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Comparative Analysis of Expressed Sequence Tag from *Picea abies* L. to Identify Dormancy Regulation-Implicated Genes in the Apical Meristem

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Abstract

The molecular basis of the plant meristem's dormancy-release is a sophisticated process and poorly understood. To find genes related to the release of the dormancy of the *P. abies* apical meristem, an expressed sequence tag analysis was used. The preliminary data for two cDNA libraries was gathered using the Harvard University database (Dormancy and dormancy-release libraries with 6987 and 6981 EST, respectively). The EGassembler software was used to assemble all EST sequences in order to find similarity between two libraries. After that, all contigs were processed using CLC bio software's X-blast against a non-redundant protein database. To detect genes with differential expression in two libraries, the IDEG6 software and the Audic-Claverie test were utilized. The GoMapMan comparative classification tool was used to categorize functional catalogs. All unigenes were grouped into 35 functional catalogs, of which 10 significantly different functional catalogs were identified, including major CHO metabolism, hormone metabolism, stress, transport, secondary metabolism, cofactors and vitamins, nucleotide metabolism, redox-regulation, mitochondrial electron transport/ATP synthesis, and fermentation. So far, there has been no report on the role of secondary metabolites in regulating plant meristem dormancy. This study provides insight into the probable function of secondary metabolites as major regulators of the apical meristem's dormancy in *P. abies*. In addition, redox and epigenetic changes downstream of hormones also appear to be involved in dormancy regulation. The potential of ROS specificity in terms of the spatio-temporal properties that characterize the expression of antioxidant enzymes allows them to be used as biomarkers in major developmental stages to develop a set of features in woody species that promote growth, wood, and fiber attributes. This research also provides information about the molecular mechanisms of the morphogenesis process in Norway spruce.

Keywords: Comparative analysis, Dormancy-release, Functional catalogs, ROS specificity, Secondary metabolites.

INTRODUCTION

P. abies L. Karst (2n = 24) is a non-native coniferous species that is planted for both ecological and economic reasons, mainly in northern Iran. It is widely utilized in the wood industry for timber construction, pulpwood for paper and furniture, as well as ornamental, due to its rapid development. Because of its shallow root system, *P. abies* is extremely vulnerable to heat and drought when planted in unsuitable provenances. Moreover, the most serious threats to *P. abies* are root rot and bark beetles (Skrøppa, 2003), so yield is expected to be severely affected. Spruce stands, on the other hand, have limited regeneration potential, making them less desirable for reforestation, and tree-breeding programs move slowly due to extended maturity times (Břiza *et al*., 2013). Biotechnological techniques provide numerous new ideas for speeding up advances in commercially important plant characteristics (Heidari *et al*., 2020). Regeneration of *P. abies* can be performed with the proper use of *in vitro* specific explants and has therefore been the subject of empirical research for decades (Lin *et al*., 2011). One of the most efficient techniques for *P. abies* micropropagation appears to be regeneration from meristematic tissues.

Plants have the unique ability to develop and produce new organs during their lifecycle owing to the activities of populations of pluripotent stem cells within their meristematic tips, known as the shoot apical meristem (SAM). Stem cells play a pivotal role in multicellular organisms' growth and development. There is a need to suppress cell proliferation and induce an inactive dormant state in perennials. So that the meristem is insensitive to signals known as growth-promoting for a period before becoming released and restarting growth. This fact is one of the most prominent aspects of the perennial lifetime of plants (Rohde and Bhalerao, 2007). Perennials are the only plants that grow in a periodic pattern, and their investigation might reveal meristem traits that are missing or less amenable to scientific investigation in annuals. The temporary transition during dormancy and reactivation is a complex process that has gained less investigation in spite of its significance for perennial characteristics that determine general vitality and annual productivity. Genetic regulatory changes, physiological and morphological control the transition (Ueno, 2013). While much progress has been made in identifying the molecular factors and physiological aspects of dormancy, fewer investigations have studied gene and protein expression patterns during meristem dormancy-release using microarray and proteomics analyses.

Using genomics and proteomics analyses, Xu *et al*. (2016) studied 132 proteins and 20 genes that were differently expressed in the shoot apical meristem in dormancy and release phases in *Cunninghamia lanceolata*. They observed that in the release stage, the gene expression linked with cell division and expansion rose dramatically, but the expression of the genes connected with the cytoskeleton, energy metabolism and abscisic acid insensitive 3 (ABI3) was substantially reduced during the dormant stage. Karlberg *et al*. (2010) propose that chromatin remodeling may be implicated in organizing ubiquitous gene expression levels in the hybrid aspen apex's activity-dormancy cycle and that transcriptional regulation may be important in hormone modulation such as ABA and GA using microarray techniques. Genomic tools, such as the EST technique, have been demonstrated to be effective, comparatively cheap, rapid, and powerful means of identifying novel genes regulated by ambient and developmental changes and clarifying the biological functions of a significant number of genes whose functions are not fully understood, particularly in organisms lacking reference genomic data (Swarbreck *et al*., 2011).

