Transcriptome Analysis Reveals Key Genes in Response to High-Temperature Stress in *Rhododendron molle*

Linshi Wu,[a,b,]* Yan Liu,[a,b,]* Xinyun Liu,[a,b] Qiaoyun Li,[a,b] Xinyu Yi,[a,b] Chan Chen,[a,b] Ling Wang,[a,b] and Juyang Liao [a,b]

Rhododendron molle is a deciduous rhododendron, a high-altitude plant prized for its medicinal and ornamental properties. A major challenge when introducing this plant to lower altitudes is understanding its response to high-temperature stress. Using transcriptome analysis, this study examined leaves under varying temperatures, identifying 344,593 transcripts, 124,901 Unigenes, and 12,089 differentially expressed genes (DEGs) at 36 °C high-temperature stress (ST). At 42 °C high-temperature stress (SY), 12,032 DEGs were found, indicating a significant impact of temperature on gene expression. A Gene Ontology analysis (GO) revealed that these DEGs are mostly involved in stress response, catalytic activity, binding, transporter activity, and immune processes. A Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis highlighted enrichment in pathways such as plant-pathogen interaction and spliceosomes, suggesting their key roles in the temperature stress response. Key genes such as Brassinosteroid-Insensitive 1-Associated Receptor Kinase (BAK1) and Pathogenesis-Related Gene Transcription Activation Factor (PTI6) were upregulated under ST stress, whereas heat shock proteins (HSP83A) and HSP90-1 were downregulated under SY. These findings offer insights into the molecular response of *Rhododendron molle* to high temperature, aiding further research in this area and potentially improving the plant's cultivation and application.

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Keywords: Rhododendron molle; High temperature stress; Transcriptome analysis; Differentially expressed genes (DEGs)

*Contact information: a: Hunan Botanical Garden, Box 4100004, Changsha P.R. China; b: Rhododendron Engineering Technology Research Center, Box 4100004, Changsha P.R. China; *These authors contributed equally to this work; Corresponding authors: Juyang Liao liaojuyang@163.com (Hunan Botanical Garden, Changsha, PR China)*

INTRODUCTION

Rhododendron molle, distinctively known as the "yellow azalea" or "noisy sheep flower," holds the singular distinction of being the only species native to China within the subgenus *Pentanthera* of the Rhododendron family (Ericaceae). Celebrated for its exceptional ornamental appeal, it plays a crucial role in enriching the color diversity of azaleas (Ureshino *et al.* 1998). This plant predominantly flourishes at altitudes of around one thousand meters, with its main habitat spread across central and southern China and a smaller presence in the southwestern region. Notably, *Rhododendron molle* is currently recognized as an endangered species (Wang *et al.* 2010), underscoring the need for conservation efforts to protect this unique and valuable botanical treasure.

Rhododendron molle, a plant with significant medicinal qualities, is enriched with compounds such as lignans in its flowers and flavonoids, rhododendron toxins, and methyl-lichenic acid in its leaves. Lignans are an important kind of natural phytoestrogen with various biological activities, including antioxidant, antiviral, and anti-tumor activities. The lignans in rhododendrons have little effect on animals, but the gray anotoxins have a strong insecticidal effect and have been widely used as a pesticide. When applied *via* contact or fumigation, they poison the stomach, interfere with feeding, and inhibit the growth and development of many kinds of pests. *Rhododendron molle's* other constituents endow it with diverse therapeutic benefits. Traditionally, it has been used for its effectiveness in dispelling wind, alleviating pain, and reducing dampness, and it also serves as an anesthetic. It is particularly effective in treating conditions such as fractures, rheumatism, and persistent skin eczema (Liu *et al.* 2007; Cheng *et al.* 2011; Zhi *et al.* 2013; Li *et al.* 2018). Intriguingly, certain toxins in *Rhododendron molle*, especially those derived from the "noisy sheep flower," have shown remarkable agricultural value. They demonstrate potent stomach-poisoning, contact-killing, and fumigation effects against various pests (Zhu *et al.* 2023). Notably, research has highlighted that these toxins are effective in deterring feeding in the larvae of pests like *Leptinotarsa decemlineata* and *Spodoptera frugiperda*, underscoring the plant's potential in natural pest control (Shin and Yu 1993). Aside from its medicinal properties, *Rhododendron molle* also stands as a pivotal resource in azalea breeding. Azalea breeders have long recognized its excellent compatibility with other azalea species, making it an ideal candidate for hybridization (Geng *et al.* 2019). Many of its traits, typically recessive in the first generation, render it a superior parent for breeding programs. Despite its critical role in azalea cultivation, the challenges presented by *Rhododendron molle* such as its low seed germination rate and the difficult rooting and low survival rates of cuttings, have hindered its use in urban landscaping (Meijón *et al.* 2009). Consequently, intensifying research to improve *Rhododendron molle* cultivation techniques and promote their broader implementation is crucial for diversifying urban tree species and holds significant implications for agricultural production and medical research (Zhou and Zhu 2020).

Rhododendron molle grows under shrubs or mixed forests on mountain ridges in mountainous grasslands or hilly areas at an altitude of 1000 meters, with the most suitable growth temperature being 15 to 20 °C; these conditions adversely affect the introduction and garden cultivation of Rhododendron molle (Arisumi *et al.* 1989). Additionally, global climate change has led to more frequent occurrences of hot weather and water shortages across the nation, impacting the normal growth of plants. This situation of high temperatures is expected to worsen in the future (Schlenker and Maximilian 2018; Yuan *et al.* 2019). Therefore, it is crucial to understand and explore the molecular regulatory mechanisms that enable Rhododendron molle to withstand high-temperature stress, which is particularly important for breeding purposes.

