A NAC transcription factor and its interaction protein hinder abscisic acid biosynthesis by synergistically repressing NCED5 in Citrus reticulata

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Abstract

Although abscisic acid (ABA) is a vital regulator of fruit ripening and several transcription factors have been reported to regulate ABA biosynthesis, reports of the effect of ABA on citrus ripening and the regulation of its biosynthesis by a multiple-transcription-factor complex are scarce. In the present study, a systematic metabolic, cytological, and transcriptome analysis of an ABA-deficient mutant (MT) of Citrus reticulata cv. Suavissima confirmed the positive effect of ABA on the citrus ripening process. The analysis of transcriptome profiles indicated that CrNAC036 played an important role in the ABA deficiency of the mutant, most likely due to an effect on the expression of 9-cis-epoxycarotenoid dioxygenase 5 (CrNCED5). Electrophoretic mobility shift assays and dual luciferase assays demonstrated that CrNAC036 can directly bind and negatively regulate CrNCED5 expression. Furthermore, yeast two-hybrid, bimolecular fluorescence complementation, and dual luciferase assays demonstrated that CrNAC036 interacted with CrMYB68, also down-regulating the expression of CrNCED5. Taken together, our results suggest that CrNAC036 and CrMYB68 synergistically inhibit ABA biosynthesis in citrus fruit by regulating the expression of CrNCED5.

Keywords: ABA, Citrus reticulata, fruit ripening, MYB transcription factor, NAC transcription factor, postharvest, synergistic transcriptional regulation.

Introduction

Fruit ripening is a complex process, and based on the patterns of respiration and ethylene biosynthesis during fruit ripening, fleshy fruits can be divided into two categories, namely, climacteric and non-climacteric fruits (Giovannoni, 2004). Abscisic acid (ABA) is a vital hormone that affects the ripening process of both categories. Knocking-down 9-cis-epoxycarotenoid dioxygenases (NCEDs) led to a significant down-regulation of the expression of genes associated with cell wall catabolism (such as pectate lyase and expansin) in tomato. Moreover, the application of exogenous ABA or treatment with an inhibitor of its biosynthesis...
can significantly promote or hinder the color change, softening, and flavor component accumulation of tomato, peach, strawberry, and grape fruits (Zhang et al., 2009a,b; Jia et al., 2011; Sun et al., 2012; Leng et al., 2014). Beside the effect on fruit ripening, exogenous ABA treatment can also induce the expression of genes associated with chlorophyll degradation (such as stay-green and pheophytinase) and promote the senescence of Arabidopsis (Yang et al., 2014). Owing to ABA’s vital role in fruit ripening, the pathway of its biosynthesis has been well characterized: violaxanthin derived from carotenoid metabolism is transformed by ABA4, NCEDs, and several other enzymes to yield ABA (Finkelstein, 2013). As the rate-limiting enzymes of ABA biosynthesis, NCEDs have attracted considerable attention and the transcriptional regulation of NCEDs is one of the hot topics in ABA research. In tomato, banana, peach, and citrus fruit, WRKYs, ERF3, and bHLH1 transcription factors can directly bind to their promoters and regulate the expression of NCEDs (Endo et al., 2016; Luo et al., 2017; Wang et al., 2019b). In the well-known model of anthocyanin biosynthesis, the synergistic transcriptional regulation of the MBW complex (MYB–bHLH–WD40) can induce higher expression of anthocyanin-associated genes than the effect of the single transcription factors separately, which demonstrated that synergistic regulation of transcription factor complexes is more efficient in the fruit ripening process (Schaart et al., 2013; Wang et al., 2019a). However, reports concerning the synergistic transcriptional regulation of multiple transcription factors on fruit NCEDs remain limited.

The regulatory mechanism of fruit ripening originated from those of carpel senescence. The carpel senescence-related NAC (NAM/ATAF/CUC) transcription factors play vital roles in different fruit ripening processes (Lü et al., 2018). SINOR-like1 and SINAC4 can act as positive regulators of tomato color formation and several NACs play important roles in apple and oil palm fruit development (Zhu et al., 2014; Tranbarger et al., 2017; Gao et al., 2018; Zhang et al., 2018). Moreover, NAC proteins can also directly control the monoterpene synthesis of kiwifruit and the lignification of postharvest loquat fruit (Nieuwenhuizen et al., 2015; Xu et al., 2015). In order to enhance the regulatory effect on the downstream genes, NAC transcription factors often form homodimers or heterodimers with other proteins. In peach, the NAC transcription factor BL can interact with NAC1 to amplify its regulatory effect on anthocyanin biosynthesis; and in banana fruit, NAC5 can interact with WRKY1/2 to enhance the induction of resistance genes (Zhou et al., 2015; Shan et al., 2016). Besides the positive effect of NAC regulators on ripening and senescence, overexpressing the NAC transcription factors JUNGBRUNNEN1 (JUB1, ANAC042) and VNI2 (ANAC083), and TaNAC-S significantly delayed the senescence of Arabidopsis and wheat seedlings (Triticum aestivum L.), respectively (Yang et al., 2011; Wu et al., 2012; Zhao et al., 2015). This fact notwithstanding, little is known concerning the negative regulatory effects of NAC on fruit ripening.

