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Full Length Article

Transcriptome Sequencing Reveals Genes Involved in Petal Spot Formation of Asiatic Hybrid Lily Cultivar 'Easy Dance'

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Abstract

Petal spot is an important characteristic for ornamental flowers. However, fewer studies have been done to uncover the molecular formation mechanism of petal spot in *Lilium* sp. In our research transcriptome sequencing of Asiatic hybrid lily 'Easy Dance' flower with large brown spot was performed. Transcriptome of brown-pigmented interior tissue of spot region (SP) was compared with un-pigmented exterior tissue of spot region (SNP) and no spot region (NS) of flower to identify structural genes and regulatory genes involved in brown spot formation. The results revealed that 11,544 unigenes exhibited significantly differential expression between NS and SP, and 12,636 unigenes between SNP and SP. Functional enrichment analysis of differential expressed genes revealed several pathways possibly involved in spot formation in *Lilium*. Some of unigenes annotated as *CHS*, *PAL*, *C4H*, *F3'H*, *F3'5'H*, *F3H*, *FLS*, *DFR* and *3GT* indicated higher expression in SP compared with NS and SNP. All expressions of carotenoid biosynthesis genes were down-regulated in spot. In addition, six transcription factors annotated as bHLH, R2R3-MYB or WD40, which regulated anthocyanin biosynthesis in spot, were identified by transcriptome sequencing and phylogenetic analysis. Our research will help to deepen the understanding of formation mechanism of petal spot in *Lilium*. © 2018 Friends Science Publishers

Keywords: *Lilium* sp.; Spots; Anthocyanin biosynthesis; bHLH; R2R3-MYB

Introduction

With the improvement of the living standards in recent years, peoples' demands for ornamental flowers are changing from yield to quality (Zhao *et al.*, 2014). Pigment spots or stripes belong to the pigmentation patterning of flower that is one of key characteristic for ornamental flowers and determines their commercial value. Ornamental plants with novel pigmentation patterning have become attractive breeding targets for some flower breeders. Thus, the mechanism of pigmentation pattern formation has attracted the attention of numerous plant biologists and breeders (Yamagishi *et al.*, 2014).

Asiatic hybrid lilies depict a variety of colors, derived from interspecific crosses of section Sinomartagon of the family Liliaceae, are famous ornamental flowers (Lim and van Tuyl, 2006). The large variations in Asiatic hybrid lilies flower colors come from the accumulation of anthocyanins and carotenoids. The yellow and orange pigmented petals are caused by carotenoids, chocolate brown and pink by anthocyanins, and red pigmented by the mixture of carotenoids and anthocyanins (Yamagishi *et al.*, 2014). Flowers of Asiatic hybrid lily cultivars have different pigmentation patterns except a lot of variation in colors (Yamagishi, 2013). Dark red or red spots often arise in the internal surfaces of petals in Asiatic hybrid cultivars (Abe *et al.*, 2002). Previous researches indicated that the major pigments in petal spots are anthocyanins in many flowering plant species (Cooley and Willis, 2009). The chemical structure of anthocyanin has been well researched, and several genes in anthocyanin biosynthesis have been identified (Winkel-Shirley, 2001). The transcription of anthocyanin biosynthetic genes is regulated by the interactions among basic-helix-loop-helix (bHLH), R2R3-MYB transcription factors and WD40 proteins (Koes *et al.*, 2005). However, the biosynthesis and regulation of anthocyanin related to petal spot have seldom been investigated in lily. Only two transcription factors, LhMYB6 and *LhMYB12-Lat*, regulating spot development in lily petal have been reported (Yamagishi *et al.*, 2010; Yamagishi *et al.*, 2014). Therefore, a more comprehensive research on biosynthesis genes and regulatory factors of pigment spot is important to better understand the mechanism on petal spot formation in *Lilium* sp.

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To uncover the mechanisms involved in spots formation in Asiatic hybrid lilies, three transcriptomes of the brown-pigmented and unpigmented tissue of spot region, and no spot region of Asiatic hybrid lily 'Easy Dance' flower were sequenced and analyzed. Some genes with different expression patterns in three samples were obtained, and some structural and regulatory genes involved in spot formation were identified.

