Transcriptome-sequencing analyses reveal flower color formation in *Strelitzia reginae*

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Abstract

*Strelitzia reginae* is a popular cut flower that has blue petals and orange sepals. Flower color is an important plant trait; however, little is known about its molecular mechanisms in *S. reginae*. In this study, cDNA libraries were constructed for blue petals and orange sepals of *S. reginae*. A total of 75 487 unigenes were obtained from transcriptome sequencing and *de novo* assembly, of which 41.86% were annotated by public databases. Ultra-high performance liquid chromatography analysis revealed that anthocyanins were the main pigment in blue petals, and that carotenoids controlled pigment formation in the orange sepals. Using a system analysis-based approach, 73 and 29 candidate genes related to anthocyanin and carotenoid biosyntheses were identified, respectively. Among these, *chalcone synthase 2*, *chalcone isomerase 1*, *flavanone 3-hydroxylase 1*, *flavonoid 3′,5′-hydroxylase 1*, *dihydroflavonol 4-reductase 1*, *anthocyanidin synthase 1*, and *anthocyanidin 3-O-glycosyltransferase 1* were considered to be important in regulating the formation of blue petals, and *phytoene synthase 1*, *phytoene desaturase 1*, *ζ-carotene desaturase 1*, *lycopene β-cyclase 3*, and *β-carotene hydroxylase 2* might play important roles in orange sepal formation. This study improves our understanding of flower color and provides evidence for future molecular breeding of ornamental plants based on flower color modifications.

Additional key words: anthocyanins, carotenoids, transcriptome sequencing.

Introduction

Flower color is one of the important characters that have been used in measuring the quality of ornamental plants. The quality of flowers is directly related to their ornamental and commercial values. In addition, flower color can attract insects to help pollination. Plant colors are mainly controlled by two pathways, namely, the anthocyanin pathway and the carotenoid pathway (Weiss 1995, Tanaka et al. 2008). Anthocyanins are the main pigments responsible for yellow, orange, red, purple, and blue colors (Tanaka et al. 1998, Iwashina 2000). Among these, blue is considered a rare color because it is difficult to obtain real blue flowers in many floricultural plants, such as carnation, chrysanthemum, rose, and lotus (Chandler and Tanaka 2007, Yang et al. 2009). Traditional breeding methods are difficult in direct breeding blue flowers, and genetic engineering shows some advantages (Sasaki and Nakayama 2015). Carotenoids comprise 40 carbon isoprenoids that are usually involved in yellow-orange coloration (Hirschberg 2001). To date, almost all of the genes related to carotenoid biosynthesis have been characterized (Pandurangaiah et al. 2016, Rezaei et al. 2016, Yang et al. 2016). However, there are few genetic data involved in carotenoid pathways of *Strelitzia reginae*.

*Strelitzia reginae*, also known as the bird of paradise, belongs to family *Strelitziaceae* and is native to southern Africa. It has a significant commercial value as cut flowers, which has blue petals and orange sepals (Paiva et al. 2004). However, pigment composition and genomic analyses in relation to flower color have not been conducted to date. The mechanisms of flower color formation in *S. reginae*, thus, remain unclear. Research studies on the regulatory mechanisms of flower color in *S. reginae* are therefore necessary.

Ribonucleic acid sequencing (RNA-seq) has become a...
particularly effective method for exploring the molecular mechanisms of certain characters, especially in non-model plants that have been used as reference genomes (Lou et al. 2014, Zhao et al. 2014, Zhang et al. 2015). In this study, we first sequenced the transcriptomes of orange sepal and blue petals of S. reginae on an Illumina HiSeq™ 4000 platform. Qualitative and quantitative analyses of anthocyanins and carotenoids were performed with ultra performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC-QTOFMS) and UPLC technologies. We analyzed the biochemical and molecular mechanisms of orange and blue color formation in S. reginae with the aim to improve understanding of flower color formation for possible future molecular breeding of ornamental plants based on flower color modifications.

