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Genomic alterations of early drought responses in eastern white pine (*Pinus strobus*)

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Abstract

Research exposing early drought adaptation is limited in trees. To elucidate the root specific responses in eastern white pine (*Pinus strobus*), we conducted illumina RNA-Sequencing and *de novo* transcriptome assembly (developing full-length transcripts and generating contigs that corresponds to paralogous genes using TRINITY tool). Early chlorophyll estimation showed a declining trend in *Chlorophyll b* under drought treatment. Further, our comprehensive transcriptome analysis (control *vs* drought) revealed key evidences for an early drought response through root functioning: (i) activation of core ABA signaling, as seen in the upregulation of *PYL1* and *RCAR11* genes; (ii) upregulation of sucrose loading proton pumps, H^+ ATPase and the downregulation of starch breakdown gene, *SBE2.2*; (iii) downregulation of ROS regulator genes, NADPH dehydrogenase and Ca^{2+} dependent protein kinases; (iv) upregulation of xylem secondary cell wall lignification genes like *PER72* and *CORD72*; (v) upregulation of an aquaporin gene *PIP3A* along with *SNARE*; (vi) upregulation of root gravitropism and hydrotropism genes, *SEC6* and *ARF7*. Further confirmation of early robust responses to severe drought was evident through the high expression of genes related to root cell wall thickening, root volatile synthesis, antioxidant defense, hormonal crosstalk, and ABA-responsive transcription factors. In conclusion, our research has identified the involvement of pivotal genes regulating photosystem complex in the drought prone tree species *P. strobus* vital for future genetic studies.

Keywords: Root; Trinity-RNA Seq; Xylem vessel; Sucrose proton pump; Abscisic acid

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Introduction

Forests face significant threats from climate extremes, particularly intense drought [1]. Drought ranks among the most damaging abiotic stresses affecting trees globally, leading to the generation of reactive oxygen species (ROS) [2, 3]. These ROS, being highly toxic and reactive free radicals, can disrupt cellular homeostasis and potentially result in tree mortality [4]. Understanding how trees respond to water shortage is crucial for predicting the

future of ecosystem functions. Water stress is primarily detected by roots, which then transmit this signal to above-ground organs. This root-localized signaling cascade triggers various phytohormone pathways, metabolite synthesis, source-sink relationships, and defense protein signals. These signals are subsequently transported through vascular systems to different plant tissues, ensuring better survival [5].

Several factors that govern the root response to drought stress are common across different plant genera, with one key factor being the involvement of the phytohormone abscisic acid (ABA) [6]. In water-deficient environments, ABA tends to accumulate in roots and is then transported to above-ground plant parts [7]. ABA plays a crucial role in stomatal closure, root-to-shoot communication, and the regulation of root formation [8]. It has been demonstrated that ABA is involved in establishing a communication channel from roots to shoots through ABC transporters under water stress conditions [9]. When facing stress, plants generally maintain a high root–shoot ratio to allocate more resources to roots, enhancing water and nutrient absorption [10]. Sucrose unloading in roots from leaves or shoots is facilitated by various sugar transporters [11]. Upregulation of sucrose transporters (SUC) increases carbon supply to roots, promoting elongated roots with an enhanced ability to capture water from deeper soil layers [12]. Roots also establish an effective antioxidant system to counteract reactive oxygen species (ROS) generated due to soil dryness by upregulating scavengers and synthesizing secondary metabolites [13]. During water stress, roots typically become metabolically more active, undergoing various changes in architecture and anatomy to enhance water and nutrient uptake [14].

When plants sense stress, a complex communication channel is initially established among root cells, which then transmit these signals to above-ground organs via ABA. Our previous study on the physiological responses of *Pinus strobus* seedlings to severe drought showed an inclining trend in root sugar and a declining trend in starch accumulation, with a significant increase in ABA content [15]

Understanding this intricate ABA coordination among root cells is crucial to ensuring plant survival under stressful conditions, particularly under drought. Although a comprehensive understanding of this complex network exists for crops, a clear comprehension of this regulation in tree species, specifically in roots, is still lacking. Investigating root signal cascades under drought stress conditions can provide deeper insights into architectural adaptations and metabolic regulations, thereby enhancing our knowledge of how plants cope with stress. This understanding is essential for identifying genes or loci that can be precisely targeted to develop stress-resistant trees. Thus, this research aims to identify genetic modulations occurring in the *P. strobus* roots subjected to short-term severe drought. Together, we present a dataset comprising root transcriptional modulations of a forest tree species *P. strobus* under severe drought exposure.

