Research article

Effect of bud burst forcing on transcript expression of selected genes in needles of Norway spruce during autumn

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Abstract

Expression of selected genes in needles of Norway spruce (Picea abies [L.] Karst) was investigated by following their transcription levels during late autumn. Transcription was assessed in mature needles which likely serve as sensor of environmental cues that enable trees in the temperate and boreal regions to change between stages of growth, frost tolerance and bud dormancy. Samples were collected from grafts kept under outdoor conditions and after bud burst forcing in greenhouse at 20 °C/14 °C (12 h darkness) for one week. Transcription was assayed with real-time RT-PCR. During the sampling period, chilling requirement was partially fulfilled, and time to bud burst after forcing was decreased. Of the 27 transcripts studied, expression of 16 was significantly affected either by forcing, sampling time, or interaction between them.

PasAP, PasACP, PasSGS3, PasWRKY, PasDIR9, PasCCCH and dehydrin genes responded drastically to forcing temperatures at all sampling points, showing no correlation with readiness for bud burst. Expression patterns of some vernalization pathway gene homologs PasVIN3, and also of PasMDC, PasLOV1 and PasDAL3 had a clear opposite trends between forcing and outdoor conditions, which could imply their role in chilling accumulation and bud burst regulation/cold acclimation. These genes could constitute putative candidates for further detailed study, whose regulation in needles may be involved in preparation towards bud burst and chilling accumulation sensing.

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1. Introduction

In forest trees adapted to the temperate and boreal regions, growth is synchronized with the seasonal climatic cycle. Bud burst in the spring and growth cessation, bud set, and induction of endodormancy in the autumn are essential features of this adaptation. Timing of these processes is related to the risk of frost damage.

Effects of environmental stimuli like temperature, photoperiod, and light quality on bud burst have been widely studied [3,4,22,27,38,42]. Even though timing of vegetative bud burst is influenced principally by temperature, photoperiod and light quality may have modifying or subordinate roles. Bud dormancy is induced during late summer and early fall. During late fall and early winter the dormant buds are subjected to chilling, and when enough chilling has been met, the buds are ready to resume growth in response to warmer temperatures in the spring [32] even in short days [38 and references therein]. With increasing chilling period, time to bud burst after a transfer to higher temperature is gradually reduced [15,19,25]. In fact, temperature ranges for chilling and for heat sum accumulation are overlapping, making it difficult to separate between these two effects, and challenge our ability to experimentally study dormancy induction, cold acclimation, heat accumulation and deacclimation. Long photoperiod can also promote bud flushing, for example, in Betula pendula/Betula pubescens [15], and Picea abies [38]. But in these species the effect of daylength is limited, and, particularly in Picea, is nullified by sufficient exposure to chilling temperatures. Exposure to low temperatures during the autumn also results in enhanced cold acclimation, which can be reversed (deacclimation) by high temperatures [30].

Timing of bud burst is under moderate to strong genetic control [2,11]. However, molecular mechanisms underlying bud burst are still unclear. Recently, Yakovlev et al. [44] reported significant
differences in transcript levels between early and late flushing Norway spruce families. Similarly, several differentially expressed genes, such as DAG2 and Zwht211, which constitute relevant candidates for signaling pathway of bud burst in oak have been identified [7]. Differential expression of dehydrins during bud burst preparation was also reported [43]. Chromatin remodeling and epigenetic regulation have also been suggested to be involved in bud burst [16].

In this study we describe transcript expression profiles of 27 candidate genes in needles of Norway spruce grafts during autumn and under experimental conditions inducing bud burst. The studied genes were chosen from suppressive subtraction hybridization (SSH) libraries. In addition, some gene homologs described to be involved in vernalization pathway were also studied. The transcript profiling data was generated using real-time reverse transcription polymerase chain reaction (RT-PCR).

