Identification of cold acclimation-responsive *Rhododendron* genes for lipid metabolism, membrane transport and lignin biosynthesis: importance of moderately abundant ESTs in genomic studies

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**ABSTRACT**

We have previously analysed expressed sequence tags (ESTs) from non-acclimated (NA) and cold-acclimated (CA) *Rhododendron* leaves, and identified highly abundant complementary DNAs (cDNAs) possibly involved in cold acclimation. A potentially significant, but relatively unexplored, application of these EST data sets is the study of moderately abundant cDNAs, such as those picked only 1–3 times from each *Rhododendron* EST library containing ~430 ESTs. Using statistical tests and Northern blots, we established that the probability of differential expression of moderately abundant cDNAs based on the EST data is, indeed, a reasonably accurate predictor of their ‘true’ upregulation or downregulation as 11 out of 13 cDNAs (85%) studied fit this criterion. The analyses also revealed four aspects of cold acclimation in *Rhododendron* leaf tissues. Firstly, the concomitant upregulation of long-chain acyl-coenzyme A (acyl-CoA) synthetase, CTP:cholinephosphate cytidylyltransferase and delta-12 fatty acid desaturase in CA leaf tissues suggests that phospholipid biosynthesis and desaturation are important components of cold hardening in *Rhododendron*. Secondly, upregulation of plastidic nicotinamide adenine dinucleotide phosphate-succinic dehydrogenase (NADP-ME) in CA tissues suggests that malate is an important source of acetyl-CoA used for fatty acid biosynthesis during cold acclimation. Thirdly, downregulation of plasma membrane intrinsic protein (PIP2-1) aquaporin and upregulation of gated outward rectifying K+ channel (GORK) in CA tissues may be associated with the protection of overwintering leaves from freeze-induced cellular dehydration. Fourthly, upregulation of coumarate 3-hydroxylase may be associated with cell wall thickening in CA tissues. Physiological implications of these results, which reveal potentially novel regulations of cold acclimation in overwintering woody evergreens, are discussed. This work highlights the importance of also investigating low/moderately abundant ESTs (in addition to highly abundant ones) in genomic studies, in that it offers an effective strategy for identifying stress-related genes, especially when large-scale cDNA sequencing/microarray studies are not possible.

**Key-words**: aquaporin; cold hardiness; coumarate 3-hydroxylase; desaturation; expressed sequence tags; gated outward rectifying K+ channel; gene expression; moderately abundant cDNAs; phospholipid biosynthesis.

**INTRODUCTION**

Woody perennials growing in temperate zones survive harsh winters through cold acclimation, a phenomenon where plants increase their freezing tolerance in response to decreasing day-length, low non-freezing temperatures, followed by subfreezing temperatures through fall and winter (Sakai & Larcher 1987). Cold acclimation results from a complex process involving a number of physiological and biochemical changes (Wisniewski, Bassett & Gusta 2003), including changes in gene expression, plant cell wall composition, membrane structure and function, and primary and secondary metabolism (Guy 1990; Thomashow 1999; Kaplan et al. 2004).

Expressed sequence tags (ESTs), which are partial, single-pass sequences from randomly selected complementary DNA (cDNA) clones, provide a valuable tool for identifying plant genes involved in stress resistance. We have previously sequenced 423 and 439 5′-ESTs generated from the cDNA libraries of non-acclimated (NA; leaf freezing tolerance of −7 °C) and cold-acclimated (CA; leaf freezing tolerance of −53 °C) *Rhododendron* leaves, respectively (Wei et al. 2005). Comparative EST analysis was performed in the identification of cold acclimation-responsive genes encoding dehydrins/late embryogenesis abundant (LEA) proteins, early light-induced proteins (ELIP), beta amylase and genes associated with photosynthesis and photorespiratory glycolate pathway. However, the aforementioned genes were all represented by relatively highly abundant cDNAs, that is, picked 4–40 times in NA or CA EST libraries, whereas the moderately abundant cDNA
clones, which were picked only 1–3 times from these EST libraries, were not analysed. The current study focuses on the analysis of this category of moderately abundant cDNAs, especially those relevant to cold acclimation, such as genes involved in cell wall composition and membrane structure and function.

The plasma membrane is considered the primary site of freezing injury (Levitt 1980). Therefore, several research groups have studied biochemical and biophysical changes that the plasma membrane undergoes during cold acclimation. A consensus has emerged from these studies that the plasma membrane phospholipid (PL) content, particularly phosphatidylcholine (PC) and phosphatidylethanolamine (PE), increases during cold acclimation (Sikorska & Kacperska-Palacz 1979; Uemura & Yoshida 1984; Lynch & Steponkus 1987; Palta, Whitaker & Weiss 1993). It has also been noted that the degree of PC and PE fatty acid unsaturation, a process known to be mediated by fatty acid desaturases (FAD), increases during cold acclimation in diverse species and tissues (Lynch & Steponkus 1987; Palta et al. 1993). The extent of lipid desaturation, which directly controls the fluidity of membranes at low temperature, is believed to affect the proper functioning of membrane-bound enzymes (Houslay & Gordon 1984). Therefore, increase in the membrane fluidity is considered an important component of cold acclimation (Guy 1990; Nishida & Murata 1996). However, little is known about the transcriptional regulation of PL accumulation and desaturation during cold acclimation in overwintering woody perennials.

Plant tissues typically experience cellular dehydration during a freezing event because of extracellular ice formation and subsequent exosmosis. Cellular dehydration is also generally associated with the cold acclimation process (Chen, Li & Burke 1975; Guy et al. 1992; Karlson et al. 2003) and might be under regulatory control of membrane transport proteins, including water channel proteins commonly referred to as ‘aquaporins’ (Schaeffer 1998; Chrispeels, Crawford & Schroeder 1999). Information regarding the altered expression of aquaporins and/or other potential dehydration-responsive membrane transport proteins during cold acclimation in woody perennials may advance our understanding of this phenomenon from a new perspective.

