Drought stress-induced *Picea abies* transcriptome changes in the context of functional interactions

Malte Mader, Heike Liesebach, Birgit Kersten*

Thünen Institute of Forest Genetics, Sieker Landstr. 2, 22927 Grosshansdorf, Germany

*Corresponding author: Birgit Kersten, E-mail: birgit.kersten@thuenen.de

Abstract

Molecular responses to drought stress have been mainly studied in deciduous tree species although conifers dominate boreal forests. Here, we analysed the transcriptional response of *Picea abies* (L.) H. Karst. needles after exposure to severe drought by quantitative RNA-sequencing. In total, 2,402 differentially expressed genes (DEGs) were identified, of which 1,186 were up- and 1,216 downregulated. The upregulated DEGs are mainly involved in responses to stress, nitrogen compound, water deprivation, and abscisic acid as well as in channel activity. Although only one bZIP was identified among the DEGs, several other transcription factors involved in ABA-dependent pathways such as MYB, bHLH and WRKY showed differential expression. AP2/EREBP transcription factors related to ABA-independent pathways were also identified as DEGs. A functional interaction network of the 40 most connected *Arabidopsis thaliana* homologs of all *Picea abies* DEGs placed the two top-hubs P5CS1 and P5CS2 in the center. P5CS1 is the key enzyme in the biosynthesis of proline known to be accumulated in plants under abiotic stress. Lignin synthesis and DNA-related processes, among others, are overrepresented in this network. Our data highlight interesting gene targets for functional studies and natural genetic variation analyses to support the future identification and selection of potential drought tolerant trees.

Keywords: *Norway spruce, RNA-seq, water deprivation, transcriptome analysis*

Introduction

Molecular responses to drought stress have been most extensively studied in deciduous tree species although conifer tree species dominate boreal forests (Shorohova et al., 2011). These forests are being exposed to more frequent and prolonged periods of drought as a result of ongoing climate change (Feller, 2016). Currently, large areas of *Picea abies* (L.) H. Karst. (Norway spruce) stands in Europe are increasingly affected by drought and heat waves (Ge et al., 2011). Moreover, early spring drought has occurred with much higher frequency (Maresova et al., 2022).

Primary abiotic stresses, such as drought, salinity and cold, are often interconnected and cause cellular damage and secondary stresses, such as osmotic and oxidative (Harfouche et al., 2014). The initial stress signals (e.g., osmotic and ionic effects or changes in temperature or membrane fluidity) trigger downstream signaling process(es) and transcriptional controls, which activate stress-responsive mechanisms to re-establish cellular homeostasis and to protect and repair damaged proteins and membranes. Inadequate responses at one or more steps in the signaling and stress-gene-activation process might ultimately result in irreversible changes in cellular homeostasis and in the destruction of functional and structural proteins and membranes, leading to cell death (Harfouche et al., 2014).

Major insights to molecular signaling processes important for plant drought stress response have been gained from studies of herbaceous plants including *Arabidopsis thaliana*, *Oryza sativa* and *Zea maize* (Daszkowska-Golec and Szarejko, 2013; Yao et al., 2021; Aslam et al., 2022). Water deficit causes various alterations in plants, such as stomatal closure, decrease of turgor and changes in the composition of the cell wall or plasma membranes (Kizis et al., 2001; Osakabe et al., 2014). Although

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relatively little is known about the mechanisms for sensing these changes, it is well established that the phytohormone abscisic acid (ABA) is an important signal. An increase in the ABA level initiates a signaling cascade to close stomata and reduce water loss (Munemasa et al., 2015). ABA signaling results in the induction of various stress responsive genes and transcriptional networks (reviewed in (Yao et al., 2021)). ABA-dependent signaling has been described to be mediated by several transcription factors. The bZIP proteins belonging to the group of ABA-responsive element (ABRE) binding proteins (AREB)/ABRE binding factor (ABF), the so called AREB/ABFs bind to ABRE-cis elements in promoters of target genes and induce their transcription. Another ABA-dependent pathway requires protein biosynthesis of the MYC and MYB transcription factors, which act cooperatively to regulate the expression of target genes. Also, members of the WRKY and NF-Y transcription factor families have been shown to regulate ABA-responsive gene expression under drought stress (Baldoni et al., 2015; Singh and Laxmi, 2015). Some NAC transcription factors also regulate drought responses through the ABA-dependent pathway, while other NACs do so through the ABA-independent pathway (Yao et al., 2021).

Signalling via the ABA-independent pathway leads to rapid responses to drought or cold. In this pathway, the dehydration-responsive element-binding protein (DREB)/C-repeat binding factor (CBF) transcription factors, a subfamily of APETALA 2 (AP2)/ethylene-responsive element-binding proteins (EREBP) transcriptional activators are involved which bind to the dehydration-responsive element (DRE) in target promoters (Yamaguchi-Shinozaki and Shinozaki, 2006).

The drought-induced transcription factors will regulate target gene expression. Target genes may be involved in detoxification (e.g., CAT), osmoprotection mechanisms (mediated, e.g., by metabolites such as sugars, proline, and amines), molecular chaperone functions (e.g., heat shock proteins and late embryogenesis abundance proteins), water- and ion movement functions (e.g., aquaporins and ion transporters), among others (Harfouche et al., 2014). Especially, the maintenance of cellular turgor by osmoprotection is of high priority to tolerate water deficit (Tabaeizadeh, 1998).

Despite progress in the dissection of the molecular basis of drought stress tolerance in trees in general (reviewed in (Harfouche et al., 2014; Yao et al., 2021)), studies on conifers are still relatively rare (Moran et al., 2017). A few studies have examined the physiological and transcriptional drought responses of some coniferous species (Lorenz et al., 2011; Behringer et al., 2015; Du et al., 2018; Fox et al., 2018; de Maria et al., 2020; Pervaiz et al., 2021), including *P. abies* (Klápste et al., 2020; Haas et al., 2021).