Materials and methods

Experimental design and chlorophyll estimation

We have explained the experimental setup and treatment groups in our earlier publication [15] (doi:10.3389/fpls.2022.1030140). Briefly, the *P. strobus* seedlings (3-year-old) grown in pots were exposed to control (100% precipitation) and severe drought (20% of total precipitation) for 32 days. The drought treatment was conducted at the end of growing season (1st November-2022 to 2nd December-2022). After 32 days of treatment (DAT), needles were collected from well-watered seedlings and drought exposed seedlings. Chlorophyll (Chlorophyll *a* and Chlorophyll *b*) estimation was carried out based on our previous study (reference). Ten samples per treatment were used for chlorophyll analysis. Chlorophyll *a* and Chlorophyll *b* content was calculated using the formula:

$$Chl\ a\ \left(\frac{mg}{FW}\right) = \frac{2.7\ x\ (A663) - \ 2.69\ x\ (A645)\ x\ Volume}{1000\ x\ W}$$

$$Chl\ b\ \left(\frac{mg}{FW}\right) = \frac{22.9\ x\ (A645)\ - \ 4.69\ x\ (A663)\ x\ Volume}{1000\ x\ W}$$

D663 = absorbance/optical density measured at 663 nm
D645 = absorbance measured at 645 nm
V = total volume of chlorophyll extract, usually in mL
W = weight of plant tissue used, usually in g

RNA extraction

After 32 DAT, we obtained total RNA from the root samples (primary root fraction: 50–100 mg each, free from soil impurities using basic filter tissue) using the Ribospin™ Plant RNA extraction kit (GeneAll; Seoul, Republic of Korea), following the manufacturer's instructions. The extracted RNA was sent to Macrogen (Seoul, South Korea) for library preparation and sequencing. The RNA integrity numbers (RIN) were checked using a Bioanalyzer RNA Pico 6000 Chip Library Preparation (Agilent Technologies, Santa Clara, CA, USA), ensuring all samples for cDNA library construction had RIN > 7. Paired-end non-directional cDNA libraries (2 × 101-bp) were created following the TruSeq Standard mRNA sample preparation guide and sequenced on an Illumina NovaSeq 6000 system (Illumina, San Diego, CA, USA) at Macrogen.

De novo assembly and differential gene expression analysis

We used Trinity v. 2.13.2 (https://github.com/trinityrnaseq/-trinityrnaseq) with default settings to perform de novo assembly on raw reads, creating a reference transcriptome. The Galaxy Genome Annotation Platform (www.usegalaxy.org) was then used for a streamlined DEG analysis workflow. Initially, raw data files were uploaded to the Galaxy server. For quality assessment and removal of low-quality reads, we employed FASTQC & TRIMMOMATIC with default parameters (Bolger et al. 2014; Brown et al. 2017). To align trimmed reads, we used HISAT2, a fast and sensitive alignment tool (Kim et al. 2015). The de novo assembled transcripts of Korean fir served as the reference genome, and trimmed datasets in fasta format were used as input files. We selected options such as 'paired end and unstranded' for the analysis. HISAT2 generated BAM files during its run. Subsequently, STRINGTIE, a fast and efficient RNA-Seq alignments assembler, was used to generate potential transcripts (Pertea et al. 2015). The BAM files from the HISAT2 run were used as inputs for STRINGTIE.

We then used the total gene structure of Arabidopsis in GTF format as the reference file to guide the assembly process, keeping all other options at their default settings. Afterward, we merged all STRINGTIE files using the STRINGTIE merge tool. Following this, we utilized the STRINGTIE tool again, using the merged file as a reference file. During this execution, we set the 'Use reference transcription' option to 'YES'. For the differential expression analysis, we selected the DESeq2 (Love et al. 2014) / edgeR (Robinson et al. 2009) / limma-voom (Ritchie et al. 2015) option as the output file. Other options were left at their default settings, and the analysis was carried out. In DEG analysis using the DESeq2 tool, we chose the 'Gene count file'. Factor levels were specified as control and waterlog separately. Count data was selected as the input data, and the remaining settings were kept at their default values.

The DESeq2 result file was annotated against the 'stringtie merge file' using the Annotate DESeq2 tool with default parameters. The annotated transcripts were then used for further analysis. DEG transcripts were filtered with a P-value < 0.05 and FDR < 0.001. Using -log2 fold change values, identified DEGs were categorized as upregulated and downregulated (refer to Table S3). The sequences corresponding to the assembled unigenes after filtering, were compared with A. thaliana cDNA transcripts using the Basic Local Alignment Search Tool for nucleotides (BLASTn) with default parameters. Gene ontology (GO) enrichment analysis was performed using the ShinyGO 0.77 classification system

(http://bioinformatics.sdstate.edu/go/). For Gene Ontology Biological Process (GOBP), a Fisher's exact test with an FDR-corrected (FDR < 0.001) P-value < 0.05 was used to determine significant enrichment.

