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Original Paper

SmCOI1 affects anther dehiscence in a male-sterile *Solanum melongena* line

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Abstract Anther indehiscence is an important form of functional male sterility that can facilitate the production of hybrid seed; however, the molecular mechanisms of anther indehiscence-based male sterility have not been thoroughly explored in eggplant (*Solanum melongena* L.). Here, we used two-dimensional gel electrophoresis to compare the protein profiles in the anthers of normally developing (F142) and anther indehiscent (S16) *S. melongena* plants. Four differentially expressed proteins were identified using matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry. Of these proteins, the transcript accumulation of the eggplant *CORONATINE INSENSITIVE1* (*SmCOI1*) was significantly downregulated in S16 relative to F142. Phylogenetic analysis showed that SmCOI1 has high amino acid sequence similarity and clustered into the same subgroup as its homologs in other members of the *Solanaceae*. Subcellular localization analysis showed that SmCOI1 localized to the nucleus. Moreover, reverse-transcription quantitative PCR revealed that the jasmonic acid pathway genes *SmJAZ1* and *SmOPR3* are upregulated in F142 relative to S16. Protein–protein interaction studies identified a direct interaction between SmCOI1 and SmOPR3, but SmCOI1 failed to interact with SmJAZ1. These findings shed light on the regulatory mechanisms of anther dehiscence in eggplant.

Key words: 2D-PAGE, anther indehiscence, protein–protein interaction, *SmCOI1*, *Solanum melongena* L..

Introduction

Eggplant (*Solanum melongena* L.), which is thought to have originated from Africa, is an important and popular vegetable crop widely cultivated in Asia, Africa, Europe, and the Near East. The use of hybrid vigor in the breeding of vegetable cultivars was first described in eggplants. Reliable male-sterile systems for eggplant could be valuable for simplifying and reducing the time, labor, and cost involved in the production of hybrid seeds (Mennella et al. 2010). In eggplant, functional genic male sterility (GMS) has been reported (Burke et al. 1984; Filippone and Lurquin 1989; Jasmin 1954; Pettigrew and Nuttall 1963). These GMS lines show anther indehiscence, in which the anthers do not open to release pollen, thereby disabling pollination. Compared to other types of plant male sterility, research addressing the mechanisms of functional male sterility and the breeding of functional male sterile cultivars is relatively scarce, especially in eggplant.

In anther dehiscence, mature pollen grains are released from the locules of the anther for pollination (Shih et al. 2014). Although the molecular mechanisms controlling anther dehiscence remain relatively unclear, it is known that the process of anther formation and pollen release is regulated by anther-specific genes. Anther dehiscence is regulated by a complex pathway and mutation or overexpression of the key genes can cause defects in anther development and male sterility. Expression of the *Arabidopsis thaliana* gene *REDUCED MALE FERTILITY* (*RMF*) is restricted to anthers and pollen grains; overexpression of *RMF* alters anther development (Kim et al. 2010). *Arabidopsis SAF1* (Kim et al. 2012), *CA2* (Villarreal et al. 2009), and *AHP4* (Jung et al. 2008) overexpression lines have anther indehiscence phenotypes that involve secondary thickening of the inner wall of anthers. The *Arabidopsis* anther defective mutant *DELAYED DEHISCENCE1* (*dde1*) has a delayed

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anther tissue degeneration phenotype related to anther indehiscence (Sanders et al. 2000).

The JA pathway is one of the most important pathways regulating anther dehiscence. (Chini et al. 2007; Devoto et al. 2002; Fonseca et al. 2009; Katsir et al. 2008; Rushton et al. 2008; Thines et al. 2007). The *Arabidopsis coronatine insensitive1* (*coi1*) mutant is susceptible to exogenous jasmonic acid (JA) and has defects in pollen and anther and restoration of fertility after application of exogenous JA (Xie et al. 1998). COI1, as the core member of the JA signal transduction pathway, associates with SKP1, CUL1, RBX1, and the SCF^{COI1} ubiquitin ligase complex to mediate JA signaling (Yan et al. 2009). *COI1* was first cloned from *Arabidopsis* in 1998 and has since been isolated and cloned from many plant species. In *Solanum lycopersicum*, downregulation of the COI1 protein causes insensitivity to JA signals, the inability of seeds to mature normally, and diminished resistance (Li et al. 2004). However, anther dehiscence research on *S. melongena* L. remains in its infancy. Thus far, it remains unclear whether COI1 physically interacts with other proteins to control anther dehiscence in *S. melongena* L.