Citrus is one of the most important fleshy fruit crops in the world. However, research on this important crop is restricted by its long juvenile phase, high heterozygosity and the difficulty in obtaining transgenic plants. Fortunately, natural mutants can help researchers to overcome these limitations. In the past decades, experiments with ABA-deficient natural mutants and ABA treatments have indicated that ABA is a vital regulator of color change and the metabolism of both sugars and organic acids (Rodrigo et al., 2003; Wu et al., 2014; Zhang et al., 2014; Rehman et al., 2018). Recently, we found a novel stay-green natural mutant (MT) in Citrus reticulata cv. Suavissima, and used it to study the transcriptional regulation of CrMYB68 on carotenoid metabolism via effects on CrBCCH2 and CrNCED5 (Zhu et al., 2017). However, research concerning comprehensive effects of ABA on the ripening process (such as chlorophyll degradation, chloroplast disassembly, and ripening-related gene expression) and synergistic transcriptional regulation of ABA biosynthesis in citrus is rare. In the present study, we comprehensively analysed the effect of ABA on citrus ripening and further demonstrated that a novel NAC transcription factor, CrNAC036, directly regulated the expression of CrNCED5 and could additionally interact with CrMYB68 to synergistically down-regulate the expression of CrNCED5, providing mechanistic insight into the low ABA content in MT.

Materials and methods

Plant materials

Fruit from Citrus reticulata cv. Suavissima (wild type, WT) and its spontaneous stay-green mutant (mutant type, MT) were harvested from trees in the same commercial orchard in Wenzhou (Zhejiang Province, P.R., China) at 120 (enlarged stage), 170 (mature green stage), 210 (commercially ripe stage), and 245 (fully ripe stage) days after flowering (DAF) in 2011 and 2012. Given that we only observed slight phenotypic differences between WT and MT at 120 DAF and intended to obtain more information about the fruit at the breaker and postharvest stages, fruit samples were collected at 170, 185 (the breaker stage), and 210 DAF and 30 d after storage (DAS) in 2013.

Treatments and sampling

Both MT and WT fruits were harvested at 170 DAF and randomly divided into two groups. MT and WT group I fruits were dipped into 100 μM ABA solution (132 mg ABA was first dissolved in 1 ml ethanol and then added to 5 liters of distilled water containing 0.1% Tween 80) for 2 min. MT and WT group II fruits were dipped into 5 liters of distilled water (containing 1 ml ethanol and 0.1% Tween 80) for 2 min. After drying at room temperature for 30 min, the fruits were transferred to a sealed chamber (temperature 20–25 °C and relative humidity 75–85%) and sampled at 0 and 6 h after treatment. The concentration and the method of ABA treatment were according to former reports (Zhang et al., 2009b; Oh et al., 2018). All sampled tissues were immediately frozen in liquid nitrogen, powdered, and stored at −80 °C until analysis.

Fruit storage and sampling

The fruits harvested at 210 DAF in 2013 were used for a storage experiment. They were packed in plastic bags and kept at the optimum cold storage temperature (15–18 °C) and relative humidity (75–85%). At 30 DAS, the flavedo was sampled, frozen in liquid nitrogen, and immediately stored at −80 °C.

Extraction and UPLC analysis of chlorophylls and chlorophyll derivatives

Chlorophylls and chlorophyll derivatives were extracted according to a method described by Minguiz-Mosquera and Garrido-Fernandez...
(1989). The separation and quantification of chlorophylls and derivatives were carried out by a UPLC (Waters, H-Class) system using a C18 column (BEH C18, 50×2.1 mm, i.d. 1.8 μm). Separation was performed using an elution gradient (0.4 ml min⁻¹) with the mobile phases (A: water; ion pair reagent: methanol (1:1.8, v/v/v); B: methanol: acetone (1:1, v/v) as described by Minguiz-Mosquera et al. (1991). The elution gradient program was optimized as follows (time, A): 0 min, 75%; 3 min, 75%; 35%; 4 min, 35%; 5 min, 35%; 6 min, 16%; 8 min, 0%; 9 min, 75%; 0 min, 75%. The on-line UV-visible spectra were recorded from 350 to 750 nm with a photodiode array detector (ε, PAD). Detection was at 654 nm for chlorophyll b and its derivatives and 664 nm for chlorophyll a and its derivatives. Data were collected and processed with Epower 3 software. Chlorophylls and their derivatives were identified by comparing their retention time and spectral characteristics with those of authentic standards. At least three independent extractions and detection were performed for each sample.

