Proteomic Study of Low-Temperature Responses in Strawberry Cultivars (*Fragaria* × *ananassa*) That Differ in Cold Tolerance[1][W][OA]

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To gain insight into the molecular basis contributing to overwintering hardiness, a comprehensive proteomic analysis comparing crowns of octoploid strawberry (*Fragaria* × *ananassa*) cultivars that differ in freezing tolerance was conducted. Four cultivars were examined for freeze tolerance and the most cold-tolerant cultivar ('Jonsok') and least-tolerant cultivar ('Frida') were compared with a goal to reveal how freezing tolerance is achieved in this distinctive overwintering structure and to identify potential cold-tolerance-associated biomarkers. Supported by univariate and multivariate analysis, a total of 63 spots from two-dimensional electrophoresis analysis and 135 proteins from label-free quantitative proteomics were identified as significantly differentially expressed in crown tissue from the two strawberry cultivars exposed to 0-, 2-, and 42-d cold treatment. Proteins identified as cold-tolerance-associated included molecular chaperones, antioxidants/detoxifying enzymes, metabolic enzymes, pathogenesis-related proteins, and flavonoid pathway proteins. A number of proteins were newly identified as associated with cold tolerance. Distinctive mechanisms for cold tolerance were characterized for two cultivars. In particular, the 'Frida' cold response emphasized proteins specific to flavonoid biosynthesis, while the more freezing-tolerant 'Jonsok' had a more comprehensive suite of known stress-responsive proteins including those involved in antioxidation, detoxification, and disease resistance. The molecular basis for 'Jonsok'-enhanced cold tolerance can be explained by the constitutive level of a number of proteins that provide a physiological stress-tolerant poise.

Strawberry (*Fragaria* × *ananassa*) cultivation predominates in regions with mild winters. In colder climates, overwintering hardiness is an essential trait for strawberry cultivation. Freezing injury of strawberry plants is one of the greatest factors in reducing crop yield and quality in temperate regions. Winter damage in Norway, for example, on average causes losses of 20%. Thus, production of cultivars with improved freezing hardiness is one of Norway's major objectives for their strawberry breeding programs. Improvement of cold hardiness is desirable for securing economic sustainability of the existing crops, and for expanding the growing regions of temperate fruit crops. Because strawberry is a representative species for the Rosaceae crops (e.g. peaches [*Prunus persica*], apples [*Malus domestica*], cherries [*Prunus avium*], blackberries [*Rubus fruticosus*], and raspberries [*Rubus idaeus*]), this knowledge is expected to be transferrable to benefit improvement of many of these related crops.

Winter hardiness, a variable trait of strawberries, depends on the overwintering crown for spring regeneration that requires that the crowns remain minimally compromised from the physiological damage of freezing. The crown is especially susceptible to ice crystal damage due to the large cells of the pith tissue. Freezing damage is readily seen as brown or black discoloration resulting from cellular injury and consequent oxidation (Marini and Boyce, 1977). 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

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hardiness observed for strawberry species is likely contributed by the proteins that accumulate in the overwintering crown to mitigate adverse effects of freezing damage. Modifying extracellular ice formation, protecting protein functions with chaperones, scavenging reactive oxygen species (ROS), and increasing cell wall integrity are important aspects for surviving low temperatures.

Physiological, biochemical, and molecular changes that occur in plants in response to low temperature have been extensively reviewed (Heino and Palva, 2004; Chinnusamy et al., 2007; Zhu et al., 2007; Guy et al., 2008; Ruelland et al., 2009). One important cold signaling pathway, controlled by C-repeat/drought-responsive element binding factors (CBFs) has been shown to enhance freezing tolerance in Arabidopsis (*Arabidopsis thaliana*; Jaglo-Ottosen et al., 1998; Gilmour et al., 2000). Genes encoding CBFs have been identified in sour cherry (*Prunus cerasus*) and strawberry (Owens et al., 2002; Kitashiba et al., 2003). However, it is likely that CBF regulons differ in different plants (Zhang et al., 2004). Crucial for surviving freezing temperatures are transcriptional activation and repression of genes, changes in metabolism, activation of scavengers for ROS, alteration of membrane composition, and accumulation of cryoprotective molecules (e.g. sugars, compatible solutes, proteins). These general freezing-tolerant mechanisms apply to a wide range of organisms and plant species and reveal important modes of defense against freezing damage. To provide practical applications for improving freezing tolerance in strawberry, more knowledge is required about the contributions provided by these different mechanisms for specific tissues, cells, and developmental stages, particularly for overwintering tissues (Wisniewski et al., 2004, 2007). Robust winter survival not only requires freezing tolerance but also involves adaptation strategies for additional abiotic stresses (e.g. desiccation, anoxia, frost heave), as well as biotic stress (Bertrand et al., 2003).

Global transcript, protein, and metabolic approaches are rapidly advancing our knowledge about cold acclimation processes (Cook et al., 2004; Kaplan et al., 2007; Kosmala et al., 2009; Maruyama et al., 2009). Cold acclimation is known to induce proteins relevant for freezing survival (Zhu et al., 2007; Thomashow, 2010); however, it is plausible that some proteins associated with cold tolerance are expressed under nonstress 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 was facilitated by using proteomics. Advantages of proteomics include detecting posttranslational 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 crown, and compare several commercial cultivars of strawberry in terms of their relative freezing tolerance and concomitant protein expression patterns. There is limited knowledge of winter hardiness in herbaceous perennials and much less is known about the most relevant overwintering crown tissue. This study provides a framework for the cold acclimation response in strawberry crown and identifies proteins that are elevated in the most freezing-tolerant strawberry cultivars. Potential protein biomarkers are identified that can be utilized to facilitate conventional breeding endeavors for cold-tolerant cultivars strawberry.

RESULTS AND DISCUSSION

Relative Cold/Freezing Tolerance of 'Jonsok' and 'Frida'

Anecdotal field observations of winter survival and subsequent yields of strawberry cultivars grown in Norway suggested that 'Jonsok' is more cold tolerant than other grown cultivars. The four strawberry cultivars 'Elsanta', 'Frida', 'Senga Sengana', and 'Jonsok' were tested for winter survival traits under controlled laboratory environments. After cold acclimation (6 weeks at 2°C), '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 I; Supplemental Table S1; Supplemental Materials and Methods S1). In particular, survival rates were significantly different after 48-h treatments at −6°C and −9°C with 'Jonsok' and 'S. Sengana' being more cold tolerant and 'Frida' and 'Elsanta' being less so. Exponential extrapolated killing curves indicated: 50% survival of 'Jonsok' at approximately −8.3°C and for 'Frida' at approximately −5.5°C. Internal browning of crowns was consistent with these results. The Norwegian-bred 'Jonsok' and 'Frida' were analyzed here in detail as representing the most and least freezing-tolerant cultivars.

Proteomic (Two-Dimensional Electrophoresis) Maps of Strawberry Crown Tissue

The major overwintering structure of strawberries, the crown, was evaluated for changes in proteins that 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; to evaluate immediate responses reflecting rapid adjustments in protein levels) and long-term (42 d) cold treatments (2°C) to evaluate protein levels after extensive acclimation. Multiple crowns (up to six) were included for each replicate thereby minimizing the biological variance. Each crown was divided and used for two-dimensional electrophoresis (2DE) analysis, or

Table I. *Freezing survival demonstrates the relative cold/freezing tolerance for strawberry cultivars*

Surviving plants, browning extent, and intensity were scored as described in the "Materials and Methods." Exponential extrapolated killing curves indicated 50% survival of 'Jonsok' at approximately −8.3°C and for 'Frida' at approximately −5.5°C. The LT50 (temperature at which 50% of plants died or 50% of maximal browning occurred), the SE, 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 Supplemental Table S1.

Cultivar	Plant Survival			Tissue Browning			Browning Intensity		
	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
'S. Sengana'	−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

for label-free quantitative proteomics (LFQP) analysis and half the crown was retained for transcript analysis (see "Materials and Methods"). A total of 168 plants from all cultivars were used to complete three 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- to 100-kD range. This first report of a 2DE protein reference map for strawberry crowns ('Jonsok') is shown with arrows indicating the 109 spots that were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS; Fig. 1).

Agglomerative Hierarchical Clustering of 2DE Data

2DE proteome profiling patterns were compared for 'Elsanta', 'Frida', 'S. Sengana', and 'Jonsok' for the 0, 2, and 42 d of cold treatment (2°C) by using agglomerative hierarchical clustering 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 agglomerative hierarchical clustering analysis is presented in Supplemental Figure S1. The replicates for each cultivar at 0 and 2 d 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. 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.

Principal Component Analysis of 'Jonsok' and 'Frida'

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 (Fig. 2). 'Jonsok' and 'Frida' are clearly distinguished from each other at all cold treatments. The scree plot (Fig. 2, inset) indicates that the first two principal components (PCs), PC1 and PC2, account for 50.75% of the total variability in protein expression profiles. The PC2 dimension indicates detectable differences in the cultivars at control and 2-d 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-d cold treatments, while the protein expression patterns tend to converge after the 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 differences in cold tolerance between the two cultivars may be significantly linked to differences in protein expression under control conditions or in the initial phase of cold treatment. To detect which spots were contributing to the difference between the cultivars, additional PCA analysis was performed separately for each cold treatment time point. The contribution of individual protein spots to the variance observed between 'Jonsok' and 'Frida' is indicated by the factor loading i.e. component loading determined for each time point listed in Supplemental Data S1.

