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Expression of grape *ACS1* in tomato decreases ethylene and alters the balance between auxin and ethylene during shoot and root formation

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ABSTRACT

Ethylene plays an important role in the grape rachis, where its production can be 10 times higher than in the berry. *VvACS1* is the only rachis-specific ACC synthase (ACS) gene, and its expression is coincident with ethylene production in the rachis of *Vitis vinifera* 'Thompson seedless'. *VvACS1* was cloned and ectopically expressed in tomato (*Solanum lycopersicum* 'Moneymaker'). Lateral buds were increased in two- or four-week-old 35s::*VvACS1* transgenic tomato plants after transplanting. Compared with wild-type (WT) plants, the transgenic tomato plants showed higher expression of the *VvACS1* gene in the flowers, leaves, rachis, and fruits. There was no obvious difference of ACS activity in the fruit of tomato, and only increased ACS activity in the rachis of tomato. Ethylene production was decreased in flowers, leaves, and fruits (seven weeks after full bloom), while the relative expression of endogenous tomato *ACS1* and *ACS6* genes was not down-regulated by the ectopic expression of *VvACS1*. These results imply that post-transcriptional or post-translational regulation of ACS may occur, resulting in lower ethylene production in the transgenic tomato plants. Moreover, expression of *VvACS1* in tomato resulted in decreased auxin and increased zeatin contents in the lateral buds, as well as reduced or delayed formation of adventitious roots in lateral bud cuttings. RNA-Seq and qRT-PCR analyses of rooted lateral bud cuttings indicated that the relative expression levels of the genes for zeatin *O*-glucosyltransferase-like, auxin repressed/dormancy-associated protein, and ERF transcription factors were higher in transgenic tomatoes than in WT, suggesting that ethylene may regulate auxin transport and distribution in shoots and that adventitious root formation employs coordination between auxin and ethylene.

1. Introduction

Ethylene plays a key role in plant growth and development (Moeder et al., 2002) and is an important mediator of plant responses to environmental stimuli (Kende, 2003). The ethylene biosynthesis pathway is well understood in higher plants (Yang and Hoffman, 1984) and is highly regulated by two key enzymes, ACS (1-aminocyclopropane-1-carboxylic acid synthase) and ACO (1-aminocyclopropane-1-carboxylic acid oxidase) (Kende, 2003). In higher plants, both ACS and ACO enzymes are encoded by multigene families, and expression and function of these genes show different spatial and temporal patterns (Barry et al., 2000; Sunako et al., 1999; Oraguzie et al., 2004, 2007; Costa, 2005; Jiang et al., 2011; Ye et al., 2017).

In climacteric fruits, ethylene synthesis and perception have been widely shown to be essential for fruit ripening (Lelievre et al., 1997; Bolitho et al., 1997; Martinis and Mariani, 1999; Barry et al., 2000). For instance, in apple, *MdACS1* plays an important role in fruit ripening, and the allele *MdACS1-2* is related to a long shelf life of fruit (Sunako et al., 1999; Oraguzie et al., 2004, 2007). In non-climacteric fruit, ethylene is involved either directly or indirectly in the regulation of fruit development and organ senescence (Chervin et al., 2008; Pech et al., 2008; Aizat et al., 2013).

Grape is a typical non-climacteric fruit, and ethylene production in the fruit is quite low (Munoz-Robredo et al., 2013). However, ethylene production in the rachis, the main axis of the grape cluster, can be quite high in some grape genotypes. For instance, in *Vitis vinifera* 'Thompson seedless,' the ethylene production level is 10 times higher in the rachis than in the berry, and a peak occurs before harvest (Ye et al., 2017). Ethylene is involved in the process of rachis browning after harvest. Ethylene production in the rachis is induced by exogenous abscisic acid and ethephon and restrained by ethylene inhibitors such as 1-methylcyclopropene (1-MCP) (Li et al., 2015). All previous research suggests

Abbreviations: ACS, 1-aminocyclopropane-1-carboxylic acid synthase; ACO, 1-aminocyclopropane-1-carboxylic acid oxidase; WAFB, weeks after full bloom; WAH, weeks after harvest; DEGs, differentially expressed genes; ZOG, zeatin *O*-glucosyltransferase-like; ERFs, ethylene response factors

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that ethylene plays an important role in rachis development and senescence.

In grape, there are at least 10 *VvACS* and 3 *VvACO* gene family members, each with distinct expression patterns during berry development (Xu and Wang, 2012; Munoz-Robredo et al., 2013; Ye et al., 2017). Among these, *VvACS1* is the only rachis-specific gene. In the rachis of 'Thompson seedless,' *VvACS1* showed expression levels coincident with ethylene production during both berry development and storage, which led to the hypothesis that expression of this organ-specific gene, *VvACS1*, plays important roles in rachis senescence (Ye et al., 2017). In order to understand the roles of the *VvACS1* gene in ethylene production during ripening and senescence of berry and rachis, the *VvACS1* gene was cloned from 'Thompson seedless' and ectopically expressed in tomato (a climacteric fruit; *Solanum lycopersicum* 'Moneymaker', an open-pollinated, indeterminate variety), which is widely used as a model plant to identify genes' functions (Kimura and Sinha, 2008). The transgenic tomato plants were evaluated for ethylene production, physiological characteristics, and phytohormone contents.