2. Materials and methods

2.1. Plant material, experimental and growth conditions

Needle samples were collected from grafts of 16- and 32-year old Norway spruce at the Punkaharju Research Station of the Finnish Forest Research Institute (61°48’ N 29°20’ E). The twigs for grafting had been collected in March 2006 from the field experiments near Punkaharju Research Unit. The 2-year old rootstocks had been grown from the seed produced in Hartola seed orchard (61°34’N, 26°05’E). The grafts were produced in a greenhouse of Punkaharju Research Unit in late April and early May 2006. They were grown in the greenhouse under average daily temperature of 17 °C (ranging from about 7 °C to about 25 °C) until October 10th, when they were moved outside to natural (chilling) conditions (Fig. 1). Later the grafts were transferred at three time points (Table 1) from outdoor to a greenhouse with 12-h photoperiod and constant temperature. The photoperiod was controlled using black curtains. The natural daylength was extended with metal halide lamps (Philips HPI-T Powertone 400 W). The curtains were off and the lamps on between 6 a.m. and 18 p.m. (standard time).

Needle samples were taken before transfer, and one week after forcing from different grafts because new shoots on the scions were short and restricted in number. During the second transfer the root balls were temporarily frozen and the seedlings were kept in dark for one week at about 4 °C. Accordingly, the second sampling after one week forcing was done on November 30th. Needles from three grafts (replicates) for each sampling date were harvested except the first harvesting where there were only two grafts per age available. In addition, at each time points, two grafts from both 16- and 32-year old Norway spruce were left in forcing conditions to observe bud burst (Table 1). Flushing of the apical bud in each graft was observed three times a week. A bud was considered to have burst when needles were visible.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted from 0.1 to 0.5 g of needles with RNAqueous-Midi™ kit (Ambion #1911, Austin, TX, USA) according to the manufacturer’s protocol. Remaining traces of DNA were removed with DNA-Free kit (Ambion #1906) according to the manufacturer’s protocol. RNA purity and quality were checked by agarose gel electrophoresis and quantified with spectrophotometric OD260.

2.3. Real-time RT-PCR

cDNA was synthesized from 300 ng of total RNA using the Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s procedure. After heat-deactivation of the enzyme, obtained ss cDNA was diluted twice and stored at ~80 °C.

Transcript levels of the studied genes (Table 2) were determined by quantitative real-time PCR and performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, #4351106) using the PCR cycling parameters: 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60°C for 1 min as recommended by the manufacturer. PCR reactions were set up in 25 µl volumes containing 2 µl of cDNA sample, 1 × SYBR Green PCR master mix...
were plotted in a three dimensional figure (Fig. 3).

To subject the relative amounts of transcripts to statistical analysis, we made the raw data from real-time PCR by obtaining the critical threshold cycle value ($dCt$) for the endogenous transcript of Actin (AT) and the corresponding threshold value for the transcript to be quantified ($TARGET$). The higher the transcription, the lower $dCt$ values. A subtraction variable $X = AT - TARGET$ was made for each sample and each target gene. To obtain a better presentation of up- and down-regulation of transcripts, the raw data $X$ was transformed. First, a mean value ($\Theta$) was calculated from all experimental observations of $X$ for a specific target gene. Then a new transformed variable was made by the formula $Yi = Xi - \Theta i$ for each sample and transcript. Note that $\Theta$ is a scalar specific for each target transcript, and $Y$ and $X$ represents variables (varying by samples) specific for each target gene. $Y$ is symmetric around its mean value, which is 0 for each target gene. Positive values represent higher and negative values lower transcription than average, and the unit is number of PCR cycles above or below zero (Fig. 2). The new variable was analyzed with the general linear model (GLM) procedure in SAS software (SAS Institute Inc., Cary, NC, USA version 9.1 for Windows) according to $Yij = \mu + Fi + Tj + Fij + ei j$, with $Fi =$ forcing effect (i = outdoor, indoor); $Tj =$ time effect ($j =$ October, November, December); $Fij =$ interaction between forcing and time effects; $eij =$ residual random error (that is, $F$, $T$, and $FT$ were regarded as fixed effects).

Principal Component Analysis (PCA) was preformed on normalized data set (i.e. all Yi) with PRINCOMP procedure in the SAS software to test for differences or patterns among treatments and similarities among observations within treatments. The purpose of a principal component analysis (PCA) is to derive a small number of linear combinations (principal components) of a set of variables that retain as much of the information in the original variables as possible. The input data comprised 32 observations (2 treatments, 3 sampling points, replicated twice for first sampling and thrice for second and third samplings) of each of the 24 transcripts included in the statistical analysis. The principal components were subjected to analyses of variation according to the model given above, and least square means from these analyses were plotted in a three dimensional figure (Fig. 3).