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

After 2DE, 900 spots were matched and analyzed using PDQuest 2DE gel analysis software for 'Elsanta', 'Frida', 'S. Sengana', and 'Jonsok'. 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's 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 cutoff to identify the 2DE spots differentially regulated upon cold treatment (threshold ratio cold-stressed versus control plants >2- or <0.5-fold).

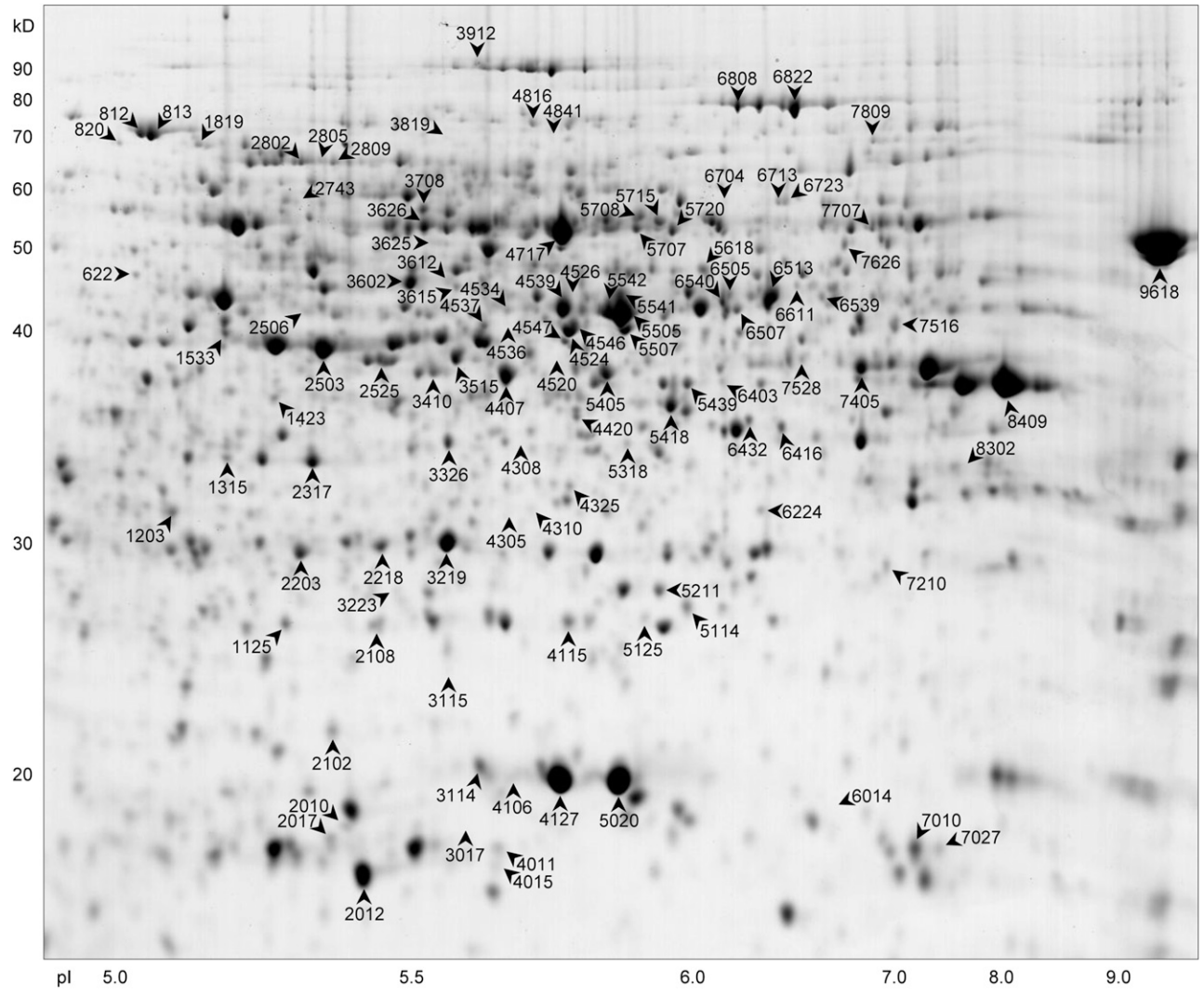

Figure 1. Representative 2DE gel of strawberry crown proteins ('Jonsok' at 2 d 2°C treated). The 109 proteins identified by LC-MS/MS (Supplemental Data S1) are indicated with spot numbers. Gel was performed with 400 μg of protein using 24-cm immobilized pH gradient strips (3–10 nonlinear) resolved on 12% SDS-PAGE and stained with colloidal Coomassie Brilliant Blue.

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 (Fig. 3A). There were 19 (2.1%) and 41 (4.6%) spots that increased in response to cold at 2 and 42 d in 'Jonsok' compared with 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 (ADH). Among the 41 proteins that decreased in both cultivars, three were identified as Glc-6-P isomerase, a putative 20S proteasome β-subunit 5, and a calcium-dependent protein kinase. Only one protein copper/zinc superoxide dismutase (Cu/Zn SOD) 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 in 'Frida' to be increasing in response to cold stress approached, but did not reach the levels of accumulation present in 'Jonsok' at 42 d. Some of these proteins include a putative protein phosphatase, pyruvate kinase, and ADH. Likewise, proteins in 'Jonsok' that were cold responsive and approached, but did not reach the levels in 'Frida' were identified as lipoxygenase, glyceraldehyde-3-P dehydrogenase, and *S*-adenosyl-Met synthase. Together, these changes partially explain the convergence in overall protein expression levels observed in the PCA analysis (Fig. 2).

Interestingly, less than half of the cold-responsive protein spots were in common between the two

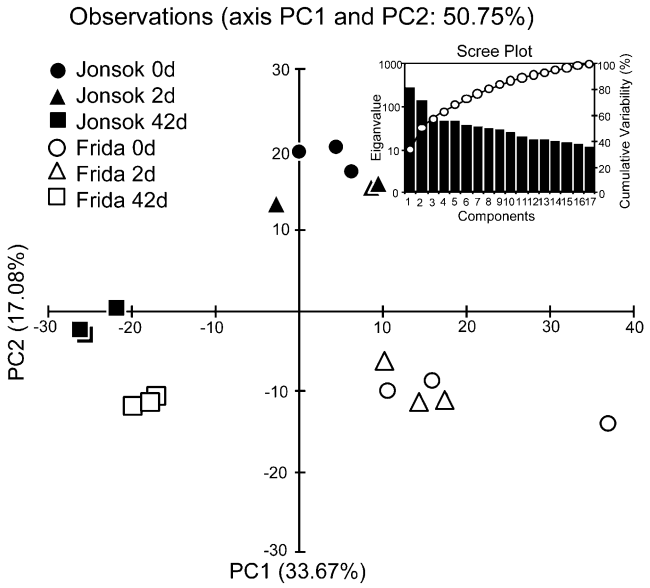

Figure 2. PCA indicates 'Frida' and 'Jonsok' protein composition are distinctive and that they respond differently to cold stress. Time (in days) of exposure to 2°C is indicated by 0, 2, and 42 d. All 900 common spots were included in this analysis. The scree plot (inset) indicates that the first two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

cultivars (Fig. 3, 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, two sided and (2) PCA factor loading with Pearson's correlation coefficient equal or better than the absolute value of 0.80. 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' (Fig. 3B), and a total of 15 proteins were consistently (at all experimental conditions) greater in 'Frida' than 'Jonsok' (Fig. 3C).

A list of the 63 most significant differentially accumulated proteins identified for 'Jonsok' (35 proteins) and 'Frida' (28 proteins) was produced based on a mixture of statistical, clustering, and PCA analysis (Table II). The protein spots that correlate to the 63 differentially expressed proteins are labeled on the reference 2DE maps for 'Jonsok' and 'Frida' (Supplemental Fig. S2). The intensity and statistical significance of these spots within the entire 2DE proteomic data were examined using volcano plots, a method commonly applied to evaluate microarray data sets (Cui and Churchill, 2003). In the 42 d proteome data set, 35 spots (>2-fold) were significant at the $P < 0.001$ (23 were identified) and 148 spots (>2-fold) were significant at the $P < 0.05$ (Supplemental Fig. S3). After applying ANOVA, a subset of these was used to create our potential protein marker list (Table II).

Functional Categories of Identified Proteins from 2DE

Of the 157 spots obtained from 2DE gels and analyzed by LC-MS/MS, a total of 109 were successfully identified with high confidence using Rosaceae and *Fragaria* databases (Supplemental Data S2). Most of the protein spots selected for identification were 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

