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Transcriptome profiling of blue leaf coloration in *Selaginella uncinata*

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Abstract

To explore candidate genes responsible for the blue color of *Selaginella uncinata* leaves, we used transcriptome sequencing to compare differential gene expression in blue and red *S. uncinata* leaves selected based on morphological observations and pigment content. In total, we obtained 30,119 UniGenes with an average length of 1,133 bp, and we identified 1,442 differentially expressed genes (DEGs). Among the 1,442 DEGs, 886 were upregulated and 556 were downregulated in blue leaf samples. After verification of expression by qRT-PCR analysis, seven key enzymes and their encoding genes were identified as being involved in the chlorophyll and flavonoid metabolism pathways. These genes included *CHLH*, *LPOR*, *CLH*, *HCT*, *CHS*, *MYB*, and *F3'H*, which may be involved in the blue leaf coloration of *S. uncinata*. Results from this study implied that the primary pathway of pigment metabolism in *S. uncinata* may be the chlorophyll metabolism pathway rather than the anthocyanin biosynthesis pathway. It is possible that chlorophyll b (Chl b) may be transformed into chlorophyll a (Chl a) by an alternative pathway in which it is converted to Chlide b by *CLH*, and then transformed to Chl a without the involvement of *CBR* or *HCAR*.

Key words Transcriptome sequencing · Blue leaf · Coloration · *Selaginella uncinata*

Abbreviations

ptDNA	chloroplast DNA
mtDNA	mitochondrial DNA
qRT-PCR	quantitative real-time polymerase chain reaction

BLAST	Basic Local Alignment Search Tool
COG	Clusters of Orthologous Groups
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
RPKM	reads per kilobase of transcript per million mapped reads
DEG	differentially expressed gene
FDR	false discovery rate
MGDG	monogalactosyldiacylglycerol
DGDG	digalactosyldiacylglycerol
CHLH	Mg-chelatase
Proto	protoporphyrin IX
Pchlde	protochlorophyllide
LPOR	light-dependent NADPH-Pchlde oxidoreductase
DPOR	light-independent (dark) Pchlde oxidoreductase
CLH	chlorophyllase (Chlase)
CS	Chl synthase
CAO	chlorophyllide a oxygenase
HMChl a	7-hydroxymethyl chlorophyll a
NOL	Non-Yellow Coloring 1-Like
CBR	Chlorophyll(ide) b reductase

HCAR	7-hydroxymethyl chlorophyll a reductase
HCT	hydroxycinnamoyltransferase
5AT	anthocyanin 3,5- <i>O</i> -diglucoside 6'''- <i>O</i> -hydroxycinnamoyltransferase
BAHD	(benzylalcohol <i>O</i> -acetyltransferase, BEAT; anthocyanin <i>O</i> -hydroxycinnamoyltransferase, AHCT; anthranilate- <i>N</i> -hydroxycinnamoyl/benzoyltransferase, HCBT; deacetylvindoline 4- <i>O</i> -acetyltransferase, DAT)
CHS	chalcone synthase
F3'H	flavonoid 3'-hydroxylase
DHK	dihydrokaempferol
DHQ	dihydroquercetin
LC-MS	liquid chromatography-mass spectrometry

Introduction

Plants with colored leaves are typically of interest because of their colorful leaves and seasonal changes in ornamental plants. Plants with blue leaves are particularly rare. *Selaginella uncinata* is a perennial herb that belongs to the Lycophytes family and is found only in China. Its leaf epidermis appears blue, but the underside of the leaf color is green. Previously, we reported that leaf color changes from blue in the shade to red under full light exposure (Li et al. 2015). The blue and red colors of *S. uncinata* leaves contribute to creating a distinct plant landscape.

Previous leaf color studies have focused on the three leaf pigments (chlorophyll, carotenoid, and anthocyanin), the effects of leaf anatomical structure and environmental factors on leaf color, and the use of transcriptome sequencing to study molecular mechanisms of leaf coloration. For example, purple and non-purple Chinese cabbages were tested to identify candidate genes responsible for the biosynthesis of anthocyanin (Zhang 2014). Yang (2015) used the transcriptome feature analysis of green and albino leaves to identify the specific expression patterns of early light-inducing protein (*ELIP*) genes during leaf development of *Pseudosasa japonica* f. *akebonosuji*. Abnormal assembly of thylakoid membranes and the subsequent blocking of chlorophyll synthesis are caused by changes in expression of *ELIP* genes. The final effect of depletion of chlorophyll from plastids results in reduced performance of the light harvesting system and in leaf color variation. However, these studies focused mainly on red or purple colors connected with anthocyanin biosynthesis, yellow color related to carotenoid biosynthesis, and brindle color associated with chlorophyll biosynthesis. There have been only a few studies on blue leaves. Related to studies focusing on *S. uncinata*, the nuclear genome of *S. moellendorffii* was sequenced completely in 2007 (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Smoellendorffii) [6 Oct. 2016]. It was chosen for sequencing because its nuclear genome, consisting of approximately 110 mega bases (Mb), is particularly small compared to the nuclear DNA complement of other land plants. Lycophytes represent an important evolutionary link between vascular plants and the nonvascular mosses, liverworts, and hornworts (Banks 2009). Genome sequencing of *S. uncinata* has not been performed, but we do know that its somatic chromosome number is $2n = 18$ (Takamiya 1993). The complete chloroplast DNA (ptDNA) sequence from *S. uncinata* has been reported. The sequence data are notable due of the

