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ORIGINAL ARTICLE

Major differences observed in transcript profiles of blueberry during cold acclimation under field and cold room conditions

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Abstract Our laboratory has been working toward increasing our understanding of the genetic control of cold hardiness in blueberry (*Vaccinium* section *Cyanococcus*) to ultimately use this information to develop more cold hardy cultivars for the industry. Here, we report using cDNA microarrays to monitor changes in gene expression at multiple times during cold acclimation under field and cold room conditions. Microarrays contained over 2,500 cDNA inserts, approximately half of which had been picked and single-pass sequenced from each of two cDNA libraries that were constructed from cold acclimated floral buds and non-acclimated floral buds of the fairly cold hardy cv. Bluecrop (*Vaccinium corymbosum* L.). Two biological samples were examined at each time point. Microarray data were analyzed statistically using *t* tests, ANOVA, clustering algorithms, and online analytical processing (OLAP). Interestingly, more transcripts were found to be upregulated under cold room conditions than under field conditions. Many of the genes induced only under cold room conditions could be divided into three major types: (1) genes associated with stress tolerance; (2) those that encode glycolytic and TCA cycle enzymes, and (3) those associated with protein synthesis machinery. A few of the genes induced only under field conditions appear to be related to light stress. Possible explanations for these differences are discussed in physiological context. Although many similarities exist in how plants respond during cold acclimation in the cold room and in the field environment, there are major differences suggesting caution should be taken in interpreting results based only on artificial, cold room conditions.

Keywords Blueberry · Cold hardiness · Cold tolerance · Freezing tolerance · Genomics · *Vaccinium*

Abbreviations

CA	Cold acclimated
EST	Expressed sequence tag
NA	Non-acclimated

Introduction

Blueberry (*Vaccinium* section *Cyanococcus*) is an important small fruit crop rich in antioxidants, often grown in acidic and imperfectly drained soils that would otherwise be considered unfit for agricultural production. Enhanced cold hardiness, including tolerance to

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winter freezing and spring frosts, are needed for genetic improvement of current cultivars (Moore 1993). Genetic evidence from numerous plants, including woody perennials, indicates that cold hardiness is a quantitative trait (Byrne et al. 1997; Arora et al. 2000; Howe et al. 2000; Jermstad et al. 2001). Considerable molecular evidence, mostly from herbaceous plants, indicates that development of cold hardiness or cold acclimation is a complex phenomenon involving changes in gene expression that result in the alteration in metabolism and composition of lipids, proteins, and carbohydrates (Guy 1990, 1999; Shinozaki and Yamaguchi-Shinozaki 1996, 2000; Thomashow 1999, 2001). Genes induced during cold stress encode several different classes of gene products: enzymes required for the biosynthesis of osmoprotectants; lipid desaturases for maintaining membrane fluidity; protective proteins such as antifreeze proteins, dehydrins, chaperones, and mRNA-binding proteins; proteins involved in protein turnover including ubiquitin, ubiquitin-associated proteins, and other proteases; detoxification proteins; and proteins involved in signal transduction like transcription factors, protein kinases, and phospholipase C (Shinozaki and Yamaguchi-Shinozaki 1996, 2000; Thomashow 1999, 2001; Xin and Browse 2000; Browse and Xin 2001).

Within the last few years, microarrays have been used in transcript profiling on a larger scale during cold and other related stresses in *Arabidopsis*. Seki et al. (2001) used microarrays containing approximately 1,300 full-length cDNAs to identify genes induced by drought and cold in *Arabidopsis*; they identified 44 cDNAs induced by drought and 19 induced by cold. Fowler and Thomashow (2002) used commercially available microarrays to examine transcript levels of ~8,000 genes in cold-exposed (up to 7 days at 4°C) *Arabidopsis* plants; 306 of these genes were cold responsive, 218 being upregulated and 88 being downregulated. At least 28% of the cold-responsive genes did not appear to be regulated by C-repeat binding factor or CBF proteins (transcription factors responsible for the upregulation of many genes in response to cold and drought stress), indicating that multiple regulatory pathways are activated during cold acclimation. Kreps et al. (2002), also using a commercially available GeneChip microarray, monitored transcriptome changes in leaves and roots of *Arabidopsis* plants in response to salt, osmotic, and cold stress and found the majority of changes to be stimulus specific. New members of the CBF regulon were identified using microarrays and transgenic plants overexpressing CBFs (Maruyama et al. 2004). A recent article by Hannah et al. (2005) summarizes the microarray data on cold stress in *Arabidopdis*.

Over the past several years, we have studied the biology of cold tolerance in blueberry with an ultimate goal of applying the information learned to develop more cold hardy cultivars. These studies, unlike those with *Arabidopsis*, have focused on cold acclimation in a woody perennial and have focused on flower bud tissue, rather than leaf tissue, because damage to flower buds directly results in reduction in fruit yield. *Arabidopsis* cold acclimates only about 5–7°C allowing for brief exposures to freezing temperatures whereas woody perennials can withstand extremely low subzero temperatures for extended periods of time. In addition, overwintering flower buds display both enhanced freezing tolerance and dormancy/relief of dormancy. Using a molecular genetic approach, we have previously identified and isolated several cold-responsive genes from blueberry flower buds including 65, 60, and 14 kD dehydrins. We found good quantitative correlation between dehydrin accumulation and cold hardiness in several different genotypes (Muthalif and Rowland 1994; Arora et al. 1997). Full-length cDNA clones encoding the 60 kD (Levi et al. 1999) and the 14 kD (Dhanaraj et al. 2005) dehydrins have been isolated and the seasonal expression of their messages found to be similar to their protein accumulation patterns.

Because of the complexity and multigenic nature of cold acclimation, we believe many other genes are likely involved in cold acclimation in blueberry, in addition to the previously described dehydrins. To gain a better understanding of cold acclimation in woody perennials and, specifically, to identify other genes involved in cold acclimation in blueberry, we recently undertook a genomics approach based on the analysis of expressed sequence tags (ESTs) (Dhanaraj et al. 2004). Two cDNA libraries, constructed from cold acclimated (CA) and non-acclimated (NA) floral buds of the *V. corymbosum* cv. Bluecrop, were used. Bluecrop was chosen because it is quite cold hardy and is the "industry standard" of cultivars. Initially, about 600 5′-end ESTs were generated from each of the libraries. Putative gene identities were assigned to the cDNAs based on homology to other genes/ESTs from Genbank, and these cDNAs were classified into functional categories (Dhanaraj et al. 2004). The cDNAs that were picked many more times from one library than from the other potentially represented differentially expressed transcripts. Northern analyses indeed confirmed the preferential expression of several selected transcripts under either cold acclimating (examples include dehydrin, senescence-associated protein, early light-inducible protein, beta amylase, and a protein of unknown function) or non-acclimating conditions

(examples include histone H3.2 and BURP-domain dehydration-responsive protein RD 22) (Dhanaraj et al. 2004).

In this paper, we report the generation of an additional 1,500 ESTs from the NA and CA libraries and their use, together with the previously characterized cDNAs, to construct blueberry microarrays. These microarrays, containing over 2,500 cDNA inserts, were used to analyze gene expression in Bluecrop at multiple times during cold acclimation under field (0–1,200 chill units) and cold room (0–1,000 chill units) conditions. Research on transcript profiling of cold-responsive genes in woody perennials is lacking in the literature as are studies comparing field responses to responses in controlled environments. Our results show that the abundance of transcripts of numerous blueberry genes change during cold acclimation including genes not found previously to be cold-responsive in *Arabidopsis*, and, interestingly, more transcripts were found to be upregulated under cold room conditions than under field conditions, suggesting caution should be exercised in evaluating transcriptome changes from controlled cold exposure versus "real world" conditions.

