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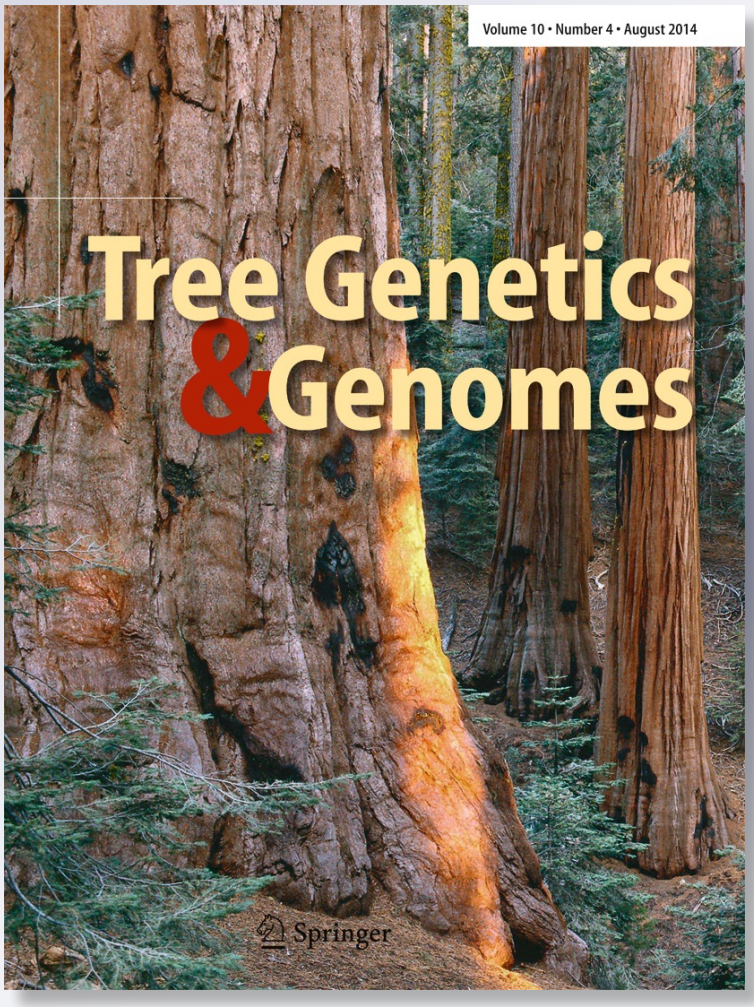

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ORIGINAL PAPER

Timing for success: expression phenotype and local adaptation related to latitude in the boreal forest tree, *Populus balsamifera*

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Abstract Timing of seasonal dormancy is among the most important traits allowing forest tree populations to span latitudinal gradients of temperature and growing season length. We used RNA-sequencing (RNA-seq) data to identify gene expression differences between latitude-adapted populations and search for elevated genetic differentiation, a signature of adaptation, between northern and southern populations of *Populus balsamifera*. RNA was extracted from leaf tissues collected from trees originating from two northern (65° N) and two southern (50° N) populations and growing in a common garden. Leaves for RNA-seq were collected on the same day in late July, when southern trees were actively growing and northern trees had set bud. Transcripts of 2,594 genes were more abundant in actively growing leaves, whereas 1,424 were more abundant in leaves of trees that had set bud. The actively growing southern trees exhibited overexpression of genes in the biological process gene ontology (GO) domain, whereas dormant northern trees exhibited overexpression of genes in the cellular component domain. We identified relatively few genes (641) bearing signatures consistent with local adaptation to latitude for three or more SNPs. Exons from differentially expressed (DE) genes were not more likely than non-DE genes to exhibit patterns of latitudinal differentiation consistent with local adaptation. These results indicate that genes associated with differences in the timing of bud set, which is correlated with initiation of dormancy, were not more likely than other categories to exhibit latitudinal differentiation in exonic regions.

Keywords Cis-regulation · Differential expression · Genomics · Poplar · Phenology · RNA-seq

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Introduction

Timing of seasonal dormancy is strongly correlated with altitude and latitude in forest trees (Pauley and Perry 1954; Hall et al. 2007; Keller et al. 2011a, b; Savolainen et al. 2011; Neale and Kremer 2011; Alberto et al. 2013). This correlation likely reflects an adaptive trade-off between maximizing growth and limiting freeze damage in seasonal environments. In perennial plants, dormancy is controlled by a complex suite of environmental cues that include photoperiod, temperature, and moisture (Howe et al. 1996, 2003; Cooke et al. 2012) and is characterized by dramatic developmental changes including growth cessation, bud set, storage of reserve metabolites, and development of cold tolerance (Rohde and Bhalerao 2007). The parallel and sometimes rapid evolution of latitudinal clines for the timing of the initiation of dormancy in northern hemisphere forest tree species (Ingvarsson et al. 2006; Savolainen et al. 2011; Olson et al. 2013) indicates that the response to selection on the timing of the initiation of dormancy can be rapid and strong, but our knowledge of the genes underlying these clines remains incomplete (Garcia-Gil et al. 2003; Luquez et al. 2008; Eckert et al. 2010; Chen et al. 2012; Olson et al. 2013).

