Global patterns of protein abundance during the development of cold hardiness in blueberry

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Abstract

To gain a better understanding of the cold acclimation progression in blueberry, we investigated the proteome-level changes that occur in flower buds with increasing exposure to chilling temperatures using the 2D-DIGE technique. From this procedure, 104 protein spots were found to be differentially expressed. These proteins, identified by mass spectrometry, were compared to those previously found on 1-D protein gels and to differentially expressed transcripts from an earlier transcriptome study. The most highly induced proteins corresponded to previously described dehydrins. Approximately half of the changes in the proteome reflected similar changes in the transcriptome. In addition, from 2D-DIGE, different quantitative patterns of protein induction and suppression were found. The largest differences occurred during the transition from the first to the second stage of cold acclimation, which corresponded to timing of the largest increase in cold hardiness. This, with qualitative differences affecting the regulation of several functional groups, suggest as a whole that plants are able to monitor changes in the environment and then respond by modulating their proteome accordingly.

Major pathways increasing in abundance included stress-related proteins, carbohydrate/energy metabolism, amino acid metabolism, biosynthesis of phenolic compounds and gene expression regulation. On the other hand, pathways decreasing in abundance consisted of stress-related proteins, photosynthetic proteins and cell growth and structural components. Their possible implication in the development of cold hardiness is discussed.

Keywords: blueberry; cold acclimation; low temperature; mass spectrometry; proteomics; 2D-DIGE

1. INTRODUCTION

Characterizing and understanding how plants adapt and acclimate to freezing temperatures during various parts of their life cycle has been the subject of study since the latter part of the 19th century (Gusta and Wisniewski, 2013). In temperate regions, many species have evolved a low temperature response, known as cold acclimation, whereby they can increase their freezing tolerance after a period of exposure to low-nonfreezing temperatures (Levitt, 1980). Scientific interest in the molecular basis of cold acclimation and freezing tolerance is driven both by a desire to understand the evolutionary mechanisms that enable plants to tolerate the environmental stress and by the prospect that such understanding might provide new strategies to improve this trait in agriculturally important crops, creating new cultivars adapted to diverse environmental conditions (Fanucchi *et al.*, 2012). Studies of gene expression changes at the transcriptome level have contributed greatly to our actual understanding of the cold

stress response and provided a detailed list of cold-responsive genes in many species (see Knight and Knight, 2012; Qin *et al.*, 2011; Thomashow, 2010 for reviews). Significant advancement has also been made in understanding how transcriptional changes during cold acclimation are reflected at the translational level (see Janská *et al.*, 2010; Renaut *et al.*, 2006 for reviews). Proteomics provides a global and integrated view of cellular processes and networks and helps to extend our knowledge from gene expression at the transcriptional level to the metabolite level and finally to phenotypic expression. In recent years rapid advances in this field have been gained due to development of a number of high throughput and sensitive quantitative proteomic techniques (Neilson *et al.*, 2010).

Undoubtedly, *Arabidopsis* has been extremely useful in elucidating the underlying processes involved in regulation of cold responses in plants (Amme *et al.*, 2006; Bae *et al.*, 2003; Goulas *et al.*, 2006). For example, the CBF/DREB transcription factors (Liu *et al.*, 1998; Stockinger *et al.*, 1997) and their transcriptional regulators, ICE (Chinnusamy *et al.*, 2003; Fursova *et al.*, 2009) and CAMTA (Doherty *et al.*, 2009), were all discovered first in Arabidopsis. Beyond this highly significant work in *Arabidopsi*s, the majority of proteomics studies on plants exposed to low temperatures have been performed with herbaceous plants (for review see Kosová *et al.*, 2011). However, woody plants exhibit a much greater level of cold tolerance, with some species readily surviving temperatures well below -40°C (Wisniewski *et al.*, 2003) and therefore may have evolved additional genomic mechanisms contributing to physical and biochemical changes that allow survival under extreme winter conditions.

Aside from its economic value and functional food importance due to health-promoting properties, blueberry (*Vaccinium* spp.) has been used as a model system for studying adaptive mechanisms for dealing with freezing stress in woody perennials and for developing strategies to improve resistance (Rowland *et al.*, 2011). Susceptibility to spring frosts and degree of winter freezing tolerance have been identified as two of the most important genetic limitations of current blueberry cultivars. Winter damage is considered the major factor limiting yields in some regions of the U.S. (Hanson and Hancock, 1990; Moore, 1994). Among the available blueberry germplasm, there is

genotypic variability for the timing and rate of cold acclimation and deacclimation, the maximum level of cold tolerance achieved, and the maintenance of cold tolerance during the winter (Rowland *et al.*, 2008), all of which determine overall cold hardiness. In the U.S., the high composition of southern-adapted germplasm in the genetic background of some newly released blueberry cultivars has raised concerns about their suitability for certain regions, if not sufficiently cold hardy (Ehlenfeldt *et al.*, 2006).

The first studies in blueberry proteomics, which were aimed to identify proteins associated with low-temperature exposure, were carried out in the mid 1990s. From these studies, several dehydrins were identified as highly induced during cold acclimation making them the most abundant proteins in flower buds during the winter (Muthalif and Rowland, 1994a). Studies to further characterize expression of the blueberry dehydrins in response to chilling, cold, and drought stress and in various tissues were also performed (Arora *et al.*, 1997; Panta *et al.*, 2001; Parmentier-Line *et al.*, 2002), finally resulting in the isolation and sequencing of cDNA clones for the major 60 (Levi *et al.*, 1999) and 14 kDa dehydrins (Dhanaraj *et al.*, 2005). Since then, little information on blueberry proteins has been obtained or published. As of August 2015, only 41 proteins from *V. corymbosum* and 16 proteins from *V. ashei* were available in the protein section of NCBI. With the near completion of the genome assembly and the development of various genetic, genomic, and bioinformatic tools, blueberry now offers many possibilities to study questions that cannot be easily addressed in *Arabidopsis* (Die and Rowland, 2013). Combining proteomic and genetic analyses will be a key component in understanding the control of cold tolerance. Having more genomic data available should also improve *in silico* protein predictions from mass spectrometry data.