This study contributes to a deeper understanding of molecular drought stress responses at the transcript level in Norway spruce focussing on a specific genotype.

Materials and Methods

Picea abies clone

Clonal plants of *P. abies* were obtained by a tissue culture method based on cytokinin-induced adventitious buds from four-week-old seedlings (Ewald and Suss, 1993; Ewald and Hu, 2007). The method was applied, among others, to seeds of the provenance 'Marienberg' originating from the Saxon Ore Mountains. One of the clones with long-term juvenile behavior and orthotropic growth was continuously propagated. Plants of this clone, which were about 15 cm high, were potted 16 months before the start of the drought stress experiment to ensure undisturbed root development and maintained in the open field according to good horticultural practice.

Drought stress experiment

The plants should be under drought stress during their flushing in order to sample the freshly sprouted needles. Therefore, eight plants of the Norway spruce clone in the dormant bud stage were individually labeled and kept in the greenhouse since March 2017. When bud burst started with the first visible green tips, the experiment started with a last watering on 04/03/2017 for all ramets. Then, watering was continued three times a week for 4 plants until a constant pot weight was reached (control), and no watering was done for the other 4 plants (stress). Plants were observed at least three times a week. Slight differences in sprouting progress of the 8 ramets were observed, but still no difference between control and stress variants at the painter's brush stage. However, sprouting then developed more slowly in the stressed plants than in the control. First slight signs of wilting were visible on 04/24/2017, and on 04/26/2017 samples were taken from the 3 most homogeneous stress and control plants as complete, freshly sprouted needles.

RNA extraction and sequencing

Total RNA from 6 ramets (3 stressed and 3 controls) was extracted individually by applying the Spectrum™ plant total RNA kit (Sigma, Saint. Louis, USA). To avoid DNA contamination, the On-Column DNase I Digest Set (Sigma, Saint. Louis, USA) was used following the manufacturer's instruction. Quantity of the RNA was determined with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The quality was measured with the Bioanalyzer Agilent 2100 (Agilent Technologies, Waldbronn, Germany). For each sample of total RNA, non-stranded cDNA libraries were created from 1 µg of RNA (GATC Biotech AG, Konstanz, Germany). All libraries were sequenced by GATC Biotech AG (Konstanz, Germany) on an Illumina HiSeq 4000 platform to create 150 bp paired-end reads.

Transcript quantification, count normalisation and differential gene expression analysis

Reads were trimmed for adapter sequences and quality with trimmomatic (Bolger et al., 2014). Read mappings were done with the STAR aligner (Dobin et al., 2013) against all contigs containing genes in the reference genome of *P. abies* (Nystedt

et al., 2013). Reads were counted with htseq-count (Anders et al., 2015). FPKM (fragments per kilobase of transcript per million fragments mapped) values were calculated from count values to normalise counts for effects of sequencing depth and transcript length. Differential gene expression analysis was done in R with the bioconductor package Deseq2 (Love et al., 2014).

MapMan annotation and pathway mapping of differentially expressed genes

Coding sequences (CDS) of all differentially expressed genes (DEGs) were extracted from all CDS annotated in the reference genome of *P. abies* v 1.0 (Nystedt et al., 2013) using the tool faSomeRecords from UCSC (http://hgdownload.soe.ucsc.edu/admin/exe/linux.x86_64/). Extracted CDS were analysed using the Mercator web application 3.6 (Lohse et al., 2014) (https://www.plabipd.de/portal/web/guest/mercator-sequence-annotation) with default parameters to assign MapMan "Bins" to each DEG. Based on the MapMan annotation, DEGs were mapped with related log2 fold changes (log2FC)-values to graphical displays of different biological processes, pathways and gene groups using MapMan 3.6.0RC1 (Usadel et al., 2009; tool downloaded from https://plabipd.de/portal/mapman).

Construction of functional protein association networks

Functional protein association networks were constructed by STRING (https://string-db.org/) using *A. thaliana* gene ID lists as input (default parameter; but with "meaning of network edges" set to confidence). Network clustering was performed using kmeans clustering.

Gene Ontology enrichment analyses

Over-representation analysis of Gene Ontology (GO) terms in different transcript groups of *P. abies* was carried out using the plugin BiNGO v3.0.3 (Maere et al., 2005) for the software package Cytoscape v3.8.0 (Shannon et al., 2003). A GO ontology file (go-basic.obo) was downloaded from http://geneontology.org/docs/download-ontology/ and used as custom ontology file. The *P. abies* whole GO annotation was used as reference set. A Cytoscape Bingo-specific GO annotation file for *P. abies* (Additional file 1) was created by an inhouse script using the GO annotation of all *P. abies* gene identifiers as an input after download from PlantGenie (https://plantgenie.org/; Sundell et al., 2015). Statistically significant GO-terms over-represented in the test set were selected according to their corrected p-value (False Discovery Rate, FDR rate ≤ 2 %) using a hypergeometric test. Multiple testing correction was performed using Benjamini-Hochberg false discovery rate correction ($p<0.05$).

Over-representation analysis of GO terms in different transcript groups of *A. thaliana* was carried out using AgriGO v2.0 (http://systemsbiology.cau.edu.cn/agriGOv2/; Tian et al., 2017) using the singular enrichment analysis tool and all TAIR genome loci (TAIR10_2017) as reference, the hypergeometric test as statistical test and Bonferroni as multi-test adjustment method with a significance level of 0.05.

PFAM-domain enrichment analysis

The PFAM-domain enrichment analysis was performed using the analysis tool "enrichment" at PlantGenie (https://plantgenie.org/) after selecting *P. abies* v1.0 as species. The list of gene identifiers of all upregulated DEGs served as input for this analysis.

Results

Transcriptome response of Picea abies to drought stress

RNA-sequencing (RNA-seq) of six ramets of a *P. abies* clone under severe drought stress of 23 days or control conditions (Figure 1) yielded an average of about 37.5 million Illumina read pairs per sample (NCBI's SRA, BioProject accession PRJNA912094). On average, approximately 82 percent of the reads per sample mapped to the *P. abies* reference genome (Nystedt et al., 2013).