Changes in gene expression, measured as steady state messenger RNA (mRNA) levels, that occur during the progression from active summer growth to dormancy have been documented across multiple species and at varied time points (Andersson et al. 2004; Ruttink et al. 2007; Holliday et al. 2008; Hoffman et al. 2010; Galindo González et al. 2012; Ueno et al. 2013). In a thorough growth chamber study conducted with wild-type and transgenic hybrid poplars (*Populus tremula*×*Pityriasis alba*), Ruttink et al. 2007 characterized how expression changed from the initial onset of dormancy to senescence. Those investigators showed that upon exposure to short-day treatments, trees first show changes in expression among genes in the light signal transduction pathways, then within 1 week of short-day (SD) treatment, there are dramatic shifts in expression of genes downstream of phytochromes A and B, such as phy-interacting factor (*PIF4*) and suppressor of phyB2 (*SHY2*), as well as genes integral to the circadian oscillator clock such as late-elongated hypocotol (*LHY*). These changes during the early transition to dormancy are similar to the changes during transition to flowering in *Arabidopsis*; approximately one third of genes differentially expressed in the light signal transduction pathway during the transition to flowering in *Arabidopisis* exhibit at least a 2-fold change in expression for *Populus* during the transition to dormancy (Ruttink et al. 2007). Changes in the light signal transduction pathway are successively followed by activation of genes in the ethylene biosynthesis pathway and abscisic acid (ABA) biosynthesis and signaling pathways. After 4 weeks of SD treatment, around the time that growth of meristems ceases, there is an increase in the expression of genes related to mobilization and storage of starch. Finally, genes associated with dehydration and cold tolerance are upregulated, including over a half of genes predicted to be targets of C-repeat binding transcription factors (Ruttink et al. 2007).

Although it is clear that there are many changes in gene expression during the transition to dormancy, it is not clear if these genes are overrepresented among targets of selection or whether they reflect downstream responses of adaptation. Association mapping studies in balsam poplar (*Populus balsamifera*) and European aspen (*P. tremula*) have identified several genes in the light signal transduction, circadian oscillator, vernalization, and integration pathways as strong candidates for controlling clinal variation in the timing of dormancy induction (Hall et al. 2007; Olson et al. 2013). These include genes such as *FRIGIDA* (*FRI*), which underlies natural variation in the timing of flowering in *Arabidopsis* (Stinchcombe et al. 2004; Riihimaki et al. 2005); *LEAFY* (*LFY*), which exhibits elevated diversity in *P. balsamifera* (Keller et al. 2011a); and *GIGANTEA* (*GI*), which partially underlies the timing of bud set in *Picea* (Holliday et al. 2010a, b; Chen et al. 2012). Several of the genes identified as controlling clinal variation in the timing of bud set also exhibit elevated levels of among-population differentiation or strong covariances between SNP frequencies and environmental properties—both signatures of local adaptation (Lewontin and Krakauer 1973; Coop et al. 2010)—in balsam poplar (Keller et al. 2012). These studies examined a relatively small number of predefined candidate genes. Genome scans to search for targets of clinal adaptation may therefore be expected to identify further genes responsible for adaptive shifts in the timing of dormancy.

Here, we use RNA-sequencing (RNA-seq) data from *P. balsamifera* to characterize differences in gene steady state mRNA levels between trees that are actively growing and those that have ceased growing and set terminal vegetative buds, which characterize the early stages of entry into dormancy. We used the same data to search for evidence of elevated population differentiation at the nucleotide level—a signature of local adaptation—among genes that are expressed in the leaves of both actively growing trees and trees that have set buds. We sampled four geographically defined populations (two northern (65° N) and southern (50° N) populations) of *P. balsamifera* growing in a common garden on the same summer day. Reflective of the local adaptation on timing of entry into dormancy, on this late July day, southern genotypes were actively growing, but northern genotypes had set bud. By sampling both southern and northern individuals under the same environmental conditions but at different developmental stages, we are able to identify a large suite of differentially expressed genes that correlate with initiation of seasonal dormancy in trees growing in seminatural field conditions.

The specific goals of this study are 3-fold. First, we compared steady state mRNA levels for different *P. balsamifera* genotypes on the same calendar day to identify differentially expressed genes that underlie developmental and population level differences between actively growing southern genotypes and northern genotypes at early stages after bud set. Second, we applied Fct-based tests (Lewontin and Krakauer 1973; Excoffier et al. 2009) to all expressed genes to identify genes that bear a signature of local adaptation. Finally, we compare results from differential expression and signatures of local adaptation in nucleotides to assess whether a pattern is present that would be consistent with differentially expressed genes having been targets of selection during *P. balsamifera*'s range expansion into more northern latitudes.

Materials and methods

Study species and sampling design

P. balsamifera is a widely distributed North American boreal tree ranging from 42° N to nearly 70° N (Olson et al. 2010). Balsam poplar is phylogenetically sister with the model species, *Populus trichocarpa*, and their divergence took place

within the Pleistocene glaciation, perhaps induced by climatic change (Levsen et al. 2012; Ismail et al. 2012). To examine gene expression differences among trees at different developmental stages, we sampled mRNA from one fully expanded leaf of 6–8 genotypes from each of four populations (30 individuals in total) of *P. balsamifera* on 26 July 2011.

