polymer cross-linking (Staiger et al., 1997). For example, the accumulation of an actin depolymerization factor protein (ADF) during the acclimation period was shown to be at a higher level and for a longer duration of time in cold hardy wheat cultivars compared with more cold sensitive one (Ouellet et al., 2001), implying that the polymerization dynamics of actin is important for adapting to growth at low temperatures. Additional evidence supports the involvement of proteins such as annexins in membrane and cytoskeleton interactions that potentially stabilize the cytoskeleton against cold-induced disruption (Hayes et al., 2004; Konopka-Postupolska et al., 2009).

The level of cold stability of microtubules has been correlated with low temperature tolerance as seen by an investigation comparing the cold stability of microtubules using immunofluorescence microscopy during seasonal active and dormant conifers (Begum et al., 2011). In some studies, the depolymerization of microtubules caused by low temperature is followed by the reappearance of more-cold stable microtubules (Abdrakhamanova et al., 2003). Thus the level of cold-tolerance that is displayed by plants may depend on the capacity to re-establish new cold stable microtubules.

1.1.3. Reactive Oxygen Species

Reactive oxygen species (ROS) encompass a broad range of molecules that include hydrogen peroxide (H_2O_2), nitric oxide (NO) and free radicals (superoxide radical, O2•−, hydroxyl

radical, OH•). ROS, such as O2•− and H_2O_2 are normal byproducts of aerobic metabolism and are also important intracellular signaling molecules (Apel and Hirt, 2004; Suzuki and Mittler, 2006). Because of their role in signaling it is not surprising that effective mechanisms have evolved to maintain the cellular redox homeostasis. Biotic and/or abiotic stresses with significant duration and/or intensity increase the risk of ROS levels exceeding the cellular capacity to control them (Einset et al., 2007b). The potential for cellular damage increases as excess ROS, are converted to hydroxyl radicals (•OH) which damage polyunsaturated fatty acids, structural proteins, enzymes, and nucleic acids. The main sources of ROS are the chloroplasts (in photosynthesizing plant cells) and the mitochondria (in non-photosynthesizing plant cells) and each have ways for initially preventing the potentially damaging ROS levels. Oxidative stress occurs when the production of ROS exceeds the capacity of enzymatic and non-enzymatic antioxidants to control ROS levels. When avoidance measures are bypassed, mechanisms such as detoxification, repair, and degradation are employed to mitigate ROS damage. With regard to freezing tolerance, the susceptible cellular constituents that are vulnerable to ROS damage include membranes and lipids which are critical for freezing tolerance. Environmental stresses common to overwintering plants include hypoxic and anoxic conditions created by ice encasement. The regulation of ROS level is important at the onset, and during, as well as in the recovery phase for stress (Blokhina et al., 2003). For this reason, cold-hardy organisms must be adapted to prevent oxidative damage following freezing and resumption of aerobic metabolism following ice encasement or de-hardening.

This review makes a distinction between 'antioxidants' and 'detoxification chemicals' based on if there is a direct or indirect mode of action with reactive oxygen species. Antioxidants are enzymes and/or chemical compounds that protect the cell from damaging oxidation levels by binding to ROS directly, thus performing redox homeostatic buffering agents. 'Detoxification chemicals', on the other hand, protect the cell from toxic molecules that are produced either as a consequence from ROS interaction with cellular components (e.g. proteins, lipids, or nucleic acids) or byproducts from metabolic activity, other than ROS.

1.1.3.1. Antioxidant

A plant's response to stress involves mechanisms to decrease the potential oxidative stress damage by controlling the steady state levels of ROS in cells. This serves to prevent damage caused by ROS and also maintain the redox state of the cell which is an integral part of the plants ability to respond effectively to additional stresses. Tolerance to any stress largely depends on the potential of the antioxidative defense system. Sources of ROS include organelles with a high oxidizing metabolic activity or with an intense rate of electron flow, such as chloroplasts, mitochondria or peroxisomes (Asada, 2006). The antioxidative defense system is comprised of protective enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidases (APX), monodehydroascobate reductases (MDAR), dehydroascorbate reductase (DHAR), glutathione reductases (GR) and low molecular weight antioxidant compounds like glutathione, ascorbate, and tocopherols.

Most subcellular compartments have SOD activity that catalyzes the superoxide radicals, O2•−, into hydrogen peroxide, H_2O_2, and oxygen which are then scavenged enzymatically by APX, or CAT. Enhanced activities of antioxidative enzymes have been correlated with increased cold tolerance in cucumber (Lee and Lee, 2000), rice (Morsy et al. 2007), maize (Hodges, 1997) and chickpea (Kaur et al., 2009). A number of transgenic studies have shown enhanced low temperature tolerance from expressing antioxidants (McKersie et al., 1999; Vinocur and Altman, 2005). Cold tolerance was increased in rice expressing a catalase from wheat (Matsumura et al., 2002). The simultaneous overexpression of both CuZnSOD and APX in transgenic tall fescue plants confers increased tolerance to a wide range of abiotic stress (Lee et al., 2007).

Glutathione plays an important role in preventing cellular damage from oxidation in several ways. It is used by other enzymes involved in removing ROS (i.e. glutathione peroxidase and glutathione S-transferase (Noctor et al., 2011) and it also directly participates in neutralizing free radicals as well as helping maintain the reduced state of important antioxidants such as ascorbate, α-tocopherol and zeaxanthin (Lee et al., 2002b). In addition, glutathione can also protects protein thiols from oxidation via glutathionylation (Rouhier et al., 2008). This activity is ascribed to the reversible redox reactions of the sulfhydryl (thiol) group of

cysteine. The reduced glutathione (GSH) can participate in numerous redox reactions. Once GSH becomes oxidized it can form disulfides with another glutathione cysteine residue producing glutathione disulfide (GSSG). The regeneration of GSH is catalyzed by glutathione reductase (GR). The ratio of GSH to GSSG can be a measure of oxidative stress whereas decreased ratios are indicative of high levels of ROS. Chilling stress tolerance has been shown to correlate with GSH concentration and GR activity in a study comparing chilling-sensitive to chilling tolerant maize (Hodges, 1997).

1.1.3.2. Detoxification

Cytotoxic biomolecules can originate when ROS interacts with lipids, or proteins or other cellular components or are produced as non-enzymatic by-products of glycolysis (Richards 1993). Examples include 4-hydroxy-nonenal, produced from oxidative degradation of lipids, and reactive ketoaldehydes (e.g. methylglyoxal) from lipid and carbohydrate metabolism (Yadav et al., 2005). Glutathione is a pivotal component of plant detoxification systems in addition to roles in antioxidative stress tolerance. Cellular toxins are targeted for removal through glutathione conjugation by GST (glutathione S-transferase) (Li, 2009). A low temperature regulated GST has been isolated in a freezing tolerant potato species, which did not accumulate in a freezing sensitive potato species (Seppänen et al., 2000). GSH is also utilized by the glyoxalase system which is a set of two enzymes (glyoxalase I and glyoxalase II) involved in detoxifying methylglyoxal. Transgenic tobacco plants overexpressing glyoxalase enzymes resist an increase in methylglyoxal and maintain higher reduced glutathione levels under salinity stress (Singla-Pareek et al., 2006; Yadav et al., 2005).

Plant aldo-keto reductases (AKRs) are enzymes that perform such functions involved in detoxification. Although members of AKRs display distinct substrate specificity, they generally reduce aldehydes and ketones into primary and secondary alcohols and their activity has been shown to lead to broad protection from lipid peroxidation (Oberschall et al., 2000). Greater tolerance to low temperature was observed in tobacco overexpressing an alfalfa aldo-keto reductase (Hegedüs et al., 2004). A distinct benefit afforded by some aldo-keto reductases, like the one studied from alfalfa, includes the ability to catalyze the

production of sugar alcohols such as sorbitol or mannitol which can scavenge ROS even at low concentration in the cell.

1.1.4. Chaperones

Chaperones assist in maintaining the proper state (e.g. structure, location, degradation) of mRNA and proteins, and perform essential functions in both normal development and during environmental stress. Increasing evidence supports that some RNA-binding proteins (RBPs) are important for enhancing plant tolerance to cold temperatures and biotic stress. RBPs are involved in key regulatory processes, such as pre-mRNA splicing, polyadenylation, mRNA transport, mRNA stability, translation and degradation (Lorkovic, 2009). There are several different types of RBPs that are classified by the presence of one or more conserved domains/motifs and binding affinity. One of the first RNA-binding motifs identified in Eukaryotes is known as the RNA Recognition Motif (RRM) which has a conserved signature domain of eight amino acids with ~80 additional amino acids creating a general topography of four antiparallel β strands interspersed with two α-helices (Adam et al., 1986; Dreyfuss et al., 1988; Nagai et al., 1990). RRMs are present in many different RBPs often in conjunction with other common motifs or domains such as, Zinc-fingers, DEAD/DEAH box, and glycine-rich regions generating diverse RNA-binding proteins.

Another RNA-binding motif is known as the cold-shock domain (CSD). Plant cold shock domain proteins (CSDPs) were initially detected based on having a region similar as the CSD present in bacteria (Manival et al., 2001). The tolerance to low temperature of bacteria is conferred by functions performed by cold shock proteins (CSP) that accumulate during low temperature (Phadtare et al., 1999; Schmid et al., 2009). These functions include facilitating efficient transcription and translation processes by destabilizing secondary structures in nucleic acids that are strengthened by low temperatures. Cold responsiveness CSDPs have been identified in plants and similar functions have been proposed for plant CSDPs (Karlson and Imai, 2003). A main feature that makes plant CSDP different than in bacteria is the presence of two or more Cys-Cys-His-Cys (CCHC)-type zinc fingers in the C-terminal region interspersed with glycine-rich regions. The length and number of zinc fingers and

glycine rich regions were recently shown to contribute to the RNA chaperone activity that was demonstrated for CSDP1 of Arabidopsis through sequence motif-swapping and deletion experiments (Park et al., 2010). Similar to the CSDPs, a glycine rich RNA binding proteins (GRPs) have two or more (CCHC)-type zinc fingers and glycine-rich regions in the C-terminal region, but instead of a CSD they have one or more RRM present at the N-terminal. The GRP, AtRZ-1a, gene expression was shown to be specifically increased by cold stress and not by drought or ABA in Arabidopsis (Kim and Kang, 2006; Kim et al., 2007b). Evidence supporting AtRZ-1a has a function for enhancing freezing tolerance was shown by overexpressing AtRZ-1a in Arabidopsis, which resulted in better growth at low temperatures than wild-type. It was also shown to complement the cold sensitivity of *E. coli* that lacks cold shock proteins. (Kim et al., 2007a; Kim et al., 2005; Kim et al., 2007b).

RNA helicases require ATP, a feature that makes them distinct from the CSDPs and GR-RBPs. Compared to other organisms, plants have the largest number of DEAD-box RNA helicase genes. In Arabidopsis low expression of osmotically responsive genes 4 (LOS4) gene, which is a DEAD-box RNA helicase, has been shown to be required for RNA export from the nucleus to the cytoplasm (Zhang et al., 2004) and also essential for plant tolerance to chilling and freezing stress (Gong et al., 2005; Gong et al., 2002).

Another group of chaperones, the heat shock proteins (HSP's) have been shown to mediate the refolding and/or degradation of trapped or misfolded proteins, and to facilitate intracellular protein transport. Low temperature accumulation has been shown for HSPs including HSP90 in *Brassica napus* (Krishna, 1995), HSP70 in spinach (Anderson et al., 1994; Guy and Li, 1998) and Arabidopsis (Sung et al., 2001) and cytosolic HSP17 in tomato (Sabehat et al., 1998).

1.1.5. Pathogenesis-Related Proteins

There are 17 groups of pathogenesis-related (PR) proteins that have been classified based on amino acid sequences and enzymatic activity (van Loon et al., 2006). Cold-induced expression has been shown for many: PR-1, PR-2 (β-1,3 glucanase), PR-3 (chitinase), PR-5

(thaumatin-like), PR-6 (proteinase-inhibitor), PR-9 (peroxidase), PR-10 (ribonuclease-like), PR-12 (defensin), PR-13 (thione), and PR-14 (lipid transfer protein). Moreover, cold-induction of these genes correlate with enhanced pathogen resistance and this has been shown for various plant species such as, wheat, rye, barley, meadow fescue, and rape (Ergon and Tronsmo, 2006; Gaudet et al., 2011; Kawakami and Abe, 2003; Koike et al., 2002; Płażek et al., 2003). Enhanced resistance against pathogens has been also been demonstrated in transgenic plants overexpressing thaumatin-like proteins or chitinase (Datta et al., 1999). In addition to increased pathogen resistance, enhanced tolerance to cold has been observed when co-expressing PR proteins such as chitinase with β-1,3 glucanase (Kalpana et al., 2006; Schickler and Chet, 1997).

Proteins detected in the apoplast of overwintering cereals are related to some PR-proteins that include thaumatin-like, chitinase, and β-1,3 glucanase (Antikainin, 1997), and have demonstrated ice-binding and antifreeze-like activities (Dave and Mitra, 1998; Fernandez-Caballero, 2009; Goñi et al., 2010; Hincha et al., 1997; Romero, 2008). Antifreeze-like properties lower the freezing point of a solution in a non-colligative manner and slow the rate of ice formation and also prevent the growth of ice crystals thus providing protection against cell and tissue damage (Griffith and Yaish, 2004; Yaish et al., 2006). In addition to these functions some PR-proteins perform functions to facilitate storage of nutrient resources in overwintering organs. Thus the contribution of these proteins to overwintering survival appears multifunctional.

1.1.6. Dehydrins

Dehydrins can be one of the most prevalent proteins induced and accumulated in response to cellular water-deficit stress in tolerant plants. Dehydrin accumulation is also associated with internal water deficit stress occurring with seed maturation. Some dehydrins exhibit constitutive expression while others are more pronounced at certain times of seed or flower development suggesting possible roles for both growth and abiotic stress tolerance. Even though we do not know the reason why plants require dehydrins, in vitro studies point to various protective roles. For instance, cold-induced dehydrins isolated or purified from

several plant species have been shown to be effective cryoprotectants (Hara et al., 2001; Kazuoka and Oeda, 1994; Wisniewski et al., 1999). The citrus dehydrin, CrCoR15, preserves enzyme activity under desiccation stress (Sanchez-Ballesta et al., 2004). Correlating dehydrin protein accumulation with enhanced stress tolerance has been supported by transgenic studies (over expressing a wheat dehydrin in strawberry improved freezing tolerance) as well as studies comparing stress tolerance with intra- and inter-specific plant populations (Danyluk et al., 1994; Houde et al., 2004; Ismail et al., 1999). A dehydrin from maize, DHN1, has been shown to preferentially bind lipid vesicles and increases helicity in the presence of lipids (Koag et al., 2003). In addition to interactions with membranes, protein interactions have been postulated. The chaperone, calreticulin, has similarities to some dehydrins with regards to having an acidic pI and ability to bind zinc and having multiple Ca^{2+} binding sites. In general, dehydrins are thought to protect the cell by preserving the integrity of cell constituents or by buffering the cell from toxic levels of ions that accumulate during times of environmental stresses (Alsheikh et al., 2003). Thus dehydrins appear to have the potential to be contributing to enhanced tolerance to cold stress in many ways based on the various protective roles they are associated with.

1.2. Significance Aspects from this Study

Strawberry cultivation predominates in regions with mild winters and overwintering hardiness is an essential trait for strawberry cultivation in colder climates. Freezing injury of strawberry plants is one the greatest factors reducing crop yield and quality in temperate regions. Consequentially, one of the major aims of low temperature tolerance research is to facilitate the development of cultivars that can withstand extreme, irregular, and harsh winter conditions thus, securing yield and profitability to the growers. Because strawberry is a representative species for the Rosacea crops (includes peaches, apples, cherries, blackberries, and raspberries) this knowledge is expected to be transferrable to benefit improvement of many of these related crops.

Low temperature tolerance studies using the model system *Arabidopsis thaliana* has greatly advanced our understanding of low temperature tolerance mechanisms and regulation.

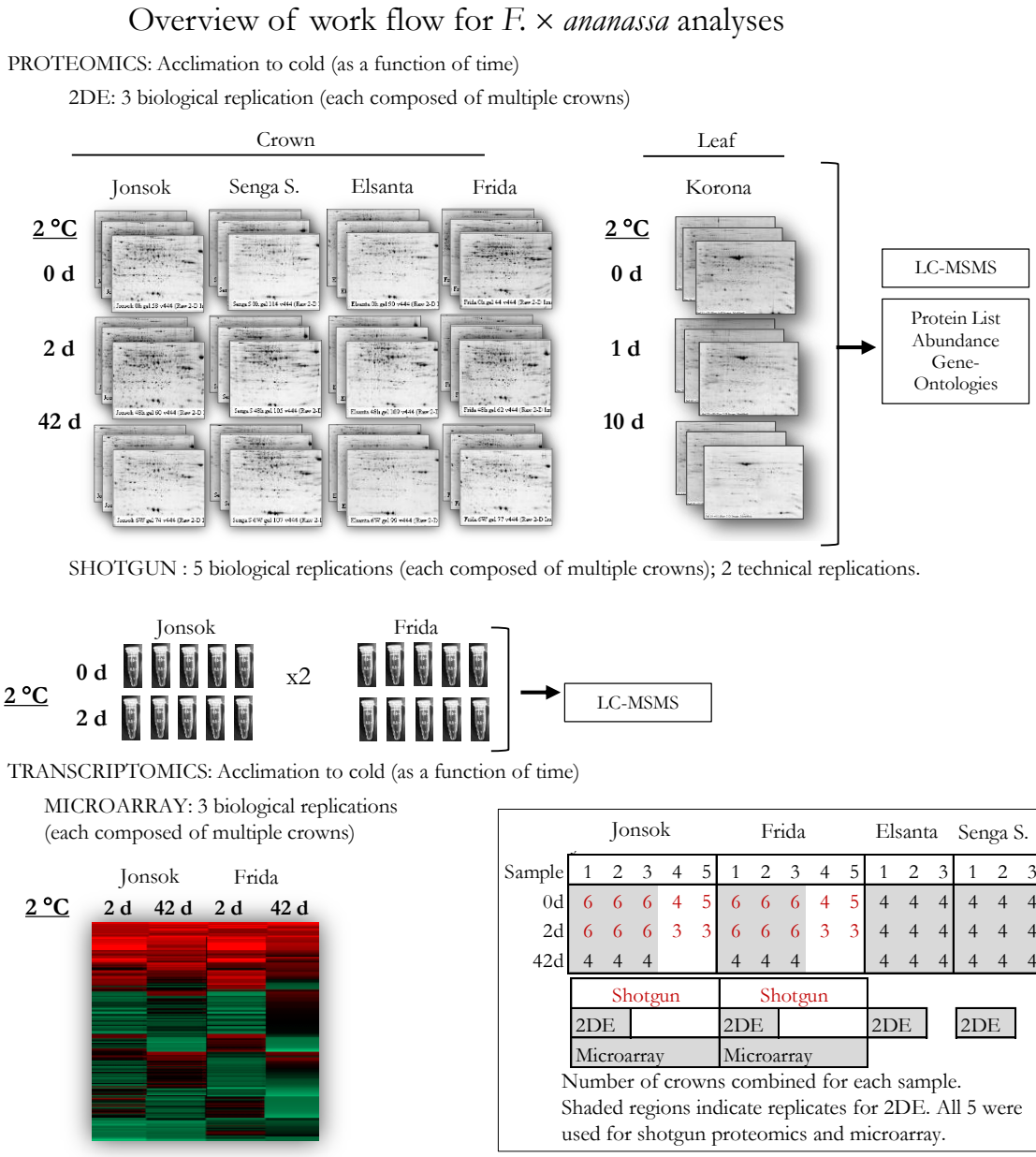
However, it remains important to study individual species and relevant overwintering structures (Wisniewski, 2007). For instance, investigations comparing tissues in the same species and/or closely related species provide important insight into the differences in protein expression in overwintering structures (Bocian et al., 2011; Kosmala et al., 2009).

Strawberry depends on the overwintering crown and root tissues for spring regeneration. This requires that the crowns and roots remain uncompromised from the physiological damage of freezing. The crown is especially susceptible to ice crystal damage due to presence of the large cells of the pith tissue. Freezing damage is readily seen as brown or black discoloration resulting from cellular damage and consequent oxidation. This damage also increases susceptibility to fungal and bacterial rot that diminish spring crop yields. Both freezing tolerant mechanisms and disease resistant mechanisms are therefore important for successful overwintering. The variability of cold hardiness observed for *F.* × *ananassa* species is likely contributed by proteins accumulated in the overwintering crown and their ability to mitigate adverse effects of freezing damage. Modifying extracellular ice formation, protecting protein functions with chaperones, scavenging reactive oxygen species, and increasing cell wall integrity are important aspects for surviving low temperatures. With the aim of developing new cultivars with improved overwintering hardiness, we describe the first proteomic map for the most relevant overwintering tissue for strawberry, the crown, and further compare several commercial cultivars of strawberry in terms of their relative freezing tolerance and concomitant protein expression patterns. This report thus identifies potential protein bio-markers which can be utilized to facilitate conventional breeding endeavors for cold tolerant cultivars of strawberries. We have developed and adopted state-of-art molecular tools to investigate cold responses in strawberry plants during the acclimation phase resulting in the identification of a large number of proteins that correlate to cold/freezing tolerance in strawberry.

1.3. Explanation of Interrelatedness of Chapters

Chapter 2 presents and compares the results of the two different protein screening methods, 2D gel electrophoresis and a shotgun approach that were applied to the overwintering

relevant structure, the crown, of strawberry to identify candidate biomarkers for cold tolerance and provide the general characteristics for the strawberry crown proteome. Chapter 3 originated from a collaboration that focused on evaluating cold tolerance for strawberry cultivars different than those introduced in Chapter 2 but focused on leaves rather than crowns. This Chapter offers the additional context of placing *F.* × *ananassa* cold responses within the existing knowledge base of low temperature stress protein changes in leaves. Chapter 4 compares the shotgun proteomic and microarray results for 'Jonsok' and 'Frida' under control (0 day) and 2 day cold acclimation. All microarray data presented in this dissertation came from work done from collaborators. The overview of the workflow for *Fragaria* × *ananassa* provides credit to individuals responsible for experiments (Figure 1.1).



Muath Alsheikh (Proteomic and Transcriptomics-Microarray Experimental Design); **Anita Sønsteby** (Freezing experiment); **Gage Koehler** (Protein extraction, 2-DE analysis, Shotgun analysis, Microarray analysis); **Xianyin Lai -Frank Witzmann lab** (LC-MS/MS for 2DE); **Jin-Sam You -Monarch** (LC-MS/MS for shotgun and 2DE); **John V. Goodpaster** (2DE Statistics: ANOVA, PCA, AHC)

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Figure 1.1 Overview of experiments for *F.* × *ananassa.* The same sample (source, combination of crowns) were used for 2DE (0, 2, 42 day), shotgun (0 and 2 day), and microarray (0, 2, 42 d).

CHAPTER 2. PROTEOME ANALYSIS OF CROWNS OF FRAGARIA × ANANASSA CULTIVARS WITH DIFFERENT FREEZING TOLERANCE

2.1. Introduction

There are many levels to evaluate the molecular responses of organisms during cold exposure including genetic, transcript, metabolites, and proteins. Because of the complexity inherent to studying plants with high ploidy, proteomic-based methods offer benefits for comparing differences among cultivars. The use of 2DE and a high through-put shotgun method applied for this study identifies proteins that make the most freezing tolerant cultivar, 'Jonsok' distinct from 'Frida', the lesser freezing tolerant cultivar. In addition, based upon the obtained results, the testable hypothesis is made that the greater freezing tolerance of 'Jonsok' is due to the proteins expressed before or in the initial phase of cold treatment.

The strawberry genus (Fragaria) is made up of 21 species that vary in ploidy with a base chromosome number of x = 7. The diploid species *Fragaria vesca* has a relatively small genome ~240 Mb and has recently been sequenced (Shulaev et al., 2011). The cultivated strawberry (*Fragaria* × *ananassa*) is an octoploid (2n = 8x = 56). Because Fragaria is positioned as a model system for the Rosaceae family there is a strong incentive for comparative mapping experiments. So far, comparative genetic mapping between octoploid and diploid Fragaria species reveals a high level of colinearity with no evidence of any chromosomal rearrangements between the diploid and octoploid strawberry (Rousseau-Gueutin et al., 2008; Sargent et al., 2009). In addition, comparative genetic mapping experiments using other member species within the Rosaceae family suggest there is sufficient level of synteny among members to support the transfer of information obtained about Quantitative Trait Loci, markers, and genes for these species (strawberry, apple, pear, and cherry) (Pierantoni et al., 2004; Rousseau-Gueutin et al., 2008; Rousseau-Gueutin et al., 2011; Vilanova et al., 2008).

The origin of the modern commercial strawberry (*Fragaria* × *ananassa*) dates back to the eighteenth century, where in Europe, a cross between two octoploid species (*Fragaria virginiana* and *Fragaria chiloensis*) gave rise to a hybrid plant that soon became popular because of the large, sweet fruits that were uncommon for European strawberries (Darrow, 1966). The systematic breeding using *F. virginiana* and *F. chiloensis* continues to this day with new cultivars being identified with superior traits such as, vigor, seed set, fruit color, fruit size, disease and pest tolerance (Hancock et al., 2010; Luby et al., 2008; Stegmeir et al., 2010). The diploids that gave rise to these two parental lines have yet to be determined but *F. vesca* is among candidates that have been suggested to be an early ancestor (Folta and Davis, 2006; Potter et al., 2000; Rousseau-Gueutin et al., 2009).

Global transcript, protein, and metabolic approaches are rapidly advancing our knowledge about cold acclimation processes (Cook et al., 2004; Kaplan et al., 2007; Maruyama et al., 2009; Sandve et al., 2011). Cold acclimation is known to induce proteins relevant for freezing survival (Thomashow, 2010; Zhu et al., 2007), however, it is plausible that some proteins associated with cold tolerance are expressed under non-stress conditions i.e., are not cold inducible (Takahashi et al., 2006). Novel insights into the most efficient freezing-tolerant mechanisms are expected to be gained from comparing closely related plants that differ in freezing tolerance. Because of the genetic complexity of commercial octoploid strawberry, the identification of potential markers linked to freezing tolerance could be facilitated by using proteomics. Advantages of proteomics include detecting post-translational modifications of proteins and revealing changes in protein levels that may not be seen utilizing transcriptomic approaches. The identification of proteins that correlate with winter survival in strawberry could expedite the establishment of new cultivars through either conventional breeding endeavors or through direct gene manipulation.

With the aim of developing new cultivars with improved overwintering hardiness, we describe a proteomic map for the most relevant overwintering tissue for strawberry, the crown, and compare several commercial cultivars of strawberry in terms of their relative freezing tolerance and concomitant protein expression patterns. Further, this chapter

identifies potential protein biomarkers which can be utilized to facilitate conventional breeding endeavors for cold tolerant cultivars of strawberries.

2.2. Methods

2.2.1. Plant Material and Experimental Design for Freezing Experiment

F. × *ananassa* runners were collected from the field and rooted in a heated greenhouse maintained at 20 ± 2 ºC and 20-h-light/4-h-dark for 2 weeks in 50 x 30 cm rooting trays (4.5 x 5.5 cm/well) in a peat-based potting compost (90% peat, 10% clay), with the addition of 1:5 v/v of granulated perlite. After rooting, the plants were transferred and grown for additional 6 weeks in 10 cm plastic pots using the same mixture as above. Throughout the experiment, the plants were regularly watered as required, and fertilized twice weekly using CALCINIT™ (15.5% N and 19% Ca) and Superba™ Rød (7-4-22 NPK plus micronutrients) from Yara International, Norway. The plants were then hardened for 6 weeks at 2 ºC and 10-h-light/14-h-dark at 90 µmol quanta m-2 s-1. After hardening, the plants were exposed to freezing temperatures ranging from -3 to -12 ºC. The freezing was performed in darkness in freezing cabinets starting at 2 ºC. Temperatures were adjusted by a cooling rate of 2 ºC h-1 and then held at the respective freezing temperatures for 48 h. Control plants were exposed to 0 °C in darkness for 48 h for comparison. After completion of the freeze and thaw cycle, the plants were thawed at 2 ºC for 24 h, whereupon the plants were moved into a greenhouse maintained at 18 ± 2 ºC and 20 h photoperiod. Plant survival and growth performance was scored 5 weeks later. Plant survival was scored visually on a scale from 1 (normal growth) to 5 (dead, no re-growth). The extent and intensity of discoloration (tissue browning) were recorded for the surviving plants from longitudinal crown sections as described by Marini and Boyce (1977) on a scale from 1 (low extent/intensity) to 5 (high extent/intensity) (Marini, 1977). All experiments were replicated with three randomized blocks of 3 to 4 plants for each population, giving a total of 9 to 12 plants of each population in each treatment. ANOVA analyses (Table 2.4) were performed by standard procedures using a MiniTab® Statistical Software program package (Release 15;

Minitab Inc., State College, PA). The freezing conditions, the scoring details and the origin and parents of the four cultivars used are summarized in Table 2.1 through Table 2.3 and Figure 2.1.

Table 2.1 Strawberry (*F.* × *ananassa*) cultivars used in the freezing experiments.

Cultivar	Origin	Parents
'Jonsok'	Norw. Univ. of Life Sciences, Norway	'Senga Sengana' × 'Valentine'
'Senga S.'	Germany	'Sieger' × 'Markee'
'Elsanta'	Inst. Hort. Plant Breeding, The Netherlands	'Gorella' × 'Holiday'
'Frida'	Norw. Univ. of Life Sciences, Norway	'Ås 98' (private collection × 'Polka') × 'Oda' ('Inga' × 'Onebor')

Table 2.2 Summary of freezing conditions for experiment 1, 2, and 3.

Exp no.	Freezing procedure
1	Plants frozen for 48 h at 0, -3, -6, -9 ºC at a freeze and thaw rate of 2 ºC/h
2	Plants frozen for 48 h at 0, -3, -6, -9, -12 ºC at a freeze and thaw rate of 2 ºC/h
3	Plants frozen for 48 h at 0, -3, -6, -9, -12 ºC at a freeze and thaw rate of 2 ºC/h

There were 3 to 4 plants of each cultivar for each experiment except for 'Senga Sengana' which was not included in experiment 3.

Table 2.3 Freeze injury in strawberry plants determined by scoring 1-5.

Plant Condition	Tissue Browning	Browning Intensity
1 - Normal growth	1 - Medulla and vascular tissue have no visible browning	**1**
2 - Survives – close to normal growth	2 - Trace of browning observed in medulla, no browning in vascular tissue	**2**
3 - Survives – weak growth	3 - Less than half of the medulla and vascular tissue are brown	**3**
4 - Survives – close to dead	4 - More than half of the medulla and vascular tissue are brown	**4**
5 - Dead – no re-growth	5 - Entire medulla and vascular tissue are brown	**5**

A score of 1 through 5 was based on the condition of the plant at re-growth, and the extent and intensity of tissue browning 5 weeks after the freezing procedure ended. Tissue browning and browning intensity were scored for the surviving plants from longitudinal crown sections as described by Marini and Boyce (1977).

Figure 2.1 An example of visible freezing damage in crown tissue. Longitudinal sections of crowns from *F.* × *ananassa* 'Elsanta' 5 weeks after a freezing procedure at 0 ºC (left) and -6.0 ºC (right). Injury from freezing is readily seen as brown or black discoloration resulting from cellular damage and consequent oxidation. Photos by Anita Sønsteby 2010.

2.2.2. Plant Material for Protein Analysis

Plant cultivation was carried out as described above (freezing experiment). The plants were cold hardened at 2 ºC and 10-h-light/14h-dark at 90 µmol quanta m-2 s-1 for either 0, 2 or 42 days. Tissue was harvested by dividing each crown longitudinally and immediately frozen in liquid nitrogen and stored in -80 ºC. Each replicate was composed of four to six crown segments. To ensure direct comparability of the protein and RNA levels, replicates were created by combining the 4 to 6 half-crowns that were cut longitudinally for proteomic experiments and the corresponding 4 to 6 half crowns for transcript experiments (transcript analysis described in later chapters).

2.2.3. Sample Preparation for 2DE

Tissue was ground to a fine powder in liquid nitrogen in the presence of polyvinylpolypyrrolidone (PVPP) at 10% of tissue weight. The powder was washed twice with cold 100% acetone with centrifugation at 8000 rpm at < 0 ºC for 20 minutes (Sorval SS-34 rotor, 7649 × g avg). The powder was then vacuum dried over dry ice (-78 ºC) to remove acetone. A phenol extraction followed by methanolic ammonium acetate precipitation was then performed as follows. Tris buffered phenol, pH 8.8 (TBP) and extraction buffer (5.0 mL each per 1 g fresh weight) were added and then tissue was

polytroned with a Brinkman homogenizer model PC 10/35 at speed setting #5 (Brinkman Instruments, Switzerland) for 30 seconds. The extraction buffer used contained 40% sucrose w/v, 2% SDS w/v, 1X Complete Roche Protease inhibitors, phosphatase inhibitors (2 mM sodium orthovanadate (5 mM NaF, 1 mM NaPPi, 1 mM 3-glycerolphosphate, and 3 μM microcystin) and 2% β-mercaptoethanol dissolved in 0.1 M Tris-HCl pH 8.8. Sample was incubated at 4 ºC with agitation for 30 minutes followed by centrifugation at 7000 rpm (Sorval-34 rotor, 5000 × g avg) for 15 minutes at 4 ºC. The upper phenol phase was removed and the lower phase was re-extracted with 5.0 mL of TBP. Back extraction was performed on the combined upper phases by adding an equal volume of extraction buffer. Following extraction, proteins were precipitated by adding 5 times the volume of 0.1 M ammonia acetate in 100% methanol overnight at -78 ºC. The pellet was recovered by centrifuging at 7000 rpm, as before and washed twice with 0.1 ammonia acetate in 100% methanol followed by two washes with 80% acetone. The pellet was resuspended by vortexing and precipitation at -20 ºC for 30 minutes between washes. The final pellet was air dried (~5 to 10 min). Pellets (~ 4.0 mg) were dissolved in ~600 μL of isoelectric focusing (IEF) buffer containing 8 M Urea, 2 M Thiourea, 2% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate w/v, 2% de-ionized Triton X-100, 50 mM DTT, and 0.5% pH 3-10 ampholytes. An Amido Black assay (Kaplan and Pedersen, 1985) was used to determine concentration of protein. One to three mg protein was obtained per gram of crown fresh weight.

2.2.4. 2DE (Two-Dimensional Gel Electrophoresis)

IEF strips (24 cm, nonlinear pH 3 to 10, Bio-Rad, Hercules, CA) were passively rehydrated with 400 μg of protein at 20 ºC for 14 hours. Rehydration buffer included IEF buffer with 0.0005% bromophenol blue. Samples were then rinsed with water and focused at 20 ºC using a Protean IEF Cell (BioRad) using the following parameters: 100 V for 300 Vhr, 300 V for 900 Vhr, 5000 V for 35000 Vhr and 8000 V for 53800 Vhr all with rapid ramps. Total Vhr was 90000 with a maximum of 50 μAmps per strip. After IEF, the strips were equilibrated with 450 μL of 6 M Urea, 0.05 M Tris/HCl pH 8.8, 4% SDS, 20% glycerol, 2% DTT w/v for 15 min (5 min × 3 changes) for the first step. Iodoacetamide (2.5% w/v)

replaced DTT for the second step for 15 min (5 min × 3 changes). Strips were then placed on a 12% SDS-polyacrylamide gel and sealed with 0.65% agarose dissolved in 1X electrode buffer. Gel electrophoresis was conducted at 600 mAmp constant in a PROTEAN plus Dodeca cell (Bio-Rad) apparatus to run 12 gels simultaneously at a constant temperature of 20 ºC.

2.2.5. 2DE Gel Imaging and Data Analysis

Gels were fixed with 40% methanol and 10% acetic acid in water for 3 h. Gels were washed 3 times in water for 15 minutes each and stained for a minimum of 72 hours with colloidal coomassie G-250 (Candiano et al., 2004). Gels were then destained in water and scanned using a GS-800 Calibrated Imaging Densitometer (Bio-Rad). Thirty-six gel images (4 cultivars, three conditions, each in triplicate) were analyzed using PDQuest version 7.1 (Bio-Rad Laboratories, Hercules, CA, USA). Molecular weights and isoelectric points (pI) were assigned to spots by performing a separate experiment running internal 2DE SDS-PAGE Standards (Bio-Rad Laboratories, Hercules, CA, USA) with the same electrophoresis parameters as described above except using 100 µg protein ('Jonsok' at 0 d) and subsequently applying the determined MW and pI values to the larger experiment. In addition to the 2DE internal standards used to determine mass and isoelectric point, one protein, strongly identified as the elongation factor 1-alpha (SPP 9618) was used as a pI standard of 9.2. A total of 900 total protein spots were matched and inspected visually to validate all automated matching. The protein spot quantities were normalized based on the total valid spots for each gel and expressed as parts per million (ppm). Average intensities, standard deviations and coefficient of variations were obtained. Significant protein spot differences between cultivars or due to cold response changes were inspected using Student's t-test (unpaired, two tailed) $p < 0.05$, analysis of variance (ANOVA), and principal component analysis (PCA). All 2DE data was normalized to unit vector length by calculating the square root of the sum of squares of all protein spot quantities for a given sample. Each protein spot quantity in that sample was then divided by this normalization factor. This pre-treatments step removed any differences between samples due to overall quantity as well as differences in detection sensitivity for a given gel. PCA and ANOVA were then carried out

using XLSTAT (AddinSoft SARL, Paris, France), an add-in to Microsoft Excel. PCA used the Pearson Product Moment to calculate correlations between variables and a Scree plot was visually inspected to determine the number of significant principal components. For ANOVA, significance was set at $p < 0.05$ and the Tukey's HSD (Honestly Significant Difference) test was used to analyze the difference between groups. Two-way ANOVA was performed using JMP version 3.1.6 for the Macintosh (SAS, Cary, NC). PCA, and ANOVA completed by Dr. John Goodpaster, IUPUI Chemistry Department).

2.2.6. 2DE Protein Identification by LC-MS/MS

2.2.6.1. Protein Confidence Values Listed as Protein Probability

The gel spots were manually cut from the wet gels. The gel plugs were destained with 50% acetonitrile (ACN) in 50 mM ammonium bicarbonate (NH_4HCO_3) twice, reduced with 10 mM DTT in 100 mM NH_4HCO_3, alkylated with 55 mM iodoacetamide in 100 mM NH_4HCO_3, and digested by trypsin for 3 h at 37 ºC. The tryptic peptides were extracted with 30, 50, and 100% ACN sequentially. The extracted peptides combined were dried by SpeedVac and reconstituted with 5% ACN in 0.1% FA (formic acid).

The peptide samples were analyzed using a Thermo-Finnigan linear ion-trap (LTQ) mass spectrometer coupled with a Surveyor autosampler and MS HPLC system (Thermo-Finnigan). Tryptic peptides were injected onto the C18 microbore RP column (Zorbax SB-C18, 1.0 mm × 50 mm) at a flow rate of 50 μL/min. The mobile phases A, B, and C were 0.1% FA in water, 50% ACN with 0.1% FA in water, and 80% ACN with 0.1% FA in water, respectively. The gradient elution profile was as follows: 10% B (90% A) for 10 min, 10-20% B (90-80% A) for 5 min, 20-70% B (80-30% A) for 35 min, and 100% C for 10 min. The data were collected in the "Data dependent MS/MS" mode with the ESI interface using the normalized collision energy of 35%. Dynamic exclusion settings were set to repeat count 2, repeat duration 30 s, exclusion duration 120 s, and exclusion mass width 1.50 m/z (low) and 1.50 m/z (high).

The acquired data were searched against NCBI protein sequence database of *F.* × *ananassa* (downloaded on 12 February 2009 from http://www.ncbi.nlm.nih.gov/, 574 entries) and Rosaceae (downloaded on 12 February 2009 from http://www.ncbi.nlm.nih.gov/, 8,926 entries) using SEQUEST (v. 28 rev. 12) algorithms in Bioworks (v. 3.3). General parameters were set as follows: peptide tolerance 2.0 amu, fragment ion tolerance 1.0 amu, enzyme limits set as "fully enzymatic - cleaves at both ends", and missed cleavage sites set at 2. The searched peptides and proteins were validated by PeptideProphet (Keller et al., 2002) and ProteinProphet (Nesvizhskii et al., 2003) in the Trans-Proteomic Pipeline (TPP, v. 3.3.0) (http:// tools.proteomecenter.org/software.php) with a confidence score represented as probability. The validated peptides and proteins were filtered using the following cut-off: (1) the confidence of protein was ≥ 90.00% (0.9000); (2) at least two peptides were identified for a protein; and (3) the confidence of peptides was ≥ 80.00% (0.8000) with at least one peptide's confidence ≥ 90.00% (0.9000). Only the peptides and proteins meeting the above criteria were chosen.

2.2.6.2. Protein Confidence Values Listed as q-values

To build the Fragaria protein database, the *Fragaria* × *ananassa* and *Fragaria vesca* protein fasta database and EST sequence databases for taxonomy id 3747 and 57918 were downloaded from NCBI. The ESTs were translated in three different reading frames and the largest protein among three reading frames was chosen. The *F.* × *ananassa* protein fasta database and the chosen translated database were concatenated, after which the same sequences were removed from the list. The final protein entry was 45793. Database search was done using Sequest and X! Tandem algorithms.

2.2.7. Shotgun Proteomics

These analyses were conducted and analyzed essentially as described in (Higgs et al., 2005) and (Wang et al., 2008). The time points used for this experiment consisted of the 0 and 2 day exposure to 2 ºC. Three to six individual crowns were used for each of five biological replications. Each biological replication was injected twice and the two technical replicate

intensity values were averaged. Tryptic peptides (< 20 μg) were injected onto an Agilent 1100 nano-HPLC system (Agilent Technologies, Santa Clara, CA) with a C18 capillary column in random order. Peptides were eluted with a linear gradient from 5%-45% acetonitrile developed over 120 minutes at a flow rate of 500 nL/min and the effluent was electro-sprayed into the LTQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Data were collected in the "Triple Play" (MS scan, Zoom scan, and MS/MS scan) mode. The acquired data were filtered and analyzed by a proprietary algorithm. The database used was the same as described for 2DE protein identification by LC-MS/MS with confidence values listed as q-values.

2.2.8. Western Blots

Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and electrophorectically transferred to nitrocellulose membrane overnight at 0.2 Amp at 4 ºC. Nonspecific binding sites on blots were blocked overnight with PBS [(phosphate buffer solution (137 mM NaCl, 2.7 mM KCL, 4.3 mM Na2HPO4, 1.5 mM KH_2PO_4)] 5% nonfat dry milk (w/v), pH 7.4. Equal amounts of protein (25 μg from same samples used for 2DE analysis for cAPX and ADH or 5 μg for dehydrin antibody) loaded for time point and probed with antibody raised against to ADH (alcohol dehydrogenase) and cAPX (cytosolic ascorbate peroxidase) proteins (Agrisera products; AS10 685 and AS06 180 respectively) or raised against the K-segment (dehydrin) overnight at 4 ºC with the first antibody at ratios 1:3000 (ADH) or 1:4000 (cAPX) or 1:4000 (dehydrin), followed by 3 washes at 30 minutes each, then followed by a 45 minute incubation with the secondary antibody (peroxidase conjugate anti-rabbit at a ratio of 1:4000). Three washes (5% nonfat milk/PBS (w/v) for 30 minutes each then followed by two washes with 1xPBS, pH7.4 for 1 hour each. Immunodetected proteins were obtained using Supersignal® West Dura (Thermo scientific) and blots were imaged with the Molecular Imager®, ChemiDoc System (BioRad).

