Comparative Transcriptome Analysis Reveals Different Mechanisms of Adaptation to Environment among Three Species of *Saussurea* DC.

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ABSTRACT

*Saussurea medusa*, *Saussurea hypsipeta* and *Saussurea obvallata* are typical alpine snowline plants growing in the Qinghai-Tibet plateau. They are characterized by a specialized morphology. *S. medusa* and *S. hypsipeta* have very dense trichomes on whole plant, whereas *S. obvallata* has transparent bracts covered in inflorescences. The different forms reflect their adaptation to cold environments. To investigate the different mechanisms of adaptation of these species to cold temperatures, transcriptome sequencing was performed in three species of *Saussurea* DC. A total of 116394, 137237 and 113879 Unigenes were identified from *S. medusa*, *S. hypsipeta* and *S. obvallata*, respectively. Of these, 55909 (48.03%), 65519 (47.74%) and 51889 (45.56%) Unigenes were matched in public databases. GO analysis identified that most of annotated Unigenes in the three species of plants were related to cellular, metabolic, and single-organism processes, and binding and catalytic activities. The differential expression of 37 genes related to environmental adaptation were discovered by pairwise comparisons. Of these, two candidate genes (*Interaptin-like and CSLB3*) related to trichome development were identified only in *S. medusa* and *S. hypsipeta*, which was consistent with their distinct morphology. Our data can provide a valuable resource for the further studies on the adaptive mechanisms of molecular and functional ecology in *Saussurea* DC.

KEYWORDS

*Saussurea* DC.; transcriptome; adaptive mechanisms

1 Introduction

*Saussurea medusa* Maxim., *Saussurea hypsipeta* Diels. and *Saussurea obvallata* (DC.) Edgew. are perennial herbaceous plants in the family Asteraceae, distributed mainly in the alpine zone of the Qinghai-Tibet Plateau at altitudes from 3800 to 5000 m [1,2]. Many of the high-altitude *Saussurea* are known for their spectacular growth forms. *S. medusa* and *S. hypsipeta*, which are called “woolly” plants, have dense layers of woolly trichomes on their stems, leaves, bracts, and inflorescences [2]. *S. obvallata* is called called “glasshouse” plant, which encloses their inflorescences in large translucent bracts [3]. These spectacular growth forms are thought to represent an evolutionary response to cold and windy environments [4,5]. However, how are the morphological differences among three the species of *Saussurea* DC. produced? What are the molecular mechanisms behind these adaptations?
Transcriptomes are the whole RNA transcripts in cells or tissues. They reflect expressed genes at different life stages, tissue types, physiological state and environmental conditions. Transcriptome-based techniques provide useful means of studying gene expression and gene structure, and revealing the molecular mechanism involved in a specific biological process [6]. Recently, this technique has been widely used in the study of molecular mechanisms of plant responses to drought [7], waterlogging [8], low temperature [9], salt [10], high light [11], and irradiation stresses [12]. By comparative transcriptome analysis, new genes are found and molecular mechanisms are revealed in these plants.

Most studies of Saussurea plants growing in the Qinghai-Tibet plateau have focused on their adaptive shapes and anatomies as well as physiological functions [1–3]. Little is known about molecular mechanisms behind these plant adaptations [13]. The present work was performed to examine the comparative transcriptome analysis by transcriptome sequencing and gene function annotation on three species of Saussurea plants growing in the Daban Mountain in the northeastern Qinghai-Tibet Plateau. The studies of molecular mechanisms in alpine plant species will contribute to further understand the adaptation of plants to alpine environments since their responses are associated with cold climates; it is probably not useful to know them in the context of a warming climate.

2 Materials and Methods

2.1 Materials Collection

*S. medusa*, *S. hypsipeta* and *S. obvallata* were collected at flowering stag from Qilian Mountains (37°5′-59°N, 100°55′-102°41′E, 4,200 m a.s.l.) in the northeastern Qinghai-Tibet plateau, China. Growth form of these species is depicted in Fig. 1. This field site features typical plateau continental climate with an annual average (a) temperature of –2°C, (b) rainfall of 482 mm, (c) solar radiation of 6.46 × 10^9 Jm^{-2}, and (d) barometric pressure of 684.2 hPa. Three plants were collected from each species. The roots, stems, leaves, and flowers obtained from one individual were pooled into a single sample. These materials were flash-frozen in liquid nitrogen.