presence of a unique 20-kb inversion and the remarkable loss of almost one half of the tRNA-coding genes found in all other ptDNAs (Tsuji et al. 2007). David Roy Smith (2009) studied the plastid genome of *S. uncinata* and found as of February 2009, that all completely sequenced plastid genomes had a GC content below 43%, except *S. uncinata*, which had a GC content of 55%. He also identified four characteristics of *Selaginella* ptDNA. Oldenkott et al. (2014) investigated the chloroplast transcriptome of *S. uncinata*. Their exhaustive cDNA studies identified 3,415 RNA-editing events, exclusive of the C-to-U types, among the 74 mRNAs encoding intact reading frames in the *S. uncinata* chloroplast. Chinese scholars, such as Zheng (2008, 2011, 2013, 2014) and Zou (2013), focused primarily on the wide use of *S. uncinata* in Chinese herbal medicine for its anti-tumor and antiviral effects. In contrast, studies of *S. uncinata* in other countries have concentrated mainly on developmental anatomy, system development, and the genome.

There have been very few reports on the blue leaves of *S. uncinata*. Glover and Whitney (2010) summarized that plants achieve color in two main ways, chemical- or pigment-based color and structural color, and they pointed out that iridescence is a unique attribute of structural color. It has been hypothesized that the iridescence of *Selaginella* species might aid the capture of photosynthetically active wavelengths in low light conditions because the leaf iridescence may act as a natural anti-reflective coating. Héban and Lee (1984) pointed out that the iridescent blue color of *S. willdenowii* and *S. uncinata* is caused by a physical effect, thin-film interference. There are also other blue iridescence plants, such as *Diplazium tomentosum*, *Lindsaea lucida*, and *Danaea nodosa*, in which the iridescent ultrastructural basis is a helicoidal arrangement of multiple layers of cellulose microfibrils in the

uppermost cell walls of the adaxial epidermis (Graham and Norstog 1993; Gould and Lee 1996). In *Begonia pavonina*, *Phyllagathis rotundifolia*, and *Elaeocarpus angustifolius*, the blue coloration is caused by parallel lamellae in specialized plastids (the iridoplast) adjacent to the abaxial wall of the adaxial epidermis (Lee 1991; Gould and Lee 1996). Additionally, the blue-green coloration of *Trichomanes elegans* is caused by the remarkably uniform thickness and arrangement of grana in specialized chloroplasts adjacent to the adaxial wall of the adaxial epidermis (Graham and Norstog 1993). In the above studies, it was declared that the extraction of blue pigment from the research objects could not be achieved, so in all cases it was considered that the blue iridescence was a structural color. However, we now know that, without the so-called blue pigment delphinidin, leaves may also appear blue because of other factors such as copigmentation (Kondo et al. 2005; Toyama-Kato et al. 2007) and metal ions (Leonard and Sam 1966; Momonoi et al. 2009). There have been no reports about blue leaf coloration at the molecular level.

In our study, we attempted to identify candidate genes responsible for the blue leaf coloration of *S. uncinata* through transcriptome sequencing and quantitative real-time polymerase chain reaction (qRT-PCR) verification of gene expression. These results will be useful for subsequent gene function verification and further studies focusing on blue leaf gene regulation mechanisms, and lay the foundation for future artificial regulation of leaf color and goal-directed cultivation of blue leaf plants.

Materials and Methods

Plant material

Potted *S. uncinata* plants, which were 6-months-old, were purchased from a flower market in Guangxi, China and then shaded using a sun-shade net. Five light intensities were established: full exposure R_0 (light transmittance 100%NS), one layer of shade R_1 (38%NS), two layers of shade R_2 (20%NS), three layers of shade R_3 (16%NS), and four layers of shade R_4 (10%NS). After 3 months, the two treatments which produced the most distinctly different colors (red under the R_0 treatment and blue under the R_3 treatment) were selected based on morphologic observations and pigment contents for subsequent transcriptome sequencing and qRT-PCR expression analysis.

Morphologic observation and measurement of leaf pigment contents

After shading for 3 months, leaf color changes of *S. uncinata* were observed. Mature, normal *S. uncinata* leaves were selected randomly to measure chlorophyll, carotenoid, and anthocyanin contents every 15 d. Chlorophyll and carotenoid, were extracted using the ethanol-acetone mixture (1:2, v/v) soaking method (Yang 1996) and concentrations were calculated using the following equation:

$$\text{Pigment concentration (mg/L): } C_a = 12.21OD_{663} - 2.81OD_{646}$$

$$C_b = 20.13OD_{646} - 5.03OD_{663}$$

$$C_{ar} = (1000OD_{470} - 3.27C_a - 104C_b)/229$$

$$\text{Pigment content (mg/g)} = C \cdot V \cdot \text{dilution ratio} / \text{fresh weight of sample}$$

where, C_a , C_b , and C_{ar} are the contents of chlorophyll a, chlorophyll b, and carotenoid, respectively; and OD_{663} , OD_{646} , and OD_{470} are the means of the optical densities of extraction solutions at 663, 646, and 470 nm, respectively.

Using the anthocyanin extraction method of Cui et al. (2008), we considered the anthocyanin concentration (per gram fresh weight in 1 mL of extraction solution, $OD_{535} = 0.1$) as one pigment unit, and the relative content of anthocyanin (pigment unit) = $OD_{535} / 0.1$.