Correlation analysis between RNA-Seq and qRT-PCR data

Total RNA samples used for the RNA-Seq analysis was also used for the qRT-PCR analysis. Complementary DNA was synthesized using iScript reverse transcriptase mix from Bio-RAD, CA, USA. Quantitative reverse-transcription PCR (qRT-PCR) was performed using the IQ SYBR Green Supermix, RT qPCR Kit (Bio-RAD, CA, USA). Eight DEGs from *P. strobus* transcriptome data were selected for qRT-PCR, conducted with a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The program included 40 cycles at 95 ◦C for 10 s, 61 ◦C for 30 s, and 72 ◦C for 30 s after an initial step at 95 ◦C for 2 min. All qRT-PCR reactions were carried out in triplicate for each biological sample. Expression ratios were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), with *ACTIN* gene as the internal control. The R statistical software was used to analyze the Pearson correlation coefficient between qRT-PCR and RNA-Seq data. Primer sequences for qRT-PCR can be found in Table S1.

Results and Discussion

When plants face water stress, effective communication between above-ground and below-ground organs becomes crucial for coordinated growth which rely on the root response. Various root developmental pathways, including hormones, peptides, proteins, hydraulic signals, and metabolites, are involved in this process. The nature of these signals can differ drastically between crops and trees. Limited information is available on these signals during short-term severe drought, particularly when compared to long-term drought in trees. Our physiological analysis of chlorophyll estimation in *P. strobus* leaves denoted, no any significant change in the Chlorophyll a content between control and drought treatments, whereas a declining trend in the Chlorophyll b content was observed between control and drought treatments (Figure S1). Declining trend in Chlorophyll b denotes that the light absorption capacity and transfer of light energy to Chlorophyll a is affected during the early days of severe drought. Chlorophyll b is necessary for stabilizing the major light-harvesting chlorophyll-binding proteins. Chlorophyll b is synthesized from chlorophyll a and is catabolized after it is reconverted to Chlorophyll a. This interconversion system between Chlorophyll a and Chlorophyll b refers to the chlorophyll cycle [16, 17]. From our results, it can be speculated that a prolonged severe drought might influence drastic changes in the functioning of photosynthetic complex in *P. strobus*. However, the molecular pathway behind this changeover in the photosynthetic system is currently unknown.

To uncover the molecular changes, we utilized RNA sequencing to analyze *P. strobus* roots exposed to severe drought for 32 days [15]. Our transcriptomic analysis (control vs drought) revealed 312 genes upregulated (-log2FC > 1; P < 0.05) and 30 genes downregulated (-log2FC < -1; P < 0.05) (Figure. 1A and Table S2-S3). According to the GOBP analysis, these DEGs were significantly involved in various metabolic pathways, such as protein phosphorylation, response to oxygen stimuli, secondary metabolic process, terpenoid metabolic process, defense response to bacterium, response to sucrose process, response to disaccharide, cellular response to sucrose process, and geranyl metabolic process (Figure. 1B and 1C). KEGG enrichment analysis indicated that the DEGs (P < 0.05) were mainly associated with secondary metabolite biosynthesis, phenylpropanoid biosynthesis, glycolysis, pyruvate metabolism, terpenoid biosynthesis, fatty acid degradation, tyrosine metabolism, and zeatin biosynthesis (Figure. 1D). These results suggest an early coordination of complex regulatory pathways in *P. strobus* root response to water stress.