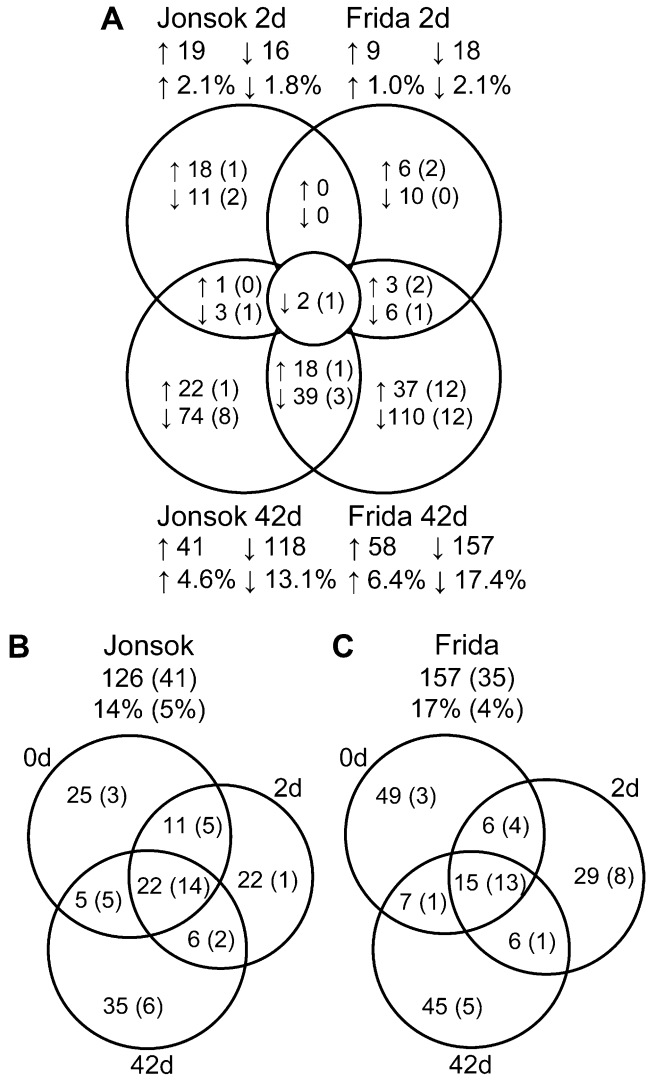

Figure 3. Proteins differentially expressed in 'Jonsok' and 'Frida'. Section A shows cold-responsive proteins at 2 d (2d) and 42 d (42d) that have changed ≥2-fold relative to control (0 d) in 'Frida' and 'Jonsok'. The number and percent of protein spots accumulating or decreasing are indicated with arrows. The number of proteins with higher levels (≥2-fold) in 'Jonsok' (section B) and 'Frida' (section 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 sections 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.

Table II. *The proteins identified in strawberry crown by LC-MS/MS that distinguish the two cultivars, 'Jonsok' and 'Frida'*

List includes 2DE spot number (SSP), protein name, number of distinct sequences from LC-MS/MS, GenBank accession code from NCBI, Arabidopsis Genome Initiative (AGI) gene index number, and the time point(s) at which the protein spot is determined significant by meeting criteria from multiple methods of analysis.

SSP	Protein Identified	Distinct Sequences	AccessionCode	Species	AGI	MeetsCriteria[a]
Proteins that are at higher levels in 'Jonsok' than 'Frida'						
4546	ADH	29	CAA33613	*F.* × *ananassa*	AT1G77120	0 d
3114	Fra a 1-A	5	ABD39049	*F.* × *ananassa*	AT1G24020	0 d
4015	Fra a 2	6	DY675259	*F. vesca*	AT1G24020	0 d
813	High M_r HSP	38	AAF34134	*Malus* × *domestica*	AT3G12580	0 d
812	Hsc70 (heat shock cognate 70 kD)	9	EX686389	*F. vesca*	AT5G02500	0 d
7626	Vacuolar sorting protein	7	EX684112	*F. vesca*	AT2G27600	0 d
3515	ANR	18	ABD95362.1	*F.* × *ananassa*	AT1G61720	0 d, 2 d
4011	Fra a 3	6	EX681634	*F. vesca*	AT1G24020	0 d, 2 d
5125	GST	3	EX662925	*F. vesca*	AT2G47730	0 d, 2 d
1819	HSP70	9	EX686887	*F. vesca*	AT5G09590	0 d, 2 d
4537	Phosphoglycerate kinase	10	EX672771	*F. vesca*	AT1G79550	0 d, 2 d
4325	Protein phosphatase type 2C	6	EX684817	*F. vesca*	AT3G15260	0 d, 2 d
622	SGT1; putative protein phosphatase	7	EX680652	*F. vesca*	AT4G11260	0 d, 2 d
6540	ADH	5	P17648	*F.* × *ananassa*	AT1G77120	0 d, 2 d, 42 d
5439	Aldo/keto reductase	13	EX674338	*F. vesca*	AT1G60710	0 d, 2 d, 42 d
6432	Annexin	9	DY668560	*F. vesca*	AT1G35720	0 d, 2 d, 42 d
2317	β-1,3-glucanase	11	EX665040	*F. vesca*	AT3G57240	0 d, 2 d, 42 d
2010	Cu/Zn SOD	4	EX670753	*F. vesca*	AT1G08830	0 d, 2 d, 42 d
3626	Enolase	7	EX680335	*F. vesca*	AT2G36530	0 d, 2 d, 42 d
1315	Glyoxalase I (lactoylglutathione lyase)	6	EX680994	*F. vesca*	AT1G11840	0 d, 2 d, 42 d
4115	GST	8	EX662925	*F. vesca*	AT2G47730	0 d, 2 d, 42 d
1423	IFR	4	CO817159	*F.* × *ananassa*	AT1G75280	0 d, 2 d, 42 d
820	Nucleoredoxin, putative	4	DY671291	*F. vesca*	AT1G60420	0 d, 2 d, 42 d
6224	Porin	8	EX688506	*F. vesca*	AT3G01280	0 d, 2 d, 42 d
6723	Pyruvate kinase	3	EX666373	*F. vesca*	AT5G08570	0 d, 2 d, 42 d
4547	rgp (α-1,4-glucan-protein synthase)	9	EX685340	*F. vesca*	AT3G02230	0 d, 2 d, 42 d
2203	Thaumatin-like protein	9	EX674730	*F. vesca*	AT1G75800	0 d, 2 d, 42 d
6505	ADH	10	CAA33613	*F.* × *ananassa*	AT1G77120	0 d, 42 d
2218	APX	14	AAD41406.1	*F.* × *ananassa*	AT1G07890	0 d, 42 d
1533	OMT	16	AF220491	*F.* × *ananassa*	AT5G54160	0 d, 42 d
2012	Pathogenesis-related protein Ypr10	10	ABX89934	*F.* × *ananassa*	AT1G24020	0 d, 42 d
5318	Aldo/keto reductase	8	EX680606	*F. vesca*	AT2G37770	2 d
4717	F1-ATPase α-subunit	40	AAW33106	*Rubus* sp. *JPM-2004*	AT2G07698	2 d
6539	3-Ketoacyl-CoA thiolase	5	EX682172	*F. vesca*	AT2G33150	42 d
7528	Cytosolic aldolase	8	AAG21429.1	*F.* × *ananassa*	AT3G52930	42 d
Proteins that are at higher levels in 'Frida' than 'Jonsok'						
6507	Acetyl-CoA acetyltransferase, cytosolic1	5	DY674748	*F. vesca*	AT5G48230	0 d
1203	Caffeoyl-CoA 3-OMT	4	CAA04769	*F. vesca*	AT4G34050	0 d
6403	Malate dehydrogenase, mitochondrial	10	P83373	*F.* × *ananassa*	AT1G53240	0 d
3708	Pyruvate decarboxylase	7	EX678086	*F. vesca*	AT5G17380	0 d
3017	40S ribosomal protein S12-2	4	EX685033	*F. vesca*	AT2G32060	0 d, 2 d
8302	Adenylate kinase	2	EX657534	*F. vesca*	AT5G63400	0 d, 2 d
7405	Cytosolic aldolase	30	AAG21429	*F.* × *ananassa*	AT3G52930	0 d, 2 d
4305	Glyoxalase II (hydroxyacylglutathione hydrolase)	11	EX665941	*F. vesca*	AT3G10850	0 d, 2 d
6704	GPI (Glc-6-P isomerase)	5	DY671931	*F. vesca*	AT5G42740	0 d, 2 d
7210	Proteasome subunit b type-4	7	EX661942	*F. vesca*	AT1G56450	0 d, 2 d
3612	SAMS	9	EX684436	*F. vesca*	AT3G17390	0 d, 2 d
3625	6-Phosphogluconate dehydrogenase	9	DY671162	*F. vesca*	AT3G02360	0 d, 2 d, 42 d
6416	Annexin	8	DY668560	*F. vesca*	AT1G35720	0 d, 2 d, 42 d
6014	Cystathionine β-synthase domain	4	EX673938	*F. vesca*	AT5G10860	0 d, 2 d, 42 d
6611	Citrate synthase, mitochondrial	4	P83372	*F.* × *ananassa*	AT2G44350	0 d, 42 d
3912	Aconitase hydratase	5	EX661092	*F. vesca*	AT2G05710	2 d
4520	ANR	15	ABG76842.1	*F.* × *ananassa*	AT1G61720	2 d
4310	Carbonic anhydrase-like	5	EX670805	*F. vesca*	AT1G19580	2 d
7707	Catalase	8	EX683064	*F. vesca*	AT4G35090	2 d

(*Table continues on following page.*)

Table II. (*Continued from previous page.*)

SSP	Protein Identified	Distinct Sequences	AccessionCode	Species	AGI	MeetsCriteria[a]
4526	CHS	13	BAE17124.1	*F.* × *ananassa*	AT5G13930	2 d
5542	DFR	14	BAA12723	*Rosa* hybrid cultivar	AT5G42800	2 d
4536	F3H	10	AAU04792.1	*F.* × *ananassa*	AT3G51240	2 d
3115	Hexokinase1	6	ABG36925	*F.* × *ananassa*	AT1G47840	2 d
3819	sti1-like (stress-inducible protein)	2	EX671298	*F. vesca*	AT1G62740	2 d, 42 d
6808	Met synthase	13	DY672153	*F. vesca*	AT5G17920	42 d
2506	Cinnamyl-ADH	3	CAC09058.1	*F.* × *ananassa*	AT3G19450	42 d
3223	Iron-binding protein	4	EX677466	*F. vesca*	AT3G11050	42 d
3615	GEM-like protein1	2	EX673973	*F. vesca*	AT2G22475	42 d

[a]The time points are given for protein spots that meet the following criteria; factor loading value of 0.80 or better, $P < 0.05$ using Student's *t* test and ANOVA, and protein levels with 2-fold or greater difference. The Arabidopsis homolog results from performing a Blast on TAIR WU-BLAST 2.0, database TAIR9 proteins.

were good anchors for the gel analysis. After identifying Arabidopsis homologs, the gene ontology terminology (cellular component, molecular function, and biological function) was evaluated for all the identified protein spots (109) and for the differentially expressed proteins identified for 'Jonsok' and 'Frida' (Supplemental Fig. S4). 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 (more than half) of proteins identified in 'Jonsok' and 'Frida' fall into the biological process categories of stress-related or stress-responsive proteins. In terms of cellular components category, the cytosol, cell wall, plasma membrane, mitochondria, and extracellular seem somewhat overrepresented. In the molecular function category, the identified proteins were underrepresented in DNA or RNA binding, transcription factor activity, nucleic acid binding, and overrepresented in enzymatic functions, perhaps not surprising as the nature of proteomics encourages identification of more abundant proteins.