Among the four sampled populations, the trees from the two southern populations were originally collected from near 50° N (Carnduff (CAR), 8 individuals and Sioux Lookout (SOU), 8 individuals), and trees from two northern populations originated near 65° N (Fairbanks (FBK), 6 individuals and Norman Wells (NWL), 8 individuals; Olson et al. 2013). At the time of sampling, all trees were growing in a common garden located on the campus of University of Alaska, Fairbanks (~65° N lat, Olson et al. 2013), the sun was above the horizon for ~18.9 h, and trees from the two southern populations were actively growing, whereas those originating from the two northern populations had set vegetative buds, a trait that is highly correlated with latitude presumably reflecting strong selection and local adaptation (Keller et al. 2011b); broad sense heritability of bud set in this common garden was estimated to be 0.83 (Olson et al. 2013), and Qct for bud set across three regions of *P. balsamifera*'s range was estimated to be 0.78 (Keller et al. 2011a). After sampling, leaves were immediately frozen in liquid nitrogen and stored at −80 °C. Within 1 week of sampling, total RNA was extracted using a Qiagen RNeasy Plant kit with the addition of 4 %*w*/*v* PVP-40 to the RLT extraction buffer. RNA was applied to MultiMACS columns to enrich mRNA prior to library preparation. Libraries were barcoded and pooled four per lane (with individuals from the same population arranged in one lane) prior to sequencing on Illumina Hi-Seq 2000 to produce a total of 36 billion 100-bp paired-end reads. Library preparation, labeling, and sequencing were conducted at the Michael Smith Genome Sciences Center (www.bcgsc.ca). Illumina reads were aligned to the annotated whole genome sequence of *P. trichocarpa* version 2.2 (www.phytozome.net/poplar.php) for quantification of mRNA using the "RNA-seq analysis" tool in CLC Genomics Workbench (CLC bio, Aarhus, Denmark). Reads from each of the 30 samples were aligned separately allowing a maximum of 2 mismatches and a minimum of 4 reads for exon discovery. Expression values were calculated based on the number of reads mapped to exons of the gene (Trapnell et al. 2012).

Differential expression

The R bioconductor package "edgeR" (McCarthy et al. 2012) was used to identify differentially expressed (DE) genes between the two developmental stages based on raw read counts per gene. This package is designed for multifactor replicated RNA-seq experiments and assumes that expression values have a negative binomial distribution; the within- and among-population variation within regions was addressed via a generalized linear model (GLM) approach. Genes with read counts <0.1 count per million (CPM) for seven or more individuals were removed prior to downstream analyses. A liberal false discovery rate (FDR) of 0.05 was used to identify DE genes.

Gene ontology (GO) enrichment tests were conducted using the web-based tool agriGO (Du et al. 2010) (bioinfo.cau.edu.cn/agriGO/analysis.php) with GO terms based on annotation of the *P. trichocarpa* genome. Within agriGO, *P* values were calculated using a hypergeometrical distribution with Hochberg FDR correction. For additional comparisons, and for comparisons with other studies, we grouped genes into six categories based on their assignment in previous studies on poplars (Andersson et al. 2004; Ruttink et al. 2007; Ibáñez et al. 2010; Rohde et al. 2011a, b): (1) light signal transduction, circadian clock, and CONSTANS/FT; (2) plant hormone-related; (3) cell proliferation; (4) adaptive response to biotic and abiotic stress; (5) metabolism pathway; and (6) transcription factors. Some of these gene categories, such as the first, reflect gene groups that are not represented as GO terms but have been identified as important signaling pathways for bud set.

SNPs

Single nucleotide polymorphisms among transcripts were identified using the Genome Analysis Toolkit (GATK) pipeline (DePristo et al. 2011), using the BAM files generated from Burrows-Wheeler Aligner (BWA) (Li and Durbin 2009). First-round SNP calling was conducted via Unified Genotyper in GATK to generate a raw VCF file. Second-round SNP calling assigned polymorphic sites in the first round as known, used a minimum phred-scaled confidence threshold of 20, and processed the BAM files through the Picard functions "AddOrReplaceReadGroups," "MarkDuplicates," "FixMateInformation," and "ValidateSam" (http://picard.sourceforge.net/) and the SAMTools function "index" (Li et al. 2009) using default values. Variant quality score recalibration (VQSR) was used to assign probability to each variant call based on a training data set, which contained only the shared SNPs called from both mpileup in SAMTools (Li et al. 2009) and Unified Genotyper in GATK. A 99 % of tranche truth sensitivity threshold was applied to select SNPs, which were further processed by a series of custom Perl scripts to filter for sites with >20 reads in >50 % of individuals in each population. This cutoff was set to increase the reliability of SNP calling at heterozygous loci and that the number of individuals in each population was sufficient for estimation of population differentiation indices.

Linkage disequilibrium (LD) was estimated as r^2 among all pairs of SNPs with a minor allele frequency >10 % within a window size of containing 50 SNPs in Tassel v. 3.0 (Bradbury

et al. 2007). This is based on the method of Hill (1974), which does not assume phase when calculating LD. LD decay was estimated by plotting the nonlinear relationship between r^2 and the physical distance between sites (DBS) (Eq. 1; Remington et al. 2001) using JMP 9 (SAS 2009).

