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VcFAS, *VcSUN* and *VcOVATE* orchestrated the fruit morphogenesis in southern highbush blueberry during the pre-anthesis and fruit development

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ABSTRACT

Fruit morphology is an important trait closely related to fruit quality and quality parameters evaluated for new cultivar breeding. *FASCIATED* (*FAS*), *SUN* and *OVATE* are characterized as key genes that regulate fruit morphology in tomato and other fruits, however, its underlying mechanism driving variation in blueberry is not known. In this study, *FAS*, *SUN* and *OVATE* genes were isolated from southern highbush blueberry 'O'Neal' (10–14 locules) and 'Bluerain' (10 locules). *VcFAS* and *VcSUN* gDNAs contained 5 introns and 4 exons, whereas no intron was detected in the *VcOVATE* gDNAs. Additionally, between two cultivars, long-fragment insertions/deletions (in/dels) were found in the 1st, 2nd and 5th introns of *VcFAS* and in the 2nd introns of *VcSUN* gDNAs. The expression levels of *VcFAS*, *VcSUN* and *VcOVATE* were significantly higher in the expanded leaves and lower in the rootlets than those in the stems. *VcFAS* and *VcSUN* mRNAs in the 'Bluerain' flower buds were dramatically higher than in those of 'O'Neal', and partially lower in the 'Bluerain' fruits than in those of 'O'Neal'. Compared with *VcFAS* and *VcSUN*, *VcOVATE* transcripts were relatively low throughout the flower bud and fruit development, although the *VcOVATE* transcripts were somewhat higher in the early flower bud and fruit stages of 'Bluerain' than in those of 'O'Neal'. The results indicated that *VcFAS*, *VcSUN* and *VcOVATE* might influence blueberry fruit morphology at pre-anthesis and post-pollination stages, but particularly at the pre-anthesis stages, and the in/dels of *VcFAS* and *VcSUN* introns might affect fruit shape and size through regulating the locule number during flower bud enlargement.

1. Introduction

Fruits surrounding the seeds of flowering plants are the unique reproductive tissues in angiosperms. The primary functions of fruits are protecting, nurturing and dispersing seeds. Similar to vegetables, fruits are also instrumental to human nutrition and health, particularly as sources of carbohydrate, dietary fiber, vitamin C, thiamine, niacin, pyridoxine, folic acid, and minerals, among others (Wargovich, 2000), and might decrease the incidence and mortality of a variety of chronic diseases (Oguntibeju et al., 2013).

Fruits display substantial morphological variation in size and shape, and fruit morphology is an important trait closely related to fruit quality and quality parameters evaluated by customer preference and for new cultivar breeding (Díaz et al., 2014; Wang et al., 2016). Fruit shape and size are typically inherited as quantitative genetic traits, but are considerably affected by non-genetic factors such as the natural environment (Rylski, 1973). In recent years, genome and cDNA sequencing, QTL mapping and other molecular techniques have been applied to characterize in more details the genes that control fruit traits such as shape and size. The considerable QTLs and/or genes involved in genetic regulation and control of fruit morphology in the Solanaceae were reviewed by Monforte et al. (2014), Tanksley (2004), van der Knaap and Ostergaard (2017), van der Knaap et al. (2014), and Wang et al. (2016). Of these genes, *FASCIATED* (*FAS*) regulates fruit shape by affecting locule number, whereas *SUN* and *OVATE* are key regulators controlling fruit elongation, an important feature that affects fruit shape.