![Table 1](https://example.com/table1)

<table>
<thead>
<tr>
<th>Transfer</th>
<th>Age</th>
<th>Date of transfer from outdoor</th>
<th>Sampling date</th>
<th>Sampled grafts</th>
<th>Observed buds</th>
<th>Days to bud burst</th>
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<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>19.10.06</td>
<td>26.10.06</td>
<td>2</td>
<td>2</td>
<td>No bud burst</td>
</tr>
<tr>
<td>2</td>
<td>16.11.06</td>
<td>30.11.06</td>
<td>3</td>
<td>3</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>14.12.06</td>
<td>21.12.06</td>
<td>3</td>
<td>3</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>32</td>
<td>19.10.06</td>
<td>26.10.06</td>
<td>2</td>
<td>2</td>
<td>No bud burst</td>
</tr>
<tr>
<td>2</td>
<td>16.11.06</td>
<td>30.11.06</td>
<td>3</td>
<td>3</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>14.12.06</td>
<td>21.12.06</td>
<td>3</td>
<td>3</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

The original normalized data of each of the 24 genes were clustered using the FASTCLUS procedure in SAS. We used a MAXCLUSTER option equal to 6, thus giving a cluster number with same size as treatment combinations (outdoor, forcing combined with three time points). This procedure groups observations with similar transcript levels together across all variables (genes), without using any previous knowledge of treatments or time points in the input data. The method uses Euclidean distances, so the cluster centers are based on least-squares estimation. Each observation will then be assigned to a cluster in the output data, and the frequency of observations from a cluster could then a posteriori be assigned to preplanned experimental combinations (Fig. 4).

Finally, we used the VARCLUS procedure in SAS to group the most responsive genes according to their similarities in expression patterns, without using any information about preplanned treatments in the analysis. We started with one cluster, containing all the variables (genes), and the splitting into new clusters continued until all clusters have at most one eigenvalue greater than one. The results were presented in Fig. 2, together with the coefficients of determination to own and nearest clusters for 16 genes (Suppl. 1).

### 2.4. Data analysis

Data acquisition and analysis were done using the 7500-system SDS software for absolute quantification and MS Excel software. We were able to detect transcription from all the twenty-seven genes studied (Table 2), but for PaSEP, PaWD40 and PaTHAU the transcripts were present at the limit of detection and therefore not included in the statistical analyses.

### 2.5. Sequences deposition

The annotated P. abies EST sequences have been submitted to the GenBank with the following accession numbers EU332972–EU332991 (Table 2).

### 3. Results

#### 3.1. Bud burst

Although the material that was used to test days to bud burst in the greenhouse was very limited, there was a clear trend of advanced bud burst during the sampling period (Table 1). Forcing in October did not result in bud burst within 76 days. The number of days to bud burst were 50–56 and 19 days for plants chilled for 37 (November) and 65 (December) days, respectively. The age of the trees grafted had no effect on timing of bud burst.

#### 3.2. Gene expression patterns

We found no significant difference in gene expression based on age of the plants so the results from 16- to 32-year old plants were pooled for further analyses. We were able to detect reliable transcription levels for 24 of the 27 examined genes. For eight genes (PaHDA5, PaSETP4, PaSTL, PaKN1, PaRib, PaTAZ, PaUBE2 and PaZZ), the difference in transcript levels between treatments was not statistically significant. Expression levels of the remaining sixteen genes – PaA2/B1, PaDN1, PaDN6, PaDIR9, PaVIN3, PaVRN2, PaLOV1, PaCCH, PaMDC, PaACP, PaSA, PaSGS3, PaWRKY, PaMYB5, PaVRN1 and PaDAR1 – were statistically significant for one or more factors in the model ($p < 0.01$) and they were considered as differentially expressed (Fig. 2).
Table 2
Candidate genes analyzed by qRT-PCR.