Drought stress induced extensive transcriptional changes in needles (Figure 2; Additional file 2: Table S1). When considering 70,736 annotated genes, 31,984 genes featured transcript expression (FPKM>1) under control condition and 32,020 under stress condition (overlap of 30,105 genes; Additional file 2: Table S1). In total, 2,402 genes were identified as DEGs, of which 1,186 were up- and 1,216 were downregulated (Figure 2b; Additional file 2: Table S2).

The 10 up- or downregulated genes each with the highest absolute values of related log2 fold change (log2FC) are presented in Additional file 2: Table S3 with their annotations. The three genes with strongest upregulation (log2FC > 14) after drought stress in needles of young *P. abies* plants are annotated as thaumatins. Thaumatin is an intensely sweet-tasting protein, 100,000 times sweeter than sucrose on a molar basis, found in berries from *Thaumatococcus daniellii*, a tropical flowering plant known as Katemfe (Edens et al., 1982). Among the downregulated genes, MA_287316g0010 was identified, which was described as potential ABA receptor PYL4 (Additional file 2: Table S3). In *A. thaliana*, this gene encodes a member of the PYR (pyrabactin resistance)/PYL (PYR1-like)/RCAR (regulatory components of ABA receptor) family proteins with 14 members. PYR/PYL/RCAR family proteins function as ABA sensors.

Influence of drought stress on cellular functions and specific pathways in Picea abies

MapMan annotation of 2,402 DEGs resulted in the assignment of MapMan "Bins" to DEGs (MapMan mapping file as Additional file 3). The display of the DEGs on the MapMan pathway "cellular functions" reveals a strong downregulation of DNA synthesis-related genes, but upregulation of RNA- and protein synthesis-related genes among others (Additional file 4). The MapMan display "cellular response" with all DEGs assigned to this pathway shows a strong response of DEGs assigned to biotic as well as to abiotic stress (Additional file 5). Moreover, genes assigned to redox processes (mainly ascorbate and

Figure 1
Photos of three control plants (upper panel) and three drought-stressed plants (lower panel). The photos were taken on the day of sampling on 04/26/2017.

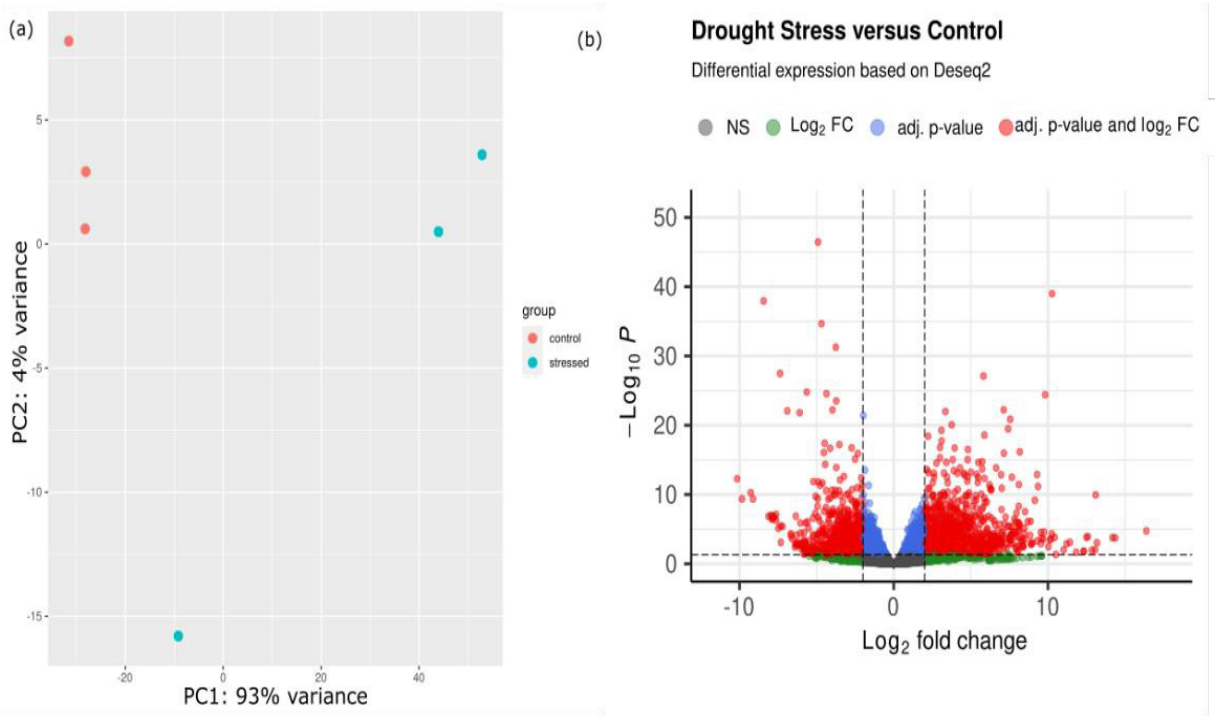
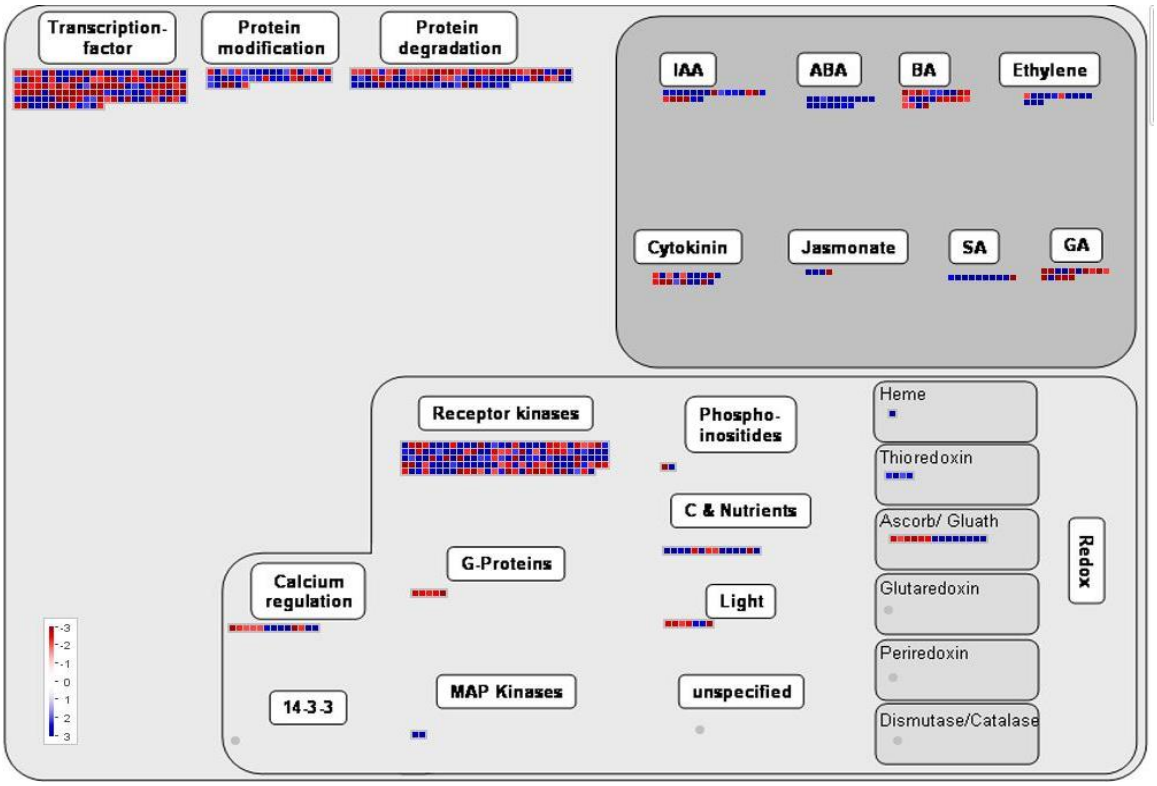