Materials and Methods

Plant Materials

Asiatic hybrid lily cultivar 'Easy Dance' was planted in the greenhouse of Beijing University of Agriculture. This cultivar has brown spot in the interior surfaces of yellow petal. Therefore, the brown-pigmented interior (SP) and un-pigmented exterior (SNP) tissues of spot region and no spot region (NS) of flower were sampled for future analysis.

RNA Isolation

The TRIzol® reagent was used for total RNA extraction of three samples. Agilent 2100 Bioanalyzer and 1% agarose gel electrophoresis were used to detect the integrity of total RNA. After treated with DNase I kit, the mRNA was isolated from the total RNA using magnetic beads with Oligo (dT).

cDNA Library Construction and Transcriptome Sequencing

The fragmentation buffer was used to break mRNA into short fragments. The mRNA fragments were used for cDNA synthesis. The Qia-Quick PCR extraction kit was used to purify the cDNA fragments. A single nucleotide A (adenine) was added to the 3' end of cDNA fragments and then the sequencing adaptor were ligated to them. PCR was used to amplify and enrich the suitable fragments. The Agilent 2100 Bioanalyzer was used to validate the sample libraries. Illumina HiSeqTM 2500 sequencing platform in the Beijing Genomics Institute (BGI) was used to sequence cDNA libraries.

De novo Assembly

Clean reads were obtained by removing empty reads, adaptor, repeated and low-quality reads, and were used for subsequent analysis. High-quality reads was used for *de novo* assembly using the Trinity software (Grabherr *et al.*, 2011). The unigenes were aligned to seven public protein and gene databases by Blast X according to method of Zhang *et al.* (2015a). After unaligned to any of the above databases, the direction of a unigene was decided by the ESTScan software (Iseli *et al.*, 1999).

Unigene Annotation and Analysis

The Blast2GO was used to retrieve associated Gene Ontology (GO) terms of unigenes by the NR annotations (Conesa *et al.*, 2005). WEGO software was used for GO functional classification (Ye *et al.*, 2006). The complex biological behavior was studied using KEGG database and the pathway of unigene was annotated.

Unigene Expression Difference Analysis

FPKM method was used to measure the transcription abundance of unigene (Mortazavi *et al.*, 2008). In this analysis, unigene expression difference was defined according to Benjamini and Yekutieli method (2001). GO functional enrichment analysis and KEGG pathway analysis were done for difference expression unigenes.

Phylogenetic Analysis

Protein sequences of anthocyanin-related R2R3-MYB and bHLH transcription factors were collected from other plant species according to the Yuan *et al.* (2014) publication. These sequences and R2R3-MYB and bHLH transcription factors in 'Easy Dance' were aligned using ClustaIW. Phylogenetic trees of transcription factors were constructed using MEGA 5 software by the neighbor-joining method with bootstrap analysis of 1000 replicates (Tamura *et al.*, 2011).

Results

Transcriptome Sequencing, *de novo* Assembly and Function Annotation

In order to elucidate the mechanism of spot formation in lily petal, three cDNA libraries generated from brown-pigmented interior tissue of spot region (SP), un-pigmented exterior tissue of spot region (SNP) and no spot region (NS) of the petal of Asiatic hybrid lily 'Easy dance' were sequenced. We obtained 73.85 Gb raw reads from every library. The Q20 percentage for three libraries was over 97.65% and the GC percentage ranged from 48.78 to 49.83%. Short reads from NS, SNP and SP were assembled into 61777, 73576 and 67814 contigs. All these contigs were linked, and then produced 41269, 48831 and 45015 unigenes whose average lengths were 788, 744 and 775 nt for NS, SNP and SP. The N50 of unigenes for NS, SNP and SP were 1496, 1441 and 1511, respectively. After removing the redundancy of unigene sequences, 60631 non-redundant unigenes were obtained. A total of 37529 non-redundant unigenes were annotated. Most of unigenes were annotated to Nr (35498, 58.55%), Nt (26460, 43.64%), Swissprot (26010, 42.90%) and Interpro (25495, 42.05%) databases.