![Figure 1: Saussurea plants in the high-elevation scree or rock fields. (A) S. medusa; (B) S. hypsipeta; (C) S. obvallata](image)

2.2 RNA Extraction and Transcriptome Sequencing

Trizol® (Invitrogen-Thermo Fisher Scientific, Carlsbad, CA, USA) was used to extract total RNA on the three species. All RNA was treated with DNase I. The NanoPhotometer® spectrophotometer
(IMPLEN, CA, USA) was used to test RNA purity; the Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA) was used to determine RNA concentrations; the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA) was used to assess RNA integrity, and RNA Integrated Number (RIN) values ≥8.

We used NEBNext Ultra™ RNA Library Prep Kit from Illumina® (NEB, USA) to construct the sequences library and the Agilent Bioanalyzer 2100 system to evaluate library quality. We sequenced library preparation to obtain raw reads by Illumina HiSeq™ 2500; clean reads were obtained by removing reads containing adapter, reads containing poly-N and low-quality reads from raw reads. At the same time, Q20, Q30, GC-content and sequence duplication level of the clean data were calculated. All the downstream analyses were based on clean data with high quality. Transcriptome was obtained by splicing clean reads with Trinity [14]. The longest transcript in each gene was taken as the Unigene for subsequent analysis.

2.3 Unigene Functional Annotation

Unigenes of *S. medusa*, *S. hypsipeta* and *S. obvallata* were searched in the public databases (Nr, Nt, Pfam, KOG/COG, Swiss-Prot, KEGG, GO), and gene function was annotated according to gene similarity. The E-value in Nr is less than or equal to 1E-5 (Nt, ≤1E-5; Pfam, ≤0.01, KOG/COG, ≤1E-3; Swiss-Prot, ≤1E-5; KEGG, ≤1E-10; GO, ≤1E-6).

2.4 Orthology Genes Screening and Analysis

Orthology genes were searched for full-length CDS sequences by OrthoMCL, and one-to-one orthology genes were screened out [15]. Nonsynonymous substitution rate (Ka), synonymous substitution rate (Ks) and Ka/Ks of orthology genes were calculated by PALM [16]. GO gene enrichment analysis was carried out for the orthology genes (Ka/Ks>1) that were positively selected by GOseq [17]. Pathway significant enrichment analysis was carried out on the orthology genes positively selected by the hypergeometric test, with KEGG Pathway as the unit [18].

2.5 Analysis of Environmental Adaptation Related Unigenes

By using the classification results of Unigene in the GO database and the functional annotations in the seven public databases, the environmental adaptation-related Unigenes of the three species were analysed by interspecies reduction.

3 Results

3.1 Transcriptome Data Assembly of the Three Species

*S. medusa*, *S. obvallata* and *S. hypsipeta* obtained 150217, 162036 and 192813 transcripts, respectively (Table 1). The average length of the transcript of *S. medusa* was 748 bp and N50 was 1292 bp. The average length of the transcript and N50 of *S. obvallata* and *S. hypsipeta* were 760 bp, 1270 bp and 698 bp, 1156 bp, respectively. The longest transcript in each gene was taken as Unigene for subsequent analysis. *S. medusa* obtained 116394 Unigenes with an average length of 623 bp and N50 of 966 bp. *S. obvallata* showed 113879 Unigenes with an average length of 635 bp and N50 of 1000 bp. *S. hypsipeta* obtained 13723 Unigenes with an average length of 581 bp and N50 of 857 bp. The results showed that the sequencing quality was sufficient for future analysis needs.
3.2 Functional Annotation and Classification of the Three Species’ Unigene

3.2.1 Unigene Annotation in the Public Database

*S. medusa*, *S. hypsipeta* and *S. obvallata* had 7620, 7245 and 9366 Unigenes, respectively, that can be matched in all databases (Table 2).

### Table 1: Transcript and Unigene assemblies of *S. medusa*, *S. hypsipeta* and *S. obvallata*

| Sequencing indicators         | *S. medusa*     | *S. hypsipeta*   | *S. obvallata*  |
|-------------------------------|-----------------|------------------|-----------------|
| Valid sequences number        | 127 827 892     | 130 802 486      | 134 018 004     |
| Base number (bp)              | 19.18 G         | 19.62 G          | 20.11 G         |
| Q30 (%)                       | 92.07           | 92.34            | 92.45           |
| GC (%)                        | 43.83           | 44.08            | 43.95           |
| Length range of transcript (bp) | 201~13 922     | 201~14 016       | 201~13 915      |
| Average length of transcript (bp) | 748           | 698              | 760             |
| N50 of transcript (bp)        | 1 292           | 1 156            | 1 270           |
| Transcript number             | 150 217         | 192 813          | 162 036         |
| Length range of Unigene (bp)  | 201~13 922      | 201~14 016       | 201~13 915      |
| Average length of Unigene (bp) | 623            | 581              | 635             |
| N50 of Unigene (bp)           | 966             | 857              | 1 000           |
| Unigene number                | 116 394         | 137 237          | 113 879         |

