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Research article

# Gene expression analysis of bud and leaf color in tea

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ABSTRACT

Purple shoot tea attributing to the high anthocyanin accumulation is of great interest for its wide health benefits. To better understand potential mechanisms involved in purple buds and leaves formation in tea plants, we performed transcriptome analysis of six green or purple shoot tea individuals from a F1 population using the Illumina sequencing method. Totally 292 million RNA-Seq reads were obtained and assembled into 112,233 unigenes, with an average length of 759 bp and an N50 of 1081 bp. Moreover, totally 2193 unigenes showed significant differences in expression levels between green and purple tea samples, with 1143 up- and 1050 down-regulated in the purple teas. Further real time PCR analysis confirmed RNA-Seq results. Our study identified 28 differentially expressed transcriptional factors and A *CsMYB* gene was found to be highly similar to *AtPAP1* in Arabidopsis. Further analysis of differentially expressed genes involved in anthocyanin biosynthesis and transportation showed that the late biosynthetic genes and genes involved in anthocyanin transportation were largely affected but the early biosynthetic genes were less or none affected. Overall, the identification of a large number of differentially expressed genes offers a global view of the potential mechanisms associated with purple buds and leaves formation, which will facilitate molecular breeding in tea plants.



## 1. Introduction

Tea (*Camellia sinensis* L.) is a popular health drink in the world and its consumption has been closely correlated with a reduced risk of a number of diseases, including cardiovascular diseases, cancers and neurodegenerative disorders (Vauzour et al., 2010). To date, the world production and apparent consumption of tea have reached 5.17 and 4.76 million ton/year, which were increased by 46% and 38% respectively in the last ten years (International Tea Committee, 2015). The popularity of tea is not only attributed to its specific aroma and taste, but also owing to the high accumulation of polyphenols, which have lots of beneficial effects to human health.

Since the beginning of this century, there has been a growing interest in purple shoot tea, such as Benibana-cha, Sunrouge tea, Zijuan tea and Ziyan tea (Terahara et al., 2001; Saito et al., 2011; Jiang et al., 2013; Lai et al., 2016). These tea cultivars having purple colored buds and leaves are closely associated with anthocyanin accumulations (Saito et al., 2011; Jiang et al., 2013; Lai et al., 2016). Meanwhile, purple shoot teas were reported to have more pharmacological benefits as compared with ordinary tea. For example, Hsu et al. (2012) found that purple shoot tea had clear antiproliferative effects on colorectal carcinoma cells. Rashid et al. (2014) reported that tea anthocyanins could cross the blood brain barrier and reinforce the brain's antioxidant capacity in mice. Furthermore, Jiang et al. (2013) identified two anthocyanin components from Zijuan tea, which had higher antioxidant activities than commercial antioxidants butylated hydroxytoluene. The beneficial effects of the purple shoot tea raised questions about how anthocyanins are biosynthesized and accumulated in tea plants.

In model plant Arabidopsis, a number of genes involved in anthocyanin biosynthesis, transportation and regulation were characterized to be involved in anthocyanin accumulation (Appelhagen et al., 2014). Among them, a TTG1/bHLH/Myb

*Abbreviations:* AE, Amplification efficiency; DEG, Differentially expressed gene; FDR, False discovery rate; FPKM, Fragments per kilobase of transcript per million reads; GO, Gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; KOG, euKaryotic Ortholog Groups; LSD, Least significant difference; NR, Non-redundant protein.

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transcriptional complex plays a key role in regulating anthocyanin biosynthetic pathway (Gonzales et al., 2008). Further studies showed that the 'late' anthocyanin biosynthetic genes, such as dihydroflavonol reductase (*DFR*), anthocyanidin synthase (*ANS*) and UDP-glucose:flavonoid 3-o-glucosyltransferase (*3-GT*) are more closely correlated with anthocyanin accumulation than 'early' genes such as phenylalanine ammonia-lyase (*PAL*) and chalcone synthase (*CHS*) (Shan et al., 2009). However, whether it is the same case for tea plants still remains elusive. A lack of global view of potential genes involved in anthocyanin accumulation in tea plants largely limits relative research.

Recently, RNA-Seq method of high-throughput sequencing technology has been widely used to explore the potential mechanisms associated with important traits in *C. sinensis* (Paul et al., 2014; Li et al., 2015; Shi et al., 2015; Tai et al., 2015; Wei et al., 2015; Wang et al., 2016; Wu et al., 2016; Zhang et al., 2016). This method allows a comparison of the whole transcriptome of samples with large difference in certain trait or under treatments. Therefore, making comparison of the transcriptomes of green and purple shoot tea will identify candidate genes associated with anthocyanin accumulation and improve insights into the anthocyanin related mechanisms in *C. sinensis*.

In this study, we carried out RNA-Seq analysis of green and purple shoot tea from six F1 hybrids (three individuals with green buds and leaves and three individuals with dark purple buds and leaves) (NCBI BioProject Accession: PRJNA312027, http://www.ncbi.nlm.nih.gov/bioproject/312027). The purposes of this work were (1) to identify candidate genes associating with bud and leaf color formation in tea; (2) to shed light on the underlying mechanisms of anthocyanin accumulation in *C. sinensis*.