Figure 2
Differentiation between samples and selection of differentially expressed genes. (a) PCA showing a clustering of the control samples (red dots) and the samples exposed to drought stress (blue dots). One sample from the stressed group seems to differ from both the other stressed samples as well as the control group. (b) Vulcano plot created with the R package EnhancedVolcano (Blighe et al., 2018) showing $\log_2$ fold changes against p-values of differentially expressed genes (stressed versus control samples). 2,402 genes showed $\log_2$ fold change values greater or less than +2 or -2, respectively with an associated adjusted p-value of less than 0.05 (red dots; differentially expressed genes listed in Additional file 2: Table S2). Blue dots show $\log_2$ fold changes between -2 and +2 with a p-value of less than 0.05. Grey dots ($\log_2$ fold change between -2 and +2) and blue dots are not significant. In total 70,736 genes were analysed.

(a)



(b)

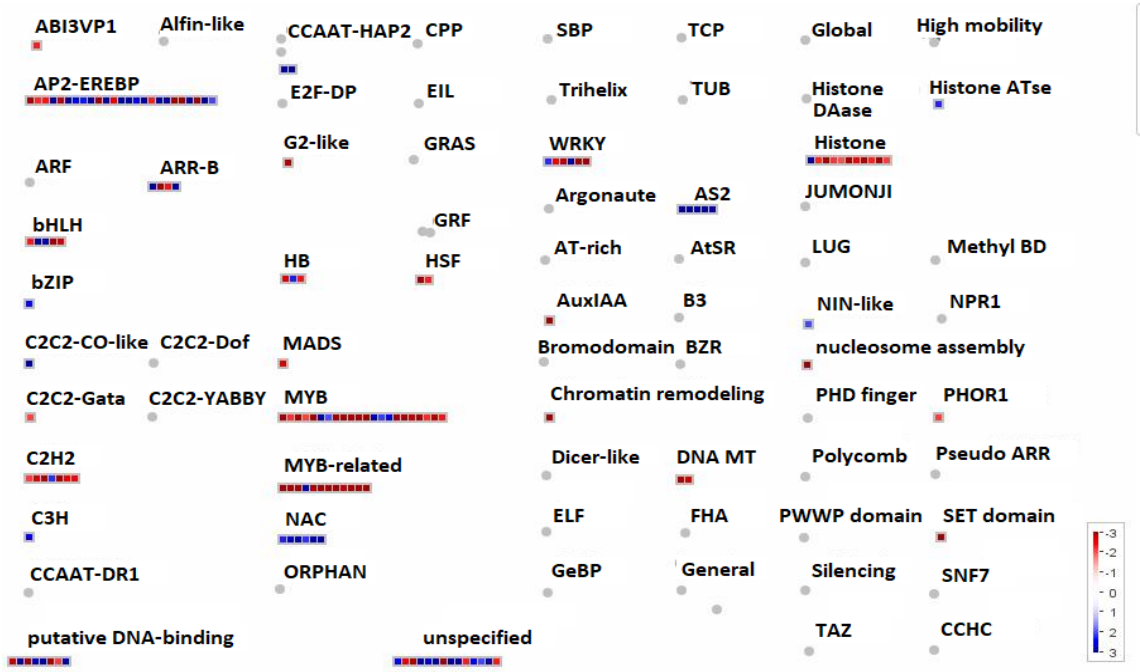


Figure 3
MapMan displays "Regulation overview" (a) and "transcription" (b) with all differentially expressed genes assigned to each pathway, respectively. The colour code represents the $\log_2$ fold change value of each displayed gene.