Cell walls play an important role in plant cold resistance (Tao, Li & Carter 1983). Cell wall thickening has been shown to occur during cold acclimation in various plant species (Chen, Li & Cunningham 1977; Huner et al. 1981; Griffith et al. 1985; Stefanowska et al. 1999), and is believed to provide resistance against cell collapse and thereby provide protection against freeze-induced mechanical stress. Lignins, the structural components of cell walls, are produced through the phenylpropanoid biosynthetic pathway involving cytochrome P450-dependent monoxygenases (Schoch et al. 2001; Nair et al. 2002; Raes et al. 2003). However, data on the expression of cytochrome P450 genes during cold acclimation are scarce, and no information exists regarding their expression in leaf tissues of overwintering evergreen woody perennials, such as Rhododendron.

The objectives of the current study were threefold; the first of which was to establish whether the calculated probability of differential expression can be used as a guide for identifying cold acclimation-responsive genes based on EST data of moderately abundant cDNAs. The recent formulation of statistical tests for the analysis of gene abundance between two (Audic & Claverie 1997) or multiple cDNA libraries (Greller & Tobin 1999; Stekel, Git & Falciani 2000) has allowed calculation of probabilities of differential expression between libraries, such as NA versus CA, in the context of the current study. The second objective was to conduct bioinformatic and comparative expression analyses (i.e. in NA versus CA leaf tissues) of Rhododendron cDNAs associated with fatty acid biosynthesis, desaturation of PC, K+ and water transport across the cell membrane, and lignin biosynthesis. The third objective was to provide new insight into mechanisms of plant cold acclimation by integrating this information with known physiological and biochemical responses of plants during cold hardening. In this study, we demonstrated a relatively less expensive yet effective means for identifying stress-related genes when large-scale cDNA sequencing or microarray studies cannot be conducted.

MATERIALS AND METHODS

Production of the unique transcript data sets

The unique transcript sets were previously compiled from 423 and 439 5′-ESTs obtained from CA and NA leaves of Rhododendron, and were deposited in the GenBank dbEST at the NCBI (GenBank accession nos. CV014938–CV015799) (Wei et al. 2005). The two sets of ESTs generated from the CA and NA libraries were clustered separately, producing a list of unique transcripts. The putative functions of the unique transcripts were assigned through a search of the PIR-NREF (Protein Information Resource: Non-Redundant Reference) protein database using BLASTX program as described by Wei et al. (2005). The expectation value (e-value) cut-off for BLASTX was set at >1e-4. The best three protein hits for each transcript were used to assign putative identities to the transcript. Detailed functional classification of all ESTs and the analysis of the most abundant cDNAs were presented by Wei et al. (2005). The current study further analyses the moderately abundant genes related to water and ion transport across plasma membrane, and the fatty acid and phenylpropanoid/lignin biosynthesis.

Plant leaf sample collection

Leaves were sampled from field-grown plants of Rhododendron catawbiense ‘Catalga’, a super cold-hardy genotype as described previously (Wei et al. 2005). Briefly, the NA and CA leaves were sampled during summer (July 2002) and winter (January 2003), respectively; their leaf freezing tolerance was −7 and −53 °C, respectively. The leaves were flash-frozen in liquid nitrogen and stored at −80 °C until RNA preparation.
RNA extraction and Northern blot analysis

Total RNA was isolated from NA and CA leaves according to the modified hot-borate method of Wilkins & Smart (1996). Equal amounts of total RNA (8 μg) from each treatment were fractionated on 1% (w/v) denaturing formaldehyde-agarose gels and photographed to confirm RNA quality and to verify equal sample loading. RNA was subsequently transferred and immobilized on BrightStar-Plus nylon membranes (Ambion, Austin, TX, USA). The preparation of DNA probes corresponding to cDNA inserts of interest were described by Wei et al. (2005). The membranes, with immobilized RNA, were probed with 32P-labelled cDNA of interest using hybridization conditions described previously (Wei et al. 2005). After the Northern blotting, the relative mRNA levels were quantified by densitometry using imaging software (NIH Image version 1.41, National Institutes of Health, Bethesda, MD).

RESULTS AND DISCUSSION

EST data sets and mathematical basis for predicting differential expression of moderately abundant cDNAs during cold acclimation

Our EST data sets from NA and CA libraries have a moderate size of ~ 430 each. Increasing the number of ESTs will enable a large-scale study of transcript profiling. However, as the generation of large numbers of ESTs can be expensive and time-consuming, the ability to fully utilize relatively smaller EST data sets in identifying differentially expressed genes would be of great practical significance, provided the following criteria are met. Firstly, the EST data sets should be of high quality; our recent study (Wei et al. 2005) showed that the average length of our ESTs is ~550 bp compared with ~350 bp for that of a 10 times larger EST data set of aspen (Populus tremula) (Bhalerao et al. 2003) generated from the cDNA libraries of summer (young) and autumn (senescent) leaf tissues. In addition, the shared percentage of ESTs between the control and stress treatments were comparable between our data sets and aspen ESTs (Wei et al. 2005), indicating that our EST data sets were representative of the whole cDNA libraries regarding abundant genes. Secondly, an appropriate mathematical analysis should be employed to increase the efficiency for identifying potentially differentially expressed genes.

Statistical analyses of the number of ESTs associated with specific cDNA libraries have been used to calculate probabilities of differential expression of genes between different plant organs or stress treatments. For example, a statistical test has been used to compare the abundance of individual genes between two cDNA libraries (i.e. EST data sets) (Audic & Claverie 1997). Bhalerao et al. (2003) applied this method to calculate the ‘confidence’ for the probability of differential gene expression based on the number of ESTs generated from two aspen cDNA libraries.