<table>
<thead>
<tr>
<th>Assemble ID/ GenBank Acc. No.</th>
<th>Name</th>
<th>Best homolog description</th>
<th>Homolog locus</th>
<th>BLASTX score/ E-value</th>
<th>Forward/reverse primers used in qRT-PCR</th>
<th>Amplicon length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con_600/EU332972</td>
<td>PaHDAC</td>
<td>Histone deacetylase HDA101 [Zea mays]</td>
<td>AAK67142</td>
<td>268/5e–71</td>
<td>TGGTATGGATGGAGGAGGCTTTC/TCACACCCAAGCTTCGAA</td>
<td>115</td>
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<tr>
<td>Con_787/EU332975</td>
<td>PaST4</td>
<td>ST4 phosphate-associate protein [Oryza sativa]</td>
<td>AAT43301</td>
<td>200/2e–7</td>
<td>TGCCGCTCTCACCACCAAAT/TCGACAATCTCAGGAATCC</td>
<td>81</td>
</tr>
<tr>
<td>Con_204/EU332978</td>
<td>PaPII</td>
<td>MADS-box protein JOINTLESS (LeMADS) [Solanum lycopersicum]</td>
<td>Q9FLY16</td>
<td>181/2e–44</td>
<td>GCTCAGCCGAGGAGGCTACGACGAGGAGGCTACGTCGA</td>
<td>100</td>
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<tr>
<td>Con_754/EU332974</td>
<td>PaMYB5</td>
<td>KNOTTED1-like homeodomain protein 2 [Picea glauca]</td>
<td>ABD60292</td>
<td>95/1e–18</td>
<td>CTCGGGCTCCGACCCAGCTCTCGGAGGTCTCTCGGAC</td>
<td>95</td>
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<tr>
<td>Con_372/EU332973</td>
<td>PaROI1</td>
<td>Transducin family protein/WD-40-repeat family protein [Arabidopsis thaliana]</td>
<td>NP_178 186</td>
<td>56/1e–6</td>
<td>TGGCCTCTCTTACGCTGACGACGAGGAAATCAAAA</td>
<td>96</td>
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<tr>
<td>Con_342/EU332988</td>
<td>PaWD40</td>
<td>Heterogenous nuclear ribonucleoprotein A2/B1 like [Oryza sativa (japonica group)]</td>
<td>BAD61233</td>
<td>76/4e–13</td>
<td>TGGACCCCCGGAGAACGATCTCAAGGCGAGGAGGACATGAA</td>
<td>108</td>
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<tr>
<td>Con_267/EU332976</td>
<td>PaDIR9</td>
<td>Dirigent-like protein [Picea engelmannii x P. glauca]</td>
<td>ABD52120</td>
<td>209/2e–6</td>
<td>CGGCCGAGGAGGACGATCCGAGAGGACTCCATGCA</td>
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<tr>
<td>Con_165/EU332977</td>
<td>PaRIP</td>
<td>Putative ripening regulated protein [O. sativa (japonica group)]</td>
<td>BAB46507</td>
<td>266/5e–70</td>
<td>TCCAGGACGACGACGACTCCTGCTTAATGGTAAGGGC</td>
<td>87</td>
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<tr>
<td>Con_403/EU332987</td>
<td>PaTATZ</td>
<td>Zinc finger TAZ-type; BTB/POZ [Medicago truncatula]</td>
<td>ABF73424</td>
<td>266/5e–70</td>
<td>TGGCCTCTCCGAGCGGGATCCTCAGGAGGTCTCTCTGTA</td>
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<tr>
<td>Con_524/EU332986</td>
<td>PaWRKY Y2</td>
<td>WRKY42 [WRKY DNA-binding protein 42]; transcription factor [Arabidopsis thaliana]</td>
<td>NP_192 354</td>
<td>131/2e–29</td>
<td>GCGCAGGAGGGACGACTCCTGCTTAATGGTAAGGGC</td>
<td>103</td>
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<tr>
<td>Con_948/EU332982</td>
<td>PaSGS3</td>
<td>SGS3 [Suppressor of Gene Silencing 3] [Arabidopsis thaliana]</td>
<td>NP_197 747</td>
<td>86/5e–16</td>
<td>TGGACGGGCTGGAAAGGTTGACGAGGTTGGGGACATGCA</td>
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<tr>
<td>Con_260/EU332990</td>
<td>PaUBE2</td>
<td>Putative ubiquitin-conjugating enzyme E2 [Oryza sativa (japonica group)]</td>
<td>AAN74837</td>
<td>238/7e–62</td>