In this work, we compare the proteomic profiles of F142 and S16 of *S. melongena* L. and investigate the protein–protein interactions of SmCOI1 with SmOPR3 and SmJAZ1. We also report the subcellular localization of SmCOI1 and the expression profiles of *SmCOI1*, *SmOPR3*, and *SmJAZ1*. These will help develop our understanding of the molecular mechanisms and biological function of anther dehiscence in eggplant.

Materials and methods

Plant materials and anther preparation

The S16 (anther indehiscent) and F142 (normal plant) *S. melongena* L. lines were grown at the Institute of Vegetables and Flowers, Chongqing Academy of Agricultural Sciences (Chongqing, China) from 2017 to 2019. The eggplant seeds were sterilized and sown in trays. Then, the seedlings on the day of flowing were transferred and grown under normal conditions. Anthers on the day of flowering were isolated for two-dimensional difference gel electrophoresis (2D-DIGE) analysis. Selected anthers of different stages before flowering were immediately frozen in liquid nitrogen and stored at −80°C until they were used for further analysis.

Two-dimensional electrophoresis (2-DE) experiment

Two-dimensional electrophoresis (2-DE) was performed using the phenol extraction protocol. Isoelectric focusing (IEF) was performed on the 2-DE system at 20°C with a current limit of 50 μA. The second dimension was performed in 1-mm-thick, 12.5% (w/v) polyacrylamide slab gels. At least three biological replicates were performed on each sample.

Silver nitrate staining

The proteins on the gel were stained with 0.2% silver nitrate and observed with the PDQuest Software.

Gel imaging and analysis

2-DE and protein extraction were performed by methanol/ammonium acetate precipitation and phenol extraction (Nian et al. 2016). The extracted sample pellet was solubilized with lysis buffer [2 M thiourea, 5 M urea, 20 mM dithiothreitol, 3.3 mM Tris-HCl (pH 7.4), 2% CHAPS, 0.002% bromophenol blue, 2% IPG buffer]. The Bradford method was used to determine protein concentrations before electrophoresis (Bradford 1976). Dry IPG strips (200×3×0.5 mm, pH 3–7 linear, GE) were hydrated for 16 h in 450 μl of lysis buffer containing 400 μg of protein. IEF was done using a 2-DE system (GE Healthcare, Piscataway, NJ, USA) at 20°C with a current limit of 50 μA. The second dimension of separation was achieved in 1-mm-thick, 12.5% (w/v) polyacrylamide gels. Three replicates were assessed for each sample. After 2-DE separation, the 2-DE gel was scanned on a gel imaging system and was subtracted, spot detected, and matched using the Image Master 2D Platinum software to obtain spot position coordinates and statistically analyze the protein spots. Four differentially expressed protein spots were excised and analyzed by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometry.

Cloning of Solanum melongena L. COI1

The sequences of *Solanaceae COI1* in the GenBank database were used to perform BLASTP. Based on the conserved regions of *COI1* in Solanaceous plants, primers were designed for cloning *Solanum melongena* L. *COI1* (Supplementary Table S1). The total RNA of eggplant was extracted using an RNA Pure Plant Kit (TIANGEN) and reverse transcribed using the Genome Walking Kit (TaKaRa) according to the manufacturer's instructions. The *SmCOI1* coding sequence was amplified by PCR using *COI1*-F and *COI1*-R primers. The target bands were isolated using agarose gel electrophoresis and retrieved using the OMEGA Gel Extraction Kit (D2500). The target fragment was cloned, transformed, and identified.