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 (Koes et al., 1994; Winkel-Shirley, 2001; Dixon and Pasinetti, 2010; Vogt, 2010).

Eighteen of the 109 2DE identified spots (not including the four *Fragaria* allergens (Fra a1s), 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 eight biosynthetic steps in the pathway and four additional proteins indirectly involved in the flavonoid pathway (Fig. 4). Flavonoid pathway proteins expressed at higher levels in 'Frida' than '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 inducible (an increase in CHS, at 2-d cold treatment was observed in both 2DE and LFQP). 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. Anthocyanidin reductase (ANR) is an oxidoreductase and competes with anthocyanidin synthase 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 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 induced in 'Frida' at 2 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 insignificant. Proteins more abundant in 'Jonsok' include *O*-methyltransferase (OMT), and isoflavone reductase (IFR)-related protein. Both proteins spots (spots 1533, 3326) identified as OMT were more abundant in 'Jonsok' at 42 d (3- and 6-fold, respectively). One of the spots, 1533, exhibited higher levels in 'Jonsok' at all time points. Two protein spots identified as IFR (spots 1423, 4420) mapped to distinct ESTs. Both were more abundant in 'Jonsok' at 0 d.

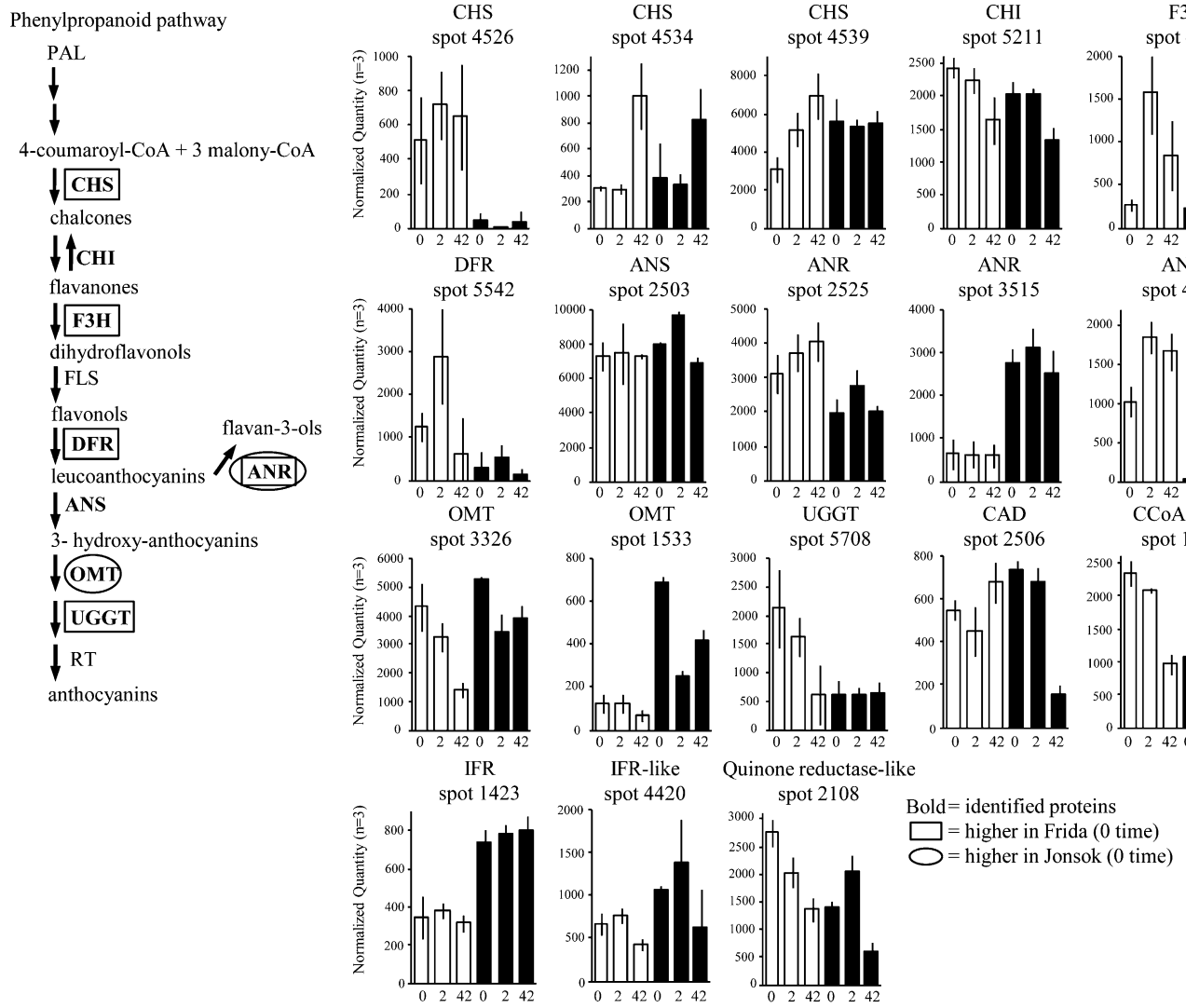

Figure 4. Proteins identified in the flavonoid pathway were most abundant in 'Frida'. Flavonoid pathway highlighting the proteins involved in this pathway in 'Frida' and 'Jonsok'. The proteins in bold indicate identified proteins. Proteins in either squares or ovals indicate that higher levels (≥2 fold, $P < 0.05$ 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 deviation for each time point (0, 2, 42 d of cold treatment at 2°C) for 'Frida' (gray bars) and 'Jonsok' (black bars). PAL, phenylalanine ammonia-lyase; RT, rhamnosyl transferase.

'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 could contribute to overwintering tolerance in 'Jonsok'. Other enzymes in this pathway did not show these large differences, e.g. chalcone isomerase while cold responsive, decreasing in the cold after 42 d (approximately 1.5-fold), was not significantly different between the cultivars. Cinnamyl-ADH, a molecular marker specific for lignification (Walter et al., 1988), increased slightly in 'Frida' at 42 d 1.24-fold (*t* test < 0.1) and UDP-Glc glucosyltranferase was approximately 3-fold greater in 'Frida' at 0- and 2-d cold treatment, but not different after 42 d. Caffeoyl-CoA 3-OMT was 2-fold higher in 'Frida' at 0 d, yet by 42 d there was no difference due to a significant decrease in 'Frida' and a significant cold response increase of 1.9-fold in 'Jonsok'. Anthocyanidin synthase did not change significantly with regard to cultivar or cold treatment.

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 RNase 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 6,000-fold higher in 'Jonsok' than 'Frida' after 42 d of cold treatment, largely due to a decrease in the amount found in 'Frida' (Fig. 5). 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.

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 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 after stress (Biemelt et al., 1998; Blokhina et al., 2003). Overall, proteins involved in antioxidative and detoxification processes were highly overrepresented in 'Jonsok' compared with 'Frida' (Fig. 6). Although 'Frida' clearly had an up-regulated 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, antioxidative activity.

The detoxification of ROS is managed through the action of SODs that catalyze the dismutation of superoxides into oxygen and hydrogen peroxide (H_2O_2), and catalases and peroxidases that further detoxify H_2O_2 to water (Apel and Hirt, 2004). In 'Jonsok', relative to 'Frida' (from 0–42 d) increased levels of Cu/Zn SOD (2- to 11-fold higher), ascorbate peroxidase (APX; 2- to 5-fold higher), annexin 1 (395- to 1,200-fold higher), and L-galactono-1,4-lactone dehydrogenase (1.2- to 1.7-fold higher) are likely key components in an increased capability to directly modulate ROS levels. SODs 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 SOD (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. APX that consumes H_2O_2, in conjunction with ascorbate, which is subsequently regenerated by the ascorbate-glutathione cycle, contributes to abiotic stress tolerance, including low-temperature stresses (Shigeoka et al., 2002).