ESTs are short sequences chosen at random from a cDNA library and sequenced and then deposited to a data bank, providing a database for researchers worldwide (Marques *et al*., 2009). Heidari *et al*. (2022) performed EST analysis of data from the Harvard University database to identify the most influential genes in the evolution of Brasica species and used IDEG6 software to identify genes with differential expression between libraries. They also classified annotated genes

into functional groups through the Mapman tool. There have been very few EST analysis studies based on large-scale cDNA sequencing to study the expression of genes in *P. abies* dormancy-release. Therefore, the comparative EST method was used to analyze the influential expression of genes in dormancy-release in the *P. abies* meristem, contributing to discerning the mechanisms that control SAM activation in *P. abies* species.

MATERIALS AND METHODS

Dormancy library data with 6987 EST (#lcl) and dormancy release library data (#lck) with 6981 EST from the *P. abies* apical meristem were gathered from the Harvard University database. The DFCI gene index web site contains ESTs and gene sequences for over 114 species. (http://compbio.dfci.harvard.edu/tgi/) (Antonescu *et al*., 2010). The characterization of the libraries is "as follows." Plants were grown and fertilized for 42 days under long day (18 h. light/6 h. dark) conditions at a temperature of 23 °C in the greenhouse. After 42 days, the plants were relocated to a climate container with a temperature of approximately 15 °C for short-day behavior (8 h. light/16 h. dark). After 35 days of short-day behavior, the temperature was reduced to 10 °C for 77 days.

Plants were exposed to a low temperature of 6 °C for 28 short days to promote dormancy release. At the time of dormancy and the first visible signs of dormancy release, samples from the plants' shoot apices were gathered. Group samples were promptly frozen in liquid nitrogen and stored at -80 °C for RNA extraction and cDNA library construction. Sequencing procedures were performed after the uncut cDNA library was generated according to the directions. The sequences were then deposited in the Harvard University database. In the preprocessing step, poor-quality sequences, or sequences with less than 100 bases, and vector sequences were trimmed from the raw single-pass sequences using the VecScreen database (https://www.ncbi.nlm.nih.gov/tools/vecscreen/) with N≥95 as a percentage of cutoff matching (Schäffer *et al*., 2018). In the processing step, the cDNA sequences were assembled into clusters using EGassembler software (http://EG assembler.hgc.jp/) for constructing contigs with the parameters set at 95% identity over 40 base-pairs. EST sequences in contigs with two or more ESTs and singletons with just one EST were divided in two groups (Masoudi Nejad *et al*., 2006). All EST sequences were assembled by the EGassembler software to find similarities between the two libraries. All contigs were then analyzed using X-blast by CLC bio protein workbench software against a non-redundant protein database with an E-value$\leq 10^{-5}$. To find genes with differential expression between libraries, researchers employ the IDEG6 program (http://telethon.bio.unipd.it/ideg6) and Audic-Claverie statistics (Romualdi *et al.,* 2003). The library's contings and singleton sequences were analyzed using the X-blast program against the *Arabidopsis* information resource downloaded from the TAIR database (ftp://ftp.arabidopsis.org) (Bassel *et al*., 2011).

The GoMapMan applied comparative classification tool (http://www.gomapman.org) was used to categorize functional catalogs. In the plant sciences, GoMapMan is an open, web-based resource for gene functional annotations. It was designed as a way to organize, integrate, and visualize gene annotations across a wide range of plant species (Ramsak *et al*., 2014). Mapman outputs are used to define a variety of library catalogs that can be beneficial in a number of different experiments. The Audic-Claverie test and IDEG6 software were used to find functional differential catalogs in libraries. The identified sequences were classified into three categories of GO gene ontologies (biological, cellular, and molecular).

RESULTS AND DISCUSSION

Sequential phases of dormancy and its release characterize the annual growth cycle of perennials. Plant dormancy modulation is a sophisticated mechanism that is influenced by a variety

of genetic and environmental variables. In perennial plants, short-day exposure causes growth to stop and dormancy to occur at the SAM, increasing chilling tolerance for survival. Chilling after that activates the SAM and enhances its chilling sensitivity even further (Shim *et al*., 2014). Gene expression adjustment specificity is requirefor the dormancy establishment and its release, which is initiated on a short day and is continued by chilling. In this research, EST analysis was performed in two *P. abies* libraries to identify and compare changes in gene expression during dormancy-release. Results showed that 970 and 1084 contigs and 3396 and 2995 singletons were formed after assembling 6987 and 6981 ESTs by EGassembler software in dormancy and dormancy release libraries, respectively (Table 1).

Table1. The number and result of analysis of EST sequences.

Library	Dormancy (D)	Dormancy release (DR)	Total number
Total EST	6987	6981	13968
Total contig	970	1084	2054
Number of EST in contig	3591	3986	7577
Singleton number	3396	2995	6391
Contigs with no certain hit	260	375	635
Singletons with no certain hit	634	693	1327

6391 ESTs failed to be assembled according to software's assembly parameters in two libraries and were considered unique ESTs in this set of data. 2470 ESTs (35.36%) and 2319 (33.23%) have a weak proportion (E-value>10^{-5}) in homology with sequences against the *Arabidopsis* database, or there were no sequences similar to them in dormancy and dormancy release libraries, respectively, so they were considered as new genes. The remaining ESTs had moderate to high homology (E-value<10^{-5}). Mapman classification revealed that nearly 77.51% and 78.40% of unigenes were classified into functional catalogs with certain activities in dormancy and dormancy release libraries, respectively (Fig. 1).