2.3. 2DE Results

Anecdotal field observations of winter survival and subsequent yields of strawberry cultivars commonly grown in Norway suggested that 'Jonsok' is more cold tolerant than other commonly grown cultivars. The four strawberry cultivars, 'Jonsok', 'Senga Sengana', 'Elsanta' and 'Frida' were tested for winter survival traits under controlled laboratory environments. 'Jonsok' was consistently more cold tolerant than 'Frida' when measured by survival as well as by browning patterns and browning intensity of the crowns after freezing (Table 2.4 and 2.5). In particular, survival rates were significantly different after 48 hour treatments at -6 and -9 ºC with 'Jonsok' and 'S. Sengana' being more cold tolerant and 'Frida' and 'Elsanta' being less so (Table 2.4). Exponential extrapolated killing curves indicated 50% survival of 'Jonsok' at approximately -8.3 ºC and for 'Frida' at approximately -5.5 ºC (Table 2.5). Internal browning of crowns was consistent with these results. The cultivars of 'Jonsok' and 'Frida' were analyzed here in detail as representing the most and least freezing tolerant cultivars after cold acclimation.

Table 2.4 Freezing survival demonstrates the relative cold/freezing tolerance of *F.* × *ananassa* cultivars.

		Plant Survival (%)					Tissue Browning (1-5)					Browning Intensity (1-5)				
Cultivar	no.	0 ºC	-3 ºC	-6 ºC	-9 ºC	-12 ºC	0 ºC	-3 ºC	-6 ºC	-9 ºC	-12 ºC	0 ºC	-3 ºC	-6 ºC	-9 ºC	-12 ºC
'Jonsok'	1	100	100	100	11.0	n.d.	1.0	2.2	3.4	5.0	n.d.	1.0	2.0	3.5	5.0	n.d.
	2	100	100	100	90.0	0.0	1.0	1.1	2.7	3.9	5.0	1.0	1.1	2.7	4.1	5.0
	3	100	100	50	0.0	0.0	1.0	1.7	4.0	5.0	5.0	1.0	1.7	4.4	5.0	5.0
Mean		100a	100a	80a	30a	0	1.0a	1.7b	3.4a	4.7a	5.0a	1.0a	1.6a	3.6a	4.7a	5.0a
'Senga S.'	1	100	100	78	0.0	n.d.	1.0	2.2	4.3	5.0	n.d.	1.0	3.1	4.1	5.0	n.d.
	2	100	100	83	8.0	0.0	1.0	1.3	2.8	5.0	5.0	1.0	1.3	2.9	5.0	5.0
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mean		100a	100a	81a	5b	0	1.0a	1.7a	3.5a	5.0a	5.0a	1.0a	2.1ab	3.4a	5.0a	5.0a
'Elsanta'	1	100	100	33	0.0	n.d.	1.0	1.8	4.4	5.0	n.d.	1.0	2.8	4.5	5.0	n.d.
	2	100	100	33	0.0	0.0	1.0	1.6	4.8	5.0	5.0	1.0	1.6	4.8	5.0	5.0
	3	100	100	17	0.0	0.0	1.0	3.3	4.8	5.0	5.0	1.0	3.0	4.9	5.0	5.0
Mean		100a	100a	27b	0b	0	1.0a	2.4b	4.7b	5.0a	5.0a	1.0a	2.6b	4.8b	5.0a	5.0a
'Frida'	1	100	100	11	0.0	n.d.	1.8	2.6	4.9	5.0	n.d.	2.8	3.3	4.9	5.0	n.d.
	2	100	100	100	67.0	0.0	1.0	1.3	2.9	4.5	5.0	1.0	1.2	3.0	4.4	5.0
	3	100	100	17	0.0	0.0	1.0	3.1	4.8	5.0	5.0	1.0	2.8	4.9	5.0	5.0
Mean		100a	100a	45ab	24ab	0	1.2b	2.3b	4.2ab	4.8a	5.0a	1.5b	2.4b	4.2ab	4.8a	5.0a

Surviving plants were recorded 5 weeks after the freezing temperature program ended. Scoring of surviving plants, the browning extent and intensity were performed as described in Table 2.3. The level of significance was determined with ANOVA. Different letters in columns next to mean values indicate significant difference between treatments ($p < 0.05$, Tukeys). n.d. denotes data not determined. This data supports Table 2.5.

Table 2.5 Exponential extrapolated killing curves indicated 50% survival of 'Jonsok' at approximately -8.3 ºC and for 'Frida' at approximately -5.5 ºC.

	Plant Survival			Tissue Browning			Browning Intensity		
Cultivar	LT50	SE	R^2	LT50	SE	R^2	LT50	SE	R^2
'Jonsok'	-8.29	1.11	0.79	-5.34	0.59	0.94	-5.19	0.53	0.94
'Senga S.'	-6.92	0.16	1.00	-5.16	0.71	0.94	-4.53	1.54	0.90
'Elsanta'	-5.58	0.05	0.99	-3.71	0.34	0.96	-3.46	0.25	0.97
'Frida'	-5.52	1.03	0.74	-4.03	0.86	0.87	-4.23	1.04	0.79

Surviving plants, browning extent and intensity were scored as described in Table 2.3. The LT50 (temperature at which 50% of plants died or 50% of maximal browning occurred), the SE (standard error) and R^2 (correlation coefficient) were calculated using a nonlinear data fit with a sigmoidal dose response mode (variable slope), using Prism 5 (GraphPad). Raw data are contained in Table 2.4.

2.3.1. 2DE Maps of *F.* × *ananassa* Crown Tissue

The major overwintering structure of strawberries, the crown, was evaluated for changes in proteins which might be associated with enhanced cold tolerance or winter survival. Clonal lines of mature strawberry plants, 6 weeks old were subjected to short (2 d) and long term (42 d) cold treatments (2 ºC). Multiple crowns (up to 6) were included for each replicate thereby minimizing the biological variance. Each crown was divided and used for 2DE analysis, or for shotgun analysis and half the crown was retained for transcript analysis described in later chapters. A total of 168 plants from all cultivars were used to complete 3 experimental time points in triplicate requiring 36 2DE gels in total. Nine hundred well resolved spots were detected by colloidal coomassie-stained gels within a range from 4 to 9 pH units and 15 to100 kDa MW range. Figure 2.2 reports the first 2DE protein reference map for strawberry crowns with arrows indicating the 110 spots that were identified by LC-MS/MS (Table 2.6). The measured MW and pI for the proteins identified in 2DE strongly matched with the MW and pI deduced from sequences (Table 2.6). One notable exception was actin which was identified in 2DE at 26 kD compared with the expected 42 kD. The 2DE protein spot identified as actin is likely due to degradation product based on having less than the expected size.

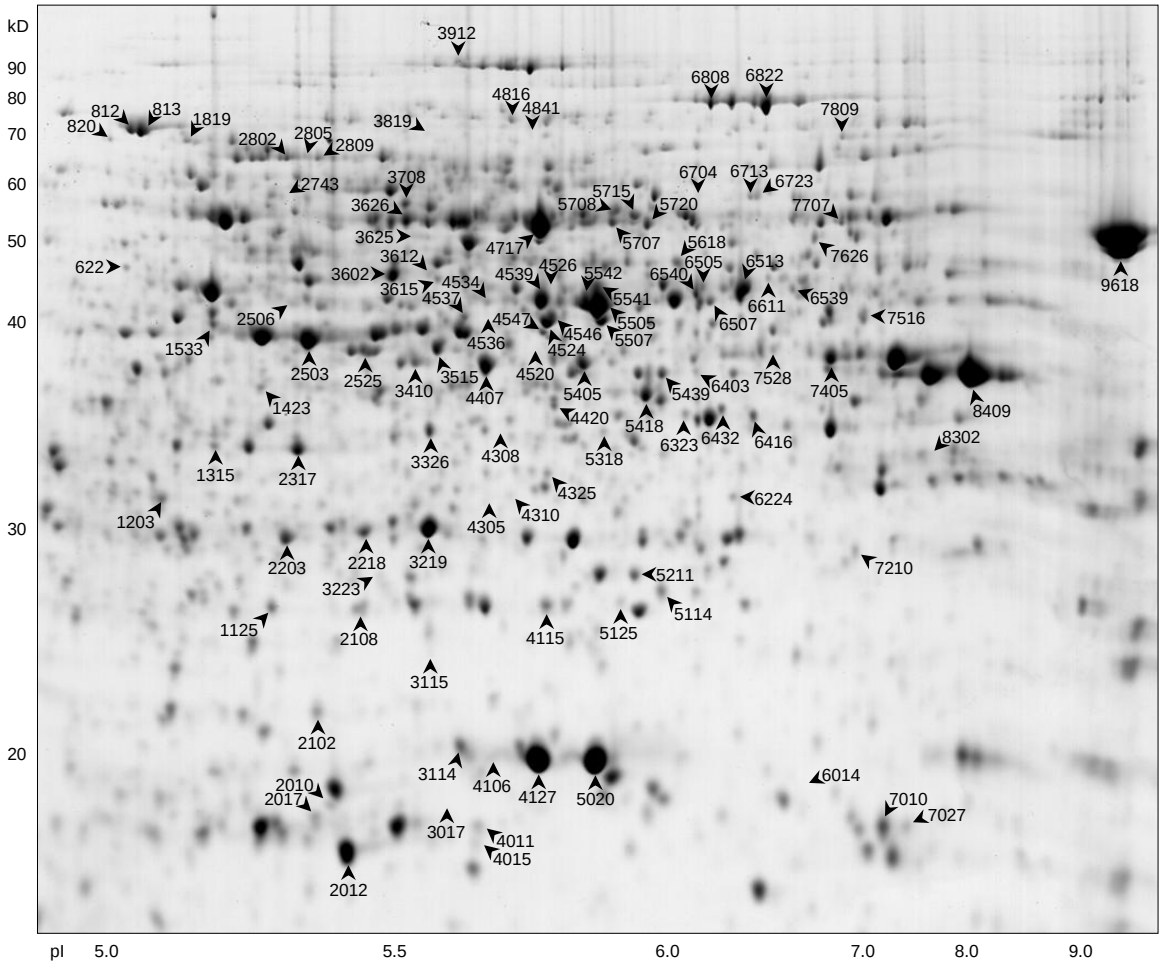


Figure 2.2 2DE gel of *F.* × *ananassa* crown proteins ('Jonsok' at 2 days 2 ºC treated). The 110 proteins identified by LC-MS/MS (Table 2.6) are indicated with spot numbers. Gel was performed with 400 µg of protein using 24 cm immobilized pH gradient strips (3 to 10 nonlinear) resolved on 12% SDS-PAGE and stained with colloidal coomassie brilliant blue.

Table 2.6 The 110 proteins from 2DE experiments of *Fragaria* × *ananassa* crown identified by LC-MS/MS. Protein identification for 110 2DE protein spots are sorted by the 2DE spot number (Spot no.) and listed with the accession code gene identifier (gi) and species corresponding to LC-MS/MS database search results. Confidence scores are either reported as probability (shaded) or by q-value (see methods). The number of peptides with different sequences (unique) and number peptides identified (no.) by LC-MS/MS are listed. Protein identifications were based from Blast searches using NCBI and TAIR. The arabidopsis homologs are listed by AGI (Arabidopsis Genome Initiative) gene index number and corresponding name. The molecular weight and isoelectric point (MW / pI) were calculated from 2DE gels (Exp.) using internal standards, and for the accession code sequences (Theor.) using Expasy.org and reported for arabidopsis gene homologs (AGI) obtained from the TAIR site (Http://www.arabidopsis.org).

Spot	Accession			Peptides		Exp.	Theor.	AGI		
no.	Code (gi)	*Species*	Confidence	unique/ no.	Putative Identification	MW / pI	MW / pI	MW / pI	AGI homolog	AGI Homolog Name
622	158372267	*Fragaria vesca*	7.14E-05	7/ 8	SGT1 (suppressor of G2 allele of SKP1)	45.4/ 5.0	33.3/ 5.1	39.8/ 4.8	AT4G11260	SGT1B; phosphatase-related
812	158378004	*Fragaria vesca*	3.11E-05	9/ 14	HSP70 (heat shock protein 70)	71.8/ 5.0	30.0/ 6.2	71.4/ 4.8	AT5G02500	HSC70-1 (heat shock cognate protein 70-1)
813	6969976	*Malus x domestica*	1.00E+00	19/ 33	HSP70 (heat shock protein 70)	71.9/ 5.1	71.2/ 5.2	71.1/ 4.9	AT3G12580	HSP70 (heat shock protein 70)
820	89549503	*Fragaria vesca*	2.65E-06	4/ 5	Nucleoredoxin	69.5/ 5.0	25.3/ 5.8	65.2/ 4.6	AT1G60420	DC1 domain-containing protein
1125	158379507	*Fragaria vesca*	7.54E-08	3/ 3	Actin	26.2/ 5.3	29.1/ 5.0	41.7/ 5.1	AT3G12110	ACT11 (actin-11)
1203	2465012	*Fragaria vesca*	1.00E+00	4/ 4	CCoAOMT (caffeoyl-CoA 3-O-methyltransferase)	31.2/ 5.1	21.1/ 5.2	29.2/ 4.9	AT4G34050	CCoAOMT1 caffeoyl-CoA 3-O-methyltransferase
1315	158372608	*Fragaria vesca*	1.08E-05	6/ 6	Glyoxalase I (lactoylglutathione lyase)	33.8/ 5.2	30.4/ 6.3	36.3/ 5.2	AT1G11840	ATGLX1 (GLYOXALASE I HOMOLOG)
1423	51047667	*F. x ananassa*	2.81E-05	4/ 5	Isoflavone reductase-like	37.1/ 5.3	30.0/ 9.0	33.7/ 5.7	AT1G75280	Isoflavone reductase
1533	6760442	*F. x ananassa*	5.15E-07	16/ 24	COMT (Caffeic acid 3-O-methyltransferase)	40.7/ 5.2	39.9/ 5.8	39.6/ 5.8	AT5G54160	ATOMT1 (O-METHYLTRANSFERASE 1)
1819	158378502	*Fragaria vesca*	3.21E-06	9/ 14	HSP70 (heat shock protein 70)	70.4/ 5.1	25.2/ 5.6	73.0/ 5.4	AT5G09590	MTHSC70-2 (MITOCHONDRIAL HSP70 2)
2010	158362366	*Fragaria vesca*	1.65E-07	4/ 6	Cu/ Zn SOD	19.1/ 5.4	15.3/ 5.8	15.1/ 5.4	AT1G08830	CSD1 (copper/ zinc superoxide dismutase 1)
2012	162424426	*F. x ananassa*	1.00E+00	4/ 10	Fra a 3	18.2/ 5.4	5.4/ 6.9	17.1/ 4.9	AT1G24020	MLP423 (MLP-LIKE PROTEIN 423)
2017	89549262	*Fragaria vesca*	9.90E-06	6/ 8	MLP (major latex-like protein)	18.9/ 5.4	24.5/ 5.9	35.6/ 5.0	AT1G70830	MLP34 (MLP-like protein 34)
2102	158374908	*Fragaria vesca*	5.51E-07	7/ 13	TPX (thioredoxin-dependent peroxidase-like)	22.2/ 5.4	15.9/ 5.9	17.4/ 5.0	AT1G65980	TPX1 (thioredoxin-dependent peroxidase 1)
2108	158361609	*Fragaria vesca*	8.81E-06	8/ 11	Quinone reductase-like	26.3/ 5.4	21.8/ 5.8	27.0/ 7.7	AT5G54500	FQR1 (flavodoxin-like quinone reductase 1)
2203	158366345	*Fragaria vesca*	1.88E-06	9/ 17	Thaumatin-like protein	29.3/ 5.3	28.0/ 6.2	34.9/ 4.7	AT1G75800	pathogenesis-related thaumatin family protein
2218	5257554	*F. x ananassa*	2.62E-07	14/ 23	APX (cytosolic ascorbate peroxidase)	29.8/ 5.4	27.3/ 5.7	27.6/ 6.0	AT1G07890	APX1 (ascorbate peroxidase 1)
2317	158356647	*Fragaria vesca*	3.44E-06	11/ 19	β-1, 3-glucanase	33.6/ 5.3	30.3/ 5.6	37.6/ 8.6	AT3G57240	BG3 (BETA-1,3-GLUCANASE 3)
2503	51872681	*F. x ananassa*	1.00E+00	3/ 4	ANS (anthocyanidin synthase)	39.7/ 5.3	42.8/ 5.3	40.4/ 5.0	AT4G22880	LDOX (leucoanthocyanidin dioxygenase)
2506	10187169	*F. x ananassa*	2.88E-06	3/ 3	CAD (cinnamyl alcohol dehydrogenase)	42.0/ 5.3	20.1/ 5.0	39.1/ 5.2	AT3G19450	ATCAD4 (cinnamyl-alcohol dehydrogenase)
2525	110564477	*F. x ananassa*	1.00E+00	9/ 13	ANR (anthocyanidin reductase)	39.1/ 5.4	36.6/ 5.7	37.9/ 6.2	AT1G61720	BAN; NAD(P)-binding Rossmann-fold superfamily protein
2743	24637539	*Prunus dulcis*	1.00E+00	4/ 4	HSP60 (heat shock protein 60)	57.0/ 5.3	57.8/ 5.3	61.3/ 5.4	AT3G23990	HSP60 (HEAT SHOCK PROTEIN 60)
2802	4165550	*Malus x domestica*	1.00E+00	4/ 7	apgm (2,3-biphosphoglycerate-independent phosphoglycerate mutase)	64.9/ 5.3	60.8/ 5.4	60.8/ 5.6	AT3G08590	Phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent
2805	4165550	*Malus x domestica*	1.00E+00	2/ 3	apgm (2,3-biphosphoglycerate-independent phosphoglycerate mutase)	65.1/ 5.3	60.8/ 5.4	60.8/ 5.6	AT3G08590	Phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent
2809	4165550	*Malus x domestica*	1.00E+00	5/ 19	apgm (2,3-biphosphoglycerate-independent phosphoglycerate mutase)	64.9/ 5.4	60.8/ 5.4	60.8/ 5.6	AT3G08590	Phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent
3017	158376648	*Fragaria vesca*	5.86E-06	4/ 4	40S ribosomal protein S12	19.4/ 5.6	13.7/ 6.8	15.3/ 5.7	AT2G32060	40S ribosomal protein S12 (RPS12C)
3114	88082485	*F. x ananassa*	1.00E+00	2/ 5	Fra a 1-A	21.2/ 5.6	17.8/ 6.0	17.1/ 4.9	AT1G24020	MLP423 (MLP-LIKE PROTEIN 423)
3115	109659933	*F. x ananassa*	1.00E+00	3/ 6	hexokinase 1	24.2/ 5.5	19.4/ 4.9	53.9/ 6.8	AT1G47840	HXK3 (HEXOKINASE 3)
3219	5257554	*F. x ananassa*	0.00E+00	15/ 30	APX (cytosolic ascorbate peroxidase)	29.6/ 5.5	27.3/ 5.7	27.6/ 6.0	AT1G07890	APX1 (ascorbate peroxidase 1)
3223	158369081	*Fragaria vesca*	9.33E-06	4/ 5	Ferritin	27.8/ 5.5	29.0/ 5.8	28.4/ 5.6	AT3G11050	FERRITIN 2

Table 2.6Continued

3326	6760443	*F. x ananassa*	1.00E+00	3/ 3	COMT (Caffeic acid 3-O-methyltransferase)	34.5/ 5.5	39.8/ 5.7	39.6/ 5.8	AT5G54160	ATOMT1 (O-METHYLTRANSFERASE 1)
3410	24638017	*F. x ananassa*	1.00E+00	2/ 3	MDH2 (malate dehydrogenase, mitochondrial)	38.3/ 5.5	35.5/ 8.7	35.8/ 8.6	AT1G53240	MITOCHONDRIAL MALATE DEHYDROGENASE 1
3515	90576646	*F. x ananassa*	1.13E-08	18/ 27	ANR (anthocyanidin reductase)	39.6/ 5.6	36.6/ 5.8	37.9/ 6.2	AT1G61720	BAN (BANYULS); oxidoreductase
3602	10187155	*F. x ananassa*	1.00E+00	2/ 11	3-ketoacyl-CoA thiolase	44.8/ 5.5	48.3/ 8.0	48.6/ 8.5	AT2G33150	PKT3 (PEROXISOMAL 3-KETOACYL-COA
3612	158376051	*Fragaria vesca*	4.03E-08	9/ 12	AdoMet synthase (S-adenosylmethionine synthetase)	44.7/ 5.5	22.0/ 8.5	42.8/ 5.6	AT3G17390	MTO3, SAMS3 (S-adenosylmethionine synthetase family)
3615	158365588	*Fragaria vesca*	4.90E-05	2/ 2	GEM-like	44.0/ 5.6	30.0/ 6.7	32.2/ 5.1	AT2G22475	GEM (GL2-EXPRESSION MODULATOR)
3625	89549254	*Fragaria vesca*	8.31E-09	9/ 15	PGD (phosphogluconate dehydrogenase)	49.8/ 5.5	22.8/ 5.2	53.6/ 7.5	AT3G02360	6-phosphogluconate dehydrogenase family protein
3626	158371950	*Fragaria vesca*	5.08E-06	7/ 15	Enolase	53.1/ 5.5	25.7/ 4.9	47.7/ 5.5	AT2G36530	LOS2, ENO2 Enolase
3708	158369701	*Fragaria vesca*	4.43E-07	7/ 9	2-HPCL (2-hydroxyacyl-CoA lyase)	55.9/ 5.5	*31.5/ 6.4*	61.5/ 5.9	AT5G17380	Thiamine pyrophosphate dependent pyruvate decarboxylase
3819	158362911	*Fragaria vesca*	6.02E-08	2/ 2	sti1 (stress-inducible protein)	70.0/ 5.5	31.5/ 5.5	64.5/ 6.1	AT1G62740	stress-inducible protein, putative
3912	158352700	*Fragaria vesca*	2.28E-07	5/ 6	Aconitate hydratase	88.7/ 5.6	32.4/ 5.7	108.2/ 7.2	AT2G05710	ACO3 (aconitase 3)
4011	158373249	*Fragaria vesca*	8.72E-13	6/ 8	Fra a 3	18.8/ 5.6	19.2/ 5.6	17.1/ 4.9	AT1G24020	MLP423 (MLP-LIKE PROTEIN 423)
4015	89557236	*Fragaria vesca*	0.00E+00	6/ 9	Fra a 2	18.3/ 5.6	19.2/ 5.6	17.1/ 4.9	AT1G24020	MLP423 (MLP-LIKE PROTEIN 423)
4106	90185686	*F. x ananassa*	1.00E+00	15/ 32	Fra a 1-C	20.8/ 5.7	17.7/ 6.0	17.1/ 4.9	AT1G24020	MLP423 (MLP-LIKE PROTEIN 423)
4115	158354533	*Fragaria vesca*	1.49E-07	8/ 10	GST (glutathione transferase)	26.5/ 5.7	25.7/ 6.3	29.2/ 8.9	AT2G47730	Glutathione S-transferase PHI 8
4127	90185686	*F. x ananassa*	1.00E+00	16/ 19	Fra a 1-C	20.9/ 5.7	17.7/ 6.0	17.1/ 4.6	AT1G24020	MLP423 (MLP-LIKE PROTEIN 423)
4305	158357552	*Fragaria vesca*	6.34E-07	11/ 17	Glyoxylase II (hydroxyacylglutathione hydrolase)	31.6/ 5.6	30.8/ 6.0	28.8/ 6.3	AT3G10850	GLY2; Metallo-hydrolase/ oxidoreductase superfamily
4308	89550344	*Fragaria vesca*	8.23E-12	9/ 13	Annexin	34.7/ 5.7	31.0/ 7.1	36.2/ 5.0	AT1G35720	ANNAT1 (ANNEXIN ARABIDOPSIS 1)
4310	158362418	*Fragaria vesca*	7.46E-09	5/ 5	Carbonate dehydratase	31.7/ 5.7	22.8/ 8.0	30.0/ 7.7	AT1G19580	GAMMA CA1 (gamma carbonic anhydrase 1)
4325	158376432	*Fragaria vesca*	9.10E-08	6/ 6	PP2C (protein phosphatase type 2C)	32.4/ 5.7	26.6/ 5.7	31.6/ 5.6	AT3G15260	Protein phosphatase 2C family protein
4407	78216493	*Malus x domestica*	1.00E+00	5/ 15	MDH1 (malate dehydrogenase, cytosolic)	38.2/ 5.6	35.6/ 6.0	35.6/ 6.5	AT1G04410	malate dehydrogenase, cytosolic, putative
4420	57896334	*Fragaria vesca*	3.64E-06	5/ 6	IFR (isoflavone reductase related protein)	36.1/ 5.8	31.2/ 8.9	34.1/ 6.8	AT4G39230	isoflavone reductase, putative
4520	110564477	*F. x ananassa*	1.04E-07	15/ 26	ANR (anthocyanidin reductase)	39.1/ 5.7	36.6/ 5.7	37.9/ 6.2	AT1G61720	BAN; NAD(P)-binding Rossmann-fold superfamily protein
4524	18453	*F. x ananassa*	1.00E+00	8/ 10	ADH (alcohol dehydrogenase)	40.7/ 5.7	41.5/ 6.6	41.2/ 6.2	AT1G77120	ADH1 (ALCOHOL DEHYDROGENASE)
4526	71979908	*F. x ananassa*	4.26E-07	13/ 19	CHS (chalcone synthase)	43.0/ 5.7	42.6/ 6.0	43.1/ 6.5	AT5G13930	naringenin-chalcone synthase
4534	71979908	*F. x ananassa*	3.60E-05	15/ 15	CHS (chalcone synthase)	42.1/ 5.6	42.6/ 6.0	43.1/ 6.5	AT5G13930	naringenin-chalcone synthase
4536	51493451	*F. x ananassa*	1.80E-05	10/ 14	F3H (flavanone 3-hydroxylase)	40.4/ 5.6	41.1/ 5.6	40.3/ 5.1	AT3G51240	F3H (FLAVANONE 3-HYDROXYLASE)
4537	158364386	*Fragaria vesca*	1.66E-06	10/ 22	PGK (phosphoglycerate kinase)	41.2/ 5.6	29.5/ 5.2	42.1/ 5.3	AT1G79550	PGK (phosphoglycerate kinase)
4539	62766610	*F. x ananassa*	1.00E+00	4/ 6	CHS (chalcone synthase)	42.3/ 5.7	42.6/ 6.0	43.1/ 6.5	AT5G13930	naringenin-chalcone synthase
4546	18453	*F. x ananassa*	1.00E+00	18/ 29	ADH (alcohol dehydrogenase)	41.5/ 5.7	41.5/ 6.6	41.2/ 6.2	AT1G77120	ADH1 (ALCOHOL DEHYDROGENASE)
4547	158376955	*Fragaria vesca*	5.84E-07	9/ 11	RGP (reversibly glycosylatable polypeptide)	40.0/ 5.7	*27.8/ 8.1*	40.6/ 5.7	AT3G02230	RGP1 (REVERSIBLY GLYCOSYLATED POLYPEPTIDE 1); cellulose synthase (UDP-forming)
4717	57115599	*Rubus sp. JPM-2004*	1.00E+00	15/ 40	F1-ATPase alpha subunit	52.2/ 5.7	14.5/ 5.9	85.9/ 5.2	AT2G07698	ATP synthase alpha chain, mitochondrial, putative
4816	14970841	*F. x ananassa*	1.00E+00	5/ 5	β-galactosidase	72.9/ 5.7	91.2/ 7.2	93.2/ 7.9	AT2G28470	BGAL8 (beta-galactosidase 8)
4841	14970841	*F. x ananassa*	1.00E+00	2/ 9	β-galactosidase	69.4/ 5.7	91.2/ 7.2	93.2/ 7.9	AT2G28470	BGAL8 (beta-galactosidase 8)
5020	90185690	*F. x ananassa*	1.00E+00	11/ 11	Fra a 1-E	20.7/ 5.8	17.8/ 6.1	17.1/ 4.6	AT1G24020	MLP423 (MLP-LIKE PROTEIN 423)
5114	16588840	*Prunus persica*	1.00E+00	2/ 3	Proteasome subunit beta type-5	26.7/ 5.9	21.8/ 6.1	32.3/ 6.7	AT1G13060	PBE1; threonine-type endopeptidase/ peptidase/
5125	158354533	*Fragaria vesca*	1.31E-06	3/ 3	GST (glutathione transferase)	26.3/ 5.9	25.7/ 6.3	29.2/ 8.9	AT2G47730	Glutathione S-transferase PHI 8
5211	71979902	*F. x ananassa*	1.00E+00	1/ 4	CHI (Chalcone isomerase 1)	27.5/ 5.9	25.4/ 5.2	26.6/ 5.2	AT3G55120	chalcone isomerase
5318	158372221	*Fragaria vesca*	1.31E-06	8/ 9	AKR (aldo/ keto reductase)	34.3/ 5.8	31.4/ 7.9	35.1/ 8.3	AT2G37770	aldo/ keto reductase family protein

Table 2.6Continued

5405	78216493	*Malus x domestica*	1.00E+00	6/ 11	MDH1 (malate dehydrogenase, cytosolic)	38.3/ 5.8	35.6/ 6.0	35.6/ 6.5	AT1G04410	malate dehydrogenase, cytosolic, putative
5418	50380153	*F. x ananassa*	1.00E+00	2/ 2	AKR (aldo/ keto reductase)	36.4/ 5.9	35.7/ 5.6	36.7/ 6.4	AT1G59960	aldo/ keto reductase, putative
5439	158365953	*Fragaria vesca*	2.23E-08	13/ 19	AKR (aldo/ keto reductase)	37.7/ 5.9	30.3/ 6.8	37.9/ 6.3	AT1G60710	ATB2; NAD(P)-linked oxidoreductase superfamily protein
5505	209360960	*Prunus dulcis*	1.00E+00	2/ 4	S-haplotype-specific F-box protein 8	42.0/ 5.8	43.3/ 5.7	41.1/ 6.9	AT3G23880	F-box family protein
5507	53988164	*F. x ananassa*	1.00E+00	2/ 3	AKR (aldo/ keto reductase)	40.8/ 5.8	36.1/ 5.8	36.7/ 6.4	AT1G59960	aldo/ keto reductase, putative
5541	15982950	*Prunus persica*	1.00E+00	5/ 10	IDH (Isocitrate dehydrogenase [NADP])	42.8/ 5.8	46.6/ 6.5	45.8/ 6.5	AT1G65930	isocitrate dehydrogenase, putative
5542	1332411	*Rosa hybrid cultivar*	1.00E+00	11/ 14	DFR (dihydroflavonol 4-reductase)	42.3/ 5.8	39.0/ 6.0	42.8/ 5.3	AT5G42800	DFR (DIHYDROFLAVONOL 4-REDUCTASE)
5618	158427470	*F. x ananassa*	1.00E+00	3/ 3	FBP (fructose-1,6-bisphosphatase)	45.8/ 6.0	44.1/ 5.2	45.1/ 5.0	AT3G54050	fructose-1,6-bisphosphatase, putative
5707	3107931	*Pyrus pyrifolia*	1.00E+00	11/ 23	UGPase (UTP-glucose-1-phosphate uridylyltransferase)	51.9/ 5.9	51.9/ 6.0	51.9/ 5.8	AT5G17310	UTP-glucose-1-phosphate uridylyltransferase 2
5708	82880416	*F. x ananassa*	1.00E+00	2/ 4	UGGT (UDP-glucose glucosyltransferase)	53.4/ 5.9	52.5/ 5.4	54.5/ 5.2	AT4G15480	UGT84A1 (UDP-Glycosyltransferase superfamily protein)
5715	2665890	*F. x ananassa*	1.00E+00	2/ 6	CDPK (calcium-dependent protein kinase)	53.2/ 5.9	59.6/ 6.2	60.9/ 6.4	AT3G57530	CPK32 (CALCIUM-DEPENDENT PROTEIN KINASE 32)
5720	3107931	*Pyrus pyrifolia*	1.00E+00	6/ 13	UGPase (UTP-glucose-1-phosphate uridylyltransferase)	51.6/ 5.9	51.9/ 6.0	51.9/ 5.8	AT5G17310	UTP--glucose-1-phosphate uridylyltransferase 2
6014	158365553	*Fragaria vesca*	1.17E-09	4/ 6	CBS domain-containing protein	20.1/ 6.6	23.6/ 8.7	22.7/ 9.5	AT5G10860	CBS domain-containing protein (cystathionine beta-synthase)
6224	158380121	*Fragaria vesca*	9.07E-07	8/ 10	Porin	31.1/ 6.2	23.8/ 5.4	29.4/ 9.2	AT3G01280	VDAC1 (voltage dependent anion channel 1)
6323	89544075	*Fragaria vesca*	2.21E-09	1/ 2	Annexin	35.3/ 6.0	29.6/ 6.9	36.2/ 5.0	AT1G35720	ANNAT1 (ANNEXIN ARABIDOPSIS 1)
6403	24638017	*F. x ananassa*	4.70E-10	10/ 13	MDH2 (malate dehydrogenase, mitochondrial)	37.6/ 6.0	35.5/ 8.7	35.8/ 8.6	AT1G53240	MITOCHONDRIAL MALATE DEHYDROGENASE 1
6416	89544075	*Fragaria vesca*	0.00E+00	8/ 9	annexin	35.4/ 6.4	29.6/ 6.9	36.2/ 5.0	AT1G35720	ANNAT1 (ANNEXIN ARABIDOPSIS 1)
6432	89544075	*Fragaria vesca*	7.32E-06	9/ 12	annexin	35.4/ 6.1	29.6/ 6.9	36.2/ 5.0	AT1G35720	ANNAT1 (ANNEXIN ARABIDOPSIS 1)
6505	18453	*F. x ananassa*	1.00E+00	7/ 10	ADH (alcohol dehydrogenase)	42.9/ 6.0	41.5/ 6.6	41.2/ 6.2	AT1G77120	ADH1 (ALCOHOL DEHYDROGENASE)
6507	89556234	*Fragaria vesca*	2.33E-07	5/ 6	Thiolase (acetyl-CoA C-acetyltransferase)	41.9/ 6.1	25.8/ 5.7	41.4/ 6.9	AT5G48230	ACAT2 (acetoacetyl-CoA thiolase 2)
6513	18453	*F. x ananassa*	1.00E+00	8/ 13	ADH (alcohol dehydrogenase)	42.7/ 6.4	41.5/ 6.6	41.2/ 6.2	AT1G77120	ADH1 (ALCOHOL DEHYDROGENASE)
6539	158373787	*Fragaria vesca*	1.63E-05	5/ 7	3-ketoacyl-CoA thiolase	42.8/ 6.7	*24.5/ 5.6*	48.6/ 8.5	AT2G33150	PKT3 (PEROXISOMAL 3-KETOACYL-COA
6540	113436	*F. x ananassa*	2.61E-07	5/ 7	ADH (alcohol dehydrogenase)	42.8/ 6.0	41.5/ 6.6	41.2/ 6.2	AT1G77120	ADH1 (ALCOHOL DEHYDROGENASE)
6611	24636275	*F. x ananassa*	9.59E-07	4/ 5	citrate synthase, mitochondrial	44.0/ 6.6	52.3/ 8.7	52.8/ 6.9	AT2G44350	ATCS; ATP binding / ATP citrate synthase/ cutrate (SI)-syntha
6704	89550793	*Fragaria vesca*	4.95E-04	5/ 6	GPI (glucose-6-phosphate isomerase)	56.1/ 6.0	28.7/ 7.2	61.7/ 6.6	AT5G42740	glucose-6-phosphate isomerase, cytosolic (PGIC)
6713	89546826	*Fragaria vesca*	0.00E+00	4/ 6	Seryl-tRNA synthetase	56.4/ 6.4	31.1/ 6.1	51.6/ 6.6	AT5G27470	seryl-tRNA synthetase / serine--tRNA ligase
6723	158357984	*Fragaria vesca*	4.57E-07	3/ 3	PK (pyruvate kinase)	57.2/ 6.5	24.8/ 8.9	55.0/ 6.2	AT5G08570	pyruvate kinase, putative
6808	89551239	*Fragaria vesca*	0.00E+00	13/ 16	ms (methionine synthase)	76.8/ 6.1	34.3/ 9.2	84.4/ 6.5	AT5G17920	ATMS1 (Colbalamin-independent methionine synthase)
6822	33235471	*F. x ananassa*	1.00E+00	4/ 10	Lipoxygenase	76.0/ 6.6	100.5/ 6.3	101.1/ 6.6	AT3G22400	LOX5; electron carrier/ iron ion binding /
7010	89553884	*Fragaria vesca*	1.38E-11	12/ 21	nsHb-1 (non-symbiotic hemoglobin class 1)	19.0/ 7.0	17.6/ 7.8	18.0/ 8.7	AT2G16060	AHB1 (ARABIDOPSIS hemoglobin 1)
7027	158379724	*Fragaria vesca*	8.95E-08	6/ 7	universal stress protein	19.1/ 7.1	17.4/ 7.9	17.3/ 8.0	AT2G47710	Adenine nucleotide alpha hydrolases-like superfamily
7210	158353550	*Fragaria vesca*	3.54E-07	7/ 10	20S proteasome beta subunit	28.8/ 6.9	28.3/ 8.4	27.7/ 6.5	AT1G56450	PBG1 (20S proteasome beta subunit G1)
7405	10645188	*F. x ananassa*	1.00E+00	17/ 30	fructose-bisphosphate aldolase	38.5/ 6.9	38.5/ 6.9	38.5/ 6.4	AT3G52930	fructose-bisphosphate aldolase, putative
7516	158351803	*Fragaria vesca*	1.03E-07	6/ 8	aminomethyltransferase	41.3/ 7.0	27.8/ 7.7	44.4/ 8.6	AT1G11860	Glycine cleavage T-protein family; aminomethyltransferase
7528	10645188	*F. x ananassa*	4.66E-12	8/ 9	FBP aldolase (fructose-bisphosphate aldolase)	39.1/ 6.6	38.5/ 6.9	38.5/ 6.4	AT3G52930	fructose-bisphosphate aldolase, putative
7626	158375727	*Fragaria vesca*	2.44E-07	7/ 11	AAA+-type ATPase	49.5/ 6.8	29.2/ 8.7	48.6/ 7.0	AT2G27600	SKD1 (AAA-type ATPase family protein)
7707	158374679	*Fragaria vesca*	6.75E-06	8/ 10	Catalase	52.0/ 6.9	29.0/ 6.2	56.9/ 7.1	AT4G35090	CAT2 (catalase 2)
7809	21396465	*F. x ananassa*	1.00E+00	3/ 5	GLDH (L-galactono-1,4-lactone dehydrogenase)	67.9/ 6.9	66.0/ 9.0	68.6/ 8.8	AT3G47930	ATGLDH (L-Galactono-1,4-lactone dehydrogenase)
8302	158349138	*Fragaria vesca*	5.50E-06	2/ 2	Adenylate kinase	33.3/ 7.5	30.0/ 9.0	26.9/ 7.5	AT5G63400	ADK1 (adenylate kinase 1)
8409	158302779	*F. x ananassa*	1.00E+00	8/ 12	GADPDH (glyceraldehyde-3-phosphate dehydrogenase)	37.5/ 7.9	17.5/ 9.3	36.9/ 7.2	AT3G04120	GAPC1 (glyceraldehyde-3-phosphate dehydrogenase C subun
9618	209922600	*Prunus persica*	1.00E+00	5/ 22	eEF1A (elongation factor 1-alpha)	50.4/ 9.2	49.3/ 9.2	49.5/ 9.6	AT1G07920	eEF-1A (elongation factor 1-alpha)

2.3.2. Agglomerative Hierarchical Clustering (AHC) of 2DE Data

2DE proteome profiling patterns were compared for *F.* × *ananassa* 'Jonsok', 'Senga Sengana', 'Elsanta' and 'Frida' for the 0, 2 and 42 days of cold treatment (2 ºC) by using agglomerative hierarchical clustering (AHC) on all 900 2DE matched spots. The Euclidean distance was used to measure the similarities between samples and Ward's algorithm was used to form clusters. The dendrogram resulting from AHC analysis is presented in Figure 2.3. The replicates for each cultivar at 0 and 2 days form clusters that are distinct from the other cultivars and from the 42 d cold treatment. After 42 d of cold treatment, three cultivars ('Jonsok', 'Frida' and 'Elsanta') form a new cluster. Each cultivar remains distinct within this 42 d cluster although one 'Jonsok' 42 d replicate formed its own branch. The 'S. Sengana' clustered separately at all time points, suggesting that this cultivar is not as responsive to cold treatments as the other cultivars. With the exception of 'Senga Sengana' cultivar, the changes in protein expression after 2 d of cold treatment are significant. Overall, the results indicate that the cultivars and their response to cold treatments can be clearly distinguished from each other based on protein expression profiles.

2.3.3. Principal Component Analysis (PCA) of 'Jonsok' and 'Frida'

PCA is a multivariate statistical method that allows a systematic way to consolidate larger multidimensional data (tables with large number of columns and rows) into a new reference system by assigning new variables called factors or principal components (PCs) (Joliffe, 2002; Pearson, 1901). PCA is thus a way to explore and identify the data (protein spots) that make the greatest contribution to the variation present in experimental samples. PCs incorporate the greatest differences observed among experimental samples and thus enable simple visualization of multidimensional data. PCs are ordered in such a way that the first PC represents the subset of data contributing largest variance and the second PC has the next largest contribution to variance and so on. For plotting purposes, the first 2 or 3 PCs are usually sufficient for visualizing the data that contributes to the majority of the variance and are plotted on the x and y-(and or Z) axis.

To determine and compare the overall cold responsive protein profiles for 'Jonsok' and 'Frida' principal component analysis (PCA) was applied to assess 2DE protein patterns

(Figure 2.4). The 2DE original data set of 5400 variables (900 protein spots for 2 cultivars at 3 time points) was reduced to two PCs that account for the majority of variation. The scree plot (Figure 2.4, inset) indicates that the first two principal components (PC), PC1 and PC2, account for 50.75% of the total variability in protein expression profiles. 'Jonsok' and 'Frida' are clearly distinguished from each other at all cold treatments. The PC2 dimension indicates differences in the cultivars at control and 2 day cold treatments. Interestingly, the long-term (42 d) cold treatment caused a large shift in the PC1 dimension and simultaneously reduced the differences between the cultivars in the PC2 dimension. This suggests the greatest overall differences in the cultivars exist under control and 2 day cold treatments, while the protein expression patterns tend to converge after long term cold treatment. The convergence of protein profiles at 42 d can be explained by the observation that many proteins in 'Frida' are increasing in abundance due to cold but do not reach levels greater than 'Jonsok' (and vice versa). This supports a hypothesis in which the difference in cold tolerance between the two cultivars may be significantly linked to the differences in protein expression under control conditions or in the initial phase of cold treatment.

The top 40 protein spots for PC1 that contribute the largest difference between the cultivars, 'Jonsok' and 'Frida' are listed, and ranked by PC score; (all better than 0.95) and are in bold when protein identification was made). The top 20 that are more abundant in 'Jonsok' than 'Frida are: **4547** rgp (alpha-1,4-glucan-protein synthase [UDP-forming], putative), 1140, **3626** (enolase), **2203** (thaumatin-like protein), **1315** (lactoylglutathione lyase), 6724, **820** (Nucleoredoxin), **2317** (β-1,3-glucanase), **5439** (aldo-keto reductase), **7027** unknown (universal stress protein), 1309, **6539** (3-ketoacyl-CoA thiolase), **5125** (glutathione S-transferase), **5318** (aldo-keto reductase family 4) , 6537, **6540** (alcohol dehydrogenase), **7626** (vacuolar sorting protein), 7306, 1127, 1223. The top 20 protein spots more abundant in 'Frida' than 'Jonsok' are **6416** (annexin-like), **6808** (methionine synthase), 4803, **3017** (40S ribosomal protein S12-2), 4607, 3020, 210, 133, 6204, **6611** (citrate synthase, mitochondrial), **6704** (glucose-6-phosphate isomerase), 4202, 4802, **4520** (anthocyanidin reductase), 3009, 2611, 5014, 2009, 3628, 5107, **3223** (Ferritin).