Materials and methods

Plants and cultivation: Strelitzia reginae Aiton was open-air cultivated at the Fujian Academy of Agricultural Sciences in Fuzhou City, China. The blue petals and orange sepal of S. reginae were collected in October 2017. A total of 12 blue petals and 12 orange sepal from each plant were pooled. The samples were frozen in liquid nitrogen and then stored at -80 °C for transcriptomic analyses.

Library construction and sequencing: Total RNA was extracted from the blue petals and orange sepal using a universal RNA extraction kit (Biotek Corporation, Beijing, China). The RNA integrity was examined with a NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). Library constructions and transcriptome sequencing (NCBI SRA accession number PRJNA513215) were performed by Biomarker Biotechnology Corporation (Beijing, China) using an Illumina HiSeq™ 4000.

Assembly and functional annotation: High-quality clean reads were obtained from the Illumina-generated raw reads by removing low-quality reads. De novo assembly of the reads into contigs was performed using Trinity (Grabherr et al. 2011) and further assembled into transcripts using paired-end reads. Finally, the transcripts were screened by identifying unigenes.

Based on sequence similarity, BLASTx analysis against public databases was performed using an E-value threshold of 10^-5, which included clusters of orthologous groups (COG) of proteins, gene ontology (GO), Kyoto encyclopedia of genes and genomes (KEGG), eukaryotic clusters of orthologous groups (KOG), protein family (Pfam), Swiss-Prot protein database, evolutional genealogy of genes: non-supervised orthologous groups (eggNOG), and NCBI non-redundant protein (Nr). A Pfam search was performed using the HMMER 3.0 program with an E-value cut-off of 10^-10 (Finn et al. 2011). Classification of GO terms was conducted using Blast2GO (Gottz et al. 2008).

Analysis of differentially expressed genes: The gene expression was estimated by RNA-Seq by expectation maximization (RSEM) (Li and Dewey 2011). The abundance of each gene was calculated using fragments per kilobase of transcript per million mapped reads (FPKM) (Trapnell et al. 2010). Differentially expressed genes (DEGs) among four different libraries were evaluated using the DESeq package (Anders and Huber 2010). A false discovery rate (FDR) < 0.01 and a fold-change value ≥ 2 were used as thresholds to confirm DEGs between two samples.

Quantitative real-time PCR analysis was performed using an ABI 7500 real-time PCR system (Applied Biosystems, Waltham, MA, USA). Primers were designed for the determination of relative expressions of the candidate genes using Primer Premier 5.0. Melting curve analysis was conducted to confirm PCR specificity. Three independent replicates of each sample were analyzed. The β-actin gene was used as an internal control. A relative quantitative computing method (2^−ΔΔCt method) was used to calculate changes in unigene expression.

Measurement of anthocyanin content: The frozen samples (100 mg) were grounded using a Tissue lyser JX-24 (Jingxin, Shanghai, China) with beads at 40 Hz for 4 min and extracted with 1 cm³ of 60 % (v/v) ethanol containing 0.1 % (m/v) hydrochloric acid at 35 °C for 2 h. After centrifugation at 4 °C and 12 000 g for 15 min, the supernatant was collected and evaporated to remove ethanol. The concentrated extracts were then loaded into an AB-8 resin column (200 × 20 mm). The anthocyanins absorbed onto the resin were washed with distilled water with 0.1 % hydrochloric acid and subsequently eluted with 60 % ethanol containing 0.1 % hydrochloric acid until colorless. The eluent was lyophilized and reconstituted in 1.5 cm³ of 50 % (m/v) acetonitrile containing 0.5 % (v/v) formic acid. Following centrifugation at 12 000 g for 15 min, 0.09 cm³ of the supernatant were combined with 0.01 cm³ of 25 μg cm⁻³ lidocaine (internal standard), and the mixture was analyzed by UPLC-QTOFMS.

