

Article

Transcriptomic Analysis Reveals the Mechanism of *Picea crassifolia* Survival for Alpine Treeline Condition

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Abstract: The physiological mechanisms driving treeline formation succession captured the attention of ecologists many years ago, yet they are still not fully understood. In this study, physiological parameters (soluble sugars, starch, and nitrogen) were investigated in combination with transcriptomic analysis in the treeline tree species *Picea crassifolia*. The study was conducted in the middle of Qilian Mountain Reserves, Gansu Province, China, within the elevation range of 2500–3300 m. The results showed that the concentrations of non-structural carbohydrates decreased with increasing elevation in the current-year needles and current-year branches, as well as in the coarse and fine roots. RNA-Seq demonstrated that 483 genes were upregulated and 681 were downregulated in the comparison of 2900 and 2500 m (2900 vs. 2500), 770 were upregulated and 1006 were downregulated in 3300 vs. 2500, and 282 were upregulated and 295 were downregulated in 3300 vs. 2900. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed that the differentially expressed genes were highly enriched in photosynthesis-related processes, carbon fixation and metabolism, and nitrogen metabolism. Furthermore, almost all photosynthesis-related genes were downregulated, whereas many genes involved in cuticle lipids and flavonoid biosynthesis were upregulated, contributing to the survival of *P. crassifolia* under the treeline condition. Thus, our study provided not only molecular evidence for carbon limitation hypothesis in treeline formation, but also a better understanding of the molecular mechanisms of treeline tree survival under adverse conditions.

Keywords: transcriptome; treeline; elevation; carbon limitation; Picea crassifolia

1. Introduction

The treeline, defined here as the highest altitude where groups of upright trees reach at least 3 m of height, is one of the most recognizable bioclimatic boundaries and it has been suggested as a critical indicator of trees' response to climate warming [1,2]. As one of the important ecological boundaries, the alpine treeline is globally determined by the soil temperature of 6.5 ± 0.8 °C at 10 cm of depth during the vegetation season [3]. Although the decrease in temperature with increasing elevations has been recognized as a major reason for treeline formation, and the mechanism by which low temperature affects the growth of treeline trees is unclear. The concordance of this treeline isotherm suggests a



common low-temperature effect on plant metabolism, either related to carbon (C) gain (source activity; photosynthesis) or sink activity (tissues formation; meristems) [4].

The physiological mechanisms driving treeline formation captured the attention of ecologists long ago, and various environmental and biophysiological hypotheses have been formulated [5,6], yet they are not fully understood. The environmental hypothesis stated that the formation of treeline was due to the following factors: climatic stress, mechanical damage, short growing season, and temperature-limited reproduction [7–10]. This hypothesis can only be used to interpret local treeline phenomena, which are related to various local site conditions [11]. However, the biophysiological hypothesis focuses on growth limitation and C supply limitation [1,2,12]. The growth limitation hypothesis (GLH) considers that colder temperatures at higher elevations limit C sink activity [5], supported by the results of trees in 13 alpine tree line regions [13] and Larix potaninii in the eastern Himalayas treeline [14]. This is contrary to the C limitation hypothesis (CLH), which states that reduced C gain with higher elevation (lower temperature) results in treeline formation [5], which is further supported by the finding that the elevational decrease in non-structural carbohydrate (NSC) concentrations in Nothofagus pumilio trees at treeline elevation [15]. However, in the birch (Betula ermanii) near the treeline on the Changbai Mountain, Northeast China, the decrease in NSCs with elevation in main storage organs supports the CLH, whereas the increasing or static trends in new developing organs support the GLH [16]. Moreover, limitation of nutrients, including P and N, is also found to contribute to the formation of the Arctic treeline, particularly in areas with cold winters [17]. So far, there is no conclusive evidence about the formation of altitudinal treelines [11–18]. Hence, these conflicting findings prompted us to further explore a new way to explain the physiological mechanism that drives high-altitude treeline formation.

Changes in temperature and water with the increasing altitudes have been shown to have significant effects on the physiological adaptations of alpine trees [6,19], which is a consequence of plant responses at the transcriptional level. Although physiological changes in trees of treelines have been reported [1,5,10,20,21], little information is available about the mechanisms regulating treeline formation at the transcriptomic level, compared with those other vegetation zones. In recent years, RNA sequencing (RNA-Seq) has enabled to detect differentially expressed genes (DEGs) because of its dynamic range of expression levels under abiotic stress, such as salinity [22] and drought [23]. In the present study, in order to verify the GLH or CLH at the molecular level, we conducted a transcriptome analysis of *Picea crassifolia* growing at different elevations, and combined it with the analysis of physiological changes in trees. Consequently, we examined the expression responses of candidate genes related to carbohydrate fixation and metabolism. In order to assess the adaptive potential of trees in the treeline environment, it is necessary to identify genes that are related to environmental responses of trees forming treelines.

2. Materials and Methods

2.1. Site Description

This study was carried out in the middle of the Qilian Mountain Reserves in the treeline ecotone of Pailugou Basin, Gansu Province (100°17′ E, 38°24′ N), with the elevation ranging from 2500 to 3400 m. The vegetation season in the treeline usually lasts from May to mid-October. The annual mean air temperature is around -0.6 °C to -2 °C, and the mean annual precipitation is 430 mm. The soil in the research site is mountain gray cinnamon soil, and the vegetation in the alpine treeline ecotone is dominated by *P. crassifolia* (ranging from 2500 to 3300 m), and treelike multi-stem shrubs such as *Potentilla fruticosa*, *Caragana jubata*, and *Salix gilashanica* (ranging from 2500 to 3400 m).

2.2. Sample Collection

Different tissues, including current-year needles, current-year branches, coarse roots, and fine roots, were collected during the early growing season (in June) at three elevations across the treeline

ecotones on north-facing slopes: closed forest stands at lower elevations (2500 m), middle forest stands between closed forest and treeline (2900 m), and treeline stands in the uppermost limit of *P. crassifolia* trees (3300 m). Sampling was performed according to the method described by Li et al. [10] and Ma et al. [24] with some modifications. Briefly, nine healthy mature trees, consisting of three individuals each repeat, were selected for repeated sampling at each elevation. The current-year needles and current-year branches were collected from non-shaded leading branches on the upslope canopy side. Simultaneously, coarse roots (0.5–1.0 cm in diameter) and fine roots (<0.5 cm in diameter) were collected. One part of the current-year needle samples was immediately stored in liquid N for RNA extraction, whereas the other part, intended for physiological measurements, was immediately stored in a cool box. After short time treatment in a microwave oven (40 s at 600 W), the sample was dried at 70 °C until it reached a constant weight after two consecutive days.

2.3. Non-Structural Carbohydrate Analysis

Soluble sugars: Approximately 0.5 g of dry powdered sample was added into a triangular bottle along with 46 mL of distilled water. After boiling the sample for 2 h in a pressure cooker and cooling it, we set the capacity of sample solution to 50 mL, and filtered the solution. The filtrate was analyzed with the Waters 2695 High Performance Liquid Chromatograph (Waters-Millipore, Milford, MA, USA) using a Sugar-Pak I column. The liquid phase was distilled water, flow rate was 0.6 mL·min⁻¹, column temperature was 70 °C; a parallax detector was used. All separated components were summed as soluble sugars [25,26].

