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Publisher's version / Version de l'éditeur:
https://doi.org/10.1111/j.1365-3040.2010.02257.x
Plant, Cell and Environment, 34, 3, pp. 480-500, 2011-01-07

Molecular events of apical bud formation in white spruce, *Picea glauca*

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Running title: Bud formation in white spruce

ABSTRACT

Bud formation is an adaptive trait that temperate forest trees have acquired to facilitate seasonal synchronization. We have characterized transcriptome-level changes that occur during bud formation of white spruce (*Picea glauca* [Moench] Voss.), a primarily determinate species in which preformed stem units contained within the apical bud constitute most of next season's growth. Microarray analysis identified 4460 differentially expressed sequences in shoot tips during short day-induced bud formation. Cluster analysis revealed distinct temporal patterns of expression, and functional classification of genes in these clusters implied molecular processes that coincide with anatomical changes occurring in the developing bud. Comparing expression profiles in developing buds under long day and short day conditions identified possible photoperiod-responsive genes that may not be essential for bud development. Several genes putatively associated with hormone signalling were identified, and hormone quantification revealed distinct profiles for ABA, cytokinins, auxin and their metabolites that can be related to morphological changes to the bud. Comparison of gene expression profiles during bud formation in different tissues revealed 108 genes that are differentially expressed only in developing buds and show greater transcript abundance in developing buds than other tissues. These findings provide a temporal roadmap of bud formation in white spruce.

Key words: White spruce, bud set, short-days, microarrays, expression analysis, growth cessation and hormone profiling

INTRODUCTION

Woody perennial species of northern latitudes have acquired a suite of adaptations that enable them to survive winter and resume growth the following spring. Development of an apical bud represents one of these seasonal adaptations. Apical bud development is a complex physiological and developmental phenomenon that comprises bud formation, cold and desiccation tolerance acquisition, and dormancy acquisition (Ruttink *et al*. 2007). Timing of these processes affects fitness: late bud development can render the tree susceptible to winter injury, while early bud development results in untimely cessation of seasonal growth and reduces overall tree productivity (Howe *et al*. 2003). Thus, a trade-off exists between growth and time of bud development for temperate zone woody perennials such as forest trees. Genetic variation has been reported in timing of bud set for a number of forest tree species (e.g. Jaramillo-Correa J.P., Beaulieu J. & Bousquet J. 2001; Frewen *et al*. 2000; Hall *et al*. 2007; Ingvarsson *et al*. 2008), which in some cases is correlated with latitudinal gradients (Hall *et al*. 2007). As such, physiological and developmental processes encompassed by bud development represent adaptive traits in temperate zone forest tree species, with successful individuals exhibiting seasonal growth patterns synchronized with their local environment.

The terminal bud of overwintering forest tree species contains preformed primordia and internodes – collectively referred to as stem units – which are initiated during bud formation. The following spring, these preformed stem units develop into the terminal shoot. Trees with an indeterminate seasonal pattern of growth can also initiate neoformed stem units during the growing season. For determinate species, these preformed stem units constitutes most or all of a season's terminal shoot growth (Kozlowski & Pallardy 1997).

Induction of bud formation and concomitant growth cessation are induced by environmental cues related to the passage into autumn, such as critical photoperiod (short days, SD) and low temperature (LT). Exposure to at least one of these environmental cues is required to initiate apical bud formation in indeterminate species. Unlike indeterminate species, SD or LT are not essential for the initiation of bud formation in determinate species. In these species, bud formation is initiated at the shoot apical meristem once elaboration of all preformed stem units is underway. Although not essential, SD and LT are sufficient to induce rapid and synchronous bud formation in determinate species, and SD or LT appear to be necessary to complete normal bud development, including passage into dormancy (Owens & Molder 1977; Heide & Prestrud 2005; Lagercrantz 2009).

Several molecular factors have been implicated in regulating bud formation. Photoperiod-induced bud formation has been shown to involve phytochrome A in the indeterminate species *Populus tremula* x *tremuloides* (Olsen, Junttila & Moritz 1997a; Olsen *et al*. 2004). *Populus* homologues of *Arabidopsis* genes that control photoperiod-induced flowering are also involved in growth cessation and bud formation (Böhlenius *et al*. 2006; Gyllenstrand *et al*. 2007; Ruonala *et al*. 2008), implying that there is overlap in molecular mechanisms that control these two developmental processes. Hormones and metabolites such as sugar are associated with regulation of both dormancy acquisition and cell cycle progression, the latter being an important factor in cessation of cell division (Anderson, Chao & Horvath 2001; Horvath *et al*. 2003; del Pozo *et al*. 2005).

Large-scale gene expression profiling in *Populus tremula* x *alba* has shed new light on processes associated with bud formation (Ruttink *et al*. 2007). Gene expression profiling has also been carried out for shoots and roots of Scots pine (*Pinus sylvestris*) seedlings (Joosen *et al*. 2006), and for foliage of Sitka spruce (*Picea sitchensis*) (Holliday *et al*. 2008) to identify genes

1 that are differentially expressed during cold acclimation. To date, however, no broad-scale
2 examination of gene expression profiles has been conducted for bud formation in a forest tree
3 with a predominantly determinate growth habit.

4 White spruce (*Picea glauca* [Moench] Voss) exhibits a primarily determinate growth
5 pattern, particularly as the tree matures (Nienstaedt 1966). Timing of bud set in white spruce
6 shows genetic variation (Jaramillo-Correa *et al*. 2001); accordingly, our long term goal is to
7 identify genes that exert genetic control over this adaptive trait. Surprisingly little is known
8 about gene expression associated with bud formation in white spruce. The objective of the
9 present study was to use microarray gene expression profiling to develop a comprehensive
10 picture of the transcriptional response that occurs in young white spruce shoot tips during the
11 transition from active growth to fully formed bud, focusing on early stages of bud formation.
12 Temporal expression patterns of functionally-related groups of genes were used to infer
13 molecular and cellular processes that are invoked during bud formation, and correlated with
14 anatomical changes that occur at the shoot apex. Genes putatively involved in hormone
15 biosynthesis and action figured prominently in the transcriptome profiles, and given that very
16 little information was available on hormone levels in developing buds of conifers, we carried out
17 quantitative hormone profiling. Transcript profiles of developing buds were compared to those
18 of leaves and stems to identify genes that are both differentially expressed specifically in buds
19 and expressed at higher levels in developing buds compared to other tissues. Gene expression
20 profiles in developing buds under SD and LD conditions were also compared to identify genes
21 that may be responding to changing photoperiod rather than participating in bud development *per*
22 *se*.
23

MATERIALS AND METHODS

Plant material and experimental design

Two-year-old white spruce derived from Quebec (Canada) provenances were used for all experiments. . Dormant one-year-old seedlings overwintered outdoors at the Valcartier Experimental Station (Quebec) were shipped via courier to Edmonton AB, where they were repotted and grown using a complete randomized block design in controlled-environment chambers at 20°C under long days (LD; 16 h days / 8 h nights) for 6 to 8 weeks. Plants were transferred to SD (8 hour days / 16 h nights) near the end of the active elongation phase, – i.e prior to initiation of apical buds as confirmed by inspection of shoot apices of a random sample of trees under a dissecting microscope – and harvested at 1 d, 3 d, 7 d, 14 d, 28 d and 70 d SD. Remaining plants were kept in SD for an additional 8-15 weeks, then transferred to LT (2 - 4°C) for 3 to 4 weeks with continuing SD prior to harvest. This final time point is referred to as LT. At each time point, shoot tips (buds), current season foliage and current season stems with well-formed secondary growth were harvested, immediately frozen in liquid nitrogen, and stored at –80°C. Four independently replicated experiments, each with multiple plants per time point, were conducted. A separate LD/SD comparison experiment was also conducted in which one group of trees was placed in SD while the second group remained in LD. Plants were harvested at the same time points as described above, minus LT.

RNA extractions, sample labelling and microarray hybridizations

Total RNA was extracted according to Pavy *et al*. (2008), and assessed with a NanoDrop 1000 (Thermo Scientific, ON, Canada) and 2100 Bioanalyzer (Agilent, ON, Canada). Four microarray experiments were conducted with an 11K white spruce low-redundancy cDNA microarray

described in Pavy *et al*. (2008): (1) a SD time course in which shoot tips from each time point were co-hybridized with shoot tips from day 0; (2) the same experiment with shoot tips from LD-treated trees; (3) a SD time course in which stems and needles from 7d, 14d, 70 d SD and LT were co-hybridized with the corresponding day 0 sample, (4) co-hybridization of SD shoot tips, needles, and secondary stems with each of the other tissues at 14d and 70 d SD (Supporting Information Fig. S1). In each case, hybridizations with four biological replicates were carried out, with two biological replicates representing dye swaps. Two micrograms of total RNA was amplified using an amino allyl antisense RNA (aRNA) procedure (Superscript Indirect RNA Amplification System, Invitrogen, Carlsbad, CA, USA). Five micrograms of aRNA was directly labeled using Alexa Fluor® 555 or 647 dyes (Invitrogen). Coupling efficiency was evaluated using the NanoDrop 1000. Hybridization and wash procedures are described in Pavy *et al*. (2008).