### Table 2: Unigene function annotation of *S. medusa*, *S. hypsipeta* and *S. obvallata*

| Database annotation          | Annotation quantity and percentage (%) of *S. medusa* | Annotation quantity and percentage (%) of *S. hypsipeta* | Annotation quantity and percentage (%) of *S. obvallata* |
|------------------------------|-----------------------------------------------------|--------------------------------------------------------|--------------------------------------------------------|
| NR database annotation       | 44606 (38.32)                                       | 43860 (38.51)                                           | 53750 (39.16)                                           |
| NT database annotation       | 26144 (22.46)                                       | 26818 (23.54)                                           | 30959 (22.55)                                           |
| KO database annotation       | 18548 (15.93)                                       | 16832 (14.78)                                           | 22152 (16.14)                                           |
| Swiss-Prot database annotation | 37253 (32)                                        | 33242 (29.19)                                           | 43245 (31.51)                                           |
| Pfam database annotation     | 34996 (30.06)                                       | 31559 (27.71)                                           | 40440 (29.46)                                           |
| GO database annotation       | 35840 (30.79)                                       | 32236 (28.3)                                            | 41307 (30.09)                                           |
| KOG database annotation      | 21053 (18.08)                                       | 17117 (15.03)                                           | 24003 (17.49)                                           |
| All databases annotation     | 7620 (6.54)                                         | 7245 (6.36)                                             | 9366 (6.82)                                             |
| At least one databases annotation | 55909 (48.03)                                    | 51889 (45.56)                                           | 65519 (47.74)                                           |

3.2.2 GO Functional Classification of Unigene

The Unigenes of *S. medusa*, *S. obvallata* and *S. hypsipeta* were divided into 55, 56 and 56 types of function respectively, and the 55 types of function were the same (Fig. 2). The Unigene of *S. obvallata* and *S. hypsipeta* found a gene related to cell aggregation. Cell agglutination is associated to the resistance of organisms to environmental UV stress [19].
3.2.3 KOG Functional Classification of Unigene

The KOG annotation results of Unigene of *S. medusa*, *S. hypsipeta* and *S. obvallata* were similar. There were 21053 Unigenes of *S. medusa*, 17117 Unigenes of *S. hypsipeta* and 24003 Unigenes of *S. obvallata* annotated on 25 KOG classifications. Firstly, the general function prediction only accounted for most of the Unigenes, which were 16.41%, 18.05% and 16.46% in *S. medusa*, *S. hypsipeta* and *S. obvallata*, respectively. Next, there were posttranslational modification, proteins turnover and chaperones. The proportion of *S. medusa*, *S. hypsipeta* and *S. obvallata* in this category was 14.32%, 14.41% and 11.64%, respectively. Signal transduction mechanisms were 8.74%, 8.54% and 7.65%, in *S. medusa*, *S. hypsipeta* and *S. obvallata*, respectively (Fig. 3). They accounted for the least Unigene in the classification of Cell motility, Extracellular structures and Nuclear structures.

3.2.4 KEGG Functional Classification of Unigene

The Unigene annotated by the three species belonged to Cellular Processes, Environmental Information Processing, Genetic Information Processing, Metabolism and Organismal Systems, including 19 secondary pathways (Fig. 4). Thereinto, 7 types of pathway Unigene accounted for a large proportion (>50%), and they included Cellular Processes, Folding and sorting and degradation, Translation, Amino acid metabolism, Carbohydrate metabolism, Energy metabolism, and Overview, respectively. The KEGG classification results indicated that the Unigene functions were mainly related to protein translation and material metabolism.
3.3 Orthology Genes Screening of the Three Species

10811 sets of orthologous genes were obtained through OrthoMCL in the three species. There were 463 sets of orthologous genes which Ka/Ks>1, indicating that these genes were rapidly divergent (Fig. 5).

The GO functional enrichment classification was carried out for the orthologous genes of positive selection in the three species. We did not find functional enrichment genes, but 78 genes were related to plant resistance. The resistance genes included peroxidase, ubiquitin, zinc-finger protein and...
acyl-CoA-binding protein, which play an important role in plant environmental stress. The KEGG gene enrichment analysis revealed that Ribosome had the largest number of genes enriched in this pathway, with a total of 14 genes, revealing that the genes related to Ribosome synthesis in these three species are subject to strong positive selection (Fig. 6).