Starch: Approximately 0.1 g of dry powdered sample was added into a plug tube with 10 mL of distilled water, and 1 mL of 2:1 hydrochloric acid, and incubated at 100 °C for 8 h. After cooling, the pH of the sample was adjusted to neutral with 40% NaOH, and the capacity of sample solution was set to 15 mL. After filtration, the filtrate was used for starch analysis. Starch content was analyzed using the same method as the determination of soluble sugars. The total concentration of NSC was defined as the sum of soluble sugar and starch concentrations [26,27].

Total N: Total N content (% d.m.) was determined in finely ground oven-dried samples with the Automatic Kjeldahl Analyzer (UK 152, Italy), using $CuSO_4$, K_2SO_4 , and H_2SO_4 for digestion, and NH_3 was determined using the indophenol-blue colorimetric method according to Li et al. [10].

2.4. RNA-Seq Library Construction, Sequencing, and Mapping

Nine libraries for RNA-Seq, including three biological replicates for 2500, 2900, and 3300 m, were constructed according to the manuals provided by Illumina and sequenced on the HiSeq2000 platform as described by Zheng et al. [22]. Briefly, the total RNA was isolated from the current-year needles using the PLANT easy extraction protocol (BLKW, Beijing, China), and mRNAs were purified, fragmented, and used for complementary DNA (cDNA) synthesis with random hexamer primers and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). After the purification of the PCR products by AMPure XP system, (Beckman Coulter, Brea, CA, USA) and assessing the library quality on the Agilent Bioanalyzer 2100 system (Agilent, Palo Alto, CA, USA), paired-end cDNA libraries were sequenced using the Illumina-Solexa sequencing platform.

The poplar reference genome and annotation files were downloaded directly from the National Center for Biotechnology Information (NCBI) website (ftp://ftp.ncbi.nih.gov/genomes/PLANTS/ Populus_trichocarpa/, accessed on 13 January 2020). An index of the reference genome was built using Bowtie v2.0.6, and single-end clean reads were aligned with the reference genome using TopHat v2.0.9 [28]. Read counts were calculated using HTSeq (www.huber.embl.de/users/anders/HTSeq, accessed on 13 January 2020) and normalized to the expected number of fragments per kilobase of transcript sequence per millions base pairs sequenced (FPKM) [29] in order to obtain the relative expression levels.

2.5. Identifying and Clustering of DEGs

The R packages of DESeq [30] were used for pairwise gene expression comparisons between two different elevations. The significance of DEGs was identified with a '*p*-value ≤ 0.05 ' and ' $|\log 2Ratio| \geq 1.0$ ' with at least one FPKM ≥ 1 within two elevations. Hierarchical clustering was performed using pheatmap package of R statistical environment in order to cluster the identified DEGs on the base of the FPKM values.

All DEGs were mapped according to the terms from Gene Ontology (GO), using the bioconductor package GoSeq [31], and Kyoto Encyclopedia of Genes and Genomes (KEGG), according to the methods of Kanehisa [32]. Significantly enriched terms were identified in comparison with the genome background.

2.6. Quantitative Real-Time PCR (qRT-PCR)

cDNA was synthesized from 1.0 µg of total RNA and digested with DNase I using the PrimeScript RT reagent kit for RT-PCR (Takara, Dalian, China), according to the manufacturer's instructions. The specific primers for poplar (Table S1) were synthesized by the Sangon Biotechnology Company (Shanghai, China). The qRT-PCR was performed using the SYBR Premix ExTaq II kit (Takara) on Roche LightCycler 480 (Roche, Penzberg, Germany). To quantify the relative expression levels of the target genes, the poplar reference gene *Actin* (GenBank accession no. AAF03692) was used as the internal control [33]. Fold changes were calculated by the relative expression of treatments compared with that of the control. Three independent biological replicates were performed for each selected gene (at least two technical repeats per biological replicate).

2.7. Statistical Analysis

Quantitative data of NSC concentration, N concentration, and transcription analysis were analyzed by one-way analysis of variance (ANOVA) according to the general linear model. Post-hoc comparison was performed by Tukey's honestly significant difference (HSD) test. Differences were accepted as statistically significant when p < 0.05. All statistical analyses were performed using SPASS 16.0.

3. Results

3.1. Changes of NSC and Total N content with Altitudes

With increasing elevation, soluble sugar content in the current-year needles did not show an obvious change (p > 0.05), although there was a decreasing trend at 3300 m (Figure 1A). However, in the current-year branches, there was an apparent increase (15.5%) in the soluble sugar content at 2900 m, but a significant decrease (11.3%) was recorded at 3300 m (Figure 1D). The starch content showed a sharp decreasing trend (70.0%–85.4%) in both needles and branches at 2900 m (Figure 1B,E), but the starch content at 3300 m was significantly higher than that at 2900 m, indicating that the conversion of starch into soluble sugars could be inhibited at 3300 m. These changes resulted in a significant decrease over 17.9%–21.9% in total NSC in the current-year needles at 2900 and 3300 m, as well as in the current-year branches at 3300 m (18.9%) with the increasing elevation. These results suggested that the conditions at higher elevation limited the supply of C in the current-year needles and branches. Similarly, with increasing elevations, soluble carbohydrates were also negatively affected in both coarse roots and fine roots (Figure 2), which resulted in a decrease in the total NSC content, especially at 3300 m (Figure 2C,F).



Figure 1. Changes of non-structural carbohydrates of *Picea crassifolia* with the increasing elevations (m). (A–C) Current-year needles; (D–F) current-year branches. Different lowercase letters represented the significance levels (p < 0.05) among the different elevations. NSC: non-structural carbohydrate.



Figure 2. Changes of non-structural carbohydrates in the roots of *Picea crassifolia* with the increasing elevations (m). (**A**–**C**), coarse roots with 0.5–1.0 cm in diameter; (**D**–**F**), fine roots with <0.5 cm in diameter. Different lowercase letters represented the significance levels (p< 0.05) among the different elevations.

The total N content increased evidently by 18.8%–38.5% at 2900 m compared with that at 2500 m in both needles and branches, whereas at 3300 m, the total N content showed a decrease, but it was still higher than that at 2500 m (Figure 3A,B). However, the total N content significantly decreased by 12.2%–15.2% in the coarse roots (Figure 3C), whereas it obviously increased by 11.3%-36.8% in the fine roots (Figure 3D). These results demonstrated that N was not a main limiting factor for the growth of *P. crassifolia* under the treeline condition.



Figure 3. Changes of total N in the different tissues of *Picea crassifolia* with the increasing elevations (m). (A) Current-year needles, (B) current-year branches, (C) coarse roots with 0.5–1.0 cm in diameter, (D) fine roots with <0.5 cm in diameter. Different lowercase letters represented the significance levels (p < 0.05) among the different elevations.

3.2. Identification of Differentially Expressed Genes in P. crassifolia at Different Elevations by RNA-Seq

A summary of raw data is shown in Table S2, and the quantitative expression of genes (estimated using the RPKM values) is shown in Table S3. A high correlation of $R^2 > 0.91$ between biological replicates was observed for three treatments at different elevations (Figure S1).

