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RESEARCH

Transcriptome Analysis Revealed Behavior Complexity of Senescence Responses in Himalayan Tree Species *Ulmus wallichiana*

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Abstract

The Himalayas present a highly contrasting environment for the organisms that inhabit it, with summer and winter being the most disparate seasons. As winter approaches, the temperature drops, and deciduous plants, such as *Ulmus wallichiana* Planch., undergo senescence. In this study, we used the RNA-seq approach to generate a de novo transcriptome assembly of *U. wallichiana*, consisting of 300,067 unigenes. We investigated the differential expression pattern of these unigenes under changing climatic conditions, with a focus on the contrast between winter and summer. We observed that 2746 unigenes were differentially expressed, with a fold change of ≥ 2 and an FDR-adjusted *p*-value of ≤ 0.001. Among these unigenes, 37 were found to be related to senescence. We also identified nine DREB unigenes in the *U. wallichiana* transcriptome, which exhibited more or less contrasting patterns between summer and winter. These DREB unigenes may play an important role in regulating the plant's tolerance to cold and/or drought stress. Our findings shed light on the complex and dynamic process of transcriptomic reprogramming that enables *U. wallichiana* to cope with seasonal changes. Furthermore, this study highlights the urgent need for further research on *U. wallichiana*, as this endangered plant species remains largely unexplored at the genomic level.

Keywords Senescence · RNAseq · Differential gene expression · DREB · *Ulmus wallichiana*

Introduction

Plants possess remarkable adaptability to thrive under challenging environmental conditions, as their adaptations are crucial for sustaining cellular functions, such as growth, stress protection, and metabolism. However, due to their sessile lifestyle, plants are constantly exposed to environmental fluctuations that trigger responses to maintain internal homeostasis. Upon sensing environmental signals, the first level of response is manifested at the transcriptomic level, where a cascade of genes is turned on/off to cope with the environmental cue. Therefore, elucidating the transcriptome response of plants to different environmental conditions can provide deeper insights into their response and adaptation to uncontrolled natural environments (López-Maury et al. 2008). Senescence is a critical developmental stage in both plant and animal cells, characterized by the cessation of growth and cellular activities, which is usually followed by cell death in plants. Gene expression is modulated by developmental and environmental signals to regulate senescence (Gamelon et al. 2014). Changes in climatic conditions may also trigger molecular events leading to senescence. Evaluating transcriptomic changes under various climatic conditions leading to senescence can elucidate the crosstalk between climatic variations and senescence. The Himalayas serve as an essential natural habitat for thousands of plant and animal species, experiencing extreme environmental variations temporally across the year and spatially across latitudinal and longitudinal gradients. The most contrasting environmental factors experienced by organisms inhabiting the Himalayas are during winter and summer seasons. The former offers low temperatures, sometimes below freezing point, while the latter exhibits relatively higher values, generally above 20 °C. Apart from temperature variations, there is a sharp variation in photoperiods, UV-B exposure, and precipitation (Majeed et al. 2020; Karlson and Werner 2001). These variations may influence various biological processes in plants, including senescence.

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Ulmus wallichiana Planch., a medicinal plant native to the Himalayas, faces extreme climatic variations throughout the year. Environmental factors such as light irradiance, photoperiods, and UV exposure have been shown to affect the development of leaves and flowers, as well as leaf senescence in plants (Heo et al. 2003; Balasaraswathy et al. 2002). To gain a deeper understanding of *U. wallichiana* genetic basis for survival, this study aimed to elucidate its transcriptomic responses under changing climatic conditions in different seasons. Using cutting-edge RNA sequencing technology, we investigated the genetic mechanisms underlying seasonal adaptation and senescence regulation in *U. wallichiana*. Our study also performed a genome-wide identification of dehydration-responsive element-binding proteins (DREBs), which are known to play a crucial role in regulating stress and adaptive responses in plants. Through this research, we shed light on the plant's adaptive strategies for survival under changing environmental conditions and gained insight into the genetic mechanisms of seasonal adaptation. Our findings reveal the combinatorial effects of various environmental factors on *U. wallichiana*'s ability to respond to changing seasons and provide new insights into the regulation of stress responses in plants.

Materials and Methods

Sample Collection and RNA Isolation

Leaf samples of the same plants were collected from Rajouri (Jammu) in liquid nitrogen in two contrasting seasons (winter and summer), to capture the impact of environmental variations on the plant's gene expression. The daily mean temperature at the sample collection sites in winter and summer was 5 °C and 26 °C, respectively. Furthermore, a 4-h difference in day length was observed in these two seasons at the time of sampling. Additionally, the average seasonal precipitation during winter and summer was 187.9 mm and 130.7 mm, respectively, as per the Meteorological Centre, Shimla. Furthermore, the UV exposure during the summer season was six times more than that in winter, which is known to affect the plant's defense mechanisms (Balasaraswathy et al. 2002). A total of 10 individual plants (5 each season) were sampled, and RNA of the collected samples was isolated using a modified version of the protocol developed by Kejani et al. (2010). Two RNA pools belonging to summer and winter were created. Each pool consisted of equimolar concentrations of the RNA from five samples. Two replicates were created for each pool to increase the statistical power of the study.