Table 1: Identification of DEGs involved in spot formation in Asiatic hybrid cultivar 'Easy Dance'

Predicted function	Number of genes	Up-regulated in SP	Up-regulated Unigene ID
Anthocyanin biosynthetic pathway			
PAL	3	3	CL8073.Contig1_All; CL2500.Contig2_All; CL2500.Contig1_All
C4H	3	3	Unigene4328_All; CL3508.Contig1_All; CL3508.Contig2_All
CHS	4	1	CL1805.Contig10_All
F3H	2	2	CL7225.Contig1_All; CL5507.Contig1_All
F3'H	8	6	CL3858.Contig3_All; Unigene33024_All; Unigene21151_All; CL2998.Contig4_All; Unigene4072_All; Unigene15105_All
F3'5'H	1	1	CL2998.Contig1_All
FLS	11	5	Unigene21082_All; CL5507.Contig2_All; CL5507.Contig1_All; Unigene6731_All; Unigene10352_All
DFR	2	1	Unigene17850_All
3GT	3	1	CL627.contig2_All
CHI	3	0	
ANS	4	0	
Carotenoid biosynthetic pathway			
PSY	1	1	Unigene15647_All
PDS	1	1	CL3926.Contig2_All
ZDS	17	17	Unigene17347_All; CL4760.Contig3_All; CL4372.Contig2_All; CL5693.Contig2_All; CL5693.Contig1_All; CL3505.Contig2_All; CL7928.Contig1_All ;Unigene8167_All; CL3351.Contig1_All; CL3505.Contig1_All; CL2217.Contig7_All; Unigene6415_All; CL163.Contig4_All; CL2217.Contig3_All; CL2217.Contig6_All; CL3926.Contig2_All; CL3351.Contig3_All
CRTISO	3	3	CL6739.Contig1_All; CL6739.Contig2_All; CL3064.Contig1_All
LCYB	1	1	Unigene8804_All
CruA	1	1	Unigene9101_All
LCYE	1	1	Unigene17497_All
CCS	1	2	Unigene8804_All;Unigene9201_All
VDE	1	1	Unigene862_All
Transcription factors families			
bHLH	32	8	Unigene19306_All; Unigene14748_All; CL6471.Contig2_All; CL3737.Contig1_All; CL7044.Contig2_All; CL3148.Contig3_All; Unigene15684_All; CL197.Contig7_All
R2R3-MYB	5	2	CL7202.Contig1_All; Unigene23782_All
WD-repeat protein40	4	4	CL4790.Contig1_All; CL4790.Contig2_All; CL4790.Contig3_All;Unigene2624_All