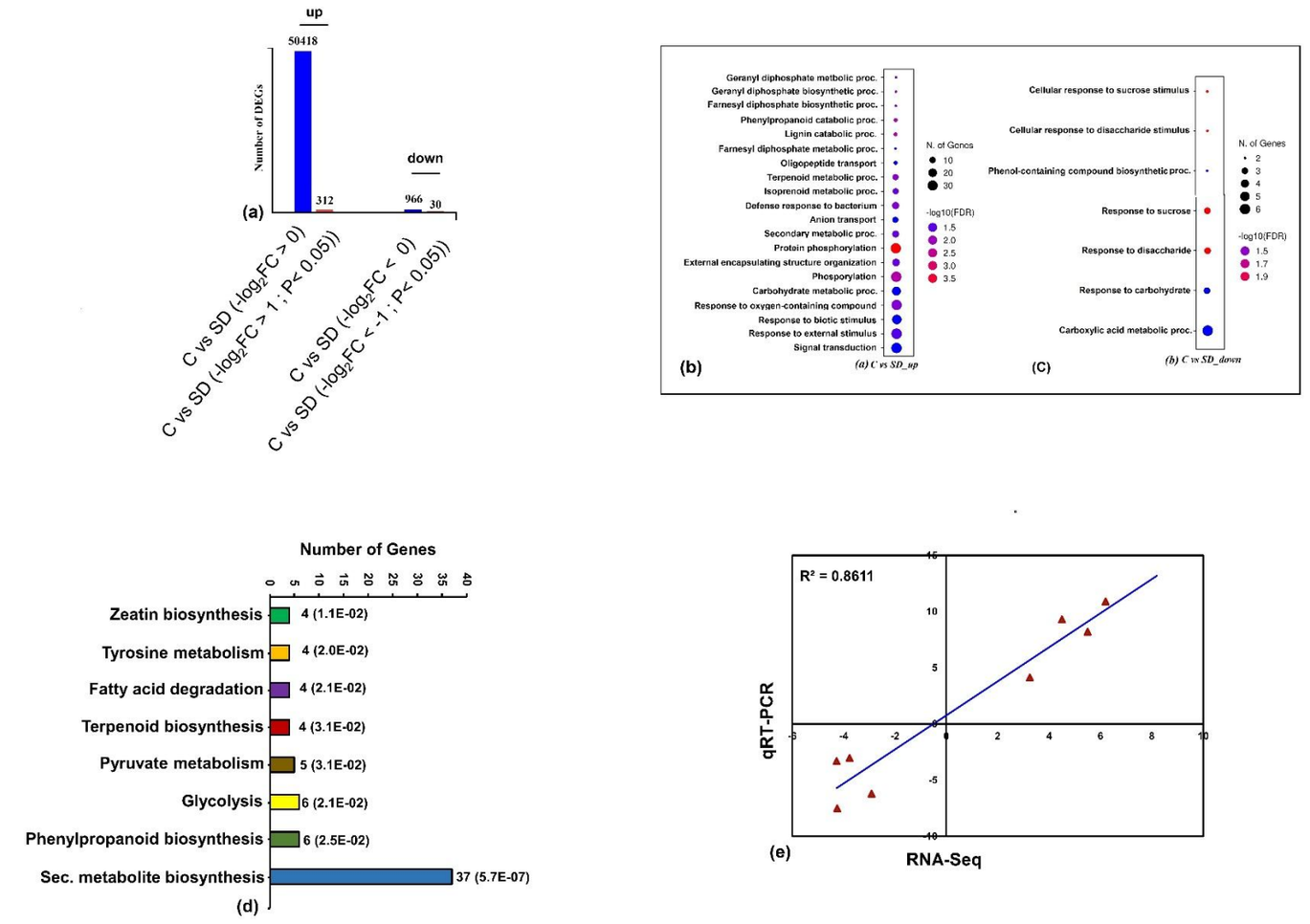

Figure.1. Identification of DEGs from *P. strobus* roots exposed to severe drought (control vs drought). (A) Number of DEGs categorized as upregulated and down regulated before filtering and after filtering (P < 0.05 and FDR < 0.001). (B) and (C) Gene ontology biological process (GOBP) analysis of differentially expressed genes, scatter chart of pathways enriched for the upregulated and downregulated DEGs. GOBP terms associated with Fisher's exact test and FDR-corrected (FDR < 0.001) P-value < 0.05 are listed. Bubble color indicates P-value (-log10 FDR). Bubble size indicates number of DEGs in GOBP terms. (D) Functional classification and pathway assembled DEGs by KEGG enrichment. (E) Validation of RNA-Seq data with qRT-PCR gene expression. X-axis indicates fold changes in RNA-Seq data and Y-axis denotes fold changes in qRT-PCR data. FDR-false discovery rate.

Severe drought in our study has caused a down-regulation of root *LHCB2*, a light-harvesting chlorophyll a/b-binding protein that plays a crucial role in photosynthesis, specifically in the light-harvesting complex II (LHCII) of photosystem II, where it binds chlorophyll a and b molecules, facilitating efficient light absorption and energy transfer [17, 18]. It has been previously reported that some exceptional plants have a modified or assimilatory roots that can perform photosynthesis, and these roots have the capacity to absorb light with the help of *LHCB2* gene [18-21]. This interesting phenomenon confirms that

P. strobus plants might have assimilatory roots that can absorb light source and the down-regulation of root *LHCB2*, further highlights that the light absorbing capacity in leaves might also have been affected, which is evidenced by a declining trend in Chlorophyll b levels in our study (Figure S1). However, the does the downregulation of root *LHCB2* has any impact on the leaf *LHCB2* expression needs further experimental elucidation.