Assuming that the two EST data sets (NA and CA) were generated by sequencing the cDNA clones that were randomly picked from two cDNA libraries, respectively, and that a given gene (represented by a cDNA) was picked ‘X’ times from the EST data set 1 with a sample size of N1 and ‘Y’ times from the EST data set 2 with a sample size of N2, the probability for false differential expression (P), according to Audic & Claverie (1997), can be calculated as follows:

\[ P(Y|X) = \left( \frac{N_2}{N_1} \right)^Y \frac{(X + Y)!}{X!Y! \left(1 + \frac{N_2}{N_1}\right)^{(X + Y)}} \]  

(1)

For two EST data sets of similar size (i.e. \( N_1 \approx N_2 \)), Eqn 1 can be simplified as

\[ P(Y|X) = \frac{(X + Y)!}{X!Y! 2^{(X + Y)}} \]  

(2)

Although Eqn 2 was originally applied to EST data sets of ~1000 ESTs (Audic & Claverie 1997), this equation could potentially be used to predict P-value for a given gene picked from NA and CA EST data sets of about half the size, that is, ~500 ESTs each, as in the current study.

Mathematically and practically, to increase the chances of identifying truly differentially expressed genes, we focused mainly on the moderately abundant genes that were picked 0 versus 1–3 times from the two EST data sets. The probability that these genes are differentially expressed can be calculated as \( 1 - P \), as illustrated in Table 1.

Case 1 in Table 1 describes a scenario where a gene is picked zero time \( (0/500) \) from one library (N1) but 1 time \( (1/500) \) from another library (N2). Because there is no-pick of a specific gene from a 500-EST data set of N1, we can hypothetically enlarge the N1 data set until that specific gene is picked once. Assuming that the EST data set may need to be increased to 1500 or to 15 000 for one-time pick, the probability for differential expression \( (1 - P) \) of this gene will be 0.7188 or 0.9396, respectively (Table 1). The corresponding fold-change in gene expression (as per Northern blots) would be expected to range from 3- to 30-fold.

Similar analyses can be made for cases 2 and 3, where the genes are picked ‘0/500 versus 2–3/500’ times from N1 and N2 EST data sets, respectively (Table 1), which shows that the probabilities \( (1 - P; 0.8945–0.9999) \) for the identification of differentially expressed genes and the predicted fold-change in their expression (6–90-fold) were higher than the values for case 1 (Table 1).

It is noteworthy that the cases where genes were picked ‘1/500 versus 2/500’ or ‘2/500 versus 3/500’ times between the two libraries were excluded from this analysis, because the expected fold-changes of expression of such genes would range from 1.5 to 2, which is less than the typical threshold (threelfold change) for the definition of ‘true differential expression’. Although, ‘1/500 versus 3/500’ pick-frequency between the two libraries can be a good case for identifying differentially expressed genes, we did not find genes with known functions to fit this category in our EST data sets.

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Validation of the mathematical basis for differential expression of moderately abundant cDNAs

Although a total of 40 genes (together from both EST libraries) fitted the ‘moderately abundant’ category (data not shown), we chose to study those that are associated with structural and functional aspects of cell wall and plasma membrane, specifically fatty acid and PC biosynthesis, PC desaturation, water and K⁺ flux across membrane, and lignin biosynthesis: a total of 15 cDNAs encoding 13 distinct genes and exclusively picked 1–2 times in one EST library but zero times from the other EST library (Table 2) were selected. Although these genes were selected based on their presumed role in cold acclimation physiology, it is noteworthy that they represent a wide spectrum of cellular functions. Moreover, the 13 genes selected for this analysis account for 30% of the entire pool of moderately abundant genes (total 40) and therefore constitute a representative sample. In addition, although only one P450 gene identified in this study is known to be involved in lignin metabolism [coumarate 3-hydroxylase (C3H); Table 2], all the five P450 genes (of the moderately abundant pool) were included in this analysis to further bolster the representative nature of the selected pool of genes.

Northern blots (as described in Figs 1–3) were performed to compare expression of these transcripts in NA and CA leaf tissues. Results indicated that of the 13 cDNAs examined, 12 were upregulated during cold acclimation, whereas one was downregulated (Table 2).

Table 1. Probability and the expected fold-change for true differential expression of a gene that is picked zero times (0/500) from one expressed sequence tag (EST) data set (N1), but 1–3 times (1–3/500) from another EST data set (N2). Assumption: if a gene is picked once from data set N2 with a size of 500 ESTs, this gene will be picked three times when the data set is increased to 1500 by sequencing more randomly picked cDNA clones from the cDNA library.