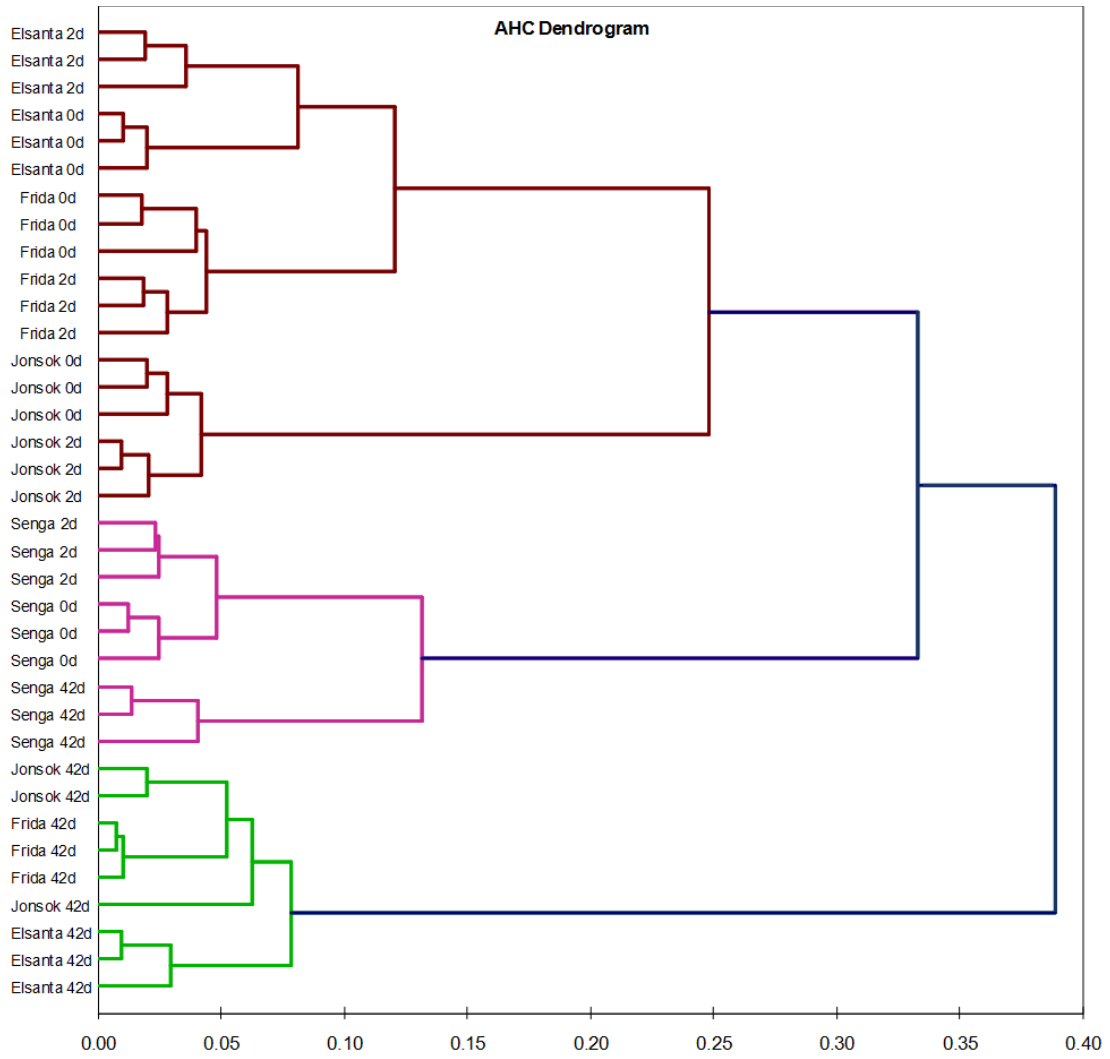


Figure 2.3 Agglomerative hierarchical clustering (AHC) indicates that cultivars and treatments group into distinct clades and subclades and thus cultivars can be distinguished from each other based on protein profiles.

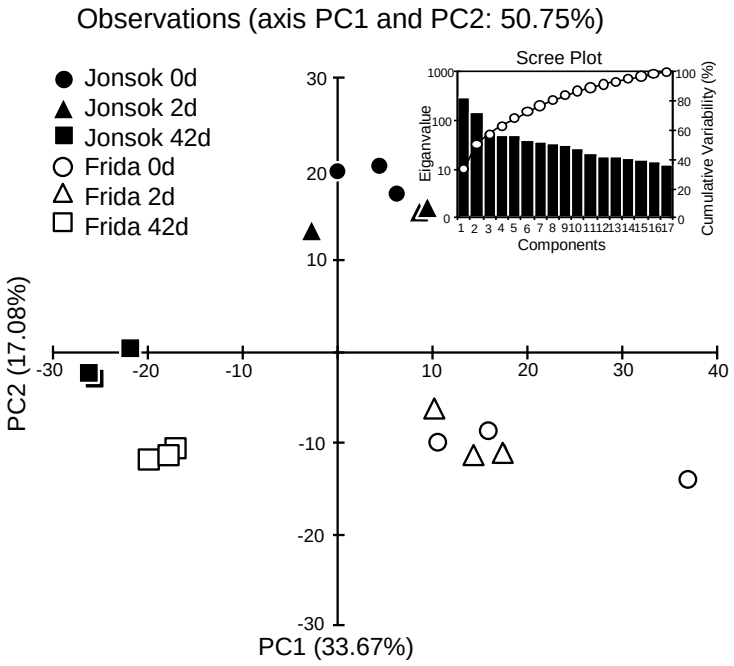


Figure 2.4 Principal component analysis (PCA) indicates 'Frida' and 'Jonsok' protein composition are distinctive and that they respond differently to cold stress. Time (in days) of exposure to 2 ºC is indicated by 0d, 2d, and 42d. All 900 common spots were included in this analysis. The scree plot (inset) indicates that the first two principal components (PC1, PC2) contribute 33.76% and 17.08% of the variance, respectively.

2.3.4. 2DE Protein Spot Comparison for 'Jonsok' and 'Frida'

After two-dimensional electrophoresis (2DE), 900 spots were matched, quantitated and analyzed using PDQuest 2DE Gel Analysis Software for 'Jonsok', 'S. Sengana', Elsanta', and 'Frida'. Significance was calculated with a two-way ANOVA, with cold treatment as one factor and cultivars as the other. All statistically significant differences between treatments were tested using the Tukey test with a confidence interval of 95%. A Student's t-test, two sided, was also performed using a p-value of 0.05 as cut-off in order to identify the 2DE spots differentially regulated upon cold treatment (threshold ratio cold-stressed vs. control plants > 2 or < 0.5 fold).

The overall trends in cold responsive proteins were specifically evaluated for 'Jonsok' and 'Frida'. Both cultivars showed a similar total number of proteins significantly increasing or decreasing during cold treatment (Figure 2.5 A). There were 19 (2.1%) and 41 (4.6%) spots that increased in response to cold at 2 and 42 d in 'Jonsok' compared to 9 (1.0%) and 58

(6.4%) spots in 'Frida'. The protein spots that decreased in response to the cold treatment at 2 and 42 d were 16 (1.8%) and 118 (13.1%) in 'Jonsok' and 18 (2.1%) and 157 (17.4%) in 'Frida'. One of the 18 proteins that increased in both cultivars at 42 d was identified as alcohol dehydrogenase. Among the 41 proteins that decreased in both cultivars, 3 were identified as glyceraldehyde-3-phosphate dehydrogenase, a putative 20S proteasome β-subunit 5, and a calcium-dependent protein kinase. Only one protein (Cu/Zn superoxide dismutase) decreased at all time points in both 'Jonsok' and 'Frida' though it remained significantly higher in 'Jonsok' at all time points. Several proteins that were observed to 'Frida' to increase in response to cold approached, but did not reach the levels that were present in 'Jonsok' at 42 d. Some of these proteins include a putative protein phosphatase, pyruvate kinase, and alcohol dehydrogenase. Likewise, proteins in 'Jonsok' that appear cold responsive and approach, but do not reach the levels in 'Frida' were identified as lipoxygenase, glyceraldehyde-3-phosphate dehydrogenase, and S-adenosylmethionine synthase. Together these changes partially explain the convergence in overall protein expression levels observed in the PCA analysis (Figure 2.4).

Interestingly, less than half of the cold-responsive protein spots were in common between the two cultivars (Figure 2.5 B and C). The protein spots, to be considered significantly different between 'Jonsok' and 'Frida', 1) differed ≥ 2 fold relative to the other cultivar with a significance of $p < 0.05$ Student's t-test) PCA factor loading with Pearson's correlation coefficient equal or better than the absolute value of 0.80). The complete data set with calculated values (t-test, PCA loading factors etc.) is appended electronically as Supplemental Data Set. From the 2DE, 283 protein spots exhibited significant differences of at least 2 fold between 'Jonsok' and 'Frida' at one or more time points. A total of 22 proteins were consistently (at all experimental conditions) greater in 'Jonsok' than 'Frida' (Figure 2.5 B) and a total of 15 proteins were consistently (at all experimental conditions) greater in 'Frida' than 'Jonsok' (Figure 2.5 C). A list of the 64 most significantly differentially accumulated proteins identified for 'Jonsok' and 'Frida' was produced based on a mixture of statistical, clustering, and PCA analysis (Table 2.7). A volcano plot visualizes how the top identified protein spots perform within the entire 2DE gel proteome dataset (Figure 2.6). The reference 2DE map illustrates the 65 differentially expressed proteins, 36 higher in 'Jonsok' and 28 higher in 'Frida' (Figure 2.7).

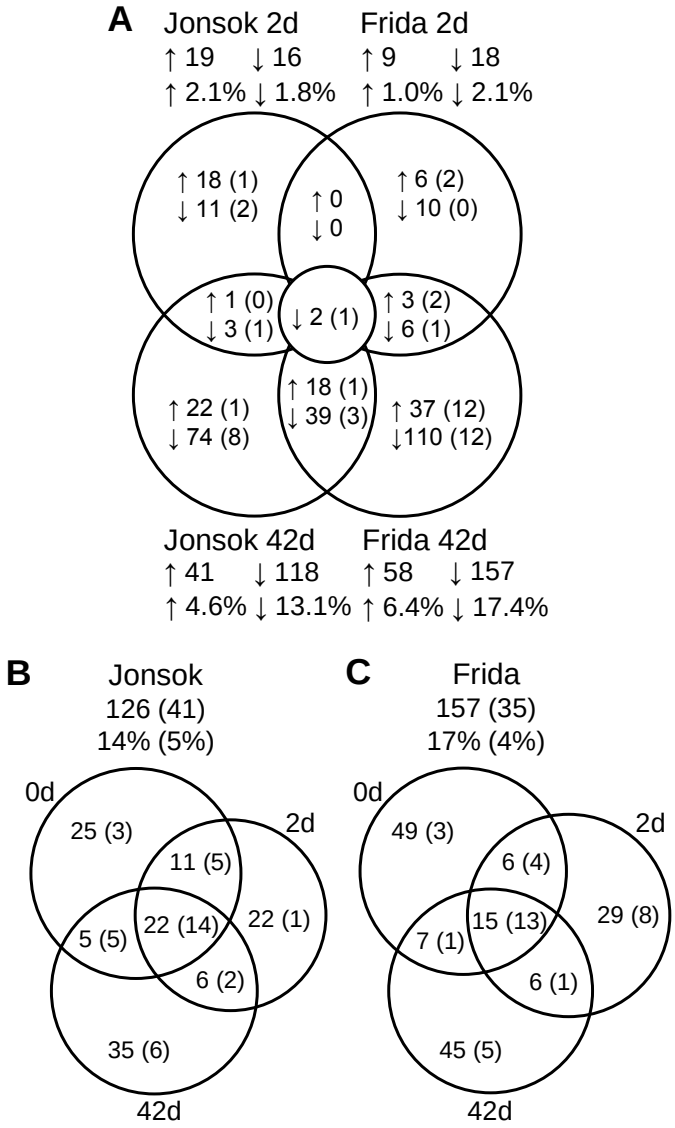


Figure 2.5 Differentially expressed proteins in 'Frida' and 'Jonsok'. Panel A shows cold responsive proteins at 2 days (2d), and 42 days (42d) that have changed ≥ 2 fold relative to control (0 d) in 'Frida' and 'Jonsok'. The number and percent of protein spots accumulating or decreasing are indicated with arrows. The number of proteins with higher levels (≥ 2 fold) in 'Jonsok' (Panel B) and 'Frida' (Panel C) with respect to the other cultivar are shown at each time point. Venn diagrams depicts the number of proteins detected at a significance of $p < 0.05$ in the Student's t-test, and for Panel B and C additionally met the criteria of better than 0.80 for factor loadings from PCA using the 900 matched spots from 2DE. The numbers within parentheses indicate the number of spots with protein identification.

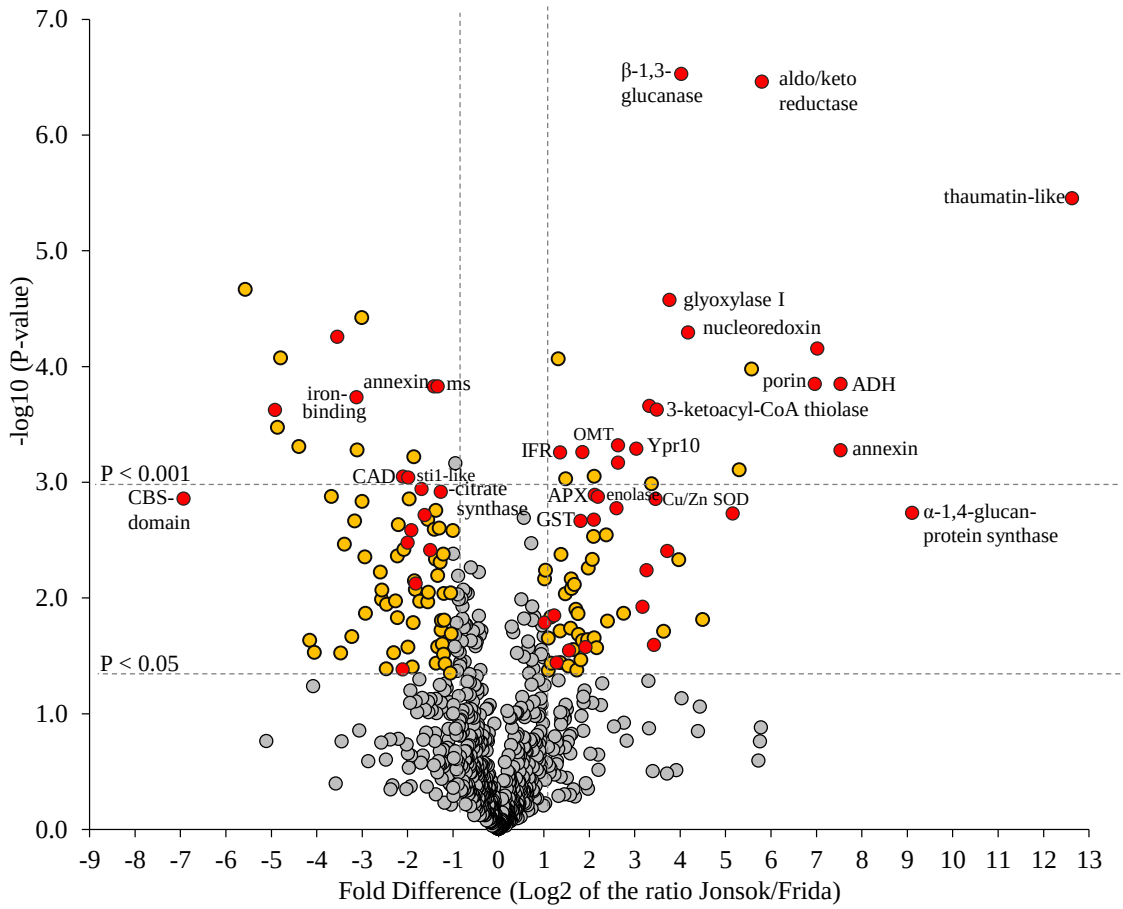


Figure 2.6 Protein differences and significances in 'Jonsok' and 'Frida' at 42 day cold treatment. Volcano plot was obtained by plotting the log2 ratio of mean values ('Jonsok'/'Frida') for the 900 matched 2DE spots at 42 day cold treatment against the negative log10-transformed P-value from the Student's t-test. Protein spots with a 2 fold difference in expression in 'Jonsok' and 'Frida' with a p-value < 0.05 are indicated by red and orange (148 spots). The red color corresponds to the 49 spots that were additionally deemed significantly different between 'Jonsok' and 'Frida' from an ANOVA analysis performed on all four cultivars at all time points (0, 2, and 42 day at 2 ºC). Twenty-four of the total 110 identified spots were labeled based on significance by ANOVA and having highest –log10 (p-value). p-values < 0.05 and < 0.001 are indicated next to y-axis. Abbreviations; ADH, alcohol dehydrogenase; APX cytosolic ascorbate peroxidase; CBS domain, cystathionine β-synthase domain; IFR, isoflavone reductase related protein; ms, methyltransferase; sti1-like, stress-induced protein; Ypr10, pathogenesis-related protein Ypr10.

Table 2.7 Identity of the proteins characterized by 2DE which distinguish the two cultivars, 'Jonsok' and 'Frida'.

A. Proteins that are at higher levels in 'Jonsok' than 'Frida'

				COLD RESPONSE								**CULTIVAR DIFFERENCE**					
			Time point(s) that meet all criteria	Log2 of the Ratio				Student's t-test $p < 0.05$				Log2 of Ratio			Student's t-test $p < 0.05$		
				Jonsok		Frida		Jonsok		Frida		Jonsok / Frida			Jonsok vs. Frida		
Spot no.	GenBank	ASSIGNED NAME		2d /0d	42d /0d	2d /0d	42d /0d	2d /0d	42d /0d	2d /0d	42d /0d	**0d**	**2d**	**42d**	**0d**	**2d**	**42d**
6539	EX682172	3-ketoacyl-CoA thiolase	42d	-0.01	-0.17	-0.16	-2.65	0.9780	0.5005	0.4655	0.0013	0.8	1.0	3.3	0.0409	0.0022	0.0002
7626	EX684112	AAA+-type ATPase	0d	0.42	0.01	0.99	0.13	0.0347	0.9314	0.1457	0.7895	1.5	0.9	1.4	0.0061	0.0274	0.0042
4546	CAA33613	ADH (alcohol dehydrogenase)	0d	-0.42	1.42	-0.01	3.17	0.3767	0.0037	0.9875	0.0102	2.5	2.1	0.7	0.0256	0.0103	0.0345
6505	CAA33613	ADH (alcohol dehydrogenase)	0d,42d	-0.21	0.74	-0.02	1.19	0.4798	0.0404	0.9680	0.0012	1.5	1.3	1.0	0.0020	0.0573	0.0164
6540	P17648	ADH (alcohol dehydrogenase)	0d,2d,42d	-0.46	-0.41	0.39	-4.03	0.0376	0.0438	0.6019	0.1644	3.4	2.5	7.0	0.0006	0.0003	0.0001
5439	EX674338	AKR (aldo-keto reductase)	0d,2d,42d	0.08	0.36	0.66	-1.97	0.6827	0.0373	0.5978	0.2287	3.5	2.9	5.8	0.0009	0.0021	0.0000
5318	EX680606	AKR (aldo-keto reductase)	2d	0.90	0.10	-2.17	-1.63	0.0808	0.8494	0.2585	0.3523	3.8	6.9	5.6	0.0538	0.0003	0.0001
6432	DY668560	Annexin	0d,2d,42d	0.22	-0.16	-1.38	0.94	0.2465	0.3557	0.2704	0.5990	8.6	10.2	7.5	0.0000	0.0005	0.0005
6323	DY668560	Annexin	0d,2d	0.10	-0.26	-0.83	-0.82	0.6205	0.3607	0.2607	0.2710	1.5	2.5	2.1	0.0182	0.0001	0.0029
3515	ABD95362	ANR (anthocyanidin reductase)	0d,2d	0.18	-0.12	-0.06	-0.10	0.3298	0.5647	0.9317	0.8797	2.1	2.3	2.1	0.0017	0.0015	0.0047
2218	AAD41406	APX (cytosolic ascorbate peroxidase)	0d,42d	-0.39	-0.48	-0.16	-1.54	0.0153	0.0104	0.5577	0.0304	1.1	0.9	2.2	0.0033	0.0011	0.0013
1533	AF220491	Caffeic acid 3-O-methyltransferase	0d,42d	-1.48	-0.73	-0.03	-0.88	0.0000	0.0011	0.9414	0.1287	2.5	1.0	2.6	0.0000	0.0145	0.0005
2010	EX670753	Cu/Zn superoxide dismutase	0d,2d,42d	-1.33	-1.13	-2.53	-3.44	0.0024	0.0011	0.0012	0.0009	1.2	2.4	3.5	0.0008	0.0155	0.0014
3626	EX680335	Enolase	0d,2d,42d	-0.04	-0.35	0.00	-0.61	0.8961	0.3336	0.9914	0.2204	1.9	1.8	2.1	0.0193	0.0038	0.0013
4717	AAW33106	F1-ATPase alpha subunit	2d	0.21	0.38	-0.30	0.99	0.3573	0.1840	0.3430	0.0159	0.6	1.1	0.0	0.0472	0.0130	0.9532
3114	ABD39049	Fra a 1-A	0d	-0.29	0.58	0.77	1.65	0.3676	0.0043	0.1081	0.0007	1.3	0.2	0.2	0.0033	0.6428	0.0950
4015	DY675259	Fra a 2	0d	-1.10	-1.66	1.98	2.06	0.0472	0.0130	0.3665	0.3962	6.5	3.5	2.8	0.0000	0.0916	0.1719
2012	ABX89934	Fra a 3	0d,42d	-0.38	-1.02	1.03	-1.53	0.0598	0.0023	0.0693	0.0524	2.1	0.7	2.6	0.0007	0.0449	0.0007
4011	EX681634	Fra a 3	0d,2d	-0.58	-3.14	-1.01	-2.83	0.1202	0.0003	0.3962	0.1509	3.9	4.4	3.6	0.0003	0.0137	0.0196
7528	AAG21429	Fructose-bisphosphate aldolase	42d	-0.27	1.65	0.47	-0.23	0.3493	0.0303	0.1062	0.6062	0.0	-0.7	1.9	0.9046	0.0279	0.0268
1315	EX680994	Glyoxalase I	0d,2d,42d	-0.55	0.82	0.71	0.89	0.0688	0.0054	0.4765	0.2890	3.8	2.6	3.8	0.0019	0.0007	0.0000
4115	EX662925	GST (glutathione transferase)	0d,2d,42d	0.31	-0.16	0.36	-0.41	0.1670	0.5412	0.3667	0.1315	1.6	1.5	1.8	0.0074	0.0018	0.0022
5125	EX662925	GST (glutathione transferase)	0d,2d	0.69	-0.29	-1.17	-2.16	0.0800	0.3427	0.4214	0.2660	2.1	4.0	4.0	0.0113	0.0035	0.0047
813	AAF34134	HSP70 (heat shock protein 70)	0d	0.07	0.92	0.72	2.47	0.1929	0.0272	0.2300	0.0006	1.8	1.1	0.2	0.0042	0.0006	0.3282
1819	EX686887	HSP70 (heat shock protein 70)	0d,2d	0.08	0.24	1.26	2.27	0.5966	0.0757	0.0206	0.0003	2.5	1.4	0.5	0.0002	0.0039	0.0103
812	EX686389	HSP70 (heat shock protein 70)	0d	-0.64	-0.01	2.55	4.76	0.0370	0.9711	0.0058	0.0000	3.9	0.7	-0.9	0.0003	0.0667	0.0297
1423	CO817159	Isoflavone reductase-like	0d,2d,42d	0.07	0.12	0.13	-0.14	0.4863	0.3244	0.6594	0.6745	1.1	1.0	1.4	0.0065	0.0006	0.0006
820	DY671291	Nucleoredoxin	0d,2d,42d	-0.46	-0.31	3.33	4.18	0.0248	0.0443	0.3554	0.1247	8.7	4.9	4.2	0.0001	0.0003	0.0001
4537	EX672771	PGK (phosphoglycerate kinase)	0d,2d	0.27	-0.94	0.29	-0.11	0.2959	0.0263	0.5779	0.7662	1.8	1.7	0.9	0.0035	0.0074	0.0744
6723	EX666373	PK (pyruvate kinase)	0d,2d,42d	0.19	0.13	0.55	0.75	0.3987	0.6808	0.3193	0.0166	2.2	1.8	1.6	0.0001	0.0079	0.0286
6224	EX688506	Porin	0d,2d,42d	0.02	-0.43	-1.97	-2.75	0.9182	0.0729	0.2191	0.1566	4.6	6.6	7.0	0.0005	0.0000	0.0001
4325	EX684817	PP2C (protein phosphatase type 2C)	0d,2d	-0.09	-0.06	0.83	0.46	0.6418	0.8302	0.3070	0.4733	2.1	1.2	1.6	0.0022	0.0292	0.0183
4547	EX685340	RGP	0d,2d,42d	-0.05	0.11	-1.00	-4.42	0.8993	0.7618	0.5952	0.3063	4.6	5.5	9.1	0.0095	0.0041	0.0018
622	EX680652	SGT1	0d,2d	-0.71	-0.69	-0.23	1.06	0.0063	0.0086	0.6059	0.0048	2.4	2.0	0.7	0.0000	0.0054	0.0452
2203	EX674730	Thaumatin-like protein	0d,2d,42d	-1.33	0.20	-1.22	-6.14	0.0007	0.0855	0.5153	0.2810	6.3	6.2	12.6	0.0001	0.0001	0.0000
2317	EX665040	β-1, 3-glucanase	0d,2d,42d	-0.14	0.33	-1.12	-1.77	0.4334	0.0194	0.0316	0.0128	1.9	2.9	4.0	0.0008	0.0007	0.0000

(Table 2.7 continues on following page)

Table 2.7 continued

B. Proteins that are at higher levels in 'Frida' than 'Jonsok'

				COLD RESPONSE								CULTIVAR DIFFERENCE					
			Time point(s) that meet all criteria[a]	Log2 of the Ratio				Student's t-test p < 0.05				Log2 of Ratio			Student's t-test p < 0.05		
				Jonsok		Frida		Jonsok		Frida		Jonsok / Frida			Jonsok vs. Frida		
Spot no.	GenBank	ASSIGNED NAME		2d/ 0d	42d /0d	2d /0d	42d /0d	2d /0d	42d /0d	2d /2d	42d /2d	**0d**	**2d**	**42d**	**0d**	**2d**	**42d**
7210	EX661942	20S proteasome beta subunit b	0d,2d	0.22	-0.54	-0.01	-0.68	0.5293	0.3402	0.9561	0.0760	-1.4	-1.1	-1.2	0.0195	0.0053	0.0251
3708	EX678086	2-HPCL (2-hydroxyacyl-CoA lyase)	0d	0.66	0.57	-0.27	-0.38	0.1124	0.1090	0.1057	0.0225	-1.4	-0.5	-0.4	0.0030	0.0726	0.0184
3017	EX685033	40S ribosomal protein S12	0d,2d	0.24	-0.78	-0.37	-2.24	0.8579	0.5697	0.0564	0.0007	-7.0	-6.4	-5.6	0.0003	0.0000	0.0000
6507	DY674748	acetyl-CoA C-acetyltransferase	0d	0.61	0.12	0.16	-1.34	0.0302	0.7285	0.4057	0.0019	-1.3	-0.9	0.1	0.0028	0.0097	0.6795
3912	EX661092	Aconitate hydratase	2d	-0.93	-0.33	0.27	0.45	0.0492	0.2491	0.1457	0.0456	0.1	-1.1	-0.7	0.8436	0.0063	0.0089
8302	EX657534	Adenylate kinase	0d,2d	0.53	0.24	-0.24	-1.36	0.1299	0.8743	0.3088	0.0079	-2.9	-2.1	-1.3	0.0002	0.0057	0.2579
6416	DY668560	annexin	0d,2d,42d	-0.35	0.05	-0.01	0.14	0.5312	0.9226	0.9636	0.3030	-1.3	-1.7	-1.4	0.0158	0.0025	0.0001
4520	ABG76842	ANR (anthocyanidin reductase)	2d	-4.63	1.22	0.85	0.71	0.3739	0.4288	0.0074	0.0218	-5.4	-10.9	-4.9	0.0009	0.0001	0.0003
2506	CAC09058	CAD (cinnamyl alcohol dehydrogenase)	42d	-0.12	-2.22	-0.29	0.31	0.2444	0.0000	0.2288	0.1005	0.4	0.6	-2.1	0.0055	0.0401	0.0009
4310	EX670805	carbonate dehydratase	2d	0.11	-0.30	1.22	-0.50	0.7049	0.6635	0.0072	0.1378	-0.9	-2.0	-0.7	0.0559	0.0014	0.2400
7707	EX683064	catalase	2d	-0.80	-0.02	0.23	0.20	0.0032	0.8544	0.0274	0.4061	-0.1	-1.1	-0.3	0.3558	0.0000	0.2055
6014	EX673938	CBS domain-containing protein	0d,2d,42d	1.31	0.52	-0.01	-0.59	0.5460	0.6957	0.9367	0.0165	-8.0	-6.7	-6.9	0.0000	0.0008	0.0014
1203	CAA04769	CCoAOMT	0d	0.92	-0.18	-0.16	-1.29	0.0026	0.0819	0.0960	0.0006	-1.1	0.0	0.0	0.0004	0.6542	0.8986
4526	BAE17124	CHS (chalcone synthase)	2d	-5.64	-0.36	0.47	0.32	0.1253	0.8227	0.3385	0.6039	-3.4	-9.5	-4.1	0.0348	0.0032	0.0297
6611	P83372	citrate synthase, mitochondrial	0d,42d	0.68	0.19	0.27	0.23	0.0580	0.5612	0.0943	0.1944	-1.2	-0.8	-1.3	0.0079	0.0016	0.0012
5542	BAA12723	DFR (dihydroflavonol 4-reductase)	2d	0.77	-0.96	1.20	-1.06	0.4642	0.5311	0.0717	0.2891	-2.0	-2.5	-1.9	0.0284	0.0237	0.4262
4536	AAU04792	F3H (flavanone 3-hydroxylase)	2d	-3.43	0.10	2.59	1.68	0.0074	0.8950	0.0089	0.0705	-0.2	-6.3	-1.8	0.5007	0.0047	0.0785
3223	EX677466	Ferritin	42d	-0.65	-2.31	0.68	1.81	0.1381	0.0173	0.3039	0.0003	1.0	-0.3	-3.1	0.0669	0.5487	0.0002
7405	AAG21429	Fructose-bisphosphate aldolase	0d,2d	0.01	0.58	0.11	-0.03	0.9706	0.1166	0.4068	0.7547	-1.1	-1.2	-0.5	0.0068	0.0022	0.0241
3615	EX673973	GEM-like	42d	-0.02	-0.64	-0.36	0.93	0.8655	0.0731	0.2457	0.1378	-0.5	-0.2	-2.1	0.0130	0.5496	0.0418
4305	EX665941	Glyoxalase II	0d,2d	-0.70	-1.81	0.60	-0.25	0.4888	0.1716	0.0356	0.2233	-1.8	-3.1	-3.4	0.0026	0.0021	0.0034
6704	DY671931	GPI (glucose-6-phosphate isomerase)	0d,2d	-0.29	-2.36	0.09	-0.70	0.6428	0.0241	0.7792	0.0023	-1.3	-1.7	-3.0	0.0040	0.0385	0.0000
3115	ABG36925	Hexokinase 1	2d	-2.09	-3.38	0.18	-4.22	0.1899	0.1283	0.4744	0.0049	-1.0	-3.3	-0.2	0.1498	0.0002	0.8513
6403	P83373	Malate dehydrogenase, mitochondrial	0d	-0.16	-0.32	-0.17	-0.50	0.7066	0.6197	0.3182	0.0299	-2.1	-2.1	-2.0	0.0016	0.0002	0.0014
6808	DY672153	Methionine synthase	42d	0.18	-0.36	0.09	0.23	0.3156	0.1731	0.5170	0.1208	-0.7	-0.7	-1.3	0.0205	0.0009	0.0001
3625	DY671162	PGD (Phosphogluconate dehydrogenase)	0d,2d,42d	-0.28	-0.56	-0.07	0.00	0.5064	0.2726	0.5935	0.9850	-1.4	-1.6	-2.0	0.0019	0.0019	0.0033
3612	EX684436	S-adenosylmethionine synthetase	0d,2d	2.25	1.26	-0.31	-1.26	0.0289	0.3824	0.0999	0.0362	-3.8	-1.2	-1.3	0.0003	0.0066	0.2794
3819	EX671298	sti1 (stress-inducible protein)	2d,42d	-0.98	0.34	-0.39	0.94	0.2455	0.5395	0.2890	0.0125	-1.1	-1.7	-1.7	0.0872	0.0088	0.0011

Crown proteins were identified by LC-MS/MS.[a] The time points are given for the protein spots that meet the following criteria; factor loading value of 0.80 or better, and $p < 0.05$ using Student's t-test and ANOVA, and protein levels with 2 fold or greater difference. The 2 and 42 d cold response of proteins is reported for 'Jonsok' and 'Frida' as log2 of the day of treatment over 0d (control). The cultivar difference is the log2 of 'Jonsok' over 'Frida' for 0, 2, and 42 days. The corresponding values obtained for Student's t-test is highlighted when $p < 0.05$.

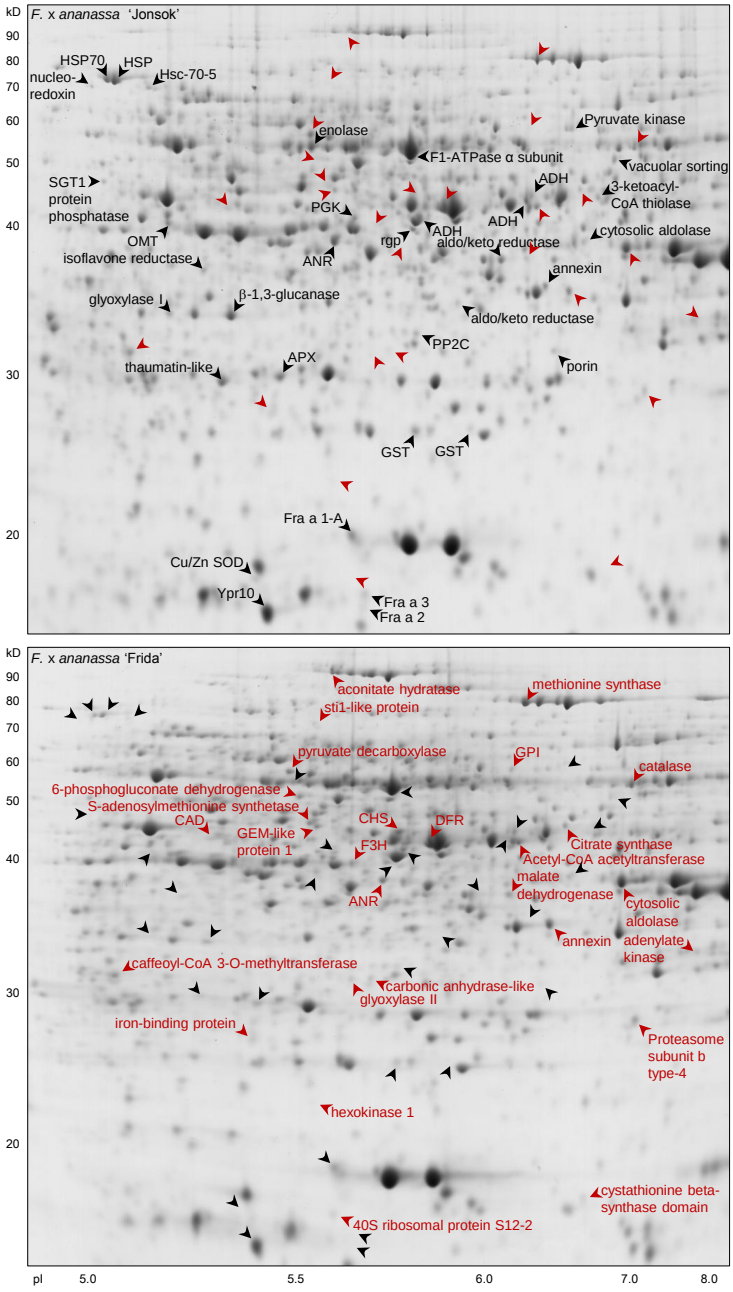
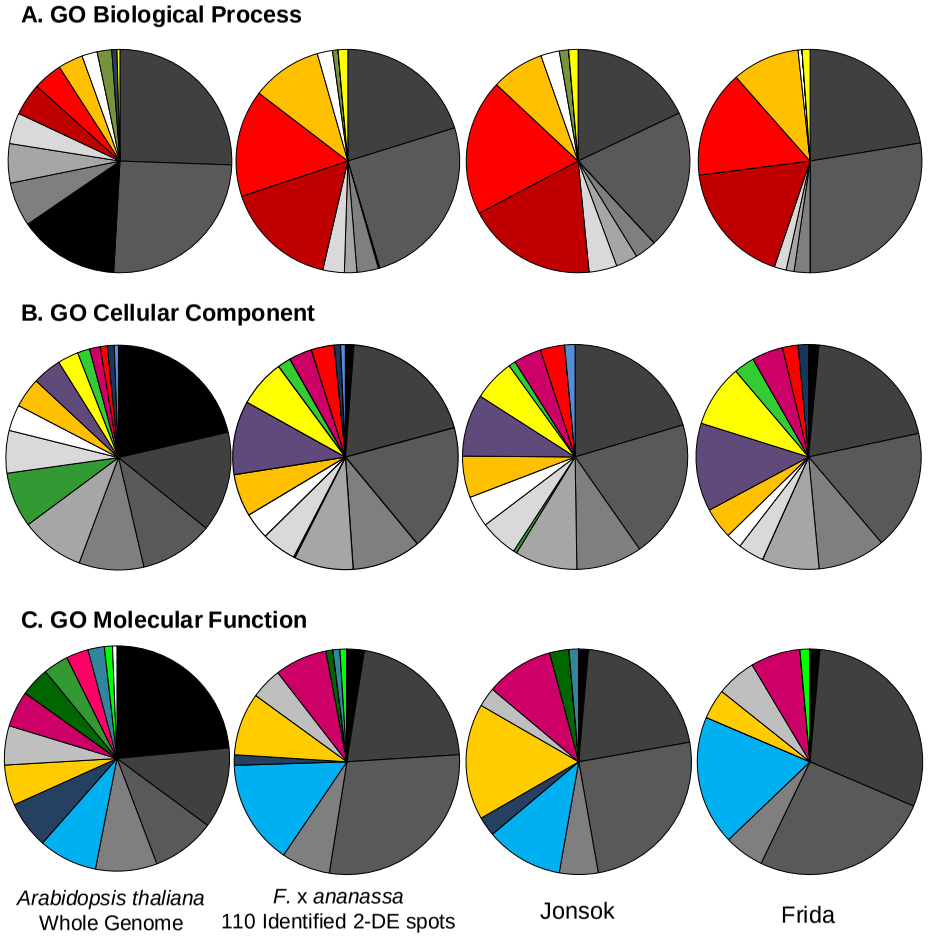


Figure 2.7 2DE maps illustrating the proteins that are differentially accumulated in 'Jonsok' and 'Frida'. 2DE gels of *F.* × *ananassa* 'Jonsok' (top) and 'Frida' (bottom) from 2 day cold treatment (2 ºC) from crown tissue. Numerous individual spot intensities differed between the cultivars and were identified with LC-MS/MS (36 and 28 for 'Jonsok' and 'Frida', respectively). Protein spots with labels indicate the identified proteins that are at higher levels ≥ 2 fold for that cultivar and detected with a significance of $p < 0.05$ (Student's t-test and ANOVA), and have a Pearson correlation coefficient of greater than the absolute value of 0.80 for factor loading values from PCA. Arrowheads without labels indicate spot location corresponding with the identified protein in the other gel. Ancillary data for these spots is summarized in Table 2.7.

2.3.5. Functional Categories of Identified Proteins from 2DE

Out of the 157 spots obtained from 2DE gels and analyzed by LC-MS/MS, a total of 110 were successfully identified with high confidence using Rosaceae and Fragaria databases. Most of the protein spots were selected for identification based on preliminary observations (raw quantity spot value difference between the cultivars), but several proteins were also chosen because they did not change and thus were good "anchors" for the gel analysis. After identifying Arabidopsis homologs, the GO terminology (cellular component, molecular function, and biological function) for all the identified (110 spots) and the differentially expressed proteins identified for 'Jonsok' and 'Frida' were obtained (Figure 2.8). The bias of our spot picking, which was based largely upon differences between the two cultivars in response to cold stress, is apparent in comparison with the overall Arabidopsis genome. The greatest proportion (almost half) of proteins identified in 'Jonsok' and 'Frida' fall into the Biological Process categories under stress-related or stress-responsive proteins. The array of stress response proteins (Table 2.8) that were displayed for 'Jonsok,' and 'Frida' differed in the amount of pathogen defense-related proteins with potential antifreeze activity (β-1,3-glucanase, thaumatin-like protein) and detoxification related proteins (AKR, GST) observed for 'Jonsok'. 'Frida' on the other hand showed more flavonoid-related proteins (F3H, and CHS). For the Cellular Components category, the cytosol, cell wall, plasma membrane, mitochondria and extracellular seem somewhat over represented. In the Molecular Function category, the identified proteins appear underrepresented in DNA or RNA binding, and transcription factor activity, and over represented in enzymatic functions, perhaps not surprising as the nature of proteomics encourages identification of more abundant proteins.