Microarray data extraction and analysis

Microarray images were obtained using a GenePix 4000B microarray scanner and analyzed with Genepix Pro 6.0 (Axon Instruments, Sunnyvale, CA, U.S.A.). Adaptive circles were used to select spots. After manual elimination of low quality spots, median background intensity was subtracted from mean spot intensity applying the local method. Data analyses were performed essentially as described in Lawrence *et al*. (2008) using the linear models for microarray data (LIMMA) (Smyth 2005) and exploratory analysis for two-color spotted microarray data (marray) (Yang & Paquet 2005) packages from BioConductor (Gentleman *et al*. 2004) in R (Ihaka & Gentleman 1996). Within-array data normalization was applied using print-tip loess. Data were then scaled to have the same median-absolute-deviation across arrays. Quality inspections of the raw and background-corrected data included minus-add (MA) plots, pairwise correlations of ratio

(M) values between slides, distribution and density plots of intensity (A) values, and box plots of M values. Linear models were fit to the normalized data using duplicate correlation for each sequence. Nonspecific filtering was then applied to reduce false discovery rate by removing invalid and low intensity sequences. An empirical Bayes statistic was applied to obtain *p*-values, which were adjusted using the Benjamini-Hockberg procedure (Benjamini & Hochberg 1995). An adjusted *p*-value cut-off of 0.01 was used to obtain statistically significant differentially expressed (DE) sequences. This list was further filtered to include only sequences exhibiting fold changes greater than 1.5 or smaller than 0.67. DE sequences were clustered using K-means (Soukas *et al*. 2000) in MeV (v4.2) (Saeed *et al*. 2003).

Sequences included on the array and their annotations are described by Pavy *et al.* (2005) and Pavy *et al*. (2008). Additionally, sequences were functionally classified according to Gene Ontology (http://www.geneontology.org/) and MIPS FunCat (http://mips.helmholtz-muenchen.de/projects/funcat). The latter were assigned using *Arabidopsis thaliana* classifications as a guide. Putative transcription factors were further classified using PlantTFDB (Guo *et al*. 2008) and PlnTFDB (Pérez-Rodriguez *et al*. 2010).

Quantitative RT-PCR

Quantitative reverse transcription PCR (qRT-PCR) was conducted for 20 DE genes chosen to represent a variety of biological functions and expression levels (Supporting Information Table S1). Gene-specific primers were designed using Primer Express (v3.0, Applied Biosystems, ON, Canada) (Supporting Information Table S1). Two micrograms of total RNA was treated with DNaseI (Invitrogen) prior to cDNA synthesis using Superscript II reverse transcriptase (Invitrogen). PCR reactions were performed in 10 µl, containing SYBR Green master mix (0.2 mM dNTPs, 0.3 U Platinum Taq Polymerase (Invitrogen), 0.25X SYBR Green, and 0.1X ROX),

20 ng of cDNA and 300 nM of each primer. Four biological and three technical replicates for each reaction was analyzed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) with a first step of 95 ºC for 2 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting curves were generated using the following program: 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s and 15 s at 60 °C. Transcript abundance was quantified using standard curves for both target and reference genes, which were generated from serial dilutions of PCR products from corresponding cDNAs. Translation initiation factor 5A (TIF5A, GenBank DR448953) was used as a reference gene, since expression of this gene did not show significant differences over the time course ($p=0.483$).

Light microscopy

Shoot-tips from plants selected at random from each independent experiment at each time point were fixed with 2% (v/v) glutaraldehyde and 1% (w/v) caffeine in 100 mM sodium phosphate buffer (pH 7.2). After successive ethanol dehydration, and infiltration the shoot-tips were embedded in JB-4 Plus® (Polysciences,Hatfield, PA, USA). Four µm sections were cut with glass knives and stained with Richardson's stain (Richardson K. C., Jarrett L. & Finke E. H. Richardson 1960).

Hormone quantification by HPLC-ESI-MS/MS

Plant hormones including abscisic acid (ABA) and its catabolites (abscisic acid glucose ester (ABA-GE), phaseic acid (PA), dihydrophaseic acid, 7' hydroxy-abscisic acid (7'OH-ABA), *neo*-phaseic acid), cytokinins (*cis*- and *trans*-zeatin-*O*-glucoside, *cis*- and *trans*-zeatin, *cis*- and trans-zeatin riboside (tZR), dihydrozeatin riboside (dhZR), isopentenyl adenosine (iPA) and isopentenyl adenine), auxins (indole-3-acetic acid (IAA), (N-indole-3-yl-acetyl)-aspartic acid

(IAA-Asp), (N-indole-3-yl-acetyl)-glutamic acid, N-(Indole-3-yl-acetyl)-alanine and N-(Indole-3-yl-acetyl)-leucine), and gibberellins (GA1, 3, 4, 7, 8, 19, 20, 24, 29 and 44) were quantified using the procedure for quantification of multiple hormones and metabolites that has been described in detail by Chiwocha *et al*. (2003, 2005). Following the tissue extraction and purification steps as described in Kong *et al.* (2008), samples were injected onto a Genesis C18 HPLC column (100 x 2.1 mm, 4 μm, Chromatographic Specialties, Brockville, ON, Canada) and separated by a gradient elution of water against an increasing percentage of acetonitrile that contained 0.04 % acetic acid. Calibration curves were generated from the MRM signals obtained from standard solutions based on the ratio of the chromatographic peak area for each analyte to that of the corresponding internal standard, as described by Ross *et al*. (2004). The quality control samples and internal standard and solvent negative controls were also prepared and analyzed along with each batch of tissue samples.

RESULTS

Visualizing bud formation in white spruce under SD

Although SD is not necessary to initiate bud formation in white spruce, it is sufficient, as SD brings about rapid and synchronous bud set relative to LD (Bigras & D'Aoust 1992). To minimize sample-to-sample variability which might have otherwise compromised the power to detect DE genes in our microarray analyses, we elected to carry out the majority of these analyses under SD conditions. A 10 week SD time course was selected based on preliminary investigations that showed few changes in bud structure after this point (data not shown). An additional time point in which trees exposed to SD for an extended period were then transferred to LT for 3 to 4 weeks was included to provide an endpoint to bud development. Dormancy

(failure to reinitiate growth after several weeks in permissive environmental conditions) was attained between the 10 wk SD and LT time points.

Microscopy analyses demonstrated that subtle anatomical changes to the shoot tip could be observed as early as 7 d SD, even though the first signs of bud formation were only visible to the eye with careful observation after 14 d SD (Fig. 1A). Developing bud scales were clearly visible by microscopy at 14 d. Morphological changes to the developing bud were quantified by measuring the height and diameter of the bud (excluding bud scales) and the apical dome, as well as the number of tiers of primordia that were visible in median longitudinal section (Fig. 1B). These quantitative measurements confirmed that changes to the shoot tip were detectable after 7 d SD, although these changes were not obvious until 14 d. Relative to day 0, the apical dome was larger at 14 d and 28 d, and smaller by 70 d. This difference in size appeared to be a function mainly of an expanded peripheral and rib zone. The number of primordia tiers corresponded well with overall bud height, and increased from 14 d until LT exposure (Fig. 2). These data indicate that stem unit initiation occurred primarily between 14 d and 70 d. By comparison, the apical dome enlarged at 14 d and 28 d and then decreased by 70 d, suggesting that the mid point of the time course was the period of maximum shoot apical meristem activity. The crown, or layer of cells with thickened cell wall, is clearly visible by 70 days.