Total RNA extraction and transcriptome sequencing

The two most different leaf colors (blue and red) were selected as samples, and three replications were established. Total RNA was extracted using the RNAiso Plus Total RNA extraction reagent (Cat#9108/9109, source), following the manufacturer's instructions. The RNA integrity number (RIN) indicating RNA quality was determined using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, US). Total RNA was further purified using an RNeasy Micro Kit (Cat#74004, QIAGEN, Germany) and an RNase-Free DNase Set (Cat#79254, QIAGEN, Germany). Six samples were sequenced using an Illumina HiSeq 2500 system at Shanghai Biotechnology.

Construction of cDNA library and sequence assembly

We processed the purified total RNA as follows: separation of mRNA or removal of tRNA, fragmentation, synthesis of the first cDNA, synthesis of the second cDNA, end-repair, addition of poly(A)

to the 3' end, adaptor connection, enrichment, and then completion of the construction of the sequencing sample library.

Sequence assembly procedures were as follows: raw reads→ clean reads→ contigs→ primary UniGene→ final UniGene. The final assembled UniGenes were used in data analysis.

Data analysis

Data analysis included functional annotation (Basic Local Alignment Search Tool [BLAST] of the UniProt database), functional classification (BLAST of the Clusters of Orthologous Groups [COG], Gene Ontology [GO], and Kyoto Encyclopedia of Genes and Genomes [KEGG] databases), differential expression analysis in terms of reads per kilobase transcript per million mapped reads (RPKM) values, GO enrichment, KEGG enrichment and cluster analysis.

Verification of expression by qRT-PCR

For further verification of the expression profiles of the genes identified in our analyses, seven differentially expressed genes (DEGs) (total of 10 contigs), all of which were known to be involved in chlorophyll or flavonoid metabolism, were selected for qRT-PCR analysis. The qRT-PCR was conducted using a Rotor-Gene 3000 real-time PCR detection system (QIAGEN) with SYBR qPCR Mix (Toyobo, Tokyo, Japan). We designed primers (listed in Table 1) using Beacon Designer software. The PCR conditions were as follows: 95°C for 5 min, 94°C for 1 min, 58°C for 50 s, 72°C for 90 s, 40 cycles, and 72°C for a final extension of 10 min. Relative mRNA levels were calculated using the $2^{-\Delta\Delta C_q}$ approach

(Gilmour 1998) relative to expression of the internal reference gene *ACTIN*. Common reference genes were searched for in the UniProt annotation list of the sequencing data, and five genes were identified. Three of these genes were *ACTIN* (Contig1769, Contig10279), *DNAJ* (Contig1949, Contig7824, Contig8368), and *MDH* (Contig4051). Their sequences were used as BLAST queries against *S. moellendorffii*, to design specific primers and compare their expression levels. Contig1769 exhibited the most consistent expression and was chosen as a reference gene.

Results

Morphologic observation and pigment contents

Selaginella uncinata plants were subjected to five light intensity treatments: full exposure R_0 (light transmittance 100%NS), one layer of shade R_1 (38%NS), two layers of shade R_2 (20%NS), three layers of shade R_3 (16%NS), and four layers of shade R_4 (10%NS). After growth under these light intensities for three months, we found that the leaves exhibited the strongest red color under treatment R_0 and the strongest blue color under treatment R_3 (Fig. 1 A).

After shading for 3 months, both the chlorophyll a and the chlorophyll b contents were highest under treatment R_3 (Fig. 1 B and C), and the anthocyanin content was relatively high under treatment R_3 (Fig. 1 E). The pigment measurement results were consistent with the morphologic observations (i.e., the leaf color was mostly blue in treatment R_3), so we inferred that the blue leaves of *S. uncinata* might be due to the color of chlorophyll or delphinidin in anthocyanin. Next, we subjected the leaves with the two most distinctly different colors, red under treatment R_0 (lowest chlorophyll and anthocyanin contents) and blue

under treatment R₃ (highest chlorophyll and higher anthocyanin contents), to transcriptome sequencing to identify candidate genes responsible for the blue leaf coloration of *S. uncinata*.

Sequence assembly

The sequence data output included 38,368,316 raw reads of red leaf samples and 34,330,760 raw reads of blue leaf samples. After sequence assembly, we obtained 30,119 UniGenes, with an average length of 1,133 bp (Table 2).

Data analysis results

1. Functional annotation

Among the assembled UniGenes, which were searched against the UniProt protein database using BLASTX, 22,158 (73.6%) UniGenes were annotated (E-value < 1e⁻⁵). The five species with the most UniGene hits were *S. moellendorffii*, *Physcomitrella patens* subsp. *patens*, *Hordeum vulgare* var. *distichum*, *Picea sitchensis*, and *Amborella trichopoda*, with 15,060, 873, 425, 290, and 207 UniProt annotated genes, respectively (Fig. 2).

2. Functional classification

We compared the assembled UniGenes to the COG database and found that 13,574 UniGenes mapped to 25 COG classifications. As shown in Figure 3, the T group 'signal transduction mechanisms' (5865 UniGenes, 19.47%) was the largest cluster, followed by the O group 'posttranslational modification, protein turnover, chaperones' (4132 UniGenes, 13.72%), and the R group 'general function prediction only' (4061 UniGenes, 13.48%). The N group 'cell motility' (17 UniGenes, 0.06%) was the smallest cluster.