	A. thaliana Genome	F x ananassa 110 spots	JONSOK 36 spots	FRIDA 28 spots
A. GO Biological Process				
other cellular processes	**25.6** 19970(12315)	**20.2** 103(53)	**17.9** 40(19)	**22.4** 39(22)
other metabolic processes	**25.3** 19803(12819)	**25.2** 128(70)	**20.2** 45(26)	**27.6** 48(26)
unknown biological processes	**14.6** 11373(11373)	**0.2** 1(1)	**0.0** 0(0)	**0.0** 0(0)
protein metabolism	**6.4** 4986(4256)	**3.1** 16(13)	**3.1** 7(5)	**2.3** 4(4)
transport	**5.7** 4451(2079)	**1.8** 9(5)	**3.1** 7(3)	**1.1** 2(2)
developmental processes	**4.5** 3501(2254)	**3.1** 16(11)	**4.0** 9(6)	**1.7** 3(2)
response to stress	**4.7** 3655(2374)	**16.3** 83(41)	**18.8** 42(16)	**17.8** 31(15)
response to abiotic / biotic stimulus	**4.2** 3301(2135)	**15.5** 79(41)	**19.7** 44(17)	**15.5** 27(15)
other biological processes	**3.6** 2815(2117)	**10.2** 52(41)	**7.6** 17(13)	**9.8** 17(13)
cell organization and biogenesis	**2.2** 1737(1288)	**2.2** 11(7)	**2.7** 6(3)	**0.6** 1(1)
signal transduction	**2.1** 1645(1354)	**0.8** 4(3)	**1.3** 3(2)	**0.0** 0(0)
DNA or RNA metabolism	**0.8** 588(382)	**0.0** 0(0)	**0.0** 0(0)	**0.0** 0(0)
electron transport /energy pathways	**0.4** 328(292)	**1.4** 7(7)	**1.3** 3(3)	**1.1** 2(2)
B. GO Cellular Component				
unknown cellular components	**21.4** 9716(9716)	**1.2** 5(5)	**0.0** 0(0)	**1.5** 2(2)
other intracellular components	**14.4** 6552(4292)	**19.6** 83(39)	**20.4** 41(16)	**20.1** 27(14)
other cytoplasmic components	**10.6** 4799(3362)	**18.2** 77(40)	**19.9** 40(18)	**17.2** 23(14)
other membranes	**9.3** 4225(3379)	**9.9** 42(28)	**9.5** 19(13)	**9.7** 13(9)
chloroplast	**9.2** 4159(2942)	**8.5** 36(26)	**9.0** 18(12)	**8.2** 11(9)
other cellular components	**7.9** 3610(3607)	**0.2** 1(1)	**0.5** 1(1)	**0.0** 0(0)
nucleus	**6.1** 2781(2497)	**5.0** 21(20)	**5.5** 11(10)	**3.7** 5(5)
plastid	**3.8** 1707(1122)	**3.8** 16(12)	**4.5** 9(6)	**2.2** 3(3)
plasma membrane	**4.3** 1952(1863)	**6.1** 26(26)	**6.0** 12(12)	**4.5** 6(6)
cytosol	**4.2** 1892(1656)	**10.6** 45(40)	**9.0** 18(16)	**12.7** 17(15)
mitochondria	**3.0** 1359(1119)	**6.8** 29(21)	**6.0** 12(8)	**9.0** 12(9)
ribosome	**1.8** 802(473)	**1.9** 8(7)	**1.0** 2(2)	**3.0** 4(3)
cell wall	**1.5** 703(600)	**3.3** 14(14)	**4.0** 8(8)	**4.5** 6(6)
extracellular	**1.0** 458(450)	**3.3** 14(14)	**3.5** 7(7)	**2.2** 3(3)
ER	**1.0** 443(416)	**0.9** 4(3)	**0.0** 0(0)	**1.5** 2(2)
Golgi apparatus	**0.6** 261(239)	**0.7** 3(1)	**1.5** 3(1)	**0.0** 0(0)
C. GO Molecular Function				
unknown molecular functions	**23.5** 10884(10884)	**2.5** 5(5)	**1.4** 1(1)	**1.4** 1(1)
other binding	**11.6** 5382(4543)	**21.5** 43(30)	**20.8** 15(10)	**30.0** 21(13)
other enzyme activity	**9.1** 4228(3219)	**28.5** 57(40)	**25.0** 18(14)	**25.7** 18(14)
hydrolase activity	**8.8** 4091(2960)	**7.0** 14(8)	**5.6** 4(3)	**5.7** 4(2)
transferase activity	**8.4** 3904(2511)	**15.0** 30(19)	**11.1** 8(6)	**18.6** 13(9)
DNA or RNA binding	**6.8** 3137(2895)	**1.5** 3(3)	**2.8** 2(2)	**0.0** 0(0)
protein binding	**5.8** 2688(2297)	**9.0** 18(17)	**16.7** 12(11)	**4.3** 3(3)
kinase activity	**5.6** 2607(1352)	**4.5** 9(5)	**2.8** 2(2)	**5.7** 4(2)
nucleotide binding	**5.2** 2387(2133)	**7.5** 15(13)	**9.7** 7(6)	**7.1** 5(5)
transporter activity	**4.2** 1939(1262)	**1.0** 2(2)	**2.8** 2(2)	**0.0** 0(0)
transcription factor activity	**3.6** 1681(1681)	**0.0** 0(0)	**0.0** 0(0)	**0.0** 0(0)
nucleic acid binding	**3.2** 1474(1474)	**0.0** 0(0)	**0.0** 0(0)	**0.0** 0(0)
other molecular functions	**2.4** 1092(1008)	**1.0** 2(2)	**1.4** 1(1)	**0.0** 0(0)
structural molecule activity	**1.2** 537(537)	**1.0** 2(2)	**0.0** 0(0)	**1.4** 1(1)
receptor binding or activity	**0.6** 293(271)	**0.0** 0(0)	**0.0** 0(0)	**0.0** 0(0)

Percent annotation count (gene count)

Figure 2.8 Gene Ontology (GO) annotation for identified proteins from 2DE analysis. GO categories are shown for Biological Process (A), Cellular Component (B), and Molecular Function (C) for the Arabidopsis thaliana genome, and for all 110 identified 2DE spots (*F.* × *ananassa* crown), and for the differentially expressed proteins identified in 'Jonsok' and 'Frida' (listed in Table 2.7). Legend includes the percent (bold) next to the number of annotations and the number genes included within each category in parenthesis

Table 2.8 The differentially expressed proteins identified in 'Jonsok' (A) and 'Frida' (B) that are included in the 'response to stress' and 'response to abiotic or biotic stimulus' categories in GO Biological Processes (Figure 2.8). Protein identification for the 2DE protein spots and spot number identifier are listed with their Arabidopsis gene homolog, AGI (Arabidopsis Genome Initiative gene index number), and AGI homolog name. The molecular weight and isoelectric point (MW/pI) for AGI's were obtained from the TAIR site (http://www.arabidopsis.org) and the 2DE MW/pI information was calculated from 2DE gels.

2DE Protein Spot Identification	Spot no.	AGI homolog	AGI Homolog Name	AGI MW/pI	2DE MW/pI
A. Jonsok					
APX (cytosolic ascorbate peroxidase)	2218	AT1G07890	APX1 (ascorbate peroxidase 1)	27.6/6.0	29.8/5.4
Cu/Zn superoxide dismutase	2010	AT1G08830	CSD1 (copper/zinc superoxide dismutase 1)	15.1/5.4	19.1/5.4
Fra a 3	2012	AT1G24020	MLP423 (MLP-LIKE PROTEIN 423)	17.1/4.9	18.2/5.4
Fra a 1-A	3114	AT1G24020	MLP423 (MLP-LIKE PROTEIN 423)	17.1/4.9	21.2/5.6
Fra a 3	4011	AT1G24020	MLP423 (MLP-LIKE PROTEIN 423)	17.1/4.9	18.8/5.6
Fra a 2	4015	AT1G24020	MLP423 (MLP-LIKE PROTEIN 423)	17.1/4.9	18.3/5.6
Annexin	6323	AT1G35720	ANNAT1 (ANNEXIN ARABIDOPSIS 1)	36.2/5.0	35.3/6.0
Annexin	6432	AT1G35720	ANNAT1 (ANNEXIN ARABIDOPSIS 1)	36.2/5.0	35.4/6.1
Isoflavone reductase-like	1423	AT1G75280	Isoflavone reductase - 3	33.7/5.7	37.1/5.3
Thaumatin-like protein	2203	AT1G75800	pathogenesis-related thaumatin protein	34.9/4.7	29.3/5.3
ADH (alcohol dehydrogenase)	4546	AT1G77120	ADH1 (ALCOHOL DEHYDROGENASE)	41.2/6.2	41.5/5.7
ADH (alcohol dehydrogenase)	6505	AT1G77120	ADH1 (ALCOHOL DEHYDROGENASE)	41.2/6.2	42.9/6.0
ADH (alcohol dehydrogenase)	6540	AT1G77120	ADH1 (ALCOHOL DEHYDROGENASE)	41.2/6.2	42.8/6.0
3-ketoacyl-CoA thiolase	6539	AT2G33150	PKT3 (PEROXISOMAL 3-KETOACYL-COA	48.6/8.5	42.8/6.7
Enolase	3626	AT2G36530	LOS2 (Enolase)	47.7/5.5	53.1/5.5
AKR (aldo-keto reductase)	5318	AT2G37770	Aldo-keto reductase family protein	35.1/8.3	34.3/5.8
GST (glutathione transferase)	4115	AT2G47730	ATGSTF8 (Glutathione S-transferase)	29.2/8.9	26.5/5.7
GST (glutathione transferase)	5125	AT2G47730	ATGSTF8 (Glutathione S-transferase)	29.2/8.9	26.3/5.9
Porin	6224	AT3G01280	VDAC1 (voltage dependent anion channel-1)	29.4/9.2	31.1/6.2
RGP (reversibly glycosylatable polypeptide)	4547	AT3G02230	RGP1 (reversibly glycosylatable polypeptide 1)	40.6/5.7	40.0/5.7
HSP70 (heat shock protein 70)	813	AT3G12580	HSP70 (heat shock protein 70)	71.1/4.9	71.9/5.1
Fructose-bisphosphate aldolase	7528	AT3G52930	Fructose-bisphosphate aldolase, putative	38.5/6.4	39.1/6.6
β-1,3-glucanase	2317	AT3G57240	BG3 (beta-1, 3-glucanase 3)	37.6/8.6	33.6/5.3
SGT1 (suppressor of G2 allele of SKP1)	622	AT4G11260	SGT1B (suppressor of G2 allele of SKP1)	39.8/4.8	45.4/5.0
HSP70 (heat shock protein 70)	812	AT5G02500	HSC70-1 (heat shock cognate protein 70-1)	71.4/4.8	71.8/5.0
HSP70 (heat shock protein 70)	1819	AT5G09590	MTHSC70-2 (MITOCHONDRIAL HSP70 2)	73.0/5.4	70.4/5.1
B. Frida					
Annexin	6416	AT1G35720	ANNAT1 (ANNEXIN 1)	36.2/5.0	35.4/6.4
Hexokinase 1	3115	AT1G47840	HXK3 (Hexokinase 3)	53.9/6.8	24.2/5.5
Malate dehydrogenase, mitochondrial	6403	AT1G53240	Malate dehydrogenase 1, mitochondrial	35.8/8.6	37.6/6.0
20S proteasome beta subunit b	7210	AT1G56450	PBG1 (20S proteasome beta subunit G1)	27.7/6.5	28.8/6.9
sti1 (stress-inducible protein)	3819	AT1G62740	stress-inducible protein, putative	64.5/6.1	70.0/5.5
Aconitate hydratase	3912	AT2G05710	ACO3 (Aconitase 3)	108.2/7.2	88.7/5.6
PGD (Phosphogluconate dehydrogenase)	3625	AT3G02360	6-phosphogluconate dehydrogenase	53.6/7.5	49.8/5.5
Ferritin	3223	AT3G11050	FERRITIN 2	28.4/5.6	27.8/5.5
S-adenosylmethionine synthetase	3612	AT3G17390	SAMS3 (S-adenosylmethionine synthetase)	42.8/5.6	44.7/5.5
CAD (cinnamyl alcohol dehydrogenase)	2506	AT3G19450	ATCAD4 (cinnamyl-alcohol dehydrogenase)	39.1/5.2	42.0/5.3
F3H (flavanone 3-hydroxylase)	4536	AT3G51240	F3H (FLAVANONE 3-HYDROXYLASE)	40.3/5.1	40.4/5.6
Fructose-bisphosphate aldolase	7405	AT3G52930	Fructose-bisphosphate aldolase, putative	38.5/6.4	38.5/6.9
Catalase	7707	AT4G35090	CAT2 (catalase 2)	56.9/7.1	52.0/6.9
CBS domain-containing protein	6014	AT5G10860	Cystathionine beta-synthase domain	22.7/9.5	20.1/6.6
CHS (chalcone synthase)	4526	AT5G13930	Naringenin-Chalcone Synthase	43.1/6.5	43.0/5.7
Methionine synthase	6808	AT5G17920	Cobalamin-independent Methionine Synthase)	84.4/6.5	76.8/6.1
GPI (glucose-6-phosphate isomerase)	6704	AT5G42740	glucose-6-phosphate isomerase, cytosolic	61.7/6.6	56.1/6.0

2.4. Shotgun Results

An alternative, high throughput proteomic method (Higgs et al., 2005) was applied to detect smaller but statistically significant differences in protein expression at 0 and 2 d cold treatments (Table 2.9). This method was also hypothesized to detect additional proteins not found by 2DE analysis, since 2DE analysis is not optimal for membrane-associated proteins or highly basic proteins. It is also important to note that the shotgun approach is better able to reflect the overall abundance of a protein unlike 2DE where posttranslational modification creates multiple spots. Three to six individual crowns were used for each of the five biological replications. Each biological replication was injected twice and the two technical replicate intensity values were averaged. This approach identified peptides corresponding to 2017 distinct ESTs or protein sequences (gene identifiers, in NCBI). Several hundred (568) of the identifications were of the highest quality indicating a peptide ID confidence value > 90% with multiple sequences identified. Out of the 2017 ESTs, 21.2% (423 ESTs) had $p < 0.05$, and 8.3% (167 SETs) had $p < 0.01$. The proteins that are differentially accumulated in 'Jonsok' and 'Frida' at 0d and/or 2d are shown in Table 2.9. This list was made by selecting the top 115 EST's (80 distinct proteins) with the best peptide identification score of 99% or better, and with significance in difference in 'Jonsok' or 'Frida' ($p < 0.05$). The confidence of assigning protein identification to the EST's reported from the shotgun was also taken into consideration.

Because the database used for shotgun LC-MS/MS was constructed from NCBI protein and nucleotide sequences, the assigned names to the EST's were determined by first translating the nucleotide sequence (when not amino acid sequence), then performing a NCBI Blast. The proteins that were 'unknown' or 'hypothetical' are not included in Table 2.9, but are accessible in the Supplemental Data Set. The identification of some proteins more than once have resulted from partial sequences existing in the NCBI database. For instance, ADH is reported 4 times with 4 different accession codes; one protein and the other 3 nucleotide sequences. Aligning the amino acid sequences of all four sequences reveals there are no observable differences in the predicted amino acids that overlap. This was observed for several other proteins (e.g., annexin, β-1,3 glucanase). In other instances, differences in sequences could be determined (e.g., CHS). These examples illustrate the limitation of

inferring or quantitating distinct gene products from these results. Regardless of this limitation, interpretations of results were simplified when proteins such as ADH, β-1,3 glucanase, enolase, thaumatin, and tropinone reductase that were identified multiple times (multiple ESTs), and were only identified as significantly more abundant in 'Jonsok'. Likewise, CHS, DFR, F3H, actin, methionine synthase, were only identified as significantly more abundant in 'Frida' (Table 2.9).

Table 2.9 Proteins which distinguish the two cultivars, 'Jonsok' and 'Frida'. This list contains the GenBank accession codes (gi), and number of peptides (and distinct peptides sequences) identified by LC-MS/MS from the "shotgun" approach for 115 proteins that were at different levels in 'Jonsok' and 'Frida'. The cultivar difference are reported as the fold values ('Jonsok' over 'Frida') for 0, and 2 days with the corresponding time points (0 d and/or 2 d) listed at which they were at different levels with significance ($p < 0.05$). The cold responses of proteins are reported for 'Jonsok' and 'Frida' as fold change (day 2 of treatment over 0 day (control). All proteins listed had confidence scores of 99% or better for the top peptide identified. All p-values are highlighted when $p < 0.05$. Assigned protein names were determined by performing NCBI Blast of EST reported from LC-MS/MS.

			CULTIVAR DIFFERENCE						COLD RESPONSE					
	Number of		FOLD		Significance; p > 0.05				FOLD		Significance; p > 0.05			
Accession	Sequences	Assigned Protein ID	Jonsok / Frida		Jonsok vs. Frida		Time point		Jonsok	Frida	Jonsok	Frida		
Code (gi)	/ Peptides	Abbreviation (name)	0 d	2 d	0 d	2 d	Jonsok	Frida	2d /0d	2d /0d	2d _0d	2d _0d	J	F
158353550	3/ 5	20S proteasome beta subunit	-1.11	-1.06	0.00575	0.08662		0d	1.03	-1.02	0.40663	0.61873		
158377351	2/ 3	20S proteasome beta subunit	-1.18	-1.11	0.03421	0.16387		0d	-1.02	-1.08	0.83125	0.29935		
158374802	2/ 2	60S acidic ribosomal protein P0	1.06	1.14	0.20071	0.01059	2d		1.07	-1.00	0.16367	0.92274		
158378367	3/ 4	60S ribosomal protein L11	-1.09	1.11	0.05752	0.02200	2d		1.12	-1.07	0.01200	0.09925	↑	
158372611	4/ 6	60S ribosomal protein L12	1.26	1.14	0.03729	0.20922	0d		-1.00	1.10	0.99602	0.35241		
158372562	2/ 3	60S ribosomal protein L9	-1.01	-1.09	0.82485	0.03038		2d	-1.10	-1.02	0.02088	0.68589	↓	
158371488	3/ 3	6PGL (6-phosphogluconate dehydrogenase)	-1.10	1.04	0.01904	0.28565		0d	1.05	-1.09	0.18764	0.03284		↓
158371946	3/ 4	6PGL (6-phosphogluconate dehydrogenase)	-1.09	-1.12	0.00688	0.00112		0d, 2d	-1.00	1.02	0.88653	0.48521		
158354600	12/ 23	Actin	-1.18	-1.17	0.04307	0.06034		0d	-1.03	-1.04	0.71032	0.58698		
158379507	4/ 5	Actin	-1.26	-1.21	0.00745	0.02197		0d, 2d	1.01	-1.03	0.86035	0.73384		
158380192	7/ 13	Actin	-1.18	-1.17	0.03756	0.04748		0d, 2d	-1.03	-1.04	0.73198	0.64524		
158379942	9/ 13	Actin	-1.18	-1.17	0.00682	0.00948		0d, 2d	1.00	-1.01	0.96422	0.91193		
158373473	9/ 13	Actin	-1.17	-1.16	0.00815	0.01283		0d, 2d	-1.00	-1.01	0.98205	0.81217		
158378957	9/ 14	Actin	-1.18	-1.16	0.01295	0.02164		0d, 2d	1.00	-1.01	0.95671	0.84688		
33563040	9/ 14	Actin	-1.15	-1.15	0.03379	0.04123		0d, 2d	-1.00	-1.01	0.95316	0.87389		
158378955	5/ 7	Adenine phosphoribosyltransferase	-1.16	-1.13	0.03830	0.09448		0d	1.02	-1.01	0.78320	0.84391		
89548637	9/ 14	Adenosine kinase	-1.11	-1.08	0.03209	0.11553		0d	1.01	-1.02	0.83324	0.64516		
89556337	4/ 8	ADH (alcohol dehydrogenase)	1.31	1.22	0.00074	0.00778	0d, 2d		-1.10	-1.02	0.16603	0.74237		
158350919	4/ 9	ADH (alcohol dehydrogenase)	1.22	1.18	0.00205	0.00823	0d, 2d		-1.07	-1.03	0.22245	0.55136		
89550819	5/ 12	ADH (alcohol dehydrogenase)	1.26	1.20	0.00065	0.00536	0d, 2d		-1.09	-1.03	0.15524	0.63197		
113436	6/ 7	ADH (alcohol dehydrogenase)	1.25	1.27	0.00448	0.00250	0d, 2d		-1.03	-1.05	0.64330	0.46553		
89541643	2/ 2	Alanine transaminase	1.17	1.11	0.04053	0.14430	0d		-1.04	1.01	0.58157	0.89740		
89544075	10/ 18	Annexin	-1.26	-1.11	0.00252	0.11570		0d	1.07	-1.05	0.28217	0.43563		
89550344	6/ 10	Annexin	-1.22	-1.10	0.00962	0.18961		0d	1.08	-1.03	0.26685	0.68078		
51047818	6/ 9	Annexin	-1.11	-1.05	0.04753	0.35319		0d	1.06	-1.01	0.29513	0.91556		

Table 2.9 continues on following page

Table 2.9. continued from previous page

Accession Code (gi)	Number of Sequences / Peptides	Assigned Protein ID Abbreviation (name)	CULTIVAR DIFFERENCE FOLD Jonsok / Frida 0 d	2 d	Significance; p > 0.05 Jonsok vs. Frida 0 d	2 d	Time point Jonsok	Frida	COLD RESPONSE FOLD Jonsok 2d /0d	Frida 2d /0d	Significance; p > 0.05 Jonsok 2d _0d	Frida 2d _0d	J	F
110564479	5/ 6	ANR (anthocyanidin reductase)	-1.25	-1.17	0.00201	0.01880		0d, 2d	1.05	-1.01	0.42162	0.80991		
90576646	6/ 6	ANR (anthocyanidin reductase)	-1.19	-1.16	0.01198	0.03245		0d, 2d	1.01	-1.02	0.86891	0.75009		
110564477	6/ 7	ANR (anthocyanidin reductase)	-1.24	-1.18	0.00311	0.01600		0d, 2d	1.04	-1.01	0.50956	0.91465		
158374331	6/ 8	Aspartate aminotransferase	1.12	1.18	0.05591	0.01024	2d		-1.04	-1.09	0.53241	0.15672		
158373368	7/ 9	Aspartate aminotransferase	1.11	1.16	0.07453	0.01170	2d		-1.03	-1.08	0.58713	0.15526		
158379523	4/ 5	ATP citrate (pro-S)-lyase	-1.02	-1.13	0.66345	0.03929		2d	1.02	1.12	0.75955	0.05071		
89544263	10/ 14	ATP synthase F1 subunit 1	-1.08	-1.03	0.03526	0.36242		0d	-1.01	-1.06	0.75834	0.11331		
158371553	4/ 4	ATP synthase F1, gamma subunit	1.21	1.34	0.07125	0.00859	2d		-1.05	-1.16	0.64776	0.14627		
158350135	2/ 3	Beta 1-3 glucanase	1.46	1.48	0.03811	0.03330	0d, 2d		-1.13	-1.14	0.48648	0.44615		
158373879	4/ 11	Beta 1-3 glucanase	1.60	2.07	0.11555	0.02057	2d		-1.15	-1.48	0.63613	0.18419		
158369226	8/ 16	Beta 1-3 glucanase	1.55	2.00	0.14000	0.02686	2d		-1.12	-1.44	0.69843	0.21923		
158356647	8/ 19	Beta 1-3 glucanase	1.55	1.93	0.12505	0.02700	2d		-1.14	-1.42	0.63760	0.21340		
89558076	2/ 2	CHI (chalcone isomerase)	-1.07	-1.21	0.09602	0.00020		2d	1.02	1.15	0.63460	0.00286		↑
158369386	10/ 19	CHS (chalcone synthase)	-1.20	-1.40	0.01102	0.00007		0d, 2d	1.08	1.26	0.25437	0.00231		↑
158370409	10/ 21	CHS (chalcone synthase)	-1.18	-1.38	0.01498	0.00008		0d, 2d	1.07	1.25	0.27795	0.00218		↑
71979908	19/ 39	CHS (chalcone synthase)	-1.18	-1.36	0.00905	0.00005		0d, 2d	1.07	1.23	0.25694	0.00215		↑
71979904	20/ 41	CHS (chalcone synthase)	-1.19	-1.37	0.00741	0.00005		0d, 2d	1.07	1.23	0.26367	0.00268		↑
1705844	4/ 9	CHS (chalcone synthase)	-1.24	-1.33	0.00183	0.00013		0d, 2d	1.08	1.16	0.21441	0.02134		↑
158367106	8/ 16	CHS (chalcone synthase)	-1.22	-1.42	0.00627	0.00004		0d, 2d	1.06	1.24	0.35654	0.00339		↑
24636275	8/ 8	Citrate synthase	-1.11	-1.05	0.02765	0.31926		0d	1.02	-1.05	0.68628	0.34011		
16303976	5/ 5	Class II chitinase	1.17	1.24	0.06106	0.01386	2d		-1.05	-1.12	0.50899	0.17367		
158376639	4/ 4	DFR (dihydroflavonol 4-reductase)	-1.06	-1.18	0.28283	0.00378		2d	-1.04	1.08	0.48150	0.14008		
158362072	5/ 5	DFR (dihydroflavonol 4-reductase)	-1.07	-1.21	0.21404	0.00210		2d	-1.07	1.06	0.22229	0.28797		
89555197	3/ 3	Dihydrolipoamide acetyltransferase	1.09	1.00	0.03357	0.91579	0d		-1.06	1.02	0.13065	0.54140		
158377954	3/ 3	Dihydrolipoamide dehydrogenase	-1.04	-1.06	0.16646	0.03623		2d	-1.06	-1.04	0.04102	0.18473	↓	
89551906	2/ 4	dtdp-glucose 4-6-dehydratase	-1.20	-1.07	0.00193	0.19198		0d	1.03	-1.09	0.54304	0.10476		
158368823	2/ 2	Elongation factor TuA (EF-TuA) chloroplast	-1.22	-1.08	0.04531	0.44678		0d	1.09	-1.05	0.37992	0.63211		
158371950	10/ 26	Enolase	1.12	1.08	0.02115	0.12765	0d		-1.02	1.03	0.71896	0.56811		
158357164	5/ 12	Enolase	1.07	1.06	0.04346	0.11391	0d		-1.01	1.00	0.69593	0.90408		
158378077	8/ 22	Enolase	1.12	1.08	0.03302	0.14547	0d		-1.01	1.03	0.83259	0.56479		
51493449	18/ 30	F3H (flavanone 3-hydroxylase)	-1.13	-1.27	0.06565	0.00113		2d	1.01	1.14	0.82861	0.04265		↑
51493451	21/ 34	F3H (flavanone 3-hydroxylase)	-1.13	-1.27	0.05587	0.00095		2d	1.01	1.14	0.83819	0.04428		↑
158377373	2/ 2	Fiber dTDP-glucose 4-6-dehydratase	-1.13	-1.01	0.00625	0.78374		0d	1.11	-1.01	0.01610	0.86200	↑	
158357398	2/ 3	Formate dehydrogenase	1.00	-1.17	0.89566	0.00081		2d	-1.00	1.17	0.90663	0.00078		↑
158372943	5/ 8	Fra a 2	1.21	1.12	0.03781	0.19511	0d		1.04	1.12	0.68761	0.20494		
89557236	6/ 9	Fra a 2	1.24	1.13	0.02076	0.16505	0d		1.03	1.13	0.73892	0.16654		
158375993	3/ 4	Fra a 4 / Profilin	-1.08	-1.09	0.07698	0.03279		2d	1.03	1.05	0.42666	0.22501		
158380206	4/ 5	Fra a 4 / Profilin	-1.31	-1.19	0.00285	0.03877		0d, 2d	1.01	-1.09	0.92205	0.25972		
85701214	4/ 6	Fra a 4 / Profilin	-1.30	-1.17	0.00388	0.05970		0d	1.00	-1.11	0.97186	0.20867		
158362529	3/ 7	Fructose-bisphosphate aldolase	1.14	1.02	0.02836	0.66915	0d		-1.02	1.09	0.67580	0.14107		
16304129	15/ 27	GADPDH (glyceraldehyde 3-phosphate dehyd	-1.07	-1.03	0.03644	0.36729		0d	1.02	-1.02	0.45753	0.56088		
158302779	20/ 43	GADPDH (glyceraldehyde 3-phosphate dehyd	-1.07	-1.02	0.03185	0.38013		0d	1.02	-1.02	0.41899	0.54454		
158361862	2/ 2	Gamma carbonic anhydrase-like	-1.15	-1.08	0.02105	0.18949		0d	-1.01	-1.08	0.89652	0.20541		
158348555	6/ 11	GDH1 (glutamate dehydrogenase 1)	-1.27	-1.17	0.00707	0.05572		0d	1.04	-1.04	0.62732	0.60408		
158378051	2/ 3	GST (glutathione S-transferase)	-1.10	-1.06	0.02878	0.15677		0d	-1.02	-1.06	0.66343	0.19269		
158378949	3/ 4	Inorganic pyrophosphatase	-1.07	-1.11	0.11570	0.02199		2d	-1.01	1.03	0.81056	0.53794		
51047667	3/ 4	Isoflavone reductase-like	-1.15	-1.12	0.00211	0.01101		0d, 2d	1.03	-1.00	0.48699	0.94071		
158372608	2/ 2	Lactoylglutathione lyase	-1.11	-1.05	0.02360	0.23774		0d	1.04	-1.02	0.41124	0.67202		
158376116	3/ 4	Mal d 1-like	-1.11	-1.07	0.03104	0.14090		0d	1.04	1.01	0.36026	0.90076		
158358695	3/ 3	Malic enzyme, putative	-1.14	-1.08	0.00815	0.10159		0d	-1.04	-1.10	0.41665	0.05038		

Table 2.9 continues on following page

Table 2.9. continued from previous page

Accession Code (gi)	Number of Sequences / Peptides	Assigned Protein ID Abbreviation (name)	CULTIVAR DIFFERENCE FOLD Jonsok / Frida 0 d	2 d	Significance; p > 0.05 Jonsok vs. Frida 0 d	2 d	Time point Jonsok	Frida	COLD RESPONSE FOLD Jonsok 2d /0d	Frida 2d /0d	Significance; p > 0.05 Jonsok 2d _0d	Frida 2d _0d	J	F
158362716	10/ 19	Methionine synthase	-1.13	-1.11	0.00497	0.01457		0d, 2d	1.04	1.02	0.34173	0.64937		
89551239	10/ 24	Methionine synthase	-1.15	-1.12	0.00408	0.01577		0d, 2d	1.04	1.02	0.31097	0.69595		
158365549	3/ 8	Methionine synthase	-1.18	-1.15	0.00375	0.01089		0d, 2d	1.05	1.02	0.34291	0.64591		
158364783	5/ 12	Methionine synthase	-1.16	-1.14	0.00420	0.01070		0d, 2d	1.03	1.01	0.46389	0.76507		
158376561	5/ 9	Methionine synthase	-1.12	-1.12	0.00396	0.00304		0d, 2d	1.04	1.04	0.29605	0.24566		
158360273	5/ 9	Methionine synthase	-1.12	-1.11	0.03209	0.03938		0d, 2d	1.04	1.03	0.45478	0.51743		
89556001	6/ 10	Methionine synthase	-1.12	-1.11	0.02921	0.03430		0d, 2d	1.04	1.03	0.43894	0.48627		
89554579	7/ 14	Methionine synthase	-1.15	-1.10	0.02513	0.11255		0d	1.06	1.02	0.29820	0.78003		
158359641	2/ 2	NADH dehydrogenase	-1.25	-1.17	0.00976	0.05632		0d	1.03	-1.03	0.67385	0.66122		
89557666	2/ 2	NADH-ubiquinone oxidoreductase 39 kD subur	-1.10	-1.18	0.07391	0.00644		2d	-1.05	1.01	0.32972	0.83342		
158363754	2/ 2	OMT (O-methyltransferase)	-1.15	-1.15	0.02444	0.02874		0d, 2d	1.04	1.04	0.48593	0.53619		
158372294	2/ 2	OMT (O-methyltransferase)	-1.17	-1.09	0.03437	0.22020		0d	-1.02	-1.09	0.78575	0.20760		
6760443	3/ 3	OMT (O-methyltransferase)	-1.16	-1.10	0.01573	0.09034		0d	1.03	-1.02	0.62949	0.68941		
84322434	3/ 4	Osmotin-like protein	1.38	1.51	0.10247	0.04130	2d		-1.04	-1.14	0.84569	0.50341		
158360959	2/ 2	Peroxiredoxin	-1.23	-1.29	0.00391	0.00068		0d, 2d	1.03	1.09	0.61301	0.19691		
89551205	2/ 2	Potassium channel beta	-1.11	-1.02	0.02865	0.65868		0d	1.04	-1.05	0.40342	0.28919		
158376406	5/ 7	Pyruvate kinase	-1.01	-1.06	0.61076	0.04667		2d	-1.01	1.04	0.72688	0.21840		
158361609	5/ 8	Quinone reductase	-1.21	-1.12	0.00649	0.07497		0d	1.03	-1.05	0.62694	0.47744		
158379027	8/ 12	Quinone reductase	-1.17	-1.18	0.03919	0.02980		0d, 2d	-1.00	1.01	0.96546	0.92540		
158375795	2/ 2	Ribosomal protein S11	1.22	1.18	0.04550	0.08875	0d		-1.11	-1.07	0.26956	0.44274		
89552266	2/ 2	Ripening-induced protein	1.21	1.13	0.01796	0.10579	0d		-1.13	-1.06	0.11334	0.46244		
2465015	5/ 5	Ripening-induced protein	1.27	1.13	0.00005	0.01587	0d, 2d		-1.14	-1.01	0.00888	0.83057	↓	
51049581	2/ 6	S-adenosylmethionine synthase	-1.23	-1.06	0.00256	0.36873		0d	1.22	1.05	0.00315	0.42087	↑	
158372548	3/ 9	S-adenosylmethionine synthase	-1.19	-1.02	0.00847	0.70326		0d	1.22	1.05	0.00333	0.41732	↑	
158378165	2/ 2	Serine hydroxymethyltransferase	1.12	-1.02	0.04871	0.76089	0d		-1.08	1.05	0.13713	0.39290		
158348545	8/ 9	Serine hydroxymethyltransferase	-1.06	-1.01	0.02891	0.75014		0d	1.03	-1.02	0.28692	0.34432		
158373569	2/ 4	Soluble inorganic pyrophosphatase	-1.25	-1.17	0.00673	0.04107		0d, 2d	1.00	-1.06	0.95864	0.41529		
89556351	2/ 3	TCP domain class transcription factor	-1.10	-1.00	0.03817	0.92687		0d	1.04	-1.05	0.36860	0.23255		
158366345	5/ 11	Thaumatin-like	1.84	2.04	0.03252	0.01460	0d, 2d		-1.14	-1.26	0.62866	0.38682		
158374908	7/ 14	TPX (thioredoxin-dependent peroxidase)	-1.19	-1.13	0.04387	0.13995		0d	1.02	-1.03	0.83475	0.67822		
158356513	3/ 3	Tropinone reductase	1.33	1.38	0.04706	0.02705	0d, 2d		-1.10	-1.14	0.47325	0.32450		
158354579	3/ 3	Tropinone reductase	1.32	1.37	0.04664	0.02769	0d, 2d		-1.10	-1.14	0.46745	0.32747		
158379484	3/ 7	Ubiquitin conjugating enzyme 2	-1.19	-1.13	0.00552	0.03889		0d, 2d	1.05	-1.00	0.39150	0.94172		
158379944	3/ 7	Ubiquitin conjugating enzyme 2	-1.19	-1.13	0.00552	0.03889		0d, 2d	1.05	-1.00	0.39150	0.94172		
158379995	2/ 2	Ubiquitin/ribosomal protein	1.14	1.04	0.00299	0.31379	0d		-1.02	1.07	0.53112	0.08824		
158355382	2/ 2	UDP-D-apiose/UDP-D-xylose synthase 2	-1.24	-1.11	0.01040	0.19603		0d	1.09	-1.03	0.24870	0.72681		
89549543	5/ 6	UDP-glucose pyrophosphorylase	-1.18	-1.12	0.00740	0.05226		0d	1.01	-1.04	0.83081	0.46345		
158373008	7/ 7	UDP-glucose pyrophosphorylase	-1.15	-1.09	0.00295	0.05255		0d	1.02	-1.04	0.70257	0.32331		
51048752	2/ 2	USP (universal stress protein family protein)	-1.16	-1.06	0.01006	0.25212		0d	1.05	-1.04	0.31474	0.49903		
89543363	2/ 2	USP (universal stress protein family protein)	-1.16	-1.06	0.01006	0.25212		0d	1.05	-1.04	0.31474	0.49903		

2.5. Discussion

2.5.1. Comparison of 2DE Protein Expression in 'Jonsok' and 'Frida'

2.5.1.1. Proteins Involved in the Phenylpropanoid Biosynthetic Pathway

The phenylpropanoid biochemical pathway results in a variety of compounds including flavonoids, tannins, lignin, stilbenes, and phenolic acids, many of which have been identified and characterized. These compounds function in pigments, regulation of plant growth, antimicrobials, cell wall modifications, and antioxidants (Dixon and Pasinetti, 2010; Koes et al., 1994; Vogt, 2010; Winkel-Shirley, 2001). Eighteen of the 110 2DE identified spots (not including the four Fra a 1's, which are only speculative participants in this pathway) correspond to proteins involved in the phenylpropanoid pathway. A significant number of these were enzymatic components contributing to the flavonoid biosynthetic process catalyzing 8 biosynthetic steps in the pathway and 4 additional proteins indirectly involved in the flavonoid pathway (Figure 2.9). Flavonoid pathway proteins expressed at higher levels in the more freezing sensitive 'Frida' than in the more freezing tolerant 'Jonsok' include three key enzymes in the flavonoid pathway, chalcone synthase (CHS), flavonoid 3'-hydroxylase (F3H) and dihydroflavonol 4-reductase (DFR). These are also cold-accumulation (an increase in CHS, at 2 d cold treatment was observed in both 2DE and shotgun). It is interesting that while several other proteins in this pathway were down-regulated in 'Frida' in response to cold stress, CHS, the first committed protein in the flavonoid pathway (Winkel-Shirley, 2001), as well as F3H are strongly up-regulated in response to cold stress. It is important to note that since both CHS and F3H have been characterized as rate-limiting enzymes (Koes et al., 1994), the data suggest a strongly enhanced ability for 'Frida' to synthesize flavonoid products. In contrast, 'Jonsok' showed a significant cold-related decrease in CHS and F3H. The overall difference in expression patterns resulted in a massive differential accumulation where CHS, DFR and F3H proteins were at 720, 5.5 and 76 fold respectively at higher levels in 'Frida' than 'Jonsok' at 2 d, and 16.6, 3.8, and 3.5 fold

respectively at 42 d. Although remaining higher in 'Frida' than 'Jonsok', both DFR and F3H decreased slightly in 'Frida'.

Anthocyanidin reductase (ANR) is an oxidoreductase and competes with anthocyanidin synthase (ANS) for the pool of flavan-3, 4-diols. It has a reported involvement in the biosynthesis of condensed tannins. ANR was identified in three spots that mapped to two distinctive ESTs. At 42 d, 'Jonsok' showed an ANR (spot 3515) increase in response to cold, reaching 4 fold higher levels than in 'Frida'. A different ANR (spot 4520) was observed to be cold accumulated in 'Frida' at 2 d and 42 d and nearly absent in 'Jonsok'. Though it is possible that the different isoforms impart different specificity for substrates; the net effect of the changes of all ANR spots was not significantly different.

Proteins more abundant in 'Jonsok' include Caffeic acid 3-O-methyltransferase (COMT), and isoflavone reductase-related protein (IFR). Both proteins spots (spot 1533, 3326) identified as OMT were more abundant in 'Jonsok' at 42 d (3 and 6 fold respectively). One of the spots, spot 1533, exhibited higher levels in 'Jonsok' at all time points. Two protein spots identified as IFR (spot 1423, 4420) appear to be different based on sequence homology. Both were more abundant in 'Jonsok' at 0 d. 'Jonsok' maintained a 2 fold or higher level of IFR (spot 1423) than 'Frida' while IFR (spot 4420) levels were not deemed significantly different at 2 and 42 d. This suggests that different flavonoid metabolites may contribute to overwintering tolerance in 'Jonsok'. Other enzymes in this pathway did not show these large differences, e.g., chalcone isomerase (CHI) while cold-responsive, decreasing in the cold after 42 d (~1.5 fold), was not significantly different between the cultivars.

Cinnamyl-alcohol dehydrogenase (CAD), a molecular marker specific for lignification (Walter et al., 1988), increased slightly in 'Frida' at 42 d 1.24 fold (t-test; $p < 0.1$) and UDP-glucose glucosyltranferase (UGGT) was approximately 3 fold greater in 'Frida' at 0 and 2 d cold treatment, but not different after 42 d. Caffeoyl-CoA 3-O-methyltransferase (CCoAOMT) was 2 fold higher in 'Frida' at 0 d, yet by 42 d there was no difference due to a significant decrease a in 'Frida' and a significant cold response increase of 1.9 fold in

'Jonsok'. Anthocyanidin synthase (ANS) did not change significantly with regard to cultivar or cold treatment.

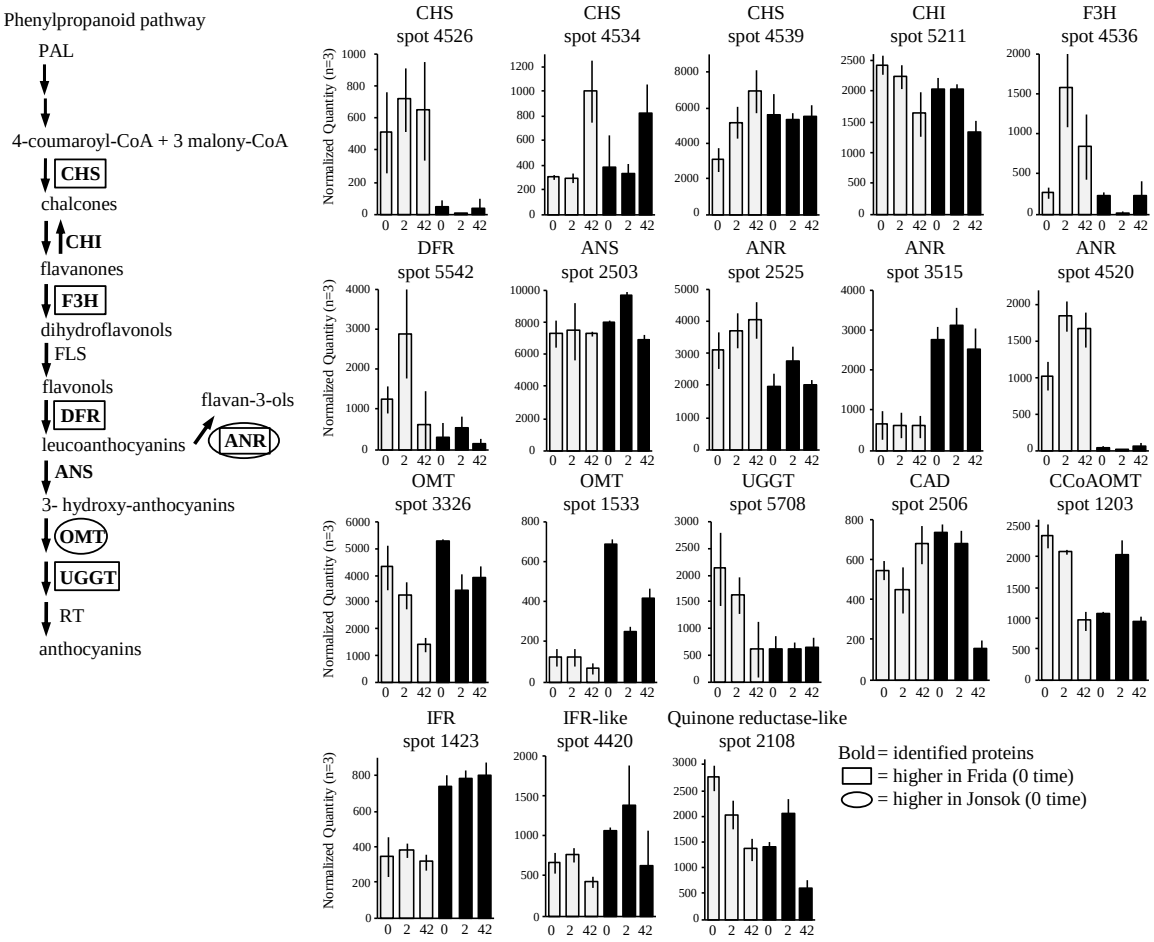


Figure 2.9 Proteins identified in the flavonoid pathway were most abundant in 'Frida. Flavonoid pathway highlighting the proteins involved in this pathway in 'Frida' and 'Jonsok'. The proteins in bold indicate identified proteins. Proteins in either squares or ovals indicate that higher levels (≥ 2 fold, $p < 0.05$ in Student's t test) are in either 'Frida' or 'Jonsok' respectively. Bar graphs show the average normalized values (from PDQuest, n=3) with standard deviations for each time point (0, 2, 42 days of cold treatment at 2 ºC) for 'Frida' (gray bars) and 'Jonsok' (black bars). Abbreviations: ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; CAD, cinnamyl alcohol dehydrogenase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3H, flavonoid 3-hydroxylase; IFR, isoflavone reductase; OMT, Caffeic acid 3-O-methyltransferase; PAL, phenylalanine; RT, rhamnosyl transferase; UGGT, UDP-glucose glucosyltransferase.