FAS is mapped to the bottom of tomato chromosome 11 and encodes a member of the YABBY-like transcription factor family (Huang and van der Knaap, 2011). The *fas* mutant, resulting from a ~294 Kb inversion with a breakpoint in the first intron of *YABBY2*, leads to increase locule number and fruit size (Cong et al., 2008; Huang and van der Knaap, 2011). FAS controls the final fruit size and regulates the shape by affecting meristem organization and boundary information (van der Knaap et al., 2014). *SUN*, named for *Solanum lycopersicum* variety Sun 1642, is located on chromosome 7 of *S. lycopersicum* and encodes a

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member of the IQD family of calmodulin-binding proteins. Mutant *sun* causes bilaterally symmetric tomato fruit elongation after pollination and during the cell division stage of fruit development (van der Knaap and Tanksley, 2001; van der Knaap et al., 2014). OVATE, mapped to tomato chromosome 2, encodes a member of OVATE Family of Proteins (OFPs) (Wang et al., 2016). The mutant *ovate*, with a G-to-T premature stop codon mutation, leads to the pear-shaped fruits in tomato, and *SlOVATE* is expressed from 10 days before pollination through 8 days after anthesis (Liu et al., 2002). As transcription repressors, OFPs could regulate fruit elongation, ovule and vascular development, DNA repair, secondary cell wall formation, firmness and sugar accumulation, among others (Liu et al., 2018; Wang et al., 2016). However, most fruit morphological genes were characterized from model plants, such as those in the Solanaceae, Brassicaceae and Curcurbitaceae (Langowski et al., 2016; Lippman and Tanksley, 2001; Perin et al., 2002; Wang et al., 2015). The molecular mechanisms regulating fruit morphology in non-model species, particularly in berries, are rarely elucidated.

Blueberry (*Vaccinium corymbosum*) belongs to Ericaceae family and genus Vaccinium, with approximately 450 species worldwide (Retamales and Hancock, 2012). The consumption of blueberry has increased substantially, because the fruits contain high contents of bioactive compounds, such as anthocyanins, flavonoids, polyphenols, and ascorbic acids, which play important roles in improving the immune system of humans and scavenging free radicals (de Souza et al., 2014; Konarska, 2015; Pescie et al., 2018). The aim of breeders is to breed commercial blueberry cultivars with high productivity, fruits of uniformly large size and vigor, improved disease resistance, intense fruit color, longer fruit storage, expanded harvest dates, and stronger tolerance to abiotic stresses (Hancock et al., 2008). Among those commercial traits, the shape and size of blueberry fruits are important factors to influence production and customer purchase (Jorquera-Fontena et al., 2017).

The present work aimed to study the effect of *FAS*, *SUN* and *OVATE* gene structures and expression levels in southern highbush blueberry 'O'Neal' (a flat-round and large fruit cultivar) and 'Bluerain' (a round and small fruit cultivar) during the floral bud and fruit development, and evaluate its biological functions to locule numbers and fruit morphogenesis. Therefore, locule numbers of flowers and fruits, cDNAs and genomic DNAs (gDNAs) structures and relative expression models of two blueberry cultivars were analyzed.

2. Materials and methods

2.1. Plant materials

The investigations were conducted in 2014 and 2015. Two cultivars of 4 (5)year-old blueberry (*V. corymbosum*) in the orchard of Zhejiang Normal University (Jinhua, Zhejiang Province, China) were used and, included the flat-round and large fruit cultivar 'O'Neal' and the round and small fruit cultivar 'Bluerain'. Young roots, stems and fully expanded young leaves, with flower buds and fruits at different stages (Fig. S1), were collected according to Dernisky et al. (2005) and Zifkin et al. (2012). The receptacles from stages IV to S0, wiped free of the corolla and petals, were used to analyze the relative gene expression levels. All samples were frozen immediately in liquid nitrogen and stored at −80 °C until further use. At least three biological replicates from each preparation were collected to minimize variation during processing.