<table>
<thead>
<tr>
<th>Case 1: a gene picked zero time from one library but 1 time from another library</th>
<th>Probability for true differential expression (1 − P)</th>
<th>Expected fold-change in differential gene expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pick-frequency</td>
<td>Y/N₂</td>
<td>X/N₁</td>
</tr>
<tr>
<td>Hypothetical frequency (1)</td>
<td>0/500</td>
<td>1/1500</td>
</tr>
<tr>
<td>1/500</td>
<td>0.7188</td>
<td>3 fold</td>
</tr>
<tr>
<td>Hypothetical frequency (2)</td>
<td>0/500</td>
<td>1/15000</td>
</tr>
<tr>
<td>1/500</td>
<td>0.9396</td>
<td>30 fold</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Case 2: a gene picked zero time from one library but two times from another</th>
<th>Probability for true differential expression (1 − P)</th>
<th>Expected fold-change in differential gene expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pick-frequency</td>
<td>Y/N₂</td>
<td>X/N₁</td>
</tr>
<tr>
<td>Hypothetical frequency (1)</td>
<td>0/500</td>
<td>1/1500</td>
</tr>
<tr>
<td>2/500</td>
<td>0.8945</td>
<td>6 fold</td>
</tr>
<tr>
<td>Hypothetical frequency (2)</td>
<td>0/500</td>
<td>1/15000</td>
</tr>
<tr>
<td>2/500</td>
<td>0.9971</td>
<td>60 fold</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Case 3: a gene picked zero time from one library but three times from another</th>
<th>Probability for true differential expression (1 − P)</th>
<th>Expected fold-change in differential gene expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pick-frequency</td>
<td>Y/N₂</td>
<td>X/N₁</td>
</tr>
<tr>
<td>Hypothetical frequency (1)</td>
<td>0/500</td>
<td>1/1500</td>
</tr>
<tr>
<td>3/500</td>
<td>0.9648</td>
<td>9 fold</td>
</tr>
<tr>
<td>Hypothetical frequency (2)</td>
<td>0/500</td>
<td>1/15000</td>
</tr>
<tr>
<td>3/500</td>
<td>0.9999</td>
<td>90 fold</td>
</tr>
</tbody>
</table>
Table 2. Moderately abundant cDNAs exclusively picked 1–2 times from either non-acclimated (NA) or cold-acclimated (CA) expressed sequence tag (EST) data sets generated from cDNA libraries prepared from leaf tissues of *Rhododendron catawbiense*. Except for NADP-ME and LACS cDNAs (which were picked twice), all other cDNAs were picked only once from one EST library and none from the other. The leaf freezing tolerance of NA and CA leaf tissues are −7 °C and −53 °C, respectively (Wei et al. 2005). The GenBank accession numbers for cDNAs can be found in the text.

<table>
<thead>
<tr>
<th>Putative gene ID&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Subcellular location&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Arabidopsis ortholog&lt;sup&gt;c&lt;/sup&gt;</th>
<th>e-value</th>
<th>NA</th>
<th>CA</th>
<th>Predicted expression in CA tissues relative to NA</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNAs for fatty acid and phosphatidycholine biosynthesis and desaturation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. NADP-ME, plastidic</td>
<td>Chl</td>
<td>At1g79750</td>
<td>5e-43</td>
<td>–</td>
<td>CA4C01</td>
<td>up (confirmed)</td>
</tr>
<tr>
<td>2. FAT</td>
<td>Chl</td>
<td>At1g08510</td>
<td>2e-43</td>
<td>–</td>
<td>CA4H06</td>
<td>up&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>3. LACS</td>
<td>Chl</td>
<td>At2g04350</td>
<td>4e-84</td>
<td>–</td>
<td>CA4C05</td>
<td>up (confirmed)</td>
</tr>
<tr>
<td>cDNAs for water and K+ transport</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. Aquaporin PIP2-1</td>
<td>PM</td>
<td>At3g54820</td>
<td>3e-74</td>
<td>NA2H11</td>
<td>–</td>
<td>down (confirmed)</td>
</tr>
<tr>
<td>8. GORK</td>
<td>PM</td>
<td>At5g37500</td>
<td>3e-71</td>
<td>–</td>
<td>CA3H11</td>
<td>up (confirmed)</td>
</tr>
<tr>
<td>cDNAs for P450s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. BAS1 (CYP72B1)</td>
<td>Unknown</td>
<td>At2g26710</td>
<td>5e-30</td>
<td>NA2A08</td>
<td>–</td>
<td>down (confirmed)</td>
</tr>
<tr>
<td>10. CYP82C4</td>
<td>Unknown</td>
<td>At4g31940</td>
<td>2e-36</td>
<td>NA5B01</td>
<td>–</td>
<td>down (confirmed)</td>
</tr>
<tr>
<td>11. C3H (CYP98A3)</td>
<td>ER</td>
<td>At2g08980</td>
<td>1e-66</td>
<td>–</td>
<td>CA1C02</td>
<td>up (confirmed)</td>
</tr>
<tr>
<td>12. CYP71B9</td>
<td>Mito</td>
<td>At2g02580</td>
<td>7e-35</td>
<td>–</td>
<td>CA1G02</td>
<td>up (confirmed)</td>
</tr>
<tr>
<td>13. CYP89A2</td>
<td>M/Cyt</td>
<td>At1g64950</td>
<td>4e-53</td>
<td>–</td>
<td>CA3B02</td>
<td>up (confirmed)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Full names of the genes: BAS1, phyB activation tagged suppressor1; C3H, p-coumarate 3-hydroxylase; CCT, CTP-cholinephosphate cytidylyltransferase; FAD2, delta-12 fatty acid desaturase; FAT, fatty acyl-ACP thioesterase; GORK, gated outward rectifying K+ channel; LACS, long-chain acyl-coenzyme A synthetase; NADP-ME, nicotinamide adenine dinucleotide phosphate-malate enzyme; PIP, plasma membrane intrinsic protein; PLD, phospholipase D.

<sup>b</sup>Symbols for the subcellular locations of proteins encoded by the genes: Chl, chloroplast; Cyt, cytosol; ER, endoplasmic reticulum; M, membrane; Mito, mitochondrion; PM, plasma membrane.

<sup>c</sup>FAT and PLD δ are the only two genes (out of 13 total) for which the predicted upregulation in cold-acclimated tissues was not confirmed by Northern blot analysis (described later).

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**Figure 2.** RNA gel blots of genes encoding aquaporin [plasma membrane intrinsic protein (PIP2-1)] (a) and gated outward rectifying K+ channel (GORK), (b) from non-acclimated (NA) and cold-acclimated (CA) expressed sequence tag (EST) data sets of *Rhododendron catawbiense* leaf tissues. A stylized diagram (a; right panel) illustrates cellular dehydration as a result of extracellular ice formation. Total RNA was isolated from NA and CA leaves and hybridized with respective cDNA probes. ‘EST# ratio’ based on the number of cDNA(s) picked from NA versus CA EST library was 1:0 for aquaporin gene and 0:1 for the GORK gene, respectively. Fold-change in the expression during cold acclimation relative to NA state (defined as ‘1’) was estimated by densitometry. Equal loading of total RNA between samples was confirmed as in Fig. 1. PM, plasma membrane.
ined actual comparative expression levels of 11 cDNAs are in accordance with the differences in their relative abundance in the two EST data sets. In other words, the cDNAs that were exclusively picked in CA EST library were found to be ‘truly’ upregulated in CA leaf tissues and those exclusively picked in NA EST library were ‘truly’ downregulated in CA leaf tissues (Table 2). Overall, the relative expression levels for 85% (11/13) of the genes studied fit with moderate ‘digital’ abundance in the EST data sets, providing a strong basis for identifying cold acclimation-responsive genes.