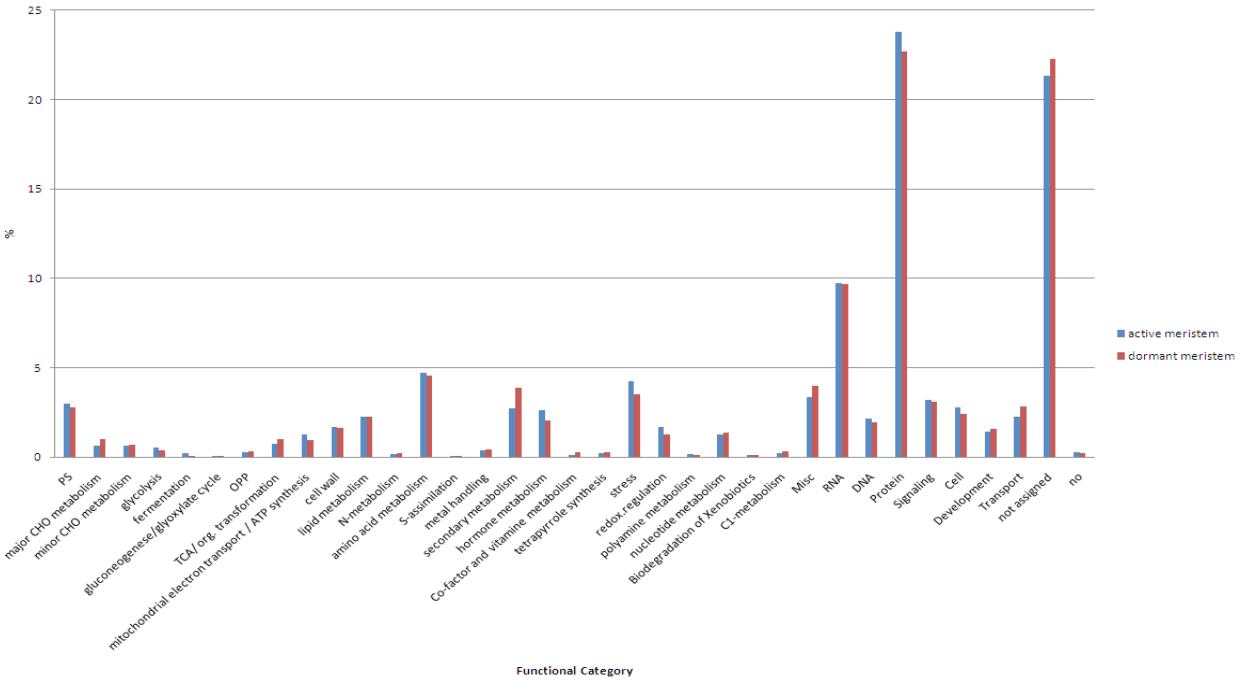

Fig. 1. Distribution of functional catalogs in libraries.

The unigenes were categorized into 35 functional groupings based on their biological process annotations. For ten different functional catalogs, including major CHO metabolism, cofactors and vitamins, hormone metabolism, transport, nucleotide metabolism, secondary metabolism, stress, redox-regulation, mitochondrial electron transport/ATP synthesis, and fermentation, the Audic-Claverie statistic showed significant differences between two libraries (Table 2).

Table 2. Differences between functional catalogs of the two libraries.

UNIQID description	DR	D	E-value
Major CHO metabolism	41	69	0.0298*
Fermentation	12	4	0.0279*
Mitochondrial electron transport/ATP synthesis	79	64	0.0447*
Secondary metabolism	170	266	0.0002*
Hormone metabolism	163	141	0.0327*
Co-factor and vitamin metabolism	6	18	0.0266*
Stress	265	244	0.0385*
Redox-regulation	104	88	0.0500*
Nucleotide metabolism	79	93	0.0442*
Transport	140	195	0.0318*

*: Significant at the 5% probability levels.

Different genes between libraries were observed from the same different functional categories as well as genes related to oxidative stress, cell wall synthesis, signalling, protein degradation, cell cycle, transcription factors, chromatin remodeling and histone synthesis. Different expressions between libraries using IDEG6 software are given in table 3.

Several phytohormones, including abscisic acid (ABA) hormone, gibberellin (GA) hormone, and cytokinins (CK) hormone, have been found to play a role in the transition from a dormant to an active state in *P. abies*. Hormones commonly cause dormant-to-active transitions by modulating gene expression patterns (Depuydt and Hardtke, 2011). EST assigned to the ABA production enzyme, 9-cis-epoxycarotenoid dioxygenase (NCED), was found to be upregulated in dormancy in the current study. NCED plays a significant role in ABA synthesis. There is ample report that ABA biosynthesis is related to the dormancy cycle in numerous species, including grapevine, pear, and peach. It is well accepted that one of the ABA's main roles is to restrict development and initiate organ abscission (Zheng *et al*., 2015; Li *et al.,* 2018; Finkelstein, 2013). As a result of ABA's function in the meristem's growth arrest, the meristem will inevitably establish dormancy (Cooke *et al.*, 2012).