Materials and methods

Plant material

To study the expression pattern of genes associated with cold acclimation in floral bud tissues of blueberry, time course experiments were set up using field-grown plants and cold room-treated greenhouse plants of the relatively cold hardy cv. Bluecrop. For woody perennials, exposure to low temperature, which plays a role in cold acclimation, is also required for breaking dormancy and resumption of growth in the spring. Bluecrop has a chilling requirement (number of hours between 0 and 7°C or chill units required for 50% bud break upon exposure to 2–3 weeks at 20–25°C) of about 1,200 chill units (Muthalif and Rowland 1994). For the field experiment, buds were collected starting in the fall of 2003 and through the winter of 2003–2004. Buds collected at the first time point in the fall (on 09/29/03) were used as the NA control. They had received 0 chill units and had a bud cold hardiness level (lethal temperature$_{50}$ or LT$_{50}$) of −10°C. In these experiments, LT$_{50}$ referred to the temperature that killed 50% of the floral buds in a controlled freeze–thaw test (Arora et al. 2004; Rowland et al. 2005). For subsequent time points, buds were collected later in the fall when they had accumulated 67 (10/20/03) and 399 chill units (12/08/03), during mid-winter when they had accumulated 779 chill units and had reached a maximum bud cold hardiness level of −27°C (02/02/04), and during late winter when they had accumulated 1,234 chill units and had partially deacclimated to a bud cold hardiness level of −18°C (03/18/04). For comparison, these collection dates of 09/29/03, 10/20/03, 12/08/03, 02/02/04, and 03/18/04 corresponded to exposure to total hours <7°C of 0′, 71′, 531′, 1,618′, and 2,306′, respectively. For the cold room experiment, floral buds from potted plants maintained in a heated greenhouse under natural photoperiod were collected on 11/25/03 before they received any chilling. Plants were then moved into a cold room maintained at 4°C with a 10-h photoperiod of 100 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) provided by cool-white fluorescent lamps (Sylvania/GTE, Danvers, MA, USA). Buds were collected when plants had accumulated 500 (12/16/03) and 1,000 chill units (01/06/04). Floral buds for each treatment were collected from two sets of either 7–10 clonally propagated field plants or greenhouse/cold room plants to represent the two biological samples from which RNA would be isolated separately and used for cDNA synthesis. Buds were collected in the early afternoon from approximately 1:00–3:00 p.m. Buds were stored at −80°C until used.

cDNA microarray clones and microarray fabrication

About 2,500 cDNA clones were used in this investigation that were generated from two Uni-ZAP^{TM} (Stratagene, La Jolla, CA, USA) cDNA libraries described previously (Dhanaraj et al. 2004), a library developed from CA floral buds collected in mid-winter and the other from NA floral buds collected in the fall before plants had received any chilling. Although some redundant clones were removed after being identified by earlier contig analysis and BLAST searches (Dhanaraj et al. 2004), many clones in this microarray are redundant, as hybridizations had to be performed before all sequencing was completed. Construction and use of microarrays were performed following the Minimum Information About Microarray Experiment (MIAME) guidelines (Brazma et al. 2001) and protocols used are described in detail below.

About 1,000 cDNA inserts generated from the two-cDNA libraries, CA and NA, from our earlier EST analysis (Dhanaraj et al. 2004) were used on the array. The 1,000 clones represented at least half the clones from each of the 875 clusters that were generated after assembling all the sequences from both libraries (Dhanaraj et al. 2004). Additionally, another ~1,500 ESTs (750 from each library) were generated and all of

these cDNA inserts were included on the array. Three full-length blueberry dehydrin cDNAs, encoding the previously characterized 60 kDa dehydrin (AF030180; gene name bbdhn1; Levi et al. 1999), the previously characterized 14 kDa dehydrin (AY660959; bbdhn6; Dhanaraj et al. 2005), and a dehydrin as yet uncharacterized at the protein level (AY660960; bbdhn7; Dhanaraj et al. 2005), were included on the microarray along with four previously isolated partial length dehydrin cDNAs (AF222738–AF222741; bbdhn2-bbdhn5; Rowland et al. 2004).

To generate the additional ~750 ESTs from each of the libraries, phage clones were first converted to plasmid clones by mass excision, according to instructions provided by Stratagene. Plasmid DNAs to be used as templates for sequencing reactions were isolated using the REAL Prep-96 plasmid kit (Qiagen, Valencia, CA, USA) and quantified spectrophotometrically. Single-pass nucleotide sequencing of recombinant plasmid DNAs was performed from the 5′ ends using Big Dye Terminator sequencing chemistry (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) by the University of Maryland, Center for Agricultural Biotechnology-DNA Sequencing Facility (College Park, MD, USA) or by Macrogen (Seoul, Korea). DNA sequences were trimmed of vector sequence manually or using the software package 'Lasergene' (DNASTAR Inc., Madison, WI, USA). Sequences were then compared with the National Center for Biotechnology Information (NCBI) non-redundant protein database (http://www.ncbi.nlm.nih.gov/BLAST/) using the BLASTX algorithm (Altschul et al. 1997) and default parameters. Sequences that had no significant similarity with sequences in the protein database were compared with the nucleotide database using BLASTN and default parameters. Individual ESTs were assembled into contigs using 'Lasergene', with parameters optimized for ESTs rather than for genomic clones. Consensus sequences from the contig analysis were also compared with the non-redundant protein database using BLASTX. The highest BLAST scores from either the individual ESTs or contigs were used to assign putative identities to the clones. In general, the sequences with BLASTX similarity scores >100 and probabilities $<10^{-5}$ were considered significant.

Clone inserts for microarrays were amplified by polymerase chain reaction (PCR) in 96-well microtiter plates using T3/T7 universal primers in 50 µl reaction volumes similar to the protocol of Hegde et al. (2000). Two microliters of PCR products were run on E-Gels (Invitrogen, Carlsbad, CA, USA) to confirm amplification and the remaining 48 µl reaction mixtures were precipitated to remove excess primers and unincorporated nucleotides by the addition of one-fifth volume 10 M ammonium acetate and 0.7 volume propanol. Pellets were washed with 70% ethanol, air dried, and dissolved in 3 µl TE. Then, 0.5 µl of each DNA sample was loaded on E-Gels again to ensure quality of the PCR products after precipitation. Finally, the concentration of PCR products was adjusted to 0.4 µg/ml in 50% DMSO/TE and samples were transferred to 384-well plates, sealed, and stored at 4°C until printed.

The standard reference design was used for these microarray experiments; the reference (control) sample was RNA extracted from NA blueberry (field or greenhouse-grown, as appropriate), and the treatment samples were RNA extracted from CA blueberry at the various time points for the field (67, 399, 779, and 1,234 chill units) and cold room-treated plants (500 and 1,000 chill units). Dye swaps were used to correct for differences in incorporation and fluorescent properties of two dyes, Alexa Fluor 555 and 647. Two replicated slides were used for each time point, one of which was the dye swap. Two biological samples for each treatment were used to account for the inherent variation in mRNA extracted from different plant materials at different times. Self-self hybridized slides were also generated for *T* test analysis, wherein a pool of RNA was split into two aliquots, one labeled with Alexa Fluor 555 and the other with Alexa Fluor 647, then hybridized to the slide. The complete list of slides printed and the probes used in their hybridization are listed on our website (http://www.psi081.ba.ars.usda.gov/BBGD/index.htm).

The PCR products suspended in 50% DMSO/TE at a concentration of 0.4 µg/ml were arrayed onto silylated Corning CMT-GAPs microscope slides. Each clone was printed in triplicate as described previously (Khan et al. 2004) using a Cartesian robot model PixSys 5500 PA workstation (Cartesian Technologies, Irvine, CA, USA) with a telechem printing head and Stealth quill pins. A total of 2,562 blueberry cDNA clones were arrayed. cDNAs from alfalfa weevil were printed at concentrations of 1, 2.5, 5, 10, 25, 50, and 100 µg/ml to serve as internal controls, and to assess labeling and detection efficiencies. The printed arrays were crosslinked to the slide by UV irradiation at 250 mJ using a UV Stratalinker 2400 (Stratagene). The slides were stored in a humidity chamber at 25°C.