Based on GO analysis, 28,078 UniGenes were assigned to three groups: biological processes (11,025 UniGenes, 39.27%), cellular components (5,872 UniGenes, 20.91%), and molecular functions (11,181 UniGenes, 39.82%). Among the biological processes, metabolic (28.84%), cellular (21.57%), and single-organism (16.75%) processes were dominant. Large numbers of UniGenes were found in the cellular components, such as cell parts (21.83%), cells (21.83%), and organelles (16.16%). Among the molecular functions, catalytic (44.58%) and binding (41.77%) activities were dominant (Fig. 4).

Through an analysis of UniGenes in the KEGG database, we assigned 22,844 (75.85% of the total) UniGenes to 305 KEGG pathways (Table 3) (Liu et al. 2014). As shown in Table 2, the three main categories of KEGG pathways were metabolic (2216 UniGenes, 9.70%), biosynthesis of secondary metabolites (1156 UniGenes, 5.06%), and ribosomes (715 UniGenes, 3.13%). Among the 305 pathways, our study focused on porphyrin and chlorophyll metabolism (49 UniGenes) and flavonoid biosynthesis (86 UniGenes) pathways, which might be related to blue leaf coloration in *S. uncinata*. These annotations could supply a new and valuable resource for investigating the functions, pathways, and processes of the coloration of *S. uncinata*.

3. Differential expression

The differences in gene expression between blue and red leaves were determined based on RPKM reads and analyzed by filtering with a false discovery rate (FDR) ≤ 0.05 and a fold change ≥ 2 . In total, we identified 1442 DEGs, of which 886 were upregulated and 556 were downregulated in blue leaf samples (Fig. 5).

4. GO enrichment and KEGG enrichment

On the basis of GO enrichment analysis relative to the red leaf samples, we found ‘iron ion binding’ (55 genes; 23 upregulated, 32 downregulated) and ‘heme binding’ (50 genes; 22 upregulated, 28 downregulated) genes, which were associated with the pathway branch from protoporphyrin IX to magnesium protoporphyrin IX that had a large proportion of downregulated genes. The vast majority of genes were involved in the ‘chloroplast thylakoid’ (17 genes; 16 upregulated, 1 downregulated), ‘lipid metabolic process’ (24 genes; 22 upregulated, 2 downregulated), ‘protochlorophyllide reductase activity’ (1 gene; upregulated), ‘chlorophyllase activity’ (1 gene; upregulated), and ‘magnesium chelatase activity’ (1 gene; upregulated), all of which were potentially involved in chlorophyll metabolism and were mostly upregulated. Genes that were expressed at higher levels in blue leaf samples included Contig577, Contig888, and Contig9403. It is likely that they play an important role in *S. uncinata* coloration.

Based on KEGG enrichment analysis relative to the red leaf samples, we found that porphyrin and chlorophyll metabolism (3 genes; upregulated) and flavonoid biosynthesis (15 genes; 1 upregulated, 14 downregulated) might be related to *S. uncinata* coloration. In which expressed higher in blue leaf samples, except that contig577, contig888, contig9403, and contig5838 were also found.

5. cluster analysis

Based on KEGG classifications, 49 UniGenes mapped to ‘porphyrin and chlorophyll metabolism,’ and 8 UniGenes might be associated with this pathway based on the protein descriptions of the UniProt annotations. In total, we chose 57 UniGenes in this classification for cluster analysis. As for flavonoid metabolism, 86 UniGenes mapped to this pathway based on the KEGG classifications. Of these, we chose

31 UniGenes according to their fold changes in expression. These were combined with another 14 UniGenes that might be related to the pathway based on the protein descriptions of the UniProt annotations. We chose 45 UniGenes in this pathway for cluster analysis. The expression patterns of the genes involved in chlorophyll metabolism differed markedly from those involved in flavonoid metabolism (total of 112 UniGenes) (Fig. 6). The vast majority of the genes involved in chlorophyll metabolism were expressed at higher levels in blue leaves, whereas the expression levels of genes involved in flavonoid metabolism varied.

Verification of gene expression by qRT-PCR

To verify further the expression of genes in our *de novo* transcriptome database, three DEGs involved in chlorophyll metabolism, including *CHLH* (Contig888), *LPOR* (Contig577), *CLH* (Contig6952 [*CLH1*], and Contig9403 [*CLH2*]), four DEGs involved in flavonoid metabolism, including *HCT* (Contig5838), *CHS* (Contig19924 [*CHS1*] and Contig20287 [*CHS2*]), *MYB* (Contig1641), and *F3'H* (Contig12143 [*F3'H1*] and Contig4811 [*F3'H2*]), a total of 10 contigs, were selected for qRT-PCR analysis, according to GO enrichment, KEGG enrichment, and fold changes in the expression of important enzyme-encoding genes that participate in chlorophyll and flavonoid metabolism. With the exception of the *F3'H* gene, the expression of the genes was higher in blue leaves, in accordance with the RNA-Seq results (Fig. 7).

Discussion

Leaf color change in ornamental plants results from the composition, content, and ratio of various leaf pigments (Chen et al. 2011). There are three main types of leaf pigments in higher plants: (1) chlorophylls, including chlorophylls a and b (the former appears blue-green and the latter is

yellow-green); (2) carotenoids, including carotene and lutein, which are orange and yellow, respectively; and (3) flavonoids, including flavonol, flavone, flavanone, flavan-3-ol, isoflavone, and anthocyanidin, for which the color ranges from red to blue (Liu 2014). Leaf color is also affected by environmental factors such as light, temperature, soil, and season (Shi 2006). In our experiment, *S. uncinata* was shaded under various light intensities. Leaf color eventually changed from blue to red in response to the different amounts of light exposure.