In this study, we compared the proteome in dormant flower buds of field-grown blueberry plants during different stages of cold hardiness development, or cold acclimation, by two-dimensional differential in-gel electrophoresis (2D-DIGE) followed by mass spectrometry. Differentially expressed proteins, during adaptation to low-temperature stress, were analyzed with the intention of identifying regulatory and

functional pathways that are responding across a time course. In the process, we tested several hypotheses: (1) that changes in the proteome could be detected during the transitions from different stages of cold acclimation (1st stage = exposure to short photoperiod, 2nd stage = exposure to short photoperiod and low, nonfreezing temperatures, and 3rd stage = exposure to subzero temperatures); (2) that the most dramatic changes detected by 2D-DIGE would be consistent with changes found previously from 1-D protein gels; (3) that proteomic changes would be congruent with previously described transcriptome changes, and (4) that proteomic changes during cold acclimation of blueberry would be similar to changes reported in other plant systems. In so doing, we aimed to better understand changes in the proteome of plants throughout the dormant period, specifically during cold acclimation, and ultimately help us develop cultivars better suited to specific environments.

2. MATERIALS and METHODS

2.1 Plant material

Flower buds were collected from multiple plants of the northern highbush blueberry cultivar 'Bluecrop' (*V. corymbosum*) grown at the USDA/ARS, Beltsville Agricultural Research Center, Beltsville, MD. 'Bluecrop' is considered to be a cold-tolerant variety (Rowland *et al.*, 2008). Samples were collected from field plants during the fall and winter of 2006-2007 at several time points with increasing exposure to chilling temperatures, measured as chill units (hours ['] between 0-7°C): 0' (7 September 2006), 397' (30 November 2006) and 789' (16 January 2007). Time points were (1) early in September before plants had been exposed to temperatures below 7ºC but exposed to shortening photoperiods (0 hours chilling, 0'), (2) late November when plants were exposed to short photoperiods and had received about 400 chill units (397' from 0-7ºC), and (3) in the middle of January when plants had received about 800 chill units (789' from 0-7ºC), had been exposed to several freezing events and attained maximum cold hardiness. These time points represent the 1st, 2nd, and 3rd stages of cold acclimation. From an average of cold hardiness measurements made that same year and the following year, these time points correspond to hardiness levels of about -10ºC (0'), -25ºC (400'),

1 and -27ºC (800') (Ehlenfeldt *et al.*, 2012). The sample pools from each time point (~10
2 g representing about 500-1000 flower buds) were made from a minimum of five plants.
3 All tissues were frozen in liquid nitrogen immediately after harvest and stored at -80°C.
4
5 **2.2 Protein extraction, CyDye labeling, and 2D-DIGE**
6 Bud samples were crushed in a pre-cooled mortar with liquid nitrogen until a fine
7 powder was formed. Proteins were extracted with TCA-phenol (Wang *et al.*, 2006) and
8 quantified according to (Esen, 1978). Two-dimensional differential in-gel
9 electrophoresis (2D-DIGE) was performed at Applied Biomics, Inc. (Hayward, CA).
10 Briefly, protein extracts from flower buds were denatured by addition of an equal
11 volume of lysis buffer containing 7M urea, 2M thiourea, 4% 3-((3-cholamidopropyl)
12 dimethyl ammonio)-1-propanesulfonate (CHAPS), followed by addition of 30 mM Tris-
13 HCl, pH 8.8. Next, each set of three samples that were to be run on a single gel were
14 labeled with a CyDye dilution of Cy2, Cy3, or Cy5 (Amersham Biosciences,
15 Piscataway, NJ) as described in Supplementary Table S1. Labeling was stopped by
16 adding 0.7 µl of 10 mM L-Lysine and incubating at 4°C for 15 min. Then, equal
17 amounts of the three labeled samples were mixed together, along with an equal volume
18 of 2X 2-D sample buffer (8 M urea, 4% CHAPS, 20 mg/ml dithiotreitol (DTT), 2%
19 pharmalytes and a trace amount of bromophenol blue) and 100 µl of destreak solution
20 (GE Healthcare Biosciences, Pittsburgh, PA). Total sample volumes were adjusted to
21 260 µl by adding rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/ml
22 DTT, 1% pharmalytes and trace amount of bromophenol blue). Each set of three labeled
23 samples were then subjected simultaneously to isoelectric focusing (IEF) on a 13-cm
24 precast non-linear immobilized pH gradient strip (pH 4-9, Amersham Biosciences).
25 Next, the samples were separated in the second dimension based on size by sodium
26 dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE).
27
28 **2.3 Image scan and data analysis**
29 Upon completion of electrophoresis, gels were scanned using a Typhoon Trioscanner
30 (Amersham BioSciences) following the manufacturer's protocol. The scanned images
31 were then processed by Image Quant software (Amersham BioSciences, v.5.0).

Differential protein expression was assessed by differential in-gel analysis (DIA). The quantitative analysis of protein spots was performed using DeCyder software (Amersham Biosciences, v.6.5). Quantitative comparisons were calculated between samples run at the same time and pair-wise volume ratios were calculated for each protein spot and used to determine relative protein expression. A Student's *t*-test was performed using the log2 normalized average spot volume ratios for all spots detected from three replicate experiments. Only statistically significant results ($P<0.05$), and differentially expressed proteins with a ratio $\geq$1.5 fold difference in one condition (increase or decrease in abundance), were chosen for mass spectrometry.