Population differentiation and local adaptation

The statistical significance of allele frequency differentiation between latitudes (Fct) and among all populations (Fst) was calculated using a hierarchical model implemented in Arlequin v. 3.5 (Excoffier and Lischer 2010). Determination of Fst outliers tests for local adaptation, as local selection elevates differentiation among populations for locally adapted SNPs or genes compared to randomly chosen reference loci from unlinked regions (Lewontin and Krakauer 1973; Beaumont and Nichols 1996; Beaumont and Balding 2004). Lack of accounting of hierarchical population structure for species such as *P. balsamifera* with known structure (Keller et al. 2010), however, can elevate the incidence of false positives (Excoffier et al. 2009). Fst and Fct are related as $(1-\text{Fst})=(1-\text{Fct})\ (1-\text{Fsc})$, where Fst is an estimate of the overall among-population differentiation, Fct is an estimate of differentiation between latitudes, and Fsc is an estimate of differentiation between populations within latitudes (Wright 1969; Excoffier et al. 2009). We focused our analyses on Fct values because there is hierarchical structuring of the sampled *P. balsamifera* populations (i.e., geographically proximate populations are far more closely related to one another than they are to geographically distal populations). *P* values for *F*-statistics were determined by simulating 50,000 replicates of the coalescent using a two-group model (corresponding to the north and south regions, respectively) with 100 demes in each group (Excoffier et al. 2009) following Keller et al. (2012). Genes were declared significant for population differentiation if they contained at least one SNP showing significant differentiation ($P<0.01$) in excess of neutral expectation. We chose this liberal cutoff to ensure inclusion of the majority of genes with true population differentiation, while also recognizing that the false positive rate is likely high. In addition, we also report the numbers of genes with ≥3 SNPs to address the technical bias caused by the potential for genes containing more analyzed SNPs having a better chance to be declared as significant (SNP numbers were correlated with the lowest *P* value for each gene; correlation coefficient −0.34, $P<2.2e{-}16$).

Results

Differential expression

Reads per genotype ranged from ~32 to 210 million (average 120 million reads) (Supplementary Table 1), of which an average of 57.2 % (range 21.6−73.7 %) were mapped to the Nisqually-1 *P. trichocarpa* reference genome (Tuskan et al. 2006) with the coverage depth ranging from 21 to 214× (average 105×; Supplementary Table 1). For the differential expression analysis, 32,466 genes passed filter cutoffs (≥0.1 CPM reads in at least seven samples). The average coefficient of biological variation (BCV) of 0.601 indicated substantial differences in per gene steady-state mRNA levels (Supplementary Fig. 1).

A total of 4,018 genes were differentially expressed (DE) between actively growing trees (southern) and trees at bud set (northern, FDR<0.05) with ~65 % of these genes (2,594) more highly expressed in actively growing trees (Fig. 1; Supplementary Table 2; 1,612 genes (62.1 %) had >2-fold DE; 390 genes (15 %) had >4-fold DE), and 1,424 genes were more highly expressed in trees that had set bud (622 genes (43.7 %) had >2-fold DE; 110 genes (7.7 %) had >4-fold DE). Far fewer genes were DE between trees from the two southern (128 DE genes) or between the two northern (52 DE genes) populations, which were at similar developmental stages at the time of sampling.

Thirty-five GO classifications were overrepresented among genes more highly expressed in the trees that were actively growing; of these, 23 (65.7 %) were classified in the cellular components domain, with ribosome exhibiting the smallest FDR, a pattern consistent with robust protein synthesis related to growth in these trees (Fig. 2b; Supplementary Table 3). Forty-three GO classifications were overrepresented among

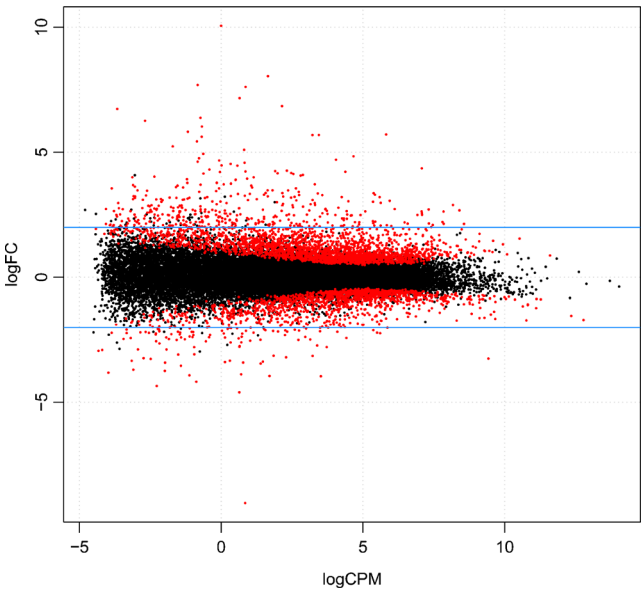

Fig. 1 Variance in differential expression between the two developmental stages of *P. balsamifera*. LogFC (logarithm of fold change)<0 represents genes overexpressed by northern genotypes, whereas logFC>0 represents genes overexpressed by southern genotypes. Genes exhibiting significant differential expression (FDR<0.05) are shown in *red*. *Horizontal blue lines* indicate the 4-fold change cutoffs. *LogCPM* logarithm value of counts per million reads (Color figure online)

