RESEARCH PAPER

Citrus CitNAC62 cooperates with CitWRKY1 to participate in citric acid degradation via up-regulation of CitAco3

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Abstract

Citric acid is the predominant organic acid of citrus fruit. Degradation of citric acid occurs during fruit development, influencing fruit acidity. Associations of CitAco3 transcripts and citric acid degradation have been reported for citrus fruit. Here, transient overexpression of CitAco3 significantly reduced the citric acid content of citrus leaves and fruits. Using dual luciferase assays, it was shown that CitNAC62 and CitWRKY1 could transactivate the promoter of CitAco3. Subcellular localization results showed that CitWRKY1 was located in the nucleus and CitNAC62 was not. Yeast two-hybrid analysis and bimolecular fluorescence complementation (BiFC) assays indicated that the two differently located transcription factors could interact with each other. Furthermore, BiFC showed that the protein–protein interaction occurred only in the nucleus, indicating the potential mobility of CitNAC62 in plant cells. A synergistic effect on citrate content was observed between CitNAC62 and CitWRKY1. Transient overexpression of CitNAC62 or CitWRKY1 led to significantly lower citrate content in citrus fruit. The combined expression of CitNAC62 and CitWRKY1 resulted in lower citrate content compared with the expression of CitNAC62 or CitWRKY1 alone. The transcript abundance of CitAco3 was consistent with the citrate content. Thus, we propose that a complex of CitWRKY1 and CitNAC62 contributes to citric acid degradation in citrus fruit, potentially via modulation of CitAco3.

Key words: Aconitase, CitNAC62, citric acid, CitWRKY1, protein–protein interaction, transcriptional regulation.

Introduction

Organic acids, including quinic, citric, malic, and oxalic acids, are present in most plants and vary among species, organ, and tissue types, developmental stages, and environmental conditions (Badia et al., 2015). In Arabidopsis, organic acids influence carbohydrate perception in germinating seedlings (Hooks et al., 2004), fumarate accumulation plays an essential role in low temperature sensing (Dyson et al., 2016), malate is involved in cellular pH regulation and stomatal movement (Hurth et al., 2005; Lee et al., 2008), and citrate contributes to metal resistance in plant roots (Wang et al., 2016).

Organic acid metabolism and degradation have been widely studied. For instance, MxCS2, a gene encoding a putative
citrate synthase in *Malus xiaojinensis*, was introduced into Arabidopsis, resulting in increased citrate content (Han et al., 2015). In contrast, inhibition of aconitase activity resulted in the accumulation of citrate (Gupta et al., 2012; Hooks et al., 2014). In addition to biosynthesis and degradation, some transporters, including a tonoplast dicarboxylate transporter (AttDT) (Hurth et al., 2005), aluminum-activated malate transporter (ALMT) (Kouvermann et al., 2007), and some V-ATPase/V-PPase genes (Li et al., 2016; Hu et al., 2016), also influence organic acid content in plants. In citrus, a vacuolar citrate/H⁺ symporter was isolated that could mediate citrate efflux and play a role in citric acid homeostasis (Shimada et al., 2006). In recent years, some transcription factors have been demonstrated to have important roles in the regulation of organic acids. In Arabidopsis, WRKY46 functions as a transcriptional repressor of *ALMT1*, regulating aluminum-induced malate secretion (Ding et al., 2013). In tomato fruits, overexpression of *SIAREBI* resulted in increased citric and malic acid contents, and the expression of the mitochondrial citrate synthase gene (*mCS*) was up-regulated (Bastias et al., 2011), while *CgDREB*-overexpressing tomato fruits showed higher levels of organic acids (Nishawy et al., 2015). However, transcriptional regulatory information is still very limited.

