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, progress in the improvement of cultivars using traditional screening methods has had limited success. Thus, the development of molecular markers for freezing hardiness would facilitate the selection 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 cultivars 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 major agricultural problems, 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 (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, see 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 cold tolerant 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 a particularly hardy and relatively cold-intolerant cultivar, 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, and 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 transcript levels by employing a microarray approach based upon genomic and transcript sequences from *Fragaria vesca*.

MATERIALS AND METHODS

Fragaria × *ananassa* from greenhouse cultivation (20^{o}C) were cold treated at 2^{o}C (90 µmol m-2 s-1), for 0 or 2 day. Crown tissues of *Fragaria* × *ananassa* cultivars ‘Jonsok’ and ‘Frida’ were collected, divided in half and immediately frozen in liquid nitrogen and stored at -80^{o}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^{o}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* × *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 Norwegian University of Science and Technology (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 proteins and transcripts with respect to ‘Frida’ and ‘Jonsok’ ratios were examined, slopes of +0.21 and -0.44 were obtained at control and 2 d cold treatment, respectively (Fig. 1). This suggests, particularly after 2 d, that the increased differences in protein 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’ (Fig. 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 cultivar 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 revealed. In general, the predominant transcript (assuming quantitative relation between hybridization signals) was in agreement with the protein response. For example, SAM synthase, UBCRx, mito 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).

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 showing potential as biomarkers for overwintering success.

ACKNOWLEDGEMENTS

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Figures

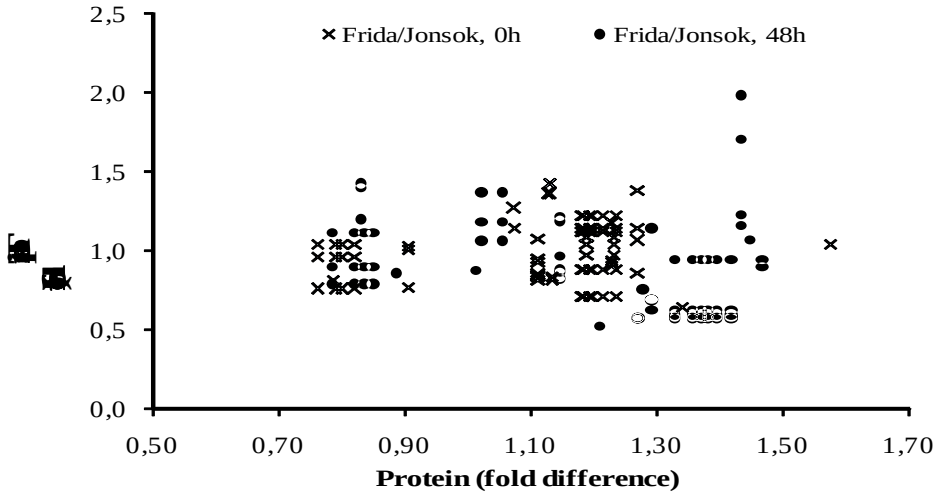


Fig. 1. Comparison of changes in protein and transcript levels in cultivars 'Frida' and 'Jonsok'. All values from Figs. 3 & 4 were used except those changes greater than 13 fold (6 data points). Fold difference of 1 indicates that 'Jonsok' and 'Frida' have equal levels of protein or transcript, greater 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.

