Running title: Cold Responses in Cultivated Strawberry

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Proteomic Study of Low Temperature Responses in Strawberry Cultivars (*Fragaria* x *ananassa* Duchesne) that Differ in Cold Tolerance

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ABSTRACT To gain insight into the molecular basis contributing to overwintering hardiness, a comprehensive proteomic analysis comparing crowns of *Fragaria* × *ananassa* (octoploid strawberry) 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 2DE analysis and 135 proteins from label-free quantitative proteomics (LFQP) were identified as significantly differentially expressed in crown tissue from the two strawberry cultivars exposed to 0, 2, and 42 day 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”.

Keywords: *Fragaria* × *ananassa*; Strawberry breeding; Cold stress; Freezing tolerance; LC-MS/MS; Proteomics; Two-dimensional electrophoresis

INTRODUCTION

Strawberry 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, apples, cherries, blackberries, and raspberries), 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 which 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, 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 hardiness observed for *F.* × *ananassa* 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, 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 (Chinnusamy et al., 2007; Guy et al., 2008; Heino and Palva, 2004; Ruelland et al., 2009; Zhu et al., 2007). One important cold signaling pathway, controlled by C-repeat/drought-responsive element binding factors (CBFs) has been shown to enhance freezing tolerance in Arabidopsis (Gilmour et al., 2000; Jaglo-Ottosen et al., 1998). Genes encoding CBF's have been identified in sour cherry (*Prunus cerasus* L) and strawberry (*F.* × *ananassa*) (Kitashiba, 2003; Owens, 2002). 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 reactive oxygen species, 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. In order 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; Wisniewski, 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; McBeath, 2002).

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

With the aim of developing new cultivars with improved overwintering hardiness, we describe a proteomic map for the 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 bio-markers are identified which can be utilized to facilitate conventional breeding endeavors for cold tolerant cultivars of strawberries.

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, and Supplemental Methods S1)**. In particular, survival rates were significantly different after 48 hour treatments at -6 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 (2DE) Maps of F. × ananassa Crown Tissue The major overwintering structure of strawberries, the crown, was evaluated for changes in proteins which might be associated with enhanced cold tolerance or winter survival. Clonal lines of mature strawberry plants, six-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 6) were included for each replicate thereby minimizing the biological variance. Each crown was divided and used for 2DE analysis, or for LFQP analysis and half the crown was retained for transcript analysis (see methods). A total of 168 plants from all cultivars were used to complete 3 experimental time points in triplicate requiring 36 2DE gels in total. Nine hundred well resolved spots were detected by colloidal Coomassie-stained gels within a range from 4 to 9 pH units and 15 to 100 kDa MW 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 LC-MS/MS (**Fig. 1**).

Agglomerative Hierarchical Clustering (AHC) of 2DE Data 2DE proteome profiling patterns were compared for *F.* × *ananassa* 'Elsanta', 'Frida', 'S. Sengana', and 'Jonsok' for the 0, 2 and 42 days of cold treatment (2 °C) by using agglomerative hierarchical clustering (AHC) on all 900 2DE matched spots. The Euclidean distance was used to measure the similarities between samples and Ward's algorithm was used to form clusters. The dendrogram resulting from AHC analysis is presented in **Supplemental Figure S1**. The replicates for each cultivar at 0 and 2 days form clusters that are distinct from the other cultivars and from the 42 d cold treatment. After 42 d of cold treatment, three cultivars ('Jonsok', 'Frida' and 'Elsanta') form a new cluster. Each cultivar remains distinct within this 42 d cluster although one 'Jonsok' 42 d replicate formed its own branch. The 'S. Sengana' clustered separately at all time points, suggesting that this cultivar is not as responsive to cold treatments as the other cultivars. 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 (PCA) 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 principle components (PC), 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 day cold treatments. Interestingly, the long-term (42 d) cold treatment caused a large shift in the PC1 dimension and simultaneously reduced the differences between the cultivars in the PC2 dimension. This suggests the greatest overall differences in the cultivars exist under control and 2 day cold treatments, while the protein expression patterns tend to converge after 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. In order 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 two-dimensional electrophoresis (2DE), nine hundred 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 cut-off in order to identify the 2DE spots differentially regulated upon cold treatment (threshold ratio cold-stressed vs. control plants > 2 or < 0.5-fold).

The overall trends in cold responsive proteins were specifically evaluated for 'Jonsok' and 'Frida'. Both cultivars showed a similar total number of proteins significantly increasing or decreasing during cold treatment (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 to 9 (1.0%) and 58 (6.4%) spots in ‘Frida’. The protein spots that decreased in response to the cold treatment at 2 and 42 d were 16 (1.8%) and 118 (13.1%) in ‘Jonsok’ and 18 (2.1%) and 157 (17.4%) in ‘Frida’. One of the 18 proteins that increased in both cultivars at 42 d was identified as alcohol dehydrogenase. Among the 41 proteins that decreased in both cultivars, three were identified as glucose-6-phosphate isomerase, a putative 20S proteasome beta subunit 5, and a calcium-dependent protein kinase. Only one protein (Cu/Zn superoxide dismutase) decreased at all time points in both ‘Jonsok’ and ‘Frida’ though it remained significantly higher in ‘Jonsok’ at all time points. Several proteins that were observed 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 alcohol dehydrogenase. Likewise, proteins in ‘Jonsok’ that were cold responsive and approached, but did not reach the levels in ‘Frida’ were identified as lipoxygenase, glyceraldehyde-3-phosphate dehydrogenase, and S-adenosylmethionine synthase. Together, these changes partially explain the convergence in overall protein expression levels observed in the PCA analysis (**Fig. 2**).

Interestingly, less than half of the cold-responsive protein spots were in common between the two cultivars (**Fig. 3B and 3C**). 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 day 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 were good "anchors" for the gel analysis. After identifying Arabidopsis homologs, the GO 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 over represented. In the Molecular Function category, the identified proteins were under-represented in DNA or RNA binding, transcription factor activity, nucleic acid binding and over-represented 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 (Dixon and Pasinetti, 2010; Koes et al., 1994; Vogt, 2010; Winkel-Shirley, 2001).

Eighteen of the 109 2DE identified spots (not including the four Fra a 1's, which are only speculative participants in this pathway) correspond to proteins involved in the phenylpropanoid pathway. A significant number of these were enzymatic components contributing to the flavonoid biosynthetic process catalyzing 8 biosynthetic steps in the pathway and 4 additional proteins indirectly involved in the flavonoid pathway (**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 (ANS) for the pool of flavan-3, 4-diols. It has a reported involvement in the biosynthesis of condensed tannins. ANR was identified in three spots that mapped to two distinctive ESTs. At 42 d, 'Jonsok' showed 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 d and 42 d and nearly absent in 'Jonsok'. Though it is possible that the different isoforms impart different specificity for substrates; the net effect of the changes of all ANR spots was insignificant. Proteins more abundant in 'Jonsok' include O-methyltransferase (OMT), and isoflavone reductase-related protein (IFR). 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 isoflavone reductase (IFR, spots 1423, 4420) mapped to distinct ESTs. Both were more abundant in 'Jonsok' at 0 d. 'Jonsok' maintained a 2-fold or higher level of IFR (spot 1423) than 'Frida' while IFR (spot 4420) levels were not deemed significantly different at 2 and 42 d. This suggests that different flavonoid metabolites could contribute to overwintering tolerance in 'Jonsok'. Other enzymes in this pathway did not show these large differences, e.g., chalcone isomerase (CHI) while cold-responsive, decreasing in the cold after 42 d (~1.5-fold), was not significantly different between the cultivars. Cinnamyl-alcohol dehydrogenase (CAD), a molecular marker specific for lignification (Walter et al., 1988), increased slightly in 'Frida' at 42 d 1.24-fold (t-test < 0.1) and UDP-glucose glucosyltranferase (UGGT) was approximately 3-fold greater in 'Frida' at 0 and 2 d cold treatment, but not different after 42 d. Caffeoyl-CoA 3-O-methyltransferase (CCoAOMT) was 2-fold higher in 'Frida' at 0 d, yet by 42 d there was no difference due to a significant decrease a in 'Frida' and a significant cold response increase of 1.9-fold in 'Jonsok'. Anthocyanidin synthase (ANS) did not change significantly with regard to cultivar or cold treatment.

Proteins Associated with Pathogen Resistance Overwintering survival requires both freezing tolerance and disease resistance against pathogens. Specific disease resistance induced by cold acclimation has been reported for several crops (Koike et al., 2002; Płażek et al., 2003), with some cold-induced pathogenesis-related proteins exhibiting both antifungal and antifreeze activities (Kuwabara and Imai, 2009). In particular, certain β-1,3-glucanases have been shown to be cold induced and have cryoprotective activity similar to other extracellular pathogenesis-related proteins (Hincha et al., 1997). β-1,3-glucanases

comprise a large and highly complex gene family involved in pathogen defense, as well as a broad range of other biological processes. YPR10 belongs to a group of pathogenesis-related proteins whose function is largely unknown although functions have been speculated to include ribonuclease and proteinase activities (Walter et al., 1996). In the cold-tolerant 'Jonsok', two different β -1,3-glucanase proteins as well as the pathogen responsive protein, YPR10 were identified. A thaumatin-like glucanase (spot 2203) is 70-fold higher in 'Jonsok' than 'Frida' constitutively and accumulated to over 6000-fold higher in 'Jonsok' than 'Frida' after 42 days of cold treatment, largely due to a decrease in the amount found in 'Frida' (**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 three-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 reactive oxygen species (ROS) signaling occurring during low temperature response (O'Kane et al., 1996; Suzuki and Mittler, 2006). Antioxidative proteins are also involved in the recovery phase following stress (Biemelt et al., 1998; Blokhina et al., 2003). Overall, proteins involved in antioxidative and detoxification processes were highly over-represented in 'Jonsok' compared to 'Frida' (**Fig. 6**). Although 'Frida' clearly had an upregulated flavonoid pathway (discussed above) that would be expected to produce a variety of antioxidant compounds; 'Jonsok' has higher levels of enzymes capable of direct, or regulation of, anti-oxidative activity.

The detoxification of ROS is managed through the action of superoxide dismutases which catalyze the dismutation of superoxides into oxygen and hydrogen peroxide, and catalases and peroxidases which further detoxify H2O2 to water (Apel and Hirt, 2004). In 'Jonsok', relative to 'Frida' (from 0 d to 42 d) increased levels of Cu/Zn superoxide dismutase (2 to 11-fold higher), ascorbate peroxidase (2 to 5-fold higher), annexin 1 (395 to 1200-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. Superoxide dismutases play a key role in virtually all organisms exposed to oxygen, and plants are no exception (Sunkar et al., 2006). Despite the observation that Cu/Zn superoxide dismutase (spot 2010) was significantly down-regulated in both 'Jonsok' and 'Frida' at 2 and 42 d, 'Jonsok' levels significantly exceeded those of 'Frida', exhibiting a 2, 5, and 11-fold greater levels at 0, 2, and 42 d, respectively.

Ascorbate peroxidase (APX) which consumes H_2O_2, in conjunction with ascorbate, which is subsequently regenerated by the ascorbate-glutathione cycle, contributes to abiotic stress tolerance; including low temperature stresses (Shigeoka et al., 2002).

The decrease in levels of SOD and APX after the cold treatment could reflect a decrease in demand for protective antioxidants as metabolism inevitably slows. Increased basal expression of SOD and APX in 'Jonsok' is expected to contribute to increased abiotic tolerance. In one study (Lee et al., 2007) simultaneously overexpression of CuZn and APX in tall fescue resulted in an increase tolerance to a wide range of abiotic stresses. In another study, comparison of chilling sensitive to a more tolerant maize 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, 1997). Thus the level of activity of these enzymes before cold stress appeared important for cold tolerance.

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

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

Other enzymes involved in redox reactions, aldo-keto reductase, 3-ketoacyl-CoA thiolase, isoflavone reductase and glutathione-S-transferase were also at higher levels or were cold-induced in 'Jonsok'. Aldo-keto reductases can detoxify lipid peroxidation products and reactive aldehydes (Bartels, 2001). Three of the 4 different aldo-keto reductases identified, corresponding to 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 beta 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 (GST) 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 following exposure to H2O2 (Chen et al., 1996) and a recent review (Dixon and Edwards, 2010) highlights evidence for the diverse functional roles of GSTs beyond "glutathione transferase" activities. Glyoxalase I (lactoylglutathione lyase) detoxifies the highly toxic methylglyoxal, a byproduct of glycolysis. Methyl glyoxal detoxification involves the glyoxalase I catalyzed formation of lactoylglutathione and subsequent conversion to lactate and glutathione by glyoxalase II. The production of methyl glyoxal dramatically increases in response to cold and other stresses and the levels of methylglyoxal are controlled by glyoxalase I (Yadav et al., 2005). Glyoxalase I (spot 1315) increased in 'Jonsok' 1.8-fold at 42 d, and levels significantly 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 isoflavone reductase-related protein (spot 1423), exhibiting a 2-fold higher levels in 'Jonsok' than 'Frida' at all time points, may act in preservation of reductants or synthesis of antioxidants (Petrucco et al., 1996).