Fluorescent probe preparation

Total RNA was extracted from blueberry floral buds using the 'hot borate' protocol outlined by Wilkins and Smart (1996). For synthesis of cDNA probes, 14 µg of total RNA was first treated with RNase-free DNase

(Ambion, Austin, TX, USA) to remove traces of contaminating DNA. Ten nanogram control poly $(A)^+$ RNA synthesized by in vitro transcription (Epicenter, Madison, WI, USA) corresponding to coding sequences from alfalfa weevil was added to the total RNA from each blueberry sample. RNA from each sample was labeled using the reverse transcription reaction as described by Hegde et al. (2000) with a few modifications (Khan et al. 2004).

Microarray hybridization and analysis

The arrays were prehybridized, hybridized, washed, and dried as described by Khan et al. (2004). Immediately after drying, microarray slides were scanned using a ScanArray 4000 (GSI Lumonics, Meriden, CT, USA). Microarrays were scanned for each fluor at a resolution of 10 μm and a PMT and laser power set to optimize the resulting fluor intensities but at the same time reduce the occurrence of saturated (white) spots and reduce background noise.

Fluorescence intensities were extracted from the scanned images using the image processing software package SPOT which is available at http://www.cmis.csiro.au/ iap/spot.htm. Background subtraction was performed before calculating ratios. The elements with either printing or hybridization artifacts were flagged (~10 to 15% of spots/slide) and discarded before analysis. The extracted data from each slide was then log transformed (using log base two) and normalized using the Lowess print-tip group normalization method followed by dye swap normalization (Yang et al. 2002).

Potential artifacts and false positives were eliminated by selecting for further analysis only those clones that exhibited similar expression patterns between the original hybridization and their dye swaps (Yang et al. 2002). These clones were determined by measuring the standard deviations between replicate slides. Only clones with a standard deviation of less than one times the mean ratio were selected as being similar across slides and biosamples. SQL procedural scripts were written to conduct such computationally intense calculations for every clone post filtration and normalization. In general there was little variation between the two biosamples.

T values on $\log_2$ expression ratios were calculated using Student's *T* test to identify genes with statistically significant expression ratios. Self-self hybridized slides with equal amounts of Alexa Fluor 555 and 647-labeled RNA from NA blueberry were used as control groups for the *T* test. Most of those clones showed a ratio close to one after Lowess print-tip normalization.

T tests were used to analyze the expression of each clone in CA versus NA buds using background corrected and normalized expression ratios. Genes that had large absolute *T*-statistic values and positive average expression levels were considered differentially induced genes, while genes that had large absolute *T*-statistic values but negative average expression levels were considered differentially suppressed genes.

Results of *T* tests were used to determine significance ($P <= 0.05$) of gene induction and gene suppression. If the gene induction or suppression passed the *T* test, then a cutoff value of 1.5-fold induction or suppression was applied. This criteria keeps the possibility of false positives to a minimum but at the same time increases the likelihood of false negatives (genes actually induced but not designated as such). This statistical method takes into account the variability within slides and between replicated slides as well as biological samples to distinguish gene expression changes caused by treatments from gene expression changes attributable to biological and measurement variability. The complete data set is available in an easy-to-search format on our website (http://www.psi081.ba.ars.usda.gov/BBGD/index.htm).

K-means and hierarchical clustering were performed using J-Express version 2.0 (from molmine; http://www.molmine.com). *K*-means was conducted setting $K = 20$, initialization method to Forgy and distance metric to Euclidian, while hierarchical clustering was conducted using the average linkage (UPGMA) method and Euclidian distance. SQL and Online Analytical Processing (OLAP) (Codd et al. 1993; Alkharouf et al. 2005) were also used to produce lists of differentially expressed genes at each of the time points. In addition, the clustering function within Analysis Services (Microsoft, Redmond, WA, USA) was used to find distinct expression profiles in the differentially expressed genes. Differentially expressed genes were classified into functional categories based on the authors' knowledge of biochemistry, plant physiology, and plant molecular biology, by reference to the BioCyc–MetaCyc: Encyclopedia of Metabolic Pathways website (http://www.MetaCyc.org/), by reference to the gene ontology (Go) database (http://www.geneontology.org/), and by searching related abstracts in PubMed (http://www.ncbi.nlm.nih.gov/PubMed/).

Northern blots

RNA for northern blot analysis was extracted from floral buds of Bluecrop field plants that had accumulated 0 (collected 9/29/03), 67 (10/20/03), 399 (12/08/03), 779 (02/02/04), and 1,234 (03/18/04) chill units and

from greenhouse/cold room-treated Bluecrop plants that had accumulated 0 (11/25/03), 500 (12/16/03), and 1,000 (01/06/04) chill units. RNA was extracted from ~600 mg frozen samples using the 'hot borate' protocol described by Wilkins and Smart (1996). Total RNA (5 μg/lane) from each time point was separated on 1% agarose/formaldehyde gels, visualized and photographed to confirm good quality and equal loading, and blotted onto Brightstar-Plus™ nylon membranes (Ambion) by capillary transfer using the NorthernMax™ blotting and hybridization kit (Ambion). RNA was bound to the membranes by UV crosslinking.

For preparation of DNA probes, cDNA inserts were amplified from plasmid clones of interest using T7/T3 primers. PCR products were quantified by electrophoresing through 2% agarose gels with known concentrations of Low DNA Mass™ ladder (Invitrogen Life Technologies). About 25 ng of each cDNA insert was ^{32}P-labeled to a specific activity of 1.0–2.0 × 10^8 cpm μg^{-1} by random priming using the Megaprime™ DNA labeling system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were prehybridized with ULTRAhyb buffer from the NorthernMax™ kit for 30 min and hybridized overnight at 42°C. The following day, blots were washed twice (15 min, 42°C) with Low Stringency wash solution from the NorthernMax™ kit and then exposed to X-ray film (Sterling Bioworld, Dublin, OH, USA) with intensifying screens at −80°C.

Results and discussion

Quality of the libraries, sequences, and contig assembly

The purpose of this research was to identify cold-responsive genes in blueberry floral buds, thus, genes potentially involved in cold acclimation of a woody perennial, and compare field versus controlled environment responses. In woody perennials of the temperate zone, cold acclimation is triggered by several environmental cues, including low temperature, and is generally considered a two-step process (Weiser 1970). The first stage is induced by short photoperiod and the timing and speed of acclimation can be affected by other factors such as available moisture. The second stage is induced by low temperature. In this study, we examined changes in gene expression associated with cold acclimation over a time course in (1) field plants under natural conditions of progressively shorter photoperiods and colder temperatures and (2) cold room-treated greenhouse plants under artificial conditions of constant 4°C and short photoperiod (10 h light/14 h dark).

The CA and NA libraries used for generation of ESTs and construction of microarrays were determined to be of good quality in our previous study in which about 600 5′-end ESTs were generated from each of the libraries (Dhanaraj et al. 2004). Quality was assessed by average insert size (≥1.0 kb), percent of clones containing a translational start site (>10%), and percent of contaminating rRNA-encoding clones (≤2%). The average read-length for the ESTs, after trimming vector sequences, was 615 bases for the CA library and 609 bases for the NA library.

The software package 'Lasergene' was used to assemble all the high quality ESTs from both libraries into contigs or clusters based on the presence of overlapping, identical, or similar sequences. The 'Lasergene' program was run with default threshold settings for clustering sequences with at least 80% similarity or with 12 or more overlapping consecutive bases. For the contig analysis, 608 ESTs generated from the CA library and 587 ESTs from the NA library from our earlier studies (Dhanaraj et al. 2004) were used along with the high quality ESTs (<4% Ns) that were generated in this study (another 679 ESTs generated from the CA library and 606 from the NA library). From these 2,480 sequences (608 + 679 + 587 + 606 = 2,480), 1,527 clusters were formed. These included 458 singletons from the CA library and 615 from the NA library, 204 contigs comprised of only CA ESTs, 116 contigs comprised of only NA ESTs, and 134 contigs comprised of ESTs from both libraries. Thus, 8.8% (134/1,527) of the total distinct transcripts were shared between the two libraries.