The biosynthesis of anthocyanin and chlorophyll is affected by light. We initially speculated that the blue leaf color of *S. uncinata* might be related to the chlorophyll metabolism pathway (i.e., the color of chlorophyll a), the flavonoid metabolism pathway (i.e., the color of delphinidin in anthocyanin, which might also be affected by co-pigmentation or metal ions), or a combination of these two pathways.

Differential gene expression analyses

The photosynthetic chlorophyll-protein complexes are embedded in a lipid matrix of thylakoid membranes composed mainly of the galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG). These lipids are required for formation of the lipid bilayer and also for the structure and function of the photosynthetic complexes. Transcriptional regulation of thylakoid galactolipid biosynthesis is coordinated with chlorophyll biosynthesis during the development of chloroplasts in *Arabidopsis thaliana* L. (Kobayashi et al. 2012, 2014). In our study, among the 886 upregulated DEGs, which were expressed higher in blue leaves, some were annotated as lipoxygenases (BLAST analysis of *S. moellendorffii*), which are associated with chlorophyll biosynthesis. The greatest

difference in expression was 264-fold (Contig19248). Many of the 886 DEGs were annotated as glycosyltransferase, alpha-galactosidase, and acyltransferase (BLAST analysis of *S. moellendorffii*), which are likely to be involved in glucosylation or acylation of flavonoids. The largest differences in expression were 61- (Contig19150), 18- (Contig21553), and 20-fold (Contig17409). The aforementioned four contigs were selected for heat-map analysis.

Chlorophyll metabolism pathway

Mg-chelatase (CHLH) catalyzes the insertion of Mg into protoporphyrin IX (Proto) until the levels of Proto, heme, and Bchl/Chl syntheses share a common pathway (Walker and Willows 1997). At the point of metal ion insertion, there are two alternatives: Mg^{2+} insertion to make Bchl/Chl (catalyzed by CHLH) or Fe^{2+} insertion to make heme (catalyzed by ferrochelatase). Thus, the relative activities of CHLH and ferrochelatase must be regulated to meet the organism's requirements for the end products (Caroline and Robert 1997).

In our study, eight UniGenes were annotated as CHLH (EC: 6.6.1.1). Their expression levels were upregulated in blue leaf samples, and these were selected for heat-map analysis. Based on the GO enrichment analysis mentioned above, the majority contained an iron ion, and the corresponding heme-binding genes were expressed in the lower parts of the blue leaf samples. These results indicate that the flux to the heme branch was decreased, causing greater CHLH activity. The highest upregulated expression level was for Contig888, which was twofold higher in blue leaves than in red leaves, as determined by qRT-PCR analysis.

There are two distinct protochlorophyllide (Pchl_{id}) oxidoreductases: light-dependent NADPH-Pchl_{id} oxidoreductase (LPOR), which requires photo-energy for catalysis and is encoded in the nucleus, and light-independent (dark) Pchl_{id} oxidoreductase (DPOR), which requires no photo-energy and whose subunits are encoded in the chloroplast genome (Ueda et al. 2014). LPOR is a monomeric protein, whereas DPOR is a multimeric protein consisting of three subunits encoded by the *chlB*, *chlL*, and *chlN* genes (Reinbothe et al. 2010). In the UniProt annotation list of our sequencing data, we found that Contig22453, Contig23235, and Contig18997 were annotated as Pchl_{id} reductase subunits ChlB, ChlL, and ChlN, respectively. They were included in the heat-map analysis, but their expression levels were lower (the highest RPKM among them was 2.898220016). Other contigs (e.g., Contig577) were annotated as LPOR, the expression of which was upregulated nearly threefold in blue leaves. LPOR was also selected for heat-map analysis and subsequent qRT-PCR verification of expression.

Zhou et al. (2007) concluded that chlorophyllase (Chlase, CLH) quite likely plays a role in converting Chl b to Chl a. The b-type to a-type transition seems to play a role in the degradation of Chl b and also during the acclimation of plants to different light regimes. Plants could potentially use the reactions of the Chl cycle to adjust the Chl a/b ratio to particular needs, under various physiological and environmental conditions (Rüdiger 2002). Based on the finding that the conversion of Chl b to Chl a occurs *in vivo* in the presence of reduced ferredoxin and ATP (Meguro et al. 2011), it is reasonable to speculate that the reduction of Fe²⁺, which competes with Mg²⁺, results in the conversion of Chl b to Chl a and the terminal blue leaf color of *S. uncinata*.

The Chl cycle starts and ends with Chlide a, and the biosynthesis of Chls a and b proceeds via Chlide a. Completion of Chl a biosynthesis requires only esterification catalyzed by Chl synthase (CS). Chl b biosynthesis from Chlide a includes a two-step reaction catalyzed by chlorophyllide a oxygenase (CAO) and the subsequent esterification (Reinbothe et al. 2010). In our study, there was no marked differential expression of CS or CAO between the blue- and red-leaf samples, so we believe that biosynthesis of both Chls was not blocked.

In the conversion of Chl b to Chl a, the first alternative via 7¹-OH-Chl a is the main pathway. The second alternative via 7¹-OH-Chlide a plays only a marginal role (Scheumann et al. 1999). In the first alternative, Chl b is converted to 7-hydroxymethyl chlorophyll a (HMChl a) by Non-Yellow Coloring 1-Like [NOL, one of the Chlorophyll(ide) b reductases (CBR)], followed by immediate conversion to Chl a by 7-hydroxymethyl chlorophyll a reductase (HCAR) (Shimoda et al. 2012), and completed with the formation of Chlide a from Chl a by CLH. In the second alternative, Chl b is converted to Chlide b by CLH, then converted to 7¹-OH-Chlide a by CBR, and completed with the conversion to Chlide a by HCAR. The Chl cycle does not function as a complete cycle. Rather, its function is believed to be to supply either Chl a or Chl b, according to the particular physiological need, and to satisfy that need at the cost of the performance of Chls (Rüdiger 2002).