Sequencing and Genome Assembly

Four cDNA libraries, two for each season, were prepared from the pooled RNA using the NEBNext® Ultra™ Directional RNA Library Prep Kit (New England BioLabs, MA, USA). The libraries were then sequenced using the Illumina HiSeq 2000 platform (Illumina, San Diego, CA, USA). The resulting paired-end raw reads were cleaned and processed to remove low-quality reads using Phred quality score ≥ 20 through Trim galore v0.4.1 (Krueger and Trimgalore 2021). The de novo assemblies from the individual libraries were generated using Trinity v2.9.1 (Grabherr et al. 2011), and a total of four different transcriptome assemblies were created. The quality of the assemblies was assessed by mapping the reads back to the corresponding de novo assembly using Bowtie2 (Langmead and Salzberg 2012) and by evaluating the assembly completeness through BUSCO v4 (Simão et al. 2015). The sequences were further refined by removing redundancy followed by clustering to generate unigenes through CD-HIT-EST using a sequence identity threshold of ≥ 95 (Li and Godzik 2006). Finally, a master assembly was generated by concatenating the unigenes from the individual transcriptome assemblies.

Differential Gene Expression Analysis

The cleaned paired-end reads were aligned against the master assembly using RSEM (Li and Dewey 2011) with Bowtie2 (Langmead and Salzberg 2012) as an internal aligner, which generated accurate count estimates of the transcripts. The resulting count matrix was used as input for DESeq2 implemented in DEBrowser (Love et al. 2014; Kucukural et al. 2019) to identify differentially expressed genes using a fold change (FC) threshold of ≥ 2 and an FDR-adjusted p-value cutoff of ≤ 0.01. Before differential gene expression analysis, we applied TMM (trimmed mean of M values) based normalization on transcript counts to ensure the robustness and reliability of the results. This normalization method is considered to be robust and powerful, allowing to control for any technical variations in the sequencing process. Additionally, the low expressed transcripts were filtered out using CPM < 1 in at least 1 sample, and the batch effect was corrected through the combat method implemented in DEBrowser.

DEGs Functional Characterization

Functional annotation of the differentially expressed genes (DEGs) was performed through WebGestalt (Liao et al. 2019) and Mercator4 (Schwacke et al. 2019). Identification

of GO terms associated with the DEGs and GO enrichment was performed through WebGestalt using *Arabidopsis thaliana* as a reference and a false discovery rate (FDR) threshold of 0.05. Mercator4 was used for pathway functional annotation of DEGs to identify the key metabolic and signaling pathways that are most affected by the changes in gene expression. The Plant TFDB (Jin et al. 2016) was used to identify the transcriptional factors that are most likely to be affected by climate changes by aligning the DEGs with the database using a *p*-value cutoff of 0.05.

Genome-Wide Identification of DREBs

DREBs are a class of transcription factors (TFs) that belong to the APETALA2/Ethylene-Responsive Factor (AP2/ERF) TF superfamily. The AP2/ERF superfamily is characterized by the AP2 domain, which is 60–70 amino acids long. We used the HMM model of the AP2 domain during HMMER search against the master assembly of *U. wallichiana*. First, full-length transcripts of *U. wallichiana* were identified through the TRAPID pipeline (Van Bel et al. 2013) using the Plaza dicot database (Van Bel et al. 2022) as a reference. Next, the HMMER model of the AP2 domain was used to search against the resulting full-length transcripts to identify AP2/ERF TFs using an *E*-value cutoff of 1e-3. The HMMER-identified AP2/ERF TFs were further verified through SMART and Expasy Prosite (https://prosite.expasy.org/) for the presence of the AP2 domain.

Quantitative Real-Time PCR (qRT-PCR) for in Silico Expression Analysis

The in silico obtained DEGs were validated through quantitative real-time PCR (qRT-PCR) of the randomly chosen 20 unigenes. We constructed cDNA from the isolated RNA using a high-quality cDNA reverse transcription kit (Applied Biosystems). Primer3Plus (Untergasser et al. 2007) was used to design primers for the selected genes. PowerUp SYBER Green Master Mix was used for qRT-PCR of the 20 unigenes with actin as housekeeping gene. Fold changes of these genes were calculated through the $2-\Delta\Delta CT$ method (Rao et al. 2013). Finally, correlation between in silico and qRT-PCR expression was evaluated to ensure the robustness and reliability of the results.