Analysis of soluble sugars and organic acids by gas chromatography

Contents of soluble sugars and organic acids were determined using gas chromatography. The samples were frozen with liquid nitrogen and powdered. A total of 1 g of frozen powder was analysed by gas chromatography as described previously (Yu et al., 2014) with minor modification. The powder was suspended in 8 ml pre-cooled 80% methanol and incubated in a 70 °C water bath for 30 min. After a 1.5 h ultrasonic extraction and centrifugation at 4000 g for 10 min, the supernatant was collected and 0.2 ml internal standard (2.5% w/v phenyl-β-D-glucopyranoside, 2.5% w/v methyl-α-D-glucopyranoside) was added. The solution was made up to 50 ml with 80% methanol, and a 1 ml aliquot of this final supernatant was vacuum-dried. The dried sample was re-dissolved in 800 μl 2% w/v hydroxylamine hydrochloride in pyridine at 70 °C for 1 h and then 400 μl hexamethyldisilazane and 200 μl trimethylchlorosilane were added for incubation at 70 °C for 2 h. 0.5 ml of the supernatant was analysed with an Agilent 6890 N device (Santa Clara, CA, USA). Then, 30 μg of total flavedo protein was resolved by SDS-PAGE (12.5%) and transferred to polyvinylidene fluoride membranes (Millipore, USA). The subsequent western blot analysis was conducted as described previously (Cao et al., 2012), using the following primary antibodies (1:3000, v/v): rabbit anti-Lhca1, anti-Lhca2, anti-Lhca3, anti-Lhca4 and anti-Lhcb1 (Agrisera, Sweden); and the following secondary antibodies (1:15 000, v/v): peroxidase-conjugated immunoperoxidase goat anti-rabbit or goat anti-mouse IgG [H+L] (Pierce, USA). Signals were detected using a Clarity Western ECL Substrate (Bio-Rad) according to the manufacturer's instructions. The chemiluminescence signal was imaged using a ChemiDoc XRS (Bio-Rad) and quantified using Quantity One software (Bio-Rad). The calculated intensity volumes were fitted with a variable slope dose–response relationship using ImageJ.

RNA isolation and comparative quantitative real time PCR analysis

Total RNA was extracted as described previously (Cao et al., 2012). The integrity of the RNA preparations was evaluated by electrophoresis and then their concentrations, A260/A280 and A230/A260 ratios were determined using a Nanodrop spectrophotometer (Agilent 2100, USA). Two biological replicates of RNA from the flavedo of MT and WT (170DAF) treated with ABA were hybridized to GeneChip® Citrus Genome Arrays (Affymetrix®; Santa Clara, CA, USA). The analyses of the gene annotation and differentially expressed genes (DEGs) were performed as described previously and DEGs were detected with restriction of P<0.01 and fold-change greater than 2 (see Supplementary Table S1 at JXB online) (Ma et al., 2014b). The transcriptional datasets generated using the GeneChip® Citrus Genome Arrays platform can be found in the Gene Expression Omnibus (GEO) with the accession number GSE113669. Genes and primers used for the quantitative reverse transcription-PCR analysis are listed in Supplementary Table S2.

Subcellular localization of CrNAC036

The subcellular localization of the CrNAC036 protein was determined as described previously (Zhu et al., 2017). Protoplasts were co-transformed with 35S:CrNAC036-pM999-GFP and the nuclear marker vector 35S:OsChl7-GFP. Fluorescence from green fluorescent protein (GFP) and cyan fluorescent protein (CFP) was observed using a confocal laser-scanning microscope (TCS SP2, Leica, Germany). The excitation and emission filters used to detect fluorescence from GFP were 488 nm and 500–530 nm, respectively. The excitation and emission filters used to detect signals from CFP were 430 nm and 470–510 nm, respectively. Chlorophyll autofluorescence was monitored using the excitation wavelength of either 488 or 514 nm and the emission wavelengths from 650 to 750 nm.

Protein preparation, identification, and electrophoretic mobility shift assay

pET15 (Novagen) was used to produce a recombinant CrNAC036 protein with a 6×His tag fused to the N-terminus. E. coli strain BL21 (DE3) was used to express the recombinant CrNAC036 protein. We purified and characterized the recombinant protein as previously described (Zhu et al., 2017) with minor modification. The recombinant protein was analysed by matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (5800 MALDI-TOF/TOF; AB Sciex) with a mass spectrometer to acquire MALDI and MS/MS spectra after trypic digestion. The MS spectra were recorded in reflector mode with a mass range of 800–4000. In MS/MS positive ion mode, for one main peak, the isolation width was 2% and the collision gas was Ar. The database search was performed using the Mascot search engine 2.2 (Matrix Science, Ltd) embedded into GPS-Explorer Software 3.6 (Applied Biosystems) against non-redundant protein databases of Citrus clementina (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Clementina). Additionally, MS/MS fragment tolerance was set to 0.4 Da. A protein confidence index ≥95% was used for further manual validation.
An electrophoretic mobility shift assay (EMSA) was performed as previously described (Zong et al., 2016; Zhu et al., 2017). Briefly, the His-tagged CrNAC036 protein and 5′-FAM-labeled oligonucleotide probes (synthesized by the Shanghai Sangon Company) were incubated in a binding solution (0.1% Nonidet P-40, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, 50 μg ml⁻¹ BSA and 100 ng μl⁻¹ poly (dl-Sc)) at 4 °C for 45 min. For the competition assays, after the protein was incubated with non-labeled probe at 4 °C for 45 min, 1 μl of the 5′-FAM-labeled probe (10 μmol l⁻¹) was added to the mixture and incubated at 4 °C for 45 min. The binding reactions were resolved using electrophoresis with 6% polyacrylamide gels at 4 °C in 0.5×TBE (Tris-Borate-EDTA) in the dark for 1 h and imaged with an Amersham TM Imager 600 (GE Healthcare).