Chromatographic separation was performed on an ACQUITY UPLC I-Class system (Waters Corporation, Milford, MA, USA) with a Waters Acquity UPLC HSS T3 column (2.1 × 100 mm, 1.7 μm) maintained at 45 °C. Chromatograms of anthocyanins were acquired at a wavelength of 525 nm. The injection volume was 3 mm³. The mobile phases consisted of water (phase A) and acetonitrile (phase B), both with 0.5 % formic acid (v/v). A linear gradient elution was performed with the following program: 0 - 2 min, 1 % B; 3 min, 5 % B; 9 min, 20 % B; 12 min, 50 % B; 15 min, 100 % B; 17 min, 100 %B; and 17.1 min, 1 % B; and held to 20 min.

The eluents were analyzed on a Vion IMS QTOF mass spectrometer (Waters Corporation) on an ESI+ mode. The capillary voltage was set to 2 kV. The sampling cone voltage and cone gas flow were 40 V and 50 dm³ h⁻¹, respectively. The desolvation gas was maintained at a flow rate of 900 dm³ h⁻¹ and a temperature of 450 °C, respectively. The ion source temperature was 115 °C. The time of flight mass spectrometry scan was operated at a high-resolution with 0.2 s survey scan time and a range of 50 - 1000 m/z in the continuum mode both for function 1 and 2. To improve the
Identification of unknown metabolites, the MS² function was also performed to obtain fragment ion information with a ramp collision energy from 20 to 45 eV. The mass accuracy calibration was performed with the lock mass, leucine-enkephalin at 250 ng cm⁻³ and 5 mm² min⁻¹, with data acquisition frequency set at 30 s. The UNIFI 1.8.1 (Waters Corporation) software was used to control the instrument and collecting data. Delphinidin 3-glucoside was used as quantification standard. Three biological replicates were employed.

**Measurement of carotenoid content:** Frozen flower samples (1 g) were extracted with acetone:petroleum ether (1:1) at 50 °C for 3 h, until the residue was whitish. The extract was evaporated to dryness at 40 °C using a rotary evaporator, dissolved in 20 cm³ of petroleum ether containing 0.1 % (m/v) butylated hydroxytoluene, then 20 cm³ of 10 % (m/v) KOH-methanol solution was added. When emulsification occurred, 10 % (m/v) NaCl was added to emulsification. The upper petroleum ether phase was collected, transferred to a separatory funnel, and the petroleum ether phase was washed repeatedly with double distilled water until the aqueous phase was neutral. Approximately 10 g of anhydrous sodium sulfate were added, filtered, and evaporated to dryness using a rotary evaporator at 40 °C. The solution was then dissolved in 2 cm³ of acetone (containing 0.1 % BHT). The carotenoids were separated and identified using an ACQUITY UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm) (Waters Corporation) with a mobile phase of acetonitrile:methanol (9:1) at a wavelength of 450 nm. Three biological replicates were performed.

**Results**

Using UPLC-QTOFMS, two anthocyanins (delphinidin 3-glucoside and hydroxycyanidin 3-glucoside) were detected in the blue petals. No anthocyanins were detected in the orange sepals (Fig. 1). The delphinidin 3-glucoside was the predominant anthocyanin, and a peak content of 54.82 μg·g⁻¹ (f.m.) was detected at the blooming stage (Table 1 Suppl.).

Using UPLC, three carotenoids (lutein, β-cryptoxanthin, and β-carotene) were detected in the orange sepals (Fig. 1). No carotenoids were detected in the blue petals. The β-carotene and β-cryptoxanthin were the predominant carotenoids, with peak content of 825 and 322 μg g⁻¹ (f.m.) detected at the blooming stage (Table 1 Suppl.).

Due to the absence of available genomic information on *S. reginae*, Illumina sequencing and de novo assembly were conducted using the blue petals and orange sepals in this study. A total of 26.16 Gb high-quality clean reads were generated. De novo assembly of these reads respectively resulted in 127 803 transcripts and 75 487 unigenes, with N50 values of 1 981 and 1 565 bp, reflecting a good assembly. Of the obtained unigenes, 22 376 were longer than 1 000 bp, accounting for 31.8 % of all of the unigenes (Table 1).