Results

Sequencing and De Novo Assembly

Around 97,581,867 paired-end raw reads were generated by Illumina Hiseq 2000 sequencing of the four cDNA libraries. Approximately 1,062,427 assembled sequences were generated from the four individual assemblies. After that, < 500 bases sequence were excluded from each assembly, which resulted into 376,157 sequences. After removal of sequence redundancy and clustering through CDHIT-EST, unigenes were generated for each assembly followed by their concatenation to create a master assembly, which consisted of 300,067 unigenes. The details of the master and individual assemblies are presented in Table 1. The GC content of the individual assemblies ranged from 40.74 to 48.09%, whereas that of the master assembly was 45%. Quality assessment of the assemblies revealed > 90% average read representation. Furthermore, BUSCO's completeness assessment showed > 80% completeness of all assemblies. Overall, the master assembly was 89% complete. Moreover, the N50 value of 1516 and the mean sequence length of 1222 for the master assembly obtained in our study were better than that of the other de novo transcriptome assemblies like those of *Taxus contorta* (Majeed et al. 2020, 2019), *Rhododendron arboretum* (Choudhary et al. 2014), and *Ulmus wallichiana* (Singh et al. 2021).

Table 1 The summary statistics of the individual and master assemblies. S1 and S2 represent the assemblies corresponding to two replicates of summer, whereas W1 and W2 represent the assemblies corresponding to two replicates of summer

Statistics	S1	S2	W1	W2	Master
Number of reads	29,745,167	36,444,917	15,434,460	15,957,323	*****
Assembled transcripts	175,208	626,188	174,881	86,150	*****
Transcripts after filtration	68,964	177,527	75,594	54,072	376,157
Number of unigenes	56,005	149,332	51,835	42,895	300,067
Mean sequence length	805	524	688	1122	1222
N50	1604	622	1041	1789	1516
L50	26,978	126,325	34,405	17,932	65,260
Seq > 1 K	43,454	62,613	36,252	36,545	113,017
GC content	41.52%	48.09%	43.29%	40.74%	45%
Completeness	87.40%	84.60%	87.80%	83.50%	89%
Read representation	96.26%	89.21%	80.40%	95.54%	90.35%

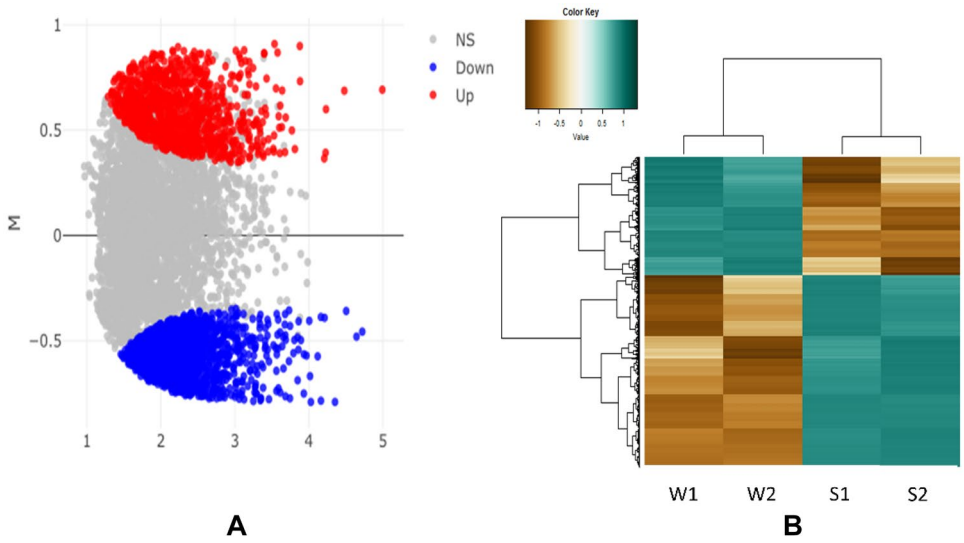

Fig. 1 Graphical representation of differential expressed genes. **A** MA plot showing DEGs with red and blue dots representing upregulated and downregulated genes, respectively. **B** Heat map of DEGs, with brown and green boxes representing downregulated and upregulated genes, respectively

Differentially Expressed Genes (DEGs)

After read count estimation and filtration of low-count unigenes, a total of 37,364 unigenes out of 300,067 were retained for differential gene expression (DGE) analysis using DESeq2. The quality of the data for downstream analysis was improved using batch effect correction and normalization. PCA revealed that 99.9% variation was explained by the principle axis 1 and 2 after normalization (Supplementary file S1). Around 2746 unigenes were found as differentially expressed at a fold change of ≥ 2 and a *p adj* value of 0.001, with 1045 unigenes upregulated and 1701 unigenes downregulated in winter as compared to summer (Fig. 1). The expression profiles of important senescence-related genes are presented in Fig. 2. Sixteen unigenes were selected for validation through qRT-PCR. The expression pattern of these unigenes from RNAseq was correlated to that of qRT-PCR. A correlation coefficient of $r = 0.61$ and $p = 0.004$ was observed, which indicates the credibility of our in silico expression analysis. GO terms were successfully transferred to 2056 DEGs. GO enrichment analysis revealed that 449 categories were enriched in biological process (BP), 118 in cellular component (CC), and 57 in molecular functions (MF) (Fig. 3). A total of 2067 DEGs were functionally annotated through Mercatoer4. A total of 64 unigenes were identified as TFs through the Plant TFDB, which were distributed among 53 families (Supplementary file S2). Among the enriched biological process, response to the temperature, light, and UV, carbohydrate, amino acid, lipid, and nucleotide metabolism, and phytohormones are worth mentioning.