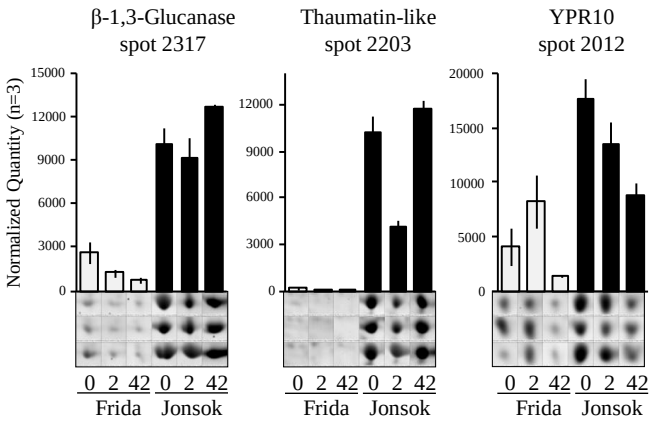


Figure 2.10 Levels of proteins associated with pathogen resistance distinguish 'Jonsok' (black bars) from 'Frida' (gray bars). Bar graphs show the average normalized values (from PDQuest, n=3) with standard deviations for each time point (0, 2, 42 days of cold treatment at 2 ºC) for 'Frida' and 'Jonsok'. The corresponding 2DE spot images are presented beneath each graphed bar.

2.5.1.2. Proteins Associated with Pathogen Resistance

Overwintering survival requires both freezing tolerance and disease resistance against pathogens. Specific disease resistance induced by cold acclimation has been reported for several crops (Koike et al., 2002; Płażek et al., 2003), with some cold-induced pathogenesis-related proteins exhibiting both antifungal and antifreeze activities (Kuwabara and Imai, 2009). In particular, certain β-1,3-glucanases have been shown to be cold induced and have cryoprotective activity similar to other extracellular pathogenesis-related proteins (Hincha et al., 1997). β-1,3-glucanases comprise a large and highly complex gene family involved in pathogen defense as well as a broad range of other biological processes. YPR10 belongs to a group of pathogenesis-related proteins whose function is largely unknown although functions have been speculated to include ribonuclease and proteinase activities (Walter et al., 1996). In the cold-tolerant 'Jonsok', two different β-1,3-glucanase proteins as well as the pathogen responsive protein, YPR10 were identified. A thaumatin-like glucanase (spot 2203) is 70 fold higher in 'Jonsok' than 'Frida' constitutively and accumulated to over 6000 fold higher in 'Jonsok' than 'Frida' after 42 days of cold treatment, largely due to a decrease in the amount found in 'Frida' (Figure 2.10). Another β-1,3-glucanase (spot 2317) was 4 fold higher than the corresponding protein in 'Frida' at control conditions and increased to about 16 fold higher than 'Frida' after 42 d of cold treatment. Interestingly this increase is due to a

slight, yet significant, increase in 'Jonsok' levels (1.3 fold) and a 3 fold decrease in 'Frida'. YPR10 (spot 2012) was constitutively higher in 'Jonsok' by approximately 4 fold, though decreasing slightly during the cold treatment, ended up being 6 fold greater than 'Frida' after 42 d of cold treatment.

2.5.1.3. Antioxidative and Detoxification Proteins

Tolerance to any stress depends significantly on the potential of the antioxidative defense system. Initially antioxidative capacity can mitigate the potentially damaging effects of reactive oxygen species (ROS) signaling occurring during low temperature response (O'Kane et al., 1996; Suzuki and Mittler, 2006). Antioxidative proteins are also involved in the recovery phase following stress (Biemelt et al., 1998; Blokhina et al., 2003). Overall, proteins involved in antioxidative and detoxification processes were highly over represented in 'Jonsok' compared to 'Frida' (Figure 2.11). Although 'Frida' clearly had an upregulated flavonoid pathway (discussed above) that would be expected to produce a variety of antioxidant compounds; 'Jonsok' has higher levels of enzymes capable of direct, or regulation of, anti-oxidative activity.

The detoxification of ROS is managed through the action of superoxide dismutases which catalyze the dismutation of superoxides into oxygen and hydrogen peroxide, and catalases and peroxidases which further detoxify H_2O_2 to water (Apel and Hirt, 2004). In 'Jonsok', (relative to 'Frida') increased levels of Cu/Zn superoxide dismutase (2-11 fold higher), ascorbate peroxidase (2-5 fold higher), annexin 1 (200-1200 fold higher), and L-galactono-1,4-lactone dehydrogenase (1.8-1.7 fold higher) are likely key components in the capability to directly modulate ROS levels and are all at higher levels in 'Jonsok'. Superoxide dismutases play a key role in virtually all organisms exposed to oxygen and plants are no exception (Sunkar et al., 2006). Despite the observation that Cu/Zn superoxide dismutase (spot 2010) was significantly down-regulated in both 'Jonsok' and 'Frida' at 2 and 42 d, 'Jonsok' levels significantly exceeded those of 'Frida', exhibiting a 2, 5, and 11 fold greater levels at 0, 2, and 42 d, respectively. Ascorbate peroxidase (APX) which consumes H_2O_2, in conjunction with ascorbate which is subsequently regenerated by the ascorbate-glutathione cycle, contributes

to abiotic stress tolerances including low temperature stresses (Shigeoka et al., 2002). Arabidopsis annexin 1 has peroxidase activity and over-expression and knock-out experiments have demonstrated a significant contribution to stress tolerance (Konopka-Postupolska et al., 2009). Interestingly, distinct annexin 1 isoforms were found in 'Jonsok' and 'Frida'. The difference in mass and charge may be due to post-translational glutathionylation as observed in Arabidopsis (Konopka-Postupolska et al., 2009).

L-galactono-1,4-lactone dehydrogenase (GLDH) catalyzes the last step in the main pathway of vitamin C (L-ascorbate acid) biosynthesis in higher plants, thus is an important player in this small molecule antioxidant pathway. At least in one case, exogenously increasing the levels of the GLDH intermediate enhanced oxidative stress tolerance (Zhao, 2005), and it has been suggested that the dehydrogenase may be an important control point in ascorbic acid synthesis (Valpuesta and Botella, 2004).

Other enzymes involved in redox reactions, aldo-keto reductase, 3-ketoacyl-CoA thiolase, isoflavone reductase and glutathione S-transferase were also at higher levels or were cold-induced in 'Jonsok'. Aldo-keto reductases can detoxify lipid peroxidation products and reactive aldehydes (Bartels, 2001). Three of the 4 different aldo-keto reductases identified, corresponding to spot 5318, 5439, and 5507, were at higher levels in 'Jonsok' and also demonstrated cold induction. 3-ketoacyl-CoA thiolase has a role in peroxisome morphology and has potential role for redox control of peroxisomal fatty and beta oxidation (Germain et al., 2001). One of the two 3-ketoacyl-CoA thiolase identified (spot 6539) reached a 10 fold higher level in 'Jonsok' at 42 d due to a significantly decreased level in 'Frida'. The one thiolase isoform (spot 3602) demonstrated a 1.3 fold cold induction in 'Jonsok' at 2 d. Glutathione S-transferases (GST) are cytosolic dimeric proteins involved in cellular detoxification by catalyzing the conjugation of glutathione with various electrophilic compounds, including oxidized lipids. Two proteins spots (spot 4415, 5125) identified as GST were more abundant in 'Jonsok' than 'Frida' at all time points. The closest homolog in Arabidopsis, GST8 (At2g47730), is strongly induced following exposure to H_2O_2 (Chen et al., 1996) and a recent review (Dixon et al., 2010) highlights evidence for the diverse functional roles of GSTs beyond "glutathione transferase" activities. Glyoxalase I

(lactoylglutathione lyase) detoxifies the highly toxic methylglyoxal, a byproduct of glycolysis. Methyl glyoxal detoxification involves the glyoxalase I catalyzed formation of lactoylglutathione and subsequent conversion to lactate and glutathione by glyoxalase II. The production of methyl glyoxal dramatically increases in response to cold and other stresses and the levels of methylglyoxal are controlled by glyoxalase I (Yadav et al., 2005). Glyoxalase I (spot 1315) increased in 'Jonsok' 1.8 fold at 42 d, and levels significantly exceeding those of 'Frida' at 0, 2, and 42 d exhibiting a 14, 6, and 14 fold higher levels respectively. Interestingly, glyoxalase II (spot 4305) was more abundant in 'Frida' at 0, 2, and 42 d exhibiting a 4, 9, and 10 fold higher levels respectively. The isoflavone reductase-related protein (spot 1423), exhibiting a 2 fold higher levels in 'Jonsok' than 'Frida' at all-time points, may act in preservation of reductants or synthesis of antioxidants (Petrucco et al., 1996).

Overall, 'Frida' relative to 'Jonsok', had a conspicuous lack of the well-known players with roles in antioxidation and detoxification. The presence of these proteins in 'Jonsok' at constitutive higher levels, before cold treatment, could prophylactically improve cold stress tolerance through reducing oxidative stress during the initial cold exposure, throughout overwintering and later, in the spring recovery phase.

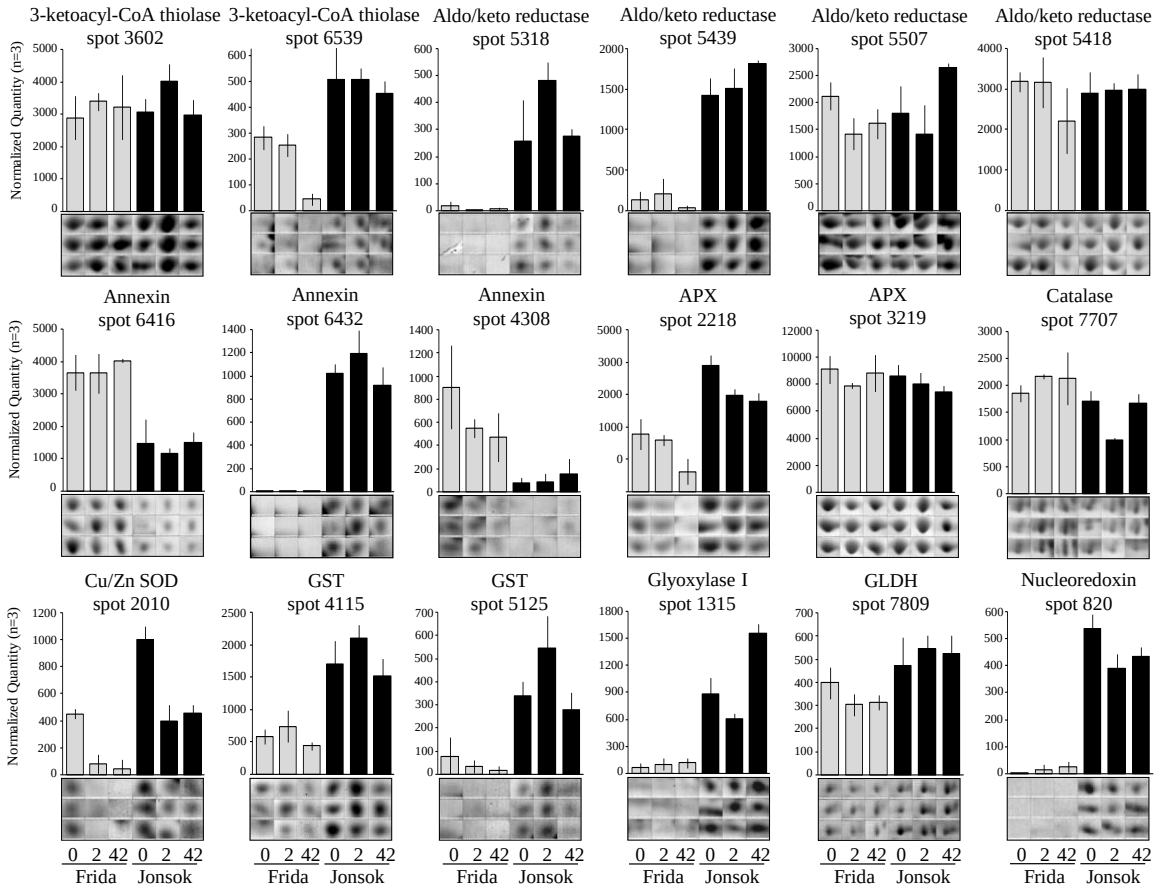


Figure 2.11 Levels of proteins associated with antioxidation and detoxification distinguish 'Jonsok' (black bars) from 'Frida' (gray bars). Bar graphs show the average normalized values (from PDQuest, n=3) with standard deviations for each time point (0, 2, 42 days of cold treatment at 2 ºC) for 'Frida' and 'Jonsok'. The corresponding 2DE spot images are presented beneath each graphed bar. Abbreviations: APX, cytosolic ascorbate peroxidase; Cu/Zn SOD, Cu/Zn superoxide dismutase; GST, glutathione S-transferase; GLDH, L-galactono-1,4-lactone dehydrogenase.

2.5.1.4. Anoxia/Hypoxia Related Proteins

A low oxygen environment is not uncommon for tissues located underground and melting snow or ice encasement can further exacerbate hypoxic environments. Accumulation of toxic end products of anaerobic metabolism (particularly lactic acid) can result in injury and compromise winter survival. A common response in plants that are highly tolerant to anaerobiosis is to increase the glycolytic fermentation pathways and to shift the endpoint away from lactate and toward ethanol (Drew, 1997). Particularly important is the role for pyruvate decarboxylase to direct flow from lactate to ethanol. In 'Jonsok', of the 7 enzymes leading from fructose-1, 6- bisphosphate to ethanol, five are either at levels higher than those found in 'Frida' or accumulate following cold treatment. Thus after 42 d cold treatment, aldolase (4 fold greater in 'Jonsok'), enolase (4 fold greater in 'Jonsok'), pyruvate kinase (3 fold greater in 'Jonsok'), pyruvate decarboxylase (0.7 fold of 'Frida' levels, but is cold induced approx. 1.5 fold compared to control), as well as alcohol dehydrogenase (ADH) are significantly greater than the corresponding enzymes in 'Frida'. Four of the five spots identified as ADH isoforms were higher than levels found in 'Frida' at 42 d (130 fold, spot 6540; 2.5 fold, spot 6513; 2.0 fold, spot 6505; 1.7 fold). An alternative process to the fermentation pathway for providing electron acceptors; a type I hemoglobin facilitating a nitrate-nitric oxide cycle, has been postulated to be critical for survival in hypoxic environments (Igamberdiev and Hill, 2004). The non-symbiotic hemoglobin class 1 protein (spot 7010), a known hypoxia induced protein increases in 'Jonsok' 1.6 fold at 2 d, and was 2 fold higher in 'Jonsok' than 'Frida' at 2 and 42 d.

The oxygen sensing mechanisms existing in plants are beginning to be elucidated from recent studies showing that some proteins that are substrates of the N-end rule pathway are subject to degradation during aerobic conditions and stabilization under hypoxia. For example, Licausi et al., (2011) have shown that the transcription factor Rap2.12 regulates hypoxia tolerance in plants and is dependent on the N-terminal amino acid sequence responsible for leading to degradation of the transcription factor, RAP2.12 under aerobic conditions. Consistent with these findings, Gibbs et al (2011), shows that plants lacking the components of the N-end rule degradation pathway, constitutively express hypoxia related genes and these plants demonstrate an increase tolerance to hypoxia. Thus the N-terminal

pathway is part of the oxygen response mechanism in *Arabidopsis thaliana*. (Gibbs et al., 2011; Licausi et al., 2011).

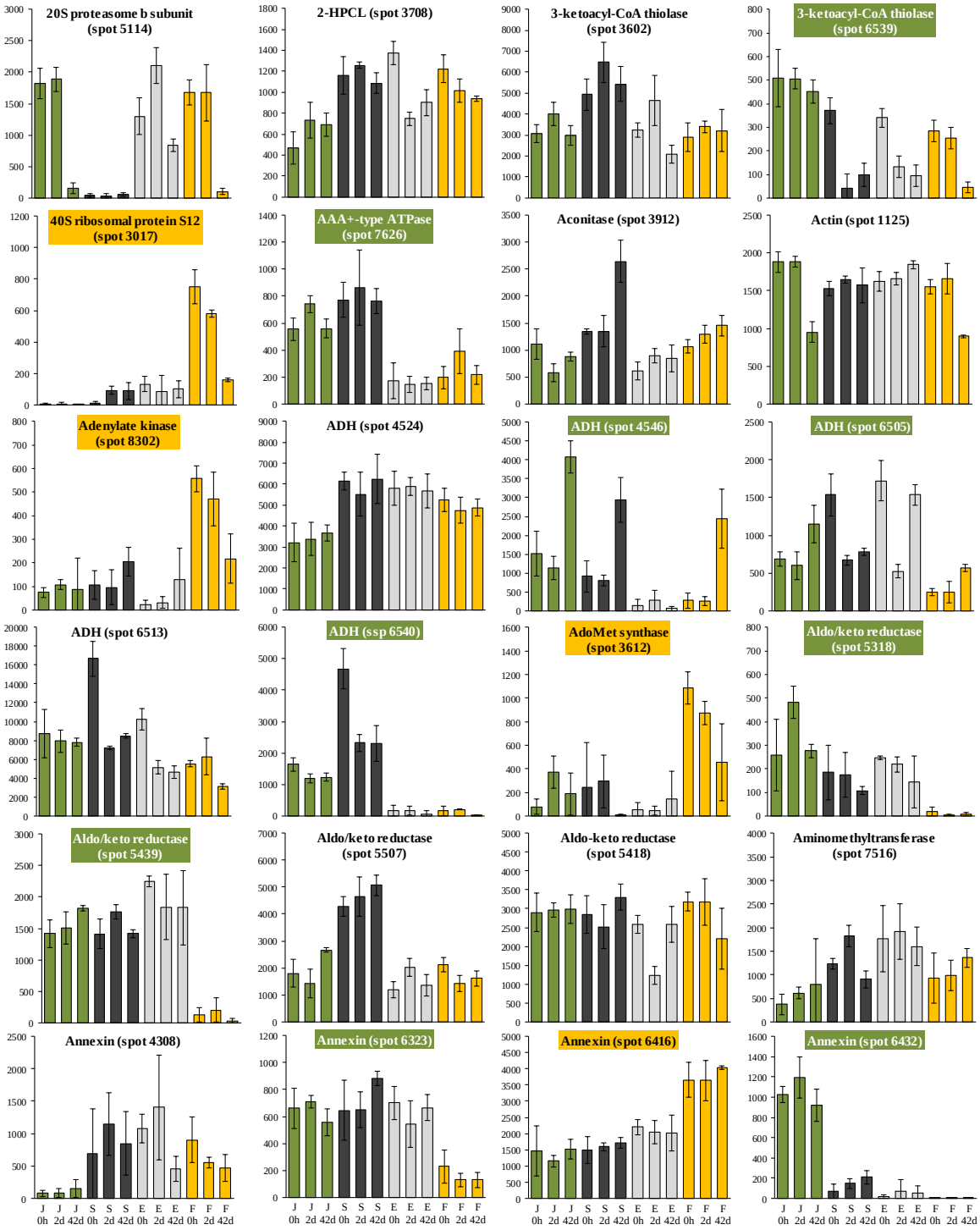
2.5.1.5. Other Proteins Associated with Freezing Stress Tolerance

Additional proteins related to freezing/cold tolerance and which distinguish the 'Jonsok' profile from 'Frida' include enolase (spot 3626), 4 distinct heat-shock proteins (HSPs) (spot 812, 813, 1819, 2743). Enolase has strong homology to the LOS2 enolase gene in *Arabidopsis thaliana* gene, a bi-functional enzyme that acts as a key enzyme in the glycolytic pathway in the cytoplasm and in the nucleus acts as a transcriptional repressor of ZAT10. ZAT10, a zinc finger protein can act either positively or negatively in regulation of abiotic stress (Mittler et al., 2006). In Arabidopsis, the chilling sensitive mutant, los2, has impaired stress-responsive gene expression which appears independent of the CBF expression pathway (LEE ET AL., 2002A). Enolase levels in 'Jonsok' were 4 fold higher than 'Frida' at all time points. It was interesting that a significant cold induction of enolase was observed in 'Senga Sengana' at 42 d (1.7 fold) but it was not cold induced in 'Jonsok'. Enolase levels, even in the absence of cold induction, have been reported to correlate with increased freezing tolerance (Takahashi et al., 2006).

Three of the 4 distinct HSPs that were identified by 2DE exhibited a significant cold induction in 'Frida' (spot 812, 813, 1819), yet 'Jonsok' had greater overall levels at all time points except for spot 812 at 42 d due to the significant induction in 'Frida'. 'Jonsok' shows a 1.9 fold cold induction of spot 813 at 42 d. Molecular chaperones present before cold stress would theoretically poise cellular processes that are requisite for cold acclimation. All HSP's identified were present at greater levels in 'Jonsok' than 'Frida' before cold treatment.

The complexity of information obtained from this 2DE analysis (as in all high throughput experiments) requires multivariate analysis such as PCA, ANOVA, and functional clustering analysis for simplification and interpretation. The significant differences in 'Jonsok' and 'Frida are placed in context of two additional cultivars for visualization. The comparison of protein expression profiles from 2DE analysis for all four cultivars at 0, 2, and 42 day time

points are presented in addition for all 110 proteins identified from 2DE as individual graphs Figure 2.12. The 2DE proteins identified as distinguishing the two cultivars 'Jonsok' and 'Frida' (Table 2.7) are presented in context with 'Senga Sengana' and 'Elsanta'. Several of the proteins identified as distinguishing 'Jonsok' from 'Frida' also distinguish 'Senga Sengana' from 'Frida'.



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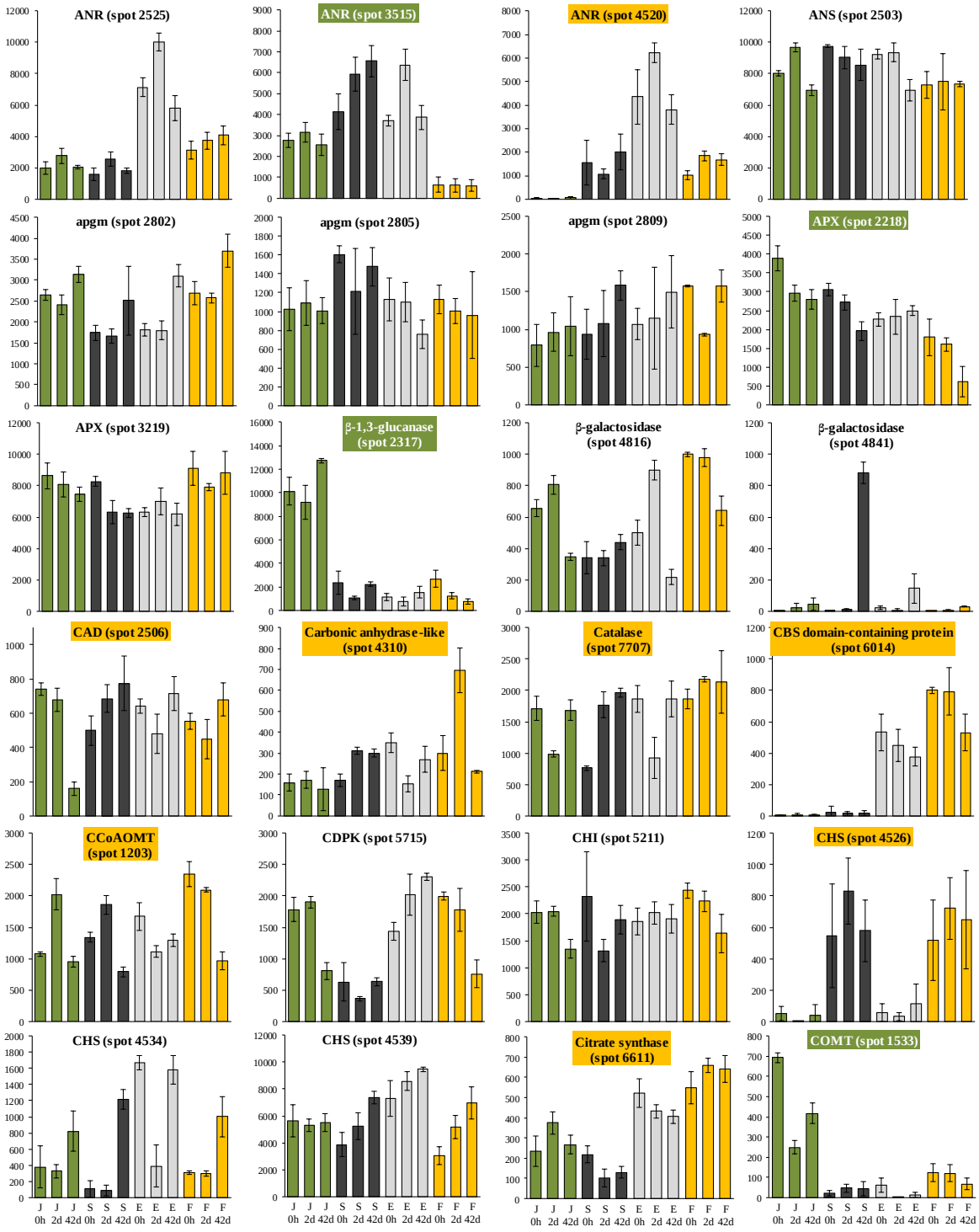

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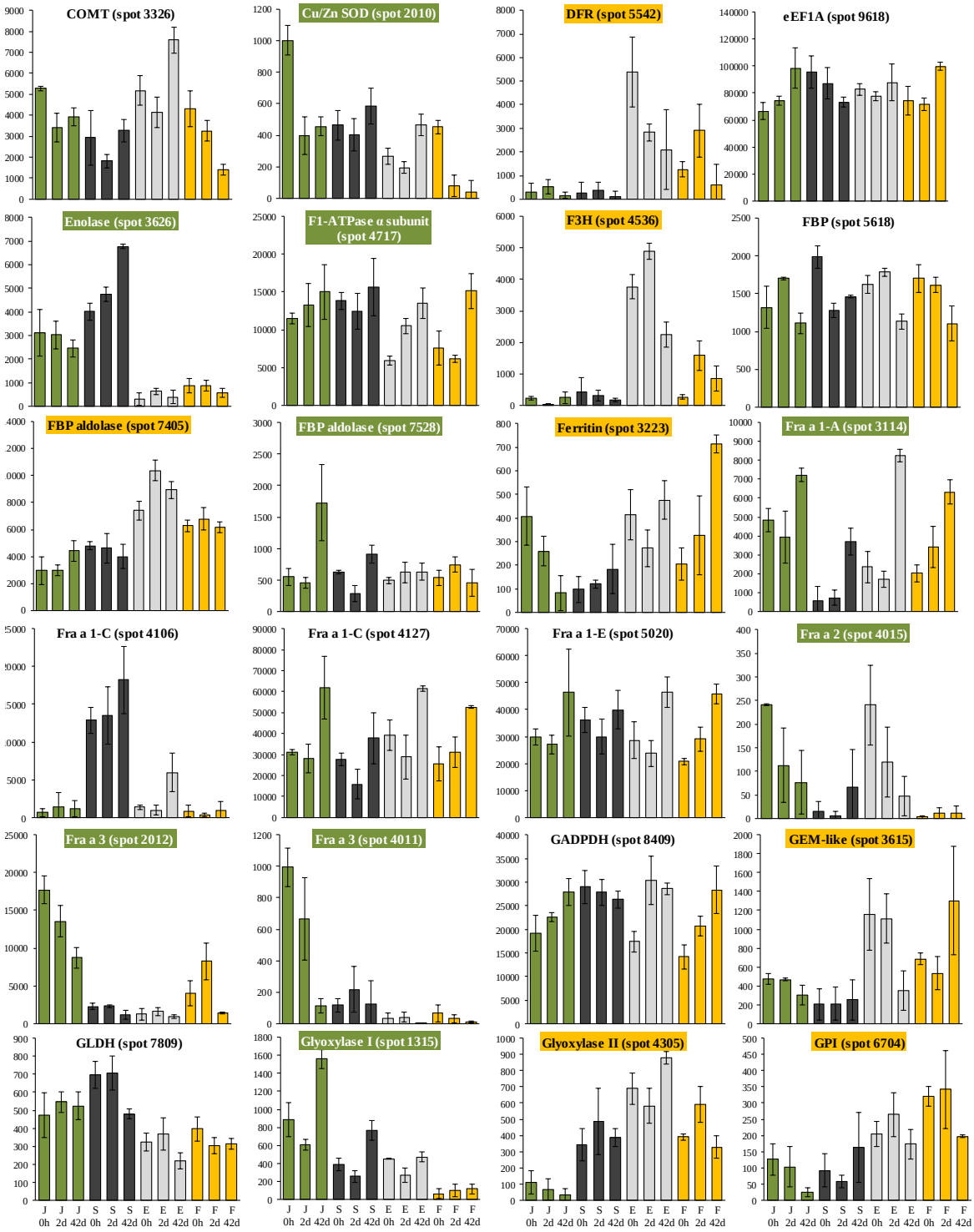

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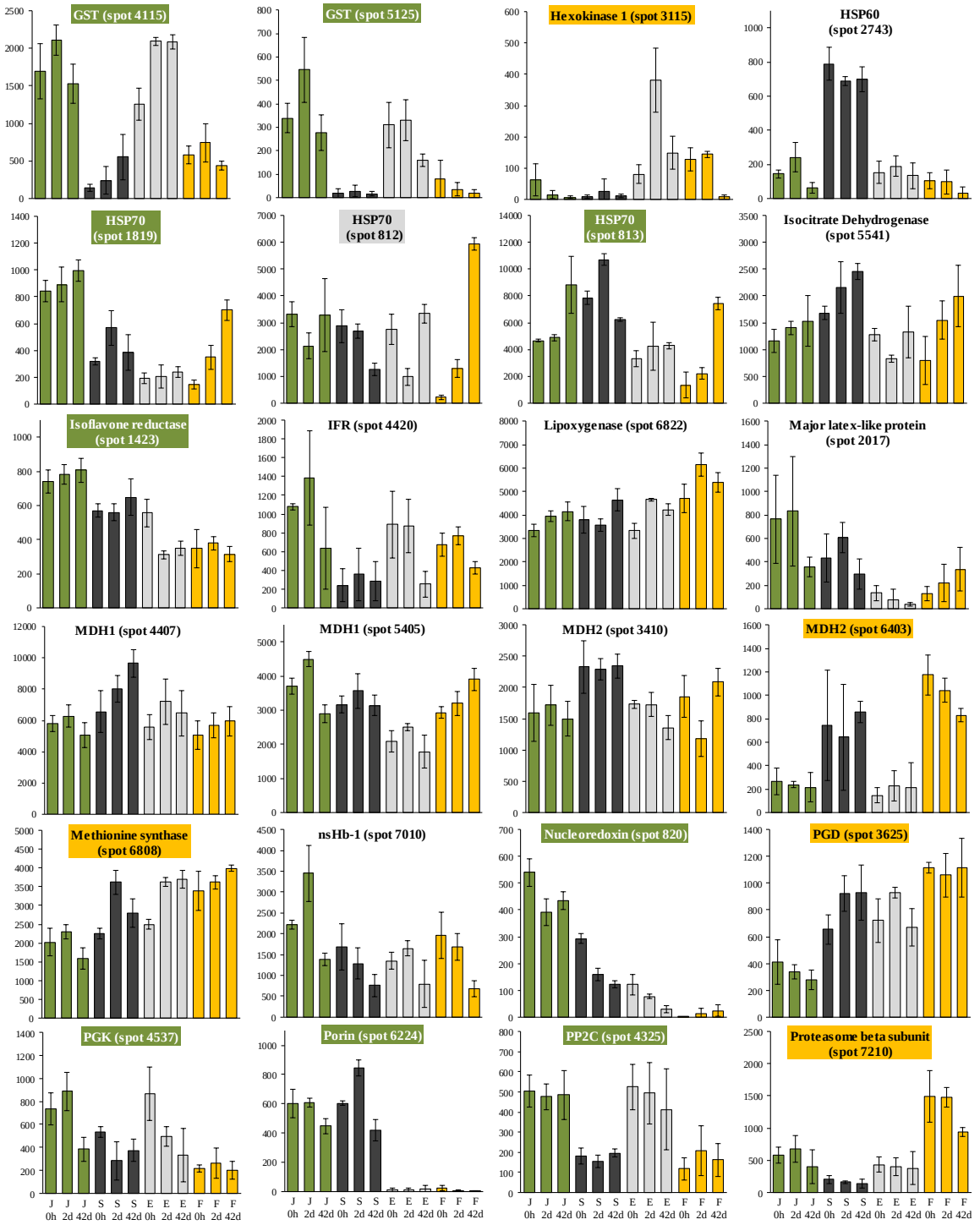

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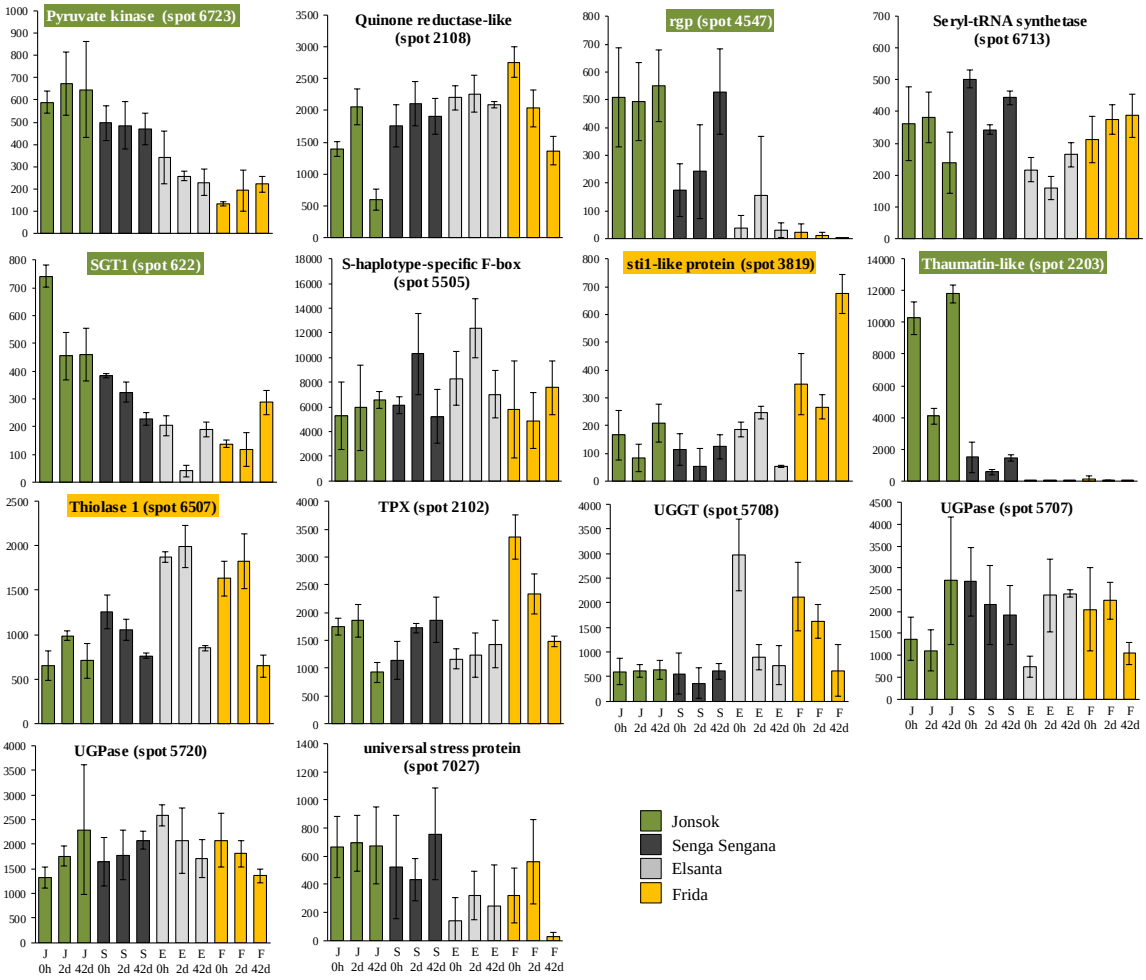


Figure 2.12 The 110 identified protein spots from 2DE analysis are illustrated for the four cultivars (in order from most to least freezing tolerant; 'Jonsok', 'Senga Sengana', 'Elsanta', and 'Frida' for the three experimental time points (0, 2, and 42 day cold treatment). The Y-axis values are the average quantity (optical density) n=3. Values were normalized to the total valid spots for each gel using PD Quest. Individual graph titles are highlighted in 'green' or 'orange' to correspond with the 2DE proteins that were identified in Table 2.7 as significantly higher levels in 'Jonsok' and 'Frida' respectively.

2.5.2. Comparison of 2DE and Shotgun-based Approaches

Two mass spectrometry-based proteomic approaches used for identification of proteins in complex mixtures include 2DE (gel based method) and shotgun-based, both used in this study. Each has distinct advantages and disadvantages. Shotgun is a very powerful tool allowing for thousands of protein identifications and quantification at one single time permitting a deeper look into biological networks that are potentially different. 2DE is generally more accommodating to identify PTM, and typically provides higher sequence coverage for proteins.

The gel-based 2DE method, by first resolving proteins by mass and pI, typically provides better sequence coverage for a specific protein (2DE protein spot). This can lead to revealing subtle difference in protein sequences present in different samples. 2DE is more labor and time intensive than shotgun to acquire proteomic data (running dimensions, staining, destaining, visualization, and quantitation). The most notable drawbacks for 2DE are limitations to visualize low abundant proteins and ineffective resolution of very acidic or basic proteins and hydrophobic proteins (e.g. integral membrane bound).

In comparison, the shotgun-based method overcomes the limits of gel-based resolution and therefore can identify proteins with a broader physiochemical range including hydrophobic and proteins with extreme pI's and proteins that are too low in abundance to detect by 2DE. Shotgun typically generates information for thousands of peptides resulting in the number of identified proteins sometimes in orders of magnitude higher than 2DE. Shotgun can be more sensitive for detecting subtle yet significant changes that would be challenging for 2DE. A drawback for the shotgun-based method can be a reduction in sequence coverage for individual proteins. This is especially relevant if the complexity of proteins in samples increase beyond the ability to completely separate peptides during chromatography.

Identifying post-translational modifications has numerous important aspects for biologists. 2DE and shotgun-base approaches can utilize similar strategies for identification of post-translational modifications (PTM) of proteins, such as immunoprecipitation of protein complexes first, but generally these two methods rely on different strategies. 2DE generally

has the advantage over shotgun-based approach by not relying on the intensive use of mass spectrometry to identify proteins that are PTM. 2DE method can be easily linked with other techniques such western blotting that can visualize global protein post-translational modifications (phosphorylation, glycosylation, degradation etc.) or by using commercially available specific stains. Because shotgun relies on mass spectrometry data to identify PTM, it requires that the type of PTM be stable and detectable. Using chemical derivatization that allows specific types of fragment ions to be observed in mass spectrometric analysis is becoming a common strategy to characterize the post-translationally modified for shotgun-based approaches (An et al., 2010; Roth et al., 1998). A major challenge for the shotgun-based approach for identifying PTMs includes the limited amount of databases with information pertaining to PTMs. Computation methods continue to advance and well annotated genomic databases increase to meet these challenges.

2.5.3. Shotgun Proteomics Approach Corroborates 2DE Findings

Many of the proteins identified by the shotgun approach corroborated the 2DE findings (Table 2.10). For example, shotgun analysis identified higher protein levels of chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), methionine synthase, and S-adenosylmethionine synthetase in 'Frida' and greater levels of alcohol dehydrogenase (ADH) in 'Jonsok'. Of the 29 identified peptides that were significantly different between 'Jonsok' and 'Frida', 9 of them correspond to CHS, F3H and chalcone flavanone isomerase (CHI) and all are higher in 'Frida' at 2 d than 'Jonsok' with CHS having the strongest cold induction at 2 d. In 'Jonsok', none of these proteins are cold induced (Table 2.9). Six of the 29 identified peptides correspond to CHS. All 6 CHS peptides exhibited a 1.2 fold increase in 'Frida' in response to cold (0 to 2 d) and exhibited an average 1.4 fold abundance over 'Jonsok' at 2 d. F3H was also observed to be more abundant in 'Frida' at 2 d (1.3 fold), consistent with an observed greater abundance by 2DE. S-adenosylmethionine synthase was significantly more abundant in 'Frida' at 0 d and 2 d in both shotgun analysis and 2DE (PCA, t-test, ANOVA). In 'Jonsok' only, S-adenosylmethionine synthase was cold induced (1.2 fold by shotgun; 4 fold by 2DE) at 2 d (Table 2.9). In terms of cold induced proteins, allene oxide cyclase ranked highest (at 2 d) with a maximum fold increase of 1.5 fold in

'Frida' and 1.28 in 'Jonsok'. This protein was not identified in the 2DE analysis. While the shotgun results were generally qualitatively in agreement with the 2DE, quantitatively smaller responses were observed. It is likely that the shotgun approach identified the summative changes in multiple isoforms of the various proteins, while the advantage of 2DE is that unique isoforms could be distinguished.

As expected, shotgun analysis did identify additional proteins beyond the 2DE analysis, such as the identification of proteins with very basic pI (pI > 10) (e.g. numerous ribosomal proteins, histones, and proteins involved in nucleotide transport) (Supplemental Data). Among the proteins significantly induced by cold in 'Jonsok' that were not identified by 2DE include a 60S ribosomal protein, and a sucrose phosphate phosphatase. Some of the protein families that were identified as significantly more abundant in 'Jonsok' at 2 d cold treatment that were not identified by 2DE include an aspartate aminotransferase, tropinone-reductase. With regards to 'Frida,' 20S proteasome subunits, and ubiquitin conjugating enzymes made 'Frida's protein profile distinct from 'Jonsok' at 2d cold treatment.

Overall, the 2DE and shotgun-based approaches were complementary methods and achieved the identification for proteins with a wide range of physiochemical properties, and detection of significant differences in protein abundance. The functional significance of the 2DE and shotgun findings are uncertain in some cases, such as annexin which was identified for 4 different 2DE spots that showed a difference in abundance for 'Frida' and 'Jonsok'. This instance can be furthered investigated using western blot analysis with specific protein antibodies and perhaps in conjunction with antibodies that can detect specific post translational modification. To investigate whether these protein spots these by using western blot analysis with specific protein antibodies, and perhaps in conjunction with antibodies that can detect specific post translational modifications.

Table 2.10 Proteins identified in both LFQP shotgun and 2DE analysis.

From LC-MS/MS based LFQP shotgun analysis, 153 ESTs were identified as significantly different between 'Jonsok' and 'Frida' at control (0d) and 2 day cold (2d) treated tissues. Twenty-one of these proteins were also identified in the 2DE approach based on EST identifiers. The GenBank accession code (gi), protein name, relative abundance levels greater in 'Jonsok' or 'Frida' detected by LFQP, and the time point at which the difference is significant is listed for LFQP or 2DE. When the relative difference in abundance for 'Jonsok' or 'Frida' agrees between LFQP and 2DE a 'yes' is indicated. In the instance that the same EST was identified for more than one 2DE spot, the 'yes' or 'no' corresponds to the 2DE spot number listed in the last column. Significance was based on $p < 0.05$, ANOVA for LFQP (shotgun) and $p < 0.05$, Student's t-test for 2DE.

