Chapter 17

THE LOW TEMPERATURE METABOLOME OF *ARABIDOPSIS*

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1 INTRODUCTION

Low temperature represents an environmental variable which significantly affects plant performance, causing losses in productivity and limiting geographical distribution of many species (Boyer, 1982). Low temperature exposure has consequences for most biological processes, and freezing temperatures often lead to severe damage due to the cellular dehydration which occurs upon ice formation (Thomashow, 1999; Xin and Browse, 2000). However, the exposure of certain plant species, including *Arabidopsis*, to low temperatures (5–10°C), initiates a series of events which, over a varying period of time, results in these plants acclimating to the lower growth temperature and becoming more freezing tolerant (Browse and Xin, 2001; Stitt and Hurry, 2002). This is referred to as cold acclimation.

Cold acclimation is complex and involves numerous molecular, physiological and biochemical changes. Due to its agricultural importance, considerable effort has been directed at understanding the phenomenon of cold acclimation at the molecular genetic level (Thomashow, 2001; Fowler and Thomashow, 2002). Equally important are the biochemical changes which occur at the level of the metabolome (Cook et al., 2004; Kaplan et al., 2004). Examination at the metabolic level offers a direct link between a gene and function, as well as the elucidation of relationships that occur through complex biochemical regulation (Fiehn, 2002).

Our goal was to examine the effects of cold acclimation on metabolome from a global perspective; incorporating changes from all metabolic pathways using an unbiased, non-targeted approach afforded us by Fourier transform ion

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cyclotron mass spectrometry (FTMS) technology (Aharoni et al., 2002; Brown et al., 2005).

2 MATERIALS AND METHODS

2.1 Plant material, growth conditions and experimental design

Seeds of *Arabidopsis thaliana* (L.) Heynh., ecotype Columbia were germinated from seed under controlled environment conditions at 23°C with an 8 h photoperiod and growth irradiance of 90 μmol quanta $m^{-2}s^{-1}$ as described previously (Gray et al., 2003). Plants were allowed to grow under these non-acclimating conditions for 27 days and then shifted to cold acclimating conditions at 4°C with the same photoperiod and irradiance as the non-acclimated control plants. Leaves were sampled in triplicate biological replicates, flash frozen in liquid nitrogen and ground to powder.

2.2 Non-targeted analyses of metabolites using FTMS

2.2.1 Sample extraction and preparation

Fifty mg of ground leaf material was triple extracted using 1 mL of 1% (v/v) formic acid and 3 × 3 mL of ethyl acetate. The aqueous fractions were centrifuged for 10 min, the supernatant removed and stored at –80°C until analysis. The combined ethyl acetate fractions were evaporated to dryness under nitrogen, reconstituted in 1 mL of 100% (v/v) methanol and also stored at –80°C prior to FTMS analysis. Samples were diluted 1:19 prior to electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) analyses. Dilution for all negative and positive ion ionization analyses occurred using methanol: 0.1% (v/v) ammonium hydroxide (50:50, v/v) and methanol: 0.1% (v/v) formic acid (50:50, v/v) as mobile phases, respectively.

2.2.2 Instrument operating conditions

All analyses were performed on a Bruker Daltonics APEX III Fourier transform ion cyclotron resonance mass spectrometer equipped with a 7.0-Tesla actively shielded superconducting magnet (Bruker Daltonics, Billerica, MA, USA). Samples were introduced separately by direct injection into ESI or APCI sources. Flow rates were 600 μL h^{-1} for both ESI and APCI. Ionization (ESI and APCI) and ion transfer/detection parameters were optimized using a standard mix of serine, tetra-alanine, reserpine, Hewlett-

Packard tuning mix, and the adrenocorticotrophic hormone fragment 4–10. In addition, the instrument conditions were tuned to optimize ion intensity and broadband accumulation over the mass range of 100 to 1000 a.m.u. according to the instrument manufacturer recommendations. A mixture of the above-mentioned standards was used to internally calibrate each sample spectrum for mass accuracy over the acquisition range of 100 to 1000 a.m.u. Using a linear least squares regression line, mass axis values were calibrated so that each internal standard mass peak had a mass error of <1 ppm compared to its theoretical mass.

2.2.3 Raw spectra processing and data alignment

Using XMASS software (v 6.0.3) from Bruker Daltonics Inc., data file sizes of 1 megaword were acquired and zero-filled to 2 megawords. A simm data transformation was performed prior to Fourier transform and magnitude calculations. The mass spectra from each analysis were integrated, creating a peak list that contained the accurate mass and absolute intensity of each peak. In order to compare and summarize data across different ionization modes and polarities, all detected mass peaks were converted to their corresponding neutral masses assuming hydrogen adduct formation.