Figure 4
Over-represented GO terms assigned to "biological process" in the set of 1,186 differentially expressed *Picea abies* genes upregulated by drought (section of all significantly overrepresented GO terms). Mainly GO terms with direct or indirect connection to "response to stimulus" were presented. All significantly over-represented GO terms assigned to "biological process" are presented in Additional file 6. Overrepresentation was tested compared to all *Picea abies* transcripts used as reference set. Coloured nodes range from yellow to dark orange, representing p-values from 5E-2 to 5E-7. White nodes represent GO terms that are not significantly over-represented in the group. The size of a node is proportional to the number of transcripts annotated to that node.

glutathione-related redox processes), development, cell division and cell cycle are differentially expressed during drought stress (Additional file 5). Among the abiotic stress-related genes, 11 genes were assigned to the sub-BIN "drought/salt" (Additional file 2: Table S4).

When considering hormone metabolism, all DEGs related to ABA and most of the ethylene- as well as salicylic acid (SA)-related DEGs were upregulated under drought stress (Figure 3a). Differential expression (up- or downregulation) was also detected for genes involved in the metabolism of the other plant hormones: brassinosteroid (BA), auxin (IAA), cytokinin, gibberelin (GA), and jasmonate. Many receptor kinases, transcription factors and genes involved in protein modification and degradation, among others, were identified as drought stress-induced DEGs in this study (Figures 3a and b).

A more detailed graphical display of the transcription factors identified as DEGs is presented in Figure 3b. Only one of the DEGs was annotated as bZIP and showed upregulation under drought stress (MA_9399348g0010; log2FC: 2.4; Additional file 2: Table S2). The putative *A. thaliana* homolog of this gene is bZIP3 (Additional file 2: Table S2).

Many gene members of other transcription factor families described to be involved in ABA-dependent pathways such as MYB, bHLH (MYC), and WRKY show also differential expression under drought stress (Figure 3b). All DEGs annotated as potential NACs were upregulated. Moreover, four potential AS2 transcription factor genes, encoding LOB (lateral organ boundaries) domain-containing proteins were upregulated. Considering AP2/EREBP transcription factors (involved in an ABA-independent pathway; see Introduction), more than 20 DEGs were identified (Figure 3b).

Overrepresented functions and protein domains in the upregulated genes

The gene set of the 1,186 *P. abies* DEGs upregulated by drought stress in this study was analysed in more detail to get an impression on cellular functions and processes induced by drought stress. Several GO terms assigned to "biological

Table 1
Significantly enriched GO terms assigned to "molecular function" in the DEGs upregulated by drought stress in *Picea abies* (adjusted p-value<0.05).

GO-ID	Description	Adjusted p-value	Test group	Reference group
22834	ligand-gated channel activity	7.7E-3	6/256	26/9821
15276	ligand-gated ion channel activity	7.7E-3	6/256	26/9821
16491	oxidoreductase activity	3.9E-2	60/256	1517/9821
47268	galactinol-raffinose galactosyltransferase activity	3.9E-2	2/256	2/9821
47896	formaldehyde transketolase activity	3.9E-2	2/256	2/9821
22836	gated channel activity	3.9E-2	6/256	42/9821
5242	inward rectifier potassium channel activity	3.9E-2	3/256	8/9821
99094	ligand-gated cation channel activity	3.9E-2	3/256	8/9821

Test group, DEGs upregulated; reference group, all genes annotated by GO in *Picea abies* 1.0. The first number in each group refers to the number of genes assigned to the related GO-ID in the group. The second number refers to the total number of genes assigned to any GO-term related to "molecular function" in the group. Statistically significant GO-terms over-represented in the test set were selected according to their adjusted p-values (False Discovery Rate, FDR rate ≤ 2 %; multiple testing correction with Benjamini-Hochberg false discovery rate correction).

Table 2
Most significantly enriched PFAM-IDs in differentially expressed *Picea abies* genes upregulated by drought stress (top 5-PFAM-IDs).

PFAM-ID	Description	Adjusted p-value	Test group	Reference group
PF00257	Dehydrin	1.0E-12	11/779	16/34399
PF00314	Thaumatin family	3.4E-9	14/779	56/34399
PF00182	Chitinase class I	2.0E-8	13/779	54/34399
PF02496	ABA/WDS induced protein	8.0E-7	11/779	49/34399
PF01501	Glycosyl transferase family 8	8.0E-7	10/779	39/34399

Test group, DEGs upregulated; reference group, all genes annotated by PFAM in *Picea abies* 1.0. The first number in each group refers to the number of genes assigned to the related PFAM-ID in the group. The second number refers to the total number of genes assigned to any PFAM-ID in the group. The PFAM-domain enrichment analysis was performed using the analysis tool "enrichment" at PlantGenie (https://plantgenie.org/) after selecting *P. abies* v1.0 as species. Results are presented as provided by PlantGenie (Fisher exact test for PFAM enrichment analysis and Benjamini-Hochberg for multiple testing correction; adjusted p-values after multiple testing correction; https://plantgenie.org/Help).

process" (Additional file 6) showed significant overrepresentation compared to the *P. abies* reference set; among them many GO terms directly or indirectly related to "response to stimulus" (Figure 4). In this group, the GO terms "response to stress" and "response to nitrogen compound" show the lowest p-values. As expected, also GO terms related to water stress, such as "response to water deprivation" and "response to abscisic acid" are overrepresented.

Eight GO terms assigned to "molecular function" - including five GO terms related to "channel activity" - showed significant over-representation in the set of upregulated DEGs (Table 1). No GO terms related to the main GO category "cellular compartment" were enriched in the upregulated DEGs. In total, 47 significantly enriched PFAM-IDs were identified in the group of upregulated DEGs (Additional file 7; see all PFAM-IDs with adjusted p-value<0.05). The five most significantly enriched PFAM-IDs are presented in Table 2.