2.4 Protein identification by mass spectrometry

Based on 2D-DIGE and data analysis by DeCyder software, spots of interest were subjected to in-gel trypsin digestion, peptide extraction, desalting, and spotting on a MALDI plate followed by MALDI-TOF/TOF to determine the protein identity. Briefly, mass spectra (MS) of the peptides in each sample were obtained using Applied Biosystems Proteomics Analyzer (Foster City, CA). The 10-20 most abundant peptides in each sample were further subjected to fragmentation and tandem mass spectrometry (MS/MS) analysis. Combined MS and MS/MS spectra were submitted for database search using GPS Explorer software equipped with the MASCOT search engine to identify proteins from primary sequence databases. The highest scoring hit from the database search for each 2D gel spot was used as the protein identification label. Candidates with protein score C.I.% or ion C.I.% > 95 were considered significant.

2.5 Functional classification and bioinformatics tools

The Gene Ontology Functional Annotation Tool Blast2GO version 2.7.1 (Conesa *et al.*, 2008; Götz *et al.*, 2008) was used to assign GO identities and enzyme commission numbers to proteins identified by mass spectrometry. For the annotation, the following configuration settings were used: BLASTP against NCBI non-redundant (nr) protein database, *E*-value filter $\leq 10^{-6}$, length cutoff of 33, maximum 10 BLAST hits per sequence, and annotation cutoff of 50. Furthermore, to improve annotation ability, InterProScan was performed and results were merged to GO annotation. The program

Blast2GO was also used to assign biological functions, cellular components, and cellular processes to the sequences. The analysis of biological processes/pathways was carried out using the KEGG (Kyoto Encyclopedia of Genes and Genomes) map module supported by the Blast2GO bioinformatics tool. Blast2GO and protein spot ratio outputs were passed through a custom pipeline built with the scripting language Python to analyze data, build clusters, and generate some figures. The open-source interface for the statistics software R, RStudio (http://www.rstudio.com/), was used to perform exploratory data analysis (EDA).

3. RESULTS

3.1 Protein profiles of flower buds at different stages of cold acclimation obtained by 2D-DIGE

To analyze the proteome response of blueberry flower buds during the different stages of cold acclimation, proteins were extracted from buds of field plants of the northern highbush cultivar ‘Bluecrop’ collected at three different time points. ‘Bluecrop’ is the industry standard for many areas of the U.S. and has a relatively high level of cold tolerance (Rowland *et al.*, 2008). Time points were (1) early in September (0 hours chilling, 0’), (2) late November (397’), and (3) in the middle of January (789’).

Proteins from the three time points were labeled with different CyDyes and fractionated simultaneously by two-dimensional differential gel electrophoresis (2D-DIGE), separating them by isoelectric point (pH of 4.0-9.0) in the first dimension and molecular mass (size of 14-150 kDa) in the second dimension. Pictures of a representative gel are shown in Fig. 1. From initial visual inspection, protein profiles appeared very similar between 379’-789’ and very different between 0’-379’ and 0’-789’ (Fig. 2). Matching of protein spots across gels allowed calculation of the relative protein expression ratios of differentially expressed proteins and their standard errors (fold changes) in relation to the other samples run at the same time (Supplementary Table S2).

Analysis of the acquired images from three replicate gels indicated a number of reproducibly differentially expressed proteins. A comparison among conditions revealed

that 106 spots were differentially expressed by a factor of at least ±1.5 (*t* test with significance $P<0.05$) in at least one condition (Fig. 3). Exploratory data analysis was done to visualize and summarize the overall quality of the data and identify general patterns (Supplementary Fig. S1). A multi dimensional scaling plot resulted in the clear separation of the buds from the three timepoint comparisons (397'-0', 789'-397', 789'-0') and grouping of buds from the three replicates of the same timepoint comparison. This is as expected if there were larger differences between timepoints than between replicates.

It is noteworthy that different patterns of protein induction or suppression were found (Fig. 4). At 397', 61 spots had increased in abundance and 31 had decreased significantly. From those 61 spots that increased, 18 spots showed a steady increase in expression over the dormancy progression (0-789') (Fig. 4A), while 8 of the 31 spots that decreased showed a steady decrease (Fig. 4E). However, most of the spots displayed an early change in expression at 397', being increased (39) or decreased (23), but maintaining a constant level at 789' (Fig. 4B and 4D). Only a few proteins showed more of a transient expression pattern (4), peaking at one stage followed by a turnabout at the next one (Fig. 4C). Moreover, we found a group of 14 spots that did not show any expression change at 397'-0' but they did when we compared their expression levels at 789'-397' (4 spots) or 789'-0' (10 spots; Supplementary Fig. S2). Table 1 summarizes the quantitative distribution of spots according to their expression patterns over the cold acclimation progression.

3.2 Mass spectrometry, GO annotation and functional classification

The 106 differentially expressed spots were excised from gels and subjected to MS/MS analysis. Two spots did not produce positive identification. Statistical and identification data for the rest of the 104 processed spots are presented in Supplementary Table S2. Gene Ontologies (GO) were assigned to proteins on the basis of Blast2GO annotation. As a result, 98 sequences could be functionally classified into one or more ontologies: 49 sequences were classified in terms of cellular components, 64 sequences by molecular functions, and 80 sequences were classified according to biological processes;

47 sequences were classified in all three ontologies. A comparative distribution analysis of the biological processes from the differently regulated proteins (397'-0' *versus* 789'-397') revealed some slight differences. In both cases, the vast majority of GO terms were assigned to metabolic process (GO:0008152) and cellular process (GO:0009987) categories. However, the contribution to the total GO terms of metabolic process was more predominant at 789'-397' (+3.1%) while other categories, such as biological regulation (GO:0065007) or response to stimulus (GO:0050896), were more abundant at 397'-0' (+3.2%) (Supplementary Fig. S3). Further detailed analysis of response to stimulus category showed that more than half of the GO terms obtained were associated with response to stress, responses to dehydration or water-related stimulus, response to metal ion and oxidative stress-related responses (Supplementary Fig. S4).