In order to determine the gene expression changes in *P. crassifolia* resulting from the variations in elevation, differentially expressed genes (DEGs) were identified in response to the increasing elevation (2500, 2900, and 3300 m). The $|\log 2Ratio| \ge 1$ was used as the threshold to screen the DEGs and to obtain more information on genes potentially involved in the response to the elevation variations. We found 483 upregulated genes and 681 downregulated genes by comparison of trees from 2900 m with trees from 2500 m (2900 vs. 2500), 770 upregulated genes and 1006 downregulated genes in 3300 vs. 2500, and 282 upregulated genes and 295 downregulated genes in 3300 vs. 2900 (Figure 4A; Table S4). Moreover, only 13 DEGs were common to 3 pairwise comparisons of different elevations, but 1146 DEGs were specific to the elevation of 3300 m (Figure 4B). Furthermore, gene expression patterns of 12 randomly selected DEGs were verified by qRT-PCR, which demonstrated similar expression trends with RNA-Seq results, indicating the reliability of DEG analysis (Figure 5). This indicated the presence of a large proportion of DEGs in *P. crassifolia* growing in the treeline environment, and these DEGs could aid the species to cope with the harsh environmental conditions.



Figure 4. Transcriptomes of *Picea crassifolia* with elevations. (**A**) Numbers of differentially expressed genes (DEGs). (**B**) Venn diagrams showing unique and shared DEGs between altitudes.

These DEGs may be involved in different functions. The analysis of GO enrichment and KEGG pathway was applied to gain an insight into their functions. DEGs in 2900 vs. 2500, 3300 vs. 2500, and 3300 vs. 2900 were annotated in GO, and categorized into three major categories (biological process, cellular component, and molecular function) (Figure 6; Table S5). GO annotation suggested that biological processes and molecular functions, including "primary metabolism", "organic substance metabolism", "biological regulation", "organic nitrogen compound biosynthesis", "cellular nitrogen compound biosynthesis", "cellular aromatic compound metabolism", "carbohydrate metabolism", and "lipid metabolism" were related to C and N metabolism at different elevations. In particular, biological processes of C and N metabolism were significantly enriched in 3300 vs. 2500.



Figure 5. Verification of RNA sequencing (RNA-Seq) results by quantitative real-time PCR (qRT-PCR).



Figure 6. Gene Ontology (GO) term enrichment in Picea crassifolia with elevations.

In the KEGG pathway analysis, 83, 96, and 56 pathways were categorized into 14 major KEGG pathways from the pairwise comparisons between 2900 vs. 2500, 3300 vs. 2500, and 3300 vs. 2900, respectively. The pathways of metabolism, mainly including secondary metabolites, carbohydrates, energy, lipid, amino acids, cofactors, and vitamins, were mostly influenced by the variations in elevation, especially for 3300 vs. 2500, rather than being influenced by signal transduction, environmental adaptation, and transport (Figure 7; Table S6). The response of these pathways to increasing elevations could be helpful for the survival of P. crassifolia. Among these responses, the metabolism of carbohydrates and energy was enriched in 23 pathways (Table S6). Compared with those at 2500 m, 39 DEGs were upregulated and 40 were downregulated at 2900 m, whereas 74 were upregulated and 44 were downregulated at 3300 m. This indicated that most of the genes were negatively regulated around the elevation of the treeline compared with the lower elevations. We found that all 40 genes enriched in photosynthesis, antenna proteins of photosynthesis, and C fixation in photosynthetic organisms were downregulated at 3300 m. Similarly, DEGs involved in glyoxylate and dicarboxylate metabolism (14), pentose phosphate pathway (9), and fructose and mannose metabolism (9) were all downregulated at 3300 m. Moreover, 26 of the 30 DEGs in the C metabolism were also downregulated at 3300 m and 19 DEGs enriched in cutin, suberine, and wax biosynthesis were upregulated at 3300 m. These results demonstrated that the increasing elevations inhibited C fixation and altered the direction of C fluxes.



Figure 7. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in Picea crassifolia with elevations.

3.3. Analysis of Gene Expression Pattern

To further explore the gene expression patterns in response to the increasing altitudes, we applied *K*-means clustering to the identified DEGs. In this analysis, 1466 DEGs with a known-function were grouped into six categories (referred to as Sub1, Sub2, Sub3, Sub4, Sub5, and Sub6), comprising 113, 616, 165, 220, 194, and 158 genes, respectively (Figure 8; Table S7). The DEGs in the Sub1 and Sub2 groups were characterized by constant upregulation with the increasing elevations (Table S7). The GO and KEGG analysis showed that these genes were mainly enriched in secondary metabolite metabolism (such as biosynthesis of secondary metabolites; flavonoid biosynthesis; and stilbenoid, diarylheptanoid, and gingerol biosynthesis), carbohydrate metabolism (cutin, suberine, and wax biosynthesis; glycolysis/gluconeogenesis; and pyruvate metabolism, and lipid metabolism (fatty acid elongation, fatty acid biosynthesis, glycerophospholipid metabolism, and fatty acid degradation) (Tables S8 and S9). The expression of DEGs in Sub3 increased at 2900 m and decreased at 3300 m (Figure 8), and these DEGs were mainly enriched in signal transduction (such as plant hormone signal transduction), environmental adaptation (plant-pathogen interaction), amino acid metabolism (beta-alanine metabolism, tryptophan metabolism, and taurine and hypotaurine metabolism),

and secondary metabolite metabolism (terpenoid backbone biosynthesis and carotenoid biosynthesis) (Tables S8 and S9). However, the DEGs in Sub4 were initially downregulated and then upregulated (Table S7), and they were mainly enriched in folding, sorting, and degradation (protein processing in the endoplasmic reticulum), secondary metabolite metabolism (stilbenoid, diarylheptanoid, and gingerol biosynthesis), and energy metabolism (carbon fixation in photosynthetic organisms) (Tables S8 and S9). The last two groups (Sub5 and Sub6) were continuously downregulated with increasing elevations, and they were mainly enriched in secondary metabolite metabolism (biosynthesis of secondary metabolites; phenylpropanoid bio synthesis; flavonoid biosynthesis; and stilbenoid, diarylheptanoid, and gingerol biosynthesis), carbohydrate metabolism (carbon metabolism, glyoxylate and dicarboxylate metabolism, pentose phosphate pathway, and fructose and mannose metabolism), energy metabolism (photosynthetic antenna proteins, photosynthesis, carbon fixation in photosynthetic organisms, and nitrogen metabolism), and amino acid metabolism (biosynthesis of amino acids, and phenylalanine, tryptophan, glycine, serine, and threonine metabolism) (Tables S8 and S9). This clustering demonstrated that the genes involved in photosynthetic C fixation, carbohydrate metabolism, and N metabolism were significantly influenced by elevation. Hence, we further explored the DEGs involved in C and N metabolism.



Figure 8. Clustering analysis by *K*-means clustering of differentially expressed genes (DEGs) in *Picea crassifolia* with elevations.