As expected, the conserved dehydrin protein family (PF00257) is enriched in the upregulated genes. This multifamily of proteins is produced in response to cold and drought stress in plants and belongs to the Group II Late Embryogenesis Abundant (LEA) family. Dehydrins include a high number of charged amino acids and are hydrophilic, reliably thermostable, and disordered. The conserved protein domain "Thaumatin family" (PF00314) is also overrepresented in the upregulated DEGs. The thaumatin family is also referred to as pathogenesis-related group 5 (PR5), as many thaumatin-like proteins accumulate in plants in response to infection by a pathogen and possess antifungal activity (Ruizmedrano et al., 1992). The proteins are involved in systemically acquired resistance and stress response in plants, although their precise role is unknown (see also InterPro entry IPR001938). The "ABA/WDS"-family (PF02496) that is also enriched in upregulated DEGs is a family of plant proteins induced by water deficit stress (WDS) (Padmanabhan et al., 1997) or ABA and ripening (Canel et al., 1995).

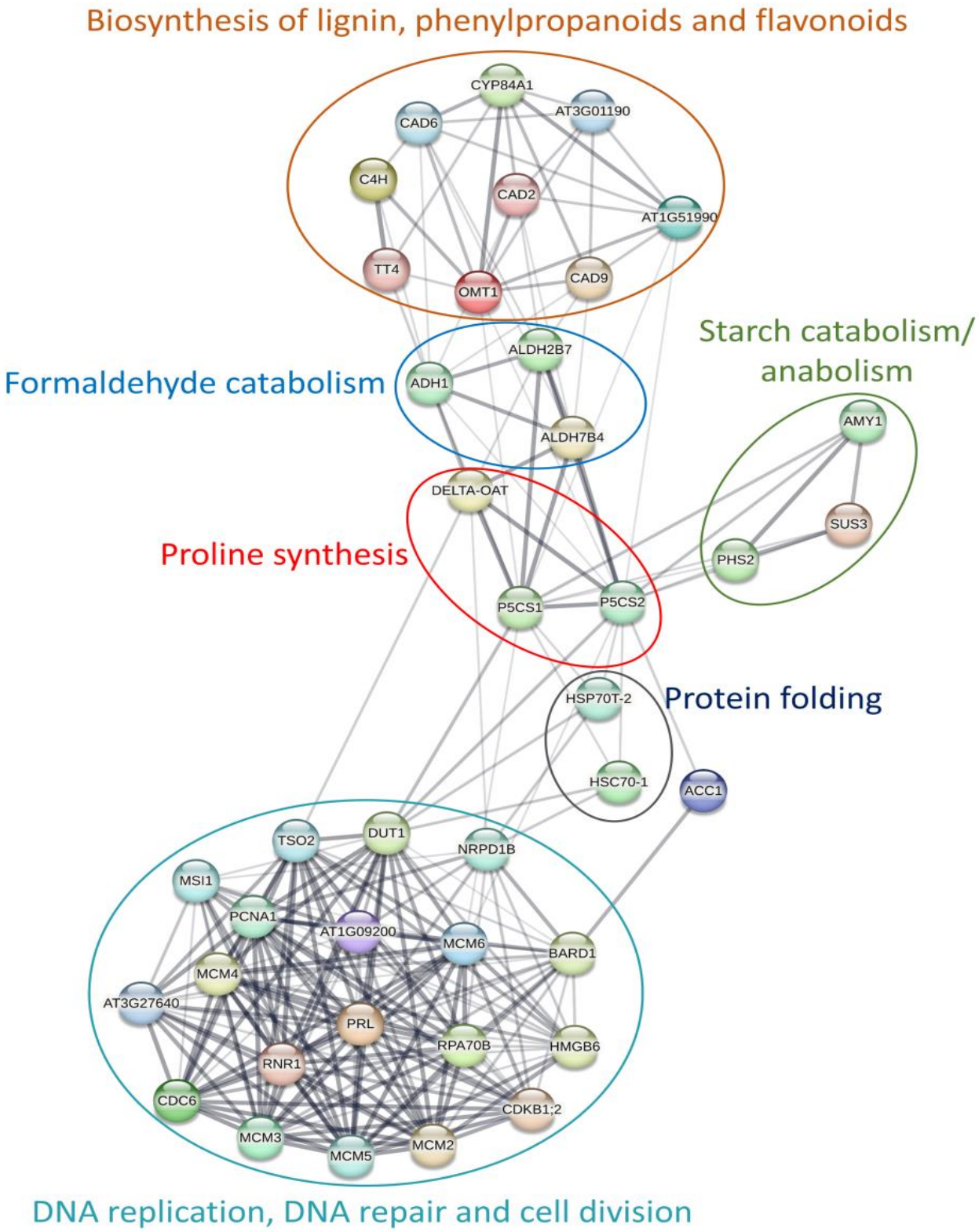


Figure 5
STRING functional interaction network of the 40 most connected *Arabidopsis thaliana* homologs of all differentially expressed *Picea abies* genes. Genes with at least 25 connections were selected based on the STRING analysis of all differentially expressed genes in *Picea abies* (Additional file 2: Table S5) and then analysed separately with STRING. The functional annotation of these hub genes is in Additional file 2: Table S6.

Transcriptome modulation by drought in the context of functional interactions

A nonredundant set of all known *A. thaliana* homologs (comprising 1,053 genes) of the 2,402 drought-induced DEGs in *P. abies* (Additional file 2: Table S2) were analysed using STRING (https://string-db.org/; Szklarczyk et al., 2019) to construct a functional protein association network (considering genomic context information, co-expression, experimental biochemical/genetic data, text-mining, and previously curated pathway and protein-complex knowledge from other different databases). Most of the analysed *A. thaliana* genes (880 genes out of 1.053) are connected with at least one other gene in this huge clustered network comprising 880 nodes and 3.396 edges (Additional file 8). The number of edges per connected node range from 1 to 62 with a mean value of 3.9 edges per node (Additional file 2: Table S5).