Results from a cursory examination of the most highly abundant cDNAs that were picked many more times from one library than from the other library were consistent with our earlier findings on the initial, smaller subset of ESTs (Dhanaraj et al. 2004). For example, dehydrin cDNAs were picked many more times from the CA library than from the NA library, while BURP-domain dehydration-responsive protein RD22 cDNAs were picked many more times from the NA library than from the CA library. In our earlier study, we had confirmed these genes, as well as others, to be cold-responsive by northern blot analyses (Dhanaraj et al. 2004).

Microarrays and identification of differentially expressed genes

Using cDNA microarrays containing approximately 2,500 cDNA inserts from the CA and NA libraries, we examined changes in abundance of gene transcripts in floral buds of blueberry at multiple times during cold

acclimation under field and cold room conditions. RNA was isolated from buds of field plants and from buds of cold room-treated greenhouse plants of Bluecrop that had received various amounts of chilling (0–1,234 chill units for field plants; 0–1,000 chill units for cold room plants). The 0 chill unit RNAs served as the controls, for duplicate hybridizations to microarrays using inverse labels (dye swaps). We examined the variation within and across slides after Lowess print-tip group normalization to ensure correct normalization and confirm reproducibility. Very little variation occurred within slides with approximately 90% of the clones having standard deviations of less than 0.5 times the mean ratio after filtration for artifacts and normalization. In addition, across slide variation was low and the reproducibility was high when data from biological replicates and dye swaps were compared. Figure 1 documents, by box plots, the low variation seen between the biological replicates of the field samples.

The number of differentially expressed genes [induced above 1.5 fold or suppressed below −1.5 fold after T tests ($P \leq 0.05$)] at each time point under field and cold room conditions is shown in Fig. 2. The number of differentially induced genes during cold acclimation in the field ranged from 36 at 67 chill units (the earliest time point examined), to a maximum of 106 at 399 chill units, to 102 at 779 chill units (when buds were maximally cold hardy), to 27 at 1,234 chill units (when plants had begun to deacclimate). The total number of genes induced at any of these time points was 134 or 5.2% of the 2,562 cDNAs that were arrayed. Surprisingly, the number of genes induced during cold acclimation under cold room conditions was much higher than under field conditions, ranging from 221 at 500 chill units to 150 at 1,000 chill units. The total number of genes induced at either time point was 241 or 9.4% of the arrayed genes, almost twice the number induced under field conditions. One of the reasons may be that plants in the cold room were exposed to a constant 4°C unlike field plants, which were exposed to fluctuating temperatures and higher daytime than nightime temperatures. Thus, the duration of the cold treatments was different. For example, to reach 500 chill units in the cold room takes 21 days whereas it took 70 days to reach 400 chill units in the field. In this sense, the cold room treatment is more severe, being applied in a quicker manner. Field plants, however, are exposed to temperatures much lower than 4°C at times. For example, between 399 and 779 chill units, the average temperature was 0.2°C and the average minimum was −4.7°C. Lowest temperature for the season was −14.4°C, recorded on 1/10/04. Field plants are also expected to be slightly more cold hardy than cold room-treated plants. Bluecrop field plants reached a maximum cold hardiness of −27°C, whereas previously we have shown that cold room-treated (4°C) Bluecrop plants reach a maximum cold hardiness of −24.6°C (Arora et al. 1997). This is an important point, indicating that the large number of genes induced under cold room conditions that are not induced under field conditions may not be directly involved in freezing tolerance, that is, they do not result in increased cold tolerance in the cold room vs. the field. Another temperature-related explanation for the higher number of induced genes may be that the relatively smaller volume of soil surrounding the roots of potted plants used in the cold room experiment, as compared to field plants, would result in a smaller difference between soil temperature and air temperature. In field plants, when the air temperature is above 0°C, the temperature in

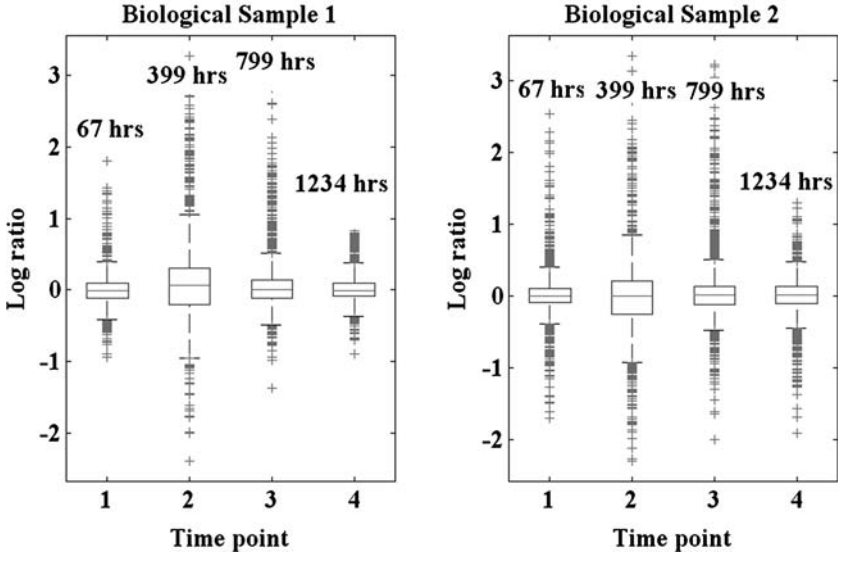

Fig. 1 *Box plots* showing the variation in the microarray experiment between the biological replicates of the field samples

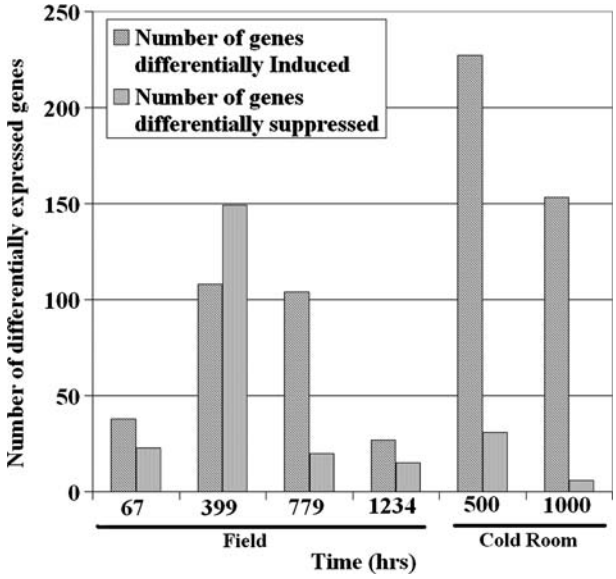

Fig. 2 The number of differentially expressed genes during cold acclimation after *T* tests ($P < =0.05$) at each time point of the field and cold room experiments using a cutoff value of 1.5 fold and selecting for clones that had standard deviations of less than or equal to one times the mean expression ratio

the plant root zone (10–20 cm below the ground) is 3–7°C higher than the air temperature (Aase and Siddoway 1979; Coulson et al. 1995; Leep et al. 2001). Since soil temperature around plant roots influences the uptake and translocation of nutrients, transpiration, and growth (Boatwright et al. 1976; Mellander et al. 2004), the difference in soil temperature in field versus cold room treatments might cause differential expression of genes in above ground plant tissues, including floral buds, between these two treatments. Still another explanation may be that plants in the cold room were exposed to a constant short photoperiod (10 h light/14 h dark) and artificial light (cool-white fluorescent lamps) unlike field plants, which were exposed to gradually decreasing and then gradually increasing photoperiod and natural light.

