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Unraveling a crosstalk regulatory network of temporal aroma accumulation in tea plant (*Camellia sinensis*) leaves by integration of metabolomics and transcriptomics

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ABSTRACT

Terpenoid volatiles are major contributors to the floral odors and are responsible for the sensory quality of teas. However, little is known about the global regulation of various networks to achieve high terpenoids production in tea leaves. Here, metabolomics and transcriptome profiles were obtained from the tea leaves harvested in five different months. Our results showed that most sesquiterpene contents were lowest when sampled in April, increased markedly in June, and declined in August, September and October. An opposite accumulation pattern was observed in the majority of monoterpenes, and similar accumulation patterns were shared among a subset of sesquiterpenes and monoterpenes. We further found expression patterns of many genes in MVA pathway strongly correlated with the accumulation of some monoterpenes; as well, expression profiles of MEP pathway genes were closely associated with a few sesquiterpenes. A crosstalk regulatory network was constructed using co-expression analysis, and 13 transcription factors genes, with possible crucial roles in the regulation of terpene metabolism, were uncovered in the network. Additionally, *CsOCS2*, one key gene involved in terpenoid biosynthesis was functionally characterized *in vitro*. These results suggest that there might be crosstalk and competition for substrates between the down-stream monoterpenes and sesquiterpenes biosynthesis, which endow tea with its unique aroma, during different growth months.

1. Introduction

Excluding water, tea (*Camellia sinensis*) is the most consumed beverage worldwide, and its popularity is due in part to its sensory qualities and health benefits (Cai et al., 2004; Daglia et al., 2014). Aroma is one of the main sensory properties affecting tea quality (Yang et al., 2013). Tea aroma comprises terpenoid volatiles, lipids, carotenoids, phenylpropanoids and their glycoside derivatives (Ho et al., 2015); terpenoid volatiles with a relatively low detection threshold have been reported as key aroma compounds in tea (Christian and Peter, 2006). Usually, formation of tea aroma can be heavily influenced by tea variety, climate and harvest season (Yang et al., 2012; Zhou et al., 2017; Fu et al., 2015). According to the growing season, green tea can be divided into "spring tea", "summer tea", and "autumn tea" in China, which refers to tea harvested and processed before late May, between early June and early July, and after mid-July, respectively (Xu et al., 2012). Teas, which are processed from fresh tea plant leaves plucked in spring, are recognized as more desirable sensory quality (taste and aroma) than these in summer and autumn (Dai et al., 2015). The spring tea commonly contains higher levels of amino acids and moderate levels of catechins (thereby yielding a heavy, mellow, and brisk flavor), whereas summer/autumn tea usually contains higher levels of catechins and lower levels of amino acids (leading to a more bitter and astringent flavor) (Liu et al., 2016). However, the distribution characteristics of terpenoid volatiles contributed to aroma in teas at different growing seasons were still unclear; and very little is known of how and by which mechanism regulate the expression of related genes in terpenoid volatiles biosynthesis.

Terpenoids constitute the most diverse family of secondary metabolites and have been well studied in plants (Dudareva et al., 2013; Hemmerlin et al., 2012); they are derived from two common interconvertible five-carbon precursors, isopentenyl diphosphate (IPP) and its allylic isomer, dimethylallyl diphosphate (DMAPP) (Mcgarvey and Croteau, 1995). These C_5 precursors are synthesized from two compartmentally separated pathways: the cytoplasmic mevalonic acid (MVA) and the plastidial methylerythritol phosphate (MEP) pathways (Lichtenthaler, 1999). The MVA pathway provides precursors to sterols, certain sesquiterpenes, and the side chain of ubiquinone (Newman and

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Chappell, 1999; Sapir-Mir et al., 2008), while the MEP pathway gives rise to monoterpenes, diterpenes, carotenoids, and the side chains of chlorophylls (Lichtenthaler, 1999; Rohmer, 1999). However, very few studies focus on whether an exchange of precursor metabolites can occur between the cytosolic MVA and plastidial MEP pathways under certain conditions (Vranová et al., 2013).

Transcription factors (TFs) play important roles in regulating the expression of genes involved in terpenoid biosynthesis (Dudareva et al., 2013; Hong et al., 2012). In *Arabidopsis* inflorescence, sesquiterpene production is transcriptionally controlled through the *AtMYC2*, a basic helix–loop–helix TF targeting the TPS21 and TPS11 synthase promoter (Hong et al., 2012). Overexpression of the R2R3-type MYB TF *PtMYB14* in transgenic *P. glauca* plantlets leads to the accumulation of sesquiterpenes, which can be partly explained by up-regulation of terpenoid pathway transcripts, although no direct evidence of promoter interaction was presented (Bedon et al., 2010). The activity of *MsGPPS·LSU* in spearmint is suppressed through *MsMYB* binding directly to its promoter, which regulates monoterpene biosynthesis (Reddy et al., 2017). Despite some studies exploring TF-mediated regulation of terpenoid biosynthesis pathways, the mechanisms of gene regulation remains unclear in tea plant.