The decrease in levels of Cu/Zn SOD and APX after the cold treatment could reflect a decrease in demand for protective antioxidants as metabolism inevitably slows. Increased basal expression of Cu/Zn SOD) and APX in 'Jonsok' is expected to contribute to increased abiotic tolerance. In one study (Lee et al., 2007) simultaneous overexpression of Cu/Zn and APX in tall fescue resulted in an increase tolerance to a wide range of abiotic stresses. In another study, comparison of

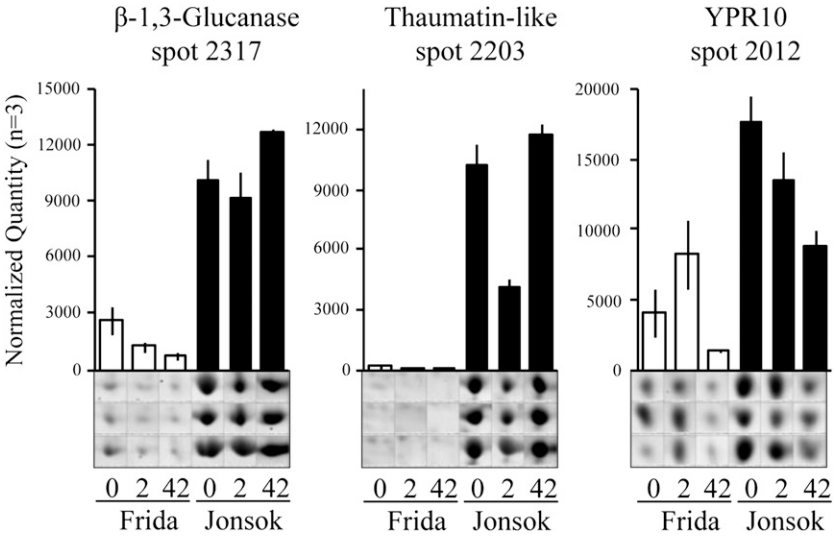

Figure 5. Levels of proteins associated with pathogen resistance distinguish 'Jonsok' (black bars) from 'Frida' (gray bars). Bar graphs show the average normalized values (from PDQuest, $n = 3$) with standard deviation for each time point (0, 2, 42 d of cold treatment at 2°C) for 'Frida' and 'Jonsok'. The corresponding 2DE spot images are presented beneath each graphed bar.

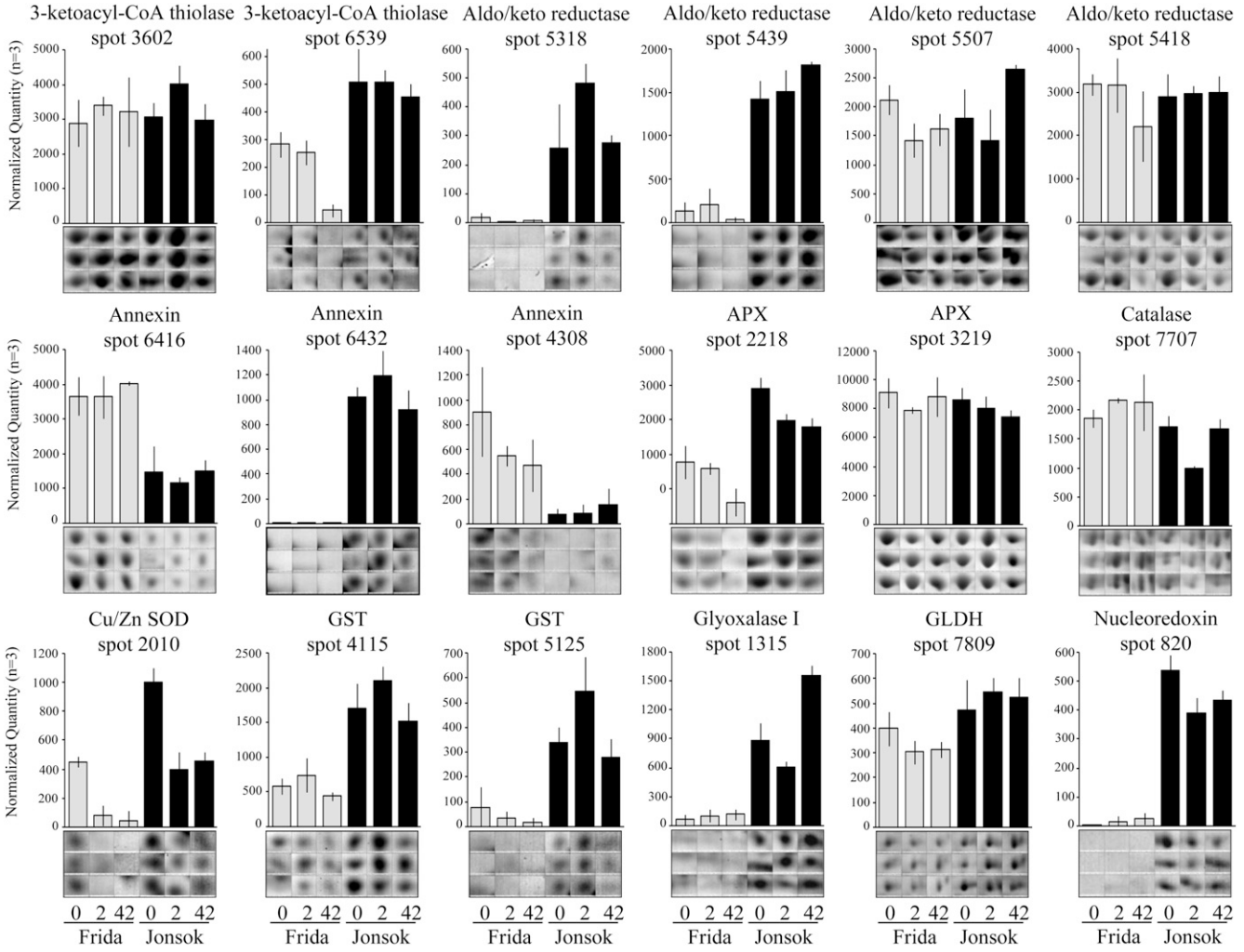

Figure 6. Levels of proteins associated with antioxidation and detoxification distinguish 'Jonsok' (black bars) from 'Frida' (gray bars). Bar graphs show the average normalized values (from PDQuest, $n = 3$) with standard deviation for each time point (0, 2, 42 d of cold treatment at 2°C) for 'Frida' and 'Jonsok'. The corresponding 2DE spot images are presented beneath each graphed bar.

chilling sensitive to a more tolerant maize (*Zea mays*) line suggested that the reduced activities of APX, catalase, and monodehydroascobate reductase may contribute to lower chilling tolerance at the early stages of development in maize (Hodges et al., 1997). Thus the level of activity of these enzymes before cold stress appeared important for cold tolerance.

Arabidopsis annexin1 has peroxidase activity and overexpression and knockout experiments have demonstrated a significant contribution to stress tolerance (Konopka-Postupolska et al., 2009). Interestingly, distinct annexin1 isoforms were found in 'Jonsok' and 'Frida'. The difference in mass and charge may be due to posttranslational glutathionylation as observed in Arabidopsis (Konopka-Postupolska et al., 2009).

L-Galactono-1,4-lactone dehydrogenase 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 L-Galactono-1,4-lactone dehydrogenase intermediate can enhance oxidative stress tolerance (Zhao et al, 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, IFR, 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 four different aldo-keto reductases identified, corresponding to spots 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 acid β-oxidation (Germain et al., 2001). One of the two 3-ketoacyl-CoA thiolases (spot 6539) reached a

10-fold-higher level in 'Jonsok' at 42 d due to a significant decrease in 'Frida'. Another thiolase isoform (spot 3602) demonstrated a 1.3-fold cold induction in 'Jonsok' at 2 d. Glutathione transferases (GSTs) are cytosolic dimeric proteins involved in cellular detoxification by catalyzing the conjugation of glutathione with various electrophilic compounds, including oxidized lipids. Two proteins (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 after exposure to H_2O_2 (Chen et al., 1996) and a recent review (Dixon and Edwards, 2010) highlights evidence for the diverse functional roles of GSTs beyond GST activities. Glyoxalase I (lactoylglutathione lyase) detoxifies the highly toxic methylglyoxal, a by-product of glycolysis. Methylglyoxal detoxification involves the glyoxalase I catalyzed formation of lactoylglutathione and subsequent conversion to lactate and glutathione by glyoxalase II. The production of methylglyoxal 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 exceeded 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 IFR-related protein (spot 1423), exhibiting 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 a reduction of oxidative stress during the initial cold exposure, throughout overwintering, and later in the spring recover phase.

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 end point 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 seven enzymes leading from Fru-1,6-bisphosphate to ethanol, five are either at levels higher than those found in 'Frida' or accumulate after 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 approximately 1.5-fold compared with control), as well as 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 nonsymbiotic 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.

Additional Proteins Identified

Additional proteins related to freezing/cold tolerance that distinguish the 'Jonsok' profile from 'Frida' include enolase (spot 3626) and four distinct heat shock proteins (HSPs; spot 812, 813, 1819, 2743). Enolase has strong homology to the LOS2 enolase gene in Arabidopsis, a bifunctional 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 Zn-finger protein can act either positively or negatively in regulation of abiotic stress (Mittler, 2006). In Arabidopsis, the chilling-sensitive mutant, los2, has impaired stress-responsive gene expression that appears independent of the CBF expression pathway (Lee et al., 2002). 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 'S. Sengana' at 42 d (1.7-fold) but it was not cold induced in 'Jonsok'. However, enolase levels, prior to cold acclimation, have been reported to correlate with increased freezing tolerance (Takahashi et al., 2006). Three of the four 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 requisite for cold acclimation. All HSPs identified were present at greater levels in 'Jonsok' than 'Frida' before cold treatment. Proteins identified in this study included those which, to our knowledge, have not been previously shown to be altered in cold-tolerant plants or responsive to cold (although in some case they might have been implicated in cold tolerance). From 2DE analysis, these include aldo/keto reductase (spot 5318), Fra a1 proteins (spots 3114, 4106) that share homology to pathogenesis-related proteins

(PR10). Since these had not been previously recognized from microarray analysis, it is possible they represent posttranscriptional regulation or posttranslational modifications (that resulted in distinct spots).