On the basis of the GO annotations and subsequent KEGG analysis, we classified the differentially expressed proteins by functional similarity in order to obtain a general overview of biological processes/pathways involved in cold acclimation (Tables 2 and 3). Because the most dramatic differences in protein profiles were seen between the first two time points, most of the proteins that increased (Table 2) and most that decreased in abundance (Table 3) showed significantly different levels at 397'-0'. Only four proteins showed no significant change at 397'-0' but significant difference at 798'-397'. Of these, three proteins significantly increased and one significantly decreased in abundance between 798'- 397'. Overall, a larger proportion of proteins showed an increase, rather than a decrease, in abundance during cold acclimation. Generally, proteins that increased included many typical stress responsive proteins (chaperones, dehydrins) as well as ROS-scavenging enzymes and proteins involved in stress signaling and transduction, like calcium binding proteins. Proteins with decreased abundance were involved in stress responses (abscisic stress ripening protein), energy (chloroplastic oxygen-evolving enhancer protein), and amino acid metabolism (polyphenol oxidase), among others.

4. DISCUSSION

We have previously characterized changes in the transcriptome of blueberry flower buds during cold acclimation using both Sanger and next generation 454 ESTs (expressed sequence tags) and microarrays (Dhanaraj *et al.*, 2004; Dhanaraj *et al.*, 2007; Die and Rowland, 2014). In this current study, we used the 2D-DIGE technique to monitor changes in the proteome of blueberry flower buds during different stages of cold acclimation as defined as: 1st stage, exposure to shortening photoperiod but not low temperatures (0’); 2nd stage, exposure to low temperatures but few, if any, freezing episodes (397’); 3rd stage, exposure to significant subzero temperatures (789’).

4.1 Hypothesis 1: Proteome changes are detected during different stages of cold acclimation

Differentially expressed proteins were detected from 2D-DIGE, and based on spot-volume profiles, they were separated into 7 specific groups depending on their expression patterns (Fig. 4). These patterns differed based on whether the proteins displayed a steady change in abundance during the different stages of cold acclimation (24.5%) or whether they displayed fluctuating changes in abundance over the time points (75.5%). The highest number of differentially regulated proteins was detected by 397’, although plants did not attain maximum cold hardiness until 789’, indicating that the preparation for winter at the molecular level begins at an early stage. Plants had experienced only one day between 0’ and 397’ when temperatures dipped below 0ºC, whereas there were 13 days between 397’ and 789’ when plants experienced subfreezing temperatures. This exposure to subfreezing temperatures is likely essential for plants to reach maximum cold hardiness levels, even though few changes were observed in the proteome between 397’ and 789’. The quality of the differentially expressed proteins did seem to be somewhat different at 397’ and 789’. When we grouped proteins on the basis of GO annotations, biological regulation and response to stimulus categories were more predominant at 397’, while metabolic process was more predominant at 789’. This may indicate that plants are responding more to environmental cues such as low temperature, photoperiod, or other stimuli between the first (0’) and second stages of cold acclimation (397’), while more metabolic adjustments may be occurring between the second (397’) and third stages (789’). By 789’, ‘Bluecrop’ plants had not only attained their maximum cold hardiness level but

were also nearing satisfaction of their chilling requirement and termination of endodormancy (Ehlenfeldt *et al.*, 2012). The quantitative and qualitative differences in the regulation of proteins over the timecourse suggest, as a whole, that the plant cells are able to monitor changes in the environment and levels of stress intensity throughout the dormant period and then respond and modulate their protein expression accordingly. By looking closely at the response to stimulus GO terms, a connection between cold acclimation and other stress responses emerges as well highlighting that the same signal pathways are used by different stress responses. Cold acclimation is a multifactoral phenomenon involving overlapping responses to cold, drought and oxidative stress.

4.2 Hypothesis 2: Changes detected by 2D-DIGE are consistent with changes observed on 1-D protein gels

Dehydrins were the first and the most abundant proteins identified as responsive to low temperature in blueberry on 1-D protein gels (Muthalif and Rowland, 1994a). Increased levels of three dehydrins of 65, 60, and 14 kDa are closely correlated with midwinter cold hardiness levels (Arora *et al.*, 1997; Muthalif and Rowland, 1994b; Panta *et al.*, 2001; Rowland *et al.*, 2005). The proposed role of dehydrins is to stabilize cell membranes and prevent protein denaturation at low intracellular water content, as well as prevent the formation of intracellular ice (Janská *et al.*, 2010). From 2D-DIGE results, the group of upregulated proteins comprising clusters 1 and 2 (Fig. 4, Table 2) are, in fact, dominated by dehydrins, and the three dehydrins previously described are the most highly induced of all the differentially expressed proteins. In addition from 2D-DIGE, it is apparent that several molecular weight isoforms of the 65 kDa (spots 24, 25, and 26), 60 kDa (spots 29, 30, and 31), and 14 kDa (spots 84, 87, and 90) dehydrins exist with varying pIs. Other dehydrins were detected as well that are upregulated with cold, spot 51 and spot 120 (high similarity to COR11).