Dye luciferase and bimolecular fluorescence complementation assays

We used rice protoplasts for the dual luciferase transcriptional activity assay as described previously (Zong et al., 2016) because of the high stability, transformation efficiency, and short growth cycle of rice. The Dual-Luciferase® Reporter Assay System (Promega) was used to measure the luciferase activity according to the manufacturer’s instructions. The relative luciferase activity was calculated as the ratio of firefly luciferase (fLuc)/Renilla luciferase (rLuc).

To prevent chlorophyll fluorescence from interfering with the bimolecular fluorescence complementation (BiFC) assay, we prepared protoplasts from etiolated rice seedlings. The coding sequence from CrNAC036 was inserted into a BiFC expression vector (pCL112) to produce the nYFP vectors. CrMYB68 was inserted into a BiFC expression vector (pCL113) to produce cYFP vectors (Bhargava et al., 2010). Florescence was observed using a confocal laser-scanning microscope (TCS SP2, Leica, Germany). All the plasmids used in the dual luciferase transcriptional activity assay and the BiFC assay were purified using the QIAGEN Plasmid Midi Kit.

Yeast two-hybrid analysis

The CrNAC036 and CrMYB68 coding sequences were inserted into pGBKTrT and pGADT7 to generate pGBKTrT-CrNAC036 and pGADT7-CrMYB68 vectors, respectively. A yeast strain (AH109, Clontech) was co-transformed with these two vectors and grown on a selective SD/-Trp/-Leu medium. The interactions were evaluated on SD/-Trp/-Leu/-His/-Ade medium containing X-gal-galactosidase.

Statistical analysis

The variance of the data was analysed using SPSS 16.0 (SPSS Inc. Chicago, IL, USA). Multiple comparisons were performed by one-way ANOVA at the significance level of P<0.05 based on Duncan’s multiple range test. Student’s paired t-test was performed to assess whether the differences between two genotypes were statistically significant.

Accession numbers

CrNAC036 (coding sequence, MH339996), CrABA4 (promoter sequence, MH339995), and CrNCED5 (promoter sequence, KY612516) are available at NCBI with the indicated accession numbers. The microarray raw data are available at NCBI’s Gene Expression Omnibus with the accession code of GSE113669.

Results

Differences in ABA-associated ripening phenotypes between WT and MT fruit

As described previously, the low ABA content and some late-ripening phenotypes such as the low color index, maturity index, softening, and rotting rate during storage in MT fruit are in accordance with those displayed by other ABA-deficient citrus mutants (Rodrigo et al., 2003; Wu et al., 2014; Zhang et al., 2014; Zhu et al., 2017). To further analyse the effect of ABA on fruit ripening process, we systematically analysed other ABA-associated phenotypes such as the levels of chlorophyll-associated metabolites and photosynthesis-related protein, the subcellular morphology of the chloroplast–chromoplast conversion, and the content of sugars and organic acids.

The WT ripening process was characterized by a decrease in the levels of chlorophyll-related metabolites, such as chlorophyll a, chlorophyll b, and chlorophyllide a (Fig. 1; Supplementary Fig. S1). At the same time, the thylakoid membranes were disassembled and the chloroplasts were gradually converted to chromoplasts, which contained plastoglobules filled with carotenoids. By contrast to WT flavedo, MT flavedo still retained chloroplasts with intact thylakoid membranes at 210 DAF (see Supplementary Fig. S2). Consistently, the MT flavedo contained higher levels of photosystem-associated proteins than the WT flavedo across the ripening process (Supplementary Fig. S3). Moreover, compared with the results of the previous work, similar increases in sugars and decreases in organic acids were also observed in the WT flavedo during fruit ripening in this study; however, the rates of these changes in the MT flavedo were slower than those in the WT flavedo (Fig. 1).

Furthermore, we observed the same trend of change in the levels of sugars and organic acids in the flesh of WT and MT fruit (Supplementary Fig. S1).

To further explore the effect of ABA on ripening-related gene expression and the regulatory mechanism underlying the ABA-deficient phenotype of MT, GeneChip Citrus Genome Arrays (Affymetrix, Santa Clara, CA, USA) were used to characterize change in the transcriptome of MT and WT fruits (170 DAF) after exogenous ABA treatment. Combining the data obtained with former microarray and digital gene expression profiling experiments (Zhu et al., 2017), we analysed the DEGs after ABA treatment and between MT and WT at different fruit stages (Fig. 2A). These analyses indicated that 21 genes were differentially expressed across all transcriptome profiling experiments and the expression of 13 DEGs was consistently higher while one gene was consistently lower in ABA-treated fruits (in comparison with that of water-treated fruit) and WT (in comparison with that of MT at different fruit stage) (Table 1). Among these genes, CrNCED5 was induced in both WT and MT fruits after ABA treatment and also highly expressed in WT fruit during the fruit development stages. Moreover, some genes involving in ABA-induced ripening process, such as cell wall degradation, were induced by ABA treatment and highly expressed in the WT fruit (Table 1). Interestingly, there were two transcription factors that co-expressed with CrNCED5 and since NAC family proteins play important roles in both ABA biosynthesis and the fruit ripening process, we inferred that CrNAC036 may be an important regulator of CrNCED5 (Table 1).