The LFQP analysis identified a phosphate abc transporter (accession 89555622), argininosuccinate synthase (accession 89545626), and a 60S ribosomal protein (L5, L16; accession 158378367).

Identification and Quantification of 'Jonsok' and 'Frida' 2-d Cold-Responsive Proteins by LFPQ Approach Corroborates and Extends 2DE Findings

An alternative, highly quantitative, high-throughput proteomic method (Higgs et al., 2005) was applied to detect smaller but statistically significant changes in protein expression after 2-d cold treatments (Supplemental Data S3). This method was also conjectured to detect additional proteins since 2DE analysis is not optimal for membrane-associated proteins or highly basic proteins. It is also important to note that the LFQP 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 five biological replications. Each biological replication was injected twice and the two technical replicate intensity values were averaged. This approach identified peptides corresponding to 2,017 distinct ESTs or protein sequences (gene identifiers, in the National Center for Biotechnology Information [NCBI]). Five-hundred seventy-one (28%) proteins were identified with the highest quality, indicating a peptide ID confidence value >90% with multiple peptides (distinct sequences) identified for each EST. Of these, 135 were found to significantly differ based on $P <$ value 0.05, ANOVA.

LFQP identified 21 ESTs that correspond to the ESTs identified by 2DE and the majority of these corroborated the 2DE findings (Table III). ADH, β-1,3-glucanase, and thaumatin-like proteins were among the highest ranked proteins for distinguishing 'Jonsok' from 'Frida' in both methods. Likewise, CHS, F3H, and Met synthase were among the highest ranked for 'Frida'. When the trends did not agree, as demonstrated by the ANR, and annexin, the differences may be due in part to posttranslational modifications. For example, the annexins have previously been shown to have an *S*-glutathionylation modification (Konopka-Postupolska et al., 2009).

Based on protein function (same protein name but different EST), LFQP identified CHS, F3H, DFR, and ANR, Met synthase, or *S*-adenosyl-Met synthetase (SAMS) as exhibiting differential accumulation in 'Frida' as seen for 2DE at one or both time points. SAMS was significantly more abundant in 'Frida' at 0 and 2 d in both LFQP analysis and 2DE. In 'Jonsok' only, SAMS was cold induced (1.2-fold by LFQP; 4-fold by 2DE) at 2 d. Similarly, LFQP, identified ADH, β-1,3-glucanase, thaumatin-like proteins, enolase, or Fra2 proteins as exhibiting greater levels in 'Jonsok' than 'Frida' (consistent with the 2DE analysis). With respect to cold-induced proteins, allene oxide cyclase ranked highest with a maximum fold increase of 1.3-fold in both 'Frida' and in 'Jonsok'. This protein was not identified in the 2DE analysis. All the proteins identified by LFQP as significantly different between 'Jonsok' and 'Frida' and those changing in response to cold treatments are shown in Supplemental Data S3. While the LFQP results were generally qualitatively in agreement with the 2DE, quantitatively smaller responses were observed. It is likely that the LFQP approach identified the summative changes in multiple isoforms of the various proteins, while the advantage of 2DE is that unique isoforms could be distinguished. Many cold-responsive proteins observed after 2 d indicate rapid adjustments of protein levels in the less-tolerant cultivars to those elevated levels found constitutively in the most cold-tolerant cultivars. For instance, an ATP synthase α-subunit shows an overall cold accumulation in all cultivars to a similar ending level for 42-d cold treatment. Interestingly, the two most freezing-tolerant cultivars, 'Jonsok' and 'S. Sengana' exhibit less cold induction due to the constitutive elevated levels existing for this protein (i.e. prior to cold exposure). The significance of this protein, as well several other proteins (e.g. enolase, Fras, and HSP70) indicate the most freezing-tolerant cultivars are poised for enduring rapid changes in temperature, consistent with cultivar differences in capacity or rate for cold acclimation. The importance of comparing closely related species that differ in cold tolerance can reveal proteins that may contribute to cold tolerance but lack significant cold induction.

Analysis of Cold-Responsive Transcripts in Strawberry

The cold acclimation process in plants is associated with the changes in expression of numerous characterized cold-responsive genes. To investigate the changes in gene expression at the mRNA level, quantitative (q)PCR was performed for six transcripts, two of which were dehydrins (Fig. 7). Dehydrin levels strongly correlate with freezing tolerance, are regulated by the CBF cold-responsive pathway, and when overexpressed, increase cold tolerance (Hara et al., 2003; Houde et al., 2004; Puhakainen et al., 2004). Strawberry dehydrins of the acidic class (a SK2 type, COR47-like dehydrin) and the basic classes (a Y2SK2 type, XERO2-like dehydrin; Koehler et al., 2007) were examined. While transcript levels of the XERO2-like dehydrin increased in both 'Jonsok' and 'Frida' in response to cold, the rate of increase was significantly greater in 'Jonsok' than 'Frida'. The highest levels occurred at the 42 d time point with an overall 447-fold increase for 'Frida' and 2,500-fold increase for 'Jonsok' compared with the 0 h control. The Cor47-like transcript (SK2) showed a rapid but transient cold response accumulation for both 'Frida' and 'Jonsok' (6- and 18-fold, respectively, at 1 d).

Table III. *Proteins identified in both LFQP and 2DE analysis*

From LC-MS/MS-based LFQP analysis, 135 ESTs were identified as significantly different between 'Jonsok' and 'Frida' at control (0 d) and 2 d cold (2 d) 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' (J) or 'Frida' (F) 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 and $P < 0.05$, Student's t test for 2DE. GADPDH, Glyceraldehyde-3-P dehydrogenase; n.s., not significant; TPX, thioredoxin-dependent peroxidase.

Code gi	Protein ID	LFQP	SigLFQP	Sig2DE	Agree	2DE Spot
113436	ADH	J	0 d,2 d	0 d, 2 d	Yes	6540
158356647	β-1,3-glucanase	J	2 d	0 d, 2 d	Yes	2317
158371950	Enolase	J	0 d	0 d, 2 d	Yes	3626
89557236	Fra a 2	J	0 d	0 d	Yes	4015
158366345	Thaumatin-like	J	0 d, 2 d	0 d, 2 d	Yes	2203
158379507	Actin	F	0 d, 2 d	0 d	No	1125
89544075	Annexin	F	0 d	0 d, 2 d	Yes, no	6416, 6432
89550344	Annexin	F	0 d	0 d, 2 d	Yes	4308
90576646	ANR	F	0 d, 2 d	0 d, 2 d	No	3515
110564477	ANR	F	0 d, 2 d	0 d, 2 d	Yes, yes	4520, 2525
71979908	CHS	F	0 d, 2 d	0 d, 2 d	Yes, no	4526, 4534
24636275	Citrate synthase	F	0 d	0 d, 2 d	Yes	6611
51493451	F3H	F	2 d	2 d	Yes	4536
158302779	GADPDH	F	0 d	n.s.	No	8409
51047667	IFR	F	0 d, 2 d	0 d, 2 d	No	1423
158372608	Glyoxalase I	F	0 d	0 d, 2 d	No	1315
89551239	Met synthase	F	0 d, 2 d	0 d, 2 d	Yes	6808
6760443	OMT	F	0 d	n.s.	No	3326
158353550	Proteasome subunit	F	0 d	0 d, 2 d	Yes	7210
158361609	Quinone reductase	F	0 d	0 d	Yes	2108
158374908	TPX	F	0 d	0 d	Yes	2102

Two additional dehydrin transcripts (most similar to the Arabidopsis Erd10 and Erd14, ESTs were DV438327 and DV439798, respectively) had virtually identical expression patterns (data not shown) to the Cor47-like transcript. Levels of *Fragaria* Cold-Regulated1 (Fcor1) and Fcor2 transcripts were previously shown to be correlated with freezing tolerance (NDong et al., 1997). Similar to the observations of NDong et al. (1997) it was found that Fcor1 accumulation was transient while Fcor2 accumulation was more sustained over a 2-week period. Consistent with the association with freezing tolerance, it was found that accumulations of these transcripts were significantly greater in the more cold-tolerant 'Jonsok' than in 'Frida'. Cold-responsive transcription factors (CBF1, -2, -3) are transiently expressed, generally peaking at less than 1 d after cold stress (Gilmour et al., 1998; Jaglo et al., 2001). Examination of a transcript most similar to the Arabidopsis CBF4 (FaCBF4, the only sequence available when this work was begun) revealed a very late response, accumulating at 42 d in both 'Frida' and 'Jonsok', and was practically undetectable at earlier time points in cold treatments. In other plant species, CBF4 is thought to be primarily associated with desiccation/drought responsiveness (Haake et al., 2002). F3H transcripts decreased significantly in response to cold treatment in 'Jonsok' but increased transiently in 'Frida'. Importantly, the changes in the levels of the F3H transcript qualitatively reflect the changes observed at the protein level; the transient accumulation of F3H protein in 'Frida' and the lower levels of F3H protein in 'Jonsok' (Fig. 4; Supplemental Data S1) are adequately explained by changes in levels of F3H transcript.