4.3 Hypothesis 3: Changes in the proteome are partly congruent with transcriptome changes

Using reciprocal blast, we examined the congruence between the significantly differentially expressed protein spots and the transcripts from one of our well-characterized transcriptome datasets (Die and Rowland, 2014). A confident match was

made for 64 of the spots (61.54% of the 104 annotated spot proteins). Out of these 64 spots, 49 corresponded to a contig in the transcriptome dataset that was significantly regulated in the same direction. Discrepancies between proteome and transcriptome data may be indicative of translational control mechanisms at work or may reflect differences in sensitivities of the techniques, among other possibilities. These discrepancies confirm the need to combine different approaches in unraveling the molecular mechanisms underlying important agronomic traits.

4.4 Hypothesis 4: Proteomic changes during cold acclimation of blueberry are similar to changes reported in other plant systems

All the proteins that were differentially expressed were classified into major functional categories (Tables 2 and 3). Major pathways increasing in abundance included stress-related proteins, carbohydrate/energy metabolism, amino acid metabolism, biosynthesis of phenolic compounds, and gene expression regulation/signaling. Pathways decreasing in abundance mostly consisted of stress-related proteins, photosynthetic proteins, and cell growth and structural component proteins. The general pathways and their relation to the literature are discussed in detail in the following sections.

Cold- responsive stress proteins

Cold acclimation resulted in a significant increase in the abundance of several well-characterized cold- and drought-related stress protective proteins such as chaperonins and dehydrins. The dehydrin proteins are described above in section 4.2. Chaperonins included two heat shock proteins (spots 9 and 86). Another upregulated protective protein, which has a chaperone function and may help stabilize partially denatured proteins or membranes in the chloroplast, is a binding protein (spot 10). We also found a filamentation temperature-sensitive protein (spot 11) that increases in abundance. The upregulation of this protein under cold stress may help to alleviate photosynthetic apparatus damage and chloroplast disorganization during photoinhibition, with a role in repair of PSII and photosynthetic adjustments (Kato *et al.*, 2012; see discussion below). It is important to note here that the flower buds used in our study included the bud scales, which are modified leaves, thus explaining the presence of chloroplast-related proteins. We have previously observed induction of several genes at the transcript level

related to light stress during cold acclimation of blueberry flower buds (Dhanaraj *et al.*, 2004; Dhanaraj *et al.*, 2007; Naik *et al.*, 2007).

The progression of cold acclimation also resulted in a decrease in abundance of several stress-related proteins including three abscisic stress ripening (ASR) proteins (spots 19, 20, and 68; Table 3). Spot 68 is, in fact, the most down-regulated of all the differentially expressed proteins. Although it is known that ASR proteins are involved in abiotic stress responses, the molecular mechanism underlying their function remains unclear. Arabidopsis plants overexpressing an ASR gene from lily exhibited enhanced cold and freezing tolerance, and a detailed analysis found that ASR mediates cold/freezing stress signaling by upregulating a group of 12 genes and down-regulating 25 genes (Hsu *et al.*, 2011). This and other work suggest a dual activity for ASR, as a chaperone-like protein and as a transcription factor (Dominguez and Carrari, 2015; Hsu *et al.*, 2011). Like the transcriptional activator CBF, whose expression has been studied previously in blueberry at the transcript level (Polashock *et al.*, 2010), the ASR proteins are expressed at the highest level at the first stage of cold acclimation (0') and decrease thereafter. Spot 68 decreased in abundance by 6.6 fold by 397' and by 15 fold by 789' of chilling.

Proteins involved in carbohydrate and energy metabolisms

Energy is necessary to drive acclimation, and mobilization of sucrose has been shown to be essential during this process (Wisniewski *et al.*, 2014). We found increased levels of several proteins involved in glycolysis (phosphoglycerate mutase, spot 12; phosphopyruvate hydratase, spot 21) and the TCA-cycle (cytosolic aconitase, spot 4) during cold acclimation of blueberry (Table 2). Presumably, these pathways, i.e., respiratory acclimation, are needed to breakdown sugars and generate the required chemical energy in the form of ATP for coping with low temperatures and/or provide substrates for some anabolic pathways needed for cold acclimation. Respiratory acclimation has been linked to co-upregulation of alternative oxidase (AOX) and NADH-dehydrogenase (Rasmusson *et al.*, 2009). Notably, one AOX (spot 75) showed a steady incremental increase in abundance over the timecourse (cluster 1). *Aox1a* Arabidopsis mutant plants show a high sensitivity to combined light and drought stress (Giraud *et al.*, 2008), and induction of AOX1a via plastid signals enhances tolerance to

1 cold stress by increasing respiration and temperature, highlighting a recently discovered
2 communication link between plastid and mitochondrion (Tang *et al.*, 2014).
3
4 We also found increases in abundance of several proteins involved in the malate
5 metabolic process, notably two NADP-dependent malic enzymes (spots 14 and 110)
6 and a phosphoenolpyruvate carboxylase protein (PEPCase, spot 18). Malate seems to
7 be an important source of acetyl-CoA used for fatty acid biosynthesis during cold
8 acclimation in *Rhododendron*, a close relative of the blueberry species (Wei *et al.*,
9 2006). Malate has been shown to accumulate in cold stressed winter rye and
10 *Arabidopsis* leaf tissues (Crecelius *et al.*, 2003; Kaplan *et al.*, 2004), and PEPCase is
11 regarded as an indicator of the cold hardiness status in genotypes of rye, citrus, and
12 cotton (Crecelius *et al.*, 2003; Vu *et al.*, 1995; Zheng *et al.*, 2012).
13
14 Another protein of interest in the "Energy metabolism" group is Rubisco, which is
15 sometimes considered to be an unconventional storage protein (Cooke and Weih,
16 2005). The observed accumulation of the large subunit of Rubisco (spots 22 and 23) is
17 in contrast to the decrease in abundance of the small subunit (spot 178; Tables 2 and 3).
18 However, this discrepancy between the two Rubisco subunits has been found in other
19 plants, for example, during dormancy transitions of the woody perennial white spruce
20 (Galindo González *et al.*, 2012). These results suggest that the large subunit may serve
21 a storage function in overwintering blueberry flower buds. It is important to note here
22 that while the blueberry flower buds are acclimating to cold, they are also transitioning
23 from dormancy induction, maintenance, and then release. Therefore, some or many of
24 the observed proteomic changes could be related to one or the other or could be
25 common to both phenological transitions, cold acclimation and dormancy.
26
27 **Proteins involved in amino acid metabolism and reinforcement of antioxidant**
28 **system**
29 Another important metabolic pathway, strongly induced with exposure to cold, is
30 methionine metabolism via methionine synthase (spots 107 and 109) (Table 2).
31 Methionine is a building block (a starting point) for protein synthesis and methionine