The types of genes, identified from BLAST searches of GenBank, that were induced at any time point under field and cold room conditions are listed in Table 1. Only those genes induced >2 fold are shown in order to save space. For a detailed listing of differentially induced genes at each time point, see Supplemental Tables or our website (http://www.psi081.ba.ars.usda.gov/BBGD/index.htm). Many of the cold induced genes identified in *Arabidopsis* (Seki et al. 2001; Fowler and Thomashow 2002; Hannah et al. 2005) were identified as being cold induced in blueberry under both field and cold room conditions, such as galactinol synthase, beta amylase, LEAs, dehydrins, and early light-inducible protein. In addition, other genes were identified as being cold-induced in blueberry under field and cold room conditions that have not been reported as cold-induced in *Arabidopsis*, such as auxin-repressed protein, protein kinase PINOID, pectate lyase-like protein, and S-adenosylmethionine decarboxylase proenzyme, among others. Of particular interest is PINOID, which is involved in auxin-mediated signaling. In *Arabidopsis* PINOID has been shown to work in concert with LEAFY to direct formation of the floral meristem (Ezhova et al. 2000; Lebedeva et al. 2005), testifying to an important role of auxin gradients in regulating expression of LEAFY. Its cold-responsiveness has perhaps not been reported before in *Arabidopsis* because most researchers have used leaf tissue or whole seedlings in cold stress experiments, not flower buds. Also, the amount of polyamines such as putrescine, spermidine, and spermine increase under environmental stress conditions (Wi and Park 2002). Overexpression of S-adenosylmethionine decarboxylase, a key enzyme in polyamine biosynthesis, has been shown to increase broad-spectrum tolerance to abiotic stresses in transgenic tobacco plants (Wi et al. 2006).

Although many of the same genes were induced under field and cold room conditions in blueberry, there were also many differences in the types of genes induced under the two conditions. Many of the genes induced under cold room conditions that were not induced under field conditions (Table 2—only those induced >2 fold are shown) can be divided into three major groups: (1) genes associated with stress tolerance; (2) those that encode glycolytic and TCA cycle enzymes; and (3) those that encode protein synthesis machinery. Examples of some of the encoded proteins associated with stress tolerance are phospholipid hydroperoxide glutathione peroxidase, glutathione-S-transferase, metallothionein-like protein type 2, ankyrin-repeat protein, and low temperature-induced 78 kD protein, among many others. The glycolytic and TCA cycle enzymes, whose messages were upregulated under cold room conditions but not field conditions, include phosphoglyceromutase, glyceraldehyde-3-phosphate dehydrogenase, enolase (induced 1.7–1.9 fold, so not shown in Table 2), alcohol dehydrogenase 2 (induced 1.5 fold), malate dehydrogenase, and succinate dehydrogenase flavoprotein alpha subunit (induced 1.8 fold). The protein synthesis machinery members include many 30S (induced 1.9 fold), 40 S and 60 S ribosomal proteins, and translation initiation factor 5A-4 (induced 1.5–1.6 fold). Perhaps the upregulation of genes involved in respiration and protein synthesis simply relates to the observation that more genes are upregulated under cold room conditions than

Table 1 Genes upregulated >2.0 fold[a] during cold acclimation in field and cold room from microarray experiments

Gene product	Accession numbers in field[b]	Fold increase in field[b]	Fold increase in cold room
Dehydrin	AF030180, AF222738, AF222739,	3.6, 3.6, 3.8	5.4, 3.7, 4.1
	AF222740, AF222741, AY660959,	3.9, 2.3, 4.1	5.5, 2.2, 8.6
	CF810618, CF811265, CF811272,	3.3, 6.1, 6.6	4.6, 9.1, 13.8
	CF811614, CF811663, CV090332,	3.8, 7.5, 2.6	11.1, 10.0, 2.5
	CV090537, CV090724, CV090829,	4.3, 2.3, 5.4	6.3, 3.1, 9.5
	CV091312, CV091466, CV826719	3.7, 2.5, 4.3	6.2, 2.5, 7.6
Early light-inducible protein	CF810877, CF810917, CV090499,	4.5, 6.5, 3.8	2.7, 2.6, 2.1
	CV091100	2.9	4.2
Probable beta-amylase	CF810476, CF810753, CV090504	4.2, 4.0, 3.7	2.6, 2.1, 2.1
Wheat endosperm cDNA library Triticum aestivum cDNA clone WHE0073_B10_D19/ cell wall protein	CF811328, CF811493, CV091278	4.3, 2.6, 3.7	6.6, 3.3, 5.9
LEA	CF810442, CV090832, CV090880	2.9, 2.5, 2.0	2.5, 7.9, 5.0
Putative methyl transferase	CV826720	4.0	7.0
Auxin repressed protein	CF811432	2.0	2.3
Protein kinase PINOID	CV090257	5.5	12.4
Pectate lyase-like protein	CF811560	2.3	3.5
DNA-binding domain protein	CV091089	3.8	4.8
Glycine-rich protein	CV090428	4.6	5.0
Galactinol synthase	CF810581	2.8	2.2
F-box family protein	CV090254	2.6	2.5
S-adenosylmethionine decarboxylase proenzyme	CV090423	2.3	2.7
Bile acid: sodium symporter family protein	CV090298	2.2	2.2
Hypothetical proteins	CV090386, CV090388, CV090398,	5.0, 2.6, 2.7	4.8, 3.6, 3.8
	CV090409, CV090627	2.4, 2.6	4.5, 3.7
Proteins with no significant similarity to other sequences in GenBank	CV091005, CV090231, CV090247,	2.2, 3.0, 3.6	4.1, 3.4, 6.4
	CV090434	2.5	3.2

[a] Only those genes induced >2.0 fold are shown in order to save space

[b] Maximum fold increase over all time points tested is shown

under field conditions, thus the need for more energy and more translation factors. The upregulation of genes encoding glycolytic and TCA cycle enzymes in blueberry is also consistent with a previous report that the pool sizes of early glycolytic intermediates and most TCA cycle intermediates in *Arabidopsis* plant tissues increase after cold room treatment (Kaplan et al. 2004). Expression of the *Adh2* gene, which is required to oxidize ethanol to acetaldehyde, is also induced by cold room treatment in several plant species (Dolferus et al. 1994; Conley et al. 1999; Minhas and Grover 1999).

A few of the genes induced under field conditions that were not induced under cold room conditions (Table 3) appear to be related to light stress. In addition to early light-inducible protein (ELIP)-mRNA, which was induced under both field and cold room conditions, photosystem II D1 transcript and photosystem II CP 47 transcript were upregulated only under field conditions, suggesting that field plants were exposed to, or more vulnerable to, light stress than cold room plants. Various protective mechanisms allow plants to survive light stress, including a rise in ELIP level, which is a nuclear-encoded thylakoid membrane protein that was originally found to be transiently induced during greening of etiolated plants (Meyer and Kloppstech 1984). ELIPs bind chlorophyll a and lutein, and are speculated to protect chloroplasts from light-induced damage by functioning as photoprotective pigment carriers or chlorophyll exchange proteins (Adamska 1997). In addition, they might provide protection against photooxidative damage through the dissipation of excessive light energy (Hutin et al. 2003). It is noteworthy that the maximum upregulation of an ELIP message under field conditions (8.8 fold) was more than two times that of the maximum upregulation under cold room conditions (4.2 fold). Moreover, the average upregulation of ELIP messages in the field was approximately fourfold compared to approximately twofold in cold room plants (see Supplemental Tables and our website).

