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Research paper

Comparative transcriptome profiling of genes and pathways involved in leaf-patterning of *Clivia miniata* var. *variegata*

Wang Qin-Mei[a,b], Cui Jianguo[b], Dai Hongyan[a], Zhou Yongbin[b], Li Na[c], Zhang Zhihong[a,*]

[a] College of Horticulture, Shenyang Agricultural University, Shenyang 110866, Liaoning, China
[b] College of Forestry, Shenyang Agricultural University, Shenyang 110866, Liaoning, China
[c] College of Land and Environment, Shenyang Agricultural University, Shenyang 110866, Liaoning, China

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ABSTRACT

Clivia miniata var. *variegata* (*Cmvv*) typically possesses yellow- and green-striped leaves. The striped plant not only has a high ornamental value but also be suitable for photosynthesis and chloroplast development research. Our previous study had revealed that yellow stripes (YSs) of *Cmvv* leaves contain chlorophyll-less ineffective chloroplasts. However, mechanism of *Cmvv* variegation is yet to be investigated. In the study, transcriptomes of both the YSs and green stripes (GSs) from single *Cmvv* leaves were compared using high-throughput sequencing. A total of 688 differential expression genes (DEGs) were identified based on biological replications. The qRT-PCR results indicated that transcriptome profiles accurately reflected global transcriptome differences between YSs and GSs. Subcellular localization analysis suggested that 56 DEG proteins were targeted to chloroplasts, and might be involved in anterograde signaling and leaf patterning. Moreover, the DEGs were mostly enriched in *photosynthesis* and *plant-pathogen interaction* KEGG pathways. Meanwhile, there should be coordination interaction between the two pathways. Seven of the eight DEGs involved in *photosynthesis* KEGG pathway were chloroplast-encoded genes and distributed among different cistrons of chloroplast DNA (cpDNA) large single copy regions (LSC) which are more prone to mutation. It was proposed that the YSs were caused by mutation(s) in cpDNA LSC. Thus, when the primary zygote of *Cmvv* was chimeric in LSC, leaf might be yellow- and green-striped. The study would give new insights into plant variegation and offers candidate genes to guide future research attempting to breed variegated plants.

1. Introduction

Photosynthesis is among the world's most important and ancient biological processes and able to convert light into the chemical energy needed by a living organism (Arnon, 1959). Photosynthesis is a highly complex multi-step process, involving the initial photosynthetic electron transport (PET, light reactions), the Calvin-Benson cycle (dark reactions), as well as subsequent steps involving the assimilation, transport, and utilization of photoassimilate (Berry et al., 2013). Almost all the green plants can carry out photosynthesis and their leaf-color mutants are common (Liu et al., 2016). Due to deficit in photosynthesis, the leaf yellow mutation is usually lethal during juvenile stage and not suitable for photosynthesis research (Yu et al., 2007). *Clivia miniata* var. *variegata* (*Cmvv*) is one of the leaf variegated mutants of *Clivia miniata*. However, *Cmvv* usually includes plants of three different phenotypes: green plant with near wild-type leaves, juvenile lethal yellow plant with full yellow leaves, and variegated plant with yellow- and green-striped leaves (Wang et al., 2016). The variegated phenotype can exhibit a marked heterogeneity in its occurrence and intensity among different striped *Cmvv* and among different leaves of a striped *Cmvv* (Fig. 1). The stable striped *Cmvv* not only has a high ornamental value but also be suitable for photosynthesis and chloroplast development research.

Abbreviations: PET, Photosynthetic electron transport; *Cmvv*, *Clivia miniata* var. *variegata*; cpDNA, Chloroplast DNA; CTP, Chloroplast transit peptide; IR, Large inverted repeat; LSC, Large single copy regions; SSC, Small single copy regions; YSs, Yellow stripes of leaves; GSs, Green stripes of leaves; Plant1, A mature striped *Cmvv* plant; Plant2, Another mature striped *Cmvv* plant; GS_1, Completely green stripe of a young striped-leaf in Plant1; YS_1, Completely yellow stripe of the same young striped-leaf in Plant1; GS_2, Completely green stripe of a young striped-leaf in Plant2; YS_2, Completely yellow stripe of the same young striped-leaf in Plant2; Nr, NCBI non redundant protein; Nt, NCBI nucleotide sequences; KOG, euKaryotic Ortholog Groups; Swissprot, Manually annotated and reviewed protein sequence database; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; CDS, Coding sequence; FDR, False discovery rate; lTP, Thylakoid lumenal transfer peptide; Indels, Insertions and deletions; PS II, Photosystem II; PS I, Photosystem I

* Corresponding author.
E-mail address: zhangz@syau.edu.cn (Z. Zhang).