RNA-Seq can be used to comprehensively obtain the sequence and expression information of the transcriptome of a particular organism under different environmental conditions and analyze the differential expression of genes at the RNA level to identify key DEGs. This, in turn, allows an in-depth study of the regulatory mechanisms and functions of differential genes in different environments (Yan *et al.* 2004). For example, 18 genes encoding heat stress proteins (HSPs) were identified in *Paeonia suffruticosa* by means of transcriptome analysis, and all of them showed significant up-regulation under high-temperature treatment. A transcriptome analysis of *Narcissus tazetta* var. *chinensis* in response to high-temperature stress identified 96 known miRNAs, 25 novel miRNAs and

52,286 predicted siRNAs that may play important roles in response to heat stress in daffodils (Yan *et al.* 2004). In this study, we analyzed the transcripts of Rhododendron molle under high-temperature stress using RNA-seq technology to obtain the differentially expressed genes. Our results can provide a reference for the later molecular breeding of Rhododendron molle variants that are robust against high temperatures.

EXPERIMENTAL

Study Sites and Plant Materials

The experimental materials were kept in Hunan Provincial Botanical Garden (113°01′30″E, 28°06′40″N) Two-year-old cuttings of *Rhododendron truncatulum* were transplanted into pots (h⩾20cm), with one cutting planted in each pot. A total of 2 high-temperature treatments (36 ℃ and 42 ℃ referred to as ST and SY, respectively) were set up, and a 25 ℃ treatment was used as the control (CK). The experimental design included lighting at 2000 lx(14 h day/10 h night) in an artificial climate chamber. Three biological replicates were planted in the control and each treatment. Under continuous high-temperature stress in the SY treatment, *Rhododendron molle* displayed symptoms of yellowing and blackening of its leaves after 10 days, with signs of death observed after 14 days. Therefore, a high-temperature stress duration of 15 days was designed (Jiang *et al.* 2023). On the 15th day, healthy leaves without pests or diseases were collected from the target plants, rapidly frozen in liquid nitrogen, and stored at -80 °C. These samples were then sent to Nanjing Jisi Huiyuan Biotechnology Co., Ltd. for further analysis.

RNA Extraction and Sequencing Library Construction

The mRNA was extracted from the biological samples using the Poly (A) structure, and the RNA quality was determined using a Nanodrop ultra-micro UV-Vis spectrophotometer and Agilent 2100 Bioanalyzer. Eukaryotic mRNA was enriched using magnetic beads with Oligo (dT), and the first cDNA strand was synthesized by adding Fragmentation Buffer to randomly interrupt the mRNA, followed by the synthesis of the double-stranded cDNA strand, purification of the cDNA, and end-repair, *etc.* Finally, the cDNA library was enriched *via* PCR.

Assembly and Functional Annotation of the Transcriptome

High-throughput sequencing (*e.g.*, Illumina HiSeq 2500) was used for experimental processing. The Unigene sequence were compared with the NR (Marchler *et al.* 2015), Swiss-Prot (Gasteiger *et al.* 2001), GO (Gene Ontology Consortium, 2004), COG (Tatusov *et al.* 2003), KOG (Jensen *et al.* 2007) and KEGG (Kanehisa *et al.* 2012) databases using BLAST v2.3.0+ (http://blast.ncbi.nlm.nih.gov/Blast.cgi) software, and the Unigene amino acid sequence were predicted and compared with the Pfam database (Mistry *et al.* 2021) using HMMER v3.4 (Finn *et al.* 2011) software to obtain the Unigene annotation information.

Functional Analysis of the DEGs

The sequenced reads were compared with the Unigene library using Bowtie2 v2.4.4 (Langmead and Salzberg 2012), and the expression level was estimated based on the comparison results in combination with RSEM (Li and Dewey 2011). The expressing abundance of the corresponding Unigene is expressed using FPKM values.

RESULTS AND DISCUSSION

Sequencing Data Assembly Results

Each sequencing sample was repeated once, and after sequencing quality control, the accuracy of each sample base was greater than 96% (corresponding to Q20), and the GC content was basically below 50%. Thus the sequencing data could be used for assembly (Moreton *et al.* 2016). The data output and assembly statistics for each sample are shown in Table 1. For the *de novo* assembly of the transcriptome, Trinity assembly software was adopted. The assembly resulted in a total of 124,901 Unigenes, with a total length of 530,057,171 nt. and an average length of 1538.21 nt, N50. All the Unigenes were arranged in order from longest to shortest, and their lengths were added. When a fragment's length is 50% of the total number of fragments, the length and number of the fragment, *i.e.*, the length and number of N50, indicate the quality of the assembly: the lower these values are for the Unigenes, the better the quality.

Table 1. Sequencing Sata Quality

Sample Group	Sample number	Clear data	Number of bases	GC(%)	Q20(%)	Q30(%)
25°C-1	CK1	30190754	9057226200	49.08	96.82	91.23
25°C-2	CK2	30615722	9184716600	49.42	97.07	91.83
25°C-3	CK3	28976792	8693037600	50.13	97.26	92.29
36°C-1	ST1	31091223	9327366900	48.74	97.08	91.85
36°C-2	ST2	32423679	9727103700	49.29	96.82	91.28
36°C-3	ST3	27283463	8185038900	48.95	96.74	91.16
42°C-1	SY1	28309288	8492786400	49.9	96.87	91.4
42°C-2	SY2	26645643	7993692900	49.77	97.45	92.68
42°C-3	SY3	30071112	9021333600	50.23	97.23	92.21

Sample numbers 1, 2 and 3 indicate three replicates of the sample; GC(%): Clean Data GC content, *i.e.* the percentage of both G and C bases in Clean Data to the total bases; N(%): N base content in Clean Data; Q20(%): percentage of bases with Clean Data quality values greater than or equal to 20; Q30(%): percentage of bases with Clean Data quality values greater than or equal to 30.