Subcellular localization

SmCOI1 was sub-cloned and ligated into pCAMBIA1300 to generate the recombinant plasmid. This plasmid was then transformed into Agrobacterium (GV3101), which was used for transfection of *Nicotiana benthamiana* and subsequent subcellular localization of the SmCOI1-GFP by confocal laser scanning microscopy.

Yeast two-hybrid assay

SmCOI1, *SmOPR3*, and *SmJAZ1* were separately subcloned into the activation domain of pGADT7 and pGBKT7 using the BamHI and XhoI sites and then ligated into pGADT7 or pGBKT7 to construct the recombinant plasmids. Using the Matchmaker Gold Yeast Two-Hybrid System, the pGADT7 or

pGBKT7 recombinants were transformed into Y187 and Y2H (Clontech) and transformed yeast strains were fused and plated on SD inducing medium containing -Ade/-His/-Leu/-Trp. In some experiments, the following chemicals was also added: 60 μM JA-Ile. Plates were incubated for up to 5 days at 30°C. Verifying the protein interactions of SmCOI1 with SmOPR3 and SmJAZ1 in yeast.

Pull-down assay

SmCOI1 was sub-cloned into the pET32a(+) vector, while *SmOPR3* and *SmJAZ1* were cloned into the pGEX-4T-1 vector. Then, the plasmids were transformed into *E. coli* Rosetta (DE3) competent cells and 1.0 mM isopropyl β-D-thiogalactoside was added before incubation at 37°C for 3.5 h. The SmCOI1-HIS protein was purified using BeaverBeads IDA-Nickel Kit-10 (Beaver). The SmOPR3-GST and SmJAZ1-GST proteins were purified by BeaverBeads GSH (Beaver). The purified SmCOI1-HIS protein was added to a 2 ml centrifuge tube, and HIS magnetic beads were added and incubated for 45 min at 22°C for 60 rmp to allow the magnetic beads to adsorb the SmCOI1 protein with HIS tag. Pour off the supernatant after magnetic separation, GST-OPR3 and GST-SmJAZ1 proteins were added to incubated for 45 min at 22°C for 60 rmp, respectively. Pour off the supernatant after magnetic separation, bounded protein eluted by imidazole solution from magnetic beads. Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to detect whether the protein with a HIS tag captures the GST-tagged protein. If a HIS-tagged protein is capable of capturing a GST-tagged protein, both the HIS-tagged protein and the GST-tagged protein will be present in the lane, otherwise only the HIS-tagged protein will be present. In addition, in some experiments, the following chemicals was also added: 60 μM JA-Ile.

Quantitative real-time PCR

Gene expression was analyzed in eggplant from 5 days before flowering to 2 days after flowering by reverse-transcription quantitative PCR (qRT-PCR). The primers used to test the transcript levels of *AT1G18080-like*, *Fe-SOD*, *PKp*, *SmCOI1*, Sm*OPR3*, and Sm*JAZ1* are shown in Supplementary Table S1, using *GAPDH* as the internal reference. The qRT-PCR mixtures contained 2 μl primers, 2 μl cDNA, 10 μl SsoFast EvaGreen Supermix (Bio-Rad), and distilled water to a final volume of 20 μl. The reaction conditions were: 95°C for 30 s, 95°C for 5 s, 59°C for 30 s, and 65°C for 5 s (39 cycles). All RT-qPCR results are presented as means±standard error (SE) of three biological replicates, and each sample was quantified in triplicate. The relative expression level of the genes was calculated using the $2^{-\Delta\Delta Ct}$ (Pfaffl 2001) equation.

Results

Morphological comparison of the F142 and S16 lines

Male sterility is an important tool for leveraging eggplant heterosis. Anther indehiscence is an important form of functional male sterility. By morphological analysis, we observed that anthers were indehiscent in S16 relative to F142 (Figure 1). On the day of flowering in F142, the anthers presented small holes to release pollen, whereas in S16, the anthers were tightly closed without dehiscence.