As mentioned below, ABA is expected to have some effects on growth arrest through several ways. Besides, EST assigned to callose synthase was up-regulated, and (1-3)-beta-glucanase was down-regulated in the dormant state. Callose is a polysaccharide found in the cell walls of a wide range of higher plants in the form of a 1,3-glucan with some 1,6-branches. The enzymes -1,3-glucanase (BG) and callose synthase regulate the level of callose in the plant cells. BG is a hydrolytic enzyme that can catalyze the endo-type cleavage of 1,3-β-D-glucosidic linkages into single β-1,3- glucan units. In the dormant state, ABA-mediated down-regulation of BG was identified to be a major cause of enhanced callose deposition (Oide *et al*., 2013). According to a recent study, ABA has a role in bud dormancy via modulating callose generation. In the ABA-insensitive mutant abi1-1, the dormancy's photoperiodic control is disrupted, which in response to short days, does not produce plasmodesmata (PD) sphincters, which is related to downregulation of transcripts involved in callose synthesis and PD degradation (Tylewicz *et al*., 2018). In plants, despite the role of callose, the underlying mechanism of ABA-mediated callose deposition in PD, which might be an essential process in the plant apical meristem's dormancy induction, has only recently begun to be discovered, peculiarly in *P. abies*.

Table 3. Most significant statistically difference contigs between libraries using ideg6 software.

Unisequence	Putative identity (MapMan)	Cellular component	Biological process	D	DR	p-value
Contig 102	9-cis-epoxycarotenoid dioxygenase (NCED)	Chloroplast stroma	Abscisic acid biosynthetic process	5	2	$1E^{-05}$
Contig 70	Callose synthase	Membrane	(1-3)-beta-D-glucan biosynthesis	9	4	$2E^{-18}$
Contig 324	(1-3)-beta-glucanase	-	Carbohydrate metabolic process	2	5	$5E^{-45}$
Contig 578	Cyclin-dependent kinase B	Nucleus	Cell cycle	3	7	$3E^{-21}$
Contig 125	Cyclin-dependent kinase inhibitor	Nucleus	Cell cycle arrest	6	2	$1E^{-27}$
Contig 14	Gibberellin 2-oxidase	Cytoplasm; nucleus	Gibberellic acid homeostasis	2	8	$1E^{-91}$
Contig 69	Peroxidases	Extracellular region	Response to oxidative stress	1	7	$2E^{-20}$
Contig 189	Amine oxidase	Extracellular region	Abscisic acid-activated signaling	2	8	$1E^{-05}$
Contig 457	Glutathione peroxidase	Cytosol	Response to oxidative stress	1	6	$2E^{-05}$
Contig 199	Catalase	Cell wall	Response to oxidative stress	3	5	$1E^{-09}$
Contig 627	Respiratory burst oxidase protein 2a	Membrane	Peroxidase activity	2	4	$2E^{-11}$
Contig 219	Respiratory burst oxidase protein 2b	Membrane	Peroxidase activity	1	4	$3E^{-06}$
Contig 443	Sucrose-phosphate synthase	Cytosol; plasma membrane	Sucrose biosynthetic process	4	9	$6E^{-17}$
Contig 681	Glycosyltransferase	Golgi membrane	Cell wall organization	2	5	$1E^{-28}$
Contig 23	Adenylate isopentenyltransferase	Cytoplasm	Cytokinin biosynthetic process	3	7	$3E^{-05}$
Contig 730	Methyltransferase	Integral component of membrane	Methylation	6	1	$1E^{-90}$
Contig 87	Histone deacetylase	Nucleus	Histone deacetylation	4	0	$3E^{-73}$
Contig 964	E3 ubiquitin protein ligase	Nucleus	Ubl conjugation pathway	5	1	$2E^{-16}$
Contig 908	Transcriptional activator DEMETER-like	Nucleus	DNA demethylation	1	4	$3E^{-08}$
Contig 391	Late embryogenesis abundant protein	Cytosol	Response to abscisic acid	14	9	$3E^{-90}$
Contig 171	DREB1/CBF transcription factor	Nucleus	Transcription regulation	4	1	$6E^{-05}$
Contig 74	APETALA2-related transcription factor 2	Nucleus	Transcription regulation	2	0	$4E^{-05}$
Contig 849	Heat shock proteins	Nucleus	Response to chilling, protein refolding	7	4	$4E^{-60}$
Contig 608	Basic leucine zipper transcription factor	Nucleus	Regulation of transcription	5	2	$2E^{-78}$
Contig 462	calmodulin	Cytosol	Calcium-mediated signaling	3	8	$6E^{-89}$
Contig 275	Isoflavone synthase	Membrane	Isoflavone biosynthesis	16	4	$1E^{-05}$
Contig 716	Shikimate kinase	-	Chorismate biosynthetic process	5	1	$9E^{-05}$
Contig 5	Phospho-2-dehydro-3-deoxyheptonate aldolase	Chloroplast	Thiamine biosynthetic process	5	3	$5E^{-12}$
Contig 784	1-deoxy-D-xylulose-5-phosphate synthase	-	Thiamine biosynthetic process	3	0	$6E^{-09}$
Contig 11	Dihydrofolate reductase-like	Cytosol	One-carbon metabolic process	4	1	$2E^{-06}$
Contig 928	V-ATPase 69 kDa subunit	Plasma membrane	Transport	3	5	$5E^{-18}$
Contig 799	Bidirectional sugar transporter SWEET	Cell membrane	Carbohydrate transport	7	4	$3E^{-09}$

Annotation and expression levels of the most different expressed EST in the libraries. The columns D and DR shows the number of ESTs for each unisequence in dormancy and dormancy release libraries, respectively. The p-value refers to the Audic-Claverie statistics for differential expression.