In citrus fruit, especially acidic varieties, citric acid is the predominant organic acid, accounting for more than 90% of total organic acids (Albertini et al., 2006; Baldwin et al., 2014). The difference in the acidity of various citrus fruits at the commercial mature stage is due to expansion of the fruit, citrate synthesis and vacuole storage, and is also largely determined by the degradation pathway, including the gamma-aminobutyric acid (GABA) shunt and the glutamine and acetyl-CoA pathways (Katz et al., 2011; Walker et al., 2011; Lin et al., 2015). Among these, the GABA shunt was considered to be the dominant pathway; the first step of this pathway is the conversion of citrate to isocitrate by aconitase (Terol et al., 2010). In citrus fruits, inhibition of mitochondrial aconitase activity contributes to acid accumulation, and increasing cytosolic aconitase activity reduces the citrate level toward fruit maturation (Degu et al., 2011; Sadka et al., 2000). Transcript analysis from multiple sources indicated that *CitAco3* is negatively correlated with citric acid content in citrus fruit and *CitAco3* may contribute to citrate degradation (Chen et al., 2012, 2013). However, understanding of the molecular basis of fruit citrate degradation has been limited to transcript analysis, including *CitAco3* and the other structural genes. Because of the difficulty of producing transgenic citrus material, the in planta roles of these genes in citrate degradation, and the in vivo mechanisms regulating their transcripts, remain unknown.

In the present research, gene expression and partial functional verification of *CitAco3* in relation to citrate degradation were studied. In order to understand the regulation of *CitAco3* expression, a set of 16 transcription factors was isolated on the basis of their co-expression with *CitAco3*. The potential regulatory roles of the transcription factors were investigated and two of them showed transactivation activity of the *CitAco3* promoter. In addition, the interaction and synergistic effects of two transcription factors, protein–protein interaction, and the possible movement of transcription factors within the plant cell were evaluated with regard to citrate degradation.

### Materials and methods

#### Plant materials

Ponkan (*Citrus reticulata* Blanco cv. Ponkan) fruits received from a commercial orchard in Quzhou, Zhejiang, China, were used in this study. Fruits of uniform size and appearance were collected at each sampling point, from six different trees. Sampling points were at 60, 90, 120, 150, and 180 days after full blossom (DAFB). The flesh was frozen in liquid nitrogen and stored at −80 °C for further experiments.

#### Citric acid measurement

The citric acid content of Ponkan fruits and leaves was measured according to Lin et al. (2015). Fruits (0.1 g) and leaves (0.05 g) were ground in liquid nitrogen and extracted with 1.4 ml methanol at 70 °C for 15 min, and then centrifuged at 10 000 g. The upper phase was removed and stored at −80 °C until analysis. Aliquots of 100 μl upper phase were dried in a vacuum. The residue was dissolved in 40 μl 20 mg ml⁻¹ pyridine methoxamine hydrochloride, and incubated at 37 °C for 1.5 h. The sample was then treated with 60 μl Bis(trimethylsilyl)trifluoroacetamide (1% trimethylchlorosilane) at 37 °C for 30 min. Ribitol (20 μl, 0.2 mg ml⁻¹) was added to each sample as an internal standard. A 1 μl aliquot of each sample was absorbed with a split ratio of 1:1 and injected into a GC-MS fitted with a fused-silica capillary column (30 m×0.25 mm internal diameter, 0.25 μm DB-5 MS stationary phase). The injector temperature was 250 °C and the helium carrier gas had a flow rate of 1.0 ml min⁻¹. The column temperature was held at 100 °C for 1 min, increased to 184 °C at a rate of 3 °C min⁻¹, then increased to 230 °C at a rate of 15 °C min⁻¹ and held for 1 min. The MS operating parameters were as follows: ionization voltage 70 eV, ion source temperature as 230 °C, and interface temperature 280 °C.

#### RNA extraction and cDNA synthesis

Total RNA was extracted from frozen tissues according to the protocol described by Chen et al. (2012). The genomic DNA in total RNA was degraded with RNase-free DNase I (Ambion). First-strand cDNA synthesis was initiated with 1.0 μg DNA-free RNA and GoScript™ Reverse Transcriptase (Promega) following the manufacturer’s protocol. Ten-fold diluted cDNA was used as the template for quantitative real-time PCR analysis. RNA extraction and cDNA synthesis were performed with three biological replicates for each sampling point.