3.4. DEGs Involved in Carbon Assimilation and Energy Metabolism

In this study, 25 of the 33 DEGs involved in C assimilation and metabolism were downregulated with the increasing elevations (Table 1; Table S10A). Among these 25 DEGs, almost all photosynthesis-related genes were inhibited by higher altitudes (3300 m), whereas *CLH2*, a gene that encodes chlorophyllase-2 (which degrades chlorophyll), significantly increased with the increasing

elevations. Correspondingly, the expression of eight in the nine LHCBs or LHCA (chlorophyll a-b binding proteins) was downregulated, which decreased the ability of capturing and delivering excitation energy to photosystems. Meanwhile, 12 of the 13 photosystem genes (photosystem I complex/PSI and photosystem II complex/PSII), excluding the 4.0-5.7-fold increase in photosystem I reaction center subunit N (PSAN) (MA_3482g0010), were negatively regulated, although ribulose bisphosphate carboxylase small chain (*RBCS*) (MA_476g0010), which is responsible for the primary event in carbon dioxide fixation, was upregulated. This indicated that photosynthetic capacity might be significantly inhibited at the altitude of 3300 m. Subsequently, central C metabolism was also diversely affected by the increasing elevations. Half of the genes were downregulated, including one F16P (MA_10432295g0010) encoding fructose-1,6-bisphosphatase, one KPRS1 (MA_10431983g0010) encoding ribose-phosphate pyrophosphokinase 1, and two G3PCs encoding glyceraldehyde-3-phosphate dehydrogenase, whereas others were upregulated, including one SDHA (MA_10427069g0010) encoding succinate dehydrogenase (ubiquinone) flavoprotein subunit, and two GADs (MA_161013g0010, MA_89828g0010) encoding glutamate decarboxylase. These results suggested that the expression of genes involved in photosynthetic C assimilation and energy metabolism was significantly altered by the increasing elevations.

3.5. DEGs Involved in Non-Struct(ural Carbon Metabolism

In order to examine the effects of elevations on NSC metabolism, three KEGG pathways (soluble sugars, fatty acid metabolism, and flavonoid biosynthesis) were examined (Table 2; Table S10B). With the increasing elevation, seven DEGs involved in soluble sugar metabolism were identified, including *AGLU* (α -glucosidase), *GOLS1* (galactinol synthase 1), three *GAOAs* (galactose oxidase), and two *RFSs* (probable galactinol–sucrose galactosyltransferase). *AGLU*, as a gene responsible for the breakdown of starch and disaccharides into glucose, was upregulated by more than 2.3-fold. Similarly, the expression of two *RFSs*, which are involved in the synthesis of raffinose, was also significantly increased at a certain elevation. However, *GOLS1*, involved in the biosynthesis of raffinose family oligosaccharides (RFOs) that function as osmoprotectants, was downregulated; three *GAOAs* were also downregulated.

Gene	Log2(2900/2500)	Log2(3300/2500)	Gene ID	Annotation	Accession		
Photosynthetic Carbon Assimilation							
CLH2	1.8	1.9	MA_337265g0010	Chlorophyllase-2	sp Q9M7I7 CLH2_ARATH		
LHCA4	-0.6	-1.0	MA_65346g0010	Chlorophyll a-b binding protein 4	sp P27521 CA4_ARATH		
LHCB4C	-0.6	-1.0	MA_129206g0010	Chlorophyll a-b binding protein CP29.3	sp Q9S7W1 CB4C_ARATH		
LHCB2A	-0.6	-1.3	MA_97315g0010	Chlorophyll a-b binding protein type 2 member 1A	sp P15193 CB2A_PINSY		
LHCB22	0.9	1.8	MA_1315g0010	Chlorophyll a-b binding protein type 2 member 1B	sp P15194 CB2B_PINSY		
LHCB21	-0.9	-1.3	MA_10431300g0030	Chlorophyll a-b binding protein type 2 member 2	sp P15192 CB22_PINSY		
LHCB21	-1.1	-1.9	MA_10431300g0010	Chlorophyll a-b binding protein type I	sp P10049 CB21_PINTH		
LHCB21	-1.0	-1.7	MA_10431300g0020	Chlorophyll a-b binding protein type I	sp P10049 CB21_PINTH		
LHCB21	-1.0	-1.7	MA_267089g0010	Chlorophyll a-b binding protein type I	sp P10049 CB21_PINTH		
PSAD	-0.5	-1.0	MA_9169733g0010	Photosystem I reaction center subunit II, chloroplastic	sp P32869 PSAD_CUCSA		
PSAF	-0.8	-1.5	MA_10090427g0010	Photosystem I reaction center subunit III, chloroplastic	sp P46486 PSAF_FLATR		
PSAN	4.0	5.7	MA_3482g0010	Photosystem I reaction center subunit N, chloroplastic	sp P49107 PSAN_ARATH		
PSAG	-0.5	-1.1	MA_100910g0020	Photosystem I reaction center subunit V, chloroplastic	sp Q9S7N7 PSAG_ARATH		
PSAL	-0.5	-1.0	MA_3403026g0010	Photosystem I reaction center subunit XI, chloroplastic	sp P23993 PSAL_HORVU		
PSAO	-0.6	-1.0	MA_893g0010	Photosystem I subunit	sp Q949Q5 PSAO_ARATH		
PSAO	-0.5	-1.0	MA_7732837g0010	Photosystem I subunit	sp Q949Q5 PSAO_ARATH		
PSBR	-1.1	-1.1	MA_282261g0010	Photosystem II 10 kDa polypeptide, chloroplastic	sp P06183 PSBR_SOLTU		
PST2	-0.6	-1.2	MA_17217g0020	Photosystem II 5 kDa protein, chloroplastic	sp P31336 PST2_GOSHI		
PSBY	-0.7	-1.2	MA_12201g0010	Photosystem II core complex proteins psbY, chloroplastic	sp P80470 PSBY_SPIOL		
PSBO	-0.4	-1.3	MA_491379g0010	Photosystem II oxygen-evolving enhancer protein 1, chloroplastic	sp O49079 PSBO_FRIAG		
PSBP	-0.7	-1.0	MA_3005g0010	Photosystem II oxygen-evolving enhancer protein 2, chloroplastic	sp Q9SLQ8 PSBP_CUCSA		
PSBQ1	-0.5	-1.0	MA_218996g0010	Photosystem II oxygen-evolving enhancer protein 3-1, chloroplastic	sp Q9XFT3 PSBQ1_ARATH		
RBCS	1.4	2.9	MA_476g0010	Ribulose bisphosphate carboxylase small chain, chloroplastic	sp P16031 RBS_LARLA		
FRO7	-0.3	-1.1	MA_79239g0010	Ferric reduction oxidase 7, chloroplastic	sp Q3KTM0 FRO7_ARATH		
Energy Me	etabolism						
F16P	-0.2	-1.0	MA_10432295g0010	Fructose-1,6-bisphosphatase, chloroplastic	sp Q07204 F16P1_BRANA		
KPRS1	-0.7	-1.1	MA_10431983g0010	Ribose-phosphate pyrophosphokinase 1	sp Q9XG98 KPRS1_SPIOL		
G3PC	-1.1	-0.1	MA_10426544g0010	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	sp P34924 G3PC_PINSY		
G3PC	-1.1	-0.7	MA_8215705g0010	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	sp P34924 G3PC_PINSY		
ALD	1.1	1.7	MA_259175g0010	Aldose 1-epimerase	sp Q5EA79 GALM_BOVIN		
SDHA	0.7	1.4	MA_10427069g0010	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	sp Q6ZDY8 SDHA_ORYSJ		
GAD	2.0	2.3	MA_161013g0010	Glutamate decarboxylase	sp Q07346 GAD_PETHY		
GAD	1.4	0.7	MA_89828g0010	Glutamate decarboxylase	sp Q07346 GAD_PETHY		

Table 1. DEGs involved in carbon assimilation and carbon metabolism.