Code gi	Protein ID	LFQP	Sig LFQP	Sig 2DE	Agree	2DE spot
113436	ADH	Jonsok	0d,2d	0d,2d	yes	6540
158356647	β-1,3-glucanase	Jonsok	2d	0d,2d	yes	2317
158371950	Enolase	Jonsok	0d	0d,2d	yes	3626
89557236	Fra a 2	Jonsok	0d	0d	yes	4015
158366345	Thaumatin-like	Jonsok	0d, 2d	0d,2d	yes	2203
158379507	Actin	Frida	0d, 2d	0d	no	1125
89544075	Annexin	Frida	0d	0d,2d	yes, no	6416, 6432
89550344	Annexin	Frida	0d	0d,2d	yes	4308
90576646	ANR	Frida	0d, 2d	0d,2d	no	3515
110564477	ANR	Frida	0d, 2d	0d,2d	yes, yes	4520, 2525
71979908	CHS	Frida	0d, 2d	0d,2d	yes, no	4526, 4534
24636275	Citrate synthase	Frida	0d	0d,2d	yes	6611
51493451	F3H	Frida	2d	2d	yes	4536
158302779	GADPDH	Frida	0d	n.s.		8409
51047667	IFR	Frida	0d, 2d	0d,2d	no	1423
89551239	Methionine synthase	Frida	0d, 2d	0d,2d	yes	6808
6760443	OMT	Frida	0d	n.s.		3326
158353550	Proteasome subunit	Frida	0d	0d,2d	yes	7210
158361609	Quinone reductase	Frida	0d	0d	yes	2108
158374908	TPX	Frida	0d	0d	yes	2102

Abbreviations: ADH, alcohol dehydrogenase, ANR, anthocyanidin reductase; CHS, chalcone synthase; F3H, flavonoid 3-hydroxylase; GADPDH, glyceraldehyde-3-phosphate dehydrogenase; IFR, isoflavone reductase; OMT, O-methyltransferase; TPX, thioredoxin-dependent peroxidase.

2.5.4. 1-DE Western Blot Analysis Validates 2DE Observations

One of the goals of this work was to identify protein candidates for molecular markers for overwintering success. A next step beyond the protein discovery is to confirm some of the likely biomarkers. With this goal in mind, we are beginning to evaluate these candidates with antibodies. Two such candidates, cytoplasmic ascorbate peroxidase (APX) and alcohol dehydrogenase (ADH) protein levels were evaluated by 1-DE western blotting in 'Jonsok' and 'Frida' at 0, 2, and 42 day cold treatment. Consistent with 2DE and shotgun, these preliminary evaluations indicate that 'Jonsok' exhibits higher levels of ADH early on compared to 'Frida' (Figure 2.13). The APX expression is similar to the APX levels observed for the 2DE analysis with 'Jonsok' having more present at 0, and 2 day. The 1-DE western blots support both the identification and differences in abundances of proteins identified in 2DE and shotgun. This method does provide direct evidence that these are good potential

biomarkers. The validation for utility of protein biomarkers will be performed in a future analysis. A preliminary validation for ADH as a marker for enhanced freezing tolerance has been accomplished in diploid strawberry *Fragaria vesca* in collaboration with our colleagues and these results have been submitted for publication ('Dehydrin, alcohol dehydrogenase, and central metabolite levels are associated with cold tolerance in diploid strawberry (Fragaria spp.); J. Davik, B. From, G. Koehler, T. Torp, J. Rohloff, P. Eidem, R. Wilson, A. Sønsteby, S. Randall and M. Alsheikh, submitted to Planta).

Dehydrins are strongly correlated with cold stress tolerance in many plant species. Because of the interest in our lab in the roles of dehydrins in low temperature tolerance, it was natural to investigate and compare dehydrin protein expression in the cultivars 'Jonsok' and 'Frida' (Figure 2.14). 'Jonsok' and 'Frida' demonstrate dehydrin accumulation strongly at 42 day cold treatment.

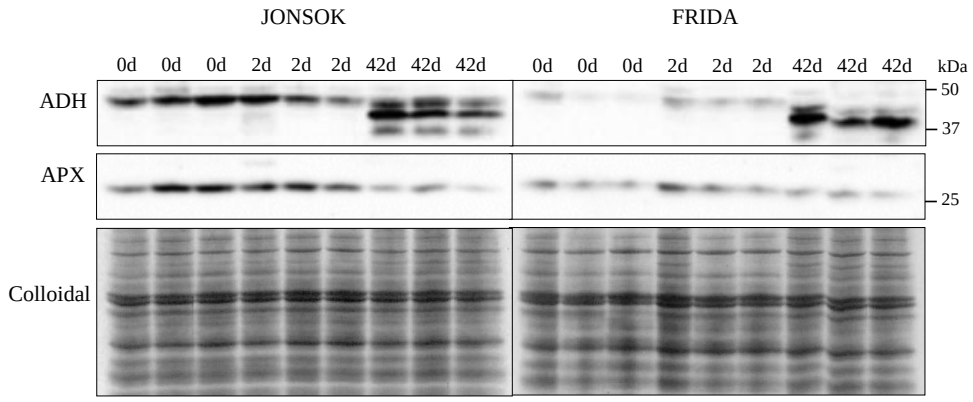


Figure 2.13 Confirmation of two potential biomarkers using 1-DE western blot analysis. 'Jonsok' and 'Frida' crown proteins (25 µg) from 0, 2, and 42 d (all in triplicate) were probed using ADH and cAPX antibody.

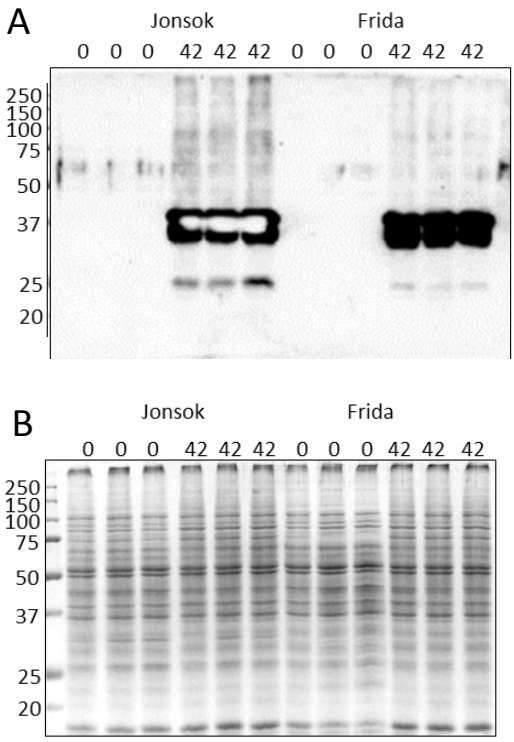


Figure 2.14 Evaluation of dehydrin levels using 1-DE western blot analysis. A)'Jonsok' and 'Frida' crown proteins (5 μg) from 0, and 42 d (all in triplicate) were probed using the antibody raised against K-segment (Dehydrin). B). Gel stained with colloidal coomassie brilliant blue for protein load comparison.

2.6. Conclusion

By comparing protein expression in the crown tissue of octoploid strawberry from the less tolerant cultivar ('Frida') to one of greater tolerance ('Jonsok'), we have noted several trends. First, 'Jonsok', appears poised for tolerating cold stress, as many known proteins related to freezing/cold tolerance are constitutively expressed at significantly greater levels than those found in 'Frida'. This poise has been observed in other species (Taji et al., 2004; Takahashi et al., 2006). Additionally, the array of cold response proteins is significantly more complex in 'Jonsok', including a large variety of proteins known to be associated with both abiotic and biotic stress tolerance. Secondly, the convergence of protein expression in the two cultivars, visualized by principal component analysis (PCA), which becomes readily apparent after 42 d, is largely due to 'Frida' "catching up" in terms of expression patterns to the more cold-tolerant cultivar (see Figure 2.13; ADH). However, one should not ignore the observation

that ‘Frida’ is a cold/freezing tolerant cultivar, just less so than ‘Jonsok’, and indeed appears to have adopted a very strong antioxidation response as evidenced by activation of the ascorbate pathway and phenylpropanoid pathway. Indeed these latter approaches may represent an alternative, perhaps lesser, but nonetheless effective response to cold stress.

Many previous approaches to understand winter hardiness have focused on molecular responses to cold acclimation in single varieties or cultivars. The present study, through the comparison of two cold tolerant cultivars, which differ in their extent of cold hardiness, has revealed a variety of differences in proteins involved in stress responses. Through the comparison of these two closely related cultivars, we have further observed differences that are largely due to alterations in constitutive expression, identifying a substantial number of proteins, many of which are known to confer stress tolerances; and which are candidates for molecular markers associated with overwintering success.

CHAPTER 3. COLD-REGULATED PROTEINS IN LEAVES OF FRAGARIA × ANANASSA 'KORONA'

3.1. Introduction

This study originated from collaboration with a focus to evaluate cold tolerance for strawberry cultivars by comparing cold-responsive metabolites and cold responsive proteins in leaves (rather than crowns). This chapter presents the proteomics portion of this work for the *F.* × *ananassa* 'Korona' (not covered in the previous chapter). This chapter offers the additional context of placing F. × ananassa cold responses within the existing knowledge base of low temperature stress protein changes in leaves, allowing one to evaluate the uniqueness or generality of Fragaria responses in photosynthetic tissues.

Cold-regulated transcripts have earlier been identified in *Fragaria* × *ananassa* leaves (Ndong et al., 1997; Yubero-Serrano et al., 2003), and recent investigations have adopted genetic engineering in the study of dehydrins (Houde et al., 2004), the CBF1 regulon (Owens, 2003; Owens, 2002), and for the introduction of fish antifreeze-proteins (Khammuang, 2005). Furthermore, the impact of polyamines (glycine betaine) in cold acclimation processes has been reported (Einset et al., 2007a; Einset et al., 2007b; Einset et al., 2008; Rajashekar et al., 1999), and newer studies have investigated stress-related ROS production and enzyme activity (Gülen, 2008), also in relation to leaf antioxidant levels (Zhang, 2008).

The previous chapter focused on the comparison of the proteome profiles in the crown of different cultivars. The rationale behind that focus was to study overwintering relevant tissues in Fragaria. While much attention has been paid to the cold regulation in vegetative tissues in annual species it was considered less relevant to understanding cold regulated responses in strawberry crown. In this chapter we discuss responses to cold at the protein level in the leaf.

Our interest in focusing on leaves include 1) In some mild climates, leaves can overwinter and provide a significant head start for regrowth in the spring, 2) to investigate whether leaves might express markers identified in crown tissue that could be used to perform non-destructive screening of cold-tolerant Fragaria lines. This ability would allow screening for cold tolerance without first obtaining clonal lines thus enabling high throughput capabilities.

3.2. Methods

3.2.1. Plant Growth and Cold Treatment

Eight weeks old runner-propagated *F* × *ananassa* (Duch. cultivar 'Korona'), was grown on fertilized soil (P-Jord; Emmaljunga Torvmull AB) in plug trays (3 x 6 cells) in a greenhouse at 18 ºC under natural light and long-day conditions. Plants were short-day adapted for 1 week at 12 ºC under artificial light (fluorescent tubes, ~90 µmol m-2 sec-1) in a conditioning room prior to transfer to a cold storage room at 2 ºC under artificial light (fluorescent tubes, ~90 µmol m-2 sec-1) and relative humidity at average of 80%. Plant sampling was carried out at the following time points, 0, 24, and 240 h after initiation of the cold treatment. Control samples (0 h) were harvested prior to the transfer to the cold room. Harvested plant material of leaf from 3 plants per time point was pooled, flash-frozen in liquid N2 and stored at -80 ºC before sample processing.

3.2.2. 2DE and Gel Imaging

First dimension focusing parameters was the same as Chapter 2 except IEF strips (BioRad, 24 cm; 3 to 10 NL) were passively rehydrated with 220 µg of leaf proteins at 20 ºC for 14 h. Protein Analysis PD Quest software was used to evaluate nine (three conditions, each in triplicate) 2DE protein gels. A total of 845 spots were matched for analysis. Significant differences are based on t-test results of 0.05 or better.

3.2.3. 2DE Protein Identification by LC-MS/MS

Methods described as in Chapter 2.

3.2.4. Western Blotting

Equal amounts of protein from total extracts were separated by 10% one-dimensional PAGE, transferred to nitrocellulose and probed with antibody raised against Arabidopsis COR47 as previously described (Alsheikh et al., 2005).

3.3. Results

3.3.1. 2DE Analysis of Total Proteins in *F.* × *ananassa* Leaves

Leaf samples of cold-treated *F.* × *ananassa* 'Korona' from the 0, 24 and 240 h time points were subjected to 2DE gel protein separation and subsequent LC-MS/MS analysis of selected spots. A representative 2DE gel is presented in Figure 3.1 indicating the 35 protein spots identified by LC-MS/MS (Table 3.1). A total of 845 spots were matched in all nine gels. Quantitative image analysis detected 39 protein spots (4.6%) that changed significantly ($p < 0.05$) by more than 2 fold at either 24 h, or 240 h compared to control. Figure 3.2 summarizes the number of the cold responsive protein spots that were detected at different levels of significance. Data indicated more down- than up-regulated spots at a ratio of 3:1 (Figure 3.2 C). Twenty-eight of the spots were selected for MS-based identification as they were the ones that appeared to be significantly different in one or more conditions (Figure 3.3). In addition, 5 spots that appeared not to be significantly changing under any conditions were identified as a ClpC (ATP-dependent clp protease), glutamine synthetase, Rieske FeS, ADH, and RuBisCO SS (not shown in Figure 3.3). Sampling was deliberately not performed in the region containing RuBisCO LS and since that spot was overloaded, it was not quantitatively evaluated. Of the 28 cold responsive proteins identified, 14 were up-regulated and 14 were down-regulated at least 1.5 fold with a minimum significance $p < 0.1$. Functional classifications for the identified proteins were obtained by finding their arabidopsis homologs and utilizing the TAIR GO resources (Figure 3.4). The number of chloroplast

associated proteins for the cold up-regulated proteins compared to the down-regulated proteins was one of the most notable differences, indicating at least 3 times more chloroplast associated proteins present in the upregulated proteins.

Among the chloroplast-associated cold-upregulated proteins were ferritin (spot 114), and a chlorophyll a/b binding protein (spot 3109), both increasing over 4 fold, the highest fold increases at 240 h. RuBisCO activase increased significantly upon cold treatment of 240 h. Several chloroplast-metabolic proteins (Figure 3.5) showed increases after 240 h in cold. Two of these proteins (ferritin and PG kinase) have isozymes in the cytosol, though these appear to be the chloroplastic isozymes. The up-regulation of the ATP-dependent Zn peptidase (240 h) is of interest because of its role in thylakoid formation and the removal of damaged D1 precursors in monomeric photosystem II reaction centre complexes. With regard to the chloroplast associated down-regulated or not changed proteins include two cytochrome b6-f genes differed slightly with one (spot 3007) decreased sharply after 24 h, but returning to starting levels after 240 h in cold. The other cytochrome b6-f FeS (spot 4004) did not change significantly. Two protein spots, identified as polyphenol oxidase both decreased. The chloroplast import chaperone ClpC levels did not change significantly. The unresponsiveness of this protein to cold treatment has been previously reported (Dutta et al., 2009). The two ATP synthase genes (mitochondrial alpha and chloroplast delta) increased 1.6 fold at 24 h and returned to control levels at 240 h. RuBisCO SS protein did not change significantly throughout the cold acclimation period (RuBisCO LS was not quantitated).

Proteins characterized as non-chloroplastic (4 metabolism-associated) showed differing expression patterns in response to cold (Figure 3.5). An auxin-binding protein (function unknown), and eIF4A both decreased significantly. The helicase eIF4A, important in translation, was strongly decreased in response to cold treatment. The eIF4A is known to be post translationally modified by phosphorylation with observed lateral shifts in 2DE gels (Gallie et al., 1997; op den Camp and Kuhlemeier, 1998; Webster et al., 1991). The observed decrease most likely represents a change in phosphorylation state that has been associated with developmental stage or stress related. The decrease of the flavanone-3-hydroxylase (F3H), a key enzyme of flavonoid biosynthesis in plants, indicates a distinct down-regulation

of secondary metabolism in strawberry leaves cv. 'Korona' upon cold acclimation. Both the glycine cleavage enzyme H and the unknown function stress-related protein increased strongly upon cold treatment after 240 h. The putative glyoxalase I has potential detoxification functions involving sulfhydrils and methylglyoxal, a byproduct of the glycolytic pathway, and increased transiently after 24 h.

Overall the comparison of the up-regulated to down-regulated protein groups revealed different metabolic pathways. The upregulated proteins represented glycolysis (PDC, PG kinase), TCA cycle (MDH, citrate synthase), and starch and sucrose metabolism (cellulose). Pyruvate metabolism was represented by MDH and glyoxalase 1, both proteins transiently increased at 24 h and returned to control levels by 240 h. The flavonoid biosynthesis pathway was represented by down regulated proteins including, PAL, F3H, and leucoanthocyanidin reductase.

3.3.2. Evaluation of Dehydrin levels in 'Korona' Leaves

Dehydrin protein levels were measured in order to verify expected cold responses in leaf tissue of *F.* × *ananassa* 'Korona' with regard to the well-characterized up-regulation of genes encoding dehydrins (Alsheikh et al., 2005). Blots probed with antibodies which specifically recognize the Arabidopsis dehydrin, COR47, revealed a significant increase in protein levels of a 53 kDa band, designated as FaCOR47 due to its cross reactivity to the antibody and its appropriate mass (Figure 3.6). Likewise, another antibody-reactive band (48 kDa) was highly expressed similarly upon cold treatment. This lower band likely represented the non-phosphorylated form of FaCOR47 (Alsheikh et al., 2005). The higher mass but minor band of 82 kDa is likely an aggregate of COR47 often detected in such blots.

3.4. Discussion

One important coping mechanism for low temperature stress in plants is the reduction of the photosynthetic capacity to prevent situations where light energy harvested by the leaves might be in excess of what can be processed by photosystems. Cold tolerant crops have

been reported to maintain leaf respiration and photosynthesis rates during exposure to cold (Yamori et al., 2009; Yamori et al., 2011) as opposed to more cold intolerant lines showing strong decrease in photosynthesis. There are several factors that influence the cold response of plants in terms of molecular changes associated with photosynthesis. Light intensity, cold intensity and duration, nutrition and metabolic interactions between organelles, or some factors that determine the type and how dynamic changes are reported to have an impact on molecular responses. In addition, diverse responses to cold temperatures are observed for different plant types, such as woody and herbaceous plants, indicating different strategies are utilized for coping with photosynthetic adjustments in low temperatures. However, despite the different methods observed in diverse plants for coping, it is recognized that optimal photosynthesis requires a balance between the rates of carbon fixation in the chloroplast and cytosolic sucrose synthesis. In this study 'Korona' exhibited up-regulation of proteins in both metabolic and photosynthetic associated proteins indicative of a more 'cold-tolerant' type of response. Protein levels increased for RCA (RuBisCO activase) and FER1 (ferritin) at 10 d, as well as with increased levels of PGK and two chlorophyll a/b binding proteins. Since cold tolerant species show a higher degree of photosynthetic homeostasis than sensitive species (Yamori et al., 2009), the overall unified photosynthetic response at the transcriptional level allow the conclusion, that the *F.* × *ananassa* 'Korona' shows a typical cold response.

An attempt was made to correlate proteins expressed in leaves and crown by comparing 2DE protein patterns in 2DE gels. Remarkably, even though the tissues and cultivars were different, the majority of protein spots show spatial cognates between crown and leaf (>60%). Forty proteins that overlapped between crown and leaf tissue had displayed a significant difference in accumulation in 'Jonsok' and 'Frida' crown and were evaluated if the cold responsiveness was similar in leaf tissue. Only one protein spot of the forty demonstrated a significant change (-1.8 fold; $p < 0.05$) in leaves as in crowns. This spot corresponded to a Thaumatin-like protein ('Jonsok' abundant protein), a protein chosen as a potential cold tolerance stress marker (see Chapter 2).

It is difficult to assess how likely the possibility is to utilize leaf tissue instead of crown tissue based on this initial analysis, since the cultivars are different, but because there is evidence that several proteins of interest, including thaumatin, were present in leaves, it is suggestive that evaluation of leaf proteins in Jonsok is warranted. Once antibodies become available that are reactive to potential cold tolerant markers this effort will be facilitated and supply more definitive evidence into the possibility of using leaf tissue instead of crown tissue.

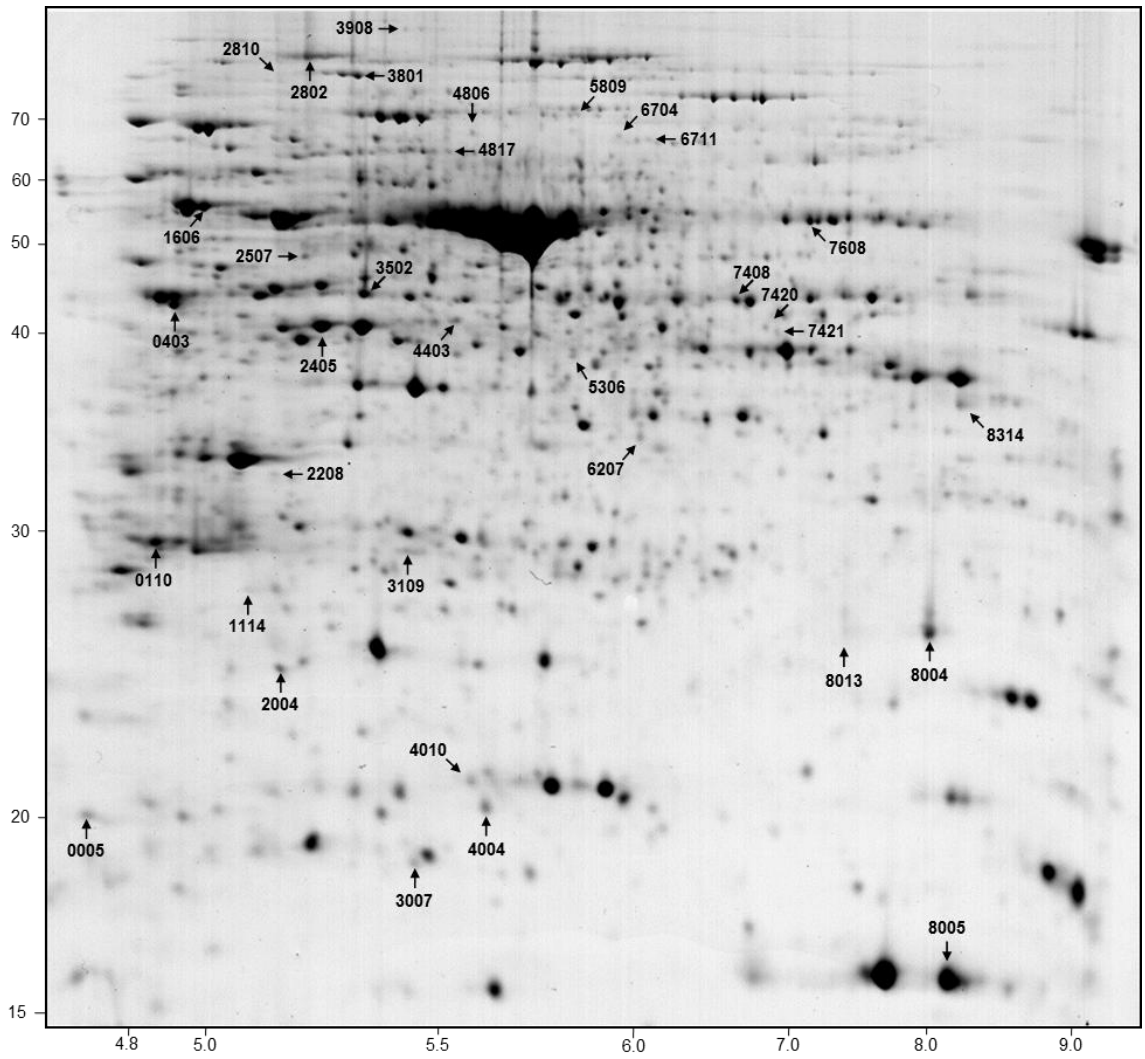


Figure 3.1 A representative 2DE gel (24 h cold treatment) of leaf tissue proteins of *F.* × *ananassa* 'Korona'. Thirty-five protein spots identified by LC-MS/MS are labeled by their spot ID's.

A

Fold changes at 24 and 240 h over 0h			
Increased	P < 0.05	P < 0.10	All spots
> 1.5	23 (11)	36 (13)	88 (14)
> 2.0	13 (4)	17 (5)	24 (5)
> 3.0	3 (2)	4 (2)	5 (2)
> 5.0	0	0	0
Decreased			
> 1.5	43 (8)	83 (12)	241 (13)
> 2.0	26 (8)	46 (12)	99 (13)
> 3.0	13 (4)	22 (6)	28 (7)
> 5.0	5 (1)	7 (3)	8 (4)

B

Percent of protein changes at 24 h and 240 h			
Increased	P < 0.05	P < 0.10	All spots
> 1.5	2.7	4.3	10.4
> 2.0	1.5	2.0	2.8
> 3.0	0.4	0.5	0.6
> 5.0	0.0	0.0	0.0
Decreased			
> 1.5	5.1	9.8	28.5
> 2.0	3.1	5.4	11.7
> 3.0	1.5	2.6	3.3
> 5.0	0.6	0.8	0.9

C

RATIO (DECREASE : INCREASE)			
Fold	P < 0.05	P < 0.10	All spots
> 1.5	2:1	2:1	3:1
> 2.0	2:1	3:1	4:1
> 3.0	4:1	6:1	6:1
> 5.0	5:1	7:1	8:1

Figure 3.2 Changes of protein spot intensities from 2DE gel analysis of leaves from *F.* × *ananassa* 'Korona' during 0, 24 and 240 h of cold acclimation at 4 C. A) The number of protein spots that increased or decreased showing significance (t-test; $p < 0.05$ or $p < 0.10$) at 24 and 240 h of cold exposure at 4 ºC. The number of these proteins with identification by LC-MS/MS is given in parenthesis. B) The percent of fold changes (magnitude and significance) observed at 24 and 240 h of cold exposure (out of 845 protein spots). C) More proteins decreased than increased during cold treatment. Ratios are given (number of proteins decrease to increase) for proteins that significantly changed at 24 and 240 h.

Table 3.1 Proteins identified from 2DE analysis of *F.* × *ananassa* 'Korona' leaf by LC-MS/MS. Thirty-five identified protein spots are ranked by spot ID (2DE identifier) with accession code (gi), confidence scores and number of distinct peptides and number of peptides corresponding to LC-MS/MS. The Arabidopsis gene homolog, AGI (Arabidopsis Genome Initiative gene index number) is listed. Protein identification was assigned based on Blast search on NCBI. The molecular weight and isoelectric point (MW/pI) for AGI's were obtained from the TAIR site (http://www.arabidopsis.org) and the 2DE MW/pI information was calculated from 2DE gels (EXP). Cold responses of proteins are given as fold changes for 24 h and 240 h from 0 h (control).

Spot	Accession	Confidence	Peptides	AGI	Putative Identification	AGI	EXP	Fold Change	
ID	gi				Abbreviation (name assigned)	MW/ pI	MW/ pI	24 h	240 h
5	89558548	8.92E-05	4/ 8	AT1G32470	Gly Clvg (Glycine cleavage system H protein)	17.9/ 4.9	20.0/ 4.7	1.05	1.99
110	158373230	5.22E-09	21/ 24	AT2G34430	Chl a/ b. II (Chlorophyll a/ b-binding, photosystem II type I)	28.2/ 4.9	29.4/ 4.9	1.77	1.82
403	158360826	9.40E-10	13/ 17	AT2G39730	RCA, (RuBisCo Activase)	52.0/ 5.9	42.7/ 4.9	-1.39	1.93
1114	89556609	9.78E-09	1/ 1	AT5G01600	Ferritin	28.2/ 6.0	28.0/ 5.1	1.75	4.78
1606	158362822	5.39E-04	1/ 2	AT2G07698	ATP synthase subunit alpha, mitochondrial	85.9/ 5.2	53.6/ 5.0	1.11	1.25
2004	158353965	2.00E-06	13/ 19	AT4G09650	ATP synthase delta chain, chloroplastic	25.7/ 9.7	24.6/ 5.2	1.62	1.16
2208	158353167	4.79E-06	2/ 3	AT1G67280	Glyoxalase 1 (lactoylglutathione lyase)	39.2/ 7.5	32.3/ 5.2	2.09	1.21
2405	158376871	1.71E-07	12/ 15	AT5G37600	Glutamine synthase	39.1/ 5.1	39.9/ 5.2	-1.19	1.04
2507	158362108	1.81E-07	6/ 7	AT1G54270	eIF4A (Eukaryotic initiation factor 4A)	46.8/ 5.4	50.4/ 5.2	-4.82	-3.08
2802	14970841	0.9992	1/ 3	AT2G28470	β-galactosidase	93.2/ 7.9	60.3/ 5.2	-1.15	-2.15
3007	158376706	4.49E-08	6/ 9	AT4G03280	Cyt b6-f FeS (Cytochrome b6-f complex iron-sulfur subunit)	24.4/ 8.7	18.7/ 5.4	-5.11	-1.42
3109	158352462	8.75E-07	9/ 14	AT4G10340	Chlorophyll A/ B binding protein	30.2/ 6.3	29.0/ 5.4	1.86	4.35
3502	158357589	2.42E-09	14/ 19	AT1G56190	PG kinase (Phosphoglycerate kinase)	50.0/ 8.6	44.5/ 5.3	1.69	1.92
3715	10121330	0.9981	2/ 4	AT5G54960	PDC (pyruvate decarboxylase)	65.9/ 5.9	56.7/ 5.4	-1.09	1.49
3801	158353156	0.00E+00	10/ 11	AT5G50920	CLPC (ATP-dependent clp protease)	103.5/ 6.7	60.2/ 5.3	-1.03	1.18
3811	12484392	0.9998	3/ 3	AT1G64390	Cellulase	68.6/ 9.9	57.9/ 5.4	1.08	1.61
4004	158376706	5.29E-06	2/ 2	AT4G03280	Cyt b6-f FeS (Cytochrome b6-f complex iron-sulfur subunit)	24.4/ 8.7	20.2/ 5.5	1.13	1.12
4010	60389902	0.9336	3/ 4	AT3G27968	Fra a 1 (Fragaria Allergen Fra a 1)	65.8/ 8.7	21.0/ 5.5	-1.53	-6.52
4403	51493451	8.25E-06	3/ 3	AT3G51240	F3H (Flavanone 3-hydroxylase)	40.3/ 5.1	40.8/ 5.5	-2.41	-1.78
4806	15131531	1.0000	3/ 4	AT2G40940	Ethylene receptor	68.3/ 6.5	58.3/ 5.5	2.28	2.50
4817	57896462	3.08E-06	1/ 4	AT1G50250	FtsH (ATP-dependent zinc metalloprotease)	76.8/ 5.5	56.3/ 5.5	-1.32	1.67
5306	155029180	1.0000	2/ 3	AT5G51970	NAD-SDH (NAD-dependent sorbitol dehydrogenase)	39.3/ 5.8	38.2/ 5.7	-1.29	-2.96
5809	157313318	1.0000	2/ 3	AT1G55020	Lipoxygenase	98.0/ 5.2	58.1/ 5.7	-1.20	-5.69
6207	171191321	0.9759	1/ 2	AT4G24730	Polyphenol oxidase	49.5/ 7.0	34.7/ 6.0	-1.37	-2.34
6704	58891689	1.0000	3/ 5	AT3G15730	PLD alpha (phospholipase D alpha)	91.9/ 5.7	57.1/ 5.9	-1.33	-2.11
6711	158379721	7.66E-02	2/ 2	AT1G67360	REF/ SRPP-like protein (rubber elongation factor)	26.4/ 9.4	56.9/ 6.1	-1.28	1.90
7408	158370565	8.67E-07	2/ 2	AT5G43940	Alcohol dehydrogenase class-3	42.0/ 6.9	43.6/ 6.7	-1.09	-1.42
7420	116292589	0.9912	2/ 2	AT1G75290	Leucoanthocyanidin reductase	35.1/ 6.1	39.9/ 6.9	-1.44	-3.09
7421	171191321	0.9998	1/ 3	AT4G24730	Polyphenol oxidase	49.5/ 7.0	39.6/ 7.0	-1.19	-6.26
7608	24636275	1.0000	2/ 4	AT2G44350	Citrate synthase, mitochondrial	52.7/ 6.9	52.9/ 7.2	-1.32	1.79
8004	158378481	2.34E-05	2/ 3	AT5G20630	Auxin-binding protein	21.8/ 6.8	25.8/ 8.0	-1.98	-2.25
8005	51048685	4.05E-06	14/ 18	AT1G67090	Ribulose bisphosphate carboxylase small chain	20.2/ 7.8	15.8/ 8.1	1.34	-1.06
8013	157041071	0.9999	2/ 2	AT3G53260	PAL (Phenylalanine ammonia-lyase)	77.9/ 6.4	25.8/ 7.5	-2.73	-3.46
8314	24638017	0.9996	1/ 8	AT1G53240	Malate dehydrogenase, mitochondrial	35.8/ 8.6	35.7/ 8.3	2.49	1.24
9504	209922600	0.9999	4/ 7	AT1G07920	eEF1A (elongation factor 1-alpha)	49.5/ 9.6	51.7/ 9.2	-1.41	-1.33

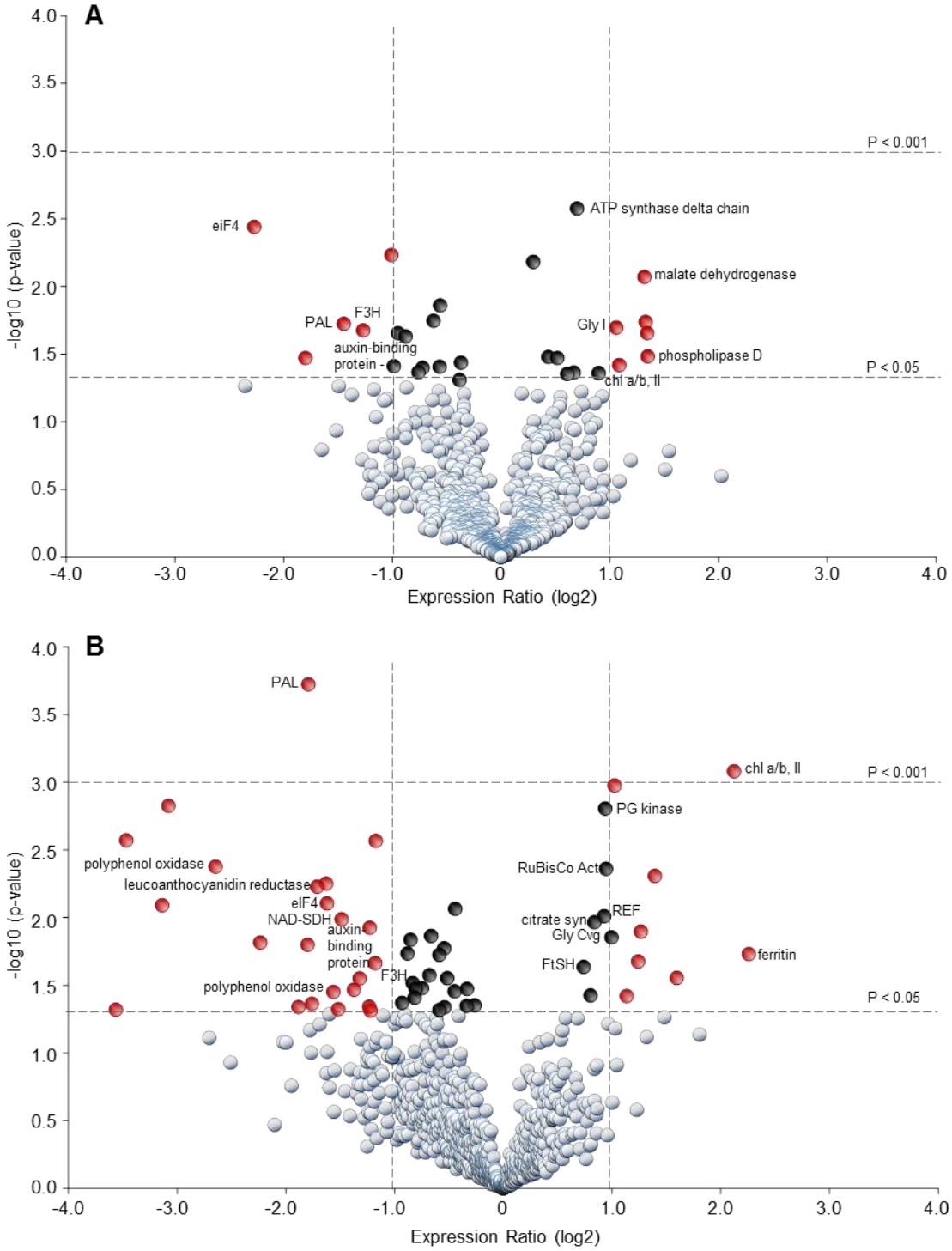
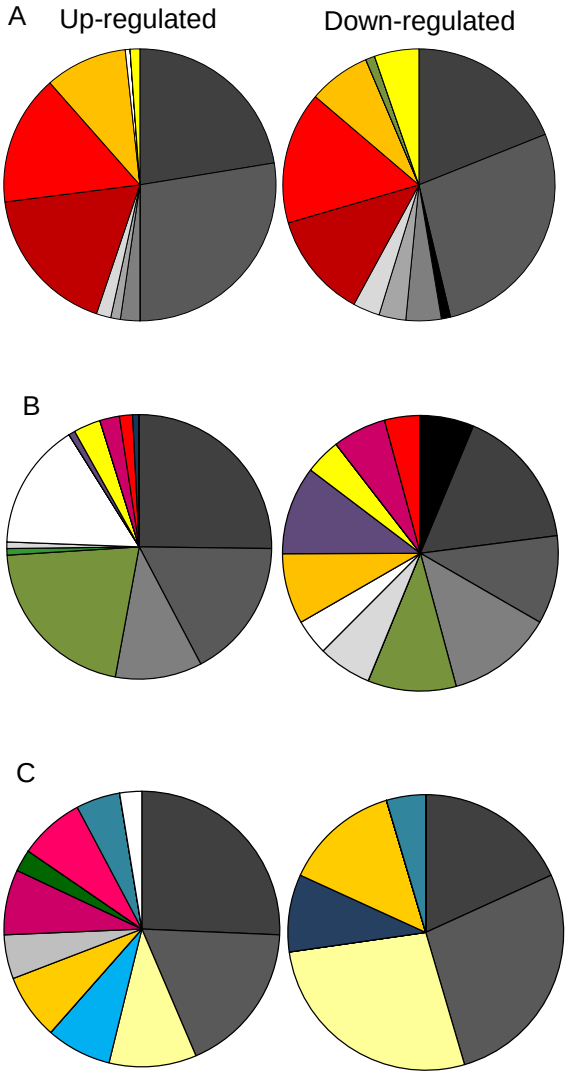


Figure 3.3 Protein expression levels in leaves of *F.* × *ananassa* 'Korona' after 24 h and 240 h of cold treatment panel A and B respectively. Volcano plot was obtained by plotting the log2 ratio of mean values (24 or 240 h cold treatment over control) for the 845 matched 2DE spots against the negative log10 of the p-value from the Student's t-test. Proteins that changed 2 fold or more with a significance of p-value < 0.05 are indicated with red. Proteins that changed significantly ($p < 0.05$) but changed less than 2 fold are indicated in black. Identified proteins are indicated.