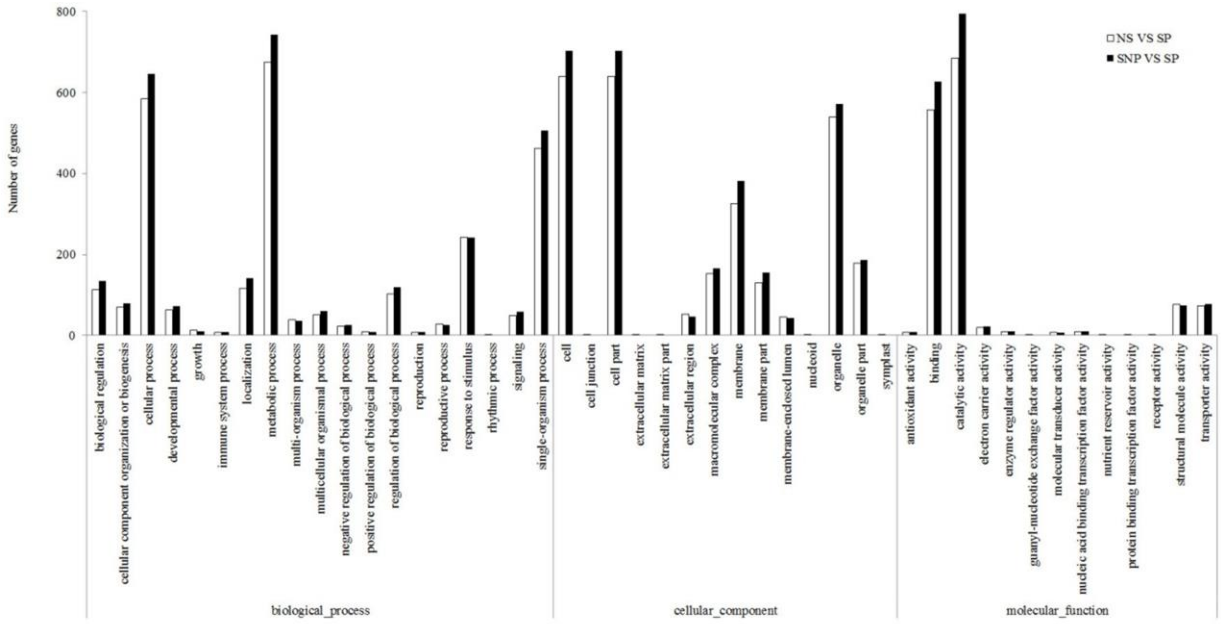

Fig. 1: Go classification of DEGs from NS-VS-SP and SNP-VS-SP

Differentially Expressed Unigenes Analysis

To identify key genes involved in spot formation, the transcriptome from SP was compared with those from SNP and NS. Total of 11544 and 12636 differentially expressed genes (DEGs) were obtained from NS-VS-SP and SNP-VS-SP, respectively (Table 1). By GO analysis, 1316 DEGs from NS-VS-SP and 1482 DEGs from SNP-VS-SP could be classified into 46 groups which belong to three categories (Fig. 1). In 'biological process', the major classifications were 'metabolic process' and 'cellular process'. In 'cellular component', cell, cell part and organelle accounted for the major proportion. The most frequent 'molecular function' terms were 'catalytic activity' and 'binding'. Using KEGG

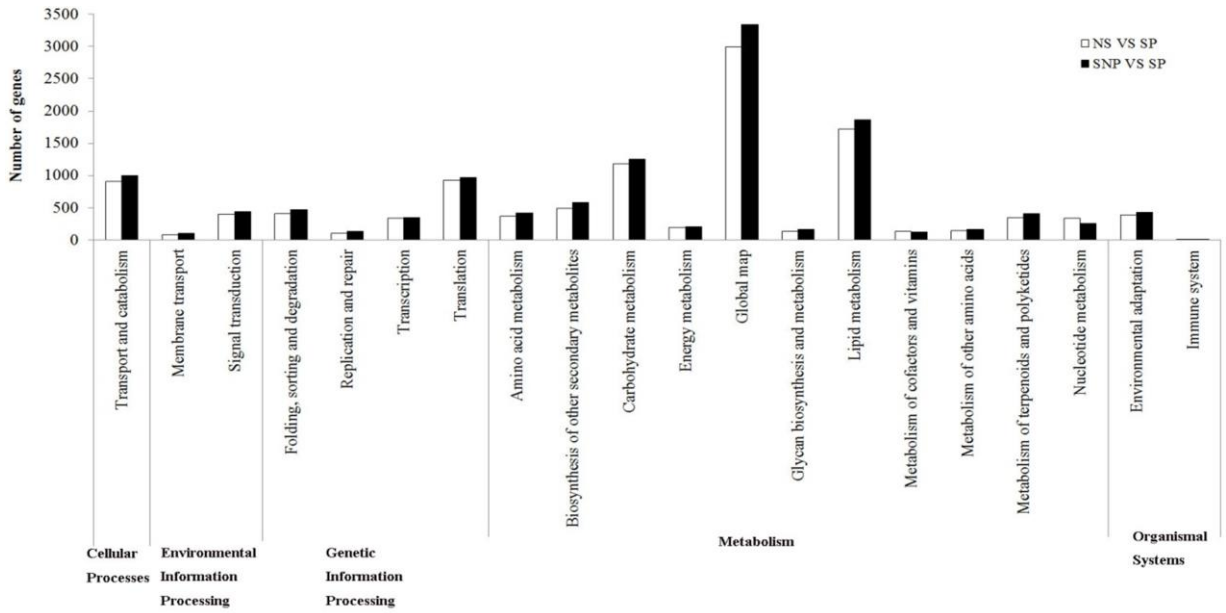

Fig. 2: KEGG pathway of DEGs from NS-VS-SP and SNP-VS-SP

analysis, 6269 DEGs (54.3%) from NS-VS-SP and 6860 DEGs (54.3%) from SNP-VS-SP were mapped to 126 KEGG pathways of 20 KEGG classes (Fig. 2). The largest class was 'global map', followed by 'lipid metabolism' and 'carbohydate metabolism'. Several KEGG pathways related to pigmentation development, including 'anthocyanin biosynthesis', 'flavonoid biosynthesis', 'isoflavonoid biosynthesis', 'flavone and flavonol biosynthesis' and 'carotenoid biosynthesis', were identified from classes of 'Metabolism of terpenoids and polyketides' and 'Biosynthesis of other secondary metabolites'.