Cluster analysis of genes DE during SD-induced bud formation

SD provoked large-scale changes in gene expression in developing buds over the 70 d time course. Further changes were observed after transfer of SD-treated plants to LT. Of the 10400 elements on the microarray (Pavy *et al*. 2008), we identified 4460 elements, putatively corresponding to genes, that were significantly DE (adjusted p value $\leq$ 0.01, fold change

threshold greater than 1.5 or less than 0.67) for at least one time point relative to day 0. The microarray data have been deposited in NCBI's Gene Expression Omnibus (Edgar, Domrachev & Lash 2002) and are accessible through GEO Series accession number GSE23519 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE23519). Twenty DE genes representing several expression patterns and functional categories were used in qRT-PCR for microarray transcript profile validation (Supporting Data Table S1, Fig. S6). There is excellent overall agreement in expression profiles between the microarray and qRT-PCR data for each gene, although in some cases the magnitude of differential expression (fold change) is greater at certain time points with one technique versus the other.

K-means clustering of the 4460 DE genes identified 10 groups of genes with distinct expression patterns that were classified according to functional categories (Fig. 3; Supporting Information Table S2), and can be related back to anatomical changes in the shoot apex (Figs. 1 and 2). Clusters (a), (d), (f) and (i) contain genes that were down-regulated relative to day 0 (Fig. 3I), and included genes involved in lipid metabolism; cell wall organization and biogenesis, especially cell wall carbohydrate polymer synthesis and modification; stress responses; cell cycle and DNA processing, including chromatin modification; secondary metabolism, including phenylpropanoid and isoprenoid pathways; protein synthesis and protein fate, including many ribosomal proteins in Cluster (f) and proteolytic machinery in Cluster (i); and cellular communication and signal transduction, including several developmental regulators. Cluster (i) additionally contained genes associated with cytoskeleton organization, photosynthesis, and cellular transport.

Clusters (c), (g) and (h) contained genes that were up-regulated during bud formation. Cluster (c) contained several stress response genes. Cluster (g) included DE genes associated with stress response; protein synthesis and protein fate; secondary metabolism, particularly lignin

biosynthesis; and cellular communication and signal transduction. Cluster (h) included genes associated with hormone action, redox, carbohydrate metabolism, stress responses, transport, protein fate, and cellular communication and signal transduction.

Clusters (b), (e) and (j) contained genes whose transcript abundance relative to day 0 increased during the mid stages of the SD time course, then returned to levels at or below day 0 levels by the end of bud development. These clusters contained a number of cell wall associated genes. Cluster (j) additionally contained genes associated with protein fate, protein synthesis and protein stability; photosynthesis; and cellular communication and signal transduction (50).

Examination of functional categories across clusters provided additional insight on coordinate expression patterns. For example, DE starch breakdown genes were represented mainly in clusters (g) and (h). DE starch biosynthesis genes (starch synthase, ADP-glucose pyrophosphorylase large subunit, starch branching enzyme) appeared primarily in cluster (h). Sucrose biosynthesis genes (e.g. sucrose phosphate synthase) appeared in Clusters (c) and (h). Invertases, involved in sucrose breakdown, were in Cluster (i), whereas sucrose synthase was in Cluster (g). Sixty-five of 87 lipid metabolism genes were downregulated (Clusters (d), (e), (f), and (i)).

Other functional categories were well represented in multiple clusters. Photosynthesis associated genes were prominent in Clusters (i) and (j) (Supplemental Information Fig. S2). A total of 187 DE cell wall associated genes were identified across most clusters (Supplemental Information Fig. S3). Several cell wall genes (65) were upregulated during the transitional phase from active growth to bud formation, while most cell wall genes (122) showed downregulation by the completion of bud development.

Putative regulatory genes DE in shoot tips during bud formation

Annotation of the differentially expressed gene list revealed 391 genes encoding proteins with putative regulatory function, including protein kinases (79), protein phosphatases (16), receptor-like kinases (41), F-box proteins (9), NBS-LRR (38), 14-3-3 proteins (3), photoreceptors (2), and transcription factors (TFs, 113) (Supporting Information Table S3). The 113 putative TFs represent 27 TF families as classified in PlantTFDB (Guo *et al*. 2008) and PlnTFDB (Perez-Rodriguez *et al*. 2010) and show diverse expression profiles (Supporting Information Fig. S4).

Several DE genes exhibited sequence similarity to regulators from other species implicated in dessication or cold tolerance, others were potentially associated with photoreception, regulation of light responses, regulation of photoperiod responses, and the circadian clock. Many other DE genes exhibit sequence similarity to genes from other species that play roles in developmental processes such as meristem maintenance, development of new organs, and flowering (Supporting Information Fig. S5). Of the regulators putatively associated with developmental processes, fourteen encoded HB-homeodomain proteins, including all three *KNOX* genes represented on the array (*KNOX1*, *KNOX3*, and *KNOX4*). Array and qRT-PCR data indicated that these three *KNOX* genes showed concerted upregulation during the mid and latter stages of bud formation, coinciding with primordia initiation (Fig. 4A, C, D). qRT-PCR expression profiling for *KNOX2*, the fourth member of the white spruce *KNOX* gene family that was not represented on the array, showed a similar pattern (Fig. 4B). qRT-PCR was also used to verify expression profiles for seven additional genes encoding putative developmental regulators (Fig. 4 E to K). Some, like MOTHER OF FT AND TF1 (Fig. 4I) show sequence similarity to genes implicated in regulating time of flowering. Other such genes include *FCA* (upregulated in SD during LT), *PHYTOCHROME AND FLOWERING TIME 1* (upregulated in SD during LT) and maize *INDETERMINATE1* (downregulated in SD at 14 and 28 d), as well as the vernalization (cold promotion of flowering) associated *VERNALIZATION INDEPENDENCE 4*

(upregulated in SD at 70 d SD and LT; Colasanti *et al*. 2006; Hedman, Källman & Lagercrantz 2009; Kim *et al*. 2009).

Hormone levels and DE genes potentially associated with hormone biosynthesis and action

The microarray dataset identified 92 DE genes potentially associated with biosynthesis, catabolism, perception and signaling processes of hormones (Supporting Information Fig. S7), including auxins (IAA), gibberellins (GAs), brassinosteroids (BRs), ethylene, jasmonates (JAs), abscisic acid (ABA) and cytokinins (CKs). Expression patterns for ten of these DE genes were confirmed by qRT-PCR (Fig. 5). We also quantified levels of ABA, IAA, GAs, CKs and some of their metabolites to compare to these patterns of gene expression (Fig. 6).

Among 18 DE genes putatively involved in auxin action, seven showed upregulation shortly after transfer to SD conditions, including genes coding for a PIN like protein, auxin-repressed protein-like protein ARP1, auxin efflux carrier, and auxin response factor (Fig.S7). qRT-PCR expression profiling of a putative auxin efflux carrier and a putative *TIR1* auxin receptor demonstrated that both genes show a peak of expression at 7d SD - coinciding with the first discernable morphological changes associated with bud set - and decline thereafter (Fig. 5). In contrast, expression of one auxin transporter-like protein declined only after exposure to low temperature. IAA levels showed a transient decrease at the mid point of SD time course (Fig. 6). IAA-Asp, IAA-Leu and IAA-Glu were generally below detectable limits, while IAA-Asp was detected in LT buds (Fig. 6).

Four genes associated with GA action were DE (Fig. S7). Expression patterns for genes encoding an F-box protein *GID2,* probable gibberellin receptor *GID1L3*, and a DELLA protein were confirmed with qRT-PCR (Fig. 5). GAs were below detectable limits in all but a few

samples, where GA_{24} was detected. In these samples, GA_{24} decreased from 178 ng g^{-1} DW at 0 day to 144 ng g^{-1} DW after 28 d (data not shown).

A small number of genes with similarities to ABA biosynthetic or signal transduction genes were differentially expressed during bud formation, including *ABI1*- and *HOMOLOGY TO ABI1*-like protein phosphatase 2Cs (Fig. S7). ABA levels showed inverse patterns with the inactive forms such as ABA-GE and 7'-OH-ABA: while ABA concentrations were lowest at 28 d, concentrations of ABA-GE were greatest at 7, 14, and 28 d, and concentrations of 7'-OH-ABA were greatest at 14 d. Phaseic acid was present in all samples at low concentrations, with lower quantities detected at the latter stages of bud formation (Fig. 6).

Only a few genes potentially associated with CK biosynthesis and metabolism were identified as DE. A putative isopentenyltransferase, response regulator 9, type-A response regulator and a zeatin O-xylosyltransferase were up-regulated at early or mid stages of bud formation (Fig. S7)**.** The CKs t-ZR ((*trans*) zeatin riboside), dhZR (dihydrozeatin riboside) and iPA (isopentenyladenosine) all showed increased concentrations at 14 d, followed by a decline. t-ZR also had elevated levels at 28 d before returning to concentrations similar to those at the beginning of the time course. iPA increased following transfer of plants to LT (Fig. 6).