In the present work, we collected fresh leaves of tea plants at five different growing months in the same tea plantation, and investigated the regulation of volatile terpenoids by integration of metabolomics and transcriptomics. It was found that fine orchestration of *bHLHs*, *MYBs* and some functional genes coding key enzymes regulates biosynthesis of monoterpenes and sesquiterpenes in various growing months in tea plant; these regulatory changes might be triggered by light and mediated through the phytohormone JA based on co-expression analysis. These finding will promote our understanding of the complicated but important biosynthesis and regulation of terpenoids, and offer some suggestions for aromatic improvement of summer-autumn tea.

2. Materials and methods

2.1. Plant growth and samples

Cuttings of 8-year-old cloned tea plants (*Camellia sinensis* cv. Shuchazao) were grown in the experimental nursery under natural daylight conditions at the 916 Tea Plantation in Shucheng County (latitude N31.3, longitude E117.2), Anhui Province, China. 20 plants were planted in an experimental plot with a 120 cm row distance and 33 cm space between plants within a row, and three experimental plots, including 60 plants, were carried out in this study (Fig. S1). The tea plants were fertilized and watered by the same standards. The tea plants, with uniform height and canopy width, and without signs of disease and insects, were selected for our experiments.

The second fully expanded leaves (position with reference to the apical bud) on the same round shoots were picked from the tea plants in an experimental plot, and used as an independent biological replicate sample with mixture in a sampling month. Three biological replicates samples were performed for each sampling month.

The leaves were collected from 9:00 to 9:30 on April 10 (April; average temperature, 16.4 °C; precipitation, 115.4 mm; duration of sunshine 181.9 h), June 6 (June; average temperature, 28.26 °C; precipitation, 176.3 mm; duration of sunshine, 245.2 h), August 5 (August; average temperature, 26.9 °C; precipitation, 147.8 mm; duration of sunshine, 176.6 h), September 15 (September; average temperature, 22.9 °C; precipitation, 41.6 mm; duration of sunshine, 163.4 h) and October 18 (October; average temperature, 17.6 °C; precipitation, 56.7 mm; duration of sunshine 158.7 h) respectively in 2015. All the samples were immediately frozen in liquid nitrogen and then maintained at −80 °C until vacuum freeze-drying.

2.2. Chemicals

Authentic standards geraniol, linalool, *trans*-nerolidol, *cis*-nerol, *cis*-3-hexen-1-ol, citral, methyl salicylate, *cis*-3-hexenyl hexanoate, beta-ionone, geranyl acetone, beta-myrcene, D-limonene, beta-ocimene, alpha-farnesene, naphthalene, indole and geranyl diphosphate (GPP) ammonium salt were purchased from Sigma-Aldrich (Shanghai, China). *cis*-jasmone was purchased from Aladdin Industrial Inc. (Shanghai, China).

2.3. GC–MS analysis of volatile compounds in leaves

Volatile collection, identification and quantification were conducted, using headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography (Agilent 7697A)/mass spectrometry (Agilent 7890A) (GC/MS), as described in the Wang et al. (2008) study with some minor modifications. The three biological replicates of vacuum-freeze dried leaf samples (1.5 g) were separately ground into uniform powders with a mortar and pestle under liquid nitrogen. Each powder sample was placed in a 25 mL solid-phase microextraction (SPME) vial containing 15 mL of boiling distilled water, then sealed and kept at 80° C to equilibrate for 10 min in a water bath. Headspace sampling was conducted immediately following equilibration with a 65 μM PDMS/DVB fibre (Supelco, Bellefonte PA). After 30 min, the SPME syringe was introduced into the injector port of the GC–MS apparatus (Agilent 7697A/Agilent 7890A) with a DB-5 capillary column (30 m × 0.25 mm × 0.25 μm; Agilent) for GC–MS analysis (Liu et al., 2014, Han et al., 2016).

All compounds were identified based on comparison to mass spectral libraries (NIST), to compounds with known retention times, and when possible, pure standards were run with the same method. Compounds were quantified based either on the relative proportions of the constituents obtained by FID peak area normalization (Lv et al., 2012) or on the calibration curves (Table S1) established using a series of diluted solutions prepared with authentic standards. The concentrations of the volatiles were expressed as $\mu g\,kg^{-1}$.

2.4. LC–MS/MS analysis of phytohormones

The extraction, identification and quantification of JA and MeJA were conducted according to the method of Chen et al. (2013). Samples from the five harvest months (with three replicates) were analyzed using LC-ESI-Q TRAP-MS/MS. JA and MeJA were quantified in the multiple reaction monitoring (MRM) mode. The Analyst 1.5 software (AB Sciex, Framingham, MA, USA) was used for data acquisition, peak integration, and calculations. JA and MeJA were quantified by calculating the area of each individual peak.

Metabolite data were $\log_2$-transformed for statistical analysis to improve normality and normalized. The comparisons among different harvest months were estimated by Fisher's least significant difference (LSD) ($P < 0.05$).

2.5. Transcriptome sequencing and data analysis

Fifteen total RNA samples (five different months with three repetitions) were isolated from the second fully expanded leaves using the RNeasy Plus Mini kit (Qiagen, Valencia, CA, USA). The total RNA was quantified and the quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Fifteen libraries constructed using 1.5 μg of total RNA per sample were subjected to an Illumina HiSeq 4000 for sequencing.

Clean reads of fifteen samples were obtained after quality control. An index of the *Camellia sinensis* genome (Wei et al., unpublished data) was built using Bowtie2-2.2.9. Differential gene expression (DGE) in