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Research article

The molecular events of IAA inhibiting citrus fruitlet abscission revealed by digital gene expression profiling

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ABSTRACT

Citrus fruits possess two abscission zones (AZ), AZ A and AZ C located at the pedicel and calyx, respectively. Early citrus fruitlet abscission (CFA) exclusively occurs at AZ A. Previous data have shown that indole-3-acetic acid (IAA) could inhibit fruitlet abscission. However, its role in CFA remains vague. In this study, we first removed the ovaries of fruitlets in order to exclude their interferences. Then, the calyxes were treated with IAA, gibberellin 3 (GA_3) and 6-benzylaminopurine (6-BA), respectively. The results have shown that IAA could prevent CFA from taking place, while either GA_3 or 6-BA could not. When IAA concentration decreased to a value between 30 mg/L and 40 mg/L, CFA occurred, showing a concentration-dependent manner. Digital gene expression analysis revealed that 2317 corresponded to IAA treatment, of which 1226 genes were closely related to CFA. The most affected genes included those related to biosynthesis, transport and signaling of phytohormones, primarily ethylene (ET), abscisic acid (ABA) and auxin as well as protein ubiquitination, ROS response, calcium signal transduction, cell wall and transcription factors (TFs). The results obtained in this study suggested that the IAA in AZ A could suppress ethylene biosynthesis and signaling, and then inhibit abscission signaling. To our knowledge, it is the first time to reveal the key role of IAA in CFA, which will contribute to a better understanding for the mechanism underlying CFA.

1. Introduction

Fruit abscission is a naturally occurring process that enables fruit trees to abandon no longer needed, infected or damaged fruits which propagates to be negative to plant survival (Xie et al., 2013). In citrus, two successive and distinguishable abscission waves are observed (Iglesias et al., 2006; Xie et al., 2015a, 2015b). The first wave is characterized by massive flower and ovary abscission from AZ A which locates at the fruit pedicel, and the second is named 'June drop' that growing fruitlets separated from abscission AZ C which locates between calyx and ovary. In practice, abscission from AZ A is a major yield reduction factor for citrus and the excessive level of CFA could result in a large economic loss for the growers.

Accumulating data have shown that CFA is coordinately regulated by hormones, such as ethylene, IAA, ABA, GAs and so on (Gómez-Cadenas et al., 2000; Iglesias et al., 2006; Xie et al., 2013, 2015a, 2015b). At present, it's widely accepted that ethylene plays a crucial role in CFA because application of an inhibitor of ethylene biosynthesis, named aminoethoxyvinyl-glycine (AVG), significantly increases citrus fruitlet set; on the contrary, an ethylene precursor, i.e. 1-aminocyclopropane-1-carboxylic acid (ACC), could effectively promote CFA (Gómez-Cadenas et al., 2000; Iglesias et al., 2006). Similar results have been obtained in other fruit crops, including apple (Stopar et al., 2007), plum (Meland and Kaiser, 2016), mango (Hagemann et al., 2015), tomato (Zhang et al., 2015) and litchi (Li et al., 2015). Many genes involved in ethylene biosynthesis (*ACO* and *ACS*) and signaling (*ERS*, *ETR*, *CTR* and *ERF*) are related to abscission (Ruperti et al., 1998; Dal Cin et al., 2005; Li et al., 2015). However, in Arabidopsis or tomato, abscission still occurs in ethylene perception mutants albeit delays (Lanahan et al., 1994; Bleecker and Patterson, 1997; Whitelaw et al., 2002), which suggests that ethylene may be critical for dictating the timing of organ shedding, but not for abscission to take place (Basu et al., 2013). Like ethylene, ABA and jasmonic acid (JA) could also enhance fruit abscission (Gómez-Cadenas et al., 2000; Xie et al., 2013; Li et al., 2015), in which ABA is considered as a synergist to ethylene by enhancing ACC content and JA seems to initiate abscission process in an ethylene-independent manner (Xie et al., 2013).