<td>TGGACCCCCAGACGACGACGACTTACCACTGCAAAAA</td>
<td>80</td>
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<tr>
<td>Con_355/EU332989</td>
<td>PaZZ</td>
<td>Zinc finger ZZ type, ubiquitin-associated [Oryza sativa (japonica group)]</td>
<td>NP_001053083</td>
<td>59/1e–07</td>
<td>TGGACCCCCAGACGACGACGACTTACCACTGCAAAAA</td>
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<tr>
<td>Con_184/EU332985</td>
<td>PaSEP</td>
<td>Secretory peroxidases, TPA: class III peroxidase 118 precursor [Oryza sativa (japonica group)]</td>
<td>CAH69360</td>
<td>154/1e–38</td>
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<td>PaSAP</td>
<td>Putative senescence-associated protein [Pisum sativum]</td>
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<td>266/5e–70</td>
<td>TGGACCCCCAGACGACGACTGCTTAATGGTAAGGGC</td>
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<td>Con_700/EU332983</td>
<td>PaTHAU</td>
<td>Thaumatin pathogenesis-related [Medicago truncatula]</td>
<td>ABE78554</td>
<td>110/2e–23</td>
<td>TGGACCCCCAGACGACGACTCCTGCTTAATGGTAAGGGC</td>
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<tr>
<td>Con_205/EU332980</td>
<td>PaCCCH</td>
<td>Putative CCCH-type zinc finger protein [A. thaliana]</td>
<td>AAN72125</td>
<td>58/2e–07</td>
<td>TGGACCCCCAGACGACGACTCCTGCTTAATGGTAAGGGC</td>
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<tr>
<td>Con_531/EU332991</td>
<td>PaMDC</td>
<td>Putative mitochondrial dicarboxylate carrier protein [Trifolium pratense]</td>
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<td>TGGAGGAGGGACGACTCCTGCTGCTGCTTGGT</td>
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<tr>
<td>Con_1071/EU332984</td>
<td>PaACP</td>
<td>Acid phosphatase [Arabidopsis thaliana]</td>
<td>NP_194 655</td>
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<td>CO480130</td>
<td>PaLOV1</td>
<td>Twin LOV protein [Arabidopsis thaliana]</td>
<td>BAB83700</td>
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<td>DR500811</td>
<td>PaVIN3</td>
<td>VIN3 (Vernalization Insensitive 3); zinc ion binding [Arabidopsis thaliana]</td>
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<td>87</td>
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<tr>
<td>CO239905</td>
<td>PaVRN1</td>
<td>VRN1 (Reduced Vernalization Response 1) [Arabidopsis thaliana]</td>
<td>NP_188 529</td>
<td>66/4e–10</td>
<td>TGGACCCCCAGACGACGACTCCTGCTTAATGGTAAGGGC</td>
<td>120</td>
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<tr>
<td>DR469772</td>
<td>PaVRN2</td>
<td>VRN2 (Reduced Vernalization Response 2); transcription factor [Arabidopsis thaliana]</td>
<td>NP_567 517</td>
<td>192/4e–48</td>
<td>TGGAGGAGGGACGACTCCTGCTGCTGCTTGGT</td>
<td>92</td>
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<tr>
<td>DR450774</td>
<td>PaDAL3</td>
<td>DAL3-like protein [Picea glauca]</td>
<td>CAJ55868</td>
<td>224/3e–47</td>
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<td>AY961924</td>
<td>PaDEH1</td>
<td>Putative dehydrogenase 1 (Picea abies)</td>
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<td>Putative dehydrogenase 6 (Picea abies)</td>
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<td>AY963918</td>
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<td>AAF03692</td>
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</table>