Transcriptome Unigene Cluster Results

Since there were no reference genomic data for *Rhododendron molle,* Clean reads were used in Trinity software for hybrid splicing. A total of 344,593 transcripts and 124,901 Unigenes were obtained. The average length of the transcripts was 1538.21, and the N50 values of the transcripts and Unigenes were 2466 and 1725, respectively (Table 2), indicating high assembly integrity. This data met the requirements for the next step of gene function annotation and classification.

Table 2. Data Assembly Statistics

Length Range (bp)	Transcripts		Genes	
	Quantity	Percentage (%)	Quantity	Percentage (%)
300-500	77742	32.39	40459	32.39
500-1000	97313	33.76	42169	33.76
1000-2000	81183	19.36	24183	19.36
＞2000	88355	14.48	18090	14.48
Total number	124901	-	344593	-
Total number of bases	530057171	-	143479714	-
Average length	1538.21	-	1148.75	-
N50 length	2466	-	1725	-

The assembled transcriptome sequences were compared with COG, GO, KEGG, KOG, Swiss-Prot, Pfam, NR, and other databases to obtain the annotation information of each database. The numbers of Unigenes annotated in the above databases were 10,973, 32,259, 51,672, 29,049, 23,701, 35,661, 62,917, respectively. The gene function annotation statistics are shown in Table 3.

Table 3. Annotation Statistics of Single Gene Clusters

Database Comments	Number of Genes	Sequence Length (300-1000)	Sequence Length (>1000)
COG	10973	3570	7403
GO	32259	14346	17913
KEGG	51672	25393	26279
KOG	29049	13694	15355
PFAM	23701	7750	15951
Swiss-prot	35661	16091	19570
NR	62917	32338	30579
All	64712	33683	31029

Analysis of Differentially Expressed Genes Under Different Heat Stresses

Based on the RPKM values, the transcript gene expression differences under the different treatments were assessed, with screening criteria of FDR≤0.05 and FC≥2. In Fig. 1, the horizontal coordinate indicates the logarithmic value of the difference multiplier between the two treatment groups, and the vertical coordinate indicates the negative log10 value of the FDR of the difference between the two subgroups. In these volcano plots of differentially expressed genes, red (up-regulated expression) and green (down-regulated expression) dots indicate differential gene expression, while gray dots indicate no difference. By comparing the differential expression of genes between high-temperature stress and the control, the volcano plot analysis (Fig. 1) showed a total of 12,089 differentially expressed genes, of which 6004 were up-regulated and 6,085 were down-regulated, in the control compared with the 36 °C high-temperature stress. Additionally there were 12,032 differentially expressed genes in the control compared to the 42 °C high-temperature stress, of which 5,431 were up-regulated and 6,601 were down-regulated. There were 4594 common differentially expressed genes between the two groups of differential genes (Fig. 2), of which 1224 were co-up-regulated and 3007 were co-down-regulated, in addition to 363 common reverse differentially expressed genes between the two. Some of the common differentially expressed genes responded to both 36 °C and 42

°C high-temperature stress. The number of down-regulated genes in *Rhododendron molle* was higher than the number of up-regulated genes with increasing temperature under the high-temperature stress treatments, indicating that high-temperature stress significantly suppressed some of the physiological activities of *Rhododendron molle*.