3.4 Analysis of Environmental Adaptation Related Unigenes

Based on the classification of the three species Unigenes in GO database, and analysis of Unigene about environmental adaptation, the results showed that the genes related to environmental adaptation in the three species were mainly molecular chaperone, ubiquitin, calmodulin, enzyme and ribosomes (Table 3). HSC82, CLPB and HSP17.9A can enhance the effect of plants on high temperature and salt stresses [20–22]. Dna J2 and osigba0134h18.3 were annotated by GO, which are involved in the adaptation of plants to high
temperature stress. \textit{PER50} is one of the enzymes with which plants respond to oxidative stress \cite{23}, while \textit{S2P} corresponds to salt stress \cite{24}. \textit{ATPK2} is involved in plant adaptation to cold and high salt stresses \cite{25}, while \textit{CRK29} and \textit{CIPK7} are in connection with plant immune responses and adaptation to cold stress \cite{26,27}. \textit{FEN1} is one of the key enzymes in DNA replication and repair in eukaryotes \cite{28}. \textit{RIN1} annotated by GO is involved in plant defensive responses to fungi. \textit{Malate oxidoreductase} can regulate plant growth and respiration \cite{29}. Gene expression is regulated by \textit{SAHH1} through gene methylation modification \cite{30}. \textit{GABA-TP1} participates in plant adaptation to temperature stress \cite{31}. \textit{SAMT} is related to plants’ defense responses to biologic stimulation \cite{32,33}. \textit{Calm3} mediates the regulation of enzymes and ion channels through calcium ions \cite{34}. \textit{IAA13} regulates growth and development of plants through ARFs \cite{35}. \textit{H0901F07.20} annotated by GO involves in the binding of lipoyl coenzyme A. \textit{ATG8} is related to the formation of autophagosomes in plants \cite{36}. \textit{NIP3} is correlated with the transport of the heavy metal arsenic in plants \cite{37}. \textit{OJ1754_E06.16} is associated with the processes of protein secretion and signal transmission. \textit{FAF} takes part in plant ABA activation signaling pathway and phosphorylation regulation. \textit{MutS} participates in DNA mismatch repair. \textit{GF14A} is connected with plant adaptation to drought stress \cite{38}. \textit{NIMIN-2} is related to the acquisition of systemic resistance in plants \cite{39}, while \textit{ATL4M} is related to abiotic stress responses \cite{40}. \textit{Interaptin-like} links the intracellular system to the cytoskeleton, which regulates the morphogenesis of the multicellular trichomes \cite{41,42}. \textit{CSLB3} is related to the formation of non-cellulosic polysaccharide skeletons in plant cell walls. \textit{GBF} is one of the transcription factors in which cells respond to signals \cite{43}. \textit{AFG2} is associated with the maturation of 60S ribosomal subunit \cite{44}. \textit{PGPS/NH15} annotated by GO participates in the regulation of oxidation-reduction enzyme activities.

\begin{table}[h]
\centering
\caption{Differential expression genes for environmental adaptation of \textit{S. medusa}, \textit{S. hypsipeta} and \textit{S. obvallata}}
\begin{tabular}{l|l|c|c|c}
\hline
Name and function of gene & Unigene annotation and expression & \multicolumn{3}{c}{Unigene annotation and expression} \\
& & \textit{S. hypsipeta} & \textit{S. medusa} & \textit{S. obvallata} \\
\hline
\textit{CLPB} & Molecular chaperone & Yes & Yes & No \\
\textit{OSIGBa0134H18.3} & Molecular chaperone & Yes & Yes & No \\
\textit{PER50} & Peroxidase & Yes & Yes & No \\
\textit{S2P} & Metalloproteinase & Yes & Yes & No \\
\textit{CIPK7} & CBL-interacting protein kinase 07 & Yes & Yes & No \\
\textit{FEN1} & Flap endonuclease 1 & Yes & Yes & No \\
\textit{RIN1} & RuvB-like helicase 1 & Yes & Yes & No \\
\textit{OJ1754_E06.16} & Ethylene-responsive small GTP-binding protein & Yes & Yes & No \\
\textit{ATL4M} & RING-H2 finger protein ATL4M & Yes & Yes & No \\
\textit{Interaptin-like} & Interaptin-like & Yes & Yes & No \\
\textit{CSLB3} & Cellulose synthase-like protein B3 & Yes & Yes & No \\
\textit{PGPS/NH15} & PGPS/NH15 & Yes & Yes & No \\
\textit{Dna J2} & Molecular chaperone & No & No & Yes \\
\textit{IAA13} & Auxin & No & No & Yes \\
\textit{RPL26B} & Ribosomal 26 subunit & No & No & Yes \\
\end{tabular}
\end{table}