Two-dimensional electrophoresis analyses

The isolation of differentially expressed proteins comparing F142 and S16 was a principal aim of this experiment. By 2-DE analysis of anther proteins, we found that F142 and S16 produce distinct protein profiles. To establish a 2D gel map of the anther proteins, a narrow-range (pH 3–10) IPG strip was used to minimize point overlap (Figure 2). Approximately 43 protein points were detected on each gel using Image Master2D Platinum software 6.0, among which four differentially expressed protein points were identified.

Identification of differentially expressed proteins by mass spectrometry

In this study, comparing the anther protein profiles of the *S. melongena* L. F142 and S16 lines, we identified four highly differentially expressed protein points by MALDI-TOF/TOF mass spectrometry (Table 1). The isoelectric points of the four differentially expressed protein points

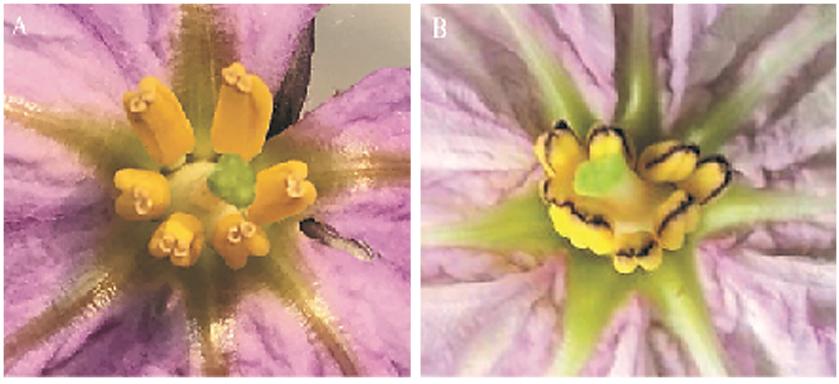

Figure 1. Morphological analysis of *Solanum melongena* L. F142 (A) and S16 (B) flowers and anthers.

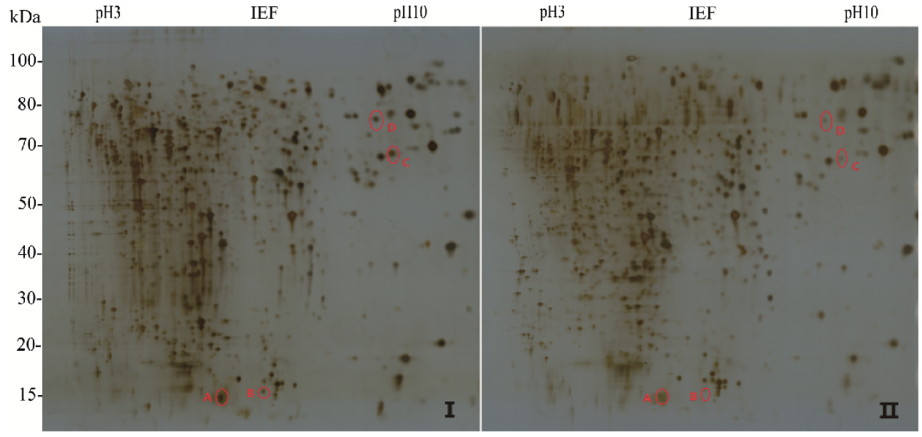

Figure 2. Two-dimensional electrophoresis maps of anther proteins from *Solanum melongena* L. F142 (I) and S16 (II). Protein (60 μg) was loaded on the 17 cm IPG strip with a linear gradient of pH 3–10. Proteins were visualized by silver staining. The letters indicated the differentially expressed proteins.

Table 1. Differentially expressed proteins identified by MALDI-TOF/TOF-MS/MS.