A self-generated 2-dimensional (mass versus sample intensity) array was then created using *DISCOVA*rray software (Phenomenome Discoveries Inc., Saskatoon, SK, Canada). The data from multiple files were integrated and this combined file was then processed to determine all of the unique masses. The average of each unique mass was determined, representing the y-axis. A column was created for each file that was originally selected to be analysed, representing the x-axis. The intensity for each mass found in each of the files selected was then filled into its representative x, y coordinate. Coordinates that did not contain an intensity value were left blank. Once in the array, the data was further processed, visualized, and interpreted, as well as a putative chemical identity assigned.

2.2.4 Statistical analyses

The array was imported as a text file into GeneLinker Gold v. 3.0 (Predictive Patterns Software Inc., Kingston, ON, Canada) for statistical analyses. An *F*-Test was used to create a list of masses that had significant intensity changes ($p \leq 0.05$) between any two sample means generated from biological triplicates. These masses were designated as separate component names, and the corresponding sample peak intensities were used for subsequent hierarchical cluster analysis (HCA) by components.

3 RESULTS

Typically, studies examining cold acclimation grow plants under non-acclimating (23°C) conditions to a certain developmental age and then shift them to the cold acclimating temperature (4°C). In the present study, we employed a similar experimental design, examining shifted leaves for an extensive time course (up to 49 days under cold acclimating conditions).

In total, 1187 compounds were found in the *Arabidopsis thaliana* leaf extracts from all sampling points. These data were filtered such that only those compounds that were observed to significantly change during the experiment were retained (*F*-test, $p \leq 0.05$). These components (593 compounds) were further subjected to pair wise analysis (data not shown).

We employed HCA to further examine the differences and similarities between the leaf putative metabolite profiles from our filtered data sets. The results of the HCA demonstrate that the cold acclimated leaves present a constantly changing metabolic phenotype and this became more distinct

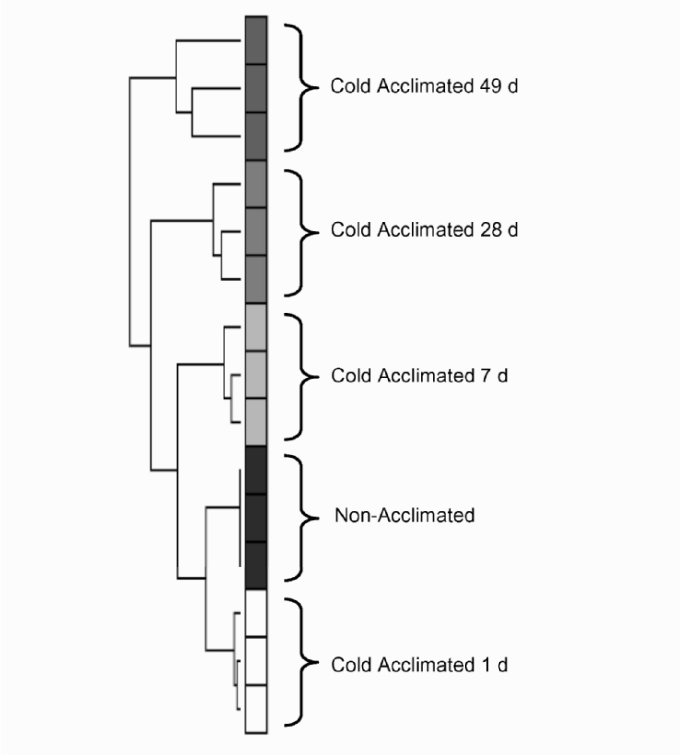

Figure 17-1. The effect of cold acclimation on the metabolic profile of shifted leaves in comparison to the non-acclimated control as determined by HCA.

from the non-acclimated control the longer the shifted leaves remained at low temperature (Figure 17-1). This is indicative of a complete reprogramming of the metablome in response to low temperature. This reorganization of the metabolome is further supported by the pair-wise comparisons of the changing compounds (data not shown).

To confirm and validate the results of our global analysis with compounds known to change during cold acclimation, we examined the metabolites associated with photosynthetic carbon metabolism. In *Arabidopsis* and numerous other cold-tolerant plant species, a reprogramming of photosynthetic carbon metabolism is frequently observed which results in the preferential accumulation of soluble sugars (Stitt and Hurry, 2002). These are thought to be an essential element for acclimation to low growth temperatures and the attainment of maximal freezing tolerance for winter survival (Stitt and Hurry, 2002; Strand et al., 2003).

The responses observed for the total hexose (Figure 17-2a), di-hexose (Figure 17-2c) and hexose-phosphate (Figure 17-2e) pools are consistent with those obtained from previous studies examining the individual compounds which would comprise these pools (Strand et al., 1997, 1999; Hurry et al., 2000). Representative spectra for these pools are presented in Figures 17-2b, d, and f and correspond to the detect ion masses obtained from the negative ESI mode of highly polar fraction in each case. These were 179.0562, 341.1083 and 259.0222 a.m.u respectively.