Eighteen genes putatively involved in ethylene biosynthesis and perception were identified. All but two of these showed upregulation relative to day 0 during bud formation, with several showing significant upregulation in buds following transfer of the plants to LT (Fig. S7), e.g. 1-aminocyclopropane-1-carboxylate synthase (Fig. 5). A putative EIN3-binding F-box protein exhibited downregulation through the mid stages of bud formation (Fig. 5).

BR biosynthetic genes were downregulated by 70 d or following transfer to LT. Transcript abundance of castasterone 26-hydroxylase increased relative to day 0 until 14 d, then decreased and was non detectable in LT buds.

Several DE genes corresponding to JA biosynthetic enzymes were identified, including lipoxygenase, allene oxide synthase and allene oxide cyclase. Most of these genes were downregulated during the later stages of bud formation (Figs. 5 and S7). In contrast, two genes encoding putative coronatine-insensitive 1 (COI1) proteins showed dramatic upregulation following transfer to LT (Fig. 5).

Comparison of gene expression profiles during SD and LD bud development

White spruce can initiate terminal bud formation even in the absence of environmental cues such as SD or LT. However, the timing of bud set under LD is not synchronous, and a proportion of these individuals will undergo a second flush from these buds without first proceeding into dormancy (N. Isabel, personal communication), suggesting that SD are required to complete bud formation. Although we used SD to induce rapid and synchronous bud formation for the above-described microarray analysis, the ability of white spruce to form buds under LD (although perhaps not complete bud set) afforded an excellent opportunity to compare gene expression in developing buds under SD and LD conditions. Genes DE under both conditions are more likely to be essential for bud formation, while genes only DE under SD - particularly early in the time course - might be responding solely to changing photoperiod, and might not be obligatory for bud development.

An experiment was carried out with LD developing buds using the same experimental design as the SD experiment described above, but lacking the LT treatment (Supporting Information Fig. S1). This experiment identified 2447 genes DE in developing buds under LD (Supporting Information Table S4). We then compared the DE gene list from this LD experiment to that of the SD experiment, since asynchronous bud initiation under LD prevented meaningful comparison by direct hybridization of LD samples with the corresponding SD samples. To

accommodate this asynchrony of bud initiation under LD, data from 1 d, 3 d, and 7 d were combined for each of the SD and LD experiments and denoted "early stage", while data from 14 d and 28 d were combined and denoted "mid stage". Data from 70 d were called "late stage". A gene was considered DE at a particular stage if the transcript abundance value was significantly different from 0 d on any day included in that stage. A gene was identified as DE in multiple stages if transcript abundance was significantly different from 0 d at time points falling into more than one stage. The analyses revealed 253, 1035 and 841 DE genes shared between LD and SD at early, mid and late stages, respectively, with some genes being included in more than one stage (Fig. 7, Supporting Information Table S5). This core set of shared genes probably includes genes necessary for bud formation. The early stages of LD and SD bud formation had the fewest DE genes in common. Supporting the idea that this analysis excluded genes from the core set that are solely light-responsive, genes found to be DE only under SD at early time points included cryptochrome, photosynthetic genes, pigment biosynthetic genes and genes involved in sugar and starch transport and metabolism. Interestingly, SD-only DE genes included several genes implicated in IAA and GA action, as well as a number of cell wall biosynthetic and stress response genes.

The early stage core set of bud formation genes included TFs implicated in developmental processes such as *KNOX3*, *KNOX4* and *APETALA2*. *KNOX4* was also included in the mid and late stage core sets. Other TFs and regulators in the mid stage core set potentially associated with organ differentiation and meristem maintenance included genes with similarity to *CYCLOIDEA*, *MUTANT CINCINNATA*, *LEUNIG*, *FIMBRIATA*, *MOTHER OF FT AND TF1*, and members of the *NAC*, *NAM*, *MADS-box*, *MYB*, *bZIP*, *APETALA2*, and *ZF-HD* TF families. For the most part, there was concordance in the direction of transcript abundance change relative to 0 d in the SD and LD datasets for the regulators included in the core set, although there was more disagreement

in the direction of transcript abundance changes in the early stage set, e.g. for *KNOX3* and *KNOX4*. Other TFs implicated in developmental processes were DE under SD only such as a gene encoding a LOB domain-containing protein and *STYLOSA* (Fig 4 and Table S.6). While MOTHER OF FT AND TF 1 was upregulated under LD at 28 d, similar to the pattern of expression in SD, a number of flowering-associated genes were only DE at 70 d SD or SD/LT, and were not DE during the LD time course, including *FCA*, *PHYTOCHROME AND FLOWERING TIME 1*, and *VERNALIZATION INDEPENDENCE 4*.

Cell wall related-genes were prominent in each of the core sets. Genes encoding proteins involved in cell cycle and DNA processing, stress response, protein fate and hormonal regulation were also represented in mid and late stage core sets. Stress response genes were particularly evident in the late stage core set. Again, there was very good overall agreement between the SD and LD expression data with respect to the direction of transcript abundance change relative to 0 d.

Tissue expression patterns of genes DE during bud formation

Genes involved in processes specific to bud formation might be expected to have a higher level of expression in developing buds than in other tissues. As an initial step towards identifying genes that function in processes unique to or more prevalent in bud formation, we used a three stage approach to identify genes that were both DE specifically in developing buds and expressed at significantly higher levels in developing buds compared with other tissues. First, we identified genes DE specifically in developing buds, needles or stems by comparing the list of 4460 DE genes in developing buds under SD to lists of DE genes from needles and stems sampled from the same plants at 3 d, 14 d, 70 d and LT and hybridized against the corresponding day 0 sample (Supporting Information Fig. S1). Of the 4460 DE genes in developing buds, 1134 were uniquely

DE in developing buds, i.e. not DE at any time point in needles or stems (Supporting Information Table S6), 439 were uniquely DE genes in stems and 1379 in needles (Fig. 8A).

Second, we determined which genes were expressed at significantly higher levels in a particular tissue relative to the other two tissues by conducting microarrays in which each tissue was hybridized directly against the other two tissues at 14 d and 70 d SD (Supporting Data Fig. S1). Comparisons between tissues (using p-value ≤ 0.01 and fold change ≥ 1.5) revealed that 479 genes were expressed at significantly higher levels in developing buds versus stems or needles (Supporting Data Table S7), 884 in stems compared to developing buds or needles, and 1005 in needles compared to developing buds and stems (Fig. 8B).

Third, we determined the intersections of these datasets to identify genes that were uniquely DE in a given tissue, and also expressed at significantly higher levels in that same tissue relative to the other two tissues. There were 108 genes uniquely DE in developing buds that were also preferentially expressed in developing buds (Supporting Information Table S8), 67 genes in stems and 191 genes in needles (Fig. 8C). More than half (61) of the 108 developing bud genes belonged to clusters (d) and (i) of Figure 3, showing downregulation in later stages of bud development. Functional categories represented included stress responses (3 genes), carbohydrate metabolism (3 genes), cell wall biosynthesis (4 genes), hormone biosynthesis and action (4 genes), cellular communication and signal transduction (8 genes), and lipid metabolism (12 genes). Lipid, carbohydrate, and cell wall metabolism genes were all downregulated in later stages of bud development. Three of the four potentially hormone associated genes, encoding an IAA-like protein, a TIR1-like protein, and a GASA-like protein, were upregulated late in bud development, while another GASA-like gene was downregulated. Six TFs were both uniquely DE and expressed at higher levels in developing buds, including one *MADS-box*, one *HB*, two

bZIP, one *PHD*-like, and one *ZF-HD*. Of the 108 genes uniquely DE and expressed at higher levels in developing buds, 57 were also found in the SD-LD core set of genes. No TFs or hormone-associated genes on this list were present in the SD-LD core set.

DISCUSSION

In this study, we examined global gene expression profiles over a time course of bud development in white spruce, focusing on the early stages of SD-induced bud formation. Our analysis, similar to the study of Ruttink *et al*. (2007) examining bud formation in *Populus*, demonstrated that extensive changes to the transcriptome take place in the shoot apex during this transition between active growth and dormancy. Other studies utilizing large-scale transcript profiling to examine autumnal changes in gene expression in foliage and cambial tissues of forest trees have observed similar large shifts in gene expression (e.g. Andersson *et al*. 2004; Druart *et al*. 2007; Holliday *et al*. 2008). We used cluster analysis and functional categorization of the 4460 genes DE during bud formation to infer cellular and molecular processes that occur in the developing bud of white spruce, and then related these changes in gene expression to hormone levels and anatomical changes that were observed as the shoot apex transitioned to a state of dormancy. This comprehensive analysis provides a temporal framework, or roadmap, of events that occur during bud formation in white spruce (Figure 9).