To obtain further information about the unigenes, all of the unigene sequences were annotated using BLAST analysis against eight public databases. Of the 75 487 unigenes, 29 505 assembled sequences were annotated, accounting for approximately 41.86 %, using an E-value ≤ 1e-5. A total of 15 936 unigenes (21.11 % of all unigenes) were functionally annotated into three major categories and 52 GO classes. In the molecular function categories, most unigenes were related to catalytic activity, binding, and transporter activity. However, a large proportion of assembled unigenes remained unannotated, which involved non-coding RNAs, untranslated regions,
or may be unique to *S. reginae*, which can be a good resource for studying novel genes (Chung *et al*. 2016, Kwenda *et al*. 2016).

**KEGG** is an important database that can be used to systematically analyze metabolic pathways and products of related genes. Using the **KEGG** pathway, the DEGs in *S. reginae* were analyzed. Among them, 1,497 were grouped into 117 **KEGG** pathways. Four functional categories (phenylpropanoid biosynthesis, phenylalanine metabolism, flavonoid biosynthesis, and carotenoid biosynthesis) involved in flower color revealed significant enrichment. The enrichment of pathways involved in the anthocyanin biosynthesis and carotenoid biosynthesis were consistent with the observed high anthocyanin content in the blue petals and carotenoid content in the orange sepals. These provided a valuable resource for gene discovery and functional analysis of *S. reginae*.

To explore the molecular mechanism of formation of blue petals and orange sepals in *S. reginae*, a comparison of gene expression was conducted between these two plant parts. A total of 5,233 unigenes were differentially expressed between blue petals and orange sepals. Among them, 2,695 unigenes were upregulated, whereas 980 were downregulated in the blue petals. Transcript abundance was revealed by hierarchical cluster analysis. The expression of the blue petals and orange sepals showed pronounced differences.

The unigenes involved in anthocyanin biosynthesis were analyzed in *S. reginae*. A total of 73 unigenes were identified (Table 2 Suppl.). To obtain the key genes related to the metabolism of blue color, transcriptional profiles of genes involved in anthocyanin biosynthesis between blue petals and orange sepals were analyzed. The expressions of 21 key unigenes showed significant differences, which included 16 upregulated and 5 downregulated unigenes (Table 2 Suppl.). These DEGs included

| Length [bp] | Transcript number | Unigene number | Percent | Percent |
|-------------|------------------|----------------|---------|---------|
| 200 - 300   | 18100            | 16478          | 14.16   | 21.83   |
| 300 - 500   | 21667            | 19436          | 16.95   | 25.75   |
| 500 - 1,000 | 30329            | 15376          | 23.73   | 22.78   |
| 1000 - 2000 | 32185            | 13376          | 25.18   | 17.72   |
| 2000+       | 25522            | 9000           | 19.97   | 11.92   |
| Total number| 127803           | 75487          |         |         |
| Total length| 161149389        | 71961475       |         |         |
| N50 length  | 1981             | 1565           |         |         |
| Mean length | 126092           | 95330          |         |         |

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Table 1. Quality parameters of the *Strelitzia reginae* transcriptome.