Water scarcity significantly affects the root vascular system (xylem and phloem), leading to a decrease in the local water potential of the roots. This change in pressure prompts the ABA accumulation in roots, signaling the closure of leaf stomata [22, 23]. We observed an increase in ABA biosynthesis genes like carotenoid isomerase, *CCR2*, and short-chain dehydrogenases/reductase, *SDR3*, confirming enhanced ABA synthesis in *P. strobus* roots under severe drought [15]. As ABA accumulates, receptor proteins, particularly *PYR/PYL/RCAR*, perceive the signal through ABA binding [24]. Coincidently, this up-regulation of genes involved in ABA biosynthesis and signaling was expected, since ABA mediates water deprivation response in plants [25, 26]. For effective stress mitigation, ABA movement between cells and tissues is crucial and involves various transporters. Notably, *ABCG17* and *ABCG18* transporters act as ABA importers, while nitrate transporter NPF4.6 assists in ABA uptake into vascular tissues [24]. Our study detected elevated transcript levels for transporters like ATP-binding cassette (*ABCG35*, *ABCG41* and *ABCG42*) and nitrate transporters (*NPF8.5* and *NPF5.12*), providing new insights into their role in drought response. Additionally, ABA-glucose ester (ABA-GE) is a class of molecules that shuttle ABA signals between plant organs [27]. The synthesis of ABA-GE requires a glucosyl-transferase, and its breakdown to ABA involves β-glucosidase, *BGLU18* [28]. Our study found high expression levels for *BGLU18* gene, suggesting its involvement in the root ABA transport. Recent research also indicates that the *bglu18* mutant exhibits increased susceptibility to drought stress due to both ABA deficiency and an elevated number of stomata, resembling de novo ABA-deficient mutants [29]. As such, further investigation is needed to understand the specific role of BGLU18 in root cells (Table. S2).

Interestingly, Raf like kinase36 (Raf36), a substrate of SnRK2 kinase, accumulates and inhibits ABA responses in optimal growth conditions. However, during abiotic stress, ABA-activated SnRK2s phosphorylate and degrade Raf36, resulting in stronger ABA responses [30]. Our study observed elevated expression of Raf36, suggesting a possible SnRK2 phosphorylation and a robust ABA response in protecting *P. strobus* roots during drought stress. In summary, the upregulation of ATP-binding cassette, nitrate transporters, and, importantly, β-glucosidase18 and Raf36 gene implies an early movement of synthesized ABA between P.strobus root cells to mediate the response to drought stress. Additionally, sulfate accumulation in xylem sap during drought stress is known [31]. Notably, sulfate acts as an early signal in maize under soil drying, preceding ABA transport and xylem sap pH increase [32]. Supporting this, two sulfate metabolism genes, O-acetylserine sulhydrylase1 and γ-glutamyl cyclotransferase2 were highly upregulated in our study. These sulfate regulations might also serve as root-derived signals influencing P.strobus leaf stomatal closure, although direct evidence is lacking (Table. S2).

Drought stress often results in an excess production of reactive oxygen species (ROS). ROS and calcium ions (Ca2+) are stress signals closely tied to the ABA signaling pathway during abiotic stress (Medeiros et al. 2020). Elevated ROS levels lead to increased intracellular Ca2+, activating anion channels through Ca2+ dependent protein kinases (CDPKs) [33]. The down-regulation of two Ca2+ dependent protein kinase genes (CDPK3 & CDPK12) and NADPH dehydrogenase, NBD2, indicates an early inhibition of ROS burst response in *P. strobus* roots [33, 34]. Further, the upregulation of SUMM2 (essential for maintaining ROS homeostasis against pathogen attacks) and CRK5 (necessary for balancing ROS-nitric oxide in mediating root gravitropism) provides further evidence for

the involvement of multiple signaling pathways in reducing ROS levels in drought affected eastern white pine roots [35, 36].