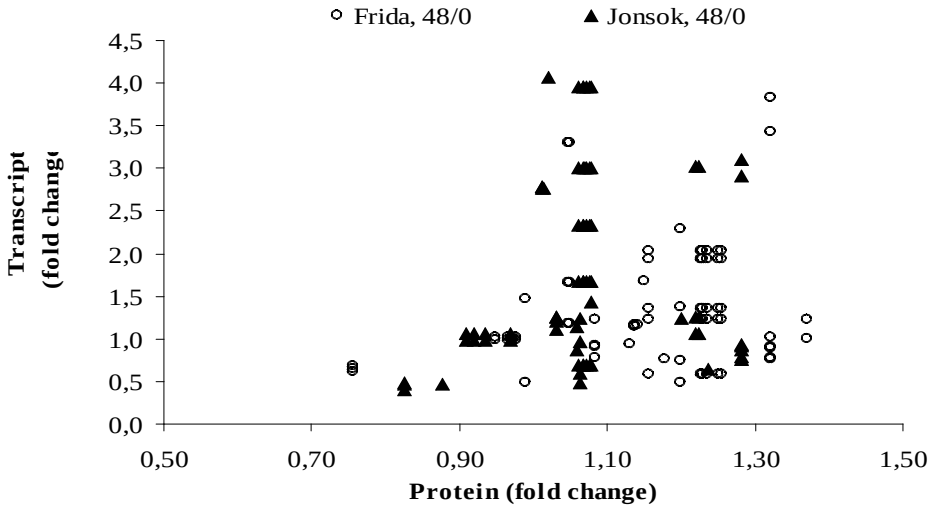
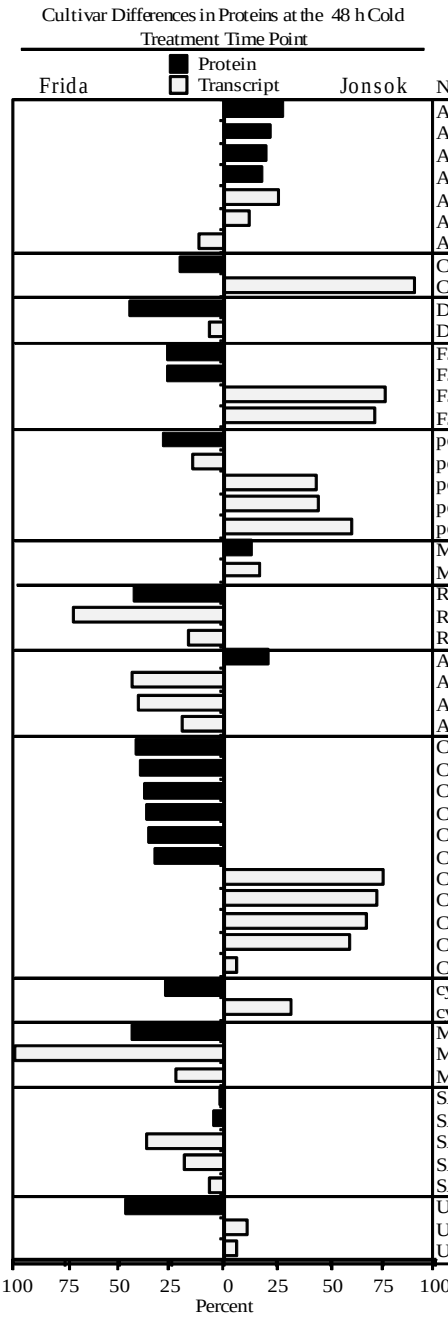
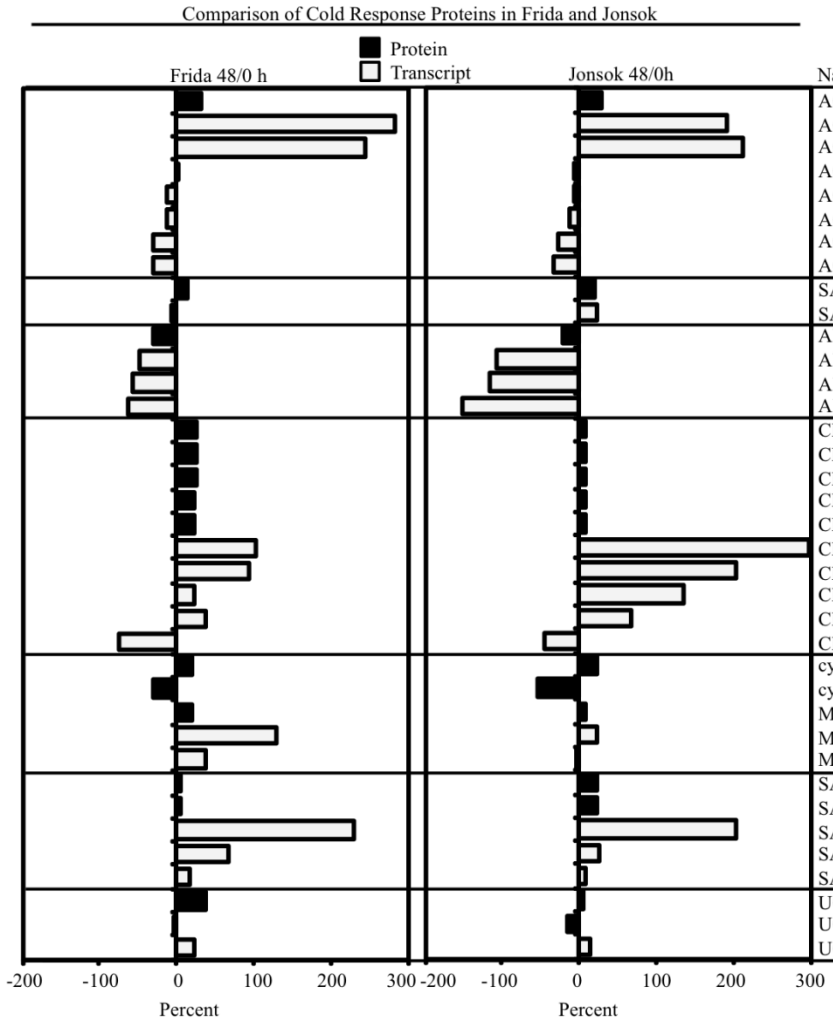


Fig. 2. Comparison of fold changes in protein and transcript levels in cultivars 'Frida' and 'Jonsok' as function of cold treatment. All values from Figs 3 & 4 were used except those changes greater than 13 fold (6 data points). A fold difference of 1 indicates that control and 48 h cold treated tissues 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

Fig 3. Cultivar differences (>20%) at the 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 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

Fig. 4. Cold response proteins. Seven proteins that change 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).