## 2. Materials and methods

### 2.1. Plant materials

A segregating F1 population using the Longjing43 (♀) and Baihaozao (♂) cultivars as parents with 327 individuals was grown in the experimental tea garden of TRICAAS in Hangzhou, China since 2012. Longjing43 is a popular cultivar for producing "Xihulongjing tea". Baihaozao is a high-production cultivar. Both cultivars are nationally registered in China. A moderately saturated SSR-based genetic linkage map was constructed based on this population (Tan et al., 2013). Further field observation showed that these F1 hybrids had a clear segregation in buds and leaves color in summer tea (Tan et al., 2016). Some kept green, but the other exhibited different degrees of purple color. These materials are useful for our research of the molecular mechanisms associating with bud and leaf color, as they have similar genetic backgrounds. According to field observation, three individuals with green buds and leaves (namely G1, G2 and G3) and three individuals with purple buds and leaves (namely P1, P2 and P3) were selected for anthocyanin and RNA-Seq analysis.

Fresh materials (one leaf and a bud) were collected from each individual on June 3, 2015 and stored at −80 °C. Parts of tea samples were ground by liquid nitrogen and subjected to anthocyanin analysis. The rest samples were used for RNA-Seq and quantitative real time PCR (qRT PCR) analysis.

### 2.2. Anthocyanin analysis

Total anthocyanin contents were measured with a spectrophotometer (UV-160 Shimadzu, Japan) by the method modified from Proctor (1974). Tea sample (0.1 g) was extracted with 10 ml of 0.1 M HCl in ethanol at 60 °C for 30 min with intermittent shaking (10 s on vortex mixer). The extract was filtered and its absorbance was determined at 530, 620 and 650 nm, respectively. The anthocyanin content measurement was based on the formula: $\Delta A = (A530 - A620) - 0.1(A650 - A620)$ (Proctor, 1974). Total anthocyanin contents were calculated by the formula: total anthocyanin (μmol/g) = $(\Delta A \times 100)/(4.62 \times$ sample weight). All data are presented as the mean ± SD (n = 3). Significance was determined via one-way analysis of variance, and for differences between groups, the least significant difference (LSD) *t*-test was employed ($P < 0.05$).

### 2.3. RNA sequencing analysis

Total RNAs of tea samples were extracted by TriReagent (Qiagen, Valencia, CA), and mRNAs were purified using the Truseq RNA Sample Prep Kit (Illumina). RNA sequencing of six tea samples (G1, G2, G3, P1, P2 and P3), de novo assembly and functional annotation were performed exactly according to the method described by Wei et al. (2014).

All tea samples were sequenced (125-bp paired-end read) according to the Illumina HiSeq 2500 instrument (Illumina, San Diego, CA, USA) using a customer sequencing service (Shanghai OE Biotech. Co., Ltd, China). The obtained sequence data were BLAST against NCBI non-redundant protein (NR), Swissprot, euKaryotic Ortholog Groups (KOG), and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases using an E-value cut-off of $10^{-5}$. Functional annotation by gene ontology (GO) terms (www.geneontology.org) was analyzed by the Blast2go software.

### 2.4. Identification of differentially expressed genes

For differential gene expression analysis, FPKM (fragments per kilobase of transcript per million reads) was used to measure the transcript abundances by RSEM follow trinity script. Cuffdiff was then used to determine differential expression (FDR ≤ 0.01) with blind dispersion methods (Trapnell et al., 2013). Furthermore,

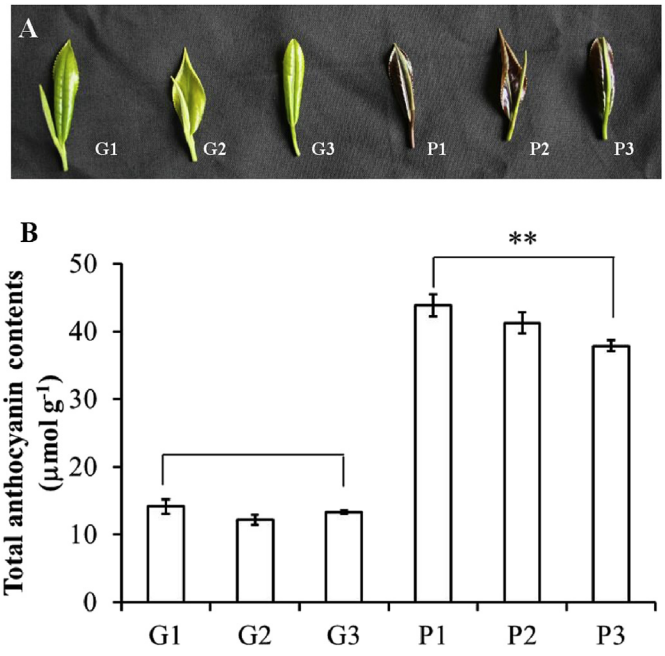


**Fig. 1.** The phenotypes and total anthocyanin contents in six tea samples. A. The phenotypes of six tea samples used in this study; B. The total anthocyanin contents in six tea samples. ** represents a highly significant difference between green and purple tea individuals ($P < 0.01$).