On the other hand, *P. strobus* seedlings exhibit a rapid antioxidant defense response in their roots to survive under drought. This is evident from the activation of both enzymatic (peroxidase PED1, monodehydroascorbate reductase MDAR3, catalase1 CAT1, copper amine oxidase CUAO1, lipoxygenase LOX1, Homeobox domain HB2, and S-nitrosylation glutathione reductase GSNOR) and non-enzymatic (terpene synthase TPS1, geranyl diphosphate synthase GPS, geranyl pyrophosphate synthase GPPS, farnesyl disphosphate synthase FPS, and α-farnesyl synthase) gene activation. Upregulation of several pathogenesis-related genes like tolerance to tobacco ringspot virus TTR1, HopW1-Interacting protein WIN2, pathogenesis-related PR2, IAA leucine-resistant like ILL5, and geminivirus REP interacting kinase GRIK2, further indicates effective prevention of pest attacks due to drought stress influenced dry soil. Notably, ABA regulates the expression of CUAO1, PR2, and GRIK2 genes [37, 38]. Whether ABA is involved in the overall defense enhancement of P.strobus plants to counter early water deficit remains to be answered. Another interesting aspect to elucidate is the identification of phosphorylation site of the GRIK2 protein kinases in response to water stress [39]. Polyunsaturated fatty acids (PUFA) can be easily degraded by ROS. When ROS affects PUFA, it destabilizes the lipid membranes (Czarnocka and Karpiński 2018). The increased expression of genes involved in PUFA synthesis, such as acyl-carrier protein ACP1, acyl-carrier transferase ACT1, and long-chain acyl coA synthase LACS5, clearly shows that there is controlled ROS levels in P.strobus roots (Table. S2).

A plant's ability to adapt to drought relies mainly on the carbon source sucrose. In many plants, sucrose unloading from leaves/shoots is carried out by sucrose transporters (SUC), which work against a concentration gradient and are powered by the plasma membrane enzyme H+ATPase [40-42]. To establish this concentration gradient, the proton pump H+ATPase needs ATP from the unloaded sucrose. This process begins with the cleavage of sucrose by the sucrose synthase gene [42]. While no sucrose transporters were upregulated in our study, we observed increased expression of two-proton pumps H+ATPase and two sucrose synthase genes (SUS3 & SUS5). This suggests a high unloading of sucrose from phloem and its breakdown into byproducts for energy in water-stressed *P. strobus* root tissues. Conversely, we noted a decreased expression of starch branching enzyme, SBE2.2, which affects starch biosynthesis [43, 44]. The transcription factors LEC1 and ABI4 (central to ABA signaling), directly bind to the promoter regions of SUS3 and SBE2.2 genes, respectively, regulating their expression [45, 46]. Surprisingly, ABA partially influences LEC1 transcription factors during root and cotyledon development [47, 48]. Investigating the role of ABA in regulating sugar metabolism through LEC1 and ABI4 during short bursts of water stress will be crucial in future studies. These results confirm our earlier findings of decreased starch and increased total sugar content in eastern white pine roots under severe drought [15] (Table. S2).

The significance of nutrient-carbon interactions (and hydraulics) during drought depends on how long and intense the water restriction is. In severe and prolonged droughts, there is a notable reduction in the allocation of carbon to roots and exudation. However, under moderate drought conditions, there is a stimulation of assimilate transport to roots and the rhizosphere belowground [49]. According to our findings, an increase in the unloading of sucrose from phloem and the breakdown of amylopectin skeletons (downregulation of SBE2.2) led to enhanced nutrient uptake and mobilization. This is reflected in the elevated expression of nitrogen transporters (NRT and NPF), phosphate transporter (PHT1.7), potassium transporter (THR1), and iron transporters (S8H, LAC12 and LPR1). The balance between carbon and nutrients in our study indicates the uninterrupted mobilization of assimilates and nutrients between root cells, suggesting

that *P. strobus* plants might consider severe drought as mild stress [50].

Understanding how xylem responds to water shortage is crucial for comprehending the impact of water scarcity on a tree's survival. When a tree is under water stress, changes in turgor pressure can cause the secondary cell walls around the xylem to break, forming pit cavities (embolism) [51]. This disrupts water transport and can lead to mortality. A plant's ability to minimize the harmful effects of embolism is an important trait for growth and survival. Some plants respond to water stress by strengthening their secondary cell walls between xylem vessels, and key components in this process are lignin and cellulose [52]. Upregulation of genes like Peroxidase72, UDP-glycosyl transferase72, involved in lignin and cellulose biosynthesis, suggest that changes in lignin content during water stress increase the hydrophobicity of xylem secondary cell walls [53, 54]. Additionally, the upregulation of genes like cellulose synthase like CSLG2 and cortical microtubule disordering CORD2 indicates adjustments in cellulose deposition, forming a strategy known as 'embolism avoidance' to transport water in *P. strobus* seedlings during severe drought [55] (Table. S2).