(Continued)
4 Discussion

Our sequencing results showed that Unigene N50 of the three species were 857~1000 bp, the base quality value Q30 was over 92%, and the GC content was over 43%. The amount of sequencing data and the number of Unigene spliced were relatively large, which showed that the sequencing quality was good, and the sequence information generated was sufficient and effective [45]. The results of Go, KOG and KEGG of three species Unigenes were very similar, indicating that their genetic relationship was very similar.

The temperature in the collecting area was between 0–30°C, UV radiation up to 7000 μW·cm⁻², and the oxygen partial pressure was 13.25 kPa for the three species. Stresses of low oxygen, strong radiation and large temperature differences made that makes the plants growing in this area formed special stress resistance mechanisms in the long-term to achieve collaborative resilience, such as trichomes, ABA signaling pathway, molecular chaperones and ubiquitin pathway. In our study, there was a gene expression related to cell aggregation in both S. hypsipeta and S. obvallata. Some research shows that

| Name and function of gene | Unigene annotation and expression |
|---------------------------|----------------------------------|
|                           | S. hypsipeta | S. medusa | S. obvallata |
| H0901F07.20 | Aceyl coenzyme | No | No | Yes |
| NIP3       | Aquaporins | No | No | Yes |
| GBF        | G-box binding factor bZIP transcription factor | No | No | Yes |
| HSC82      | Molecular chaperone | No | Yes | No |
| HSP17.9A   | Molecular chaperone | No | Yes | No |
| UBC1       | Ubiquitin ligase | No | Yes | No |
| KCTD9      | Ubiquitin | No | Yes | No |
| SAMT       | Salicylate O-methyltransferase | No | Yes | No |
| ATPK2      | Protein kinase | Yes | No | Yes |
| CRK29      | Protein kinase | Yes | No | Yes |
| Malate oxidoreductase oxidoreductase | Yes | No | Yes |
| GABA-TP1   | Gamma-aminobutyrate transaminase 1 | Yes | No | Yes |
| SAMT       | Salicylic acid carboxy methyl transferase | Yes | No | Yes |
| ATG8       | Autophagy | Yes | No | Yes |
| NMIN-2     | Protein NIM1-INTERACTING 2 | Yes | No | Yes |
| UBE2J2     | Ubiquitin ligase | Yes | No | No |
| SAHH1      | Hyperhomocysteinase | Yes | No | No |
| Calm3      | Calmodulin | Yes | No | No |
| AFG2       | ATPase family gene 2 protein | Yes | No | No |
| FAF        | Protein FAF | No | Yes | Yes |
| MutS       | DNA mismatch repair mutS | No | Yes | Yes |
| GF14A      | 14-3-3-like protein GF14-A | No | Yes | Yes |
Gloeocapsa sp. cells with low metabolic activity aggregate to form a lamellar biofilm to protect organisms against ultraviolet radiation [19]. Then, we compared leaf structure among the three species; *S. medusa* had the longest, thickest and white trichomes on the leaf epidermis. This structure can effectively resist and reflect ultraviolet radiation. *S. hypsipeta* and *S. obvallata* may resist the ultraviolet radiation stress through cell aggregation, but the specific mechanism needs to be verified in future experiments. Interaptin protein was the binding protein between the components of the inner membrane and the actin of the cytoskeleton, Microtubules and actin promote the development of trichomes [41,42]. CSLB3 participates in the formation of the noncellulose polysaccharide skeleton in plant cell wall. In this study, we found that the *Interaptin-like* and *CSLB3* genes were both expressed in *S. hypsipeta* and *S. medusa*. It is speculated that the expression of these two genes can promote the occurrence of trichomes and improve the resistance of these two plant species to radiation and temperature stresses. This finding was also consistent with the structural characteristics of the three species. *S. medusa* and *S. hypsipeta* are densely covered with trichomes. The ground tissues of *S. obvallata* showed no trichomes, and the inflorescences were enclosed in translucent bracts. At the same time, we found that the three plant species expressed different molecular chaperones, ubiquitin, protease and other genes to allow the adaptation of plants to the environmental stresses, and their mechanisms need further study.

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