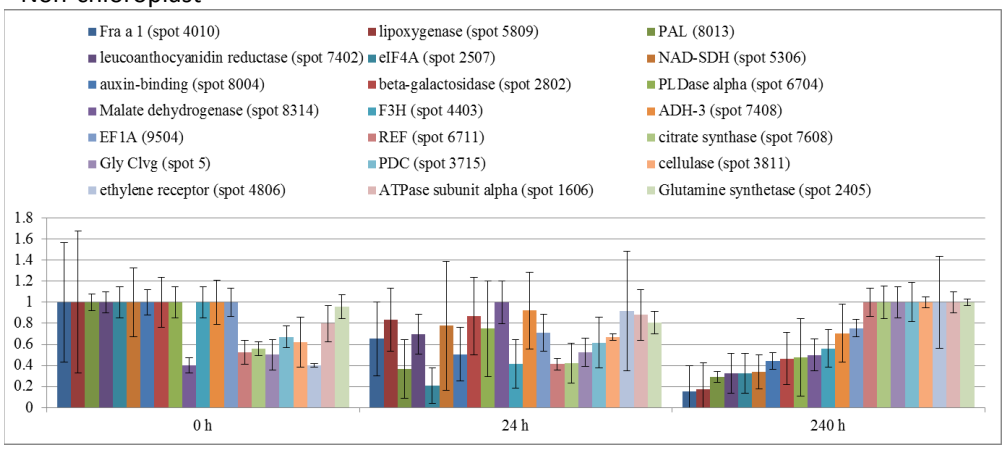


	Korena up-regulated		down-regulated	
A. GO Biological Process				
other cellular processes	**19.0**	18(9)	**20.3**	13(9)
other metabolic processes	**27.4**	26(13)	**31.3**	20(12)
unknown biological processes	**1.1**	1(1)	**1.6**	1(1)
protein metabolism	**4.2**	4(3)	**4.7**	3(3)
transport	**3.2**	3(2)	**0**	0
developmental processes	**3.2**	3(2)	**4.7**	3(2)
response to stress	**12.6**	12(6)	**12.5**	8(4)
response to abiotic / biotic stimulus	**15.8**	15(6)	**11.0**	7(5)
other biological processes	**7.4**	7(5)	**9.4**	6(4)
cell organization and biogenesis	**0**	0	**1.6**	1(1)
signal transduction	**1.1**	1(1)	**1.6**	1(1)
DNA or RNA metabolism	**0**	0	**0**	0
electron transport /energy pathw ays	**5.3**	5(4)	**1.6**	1(1)
B. GO Cellular Component				
unknown cellular components	**0.0**	0	**6.3**	3(3)
other intracellular components	**25.2**	31(9)	**16.7**	8(4)
other cytoplasmic components	**17.1**	21(9)	**10.4**	5(4)
other membranes	**10.6**	13(6)	**12.5**	6(4)
chloroplast	**21.1**	26(10)	**10.4**	5(3)
other cellular components	**0.8**	1(1)	**0**	0
nucleus	**0.8**	1(1)	**6.3**	3(3)
plastid	**15.5**	19(7)	**4.2**	2(1)
plasma membrane	**0**	0	**8.3**	4(4)
cytosol	**0.8**	1(1)	**10.4**	5(5)
mitochondria	**3.3**	4(4)	**4.2**	2(2)
ribosome	**0**	0	**0**	0
cell wall	**2.4**	3(3)	**6.3**	3(2)
extracellular	**1.6**	2(2)	**4.2**	2(1)
ER	**0.8**	1(1)	**0**	0
Golgi apparatus	**0**	0	**0**	0
C. GO Molecular Function				
unknown molecular functions	**0.0**	0	**0**	0
other binding	**27.8**	10(8)	**18.2**	4(3)
other enzyme activity	**19.4**	7(5)	**27.3**	6(6)
hydrolase activity	**11.1**	4(2)	**27.3**	6(4)
transferase activity	**8.3**	3(3)	**0**	0
DNA or RNA binding	**0**	0	**9.1**	2(2)
protein binding	**8.3**	3(3)	**13.6**	3(3)
kinase activity	**5.6**	2(2)	**0**	0
nucleotide binding	**8.3**	3(2)	**0**	0
transporter activity	**2.8**	1(1)	**0**	0
transcription factor activity	**0**	0	**0**	0
nucleic acid binding	**8.3**	0	**0**	0
other molecular functions	**5.6**	2(1)	**4.6**	1(1)
structural molecule activity	**0**	0	**0**	0
receptor binding or activity	**2.8**	1(1)	**0**	0

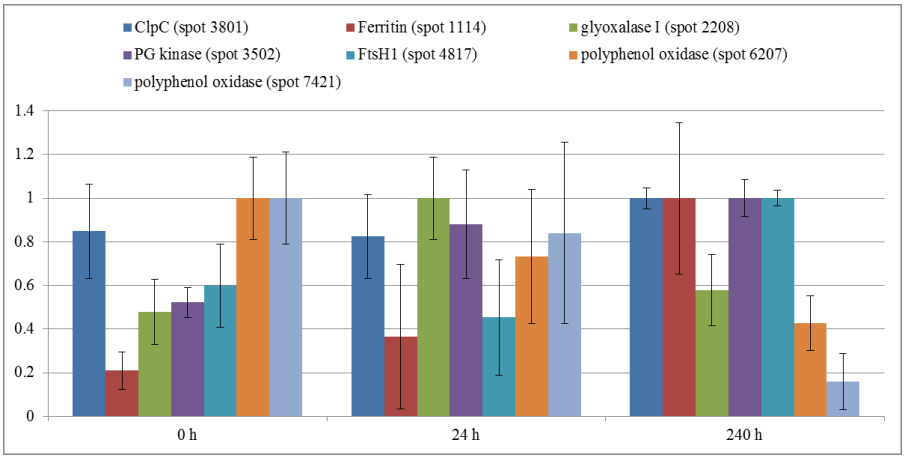
Percent annotation count (gene count)

Figure 3.4 Gene Ontology (GO) annotation for the differentially expressed proteins from 2DE analysis (homologous to Arabidopsis genes) in *F.* × *ananassa* 'Korona'. GO categories for Biological Process (A), Cellular Component (B), and Molecular Function (C) for the differentially expressed proteins that were up-regulated (14 spots) or down-regulated (14 spots) greater than 1.5 fold after treatment at 4 ºC at either 24 or 240h (listed in Table 3.1). Legend includes the percent (bold) next to number of annotations and number genes included within each category

Non-chloroplast



Chloroplast: Metabolic



Chloroplast: Photosynthetic

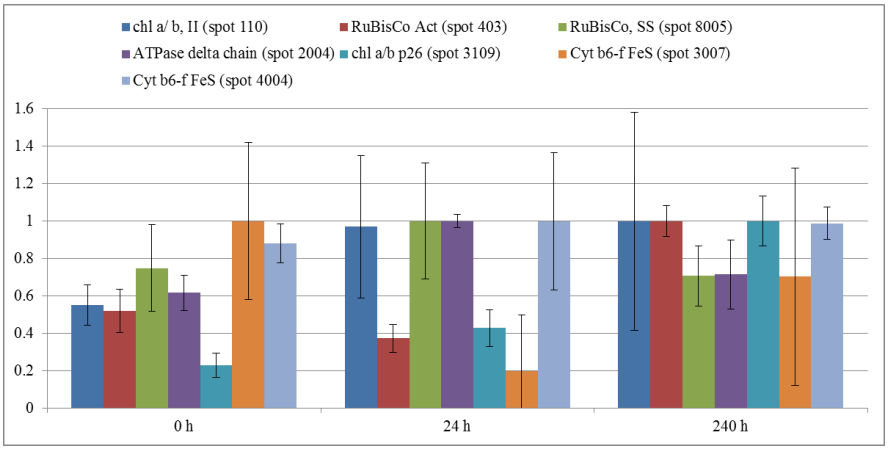


Figure 3.5 Data represent average values of 3 gels (3 replicate experiments) normalized to the greatest value, error bars indicate standard deviations. Diagrams were categorized into A. non-chloroplastic, B. chloroplastic metabolism, and C. photosynthesis-related chloroplast proteins. Abbreviation defined in Table 3.1.

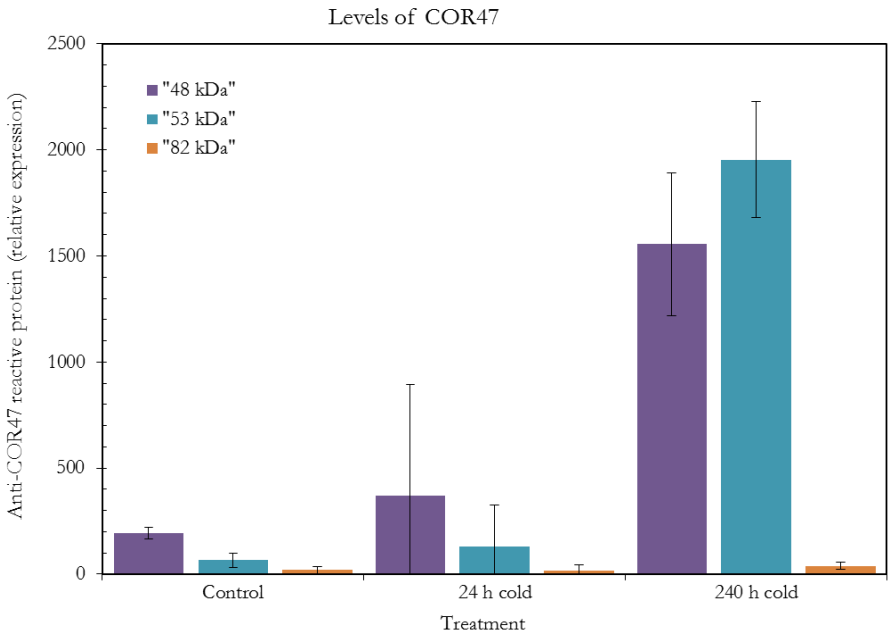


Figure 3.6 COR47-reactive bands in 'Korona' 1-DE western blot. COR47 protein levels increase with extended cold treatment. Western blots were probed with antibody raised against arabidopsis COR47. Two major bands were detected at 53 and 49 kDa, likely corresponding to the Fragaria COR47 homolog. The 53 kDa and the 49 kDa bands correspond to the phosphorylated and non-phosphorylated proteins respectively. The upper minor protein (82 kDa) is likely an often observed aggregate of COR47.

CHAPTER 4. SUMMARY

The subject of this dissertation is the overwintering tolerance in strawberry cultivars. From the proteomic analyses and preliminary microarray results, the differences in gene expression displayed by the cultivars, 'Jonsok', 'Senga Sengana', 'Elsanta', and 'Frida' suggest they have different strategies for overwintering, as interpreted from the short and long exposures to cold. This work presents a list of potential biomarkers, candidates for use in the development of cultivars with enhanced winter survival.

By comparing expression of proteins and transcripts in the crown tissue of octoploid strawberry from the less tolerant cultivar ('Frida') to one of greater tolerance ('Jonsok') we have noted several trends. First, 'Jonsok', appears poised for tolerating cold stress, as several known proteins related to freezing/cold tolerance are constitutively expressed at significantly greater levels than those found in 'Frida'. Although this poise has been observed in other species and to contributes to low temperature tolerance and other tolerances (Taji et al., 2004; Takahashi et al., 2006) the unique contribution distinguishing our work includes information that is relevant, and unreported before, for the strawberry cold tolerance. The array of proteins associated with low temperature stress tolerance is significantly more complex in 'Jonsok' than in 'Frida', including a large variety of proteins known to be associated with both abiotic and biotic stress tolerance. Secondly, the convergence of protein expression in the two cultivars, visualized by principle component analysis (PCA), which becomes readily apparent after 42 d, is largely due to 'Frida' "catching up" in terms of expression patterns to the more cold-tolerant cultivar. However, one should not ignore the observation that 'Frida' is a cold/freezing tolerant cultivar, just less so than 'Jonsok', and indeed appears to have adopted a very strong antioxidation response as evidenced by activation of the ascorbate pathway and phenylpropanoid pathway. Indeed these latter

approaches may represent an alternative, perhaps lesser, but nonetheless effective response to cold stress.

4.1. Summary of Results

(1) Protein was extracted from crown structures from plants exposed to cold (0, 2, 42 day at 2 ºC) with Tris-buffered Phenol followed by ammonia acetate/methanol precipitation (Appendix A) and resolved by 2D electrophoresis. Protein patterns (900 spots) from coomassie stained gels and were compared for four cultivars. The cultivars, 'Jonsok', 'Senga Sengana', Elsanta' and 'Frida' can be distinguished based on protein profiles. Identification was achieved for 110 spots by LC-MS/MS. There were 94 unique proteins based on sequence analysis out of the 110 identified proteins.

(2) 'Jonsok' and 'Frida' extracted proteins from (0, 2 days at 2 ºC) were submitted for Shotgun (LC-MS/MS) analysis. Based on the comparison of protein abundance displayed in 'Jonsok' and 'Frida', the number of significant differences between the cultivars were 326 (16%) at 0 d (control), and 216 (10%) at 2 d, out of the 2017 total ESTs reported (p-value < 0.05).

(3) Preliminary analysis of microarray (43000 probes) examination from the same crown structures exposed to cold (0, 2, 42 day at 2 ºC) supported protein experiment findings such as, that defense-related processes (Thaumatin, Fra a2, Chitinase etc) and proteins associated with detoxification (aldo-keto reductase, GST, ADH) were represented more strongly in 'Jonsok'.

(4) As part of collaboration (Dr. Jens Rohloff), 2DE protein expression was investigated in leaf during exposure to cold. Leaf proteomic 2DE results for 'Korona' appear different than crown. Proteins associated with cold tolerance have been shown to have tissue and organ specificity. Not uncommonly reported are the photosynthetic associated proteins showing cold response that were observed for 'Korona'. There were interesting findings with relevance to the 2DE protein expression profiles observed in crowns. Very similar spatial

patterns of 2DE protein spots exist between crown and in leaf (i.e. 2DE maps were overlaid). Even though the 2DE maps have very similar spatial patterns, the proteins that were altered in response to cold in crown were not significantly changed in leaf.

(5) Evaluation of dehydrins in strawberry show strawberry cultivars 'Jonsok' and 'Frida' accumulate dehydrins in response to cold. The 1D western blot analysis shows similar levels of dehydrin accumulation with slightly higher levels in 'Jonsok'. In a comprehensive study, the accumulation of dehydrins (and alcohol dehydrogenase) in diploid strawberry correlated to low temperature tolerance (Davik et al., submitted 2011).

Our data suggest the relative cold tolerance among cultivars may be attributed to some proteins with constitutive or base-line expression before cold exposure. Plants indigenous to cold climates or regions with frequent cold stress exposure do indeed exhibit a higher chilling tolerance compared to tropical plants. This brings into question what mechanisms are present before cold exposure that are attributing to higher tolerance. Based on cold-accumulated proteins and absolute levels (basal) of proteins represented in the most freezing tolerant cultivar 'Jonsok' compared to 'Frida' several of proteins appear multifunctional such as the pathogen-related proteins that have antifungal and/or antifreeze properties. It is likely that these proteins have contributions to overwintering that are inherent in their multifunctional properties. It will be interesting to see if other proteins have undiscovered properties that contribute to overwintering success.

CHAPTER 5. FUTURE WORK

Validate candidate markers: The identification of candidate proteins associated with freezing tolerance in Fragaria cultivars is an initial step towards integrating these potential molecular markers into a program for developing new cultivars with enhanced overwintering success. An important next step in this development of freezing tolerance bio markers is to validate them. Candidate molecular markers will be validated by evaluating how well they correlate with freezing tolerance in *F.* × *ananassa* crosses (e.g. 'Jonsok' × 'Frida', or 'Jonsok' × 'Elsanta'). Correlation between phenotype variations (freezing tolerance) with candidate proteins will provide strong evidence for their association in overwintering survival.

De-acclimation experiments: Reliable overwintering survival in temperate climates is dependent on the adaptive processes during cold acclimation, dormancy, and recovery during de-acclimation. Different molecular mechanisms that are associated with each process potentially account for the differences in overwintering hardiness displayed in plant species. De-acclimation is an important aspect of successful overwintering that emphasizes molecular mechanisms involved in recovery. This presents the opportunity to compare cultivars in the future to evaluate differences in molecular processes that may be contributing to reliable survival, such as those mechanisms related to antioxidant capacity.

Leaf and crown comparison: The potential to use leaf tissue instead of crown for accessing candidate markers for cold tolerance that were identified in 'Jonsok' will be evaluated. The ability to use leaf tissue instead of crowns to assay biomarkers has several benefits including saving valuable time. This endeavor will be facilitated with the use of antibodies that are reactive to candidate cold tolerance markers.

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Appendix A. Protein Extraction from Strawberry Crown Tissue and Protein Preparation for 2DE Proteomic Analysis

The following procedure for protein extraction was designed based on Hurkman and Tanaka 1986 protocol with modifications to optimize protein extraction from crown tissue for label free protein quantification protein methods, 2DE and shotgun.

SOLUTIONS:

1. Tris-Buffered Phenol (TBP), pH 8.8: Phenol with 0.1% w/v 8-Hydroxyquinoline: Equilibrate with equal volumes of 1.0 M Tris-HCl, then equilibrate with equal volumes of 0.1 M Tris-HCl, pH 8.8 twice. For 27 g tissue it is estimated that 270 mL equilibrated phenol will be needed.

2. Extraction Buffer (EBA). For 1.0 g of tissue, 10 mLs of extraction buffer is needed. Dissolve the following in 0.1 M Tris-HCl pH 8.8:

	10 mL	100 mL	(10g)	350 mL (~27g)
Sucrose 40%	4g	40g	140g	
SDS 2%	0.2g	2.0g	7g	

Immediately before use add to 94.0 mL of EBA:	Final conc:	100 mL
Complete Roche Protease Inhibitors (50X stock)	1X	2 mL
Phosphatase Inhibitor Cocktail	1X	2 tabs in 2 mL H2O
β-mercaptoethanol (100% stock)	2%	2 mL
microcysteine (0.3 mM stock)	3uM	10.0 ul

Note: Protease inhibitors were added immediately before processing samples

3. 0.1M ammonium acetate in 100% methanol (store at -20 ºC)

4. 80% acetone (store at -20 ºC)

5. Isoelectric focusing buffer (IEF)	Final conc:	20 mL
urea	8 M	9.62 g
thiourea	2 M	3.04 g
CHAPS	2% (w/v)	0.40 g
deionized Triton X-100 (10% stock)	2%	4.0 mL
pH 3-10 ampholytes (40% stock)	0.5%	0.625 mL
DTT	50 mM	155 mg

Stored at -80 ºC in 1.0 mL aliquots.

APPENDICES

6. Equilibration Buffer Base:	Final conc:	25 mL	360 mL
urea	6 M	9.0 g	129.6 g
1.5M Tris/HCl 8.8pH (filter Sterilized)	0.05 M	825 uL	11.88 mL
20% SDS filter sterilized	4%	5 mL	72 mL
50% glycerol	20%	10 mL	144 mL

Buffer I: 2% w/v DTT (3.6 g DTT in 180 mL Equilibration Buffer; enough for 12 strips). Use 5 mLs for each strip (equilibrate 5 min x 3).

Buffer II: 2.5% w/v Iodoacetamide (4.5 g in 180 mL Equilibration Buffer 180 mL for 12 strips). Use 5 mLs for each strip (equilibrate 3 min x 3).

7. Agarose (0.55%) in 1 X Electrode Buffer w/ Bromophenol Blue (0.275 g agarose for 50 mL 1x electrode buffer (or 0.825 g for 150 mLs).

PROCEDURE STEPS

1. Grind 1.0 g tissue in mortar and pestle with liquid nitrogen; adding 10% PVPP of tissue weight while grinding (~0.1 g).

2. Cold Acetone Wash. Transfer frozen sample into 30 mL glass corex centrifuge tube. Use cold acetone 10 mL to rinse tissue out from mortar and pestle. Vortex at setting #5. Spin 8000 rpm for 20 min at ~ 0 ºC; (SS-34; 7649 xg ave)

3. Discard supernatant and repeat step 2; use liquid nitrogen briefly to cool acetone.

4. Dry under vacuum with dry ice to remove acetone. Submit ~120 -150 µL for LC-MS/MS.

5. Transfer tissue to 50 mL Falcon tube. Add 5 mL of Extraction Buffer (EBA) and add 5 mL Phenol (TBP) per 1g tissue weight.

6. Use Polytron for 30 sec at #4 and then incubate with agitation for 30 min at 4 ºC.

7. Transfer to 30 mL glass corex centrifuge tube and spin at 7000 rpm for 15 min at 4 ºC; (SS-34; 5000 xg ave)

8. Remove upper Phenol phase with glass pasteur pipette into 30 mL glass corex tube.

9. Re- extraction: Add 5.0 mL of fresh phenol (TBP) to aqueous phase and transfer back to 50 mL falcon tube; Vortex then incubate with agitation for 30 min at (~8 ºC; spin as above). The phenol phase (upper) is retrieved and combined to with the first.

10. Back-extract combined phenol phases with equal volume of extraction buffer (EBA); transfer to 50 mL falcon tube; vortex, and then incubated with agitation for 30 min at ~8 ºC; spin as before. Weigh volume of resulting phenol phases.

11. Precipitate proteins overnight at -80 ºC by adding 5 volumes of 0.1 M ammonium acetate in 100% methanol. Recover pellet by spinning 7000 rpm for 15 min at 4 ºC; (SS-34, 5856 xg ave)

12. Wash pellet twice in 10 ml 0.1 M ammonium acetate in methanol, spinning as before. Resuspend pellet with careful vortexing and pipetting; place in -20 ºC in between spins for ~20 min.

13. Wash pellet twice in ice-cold 80% acetone, spinning as before. Store suspended pellet in last wash at -80 ºC until the other samples that are to be analyzed catch up; then proceed with the following after pelleting protein.

14. Resuspend pellet after air drying (~ 5 to 10 min) in ~600 µL IEF buffer.

15. Incubate samples for 30 min at room temperature with agitation.

16. Spin samples in ultracentrifuge tubes 49k rpm for 30 min (100000 xg). Aliquots are taken for protein assay (Amido Black method). The remainder is stored at -80 ºC until gel run.

17. Prepare samples (2.05 µg in 450 uL with IEF buffer). Rehydrate 220 µg per strip in apparatus at 20 ºC for ~14 hrs.

18. Focus (~20 hrs)

3 to 10NL; 24cm IEF strips; max 50 mAmps per strip

STEP	CONDITION	RAMP
1	100V, 300Vhr	rapid
2	300V, 900Vhr	rapid
3	5000V, 35000Vhr	rapid
4	8000, 53800Vhr	rapid

19. Equilibrate IEF strips; Run gel

20. Fix overnight and follow with staining and detaining protocols.

Appendix B. Permissions for Publications

7 September 2011

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Dear Gage

There is no problem about including your Acta Hort manuscript in your dissertation.

Sincerely
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VITA

VITA

GAGE KOEHLER

EDUCATION

Ph.D. in Biology, IUPUI, Indianapolis, IN. 2011. Advisor: Dr. Stephen Randall. Dissertation title: Overwintering Survival of Strawberry (*F.* × *ananassa*): Proteins Associated with Low Temperature Stress Tolerance during Cold Acclimation in Cultivars.
Research focus: Assisting in the identification and characterization of freezing related biomarkers for developing strawberry cultivars with enhanced winter survival.
Techniques: design and implementation of tissue extraction protocols, running; imaging and analyzing large 2DE format gels; and protein identification from LC-MS/MS 2DE and shotgun experiments.

M.S. in Biology, Purdue, Indianapolis, IN. 2005. Master's Thesis title: Characterization of Dehydrins in Arabidopsis thaliana. Thesis Summary: Subcellular location of dehydrin proteins was determined with sucrose density gradient analysis and western blotting. A global view was presented of protein changes (abundance and phosphorylation pattern) that occurred in plants responding to cold stress by applying 2D gel analysis. Advisor: Dr. Stephen Randall.

B.S. in Horticulture, Texas A&M University; College Station, TX. 1997

Radiation Safety Certificate, Indiana University Purdue University Indianapolis, IN. 2005

PUBLICATIONS

J. Davik, B. From, G. Koehler, T. Torp, J. Rohloff, P. Eidem, R. Wilson, A. Sønsteby, S.K. Randall and M. Alsheikh. Dehydrin, alcohol dehydrogenase, and central metabolite levels are associated with cold tolerance in diploid strawberry (Fragaria spp.). submitted to Planta

G. Koehler, S.K. Randall, P. Winge, J. Rohloff, R.C. Wilson, and M. Alsheikh (2010). Molecular cold responses for two strawberry cultivars: comparison of proteomic and microarray analysis. Acta Hort. 2010.

D. H. Huizinga, R. Denton, K. G. Koehler, A. Tomasello, L. Wood, S. E. Sen, and D. N. Crowell. (2010). Farnesylcysteine lyase is involved in negative regulation of abscisic acid signaling in Arabidopsis. Molecular Plant Vol 3 Pages143-155.

G. Koehler, T. J. Weisel, S. K. Randall. (2007). Transcript expression analysis indicates distinct roles for dehydrin subclasses. Current Topics in Phytochemistry. Vol 8 Pages73-83.

TEACHING EXPERIENCE

Teaching Assistant, Indiana University Purdue University, Indianapolis, IN. 2002 -2005
Chemistry Mentor, Indiana University Purdue University, Indianapolis, IN. 2001 -2002

WORKSHOPS / CONFERENCES

International Horticultural Congress (IHC):
2010*-Lisbon, Portugal *Identification of molecular markers associated with winter survival in the cultivated strawberry by integration of "omics" technologies*

American Society of Plant Biologists (ASPB):
2009*-Honolulu, Hawaii *Comparative Analysis of Protein and Transcripts Associated with Cold Response in Cultivated Strawberry*
2007*-Chicago, IL *Evaluating dehydrin expression in Arabidopsis thaliana*
2006*-Boston, MA *Characterizing the Kinase Responsible for Phosphorylating the Dehydrin Erd14*
2005*-Seattle, WA
2004*-Ohio State University Plant Molecular Biology and Biotechnology Symposium

Plant Phosphorylation Workshops:
2006-Pacific Grove, CA *Characterizing the Kinase Responsible for Phosphorylating the Dehydrin Erd14*
2005-Sanibel Island, FL *Comparison of dehydrins in Arabidopsis thaliana*

Indiana Academy of Science (IAS):
2011*-Indianapolis, IN *A Proteomic Study of Low Temperature Responses in Strawberry Cultivars (F. × ananassa Duchesne) that Differ in Cold Tolerance.*
2009*-Kokomo, IN *Comparative Analysis of Proteins in Two Strawberry Varieties that Differ in Freezing Tolerance.*
*Presented Posters

AWARDS

Elizabeth Steele Creveling Scholarship (2009)
Grants in Aid of Research, Sigma Xi (2004)

PUBLICATIONS

Current Topics in Phytochemistry
Vol. 8, 2007

Review

Transcript expression analysis indicates distinct roles for dehydrin subclasses

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ABSTRACT

Dehydrins are plant specific proteins, distinguished by the presence of a consensus sequence, known as the K-region or K-peptide. Expression patterns of dehydrins in *Arabidopsis thaliana* divide the dehydrins into two highly correlative groups; however, unique patterns of expression are found within these groups. Some dehydrins are more responsive during development than to abiotic stresses. The KS-type dehydrin transcripts are highly expressed but are unresponsive to various stresses. The KS-type dehydrins are not enriched in promoter cis-elements similar to the other dehydrins suggesting they may be regulated through distinct pathways from the rest of the dehydrin family. In general, the three most acidic dehydrins have the greatest transcript levels in vegetative tissues (both roots and aerial tissues) and levels sharply decline during seed development. RAB18 and the basic dehydrins are at highest levels in the seed. A finding of a concomitant pattern of expression of the seed dehydrins on one hand and the vegetative dehydrins on the other, suggest a unique coordinated role for dehydrins in seedling development. In general, the alteration of dehydrin proteins appears to be necessary during development, as well as, during abiotic stress. While the majority of focus in the past has centered on identifying the role of dehydrins in environmental stresses, it is worth considering what functions are being accomplished by some dehydrins during development that may increase in demand during abiotic stress.

KEYWORDS: dehydrin, Arabidopsis, expression, transcripts, microarray, abiotic stress, development

INTRODUCTION

Plants are elegantly equipped to monitor light quality, temperature, and water availability, and many other environmental stimuli to optimize their growth under a variety of environmental conditions. Despite these attributes, crop damage due to environmental stress is a significant constraint and economic cost to growers. Understanding mechanisms of stress tolerance are important for improving agriculture [1, 2, 3]. There are many cellular modifiers and metabolic alterations that contribute to the optimization of growth during stress conditions. Dehydrins have functions important for plant growth and abiotic stress tolerance [4].

Dehydrins defined: The sole criterion for defining dehydrins [5] is at least one K-segment, a conserved 15 amino acid, lysine rich sequence (EKKGIMDKIKEKLPG). Interestingly, this criterion delineates organisms that are predominately photosynthetic from non- photosynthetic; thus dehydrins are found only in photosynthetic organisms. Other conserved domains, often found in dehydrins, include a serine rich sequence referred to as the S-segment ($LHRSGS_{4\text{-}10}(E/D)_3$) and a Y-segment (DEYGNP). Common characteristics

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of dehydrins include being very hydrophilic, and remaining soluble after boiling. It has been suggested that dehydrins be grouped into classes based on their respective pIs [6], a practice we have adopted. Biochemical characteristics are shown to be different between the subclasses. The acidic dehydrins, Cor47, Erd10, and Erd14 exhibit phosphorylation dependent ion-binding. A neutral dehydrin, Rab18, is able to be phosphorylated but does not exhibit ion-binding capacity. Xero2, a basic dehydrin has neither ion-binding capacity nor the ability to be phosphorylated [7]. In Arabidopsis, the acidic dehydrins include At1g20440 (COR47), At1g20450 (ERD10), At1g76180 (ERD14), and At4g38410 (Q9T), the neutral dehydrins (pI 6.4-7.6) include At4g39130 (Q9S), At2g21490 (X91920), At1g54410 (smKS) and At5g66400 (RAB18); the basic dehydrins include At3g50980 (XERO1), At3g50970 (XERO2), and At1g59910 (lgKS).

Functional implications: Dehydrins can be one of the most prevalent proteins induced and accumulated in response to either cellular water-deficit stress in tolerant plants or with internal water deficit stress occurring with seed maturation. In general, dehydrins are thought to protect the cell by preserving the integrity of cell constituents or by buffering the cell from toxic levels of ions that accumulate during times of environmental stresses [8, 9]. Many studies have implicated biological roles for dehydrins. In vitro studies point to various protective roles such as cryoprotectants [10, 11, 12] or protection from desiccation damage [13]. Over expression of several dehydrins provides enhanced stress tolerance [6, 14, 15, 16, 17, 18].

There is a wealth of information regarding regulation, localization, and physio-chemical properties of dehydrins. While the understanding of molecular/biochemical functions of dehydrins is crucial to understanding the physiological role of dehydrins, this present analysis does not focus on this aspect. Rather the goal of this analysis is to extract information from the microarray experiments and other arabidopsis databases which may allow us to categorize not only the myriad biological roles of the dehydrins in arabidopsis but to elucidate novel physiological roles likely applicable both to arabidopsis and to other plant species.

METHODS FOR COMPUTATIONAL DATA ANALYSIS

Gene correlation data was computed using Genevestigator (https://www.genevestigator.ethz.ch/at/), The *Arabidopsis thaliana* microarray database and analysis toolbox [19]. The web-based Gene Correlator application was used to determine the magnitude of co-expression between two genes over the ATH1: 22k array, wild-type only chip, with all chip source parameters enabled. Varying combinations of gene identifier codes (At1g76180, At5g66400, At1g20440, At1g20450, At3g50980, At3g50970, At2g21490, At4g38410, At4g39130, At1g54410, and At1g59910) were inputted into the x-axis and y-axis form, and then a linear plot and the Pearson's correlation coefficient (i.e. the r^2 value) were computed. The corresponding r^2 values for each set of genes were then plotted together to compare the overall correlation in gene expression. For the microarray analysis, all data and complete description of experimental design and procedures are available at http://Arabidopsis.org/info/expression/ATGenExpress.jsp. Promoter motifs from dehydrin groups were screened using www.bioinformatics2.wsu.edu/Athena/ [20].

RESULTS OF ANALYSIS

General characteristics of the dehydrins: Phylogenetic analysis based upon protein sequences [7] shows that two major groups of Arabidopsis dehydrins are found. The Cor47, Erd10, Erd14, and Q9T protein sequences are most closely related to each other, while Xero1, Rab18, X91920, and Xero2 protein sequences are most closely related to each other. Much more distantly related are the remaining dehydrins that show little similarity to each other as a group. A comparison of the protein encoding regions is visualized in Figure 1. The gene arrangement of the protein coding region is two exons that are most often delimited by the S-segment (contributed by exonic sequences on both sides of the intron). The dehydrins can be considered to be composed of either acidic coding exons, basic coding exons or combinations of the two; yielding acidic, neutral, or basic dehydrins. Sequence homology based multiple alignments (e.g., ClustalW, not shown) confirm the conclusions drawn from the pI's of the exons. There is little homology between the basic

Figure 1. Coding regions of dehydrins from *Arabidopsis thaliana*. Dehydrin common name and chromosome location are indicated to the left of schematic. Calculated masses (kDa) and isoelectric point (pI) are shown. The pI's were estimated using the program located at http://ca.expasy.org/tools/pi_tool-doc.html. It should be noted that this program uses primarily pK values for the individual amino acids and some dehydrin pI estimates differ depending on which program is used. The Y-segment is designated as triangles. The S-segment is designated as open boxes. The K-segment is designated as circles. Exons with a pI ranging between 4 and 6 are hatched. Exons with a pI between 7 and 8 are dotted. Exons with a pI greater than 9 are designated in solid black. Introns are designated with lines. LgKS is not drawn to scale and is approximately three times larger than depicted above.

and acidic regions, though the K-segments can be found in either. With the exception of the lgKS, the first exons of all the dehydrins cluster together, while all the second exons of the dehydrins form a distinct cluster. The exon arrangement of lgKS is inverted, where its second exon clusters with all other exon 1's and it's first exon clusters with all exon 2's. The single exon of KS groups with exon 2 of all dehydrins. If one deletes the K regions from all the dehydrins and then performs the same alignment, essentially the same results are obtained except that the lgKS and KS form a distinct third cluster.

Overall correlation of expression in dehydrins: To determine an overall comparison of expression patterns in dehydrins, microarray experiments on the entire NASC information array were analyzed in a one-by-one comparison (Figure 2). COR47, ERD10 and ERD14 had over all high correlations of expression, and likewise Q9SVE4, X91920, RAB18, and XERO1 are highly correlated. The similarity in expression patterns mirrors the phylogenic relationships between these proteins. However, neither Q9T (an acidic dehydrin) nor the the KS-type dehydrins show a significant correlation in expression to any other dehydrin. This lack of correlation can be explained by Q9T's restricted expression in the roots and seed, and in the KS-type to constant expression in all tissues with neither being particularly responsive to abiotic stress (see Figure 4). Interestingly XERO2, a basic dehydrin shows greater correlation in expression to the acidic dehydrins, than to the other basic dehydrins.

Microarray patterns: responses to ABA and development

The developmental data set [21] was analyzed. The dehydrin transcripts that exhibit the greatest response to ABA in seedlings include COR47, ERD10, ERD14, RAB18, and XERO2 (Table 1). These are the most well characterized "core"

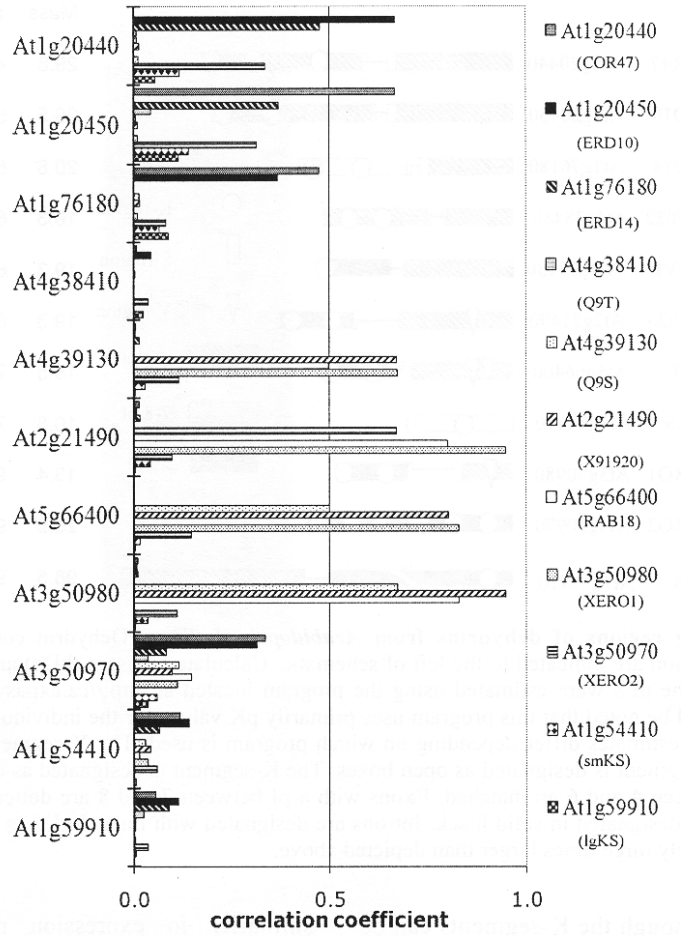

Figure 2. Correlation of expression in dehydrins. Pearson's correlation coefficient of mRNA levels were obtained using Genevestigator (https://www.genevestigator.ethz.ch/at/) *Arabidopsis thaliana* microarray database and analysis toolbox, probing the ATH1 (22K array) chip. Correlations were obtained by pairwise comparing each dehydrin. Correlations of expression for each dehydrin (on X-axis) are made to each individual dehydrin; correlations against themselves (=1.0) are not shown, instead a space is present in the figure. Vertical lines on the x-axis separate each set of correlations. The bars in each set are in the same order as the dehydrins along the x-axis. Both common names and gene identifiers are given.

dehydrins. These dehydrins also tend to exhibit relatively higher transcript levels during development (i.e., in seed imbibition and in young seedlings). The dehydrins, Q9S, X91920, XERO1, and Q9T do not exhibit an ABA response in seedlings like the 'core dehydrins' (Table 1). Alternatively, transcripts of Q9S, X91920, RAB18, XERO1, and XERO2 are strongly increased following ABA treatment of imbibed seeds, while COR47, ERD10, and ERD14 are not. Thus all dehydrins are strongly regulated during seed development and seed imbibition. The acidic dehydrins increase 10 to 100-fold during seed imbibition (Table 1). Q9S and X91920 increase during seed formation (100- and 1000-fold, respectively) and during stage 12 of flower development (Figure 3). XERO1 exhibits the greatest fold increase of all dehydrins (> 1000-fold) during the latter stages of seed development. Q9T, though transcripts are present at relatively low levels, is the only dehydrin that exhibits a transient increase (23 fold) during seed development, declining after stage 7. X91920, XERO1, Q9S,

Table 1. Dehydrin transcript responses to ABA. Changes in transcript levels of the dehydrins, in seeds following 24 hours of imbibition in the presence or absence of 3 uM ABA; and in seedlings following 3 hour treatment in the presence or absence of 10 uM ABA. Transcript change is the difference in levels in the presence of ABA minus that in the absence of ABA.

	Seeds				7 day old seedlings		
	Dry seed	24 hr Imbibed seeds (H_20)	24 hr Imbibed seeds (ABA)	Transcript change	3 hr (H_2O)	3 hr (ABA)	Transcript change
COR47	9	6720	5968	-752	15830	86505	70675
ERD10	12	162	133	-29	6695	64638	57943
ERD14	11	1063	1788	725	14605	68926	54321
Q9T	21	37	37	0	87	151	64
Q9S	9287	89	2742	2654	3	3	0
X91920	80148	13775	51791	38016	5	5	0
RAB18	51986	29724	38034	8310	265	45908	45643
smKS	685	10510	6791	-3719	40049	44287	4238
XERO1	53831	3923	13593	9669	3	4	1
XERO2	88950	1114	5015	3900	2506	92362	89857
1gKS	3321	2391	2308	-83	1538	2265	727

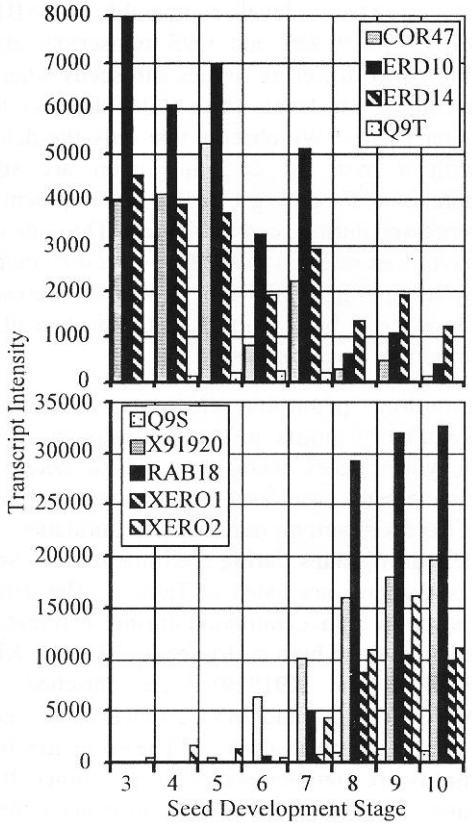

Figure 3. Changes in dehydrin levels during seed development. Left to right represents increased time of seed development, seed stages as follows. Siliques, w/ seeds stage 3; siliques, w/ seeds stage 4; siliques, w/ seeds stage 5; seeds w/o siliques, stage 6; seeds w/o siliques, stage 7; seeds w/o siliques, stage 8; seeds w/o siliques, stage 9; seeds w/o siliques, stage 10. Raw transcript levels are shown.

RAB18 and XERO2 accumulate to their highest levels during seed development. While the four most acidic dehydrins (COR47, ERD10, ERD14 and Q9T) are present during early stages (e.g., stage 7), they exhibit a sharp decline in the later stages of seed maturation (stages 8-10) (Figure 3). In contrast, during the first 24 hours of seed imbibition the dehydrin transcripts that are highest in the seed (in order of transcript expression value: RAB18, X91920, XERO2, XERO1, and Q9S) decline sharply, while the transcripts of COR47, ERD10, ERD14, and Q9T substantially increase (Table 1). The lgKS and smKS are expressed at relatively high levels regardless of developmental stage or abiotic stress and do not often vary greater than ~10 fold throughout development or abiotic stresses. The smKS exhibits its largest change in transcript intensity during seed imbibition increasing ~15 fold. In summary, during both seed development and seed imbibition the transcript profiles of X91920, XERO1, Q9S, RAB18, and XERO2 have a concomitant but opposite accumulation trends to the acidic dehydrins COR47, ERD10, ERD14 and Q9T (Figure 3, Table 1). This suggests the dehydrin subfamilies have distinct roles in seeds and in vegetative tissues.

Microarray patterns: abiotic stress responses: The abiotic stress set [22] was analyzed. The three most acidic dehydrins and the basic dehydrin, XERO2, have very similar expression patterns during abiotic stress (Figure 4, Panel A). XERO2 has the greatest increase in response to cold and osmotic stress. The three DREB1A/CBF binding motifs present in XERO2 promoter (the greatest amount of the dehydrins) perhaps contribute to this responsiveness (Table 2). While this similarity in expression patterns of XERO2 and the acidic dehydrins in response to abiotic stresses are clear; the expression of XERO2 in comparison with the acidic dehydrins during seed maturation could not be more dissimilar (Figure 3). In aerial tissues, Q9T is unresponsive to abiotic stress yet in roots mimics the other acidic dehydrins response to abiotic stress. RAB18 and it's closest homologs (XERO1 and X91920) are shown in Figure 4B. RAB18 and XERO1 responses are essentially restricted to osmotic and salt in both aerial portions and in roots. X91920 responds only slightly to osmotic stress in root tissues, but has a greater response in aerial tissues. The dehydrins with the least response to abiotic stress are shown in Figure 5C. This includes both KS-type dehydrins and Q9S. Of all the dehydrins the smKS has the highest transcript levels in all tissues yet is minimally responsive to stress. It is interesting that the smKS modulation in transcript levels is generally in the opposite direction of the lgKS. Q9S transcripts are not modulated at all during abiotic stress but accumulate to high levels during seed development/maturation.

Microarray patterns: anatomical distribution: In general the acidic dehydrins are present in a variety of vegetative tissues, with the lowest levels of transcripts appearing in shoot apices (Figure 5). Likewise, XERO2 and RAB18 are also at their lowest transcript level in apices. Q9T appears to be fairly restricted in its expression to roots and to early stages of seed development. In seeds, XERO1 and X91920 transcripts accumulate greater than 1000-fold relative to vegetative tissues, reaching levels comparable to RAB18 and XERO2. X91920 and Q9S transcripts are also limited to flowering tissues and seeds where they accumulate to levels 10 to 100- fold more than in other tissues. We observe that only the dehydrins with a basic pI encoding exon are strongly upregulated during seed development and repressed during seed imbibition. These dehydrins have been referred to as "seed-specific" dehydrins by Illing *et al.*, 2005 [23]. It will be interesting to know if the basic portion of the protein plays an important function in seeds.