For many of the genes identified in this roadmap, this study provides important first evidence for a role in bud formation of a conifer. Annotation of the white spruce genes is based primarily on sequence similarity to angiosperm genes. The availability of genomic resources for an ever-increasing number of "non-model" plant species has enabled functional and comparative analyses of several gene families, which demonstrate conservation of overall function for many

genes across a broad spectrum of plant species (Rosin & Kramer 2009). The genes identified in this study represent attractive candidates for functional characterization to further validate their role in processes inherent to bud development.

Temporal expression patterns of genes associated with developmental processes coincide with anatomical changes to the shoot apex

Several hallmarks of bud development were observed in the anatomical study, including bud scale initiation, shoot apical meristem (SAM) expansion, stem unit initiation, crown development, bud scale maturation, and cessation of stem unit initiation. The chronology of these events is depicted in Figure 9, and is similar to that documented for other north-temperate conifer species (e.g. MacDonald & Owens 1993).

A number of DE genes exhibiting patterns of expression coincident with organogenesis showed similarity to known TFs and other regulators of developmental processes in *Arabidopsis* and other annuals. SAM activity is presumably vital to bud formation. (;Dodsworth 2009). *WUSCHEL* expression is required for maintenance of SAM stem cell populations, CLAVATA3 represses *WUSCHEL* expression, and CLAVATA1 is one factor mediating CLAVATA3 perception (Clark *et al*. 1996, Schoof *et al*. 2000; Dodsworth 2009). In our microarray analysis of SD-induced bud formation, a *CLAVATA1*-like gene was downregulated following transfer of the seedlings to LT, coincident with completion of cell proliferation from the SAM. *KNOX* genes, belonging to the homeodomain class of transcription factors, play overlapping roles in controlling cell fate in the SAM by preventing cell differentiation (Hake *et al*. 2004). Four class I *KNOX* genes show concerted upregulation during the mid to latter stages of bud formation, consistent with the notion that cellular differentiation slows as the rate of primordia initiation diminishes. In *Arabidopsis*, *KNOX* gene products interact with other homeodomain proteins such

as *BEL1* (Hay & Tsiantis 2009); a gene encoding a *BEL1*-like protein was upregulated at 14 d in our dataset. Two genes similar to *LATERAL ORGAN BOUNDARIES (LOB)* domain genes of the *ASYMMETRIC LEAVES2* family which are regulated by *KNOX* and whose products, in turn, modulate organogenesis (Dodsworth 2009) were upregulated at 14 and 28 d. Members of the *HD-ZIPIII* subfamily of TFs are also associated with SAM function, as well as defining lateral organ polarity. Two genes encoding putative *HD-ZIPIII*s with similarity to *PHABULOSA* were downregulated at 14 d SD, coincident with the observed increase in SAM dimensions. Morover, two *APETALA2*-related genes, which show high similarity to Norway spruce genes expressed in spruce seedling SAM and primordia, and capable of misregulating floral development in transgenic *Arabidopsis* (Nilsson *et al*. 2007) were downregulated at 14 and 28 d. *APETALA2* is also suggested in modulating WUSCHEL/CLAVATA feedback loop (Dodsworth 2009).

Other DE genes exhibited sequence similarity to TFs regulating diverse facets of organogenesis such as cellular identity, polarity, and patterning, including *LEUNIG*, *KANADI*, *ROOT GROWTH DEFECTIVE3*, *CYCLOIDEA,* and *MUTANT CINCINNATA* (Martín-Trillo & Cubas, 2009; Tamaki *et al*. 2009; Barton 2010). In our study, *CYCLOIDEA, MUTANT CINCINNATA* and *ROOT GROWTH DEFECTIVE3* genes, are DE in developing buds under both SD and LD conditions, indicating their central role in bud formation. A number of other TFs regulating developmental processes are DE under both SD and LD conditions. For example, two genes encoding homeodomain-like proteins are both specifically and preferentially DE in developing buds suggesting that they might function in process(es) more prevalent in developing buds than in other tissues.

Two distinct *MADS-box* genes, both downregulated at 28 d, are similar to *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), an *Arabidopsis* gene necessary for flowering (Bernier & Périlleux 2005). Recent studies implicate orthologues of other regulators of flowering

time in forest tree dormancy acquisition or release (e.g. Böhlenius *et al*. 2006; Gyllenstrand *et al*. 2007; Ruonala *et al*. 2008; Mohamed *et al*. 2010). One *SOC1*-like gene is both DE only in developing buds and also expressed at higher levels in developing buds relative to other tissues, implying that this *MADS-box* gene could play a specialized role in bud development. *CONSTANS* is a key player in the photoperiodic flowering pathway, regulating *SOC1* and *FLOWERING LOCUS T*. Genes putatively encoding both *CONSTANS* and *CONSTANS*-interacting proteins were DE during SD-induced bud formation. *CONSTANS* is regulated by the circadian clock, which plays a pivotal role in the transition from vegetative to reproductive growth in annuals (Imaizumi 2010). Emerging evidence suggests that the circadian clock is also important in the transition from active growth to dormancy in perennials (Allona *et al*. 2008; Kozarewa *et al*. 2010). Consistent with these studies, two genes similar to *PSEUDO-RESPONSE REGULATOR7*, a component of the core oscillator of the circadian clock (Miwa *et al*. 2006; Farré & Kay 2007; Ibanez *et al*. 2008), were DE during SD bud formation, but not significantly during LD bud formation.

Photoreceptors such as phytochrome play a key role in the photoperiodic flowering pathway, and control light signalling to the circadian clock (Bernier & Périlleux 2005; Harmer 2009). Phytochromes have also been shown to regulate bud formation for those species with a photoperiod requirement such as *Populus* (Olsen *et al*. 1997b). Two distinct phytochrome genes, both encoding *PHYA*-like genes and one showing strong similarity to the *Picea abies PHYO* gene (Clapham *et al*. 1999), were DE during SD-induced bud formation. One was upregulated at 70 d SD and was also DE in LD conditions, indicating that expression of this gene is not regulated by photoperiod. The other PHYA-like gene was downregulated at 14 d SD. Phytochromes are thought to be the primary regulators of initiation of autumn cold acclimation in perennials (Howe

et al. 1996; Horvath *et al*. 2003), and the expression pattern of this late-upregulated phytochrome is suggestive of a role in this process.

As expected, a number of genes associated with the cell cycle were DE during bud formation. Rohde and Bhalerao (2007) found that a cyclin was downregulated in *Populus* apices during the transition from active growth to dormancy under SD, while Espinosa-Ruiz *et al*. (2004) observed downregulation of cyclin-dependent kinases coincident with termination of cell division in *Populus* cambium subjected to SD. Most of the five DE cyclins and nine DE cyclin-dependent kinases showed progressive downregulation over the course of bud formation, and were also downregulated during bud formation in LD. A number of histones were also downregulated under both SD and LD during bud formation, as were genes associated with DNA replication. *FASCIATA1* is only DE under SD conditions, and putatively encodes a subunit of the chromatin assembly factor that facilitates incorporation of histones into newly synthesized DNA (Inagaki, Nakamura & Morikami 2009). *Arabidopsis fas1* mutants show aberrant development as well as impaired heterochromatin formation and transcriptional gene silencing (Inagaki, Nakamura & Morikami 2009), illuminating the link between DNA replication, chromatin structure and development.

A suite of genes implicated in chromatin remodelling and chromatin modifications were also DE during bud formation, in agreement with Karlberg *et al*. 2010, who showed that genes associated with chromatin remodelling are DE during *Populus* shoot apex dormancy acquisition. In *Arabidopsis*, chromatin remodelling via chromatin methylation and/or histone acetylation has been demonstrated to regulate aspects of both organogenesis and flowering (Jarillo *et al*. 2009), as well as mediate responses to environmental stresses (Chinnusamy & Zhu 2009). Chromatin methylation is associated with gene silencing, while histone acetylation is associated with transcriptional activation. A CMT-type DNA-methyltransferase putatively involved in DNA

methylation was downregulated throughout most of bud formation. A histone deacetylase was downregulated during the early phase of bud formation, while a histone acetyltransferase was upregulated during the latter stages. The histone acetyltransferase is DE under LD conditions as well. Several chromatin assembly factors were also DE, as well as a putative chromodomain-helicase-DNA-binding protein similar to *PICKLE* in *Arabidopsis*. This *PICKLE*-like gene was not DE under LD conditions. *PICKLE* and *PICKLE RELATED2* interact with polycomb group proteins to affect chromatin structure, leading to repressed expression of developmental regulators such as *LEAFY COTYLEDON1* (Aichinger *et al*. 2009; Kohler & Aichinger 2010). Together, these gene expression patterns suggest that chromatin remodelling plays a role in transcriptome reconfiguration during bud formation in white spruce.