Gene	Log2(2900/2500)	Log2(3300/2500)	Gene ID	Annotation	Accession
Soluble Su	agars				
AGLU	2.3	2.5	MA_10431114g0020	Alpha-glucosidase	sp O04893 AGLU_SPIOL
AGAL	2.7	3.5	MA_10434513g0010	Alpha-galactosidase mel1	splQ9URZ0 AGAL_SCHPO
GOLS1	-1.0	-1.9	MA_67331g0010	Galactinol synthase	sp Q9XGN4 GOLS1_AJURE
GAOA	-0.4	-1.4	MA_10429735g0020	Galactose oxidase	sp I1S2N3 GAOA_GIBZE
GAOA	-0.6	-2.9	MA_10429735g0010	Galactose oxidase	sp I1S2N3 GAOA_GIBZE
GAOA	-1.4	-1.8	MA_10437233g0040	Galactose oxidase	sp P0CS93 GAOA_GIBZA
RFS5	-0.1	1.4	MA_5981g0010	Probable galactinol-sucrose galactosyltransferase 5	sp Q9FND9 RFS5_ARATH
RFS6	1.5	0.0	MA_18317g0010	Probable galactinol-sucrose galactosyltransferase 6	sp Q8RX87 RFS6_ARATH
Fatty Acid	S				
TSC10A	2.9	6.5	MA_18495g0010	3-dehydrosphinganine reductase TSC10A	sp Q0WRJ2 TSC10A_ARATH
TSC10B	1.6	3.0	MA_681548g0010	3-dehydrosphinganine reductase TSC10B	sp F4JZN6 TSC10B_ARATH
KCS1	1.8	3.1	MA_10426663g0020	3-ketoacyl-CoA synthase 1	sp Q9MAM3 KCS1_ARATH
KCS10	2.3	3.2	MA_91011g0010	3-ketoacyl-CoA synthase 10	sp Q570B4 KCS10_ARATH
KCS4	2.2	4.0	MA_56709g0010	3-ketoacyl-CoA synthase 4	sp Q9LN49 KCS4_ARATH
KCS6	0.9	1.7	MA_10605g0020	3-ketoacyl-CoA synthase 6	sp Q9XF43 KCS6_ARATH
KCS9	1.8	2.0	MA_339700g0010	3-ketoacyl-CoA synthase 9	sp Q9SIX1 KCS9_ARATH
KASC1	1.4	2.2	MA_127500g0010	3-oxoacyl-[acyl-carrier-protein] synthase I, chloroplastic	sp P52410 KASC1_ARATH
FAR2	3.9	4.9	MA_59202g0010	Fatty acyl-CoA reductase 2	sp Q08891 FAR2_ARATH
FAR2	2.9	5.7	MA_95531g0010	Fatty acyl-CoA reductase 2	sp Q08891 FAR2_ARATH
FAR2	2.6	3.5	MA_182956g0010	Fatty acyl-CoA reductase 2	sp Q08891 FAR2_ARATH
AOC3	1.4	1.1	MA_137604g0010	Allene oxide cyclase 3, chloroplastic	sp Q9LS01 AOC3_ARATH
DOX1	-1.9	0.1	MA_879270g0010	Alpha-dioxygenase 1	sp Q9SGH6 DOX1_ARATH
AGAL	2.7	3.5	MA_10434513g0010	Alpha-galactosidase mel1	sp Q9URZ0 AGAL_SCHPO
ACCC1	0.9	1.4	MA_8514g0010	Biotin carboxylase 1, chloroplastic	sp B9HBA8 ACCC1_POPTR
GONS3	1.7	2.8	MA_10432234g0010	GDP-mannose transporter GONST3	sp Q9S845 GONS3_ARATH
GDL6	-0.8	2.1	MA_405425g0010	GDSL esterase/lipase 6	sp Q9C996 GDL6_ARATH
GDL1	1.3	2.1	MA_27394g0010	GDSL esterase/lipase At1g06990	sp Q9LMJ3 GDL1_ARATH
GDL11	-2.5	-0.6	MA_108996g0010	GDSL esterase/lipase At1g28650	sp Q3E7I6 GDL11_ARATH
GDL34	1.7	2.8	MA_502586g0010	GDSL esterase/lipase At2g04570	sp Q9SJB4 GDL34_ARATH
GDL34	1.0	2.0	MA_127753g0010	GDSL esterase/lipase At2g04570	sp Q9SJB4 GDL34_ARATH
GDL42	3.0	4.0	MA_10200362g0010	GDSL esterase/lipase At2g30310	sp O22927 GDL42_ARATH
GDL59	-1.5	-2.2	MA_569398g0010	GDSL esterase/lipase At3g53100	sp Q0WPI9 GDL59_ARATH

Table 2. DEGs involved in non-structural carbon metabolism.

Table 2. Cont.