2. Material and methods

2.1. Plant material and growth conditions

The full-length cDNA of the grape *ACS1* gene (*VvACS1*, GenBank number: GSVIVT01019920001) was prepared from grape leaf tissues (*V. vinifera* 'Thompson seedless') using the Smart RACE kit (Clontech, USA) according to the manufacturer's protocol (Forward primer: cacc ATGAGAGTGATAGTTCCTTTAC, reverse primer: TCATCTGGGGGATA TACAG). The *VvACS1*gene was then cloned into an entry vector (pENTR/D-TOPO) with pENTR Directional TOPO cloning kits (Invitrogen, USA) and was then verified with sequencing. The VvACS1 gene was transferred from the entry vector into the destination vector (pCHF3-GW1) with an LR recombination reaction using Gateway LR Clonase Enzyme Mix (Invitrogen, USA) (Lin et al., 2013). The *VvACS1* gene in the binary expression vector was under the control of the CaMV 35S promoter and the nopaline synthase (Nos) terminator. Cotyledons of *in vitro* tomato plants (*Solanum lycopersicum* 'Moneymaker') were used as explants for transformation, and transgenic plants were obtained using published methods (Wang and Bouzayen, 2005). All experiments were carried out using transgenic lines from T1 generations.

Tomato seeds from wild-type (WT) and transgenic lines were sown in a peat matrix in a seedling tray. Leaf samples were collected three weeks later for screening by PCR. Four-week-old tomato seedlings were transplanted into a greenhouse at the Henan Agricultural University Experimental Station from March to June of 2015 under natural conditions. Eighty-four tomato plants for each transgenic line or WT were grown in three plots. Lateral buds, plant height, and ethylene production were investigated once a week. All plants grew well without disease or insect infestations, with no special measures taken during the experiments.

For the analysis of ACC synthase activity, ethylene production, and auxin and zeatin levels, 20 leaves, flowers, inflorescence axes, mature fruits, rachis, or lateral buds were collected from 20 plants in each experiment, and each experiment was repeated three times.

2.2. Analysis of lateral bud formation

During the preliminary test, we observed differences in formation time and number of lateral buds between transgenic tomato plants and WT. To analyze the differences, the average number of lateral buds at each node was calculated at four weeks after transplanting. Twenty similarly grown tomato plants were analyzed in each experiment, and each experiment was repeated three times.

2.3. VvACS1 gene expression in various organs

To analyze expression of *VvACS1* in transgenic tomato plants, young leaves (the first to the third node), flowers (full blooming), and mature fruits (eight weeks after full blooming, 8 WAFB) were collected at the tenth week after transplanting. Total RNA was isolated from 100 to 200-mg samples using the CTAB method (Gambino et al., 2008). RNA quality and quantity were assessed in a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA) and by electrophoresis in a 1.5% agarose gel.

Total RNA (1–2 μg) was purified with DNaseI treatment and reverse transcribed using PrimeScript™ RT Reagent Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The first-strand cDNA reaction was diluted to 80–100 μL with RNase-free water. Quantitative real-time RT-PCR of *VvACS1* was carried out with SYBR® Premix Ex Taq™ II kit (TaKaRa, Dalian, China) in an ABI PRISM 7500 FAST Sequence Detection System (Applied Biosystems, USA) using the following primer sequences: forward primer: CGTGGCAGGGTTGAAACA ATTAGGA, reverse primer: CCCTTTCTCACTGTAAGAGCGGATT (Ye et al., 2017). Reactions (20 μL total volume) contained 1 μL cDNA template, 2 μL primers (0.4 μM each forward and reverse primer), 10 μL SYBR® Premix Ex Taq ™ II solution, and 7 μL water. The amplification reaction was conducted at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 31 s, and a final dissociation step at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Each experiment was repeated three times with three biological replicates.

To compare expression of *VvACS1* in various organs and tissues of the transgenic tomato plants, GAPDH was used as a reference gene. For each sample, relative expression was calculated using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001).

2.4. Total enzymatic activity analysis of ACC synthase

Young leaves (the first to third node), mature leaves (the seventh to eighth node), flowers (full blooming), inflorescence axes, immature (4 WAFB) and mature fruits (8 WAFB) were collected from 60 tomato plants at the tenth week after transplanting to assay total ACC synthase enzymatic activities. After harvest, ACC synthase activities of tomato fruits and rachis were assessed at the first, second, third, and fourth week. ACC synthase extraction and activity measurement were performed according to the method of Zheng et al. (2005). Briefly, frozen samples (2 g) were homogenized with 2 mL extraction buffer (4 mM dithiothreitol, 10 μM pyridoxal phosphate, 10 mM ethylenediaminetetraacetic acid) and then centrifuged at 2500*g* for 20 min at 4 °C. The supernatant was collected, from which a 0.8 mL aliquot was added to a 1.2-ml reaction mixture (0.25 mM s-adenosyl methionine, 30 μM pyridoxal phosphate), sealed in a 25-ml vial, and incubated for 60 min at 30 °C. To stop the reaction, 0.1 mL $HgCl_2$ (25 mmol/L) was added. Afterward, 0.2 mL of a cold mixture of NaOCl and NaOH (2:1, v/v) was injected into the vial, and the solution was vortexed and incubated on ice for 3 min. Finally, 1 mL of gas was taken and analyzed in a gas chromatograph (GC 2010PLUS, Shimadzu, Japan) as described (Li et al., 2015). Each experiment included three samples, and each sample was measured two or three times.

2.5. Ethylene production in tomato plants

Ethylene levels in leaves (including young and mature leaves), flowers (full blooming), inflorescence axes, and mature fruits (8 WAFB) were analyzed at the tenth week after transplanting. In addition, tomato fruits and rachis at different developmental stages (1, 3, 5, 7, and 8 WAFB) and different storage periods (one, two, three, and four weeks after harvest, WAH) were collected for analysis.

Ethylene analysis of each sample was performed using gas chromatography (GC; GC 2010PLUS, Shimadzu, Japan) with a flame ionization detector as described (Li et al., 2015). Each sample was collected