Overall, 'Frida' relative to 'Jonsok', had a conspicuous lack of the well-known players with roles in antioxidation and detoxification. The presence of these proteins in 'Jonsok' at constitutive higher levels, before cold treatment, could prophylactically improve cold stress tolerance through 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 endpoint away from lactate and toward ethanol (Drew, 1997). Particularly important is the role for pyruvate decarboxylase to direct flow from lactate to ethanol. In 'Jonsok', of the 7 enzymes leading from fructose-1,6- bisphosphate to ethanol, five are either at levels higher than those found in 'Frida' or accumulate following cold treatment. Thus after 42 d cold treatment, aldolase (4-fold greater in 'Jonsok'), enolase (4-fold greater in 'Jonsok'), pyruvate kinase (3-fold greater in 'Jonsok'), pyruvate decarboxylase (0.7-fold of 'Frida' levels, but is cold induced approx. 1.5-fold compared to control), as well as alcohol dehydrogenase (ADH) are significantly greater than the corresponding enzymes in 'Frida'. Four of the five spots identified as ADH isoforms were higher

than levels found in 'Frida' at 42 d (130-fold, spot 6540; 2.5-fold, spot 6513; 2.0-fold, spot 6505; 1.7-fold). An alternative process to the fermentation pathway for providing electron acceptors; a type I hemoglobin facilitating a nitrate-nitric oxide cycle, has been postulated to be critical for survival in hypoxic environments (Igamberdiev and Hill, 2004). The non-symbiotic hemoglobin class 1 protein (spot 7010), a known hypoxia induced protein increases in 'Jonsok' 1.6-fold at 2 d, and was 2-fold higher in 'Jonsok' than 'Frida' at 2 and 42 d.

Additional proteins identified Additional proteins related to freezing/cold tolerance that distinguish the 'Jonsok' profile from 'Frida' include enolase (spot 3626) and 4 distinct heat-shock proteins (HSPs) (spot 812, 813, 1819, 2743). Enolase has strong homology to the LOS2 enolase gene in *Arabidopsis thaliana* gene, a bi-functional enzyme that acts as a key enzyme in the glycolytic pathway in the cytoplasm and in the nucleus acts as a transcriptional repressor of ZAT10. ZAT10, a zinc finger protein can act either positively or negatively in regulation of abiotic stress (Mittler, 2006). In Arabidopsis, the chilling sensitive mutant, los2, has impaired stress-responsive gene expression which appears independent of the CBF expression pathway (Lee et al., 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 'Senga 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 4 distinct HSPs that were identified by 2DE exhibited a significant cold induction in 'Frida' (spot 812, 813, 1819), yet 'Jonsok' had greater overall levels at all time points except for spot 812 at 42 d, due to the significant induction in 'Frida'. 'Jonsok' shows a 1.9-fold cold induction of spot 813 at 42 d. Molecular chaperones present before cold stress would theoretically poise cellular processes requisite for cold acclimation. All HSP's 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) which share homology to pathogenesis-related proteins (PR10). Since these had not been previously recognized from microarray analysis, it is possible they represent post-transcriptional regulation or post-translational modifications (that resulted in distinct spots).
The LFQP analysis identified a phosphate abc transporter (accession 89555622), argininosuccinate synthase (accession 89545626), a 60S ribosomal protein (L5, L16) (accession 158378367).

Identification and Quantification of 'Jonsok' and 'Frida' 2 Day 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 post-translational 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 2017 distinct ESTs or protein sequences (gene identifiers, in 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 corresponded 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 methionine 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 post-translational 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, methionine synthase, or S-adenosylmethionine 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 d 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 Fra 2 proteins as exhibiting greater levels in 'Jonsok' than 'Frida' (consistent with the 2DE analysis. With regards 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 days 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 alpha-subunit shows an overall cold accumulation in all cultivars to a similar ending level for 42 day cold treatment. Interestingly, the two most freezing tolerant cultivars, 'Jonsok' and 'Senga' 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, Fra's, 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 F. × ananassa 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, qPCR 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 over- expressed, 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 2500-fold increase for 'Jonsok' compared to the 0 hours control. The Cor47-like transcript (SK2) showed a rapid but transient cold response accumulation for both 'Frida' and 'Jonsok' (6-fold and 18-fold respectively, at one day).

Two additional dehydrin proteins (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 dehydrin. Levels of Fcor1 and Fcor2 (Fragaria Cold-Regulated) transcripts were previously shown to be correlated with freezing tolerance (Ndong et al., 1997). Similar to the observations of Ndong et al., it was found that Fcor1 accumulation was transient while Fcor2 accumulation was more sustained over a two-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 one day following 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, and 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; Peng et al., 2008; Renaut et al., 2004) 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, in order to address dehydrin protein levels, 1-DE western blot analysis using an anti-K peptide (diagnostic for dehydrin was performed on the strawberry crown tissues (**Supplemental Figure S5**). A strong accumulation of dehydrin protein band at the 42 day cold treatment was observed (no detectable band at 0 or 2 day 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 one day 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 Figure 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 Fragaria crown tissue (**Supplemental Figure S5** and Davik et al, unpublished) 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 non-photosynthetic 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 to 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.

CONCLUSIONS

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. Secondly, 'Jonsok' responds to cold more rapidly, particularly noticeable when examining mRNA responses. Lastly, the convergence of protein expression in the two cultivars, visualized by principle component analysis (PCA), which becomes readily apparent after 42 d, is largely due to 'Frida' "catching up" in terms of expression patterns to the more cold-tolerant cultivar. However, one should not ignore the observation that 'Frida' is a cold/freezing tolerant cultivar, just less so than 'Jonsok', and indeed appears to have adopted a very strong antioxidation response as evidenced by activation of the ascorbate pathway and phenylpropanoid pathway. Indeed these latter approaches may represent an alternative, perhaps lesser, but nonetheless effective response to cold stress.

Most previous approaches to understand winter hardiness have focused on molecular responses to cold acclimation. The present study, through the comparison of two cold tolerant cultivars, which differ in

their extent of cold hardiness, has revealed a variety of differences in 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 which are candidates for molecular markers associated with overwintering success.

MATERIALS AND METHODS

Plant Material and Experimental Design for Freezing Experiment - Fragaria × ananassa runners were collected from the field and rooted in a heated greenhouse maintained at 20 ± 2 °C and 20-h-light/4-h-dark for 2 weeks in 50 x 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% N and 19% Ca) and Superba™ Rød (7-4-22 NPK plus micronutrients) from Yara International, Norway. The plants were then hardened for 6 weeks at 2 °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 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 ± 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 re-growth). The extent and intensity of discoloration (tissue browning) were recorded for the surviving plants from longitudinal crown sections as described by Marini and Boyce (1977) on a scale from 1 (low extent/intensity) to 5 (high extent/intensity). All experiments were replicated with three randomized blocks of 3 to 4 plants for each population, giving a total of 9 to 12 plants of each population in each treatment. ANOVA analyses (**Supplemental Table S1**) were performed by standard procedures using a MiniTab® Statistical Software program package (Release 15; Minitab Inc., State College, PA). The freezing conditions, the scoring details and the origin and parents of the four cultivars used are summarized in **Supplemental 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 days. 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 4 to 6 half-crowns that were cut longitudinally for proteomic experiments and the corresponding 4 to 6 half crowns for RT-qPCR.

Sample Preparation for 2DE - Tissue was ground to a fine powder in liquid nitrogen in the presence of polyvinylpolypyrrolidone (PVPP) at 10% of tissue weight. The powder was washed twice with cold 100% acetone with centrifugation at 8000 rpm at < 0 °C for 20 minutes (Sorval SS-34 rotor, 7649 × g ave). 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 (TBP) and extraction buffer (5.0 mL each per 1 g fresh weight) were added and then tissue was polytroned with a Brinkman homogenizer model PC 10/35 at speed setting #5 (Brinkman Instruments, Switzerland) for 30 seconds. The extraction buffer used contained 40% sucrose w/v, 2% SDS w/v, 1X Complete Roche Protease inhibitors, Phosphatase inhibitors (2 mM Sodium orthovanadate (5 mM NaF, 1 mM NaPPi, 1 mM 3-glycerolphosphate, and 3 µM microcystin) and 2% ß-mercaptoethanol dissolved in 0.1 M Tris-HCl pH 8.8. Sample was incubated at 4 °C with agitation for 30 minutes followed by centrifugation at 7000 rpm (Sorval-34 rotor, 5000 × g ave) for 15 minutes at 4 °C. The upper phenol phase was removed and the lower phase was re-extracted with 5.0 mL of TBP. Back extraction was performed on the combined upper phases by adding equal volume of extraction buffer. Following extraction, proteins were precipitated by adding 5 times the volume of 0.1 M ammonia acetate in 100% methanol overnight at -78 °C. The pellet was recovered by centrifuging at 7000 rpm, as before and washed twice with 0.1 ammonia acetate in 100% methanol followed by two washes with 80% acetone. The pellet was resuspended by vortexing and precipitation at -20 °C for 30 minutes between washes. The final pellet was air dried (~5 to 10 min). Pellets (~ 4.0 µg) were dissolved in ~600 µL of isoelectric focusing (IEF) buffer containing 8 M Urea, 2M Thiourea, 2% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate w/v, 2% deionized Triton X-100, 50 mM DTT, and 0.5% pH 3-10 ampholytes. An Amido black assay (Kaplan and Pedersen, 1985) was used to determine concentration of protein. One to three mg protein was extracted per gram of crown fresh weight.

2-D Gel Electrophoresis - IEF strips (24 cm, nonlinear pH 3–10, Bio-Rad, Hercules, CA) were passively rehydrated with 400 µg of protein at 20 °C for 14 hours. 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, 5000 V for 35000 Vhr and 8000 V for 53800 Vhr all with rapid ramps. Total Vhr was 90000 with a maximum of 50 µAmps per strip. After IEF, the strips were equilibrated with 450 µL of 6 M Urea, 0.05 M Tris/HCl pH 8.8, 4% SDS, 20% glycerol, 2% DTT w/v for 15 min (5 min × 3 changes) for the first step. Iodoacetamide (2.5% w/v) replaced DTT for the second step for 15 min (5 min × 3 changes). Strips were then placed on a 12% SDS- polyacrylamide gel and sealed with 0.65% agarose dissolved in 1X electrode buffer. Gel electrophoresis was conducted at 600 mAmp constant in a PROTEAN plus Dodeca cell (Bio-Rad) apparatus to run 12 gels simultaneously at a constant temperature of 20 °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 3 times in water for 15 minutes each and stained for a minimum of 72 hours with colloidal Coomassie G-250 (Candiano et al., 2004). Gels were then destained in water and scanned using a GS-800 Calibrated Imaging Densitometer (Bio-Rad). Thirty-six gel images (4 cultivars, three conditions, each in triplicate) were analyzed using PDQuest version 7.1 (Bio-Rad Laboratories, Hercules, CA, USA). Molecular weights and isoelectric points (pI) were assigned to spots by performing a separate experiment running internal 2DE SDS-PAGE Standards (Bio-Rad Laboratories, Hercules, CA, USA) with the same electrophoresis parameters as described above except using 100 µg protein ('Jonsok' at 0 d) and subsequently applying the determined MW and pI values to the larger experiment. In addition to the 2DE internal standards used to determine mass and isoelectric point, one protein, strongly identified as the elongation factor 1- alpha (SPP 9618) was used as a pI standard of 9.2. A total of 900 total protein spots were matched and inspected visually to validate all automated matching. The protein spot quantities were normalized based on the total valid spots for each gel and expressed as parts per million (ppm). Average intensities, standard deviations and coefficient of variations were obtained. Significant protein spot differences between cultivars or due to cold response changes were inspected using Student's t-test (unpaired, two tailed) $P < 0.05$, analysis of variance (ANOVA), and principal component analysis (PCA). All 2DE data was normalized to unit vector length by calculating the square root of the sum of squares of all protein spot quantities for a given sample. Each protein spot quantity in that sample was then divided by this normalization factor. This pre-treatments step removed any differences between samples due to overall quantity as well as differences in detection sensitivity for a given gel. PCA and ANOVA were then carried out using XLSTAT (AddinSoft SARL, Paris, France), an add-in to Microsoft Excel. PCA used the Pearson Product Moment to calculate correlations between variables and a Scree plot was