Spot No.	Accession No.	Protein name	Protein PI	Protein MW	Protein score	Coverage rate
A	gi|295828462	AT1G18080-like protein	8.50	20221.32	228	49.45%
A	gi|295828464	AT1G18080-like protein	8.50	20221.32	228	49.45%
A	gi|295828466	AT1G18080-like protein	8.50	20221.32	228	49.45%
B	gi|312837924	Fe superoxide dismutase 1	6.26	23018.98	199	18.71%
B	gi|334701491	Fe superoxide dismutase 1	6.26	23018.98	199	18.71%
C	gi|350535701	coronatine-insensitive 1	5.01	148068.57	220	44.99%
C	gi|350535701	coronatine-insensitive 1	5.01	148068.57	220	44.99%
C	gi|350535701	coronatine-insensitive 1	5.01	148068.57	220	44.99%
D	gi|332645501	pyruvate kinase family protein	7.12	57915.32	123	44.21%
D	gi|184097402	pyruvate kinase family protein	7.12	57915.32	123	44.21%
D	gi|297322016	pyruvate kinase family protein	7.12	57895.31	123	44.21%

was predicted to be in the range 5.01–8.50, and the protein molecular weight was predicted to be 13886.9–148068.57 Da. The protein score was predicted to be 123–242, and the coverage rate was predicted to be 17.92–60.44%. Through database retrieval, these differentially expressed protein points were identified as a Guanine nucleotide-binding proteins (G-protein), iron superoxide dismutase (Fe-SOD), CORONATINE INSENSITIVE1 (COI1), and pyruvate kinase (PKp). All four proteins were downregulated in S16 relative to F142.

Analysis of transcript accumulation

Next, we tested the transcript levels of the genes encoding the four differentially expressed proteins. The *COI1* transcript levels were higher in F142 than in S16 and peaked at Stage 6 (Figure 3C). We detected no significant differences in the transcript abundance of *AT1G18080-like*, *Fe-SOD*, and *PKp* when comparing F142 and S16 (Figure 3A, B, D). Consequently, we inferred that anther dehiscence could be regulated by *SmCOI1*.

Sequence analysis of SmCOI1

The 1653 bp *SmCOI1* cDNA fragment was amplified by PCR, sequenced, and found to encode a protein of 551 amino acids with a predicted molecular mass of about 62.8 kD and an isoelectric point of 5.01. The *SmCOI1* alignment and phylogenetic analysis were performed using Geneious software. We found that the *SmCOI1* of *S. melongena* L. had high similarity with *COI1* homologs from other Solanaceous plants (Supplementary Figure S1). These results demonstrate the accuracy of *SmCOI1* cloning in this study.

Subcellular localization

To further verify the function of SmCOI1, we next investigated the subcellular localization of SmCOI1 through transiently expressing 35S::GFP-SmCOI1 in *Nicotiana benthamiana* inner epidermal cells. In this assay, GFP-SmCOI1 was localized in the nucleus (Figure 4). This is consistent with previous observations in *Arabidopsis* (Yan et al. 2018).

Expression analyses of OPR3 and JAZ1 in different stages in S. melongena L

Anther dehiscence is regulated by JA (Ishiguro et al. 2001; Xiao et al. 2014). Mutations in the genes involved in JA biosynthesis typically cause a delay or failure