Genome-Wide Identification of DREBs/ERFs

Using the TRAPID pipeline, from a total of 300,067 unigenes, 61,583 were identified as full-length unigenes having

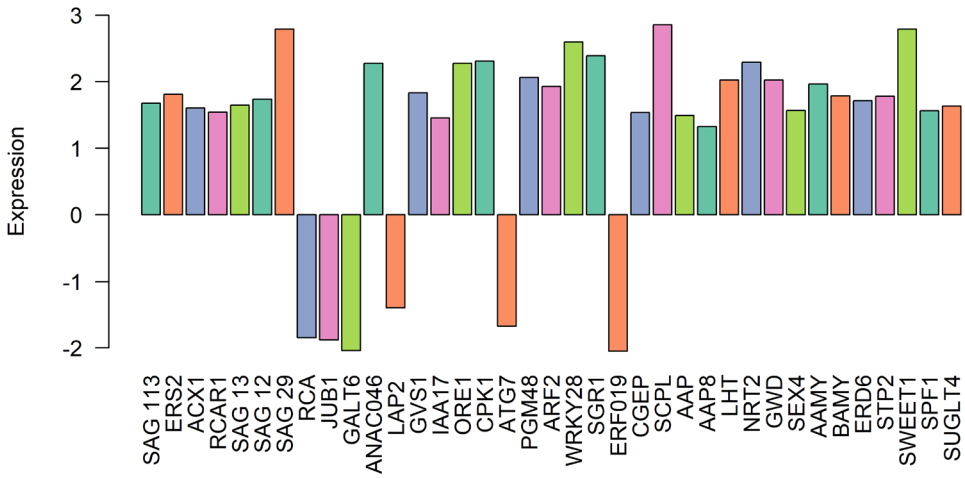

Fig. 2 Expression profile of putative unigenes involved in senescence. The bars on X-axis represent unigenes and their height corresponds to the expression values on log2 scale (Y-axis)

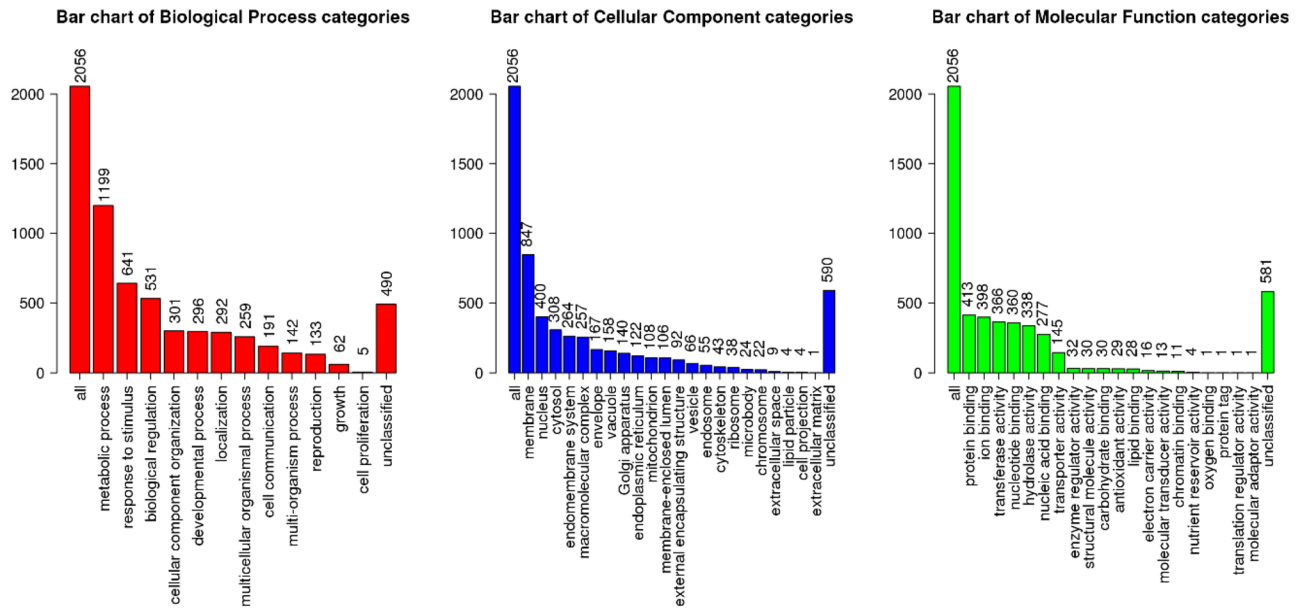

Fig. 3 GO enrichment categories were enriched in biological process (BP), cellular component (CC), and in molecular functions (MF)