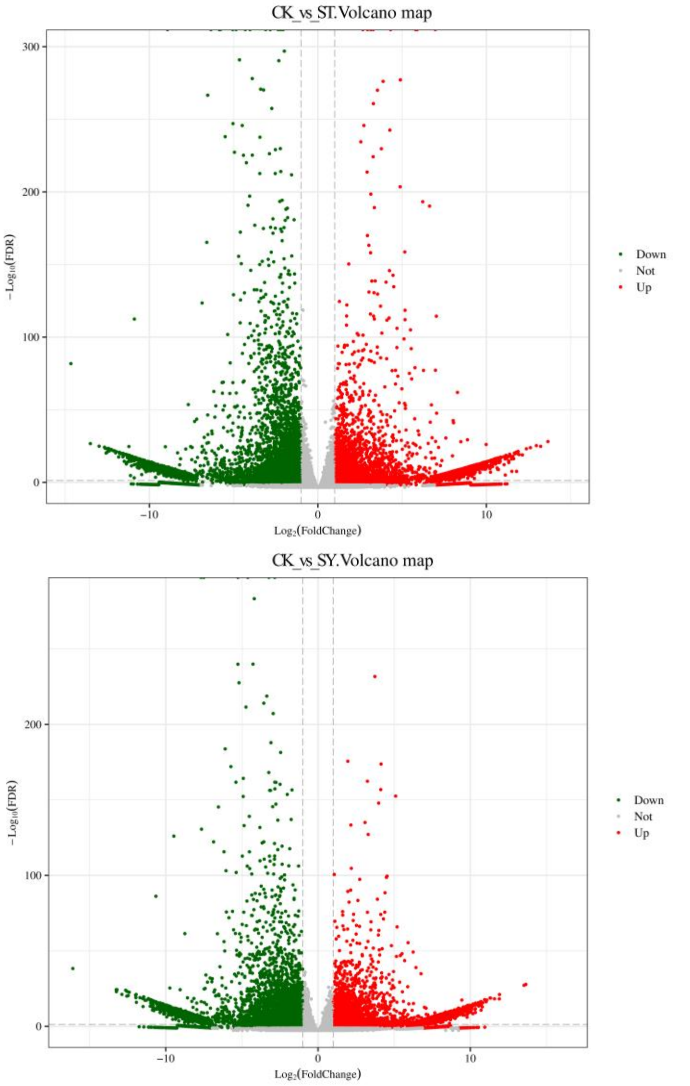


Fig. 1. Volcano plot of the number of differentially expressed genes. Each point in the differential expression volcano plot represents a gene, and the horizontal coordinate indicates the logarithmic value of the expression ploidy of a gene in the two samples; the vertical coordinate indicates the negative logarithmic value of FDR. The larger the absolute value of the horizontal coordinate, the greater the difference in expression ploidy between the two samples; the larger the value of the vertical coordinate, the more significant the differential expression and the more reliable the differentially expressed genes obtained from the screening. The green dots represent down-regulated differentially expressed genes, the red dots represent up-regulated differentially expressed genes, and the gray dots represent non-differentially expressed genes.

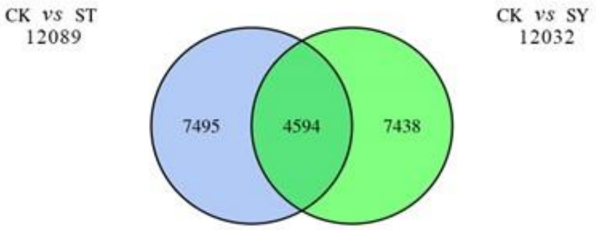
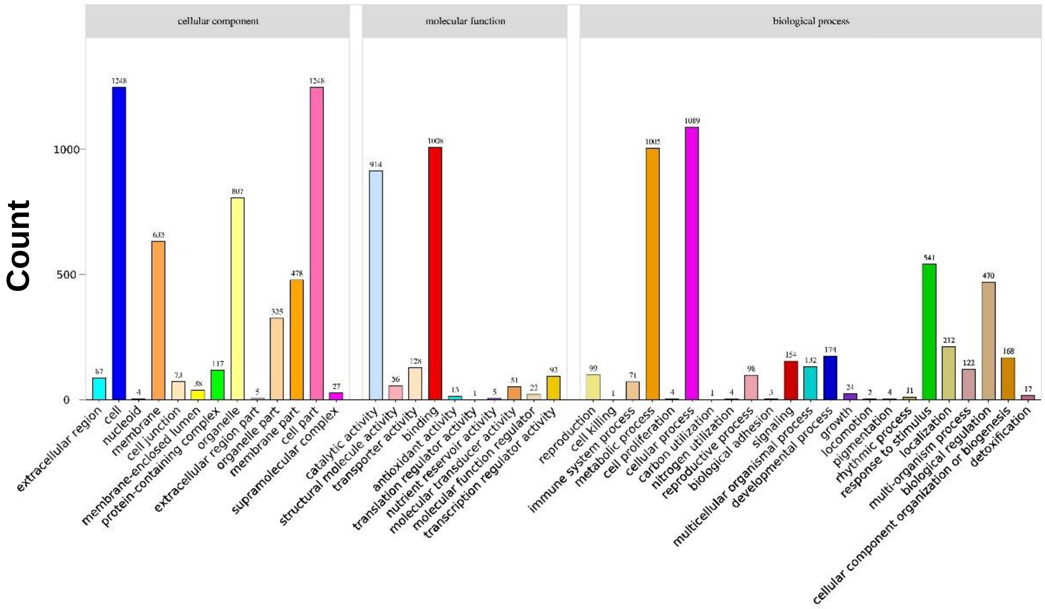


Fig. 2. Venn diagram of the number of differentially expressed genes

GO Classification and Enrichment Analysis of Significantly Different Genes

The obtained target genes were subjected to GO enrichment analysis. The biological functions of the differentially expressed genes were mainly analyzed in terms of their molecular functions, cellular components, and biological processes. The biological processes enriched on metabolic processes, cellular processes, stimulus responses, and biological regulation; the cellular component part enriched on cells, cellular components, organelles, organelle components, membranes, membrane components, and protein complexes; and the molecular function part enriched on binders and catalytic activity. The GO enrichment results for *Rhododendron molle* under different high-temperature stresses are displayed in Fig. 3. The significantly enriched GO term categories did not change, but the numbers of enriched genes all increased, indicating that high-temperature stress led to increased plant cell destruction.