609 visually inspected to determine the number of significant principal components. For ANOVA,
610 significance was set at $p < 0.05$ and the Tukey's HSD (Honestly Significant Difference) test was used to
611 analyze the difference between groups.
612
613 *Protein Identification by LC-MS/MS (Confidence Values Listed as Protein Probability)* - The gel spots
614 were manually cut from the wet gels. The gel plugs were destained with 50% acetonitrile (ACN) in 50
615 mM ammonium bicarbonate (NH_4HCO_3) twice, reduced with 10 mM DTT in 100 mM NH_4HCO_3,
616 alkylated with 55 mM Iodoacetamide in 100 mM NH_4HCO_3, and digested by trypsin for 3 h at 37 °C. The
617 tryptic peptides were extracted with 30, 50, and 100% ACN sequentially. The extracted peptides were
618 dried by SpeedVac and reconstituted with 5% ACN in 0.1% FA (formic acid). The peptide samples were
619 analyzed using a Thermo-Finnigan linear ion-trap (LTQ) mass spectrometer coupled with a Surveyor
620 autosampler and MS HPLC system (Thermo-Finnigan). Tryptic peptides were injected onto the C18
621 microbore RP column (Zorbax SB-C18, 1.0 mm × 50 mm) at a flow rate of 50 µL/min. The mobile
622 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%
623 FA in water, respectively. The gradient elution profile was as follows: 10% B (90% A) for 10 min, 10-
624 20% B (90-80% A) for 5 min, 20-70% B (80-30% A) for 35 min, and 100% C for 10 min. The data were
625 collected in the "Data dependent MS/MS" mode with the ESI interface using the normalized collision
626 energy of 35%. Dynamic exclusion settings were set to repeat count 2, repeat duration 30 s, exclusion
627 duration 120 s, and exclusion mass width 1.50 m/z (low) and 1.50 m/z (high). The acquired data were
628 searched against NCBI protein sequence database of *Fragaria vesca* and *Fragaria* × *ananassa*
629 (downloaded on 12 February 2009 from http://www.ncbi.nlm.nih.gov/, 574 entries) and Rosaceae
630 (downloaded on 12 February 2009 from http://www.ncbi.nlm.nih.gov/, 8,926 entries) using SEQUEST
631 (v. 28 rev. 12) algorithms in Bioworks (v. 3.3). General parameters were set as follows: peptide tolerance
632 2.0 amu, fragment ion tolerance 1.0 amu, enzyme limits set as "fully enzymatic cleaves at both ends", and
633 missed cleavage sites set at 2. The searched peptides and proteins were validated by PeptideProphet
634 (Keller et al., 2002) and ProteinProphet (Nesvizhskii et al., 2003) in the Trans-Proteomic Pipeline (TPP,
635 v. 3.3.0) (http:// tools.proteomecenter.org/software.php) with a confidence score represented as
636 probability. The validated peptides and proteins were filtered using the following cut-off: (1) the
637 confidence of protein was ≥ 90.00% (0.9000); (2) at least two peptides were identified for a protein; and
638 (3) the confidence of peptides was ≥ 80.00% (0.8000) with at least one peptide's confidence ≥ 90.00%
639 (0.9000). Only the peptides and proteins meeting the above criteria were chosen.
640
641 *Protein Identification by LC-MS/MS (Confidence Values Listed as q-values)* - To build the Fragaria
642 protein database, the *Fragaria* × *ananassa* and *Fragaria vesca* protein Fasta database and EST sequence

643 databases for taxonomy id 3747 and 57918 were downloaded from NCBI. The ESTs were translated in
644 three different reading frames and the largest protein among three reading frames was chosen. The *F.* ×
645 *ananassa* protein Fasta database and the chosen translated database were concatenated, after which the
646 same sequences were removed from the list. The final protein entry was 45793. Database search was done
647 using Sequest and X!Tandem algorithms.
648
649 *LFQP (Label-free Quantitative Proteomics)* - These experiments were conducted and analyzed essentially
650 as described in (Higgs, 2005) and (Wang et al., 2008).The time points used for this experiment consisted
651 of the 0 and 2 day exposure to 2 °C. Three to six individual crowns were used for each of five biological
652 replications. Each biological replication was injected twice and the two technical replicate intensity values
653 were averaged. Tryptic peptides (< 20 µg) were injected onto an Agilent 1100 nano-HPLC system
654 (Agilent Technologies, Santa Clara, CA) with a C18 capillary column in random order. Peptides were
655 eluted with a linear gradient from 5%-45% acetonitrile developed over 120 minutes at a flow rate of 500
656 nL/min and the effluent was electro-sprayed into the LTQ mass spectrometer (Thermo Fisher Scientific,
657 Waltham, MA). Data were collected in the "Triple Play" (MS scan, Zoom scan, and MS/MS scan) mode.
658 The acquired data were filtered and analyzed by a proprietary algorithm. The database was the same as
659 described for 2DE protein identification by LC-MS/MS with Confidence Values determined as q-values
660 (Higgs et al., 2005). LFQP raw data for the 2017 identified proteins and additional information for the top
661 135 proteins (significantly different identified with the highest confidence) as compared with the 2DE
662 analysis is available in **Supplemental Data Set S3**.
663
664 *RT-qPCR* - Total RNA was isolated from frozen crown tissues (~100 mg) dissected from control (20 °C
665 at 0 day) and cold-treated (2 °C, time points 1, 2, 14, and 42 days) *F.* × *ananassa* plants 'Jonsok' and
666 'Frida', each with three to five biological replicates per time point) according to a modified CTAB
667 protocol Total RNA (1 µg) of each sample was treated with Amplification Grade DNase I (Invitrogen,
668 Carlsbad, CA., USA) according to manufacturer's instructions. Reverse transcription (RT) of the samples
669 was performed using a mixture of random hexamers and oligo-dT12-18 primers and the SuperScript III
670 First-Strand Synthesis System (Invitrogen, Carlsbad, Ca., USA) according to the manufacturer's
671 instructions. To control for non- degraded, contaminating genomic DNA, selected replicate samples were
672 included in which H_2O replaced the Superscript III enzyme in the RT reactions. The resulting single-
673 stranded template cDNAs were used in two independent qPCR reactions with gene-specific primers
674 designed to produce 100-300 bp amplicons representing the target transcripts Rab18-like (CX661424; F.
675 vesca ortholog), Cor47-like (C0817504), Fcor1 (HQ910514, sequence from NDong et al., 1997), Fcor2
676 (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 cDNA template, 1 X 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 – 63 °C) for 40 cycles using the 7500 Real-Time PCR System (software version 1.3; Applied Biosystems, Carlsbad, CA., USA). 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; FaActin, GGGTTTGCTGGAGATGATG, CACGATTAGCCTTGGGATTC

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900 **FIGURE LEGENDS**

901

902 **Figure 1.** Representative 2DE gel of F. × ananassa crown proteins (‘Jonsok’ at 2 days 2 °C treated). The
903 109 proteins identified by LC-MS/MS (**Supplemental Data S1**) are indicated with spot numbers. Gel was
904 performed with 400 µg of protein using 24 cm immobilized pH gradient strips (3 to 10 nonlinear)
905 resolved on 12% SDS-PAGE and stained with colloidal Coomassie Brilliant Blue.

906

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

Figure 3. Proteins differentially expressed in 'Jonsok' and 'Frida'. Panel A shows cold responsive proteins at 2 days (2d), and 42 days (42d) that have changed ≥ 2-fold relative to control (0 d) in 'Frida' and 'Jonsok'. The number and percent of protein spots accumulating or decreasing are indicated with arrows. The number of proteins with higher levels (≥ 2-fold) in 'Jonsok' (Panel B) and 'Frida' (Panel C) with respect to the other cultivar are shown at each time point. Venn diagrams depicts the number of proteins detected at a significance of $P < 0.05$ in the Student's t-test, and for Panel B and C additionally met the criteria of better than 0.80 for factor loadings from PCA using the 900 matched spots from 2DE. The numbers within parentheses indicate the number of spots with protein identification.

Figure 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 deviations for each time point (0, 2, 42 days of cold treatment at 2 °C) for 'Frida' (gray bars) and 'Jonsok' (black bars). Abbreviations: ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; CAD, cinnamyl alcohol dehydrogenase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3H, flavonoid 3-hydroxylase; IFR, isoflavone reductase; OMT, Caffeic acid 3-O-methyltransferase; PAL, phenylalanine; RT, rhamnosyl transferase; UGGT, UDP-glucose glucosyltransferase

Figure 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 deviations for each time point (0, 2, 42 days 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 deviations for each time point (0, 2, 42 days of cold treatment at 2 °C) for 'Frida' and 'Jonsok'. The corresponding 2DE spot images are presented beneath each graphed bar. Abbreviations: APX,

cytosolic ascorbate peroxidase; Cu/Zn SOD, Cu/Zn superoxide dismutase; GST, glutathione S-transferase; GLDH, L-galactono-1,4-lactone dehydrogenase.

Figure 7. Transcript levels of 6 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 days 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.

SUPPLEMENTAL DATA

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

Supplemental Figure S1. Agglomerative hierarchical clustering (AHC) indicates that cultivars and treatments group into distinct clades and subclades.

Supplemental Figure S2. Representative 2DE maps illustrating the proteins differentially accumulated in 'Jonsok' and 'Frida' (Table II).

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

Supplemental Figure S4. Gene Ontology (GO) annotation for identified proteins.

Supplemental Figure S5. Dehydrin accumulation in *Fragaria* × *ananassa* 'Jonsok' and 'Frida'.

Supplemental Table S1. Freezing survival demonstrates the relative cold/freezing tolerance of *F.* × *ananassa* cultivars.

Supplemental Table S2. Freezing survival demonstrates the relative cold/freezing tolerance of *F.* × *ananassa* cultivars prior to a cold acclimation treatment.

Supplemental Data S1. List of all (900) spots matched between 'Elsanta', 'Frida', 'S. Sengana' and 'Jonsok' (Excel file).

Supplemental Data S2. List of all (109) spots from the 2DE identified by LC-MS/MS (Excel file).

Supplemental Data S3. LFQP data (Excel file).

Supplemental Methods S1. Physiological freezing experiment details.

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

Surviving plants, browning extent and intensity were scored as described in methods. Exponential extrapolated killing curves indicated 50% survival of 'Jonsok' at approximately -8.3 ℃ and for 'Frida' at approximately -5.5 ℃. The LT50 (temperature at which 50% of plants died or 50% of maximal browning occurred), the SE (standard error) and R2 (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.

	Plant Survival			Tissue Browning			Browning Intensity		
Cultivar	LT50	SE	R^2	LT50	SE	R^2	LT50	SE	R^2
'Jonsok'	-8.29	1.11	0.79	-5.34	0.59	0.94	-5.19	0.53	0.94
'Senga 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

Table II. *The proteins identified in F. × ananassa crown by LC-MS/MS which distinguish the two cultivars, 'Jonsok' and 'Frida'*

List includes 2-DE 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.

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

SSP	Protein Identified	Distinct Sequences	Accession Code	Species	AGI	Meets Criteria [a]
4546	ADH (alcohol dehydrogenase)	29	CAA33613	*Fragaria × ananassa*	AT1G77120	0d
3114	Fra a 1-A	5	ABD39049	*Fragaria × ananassa*	AT1G24020	0d
4015	Fra a 2	6	DY675259	*Fragaria vesca*	AT1G24020	0d
813	High molecular weight heat shock protein	38	AAF34134	*Malus × domestica*	AT3G12580	0d
812	Hsc70 (heat shock cognate 70 kDa)	9	EX686389	*Fragaria vesca*	AT5G02500	0d
7626	Vacuolar sorting protein	7	EX684112	*Fragaria vesca*	AT2G27600	0d
3515	ANR (anthocyanidin reductase)	18	ABD95362.1	*Fragaria × ananassa*	AT1G61720	0d,2d
4011	Fra a 3	6	EX681634	*Fragaria vesca*	AT1G24020	0d,2d
5125	GST (glutathione transferase)	3	EX662925	*Fragaria vesca*	AT2G47730	0d,2d
1819	HSP70 (heat shock protein 70)	9	EX686887	*Fragaria vesca*	AT5G09590	0d,2d
4537	PGK (Phosphoglycerate kinase)	10	EX672771	*Fragaria vesca*	AT1G79550	0d,2d
4325	PP2C (protein phosphatase type 2C)	6	EX684817	*Fragaria vesca*	AT3G15260	0d,2d
622	SGT1; putative protein phosphatase	7	EX680652	*Fragaria vesca*	AT4G11260	0d,2d
6540	ADH (alcohol dehydrogenase)	5	P17648	*Fragaria × ananassa*	AT1G77120	0d,2d,42d
5439	Aldo/keto reductase	13	EX674338	*Fragaria vesca*	AT1G60710	0d,2d,42d
6432	Annexin	9	DY668560	*Fragaria vesca*	AT1G35720	0d,2d,42d
2317	Beta-1,3-glucanase	11	EX665040	*Fragaria vesca*	AT3G57240	0d,2d,42d
2010	Cu/Zn superoxide dismutase	4	EX670753	*Fragaria vesca*	AT1G08830	0d,2d,42d
3626	Enolase	7	EX680335	*Fragaria vesca*	AT2G36530	0d,2d,42d
1315	Glyoxylase I (lactoylglutathione lyase)	6	EX680994	*Fragaria vesca*	AT1G11840	0d,2d,42d
4115	GST (glutathione transferase)	8	EX662925	*Fragaria vesca*	AT2G47730	0d,2d,42d
1423	IFR (isoflavone reductase related protein)	4	CO817159	*Fragaria × ananassa*	AT1G75280	0d,2d,42d
820	Nucleoredoxin, putative	4	DY671291	*Fragaria vesca*	AT1G60420	0d,2d,42d
6224	Porin	8	EX688506	*Fragaria vesca*	AT3G01280	0d,2d,42d
6723	Pyruvate kinase	3	EX666373	*Fragaria vesca*	AT5G08570	0d,2d,42d
4547	rgp (alpha-1,4-glucan-protein synthase)	9	EX685340	*Fragaria vesca*	AT3G02230	0d,2d,42d
2203	Thaumatin-like protein	9	EX674730	*Fragaria vesca*	AT1G75800	0d,2d,42d
6505	ADH (alcohol dehydrogenase)	10	CAA33613	*Fragaria × ananassa*	AT1G77120	0d,42d
2218	APX (cytosolic ascorbate peroxidase)	14	AAD41406.1	*Fragaria × ananassa*	AT1G07890	0d,42d
1533	OMT (O-methyltransferase)	16	AF220491	*Fragaria × ananassa*	AT5G54160	0d,42d
2012	Pathogenesis-related protein Ypr10	10	ABX89934	*Fragaria × ananassa*	AT1G24020	0d,42d
5318	Aldo/keto reductase	8	EX680606	*Fragaria vesca*	AT2G37770	2d
4717	F1-ATPase alpha subunit	40	AAW33106	*Rubus sp. JPM-2004*	AT2G07698	2d
6539	3-ketoacyl-CoA thiolase	5	EX682172	*Fragaria vesca*	AT2G33150	42d
7528	Cytosolic aldolase	8	AAG21429.1	*Fragaria × ananassa*	AT3G52930	42d