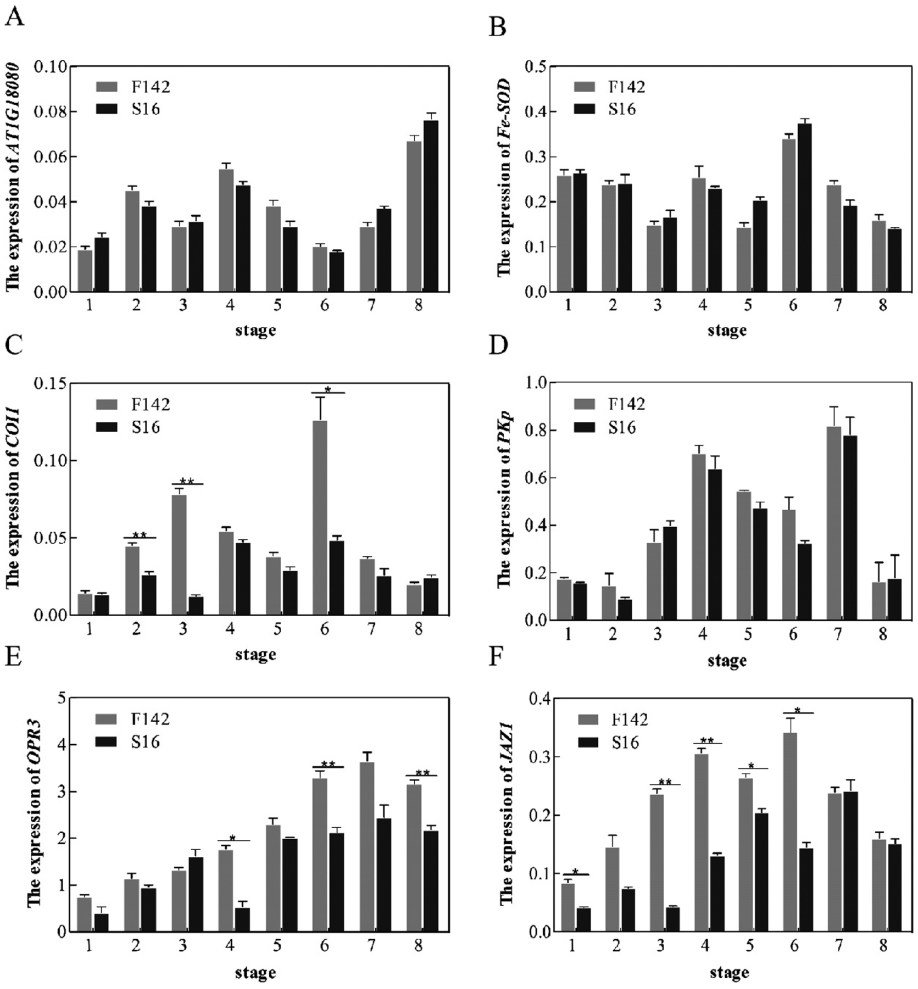

Figure 3. The transcript accumulation of *AT1G18080* (A), *Fe-SOD* (B), *COI1* (C), *PKp* (D), *OPR3* (E), and *JAZ1* (F). 1–8: 5 days before flowering to 2 days after flowering. Total RNA was extracted at each stage and used for RT-qPCR analyses. Three biological replicates (each including three technical repeats) were assessed. Paired *t*-tests, * $p<0.05$, ** $p<0.01$.

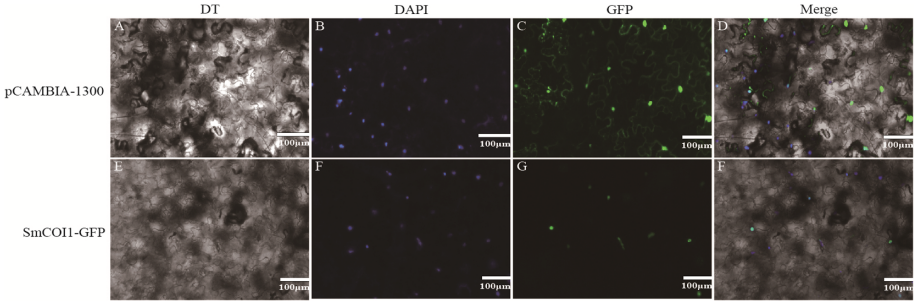

Figure 4. Subcellular localization of SmCOI1-GFP localized in the nucleus of *Nicotiana benthamiana* epidermal cells. (A–D) pCAMBIA1300 expressed in *N. benthamiana* epidermal cells. (E–H) SmCOI1-GFP expressed in *N. benthamiana* epidermal cells. pCAMBIA1300, the mock vector (no SmCOI1 inserted). SmCOI1, the target vector (SmCOI1 inserted). Cells were analyzed for fluorescence by confocal microscopy. GFP, green fluorescent signal; DAPI, nucleus dye stuff; DT, bright field observations. Merge, overlay of the DT, GFP, and DAPI signals.