2.2. Paraffin section preparation and microscopy observation

3–5 flower buds and fruits from different developmental stages of 'O'Neal' and 'Bluerain' were randomly selected for microscopic analysis. Samples were fixed in FAA (5% formaldehyde, 5% glacial acetic acid and 50% ethanol) solution in the vacuum for more than 3 d, then dehydrated, cleared and embedded according to the procedures developed by Wang et al. (2014). Cross sections of fruits ranged from 10 to 25 μm in thickness, whereas flower buds ranged from 8 to 10 μm. All sections were rehydrated and stained with saffranin and fast green (Wang et al., 2014). Images of the sections were scanned with Virtual Slide Microscopy VS120 (Olympus Co., Ltd, Shanghai, China). The locule numbers were analyzed following the standard developed by Blaker (2013).

2.3. gDNA and RNA extraction and cDNA synthesis

gDNA was extracted from young leaves using a modified cetyltrimethylammonium bromide (CTAB) method (Levi and Rowland, 1997), and residual RNA was removed using RNase A (TakaRa Biotechnology Co., Ltd., Dalian, China). Total RNA was isolated using the CTAB method developed by Chang et al. (1993). The quality and concentration of gDNA and total RNA were assessed using 1.5% agarose gel electrophoresis and spectrophotometric analyses, respectively. Purified RNA (1.0 μg) was used for reverse transcription using a PrimeScript™ RT Reagent kit with gDNA Eraser (TakaRa Biotechnology Co., Ltd., Dalian, China). Synthesized 1st-strand cDNAs were diluted 3-fold and stored at −80 °C for further use.

2.4. Isolation of gDNAs and cDNAs of VcFAS, VcSUN and VcOVATE

Specific primers were designed based on partial/full-length sequences (*VcFAS*: scaffold377; *VcSUN*: scaffold1030; *VcOVATE*: scaffold199) from the website GDV (Genome Database for *Vaccinium*, https://www.vaccinium.org/) and the software IGB (Integrated Genome Browser) for *V. corymbosum*. Table 1 showed the primer information. The genomic fragments of *VcFAS* were too long to be amplified directly, and were assembled using overlapping partial segments with internal primer pairs (Table S1). 3′ RACE was performed using a 3′-Full RACE Core Set with a PrimeScript™ RTase kit (TakaRa Biotechnology Co., Ltd., China) according to the manufacturer's protocol. To validate the accuracy of the open reading frame (ORF) sequence, high-fidelity enzyme PrimeSTAR® HS DNA Polymerase (TakaRa Biotechnology Co., Ltd., Dalian, China) was used to amplify the full-length cDNAs of target genes. All desired fragments were purified by an AxyPrep DNA Gel Extraction Kit (Axygen Biotechnology Co., Ltd., Hangzhou, China), and sequenced (Thermo Fisher Scientific Inc., Shanghai, China).

Similarity analyses of nucleotide and amino acid sequences were conducted using the BLAST program at the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). The online bioinformatics tool ProtParam (http://www.expasy.org) was used to analyze the deduced amino acid sequences. The deduced amino acid sequences of *VcFAS*, *VcSUN*, *VcOVATE* and other plant homologous sequences were aligned by default parameters and used to generate a phylogenetic tree using MEGA version 4.0 software. The phylogenetic tree was constructed with a distance matrix using the neighbor-joining method.

2.5. Relative expression analysis of VcFAS, VcSUN and VcOVATE genes in various organs and developmental stages

The relative expression levels of *VcFAS*, *VcSUN* and *VcOVATE* genes in the vegetative tissues and, flower bud and fruit developmental stages were analyzed using real-time quantitative reverse transcription PCR (qPCR). *VcGAPDH* was used as the internal control to normalize the amount of cDNA in the samples (Die and Rowland, 2013). Based on isolated cDNA sequences, specific primers were designed for qPCR analysis (Table 1). qPCR reactions were performed in an ABI StepOne Plus™ RT-PCR system (Applied Biosystems Co., Ltd., Beijing, China). The PCR reaction system, procedures and data analysis were referred to Liu et al. (2016). The *VcFAS* transcripts at stage S0 was chosen to normalize the gene expression models in various organs and developmental stages. The statistical significance of relative expression levels