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RNA-seq based transcriptomic analysis of CPPU treated grape berries and emission of volatile compounds

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ABSTRACT

Grapevine (*Vitis vinifera* L.) is considered to be one of the most popular and widespread fruit crops in the world. Numerous value added products are prepared from grape fruit and investments are being made to establish new viticulture region (Hoff et al., 2017; Imran et al., 2017). CPPU [forchlorfenuron N-(2-chloro-4-pyridyl)-N-phenylurea] is a synthetic cytokine-like plant regulator which promotes grape berry set and development. The influence of CPPU [forchlorfenuron N-(2-chloro-4-pyridyl)-N-phenylurea] on berry development of 'Shine Muscat' (*Vitis labruscana* Bailey × *V vinifera* L.) grapes was evaluated under field conditions. A concentration response was observed over a range of 0, 5, and 10 mg L^{-1} CPPU that was applied to fruitlets (mean diameter 6 mm) at 2 weeks after full bloom. Gas-chromatography mass-spectrometry (GC–MS) revealed that volatile compounds such as terpenoids and aromatics; especially linalool, geraniol and benzyl alcohols, were greatly reduced in CPPU-treated grapes. In contrast, aliphatics, such as hexanol, were increased in CPPU-treated berries. RNA sequencing (RNA-Seq) was conducted to identify differentially expressed genes (DEGs) that were induced by CPPU, especially those related to volatile biosynthesis. A total of 494, 1237, and 1085 DEGs were detected in CPPU0-vs-CPPU5, CPPU0-vs-CPPU10, and CPPU5-vs-CPPU10 treatments, respectively. The results were compared against two databases (Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG)) to annotate gene descriptions and assign a pathway to each gene. GO covers three domains: biological processes, molecular functions and cellular components. Pathway enrichment annotation demonstrated that highly ranked genes were associated with the fatty acid degradation and biosynthesis, phenylpropanoid metabolism and biosynthesis, carotenoid biosynthesis, and plant hormone signal transduction. Analysis with qRT-PCR of twelve selected transcripts validated the data obtained by RNA-seq. Additionally, we also found that genes such as *CCDs* (carotenoid cleavage dioxygenase), *LOX* (lipoxygenase), *GGDP reductase* (geranylgeranyl diphosphate reductase), *PAL* (phenylalanine ammonia-lyase) and some hormones related genes, were closely involved in the formation of volatiles compounds in CPPU treated berries. In summary, our results provide the first sequential transcriptomic atlas of CPPU treated grape berries which significantly increases our understanding of volatile metabolites and biosynthesis pathways in grape affected by CPPU.

1. Introduction

The grapevine (*Vitis vinifera* L.) is one of the most widely grown fruit species worldwide, which is consumed fresh as well as in the form of several value added products (Imran et al., 2017; Khalil-Ur-Rehman et al., 2017a,b). Production of grapes for fresh consumption is gradually increasing (Rolle et al., 2011) and plant growth regulators are an important tool in grape production. Gibberellic acid (GA) was first used to increase the size of table grape berries approximately 40 years ago (Weaver and MacCune, 1961); and it continues to be extensively used by growers. Plant hormones like GAs and ABA play an important role in different stages of plant growth and development (Khalil-Ur-Rehman et al., 2017a,b). CPPU [forchlorfenuron,N-(2-Chloro-4pyridinyl-N-phenylurea] is a synthetic cytokine-like plant regulator that promotes grape berry set and development at a low concentration (Nickell, 1987; Reynolds et al., 1992; Retamales et al., 1994; Dokoozlian et al., 2000).

Abbreviations: GC–MS, gas-chromatography mass-spectrometry; DEGs, differentially expressed genes; GO, gene ontology; KEGG, kyoto encyclopedia of genes and genomes; GA, gibberellic acid; PHIs, pre-harvest intervals; MRLs, maximum residue limits; TA, titratable acidity; TSS, total soluble solids; RPKM, reads per kilobase of exon model per million mapped reads; FDR, false discovery rate

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The market value of fruit crops is dependent on their quality (taste and aroma) and consumers prefer high quality fruit with an attractive appearance, high nutritional value, and good taste (Bruhn et al., 1991). Aroma is one of the most appreciated fruit characteristics and is a complex trait which is comprised of a large number of volatile compounds; whose composition is specific to species and often to the variety of fruit (Tomás-Barberán and Robins, 1997; Schwab et al., 2008). Different fruits often share several aromatic characteristics. Depending upon the combination of volatiles and the concentration and perception threshold of individual volatile compounds, each fruit has a distinctive aroma (Seymour et al., 2012).