in anther dehiscence (e.g., mutations affecting *OPR3* and *JAZ1*) (Grunewald et al. 2009; Stintzi and Browse 2000). qRT-PCR analysis was performed to detect the expression patterns of *SmOPR3* and *SmJAZ1* at multiple developmental stages (Figure 3E, F). The accumulation of *SmJAZ1* transcript steadily increased and peaked in Stage 6, before sharply declining after Stage 8. By contrast, *SmOPR3* expression peaked in Stage 7. The transcript abundance of *SmOPR3* and *SmJAZ1* was at its lowest during Stage 1. We also observed that the transcript abundance of *SmOPR3* and *SmJAZ1* was always lower in S16 relative to F142. These results indicate that anther dehiscence is affected by *SmOPR3* and *SmJAZ1* expression in eggplant.

SmCOI1 interacts with SmOPR3 and SmJAZ1

Thus far, it is unclear whether SmCOI1 mediates anther dehiscence by interacting with other proteins involved in the JA pathway. Using yeast two-hybrid assays, we found that SmCOI1 directly interacts with SmOPR3, but not SmJAZ1 without JA-Ile (Figure 5A). We next used Pull-down assays to test whether SmCOI1 interacts with SmOPR3 and SmJAZ1 (Figure 5C). We found that SmOPR3-GST could be pulled down by SmCOI1-HIS but SmJAZ1-GST could not . Our yeast two-hybrid results were replicated in Pull-down assays. However, COI1 interacts with JAZ1 in the presence of JA-Ile (Figure 5B).

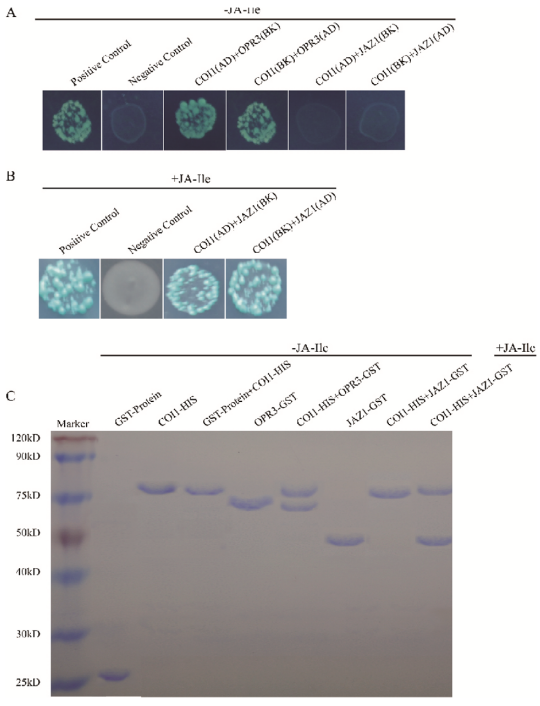

Figure 5. Protein interactions of SmCOI1 with SmOPR3 and SmJAZ1. (A) Detecting interactions of SmCOI1 with SmOPR3 and SmJAZ1 by yeast two-hybrid assay. Transformed yeast cells were plated on SD/-Ade/-His/-Leu/-Trp/X-a-Gal medium to grow at 30°C for 3–5 days. The pGBKT7-T53 and pGBKT7-lam, combined with pGADT7-T, were used as positive and negative controls, respectively. (B) Detecting interactions of SmCOI1 with SmJAZ1 by yeast two-hybrid assay. Transformed yeast cells were plated on SD/-Ade/-His/-Leu/-Trp/X-a-Gal/JA-Ile medium to grow at 30°C for 5 days. The pGBKT7-T53 and pGBKT7-lam, combined with pGADT7-T, were used as positive and negative controls, respectively. (C) Examining the interactions of SmCOI1 with the other two proteins by Pull-down. The HIS-tagged SmCOI1 protein was generated by cloning into the pET32a (+) vector (19 kDa). The GST-tagged proteins of SmOPR3 and SmJAZ1 were generated by cloning into the pGEX-4T-1 vector (26 kDa). Bound proteins were eluted and stained with Coomassie Brilliant Blue 250, then separated by 12.5% SDS-PAGE. The lanes of "GST-protein", "OPR3-GST" and "JAZ1-GST" showed only purified GST-tagged proteins. The "HIS-COI1" lane was only a purified HIS-tagged protein. The lanes of "GST-protein + COI1-HIS", "COI1-HIS+OPR3-GST" and "COI1-HIS+JAZ1-GST" indicated the results of capturing GST-protein, OPR3-GST and JAZ1-GST using COI1-HIS.