Dehydrin Protein Accumulation in 'Jonsok' and 'Frida'

The expression of dehydrins is highly correlated with cold-stress tolerance in a number of plant species (Hara et al., 2003; Renaut et al., 2004; Peng et al., 2008) including strawberry (Houde et al., 2004). The levels of some of these proteins are controlled by the cold-response pathway CBF transcription factors (Lee et al., 2005). Dehydrin transcript analysis, discussed above, revealed dehydrin accumulation at higher levels in 'Jonsok' than 'Frida' at several time points (particularly Y2SK2, the XERO2-like dehydrin). As no dehydrin was identified in either of the proteomic approaches, to address dehydrin protein levels, one-dimensional electrophoresis western-blot analysis using an anti-K peptide (diagnostic for dehydrin) was performed on the strawberry crown tissues (Supplemental Fig. S5). A

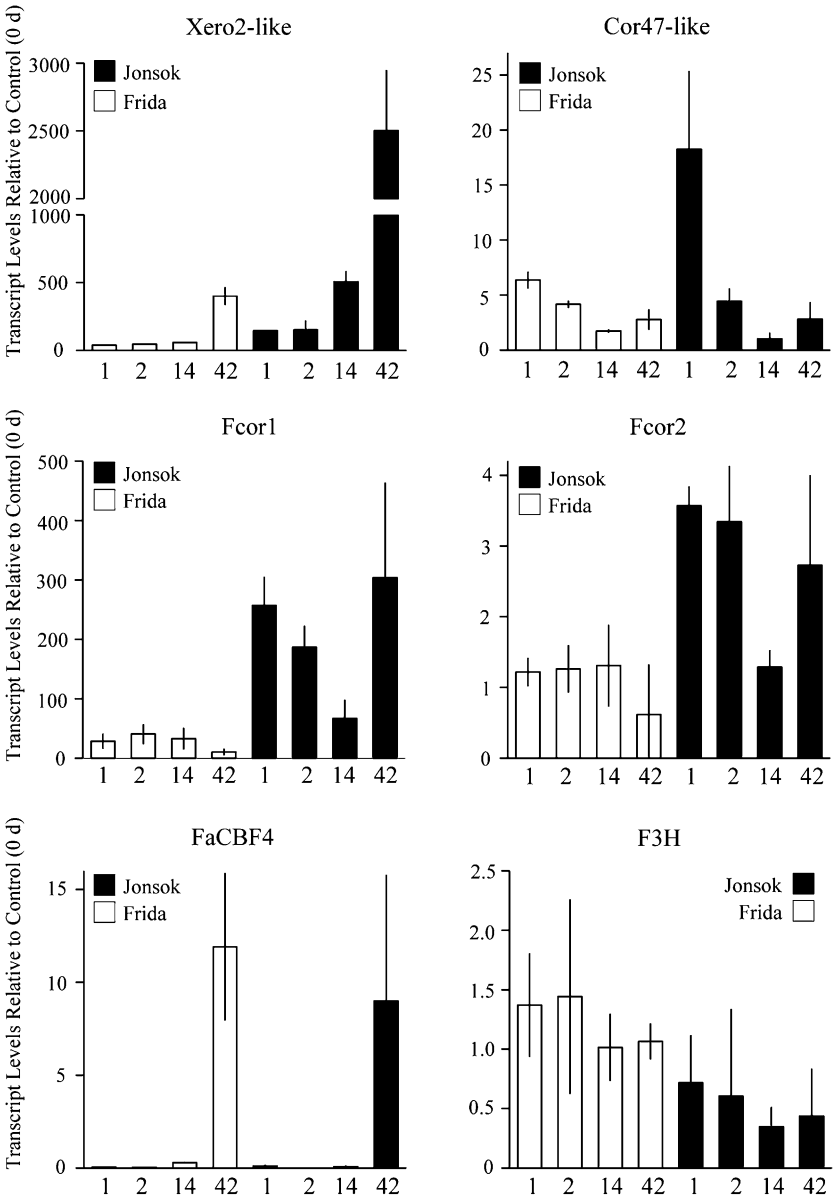

Figure 7. Transcript levels of six distinct genes from strawberry crown tissue during cold acclimation as measured by qPCR. Data are expressed transcript levels at cold-treated time points (1, 2, 14, and 42 d at 2°C) relative to control (0 d). Actin was utilized as a reference transcript for normalization in two independent qPCR reactions, each with three to five biological replicates per time point.

strong accumulation of dehydrin protein band at the 42-d cold treatment was observed (no detectable band at 0 or 2 d cold). The different dynamics of expression of the two dehydrins transcripts, COR47-like and XERO2-like, suggest different temporal roles for these proteins. While the XERO2-like dehydrin shows a consistent increase over the duration of the cold treatment, the increase in levels of the COR47-like dehydrin transcript is greatest after only 1 d of cold. It should also be considered that transcript and protein levels accumulation are not always concomitant. In one study dehydrin protein was shown to increase 10-fold when there was no apparent increase of mRNA (Gao et al., 2009). We observed a strong increase in levels of a dehydrin protein that we can only speculate is the XERO2-like dehydrin (Supplemental Fig. S5). The observed decline of a COR47-like transcript in *Fragaria* crowns is not a typical finding with respect to other plants and may reflect specificity for crown tissue. Dehydrin proteins do appear to accumulate significantly slower in *F. vesca* crown tissue (Supplemental Fig. S5; J. Davik, B. From, G. Koehler, T. Torp, J. Rohloff, P. Eidem, R. Wilson, A. Sønsteby, S. Randall, and M. Alsheikh., unpublished data) than what has been observed for other known and well-studied model systems like Arabidopsis. This might be a consequence of the strawberry crown; a largely nonphotosynthetic and exceptionally less-studied plant tissue. Since some dehydrins have been shown to have light-regulated accumulation, this factor could conceivably impact dehydrin accumulation in these partially subterranean tissues. This finding reiterates the importance of studying cold responses for specific tissues in different plants.

Is 'Jonsok' Poised Physiologically for Cold Tolerance?

In 'Jonsok', elevated constitutive levels of many proteins associated with cold tolerance suggested that this cultivar might be physiologically poised for stress tolerance in general and cold tolerance in particular. To address this possibility we performed a freezing-tolerance experiment where we compared the four cultivars of domestic strawberry for their cold tolerance (freezing) prior to a cold acclimation treatment (Supplemental Table S2). All cultivars were much more susceptible to cold damage (all plants died at temperatures less than −3°C) than the cold-acclimated plants. By comparing the results prior to and after acclimation (Supplemental Tables S1 and S2), the acclimation treatment is seen to be crucial for adaptive cold tolerance (an increase of 5°C–8°C in cold tolerance as measured by plant survival was achieved by cold acclimation). 'Jonsok' was more cold tolerant than 'Frida' (and 'Elsanta'), even prior to acclimation. This finding supports the hypothesis that the 'Jonsok' cultivar (and 'S. Sengana', a parent of 'Jonsok') is physiologically poised for cold tolerance.

CONCLUSION

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', is poised for tolerating cold stress (Supplemental Table S2), and this is contributed by proteins related to freezing/cold tolerance that are constitutively expressed at significantly elevated levels than those in 'Frida'. This poise has been observed in other species (Taji et al., 2004; Takahashi et al., 2006). We speculate the elevated levels of enolase in 'Jonsok', may contribute to this physiological stress poise. Enolase is a negative regulator of ZAT10 (Mittler et al., 2006), which itself can be a negative regulator of the CBF pathway. 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. Second, 'Jonsok' responds to cold more rapidly, particularly noticeable when examining mRNA responses. Lastly, the convergence of protein expression in the two cultivars, visualized by 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.

Most previous approaches to understand winter hardiness have focused on molecular responses to cold acclimation. This study, through the comparison of two cold-tolerant cultivars, which differ in their extent of cold hardiness, has revealed a variety of differences in expression of proteins involved in stress responses. Interestingly, both varieties showed similar CBF responses, though different in extent and perhaps timing. These cold acclimation responses, we believe, are illustrated by the convergence of expression patterns visualized by PCA analysis. 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 that are candidates for molecular markers associated with overwintering success.

MATERIALS AND METHODS

Plant Material and Experimental Design for Freezing Experiment

Strawberry (*Fragaria* × *ananassa*) runners were collected from the field and rooted in a heated greenhouse maintained at 20°C ± 2°C and 20-h light/4-h dark for 2 weeks in 50 × 30 cm rooting trays (4.5 × 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% nitrogen and 19% calcium) and Superba Rød (7-4-22, nitrogen-phosphorus-potassium plus micronutrients) from Yara International. The plants were then hardened for 6 weeks at 2°C and 10-h light/14-h dark at 90 μmol quanta m^{-2} s^{-1}. This low light level was chosen to simulate light exposures in the field as the crown is partially subterranean and when grown in temperate climates are often covered by straw or snow. After hardening, the plants were exposed to freezing temperatures ranging from −3°C to −12°C. The freezing was performed in darkness in freezing cabinets starting at 2°C. Temperatures were adjusted by a cooling rate of 2°C 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°C for 24 h, whereupon the plants were moved into a greenhouse maintained at 18°C ± 2°C 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 regrowth). 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). All experiments were replicated with three randomized blocks of three to four plants for each population, giving a total of nine to 12 plants of each population in each treatment. ANOVA analyses (Supplemental Table S1) were performed by standard procedures using a MiniTab statistical software program package (Release 15; Minitab Inc.). The freezing conditions, the scoring details, and the origin and parents of the four cultivars used are summarized in Supplemental Materials and Methods S1.

Plant Material for Protein and Transcript Analysis

Plant cultivation was carried out as described above (freezing experiment). The plants were cold hardened at 2°C and 10-h light/14-h dark at 90 μmol quanta m^{-2} s^{-1} for either 0, 2, or 42 d. Tissue was harvested by dividing each crown longitudinally and immediately frozen in liquid nitrogen and stored in −80°C. 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 four to six half crowns that were cut longitudinally for proteomic experiments and the corresponding four to six half crowns for reverse transcription (RT)-qPCR.