Northern blot data (Figs 1–3) indicated a 3- to 60-fold change in expression of various genes, whereas the frequency at which these genes were picked from the two EST data sets was 0 versus 1–3. Probability analysis and the expected fold-change for differential gene expression, as illustrated in Table 1, support this observation. Although a rather broad range of the fold-change in gene expression (3- to 60-fold) does not numerically reflect the EST abundance (0 versus 1–3) because the ‘baseline control’ was set at zero (< 1/500), it is a mathematical possibility, as illustrated in Table 1. However, it should be noted that the demonstration of direct correlation between the fold-change in gene expression and the EST abundance is not the goal of this study.

For the two exceptions (items 2 and 6, Table 2), the expression profiles of cDNAs did not fit the predicted differential expression based on the probability analysis [i.e. no significant difference in the expression levels between NA and CA tissues was noted on RNA gel blots (Fig. 1)]. This can be explained as follows: for example, in case 1 of Table 1, if a specific gene was not picked until the size of N1 EST data set was increased to 600–800, which is mathematically possible, the fold-change in the expression of this gene would be 1.2–1.6 (600/500–800/500). This change is essentially non-discernible from the control and below a typical threshold of threefold for defining significant change in gene expression. We analysed each of the 11 differentially expressed genes as components of biochemical pathways potentially involved in cold acclimation.

**Cold acclimation and PL metabolism: fatty acid biosynthesis genes**

PL biosynthesis in the leaves of higher plants has two components: (1) the de novo fatty acid synthesis from acetyl-CoA in chloroplast, and (2) glycerolipid synthesis in both the chloroplast (‘prokaryotic’ pathway) and the endoplasmic reticulum (ER; ‘eukaryotic’ pathway). Figure 4 illustrates a simplified pathway for fatty acid and PL biosynthesis and subsequent PL desaturation. Two cDNAs encoding the enzymes involved in the chloroplast portion of this pathway, namely, nicotinamide adenine dinucleotide phosphate-malic enzyme (NADP-ME) and fatty acyl-ACP thioesterase (FAT), were identified in our analysis of *Rhododendron* ESTs (items 1–2, Table 2).

**NADP-ME**

In addition to its role in C4 metabolism, the plastidic isoform of NADP-ME is also involved in the generation of pyruvate and reducing power (i.e. NADPH) for fatty acid biosynthesis (Colombo, Andreo & Podesta 1997) (Fig. 4). The two cDNAs for NADP-ME identified in the current study belong to the plastidic type (item 1, Table 2; clone nos. CA4C01 and CA5C12; GenBank accession nos. CV015221 and CV015315, respectively). Northern blot analysis showed a threefold expression of this gene in CA leaves relative to NA controls (item 1, Fig. 1). Crecelius, Streb & Feierabend (2003) reported that NADP-ME activity was almost absent in NA leaves but substantially increased in CA winter rye (*Secale cereale* L.) leaf tissues.
Another study showed that the maize plastidic NADP-ME was regulated at the transcriptional or post-transcriptional level by effectors related to plant defence responses (Maurino et al. 2001).

Malate can function as a sink for the excess reducing equivalents in chloroplasts (Scheibe 2004). It has also been shown to accumulate in cold-stressed winter rye and Arabidopsis leaf tissues (Crecelius et al. 2003; Kaplan et al. 2004); the latter group (Kaplan et al.) attributed it to an increased pool of oxaloacetate-derived amino acids in cold-stressed leaves, with malate being an oxaloacetate precursor. The upregulation of Rhododendron NADP-ME suggests that in CA tissues malate might be an important source of acetyl-CoA, the precursor for fatty acid biosynthesis (Fig. 4).

**FAT**

FAT hydrolyses acyl-ACP and releases ACP and free fatty acid (Fig. 4). In this study, 1 FAT cDNA was identified in the CA but not in the NA EST data set (item 2, Table 2; GenBank accession no. CV015281). RNA gel blots indicated similar expression levels of this gene in NA and CA tissues (item 2, Fig. 1). Although this result does not fit the mathematical prediction of this gene being upregulated in CA tissues (probability level 72–94%; case 1, Table 1), it is consistent with a previous report that an Arabidopsis ortholog of this gene (At3g25110) was unchanged in the leaf tissues after 3–27 h of cold, dehydration or salinity treatments (Kreps et al. 2002). These results indicate that Rhododendron FAT is not a cold acclimation-responsive enzyme at the transcript level. However, whether it undergoes post-transcriptional or translational changes remains an open question.