Analysis of Candidate DEGs Related to Spots Formation

As previous reports, the major pigments in petal spots for many plant species are anthocyanins (Cooley and Willis, 2009). According to the KEGG analysis, 23 up-regulated unigenes related to anthocyanin biosynthetic pathway were probably involved in pigment development in petal spots (Table 1). Among them, unigenes annotated as *PAL*, *C4H*, *CHI*, *CHS*, *F3H*, *FLS*, *F3'H*, *ANS* and *DFR* have been reported in *Lilium* (Lai *et al.*, 2012). However, *F3'5'H* (CL2998.Contig1_All) and *3GT* (CL627.contig2_All) were firstly identified from *Lilium*. Some of unigenes annotated as *PAL*, *CHS*, *C4H*, *F3'H*, *F3H*, *F3'5'H*, *DFR*, *FLS* and *3GT* have high expression in SP compared with SNP and NS.

The carotenoid pathway produces yellow and orange and pink lily flowers (Yamagishi *et al.*, 2014). The petal region without spot in Asiatic hybrid 'Easy dance' was yellow. Therefore, the DEGs involved in carotenoid biosynthetic pathway were analyzed. We found that all DEGs annotated as *PDS*, *PSY*, *CRTISO*, *ZDS*, *LCYB*, *CruA*, *LCYE*, *CCS* and *VDE* had down-regulated expressions in SP (Table 1).

The anthocyanin biosynthesis genes in higher plants could be regulated by transcriptional factors, including R2R3-MYB, bHLH and WD40 classes (Xu *et al.*, 2015). In this study, 41 transcriptional products were identified as three transcription factor families, including 5 R2R3-MYBs, 32 bHLHs and 4 WD40s (Table 1). In these transcription factors, 2 R2R3-MYBs, 8 bHLHs and 4 WD40s were highly expressed in SP compared with SNP and NS.

Phylogenetic Analysis of Transcription Factors

In order to study transcription factors involved in regulation of anthocyanin biosynthesis in spot, 2 R2R3-MYBs and 8 bHLHs up-regulated in the spot of 'Easy dance' were used for phylogenetic analysis. The results showed that Unigene19306_All identified as a bHLH transcriptional factor was grouped with LhbHLH1 (Fig. 3). In the dendrogram of R2R3-MYB transcriptional factors, CL7202.Contig1_All was in the same cluster as plant anthocyanin-promoting R2R3-MYBs, including LhMYB12, LhMYB6 and LrMYB15 (Fig. 4).

Discussion

Lily with variable flower colors and shapes, and pleasant fragrances is popular ornamental plant worldwide. It can be used as fresh-cut flower and for garden decoration because

Fig. 3: Phylogenetic tree of bHLH transcription factors involved in the regulation of the flavonoid pathway from a range of species

The sequences of Asiatic hybrid cultivar 'Easy Dance' are highlighted in dot. Sequences of other plants were retrieved from Genbanks according to Yuan (2014) publication