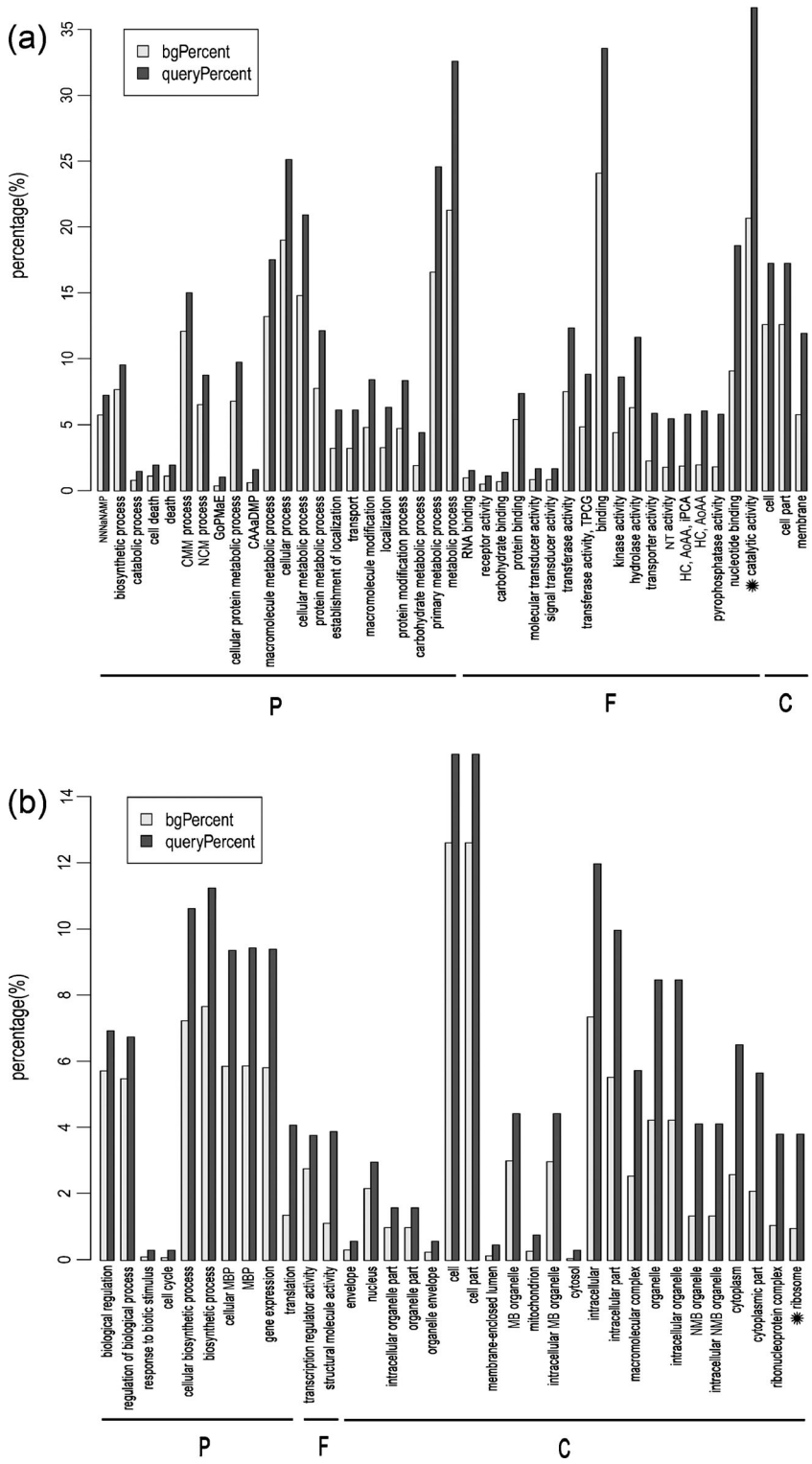

Fig. 2 Overrepresented gene ontology (GO) categories (FDR< 0.05) of **a** genes upregulated in the northern genotypes and **b** genes upregulated in the southern genotypes. The *vertical gray bars* indicate the percentage of *Populus* genes belonging to the specific GO term (bgPercent), whereas the *black bar* represents the percentage of genes in our samples that were categorized within each GO term (queryPercent). The *P* and FDR values of each GO term were described in Supplementary Table 3. The *starburst* indicates the category most highly overrepresented in the northern and southern genotypes. *C* cellular component, *F* molecular function, *P* biological process, *AoAA* acting on acid anhydrides, *CAAaDMP* cellular amino acid and derivative metabolic process, *CMM* cellular macromolecule metabolic, *GoPMaE* generation of precursor metabolites and energy, *HC* hydrolase activity, *iPCA* in phosphorus-containing anhydrides, *MB* membrane-bounded, *MBP* macromolecule biosynthetic process, *NCM* nitrogen compound metabolic, *NT* nucleoside-triphosphatase, *NMB* nonmembrane-bounded, *TPCG* transferring phosphorus-containing groups (Color figure online)

upregulated genes in trees that had set bud; of these, 22 (51.2 %) were classified in the biological processes domain, with catalytic activity exhibiting the highest significance, consistent with growth cessation as well as breakdown and reallocation of nutrients in northern trees (Fig. 2a, Supplementary Table 3).

Targets of latitudinal adaptation and overlap with differential expression

After filtering for read depth and evenness across populations (20+ reads per site from ≥50 % of individuals from each population), 304,668 high-quality biallelic SNPs representing

18,956 genes were selected for downstream analyses. For most chromosomes, r^2 did not drop below 0.2 until after SNPs were separated by ~7 kb (Supplementary Figs. 2 and 3; LD among all pairs of SNPs within a gene averaged 0.393 (95 % CI 0.389–0.397)).

Latitudinal differentiation in SNP frequencies was low (Fct=0.011) but about twice as large as the difference between populations within latitudes (Fsc=0.005 on average; including all populations Fst=0.016). Population differentiation estimated from the Arlequin hierarchical model (Excoffier et al. 2009) revealed that 15.7 % of sampled genes (2,983/18,981) contained at least one SNP with allele frequencies that were significantly diverged across latitude in excess of neutral expectation (Figs. 3 and 4; Fct>0.11, $P<0.01$); of these, 614 genes (3.2 %) had ≥3 significant SNPs. Catalytic activity was the top overrepresented GO term among genes with latitude-diverged SNPs (FDR=5.40E−13) (Supplementary Table 3, Supplementary Fig. 4). Genes with significant Fct SNPs were not overrepresented among DE genes; <2 % (358) of genes were both DE and had an SNP with significant Fct (Fig. 3), a number consistent with random expectations ($\chi^2=0.20$, $P>0.6$). As such, we find no evidence that DE genes are more likely to have been targets of selection than genes that are not DE.