Gene	Log2(2900/2500)	Log2(3300/2500)	Gene ID	Annotation	Accession
Fatty Acid	S				
GDL61	4.3	4.6	MA 9056794g0010	GDSL esterase/lipase At4g01130	sp O9M153 GDL61 ARATH
GDL61	1.4	2.1	MA 7763761g0010	GDSL esterase/lipase At4g01130	sp 09M153 GDL61 ARATH
GDL61	2.4	2.4	MA 10426064g000	GDSL esterase/lipase At4g01130	sp 09M153 GDL61 ARATH
GDL62	-1.6	-0.4	MA 829166g0010	GDSL esterase/lipase At4g10955	splO680C0lGDL62 ARATH
GDL62	1.4	0.8	MA 10212g0010	GDSL esterase/lipase At4g10955	sp O680C0 GDL62 ARATH
GDL64	1.5	2.8	MA 10432722g000	GDSL esterase/lipase At4g16230	sp O23470 GDL64 ARATH
GDL66	1.7	2.2	MA_127685g0010	GDSL esterase/lipase At4g26790	sp Q8VY93 GDL66_ARATH
GDL71	-2.2	-0.8	MA_5838g0010	GDSL esterase/lipase At5g03610	sp Q9LZS7 GDL71_ARATH
GDL72	4.4	5.1	MA_152453g0010	GDSL esterase/lipase At5g03810	sp Q9FFN0 GDL72_ARATH
GDL73	1.0	1.6	MA_429323g0010	GDSL esterase/lipase At5g03820	sp Q9LZC5 GDL73_ARATH
GDL73	5.8	6.1	MA_101465g0010	GDSL esterase/lipase At5g03820	sp Q9LZC5 GDL73_ARATH
GDL78	2.4	3.6	MA_95656g0010	GDSL esterase/lipase At5g22810	sp Q9FFC6 GDL78_ARATH
GDL79	1.3	1.7	MA_10426867g000	GDSL esterase/lipase At5g33370	sp Q8LB81 GDL79_ARATH
GDL79	1.5	2.2	MA_65830g0010	GDSL esterase/lipase At5g33370	sp Q8LB81 GDL79_ARATH
LOX1	-1.6	-1.4	MA_6186g0010	Linoleate 9S-lipoxygenase 1	sp Q76I22 LOX1_ORYSJ
LOX5	-1.5	-3.1	MA_10436100g000	Linoleate 9S-lipoxygenase 5, chloroplastic	sp Q9LUW0 LOX5_ARATH
LACS2	1.1	2.1	MA_113173g0010	Long chain acyl-CoA synthetase 2	sp Q9XIA9 LACS2_ARATH
LACS4	2.4	3.0	MA_10432761g000	Long chain acyl-CoA synthetase 4	sp Q9T0A0 LACS4_ARATH
LACS5	2.3	3.0	MA_839621g0010	Long chain acyl-CoA synthetase 5	sp Q9T009 LACS5_ARATH
WAXS1	-0.8	-2.3	MA_185166g0020	Long-chain-alcohol O-fatty-acyltransferase	sp Q9XGY6 WAXS1_SIMCH
HHT1	-1.1	1.7	MA_7233g0010	Omega-hydroxypalmitate O-feruloyl transferase	sp Q94CD1 HHT1_ARATH
HHT1	1.4	2.7	MA_56269g0010	Omega-hydroxypalmitate O-feruloyl transferase	sp Q94CD1 HHT1_ARATH
FATB	1.7	1.5	MA_10197220g0010	Palmitoyl-acyl carrier protein thioesterase, chloroplastic	sp Q39513 FATB_CUPHO
AIM1	-1.2	-1.5	MA_17817g0010	Peroxisomal fatty acid beta-oxidation multifunctional protein	sp Q9ZPI6 AIM1_ARATH
MFP	-1.1	-1.4	MA_92062g0010	Peroxisomal fatty acid beta-oxidation multifunctional protein	sp Q8W1L6 MFP_ORYSJ
SDR1	-1.7	-0.6	MA_8431623g000	Short-chain type dehydrogenase/reductase	sp Q08632 SDR1_PICAB
HACD	1.8	2.9	MA_57869g0010	Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase 2	sp Q8VZB2 HACD_ARAH
HACD	1.6	2.6	MA_125121g0010	Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase 2	sp Q8VZB2 HACD_ARAH
ADH3	1.2	1.9	MA_30381g0010	Alcohol dehydrogenase 3	sp P14675 ADH3_SOLTU
ADH	1.3	1.8	MA_10430026g0010	Alcohol dehydrogenase	sp P48977 ADH_MALDO
ADHL3	2.1	2.8	MA_173093g0010	Alcohol dehydrogenase-like 3	sp A1L4Y2 ADHL3_ARATH
ADHL3	2.3	1.0	MA_10430588g0010	Alcohol dehydrogenase-like 3	sp A1L4Y2 ADHL3_ARATH
ADHL4	2.1	0.2	MA_9998439g0010	Alcohol dehydrogenase-like 4	sp Q8VZ49 ADHL4_ARATH

Table 2. Cont.

Gene	Log2(2900/2500)	Log2(3300/2500)	Gene ID	Annotation	Accession
Fatty Acids	5				
ADHL6	1.2	1.7	MA_409626g0010	Alcohol dehydrogenase-like 6	sp Q8LEB2 ADHL6_ARATH
AL2B7	1.9	1.8	MA_10436762g0010	Aldehyde dehydrogenase family 2 member B7	sp Q8S528 AL2B7_ARATH
AL3F1	1.0	1.8	MA_10014137g0010	Aldehyde dehydrogenase family 3 member F1	sp Q70E96 AL3F1_ARATH
AL3F1	1.2	2.1	MA_97566g0010	Aldehyde dehydrogenase family 3 member F1	sp Q70E96 AL3F1_ARATH
Flavonoids	6		0		
PALY	-0.4	-1.0	MA_10429279g0010	Phenylalanine ammonia-lyase	sp Q42667 PALY_CITLI
ТСМО	-1.7	-2.2	MA_202753g0010	Trans-cinnamate 4-monooxygenase	sp P48522 TCMO_CATRO
ТСМО	-1.6	-1.8	MA_130482g0010	Trans-cinnamate 4-monooxygenase	sp Q96423 TCMO_GLYEC
ТСМО	2.4	0.7	MA_10435536g0010	Trans-cinnamate 4-monooxygenase	sp Q43054 TCMO_POPKI
COMT1	-0.5	-1.2	MA_15892g0020	Caffeic acid 3-O-methyltransferase	sp Q8W013 COMT1_CATRO
COMT1	-0.5	-1.0	MA_15892g0010	Caffeic acid 3-O-methyltransferase	sp Q43239 COMT1_ZINVI
CAMT	1.3	1.7	MA_9065834g0010	Caffeoyl-CoA O-methyltransferase	sp O04854 CAMT_EUCGU
CAMT	-1.1	0.1	MA_4674g0010	Caffeoyl-CoA O-methyltransferase	sp Q41720 CAMT_ZINVI
CSE	1.2	2.6	MA_93032g0010	Caffeoylshikimate esterase	sp Q9C942 CSE_ARATH
CSE	-0.4	-2.3	MA_38618g0020	Caffeoylshikimate esterase	sp Q9C942 CSE_ARATH
CHSY	-1.0	-1.8	MA_401848g0010	Chalcone synthase	sp O65872 CHSY_PINST
F3'H	5.1	5.0	MA_10434848g0010	Flavonoid 3'-monooxygenase	sp Q9SD85 F3′H_ARATH
F3'H	-0.1	2.0	MA_76780g0010	Flavonoid 3'-monooxygenase	sp Q9SBQ9 F3′H_PETHY
F3'H	-1.3	-1.4	MA_10430254g0010	Flavonoid 3'-monooxygenase	sp Q9SD85 F3′H_ARATH
F3'H	-1.5	-5.5	MA_252610g0010	Flavonoid 3'-monooxygenase	sp Q9SBQ9 F3′H_PETHY
F3′5′H	-4.0	-1.4	MA_281597g0010	Flavonoid 3',5'-hydroxylase	sp Q96418 F3'5'H_EUSER
F3′5′H	-0.3	1.9	MA_633198g0010	Flavonoid 3',5'-hydroxylase	sp P37120 F3'5'H_SOLME
DFRA	0.0	-2.3	MA_6840966g0010	Dihydroflavonol-4-reductase	sp P51104 DFRA_DIACA
DFRA	1.5	1.8	MA_10430209g0020	Dihydroflavonol-4-reductase	sp P51105 DFRA_GERHY
DFRA	1.3	1.8	MA_23704g0010	Dihydroflavonol-4-reductase	sp P51105 DFRA_GERHY
DFRA	2.5	3.4	MA_10433570g0010	Dihydroflavonol-4-reductase	sp P51105 DFRA_GERHY
DFRA	-1.7	-1.2	MA_10432784g0010	Dihydroflavonol-4-reductase	sp P51106 DFRA_HORVU
DFRA	0.0	-1.2	MA_10432862g0010	Dihydroflavonol-4-reductase	sp P51106 DFRA_HORVU
FLS	-0.8	-2.1	MA_10431216g0010	Flavonol synthase/flavanone 3-hydroxylase	sp Q9M547 FLS_EUSER
LDOX	-2.3	0.7	MA_4447g0010	Leucoanthocyanidin dioxygenase	sp P51091 LDOX_MALDO
5GT1	-0.9	-1.4	MA_5871698g0010	Anthocyanidin 3-O-glucoside 5-O-glucosyltransferase 1	sp Q9ZR27 5GT1_PERFR
UFGT	2.1	3.2	MA_477384g0010	Anthocyanidin 3-O-glucosyltransferase	sp Q43716 UFGT_PETHY
ANR	0.5	2.1	MA_927736g0010	Anthocyanidin reductase	sp Q9SEV0 ANR_ARATH
ANR	-0.5	-1.5	MA_6784553g0010	Anthocyanidin reductase	sp Q9SEV0 ANR_ARATH