a start and a stop codon; 31,567 as quasi-full lengths; and 42,152 as partial unigenes. The amino acid sequences of the 61,583 full-length unigenes were used for screening DREBs through HMMER. A total of 183 proteins were identified to contain the AP2/ERF domain. The AP2/ERF superfamily is divided into different subfamilies, like AP2, RAV, ERF, and DREB. The AP2 subfamily harbors two AP2 domains, and the RAV subfamily contains an AP2 domain and an additional B3 domain. The members of ERF and DREB subfamilies possess a single AP2 domain (Cao et al. 2020; Li et al. 2017; Liu et al. 2013). Based on these criteria, out of the 183 AP2/ERF domain-containing proteins, 99 were classified as AP2 subfamily, 44 as RAV subfamily, and 39 as ERF/DREB (Fig. 4). ERF and DREB subfamilies differ in the amino acid composition at the positions 14th and 19th. In the case of ERF, there are alanine (A)

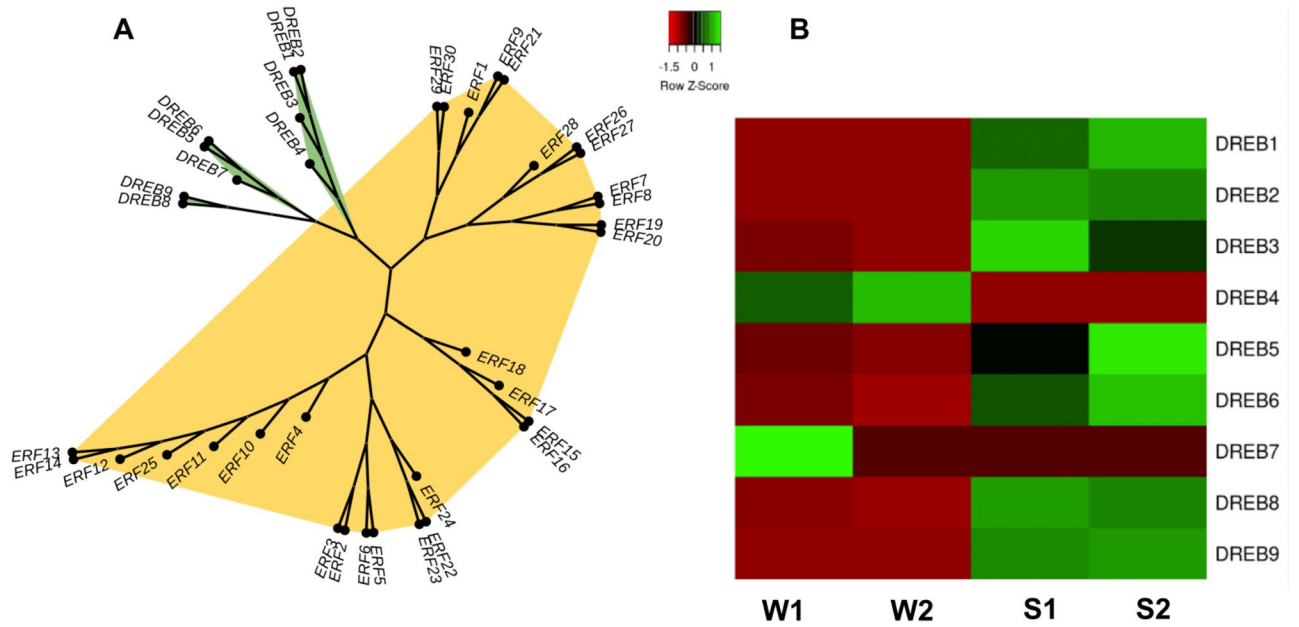

Fig. 4 **A** Unrooted Neighbour Joining Tree of DREBs (green shade) and ERFs (orange shade) based on their functional domains. **B** Heatmap showing expression pattern of DREBs, based on FPKM values

and aspartate (D) at the 14th and 19th positions, respectively. The DREBs possess valine (V) and glutamic acid (E) at the 14th and 19th positions, respectively (Sakuma et al. 2002). Based on these criteria, we obtained a set of 31 ERF proteins and 4 DREB proteins having V and E at the positions 14th and 19th. In addition, two proteins had glutamine (Q), and three proteins had leucine (L) at the 19th position instead of E. The discrepancies at the 19th position have also been observed by Konzen et al. (2019), Hassan et al. (2021), Konzen et al. 2019), and Kui et al. (2023), but V and A at the position 14th are the prominent distinguishing amino acids between DREBs and ERFs, respectively. Therefore, we selected 9 DREBs having valine at the position 14th. The expression analysis of these 9 DREB genes revealed a contrasting pattern in winter and summer seasons (Fig. 4), hence may be essential regulator transcription factors under changed environmental conditions in *U. wallichiana*. Most of the DREB genes were downregulated in winter, except *DREB4* and *DREB7*. Up-regulation of *DREB4* and *DREB7* in winter suggests their role in activating cold tolerance regulon to provide resistance to *U. wallichiana* against cold temperature in winter. Other DREBs such as *DREB1*, *DREB2*, *DREB3*, *DREB5*, *DREB6*, *DREB8*, and *DREB9*, which showed up-regulation in summer, might be involved in providing resistance to heat and/or water stress during summer. Further research is required to evaluate the precise functional role of these *U. wallichiana* DREB genes under different stress conditions.