All primers (listed in 5’–3’ orientation) were designed with Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi#disclaimer) (Rozen, Skaletsky, 2000) and synthesized by OPERON Biotechnologies GmbH (http://www.operon.com/index.php).
Some general trends in the relative transcript patterns of these 16 genes could be observed. For the genes PaDHN1, PaDHN6, PaDIR9, PaCCCH, PaVRN2 and PaDAL3, expression was down-regulated by forcing, compared to outdoor samples. Transcript levels of PaSAP, PaACP, PaSGS3, were upregulated by forcing, particularly at the first sampling time in October, slowly decreasing towards December. For PaWRKY, PaMDC and PaVIN3 transcript levels were increasing in forcing conditions from October to December. The pattern of transcript expression of PaLOV1, PaMDC and PaVIN3 after forcing was the opposite of that under natural winter (outdoor) conditions.

In samples collected outdoors, there was a decreasing trend from October to December in transcript levels of a number of the studied genes (Fig. 2). Particularly, PaSAP, PaACP, PaSGS3, PaMDC, PaVIN3, PaLOV1, PaVRN1, PaVRN2 and PaDAL3 transcripts greatly decreased in December compared to earlier sampling times. PaWRKY was down-regulated in outdoor samples, compared to forced samples, but less so in December than in October or November. Only minor seasonal changes in expression of PaA2/B1 and PaMYB5 were observed.

Clustering of the 16 most responsive genes according to their similarities in expression patterns revealed 4 disjoint clusters. Clusters numbers were presented in Fig. 2. Cluster 1 includes 5 genes down-regulated after forcing and generally decreasing transcript levels towards December, cluster 2 includes 4 genes upregulated after forcing and decreasing transcript levels towards December, cluster 3 includes 5 having opposite trends for outdoor and forcing conditions and cluster 4 includes just two genes upregulated after forcing and increasing transcript levels towards December (Suppl. 1).

### 3.3. Multivariate analyses of transcripts

In order to study the underlying relationships between studied treatments, principal component analysis (PCA) for 24 studied genes was done. Three principal components explained 72% of the total original variation for all the 24 gene products, indicating strong correlation between groups of transcripts (Fig. 3). The first component distinguished mainly between the temporal difference in transcription, the second component between outdoor and forcing conditions, while the third the interaction between time and temperature treatments. The combined PCA and analyses of variation permitted complete separation of effects of outdoor and
forcing treatments (Fig. 3). Samples from forcing treatment were tightly grouped together reflecting a similarly induced change in expression of the genes, while the samples from outdoor showed larger distance and thus a larger difference in gene expression changes between the time points.

3.4. Clustering observations for similarity in transcription

Observations belonging to forcing treatment versus outdoor conditions fell into well separated clusters, three for each treatment (Fig. 4). Under forcing conditions, 75%–83% of the observations showed similar expression patterns (cluster 5) at all time points. This indicates a remarkable homogenous response at gene regulation level in the needles under bud burst forcing conditions.

In the outdoor samples there was a marked change in expression patterns from October to December. Cluster 2 comprised the majority of observations in October, but was surpassed by cluster 6 in December.

4. Discussion

This study describes transcript expression profiles of 27 candidate genes in needles of Norway spruce grafts during natural autumn (October–December) and under experimental conditions inducing bud burst (forcing). By studying genes with previously reported functions, we envisage to gain insight into how the inwintering process is regulated when transcription in needles is related to bud dormancy release in early winter, and rise in spring temperatures that results in loss of frost hardiness in both needles.
Candidate genes for the study were selected from suppressive subtraction hybridization (SSH) libraries constructed principally to study genes whose regulations precede events leading to bud set and bud burst [44]. In addition, some of the genes for the study were selected based on similarity to genes in the described vernalization pathway in Arabidopsis. The role of needles in the control of bud burst in Norway spruce is not fully known. However, they play important role in sensing and responding to environmental changes and may therefore be an appropriate tissues for such a study. Needles of Norway spruce have also been used in studies of the molecular mechanisms for growth rhythms [13].