Interestingly, with the increasing elevation, we observed 60 DEGs involved in lipid metabolism, and 54 of them were upregulated, which might contribute to the survival in cold conditions at high altitudes. Moreover, flavonoids are important C-based secondary metabolites, which play a crucial role in the survival of trees under high altitude conditions. In this study, 29 genes involved in flavonoid biosynthetic pathway were identified as DEGs. Twelve of these genes were upregulated, including *C4H* (trans-cinnamate 4-monooxygenase), *CAMT* (caffeic acid 3-O-methyltransferase), *CSE* (caffeoylshikimate esterase), three *DFRs* (dihydroflavonol-4-reductase), *F3'5'H* (flavonoid 3',5'-hydroxylase), two *F3'Hs* (flavonoid 3'-monooxygenase), *UFGT* (anthocyanidin 3-O-glucosyltransferase) and *BAN* (anthocyanidin reductase), and they triggered the biosynthesis of anthocyanidin or condensed tannins, contributing to the resistance of trees to UV and other stresses.

3.6. DEGs Involved in Nitrogen Metabolism

With the increasing elevation, eight DEGs were found to be involved in N assimilation (Table 3; Table S10). Three *NIAs* (nitrate reductases), which are key genes involved in the first step of nitrate assimilation in plants, were downregulated. Meanwhile, one *NIR* (ferredoxin-nitrite reductase), which catalyzes the six-electron reduction of nitrite into ammonium, was downregulated. Moreover, two *NRTs* (nitrate transporters) were also downregulated, although one *NRT* and one *AMT* (ammonium transporter) were found to be upregulated. These results suggested that high altitude conditions blocked the uptake, assimilation, and transport of N.

Moreover, 18 DEGs were found to be closely associated with amino acid metabolism. Thirteen of them were downregulated with the increasing elevations, including *GLMS* (glutamine–fructose-6-phosphate aminotransferase) for glutamine, *METE* (5-methyltetrahydropteroyltriglutamate–homocysteine methyltransferase) and *HMT* (homocysteine S-methyltransferase 1) for methionine, two *ARODs* (arogenate dehydratase/prephenate dehydratase), three *AROFs* (phospho-2-dehydro-3-deoxyheptonate aldolase) and *PAL* (phenylalanine ammonia-lyase) for phenylalanine, *AAT* (aspartate aminotransferase) for aspartate, *CAS* (L-3-cyanoalanine synthase) for cysteine, two *GLYM* (serine hydroxymethyltransferase) for serine. The other five DEGs were upregulated, including two *ARGI* (arginase) and *SPD* (serine decarboxylase) for serine. This also indicated that high altitude conditions mostly had a negative effect on amino acid metabolism.

Gene	Log2(2900/2500)	Log2(3300/2500)	Gene ID	Annotation	Accession		
Nitrogen Assimilation							
NR	-0.5	-2.1	MA_2389043g0010	Nitrate reductase	sp P27969 NR_HORVU		
NR	-0.8	-1.6	MA_10432360g0020	Nitrate reductase [NADH] (Fragment)	sp P43101 NR_CICIN		
NR	-1.0	-1.0	MA_10432360g0010	Nitrate reductase [NADH]	sp P17569 NR_CUCM		
NIR	-1.6	-3.4	MA_22510g0010	Ferredoxin-nitrite reductase, chloroplastic	sp Q39161 NIR_ARATH		
NRT1	-0.7	-1.5	MA_4273216g0010	Protein NRT1/ PTR FAMILY 2.13	sp Q8RX77 PTR21_ARATH		
NRT1	-0.5	-2.0	MA_167141g0010	Protein NRT1/ PTR FAMILY 2.13	sp Q9SX20 PTR18_ARATH		
NRT1	-0.2	1.2	MA_122722g0010	Protein NRT1/ PTR FAMILY 3.1	sp Q9SX20 PTR18_ARATH		
AMT31	1.4	0.7	MA_55379g0010	Ammonium transporter 3 member 1	sp Q84KJ6 AMT31_ORYSJ		
Amino Ac	id Metabolism						
GLMS	0.8	-1.2	MA_46900g0010	Glutamine-fructose-6-phosphate aminotransferase	sp Q8AAB1 GLMS_BACTN		
METE	-1.1	-1.1	MA_10436788g0010	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	sp Q42662 METE_PLESU		
ARGI1	0.4	1.6	MA_10426056g0010	Arginase 1, mitochondrial	sp P46637 ARGI1_ARATH		
ARGI1	0.8	1.8	MA_10435722g0010	Arginase 1, mitochondrial	sp B8AU84 ARGI1_ORYSI		
AROD2	-1.5	-2.0	MA_864553g0010	Arginase 1, mitochondria6	sp Q9SSE7 AROD2_ARATH		
AROD6	-1.9	-2.9	MA_43667g0010	Arogenate dehydratase/prephenate dehydratase 6,	sp Q9SGD6 AROD6_ARATH		
AAT5	-0.8	-2.0	MA_17674g0010	Aspartate aminotransferase 5, chloroplastic	sp P46248 AAT5_ARATH		
GAD	1.4	0.7	MA_89828g0010	Glutamate decarboxylase	sp Q07346 GAD_PETHY		
HMT1	-0.2	-0.7	MA_7286g0010	Homocysteine S-methyltransferase 1	sp Q9SDL7 HMT1_ARATH		
CAS1	-1.1	-1.4	MA_6291g0010	L-3-cyanoalanine synthase 1, mitochondrial	sp Q1KLZ2 CAS1_MALDO		
PALY	-0.4	-1.0	MA_10429279g0010	Phenylalanine ammonia-lyase	sp Q42667 PALY_CITLI		
AROF	-0.3	-1.2	MA_10437122g0020	Phospho-2-dehydro-3-deoxyheptonate aldolase 1	sp P27608 AROF_TOBAC		
AROF	-0.8	-1.4	MA_10142969g0010	Phospho-2-dehydro-3-deoxyheptonate aldolase 1	sp P21357 AROF_SOLTU		
AROF	-1.0	-1.2	MA_10436001g0020	Phospho-2-dehydro-3-deoxyheptonate aldolase 1	sp P21357 AROF_SOLTU		
SDC1	1.3	1.5	MA_39829g0020	Serine decarboxylase 1	sp Q6ESZ9 SDC1_ORYSJ		
GLYM1	-0.6	-1.2	MA_7027365g0010	Serine hydroxymethyl transferase 1, mitochondrial	sp Q9SZJ5 GLYM1_ARATH		
GLYM	-0.6	-1.3	MA_905984g0010	Serine hydroxymethyltransferase, mitochondrial	sp P34899 GLYM_PEA		
SPD1	2.5	2.4	MA_136411g0010	Spermidine synthase 1	sp Q96556 SPD1_DATST		

 Table 3. DEGs involved in nitrogen metabolism.