Discussion

Leaf senescence represents the last stage of development whose progression is determined by both internal and external factors including age, phytohormones, and environmental stresses. The process is characterized by degenerative events culminating in leaf abscission (Guo et al. 2021). Catabolic processes like chlorophyll degradation and breakdown of nucleic acids, proteins, carbohydrates, and lipids predominate during senescence. Senescence ensures that the materials like proteins, carbohydrates, lipids, and nucleic acids are not lost with the dying organs. Instead, these materials are enzymatically degraded in a process, and the products are transported back and loaded within the storage organs like seeds (Lim et al. 2007). In the case of perennial deciduous trees, the resulting products from the catabolism of senescent leaves are relocated to form bark storage proteins (BSP) in the phloem, stored during winter, and finally remobilized to the developing shoots or flowers during the next growing season to fulfill their growth requirements (Keskitalo et al. 2005). Efficient senescence has been suggested to promote better growth, whereas the faulty senescence may cause reduced productivity, indicating the prominent biological significance of senescence (Guo et al. 2021). Understanding the molecular details of senescence would, therefore, provide detailed insights about the plant fitness and basis to manipulate senescence in agronomically important plants for better productivity (Keskitalo et al. 2005).

SENESCENCE-ASSOCIATED GENES (*SAGs*) play a crucial role in regulating senescence. Our analysis identified two unigenes corresponding to *SAG 113*, which showed up-regulation in winter. *SAG* expression has been found to increase with age (Guo and Gan 2014). In *Arabidopsis*, *SAG 113* over-expression negatively regulated abscisic acid (ABA) signal transduction leading to lower sensitivity of stomatal movement, rapid water loss, and leaf senescence (Zhang et al. 2012). Besides, we also observed winter-specific over-expression of *SAG12* that promotes leaf senescence and remobilizes nitrogen from the senescent leaves (James et al. 2018), *SAG13* that is involved in dark-induced senescence (Dhar et al. 2020), and *SAG29* that is induced by osmotic stresses to promote senescence. These observations corroborate our finding of higher *SAG 113*, *SAG 13*, *SAG 12*, and *SAG29* expression during winter, when leaves are at the last stage of their growth. Although leaf senescence is a genetically highly regulated process with multiple layers of regulation at the transcriptional, post-transcriptional, translational, and post-translational stages (Woo et al. 2019), the initiation is determined by external and internal signals. Among the internal signals, phytohormones stand at the forefront, which can be categorized into two groups: those promoting senescence (ethylene, jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA)) and those delaying senescence (cytokinins (CKs), gibberellic acid (GA), and auxin) (Guo et al. 2021). The external signals leading towards senescence include abiotic stresses such as drought, salt, high or low temperature, and nutrient deficiency and the biotic factors like pathogen attack (Guo et al. 2021). Our analysis revealed up-regulation of a unigene corresponding to *ETHYLENE RESPONSE SENSOR 2* (*ERS2*) in winter. This gene encodes an ethylene receptor related to bacterial two-component histidine kinases and possesses binding sites for ethylene with similar affinities like that of *ETR1* (Hall et al. 2000). Furthermore, over-expression of *ACYL-COA OXIDASE 1* (*ACX1*) that is involved in the biosynthesis of JA and *REGULATORY COMPONENT OF ABA RECEPTOR 1* (*RCAR1*) that acts as abscisic acid sensor were observed in winter, suggesting the role of ethylene and ABA in leaf senescence of *U. wallichiana*. In JA-induced leaf senescence, repression of *RUBISCO ACTIVASE* (*RCA*) by JA in a COI1-dependent manner is suggested to promote leaf senescence. Furthermore, loss of *RCA* promotes typical senescence-associated features, suggesting *RCA* as a negative regulator of leaf senescence. JA induces leaf senescence through repression of *RCA* (Shan et al. 2011). We identified six unigenes corresponding to *RCA* showing down-regulation in winter. Although we did not observe differential expression of any JA biosynthesis or signaling pathway genes, the down-regulation of *RCA* suggests the possible involvement