**PL biosynthesis and desaturation genes**

PC is the most abundant membrane PL in plants. It is a structural component of various non-plastidic membranes and is also a precursor for PL and glycolipid synthesis in plastids (Ohlrogge & Browse 1995). In addition to the elevated level of PC, the molecular species of PC have also been reported to undergo substantial change during cold acclimation. Evidence shows that polyunsaturated fatty acids, especially tri-unsaturated molecular species of PC (linolenic acid, 18:3), increase significantly in various herbaceous and woody species during cold acclimation (Smolenska & Kuiper 1977; Clarkson, Hall & Roberts 1980; Uemura & Yoshida 1984; Lynch & Steponkus 1987; Palta et al. 1993; Cyril et al. 2002). Thus, the synthesis of polyunsaturated PC is an essential component of plant cold acclimation. In the current study, four cDNAs – encoding long-chain acyl-CoA synthetase (LACS), CTP:cholinephosphate cytidylyltransferase (CCT), delta-12 fatty acid desaturase (FAD2) and phospholipase D (PLD) – related to PC biosynthesis, desaturation and turnover were picked from the CA but not in the NA EST library. We analysed these cDNAs (genes) in the context of cold acclimation physiology.
**LACS and PL biosynthesis**

LACS is involved in the provision of activated acyl groups as substrates for several fatty acid-derived metabolic pathways (Shockey, Fulda & Browse 2002), including PL biosynthesis in ER (Fig. 4). We identified two cDNAs encoding *Rhododendron* LACS in the CA but none in NA EST data set (item 3, Table 2; clone nos. CA4C05 and CA4H05, GenBank accession nos. CV015225 and CV015280, respectively). RNA gel blots showed this gene to be indeed upregulated in CA tissues (item 3, Fig. 1). An earlier report showed an *Arabidopsis* LACS ortholog (At2g04350) to be upregulated in the leaf tissues after 3–27 h of exposure to cold (Kreps et al. 2002). It is therefore reasonable to speculate that LACS facilitates the fatty acid flux from chloroplast to ER for de novo PL (PC, PE, etc.) synthesis during cold acclimation in *Rhododendron* leaf tissues. This hypothesis is consistent with previous observations on the development of frost tolerance being related to increased PL in winter rape leaves (Sikorska & Kacperska-Palacz 1979) and an increase in PC fraction of the total plasma membrane PL of CA winter rye leaves compared to NA samples (Lynch & Steponkus 1987).

**CCT and PC biosynthesis**

PC is mainly synthesized via the cytidyldiphosphate (CDP)-choline pathway (Ohlrogge & Browse 1995; Kent 1997), which is catalysed by three enzymes sequentially: choline kinase, CCT and CDP-choline:diacylglycerol cholinephosphotransferase (Fig. 4). Among them, CCT, which converts PCho (phosphocholine) and CTP to CDP-choline, is generally accepted as the rate-limiting enzyme (for a review, see Kent 1997) and has two forms: the inactive cytosolic form and the active membrane-bound form; the latter can be promoted by anionic lipids such as phosphatidic acid (PA; illustrated in Fig. 4) (Clement & Kent 1999). One cDNA encoding CCT was picked from the CA but not from the NA EST library (item 4, Table 2; GenBank accession no. CV015210). Northern blot analysis showed a 3.5-fold expression of CCT in CA leaf tissues relative to NA samples (item 4, Fig. 1). This result is consistent with previous reports on increased CCT expression in cold-treated *Arabidopsis* plants (Choi, Lee & Cho 2001; Inatsugi, Nakamura & Nishida 2002) and increased CCT activity in rye roots exposed to a 5 °C treatment (Kinney, Clarkson & Loughman 1987).

**FAD2 and PC desaturation**

As illustrated in Fig. 4, the 18:1 PC can be converted to 18:2 PC (di-unsaturated) by FAD2, which can be further converted to 18:3 PC (tri-unsaturated) by omega-3 fatty acid desaturase (FAD3). The coordinated functioning of FAD2 and FAD3 is therefore essential for the synthesis of tri-unsaturated fatty acids in plants during cold acclimation. In the current study, a *Rhododendron* cDNA encoding FAD2 was picked from only the CA EST library (item 5, Table 2; GenBank accession no. CV015233). Its expression in CA leaf tissues was 15-fold of that in NA leaf tissues (item 5, Fig. 1), confirming its expected upregulation based on probability analysis.

Expression pattern of *Rhododendron* FAD2 in the current study is consistent with the increased level of FAD proteins in cold-treated *Arabidopsis* (Somerville et al. 2000). Previous research has shown that di-unsaturated and tri-unsaturated molecular species (18:2 and 18:3) of plasma membrane and endomembrane PL increased after cold acclimation of herbaceous and woody plants (Uemura & Yoshida 1984; Lynch & Steponkus 1987), in some cases at the expense of saturated (16:0) species (Palta et al. 1993). Saturated fatty acids have high melting points and undergo phase transition at relatively higher temperatures, which is often considered one of the causes of cold injury in NA or cold-sensitive plants; phase transition temperature of 18:0/18:2 PC is −16 °C (Marsh 1990), whereas it is −53 °C for 18:2/18:2 (Lynch & Steponkus 1989).

**PLD and PC turnover**

PLD is the most dominant type of phospholipase in plant tissues. Its role in signal transduction during cold stress has been well documented (Wang 1999; Ruelland et al. 2002). One cDNA encoding PLD was picked from the CA EST library (item 6, Table 2; GenBank accession no. CV015338). RNA gel blot showed no difference in its expression level in CA versus NA leaf tissues (item 6, Fig. 1), a result that was unexpected based on the mathematical probability of differential expression for this gene (Tables 1 & 2). However, the steady expression, as opposed to upregulation, of PLD might be conducive to the plant’s energy budget in the CA state, since this may ensure that a potential increase in PC synthesis in CA tissues will not be offset by its breakdown via elevated PLD amount or activity (Fig. 4).