The numbers of genes with an SNP with significant Fct differed among the six gene categories that we assessed (likelihood ratio $\chi^2=22.1$, $P>0.0005$) (Supplementary Table 5), but this was not the case for DE genes (likelihood ratio $\chi^2=8.0$, $P>0.15$) (Supplementary Table 4). Genes related to cell proliferation were more likely to exhibit differentiation in SNP frequencies across latitude than other categories (likelihood ratio $\chi^2=5.0$, $P<0.03$), whereas transcription factors were less likely to exhibit differentiation in SNP frequencies across latitude than other categories (likelihood ratio $\chi^2=10.4$, $P<0.002$). The DE genes within each category that exhibited SNPs with the highest Fct values are presented in Table 1.

Fig. 3 Venn diagram showing the numbers of differentially expressed (DE) genes between actively growing trees (southern genotypes) and those at early stage of winter dormancy (northern genotypes), numbers of genes showing elevated population differentiation (significant Fst), and numbers showing latitudinal differentiation (significant Fct)

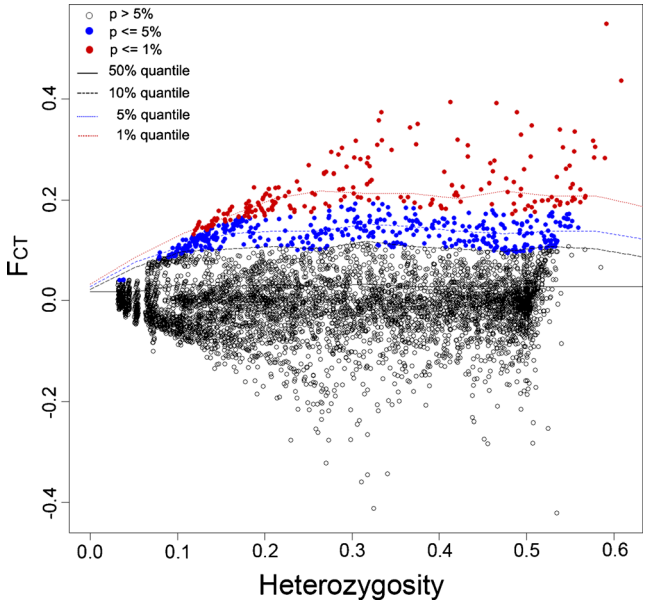

Fig. 4 Differentiation in allele frequencies between latitudes (Fct) for all SNPs on chromosome 19. SNPs significant at a 5 % critical value are plotted as *blue dots* and those significant at 1 % level are plotted as *red dots*. Other chromosomes exhibited similar patterns (Color figure online)

Discussion

Although the seasonal timing of entry into dormancy and its associated physiological changes remain one of the most striking and repeatedly identified adaptive clines in trees (Pauley and Perry 1954; Holliday et al. 2010b; Keller et al. 2011b; Savolainen et al. 2011), much remains unknown about the genetic changes responsible for generating these clines. Three main results are derived from this genome-wide assessment of the relationships between differential expression across growing season developmental stages and nucleotide differentiation across latitude in wild populations of balsam poplars. First, we did not detect that genes that are DE between active growing southern genotypes and northern genotypes at early stages of bud set in *P. balsamifera* are more likely to contain exons that are targets of local adaptation across latitude (significant Fct) than genes that are not DE. Second, among gene categories previously identified as important for the transition from active growth to dormancy (Ruttink et al. 2007), genes related to cell proliferation were more likely to exhibit elevated nucleotide differentiation across latitude and thus as candidate targets for local adaptation than other classes of genes. Finally, the differences in

Table 1 The three differentially expressed genes that contain SNPs showing the highest Fct values in each of five functional categories

Gene ID	Abbreviation	Function	NSS (NTAS)	Highest Fct	Mean Fct	logFC	FC	Category
POPTR_0007s08330	ABA3, ACI2, ATABA3, LOS5, SIR3	Molybdenum cofactor sulfurase (LOS5) (ABA3)	2 (6)	0.266	0.196	−0.630	1.548	Plant hormone
POPTR_0001s38760	ATGA2OX1, GA2OX1	*Arabidopsis thaliana* gibberellin 2-oxidase 1	1 (3)	0.213	0.213	−1.380	2.603	Plant hormone
POPTR_0019s15190	CRF4	Cytokinin response factor 4	1 (8)	0.209	0.209	−0.717	1.644	Plant hormone
POPTR_0017s11710	–	NAP1-related protein 2	1 (14)	0.297	0.297	0.704	1.630	Cell proliferation
POPTR_0006s29400	–	DNA topoisomerase, type IA, core	1 (10)	0.226	0.226	−0.635	1.553	Cell proliferation
POPTR_0003s01440	–	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	1 (16)	0.218	0.218	0.748	1.679	Cell proliferation
POPTR_0005s11600	F3H[a]	Flavanone 3-hydroxylase; naringenin 3-dioxygenase	3 (19)	0.608	0.347	0.802	1.743	Adaptive response
POPTR_0008s13590	ATTPS6, TPS6	UDP-glycosyltransferase/trehalose-phosphatase family protein	2 (10)	0.366	0.328	−0.553	1.467	Adaptive response
POPTR_0006s13070	–	–	2 (25)	0.336	0.351	0.610	1.526	Adaptive response
POPTR_0005s11600	F3H[a]	Flavanone 3-hydroxylase; naringenin 3-dioxygenase	3 (19)	0.608	0.347	0.802	1.743	Metabolism pathway
POPTR_0016s08310	ATBBC1	*Arabidopsis thaliana* breast basic conserved 1; structural constituent of ribosome	2 (7)	0.573	0.550	1.665	3.172	Metabolism pathway
POPTR_0017s13240	FER	Malectin/receptor-like protein kinase family protein	1 (43)	0.536	0.536	−0.863	1.819	Metabolism pathway
POPTR_0006s23390	JAZ12, TIFY3B	Jasmonate-zim-domain protein 12	1 (12)	0.343	0.343	0.606	1.522	Transcription factor
POPTR_0001s11100	ATMYB111, MYB111, PFG3	MYB domain protein 111	3 (12)	0.323	0.260	1.786	3.448	Transcription factor
POPTR_0007s01230	ATGLK1, GLK1, GPRI1	GBF\'s pro-rich region-interacting factor 1	4 (31)	0.232	0.195	−0.727	1.655	Transcription factor