Additionally, xylem parenchyma cells play a crucial role in adjusting to embolism by providing energy, followed by the uptake of water to sustain xylem function during water deficit conditions. The energy source is derived from breaking down starch in parenchyma cells next to xylem vessels, leading to an increase in sucrose content [52]. The H^+ ATPase proton pumps in parenchyma cells actively transport synthesized sucrose into drought-affected xylem vessels, as suggested by the increased expression of two H^+ ATPase genes in our study. This rise in sucrose (upregulation of H^+ ATPase) not only offers osmotic protection from stress but also alters the sucrose gradient across the membrane in *P. strobus* root cells. Subsequently, xylem parenchyma cells contribute a significant portion of the water needed to fill xylem vessels [52]. Aquaporins are traditional water channels that regulate radial water transport through roots, influencing hydraulic conductivity [56]. In support to this, our study observed the upregulation of an aquaporin gene PIP3A and 'PIP' trafficking receptor gene SNARE [57], indicating the chances for continuous water supply to xylem vessels during the early stages of severe drought in *P. strobus* plants. Considering the reported post-translational modifications (PTM) of aquaporin proteins in response to abiotic stress, it is crucial to identify the PTM and the SNARE receptor mediated post-golgi trafficking in the PIP3A protein (Table. S 2).

Auxin and cytokinin are crucial for root gravitropism and hydrotropism under abiotic stress. The high upregulation of the *SEC6* gene suggests an excess flow of polar auxin transport and recycling of PIN proteins, promoting root gravitropism [58]. Similarly, the upregulation of auxin responsive factor 7 (*ARF7*), a key player in root hydrotropism, indicates that *P. strobus* roots might be bending towards region with high moisture availability in soil [59]. ARF7 responds to stress signals from the shoot by activating the HY5 transcription factor [60]. Auxin and ethylene collaborate to regulate root hair development through an ethylene precursor, 1-Aminocyclopropane-1-carboxylic acid (ACC) [61]. The upregulation of a histidine transporter *LHT3* gene, involved in transporting the ethylene precursor, ACC, indicates a potential crosstalk with auxin in the continuous growth of root hairs in water-stressed *P. strobus*. Moreover, a highly upregulated ABA-induced protein, HVA22, known to inhibit gibberellic acid (GA)-mediated programmed cell death (PCD), suggests the suppression of PCD as an early response to drought in *P. strobus* root cells [62]. Balance between root cell division and elongation is well maintained by the upregulation of RGI5, a root meristem growth gene in our study. Further, ABA plays a role in regulating the NIGT 1.4 gene, which is responsible for root elongation under salt stress. This regulation involves the phosphorylation of SnRK2.2/2.3 kinases [63]. The mechanism by which ABA regulates the phosphorylation of NIGT1.4 (upregulated in our study) as an early response to severe drought requires further elucidation. Interestingly, ABA appears to regulate

several genes in our study, suggesting an ABA-dependent mode of drought stress response. Apart from this, ABA might have also involved in the regulation of MYC/MYB transcription factors as seen through the upregulation of six MYB transcription factors (three were ABA responsive). The significance of these transcriptional regulations, mediated by core ABA signaling (PYL-RCAR), for the long-term survival of eastern white pine under water stress remains crucial. Taken together, we speculate that ABA might be the master regulator in coordinating early root responses to severe drought in *P. strobus*. However, this hypothesis requires further confirmation through promoter analysis for ABRE elements in all identified genes (Table. S1).

Correlation coefficient analysis further confirms the reliability of our RNA-Seq analysis validated through qRT-PCR gene expression (Figure. 1E). Overall, our results suggest an early activation and coordination of complex pathways in eastern white pine, enabling these conifers to survive severe drought stress (Figure. 2). Beyond characterizing early drought mediated root signals, our transcriptome results highlight pivotal candidates (e.g., Raf36, H+ATPase, CRK25, HB1, PIP3A, and RGI5) for future genetic engineering studies. Genetic modifications of these candidates could play a vital role in achieving early drought tolerance in sensitive forest tree species, providing new insights into tree genetics.