#### Real-time PCR

The PCR mixture (20 μl total volume) comprised 10 μl Lightcycler® 480 SYBR Green I Master (Roche), 1 μl of each primer (10 mM), 2 μl diluted cDNA and 6 μl PCR-grade H₂O. PCR was performed on a Lightcycler® 480 instrument (Roche), initiated by 5 min at 95 °C, followed by 50 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 15 s, and completed with a melting curve analysis program. No-template controls and melting curve analyses were included in every reaction. *Citrus actin* (XM_006464903) was used as a control to quantify cDNA abundance (Chen et al., 2012). The sequences of the primers used are described in Supplementary Table S1 at *JXB* online.

#### Dual luciferase assays

Dual luciferase assays were performed as described in our previous reports (Xu et al., 2014). The promoter of the *CitAco3* gene was amplified with the primers described in Supplementary Table S2. Full-length transcripts of the transcription factors (Table 1) were
activations were indicated by the presence of colonies. Protein–protein interactions were tested with empty pPR3-N vectors and target genes with pDHB1, with 60 mM 3-amino-1,2,4-triazole (QDO+3AT). Auto-activations (DDO); (ii) SD medium lacking Trp, Leu, His, and Ade (QDO); and (iii) SD medium lacking Trp and Leu (QDO). The assays were performed with co-transformation of CitNAC62 in pPR3N and CitWRKY1 in pDHB1. The presence of colonies in QDO and QDO+3AT indicated a protein–protein interaction.

Bimolecular fluorescence complementation assay
Full-length CitNAC62 and full-length CitWRKY1 were cloned into either C-terminal or N-terminal fragments of yellow fluorescent protein (YFP) vectors (Sainsbury et al., 2009). Primers used are listed in Supplementary Table S4. All constructs were transiently expressed in tobacco leaves by Agrobacterium-mediated infiltration (GV3101) based on previous reports with some modifications (Li et al., 2016). The YFP fluorescence of tobacco leaves was imaged 3 d after infiltration using a Zeiss LSM710NLO confocal laser scanning microscope.

Transient overexpression in citrus leaves and fruits
Full-length coding sequences of target genes (CitAco3, CitNAC62, and CitWRKY1) were amplified with primers (listed in Supplementary Table S5) and inserted into the SK vector. Information regarding the SK vector is given in Hellens et al. (2005). The constructs were electroporated into Agrobacterium GV3101. For transient overexpression, firefly luciferase and renilla luciferase were assayed using dual luciferase assay reagents (Promega). For each transcription factor–promoter interaction, three independent experiments were performed (five biological replicates in each experiment).

Statistical analysis
Least significant difference (LSD) was calculated by using DPS 7.05 (Zhejiang University, Hangzhou, China). The statistical significance of differences was calculated using Student’s t-test. Figures were drawn using Origin 8.0 (Microcal Software Inc.).

Results
Association between CitAco3 and citrate degradation
The correlation of CitAco3 expression and citric acid degradation has been widely supported (Chen et al., 2012; Lin et al., 2014). The dual luciferase assays were performed with co-transformation of CitNAC62 in pPR3N and CitWRKY1 in pDHB1. The presence of colonies in QDO and QDO+3AT indicated a protein–protein interaction.
In the present study, we found that CitAco3 is more abundant in late developmental stages (150 and 180 DAFB), when the fruit citric acid decreased from a peak of 32.07 mg g⁻¹ at 120 DAFB to 6.51 mg g⁻¹ at 180 DAFB (Fig. 1A, B).

To directly investigate CitAco3 function, we introduced a cDNA, under the control of the constitutive CaMV 35S promoter, into citrus leaves and fruits using Agrobacterium-mediated transient transformation (Hellens et al., 2005). Compared with the control (empty vector), transient overexpression of CitAco3 significantly reduced the citric acid content in citrus leaves and fruits. In leaves transformed with CitAco3 or the empty vector, citric acid contents were 1.16 and 1.74 mg g⁻¹, respectively (Fig. 2A). Similar results were observed in citrus fruits, where transient overexpression of CitAco3 significantly reduced citric acid content to 12.11 mg g⁻¹, compared with the empty vector, at 15.52 mg g⁻¹ (Fig. 2B).

In vivo regulatory effects of transcription factors on CitAco3 promoter

In order to study the transcriptional regulation of CitAco3, we searched the RNA-Seq data from our previous report (Lin et al., 2015) to identify 16 transcription factors whose abundance was highly correlated with CitAco3 (Table 1). Dual luciferase assays indicated that in the presence of CitNAC62 or CitWRKY1, CitAco3 promoter activity was significantly enhanced, with approximately 2.4- and 2.0-fold induction, respectively (Fig. 3).