Sample Preparation for 2DE

Tissue was ground to a fine powder in liquid nitrogen in the presence of polyvinylpolypyrrolidone at 10% of tissue weight. The powder was washed twice with cold 100% acetone with centrifugation at 8,000 rpm at <0°C for 20 min (Sorval SS-34 rotor, 7,649*g* average). The powder was then dried under vacuum (−78°C) to remove acetone. A phenol extraction followed by methanolic ammonium acetate precipitation was then performed as follows. Tris-buffered phenol, pH 8.8 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 number 5 (Brinkman Instruments) for 30 s. The extraction buffer used contained 40% Suc w/v, 2% SDS w/v, 1× 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°C with agitation for 30 min followed by centrifugation at 7,000 rpm (Sorval-34 rotor, 5,000*g* average) for 15 min at 4°C. The upper phenol phase was removed and the lower phase was reextracted with 5.0 mL of Tris-buffered phenol. Back extraction was performed on the combined upper phases by adding equal volume of extraction buffer. After extraction, proteins were precipitated by adding 5 times the volume of 0.1 M ammonia acetate in 100% methanol overnight at −78°C. The pellet was recovered by centrifuging at 7,000 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°C for 30 min between washes. The final pellet was air dried (approximately 5 to 10 min). Pellets (approximately 4.0 μg) were dissolved in approximately 600 μL of isoelectric focusing (IEF) buffer containing 8 M urea, 2 M thiourea, 2% CHAPS, 2% deionized Triton X-100, 50 mM dithiothreitol (DTT), and 0.5% pH 3 to 10 ampholytes. An Amido black assay (Kaplan and Pedersen, 1985) was used to determine concentration of protein. One to three milligrams of protein was extracted per gram of crown fresh weight.

2DE

IEF strips (24 cm, nonlinear pH 3–10, Bio-Rad) were passively rehydrated with 400 μg of protein at 20°C for 14 h. Rehydration buffer included IEF buffer with 0.0005% bromophenol blue. Samples were then rinsed with water and focused at 20°C using a Protean IEF cell (BioRad) using the following parameters: 100 V for 300 Vhr, 300 V for 900 Vhr, 5,000 V for 35,000 Vhr, and 8,000 V for 53,800 Vhr all with rapid ramps. Total Vhr was 90,000 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, and 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 1× 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°C.

2DE Gel Imaging and Data Analysis

Gels were fixed with 40% methanol and 10% acetic acid in water for 3 h. Gels were washed three times in water for 15 min each and stained for a minimum of 72 h 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 (four cultivars, three conditions, each in triplicate) were analyzed using PDQuest version 7.1 (Bio-Rad Laboratories). Molecular mass (kD) and isoelectric point (pI) values were assigned to spots by performing a separate experiment running internal 2DE SDS-PAGE standards (Bio-Rad Laboratories) with the same electrophoresis parameters as described above except using 100 μg protein ('Jonsok' at 0 d) and subsequently applying the determined molecular mass and pI values to the larger experiment. In addition to the 2DE internal standards used to determine mass and pI, one protein, strongly identified as the elongation factor 1-α (spot 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. Average intensities, standard deviation 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) $P < 0.05$, ANOVA, and PCA. All 2DE data were 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 pretreatments 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), 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 PCs. For ANOVA, significance was set at $P < 0.05$ and the Tukey's honestly significant difference test was used to analyze the difference between groups.

Protein Identification by LC-MS/MS (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°C. The tryptic peptides were extracted with 30%, 50%, and 100% ACN sequentially. The extracted peptides were dried by SpeedVac and reconstituted with 5% ACN in 0.1% formic acid (FA). 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 × 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% to 20% B (90%–80% A) for 5 min, 20% to 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 electrospray ionization 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 mass-to-charge ratio (low) and 1.50 mass-to-charge ratio (high). The acquired data were searched against NCBI protein sequence database of *Fragaria vesca* and strawberry (downloaded on February 12, 2009 from http://www.ncbi.nlm.nih.gov/, 574 entries) and Rosaceae (downloaded on February 12, 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 (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 cutoff: (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.

Protein Identification by LC-MS/MS (Confidence Values Listed as *q* Values)

To build the *Fragaria* protein database, the strawberry and *F. vesca* protein Fasta database and EST sequence databases for taxonomy ID 3,747 and 57,918 were downloaded from NCBI. The ESTs were translated in three different reading frames and the largest protein among three reading frames was chosen. The strawberry 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 45,793. Database search was done using Sequest and X!Tandem algorithms.

LFQP

These experiments were conducted and analyzed essentially as described in Wang et al. (2008). The time points used for this experiment consisted of the 0 and 2 d exposure to 2°C. 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) with a C18 capillary column in random order. Peptides were

eluted with a linear gradient from 5% to 45% ACN developed over 120 min at a flow rate of 500 nL/min and the effluent was electro sprayed into the LTQ mass spectrometer (Thermo Fisher Scientific). 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 was the same as described for 2DE protein identification by LC-MS/MS with confidence values determined as q values (Higgs et al., 2005). LFQP raw data for the 2,017 identified proteins and additional information for the top 135 proteins (significantly different identified with the highest confidence) as compared with the 2DE analysis is available in Supplemental Data S3.

RT-qPCR

Total RNA was isolated from frozen crown tissues (approximately 100 mg) dissected from control (20°C at 0 d) and cold-treated (2°C, time points 1, 2, 14, and 42 d) strawberry plants 'Jonsok' and 'Frida' (each with three to five biological replicates per time point) according to a modified cetyl trimethylammonium bromide protocol. Total RNA (1 μg) of each sample was treated with amplification grade DNase I (Invitrogen) according to manufacturer's instructions. RT of the samples was performed using a mixture of random hexamers and oligo-dT12-18 primers and the SuperScript III first-strand synthesis system (Invitrogen) according to the manufacturer's instructions. To control for nondegraded, contaminating genomic DNA, selected replicate samples were included in which water replaced the Superscript III enzyme in the RT reactions. The resulting single-stranded template complementary DNAs were used in two independent qPCR reactions with gene-specific primers designed to produce 100 to 300 bp amplicons representing the target transcripts Rab18-like (CX661424; *F. vesca* ortholog), Cor47-like (CO817504), Fcor1 (HQ910514, sequence from NDong et al., 1997), Fcor2 (sequence from NDong et al., 1997; represented by CO817469), F3H (AB201760), and FaCBF4 (HQ910515, amplified by degenerate PCR using the sense primer 5′-ATGCTYGAGTCTTCTTCTCAYTC-3′ and the antisense primer 5′-CATRTCRTCTCCTCCGTATCC-3′, cloned and sequenced using BigDye terminator chemistry v3.1, Applied Biosystems). Actin (AB116565) was utilized as a reference transcript for normalization. The qPCR reactions contained 12 μL 10-2 diluted complementary DNA template, 1× SYBR green power master mix, and 0.1 μM of each primer in a final volume of 25 μL and were subjected to standard two-step PCR (annealing/extension temperatures between 60°C and 63°C) for 40 cycles using the 7500 real-time PCR system (software version 1.3; Applied Biosystems). Each gene-specific primer pair generated a single PCR product of expected size and sequence as determined by post-PCR dissociation analyses, agarose gel electrophoresis, and direct sequencing using BigDye terminator chemistry (v3.1, Applied Biosystems). Raw fluorescence data from the qPCR were analyzed using the LinRegPCR program (Ruijter et al., 2009) to determine fluorescence baseline correction and threshold values; these values were used to calculate the fold change in steady-state target transcript levels according to Pfaffl (2001). The following primers are listed as oligo sequences in order of sense and the antisense 5′ to 3′: FaFcor1, GCCTATGTAGTTTTCACCGTTG, TCATAAAGCTAGTGATACCCTCCA; Fcor2, GGGTGGCTCTCGTCAACTAC, CCAAGAGCTACTTTCCCACTTC; Rab18-like, CGTCACTACTCTCACTCGCTC, TCACTCCCCTATCCTGCTG; FaCOR47, GAGGAAGGAGACGATGAAGAG, CCTTCTTCTGCTCCTCTGTGTAG; FaF3H, ACCTCACTCTCGGACTCAAAC, GAGCTGGGTTCTGGAATGTC; FaCBF4, TTCAAGGAGACGAGGCAC, CGCAGCCATTTCGGTA; and FaActin, GGGTTTGCTGGAGATGATG, CACGATTAGCCTTGGGATTC.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers HQ910514 (Fcor1), CO817469 (Fcor2), CX661424 (Rab18-like), CO817504 (FaCOR47), AB201760 (FaF3H), HQ910515 (FaCBF4), and AB116565 (FaActin).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Agglomerative hierarchical clustering (AHC).

Supplemental Figure S2. 2DE maps illustrating the proteins differentially accumulated in 'Jonsok' and 'Frida'.

Supplemental Figure S3. Volcano plot of 2DE proteomic data set for 'Jonsok' and 'Frida' at 42 d.

Supplemental Figure S4. Gene ontology annotation for identified proteins.

Supplemental Figure S5. Dehydrin accumulation in strawberry 'Jonsok' and 'Frida'.

Supplemental Table S1. Freezing survival demonstrates the relative cold/freezing tolerance of strawberry cultivars.

Supplemental Table S2. Freezing survival demonstrates the relative cold/freezing tolerance of strawberry cultivars prior to cold acclimation.

Supplemental Data S1. List of 2DE spots (900) matched between all cultivars.

Supplemental Data S2. List of 2DE spots (109) identified by LC-MS/MS.

Supplemental Data S3. LFQP data.

Supplemental Materials and Methods S1. Physiological freezing experiment details.

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