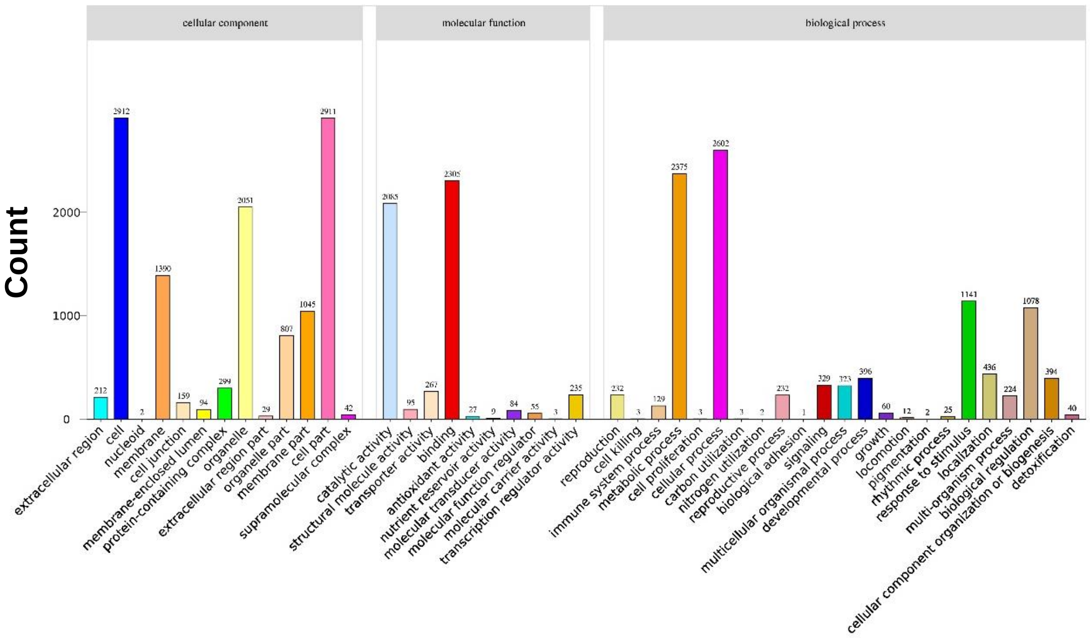


Fig. 3. Differential gene GO functions. Cellular component 1: Extracellular region; 2: Cell; 3: Nucleoid; 4: Membrane; 5: Cell junction; 6: Membrane-enclosed lumen; 7: Protein-containing complex; 8: Organelle; 9: Other organism; 10: Other organism part; 11: Extracellular region part; 12: Organelle part; 13: Membrane part; 14: Cell part; 15: Supramolecular complex . molecular function 1: Catalytic activity; 2: Structural molecule activity; 3: Transporter activity; 4: Binding; 5: Antioxidants activity; 6: Nutrient reservoir activity; 7: molecular transducer activity; 8: molecular function activity; 9: molecular carrier activity; 10: Transcription regulator activity. Biological process: 1: Reproduction; 2: Cell killing; 3: Immune system process; 4: Metabolic process; 5: Cell proliferation; 6: Cellular process; 7: Carbon utilization; 8: Nitrogen utilization; 9: Reproductive process; 10: Biological adhesion; 11: Signaling; 12: Multicellular organismal process; 13: Developmental process；14: Growth；15: Locomotion；16: Pigmentation；17: Rhythmic process; 18: Response to stimulus; 19: Localization; 20: multi-organism process; 21: Biological regulation; 22: Cellular component organization; 23: Detoxification.

KEGG Enrichment Analysis of Significantly Different Genes

The KEGG database was used to analyze the enrichment pathways of the differentially expressed genes detected in *Rhododendron molle* under different high-temperature stress treatments. As a result 4594 differentially expressed genes (DEGs) were found to be enriched in 125 pathways. The KEGG metabolic pathways for the control in comparison to 36 °C high-temperature stress mainly involved sulfur metabolism, astragalus, diarylheptane and gingerol biosynthesis, starch and sucrose metabolism, plant-pathogen interactions, phytohormone signaling, photosynthesis tentacle protein, phenylpropanoid biosynthesis, and other pathways. Among the top 20 metabolic pathways with the most reliable enrichment significance, as shown in Table 4, the plant-pathogen interaction pathway was annotated to a maximum of 88 genes; followed by spliceosomes (annotated to 60 differentially expressed genes), plant hormone signaling (annotated to 59 differentially expressed genes), ribosomes (annotated to 58 differentially expressed genes), and other pathways. The KEGG metabolic pathways for the control in comparison to 42 °C high-temperature stress mainly involved in plant-pathogen interactions, ribosomes, spliceosomes, endoplasmic reticulum protein processing, and other pathways. Among the top 20 metabolic pathways with the most reliable enrichment significance, the plant-pathogen interaction pathway was annotated to the most genes (100), followed by the ribosomal (annotated to 83 differentially expressed genes), ribosomal (annotated to 73 differentially expressed genes), and endoplasmic reticulum protein processing (annotated to 73 differentially expressed genes) pathways.

Table 4. KEGG Pathway Enrichment Information for the Top 10 Differentially Expressed Genes

ST-enriched KEGG Pathway Name	**# of EDGs**	**SY-enriched KEGG Pathway Name**	**# of EDGs**
Plant-pathogen interaction	88	Plant-pathogen interaction	100
Spliceosome	60	Ribosome	83
Plant hormone signal transduction	59	Spliceosome	73
Ribosome	58	Protein processing in endoplasmic reticulum	73
Starch and sucrose metabolism	56	Plant hormone signal transduction	47
Protein processing in endoplasmic reticulum	51	Endocytosis	43
Endocytosis	48	MAPK signaling pathway - plant MAPK	37
Glyoxylate and dicarboxylate metabolism	47	RNA transport RNA	35
Peroxisome	42	Oxidative phosphorylation	34
Phenylpropanoid biosynthesis	42	Amino sugar and nucleotide sugar metabolism	34