synthase is part of the "activated methyl cycle" which is important for synthesis of major metabolites such as methylated polyols and polyamines needed during cold stress (Bohnert and Jensen, 1996; Narita *et al.*, 2004). These compounds have been shown to accumulate in several plants exposed to chilling, including potato and poplar (Sergeant *et al.*, 2014). Cuevas et al. (2008) showed that Arabidopsis plants defective in biosynthesis of one of the polyamines, putrescine, is more freezing sensitive.

Other proteins associated with amino acid metabolism, specifically phenylalanine metabolism, were upregulated during cold acclimation as well. These include aspartate aminotransferase 2 family protein (spot 34) and phenylalanine ammonia-lyase (PAL, spot 76; Table 2). PAL is the first enzyme of the biosynthetic pathway of phenylpropanoids, and its activity has been shown to intensify with exposure to cold and freezing stress (Graham and Patterson, 1982; Janas *et al.*, 2000; Ortega-García and Peragón, 2009). One particular type of protective phenolic compound is flavonoids, which have radical-scavenging activity (Gill and Tuteja, 2010). It has been proposed that when plants are exposed to an excess of light, or an excess of excitation energy, biosynthesis of flavonoids may serve as a "secondary" antioxidant system in the chloroplasts (Close and McArthur, 2002; Fini *et al.*, 2012; Pollastri and Tattini, 2011) and as UV screens (Emiliani *et al.*, 2013). It is interesting that levels of flavanone 3-hydroxylase (spots 48 and 49) and chalcone isomerase (spot 154), both of which are involved in flavonoid biosynthesis also, increase during cold acclimation of blueberry (Table 2). A proteomic study has recently reported similar upregulation of these proteins in strawberry during cold acclimation (Koehler *et al.*, 2013). In contrast, levels of two reductases, isoflavone reductase (spots 43 and 44) and anthocyanidin reductase (spot 45) decrease during cold acclimation (Table 3). Another phenylpropanoid regulated by PAL is lignin, which is needed for cell wall thickening during cold acclimation (Stefanowska *et al.*, 1999).

Proteins involved in lowering photosynthetic activity, reduction of oxidative damage, and accumulation of phenolic compounds

The decrease in activity of photosynthetic enzymes with cold treatment can potentially result in the absorption of light energy by chloroplasts (in this case, in blueberry flower bud scales) in excess of what can be processed. This may cause inhibition of PSII reaction centers and/or photo-oxidative damage (Öquist and Huner, 2003). To prevent the formation of reactive oxygen species (ROS) under these conditions, enzymatic systems are activated (Apel and Hirt, 2004; Suzuki *et al.*, 2012). We found a significant decrease in the abundance of proteins related to the light phase of photosynthesis, such as oxygen-evolving enhancer proteins (spots 71, 138, and 160) and transporter proteins, located in the chloroplast stroma (arsenical pump-driving ATPase-like, spots 72 and 74; Table 3). We found increases in levels of different ROS-scavenging enzymes, superoxide dismutase (spot 62) and peroxidase (spot 126). Moreover, the level of a phospholipase D, with GO terms associated with response to oxidative stress, increased with cold acclimation as well (spot 61; Table 2).

Polyphenol oxidase (PPO) is another enzyme involved in plant defenses against biotic (Mayer, 2006; Thipyapong *et al.*, 2004a) and abiotic (Thipyapong *et al.*, 2004b) stress conditions. PPO catalyzes the oxygen-dependent oxidation of phenols to quinones. Therefore, PPO is another oxidase that potentially could play a role in ROS-scavenging. For example, a high level of PPO activity has been related to reduced free-oxygen levels available for ROS production during cold stress in olive trees (Ortega-García and Peragón, 2009). In our experiment, we identified three spots as PPO (spots 130, 131, and 132), which, however, decreased in abundance (cluster 4) with cold acclimation (Table 3). One explanation may be found in the connection between photo-oxidation, PPO activity and phenylpropanoid metabolism. Zhan *et al.*, (2013) have shown that light exposure results in suppressed PPO activity in fresh-cut celery, inhibition of tissue browning, and improved antioxidant capacity (increased activity of PAL). Llorente *et al.*, (2014) working with PPO-silenced potato lines showed that down-regulation of PPO leads to accumulation of defensive phenolic compounds via redirection of phenylpropanoid metabolism. Using a reverse genetic approach to silence the PPO gene in walnut, Araji *et al.*, (2014) have also demonstrated the important role of PPO in the

metabolism of phenolic compounds. Thus, down-regulation of PPO in blueberry may allow the accumulation of defensive phenolic compounds that enhance cold tolerance.