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Fig. 1. The stable striped *Cmvv*. YS, yellow stripe; GS, green stripe. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Plant chloroplasts were established by endosymbiosis from free-living *Cyanobacteria* whose genome contains 5000–7000 coding genes (Nowack and Grossman, 2012). Plastidogenesis was accompanied by transfer of many endosymbiont genes to the host nuclear genome (Timmis et al., 2004) and there are only 20–200 genes in plant chloroplast DNA (cpDNA) now (Jansen et al., 2011). As a result, plant chloroplast has lost ability to live alone (Martin, 2003) and most of the proteins function in chloroplasts are encoded by nuclear genes and transported into chloroplasts via a chloroplast transit peptide (CTP) (Allen, 2003). The cpDNA organization is generally highly conserved among seed plants. Most cpDNA of seed plant have a quadripartite structure with two copies of a large inverted repeat (IR) separated by large single copy regions (LSC) and small single copy regions (SSC) (Jansen and Ruhlman, 2012). Chloroplast-encoded genes are regulated at the level of transcription and post-transcription (Mayfield et al., 1995). However, post-transcriptional regulation (control of mRNA stability and translation) is the major regulatory mechanism for chloroplast-encoded genes, while most nuclear-encoded, chloroplast-targeted genes are regulated at the level of transcription (Berry et al., 2013). There are crosstalk between chloroplast-encoded genes and chloroplast-related nuclear genes. Chloroplast-encoded genes and chloroplast-related nuclear genes are regulated via nucleus-to-plastid anterograde signaling and plastid-to-nucleus retrograde signaling (Oh and Montgomery, 2014). Since the expression products are targeted to and function in chloroplasts, why are so many genes transferred from endosymbiont/chloroplast to nuclear genome? This is probably because that the higher evolutionary rate and more complex gene regulation of nuclear genes could confer stronger adaptive capacity and phenotypic plasticity to plants (Wang and Zhang, 2015). Notably, nuclear-encoded genes are usually regulated by cytosine methylation/demethylation of the corresponding DNA CCGG sites (Wang and Wang, 2012), but cytosine of chloroplast-encoded genes are not methylated (Rousseau-Gueutin et al., 2011). Nevertheless, it was found that a *Cmvv* sequence, which was methylated at internal cytosine of a CCGG site in yellow stripes (YSs) but unmethylated in green stripes (GSs) of the same leaves, was highly similarity to the chloroplast-encoded gene *ycf2* (Wang et al., 2016). It is interesting to reveal whether *ycf2* of *Cmvv* has transferred from cpDNA (plastome) to nuclear genome and is regulated by cytosine methylation to affect leaf color.

Leaf variegation could arise from any mutation or down regulation of a large number of genes involved in the chlorophyll biosynthesis or/and chloroplast biogenesis (Li et al., 2017; Yu et al., 2007). It is very difficult to reveal the mechanism of plant variegation. It was found that some genes might be related to leaf patterning of *Cmvv* but, major genes of leaf patterning are yet to be determined (Wang et al., 2016). Transcriptome sequencing method is an efficient solution for discovering major genes, and therefore transcriptomes of YSs and GSs were compared using high-throughput sequencing in the study. Similar transcriptome analysis had been carried out on gold-colored mutant and wild-type leaves of *Ginkgo biloba* (Li et al., 2018), on white and green leaves of *Ananas comosus* var. *bracteatus* (Li et al., 2017), and on etiolated mutant, albino mutant and wild-type leaves of *Anthurium andraeanum* 'Sonate' (Yang et al., 2015). The study aim is to reveal the mechanism of *Cmvv* variegation, which can provide theoretical basis for transmitting the striped phenotype of *Cmvv* and molecular breeding of variegation.

2. Materials and methods

2.1. Plant material and RNA preparation

Two mature striped *Cmvv* plants (Plant1 and Plant2) grown in the Shenyang Agricultural University in Shenyang of China, were used as donor plants. However, Plant2 is a ramet derived from Plant1, thus, the two donor plants should have the same genetic composition. The completely yellow and green stripes of young striped-leaves were collected from both Plant1 and Plant2. Total RNA was extracted separately from the four leaf-stripe samples using Spin Column Plant total RNA Purification Kit (Sangon Biotech, China) following the manufacturer's protocol. Thereafter 1% agarose gels stained with ethidium bromide were used to assess RNA purity and integrity. The RNA purity was checked using the NanoPhotometer®spectrophotometer (IMPLEN, CA, USA) furtherly. RNA was quantified using Qubit® RNA Assay Kit in Qubit®2.0 Flurometer (Life Technologies, CA, USA). Furtherly, RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Two replicate RNA extractions from each of the four leaf-stripe samples were performed. Equal amounts of total RNA from each replication of a sample were pooled together for the following sequencing procedures (Huang et al., 2014). For convenience, the four leaf-stripe samples were renamed as follows: GS_1 (completely green stripe of a young striped-leaf in Plant1), YS_1 (completely yellow stripe of the same young striped-leaf in Plant1), GS_2 (completely green stripe of a young striped-leaf in Plant2), YS_2 (completely yellow stripe of the same young striped-leaf in Plant2). The subsamples of leaf stripes were collected for qRT-PCR verification. All subsamples were frozen in liquid nitrogen and stored at −80 °C (Yu et al., 2016).

2.2. cDNA library construction and sequencing

Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from 3 μg total RNA (per sample) using poly-T oligo-attached magnetic beads. Then the purified mRNA was broken into short fragments using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). These short fragments were used as templates to synthesize first strand cDNA. The second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. The remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3′ ends of DNA fragments, NEBNext adapter with hairpin loop structure were ligated to prepare for hybridization. The library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 μl USER Enzyme (NEB, USA) was used with size-selected, adapter-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. Subsequently, PCR was performed with Phusion High-Fidelity DNA polymerase using universal PCR primers and index (X) primer. The PCR products were also purified using AMPure XP system. Finally, after validating the library quality on the Agilent Bioanalyzer 2100 system, four paired-end cDNA libraries with an insert size of 300 bp were sequenced on Illumina