Protein turnover also appears to be an important process invoked during bud formation, as a number of genes encoding proteases as well as components of the ubiquitin-proteasome system were DE, particularly during the latter stages of bud formation. This is similar to the findings of Holliday *et al*. (2008), who reported differential expression of multiple proteolysis-associated genes during cold acclimation in foliage of Sitka spruce. Perhaps because genes of the ubqiuitin-proteasome system were DE late in bud formation, relatively few are DE under LD conditions. Several proteases were co-ordinately upregulated at 14 and 28 d, coincident with bud scale maturation, and were also DE under LD. The final step in bud scale maturation is cell death, and it is possible that some of these proteases participate in this process. In support of this notion, a small suite of genes putatively implicated in programmed cell death are downregulated earlier in the time course. Amongst the DE genes associated with the ubiquitin-proteasome system, a number of genes encoding putative F-box proteins were upregulated during bud formation, including a gene similar to *ARABIDILLO1*, implicated in lateral root formation (Coates, Laplaze & Haseloff 2006). Two genes show similarity to *TIR1*, the auxin receptor in Arabidopsis

(Dharmasiri, Dharmasiri & Estelle 2005). This suggests that shifts in targets for proteasome-mediated proteolysis take place during bud formation, including the targets of a potential $SCF^{TIR1\text{-}like}$ complex. One *TIR1*-like gene is both exclusively DE in developing buds and expressed at higher levels in buds relative to other tissues, suggesting a specialized role for this particular *TIR1*-like gene in bud formation. Interestingly, there do not appear to be any *TIR1*-like genes that are DE under LD.

Changes in hormone levels and the expression of genes associated with hormone action coincide with developmental events in the developing bud

We observed that a number of genes associated with hormone action were DE during bud formation. Very few studies have been carried out to quantify endogenous hormone levels in developing apical buds of conifers (e.g. Chen, Bollmark & Eliasson 1996). Accordingly, we examined steady state profiles of hormones and selected metabolites previously linked to bud formation to determine whether relationships could be described between cellular processes, hormone levels and expression of genes involved in hormone biosynthesis and signalling.

IAA and CKs, which showed contrasting patterns of accumulation in our study, have been implicated in bud dormancy, although their roles in apical bud formation are not clear (Rohde *et al*. 2000). IAA has a well known role in primordia initiation in *Arabidopsis*, but concentrations of IAA in developing buds were lowest during primordia initiation, suggesting that IAA is playing additional roles in the developing bud. This observed pattern of IAA levels differs from data reported for shoot apices of willow and birch, where IAA levels decreased under SDs (Li *et al*. 2003; Olsen *et al.* 1997a). There is a large increase in conjugated IAA levels following transfer of the plants to LT, implying that this reserve of inactive IAA can be used to generate active IAA during the winter or the following spring. Some putative auxin transport genes were

upregulated at 7 and 14 d, coincident with the onset of primordia formation and preceding the decline in IAA levels, while other putative auxin transport genes were downregulated late in bud development in parallel with increasing IAA levels. Several auxun transport genes were also DE under LD conditions. Similarly, putative *TIR1* receptor genes displayed distinct patterns of expression as described above. These results show that changes in IAA levels and shifts in IAA transport and perception occur during bud formation, and that different zones of the developing bud are likely to have distinct auxin levels and auxin signal transduction capacity at different times of bud formation.

CKs are known to promote axillary bud outgrowth (Cline 1994; Nordstrom *et al*. 2004). IAA inhibits CK synthesis and reduces concentrations of transported CKs (Eklof *et al*. 1997; Nordstrom *et al*. 2004). The observed increase in CK concentrations during active primordia initiation, when IAA levels are decreased, is consistent with IAA exerting a negative effect on CK synthesis and transport. Elevated CK levels during organogenesis are also consistent with published reports of CK levels in developing buds of Norway spruce (Chen, Bollmark & Eliasson 1996). These authors provide circumstantial evidence that CKs control final bud size, which is largely determined by the number of initiated primordia. In *Arabidopsis*, CKs interact with the WUSCHEL/CLAVATA feedback loop through Type A response regulator *ARR7* (Dodsworth 2009). A putative Type A response regulator with most similarity to *ARR7* in *Arabidopsis* was upregulated under both LD and SD.

Decreased GA levels are correlated with a decline in subapical cell divisions and SD-induced growth cessation in forest tree species (Dunberg and Oden 1983; Olsen *et al*. 1995; Olsen *et al.* 1997a), and are probably necessary to necessary to prevent stem unit elongation (Ruttink *et al*. 2007). GAs were at low or non-detectable levels throughout bud formation in our study, suggesting that levels of these hormones had already decreased prior to day 0. Supporting

this conjecture, gibberellin 3-hydroxylase, which converts GA_9 to bioactive GA_4 (Yamaguchi & Kamiya 2000), is downregulated throughout SD bud development. A *DELLA* negative regulator of gibberellin signalling is upregulated during the mid phase and downregulated at 70 d under both SD and LD conditions, while two positive regulators of gibberellin signalling – an F-box protein *GID2* and receptor *GID1C* – are also upregulated (Jiang & Fu 2007). The latter do not appear to be DE under LD conditions. Use of in situ localization or other visualization technique may resolve whether these genes are co-expressed in the same cell types or in different portions of the developing bud.

ABA is hypothesized to contribute to the suppression of growth that occurs concomitantly with bud formation (Ruttink *et al*. 2007). Rhode *et al*. (2002) reported that ABA concentrations in apical buds of poplar increased after 24 to 27 d SD, coincident with the cessation of growth, while increased levels of ABA in apical and lateral buds under SD preceded or correlated with induction of endodormancy (Rinne, Saarelainen & Junttila 1994; Li *et al*. 2003). In our study, active ABA levels were lowest during the phase of most active organogenesis, and increased somewhat during the latter phases of bud formation, providing limited support for this hypothesis. The late increase in ABA could also be linked to acquisition of desiccation tolerance (Cutler *et al*. 2010). We also examined ABA metabolites to determine the routes by which ABA is metabolized in developing buds. Three major ABA catabolic pathways include: (1) the 8'-hydroxylation pathway leading to the formation of PA, (2) the 7'-hydroxylation pathway leading the formation of 7'-OH-ABA and (3) the catabolic pathway through the conjugation of ABA with glucose to form ABA-GE (Nambara & Marion-Poll 2005). All three metabolites were detected, indicating the three pathways all function during bud formation, but the high levels of ABA-GE observed over the time course of SD relative to PA or 7'-OH-ABA suggest that ABA is metabolized primarily through glucosylation in developing buds. This is consistent with other

conifer studies (e.g. Kong *et al.* 2009) but contrasts with what is found in angiosperms where phaseic acid and dihydrophaseic acid are the major catabolites. The significance of the differences is not yet known. During development of long shoots of Douglas-fir (*Pseudotsuga menziesii*; Kong *et al.* 2009), levels of ABA, ABA-GE and 7'OH-ABA were high in closed buds. Catabolite levels dropped during bud flush, and again increased as the shoot reached near full length. PA concentrations were low at all stages. Profiles of ABA and its metabolites have also been studied in western white pine (*Pinus monticola* Dougl. Ex D. Don) seeds during dormancy breakage (moist chilling) and germination (Feurtado *et al*. 2004). ABA levels decline in seed coats, embryos and megagametophytes during moist chilling, concurrent with an increase in germination capacity. ABA was mainly biotransformed to PA and its further reduced metabolite dihydrophaseic acid, although ABA-GE and 7'OH-ABA also accumulated in the embryo.

In *Arabidopsis*, a number of protein phosphatases (PPs), including PP2Cs such as *ABSCISIC INSENSITIVE1* (ABI1), act as negative regulators of ABA signalling (Cutler *et al.* 2010). Putative *ABI1* homologues have been implicated in bud dormancy in *Populus* (Ruttink *et al*. 2007). Several genes encoding PP2Cs were DE under both SD and LD, including two that showed highest sequence similarity to Arabidopsis *ABI1* and *HOMOLOGY TO ABI1* (Robert *et al.* 2006). Both genes were upregulated early in bud development. Protein kinases such as SnRK2s, SnRK3s, and CDPKs are also implicated in ABA signalling (Cutler *et al.* 2010); interestingly, *SnRK2*- and *CDPK*-like genes were DE during bud development.