Cell wall remodeling and signal transduction

We found down-regulation of several structural or cytoskeletal components such as the mitotic motor protein kinesin (spot 162) and the cell wall-related pectin acetylesterase (spot 38). Another down-regulated protein is ubiquitin ligase Bub3 (spot 41), which forms part of the culling 4 RING complex and might suggest the inhibition of cell division. This might be part of an essential mechanism to confer cold hardiness because this gene is one of the only two that have been found to be down-regulated over several time-points and accessions in *Arabidopsis* when studying the transcriptional regulation of sub-zero responsive genes (Le et al., 2015). Interestingly, we found an increase in abundance of three Ca^{2+}-binding proteins (spots 112, 113, and 114; Table 2), which is consistent with Ca^{2+}-mediated signaling. Other putative regulators of gene expression were identified, some of which increase and some that decrease with cold acclimation. For example, the RNA polymerase B transcriptional factor 3 protein (Btf3, spot 70) decreased in abundance during cold acclimation (cluster 5). The same protein has been found to be down-regulated in a tolerant potato genotype in response to chilling temperature (Folgado et al., 2013).

5. CONCLUSIONS

Understanding the molecular mechanisms by which plants respond to low temperature is of fundamental importance to plant biology. Knowledge of these mechanisms is crucial for development of rational breeding and biotechnological strategies to improve stress tolerance in crops with practical implications regarding their geographical distribution. Cold acclimation is a complex phenomenon involving the alteration of metabolism, including synthesis of specific metabolites, proteins, lipids and carbohydrates, and changes in membrane compositions. The proteomic data presented here may represent only the major changes in total proteins during cold acclimation. However, from this we can conclude that plants are able to monitor changes in the environment and then

1 respond by modulating their proteome accordingly. Most of the significantly regulated
2 proteins were detected between 0' and 397', when plants transition from the 1st to the
3 2nd stage of cold acclimation. Sixteen of the regulated spots showed a steady increase or
4 decrease in expression over the dormancy progression (0'-789'). These, and some other
5 potential regulatory proteins, would be interesting candidates for further functional tests
6 to elucidate their roles in the acquisition of plant cold hardiness.
7

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Table 1. Quantitative distribution of differentially expressed spots

	Increasing	Decreasing
397’-0’	61 spots (57.5%)	31 spots (29.2%)
789’-397’	21 spots (19.8%)	13 spots (12.3%)
397’-0’ + 789’-397’	18 spots (17.0%)	8 spots (7.5%)

Table 2. Differentially expressed proteins with increased abundance. The proteins are grouped by their functional similarity. Cluster number for proteins changing at 397'-0' is shown. Cluster symbol (*) denotes proteins changing only at 789'-397'.

Spot Id.	Name / Annotation	Biological function / Pathway	Cluster
Stress-related proteins / Defense			
spot 9	heat shock protein sti-like	protein folding	2
spot 11	filamentation temperature-sensitive h 2b	abiotic/biotic stress response	2
spot 24	dehydrin 1	dehydration protection	2
spot 25	dehydrin 1	dehydration protection	2
spot 26	dehydrin 1	dehydration protection	2
spot 29	dehydrin 1	dehydration protection	2
spot 30	dehydrin 1	dehydration protection	2
spot 31	dehydrin 1	dehydration protection	2
spot 33	alcohol dehydrogenase class III	xenobiotics biodegradation	1
spot 51	dehydrin	dehydration protection	1
spot 61	phospholipase d-gamma	response to oxidative stress	3
spot 62	superoxide dismutase	superoxide metabolic process	2
spot 64	mlp-like protein 328-like	abiotic/biotic stress response	2
spot 67	temperature-induced lipocalin	abiotic/biotic stress response	*
spot 69	adenine nucleotide alpha hydrolases-like	response to cold	2
spot 84	dehydrin 14 kDa	dehydration protection	2
spot 86	ATPase aa ClpB/HSp100 family	chaperone	2
spot 87	dehydrin	dehydration protection	1
spot 90	dehydrin	dehydration protection	1
spot 120	dehydrin cor11	dehydration protection	2
spot 126	peroxidase 12-like	oxidative stress protection	2
Regulation / Signalling			
spot 1	self-incompatibility associated ribonuclease	regulation of transcription	2
spot 2	elongation factor 2-like	translational elongation /GTP catabolic process	2
spot 3	elongation factor ef-2	translational elongation /GTP catabolic process	1
spot 32	retrotransposon ty1-copia	DNA integration / Proteolysis	1
spot 47	aintegumenta-like protein	regulation of transcription	1
spot 65	btb poz domain containing protein		2
spot 66	b3 domain-containing		3
spot 112	calcium-binding protein	abiotic/biotic stress response	2
spot 113	calcium-binding protein	abiotic/biotic stress response	2
spot 114	calcium-binding protein	abiotic/biotic stress response	2
Amino acid metabolism			
spot 8	glycyl-trna synthetatse glycine--trna ligase	glycine, serine and threonine metabolism	2
spot 13	ketol-acid reductoisomerase	valine, leucine and isoleucine biosynthesis	2
spot 34	aspartate aminotransferase 2 family protein	phenylalanine_metabolism	*
spot 76	phenylalanine ammonia-lyase	phenylalanine_metabolism	1
spot 107	methionine synthase	methionine metabolism	2