4. Discussion

4.1. Response of NSC and N to the Increasing Elevations

The elevational trend in plant tissue NSC (Figure 1) indicated that the tissue NSC concentrations of trees growing at the alpine treeline were lower than that of trees at lower elevations, in terms of the early growing season. This result suggested that the treeline trees may suffer from a 'C limitation' in the early growing season. In the evergreen trees, branch storage and remobilization were found to be crucial for new shoot growth in spring [34]. We speculated that the concentrations of NSC in tissues were decreased in the early growing season, indicating that the mobile carbohydrates stored in tissues were utilized for shoot growth. On the other hand, plants grown under environmental stress conditions have lower tissue NSC concentrations than those grown under normal conditions. Many studies have shown that trees' hardness and ability to withstand cold are positively related to soluble carbohydrate concentrations in the tissues [10,35,36]. Alpine trees grown under a cold environment consume large amounts of stored carbohydrates to maintain normal growth and resist to a low temperature stress. Our result is consistent with that work of Dang et al. [11], which showed that the NSC concentrations in tissues were highest in the late growing season and decreased from early to late growing season on both slopes, indicating that the mobile carbohydrates stored in tissues were utilized from bud break and shoot growth in the early growing season [11].

In this study, the increasing trends of total N in the needles and stems suggested that N availability did not constrain sink activity at higher altitudes, which is why the treeline trees had no sufficient nutrient supply. This result was in agreement with those of previous studies, which showed that the tissue N content in treeline trees is higher than that in lower elevation trees [10,20,37–39]. Reich et al. [40] also reported that plants at high elevations exhibit N-physiological adaptation strategies to compensate for lower efficiency of physiological processes in low temperature environments. Therefore, these studies indicated that N seemed not to be a limiting factor for tree growth and development around the alpine treelines. Our results also showed that N concentrations in the needles were higher than those in roots at all elevations, supporting the fact that N allocation to storage tissues and remobilization for reuse in trees are seasonally programmed, and that the annual growth of many tree species heavily relies on the internal cycling of N, by which N is stored over winter and subsequently remobilized in spring for new growth [41,42]. However, whether N indeed limits the growth of treeline trees and determines the treeline formation needs to be further studied.

4.2. Changes of Genes Involved in C Assimilation and Metabolism in P. crassifolia with Increasing Elevations

In this study, many genes were found to be differentially expressed with the changes of elevation, including most of the downregulated genes involved in photosynthesis and carbohydrate metabolism. This result was consistent with the decrease in NSC content with the increasing elevation. All aspects of photosynthesis including the light reactions, the Calvin cycle, and photorespiration can be affected by low temperature stress [43,44], as well as the light-harvesting chlorophyll a/b-binding proteins, which are major constituents of the light harvesting complex in the thylakoid membranes [45]. This study showed that the expression of six chlorophyll a-b binding protein genes were significantly downregulated with the increasing elevations, which helped to explain the decreased C fixation, also suggesting that cold soil and ultraviolet-B radiation (UV-B) radiation leads to the downregulation of several transcripts encoding key photosynthetic proteins.

RFOs, particularly raffinose, play a role in the acquisition of cold tolerance and other stress responses in many plant species [46,47]. α -Galactosidase (AGAL) is a key enzyme involved in the breakdown of RFOs [47], and plays a role in producing osmoprotectants that stabilize the membranes and protect cells against oxidative stress [48]. Moreover, α -glucosidase (AGLU) digests carbohydrates, including starch and stable sugar [46]. Thus, the increase in *AGAL* and *AGLU* expression observed in this study might help *P. crassifolia* to resist the cold stress at the higher elevations. However, the expression of *GOLS1* (encoding galactinol synthase 1), which is the first committed enzyme in the

RFO synthesis pathway [46], decreased with the increasing elevations. These results indicated that the RFO pathway might play a crucial role in the response of *P. crassifolia* to elevation changes.

Plant cuticle lipids form outer protective layers to resist environmental stresses, including water loss, UV radiation, and low temperature [49,50], and many of these related genes are linked by their biological function to a specific response to low temperature and strong UV [51,52]. For example, 3-ketoacyl-CoA synthases (KCSs) belong to the group of "very long chain fatty acids", which are required for wax synthesis [53,54]. Moreover, fatty acyl-coA reductases (FARs), which are reported to be involved in cuticular wax production, i.e., involved in wax synthesis [49], and long-chain acyl-CoA synthetases (LACSs), have important roles in lipid synthesis and storage, synthesis of cutin and wax, and stress responses [55]. In this study, six *KCSs*, three *FARs*, and three *LACSs* were identified to be significantly induced with the increasing elevation, which might contribute to the survival of *P. crassifoliain* in the alpine environment. Furthermore, a significant enhancement in 17 of the 21 *GDLs*—encoding GDSL esterase/lipases, which play important roles in plant physiological processes, such as development, and stress responses [56]—would benefit the survival of *P. crassifolia* in the alpine environment.

Flavonoids are a large class of plant secondary metabolites that exert diverse biological and pharmacological effects, including the protection of plants against abiotic stresses [57]. With respect to enzymatic coding genes involved in the flavonoid or anthocyanin biosynthetic pathway, in this study, 12 of the 29 DEGs were upregulated at different elevations. For example, *F3Hs* and *F3'5'Hs*, encoding flavanone 3-hydroxylases and flavonoid 3',5'-hydroxylase, respectively, are involved in an early step in flavonoid metabolism leading to the formation of dihydroflavonols from flavanones [57,58]. In grape berries, UV-B increases the expression of these two genes, resulting in increased flavonol and anthocyanin content [59]. Our study showed that half of these genes were upregulated, indicating their roles in the resistance to cold stress and UV stress at higher elevations. Meanwhile, the increase of elevations also significantly promoted the transcript of anthocyanin downstream pathway genes, including 3 *DFRs* (dihydroflavonol-4-reductase), 1 *UFGT* (anthocyanidin 3-O-glucosyltransferas), and 1 *ANR* (anthocyanidin reductase), and these would contribute to the resistance of trees to UV or other stresses [57]. Therefore, the present study results indicated that flavonoids might play an important role in the survival of *P. crassifoliain* in the alpine environment.

5. Conclusions

This study demonstrated that the concentration of NSC significantly decreased in the current-year needles of *P. crassifolia* with increasing elevations, especially starch, indicating C limitation in trees around the treeline environment in the early growing season. This was further supported by the results of RNA-Seq, which showed almost all the photosynthesis-related genes were downregulated by the increasing elevations. Meanwhile, we found that large amounts of differentially expressed genes were involved not only in the central C metabolism, but also in the C-based secondary metabolism, including wax and flavonoid biosynthesis, contributing to the survival of *P. crassifolia* in the alpine environment. Thus, our study provided molecular evidence of C limitation hypothesis in treeline formation, as well as provided a better understanding of molecular mechanism of treeline tree survival in severely adverse conditions.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/11/2/156/s1.

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Conflicts of Interest: We declare no conflict of interest between the authors.

Deposited Data: The RNA-Seq datasets by using Illumina-Solexa platform are available from the NCBI Sequence Read Archive database (SRA; http://www.ncbi.nlm.nih.gov/sra) under submission number SUB6512905. The cDNA libraries were constructed from the elevations 2500, 2900, and 3300 m samples, respectively, with three biological replicates performed.

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