Table 2 Genes upregulated >2.0 fold[a] during cold acclimation in the cold room but not during cold acclimation in the field from microarray experiments

Gene product	Accession numbers
Cofactor-independent phosphoglyceromutase	CF810895, CF811173, CV090310
Phospholipid hydroperoxide glutathione peroxidase	CF811238, CF811306
Metallothionein-like protein type 2	CV091370
Proteinase inhibitor	CF810438
Water stress-induced ER5 protein	CV090946
Putative ankyrin-repeat protein	CV091043
Glyceraldehyde 3-phosphate dehydrogenase	CV091251
Glutathione S-transferase	CV090383
Putative 60S ribosomal protein L22	CF811546
60S ribosomal protein L38	CF811222
Low-temperature induced 78 kD protein	CF810862
Stretch-regulated skeletal muscle protein	CV090605
ADP-ribosylation factor-like protein	CV090555
Zinc finger (C3HC4-type RING finger) family protein	CV090382
Chaperonin gamma chain like protein	CF810678
GAMYB-binding protein	CV090414
40S ribosomal protein	CF810599
Polygalacturonase beta chain	CF811581
Putative glycerophosphodiester phosphodiesterase	CV090840
Malate dehydrogenase, glyoxysomal	CF811185
Caltractin (centrin)	CV091493
Hypothetical proteins	CV810679, CF811329, CF811402, CF811413, CV090235, CV090289, CV090483, CV090503, CV090604, CV090820, CV091003, CV091055
Proteins with no significant similarity to other sequences in GenBank	CF811282, CV090539, CV090607

[a] Only those genes induced >2.0 fold are shown in order to save space

Table 3 Genes upregulated during cold acclimation in the field but not during cold acclimation in the cold room from microarray experiments

Gene product	Accession numbers
DnaJ protein	CF810527, CF810686
Sgs1 gene product-Rec Q family of ATP-dependent DNA helicases	CV090482, CV090492
Granule-bound glycogen (starch) synthase	CF810992, CV090603
Photosystem II D1 protein	CF810742
Photosytem II CP 47 protein	CF810690
MutF	CV090600
L-ascorbate peroxidase	CF810947
Putative deoxyguanosine kinase	CF810718
Putative aldo/keto reductase	CV091316
DNA directed RNA polymerase I 190 K	CF810490
Lysine decarboxylase-like protein	CF810885
RNA polymerase I, II, and III 24.3 kDa subunit	CF811571
Putative transposase	CF810529
Probable membrane protein YLR162w	CV090514
Membrane bound *O*-acyl transferase (MBOAT) family protein	CV090287
Hypothetical proteins	CF810483, CF810521, CF810524, CF810733, CF810744, CF810805, CV090718, CV090745, CV091113, CV091301
Proteins with no significant similarity to other sequences in GenBank	CF810595, CF811188, CV090730

D1 and CP47 are subunits of the photosystem II reaction center and function as binding electron transfer prosthetic molecules and chlorophyll a antenna pigments, respectively. High light-induced damage mainly targets the photosystem II reaction center and leads to inactivation of electron transport and the degradation of D1 protein (Aro et al. 1993; Rintamaki et al. 1996). Salt-tolerant rice plants have higher transcript levels of chloroplast genes encoding D1 and CP47, under salinity stress conditions, than salt-sensitive plants (Chattopadhayay et al. 2002). Under field conditions in winter, blueberry floral buds are likely exposed to excessive light. If photosynthetic metabolism is downregulated in blueberry in winter, as has been suggested in over-wintering rhododendron leaves (Wei et al. 2005), the light energy harvested by floral buds might exceed what can be processed by photosystems, making them particularly vulnerable to photoinhibition and photooxidative damage, thus explaining the observed upregulation of genes encoding ELIP, D1 and CP 47 proteins.

As shown in Fig. 2, the number of differentially suppressed genes during cold acclimation in the field ranged from 23 at 67 chill units, to a maximum of 143 at 399 chill units, to 18 at 779 chill units, to 15 at 1,234 chill units. The total number of genes suppressed at any of these time points was 162 (6.3% of arrayed genes). In contrast to the number of induced genes under field and cold room conditions, the number of genes suppressed during cold acclimation in the cold room was much lower than under field conditions, only 30 at 500 chill units and 5 at 1,000 chill units. The total number of genes suppressed at either time point was 32 (1.2% of arrayed genes). The types of genes suppressed at any time point under field conditions but not under cold room conditions are listed in Table 4. Only those genes suppressed by more than twofold are shown in order to save space. Again, detailed listings of differentially suppressed genes at each time point are provided in Supplemental Tables and on our website. It is interesting that many of the genes that were suppressed during cold acclimation in the field were, in fact, induced during cold acclimation in the cold room. Examples include genes encoding ubiquitin-conjugating enzyme, heat shock proteins, phospholipid hydroperoxide glutathione peroxidase, and low temperature-induced 78 kD protein. Most of the genes suppressed in the field reached maximum suppression at the 399 h time point. An examination of the weather data revealed that, in the week preceding this collection point, the average temperature had dropped below freezing, to −2.1°C from an average temperature of 6.3°C the week before. Thus, many of these genes may have been suppressed by freezing temperatures rather than by low temperature, possibly explaining why they were not suppressed in the cold room experiment.

The point should be stressed here that, although there are similarities in the types of genes that respond during cold acclimation in the cold room and in the field

Table 4 Genes downregulated >2.0 fold[a] during cold acclimation in the field but not during cold acclimation in the cold room from microarray experiments

Gene product	Accession numbers
Dehydration-induced RD22-like protein/ BURP domain-containing protein	CF810541, CF811246, CF811485, CF811494, CF811563, CF811598
Lipid transfer protein precursor	CV090572, CV090838
Lipid transferase protein precursor	CF810421
Major latex-like protein	CF811356, CF811472, CV090991
Ubiquitin-conjugating enzyme UBC2	CV091255
Putative heat shock protein	CF810579, CF811441, CV090571
Heat shock protein 82	CF810427
Heat shock protein hslJ	CV090544
Putative aquaporin PIP2-1	CV090435
Phospholipid hydroperoxide glutathione peroxidase	CF811238
Ascorbate peroxidase	CV090907
Zinc finger (CCCH-type) family protein	CV090253
Nam-like protein 1	CF811419
60S acidic ribosomal protein P2	CF811387
14-3-3-like protein	CF811371
UDP-glucose dehydrogenase-like protein	CV090601
26S proteasome regulatory subunit S2	CV090400
Low-temperature induced 78 kD protein	CF810862
Hypothetical proteins	CF811182, CF811517, CF811535, CV090580
Proteins with no significant similarity to other sequences in GenBank	CF811414, CV090849, CV091383

[a] Only those genes downregulated >2.0 fold are shown in order to save space

environment, there are major differences. In the case of blueberry, many of the genes that are induced in the cold room are indeed suppressed in the field during cold acclimation. Certainly, most of the studies to date to identify cold-responsive genes in plants have been done using artificial, cold room conditions. Our results suggest that many of the genes identified as cold-induced under these conditions may, in fact, not function during cold acclimation under natural, field conditions.

Functional classification of differentially expressed genes

Differentially expressed genes were classified into 12 functional categories similar to the system used previously by us for blueberry (Dhanaraj et al. 2004) and Ablett et al. (2000) for grape. The numbers of genes induced or suppressed at any of the time points under field or cold room conditions in each of these categories are shown in Fig. 3. Regarding the upregulated genes, only 26.9% of the genes that were induced in the field and 25.3% of the genes induced in the cold room have unknown and/or unclassified functions. Regarding the downregulated genes, however, 31.9% of the genes that were suppressed in the field and 46.9% of the genes that were suppressed in the cold room have unknown and/or unclassified functions. Of those genes that could be classified and were induced in the field, more fell into the stress/defense category, followed in descending order by primary metabolism, cell structure, and protein destination and storage categories. Of the classified genes that were induced in the cold room, more fell into the stress/defense category, like those induced in the field, followed in descending order by primary metabolism, protein destination and storage, protein synthesis, and then cell structure categories. Of the classified genes that were suppressed in the field, more fell into the stress/defense category, followed by primary metabolism, transport, photosynthesis, and protein destination and storage. The few classified genes that were suppressed in the cold room were more evenly divided between first stress/defense, signal transduction, and transport, followed by transcription and cell structure, and finally by primary metabolism, photosynthesis, and cell growth/division categories.

Expression profiling

SQL queries and OLAP (Alkharouf et al. 2005) were used to produce lists of differentially expressed genes at each of the time points from the field experiment. These lists were then fed into the clustering algorithm of Analysis Services, which is provided with SQLServer2000 (Microsoft, Redmond, WA, USA). The clustering algorithm was used mainly to graphically view the OLAP results and group genes with distinct expression profiles. Five distinct expression profiles resulted from this analysis (Fig. 4). A list of some of the genes representing each of the profiles is given in Table 5, along with their function or functional category.