Discussion

Anther dehiscence is crucial for pollination. Although morphological changes in anthers during dehiscence have been described (Goldberg et al. 1993; Wang et al. 2004), the molecular mechanisms controlling anther dehiscence remain relatively unclear. Many researchers have identified regulators of anther dehiscence in *Arabidopsis*, including *AtMYB24*, *MYB26*, *NST1*, and *NST2* (Mitsuda et al. 2005; Steiner-Lange et al. 2003; Yang et al. 2007; Zhong and Ye 2007).

Plant development and responses to environmental signals are coordinated by complex multicomponent signaling networks. JA, which is derived from fatty acids, is an important component of the regulatory system. The JA biosynthesis and signal transduction pathways have been studied in plants (Feussner and Wasternack 2002; Schaller and Stintzi 2009; Wasternack et al. 2017). JA plays a crucial role in anther dehiscence (Ishiguro et al. 2001; Xiao et al. 2014). Mutations in JA biosynthesis or signaling genes (e.g., *OPR3* and *JAZ1*) typically result in a delay or failure in anther dehiscence. The OPR protein 12-oxo-phytodienoic acid reductase was encoded, and linoleic acid was catalyzed to form linolenic acid by OPR3 in the JA pathway (Schaller et al. 2000; Stintzi and Browse 2000). JAZ proteins have been identified as negative regulators in JA-decreased gene transcript accumulation. Three research groups have independently detected the JAZ proteins as targets of the SCF^{COI1} complex, where COI1 acts as an F-box protein as part of the Skp1/Cullin/F-box protein complex that functions as an E3 ubiquitin ligase (Chini et al. 2009; Thines et al. 2007; Yan et al. 2018). In this study, we succeeded in identifying the COI1 protein in *S. melongena* L. using a proteomics approach. SmCOI1 was shown to localize to the nucleus. By qRT-PCR, *SmCOI1* was shown to be expressed at lower levels in S16 than in F142. Thus, we speculate that *SmCOI1* regulates an important molecular event during anther dehiscence. These findings are consistent with previous research in *Arabidopsis* (Xie et al. 1998).

Anther dehiscence in higher plants is a complex and elaborate process involving a specific series of gene inductions and inhibitions. In this study, by qRT-PCR, we found that the transcript abundance of *SmJAZ1* and *SmOPR3* are lower during S16. However, it is unclear whether there is a direct interaction between SmCOI1 and SmJAZ1 or SmOPR3. Previous studies have found that PACOR can bind to OsCOI1 but not to OsJAZ1 (Yan et al. 2018), while in woodland strawberry, it was found that the combination of FvCOI1 and JA-Ile enabled the interaction between COI1–JA-Ile and FvJAZ1 for further signal transduction (Valenzuela-Riffo et al. 2018). However, in this study, by yeast two-hybrid and Pull-down assays, we show that there is no

interaction between SmCOI1 and SmJAZ1 without JA-Ile in eggplant. By contrast, there was an interaction between SmCOI1 and SmOPR3. Overexpression of *AtOPR3* elevated basal levels of JA and up-regulated the expression of *AOS* and *COI1*, thereby establishing a positive feedback loop that maintains and boosts JA levels. Our findings are in line with those of previous studies reporting that JA expression is elevated by the synergistic effects of COI1 and OPR3. We conclude that COI1 interacts with OPR3 during their transcription, thereby increasing JA levels to regulate anther dehiscence (Pigolev et al. 2018). However, how these genes regulate anther dehiscence remains unclear and should be addressed in future work.

Acknowledgements

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