(Table II continues on following page)

Table II. *(Continued from previous page)*

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

SSP	Protein Identified	Distinct Sequences	Accession Code	Species	AGI	Meets Criteria [a]
6507	Acetyl-CoA acetyltransferase, cytosolic 1	5	DY674748	*Fragaria vesca*	AT5G48230	0d
1203	CCoAOMT (caffeoyl-CoA 3-O-methyltransferase)	4	CAA04769	*Fragaria vesca*	AT4G34050	0d
6403	Malate dehydrogenase, mitochondrial	10	P83373	*Fragaria × ananassa*	AT1G53240	0d
3708	Pyruvate decarboxylase	7	EX678086	*Fragaria vesca*	AT5G17380	0d
3017	40S ribosomal protein S12-2	4	EX685033	*Fragaria vesca*	AT2G32060	0d,2d
8302	Adenylate kinase	2	EX657534	*Fragaria vesca*	AT5G63400	0d,2d
7405	Cytosolic aldolase	30	AAG21429	*Fragaria × ananassa*	AT3G52930	0d,2d
4305	Glyoxylase II (hydroxyacylglutathione hydrolase)	11	EX665941	*Fragaria vesca*	AT3G10850	0d,2d
6704	GPI (glucose-6-phosphate isomerase)	5	DY671931	*Fragaria vesca*	AT5G42740	0d,2d
7210	Proteasome subunit b type-4	7	EX661942	*Fragaria vesca*	AT1G56450	0d,2d
3612	SAMS (S-adenosylmethionine synthetase)	9	EX684436	*Fragaria vesca*	AT3G17390	0d,2d
3625	6PGD (6-phosphogluconate dehydrogenase)	9	DY671162	*Fragaria vesca*	AT3G02360	0d,2d,42d
6416	Annexin	8	DY668560	*Fragaria vesca*	AT1G35720	0d,2d,42d
6014	Cystathionine beta-synthase domain	4	EX673938	*Fragaria vesca*	AT5G10860	0d,2d,42d
6611	Citrate synthase, mitochondrial	4	P83372	*Fragaria × ananassa*	AT2G44350	0d,42d
3912	Aconitase hydratase	5	EX661092	*Fragaria vesca*	AT2G05710	2d
4520	ANR (anthocyanidin reductase)	15	ABG76842.1	*Fragaria* x *ananassa*	AT1G61720	2d
4310	Carbonic anhydrase-like	5	EX670805	*Fragaria vesca*	AT1G19580	2d
7707	Catalase	8	EX683064	*Fragaria vesca*	AT4G35090	2d
4526	CHS (chalcone synthase)	13	BAE17124.1	*Fragaria × ananassa*	AT5G13930	2d
5542	DFR (dihydroflavonol 4-reductase)	14	BAA12723	*Rosa hybrid cultivar*	AT5G42800	2d
4536	F3H (flavanone 3-hydroxylase)	10	AAU04792.1	*Fragaria x ananassa*	AT3G51240	2d
3115	Hexokinase 1	6	ABG36925	*Fragaria x ananassa*	AT1G47840	2d
3819	sti1-like (stress-inducible protein)	2	EX671298	*Fragaria vesca*	AT1G62740	2d,42d
6808	Methionine synthase	13	DY672153	*Fragaria vesca*	AT5G17920	42d
2506	CAD (cinnamyl-alcohol dehydrogenase)	3	CAC09058.1	*Fragaria × ananassa*	AT3G19450	42d
3223	Iron-binding protein	4	EX677466	*Fragaria vesca*	AT3G11050	42d
3615	GEM-like protein 1	2	EX673973	*Fragaria vesca*	AT2G22475	42d

[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, a protein levels with two fold or greater difference. The Arabidopsis homolog results from performing a Blast on TAIR ITAIR WU-BLAST 2.0, database TAIR9 Proteins)

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

From LC-MS/MS based LFQP analysis, 135 ESTs were identified as significantly different between 'Jonsok' and 'Frida' at control (0d) and 2 day cold (2d) treated tissues. Twenty-one of these proteins were also identified in the 2-DE 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 2-DE. When the relative difference in abundance for 'Jonsok' or 'Frida' agrees between LFQP and 2-DE a 'yes' is indicated. In the instance that the same EST was identified for more than one 2-DE spot, the 'yes' or 'no' corresponds to the 2-DE 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 2-DE.

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

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

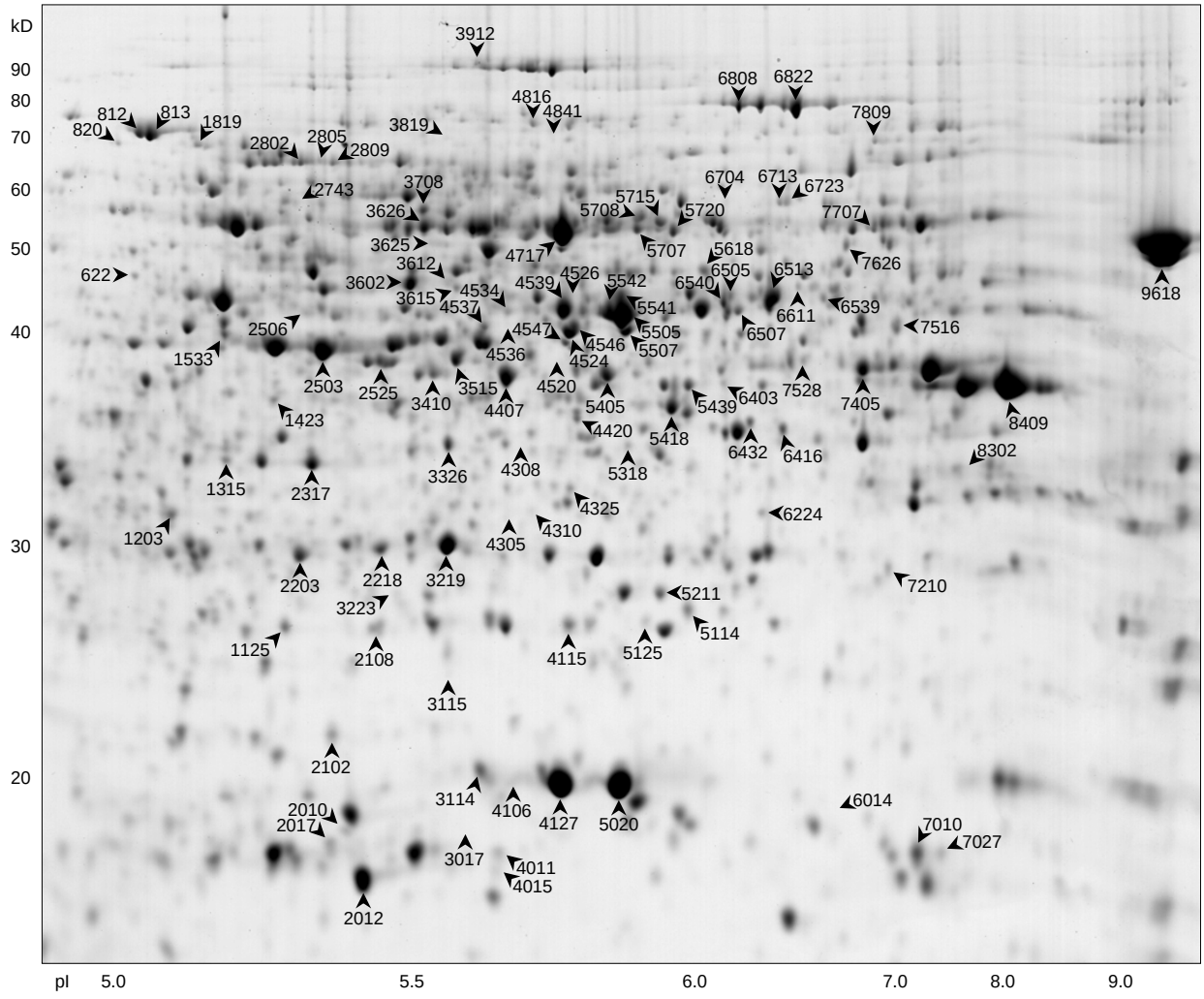


Figure 1. Representative 2DE gel of *F.* × *ananassa* crown proteins (‘Jonsok’ at 2 days 2 °C treated). The 109 proteins identified by LC-MS/MS (Supplemental Data S1) are indicated with spot numbers. Gel was performed with 400 µg of protein using 24 cm immobilized pH gradient strips (3 to 10 nonlinear)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

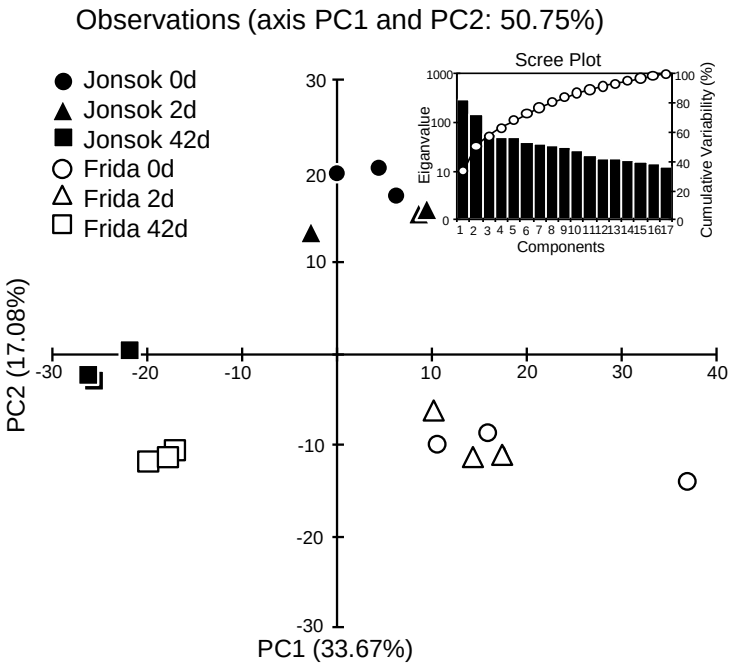


Figure 2. Principal component analysis (PCA) indicates 'Frida' and 'Jonsok' protein composition are distinctive and that they respond differently to cold stress. Time (in days) of exposure to 2 °C is indicated by 0d, 2d, and 42d. All 900 common spots were included in this analysis. The scree plot (inset)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

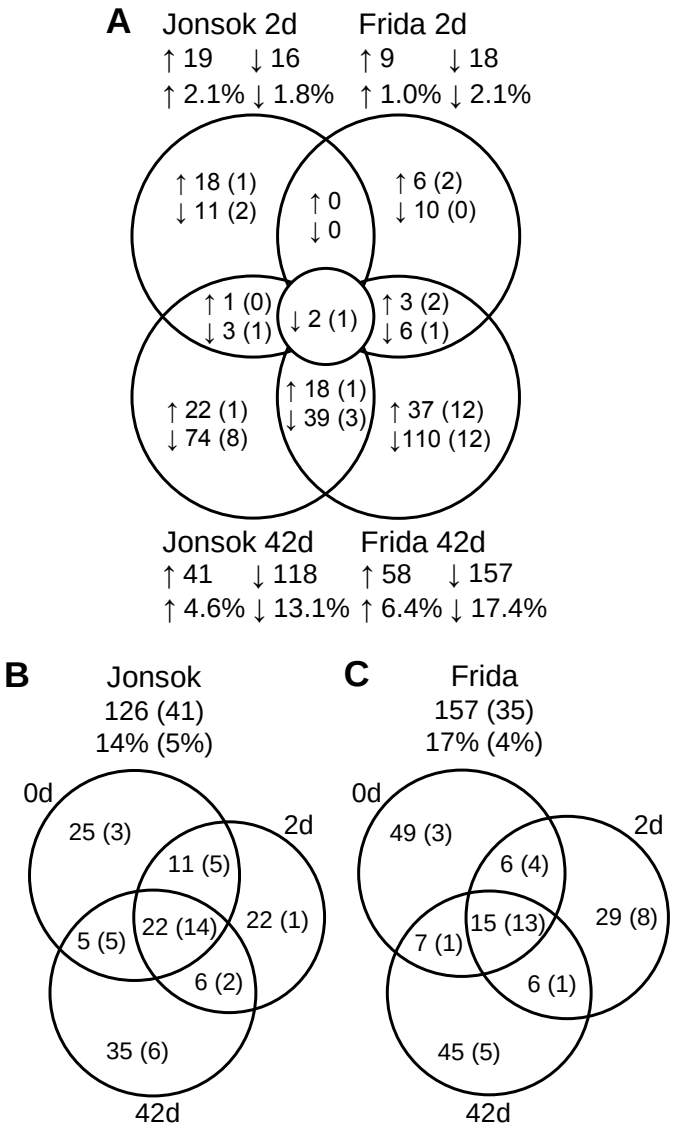


Figure 3. Proteins differentially expressed in 'Jonsok' and 'Frida'. Panel A shows cold responsive proteins at 2 days (2d), and 42 days (42d) that have changed ≥ 2-fold relative to control (0 d) in 'Frida' and 'Jonsok'. The number and percent of protein spots accumulating or decreasing are indicated with arrows. The number of proteins with higher levels (≥ 2-fold) in 'Jonsok' (Panel B) and 'Frida' (Panel C) with respect to the other cultivar are shown at each time point. Venn diagrams depicts the number of proteins detected at a significance of $P < 0.05$ in the Student's t-test, and for Panel B and C additionally met the criteria of better than 0.80 for factor loadings from PCA using the 900 matched spots from 2DE. The numbers within parentheses indicate the number of spots with protein identification.