Interestingly, the top-1 hub with 62 connections is P5CS1 (AT2G39800) and the top-2 hub with 56 connections P5CS2 (AT3G55610). Both of the related putative *P. abies* homologs, MA_10437026g0010 (P5CS1) or MA_133858g0010 (P5CS2) show an upregulation under drought stress (log2FC of 2.8 or 2.4, respectively; Additional file 2: Table S2). The P5CS1 gene encodes a delta1-pyrroline-5-carboxylate synthase in *A. thaliana*. This enzyme catalyses the key step in the biosynthesis of proline. The gene is known to be expressed in reproductive organs and tissues under non-stress conditions but in the whole plant under water-limiting condition. Expression is also induced by ABA and salt stress in a light-dependent manner. P5CS1 appears to be involved in salt stress responses related to proline accumulation, including protection from reactive oxidative species (https://www.arabidopsis.org/). P5CS2 encoding delta 1-pyrroline-5-carboxylate synthetase B is also known to be expressed under conditions of dehydration, high salt and ABA in *A. thaliana*.

In total, 40 *A. thaliana* genes with at least 25 edges in the entire STRING network (Additional file 2: Table S5) were selected (annotation of these genes in Additional file 2: Table S6) and analysed separately by STRING. In the resulting smaller network, all nodes are connected (Figure 5). P5CS1 and P5CS2 (see above) are in the center of this network, both connected to another gene potentially involved in proline metabolism: DELTA-OAT. This gene encodes an ornithine delta-aminotransferase that is transcriptionally upregulated in young plants and in response to salt stress in *A. thaliana*. The potential *P. abies* homolog of DELTA-OAT was upregulated under drought in this study (MA_10428453g0010; Log2FC: 2.3; Additional file 2: Table S2).

In the set of 40 top-hubs (Figure 5), several GO-terms are overrepresented (most significant GO terms assigned to "biological process" in Additional file 9). These GO terms include among others two terms related to lignin synthesis. Genes related to lignin synthesis appear in the upper cluster in Figure 5, e.g., OMT, annotated as caffeic acid 3-o-methyltransferase and flavone 3'-O-methyltransferase. This enzyme catalyses the methylation of monolignols, the lignin precursors. Other examples are the cinnamyl alcohol dehydrogenases CAD2, CAD6, and CAD9, which (probably) catalyse the final step specific for the production of lignin monomers (https://www.arabidopsis.org/). Also GO-terms related to DNA and cell cycle are overrepresented in the set of 40 hubs (Additional file 9; e.g., "cell cycle", "DNA replication", "DNA metabolic process", "DNA conformation change"). Related genes are mainly included in the lower cluster in Figure 5.

Discussion

Long-living plants such as trees are increasingly and repeatedly exposed to drought stress together with other biotic and abiotic stresses as a result of increasing climate change. Among all of the abiotic stresses, drought stress is one of the detrimental stresses inhibiting plant growth and productivity. Plants respond to drought stress through complex regulatory networks inducing molecular, physiologic and morphologic responses enabling plants to survive periods of limited water availability (Harfouche et al., 2014; Estravis-Barcala et al., 2020; Ranjan et al., 2022). In drought stress-induced signalling cascades, transcription factors play key roles by regulating the expression of large numbers of downstream genes (Yao et al., 2021).

In this study, drought-induced transcriptional changes in needles of Norway spruce clonal plants were analysed by subjecting the plants to severe drought stress of 23 days, in comparison to control plants (normal watering). With our experimental approach of using ramets of one clone from adventitious bud culture, we avoided different genetic backgrounds or even combinations with various genotypes in the case of grafted plants.

Drought stress induced extensive transcriptional changes comprising 2,402 DEGs, of which 1,186 were up- and 1,216 downregulated (Figure 2). Both, extensive up- or downregulation was also obvious in response to drought stress in a recent RNA-seq-study in Norway spruce (Haas et al., 2021); although fewer DEGs have been identified in needles here, when analysing different drought stress conditions (871 up- and 433 downregulated DEGs). In total, 116 overlapping DEGs were identified when comparing our DEG list (Additional file 2: Table S2) with 689 DEGs identified by Haas et al. (2021) under severe drought stress in needles.

DEGs potentially related to ABA-dependent responses were analysed in more detail because ABA is the best known trigger of the cascade of drought signaling (reviewed in (Daszkowska-Golec and Szarejko, 2013)). We identified several DEGs (all upregulated) predicted to be involved in ABA metabolism (Figure 3A), including among others a potential *P. abies* homolog (MA_10434448g0010; Additional file 2: Table S2) of *A. thaliana* NCED3, a 9-cis-epoxycarotenoid dioxygenase, that is involved in ABA synthesis and known to be upregulated by drought in several plant species (Daszkowska-Golec and Szarejko, 2013). The core components in ABA signalling include ABA receptors (PYR/PYL/RCAR), PP2C phosphatases (negative regulators), and SnRK2 kinases (positive regulators). We identified among others the putative PYL4 homologs MA_287316g0010 and MA_10427386g0010 as down-regulated DEGs (Additional

file 2: Table S2). In agreement with Haas et al., (2021), we did not identify any potential homolog of the *A. thaliana* PP2C SnRK2.6 (OST1) as DEG. This gene showed high and constant expression under drought in needles (Haas et al., 2021). Also homologs of the other two key PP2Cs in drought signalling in *A. thaliana* (SnRK2.2 and SnRK2.3; Bhaskara et al., 2012) were not identified in our study as DEGs. However, we found three potential *P. abies* homologs of HAI3 (AT2G29380; highly ABA-induced PP2C protein 3) as upregulated DEGs (Additional file 2: Table S2; MA_906876g0010 as homolog with highest upregulation). In *A. thaliana*, HAI3 is described to function as a negative regulator of osmotic stress and ABA signaling by attenuating low water potential signaling controlling proline and osmoregulatory solute accumulation (Bhaskara et al., 2012).