Another mechanism through which ABA can regulate dormancy is ABA-mediated suppression of cell cycle genes, which is usually induced via short-day. In the present study, while EST annotated to cyclin-dependent kinase B was downregulated, cyclin-dependent kinase inhibitor was upregulated under dormancy. Recently, in grapevine, Vergara *et al*. (2017) discovered that ABA decreases CDK expression (e.g., VvCDKB1 and VvCDKB2) while increasing ICK expression (e.g., VvICK5). Wang *et al*. (1998) found that ABA induces ICK1, implying a link between a phytohormone correlated to dormancy and a protein that prevents transit into the cell cycle (Wang *et al*., 1998).

The EST was attributed to gibberellin 2-oxidase, which is concerned with the production of gibberellin (GA), was enhanced during dormancy release. It has been suggested that chilling can activate genes of GA synthesis at the shoot apex in the activation of dormancy (Zanewich and Rood, 1995). High expression of A2ox, reported by Zawaski *et al*. (2011), promotes rapid dormancy release. Furthermore, during dormancy release ESTs implicated in antioxidant defense and detoxifying processes were enhanced. The effect of GA may lead to significant expression of genes implicated in these two pathways. It has been shown that GA effects on dormancy release through different pathways. GA has been demonstrated to enhance reactive oxygen species (ROS) generation, which is essential for bud release (Zhuang *et al*., 2015). The antioxidant defense machinery is related to ROS scavenging in plant cells, and ROS generation is a consequence of redox-regulation. In the current investigation, an overall increase in gene expression implicated in antioxidant enzymatic activities was observed in dormancy-releasing conditions. In controlling ROS levels, dormancy release resulted in large synthesis of peroxidases, amine oxidase, and glutathione peroxidase (GPX) activities, along with modestly enhanced catalase activities.

In the superfamily of GPX, many enzymes are subcellular localized differently as well as tissue-specific expression patterns harboring various ambient stress responses. Presently, the function of GPX superfamily in plants is not totally understood. According to Bela *et al*. (2015) research, plant GPXs protect cells from stress-induced oxidative damage, whilst they may also promote processes of growth and development. Polyamines are a group of compounds that play a role in plant growth by promoting cell proliferation. The oxidative deamination of polyamines is catalyzed by amino oxidases. Amine oxidases engage in various physiological processes via their reaction products, for instance by cellular polyamines degradation and so helping to polyamine homeostasis. The generation of H_2O_2 derived of polyamine oxidation is linked to the maturation of the cell wall and the lignification in plants' development, and is also a signal molecule that promotes the expression of defense genes. Polyamine oxidation derivatives have been related to secondary metabolite synthesis and abiotic stress tolerance (Cona *et al*., 2006). Peroxidases are involved in a variety of cell functions in plants, i.e., stress responses and development. They modulate hormone metabolism and organization of the cell wall, as well as antioxidant defense, to promote growth regulation (Jouili *et al.*, 2011).

Furthermore, in this study, ESTs annotated to NADPH oxidase/respiratory burst oxidase proteins homolog (RBOH) 2a and 2b were found to be upregulated when dormancy is released. RBOH proteins mediate diverse processes viz. stress responses, growth, as well as developmental in plants by releasing localized ROS bursts (Chapman *et al*., 2019). NADPH oxidases (RbohA and RbohB) were found to be expressed in potato when dormancy was released (Liu *et al*., 2017). Diverse activity signatures of ROS with variable temporal dynamics and, as a result, different oxidative stress-related enzymatic responses could be the result of hormonal and ambient influences. According to Liu *et al*. (2017), when O_2 levels are suppressed, tuber bud development is postponed, and potato tuber dormancy is released when treated with exogenous H_2O_2. This demonstrates that multiple ROS perform distinct functions as the potato tuber dormancy progresses. Likewise, Zafra

et al. (2010) found that the level of H_2O_2 fluctuates actively during the process of bud formation by fertilization in olives, implying that the disparity in H_2O_2 level has essential physiological relevance for the development of distinct organs. Based on these results, the present study suggests that distinct activity signatures of ROS may have been related to development state and the maintenance or release of dormancy. As a result, studying ROS metabolism during the dormant-active meristem transition may become increasingly crucial for monitoring and predicting plant performance in many aspects of morphogenesis, particularly growth and development of cells cultured in vitro.

Despite the change in the expression of more genes related to the major CHO metabolism in the meristem's dormancy (Table 2), the expression of sucrose phosphate synthase and glycosyltransferase genes increased in dormancy release. The increased expression of these genes may arise from the GA hormone leading to dormancy release, as shown by Zhuang *et al*. (2015) in *P. mume*, where GA_4 treatment resulted in the activation of a number of energy metabolism pathways. GA-induced soluble sugars may function as a source of energy for releasing dormancy in light of the aforementioned. By serving as osmoprotectants, even soluble sugars may effectively protect against damage caused by chilling, which is important for dormancy release. Sugars, on the other hand, may regulate the expression of other genes and hence contribute to signaling that activates meristem dormancy. Skylar *et al*. (2011) reported that sugars directly induce the expression of cyclins and cyclin-dependent kinases. For instance, Riou-Khamlichi *et al*. (2000) showed sucrose limitation results in arrest in the G1 phase of the cell cycle, while sucrose availability induces D-type cyclins, enabling progression. Glycosyltransferases (GT) are enzymes associated with the biosynthesis of cell walls. Growing evidence suggests that GT plays a significant role in plant development and stress adaptation. Furthermore, in the bud dormancy release phase, GT is engaged in carbohydrate metabolism (Zhang *et al*., 2018).