Notes: ST: 36 °C high-temperature stress; SY: 42 °C high-temperature stress

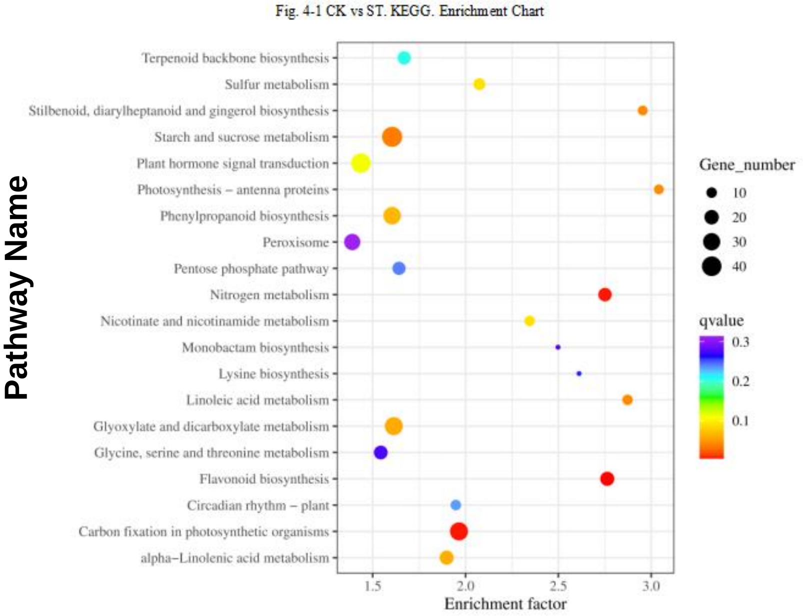


Fig. 4-1 CK vs ST. KEGG. Enrichment Chart

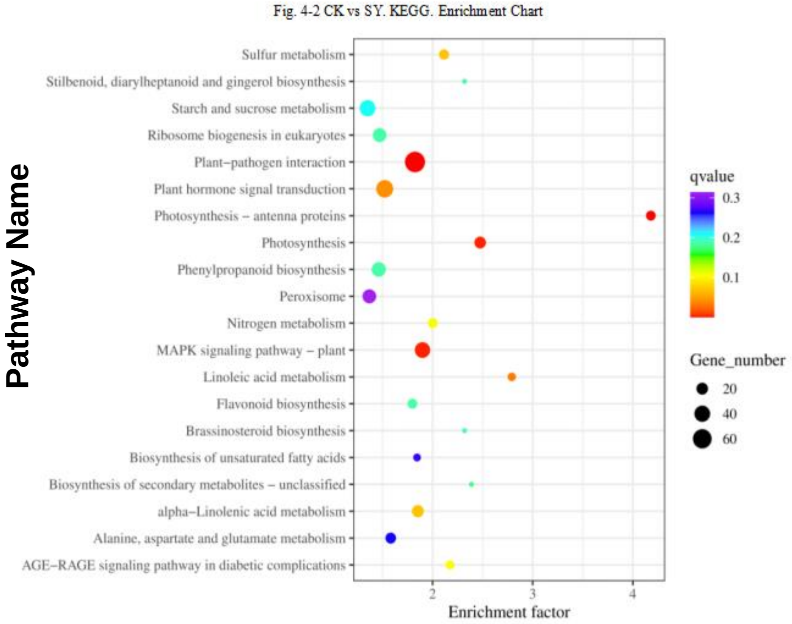


Fig. 4-2 CK vs SY. KEGG. Enrichment Chart

Fig. 4. KEGG functional enrichment analysis of differentially genes

Functional Identification of Differentially Expressed Genes

From the enrichment results for differentially expressed genes in the KEGG signaling pathways, the related genes that were up-regulated or down-regulated in all metabolic pathways of lambda *R. molle* under high-temperature stress were identified. Two significantly up-regulated genes were screened from among the most enriched genes in the plant-pathogen interactions pathway under the ST high-temperature treatment(36 °C): oleuropein receptor protein-associated receptor kinase (BAK1) and disease process-associated gene transcriptional activator (PTI6).Four genes were significantly down-regulated, including mitogen-activated protein kinase kinase 1a (MKK1a), mitogen-activated protein kinase kinase kinase 5 (MKK5), and retroviral-associated Pol polyprotein (RE1) (Tables 5 through 7). Screening under the spliceosome pathway showed significant upregulation of the sulfate glycoprotein (RH14) and RNA unwinding helicase (SMD1A) and significant downregulation of a small nuclear ribonucleoprotein (STA1). Under the phytohormone signal transduction pathway, indole-3-acetic acid amide synthase (GH3.10), two-component response regulator (RR22), and DELLA protein (GAI1) were the three up-regulated genes and gene erythromycin receptor (GID1B) and oleuropein receptor protein-associated receptor kinase (BAK1) were the two down-regulated genes. Oleuropein receptor protein-related receptor kinase (BAK1), WRKY transcription factor (WRKY22), and transcriptional activator of disease process-related genes (PTI6) were significantly up-regulated in the phytopathogen interactions pathway under the SY high-temperature treatment (42 °C), while heat shock protein HSP83A, heat kinin (HSP90-1), and mitogen-activated protein kinase kinase 5 (MKK5) were significantly downregulated. The ribosomal proteins RPS3C and RPL24 showed up-regulation while RPS20B RPL10A and showed down-regulation under the ribosomal pathway. Protein homologs (MAGO1) showed up-regulation and serine-arginine-rich splicing factors (SC35, SR34, SR34B, SCL33) all showed down-regulation under the spliceosomal pathway (Tables 8-10). Genes in the metabolic pathway exhibited different levels of coordination involving multiple pathways such as photosynthesis, respiration, redox, phytohormone signaling, and gluconeogenesis, which work together to combat high-temperature stress.