The upregulation of PLD at either the mRNA or the protein level was observed in *Arabidopsis* leaf tissues within 1–3 d of a cold treatment (2–4 °C) (Katagiri, Takahashi & Shinozaki 2001; Kawamura & Uemura 2003). However, the protein amount returned to ‘constitutive’ level after 7 d of this treatment (Kawamura & Uemura 2003). Another report showed an increased PLD enzyme activity in *Arabidopsis* suspension cells within 10 min of a cold treatment (Ruelland et al. 2002). These observations support PLD’s role as a signal transduction factor during the early stages of cold stress. In the current study, PLD transcript abundance was measured in NA (August/summer-collected) and CA (January/winter-collected) leaves. However, no time-course study was undertaken; CA leaves were exposed to natural variations of temperature (from > 20 °C in August to < −10 °C in January) over a 5-month period. Our observations that there was no change in the PLD transcript abundance in NA and CA leaf tissues seems to support aforementioned results regarding the PLD accumulation pattern during cold stress, and also the stability of PLD transcripts at low (subfreezing) temperatures. However, this observation does not exclude the possibility of change in PLD transcript abundance in the early
stages of cold acclimation. A time-course seasonal study is currently underway to investigate transcript profiles of PLD and several other cold acclimation-responsive genes in *Rhododendron*.

In summary, the concomitant upregulation of several PL/PC biosynthesis and desaturation-related genes, as already noted, suggests that the fatty acid synthesis in plastids, and the PC biosynthesis and desaturation in ER are indeed important mechanistic components of *Rhododendron* cold acclimation process.

**Cold acclimation and membrane transport: aquaporin and gated outward rectifying K⁺ channel (GORK)**

Our analysis of *Rhododendron* ESTs from NA and CA leaf tissues resulted in the identification of 1 cDNA encoding a plasma membrane aquaporin only from NA ESTs (item 7, Table 2; GenBank accession no. CV015534). RNA gel blot analysis showed that this gene is indeed downregulated in CA tissues wherein its expression was only one-tenth of that in the NA tissues (Fig. 2a). In contrast, 1 cDNA identified as a GORK of guard cell membrane (item 8, Table 2; GenBank accession no. CV015196) was picked only from the CA EST library but not from the NA ESTs. Northern analysis indicated GORK expression in CA leaf tissues to be upregulated, fourfold as compared with NA samples (Fig. 2b). The question then arises: what might be the relevance of differential expression of these two genes to the cold acclimation process?

**Downregulation of aquaporin**

It has been well established that water transport along transmembrane water potential gradients is regulated by aquaporins (aquaporins) (Vera-Estrella et al. 2004), which include plasma membrane intrinsic proteins (PIPs) and tonoplast intrinsic proteins (TIPs); the PIPs are further divided into subfamilies PIP1 and PIP2 (Schaeffner 1998). Several reports have provided evidence for a direct role of aquaporins in plant water relations whereby overexpression of PIP transcripts (Aharon et al. 2003), gene silencing by antisense suppression (Kaldenhoff et al. 1998; Martre et al. 2002; Siefritz et al. 2002) or T-DNA insertion (Javot et al. 2003) resulted in changes in root hydraulic conductivity, transpiration rates and cellular osmotic water potential. Gao et al. (1999) have demonstrated an indirect role of *Brassica napus* aquaporin (BsPIP1) in improved seed germination under osmotic and low-temperature stress. Dehydration and rise in endogenous abscisic acid (ABA), both of which are known to induce cold acclimation (Guy 1990), have been shown to regulate PIP expression (Maurel et al. 2002).

During a frost episode, plant tissues typically freeze extracellularly as a result of a heterogeneous ice nucleation event (Levitt 1980). Water potential of ice is less than that of unfrozen water; consequently, cellular water moves down the gradient to extracellular spaces, resulting in cellular dehydration (Fig. 2a). Therefore, it is conceivable that the cold acclimation process, that is, induced freezing tolerance, should include mechanisms for resisting freeze-induced cellular dehydration, and the downregulation of aquaporins may be one component of this strategy. The observed downregulation of *Rhododendron* aquaporin PIP2-1 cDNA in CA leaves (Fig. 2a) is consistent with an *Arabidopsis* microarray study which showed downregulation of PIP2-2 in CA leaf tissues (Fowler & Thomashow 2002). A recent suggestion by Sakr et al. (2003) that walnut (*Juglans regia*) PIP2 of xylem-associated cells could be involved in refilling of embolized vessels resulting from freezing during winter supports their role in low-temperature stress in plants. In the current study, both NA and CA leaves were taken from fully expanded (developed), current year’s growth. Therefore, PIP2-1 downregulation in *Rhododendron* leaves was, presumably, not associated with reduced cell enlargement, which is often exhibited in tissues that actually develop at low temperatures.

**Upregulation of GORK**

Two classes of voltage-dependent K⁺ channels have been described in the plasma membrane of plant cells: hyperpolarization-activated inward-rectifying K⁺ channels and depolarization-activated outward-rectifying K⁺ channels that mediate K⁺ influx and efflux, respectively; *Rhododendron* GORK cDNA belongs to the latter type. Plants keep their water balance by adjusting the water conductance of their tissues; vascular tissues and guard cells play important roles in this process. GORKs are located in the guard cell membrane, where they are involved in stomatal closure (Hosy et al. 2003). Our data indicated that the expression of a *Rhododendron* GORK in CA leaf tissues was 3.9-fold of NA samples (Fig. 2b). Becker et al. (2003) reported upregulation of an *Arabidopsis* GORK ortholog in response to water-stress and ABA treatments. Previous studies have shown that ABA accumulation in plant tissues is triggered by dehydration or cold stress and regulates the expression of many stress-related genes during cold acclimation (Lang et al. 1994; Shinozaki & Yamaguchi-Shinozaki 2000). Since cold acclimation should include mechanisms for resisting cellular dehydration, we speculate that upregulation of *Rhododendron* GORK in CA tissues may be involved in how plants, particularly woody evergreens, regulate cellular hydration during cold and often desiccating winters. In a recent study, a gork-1 knockout *Arabidopsis* mutant increased transpirational water loss, highlighting the role of GORK in water conservation (Hosy et al. 2003).