Analysis of CitNAC62 and CitWRKY1 expression indicated that both transcription factors had expression patterns similar to that of CitAco3, being more abundant at the late stages of fruit development (Fig. 4).

Subcellular localization and interaction of CitNAC62 and CitWRKY1

To visualize the subcellular locations of the two transcription factors, we performed a subcellular localization assay in tobacco leaves by using GFP tagging. CitWRKY1 gave strong signals in the nucleus (Fig. 5); CitNAC62 was not located in the nucleus and the signals indicated that its subcellular location was within plastids (Fig. 5). Despite the different locations of the two transcription factors, protein–protein interactions were observed between CitNAC62 and CitWRKY1 in yeast two-hybrid assays (Fig. 6A). This interaction was also verified by bimolecular fluorescence complementation assays (BiFC) using tobacco leaves. The results showed that negative combinations, such as YFPN/CitNAC62-YFPC, CitWRKY1-YFPN/YFPC and YFPN/YFPC did not produce any detectable fluorescence signal, while co-expression of CitNAC62-YFPC and CitWRKY1-YFPN gave strong signals in the nucleus (Fig. 6B).

Citric acid content is negatively regulated by CitNAC62 and CitWRKY1

CitNAC62 and CitWRKY1, under the control of the CaMV 35S promoter, were introduced into citrus fruits using

![Fig. 1](image1.png)

**Fig. 1.** Changes in (A) the citric acid content and (B) the expression of CitAco3 in the flesh of Ponkan fruits during fruit development. DAFB, days after full blossom. Error bars represent SE (n=3).

![Fig. 2](image2.png)

**Fig. 2.** Transient overexpression of CitAco3 in (A) citrus leaves and (B) fruits. The CitAco3 gene was driven by the CaMV 35S promoter. SK represents empty vector. Citric acid was analyzed at 5 d after infiltration. Error bars indicate SE from five biological replicates. *Significant differences (P<0.05).
CitNAC62 and CitWRKY1 regulate citric acid degradation (Hellens et al., 2005). Compared with an empty vector control, transient overexpression of CitNAC62 and CitWRKY1 significantly decreased the citric acid content in citrus fruits, with values of 13.61 and 13.98 mg g⁻¹, respectively, compared with 18.37 mg g⁻¹ for the empty vector control. Transient overexpression of

Fig. 3. In vivo interaction of transcription factors with the promoter of the CitAco3 gene from Ponkan fruit. In vivo associations of the transcription factors and promoter were obtained from transient expression assays in tobacco leaves. The ratio of firefly luciferase (LUC) and renilla luciferase (REN) of the empty vector (SK) plus promoter was set at 1. Error bars indicate SE from at least five replicates. **Significant differences (P<0.01).

Fig. 4. Expression of the CitWRKY1 and CitNAC62 genes in flesh of Ponkan fruits during fruit development, DAFB, days after full blossom. Error bars represent SE (n=3).

Fig. 5. Subcellular localization of CitNAC62-GFP and CitWRKY1-GFP in tobacco leaves transformed by agroinfiltration. GFP fluorescence of CitNAC62-GFP and CitWRKY1-GFP is indicated. Bars=25 µm.
combination of CitNAC62 and CitWRKY1 resulted in lower citric acid content in citrus fruits, at 10.59 mg g⁻¹ (Fig. 7A). Transient overexpression of CitNAC62 or CitWRKY1 significantly increased CitAco3 abundance (Fig. 7B). Furthermore, co-introduction of both CitNAC62 and CitWRKY1 resulted in even lower citric acid content and higher CitAco3 expression (Fig. 7), indicating that the two transcription factors can act in combination to increase the level of CitAco3 and decrease the citric acid content.