In our study, there were three UniGenes annotated as CLH (EC: 3.1.1.14). Their expression levels were all upregulated in blue leaf samples, and they were selected for heat-map analysis. Differential expression of CBR was not obvious between blue- and red-leaf samples, and no UniGenes mapped to HCAR. However, CLH expression was enhanced in the blue leaves. The highest upregulated expression

level was exhibited by Contig6952 (CLH1), which was nearly 10-fold higher in blue leaves than in red leaves, followed by Contig9403 (CLH2, nearly 8-fold). Both were chosen for qRT-PCR analysis of expression. Both CLHs play a role in the above two alternatives, but they are distinguished by their action in the first and last steps. The following questions arise: Which of the alternatives is the main pathway in *S. uncinata*? Is there another route? Is it possible that Chl b is transformed to Chlide b by CLH and then transformed to Chl a without CBR or HCAR (Fig. 8)?

As shown above, we hypothesize that there is a special chlorophyll metabolism pathway in *S. uncinata*, Chl b is transformed to Chlide b by CLH and then transformed to Chl a without CBR or HCAR. On one hand, comparing Chl a and b in the blue leaves, if a substantial amount of Chl b is transformed to Chl a, the ratio of chlorophyll a/b will be larger than 3:1, which is the ratio in normal green leaves (Chen et al. 2011); however, according to our previous measurement results (Fig 1 D), the ratio of chlorophyll a/b in the R₃ treatment varied between 1.5 and 3.2 and was lower than 3:1 most of the time. On the other hand, comparing blue leaves with red leaves, the ratio of chlorophyll a/b in blue leaves would be higher than that of red leaves, but the measurement results from 8.30 were just the reverse (Fig. 1 D). Nevertheless, these were just the initial measurement results. We will next design corresponding experiments using a visible light spectrophotometer to measure the contents of intermediates in chlorophyll biosynthesis (Yang 2015) and, by combining with data from liquid chromatography-mass spectrometry (LC-MS), analyze in detail the chlorophyll metabolism pathway in *S. uncinata*.

Flavonoid metabolism pathway

Flavonoids and anthocyanins often occur as highly glycosidated and acylated compounds. Different types of acids, including hydroxycinnamoyl units, can be attached. Several hydroxycinnamoyltransferases (HCT) involved in anthocyanin modification have been described (Petersen 2015). For example, a hydroxycinnamoyl-CoA: anthocyanin 3,5-*O*-diglucoside 6'''-*O*-hydroxycinnamoyltransferase (5AT), has been cloned and characterized in *Gentiana triflora* (Fujiwara et al. 1997, 1998a). This enzyme can transfer either 4-coumaroyl or caffeoyl moieties from CoA to cyanidin, pelargonidin, or delphinidin 3,5-diglucosides and, together with other glucosyl- and acyltransferases, establish the specific pigments of *Gentian* flowers. Enzymes involved in flavonoid and anthocyanin biosynthesis in *A. thaliana* L., including HCT, have been reviewed by Saito et al. (2013). Most BAHD (benzylalcohol O-acetyltransferase, BEAT; anthocyanin O-hydroxycinnamoyltransferase, AHCT; anthranilate-N-hydroxycinnamoyl/benzoyltransferase, HCBT; deacetylvindoline 4-O-acetyltransferase, DAT) HCTs catalyze the transfer of 4-coumaroyl or caffeoyl units from the CoA-thioester to the acceptor substrates shikimic or quinic acid (Maize 2015). In *Hydrangea*, delphinidin 3-glucoside gives a stable blue solution, coexisting with 5-O-caffeoyl and/or 5-O-p-coumaroylquinic acid and Al³⁺ (Takeda et al. 1985a, 1985b, 1990; Yoshida et al. 2002). In our study, 13 UniGenes were annotated as HCT (EC: 2.3.1.133), among which six were upregulated and seven were downregulated in blue-leaf samples. Five UniGenes were selected for heat-map analysis according to their RPKM. The highest upregulated expression level was exhibited by Contig5838, being nearly 10-fold higher in blue leaves than in red

leaves, and it was chosen for qRT-PCR analysis. The intermediate product of the reaction is caffeoylquinic acid, which might be related to the coloration of *S. uncinata*.

Chalcone synthase (CHS) catalyzes the formation of chalcones by condensing one *p*-coumaroyl-CoA and three malonyl-CoAs (Ferrer et al. 1999). Different combinations of thioesters and three malonyl-CoAs are catalyzed by CHS and eventually produce different chalcones. For example, a condensation reaction of *p*-coumaroyl-CoA produces naringenin chalcone, while condensation of cinnamoyl-CoA produces pinocembrin chalcone (Austin and Noel 2003). It was reported, based on *in vitro* determination of relative CHS activities, that each CHS has a different substrate preference (Yamazaki et al. 2001; Md-Mustafa et al. 2014). In our study, there were six UniGenes that were annotated as CHS (EC: 2.3.1.74). They use *p*-coumaroyl-CoA and caffeoyl-CoA as substrates and synthesize naringenin chalcone and pentahydroxy chalcone, respectively. Their expression levels were both upregulated (five UniGenes) and downregulated (one UniGene) in blue leaves. Among those expressed higher in blue-leaf samples, the fold change of Contig19924 (CHS1) was highest (29.51 times), followed by Contig20287 (CHS2, 7.55 times), both of which were chosen for heat-map analysis and subsequent qRT-PCR analysis.