Owing to the slight differences between MT and WT fruit at 120 DAF and in order to acquire more information about the fruit at the breaker and postharvest stages, we analysed the expression of genes involved in ABA biosynthesis and related
process (such as chlorophyll and cell wall degradation) using qRT-PCR at 170, 185 and 210 DAF and 30 DAS (Fig. 2B). At 210 DAF, the expression levels of CrABA4, CrPPH (CrPheophytinase), CrSGR (CrStay Green), CrPEL (CrPectin Lyase), and CrExpansin A8 were 4.02-, 1.15-, 1.48-, 27.04-, and 8.74-fold higher in WT fruit than in MT fruit, respectively. By contrast, the expression level of CrNAC036 in MT fruit was 16.13-fold higher than that in WT fruit.

Amino acid sequence alignment, subcellular localization, and prokaryotic expression of CrNAC036

To further analyse the potential function of CrNAC036, we compared the amino acid sequence of CrNAC036 with that of 105 Arabidopsis NAC transcription factors. In the phylogenetic analysis, CrNAC036 grouped with the clade containing At2G17040.1, At2G02450.1, At2G02450.2, At5G39820.1, and At2G43000.1 (see Supplementary Fig. S4). Moreover, subcellular localization experiments demonstrated that the CrNAC036–GFP fusion protein was co-localized with a nuclear marker protein (OsGhd7–CFP), indicating that the CrNAC036–GFP fusion protein was accumulated in the nucleus (Fig. 3A).

To determine the DNA binding activity of the CrNAC036 protein, it was expressed in Escherichia coli as a 6×His–CrNAC036 fusion protein and purified. We obtained a protein with a molecular mass of ca. 25 kDa (Fig. 3C). MS data indicated that the band corresponded to the N-terminus of the CrNAC036 protein and the a, c and d subdomains that are conserved among NAC transcription factors could all be identified in the fusion protein (see Supplementary Table S4; Supplementary Figs S5, S6). The c and d subdomains are responsible for the specific DNA binding activities of NAC transcription factors (Ooka et al., 2003). Therefore, the purified N-terminus of the CrNAC036 protein could be utilized for analysing the DNA binding activity of CrNAC036.

CrNAC036 specifically repressed the expression of CrNCED5

Previous studies reported that the senescence-associated NAC transcription factor family can bind to the DNA sequences containing the conserved sequence motif CGT/ACG (Podzimska-Sroka et al., 2015). We found the CGT/ACG motif in the promoters of CrNCED5 and CrABA4 (Fig. 4A). To test whether CrNAC036 can bind to the promoters of CrNCED5 and CrABA4, the purified 6×His–CrNAC036 fusion protein was incubated with 20-bp probes containing the CGT/ACG sequence from the promoter regions of CrNCED5 and CrABA4. It was found that the CrNAC036 protein did not
bind to the two probes containing the CGT/ACG motifs from the *CrABA4* promoter in the EMSA. However, the P1 probe from the *CrNCED5* promoter was specifically bound by the *CrNAC036* protein, as indicated by the retardation of its mobility in the EMSA. Furthermore, although the *CrNAC036* protein bound the mutant probe derived from P1, the binding...
Table 1. Consistently differentially expressed genes under ABA treatments and at different ripening stages