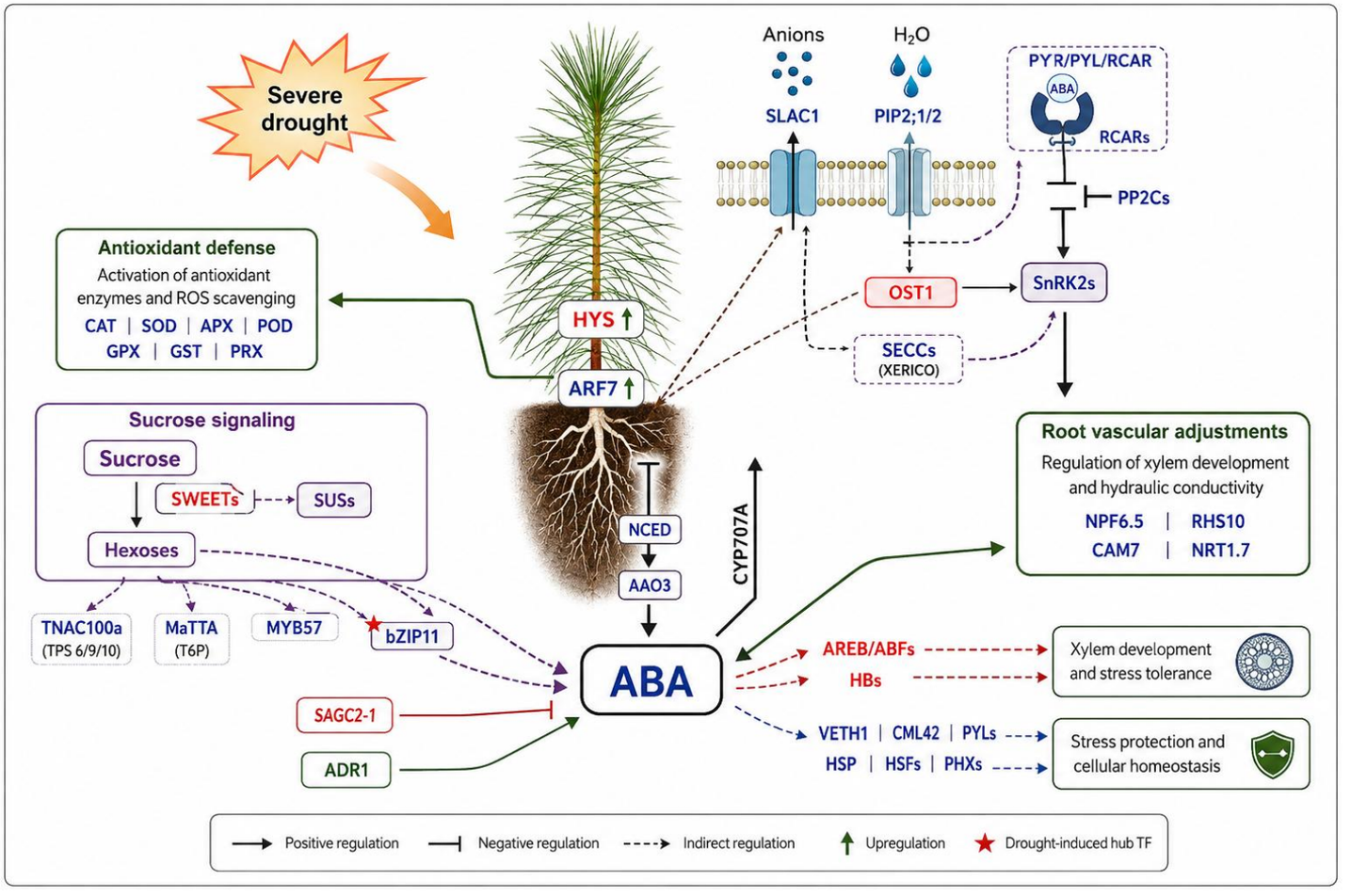

Figure. 2. Co-ordination of complex pathways in response to severe drought stress in *P. strobus* roots (control vs drought). Dotted lines indicate a possible role of ABA in regulating the genes in multiple pathways. Blue color font indicates

upregulated genes and red color font indicates downregulated genes. Signaling cascades to look for: ABA-LEC1-SUS3; HY5-ARF7; ABA-?-NIGT1.4; ABA-Raf26-PTM; ABA-SUMM2-PTM; ER to golgi trafficking of PIP3A through SNARE receptors under drought. Star shaped symbols represent the pest pathogen attack in the drought-affected roots. SD-severe drought; ROS- reactive oxygen species; HY5-hypocotyl elongation factor; ARF7- auxin responsive factor; ABI4-abscisic acid insensitive; PTM-post-translational modifications; ER-endoplasmic reticulum. Abbreviations for the rest of the genes are denoted in the main manuscript.

Data availability

Supplementary materials associated with this article include Figure S1. Leaf chlorophyll content, Table S1. List of all the primers used in this study, Table S2. Number of genes upregulated under drought stress in *Pinus strobus*, Table S3. Number of genes upregulated under drought stress in *Pinus strobus*.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author Contribution

The author(s) confirm contributions to the paper as follows: CU: Conceptualization, Data analysis, Experimental validation, writing—original draft preparation, writing—review and editing. KK: Data curation and writing—original draft preparation. HSK: Supervision and writing—review and editing. All authors contributed to the interpretation of the literature, critically revised the manuscript for important intellectual content, read and approved the final version of the manuscript, and agree to be accountable for all aspects of the work.

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