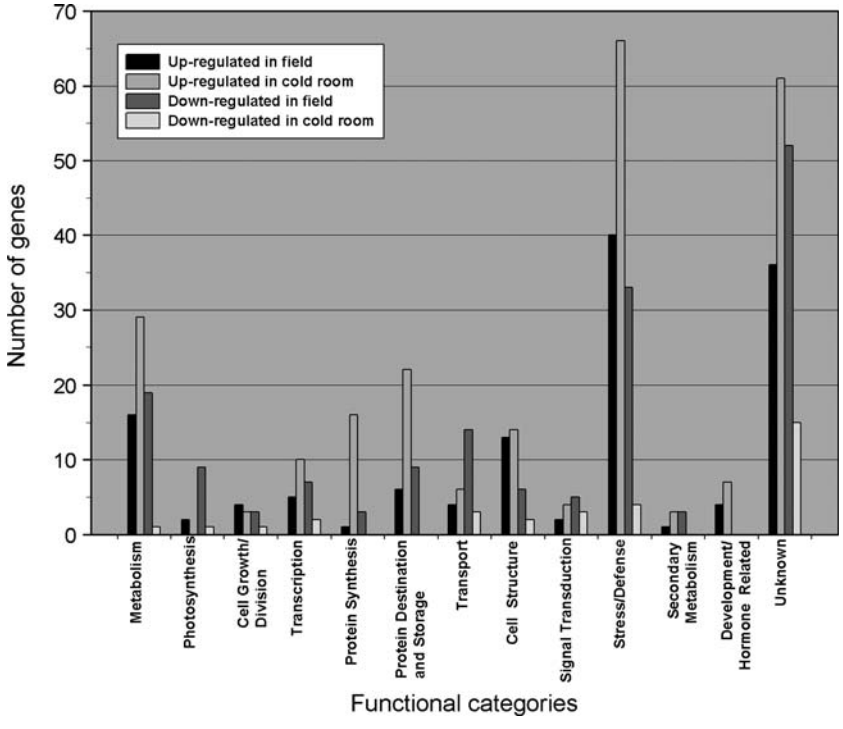

Fig. 3 Functional categorization of genes that were differentially induced or suppressed during cold acclimation in blueberry flower buds at any of the time points under field or cold room conditions. The numbers of induced or suppressed genes in each functional category are given. Many of the genes are unknown and/or have no functional classification

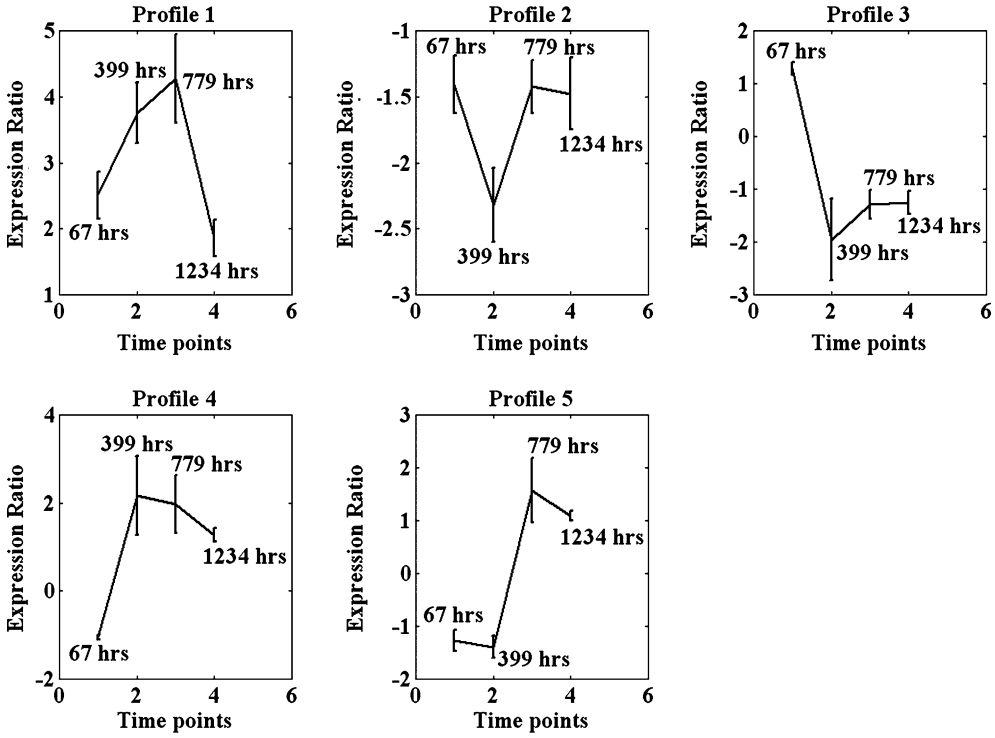

Fig. 4 Five distinct expression profiles of genes differentially expressed in blueberry flower buds during cold acclimation under field conditions. The expression ratios were calculated from normalized, rescaled ratios of the fluorescence intensity in the red and green channels. Ratios represent the average from the two biological samples that were used. *Error bars* show the standard deviation of expression levels at each time point. Basically, profiles 1, 4, and 5 represent upregulated genes while profiles 2 and 3 represent genes downregulated with cold acclimation

Basically, profiles 1, 4, and 5 represent upregulated genes while profiles 2 and 3 represent genes downregulated with cold acclimation. The first profile (profile 1 in Fig. 4) represents a number of genes whose expression correlates very well with cold hardiness levels, increasing steadily during cold acclimation, peaking at 779 chill units, when plants are maximally cold hardy, and then declining sharply by 1,234 chill units, as plants begin to deacclimate. These genes encode dehydrins, glycine-rich proteins, cell wall proteins, and protein kinase PINOID (Table 5). This is consistent with our earlier findings that dehydrin levels correlate closely with cold hardiness levels in blueberry (Muthalif and Rowland 1994; Arora et al. 1997). These genes may be predominantly cold responsive. Profile 4 represents a number of genes whose expression levels peak earlier, at 399 chill units, and then decline slightly through 779 and 1,234 chill units. They include genes encoding various LEAs, low temperature and salt-responsive proteins, and beta amylase, among others. These genes may be responsive to other factors, in addition to cold, such as short photoperiods and ABA levels. Profile 5 represents genes whose expression declines between 0 and 399 chill units, rises and peaks at approximately 779 chill units, and then declines by 1,234 chill units. Some of these genes encode granule-bound starch synthase, Sgs1 protein, cytochrome P450 monooxygenase, calmodulin, and protein phosphatase 2C. These genes could relate to breaking of dormancy and resumption of growth. Since Bluecrop has a chilling requirement of approximately 1,200 chill units (Muthalif and Rowland 1994), it would make sense that Bluecrop plants would be in a deep state of endodormancy between 0 and 400 chill units, would be starting to come out of endodormancy by about 800–1,000 chill units, and would be beginning to deacclimate and resume growth by about 1,200 chill units.