Table 5. Information on Relevant DEGs Under the Interactions Pathway in Plants

Access ID	Gene ID	Gene Naming	Gene Expression		Genetic Status	$\log_2$FC
			CK	ST		
K13416	TRINITY_DN8774_c0_g1	BAK1	7.333	16.803	up	1.388719556
K13434	TRINITY_DN4510_c1_g3	PTI6	10.928	54.169	up	2.455158812
K04368	TRINITY_DN10166_c0_g1	MKK1a	1.332	0.106	down	-3.578296933
K13413	TRINITY_DN464_c0_g2	MKK5	9.092	3.143	down	-1.417724176
K18835	TRINITY_DN4811_c0_g1	RE1	75.994	24.057	down	-1.70041001
K18835	TRINITY_DN52716_c0_g1	RE1	6.221	3.028	down	-1.297825338

Table 6. Information on Relevant DEGs Under the Spliceosome Pathway

Access ID	Gene ID	Gene Naming	Gene Expression		Genetic Status	$\log_2$FC
			CK	ST		
K12823	TRINITY_DN4293_c0_g1	RH14	23.008	44.163	up	1.009204081
K11087	TRINITY_DN49163_c0_g5	SMD1A	30.548	49.555	up	1.018533564
K12855	TRINITY_DN36300_c0_g1	STA1	0.285	0.231	down	-2.98443391

Table 7. Information on Relevant DEGs Under the Phytohormone Signaling Pathway

Access ID	Gene ID	Gene Naming	Gene Expression		Genetic Status	$\log_2$FC
			CK	ST		
K14487	TRINITY_DN1300_c2_g1	GH3.10	66.941	10.851	down	-2.627030079
K14491	TRINITY_DN16346_c0_g1	RR22	1.474	0.019	down	-3.488003388
K14494	TRINITY_DN27355_c0_g3	GAI1	12.222	5.027	down	-1.21887678
K14493	TRINITY_DN2929_c1_g1	GID1B	7.152	16.31	up	1.18489997
K13416	TRINITY_DN8774_c0_g1	BAK1	7.333	25.181	up	1.388719556

Table 8. Information on Relevant DEGs Under the Plant-Pathogens Interactions Pathway

Access ID	Gene ID	Gene Naming	Gene Expression		Genetic Status	$\log_2$FC
			CK	SY		
K13416	TRINITY_DN22606_c0_g1	BAK1	5.57	36.297	up	2.179270164
K04079	TRINITY_DN27826_c0_g1	HSP83A	11.465	0.237	down	-5.170365859
K04079	TRINITY_DN30406_c0_g1	HSP90-1	7.292	0	down	-9.94791215
K13413	TRINITY_DN464_c0_g2	MKK5	11.87	6.213	down	-0.51872317
K13425	TRINITY_DN25668_c0_g1	WRKY22	3.879	7.062	up	1.328463743
K13434	TRINITY_DN4510_c1_g2	PTI6	3.808	4.216	up	0.311380908

Table 9. Information on Relevant DEGs Under the Ribosomal Pathway

Access ID	Gene ID	Gene Naming	Gene Expression		Genetic Status	$\log_2$FC
			CK	SY		
K02985	TRINITY_DN1732_c0_g1	RPS3C	0	1.148	up	6.617789613
K02895	TRINITY_DN6795_c0_g1	RPL24	73.358	158.02	up	1.255865906
K02969	TRINITY_DN49353_c0_g1	RPS20B	0.577	0	down	-7.449425472
K02865	TRINITY_DN4437_c0_g1	RPL10A	8.062	2.804	down	-2.254091851

Table 10. Information on Relevant DEGs Under the Ribosomal Pathway

Access ID	Gene ID	Gene Naming	Gene Expression		Genetic Status	$\log_2$FC
			CK	SY		
K12877	TRINITY_DN8886_c0_g1	MAGO1	1.499	10.923	up	1.974476617
K12891	TRINITY_DN1547_c2_g1	SC35	53.243	6.944	down	-2.634410879
K12890	TRINITY_DN13178_c0_g1	SR34	61.275	28.867	down	-1.098363443
K12890	TRINITY_DN547_c3_g1	SR34B	2.978	1.231	down	-1.955287651
K12900	TRINITY_DN3908_c0_g1	SCL33	44.908	13.525	down	-1.423847576

Discussion

High-temperature stress alters plant morphology, physiological and biochemical processes, and gene expression, impeding normal growth and development (Wang *et al.* 2023). This not only alters physiological metabolic processes such as photosynthesis, osmoregulation, antioxidant metabolism, phytohormones, and sugar metabolism but also affects a variety of signaling factors, transcription factors, and functional genes (Hasanuzzaman *et al.* 2013). The response of plants to high-temperature stress is a complex biological process (Kotak *et al.* 2007). In a previous study, the heat tolerance of five species of rhododendron was studied, and the catalase (CAT) activities, ascorbate peroxidase (APX), proline (Pro) content, hydrogen peroxide (H_2O_2), and malondialdehyde (MDA) in the leaves of *R. molle* increased with increasing stress temperature. The superoxide dismutase (SOD) activity increased slightly under mild stress at 30 °C but decreased under severe stress at 38 °C (Zhang *et al*. 2011). In a study on the effect of ERM fungi on the heat resistance of *Rhododendron molle*, in the control group without any fungi added, the electrical conductivity and MDA of R. molle increased with the temperature, while proline (Pro), POD, and SOD decreased with increasing stress (Luo 2016).

Rhododendrons grow best in cool environments and do not tolerate high temperatures. Thus, the high temperatures of China's summer climate make it difficult for transplanted *Rhododendron molle* to survive. Under the same watering conditions, rhododendrons with high heat resistance were found to have a higher water utilization rate, CO_2 utilization rate, and light energy utilization rate; stronger photosynthetic capacity and antioxidant enzyme activity; and higher Pro and chlorophyll contents than heat-resistant rhododendrons in the same period (Li 2019). In our next steps, we will continue to study the differences in the expression levels of relevant genes in rhododendrons under water stress conditions, in combination with the gene changes that occur under high-temperature stress, to comprehensively analyze the key genes regulating stress resistance in rhododendrons in hot summers, so as to provide references for later rhododendron research.