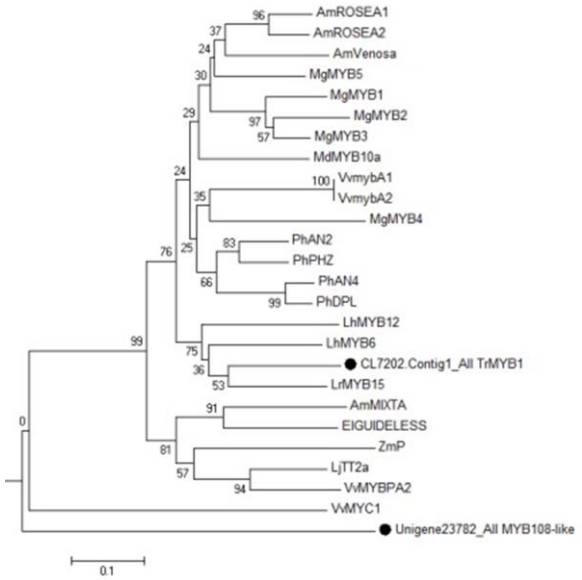

Fig. 4: Phylogenetic tree of R2R3-MYB transcription factors involved in the regulation of the flavonoid pathway from a range of species

The sequences of Asiatic hybrid cultivar 'Easy Dance' are highlighted in dot. Sequences of other plants were retrieved from Genbanks according to Yuan (2014) publication

of high ornamental value. However, the study on molecular mechanism of *Lilium* sp. was limited for their large genome (~36 Gb). High-throughput transcriptome sequencing is an efficient method for study on plant without genome information. In several researches, transcriptome sequencing has been used to estimate genetic divergence (Shahin *et al.*, 2014), and identify genes involved in the flavonoid biosynthesis (Zhang *et al.*, 2015b), carbohydrate metabolism (Li *et al.*, 2014), cold response (Wang *et al.*, 2014) and vernalization (Huang *et al.*, 2014) in *Lilium* sp. In our study, genes involved in spot development were identified from *Lilium* sp. using high-throughput transcriptome sequencing. About 73 Gb total raw reads were obtained from each library of SP, SNP and NS. And total of 11,544 and 12,636 DEGs were obtained from SP compared with NS and SNP, respectively. All these results in this study could provide a large amount of information for uncovering molecular mechanism of *Lilium* sp.

Petal spots are important characteristics for ornamental flowers and affect their value. However, there are very few researches about the molecular mechanism of spot formation. It has been reported that the main pigment in petal spots was anthocyanins (Zhang *et al.*, 2015b). Their biosynthesis are controlled by some well-characterized structure genes, including *CHS*, *F3'5'H*, *F3'H*, *ANS* and *DFR* (Li *et al.*, 2014). In our research, the results from transcriptome sequencing showed that expressions of *PAL*, *CHS*, *C4H*, *F3'H*, *F3H*, *FLS*, *F3'5'H*, *DFR*, and *3GT* were raised in petal spot. We still found that all of unigenes related to carotenoid biosynthesis pathway have lower expression in pigmented spot region. Therefore, we speculate that the low expression of genes involved in carotenoid biosynthesis was necessary for brown spot formation.

The transcriptions of key anthocyanin biosynthesis genes are mainly regulated by the interactions among bHLH, R2R3-MYB and WD40 transcription factors (Koes *et al.*, 2005). R2R3-MYB transcription factor has been reported to regulate spot formation, such as *LhMYB21-Lat* and *LhMYB6* from *Lilium* (Yamagishi *et al.*, 2010; Yamagishi *et al.*, 2014), *NEGAN* from monkey flowers (Yuan *et al.*, 2014) and *PeMYB11* from Orchidaceae (Hsu *et al.*, 2015). In this study, 14 bHLH, R2R3-MYB and WD40 transcription factors up-regulated in petal spot were identified. They are likely to participate in the regulation of spot formation in *Lilium* by regulating expressions of anthocyanin biosynthesis genes. The results from phylogenetic analysis showed that Unigene19306_All and CL7202.Contig1_All annotated as R2R3-MYB and bHLH transcription factors are the main anthocyanin-promoting protein that determines the distribution of anthocyanin pigments in Asiatic hybrid lily spot.

Conclusion

The expressions of nine structural genes in anthocyanin biosynthetic pathway are the main contributors to the pigmentation of petal spot of Asiatic hybrid lily 'Easy Dance'. Six transcription factors annotated as bHLH, R2R3-MYB and WD40 regulated anthocyanin biosynthetic pathway are involved in spot formation of 'Easy Dance'. The decline in the expression of carotenoid biosynthesis gene is necessary to brown spot formation of Asiatic hybrid lily cultivar 'Easy Dance'. These results will further our knowledge about formation mechanism of petal spot in *Lilium*.

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