4 DISCUSSION

FTMS allows for the separation of metabolites in a sample solely by mass resolution (Brown et al., 2005). Based on accurate mass determination, the elemental composition is determined which can then lead to the putative identification of the metabolite. Relative quantification is achieved by comparing absolute intensities of each mass (Aharoni et al., 2002). This technology does not allow us (in most cases) to unequivocally identify specific metabolites. However, it does allow us to detect a comprehensive list of masses (based on *m/z* values) which are reflective of individual components (or putative metabolites).

The shift from non-acclimating growth conditions to cold acclimating temperatures is characterized by transient, physiological, biochemical and molecular perturbations. These transient stress responses lead to stable, long-term adjustments that reflect developmental responses to the new growth temperature (Huner et al., 1993). Leaves shifted to low temperature present putative metabolite profiles which are constantly changing in an attempt to reach a cold acclimated metabolic state. Thus, metabolome analysis indicates that the metabolic alterations which occur in *Arabidopsis* leaves

subjected to low temperature are representative of a global reprogramming of metabolism.

Our results are consistent with the notion that photosynthetic carbon metabolism is reprogrammed in response to low temperature (Stitt and Hurry, 2002). Whereas previous conclusions were the result of studies which examined each metabolite in isolation, our data sets are reflective of the entire metabolome. By measuring an entire spectrum of compounds versus an individual or group of metabolite(s), a global unbiased assessment of metabolic processes relative to cold acclimation was determined. Clearly, the

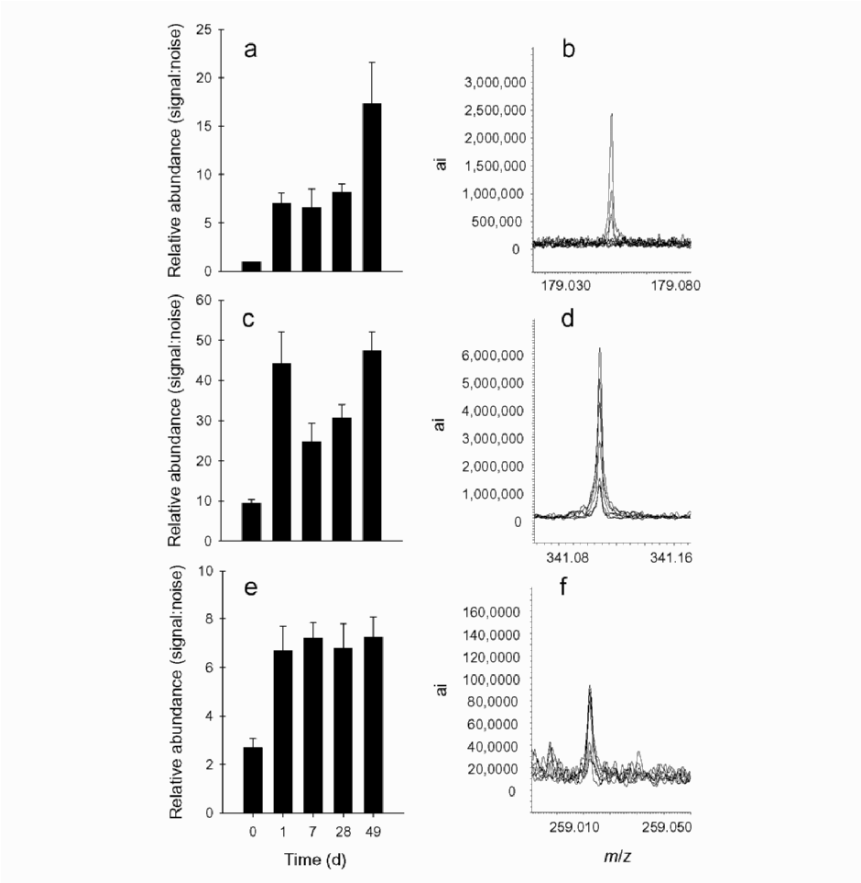

Figure 17-2. Abundance of the total hexose pool (*a*), total di-hexose pool (*c*), and total hexose-phosphate pool (*e*) in leaves shifted to cold acclimating conditions. Values represent means ± SD (n = 23). Representative spectra from (*a*), (*c*), and (*e*) are shown in (*b*), (*d*), and (*f*) respectively.

regulation or reprogramming of metabolism within the leaf during cold acclimation extends beyond that of photosynthetic carbon metabolism.

Techniques allowing a full description of the metabolome status of an organism can strongly complement existing functional genomic approaches (Sumner et al., 2003). Several studies relate stress conditions to changes in gene expression patterns at the mRNA (or protein) level (Fowler and Thomashow, 2002). However, care must be taken in the interpretation of these studies as our data demonstrate that there are fundamental differences at the level of the metabolome, which are dependent on the duration of low temperature exposure. Our results highlight the importance of proper experimental design and the significance of leaf prehistory (Krol et al., 1984; Huner et al., 1993; Gray et al., 2003) when studying complex environmental stress responses.

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