In the dormancy release library, EST assigned to adenylate isopentenyltransferase (IPT), which is involved in cytokinin (CK) biosynthesis, was upregulated. The generation of isopentenyladenosine 5'-monophosphate (iPMP) from dimethylallylpyrophosphate (DMAPP) and AMP, which is mediated by adenylate isopentenyltransferase, is the first stage in the synthesis of cytokinin in plants (Hua *et al*., 2006). Cytokinin is a crucial phytohormone that modulates many important developmental processes, including embryogenesis, seed development, organogenesis, vascular patterning, stress tolerance, cell division, and stem-cell specification. The findings of Li *et al*. (2013) reveal a new mechanistic understanding of cytokinin signaling's involvement in cell morphogenesis. CK is thought to have a role in the regulation of dormancy release via regulating cyclins. According to Depta *et al*. (2006), cytokinins have a function in meiosis promotion by regulating specific genes, including cyclin D.

Epigenetic modification, in addition to environmental and hormonal factors, has been discovered to have a critical role in controlling the dormant-active transition. EST annotated to methyltransferase, histone deacetylase and E3 ubiquitin protein ligase were induced during dormancy. While EST annotated to transcriptional activator DEMETER-like was induced in dormancy release stage. DNA methylation variations influence important physiological traits, i.e., flowering time, seed germination, seed dormancy, yield, and tolerance to various stressors. (Vanyushin, 2006). Several studies have reported modified DNA methylation during dormancy-growth cycles. Kumar *et al*. (2016) using RNA-Seq analysis in *M. domestica* showed increased expression of DNA methyltransferases during dormancy. In *C. sativa*, Santamara *et al*. (2009) reported that dormant apical buds had higher DNA methylation factors than actively growing apices. According to this, in *P. nigra* stems, Conde *et al*. (2013) revealed that during winter dormancy, DNA methylation levels are higher than during active growth. In addition, Prudencio *et al*. (2018) found a larger number of hypermethylated sequences in dormant buds than in dormancy-released in *P. dulcis*. Overall

methylation levels in dormant buds of *P. officinalis* were found to be more significant and were steadily enhanced by short-term chilling, according to Zhang *et al*. (2018). According to Ma *et al*. (2013), histone deacetylase (HDACs) regulate plant growth, development, and stress responses by controlling dynamic and reversible histone acetylation, which modifies the chromatin structure and functions and gene transcription modulation. So, this could provide new insights into the function of histone deacetylase in affecting the dormancy cycle of *P. abies*. Molecular research is needed to identify genes that are the targets in this regulatory pathway.

The E3 ubiquitin protein ligase, which is required for histone monoubiquitination, is a chromatin regulator. Histone ubiquitination's regulatory functions and basic mechanisms have only recently been discovered. Histone monoubiquitination, in combination with other post-translational modifications, especially acetylation and methylation can modulate the chromatin structure and thus modulate a number of functions such as transcription, repair, and genome replication (Pinder *et al*., 2013). Histone monoubiquitination1 (AtHUB1) and (AtHUB2) are homologs in *Arabidopsis* that serve as monoubiquitination of histone H2B and regulate a variety of biological functions, including seed dormancy and the circadian clock (Ménard *et al*., 2014). According to this investigation, DNA demethylation is also related to modulation of dormancy. Conde *et al*. (2017) described the expression of chilling-dependent demeter-like 10 (PtaDML10) in poplar apex tissue, as well as the reactivation of growth in the apical shoot during spring bud release, followed by a progressive reduction of gDNA methylation.

In temperate areas, perennial species are capable of increasing their chilling tolerance in response to low temperature perceived during dormancy, which is called chilling acclimation, to survive plant tissue and cells. While stress-inducible genes are regulated by low temperature, the majority of them in plants are known to respond to both ABA and water deprivation, implying a significant interaction between the two pathways during dormancy. Besides, analysis of comparative functional identified numerous genes related to the stress response, encompassing the late embryogenesis abundant protein (LEA). During dormancy, the LEA gene was dramatically increased, which is probably followed by a response to abiotic stress such as chilling (Pedrosa *et al*., 2015). The LEA expression pattern in the present investigation is consistent with a previous report which showed that LEA was regulated by short photoperiod and downregulated as dormancy progressed (Leida *et al*., 2012). Upregulation of the DREB1/CBF transcription factor during dormancy was identified. Wisniewski *et al*. (2011) suggested the participation of the DREB1/CBF regulon during the maintenance of the apple bud dormant state. The dehydration-responsive element-binding (DREB) protein/C-repeat binding factors (CBFs) that exist in many LEA genes belong to the APETALA2 (AP2) family of transcription factors that bind to DRE/CRT cis-element and modulate the expression of stress-responsive genes. The DREB1/CBF genes have a contribution to stress tolerance (Agarwal *et al*., 2006).