**Cold acclimation and cytochrome P450 genes**

Cytochrome P450-dependent monoxygenases (P450s) are heme-containing, membrane-bound enzymes involved in an array of metabolic functions, for example, biosynthesis of diverse metabolites – phenylpropanoids, alkaloids, hormones (GA) and terpenoids – and the detoxification of
herbicides and pesticides (for a review, see Chapelle 1998). About 270 P450 genes have been identified in the Arabidopsis genome and categorized into 45 distinct P450 families (http://drnelson.utmem.edu/CytochromeP450.html), indicating the diversity and complexity of the P450 superfamily and the challenges for the analysis of P450 genes (Bhalerao et al. 2003).

So far, only a few attempts have been made to ascribe functions to P450 genes. For example, P450 genes have been found to respond to cold acclimation, drought and salinity in Arabidopsis leaf tissues, but their functional annotation has usually been vague: either as being involved in detoxification or related to secondary metabolism (Fowler & Thomashow 2002; Kreps et al. 2002). Because of the rapid development in the discovery of new P450 genes and their functions, a manual similarity search of putative P450 ESTs, combined with in-depth literature search, has become necessary to increase the accuracy of the annotation for P450 genes.

The current study identified a total of five Rhododendron P450 cDNAs: two in the NA EST data set only and three from CA ESTs only (items 9–13, Table 2). All those exclusively picked from the NA EST library were downregulated, whereas those from the CA EST library were upregulated during cold acclimation (Fig. 3). Their family categories and putative functions were determined based on their similarity with Arabidopsis orthologs. Among them, one P450 gene was identified as BAS1 (phyB activation tagged suppressor1) (item 9, Table 2; GenBank accession no. CV015464), and another as C3H, an ER-localized enzyme involved in the phenylpropanoid and lignification pathways (item 11, Table 2; GenBank accession no. CV014961). The other three Rhododendron P450 genes have no known function as yet (Table 2).

**Downregulation of BAS1 and avoidance of light-induced stress?**

Northern analysis revealed that BAS1 (CYP72B1) cDNA was downregulated in CA leaf tissues where its expression was 0.3-fold of that in NA samples (item 1, Fig. 3). It is worth noting that Arabidopsis seedlings with reduced BAS1 expression have lower sensitivity to light-induced stress (Neff et al. 1999). Rhododendron plants are often exposed to a combination of freezing temperatures and high light in their natural habitat as understory plants in deciduous forests. Recently we observed that the photosynthetic metabolism in CA Rhododendron leaves is downregulated (Wei et al. 2005). This could potentially result in the light energy harvested by the leaves to exceed what can be processed by photosystems, thus making these plants vulnerable to high light stress and photooxidative damage. We hypothesize that reduced BAS1 expression in CA leaf tissues, together with previously reported upregulation of ELIP in CA Rhododendron leaves (Wei et al. 2005), may be components of light stress-avoidance strategy, which is particularly important for woody evergreens; ELIP (early light-induced protein) are nuclear-encoded thylakoid mem-

**Upregulation of C3H and cell wall lignification**

Northern analysis revealed that C3H (CYP98A3) was upregulated in CA leaf tissues where its expression was 47-fold of that in NA samples (item 3, Fig. 3). Such a substantial increase in C3H expression indicates its potential importance in the cold acclimation process.

The composition of lignins and the amount produced are known to be regulated by developmental and environmental cues (Campbell & Sederoff 1996). Lignins are derived by oxidative polymerization of subunits known as monolignols. Three monolignols – p-coumaryl alcohol, coniferyl alcohol or sinapyl alcohol – give rise to three subunits: p-hydroxyphenyl (H), guaiacyl (G) or syringyl (S) (Boerjan, Ralph & Baucher 2003), which are incorporated into the lignin polymer.

The phenylpropanoid/lignin biosynthetic pathway involves a total of 34 monolignol biosynthesis genes in Arabidopsis (Raes et al. 2003). An abbreviated version of this pathway is illustrated in Fig. 5 wherein C3H catalyses the conversion of p-coumaroyl-shikimate or p-coumaroyl-quinate to chlorogenic acid (caffeoyl-shikimate or caffeoyl-quinate), a key enzymatic step of this biosynthetic pathway. Our observation of the upregulated C3H gene in Rhododendron CA leaf tissues is the first evidence, to our knowledge, suggesting transcriptional regulation of lignin biosynthesis during cold acclimation. In support of our observations, a recent study of cold-stressed Arabidopsis

![Figure 5. A simplified phenylpropanoid pathway showing hydroxylation of p-coumaroyl-shikimate or p-coumaroyl-quinate (Schoch et al. 2001; Franke et al. 2002) by coumarate 3-hydroxylase (C3H) as the sole enzyme. This eventually leads to the production of guaiacyl (G) and syringyl (S), two of the three subunits of lignin polymers. The solid arrows represent a one-step only reaction, while dashed arrows represent multiple-step reactions.](image-url)
leaf-metabolome indicated a general increase in the pool size of phenylpropanoid pathway intermediates (Kaplan et al. 2004).

Previous studies have observed pronounced thickening of cell walls in CA plants of various species (Chen et al. 1977; Huner et al. 1981; Griffith et al. 1985; Stefanowska et al. 1999), suggesting an increased lignin production during cold acclimation. An increase in insoluble precursors of lignin such as sinapinic and p-coumaric acids has also been observed in cold-exposed Arabidopsis leaves (Vaughan Hurry; personal communication). The Arabidopsis mutant ref8, defective in C3H gene, is reported to have reduced lignin content, particularly those predominantly composed of G and S subunits, in stem cell walls (Franke et al. 2002). It is conceivable that the enhanced expression of C3H gene may result in a higher proportion of G and S subunits of lignin polymers in CA tissues. The resultant change in lignin composition may alter cell wall rigidity and/or water permeability in CA Rhododendron leaf tissues and thereby have implications in winter adaptation. The Arabidopsis mutant ref8 may be a useful system for investigating the role of C3H gene in cold acclimation.

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