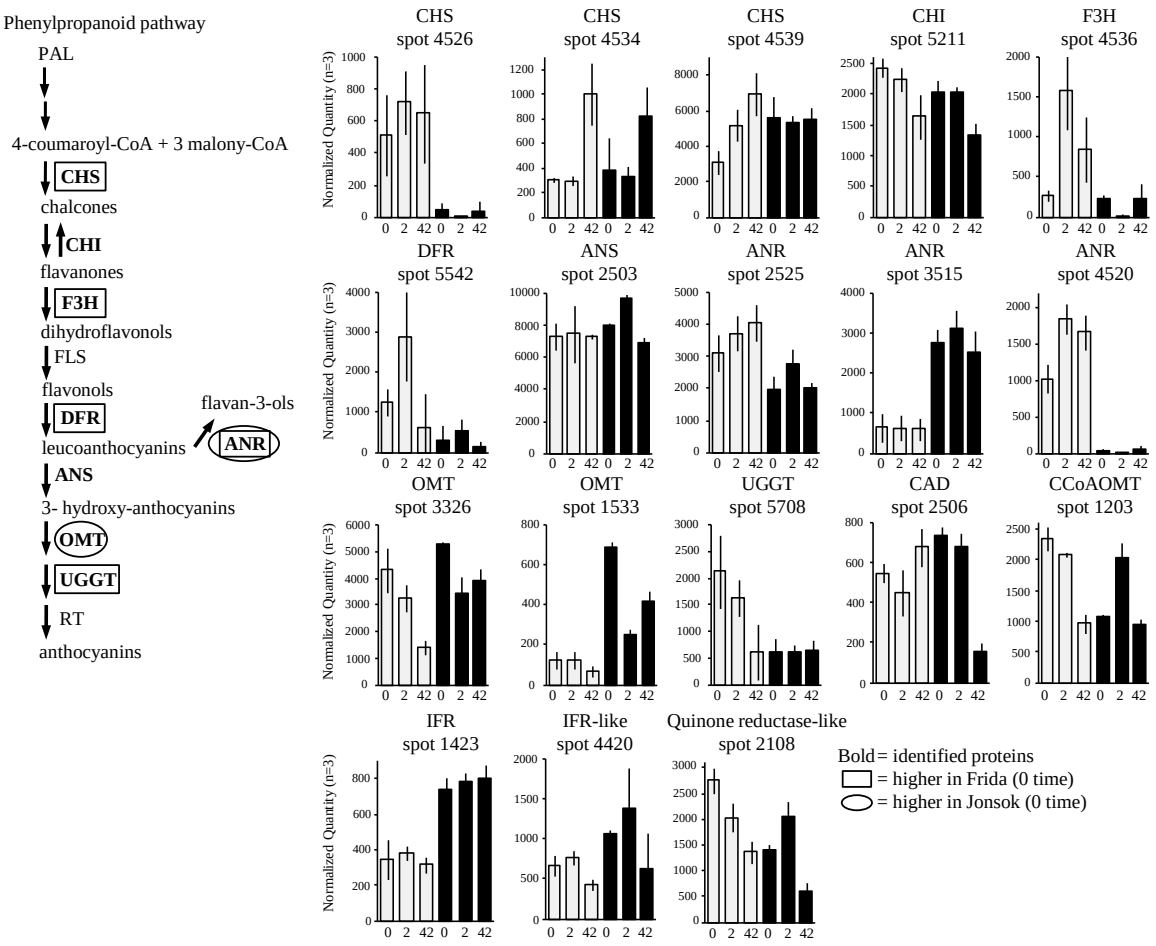


Figure 4. Proteins identified in the flavonoid pathway were most abundant in 'Frida. Flavonoid pathway highlighting the proteins involved in this pathway in 'Frida' and 'Jonsok'. The proteins in bold indicate identified proteins. Proteins in either squares or ovals indicate that higher levels (≥ 2 fold, $p < 0.05$ 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 deviations for each time point (0, 2, 42 days of cold treatment at 2 °C) for 'Frida' (gray bars) and 'Jonsok' (black bars). Abbreviations: ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; CAD, cinnamyl alcohol dehydrogenase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3H, flavonoid 3-hydroxylase; IFR, isoflavone reductase; OMT, Caffeic acid 3-O-methyltransferase; PAL, phenylalanine; RT, rhamnosyl transferase; UGGT, UDP-glucose glucosyltransferase.

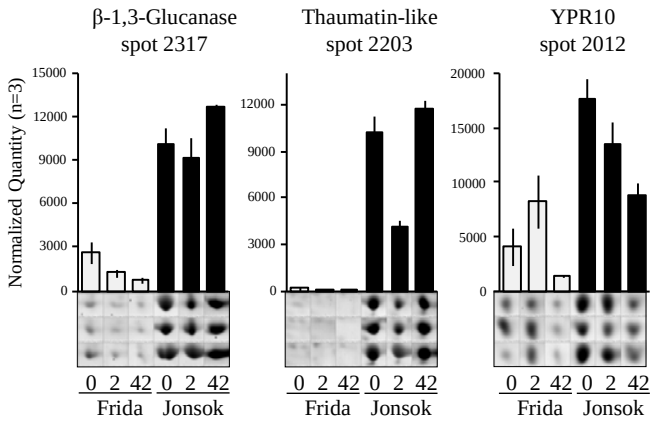


Figure 5. Levels of proteins associated with pathogen resistance distinguish ‘Jonsok’ (black bars) from ‘Frida’ (gray bars). Bar graphs show the average normalized values (from PDQuest, n=3) with standard deviations for each time point (0, 2, 42 days of cold treatment at 2 °C) for ‘Frida’ and ‘Jonsok’.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

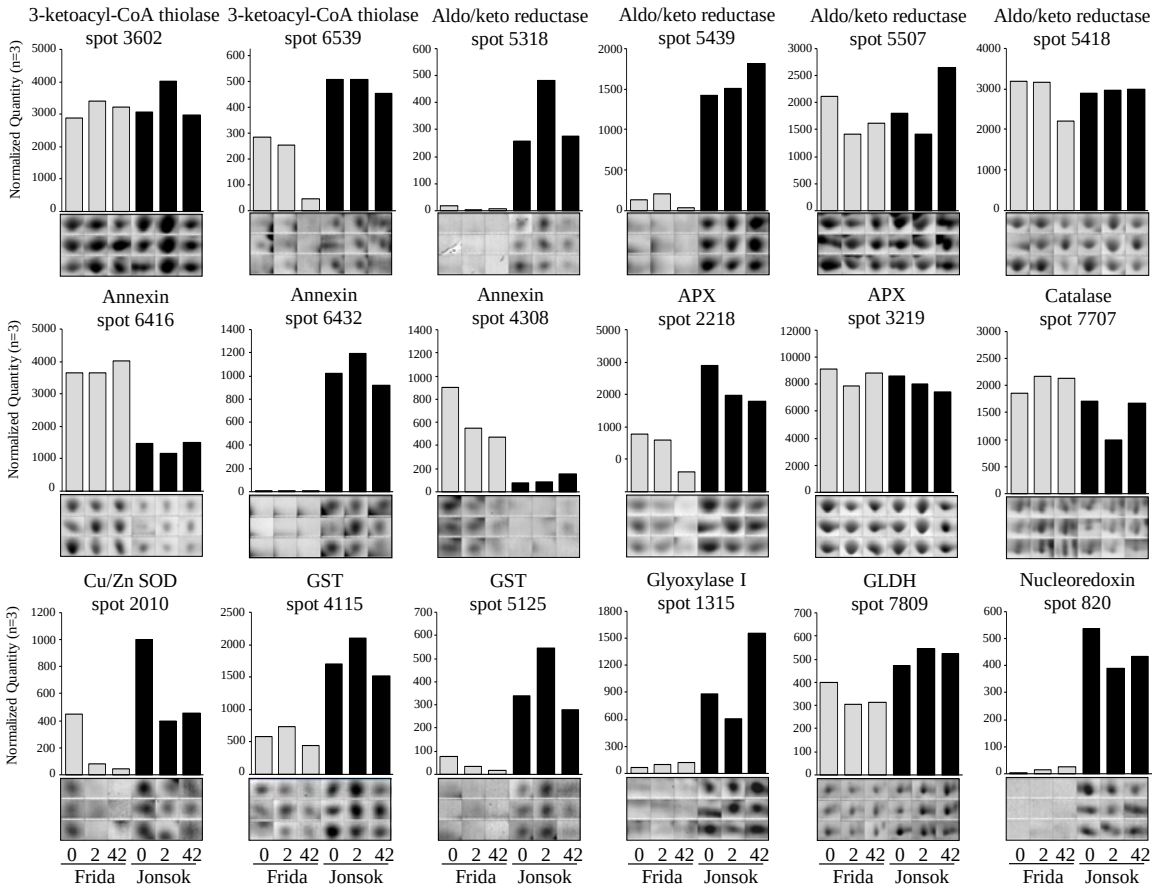


Figure 6. Levels of proteins associated with antioxidation and detoxification distinguish 'Jonsok' (black bars) from 'Frida' (gray bars). Bar graphs show the average normalized values (from PDQuest, n=3) with standard deviations for each time point (0, 2, 42 days of cold treatment at 2 °C) for 'Frida' and 'Jonsok'. The corresponding 2DE spot images are presented beneath each graphed bar. Abbreviations: APX, cytosolic ascorbate peroxidase; Cu/Zn SOD, Cu/Zn superoxide dismutase; GST, glutathione S-transferase; GLDH, L-galactono-1,4-lactone dehydrogenase.