**Fig. 2.** Expression patterns of genes involved in anthocyanin biosynthesis. The abbreviated name of enzyme in each catalytic step is shown in *bold*. Gene expression - log2 of fragments per kilobase of transcript per million mapped reads (FPKM) in sepals and petals are represented by *color gradation*. The full names of the enzymes are listed in Table 2 Suppl.
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both upstream genes [e.g., chalcone synthase (CHS), chalcone isomerase (CHI), and flavanone 3-hydroxylase (F3H)] and downstream genes [e.g., anthocyanidin 3-O-glucosyltransferase (UA3GT)] (Fig. 2). Among these, seven structural unigenes SrCHS2 (c78787.graph_c0), SrCHI1 (c35619.graph_c0), SrF3H1 (c83775.graph_c0), flavonoid 3′,5′-hydroxylase 1 (SrF3′5′H1 c98907.graph_c0), dihydroflavonol 4-reductase 1 (SrDFR1 c75504.graph_c0), anthocyanidin synthase 1 (SrANS1 c76124.graph_c0), and UA3GT1 (c95500.graph_c0) were upregulated in blue petals. And real-time quantitative PCR analysis showed that the expression of these seven genes gradually increased during flowering and peaked at the blooming stage, suggesting that these play important roles in the accumulation of "blue" pigments (Fig. 4).

To identify the key genes involved in the formation of orange pigment, 28 unigenes related to carotenoid biosynthesis were obtained (Table 2 Suppl.). Among these, 11 genes revealed significantly different expressions, and all of the DEGs were upregulated in the orange sepals (Fig. 3). Five genes, phytoene synthase 1 (SrPSY1, c81232.graph_c0), phytoene desaturase 1 (SrPDS1, c94309.graph_c0), ζ-carotene desaturase 1 (SrZDS1, c80541.graph_c0), β-carotene hydroxylase 2 (SrCHYB2, c76929.graph_c0) showed high transcript abundances. Real-time quantitative PCR analysis revealed that the expression patterns of five genes were relatively low at the bud stage and high at the blooming stage, reflecting that these play a crucial role in the formation of orange pigments (Fig. 4).

To verify the reliability of our transcriptome data, real-time quantitative PCR analyses were conducted based on the sequences of 24 core unigenes using the designed primers. The results revealed a positive correlation between quantitative PCR and the transcriptome sequence data ($R^2 = 0.8983$) (Fig. 1 Suppl.).
Discussion

*S. reginae* is a popular ornamental plant that has blue petals and orange sepals. Blue flowers are considered rare, and many other important ornamental plants lack blue flowers, such as *Tulipa* and *Phalaenopsis*. To breed plants with blue-colored flowers, the mechanism of blue coloration should be elucidated. In addition, the formation of orange/yellow flowers is controlled by two pathways, namely the carotenoid and anthocyanin pathways. Investigating orange sepal formation in *S. reginae* may thus facilitate the identification of major pigmentation pathways in specific plant parts.

As the genome sequence of *S. reginae* has not been reported to date, it is difficult to determine the mechanism underlying blue and orange color formation. In this study,
transcriptome sequencing of blue and orange sepal was conducted. Approximately 75,487 unigenes were obtained from four cDNA libraries. A total of two anthocyanins were detected in blue petals, in which delphinidin-3-glucoside showed the highest content. Among DEGs related to anthocyanin biosynthesis, SrCHS2, SrCHI1, SrF3H1, SrF3′5′H1, SrDFR1, SrANS1, and SrUA3GT1 were upregulated in blue petals and are involved in delphinidin-3-glucoside biosynthesis. The formation of blue petals is regulated by multiple genes. Three carotenoids were detected in the orange sepal, in which β-carotene and β-cryptoxanthin had high content. Among the DEGs involved in carotenoid biosynthesis, SrPSY1, SrPDS1, SrZDS1, SrLCYB3, and SrCHYB2 were upregulated in the orange sepal. These unigenes were upstream of the carotenoid pathway, mainly regulating β-carotene and β-cryptoxanthin biosynthesis.