In this study, high-temperature stress affected the physiological metabolism and growth of *Rhododendron molle*, and the transcriptome sequences of *Rhododendron molle* leaves under high-temperature stress and a control treatment were compared to reveal the response mechanism of *Rhododendron molle* to high-temperature stress at the molecular level. A study carried out in Hunan Provincial Botanical Garden showed that with an increase in the temperature and duration of high-temperature stress, the physiological and biochemical responses of *Rhododendron molle* leaves were enhanced, including a gradual decrease in SOD, MDA, and POD activities (Ying *et al.* 2018). To elucidate the biological mechanisms that occur in Rhododendron in response to high-temperature stress, the present study was conducted to analyze the transcriptome of *Rhododendron molle* leaves treated under high-temperature stress by means of Illumina high-throughput sequencing technology. The total number of genes in the sample was 124901, with a total length of 530057171 nt and an average length of 1538.21 nt. A total of 344593 transcripts and 124901 Unigenes were obtained after mixed splicing. A GO functional annotation analysis showed that the DEGs under high-temperature stress were mainly concentrated in metabolic processes, cellular processes, cells, cellular components, binders, and catalytic activity, and the most enriched DEGs were found in metabolic processes, This indicates that a large number of transcription factors were activated in *Rhododendron molle* to regulated its response to high-temperature stress by inducing the expression of high-temperature stress genes; in particular, cell destruction was severely damaged (Yu *et al.* 2011). An analysis of KEGG metabolic pathways revealed that the most enriched metabolic pathways for the DEGs were plant-pathogen interactions, spliceosomes, ribosomes, phytohormone signaling, and endoplasmic reticulum protein processing. This indicates that the metabolic pathway plays an important role in the response of *Rhododendron molle* to high-temperature stress. The enrichment pathways screened for transcription factors such as WRKY were previously found to be involved in the synthesis of plant terpenoids to withstand adversity (Singh *et al.* 2017; Alfieri *et al.* 2018; Zhan *et al.* 2022).

Brassinolide (BR), also known as brassinosteroid, is a naturally occurring compound with biological activity, characterized by a steroidal structure. It represents the sixth type of plant hormone, following gibberellins, auxins, cytokinins, abscisic acid, and ethylene (Sasse 1997). The discovery of BR was one of the most significant findings in the field of plant hormones, following that of gibberellins. This hormone is involved in regulating various processes in plants, including seed germination, nutritional growth, reproductive growth, and responses to external environmental stressors (Zheng *et al.* 2018; Rozhon *et al.* 2019). Cao and Zhao (2008) found that BR can enhance the activity of superoxide dismutase (SOD) and peroxidase (POD), increase the expression of their isoenzymes, eliminate free radicals generated within cells under high-temperature conditions, stabilize membrane structures, reduce electrolyte leakage, and increase the protein content in plants. This enables normal plant growth in high-temperature stress conditions. A study by Jin *et al*. (2015) confirmed that BR can alleviate the damage caused by high-temperature stress in banyan tree seedlings. Furthermore, a study conducted by Yang *et al*. (2019) suggested that brassinosteroid (BR) treatment can alleviate the inhibitory effects of high-temperature stress on the growth of *Toona sinensis* seedlings. In our research, we found that brassinosteroid (BR) receptor protein-associated receptor kinase (BAK1) was significantly upregulated during the heat-stress treatment (ST). This suggests that brassinosteroids play a crucial role in the response of *Rhododendron molle* to high-temperature stress.

A particular group of plant-coded proteins induced by different stress stimuli, named “pathogenesis-related proteins” (PRs), is assigned an important role in plant defense against pathogenic constraints and in general adaptation to stressful environments, Upon investigation, it was discovered that these genes play a pivotal role in responding to both biotic and abiotic stressors, underscoring their critical importance in the survival of plants (Edreva 2005). In previous studies, there was no mention of PRs being involved in plant responses to heat stress. In our research, we discovered that Pathogenesis-Related gene Transcriptional Activator (PTI6) was significantly upregulated during heat treatment (ST), indicating that PRs may play a crucial role in the response of sheep sorrel to high-temperature stress. This finding provides important theoretical support for the involvement of PRs in the response to heat stress. Therefore, PTI6 emerged as a novel and advantageous candidate gene in our study, laying a solid foundation for future in-depth investigations into *Rhododendron molle*'s adaptation to high-temperature stress.

Key heat-stimulated transcription factors (Hsf) play an important role in the response to high temperature (Pirkkala *et al.* 2001; Mittal *et al.* 2009; Liu *et al.* 2015). Hsf genes, as heat shock transcription factors, often bind to heat-excited elements in the heat shock protein (HSP) promoter and play important roles in the regulation of plant development and various stress responses (Li *et al.* 2023). In our study, we observed a significant downregulation of heat shock proteins (HSP83A and HSP90-1) under the SY treatment. This contradicts the typical plant response to combat high-temperature stress, suggesting that this downregulation may be one of the reasons for temperature sensitivity in sheep sorrel.

CONCLUSIONS

1. A transcriptome analysis was conducted on the leaves of *Rhododendron molle*. Leaves from plants kept at 25 °C were used as the control group and were compared with leaves from plants kept at 36 °C or 42 °C.
2. According to the GO enrichment, KEGG enrichment, and expression trend analyses of the DEGs, four candidate genes expressed in response to HTS were identified. BAK1, PTI6, HSP83A, and HSP90-1. BAK1 and PTI6 exhibited significant upregulation under the ST treatment, while HSP83A and HSP90-1 showed significant downregulation under the SY treatment.
3. The results of this study will be helpful for further studies of the molecular mechanisms of *Rhododendron molle* in response to HTS. The results provide a theoretical basis for exploring these molecular mechanisms and selecting high-quality heat-tolerant varieties.

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