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# 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.

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## 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

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*Arabidopsis*. Beyond this highly significant work in *Arabidopsis*, 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 *Vaccinium corymbosum* and 16 proteins from *Vaccinium* 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 and 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 7 °C but exposed to shortening photoperiods (0 h chilling, 0′), (2) late November when plants were exposed to short photoperiods and had received about 400 chill units (397′ from 0 to 7 °C), and (3) in the middle of January when plants had received about 800 chill units (789′ from 0 to 7 °C), 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 −10 °C (0′), −25 °C (400′), and −27 °C (800′) (Ehlenfeldt et al., 2012). The sample pools from each time point (∼10 g representing about 500–1000 flower buds) were made from a minimum of five plants. All tissues were frozen in liquid nitrogen immediately after harvest and stored at −80 °C.

### 2.2. Protein extraction, CyDye labeling, and 2D-DIGE

Bud samples were crushed in a pre-cooled mortar with liquid nitrogen until a fine powder was formed. Proteins were extracted with TCA-phenol (Wang et al., 2006) and quantified according to (Esen, 1978). Two-dimensional differential in-gel electrophoresis (2D-DIGE) was performed at Applied Biomics, Inc. (Hayward, CA). Briefly, protein extracts from flower buds were denatured by addition of an equal volume of lysis buffer containing 7 M urea, 2 M thiourea, 4% 3-((3-cholamidopropyl) dimethyl ammonio)-1-propanesulfonate (CHAPS), followed by addition of 30 mM Tris–HCl, pH 8.8. Next, each set of three samples that were to be run on a single gel were labeled with a CyDye dilution of Cy2, Cy3, or Cy5 (Amersham Biosciences, Piscataway, NJ) as described in Supplementary Table S1. Labeling was stopped by adding 0.7 μl of 10 mM L-lysine and incubating at 4 °C for 15 min. Then, equal amounts of the three labeled samples were mixed together, along with an equal volume of 2 × 2-D sample buffer (8 M urea, 4% CHAPS, 20 mg/ml dithiotreitol (DTT), 2% pharmalytes and a trace amount of bromophenol blue) and 100 μl of destreak solution (GE Healthcare Biosciences, Pittsburgh, PA). Total sample volumes were adjusted to 260 μl by adding rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/ml DTT, 1% pharmalytes and trace amount of bromophenol blue). Each set of three labeled samples were then subjected simultaneously to isoelectric focusing (IEF) on a 13 cm precast non-linear immobilized pH gradient strip (pH 4–9, Amersham Biosciences). Next, the samples were separated in