The identification of putative HAI3, which is known to be expressed later on in the signalling cascade (after ABA-induced activation of ABF transcription factors (Bhaskara et al., 2012)) could indicate that the 23 days-drought stress applied in our study represent a relatively late time point, potentially occurring after transcriptional induction of PP2Cs and SnRK2s. Under this assumption it is not unexpected that we did not identify any of the above mentioned three SnRK2s described in *A. thaliana* (Fujita et al., 2009) as DEGs in agreement with the study of (Haas et al., 2021). It could also be that transcriptional regulation plays a subordinate role for the above mentioned key PP2Cs and SnRK2s, since activation of the related proteins takes place at the post-transcriptional level by interactions and phosphorylation (Bhaskara et al., 2012).

Interestingly, also members of the bZIP transcription factors of the ABA-dependent pathway known from *A. thaliana* (AREB1, AREB2, ABF1, and ABF3 (Yoshida et al., 2010; Singh and Laxmi, 2015; Yoshida et al., 2015)) did not show differential expression under drought stress in Norway spruce in our study (Additional file 2: Table S2) in accordance to Haas et al. (2021). We identified only one potential bZIP as DEG, the gene MA_9399348g0010 with upregulation under drought (Figure 3b; Additional file 2: Table S2). The related putative *A. thaliana* homolog bZIP3 was described as a novel sugar-responsive transcription factor whose expression was modulated by the SNF1-RELATED KINASE 1 (SnRK1) pathway (Sanagi et al., 2018).

However, the identification of several members of transcription factor families known to be involved in the ABA-dependent pathway (Yao et al., 2021) as DEGs in our study (Figure 3b, e.g., MYBs, MYB-related, WRKY), the overrepresentation of the GO term "response to abscisic acid" (Figure 4) and of the "ABA/WDS"-family (PF02496; Table 2) in the upregulated DEGs point to the regulation of some genes via the ABA-dependent pathway among drought in *P. abies*.

Beside ABA, several other phytohormones including ethylene were reported to be involved in drought stress responses in different plant species (Salvi et al., 2021). The results in Figure 3a further support these findings for *P. abies*. Several AP2/EREBP transcription factors, known to be involved in ABA-independent drought response pathways (Yao et al., 2021) were identified as DEGs (Figure 3b), among them two upregulated DEGs (MA_19420g0010, MA_16778g0010; Additional file 2: Table S2) potentially encoding DREB2 proteins (DREB2C, DREB2A).

Observed cross-talks between ABA-dependent and -independent drought response pathways (Daszkowska-Golec and Szarejko, 2013; Yao et al., 2021) could eventually support combined stress responses to different types of abiotic stress as also indicated by our results (Additional file 5; DEGs assigned to MapMan bin "abiotic stress"). Interestingly, we found the GO term "heat acclimation" enriched among the upregulated DEGs (Figure 4). Shared heat and drought responsive genes were, e. g. identified in *Populus simonii* (Jia et al., 2017). Such shared responses between drought and heat could be vital for trees to survive because high temperatures are the most common stress occurring simultaneously with drought in forests.

The GO enrichment analysis in the upregulated DEGs provided additional relevant GO terms such as "response to abscisic acid" and "channel activity"-related terms among others (Figure 4; Table 1). However, insights in more specific molecular processes potentially involved in drought stress signalling were gained from the analysis of DEGs in the context of functional interactions using STRING (Figure 5). Functional gene association networks of DEGs using the STRING database were also constructed in a few other plant studies to interpret transcriptome changes (Davin et al., 2016; Das et al., 2022). Our functional STRING interaction network of the 40 most connected *A. thaliana* homologs of the *P. abies* DEGs placed the two top-hubs P5CS1 (MA_10437026g0010) and PCS2 (MA_133858g0010) in the center of this network (Figure 5). Hass et. al (2021) also identified MA_10437026g0010 as an upregulated DEG in root (severe drought stress). P5CS1 is the key enzyme in the biosynthesis of proline which is known to be accumulated in plants in response to many abiotic stresses. Selected haplotypes of the P5CS1 promoter in barley, e.g., had quantitative variation in P5CS1 gene expression and proline accumulation under drought stress (Shrestha et al., 2022). The introgression of a P5CS1 allele from a high proline accumulating wild barley accession into cultivar Scarlett by the authors resulted in a near-isogenic line which accumulated higher proline concentrations and featured a better performance under drought and a higher drought stress recovery compared to Scarlett.

Another member of the interaction network of the top hubs (Figure 5), DELTA-OAT (MA_10428453g0010) which was upregulated under drought in our study was shown to enhance drought and osmotic stress tolerance when overexpressed in rice (OsOAT; You et al., 2012). Some genes involved in lignin synthesis identified as members of the top-hub-network in Figure 5 were described to confer or contribute to drought tolerance in other plant species, e.g. OMT in Tobacco (Song et al., 2022), CmCAD2 and CmCAD3 in *Cucumis melo* (Liu et al., 2020). A recent study shows, that one could create and/or select plants that can better recover from drought by genetically modifying their lignin chemistry to get plants with aldehyde-rich instead of alcohol-rich lignin (Menard et al., 2022). The authors showed that higher accumulation of aldehyde-rich guaiacyl subunits of lignin (G_{CHO}) in a cad4 cad5 double mutant increased drought tolerance in *A. thaliana*.

The analysis of natural intra-specific variation in the DEGs identified in our study (especially in the top-hubs), in their related promoters and transcriptional regulators, the identification of related SNPs and the further analysis of their potential association with drought-related environmental variables or drought tolerance-related phenotypes may contribute to the dissection of the genetic bases of drought adaption and drought tolerance in *P. abies*. Exome sequencing of a first subset of DEGs from our study in individuals phenotyped for drought tolerance resulted in the identification of a few potential adaptive SNPs which were included in a novel and diverse set of SNP markers for range wide genetic studies in *P. abies* (Mader et al., 2022).

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