In birch, ethylene facilitates SD-induced terminal bud formation and normal endodormancy development (Ruonala *et al*. 2006). There may be an interaction between ABA and ethylene signalling in bud formation. Unlike wild-type, ethylene insensitive mutants of birch did not accumulate ABA in response to SD (Ruonala *et al.* 2006), while microarray analyses of *Populus* bud formation suggest that ethylene signalling precedes ABA signalling (Ruttink *et al.*

2007). Ethylene may be required for ABA accumulation that facilitates bud formation and dormancy acquisition. In our study, expression patterns of ethylene biosynthetic genes suggest that increased ethylene synthesis occurs in white spruce when primordia initiation is well underway, consistent with a role for ethylene in mediating dormancy. However, this signal does not apparently precede ABA signalling. Only a small number of these ethylene biosynthetic genes are DE under LD conditions, consistent with the notion that ethylene is implicated in later stages of bud formation that perhaps do not occur under LD.

Nothing is known about the roles of other hormones such as BRs or JA in apical bud formation, although recent research suggests that BRs may function to modulate auxin responses in the SAM of annuals (Viet 2009). The results from our microarray analyses suggest that these hormones could play some role in bud formation, and future research will explore these potential roles.

Gene expression patterns suggest modification of both symplastic and apoplastic connections occur during bud formation

Regulatory proteins such as KNOX and CLAVATA are thought to move symplastically via plasmodesmatal connections within the SAM, and that this movement is vital to maintaining meristem identity and organization. Symplastic connections also facilitate movement of resources such as photosynthates and amino acids. Rinne & van der Schoot (1998) demonstrated that cells within the SAM of birch seedlings exposed to SD became symplastically isolated due to plasmodesmata plugging with carbohydrates and proteins, including callose (1,3-β-D-glucan). 1,3-β-D-glucan synthase is postulated to play an important role in blocking plasmodesmatal connections (Rinne & van der Shoot, 2003). A gene coding for 1,3-β-D-glucan synthase was

upregulated at 14 d SD, while several genes encoding 1,3-β-D-glucanases were progressively downregulated through bud formation. These data suggest that symplastic isolation within the SAM may also occur during white spruce bud formation. Interestingly, none of these genes are DE during LD bud development, suggesting that formation of these buds does not reach the point of symplastic isolation.

Cell wall biosynthesis is major process implicated in cell development, and cell wall properties have been shown to change as developing buds transition into dormancy (Rinne, Kaikuranta & van der Schoot 2001). Many genes encoding cell wall biosynthesis or modification proteins were DE during bud formation. Some of these genes are undoubtedly involved in cell wall synthesis and modification within bud scales. For example, there was a marked peak in the expression of several lignin biosynthesis genes at 14 d, when maturation of the bud scales is commencing. Bud scale cell walls are likely to be highly modified, given the protective function of these specialized organs. Some of the cell wall and lipid associated genes found to be DE only in developing buds and expressed at higher levels in buds compared to stems and needs are likely to be implicated in bud scale development. Other cell wall-associated DE genes are probably involved in development of crown cells, which occurs mainly between 28 d and 70 d, as well as cell wall biosynthesis and modification for cells making up the SAM and subtending stem units. The timing of the expression of these genes is likely to be different than those involved in cell wall biosynthesis in the bud scales.

Most genes associated with cell wall expansion, such as xyloglucan endotransglycosylases and expansins, were upregulated during active organogenesis, downregulated at the completion of organogenesis, or both. Similarly, most genes associated with cellulose and matrix glycan biosynthesis were downregulated in the latter stages of bud

development, although some pectin and matrix glycan modification genes such as beta-xylosidase – an enzyme involved in hydrolysis of the xylan component of matrix glycans (Goujon *et al*. 2003) – were upregulated. Beta-xylosidases were also upregulated in apices of birch under SD (Ruonala *et al*. 2006). Histological studies also suggest that cell walls of cells within dehydrated, dormant buds become less permeable to water (and presumably other substances) due to deposition of hydrophobic materials such as suberin or wax-like substances (Rinne & van der Schoot 2003). Many of the DE genes encoding fatty acid biosynthetic genes were downregulated at 70 d SD and/or after transfer to LT, consistent with a role in cutin or suberin biosynthesis. This group includes two genes similar to *FIDDLEHEAD*, an *Arabidopsis* gene involved in synthesis of long-chain lipids that affects epidermal cell interactions (Pruitt *et al*. 2000). Other genes potentially associated with cutin and wax biosynthesis, such as *GLOSSY1* (Sturano *et al*. 2005), are also downregulated in latter stages of bud formation. Suberin also contains phenylpropanoid compounds such as ferulate (Pollard *et al*. 2008). Ferulate biosynthetic genes show expression patterns coincident with the fatty acid biosynthetic genes. Twelve of these DE lipid metabolism genes are both DE only in developing buds and preferentially expressed in developing buds compared to other tissues, suggesting that they are involved in biosynthesis of lipids that are more prevalent in buds than in other tissues.

Cells of the SAM represent an important sink for carbon resources. Genes of both the photosynthetic electron transport apparatus and carbon reactions showed concerted downregulation in buds only after transfer of the plants to LT, suggesting that photosynthetic capacity is maintained until this late stage. Several genes associated with chlorophyll and the light harvesting antennae were upregulated during the first week after transfer of the plants to SD and were not DE during LD bud formation, and likely represent a compensatory response by the plants to the decreased duration of light. Rubisco transcript abundance increases during the mid

phase of bud formation. Rubisco is considered by some to act as a storage protein, since it is often present in quantities excessive of what is required to achieve maximum photosynthesis (Cooke & Weih 2005). These data suggests a role for Rubisco as a storage reserve in apical buds; accumulation of Rubisco could also assist the plant to begin carbon fixation early in the spring.

Sugars and starch are important both as real-time resources for bud formation, and as stored reserves to support growth the following spring (Lipavska, Svobodova & Albrectova 2000). Sucrose and other sugars also act as signalling molecules, and have been linked to flowering (Heyer *et al*. 2004; Bernier & Périlleux 2005). Nonreducing sugars such as sucrose and raffinose have been shown to increase in conifers during the autumn (Pomeroy, Siminovitch & Wightman 1970), while starch is broken down (Alscher *et al*. 1989). Increased sucrose levels occurred in response to SD in conifers (Aronsson, Ingestadt & Lööf 1976) and willows (Ögren 1999), while raffinose levels rose in response to LT (Hinesley *et al*. 1992; Stushnoff, Seufferheld & Creegan 1998; Ögren 1999; Cox & Stushnoff 2001). could compensate for decreased photosynthetic activity, while ncreased disaccharides could provide protection against dehydration and freezing damage during the cold acclimation process (Zwiazek *et al*. 2001). Our data suggest that starch breakdown occurs throughout bud formation in white spruce, and that starch biosynthesis occurs principally following transfer of seedlings to LT. Sucrose metabolic patterns are more complex. Genes for a sucrose phosphate synthase (SPS) and a sucrose synthase were upregulated at 7 d SD, suggesting a role in providing sucrose to support metabolic activities within the developing bud, concomitantly increasing the relative sink strength of this tissue. A second SPS gene was upregulated later in bud development, implying that this gene is involved in synthesizing sugars for storage and/or osmotic regulation. We also observed a late increase in transcript levels of galactinol synthase, consistent with the synthesis of raffinose as a compatible

solute during cold and dessication acclimation. Cell wall invertases, which convert sucrose to fructose and glucose and are associated with accelerated flowering (Heyer *et al*. 2004) and active cell division during seed development (Weber *et al*. 2005), are downregulated later in development.