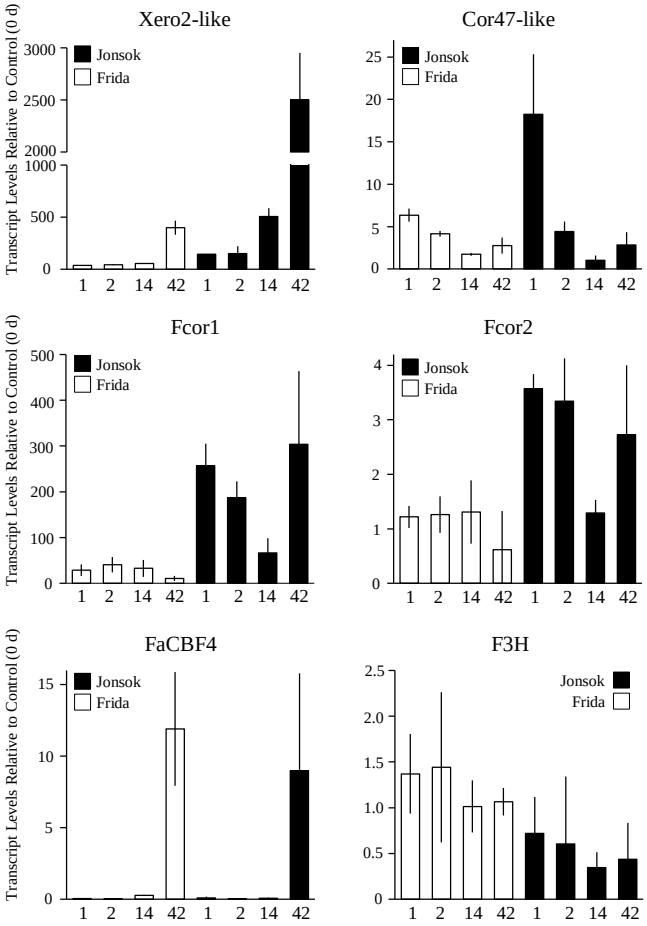
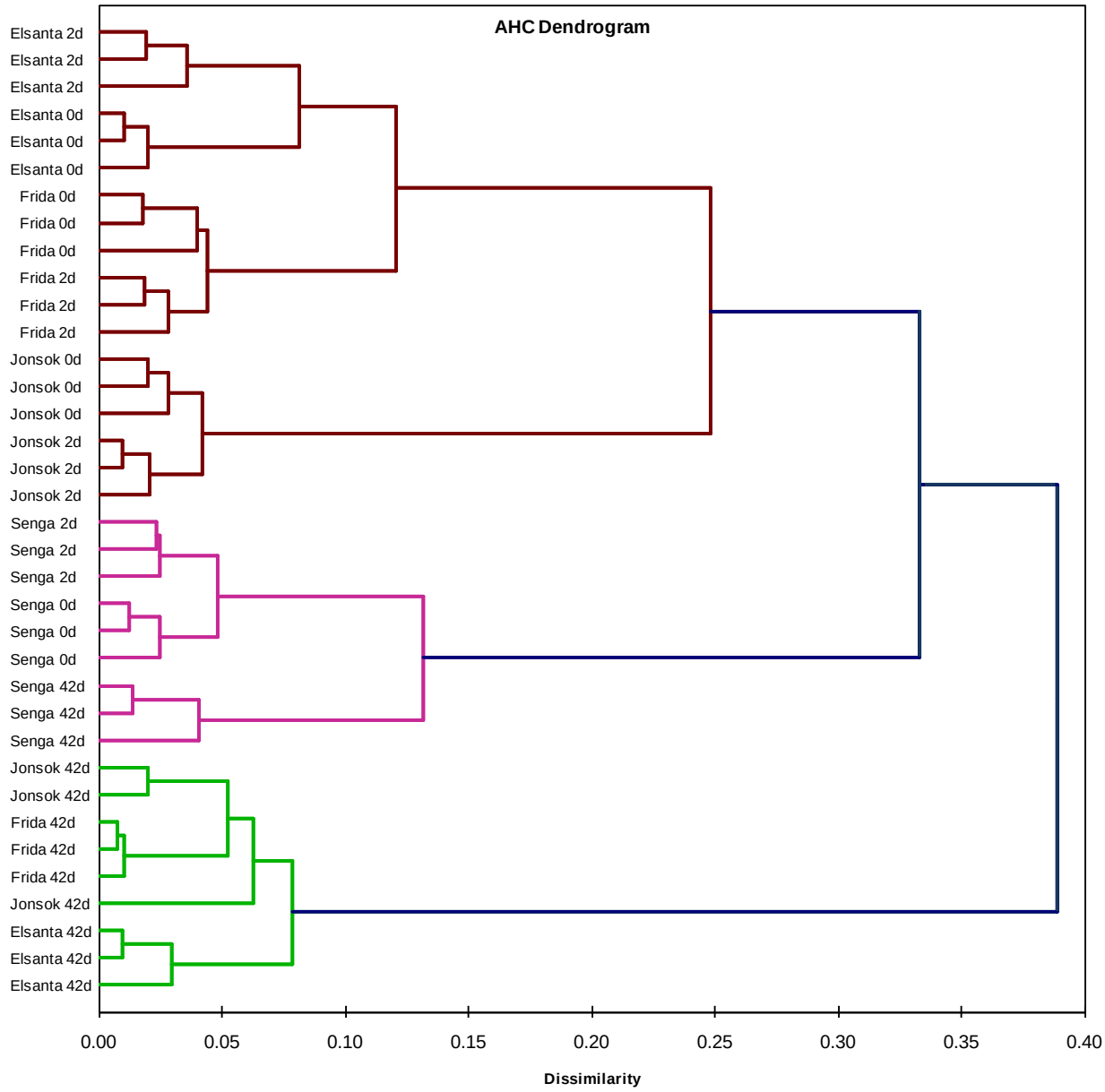
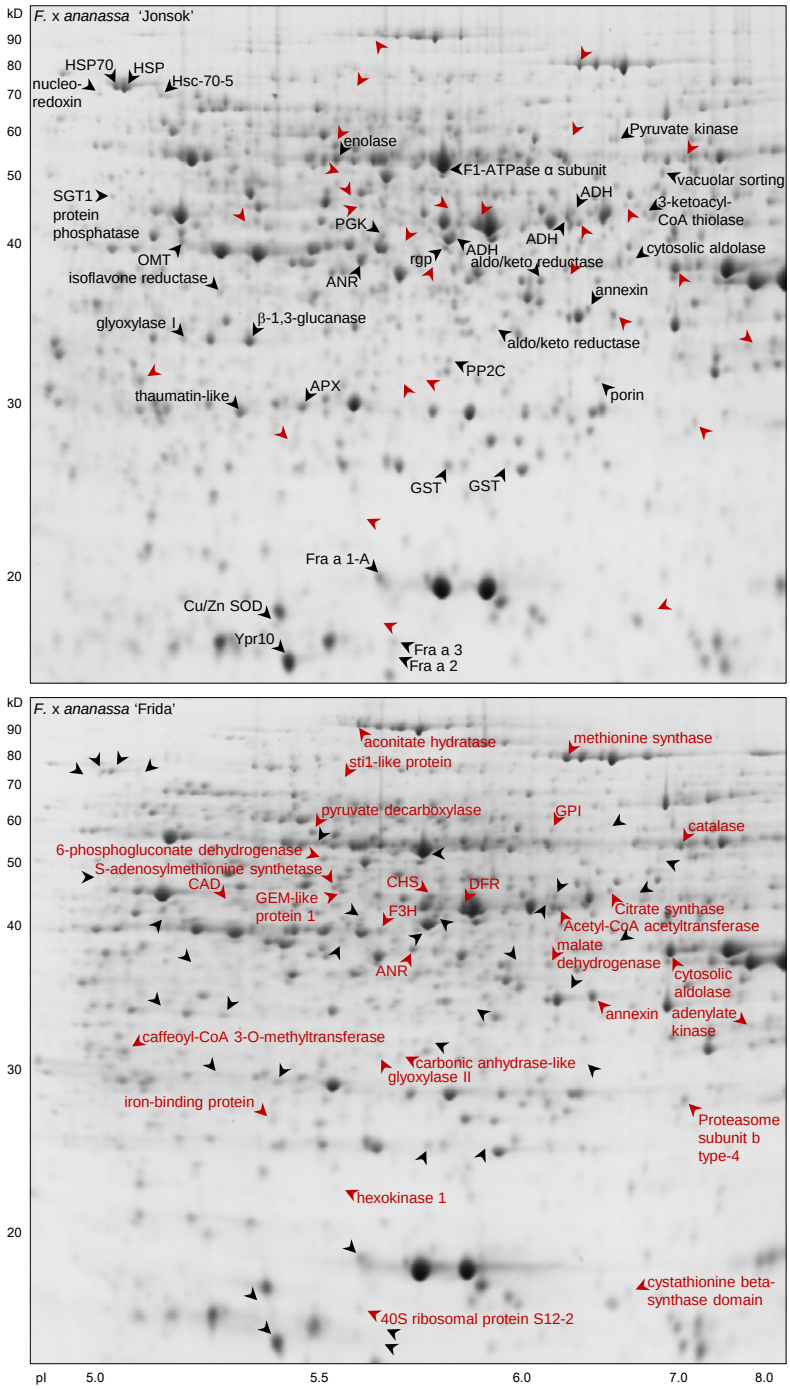


Figure 7. Transcript levels of 6 distinct genes from strawberry crown tissue during cold acclimation as measured by qPCR. Data are expressed transcript levels at cold treated time points (1, 2, 14, and 42 days at 2 °C) relative to control (0 d). Actin was utilized as a reference transcript for normalization in two independent qPCR reactions, each with three to five biological replicates per time point.

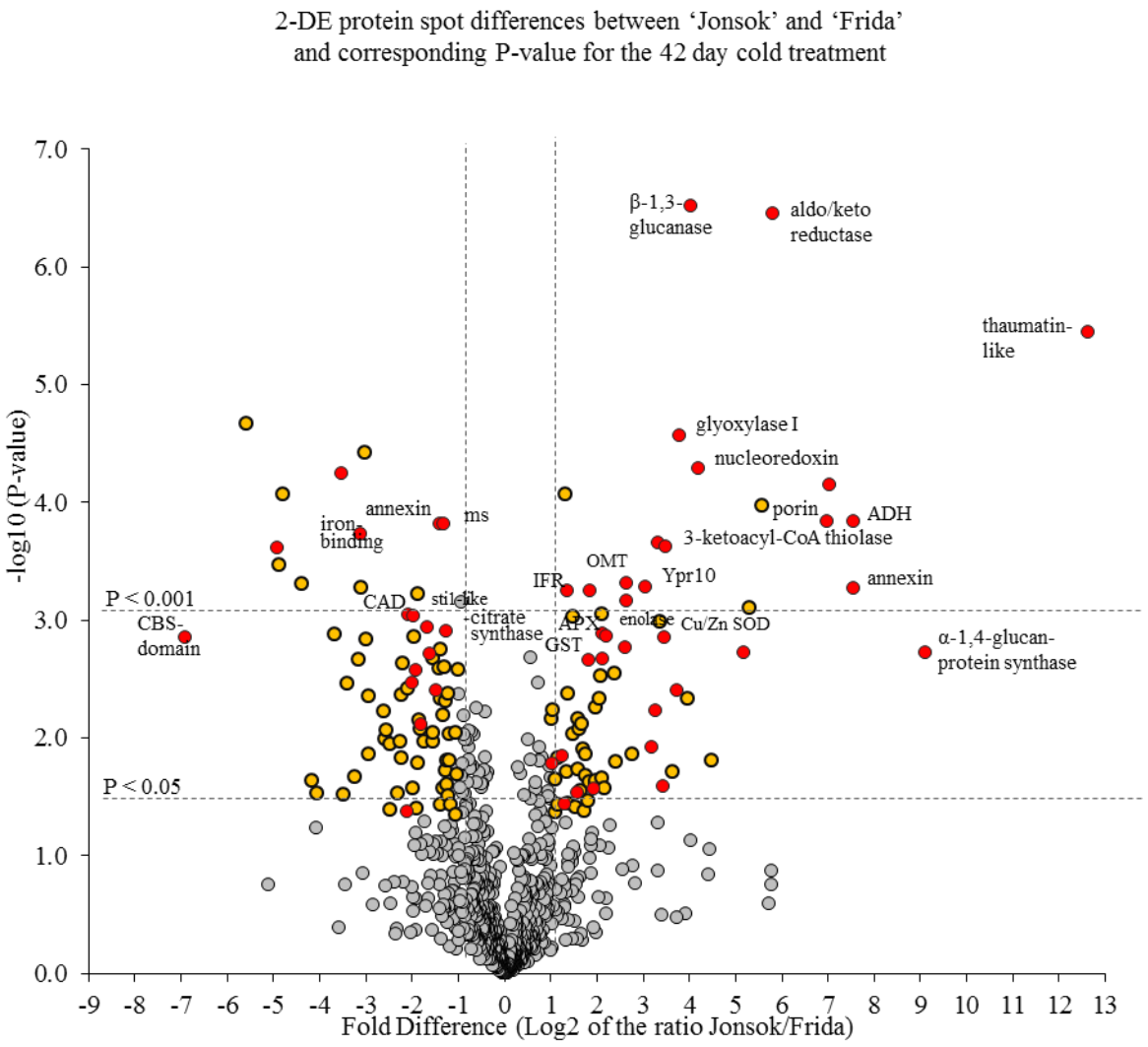


Supplemental Figure S1. Agglomerative hierarchical clustering (AHC) indicates that cultivars and treatments group into distinct clades and subclades and thus cultivars can be distinguished from each other based on protein profiles. Dendrogram representing all of the 36 2DE gels (900 common SSPs) for *Fragaria* × *ananassa* 'Elsanta', 'Frida', 'Jonsok' and 'Senga Sengana' (as 'Senga') for the 0, 2, and 42 d of cold treatment (2 °C).

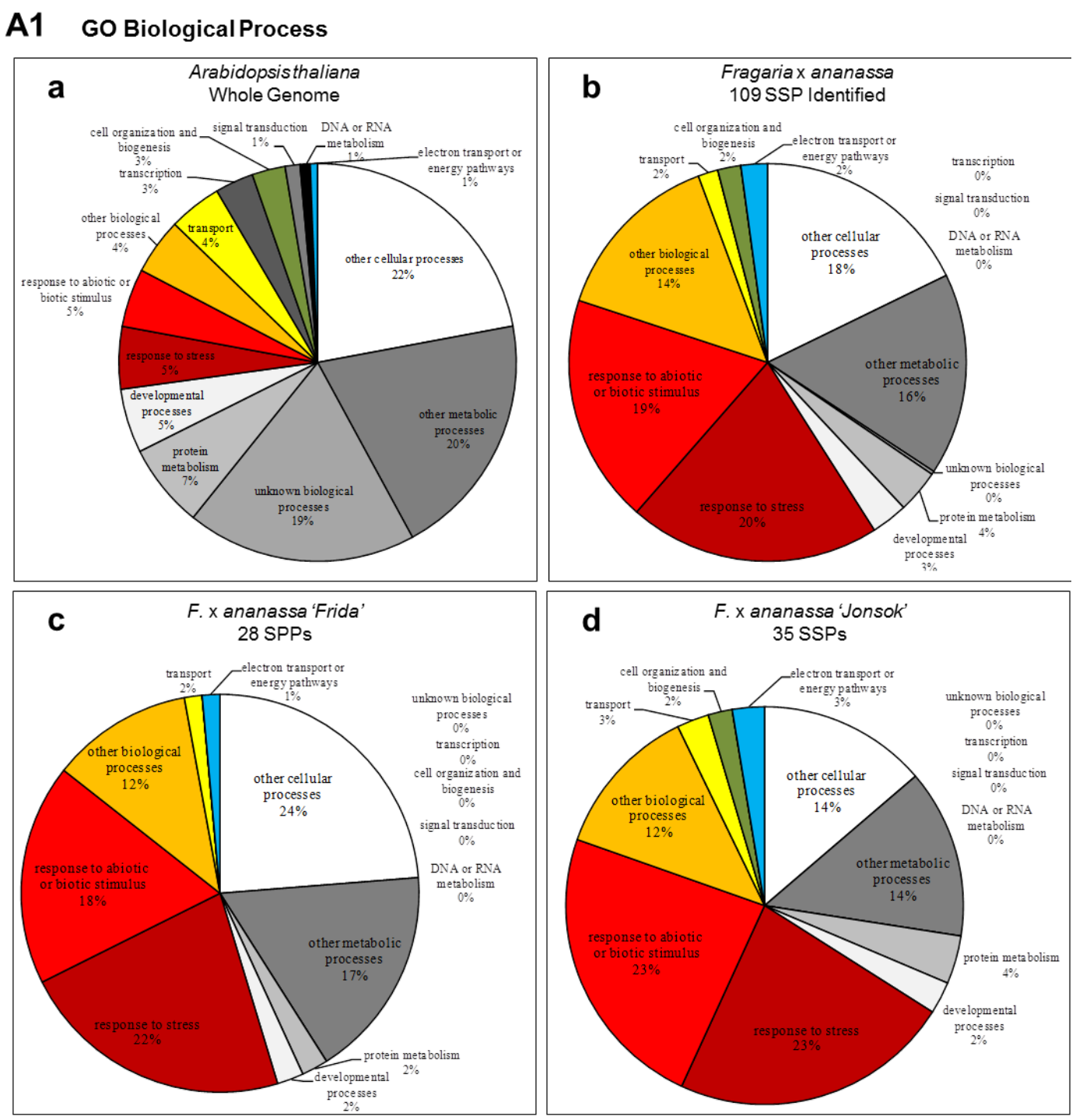
Supplemental Figure S1



Supplemental Figure S2. Representative 2DE maps illustrating the proteins that are differentially accumulated in 'Jonsok' and 'Frida'. Representative 2DE gels of *F.* × *ananassa* 'Jonsok' (top) and 'Frida' (bottom) from 2 day cold treatment (2 °C) from crown tissue. Numerous individual spot intensities differed between the cultivars and were identified with LC-MS/MS (35 and 28 for 'Jonsok' and 'Frida', respectively). Protein spots with labels indicate the proteins are at higher levels ≥ 2 fold for that variety and detected with a significance of $P < 0.05$ (Student's t-test and ANOVA), and having a Pearson correlation coefficient of greater than the absolute value of 0.80 for factor loading values from PCA. Arrowheads without labels indicate spot location corresponding with the identified protein in the other gel. Ancillary data for these spots is summarized in Table II