The anthocyanin pigment pathway is regulated by a suite of transcription factors that includes MYB, bHLH, and WD-repeat proteins (Gonzalez et al. 2008). The R2R3 MYB family plays a major role in regulating sets of genes responsible for secondary metabolite biosynthetic pathways in plants, especially for synthesizing flavonoids in the phenylpropanoid pathway (Endt et al. 2002). In maize (*Zea*

mays L.), the C1 MYB transcription factor regulates PAL, CHS, F3H, DFR, ANS, and UDP

glucose-flavonol glucosyltransferase in the flavonoid pathway (Vom et al. 2002).

In our study, eight UniGenes were annotated as transcription factors involved in flavonoid synthesis based on UniProt annotation, including seven MYB families and one WD40 family. Among the seven MYB families, three UniGenes were upregulated and four were downregulated in blue-leaf samples. All eight UniGenes were included in heat-map analysis. The most highly upregulated expression level was for Contig1641, which was 2.29-fold higher in blue leaves than in red leaves, and it was chosen for qRT-PCR analysis.

Flavonoid 3'-hydroxylase (F3'H, EC: 1.14.13.21) is a member of the cytochrome P₄₅₀ superfamily, which catalyzes monooxygenase reactions dependent on NADPH and O₂ (Zhou et al. 2012). A crucial component of the plant's protection mechanism against UVB damage might be the biosynthetic conversion of the B-ring mono-hydroxylated flavonoids to their ortho-dihydroxylated equivalents. This conversion is catalyzed by the cytochrome P₄₅₀ enzyme F3'H (Graham 1998). In our study, 32 UniGenes were annotated as F3'H, among which 19 were selected for heat-map analysis according to their RPKM. The majority of the 32 UniGenes (27 UniGenes) were upregulated in red-leaf samples. These results implied that F3'H likely plays a role in protection against intense light stress in red leaves. The highest upregulated expression level was for Contig12143 (F3'H1), which was 46.36-fold higher in red leaves than in blue leaves, followed by Contig4811 (F3'H2, 26.01-fold). Both were chosen for qRT-PCR analysis.

In anthocyanin biosynthesis, F3'H hydroxylates the 3' position of the B-ring of naringenin and dihydrokaempferol (DHK) to generate eriodictyol and dihydroquercetin (DHQ), respectively. These are important intermediates for the biosynthesis of anthocyanins and proanthocyanidins with a 3',4'-dihydroxylation pattern, representing the majority of the pigments of flowers and seed coats (Winkel-Shirley 2001).

In our study, the enzymes downstream of F3'H in anthocyanin biosynthesis, such as F3H, DFR, and ANS, were undetectable (Fig. 9). Similarly, many enzymes participating in anthocyanin biosynthesis in *S. moellendorffii* were also undetected in the KEGG pathway database, so we inferred that anthocyanin biosynthesis might not be the primary pathway of pigment metabolism in *S. uncinata*.

In the above-mentioned analysis, we selected only the UniGenes that are expressed at higher levels in blue leaves, with the exception of F3'H. Those UniGenes that are expressed at lower levels in blue leaves might also play a role in the blue coloration of *S. uncinata* by virtue of their negative regulation. In-depth molecular exploration of blue-leaf genes and their regulatory mechanisms is needed to obtain a comprehensive understanding of the coloration mechanisms of *S. uncinata*.

Conclusion

Based on an analysis of transcriptome sequencing and verification of expression by qRT-PCR, we identified seven key enzymes and their encoding genes that participate in chlorophyll and flavonoid metabolism pathways, including *CHLH*, *LPOR*, *CLH*, *HCT*, *CHS*, *MYB*, and *F3'H*, and that may be involved in blue leaf coloration. Our results imply that the primary pathway of pigment metabolism in *S.*

uncinata might not be the anthocyanin biosynthesis pathway, but rather the chlorophyll metabolism pathway. It is possible that Chl b is transformed into Chl a by another route, such as transformation to Chlide b by CLH, and then conversion to Chl a without the involvement of CBR or HCAR. This conclusion did not coincide exactly with our initial speculation based on our measurements of leaf pigment contents. We had inferred that the blue leaves of *S. uncinata* might be due to the color of chlorophyll or delphinidin in anthocyanin. Thus, our next step is to identify the dominant components of leaf pigments using LC-MS. This study establishes a basis for subsequent gene functional verification and further studies of blue-leaf gene regulation mechanisms. The results here provide a foundation for future artificial regulation of leaf color and goal-directed cultivation of blue-leaf plants.

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Author Contributions

Lin Li and Yingmin Lu conceived and designed the research. Lin Li conducted experiments and wrote the manuscript. Qing Wang and Rongyan Deng helped conduct the experiment. Shuimu Zhang helped produce several of the figures. All authors read and approved the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Figures

Fig. 1. Appearance and pigment contents of *Selaginella uncinata*. A: Leaves under different treatments. B,C: Contents of chlorophyll a and b, respectively. D: Ratio of chlorophyll a/b. E: Content of anthocyanin.