The readiness for bud burst changed significantly during the experimental period (Table 1). Exposure to low temperatures (chilling) induces cold acclimation and significantly advances timing of bud burst and removed the long photoperiod requirement for “dormancy” release [38], so forcing efficiency for bud burst initiation was based on the amount of chilling the plants have got in outdoor conditions before transferring into forcing. It is an established tenet that perennial plants lose their frost hardness when growth start in the spring/early summer [21], and this is also the case for Norway spruce [4,8,34,35]. Forcing at high temperature in greenhouses also reduces hardness in needles stem and buds of Norway spruce when seedlings are taken directly out from cold store [18,39]. It is thus likely that the forcing in our study deacclimates the old needles and reduces their frost hardness, and the new succulent flushing shoots’ buds are very sensitive to frost [6]. Thus, the observed changes in expression of the sixteen genes in forcing could well be directly related to deacclimation. However, the change in gene expression could equally well be associated to the change in genetic programming that occurs when tissues are induced to grow actively, related to other processes than the gradual loss of hardness [44,45].

Although the exact functional roles of the studied genes in needles of Norway spruce remain unknown, effects of the applied treatments, amount of chilling and forcing, were clearly separated. Congregation of forcing treatment on one side and outdoor treatment on the other side, as revealed by the PCA (Fig. 3) and the clustering of observations (Fig. 4), reflect different responses of plants to contrasting temperature treatments. This pinpoints that an expression pattern of genes, specifically selected from subtractive libraries isolated from genotypes with contrasting timing of bud burst in the spring [44], could “fingerprint” responses which take place in needles during chilling/cold acclimation and forcing/deacclimation. For the forcing treatments, the relatively dense cluster from the PCA and similarity over time in transcription pattern in the observation clusters may reflect a common response of the plants to high temperature. In general, the effect of forcing treatment was strongest in December, when chilling requirement was almost satisfied and bud burst took place within 19 days of forcing. From the PCA, the widespread cluster for the outdoor-grown plants could reflect differences in the depth of hardness and/or accumulated chilling.

Transcription profiles for the 16 most responsive genes were subdivided into 4 disjoint clusters (Fig. 2). The default VARCLUS procedure divides a set of numeric variables into disjoint clusters, based on a linear combination of the variables in the cluster (the first principal component). VARCLUS tries to maximize the variance that is explained by the cluster components, summed over all the clusters. As shown, the first principal component distinguished mainly between the temporal differences in transcription. Therefore, certain clusters quite clearly distinguish genes by their transcript profiles at different time points and simplify their further analyses (Suppl. 1).

Temperature effect on gene expression was particularly clear for dehydrin genes. Kalberer and coauthors [20] stated that dehardening (deacclimation) may be a relatively low energy-intensive process and down-regulation of gene expression and biosynthesis dominates over up-regulation. Possibly, this is accounted for the down-regulation or decreased transcript levels of dehydrins (PaDHn1, PaDHn6), PaVRN2, PaLOV1, PaCCCH (putative CCCH-type zinc finger protein) and PaDIR9 (dirigent-like protein PDIR9) by high temperature during forcing. Dirigent proteins act as an initiation site for lignin polymerization, whereas some are involved in lignin formation for plant defense [29]. Down-regulation of dehydrins after forcing is consistent with findings made by Yakovlev et al. [45], who recorded a similar trend during bud burst in Norway spruce, and by Derory et al. [7], who observed drastic decline in transcript levels of Late Embryogenesis Abundant proteins during bud burst in Senoise oak. We suspect that dehydrins play a protective role during winter, and they could influence frost hardness in the tissues.

Five of the sixteen genes (PaSGS3, PaWRKY, PaCCCH, PaMYB5 and PaA2/B1) are involved in transcriptional regulation. Role of WRKY proteins in various physiological processes has been suggested, including embryogenesis, senescence, different abiotic stress responses, regulation of biosynthetic pathways and hormonal signaling [24,31,43,44]. WRKY71 was identified as one of the putative regulators of dormancy induction in poplar [33]. WRKY is also shown to be involved in starch biosynthesis [40]. Since WRKY genes play a variety of developmental and physiological roles in plants, strong up-regulation of PaWRKY under forcing conditions, increasing towards December, could be linked to several bud burst related processes enhanced by high temperature.

The almost stably down-regulated transcript level of PaCCCH after forcing at each time point indicates temperature-dependent regulation. Thus, CCCH proteins could participate in temperature sensing or act downstream in a temperature-regulated pathway in spruce. CCCH protein was implicated in conferring of vernalization requirement and is specifically required for the up-regulation of FLC expression in Arabidopsis [36].

Heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 is an abundant and important pre-mRNA-binding protein involved in mRNA subcellular localization, stability, translation, and in particular alternative splice site selection during pre-mRNA processing, not yet described in plants [9,10]. There are slight down-regulation of PaA2/B1 after forcing at each time point that partly correlates with amount of chilling days which plants have been subjected to prior to forcing.

PaSAP (putative senescence-associated protein), PaACP (acid phosphatase) and PaSGS3 (suppressor of gene silencing 3) all show similar expression patterns. Forcing led to strong up-regulation, especially in October when the plants had been under fewer days of outdoor chilling conditions. Transcript levels were, on the other hand, lower in outdoor conditions and levels continued to decrease with time. SGS3 is shown to fulfill both defense and developmental functions in Arabidopsis, regulate vegetative phase change and floral development [28] and also protects cleaved TAS gene transcripts which are required for proper leaf development against degradation [1]. Acid phosphatase participated in protein phosphorylation (counter-balancing the protein kinases), signal transduction during development (e.g. ABA [23]) and may be used by plants to scavenge phosphate from organic sources under phosphate-limited conditions, pathogen and salt stress, and water deficit [37]. Though we do not know the exact roles of PaSGS3 and PaACP, increased transcript levels for these genes after forcing could infer their participation in cold deacclimation, even if not related directly to bud burst processes. Putative senescence-associated protein homolog (PaSAP) shared high sequence similarity with ribosomal proteins from pea, rice and sunflower, whose function is believed to bind 5S RNA. It down-regulated during senescence of pea pods, so up-regulation during bud burst forcing could imply...
increasing synthesis of ribosomal protein genes in order to maintain the assembly of ribosome and its translational activity [26]. Transcript levels of PaVIN3 (Vernalization Insensitive 3), PaLOV1 (twin LOV protein 1), PaDAL3 (DAL3 protein), and PaMDC (mitochondrial dicarboxylate carrier protein) were related to amount of chilling naturally received outdoor, upregulated in October and reduced in December when buds have reached the readiness to flush provided favorable temperature conditions. The lower the transcript levels of these genes under outdoor conditions, the shorter the days to bud burst in forcing conditions. Forcing in December led to drastic increase of these genes transcript levels. This pattern of expression might well indicate involvement of these genes in the molecular mechanism(s) regulating bud burst in spruce.

PaMDC is involved in transport of dicarboxylates across the mitochondrial membrane that connects the Krebs cycle with diverse metabolic processes [14], and the enhanced expression of PaMDC by forcing in December might be related to increased metabolic activity. PaLOV1 contains twin LOV domains with significant homology to domains in a number of proteins that are involved in sensing light oxygen and voltage [17]. All LOV domains containing proteins that have been investigated to date act as photoreceptors [5]. Considering the nearly opposite expression pattern of PaLOV1 after forcing compared to outdoors, we may speculate that PaLOV1 does not participate only in light sensing but could also be involved in detecting accumulated days of low temperatures (through any voltage accumulating mechanisms). This should be considered in further studies. Accumulating of low temperatures in Norway spruce needles did not lead to increase of PaVIN3 transcript level as has been reported for vernalization temperatures in Norway spruce needles did not lead to increase of PaVIN3 transcript level as has been reported for vernalization treatment. Forcing in December might be related to increased metabolic processes [14], and the enhanced expression of PaLOV1 (mitochondrial dicarboxylate carrier protein) were related to chilling and forcing treatment. These genes could constitute putative candidates whose regulation in needles may be involved in preparation towards bud burst and chilling accumulation sensing. Interestingly, several vernalization pathway gene homologs had strong relation with the bud burst ability of Norway spruce plants, but more detailed studies of both buds and needles during the onset and release of bud dormancy are needed to get a better understanding of processes underlying bud burst in Norway spruce.

Acknowledgements

Financial support from The Norwegian Research Council (project 155873 and 1565041/140) is acknowledged. We thank Heikki Hänninen and Risto Hakkinen for their contributions in planning of the graft production in Finland. We are also grateful to Olavi Junttila for his valuable comments on this manuscript.

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plaphy.2009.03.004.