These gene categories were identified in previous studies (Andersson et al. 2004; Ruttink et al. 2007; Ibáñez et al. 2010; Rohde et al. 2011a, b). Highest Fct indicates the highest Fct achieved by an SNP within the gene. Mean Fct indicates the mean Fct across all SNPs

FC fold change, *NSS* number of significant SNPs, *NTAS* number of total analyzed SNPs

[a] F3H was included in two functional categories

expression that we observed were similar to those observed in studies performed in more controlled environmental settings and on different *Populus* species (Jeukens and Bernatchez 2012; Le Provost et al. 2012; Cooke et al. 2012; Ekblom et al. 2012; Bougas et al. 2013), suggesting that these gene networks are both evolutionarily relatively stable and are robust to environmental conditions.

Relationships between gene expression variation and adaptive population differentiation

Several across- and within-species comparisons in humans and *Drosophila* have reported significant associations between variation in gene features (i.e., nucleotide plus other classes of variation) and gene expression (Nuzhdin et al. 2004; Lemos et al. 2005; Khaitovich et al. 2005; Lawniczak et al. 2008), suggesting that *cis*-acting regulatory elements are commonly responsible for differentiation in expression. Nonetheless, studies in yeast, *Drosophila*, and whitefish found that expression divergence was largely uncoupled from protein divergence (Larracuente et al. 2008; Tirosh and Barkai 2008; Jeukens et al. 2010). A comparison of domesticated and progenitor species of *Zea mays* has revealed significant overlap between genes showing differential expression between species and genes bearing signatures of selection (Swanson-Wagner et al. 2012). We found no support for a significant association between expression and coding sequence differentiation across latitude in *P. balsamifera*.

There are several possible explanations for this lack of association. The most likely explanation is that the genes with differential expression are overwhelmingly downstream of genes responsible for differences in the timing of initiation into the early stages of dormancy. In other words, the assayed genes reflect the phenotype and not the genetic changes responsible for differences in timing of bud set. Even if some of the genes showing DE cause differences in the timing of bud set, it is quite likely that the nucleotide changes responsible for DE are in *cis*-regulatory regions, and these may not have been high enough in LD with coding regions to have been identified through our Fct scan. It is also possible that adaptive timing of gene expression is regulated by changes in methylation caused by transacting factors (Scoville et al. 2011; Conde et al. 2012). Finally, our selection analyses may have had limited power to identify targets of adaptation—either because selection acted on standing variation generates weaker among-population differentiation in linked sites than hard selection does (Raquin et al. 2008; Pritchard et al. 2010; Hohenlohe et al. 2011) or because of the limited sample sizes in this study.

Targets of local adaptation

Genes in the light signal transduction and circadian clock pathways have garnered most of the attention as candidate targets of local adaptation across latitude (Ingvarsson et al. 2006; Keller et al. 2012; Olson et al. 2013). For this reason, we were surprised that among the 2,983 out of 18,981 genes that were identified as harboring SNPs with elevated latitudinal differentiation consistent with local adaptation, the most commonly identified gene category was cell proliferation (21.2 %). This result suggests that cell cycle regulation may be an underappreciated factor allowing species range expansion and latitude-related climate adaptation in trees. Cell cycle regulation may be important because it allows for earlier growth cessation in the north, which also is a key developmental step leading to cold acclimation that is associated with the onset of dormancy, or in northern climates, growing seasons are short and higher daily growth rates may be selectively advantageous (Soolanayakanahally et al. 2009).

The results of our Fct-based analyses of local adaptation come with several caveats that limit the completeness of our analyses. First, because we used RNA-seq data, we could only analyze data from genes that were expressed in both northern and southern populations. Second, similar to most if not all genomic data sets, duplicated genes or genes with copy number variation (CNV) among populations would confound assemblies and align multiple paralogs to a single reference gene. We have previously found evidence for extensive CNV in a poplar protease inhibitor involved in pathogen defense (Neiman et al. 2009), but the extent to which CNV is segregating for latitudinal clines related to growth phenology is not known. Finally, our analyses are based on a relatively small number of individuals which limits our statistical power to detect SNPs and genes that are differentiated among populations. On the other hand, because of this small sample size, we used a liberal criterion for identifying putative targets of selection (i.e., SNP-based tests with $P<0.01$), and it is possible that these criteria allowed false positives to dilute true signals of adaptation.