of JA in leaf senescence of *U. wallichiana*. *JUNGBRUNNEN1* (*JUB1*), a hydrogen peroxide-induced NAC transcription factor, improves longevity in *Arabidopsis thaliana*. Over-expression of *JUB1* diminishes intracellular hydrogen peroxide levels, enhances abiotic stress tolerance, and delays senescence (Wu et al. 2012). We observed down-regulation of three unigenes corresponding to *JUB1* in winter. Early onset of senescence, manifested in the form of leaf yellowing, chlorophyll, and protein reduction, was observed in mutants of *GALACTOSYLTRANSFERASE 6* (*GALT*6) (Basu et al. 2015). Our analysis also showed reduced expression of unigenes corresponding to *GALT6* in winter. There is a crosstalk between SA and JA signaling in the regulation of senescence. Exogenous JA application leads to loss of chlorophyll content (Yue et al. 2012), whereas the SA concentration is found to increase during senescence to regulate specific genes (Morris et al. 2000). There occurs a delay in senescence in methyl-jasmonate (MeJA)-induced senescence when low concentrations of SA are applied; however, senescence is accelerated at higher SA concentrations (Ji et al. 2016). It appears that SA functions as a growth developmental regulator at lower concentrations in contrast to stress hormone at higher concentrations (Chai et al. 2014). A complicated relationship exists between autophagy and reactive oxygen species (ROS), and while the ROS can induce autophagy, the latter in turn may lead to reduction of ROS. Lower concentrations of SA diminish ROS in MeJA-treated leaves (Ji et al. 2016). An autophagy-related (ATG) gene module was observed to be induced by MeJA together with low concentration SA. Here, the number of autophagic bodies accumulated in vacuoles increased due to low concentration SA during MeJA-induced leaf senescence (Yin et al. 2020), suggesting the up-regulation of autophagy by low SA concentration to alleviate senescence induced by MeJA. Experimental evidences suggest a suppressive role of auxin in leaf senescence. We identified two unigenes corresponding to *AUXIN RESPONSIVE FACTOR 2* (*ARF2*) showing over-expression in winter. ARF2 was found to act as a repressor of auxin signaling. In *Arabidopsis*, it was observed that the mutations in *ARF2* reduce the repression of auxin signaling and enhance auxin sensitivity, which delay senescence. These results suggest that *ARF2* functions as a positive regulator of leaf senescence (Lim et al. 2010), which corroborates our results. Besides, external factors like light quality also affect senescence, for example, a low ratio of red (R) to far-red (FR) and FR promotes senescence. The FAR-RED ELONGATED HYPOCOTYL3 (FHY3) binds to the WRKY28 promoter to cause its repression due to which SA biosynthesis and senescence are negatively regulated (Tian et al. 2020). Although we did not identify *FHY3*, over-expression of *WRKY28* in winter agrees with (Tian et al. 2020). Furthermore, autophagy-defective mutants (*atg2*, *atg5*, and *atg7*) exhibited early yellowing leaf symptoms (Yin et al. 2020; Doelling et al. 2002). Our analysis revealed lower expression of *ATG7* in winter corroborating the above findings.

Degradation of chlorophyll is an inherent process associated with leaf senescence. Several genes are involved in this catabolism including *NON-YELLOW COLORING 1* (*NYC1*) that encodes Chl-b reductase. The latter reduces Chl-a to Chl-b, a first step involved in chlorophyll breakdown. Other genes include *NYC1-like* (*NOL*) that catalyzes Chl-b to 7-hydroxymethyl Chl-a (Kusaba et al. 2007; Morita et al. 2009). Removal of the central Mg atom from Chl-a generates pheophytin-a, from which the phytol tail is removed by Pheophytinase (PPH) to form pheophorbide-a (Oda-Yamamizo et al. 2016). This is followed by the formation of red chlorophyll catabolite (RCC) by pheophorbide-a oxygenase (PaO) and nonfluorescent chlorophyll catabolite (NCC) and nonfluorescent dioxobilin-type chlorophyll catabolite (NDCC) by RCC reductase (RCCR), MES16, and CYP89A9. Furthermore, three homologs of *STAY GREEN* (*SGR*), viz., *SGR1*, *SGR2*, and *SGR-like*, in *Arabidopsis* also regulate senescence. During senescence, while the *SGR1* and *SGR-like* positively regulate chlorophyll degradation, *SGR2* exhibits negative regulation on this process (Ren et al. 2007). There is a coordinated regulation of these chlorophyll-degrading genes through a NAC transcription factor ANAC046 that directly binds to the promoters of *NYS1*, *SGR1*, *SGR2*, and *PaO* to exert a common regulation of these genes. Early senescence and delayed senescence were observed by over-expression and loss of function mutation of *ANAC046*, respectively (Ren et al. 2007). We observed over-expression of unigenes corresponding to *ANAC046*, which agrees with the positive regulation of *Arabidopsis* leaf senescence by *ANAC046* through the activation of chlorophyll catabolism *ANAC046* (Ren et al. 2007). Furthermore, over-expression of *SGR1* (also *NON-YELLOWING 1* (*NEY1*)) was observed during winter, which corroborates with findings of its higher expression during senescence by Ren et al. (2007) (Lundquist et al. 2012). Plastids of most plants contain lipoprotein particles known as plastoglobuli (PG), which in chloroplasts remain attached to the outer lipid leaflet of the thylakoid membrane. PGs show a dramatic increase in size during senescence. In addition to prenyl lipids, quinones, carotenoids, fatty acid esters, and triacylglycerols, PGs contain a specific core proteome of ~30 proteins (Bhuiyan et al. 2016), among which PGM48 positively regulates leaf senescence (Balazadeh et al. 2010). Our finding of over-expression of *PGM48* agrees well with these observations.