| Gene | Probe set ID | Transcriptome analysis (log2(fold change)) | Function | Arabidopsis ortholog |
|------|--------------|------------------------------------------|----------|----------------------|
|      |              | CK-WT-ABA | CK-MT-ABA | MT-170DAF-WT | MT-210DAF-WT | MT-30DAS-WT |          |
| Ciclev10014639m | Cit.17235.1.S1_s_at | -3.11 | -1.07 | -1.13 | -1.71 | -7.81 | ABA biosynthesis | AT1G30100 (AtNCED5) |
| Ciclev10029007m | Cit.31377.1.S1_at | 1.88 | 1.98 | 1.12 | 3.32 | 5.50 | Transcription factor | AT2G17040 (AtNCED3) |
| Ciclev10029283m | Cit.10057.1.S1_s_at | -1.63 | -2.47 | -1.52 | -1.13 | -2.68 | Transcription factor | AT2G28500 (AtLBDB1) |
| Ciclev10031249m | Cit.55568.1.S1_s_at | -1.25 | -1.56 | -1.74 | -3.39 | -4.91 | Cell wall degradation | AT1G67750 (AtPEL) |
| Ciclev10032524m | Cit.20839.1.S1_s_at | -1.02 | -1.18 | -1.11 | -1.81 | -3.95 | Cell wall degradation | AT2G40610 (AtEXP58) |
| Ciclev10019301m | Cit.2945.1.S1_s_at | -1.59 | -2.06 | -1.09 | -2.42 | -4.74 | Cell wall degradation | AT1G64390 (AtIGi69C2) |
| Ciclev10012984m | Cit.8763.1.S1_s_at | -2.55 | -3.60 | -1.86 | -2.59 | -8.00 | Water metabolism | AT4G00430 (AtPP1:4) |
| Ciclev10004103m | Cit.1002.1.S1_s_at | -1.09 | -0.96 | -1.81 | -1.08 | -3.00 | Glucosinolate metabolism | AT1G35100 (AtCYP83B1) |
| Ciclev10025900m | Cit.15742.1.S1_at | -1.72 | -3.34 | -1.43 | -1.79 | -2.16 | Lipid metabolism | AT1G75900 (AtEXL3) |
| Ciclev10011714m | Cit.1700.1.S1_at | -1.80 | -1.79 | -1.88 | -3.36 | -7.47 | Secondary metabolism | AT3G26040 (AtNCED5) |
| Ciclev10025900m | Cit.22427.1.S1_s_at | -1.93 | -2.49 | -1.40 | -1.19 | -3.32 | Development, unspecified | AT4G15920 |
| Ciclev10039996m | Cit.26052.1.S1_s_at | -1.06 | -1.67 | -2.05 | -1.72 | -4.77 | Unknown | AT2G39855 |
| Ciclev10032283m | Cit.21497.1.S1_at | -1.87 | -2.26 | -1.72 | -1.63 | -4.12 | Unknown | AT2G39805 |
| Ciclev10028078m | Cit.10062.1.S1_at | -1.88 | -2.08 | -1.14 | -1.15 | 2.91 | Amino acid metabolism | AT3G47340 (AtASS1) |
| Ciclev10008993m | Cit.1718.1.S1_s_at | 3.34 | 4.15 | 1.04 | 1.35 | -1.25 | Ethylene biosynthesis | AT2G19690 (AtACO1) |
| Ciclev10029695m | Cit.9890.1.S1_s_at | 1.80 | 1.01 | -1.17 | -1.53 | -1.11 | Gibberelin-regulated family protein | AT2G14900 |
| Ciclev10005627m | Cit.28626.1.S1_s_at | -1.29 | -2.11 | -1.83 | -1.78 | 1.72 | Cell wall modification | AT1G65680 (AtEXP52) |
| Ciclev10031099m | Cit.4425.1.S1_s_at | -1.32 | 2.21 | 1.54 | -1.43 | 2.25 | Cytochrome P450 | AT5G05260 (AtCYP79A2) |
| Ciclev10002768m | Cit.12040.1.S1_s_at | -1.36 | -2.81 | -2.02 | -1.81 | 1.06 | Metal handling | AT4G8570 |
| Ciclev10006006m | Cit.165.1.S1_s_at | -1.84 | -2.18 | -1.23 | -3.96 | 1.76 | Light signalling | AT3G22840 (AtELI1) |
| Ciclev10011713m | Cit.10672.1.S1_s_at | -3.78 | -3.28 | 1.17 | -2.02 | 3.22 | Light signalling | AT3G26740 (AtACCL) |

CK-MT-ABA and CK-WT-ABA indicate the DEGs of MT and WT between water treatment and ABA treatment, respectively. MT-170DAF-WT, MT-210DAF-WT, and MT-30DAS-WT indicate the DEGs at 170 DAF, 210 DAF, and 30 DAS between MT and WT, respectively.

activity was considerably lower than that observed for the wild-type P1 probe (Fig. 4B).

To further evaluate whether CrNAC036 affected the activities of the CrNCED5 and CrABA4 promoters, we fused the CrNCED5 and CrABA4 promoters including the core-binding motif of NAC family (i.e., the CGT/ACG sequence) to the firefly luciferase reporter gene, and these were then transiently co-expressed in protoplasts. Results indicated that CrNAC036 significantly repressed the activity of the CrNCED5 promoter but did not affect that of the CrABA4 promoter (Fig. 4C). Based on the results of EMSA and dual-luciferase experiments, it can thus be inferred that CrNAC036 can specifically bind to the promoter of CrNCED5 and represses its activity.

CrNAC036 interacted with CrMYB68 to synergistically down-regulate the expression of CrNCED5

NAC proteins usually enhance their own transcriptional activities via interaction with other transcription factors (Olsen et al., 2005). Our previous study indicated that CrMYB68 displays a similar expression pattern to CrNAC036 in the MT fruit and can also directly regulate CrNCED5 (Zhu et al., 2017). In order to determine whether CrNAC036 can interact with CrMYB68 to synergistically regulate the expression of CrNCED5, we first tested whether CrMYB68 and CrNAC036 can interact in the yeast two-hybrid system and BiFC assays. The yeast strains harboring both the pGADT7-CrMYB68 and the pGBK7-CrNAC036 vectors could grow and exhibit blue color on a medium containing X-gal without leucine, tryptophan, histidine, and adenine. These results indicated that CrNAC036 can interact with CrMYB68 in the yeast two-hybrid system (Fig. 5A). The ability of these proteins to interact with each other was independently verified by BiFC experiments. As shown in Fig. 5B, the interaction between CrNAC036 with the C-terminus and CrMYB68 with the N-terminus of yellow fluorescent protein (YFP) yielded a fluorescence signal in the nucleus (Fig. 5B). Secondly, we performed a dual-luciferase experiment by transiently co-expressing the two effector vectors and a luciferase reporter gene in protoplasts. As a result, the combinations of ‘None (empty vector) + CrNAC036-None’ and ‘None (empty vector) + CrMYB68-None’ significantly repressed the activity of the promoter from CrNCED5. However, the activity of the CrNCED5 promoter was the most obviously reduced when
‘CrNAC036-None’ and ‘CrMYB68-None’ were co-expressed (Fig. 5E). These results indicated that the protein–protein interactions between CrNAC036 and CrMYB68 do synergistically affect the expression of CrNCED5.