In *P. abies*, ESTs annotated to AP2 were also upregulated during dormancy. Varkonyi-Gasic *et al*. (2012) observed that kiwifruit AP2 accumulated to high levels in dormant buds but that expression decreased before bud bursting. In poplars, AP2 is linked to a critical phase in the induction and maintenance of dormancy (Rohde *et al*., 2007). In contrast, Zhang *et al*. (2014) found that the expression pattern of AP2 may participate in breaking the dormancy of tree peony. As a result, more research is required to fully understand the role of AP2 and the transcription factor DREB1/CBF during the dormancy phase. Heat shock proteins are another increased gene related to stress response in the dormant state (HSPs). These molecular chaperones, also known as stress proteins, participate in protein refolding and contribute to cellular protection, protein homeostasis, and cell survival in the face of a variety of environmental and metabolic stresses. The actions of HSPs may be the mechanisms that plants use to survive unfavorable conditions in

the bud dormancy. The generation and accumulation of HSPs can lead to greater chilling injury tolerance during dormancy, which suggests that these proteins play a central role in acquiring tolerance to chilling conditions (Takemura and Tamura, 2016). Another transcription factor, that was upregulated in the dormancy state was basic leucine zipper (bZIP), located downstream of the ABA signaling pathway, which plays a key role in dormancy. In plants, bZIPs are mediators of several fundamental developmental and physiological processes such as photomorphogenesis, leaf and seed formation, energy homeostasis, and stress responses, which contribute to the adaptation of plants to the dynamic environment (Deppmann, 2006). Bryant *et al*. (2019) showed that bZIP67 overexpression enhances dormancy in *Arabidopsis*. Sun *et al*. (2016) indicated that PpbZIPs genes may be involved in regulating dormancy in *P. persica*.

Calmodulin indicated higher expression level in dormancy release. Calmodulin (CaM) is an important calcium-binding protein which make a contribution to both regulating plant growth and development, as well as in the resistance mechanisms to various stresses with high affinity and specificity. It functions as a key component of stress signalling in the form of an intracellular Ca^{2+} receptor and mediates Ca^{2+} regulation (Antony, 2010). The increase of calmodulin indicated participation of calcium signaling during tree peony dormancy release, and calcium and calmodulin activate several proteins, including many calcium/calmodulin-dependent enzymes (Zhang *et al*., 2014). In grape bud dormancy release, several calcium signaling-related genes, including calmodulin were induced and it was suggested that calcium signaling was involved in the mechanism of grape bud dormancy release (Pang *et al*., 2007).

There were up-regulated ESTs which functionally annotated to secondary metabolism catalog i.e., phenolic acid and isoflavone biosynthesis (Shikimate kinase - Phospho-2-dehydro-3-deoxyheptonate aldolase - Isoflavone synthase) and specifically expressed more than fourfold difference in dormancy. For years, plant biology theory has been based on the functional dichotomy of plant metabolism, and the major roles of secondary metabolites in development, photosynthesis, reproduction, and other primary activities have remained unknown (Castellano and Sablowski, 2010). This dichotomy includes primary metabolites as highly conserved molecules that are directly required for the plants' growth and development, as well as secondary metabolites as mediators in plant-environment interactions such as phenolics, terpenes and nitrogen-containing chemicals that assist plants to cope with diverse stresses (Hartmann, 2007). The specific molecular boundary between these dichotomies, however, has never been thoroughly defined. Harborne (2005) suggested a role for phenolic acid as a blocker of ion cannals, an inhibitor of some enzymes and a suppressor of meiosis. At present, increasing genomics and proteomics research further obliterates these boundaries by specifying that secondary metabolites are multifaceted, i.e., they can act as primary metabolites as regulators of plant growth and development. Signaling capability and worthwhile storage and recycling make secondary metabolites favoured as multifunction molecules (Erb and Kliebenstein, 2020). The present study provides insight into the probable function of secondary metabolites as regulators of the apical meristem's dormancy in *P. abies.*

Another functional catalog that was differentially expressed between libraries was related to co-factors and vitamins. In a dormant phase, ESTs attributed to co-factors and vitamins were upregulated (1-deoxy-D-xylulose-5-phosphate synthase DXS2B and Dihydrofolate reductase-like). DXS2B is an enzyme involved in the thiamine biosynthetic process. In one-carbon metabolism, folates (vitamin B9) are crucial cofactors. Folates are essential for all living organisms because C1 transfer processes are involved in the synthesis of nucleic acids, proteins, lipids, and other macromolecules, as well as epigenetic regulation. Therefore, the change in the expression of genes involved in this functional catalog may be related to the change in the expression of genes involved in nucleotide metabolism in the meristem dormancy process. Dihydrofolate reductase is a gene family

that implements one step in folate biosynthesis. Gorelova *et al*. (2017) demonstrated the newly discovered role of folate metabolism in the maintenance of the redox balance by contributing to NADPH production through the reaction catalyzed by methylenetetrahydrofolate dehydrogenase, thus allowing plants to cope with oxidative stress. Therefore, regarding the redox regulation that has been previously described, dihydrofolate reductase may play a role in scavenging ROS. In recent years, the pathways for the biosynthesis of many vitamins have been clarified in plants at the molecular level, and several unique features are emerging. For instance, the mitochondrion has a major role in the synthesis of vitamins, and perhaps thiamin or the production of several of these co-factors is regulated by developmental signals and, perhaps more unexpectedly, by environmental signals such as light and salinity (Smith *et al*., 2007). However, much remains to be elucidated about the role of metabolites, vitamins, and cofactors in the transition of dormancy release.