spot 109	methionine synthase	methionine metabolism	2
spot 121	serine hydroxymethyltransferase 4	glycine, serine and threonine metabolism	2
Energy metabolism			
spot 22	ribulose-bisphosphate carboxylase oxygenase large subunit	photosynthesis	2
spot 23	ribulose bisphosphate carboxylase oxygenase large subunit	glyoxylate_and_dicarboxylate_metabolism / photosynthesis	2
spot 27	ATPase subunit1	ATP binding	2
spot 75	alternative oxidase	electron transport chain	1
spot 101	cell division cycle protein 48 homolog	ATP synthesis	2
spot 152	20 kda chloroplastic-like	photosynthesis	2
spot 174	ribulose bisphosphate carboxylase small chain	photosynthesis	2
Carbohydrate- / Energy metabolism			
spot 4	cytosolic aconitase	TCA cycle	1
spot 6	transketolase family protein	pentose phosphate pathway / photosynthesis	2
spot 12	phosphoglycerate mutase	glycolysis	1
spot 14	NADP-dependent malic enzyme	malate metabolic process/ pyruvate metabolism /	1
spot 18	phosphoenolpyruvate carboxylase family	malate metabolic process/ pyruvate metabolism /	1
spot 21	phosphopyruvate hydratase	glycolysis / photorespiration	*
spot 110	NADP-dependent malic enzyme	malate metabolic process / pyruvate metabolism	2
Protein metabolism			
spot 10	large subunit-binding protein subunit	chaperone activity	2
spot 102	ATP-dependent chloroplastic	proteasome	2
spot 155	proteasome subunit beta	proteasome	2
Secondary Metabolism			
spot 48	flavanone 3-hydroxylase	flavonoid biosynthesis	2
spot 49	flavanone 3-hydroxylase	flavonoid_biosynthesis	3
spot 154	chalcone isomerase	flavonoid_biosynthesis	3
Lipid metabolism			
spot 5	phospholipase D alpha	phospholipid metabolism	1
spot 55	hypothetical protein	lipid metabolic process	1
Structural Components			
spot 176	histone h4-like	nucleosome	2
Transport			
spot 165	probable metal-nicotianamine transporter	transmembrane transport	2
Unknown function			
spot 52	hypothetical protein		1

Table 3. Differentially expressed proteins with decreased abundance. The proteins are grouped by their functional similarity. Cluster number for proteins changing at 397'-0' is shown. Cluster symbol (*) denotes proteins changing only at 789'-397'.

Spot Id.	Name / Annotation	Biological function / Pathway	Cluster
Stress-related proteins / Defense			
spot 19	abscisic stress ripening protein	response to stress	5
spot 20	abscisic stress ripening protein	reponse to stress	5
spot 46	aspartic proteinase nepenthesin-1	response to abscisic acid stimulus / proteolysis	4
spot 63	minor allergen alt a 7-like	xenobiotics biodegradation and metabolism	4
spot 82	n-superoxide dismutase 2	oxidative stress protection	4
spot 68	abscisic stress ripening	response to stress	5
spot 78	phospholipase d-gamma	response to oxidative stress	5
Energy metabolism			
spot 71	oxygen-evolving enhancer protein chloroplastic-like	photosystem II stabilization	5
spot 138	oxygen-evolving enhancer protein chloroplastic	photosystem II assembly	4
spot 160	oxygen-evolving enhancer protein 2-chloroplastic	photosystem II assembly	4
spot 178	ribulose- -bisphosphate carboxylase oxygenase small subunit	photosynthesis	4
Amino acid metabolism			
spot 130	polyphenol oxidase	tyrosine metabolism	4
spot 131	polyphenol oxidase	tyrosine metabolism	4
spot 132	polyphenol oxidase	tyrosine metabolism	4
Regulation / Signalling			
spot 35	60s ribosomal protein l1	translation regulation	4
spot 70	transcription factor btf3 homolog 4-like		5
spot 170	nucleic acid binding	plasma membrane	4
Secondary Metabolism			
spot 43	isoflavone reductase-like protein	isoflavonoid biosynthesis	4
spot 44	isoflavone reductase homolog	isoflavonoid biosynthesis	4
spot 45	anthocyanidin reductase	flavonoid biosynthesis	4
Structural components			
spot 38	notum pectin acetylesterase	cell wall	4
spot 85	histone h2a	nucleosome	4
spot 162	kinesin-like protein kif11-like	microtubule-based movement	4
Carbohydrate metabolism			
spot 15	l-ascorbate oxidase homolog	ascorbate_and_aldarate_metabolism	4
spot 161	granule-bound starch synthase chloroplastic amyloplastic	starch and sucrose metabolism	4
Protein metabolism			

spot 77	lipid binding	proteolysis	5
Transport			
spot 72	arsenical pump-driving ATPase-like	cation transport	5
spot 74	arsenical pump-driving ATPase-like	cation transport	4
Lipid metabolism			
spot 88	Lipid transfer protein precursor	lipid transport	4
Cell growth			
spot 41	cell cycle arrest protein bub3	regulation of cellular process	4
Unknown function			
spot 83	hypothetical protein OsJ_09606		4
spot 137	hypothetical protein ARALYDRAFT_916149		*

Figure Legends

Figure 1. Representative gels of the experiment. 2DE gel analyses of proteins extracted from flower buds with increasing exposure to chilling temperatures (A) 0’, (B) 397’, (C) 789’.

Figure 2. 2D-DIGE analyses of flower bud proteins with different exposures to chilling temperatures. (A) Samples from 0’ (labeled with Cy2) and samples from 397’ (labeled with Cy3). (B) Samples from 397’ (labeled with Cy3) and samples from 789’ (labeled with Cy5).

Figure 3. 2D-DIGE analyses of flower bud proteins with different exposures to chilling temperatures. Protein extracts from samples from 0’ (labeled with Cy2-green) and samples from 397’ (labeled with Cy3-red). The positions of 90 differentially abundant protein spots selected for protein identification are indicated.

Figure 4. Expression profiles of the differentially expressed proteins. The protein expression average of each cluster is shown in red.