Discussion

ABA is one of the key regulators of the fruit ripening process. The transcriptional regulation of ABA biosynthetic genes has been well studied in fruits (Endo et al., 2016; Luo et al., 2017; Wang et al., 2019b). However, comprehensive details of the effect of ABA on citrus ripening and the regulation of ABA biosynthesis in citrus by a multi-transcription factor complex remain largely unknown. Here, we performed metabolic, cytological, and transcriptome analysis of an ABA-deficient mutant from Citrus reticulata cv. Suavissima. It was found that ABA served as an important regulator of citrus ripening and its biosynthesis was under the synergistic regulation of CrNAC036 and CrMYB68 by suppressing the expression of CrNCED5.

ABA plays an important role in the ripening process of citrus fruit

In MT fruit, some ripening parameters (such as color index, maturity index, and softening) were significantly delayed, which was in accordance with those displayed by other ABA-deficient citrus mutants (Rodrigo et al., 2003; Wu et al., 2014; Zhang et al., 2014; Zhu et al., 2017). Moreover, in this study, the delay of chlorophyll degradation, the chloroplast–chromoplast transition, and sugar and organic acid metabolism in MT further indicated that ABA can systematically affect the citrus fruit ripening process (Fig. 1; Supplementary Fig. S1). Furthermore, many studies have reported that ABA can directly promote ripening-related process, such as chlorophyll degradation, carotenoid biosynthesis and softening, by regulating gene expression (Zaharah et al., 2013; Gao et al., 2016; Rehman et al., 2018). These effects of ABA could also be confirmed in MT fruit, with the expression of genes that contribute to chlorophyll degradation and cell wall modification (i.e. CrPEL, CrExpansin A8, CrPPH, and CrSGR) being consistently lower in MT fruit than in WT fruit during the ripening process and the demonstration that the expression of CrPEL and CrExpansin A8 could be significantly induced by exogenous ABA treatment (Fig. 2). Additionally, injection of ABA caused an increase in the glucose and fructose levels of citrus fruit and exogenous ABA treatment can accelerate fruit coloring of citrus (Kojima et al., 1995; Wang et al., 2016; Rehman et al., 2018). Taken together, all of the previously published work and our own findings indicate that ABA is an essential positive regulator of ripening of citrus fruits.

CrNAC036 plays an important role in ABA-deficient phenotype of MT

Our results from the transcriptome and qRT-PCR experiments indicate that the expression of CrNCED5 and CrABA4 was robustly lower in MT than that in WT (Fig. 2; Table 1; Zhu et al., 2017). We also checked the expression of other genes involved in ABA biosynthesis but found that their expression did not vary robustly (Zhu et al., 2017). Thus, as NCED5 was the dominant NCED for the ABA biosynthesis in C. clementina flavedo compared with other NCEDs (Agustí et al., 2007), the low expression of CrNCED5 and CrABA4 may lead to the low ABA level in MT. Moreover, we observed, in the transcriptome data, that the expression of CrNAC036 displayed a significantly negative correlation to that of CrNCED5 and CrABA4 (Table 1; Fig. 2). Additionally, we found that the
amino acid sequence of CrNAC036 is similar to that of JUB1, which inhibits Arabidopsis senescence (see Supplementary Fig. S4), and note that the ABA content of jub1-1 plants was significantly increased compared with that of the wild type (Wu et al., 2012). Furthermore, DAP-seq analysis indicated that JUB1 can directly bind to the promoter of AtNCED5 (O’Malley et al., 2016). When taken together these data suggest that CrNAC036 may negatively regulate ABA biosynthesis by down-regulating the expression of CrNCED5 or CrABA4. However, whilst conserved NAC-recognition sequences were recognized in the promoters of both CrNCED5 and CrABA4, the data from the EMSA indicated that CrNAC036 can specifically bind to the promoter of CrNCED5 but not to that of CrABA4 (Fig. 4). Moreover, the data from the dual luciferase assay indicated that CrNAC036 can repress the activity of the CrNCED3 promoter while not affecting the activity of the CrABA4 promoter (Fig. 4). Based on these results, it can be concluded that CrNAC036 is an important regulator of ABA biosynthesis in citrus fruit, a task it achieves by specific down-regulation of the expression of CrNCED5.