Supplemental Figure S3. Volcano plot was obtained by plotting the log2 ratio of mean values ('Jonsok'/ 'Frida') for the 900 matched 2DE spots at 42 day cold treatment against the negative log10-transformed P-value from the Student's t-test. Protein spots with a two fold difference in expression between 'Jonsok' and 'Frida' with a P-value < 0.05 are indicated by red and orange (148 spots). The red color corresponds to the 49 spots that were additionally deemed significantly different between 'Jonsok' and 'Frida' from an ANOVA analysis performed on all four cultivars at all time points (0, 2, and 42 day at 2 °C). Due to space constraints, only 24 of the total 109 identified spots were labeled based on significance by ANOVA and having highest –log10 (P-value). P-values < 0.05 and < 0.001 are indicated next to y-axis. Abbreviations; ADH, alcohol dehydrogenase; APX cytosolic ascorbate peroxidase; CBS domain, cystathionine beta-synthase domain; IFR, isoflavone reductase related protein; ms, methyltransferase; sti1-like, stress-induced protein; Ypr10, pathogenesis-related protein Ypr10.



Supplemental Figure S4-A1. Gene Ontology (GO) annotation for identified proteins. GO categories for Biological Function (Panel A1) for the Arabidopsis thaliana genome (a), and for all the 109 identified spots (b) and the differentially expressed proteins identified in 'Frida' (c) and 'Jonsok' (d) (Table II) were obtained and are shown as pie charts. The list of genes under the 'Response to Stress' and 'Response to Abiotic or Biotic Stimulus Categories' for 'Frida' and 'Jonsok' are different and listed in Panel A2.

A2

List of Genes in the Categories of 'Response to Stress and Response to Abiotic or Biotic Stimulus' for GO Biological Processes

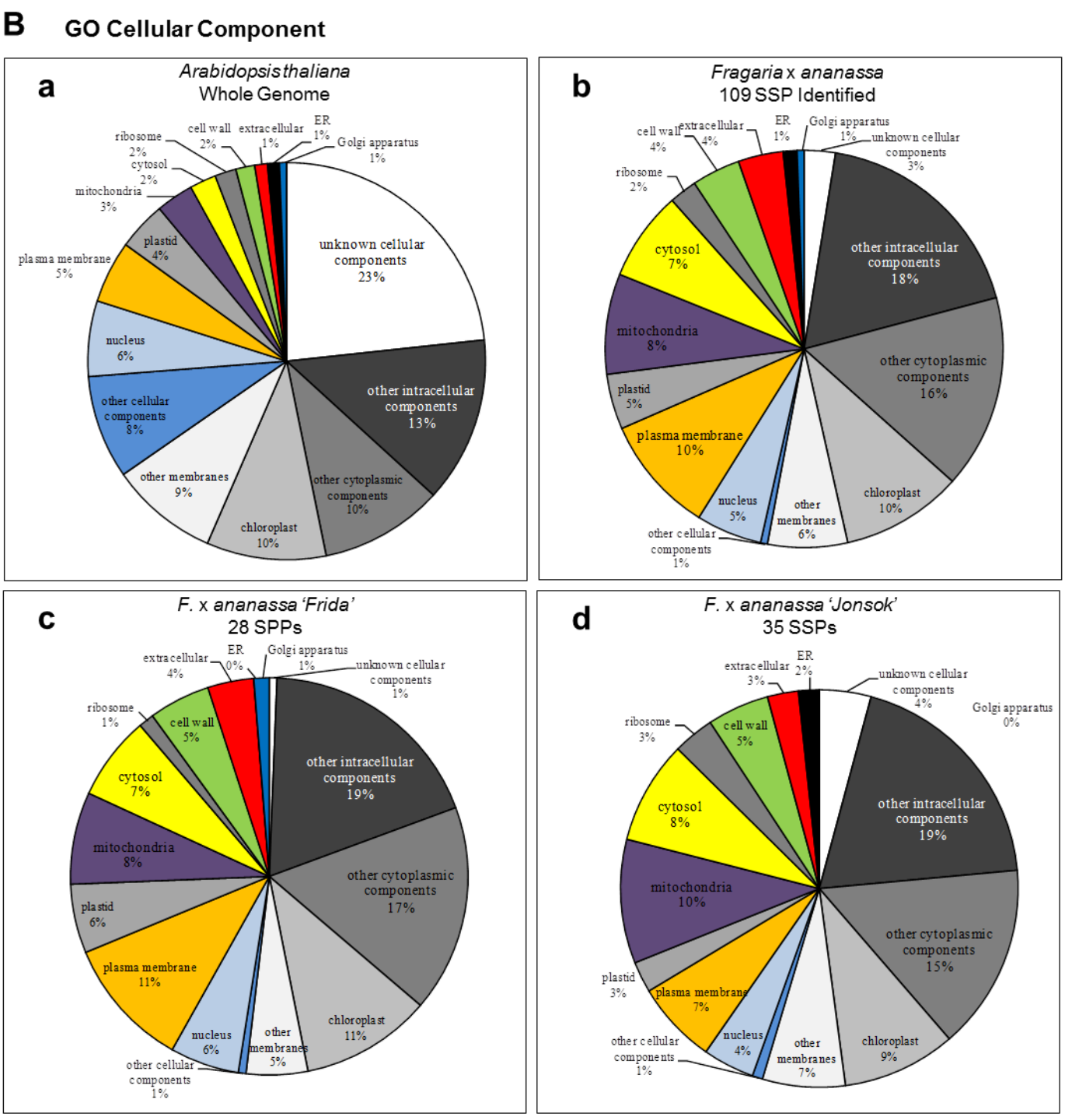
JONSOK

AGI	Protein ID	Spot no.
AT2G33150	3-ketoacyl-CoA thiolase	6539
AT1G77120	ADH (alcohol dehydrogenase)	4546, 6505, 6540
AT1G35720	Annexin	6432
AT1G07890	APX (cytosolic ascorbate peroxidase)	2218
AT3G57240	Beta-1,3-glucanase	2317
AT1G08830	Cu/Zn superoxide dismutase	2010
AT3G52930	Cytosolic aldolase	7528
AT2G36530	Enolase	3626
AT1G24020	Fra a 1-A, Fra a 2, Fra a 3	3114, 4015, 4011
AT2G47730	GST (glutathione transferase)	4115, 5125
AT3G12580	High molecular weight heat shock protein	813
AT5G02500	Hsc70 (heat shock cognate 70 kDa)	812
AT5G09590	HSP70 (heat shock protein 70)	1819
AT1G75280	IFR (isoflavone reductase related protein)	1423
AT3G01280	Porin	6224
AT3G02230	RGP (reversibly glycosylated polypeptide 1)	4547
AT4G11260	SGT1; putative protein phosphatase	622
AT1G75800	Thaumatin-like protein	2203

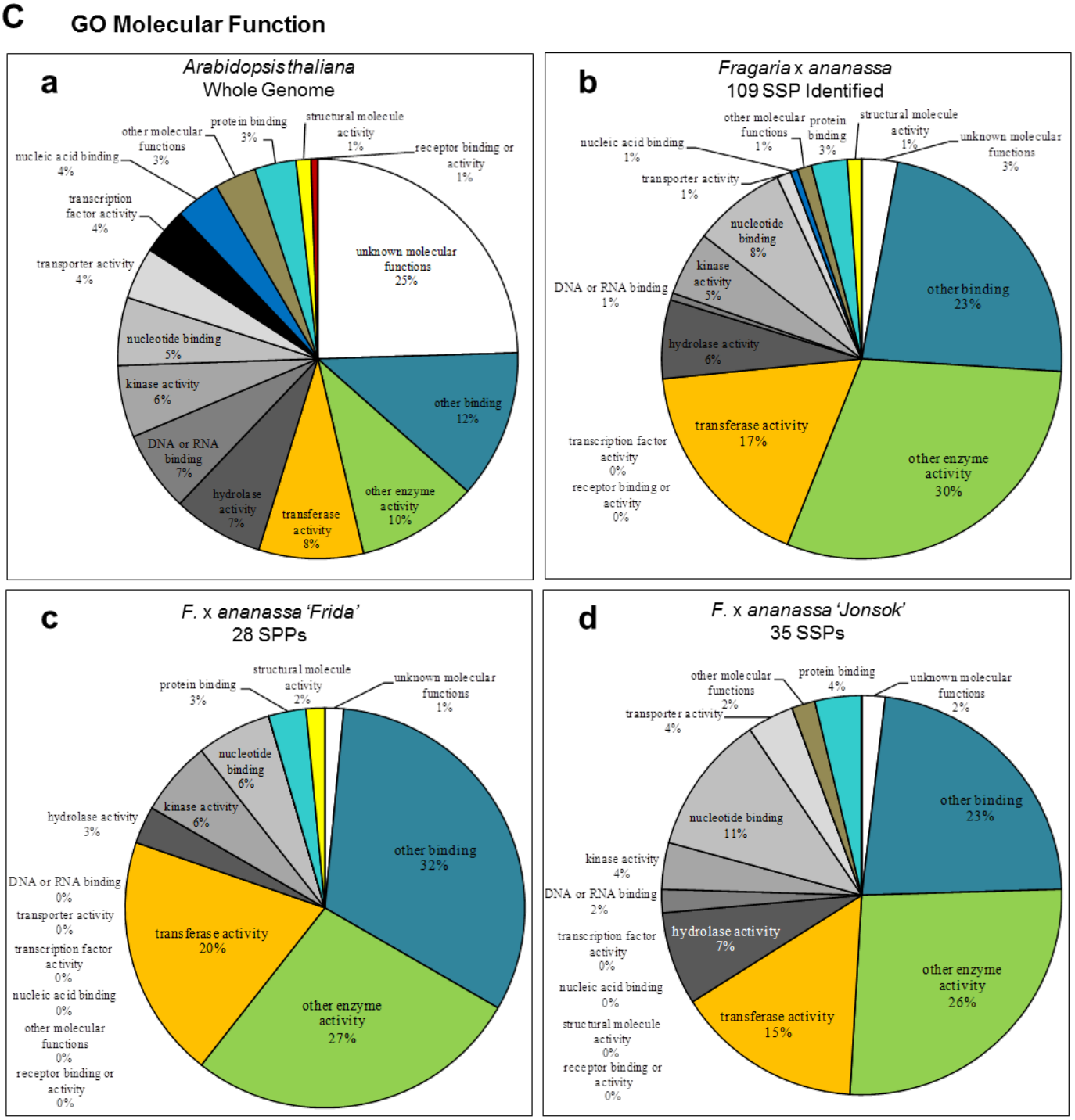
FRIDA

AGI	Protein ID	Spot no.
AT3G02360	6PGD (6-phosphogluconate dehydrogenase)	3625
AT2G05710	Aconitate hydratase	3912
AT1G35720	Annexin	6416
AT4G35090	Catalase	7707
AT5G13930	CHS (chalcone synthase)	4526
AT5G10860	Cystathionine beta-synthase domain	6014
AT3G52930	Cytosolic aldolase	7405
AT3G51240	F3H (flavanone 3-hydroxylase)	4536
AT5G42740	GPI (glucose-6-phosphate isomerase)	6704
AT1G47840	Hexokinase 1	3115
AT3G11050	Iron-binding protein	3223
AT1G53240	Malate dehydrogenase, mitochondrial	6403
AT5G17920	MS (methionine synthase)	6808
AT1G56450	Proteasome subunit b type-4	7210
AT3G17390	SAM (S-adenosylmethionine synthetase)	3612
AT1G62740	sti1-like (stress inducible-like protein)	3819

Supplemental Figure S4-A2. Gene Ontology (GO) annotation for identified proteins. The list of genes under the 'Response to Stress' and 'Response to Abiotic or Biotic Stimulus Categories' for 'Frida' and 'Jonsok' are different. The 2-DE Arabidopsis homolog is listed under the Arabidopsis Genome Initiative (AGI) gene index number, Protein identification and 2-DE identifier number are listed (Panel A2).



Supplemental Figure S4-B. Gene Ontology (GO) annotation for identified proteins. GO categories for Cellular Component (Panel B) for the *Arabidopsis thaliana* genome (a), and for all the 109 identified spots (b) and the differentially expressed proteins identified in 'Frida' (c) and 'Jonsok' (d) (Table II) were obtained and are shown as pie charts.