Flavonoid 3′-hydroxylase and F3′5′H determine the hydroxylation type of the B-ring of anthocyanins and are the key enzymes for cyanidin and delphinidin production, respectively. Thus, flavonoid 3′-hydroxylase and F3′5′H are essential for the structural organization of anthocyanins and are as considered as the “red gene” and “blue gene” (Tanaka 2006). Flowers of numerous ornamental plants lack blue color because these do not possess the F3′5′H gene. The heterologous overexpression of the F3′5′H gene in carnations (Chandler and Bruglieri 2011), roses (Katsumoto et al. 2007), and chrysanthemums (Bruglieri et al. 2013, Noda et al. 2013) results in a violet/blue color of their flowers. A total of eight F3′5′H genes were identified in S. reginae. One F3′5′H gene is upregulated in blue petals (log FC = 11.23) and upregulated during flower development, and peaked during the early flowering stage (Fig. 4). Delphinidin-3-glucoside is the predominant anthocyanin, which was in a relatively low content during the bud period and peaked during the early flowering stage (Fig. 1). This indicated that F3′5′H is a key gene that is related to delphinidin-3-glucoside content.

Dihydroflavonol 4-reductase uses one or three of dihydroflavonols (dihydroxyricetin, dihydrokaempferol, or dihydroquercetin) to create leucoanthocyanidin, which is a key step to anthocyanin synthesis. The structure of the three substrates is similar and only differs in terms of the B phenyl ring, particularly in terms of the number of hydroxyl groups, in which there is no enzymatic action (Wu et al. 2016). Thus, DFRs can catalyze all three substrates in various plant species. However, in some species, such as Petunia hybrida and Cymbidium hybrida, DFR has strict substrate specificity (Johnson et al. 2001). In this study, only delphinidin and cyanidin-based anthocyanins were detected, and no pelargonidin-based anthocyanins were found. One possible explanation for this observation is that DFRs in S. reginae cannot utilize dihydrokaempferol or DFRs can utilize three dihydroflavonols, but flavonol synthase preferentially utilize dihydroxykaempferol. In this study, DFR had a higher expression than flavonol synthase and, therefore, DFR had a greater priority. It is possible that DFR has substrate specificity and cannot utilize dihydrokaempferol in S. reginae.

Anthocyanins are modified by glycosylation, acylation, or methylation. These modifications keep the anthocyanins stable and soluble and facilitate the generation of different colors (Grotewold 2006). We hypothesize that acylation and methylation may not be as important as glycosylation during the formation of blue petals in S. reginae because acylated and methylated anthocyanins were not detected, and the genes involved in acylated and methylated modifications were downregulated. Delphinidin-3-glucoside and hydroxycyanidin 3-glucoside are major anthocyanins, reflecting that the modification of the 3-position is essential to blue petal color formation. Therefore, 3-glycosyltransferases (3GTs) might play a crucial role in the formation of blue petals in S. reginae. One 3GT has been identified, namely, UA3GT. Eleven UA3GT homologous unigenes were found in S. reginae. The expression of SrUA3GT1 is significantly upregulated from bud stage to blooming stage, suggesting that SrUA3GT1 could be the most important enzyme in delphinidin-3-glucoside accumulation. This study has improved our understanding of the molecular mechanism underlying blue flower pigmentation and provides a resource for the molecular breeding of color-modified ornamental plants.

In Camellia nitidissima, carotenoid synthesis serves as the main pathway for color formation in petals (Miyajima et al. 1985, Zhou 2012), which is basically controlled at the transcriptional level (Ohmiya 2013). In chrysanthemums, the expression of most genes in the carotenoid pathway, including PSY, PDS, ZDS, carotenoid isomerase, LCYB, lycopene cyclase, and CHYB, are upregulated during flower development (Ohmiya et al. 2006). The expression patterns of PSY, ZDS, carotenoid isomerase, and CHYB were similar in the Asiatic hybrid lily (Yamagishi et al. 2010). In this study, the content of β-carotene and β-cryptoxanthin, as detected by UPLC-TOFMS, increased from the bud to the blooming stages, as well as those of SrPSY1, SrPDS1, SrZDS1, SrLCYB3, and SrCHYB2. These results indicate that the expression of these genes is crucial for carotenoid accumulation in sepal formation in S. reginae, and that the carotenoid pathway is mainly regulated at the transcriptional level.

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