Plant calcium-dependent protein kinases (CDPKs) possess a sensor calcium binding domain and an effector phosphorylation domain. The sensor domain binds to calcium signal, whereas the effector domain through its kinase activity phosphorylates diverse substrates. By acting as calcium sensors, CDPKs have an important role in signaling pathways. Due to abiotic and biotic stress signal, intracellular calcium levels may rise. This calcium signal is perceived and translated to a

phosphorylation signal by CDPKs. ORESARA1 (ORE1) has been shown to regulate senescence and is probably the best-characterized senescence regulatory NAC type transcription factor. Besides senescence, *ORE1* is also involved in programmed cell death (PCD). *ORE1* mutants exhibit delayed senescence, whereas over-expression enhances senescence (Matallana-Ramirez et al. 2013; Durian et al. 2020). Furthermore, CDPKs also have been found to be involved in leaf senescence, for example, *CALCIUM-DEPENDENT PROTEIN KINASE 1* (*CPK1*) of *Arabidopsis* was found to phosphorylate and activate ORE1 to positively regulate senescence (Lyu et al. 2019). Besides, CPK1-ORE1 activity promotes cell death. Our analysis revealed over-expression of both *CPK1* and *ORE1* in winter, which corroborate with the findings of Matallana-Ramirez et al. (2013), (Durian et al. 2020) and (2020) (Lyu et al. 2019). These results suggest the importance of calcium signaling in leaf senescence in *U. wallichiana*. Other genes involved in promoting leaf senescence include *GENETIC VARIANTS IN LEAF SENESCENCE* (*GVS1*) (Shi et al. 2015) and *INDOLE-3-ACETIC ACID INDUCIBLE 17*(*IAA17*) (Scarpeci et al. 2017), which were over-expressed in *U. wallichiana* during winter, whereas down-regulation of *ERF019* that is involved in delaying plant growth and senescence (Smalle and Vierstra 2004) was observed in winter.

Furthermore, proteolysis is an inhering process during senescence. Proteases play an important role in protein degradation in senescent organs. Peptidases are ubiquitous proteins playing an important role in protein intracellular turnover. They may degrade specific regulatory gene products, maintain free amino acid pool, and also eliminate malfunctioned proteins. Besides, they may also be involved in nutrient recycling (Waditee-Sirisattha et al. 2011). We observed down-regulation of *LEUCYL AMINOPEPTIDASE 2* (LAP2) unigenes in winter. Genetic experiments have shown that loss of function mutation in *LAP2* enhances senescence (Schmidt et al. 2007), thereby corroborating our results. This suggests that LAP2 that exhibits exopeptidase activity may release amino acid from the N-terminal end of proteins/peptides during leaf senescence in *U. wallichiana*. Over-expression of other peptidases was also observed in winter like CHLOROPLAST GLUTAMYL PEPTIDASE (CGEP), serine carboxypeptidase (SCPL), leucyl aminopeptidase (LAP), and aspartyl aminopeptidase (AAP). The degraded products mostly in the form of amino acids are transported to the developing/sink organs through specific transporter proteins. In this regard, we observed winter-specific over-expression of amino acid permease 8 (AAP8) that is an amino acid-proton symporter, exhibiting specificity for glutamate, aspartate, and neutral and acidic amino acids (Quirino et al. 2001), lysine histidine transporter 2 (LHT2), and nitrate transporter (NRT2). Among the genes involved in the breakdown of the carbohydrates and lipids, we observed winter-specific over-expression glucan water dikinase (GWD), STARCH-EXCESS 4 (SEX4) alpha-amylase (AAMY), beta amylase (BAMY), and isoamylase (IAMY), which are involved in starch catabolism. Among the genes involved in the transport of carbohydrates, we observed winter-specific over-expression of a major facilitator superfamily monosaccharide transporter, *SPF1*, which is found to be induced during senescence (Kui et al. 2023), bidirectional sugar transporter SWEET1, sugar transport protein 2 (STP2), and sugar transporter ERD6-like 1 (SUGLT4).

Conclusion

The study explored the transcriptomic dynamics of *U. wallichiana* under changing climatic seasons. Among the biological effects of this change, senescence is most apparent in *U. wallichiana*. We explored the genetic architecture involved in controlling leaf senescence in *U. wallichiana*. Furthermore, the DREB genes identified in this study may reflect their role in conferring tolerance to low temperature or drought stress.

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Author Contribution PB conceived the idea, arranged the funding, and organized the overall study. AS and AM collected the sample, performed wet experiment and computational analysis, and wrote the manuscript. VS assisted in computational analysis and manuscript editing. HSG assisted in wet lab experiments and manuscript editing. AC contributed in wet lab experiments. PB further edited and finalized the manuscript. All authors read and approved the final version manuscript.

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Data Availability The data files are submitted to NCBI under the accession no. SAMN32907997 and SAMN32909170.

Declarations

Ethics Approval No part of this research involved human or animal samples.

Conflict of Interest The authors declare no competing interests.

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