Supplemental Figure S4-C. Gene Ontology (GO) annotation for identified proteins. GO categories for Molecular Function (Panel C) for the *Arabidopsis thaliana* genome (a), and for all the 109 identified spots (b) and the differentially expressed proteins identified in 'Frida' (c) and 'Jonsok' (d) (Table II) were obtained and are shown as pie charts.

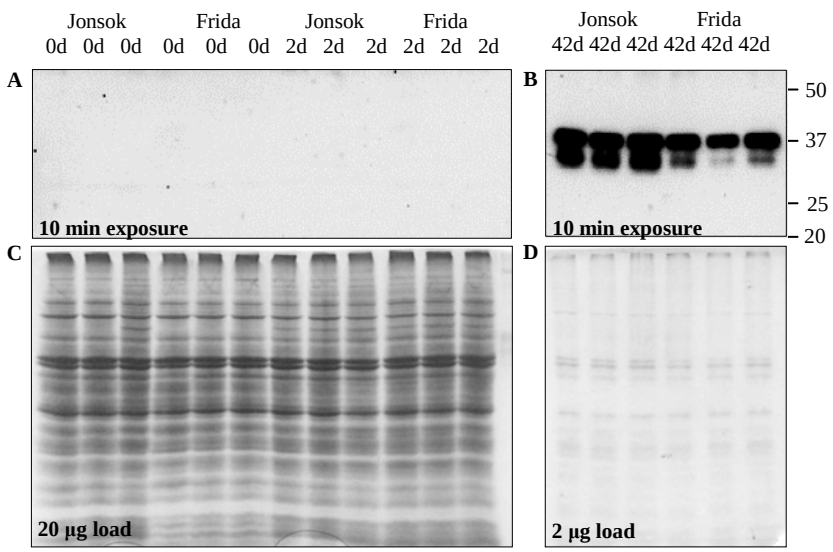
Supplemental Figure S4 *continues on following page*

D

Functional Category	*A. thaliana* Genome Annotation Count	*A. thaliana* Genome Gene Count	*F. x ananassa* 109 SSPs Annotation Count	109 SSPs Gene Count	'Frida' Annotation Count	'Frida' Gene Count	'Jonsok' Annotation Count	'Jonsok' Gene Count	Tranformed to percentage *A. thaliana* Genome	All ID'ed 109 SSPs	'Frida' 28 SSPs	'Jonsok' 35 SSPs
GO Cellular Component												
unknown cellular components	9939	9939	9	9	5	5	1	1	**23**	**3**	**4**	**1**
other cellular components	3594	3594	2	2	1	1	1	1	**8**	**1**	**1**	**1**
other membranes	3762	3103	23	20	8	6	8	8	**9**	**6**	**7**	**5**
nucleus	2623	2396	19	18	5	5	9	8	**6**	**5**	**4**	**6**
Golgi apparatus	248	230	2	1	0	0	2	1	**1**	**1**	**0**	**1**
chloroplast	4150	2942	35	25	11	9	17	11	**10**	**10**	**9**	**11**
ER	414	389	4	3	2	2	0	0	**1**	**1**	**2**	**0**
ribosome	739	465	8	7	4	3	2	2	**2**	**2**	**3**	**1**
plastid	1690	1115	16	12	3	3	9	6	**4**	**5**	**3**	**6**
cell wall	656	554	14	14	6	6	8	8	**2**	**4**	**5**	**5**
extracellular	440	433	13	13	3	3	6	6	**1**	**4**	**3**	**4**
plasma membrane	2165	2081	34	34	8	8	17	17	**5**	**10**	**7**	**11**
other intracellular components	5758	3927	65	35	23	12	30	14	**13**	**18**	**19**	**19**
mitochondria	1302	1101	29	21	12	9	12	8	**3**	**8**	**10**	**8**
cytosol	920	784	26	23	10	9	11	10	**2**	**7**	**8**	**7**
other cytoplasmic components	4281	3094	56	34	18	12	27	15	**10**	**16**	**15**	**17**
	total	total	total	total	total	total	total	total				
GO Cellular Component	42681	36147	271	119	93	160	116	116				
GO Molecular Function												
unknown molecular functions	10929	10929	5	5	1	1	1	1	**25**	**3**	**2**	**2**
DNA or RNA binding	2910	2756	1	1	0	0	1	1	**7**	**1**	**0**	**2**
transcription factor activity	1680	1680	0	0	0	0	0	0	**4**	**0**	**0**	**0**
nucleic acid binding	1611	1594	1	1	0	0	0	0	**4**	**1**	**0**	**0**
transporter activity	1864	1248	2	2	0	0	2	2	**4**	**1**	**0**	**4**
other molecular functions	1504	1372	2	2	0	0	1	1	**3**	**1**	**0**	**2**
hydrolase activity	3272	2517	11	8	2	2	4	3	**7**	**6**	**3**	**8**
kinase activity	2598	1343	9	5	4	2	2	2	**6**	**5**	**6**	**4**
receptor binding or activity	240	233	0	0	0	0	0	0	**1**	**0**	**0**	**0**
protein binding	1489	1348	5	5	2	2	2	2	**3**	**3**	**3**	**4**
structural molecule activity	538	538	2	2	1	1	0	0	**1**	**1**	**2**	**0**
nucleotide binding	2456	2201	13	11	4	4	6	5	**6**	**8**	**6**	**11**
transferase activity	3764	2483	30	19	13	9	8	6	**8**	**17**	**20**	**15**
other binding	5355	4529	40	28	21	13	12	8	**12**	**23**	**32**	**23**
other enzyme activity	4364	3336	52	39	18	14	14	13	**10**	**30**	**27**	**26**
	total	total	total	total	total	total	total	total				
GO Molecular Function	44574	38107	128	66	48	53	44	44				
GO Biological Process												
unknown biological processes	11476	11476	1	1	0	0	0	0	**19**	**0**	**0**	**0**
other cellular processes	13542	10481	66	43	33	20	21	16	**22**	**18**	**24**	**14**
other metabolic processes	12281	9788	61	44	24	18	21	16	**20**	**16**	**17**	**14**
protein metabolism	4205	3845	13	11	3	3	6	5	**7**	**4**	**2**	**4**
transcription	1921	1734	0	0	0	0	0	0	**3**	**0**	**0**	**0**
transport	2648	1824	6	5	2	2	4	3	**4**	**2**	**1**	**3**
developmental processes	3200	2088	11	8	3	2	4	3	**5**	**3**	**2**	**3**
signal transduction	743	728	0	0	0	0	0	0	**1**	**0**	**0**	**0**
DNA or RNA metabolism	504	368	0	0	0	0	0	0	**1**	**0**	**0**	**0**
cell organization and biogenesis	1660	1390	7	7	0	0	3	3	**3**	**2**	**0**	**2**
electron transport or energy pathways	362	322	8	8	2	2	4	4	**1**	**2**	**1**	**3**
other biological processes	2800	2032	53	41	16	13	19	14	**5**	**14**	**12**	**12**
response to abiotic or biotic stimulus	2916	1923	69	40	25	14	36	17	**5**	**19**	**18**	**24**
response to stress	3114	2077	76	40	31	15	35	15	**5**	**20**	**22**	**23**
	total	total	total	total	total	total	total	total				
GO Biological Process	61372	50076	248	139	89	153	96	96				

Supplemental Figure S4-D. Gene Ontology (GO) annotation for identified proteins. The data used to produce the pie charts for GO categories for Biological Function (Panel A1), Cellular Component (Panel B), and Molecular Function (Panel C), using TAIR (http://www.arabidopsis.org/tools/bulk/go/index.jsp in January 2011). The last four columns report the percentages obtained by dividing the gene count by total gene count listed for each class the *Arabidopsis thaliana* genome, and for the 109 identified spots and the differentially expressed proteins identified for 'Jonsok' and 'Frida' (Table II).

Supplemental Figure S4



Supplemental Figure S5. Dehydrin accumulation in *Fragaria* × *ananassa* ‘Jonsok’ and ‘Frida’.
A. Dehydrin accumulation (bands ~37 kDa) is observed at 42 day cold treatment (2 °C). The 0 and 2 day (Panel A, C) were loaded with 10 times more protein (20 ug) than 42 d (2 ug) (Panel B, D). Colloidal coomassie stained gels (Panel C, D) are presented for protein load comparison. The presence of dehydrins was not observed for control or 2 day cold treatment even though 10 x more protein was loaded. Antibody (anti-K-domain) was obtained from Agrisera and used at a 1:1000 dilution. Three lanes represent biological replicates. We speculate the dehydrin band (slightly larger than 37 kDa) is likely to be a Xero2-like dehydrin and would thus correspond to the transcript levels shown in Fig 7.

Supplemental Table S1. *Freezing survival demonstrates the relative cold/freezing tolerance of Fragaria × ananassa cultivars after cold acclimation*

Surviving plants were recorded 5 weeks after the freezing temperature program ended. Scoring of surviving plants, the browning extent and intensity were performed as described in Supplemental Methods S1. The level of significance was determined with ANOVA. Different letters in columns next to mean values indicate significant difference between treatments ($P < 0.05$, Tukeys). n.d. denotes data not determined**.** This data supports Table I.

	Exp	Surviving Plants (%)					Tissue Browning (1-5)					Browning Intensity (1-5)				
Cultivar	no.	0 °C	-3 °C	-6 °C	-9 °C	-12 °C	0 °C	-3 °C	-6 °C	-9 °C	-12 °C	0 °C	-3 °C	-6 °C	-9 °C	-12 °C
'Jonsok'	1	100	100	100	11.0	n.d.	1.0	2.2	3.4	5.0	n.d.	1.0	2.0	3.5	5.0	n.d.
	2	100	100	100	90.0	0.0	1.0	1.1	2.7	3.9	5.0	1.0	1.1	2.7	4.1	5.0
	3	100	100	50.0	0.0	0.0	1.0	1.7	4.0	5.0	5.0	1.0	1.7	4.4	5.0	5.0
Mean		100 a	100 a	80 a	30 a	0	1.0 a	1.7 b	3.4 a	4.7a	5.0 a	1.0 a	1.6 a	3.6 a	4.7 a	5.0 a
'Senga Sengana'	1	100	100	78	0.0	n.d.	1.0	2.2	4.3	5.0	n.d.	1.0	3.1	4.1	5.0	n.d.
	2	100	100	83	8.0	0.0	1.0	1.3	2.8	5.0	5.0	1.0	1.3	2.9	5.0	5.0
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mean		100 a	100 a	81 a	5 b	0	1.0 a	1.7 a	3.5 a	5.0 a	5.0 a	1.0 a	2.1 ab	3.4 a	5.0 a	5.0 a
'Elsanta'	1	100	100	33.0	0.0	n.d.	1.0	1.8	4.4	5.0	n.d.	1.0	2.8	4.5	5.0	n.d.
	2	100	100	33.0	0.0	0.0	1.0	1.6	4.8	5.0	5.0	1.0	1.6	4.8	5.0	5.0
	3	100	100	17.0	0.0	0.0	1.0	3.3	4.8	5.0	5.0	1.0	3.0	4.9	5.0	5.0
Mean		100 a	100 a	27 b	0 b	0	1.0 a	2.4 b	4.7 b	5.0 a	5.0 a	1.0 a	2.6 b	4.8 b	5.0 a	5.0 a
'Frida'	1	100	100	11.0	0.0	n.d.	1.8	2.6	4.9	5.0	n.d.	2.8	3.3	4.9	5.0	n.d.
	2	100	100	100	67.0	0.0	1.0	1.3	2.9	4.5	5.0	1.0	1.2	3.0	4.4	5.0
	3	100	100	17.0	0.0	0.0	1.0	3.1	4.8	5.0	5.0	1.0	2.8	4.9	5.0	5.0
Mean		100 a	100 a	45 ab	24 ab	0	1.2 b	2.3 b	4.2 ab	4.8 a	5.0 a	1.5 b	2.4 b	4.2 ab	4.8 a	5.0 a

Supplemental Table S2. Freezing survival demonstrates the relative cold/ freezing tolerance of *Fragaria* × *ananassa* without cold acclimation. Experiment was performed and evaluated as described in Supplemental Table S1. No surviving plant as determined by regrowth occurred in plans treated at -6, or -9 °C.

0 °C

Cultivar	Plant Survival (%)	Re-growth (new leaves)
'Senga S'	100	10.3
'Jonsok'	100	5.1
'Frida'	100	4.3
'Elsanta'	100	2.9
p-value	n.s	0.004

-3 °C

Cultivar	Plant Survival (%)	Re-growth (new leaves)
'Senga S'	100	14.8
'Jonsok'	30	2.3
'Frida'	0	0
'Elsanta'	0	0
p-value	0.003	0.001

Supplemental Methods S1

Table I. *Strawberry (Fragaria × ananassa) cultivars used in the freezing experiments*

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

Supplemental Methods S1

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

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

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

Supplemental Methods S1

Table III. *Freeze injury in strawberry plants determined by scoring 1-5, based on the condition of the plant at re-growth, and the extent and intensity of tissue browning*

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

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

Supplemental Methods S1

Figure 1. An example of visible freezing damage in crown tissue. Longitudinal sections of crowns from *F.* × *ananassa* 'Elsanta' 5 weeks after a freezing procedure at 0 °C (left) and -6.0 °C (right). Injury from freezing is readily seen as brown or black discoloration resulting from cellular damage as a consequent oxidation. Photos by Anita Sønsteby 2010.