Contrary to abscission-promoting hormones, IAA is able to inhibit fruit abscission. Perturbing polar auxin transport (PAT) with a PAT inhibitor (2, 3, 5-triiodobenzoic acid) obviously promotes sweet cherry

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fruit abscission (Else et al., 2004; Blanusa et al., 2005). However, application of IAA to the distal end of bean leaf explants delays cell separation (Jacobs, 1962), showing the level of IAA within AZ tissues might play a more crucial role in organ abscission than ethylene does (Basu et al., 2013). A hypothesis proposed by several reports suggests that auxin could decrease the sensitivity of AZ tissues to ethylene, thereby inhibiting fruit shedding (Bangerth, 2000; Iglesias et al., 2006; Smith and Whiting, 2010; Botton et al., 2011). Taylor and Reed (2001) also thought that the auxin-ethylene balance within AZ tissues decided the timing of shedding, and high level of IAA could prevent AZ cells from being separated. While, in pea the accumulating IAA at a node or above the junction could promote ethylene production (Bangerth, 1989). A few years later, Abel et al. (1995) also have revealed that Arabidopsis *ACS4* is specifically induced by IAA, supporting this conclusion at the molecular level. These results prompt us to investigate the molecular mechanism on how auxin decreases the sensitivity of AZ tissues to ethylene in citrus.

In Arabidopsis, auxin signaling as a prerequisite for organ shedding have been reported (Basu et al., 2013). Arabidopsis lines carrying T-DNA insertions in *AUXIN RESPONSE FACTOR 2* (*ARF2*) show delay in floral organ abscission, which further adds weight to the hypothesis that IAA may act within the AZ tissues to regulate the timing of organ shedding (Ellis et al., 2005; Basu et al., 2013). Although GA_3 and 6-BA are widely used for increasing citrus fruitlet set (García-Martínez and García-Papí, 1979), Xie et al. (2015a, b) reported that IAA could inhibit CFA and several genes related to auxin degradation and signaling involved in CFA. However, the molecular mechanism of IAA inhibiting CFA remains unknown.

Prior to abscission, the cell wall need to be degraded by several hydrolytic enzymes such as polygalacturonase (PG), cellulases, pectate lyase and β-1,4-glucanase (Wu and Burns, 2004; Zhu et al., 2011; Basu et al., 2013). Silencing of an abscission-related *PG* gene using a T-DNA insertion obviously delayed floral organ shedding, showing the involvement of PG in cell separation (González-Carranza et al., 2007). In tomato, three PG genes, including *TAPG1*, *TAPG2*, and *TAPG4*, each with different spatial and temporal expression profiles, are involved in fruit abscission (Kalaitzis et al., 1997). Expansins are specifically involved in abscission (Estornell et al., 2013). In Arabidopsis, *AtEXP10* was expressed in petiole, midrib, leaves and vestigial pedicel AZ, and the transgenic plants over-expressing *AtEXP10* show higher incidence of complete breakage of pedicels compared to control plants (Estornell et al., 2013). In citrus, the hydrolytic enzymes involved in AZ A cell wall degradation remain to be identified.

In this study, we demonstrated that IAA played a decisive role in CFA. Moreover, the molecular mechanism auxin inhibiting CFA was also investigated with digital gene expression profiling. The findings obtained in this study lay a basis for in-depth understanding for CAF from AZ A.