Some genes associated with autumnal acclimation are DE in developing buds even in the absence of SD or LT

Overwintering preparations at the shoot apex also involve cold/desiccation acclimation and dormancy acquisition. TFs classically associated with cold and desiccation were DE during bud formation, including *DREB*s and an osmosensor histidine-aspartate kinase most similar to *Arabidopsis*, *ATHK1*. *ATHK1* acts both as a sensor of osmotic stress in vegetative organs and as a regulator of the desiccation process during seed maturation (Wohlbach, Quirino & Sussman 2008), supporting the notion that this gene could play a role in regulating desiccation tolerance during bud formation. Many genes associated with stresses such as cold and desiccation showed upregulation during the later stages of SD-induced bud formation, as were genes encoding proline biosynthetic enzymes, a compatible solute. These findings are consistent with those of Holliday *et al*. (2008), who examined autumn acclimation in foliage of Sitka spruce. A few genes associated with cold and desiccation acclimation were downregulated during the later stages of bud formation, suggesting that these genes were already being actively transcribed at Day 0, i.e. during active growth, and were subsequently downregulated as growth cessation occurred. Because of the intrinsic role that SD and LT play in the cold and desiccation acclimation process, it is often assumed that lengthening nights and/or diminishing temperatures are required for expression of genes associated with hardening off. In fact, a number of these genes were DE in developing buds under LD. This suggests that a subset of genes associated

with cold and/or desiccation tolerance are regulated by factors internal to the plant, rather than by environmental cues. The upregulation of these genes in the absence of environmental cues may represent a "safeguard", whereby the plant can put in place some degree of protection against inclement conditions, for example in the event of an unseasonably early frost date.

CONCLUSIONS

This comprehensive microarray analysis of bud formation in white spruce demonstrates that substantial transcriptional reprogramming takes place during this developmental and physiological transformation from active growth to dormancy. Many of the regulons representing developmental and physiological processes identified through these microarray analyses occur in buds that are initiated under both SD and LD, indicating that photoperiod is not a proximal factor in their regulation. Virtual subtraction of DE genes of stems and needles from the DE genes identified in developing buds identified a small number of genes that may play more prominent roles in bud-associated processes than processes that also occur in other tissues, including TFs associated with developmental processes.

Together, the DE genes identified in this study constitute a roadmap of bud formation in white spruce. The timing of bud formation in white spruce is under genetic control (Jaramillo-Correa *et al.* 2001). As such, it is conceivable that one or more of the DE genes identified in this study may represent cis- or trans-acting regulators of bud formation in white spruce. Indeed, several of these genes fall within robust QTLs identified for time of bud set (B. Pelgas and N. Isabel, personal communication). Future studies will seek to establish whether any of the DE genes identified in this study exert genetic control over time of bud formation.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Nathalie Isabel and Marie-Claude Gros-Louis for the white spruce seedlings, Eric Fedosejevs, Kimberly Lam, Leonardo Galindo, Marie Gorda, Susan Koziel and Dr. John Vidmar for technical assistance with plant experiments, Dr. Enrico Scarpella and Randy Mandryk for advice on microscopy, Dr. Stephane LeBihan and staff of the BC Microarray Facility for microarray production, and Troy Locke for advice on qRT-PCR. This work was supported by grants from Genome Canada, the Province of Alberta, and the Natural Sciences and Engineering Research Council of Canada (NSERC) to JEKC.

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4

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Experimental design of the four microarray studies.

Table S1. Genes and gene-specific primers used for qRT-PCR.

Table S2. K-means clustering of DE genes in developing buds under SD.

Figure S2. Cluster analysis of photosynthesis-related genes DE in developing buds under SD.

Figure S3. Cluster analysis of cell wall-related genes DE in developing buds under SD.

Table S3. Regulatory genes, including transcription factors, DE in developing buds under SD.

Figure S4. Cluster analysis of DE TFs in developing buds under SD.

Table S4. DE genes in developing buds under LD.

Figure S5. Cluster analysis of DE regulators implicated in developmental processes in buds under SD.

Table S5. Genes DE in developing buds under both LD and SD.

Figure S6. Comparison of the expression pattern of selected genes by qPCR and on the microarrays.

Table S6. Genes uniquely DE in developing buds under SD.

Figure S7. Cluster analysis of hormone associated genes DE in developing buds under SD.

Table S7. Genes expressed at significantly higher levels in developing buds relative to stems and needles under SD.

Table S8. Genes both uniquely DE and expressed at significantly higher levels in developing buds relative to stems and needles under SD.

FIGURE LEGENDS

Figure 1. The progression to bud set in *P. glauca* apical shoot tips under SD. Two-year-old seedlings nearing the end of the active growth cycle and growing in LD (16 h light / 8 h dark) were subjected to SD (8h light / 16 h dark) in order to induce rapid and synchronous bud set. Plants were harvested over a 10-week SD time course, and also after transfer to LT. (A) Macroscopic images of apical shoot tips, obtained using a dissecting microscope. (B) Median longitudinal sections of developing buds stained with Richardson's stain. Scale bar = 100mm. Number in the bottom right corner indicates the days after the transfer to SD.

Figure 2. Quantitative changes to bud morphology over the course of bud development in SD, and after transfer to LT. A. Dimensions of both the whole bud and the shoot apex (apical dome) were measured in median longitudinal sections. I1 and I2 represent the whole bud (from the base of the primordia to the apex of the apical dome) whereas II1 and II2 are measurements of the apical dome. B. Changes to total bud diameter and bud height. C. Changes to the shoot apical region of the bud. D. Increase in the number of primordia tiers during bud development. Each of the measurements was taken from the same three independent buds.

Figure 3. Cluster analysis of 4460 differentially expressed sequences during SD-induced bud formation. (I) Expression values obtained by microarray analysis across the time course. The 4460 genes were classified into 10 groups by K-means clustering, using Euclidean distance. The number of genes grouping within each cluster is indicated for each graph. The x-axis represents the time course of SD treatment, as described in Materials and Methods. The y-axis represents the change in transcript abundance at each time point relative to Day 0, expressed as $\log_2$ of the fold

change. (II) The most prominent functional categories represented in each cluster. Letters used to designate each bar correspond to the letters for each cluster in (I). Only ESTs that had been assigned an informative annotation were included in this functional categorization.

Figure 4. Transcript abundance measured by qRT-PCR of eleven genes putatively involved in organ development and meristem maintenance in developing buds under SD. All genes had been identified as DE in developing buds by microarray analysis for at least one time point relative to day 0 except Knox4, which was not represented on the microarray. Expression was determined relative to translation initiation factor 5A (*TIF5A*); both the targets and *TIF5A* were quantified using standard curves. n=4 ± SE.

Figure 5. qRT-PCR quantification of transcript abundance for ten hormone associated genes during SD-induced bud formation. Genes were identified as DE in developing buds under SD by microarray analysis for at least one time point relative to day 0. Expression was determined relative to translation initiation factor 5A (*TIF5A*); both the targets and *TIF5A* were quantified using standard curves. n=4 ± SE.

Figure 6. Changes in concentrations of abscisic acid, auxins, cytokinins and their metabolites in developing buds under SD. One way ANOVA was performed on transformed (*) or untransformed (unmarked) data followed by parametric multiple comparison testing using Tukey's method. IAA-Asp data were not normal (~) even after transformation. For this we used

the nonparametric Kruskal-Wallis test4 and a nonparametric multiple comparison method with unequal sample sizes. Hormone and metabolite levels significantly different between the time points at $a \leq 0.05$ are indicated by different letters.

Figure 7. Comparison of DE genes in developing buds under SD or LD. To accommodate the asynchrony encountered in the initiation of bud formation under LD, data from the SD and LD datasets were combined before analysis to generate early stage, mid stage, and late stage datasets for each condition, better facilitating comparison of changes in gene expression that occur during bud formation under SD and LD photoperiods. Early stage represents genes identified as DE in developing buds under either LD or SD conditions at 1 d, 3 d, and 7 d relative to day 0; mid stage represents DE genes at 14 d and 28 d, and late stage represents DE genes at 70 d.

Figure 8. Venn diagram analysis to identify genes that are both DE specifically in a given tissue during bud formation, and also expressed at higher levels in that tissue relative to other tissues. (A) Genes significantly DE (p-value ≤ 0.01 and fold change ≥ 1.5 or ≤ 0.67) in at least one time point in developing buds, stems and needles during SD-induced bud formation, and the intersections between these datasets. Data were obtained at 3 d, 14 d and 70 d SD. (B) Genes significantly DE ($p \leq 0.01$, fold-change ≥ 1.5) in a direct 3 way tissue comparison experiment between developing buds, stems, and needles and stems at 14 d and 70 d SD. Abbreviations: B, developing bud; S, stems; N, needles. (C) The intersection between the datasets in A and B, identifying genes that are both DE only in developing buds, stems or needles, and also show significantly higher expression in that same tissue. The list of genes both specifically DE in

1 developing buds and also expressed at higher levels in developing buds compared to stems and
2 needles is found in Supporting Information Table S8.
3
4 Figure 9. Synthesis of morphological and molecular events that occur during bud formation in
5 white spruce. Blue, majority of genes upregulated; orange, majority of genes downregulated,
6 green, both up- and downregulated genes present.
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