Discussion

CitAco3 is a contributor to citric acid degradation

Multiple reports have correlated gene expression with citric acid degradation in citrus fruit, including an aconitase gene, CitAco3 (Chen et al., 2013; Lin et al., 2015). In the present study, the association of CitAco3 and citric acid degradation was confirmed during Ponkan fruit development. However, owing to the difficulty of transformation in perennial fruit such as citrus, validation of the function of CitAco3 has not been performed. With the development of a citrus transformation system (Shen et al., 2016; Yin et al., 2016), we have now shown that transient overexpression of CitAco3 led to lower citric acid content in citrus fruit and leaves, supporting a role for CitAco3 in citric acid degradation. A similar function for Aco3 has been reported in other plants, including Arabidopsis (Hooks et al., 2014) and tomato (Morgan et al., 2013). The present results support the potential role of CitAco3 in citric acid degradation in citrus fruit.

Transcription factors CitNAC62 and CitWRKY1 up-regulate CitAco3 transcript abundance and decrease citric acid content

In recent years, molecular and genetic studies have identified numerous transcription factors participating in the regulation of fruit quality (Xie et al., 2016). For instance, AP2/ERF transcription factors are involved in citrus fruit degreening (CitERF13; Yin et al., 2016) and volatile metabolism (CitAP2.10; Shen et al., 2016); and PavMYB10.1 is involved in anthocyanin biosynthesis in sweet cherry fruit (Jin et al., 2016). For organic acid metabolism, an EIN3-like transcription factor was characterized as the regulator of the ALMT1-like protein in apples (Bai et al., 2015). In addition,
MdMYB1 in apple fruits could activate the expression of two vacuolar H^+ -ATPase genes (MdVHA-B1 and MdVHA-B2), affecting malate accumulation (Hu et al., 2016). However, transcriptional regulation of citrate-related genes is largely unexplored. Here, we showed that CitNAC62 and CitWRKY1 regulate CitAco3 transcript abundance in vivo. Furthermore, transient overexpression of CitNAC62 and CitWRKY1 resulted in lower citric acid content in citrus fruit. Thus, we propose that CitNAC62 and CitWRKY1 are negative regulators of citric acid content, acting via up-regulation of the CitAco3 promoter.

**Protein–protein interaction between CitNAC62 and CitWRKY1 also involves translocation**

An interesting finding was the protein–protein interaction between CitNAC62 and CitWRKY1, which suggests that the complex of transcription factors may contribute to citric acid degradation. Protein–protein interaction between transcription factors has been widely demonstrated in many plants, including fruit species. For example, MYBs, bHLHs, and WD40s have been shown to act together in a ternary regulatory MYB-BHLH-WD40 complex in order to regulate target genes, especially in anthocyanin biosynthesis (Schaart et al., 2013), and EjAP2-1 regulates lignin biosynthesis via interaction with EjMYB1 and EjMYB2 in loquat fruits (Zeng et al., 2015). However, such an interaction has not been reported for the regulation of organic acid metabolism. Thus, the impact of the interaction of CitNAC62 and CitWRKY1 on citric acid degradation may be only moderate (according to the transient overexpression data), but the interaction provides a novel clue about citric acid regulation.

BiFC analysis indicated that interaction between CitNAC62 and CitWRKY1 happens in the nucleus, but subcellular localization analysis indicated that only CitWRKY1, and not CitNAC62, is located within the nucleus. These results suggested that CitWRKY1 may translocate CitNAC62 to the nucleus. Translocation of genes by protein–protein interactions plays important roles in plants. In Arabidopsis, AtEBP may move from the nucleus to the cytoplasm via protein–protein interaction with ACBP4 (Li et al., 2008); in rice, OsSPX4 could prevent OsPHR2 from being targeted to the nucleus through its interaction with OsPHR2 when phosphate is sufficient (Lv et al., 2014). The present findings suggest that translocation of CitNAC62 may also contribute to citric acid degradation; however, the specific role of this translocation requires further investigation. In particular, the role and mechanism of CitWRKY1 for translocation, as well as the triggers of translocation, are unclear, and it is important to evaluate the function of such translocation in citric acid degradation.

**Supplementary data**

Supplementary data are available at JXB online. Table S1. Primers for real-time quantitative PCR analysis. Table S2. Primers used for amplification of the promoter of CitAco3 and SK construction.

Table S3. Primers used in subcellular localization analysis. Table S4. Primers for yeast two-hybrid and BiFC assays. Table S5. Primers used in transient overexpression analysis.

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