Common promoter elements in dehydrins: Regulatory motifs in the 5' upstream region of dehydrin genes were analyzed to determine if cis-element promoter motifs could explain the differences among transcript accumulation among dehydrin groups during seed maturation. Selected cis-elements are listed in Table 2. The dehydrins that show an accumulation during seed maturation and remained high in dry seeds (XERO1, XERO2, RAB18, and X91920) were enriched in the MYCATERD1 and MYC2ATRD22 (cis-element enrichment, p-value 10e-3). The sequences of these motifs are complementary to each other. It is not known if these motifs function exactly the same

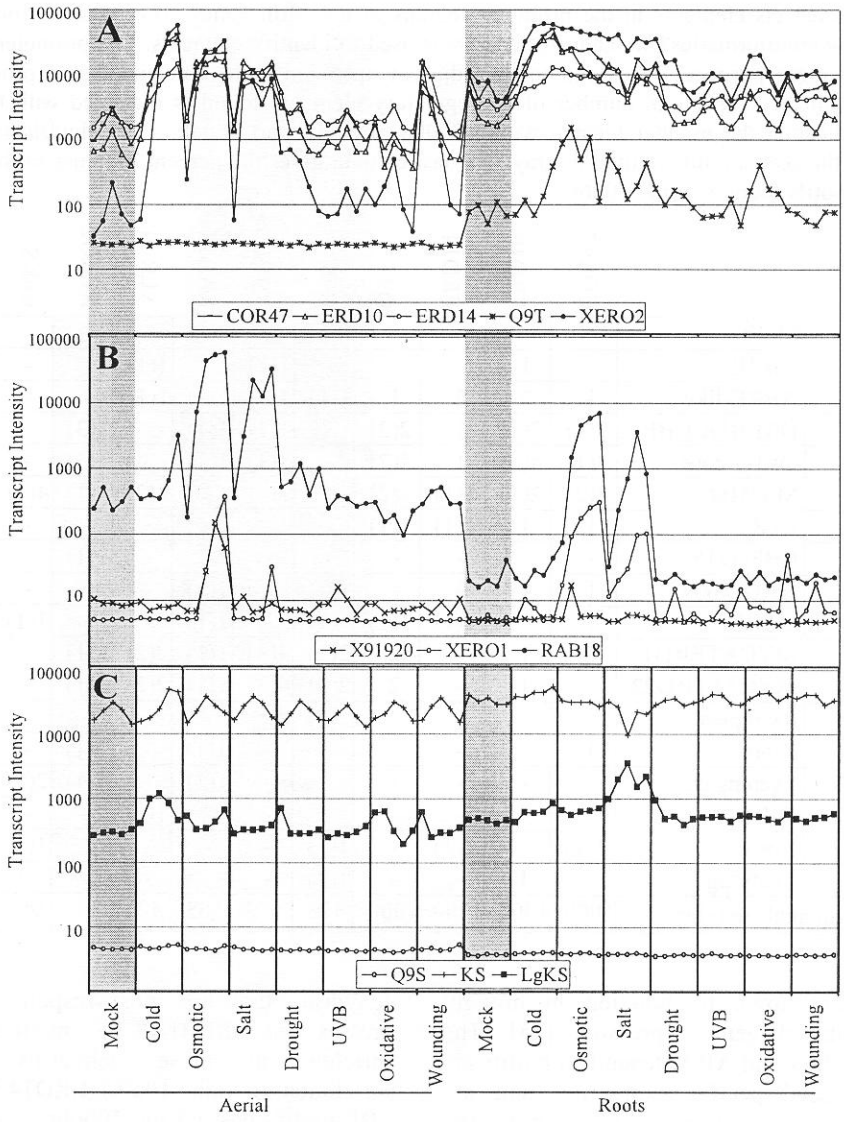

Figure 4. Comparing dehydrin transcripts during abiotic stress. Transcript intensity from the aerial portions and roots are separated on the panels, left and right respectively. The Y-axis indicates transcript values expressed logarithmically. The X-axis indicates the abiotic stress condition. Within each condition 5 time points corresponding to 1, 3, 6, 12 and 24 hr are indicated. The experimental conditions were Mock (controls, highlighted in gray), cold stress (4 C), osmotic stress (300 mM mannitol), salt stress (150 mM NaCl), drought stress (10% loss of fresh plant weight), oxidative stress (10 uM methyl viologen), UV-B stress, and wound stress (puncture of leaves by needles).

so they are listed separately. The MYCATERD1 motif is a recognition sequence in ERD1 (from -466 to -461) and necessary for the induction of expression during dehydration stress [24]. The dehydrins with this motif (within 500bp of the ATG) show the strongest accumulation during seed maturation. Erd1 (At5g51070) also accumulates transcripts in seed although not to the same degree as dehydrins. The MYCATRD22 motif is part of the promoter cis-acting elements present in RD22

Table 2. Predicted cis elements in the promoter regions of dehydrin genes. The Athena promoter analysis program (www.bioinformatics2.wsu.edu/Athena/) was used to identify elements. The promoter regions were considered for these purposes to extend to the adjacent upstream gene. The lengths of promoter regions analyzed are indicated. The total number of cis-regulatory elements found is indicated with the number in parenthesis indicating the number located within 500bp of ATG start codon. The dehydrin transcripts that accumulate in the seed are highlighted in gray. The last column gives the percent of genes in the Arabidopsis genome with motifs found within 1000bp.

		COR47	ERD10	ERD14	Q9T	Q9S	X91920	RAB18	XERO1	XERO2	smKS	LGkS	Genome
Sequence	Motif												
YACGTGC	ABRE	-	1	-	-	-	1(1)	-	1(1)	-	-	-	4%
BACGTGKM	ABRE-like	1	3	2	1	1(1)	1(1)	1(1)	1(1)	-	-	1(1)	20%
RCCGACNT	DREB1A/CBF	2(1)	2(1)	1	2(2)	-	1(1)	1(1)	-	3(3)	-	-	7%
RCCGAC	DRE - core	3(1)	4(3)	1	5(2)	-	1(1)	1(1)	-	3(3)	-	-	23%
WAACCA	MYB1at	2(2)	2(1)	3(1)	2(2)	-	3(3)	-	2(2)	1(1)	4(1)	3(1)	85%
ACCGACA	LTRE	1	1	1(1)	1(1)	-	-	-	-	-	-	-	5%
TGGCCGAC	CBFcor15	-	-	-	-	-	-	-	-	1(1)	-	-	0.3%
ACGTGTC	GADOWN	1	1	3	-	1(1)	-	1(1)	-	-	-	1(1)	8%
TAACAAR	GARE	1	-	1(1)	1(1)	1(1)	-	1(1)	-	-	1(1)	1	55%
CATGTG	MYCATERD1	1	1	-	2	2(2)	4(4)	1(1)	2(2)	1(1)	-	1	35%
CACATG	MYC2ATRD22	-	1	-	2	2(2)	4(4)	1(1)	2(2)	1(1)	-	1	35%
CATGCATG	Ry repeat	-	-	-	-	2(2)	-	-	-	-	-	-	3%
ATACGTGT	Z-box	1	-	-	-	-	-	1(1)	-	1(1)	-	-	2%
AAAATATCT	Evening element	-	-	-	-	-	-	1(1)	-	1(1)	2(2)	-	6%
MCACGTGGC	G-BOX	-	-	-	-	-	-	-	1(1)	-	-	-	2%
GATAAG	I-box	1(1)	-	2(1)	-	1(1)	-	-	-	-	1(1)	2(1)	40%
CCAATGT	Leafytag	-	1	-	-	1(1)	-	-	-	-	1(1)	1(1)	10%
Promoter length analyzed (bp):		1000	1000	1000	1000	478	516	563	421	392	952	1000	

that has been shown to function in drought and ABA-induced gene expression [25]. The RY-repeat, G-Box and ABA dependent motifs are common to seed-specific expression and are present within this select group of dehydrins. The ABRE element is a prominent upstream element in the dry seed transcriptome and common in other genes that exhibit a strong repression during imbibition [26]. The GADOWN motif (within -500bp, as described in Ogawa *et al.* 2003 [27] is present in the "seed" dehydrins Q9S and in RAB18. The presence of this promoter in the acidic dehydrin is at a significant distance from the transcription start site (~800 to 900bp). The "seed" dehydrins have relatively smaller promoter regions than the vegetative dehydrins examined. Promoter elements responsible for abiotic stress induction are known and were present for the dehydrins that are most responsive to abiotic stresses. The DREB1A/CBF3 motif is significantly enriched in these dehydrins (cis-element enrichment, p-value 10e-6). ERD14 has additional CBF motifs beyond the 1000bp promoter region. Also notable was the presence of ABA dependent (ABRE-like) and ABA-independent motifs (LTRE, and CBFcor15).

While the focus of this review has been the transcript accumulation of certain dehydrins; it is important to note evidence indicating that protein levels of these dehydrins are not always consistent with transcript levels. For example, the Rab18 protein is the predominant dehydrin protein accumulated in arabidopsis seed [28]; Koehler and Randall, *data not shown*) consistent with its high transcription levels, yet the X91920, Xero1, and Xero2 proteins are underrepresented or entirely

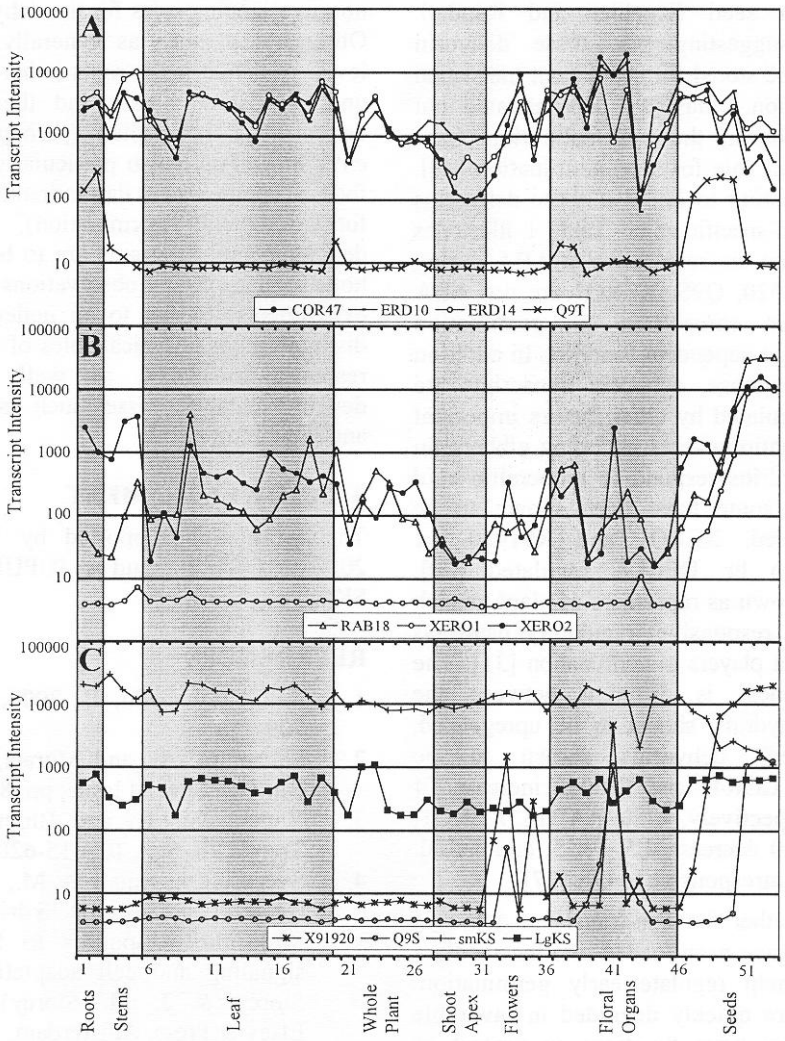

Figure 5. Anatomical distribution of dehydrin transcripts. Transcript intensity of the dehydrins in various tissues. Dehydrins were grouped in different panels (A-C) to demonstrate similar distribution and to best show individual variation. **1)** roots, 7 d. **2)** roots, 17 d. **3)** stem, hypocotyls 7 d. **4)** stem, 1st node 21+ d. **5)** stem, 2nd internode, 21+ d. **6)** leaf, cotyledons 7 d. **7)** leaf, leaves 1 + 2, 7 d. **8)** rosette leaf #4, 1 cm long, 10 d. **9)** rosette leaf # 2, 17 d. **10)** rosette leaf # 4, 17 d. **11)** rosette leaf # 6, 17 d. **12)** rosette leaf # 8, 17 d. **13)** rosette leaf # 10, 17 d. **14)** rosette leaf # 12, 17 d. **15)** leaf 7, petiole, 17 d. **16)** leaf 7, proximal half, 17 d. **17)** leaf 7, distal half, 17 d. **18)** leaf, 15 d. **19)** senescing leaves, 35 d. **20)** cauline leaves, 21+ d. **21)** seedling, green parts,7 d. **22)** seedling, green parts, 8 d. **23)** seedling, green parts, 21 d. **24)** vegetative rosette, 7 d. **25)** vegetative rosette, 14 d. **26)** vegetative rosette 21 d. **27)** shoot apex, vegetative + young leaves 7 d. **28)** shoot apex, vegetative,7 d. **29)** shoot apex, transition (before bolting) 14 d. **30)** shoot apex, inflorescence (after bolting), 21 d. **31)** flowers stage 9, 21+ d. **32)** flowers stage 10/11, 21+ d. **33)** flowers stage 12, 21+ d. **34)** flowers stage 15, 21+ d. **35)** flower 28 d. **36)** flowers stage 15, pedicels21+ d. **37)** flowers stage 12, sepals, 21+ d. **38)** flowers stage 15, sepals. **39)** flowers stage 12, petals. **40)** flowers stage 15, petals. **41)** flowers stage 12, stamens. **42)** flowers stage 15, stamen. **43)** mature pollen. **44)** flowers stage 12, carpels. **45)** flowers stage 15, carpels **46)** siliques, w/ seeds stage 3; mid globular to early heart embryos. **47)** siliques, w/ seeds stage 4; early to late heart embryos. **48)** siliques, w/ seeds stage 5; late heart to mid torpedo embryos. **49)** seeds, stage 6, w/o siliques; mid to late torpedo embryos. **50)** seeds, stage 7, w/o siliques; late torpedo to early walking-stick embryos. **51)** seeds, stage 8, w/o siliques; walking-stick to early curled cotyledons embryos. **52)** seeds, stage 9, w/o siliques; curled cotyledons to early green cotyledons embryos. **53)** seeds, stage 10, w/o siliques; green cotyledons embryos

undetectable in seed (Koehler and Randall, unpublished), suggesting that these dehydrin transcripts may be stored for subsequent translation during germination. It has been demonstrated that mRNAs stored during the seed maturation phase play an important role for seed germination [29]. It will be interesting to know if these dehydrins have germination-specific roles. Table 1 illustrates the dehydrins that are responsive to ABA during imbibition, X91920, Q9S, XERO1 are not ABA responsive after completion of germination suggesting a stage dependent function. In addition to the ABA response, dehydrin transcripts are shown to be regulated by other factors important for early germination events including gibberellin and light. Dehydrins respond to gibberellin acid differently and some have been shown to be DELLA regulated. XERO1 and X91920 are demonstrated to be DELLA regulated [30]. DELLAs are known as repressors of plant growth (gibberelic acid responsive) regulatory pathways and are important players in germination [31]. The GA responsiveness is different between the "vegetative" dehydrins, shown to be upregulated, and the "seed" dehydrins shown to be downregulated. COR47 and ERD10 increase 14 and 20-fold, respectively, while RAB18, XERO1, XERO2, X91920 decrease 4.5, 13, 5, and 4-fold. The smKS was unresponsive to GA [27].

We wonder whether the non-translated dehydrin transcripts that have been loaded into the seed are positioned to help regulate early germination. Whether they are quickly degraded in favorable conditions and/or have functions to protect or regulate germinating seeds is yet to be determined. In addition, a finding of inverse trends in transcript accumulation of the seed dehydrins on one hand and the vegetative dehydrins on the other, suggest a unique coordinated role for dehydrins in seedling development.

CONCLUSION

Though considerable variations in dehydrin expression patterns are observed, certain trends become apparent. The distinctive and opposite patterns of transcript accumulation that occurs in the acidic versus the basic dehydrins during various stages of seed development and seed germination are intriguing. This pattern suggests unique and non-overlapping roles for the dehydrin subgroups. Other trends, such as generally high transcript levels of dehydrins in roots (where tissues may be under constant stress) and the low levels of dehydrins in shoot apices (perhaps a less-stressed environment) are also particularly interesting. We focused on microarray data currently available (seed formation and germination), yet numerous developmental programs are to be explored. It is hoped that these observations may provoke experiments leading to an understanding of the distinctive physiological roles of the dehydrins in response to stress, as well as, in crucial developmental processes such as seed formation and seed germination.

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Molecular Cold Responses for Two Strawberry Cultivars: Comparison of proteomic and microarray analysis

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Abstract

A crucial consideration for strawberry producers in Norway and other northern countries is winter freezing damage. A long-term goal of the Norwegian strawberry breeding is to increase winter hardiness and to improve fruit quality. Due to the complexity involved in regulating and enhancing freezing tolerance, the progress in the improvement of cultivars using traditional screening methods have had limited success. Thus, the development of molecular markers for freezing hardiness would facilitate the selection work for this trait. We have developed and adopted state-of-art molecular tools to investigate cold response in strawberry plants during the acclimation phase resulting in the identification of a large number of genes, proteins, and distinct metabolites that correspond to cold/freezing tolerance in strawberry. To identify proteins responsible for freezing tolerance in strawberry we have examined alterations in protein levels in strawberry varieties that differ in cold tolerance following a 2 day cold exposure, using a shotgun LC-MS/MS approach and a microarray approach. Proteomic analysis suggested potential biomarkers that showed significant changes in the cultivated strawberry in early responses to cold. While it was difficult to directly correlate the identified protein with their corresponding transcript, by factoring transcript abundances, one could address whether any transcript change could account for changes in protein levels. In many cases a plausible correlation could be established. The knowledge attained from these endeavors is expected to expedite breeding of strawberries to achieve freezing tolerant lines and provide an integrative understanding of the molecular pathways that underlie this characteristic.

INTRODUCTION

Environmental stresses, including low temperature and frost, can be a major agricultural problem, resulting in catastrophic economic and productivity losses. Low temperature injury (i.e., freezing damage) is one of the primary factors that most influence strawberry production in Norway (Nestby and Bjørgum, 1999). It has been estimated that winter damage, on average, can cause

strawberry growers a yield loss of at least 20 percent (i.e., 40 mill NKr annually at the national level) (Davik *et al*., 2000). Consequentially, one of the major objectives of the Norwegian strawberry breeding programs is to generate cultivars that can withstand extreme, irregular, and harsh winter conditions; while maintaining or improving desirable traits (e.g., disease resistance, fruit quality, etc.) and therefore, improve yield and add to the profitability to the growers.

Cold hardiness of plants is a very complex phenomenon. In general, plants vary dramatically in their ability to withstand freezing temperatures and plants respond and adapt in various ways through a series of physiological, biochemical and molecular changes (for review; Shinozaki *et al*., 2003). The development of molecular markers that can assist conventional breeding techniques for winter hardiness would dramatically facilitate the selection for this trait and secure that each selection cycle is subjected to the appropriate pressure. One goal of our work is to develop molecular markers that can be used as tools that will allow identification of plants possessing the low temperature survival trait. With such markers in hand breeders will be able to select resistant genotypes more effectively. Due to the molecular, physiological, and genetic complexities of cold tolerance mechanism in plants; we have adopted two distinct but closely related approaches to identify molecular markers associated with cold tolerance in strawberry. These approaches will identify both protein and RNA markers. To begin to evaluate molecular markers, we have chosen to examine two cultivars, "Jonsok" and "Frida", which in preliminary studies have been shown to be particularly hardy and relatively cold-intolerant, respectively.

For the proteomic approach shown here we have utilized a quantitative, high through-put method developed by Higgs et al. (2005). We have focused on two days after initiation of cold treatment and compared expression levels with the 0 time controls. While changes in transcript, and particularly protein, can be relatively small after this short time, sufficient replication can allow significance at 20% changes. Thus this approach was used to address early response to cold both as a means to understand cold stress tolerance in the two cultivars, but also to support the results obtained in 2DE experiments (not shown) where longer term responses were measured. With a goal to understand regulation of protein accumulation we measured transcripts levels by employing a microarray approach based upon genomic and transcript sequences from Fragaria vesca.

MATERIALS AND METHODS

Fragaria x ananassa from greenhouse cultivation (20 C) were cold treated at 2 C (90 μmol m-2 s-1), for 0 or 48 h. Crown tissues of Fragaria × ananassa cultivars "Jonsok" and "Frida" were collected divided in half and immediately frozen in liquid nitrogen and stored at -80 C until use. Replicates were created by combining 6 half-crowns for the proteomic experiments and the corresponding other half of the crowns were used for RT-PCR and microarray experiments.

Tissue was ground to a fine powder in liquid nitrogen and then phenol extracted in presence of protease and phosphatase inhibitors and then precipitated with methanolic ammonium acetate. Pellets were dissolved in isoelectric focusing buffer. To normalize the protein loads, an amido black assay (Kaplan and Pederson 1985) was used to determine concentration of protein.

For shotgun analysis and identification of peptides (0 and 2 day exposure to 2 C) five replicates were utilized. These experiments were conducted and analyzed essentially as described in Higgs et al, 2005, with assistance of Monarch Life Sciences (now The Protein Analysis and Research Center-Indiana University), Indianapolis, IN. The peptide samples were analyzed using a Xbridge C18 2.5um (2.1mm x 5cm) column coupled to a Thermo-Finnigan linear ion-trap (LTQ) mass spectrometer coupled with a Surveyor autosampler and MS HPLC system (Thermo-Finnigan). The acquired data were searched against NCBI protein sequence database of Fragaria x ananassa (downloaded on 12 February 2009 from http://www.ncbi.nlm.nih.gov/, 574 entries) and Rosaceae (downloaded on 12 February 2009 from http://www.ncbi.nlm.nih.gov/, 8,926 entries) using SEQUEST (v. 28 rev. 12) algorithms in Bioworks (v. 3.3). The Arabidopsis homologs were retrieved by using the protein sequence of the gi from TAIR WU-Blast 2.0 (database TAIR9 Proteins). In the instances when the gi was a nucleotide sequence it was first translated to amino acid sequence. The

top hit and/or most prevalent identifiers resulting from BLASTP were cross-examined for the top ranked gi resulting from the blast on TAIR.

A customized *Fragaria* microarray chip was developed as a joint collaboration between Graminor Breeding Ltd. and NTNU. In total, 43723 unique 60 mer probes were designed and the Agilent eARRAY tool was used to produce a 4×44k format microarray chip. In addition to the available NCBI sequences, *Fragaria* cDNA sequences were provided by the Strawberry Genome Sequencing Consortium and The Center for Genomics and Bioinformatics (Indiana University).

RESULTS AND DISCUSSION

To detect statistically significant changes in protein expression after 2 d cold treatments, a highly quantitative proteomic method (Higgs et al, 2005) was applied. It is important to note that the shotgun approach is better able to reflect the overall abundance of a protein as post-translation modifications are less likely to impact protein identification. Each biological replication (see methods) was injected twice and the two technical replicate intensity values were averaged. This approach identified peptides corresponding to 2017 distinct ESTs or protein sequences (gene identifiers, in NCBI). Five-hundred sixty-eight of the identifications were of the highest quality (indicating a peptide ID confidence value >90% with multiple sequences identified). Of these, 29 (14 distinct proteins) were found to vary significantly based on a combined technical and biological Coefficient of Variation (CV) and by applying an ANOVA statistical model for each protein (data not shown).

When overall fold differences of protein and transcripts with respect to "Frida" and "Jonsok" ratios were examined, slopes of +0.21 and -0.44 were obtained at control and 48 h cold treatment, respectively (Figure 1). This suggests, particularly after 48 h, that the increased differences in protein levels between "Frida" and "Jonsok" were related to decreases in transcripts; suggesting translational or post-translation regulation may distinguish the two cultivars. In contrast, when comparing the cold responses for proteins and transcript in "Frida" to "Jonsok" (Figure 2), slopes of 1.13 were obtained for both cultivars; indicating a similar magnitude of protein changes and changes in transcript levels. Thus in terms of cold responses, changes in relative levels of proteins examined in "Frida" and "Jonsok" can be largely explained by changes in transcripts.

While in general transcript levels and changes reflected well the changes and levels of proteins, in some cases, where multiple transcripts were probed, some transcript responses did not reflect the changes found in protein levels (e.g., ubiquinol-cyt c reductase, cysteine protease, zinc ring finger protein, allene oxide cyclase) suggesting isoform-specific regulation or translational/post-translational regulation.

Several proteins had levels significantly different under control conditions in the two cultivars with chalcone synthase, peroxiredoxin, cysteine protease, DNA 3-methyladenine glycosylase, mitochondrial carrier protein, and a zinc ring finger protein being significantly higher in "Frida"; while alcohol dehydrogenase (ADH) and ripening induced protein (not shown) were higher in "Jonsok". Cold induction was not observed for ADH in either variety but an average maximum 1.3 fold of "Jonsok" over "Frida" was maintained. In "Frida" the mitochondrial carrier protein and ubiquinol-cytochrome c reductase were strongly cold-induced. In both "Jonsok" and "Frida" allene oxide cyclase and a cysteine protease were strongly cold-induced. In terms of cold induced proteins, allene oxide cyclase ranked highest (at 2 day) with a maximum fold increase of 1.47 fold in "Frida" and 1.28 in "Jonsok". In "Jonsok" three distinct SAM synthetase proteins were similarly cold-induced, although the enzyme was significantly less abundant than in "Frida" at both 0 d and 2 d. In both "Frida" and "Jonsok" levels of an aluminum-induced protein (AILP1) was reduced in response to cold.

It was interesting that of the 29 identified peptides that were significantly different between "Jonsok" and "Frida", 6 of them corresponded to the flavonoid pathway enzyme CHS (Chalcone synthase), and all were higher in "Frida" at 2 d than "Jonsok" with all CHS isoforms having a strong cold induction at 2 d. All 6 chalcone synthase peptides exhibited a 1.2 fold increase in "Frida" in

response to cold (0 to 2 day) and exhibited an average 1.4-fold abundance over "Jonsok" at 2 d. Flavonone 3-hydroxylase was also observed to be more abundant in "Frida" at 2 d (1.3 fold, not shown).

Significant changes were also observed in the microarray evaluation, with the transcript levels and changes often corresponding to the protein function. Where one or only several proteins were identified by LC-MS/MS, often multiple transcripts correlating to the same function were probed. In general, the predominant transcript (assuming quantitative relation between hybridization signals) was in agreement with the protein response. For example, SAM synthase, UBCRX, mitochondrial carrier protein, Ring finger, MLP-like, AILP1, and ADH proteins all had changes in protein and transcript that correlated well. The F3H protein and transcript were similarly up regulated in Frida; but there was a discrepancy in the transcripts and protein responses in "Jonsok", suggesting translational or post-translational regulation of protein in response to cold. The cold induced accumulation of F3H transcript in "Frida" was confirmed by qRT-PCR (approximately 2-fold increase in transcript). A similar phenomenon appeared to be effective for chalcone isomerase in "Jonsok".

CONCLUSIONS

The results emphasize the difficulty of determining which transcripts are responsible for changes in protein levels and further supports the necessity for analysis and development of proteomic molecular markers for cold tolerance. The lack of correlation between some protein and transcript levels is especially well illustrated by the CHS data (Fig. 3); where "Jonsok" showed high levels of transcripts (all 5), but low protein levels and "Frida" had high levels of protein, but low transcript levels. Most of the small, but statistically significant, changes in proteins we observed after two days of cold treatment are supported by 2DE analysis for longer cold treatments (data not shown), where much greater responses were seen. Constitutive levels of several proteins as well as their response to cold treatment distinguished the two cultivars, thus have potential for biomarkers.

ACKNOWLEDGEMENTS

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Figures

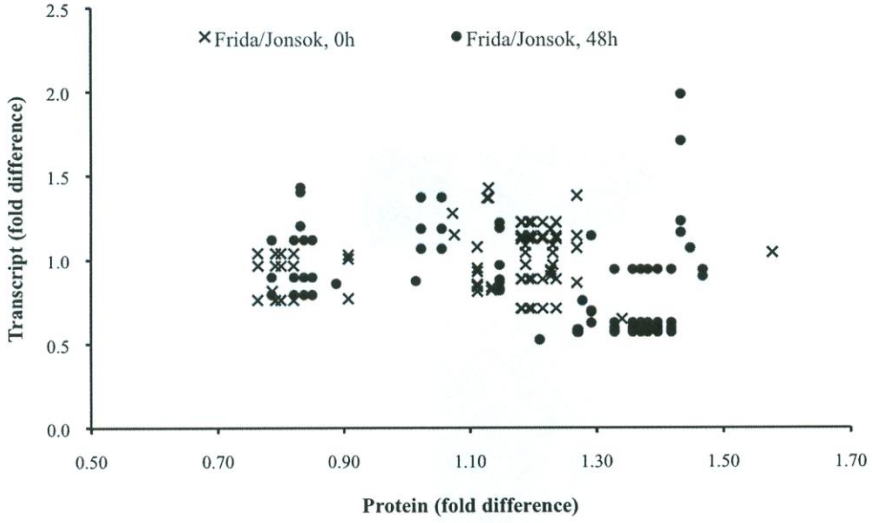


Figure 1. Comparison of changes in protein and transcript levels in cultivars "Frida" and "Jonsok". All values from Figs 3 & 4 were used except those changes greater than 13 fold (6 data points). Fold difference of 1 indicates that "Jonsok" and "Frida" have equal levels of protein or transcript, greater than 1 indicates higher levels in "Frida" than "Jonsok", less than 1 indicates lower amount in "Frida" than "Jonsok". Slope for 0 time linear fit was +0.21, for 48 h it was -0.41.

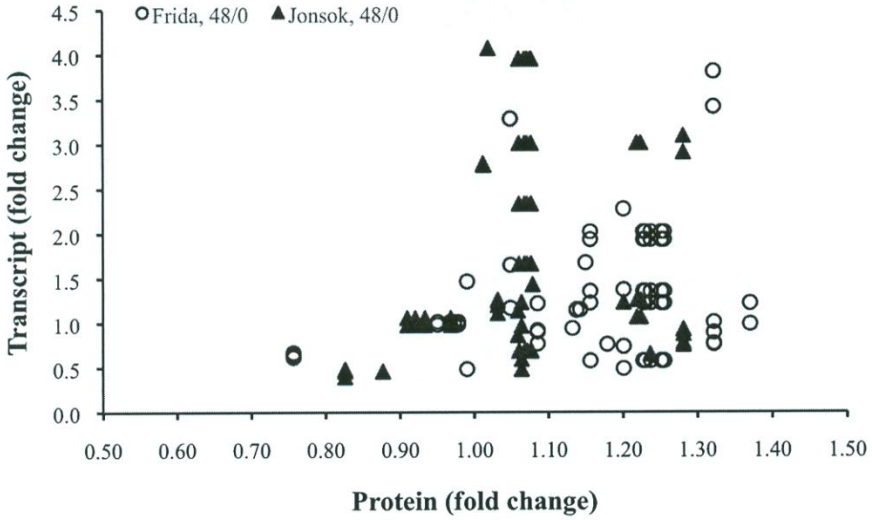
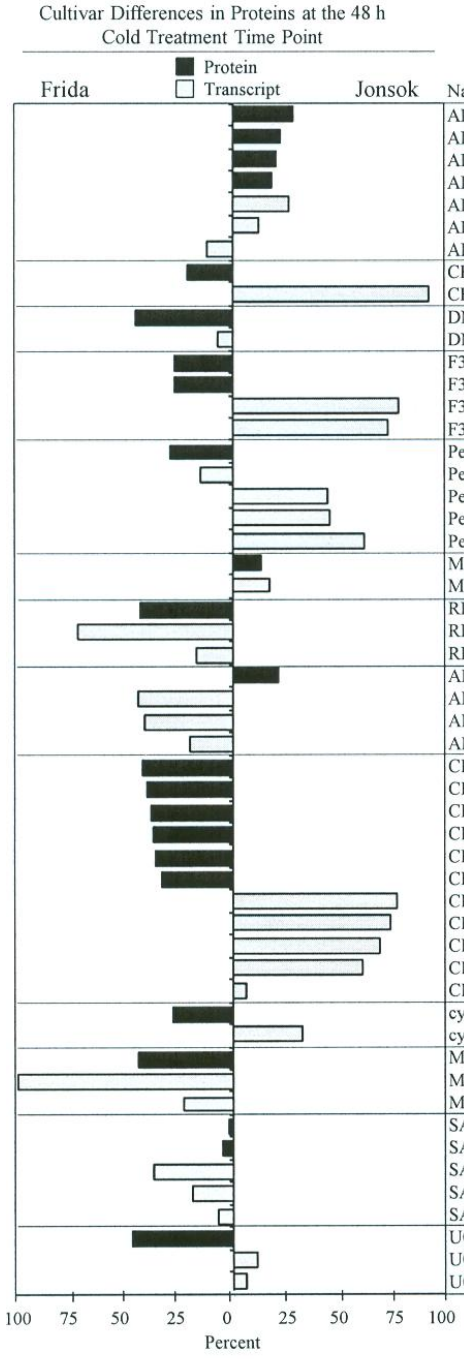
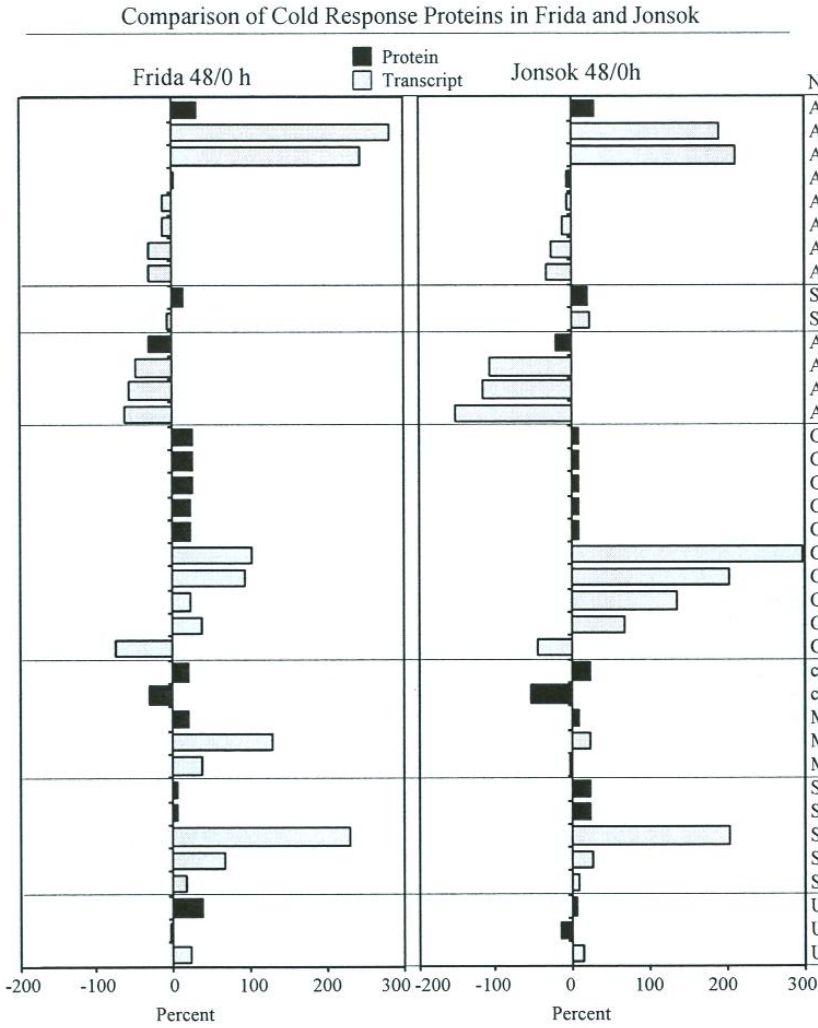


Figure 2. Comparison of fold changes in protein and transcript levels in cultivars "Frida" and "Jonsok" as function of cold treatment. All values from Figs 3 & 4 were used except those changes greater than 13 fold (6 data points). A fold difference of 1 indicates that control and 48 h cold treated tissues have equal levels of protein or transcript, greater than 1 indicates higher levels at 48 h cold treatment, less than 1 indicates lower amount at 48 h cold treatment. Slope for both "Jonsok" and "Frida" linear fit data were +1.13.



Name	Protein ID or Fragaria Probe	Cultivar Difference Fold Difference J/F 0 h	J/F 48 h	Cold Response Fold Change F 48/0 h	J 48/0 h	AGI no.
ADH	113436	1.3	1.3	-1.1	-1.0	AT1G77120
ADH	89556337	1.3	1.2	-1.0	-1.1	AT1G77120
ADH	89550819	1.3	1.2	-1.0	-1.1	AT1G77120
ADH	158350919	1.2	1.2	-1.0	-1.1	AT1G77120
ADH	Frag009853	1.3	1.3	1.0	-1.0	AT1G77120
ADH	Frag009854	1.0	1.1	-1.0	1.1	AT1G77120
ADH	Frag009855	-1.0	-1.1	15.1	14.0	AT1G77120
CHI	89558076	-1.1	-1.2	1.2	1.0	AT5G05270
CHI	Frag032039	-1.3	1.9	1.7	4.1	AT5G05270
DNA glycosidase II	158380119	-1.6	-1.4	-1.0	1.1	AT5G57970
DNA glycosidase II	Frag039343	-1.0	-1.1	1.5	1.4	AT5G57970
F3H	51493449	-1.1	-1.3	1.1	1.0	AT3G51240
F3H	51493451	-1.1	-1.3	1.1	1.0	AT3G51240
F3H	Frag022880	-1.4	1.8	1.2	2.8	AT3G51240
F3H	Frag022879	-1.4	1.7	1.1	2.8	AT3G51240
Peroxiredoxin	158360959	-1.2	-1.3	1.1	1.0	AT3G52960
Peroxiredoxin	Frag023165	-1.2	-1.1	1.2	1.3	AT3G52960
Peroxiredoxin	Frag023166	1.1	1.4	-1.1	1.2	AT3G52960
Peroxiredoxin	Frag023163	1.1	1.4	-1.1	1.3	AT3G52960
Peroxiredoxin	Frag023164	1.1	1.6	-1.3	1.1	AT3G52960
MLP-like protein 329	2465015	1.3	1.1	-1.0	-1.1	AT2G01530
MLP-like protein 329	Frag010632	1.2	1.2	-2.1	-2.2	AT2G01530
RING-H2 finger	49513383	-1.3	-1.4	1.1	1.0	AT5G07040
RING-H2 finger	Frag032364	-1.4	-1.7	-1.4	-1.7	AT5G07040
RING-H2 finger	Frag032365	-1.1	-1.2	-2.0	-2.1	AT5G07040
AILP1	158372623	1.1	1.2	-1.3	-1.2	AT5G19140
AILP1	Frag034444	-1.0	-1.4	-1.5	-2.1	AT5G19140
AILP1	Frag034446	-1.0	-1.4	-1.6	-2.2	AT5G19140
AILP1	Frag034445	1.3	-1.2	-1.6	-2.5	AT5G19140
CHS	158367106	-1.2	-1.4	1.2	1.1	AT5G13930
CHS	158369386	-1.2	-1.4	1.3	1.1	AT5G13930
CHS	158370409	-1.2	-1.4	1.3	1.1	AT5G13930
CHS	71979904	-1.2	-1.4	1.2	1.1	AT5G13930
CHS	71979908	-1.2	-1.4	1.2	1.1	AT5G13930
CHS	1705844	-1.2	-1.3	1.2	1.1	AT5G13930
CHS	Frag033515	1.1	1.8	1.9	3.0	AT5G13930
CHS	Frag033513	1.4	1.7	1.4	1.7	AT5G13930
CHS	Frag033512	-1.1	1.7	1.2	2.3	AT5G13930
CHS	Frag033516	-1.2	1.6	2.0	4.0	AT5G13930
CHS	Frag033514	-1.1	1.1	-1.7	-1.5	AT5G13930
cysteine protease	158358730	-1.3	-1.3	1.2	1.2	AT3G45310
cysteine protease	Frag021997	1.5	1.3	-1.3	-1.5	AT3G45310
Mito carrier protein	158362466	-1.3	-1.4	1.2	1.1	AT1G72820
Mito carrier protein	Frag009144	-1.1	-2.0	2.3	1.2	AT1G72820
Mito carrier protein	Frag009145	1.2	-1.2	1.4	-1.0	AT1G72820
SAM	158372548	-1.2	-1.0	1.0	1.2	AT3G17390
SAM	51049581	-1.2	-1.1	1.0	1.2	AT3G17390
SAM	Frag019727	-1.0	-1.4	1.7	1.3	AT3G17390
SAM	Frag019726	-1.1	-1.2	3.3	3.0	AT3G17390
SAM	Frag019728	1.0	-1.1	1.2	1.1	AT3G17390
UQCRX/QCR9-like	51047468	-1.1	-1.5	1.4	1.1	AT3G52730
UQCRX/QCR9-like	Frag023121	1.2	1.1	1.2	1.1	AT3G52730
UQCRX/QCR9-like	Frag023122	1.2	1.1	-1.0	-1.2	AT3G52730

Figure 3. Cultivar differences (>20%) at 48 h cold treatment. Transcripts with matching arabidopsis protein homologs are graphed for comparison. The top 7 proteins are different with respect to cultivar; the bottom 5 proteins additionally show a cold response. The proteins (black bar) and transcripts (grey bar) are graphed as a percent difference in either "Frida" or "Jonsok". The names to the right of the graph corresponds to Genbank accession code (Protein ID) or the microarray identifier (Fragaria ID) for each bar.



Name	Protein ID or Fragaria Probe	Cultivar Difference Fold Difference J/F 0 h	J/F 48 h	Cold Response Fold Change F 48/0 h	J 48/0 h	AGI no.
AOC4	158379651	-1.11	-1.15	1.32	1.28	AT1G13280
AOC4	Frag002191	1.07	-1.22	3.82	2.91	AT1G13280
AOC4	Frag002192	-1.08	-1.19	3.42	3.10	AT1G13280
AOC4	Frag002187	1.23	1.13	1.01	-1.07	AT1G13280
AOC4	Frag002190	1.18	1.22	-1.12	-1.08	AT1G13280
AOC4	Frag002189	1.05	1.03	-1.12	-1.14	AT1G13280
AOC4	Frag002193	1.19	1.20	-1.30	-1.28	AT1G13280
AOC4	Frag002188	1.17	1.15	-1.31	-1.33	AT1G13280
SAM2	158372024	-1.08	-1.01	1.13	1.20	AT4G01850
SAM2	Frag025090	-1.15	1.14	-1.06	1.23	AT4G01850
AILP1	158372623	1.10	1.20	-1.32	-1.21	AT5G19140
AILP1	Frag034444	-1.03	-1.43	-1.50	-2.08	AT5G19140
AILP1	Frag034446	-1.01	-1.40	-1.56	-2.17	AT5G19140
AILP1	Frag034445	1.29	-1.20	-1.62	-2.53	AT5G19140
CHS	158369386	-1.20	-1.40	1.26	1.08	AT5G13930
CHS	158370409	-1.18	-1.38	1.25	1.07	AT5G13930
CHS	158367106	-1.22	-1.42	1.24	1.06	AT5G13930
CHS	71979908	-1.18	-1.36	1.23	1.07	AT5G13930
CHS	71979904	-1.19	-1.37	1.23	1.07	AT5G13930
CHS	Frag033516	-1.22	1.59	2.02	3.95	AT5G13930
CHS	Frag033515	1.13	1.75	1.94	3.01	AT5G13930
CHS	Frag033512	-1.14	1.67	1.23	2.34	AT5G13930
CHS	Frag033513	1.40	1.72	1.36	1.66	AT5G13930
CHS	Frag033514	-1.13	1.06	-1.74	-1.46	AT5G13930
cysteine protease	158358730	-1.34	-1.28	1.18	1.24	AT3G45310
cysteine protease	Frag021997	1.54	1.32	-1.32	-1.54	AT3G45310
Mito carrier protein	158362466	-1.27	-1.43	1.20	1.06	AT1G72820
Mito carrier protein	Frag009144	-1.07	-1.99	2.28	1.23	AT1G72820
Mito carrier protein	Frag009145	1.16	-1.23	1.37	-1.04	AT1G72820
SAM	51049581	-1.23	-1.06	1.05	1.22	AT3G17390
SAM	158372548	-1.19	-1.02	1.05	1.22	AT3G17390
SAM	Frag019726	-1.09	-1.19	3.29	3.02	AT3G17390
SAM	Frag019727	-1.05	-1.37	1.65	1.26	AT3G17390
SAM	Frag019728	1.03	-1.07	1.17	1.06	AT3G17390
UQCRX/QCR9-like	51047468	-1.13	-1.47	1.37	1.06	AT3G52730
UQCRX/QCR9-like	Frag023122	1.22	1.06	-1.01	-1.16	AT3G52730
UQCRX/QCR9-like	Frag023121	1.19	1.11	1.22	1.14	AT3G52730

Figure 4. Cold responsive proteins. Seven proteins that changed at least 20 percent in response to 2 days cold (percent increase or decrease). The transcripts with matching protein arabidopsis homologs are graphed for comparison. The cold response is expressed as fold change (48/0 h) for "Frida" and "Jonsok". Proteins (black bars); Transcripts (grey bars).