Transcription factors and hormones such as ABA and ethylene substantially influence fruit ripening. Several transcription factor families have been proven to promote ripening through affecting ABA (ABF2 in grape) or ethylene (RIN, CNR in tomato and MdMYC2 in Malus domestica) metabolism and signaling (Klee and Giovannoni, 2011; Nicolas et al., 2014; Li et al., 2017). However, until recently no transcription factor family had been reported to regulate both fruit ABA and ethylene metabolism. Recently, Lü et al. (2018) reported that carpel senescence-related NAC transcription factors play vital roles in the ripening process of both climacteric fruit without recent whole-genome duplication and non-climacteric fruit.
Fig. 5. Interactions between CrNAC036 and CrMYB68 and their cooperative regulation of the CrNCED5 promoter. (A) Interactions between CrNAC036 and CrMYB68 in the yeast two-hybrid assays. Blue colonies growing on a synthetic drop-out medium lacking Trp, Leu, His, and Ade (SD/−Trp/−Leu/−His/−Ade) and containing X-α-galactosidase indicate protein–protein interactions. Co-transformation of pGBK7-p53 and pGAD7-RecT was used as a positive control. (B–D) Nuclear interactions of CrNAC036 with CrMYB68. Confocal images of transiently transformed nYFP-CrNAC036 and cYFP-CrMYB68 generating YFP signal in nucleus (OsGhd7–CFP was used as a nuclear marker) (B). Interaction with empty vectors was not observed (C, D). Scale bars: 10 μm. (E) Dual luciferase assays in protoplasts co-expressing CrNAC036 and CrMYB68. The fLUC/rLUC ratio represents the relative activity of the CrNCED5 promoter (P2). The sequence of P2 is indicated in Fig. 4A. The values in each column are the means of three biological replicates. Error bars indicate SD. Different letters indicate significant differences according to Duncan’s test (P<0.05). (This figure is available in color at JXB online.)
In tomato, SINAC19/48 can directly induce the expression of ACO1 and ACS2 and in *Musa acuminate*, MaNAC1/2 can affect ethylene signaling by interacting with EIN3 (Shan et al., 2012; Ma et al., 2014a; Kou et al., 2016). In the present study, our findings concerning the transcriptional regulation of CrNAC036 greatly improves our understanding of the transcriptional regulation of ABA metabolism in fruit. They additionally enrich our knowledge concerning the importance of regulators of the NAC transcription factor family on the biosynthesis of two hormones known to be vital for fruit ripening.

**CrNAC036 and CrMYB68 synergistically inhibit ABA biosynthesis by down-regulating the expression of CrNCED5**

Owing to their sessile nature, plants have evolved complex mechanisms to activate or repress gene expression in order to adapt to their prevailing environments. As important regulators of gene expression, besides working alone, different transcription factors can form complexes and recognize cis-regulatory elements within a target gene promoter for synergistic or antagonistic regulation of gene transcription. Given that ABA is an important regulator of stress tolerance and the fruit ripening process, the transcriptional regulation of NCEDs by a single transcription factor has been reported in many crops and fruits (Jiang et al., 2012; Finkelstein, 2013; Jensen et al., 2013; Endo et al., 2016; Zong et al., 2016; Luo et al., 2017; Ma et al., 2018; Wang et al., 2019b). However, to date research concerning synergistic regulation of fruit NCEDs by transcription factor complexes remains rare.

In previous publications, transcription factors of the NAC family have been reported to interact with distinct types of transcription factors including NAC family proteins, WRKY family proteins, TCP family proteins, and zinc finger-containing transcription factors (Olsen et al., 2004; Tran et al., 2007; Wang et al., 2015; Zhou et al., 2015; Li et al., 2017a). Moreover, our earlier research demonstrated that CrMYB68 was highly expressed in the MT fruit and the protein can directly repress the expression of CrNCED5 (Zhu et al., 2017). In the present study, CrNAC036 was revealed to be highly co-expressed with CrMYB68, and Y2H and BiFC results indicated that CrNAC036 physically interacted with CrMYB68. Given that both CrNAC036 and CrMYB68 are represors of CrNCED5, the dual luciferase assay co-expressing these two transcription factors showed lower promoter activity of CrNCED5 compared with that of the single-expression dual luciferase assay, which indicated that CrNAC036 interacts with CrMYB68 in order to synergistically repress the expression of CrNCED5 (Fig. 5).

When taken together, the results of our study thus highlight the powerful effect of ABA on the citrus fruit ripening process as well as providing considerable insight into the regulation of CrNCED5 by CrNAC036 either singularly or synergistically with CrMYB68. These results broaden our understanding of the function of NAC family transcription factors with regard to the key regulators (ABA and ethylene) of non-climacteric and climacteric fruit ripening processes and of the intricate regulatory network of transcription factor complexes in ABA biosynthesis.

**Supplementary data**

Supplementary data are available at JXB online.

**Fig. S1.** Phosphorhizide a and phoephytin a levels in the flavedo and sugar and organic acid levels in the flesh of WT and MT.

**Fig. S2.** Transmission electron microscopy analysis of the morphological changes in the flavedo plastids from WT and MT.

**Fig. S3.** Levels of photosystem–associated proteins in the flavedo at four developmental stages.

**Fig. S4.** Phylogenetic analysis of CrNAC036 and NAC proteins from Arabidopsis.

**Fig. S5.** Peptides from CrNAC036 identified using MALDI–TOF/TOF MS.

**Fig. S6.** The MS/MS spectrum of the identified peptides of CrNAC036 protein.

**Table S1.** The DEGs of MT and WT after ABA treatment

**Table S2.** Primers used in this study.

**Table S3.** Names and TAIR ID numbers of 105 NAC transcription factors from Arabidopsis.

**Table S4.** Peptides identified by 6×His–CrNAC036 protein using the MALDI-TOF/TOF MS method.

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