Phenotypic status as estimated by gene expression

The *P. balsamifera* genotypes that were actively growing (southern genotypes) did not exhibit elevated expression of genes identified by Ruttink et al. (2007) as characterizing the first transition in expression profiles after short-day exposure (e.g., upregulation of *PIF4* and downregulation of *SHY2*), indicating that the southern genotypes likely had not yet experienced a critical photoperiod required to initiate bud formation on the late July sampling date. These actively growing genotypes did, however, exhibit elevated expression of a suite of genes identified in *Arabidopsis* as contributing to onset of flowering (Mouradov et al. 2002; Böhlenius et al. 2006; Ruttink et al. 2007; Jackson 2009; Lagercrantz 2009; Flowers et al. 2009; Horvath 2009; Kemi et al. 2013), including homologs of *Arabidopsis ELF3*, *FLC*, and *PHYA*. Notably, *CAULIFLOWER*, a paralog of *AP1* that is expressed in young flower primordial of *Arabidopsis thaliana* (Kempin et al. 1995), exhibited the strongest differential expression

among all genes, with 16-fold overexpression by southern genotypes (Supplementary Tables 2 and 4).

The downregulation of a majority of cell proliferation genes in northern genotypes was consistent with growth cessation and dormancy initiation. DE genes discovered in our study, such as homologs of *Arabidopsis ANT*, *CYCA1*, *CYCB2*, *CYCD3*, *CYCP4*, *PCK1* encoding phosphoenolpyruvate carboxykinase 1, and *H2B* encoding core histones in the nucleosome assembly (Bhasin et al. 2006), were also reported in Ruttink et al. (2007) during growth cessation and dormancy initiation. We also identified several additional DE genes, including homologs of *Arabidopsis* NAP1-related protein 2 (*NRP2*), a chaperon for *H2B* that has been reported as influencing the arrest of cell cycle progression at G2/M in *Arabidopsis* (Zhu et al. 2006), and a few members of the homologs of *Arabidopsis CLAVATA* and *WUSCHEL* gene families (*CLAVATA3/ESR-related 16 & 26*, *WOX1*, *WOX4*, and *WOX5*), which play crucial roles in the proliferation of undifferentiated cells of shoot meristems and are required for maintenance of growth (Clark 1997; Horvath et al. 2003) (Supplementary Tables 2 and 4).

Several genes in the adaptive response to stress category were also DE including ABA-dependent stress response genes, components of cold tolerance pathways, and gene coding for cell wall-modifying proteins. ABA-dependent genes such as homologs of *Arabidopsis* genes encoding universal stress protein (USP), early-responsive to dehydration (ERD), and heat shock protein (HSP) usually exhibited increased expression in northern genotypes, a pattern that is consistent with previous results (Ruttink et al. 2007). Homologs of *Arabidopsis CBF1* and *ESK1*, both components of cold tolerance pathways (Xin and Browse 1998; Thomashow 1999), exhibited more than 2-fold greater expression in northern than southern genotypes, but neither was reported as DE across the different time point of short-day length treatment by Ruttink et al. (2007). Several gene coding for cell wall-modifying proteins which mediate plant acclimatization to biotic and abiotic stresses (Sasidharan et al. 2011), such as homologs of *Arabidopsis* expansins (all located in the cell wall), xyloglucan endotransglucosylase/hydrolase (XTHs), endo-β-1,4-glucanases (EGases), and pectin methylesterases (PMEs), exhibited significantly different expression between northern and southern genotypes, while they were not revealed as DE in Ruttink et al. (2007). For example, *Arabidopsis THALIANA EXPANSIN 11* (*ATEXPA11*; >8-fold change), *ATEXPB3* (>2-fold change (FC)), *EXPANSIN-LIKE B2 PRECURSOR* (*EXLB2*; >2 FC), two *EXLB3* (>2 FC), and *EXPANSIN A5* (*EXPA5*; <2 FC) were all downregulated in northern populations, which were consistent with chilling-induced reduction in transcript levels of the expansin genes in sweet potato (Noh et al. 2009). Decreased expression of these genes is thought to enhance cell wall extensibility and increase freeze tolerance (Sasidharan et al. 2011). Finally, homologs of *Arabidopsis FATTY ACID DESATURASE 2* (*FAD2*) exhibited 3-fold decreased expression in northern than southern genotypes, which echoes previous report on its role of freezing tolerance in poplars (Zhou et al. 2010).

Conclusions

Latitudinal clines in the timing of bud set and dormancy initiation with respect to photoperiod have evolved independently among multiple tree species and likely have evolved multiple times in the same species in response to glaciation and deglaciation events of the Pleistocene (Savolainen et al. 2011; Breen et al. 2012). Understanding the genes as well as the adaptive history of genes controlling the timing of seasonal dormancy initiation traits is an important goal for tree genetics, as it will provide insight into the nature of quantitative trait adaptation and identify genes with potential economic value. Reaching this goal will require integration of results from complementary experiments using varied designs and sampling methods. Here, we have documented expression phenotypes for actively growing southern genotypes and northern genotypes that were initiating dormancy for *P. balsamifera* growing in a common garden in Fairbanks, Alaska. Many genes that were DE across the developmental times also exhibited exon SNP frequencies that differed across latitude, a pattern consistent with local adaptation, but there was no significant association between expression and local adaptation of exons. The large number of genes identified suggests that latitudinal clines in forest trees are likely governed by many gene variants, most of which have small effects, whereas the lack of association suggests that the types of changes required for local adaptation in expression are varied, including both *cis*-acting and *trans*-acting factors.

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