Results of this study indicate that some transporter family proteins (V-ATPase transporter; H+ ATPase exporter; amino acids, phosphates and peptides transporters, mitochondrial membrane transporters, unknown membrane system transporters and miscellaneous transporters) were upregulated at the dormancy release stage (V-ATPase 69 kDa subunit), while in the dormant meristem library ESTs were more involved in sugars, amino acids and peptides transporters and miscellaneous transport (bidirectional sugar transporter sweet), which coincides with changes in the expression of further ESTs in the major CHO metabolism catalog during dormancy. This functional catalog has a high proportion of ESTs in both libraries. The vacuolar type H+ATPase (VATPase) is found in the membranes of the secretory pathway of plants. Because of its involvement in energizing secondary transport, maintaining solute homeostasis, and possibly promoting vesicle fusion, the VATPase is required for plant development under normal conditions. Under stress conditions such as salinity, drought and chilling stress, the survival of the cells depends strongly on maintaining or adjusting the activity of the V-ATPase (Dietz *et al*., 2001). Increased gibberellin during dormancy release may cause increased expression of this gene family. According to Cooley *et al*. (1999) VATPase is generated in response to gibberellin during tomato seed germination. Similar to our result, Xu *et al*. (2016) found that the expression of sugar transporter family genes in the dormant bud was higher than in the reactivating and active buds, confirming the requirement for adequate energy in dormancy. In contrast, Murcia *et al*. (2016) reported that ABA, which participates in dormancy maintenance, inhibits certain isoforms of sucrose transporters in grapevine.

In addition, there was a significant difference in the functional catalogs of fermentation and mitochondrial electron transport/ATP synthesis in dormancy-release, indicating the importance of energy release at this stage, which somehow uses the fermentation process to make ATP without oxygen to achieve more energy. The necessity of using the fermentation process in active state is a fact that needs to be further investigated.

CONCLUSION

In plants, a wide range of trait variation provides an important potential for selection to identify superior genotypes and use them as the genetic source for breeding purposes such as yield improvement and commercial cultivar introduction under various conditions (Heidari *et al.*, 2019). Traditional breeding methods are used to produce superior trees with improved growth rates as well as other desirable qualities, including stem straightness and wood and fibre properties. After selection of the best offspring during the sequential cycle of the breeding program, these trees are then introduced into breeding orchards, which is a very time-consuming process. Today, faster and more sophisticated large-scale techniques are in place that offer the potential for further improvement from the meristematic culture of superior plants. The culture of meristem tissue has

begun to be a useful technique for plant breeders. Most exploit the rapid rates of multiplication and the assured health status that can be attained in culture; there is also the possibility of manipulating the genetics of these tissues. The existence of diversity in the culture medium under *in vitro* conditions will not only accelerate the selection process of the best genotypes at the cellular level but also enhance the selection efficiency of desirable traits at the plant tolerance level. Such efforts ultimately enable the manipulation of specific genes for targeted engineering for different industrial uses (Heidari *et al.*, 2021). This enhances the efficiency of industrial wood production as well as the quality of the forest's wood supply.

Bud dormancy, particularly dormancy release, is one of plant biology's less well-understood processes. This delay in relation to other well-characterized plant phenomena can be due to methodological issues with the study of dormancy itself, as it is one of the most complicated subjects in empirical research. In temperate areas, the perennial and periodic nature of growth evolve into a unique trait through the recruitment of photoperiodic and hormone-based timing mechanisms that plan the growth cessation and arrest of meristematic tissues. In the present study, redox regulation downstream of hormones appears to be involved in the dormancy regulation of perennial plants. Moreover, the characteristics of these mechanisms are unknown at this time. More research is needed on whether there is a specificity in the production of ROS types by different subcellular compartments or NADPH oxidase in membranes during the transition of the dormant to active meristem. The ROS spatio-temporal specificity that characterizes antioxidant enzyme expression provides the potential to use them as biomarkers of crucial developmental processes in woody species such as *P. abies*.

Besides, in the light of this result, it is suggested that changes in the amount or type of secondary metabolites may play an indicative role in maintaining dormancy. According to recent research, several classes of plant secondary metabolites are integrated into plant metabolism and can act as both regulators and primary metabolites. In this regard, further empirical studies are needed to first confirm the effect of these metabolites on meristem transition and, second, to determine which processes are affected by these metabolites.

The results also indicate epigenetic regulation plays a role in dormancy-release in perennial horticultural and forestry plants. Further research, such as dormancy-release stage-specific genome-wide DNA methylation and histone acetylation analysis in perennial trees, is required to identify the main components. Increased knowledge provides a better understanding of the dormancy process and, as a result, more precise manipulation of dormancy-related forestry features. In addition, study of gene expression in dormancy-release has an important role in identifying genes involved in the organogenesis process. By considering the identification of key genes associated with dormancy-release and organogenesis processes, we can proceed to use these genes in order to micro-propagate woody species in gene transfer programs.

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