Fig. 2. Frequency distribution of the top 20 specie

Fig. 3. COG classification of *Selaginella uncinata* UniGenes

Fig. 4. Gene Ontology classification of *Selaginella uncinata* UniGenes

Fig. 5. Comparison of gene expression in blue and red leaves

Fig. 6. Heat map of 102 differentially expressed genes related to chlorophyll and flavonoid metabolism in *Selaginella uncinata*. Red indicates upregulation, green indicates downregulation, and black indicates no expression or no change.

Fig. 7. Expression profiles of seven differentially expressed genes involved in chlorophyll and flavonoid metabolism in *Selaginella uncinata* as determined by qRT-PCR analysis. The resulting mean values are relative to the expression of *ACTIN*.

Fig. 8. Pathway of chlorophyll metabolism in *Selaginella uncinata*. Red boxes with red borders indicate upregulated UniGenes in blue leaf samples. Black boxes with dotted borders indicate that no UniGenes mapped to the corresponding enzymes.

Fig. 9. Pathway of flavonoid biosynthesis in *Selaginella uncinata*. Red boxes with red borders indicate upregulated UniGenes, while green boxes with green borders indicate downregulated UniGenes in blue leaf samples. Black boxes with a dotted border indicate that no UniGenes mapped to the corresponding enzymes.

Tables

Table 1. Primers used in qRT-PCR analysis of UniGene expression in *Selaginella uncinata*.

UniGene Id	Gene Name	Enzyme or Protein Description	Forward Primer Sequence (5'–3')	Reverse Primer Sequence (5'–3')	Note or GenBank accession number
Contig1769	<i>ACTIN</i>	Actin related protein 2	ACATGGTACTTCCGCCA CTTAG	CCAGATGGACGGGTG ATAAAA	Reference Gene
Contig888	<i>CHLH</i>	Magnesium chelatase H subunit	CGTCGCACTCCATCCCATA T	AACCACGGCTAACGC ACAG	KX255717
Contig577	<i>LPOR</i>	Protochlorophyllide reductase	TCTTCCTCGGAAACAAA CCC	CGTCTTCTCATCGCTCTKX255718 ACCC	
Contig6952	<i>CLH1</i>	Chlorophyllase	CGTCGGAAATGGAATGT GG	CTGCGTTCGCTTTAGC TCTG	no submit
Contig9403	<i>CLH2</i>	Chlorophyllase	GCTCAGACGGTCATTCA GGA	CACCAAAGAAGTTTCG GGCATA	no submit
Contig5838	<i>HCT</i>	Shikimate O-hydroxycinnamoyl-transferase	CAGGTGACTCAACTGGA AGGC	GGTTGGGGACGAATA AAAGC	KX255719
Contig19924	<i>CHS1</i>	Chalcone synthase-like polyketide synthase	GCTTTGCGAGAACCTTC CTAC	ATTCCCCACCATCACC ACA	no submit
Contig20287	<i>CHS2</i>	Type III polyketide synthase	CGCTTGAGGTGGCGAAACTCCTG A	CCCCAACAACG AAT	no submit
Contig1641	<i>MYB</i>	R2R3-MYB transcription factor MYB8	TCAAGTCTGGCCGAAGG TAGT	TGGACACGGTTGCTG GAGTA	KX255720
Contig12143	<i>F3'H1</i>	Cytochrome P450-dependent monooxygenase	AGTCCTGGAGCGAGGCGAGCCTCGT TGGA	GCAACGG AGTCTTACC	KX255721
Contig4811	<i>F3'H2</i>	Putative uncharacterized protein CYP794A1	GCCTTGGCTAGAGTCAG AACCAGC	TCATCACCGTAAAGCA TGCCTCGT	KX255722

Table 2. Results of sequence assembly in *Selaginella uncinata*.

Statistics	Count	Total	N25	N50	N75	Average	Longest	N%	GC
		Length (bp)	(bp)	(bp)	(bp)	Length	(bp)		
Contigs	52,325	33,891,340	1,613	890	481	648	9,754	0.0	49.2
Primary									
UniGene	31,094	34,331,406	2,786	1,630	800	1,104	26,120	1.2	49.2
Final									
UniGene	30,119	34,110,082	2,850	1,670	833	1,133	26,120	1.3	49.1

Table 3. KEGG pathway categories of *Selaginella uncinata* UniGenes.

KEGG Category	UniGene_NUM	Ratio of Total	Pathway_ID
Metabolic pathways	2216	9.70	ko01100
Biosynthesis of secondary metabolites	1156	5.06	ko01110
Ribosome	715	3.13	ko03010
Biosynthesis of antibiotics	615	2.69	ko01130
Microbial metabolism in diverse environments	544	2.38	ko01120
Protein processing in endoplasmic reticulum	486	2.13	ko04141
Carbon metabolism	342	1.50	ko01200
Cell cycle	313	1.37	ko04110
Plant hormone signal transduction	291	1.27	ko04075
HTLV-I infection	290	1.27	ko05166
Biosynthesis of amino acids	282	1.23	ko01230
Glycolysis/Gluconeogenesis	239	1.05	ko00010
RNA transport	231	1.01	ko03013
Ribosome biogenesis in eukaryotes	213	0.93	ko03008
Epstein-Barr virus infection	197	0.86	ko05169
Oxidative phosphorylation	197	0.86	ko00190
Spliceosome	189	0.83	ko03040
Purine metabolism	182	0.80	ko00230
Phenylalanine metabolism	177	0.77	ko00360
Phenylpropanoid biosynthesis	173	0.76	ko00940
Others	13796	60.39	