Profile 2 represents genes whose expression is downregulated during cold acclimation, with significant suppression already occurring by 67 chill units, maximum suppression occurring at 399 chill units, and expression at 779 chill units coming back up to about the 67 chill unit level. These genes encode heat shock proteins, aquaporin PIP2-1, and two specific transcription factors NAM and SCARECROW, involved in development of the shoot apical meristem, among others. Maximum suppression of these transcription factors at 399 chill units is consistent with plants being in a deep state of dormancy at this time point. Finally, profile 3

Table 5 Selected genes from profiles 1 through 5 in Fig. 4 that showed differential expression in blueberry flower buds during cold acclimation in the field

Profile, functional category	Putative protein name
Profile 1	
Stress/Defense-related	Dehydrins
	GRPF1
Cell structure	Cell wall protein
Auxin-mediated signaling pathway	Protein kinase PINOID
Various hypothetical proteins and proteins with no significant similarity	Unknown
Profile 2	
Stress/Defense-related	Heat shock proteins
Metabolism	UDP-glucose dehydrogenase
Water transport	Aquaporin PIP2-1
General transcription factor	Zinc finger (CCCH-type) family protein
Detoxification/Antioxidants	Phospholipid hydroperoxide glutathione peroxidase
Development of shoot apical meristem/Specific transcription factor	NAM-like protein 1
Development, radial patterning of shoots and roots/Specific transcription factor	SCARECROW
Proteolysis	Ubiquitin-conjugating enzyme UBC2
Various hypothetical proteins and proteins with no significant similarity	Unknown
Profile 3	
Stress/Defense-related	BURP domain-containing protein
	Dehydration-responsive protein RD22 precursor
Energy	ATP synthase alpha chain
Cell structure	Similarity to nuclear protein
Protein synthesis	Translation intiation factor 5A
Lipid transport	Lipid transfer protein precursor
Proteolysis	Cysteine protease
Metabolism	Membrane-bound *O*-acyl transferase
Various hypothetical proteins and proteins with no significant similarity	Unknown
Profile 4	
Stress/Defense-related	LEA proteins
	Low temperature and salt-responsive protein
Metabolism	Beta amylase
General transcription factor	Myb-related transcription factor
Proteolysis	Polyubiquitin (UBQ3) 20S proteasome alpha subunit B SINA2
Lipid transport	Acyl-CoA binding family protein
mRNA processing	RNA helicase
Various hypothetical proteins and proteins with no significant similarity	Unknown
Profile 5	
Metabolism	Granule-bound starch synthase
Chromatin modification	Sgs1 gene product
Electron transport	Cytochrome P450 monooxygenase
Biological function unknown/Linked to Self-incompatibility locus	SLL2-S9 protein
Calcium-related	Calmodulin
Signal transduction/Phosphatase	Protein

represents genes whose expression is induced slightly by 67 chill units, then declines sharply to maximum suppression by 399 chill units, and remains low through 779 and 1,234 chill units, although slightly higher expression level than at 399 chill units. These genes encode BURP-domain dehydration-responsive protein RD 22, lipid transfer protein precursor, and membrane-bound-*O*-acyl transferase, among others. It has been shown that drought induction of RD 22 in *Arabidopsis* is mediated by ABA and that RD 22 is not

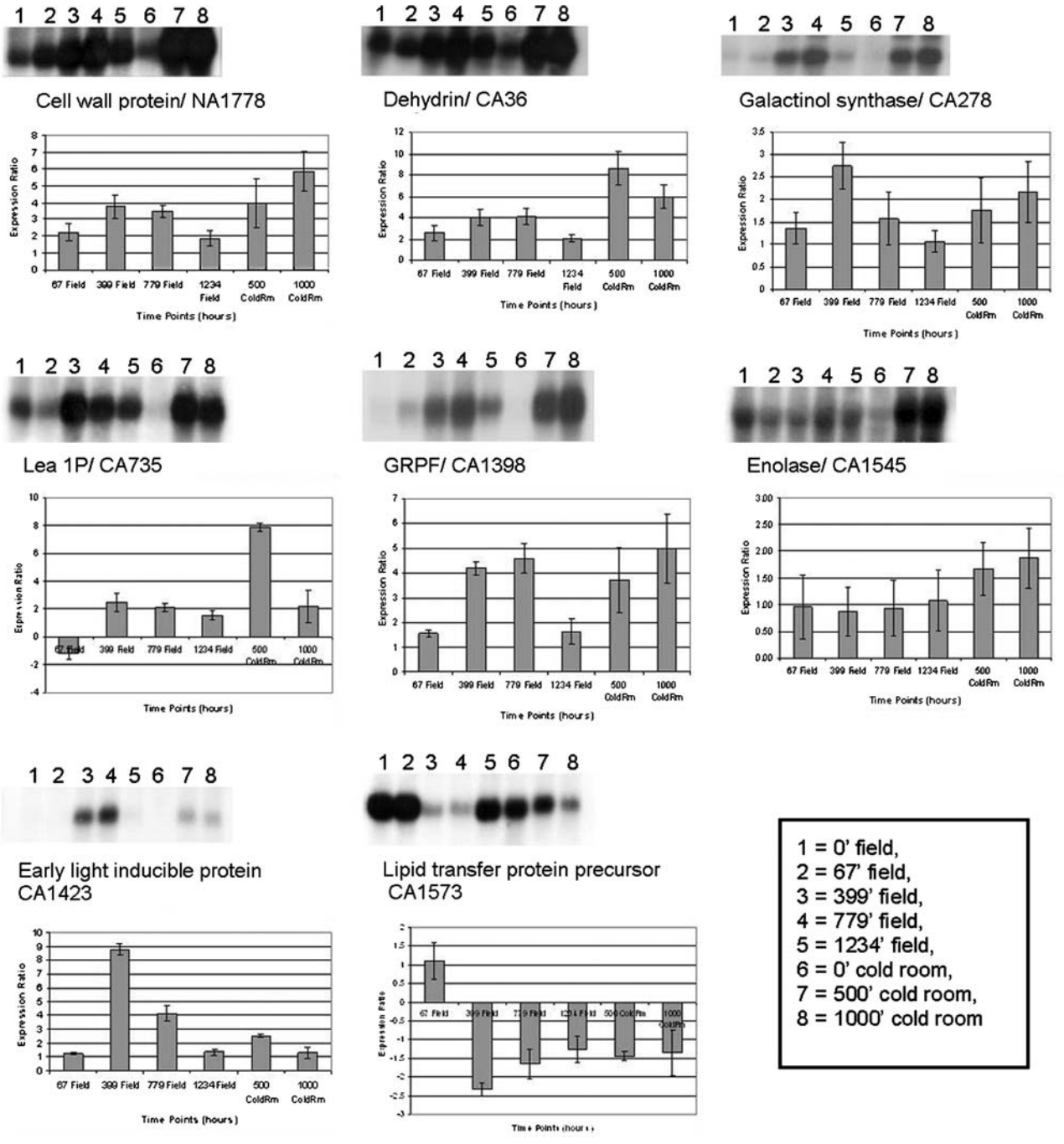

Fig. 5 Comparison of northern blot results with cDNA-microarray data. The transcript level values from the microarray data are expressed as fold changes in relation to the 0′ time points (for field or cold room, as appropriate)

induced by cold (Yamaguchi-Shinozaki and Shinozaki 1993). Thus, this profile may include genes that are predominantly induced by ABA, explaining the rise in expression early in chilling accumulation, and are not induced and, indeed, are suppressed by cold. Also consistent with this idea, is the recent finding that a non-specific lipid transfer protein from sunflower is induced by salt stress and ABA treatment (Gonorazky et al. 2005).

Validation of microarray data

Northern blots were performed to confirm the differential expression of several selected genes, which exhibited significantly different expression patterns based on the microarray data. For example, messages for a cell wall protein, dehydrin, galactinol synthase, GRPF, and Lea 1P all appeared to be induced under field and cold room conditions. Enolase transcript was induced under

cold room but not field conditions, ELIP transcript was induced to much higher levels under field than cold room conditions, and lipid transfer protein precursor transcript was suppressed under both field and cold room conditions. In Fig. 5 results from northern blots are compared with microarray expression data. For all eight genes examined, trends in expression from northern blots agreed remarkably well with trends observed from microarray hybridizations.

Although transcript profiles in NA plants grown either under field or greenhouse conditions were not compared in these microarray experiments, some differences were visible from the northern blot data and are worth noting. For example, transcript levels of LEA 1P, enolase, and lipid transfer protein precursor were all higher in NA field plants than in NA greenhouse plants (Fig. 5). Consequently, these results indicate that not only are there differences in cold-responsive genes between the CA field and cold room plants, when compared to their NA counterparts, there are also significant differences in gene expression between the initial NA field and greenhouse plants. Both NA field and greenhouse plants were exposed to natural photoperiods but the NA field plants were exposed to gradually cooler temperatures (although not reaching below 7°C) while the NA greenhouse plants were kept in a heated environment. This may explain some of the different gene expression patterns as could differences in soil conditions.

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