CPPU is used for the production of grapes on a commercial scale to increase berry size. CPPU affects the fruit size by acting synergistically with endogenous auxins, inducing parthenocarpy, and promoting cell division and lateral growth (Moriyama et al., 1994; Zhang and Whiting, 2011). The residues of all plant growth regulators are considered safe when applied in viticulture at the recommended dose and frequency when pre-harvest intervals (PHIs) are strictly followed (Ugare et al., 2013). CPPU has been registered in many countries with maximum residue limits (MRLs) that are established in a range between 0.01 and 0.1 $mgkg^{-1}$ (Ainalidou et al., 2015). However, selection for important agricultural traits often results in the loss of aroma and taste in grape and other fruits. It is plausible that CPPU may reduce grape quality by reducing the release of flavor-associated volatiles from the berries at maturity. Volatile flavor compounds are likely to play a key role in determining the perception and acceptability of products by consumers. Being the principal sensory identity and characteristic flavor of the fruit, the identification of key volatile flavor metabolites is essential because they provide the unique character of the natural fruit (Cheong et al., 2010). Most fruits produce numerous types of aroma volatiles as they ripen. Even though many of these volatile compounds are produced in trace amounts, below the threshold of detection for most analytical instruments; they can still be detected by human olfaction (Goff and Klee, 2006). Various fresh fruits often share many aroma characteristics and many volatile compounds have been identified in apple, pear, strawberry and grapes (Schwab et al., 2008; Nijssen et al., 2012; Kahle et al., 2005; Diéguez et al., 2003; Rosillo et al., 1999). In grapes, the emission of aromatic and non-aromatic volatile compounds, such as monoterpenes, alcohols, esters and carbonyls, plays a key role in determining consumer choice (Retamales et al., 1994; Diéguez et al., 2003). For example, free terpenols such as linalool and geraniol, have been identified as major aroma compounds in both red and white grapes (Rosillo et al., 1999). 'Shine Muscat' and other muscat grapes are aromatic cultivars and contain an abundance of volatiles such as terpenoids, aromatics and aliphatics (Fenoll et al., 2009).

Studies have shown that the most convenient and efficient methods to identify the genes related to secondary metabolic pathways are transcriptomics combined with metabolic analyses after treatment with stress or exogenous elicitors. Plants have the capacity to synthesize, accumulate and emit low molecular weight secondary metabolites that are mostly derived from carbohydrate compounds, saturated and unsaturated fatty acids and some amino acids (Bohlmann and Keeling, 2008; Wink, 2011). Although a large number of emitted volatiles have been detected from grape, only a fraction of these compounds have been identified as components that are involved with the perception of fruit aroma and flavor based on their quantitative abundance and olfactory thresholds. Previous studies have investigated the physiological and molecular mechanisms of plant growth regulators, on volatile compounds in different crops (Zabadal and Bukovac, 2006; Antognozzi et al., 1996; Shi et al., 2015). To the best of our knowledge, however, no attempt has yet been made to study the effect of CPPU on volatile compound of grapes using a transcriptomic approach.

Limited data is available pertaining to the specific metabolic pathways that are involved in the biosynthesis of these aroma volatiles. The recently developed deep sequencing technologies represent the most efficient transcript profiling methods that are available to date. Among these, RNA-seq allows a comparison of the transcriptome of grape with and without CPPU treatment to enable the identification of candidate genes for the biosynthesis of volatiles related metabolites. Hence, we performed transcriptomic analysis using high throughput Illumina sequencing and volatile metabolite analysis using gas chromatography mass spectrometry (GC–MS) to identify the CPPU responsive volatile secondary metabolic pathways. The identified genes were used for subsequent annotation analysis to provide a platform of transcriptome information for grape genes. In this study, we focused on the identification of terpenoids and other certain volatile metabolism related genes in grape berries that are induced by CPPU. The transcript expression profiling data generated through the RNA-seq approach will provide a foundation of information that will be impactful for the grape industry by serving as a reference point to enable future strategies which aim to improve fruit quality.

2. Materials and methods

2.1. Plant materials

In the present study, five-year-old 'Shine Muscat' grape plants were grown under a rain shelter covered with polyvinyl film and supplemented with drip irrigation at the Nanjing Agricultural University Vineyard located in Tangshan Valley, Nanjing, Jiangsu province, China. Plants were spaced 3 m apart within rows and 5 m apart between rows. On May 15, 2015, clusters of fruitlets on three separate grape vines were selected at random and divided into three groups. The first and second group of clusters were sprayed @ 5 and 10 $mg\,L^{-1}$ of CPPU, respectively. The remaining group of clusters was sprayed with water and served as a negative control. Berries were harvested twice, with the first harvest for RNA-sequencing on July 15, 2015 (early-maturity). Each collection was performed with three biological replicates and RNAs isolated from the three replicates were mixed at 1:1:1 ratio for library construction and sequencing. All of the samples were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction. The second harvest occurred on August 05, 2015 (maturity approximately 80 days after treatment) and these samples were immediately transported to the lab and observed at 4 °C. They were then used for the determination of volatile compositions and the characterization of other physiological parameters. The second harvest date was incorporated into the study to ensure that the balance between sweetness, acidity, and flavor contents was optimized.

2.2. Total soluble solids (TSS) and titratable acidity (TA)

Fresh grape berries were used for the determination of TSS and TA. Berry pulp was homogenized with a laboratory blender (Grindomix GM 200; Retsch, Haan, Germany) at 8500 rpm for 30 s. Total soluble solids (TSS) were determined using an Abbé refract meter (type Rx-5000; Atago, Tokyo, Japan). Titratable acidity (TA), which was expressed as tartaric acid in grams per 100 g of fresh weight, was determined by titration with 0.1 N NaOH to a final pH of 8.1 using an automatic titration system according to previously described methods (Titrino 702 SM; Metrohm, Herisau, Switzerland) (Steingass et al., 2014). TSS and TA were analyzed in triplicate for each berry and the data were subjected to analysis of variance. Differences between the means were tested at the 5% level using a Duncan's multiple range test.

2.3. Determination of volatile compounds by GC–MS

Each berry sample was finely cleaned with distilled water, seeds were manually removed and juice was extracted. After centrifugation at 4000 rpm for 5 min, 8 ml of supernatant was placed into a headspace bottle with NaCl (3.0g) and 3-octanol (818 $mg{\cdot}L^{-1}$, 5 μl) was used as an internal standard. Each container was placed in a 50 °C water bath for 30 min. Extracts were adsorbed onto SPME fiber (Supelco, USA) on a