2. Materials and methods

2.1. Plant material and treatments

This experiment was carried out in a citrus orchard which is located at the Citrus Research Institute, Southwest University in Chongqing city, China. Sixteen twelve-year-old citrus trees (*Citrus sinesis* L. cv. 'Cara Cara') grafted on *Poncirus trifoliate* (L.) Raf. were used. All fruitlets used in this study were at 7 days postanthesis (DPA) and developed from leafy single-flowered inflorescence. In 2013, we have randomly tagged 400 fruitlets and removed their ovaries from calyxes (Fig. 1A). Then, 3 droplets, about 0.15 ml, of GA_3 (120 mg/L) (García-Martínez and García-Papí, 1979; Nawaz et al., 2008), 6-BA (110 mg/L) (García-Martínez and García-Papí, 1979), IAA (100 mg/L) (Xie et al., 2015a) and water (control 1) were dropped on calyxes using a dropping pipette, once per day and 100 calyxes for each treatment. In parallel, 100 calyxes with ovaries severed as control 2, and the calyxes were also treated with water. The abscission rate was calculated by formula as follows:

Abscission rate = (Number of abscising calyxes/Total number of calyxes)*100% (1)

In 2014, to unravel the molecular mechanism that IAA inhibits CFA from AZ A (Fig. 1B), we randomly selected 1200 fruitlets and removed their ovaries. After that, 300 calyxes were treated by IAA (100 mg/L) and 900 by water once per day, with the method mentioned above. At 0, 2, 4 days after water treatment, AZ A tissues were collected by manually cutting at about 1 mm on each side of the abscission fracture plane, 300 calyxes for each time and the samples were referred as to ROF_0d, ROF_2d and ROF_4d, respectively. At 4 days after IAA treatment, the AZ tissues, referred as to ROF_IAA, were collected the same as described above. All the samples with three biological replicates were quick frozen using liquid nitrogen and stored at −80 °C until used. To understand whether IAA inhibits CFA in a concentration-dependent manner, 360 fruitlets were randomly selected. After ovaries removed, 30 calyxes for each treatment were treated with 12 different concentrations of IAA with the method mentioned above, once per day. The concentrations included 0, 1, 5, 10, 20, 30, 40, 60, 80, 100, 150 and 200 mg/L. The abscission rate was calculated by the abovementioned formula (1).

2.2. RNA isolation and cDNA library preparation

Based on manufacturer's instruction, total RNAs from three replicates of ROF_0d, ROF_2d, ROF_4d and ROF_IAA were isolated with Trizol reagent (TransGen Biotech, Beijing, China). RNA degradation and contamination we assessed on 1% agarose gel. The concentration was measured with DeNovix DS-11 Spectrophotometer (DeNovix, Wilmington, USA). Then, total RNAs from three replicates were mixed in equal amount to produce ROF_0d, ROF_2d, ROF_4d and ROF_IAA pools for RNA-Seq quantification. Using oligo (dT)-attached magnetic beads, messenger RNA (mRNA) was separated and then 1 mg mRNA per sample was fragmented with divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). Then, the cleaved mRNA fragments were synthesized into first-strand cDNA using random hexamer primer and M-MuLV Reverse Transcriptase. DNA polymerase I and RNase H were used to produce second strand cDNA which followed by end repair and ligation of the adapters. After that, cDNA fragments with 150–200 bp in length were isolated with AMPure XP system (Beckman Coulter, Beverly, USA). These fragments, then, were purified and enriched by PCR to produce the final cDNA libraries. Before sequenced on an Illumina Hiseq 2000/2500 platform (Illumina, San Diego, USA), the Library quality was assessed on the Agilent Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA, USA).

2.3. Reads quality control and reads mapping

In-house perl scripts were written to remove adapter sequences and reads containing ploy-N and low-quality bases. After that, clean reads were obtained and their Q20, Q30 and GC contents were also calculated. After pre-processing, four libraries with clean reads were obtained, which were the basis for all the subsequent analyses.

Reference genome (Sweet orange) and gene model annotation files were all downloaded directly from genome website (http://citrus.hzau.edu.cn). Bowtie v2.0.6 (Langmead and Salzberg, 2013) was used to build the index of reference genome and TopHat v2.0.9 (Kim et al., 2013) aligned clean reads to the reference genome. For unigene DEG, Bowtie v0.12.9 was used to aligned single-end clean reads to the unigene sequences.