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SNAC4 and *SNAC9* transcription factors show contrasting effects on tomato carotenoids biosynthesis and softening

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ABSTRACT

Color change due to lycopene accumulation and decreasing β-xanthophyll and chlorophyll concentrations occurs during ripening of tomatoes (*Solanum lycopersicum*). The functions of the transcription factors *SNAC4* (*SlNAC48*, Accession number: NM 001,279,348.2) and *SNAC9* (*SlNAC19*, Accession number: XM 004,236,996.2) during ripening have been studied using virus-induced gene silencing (VIGS) to obtain *SNACs*-silenced fruit. Silencing of *SNACs* led to delayed ripening associated with reduced ethylene production, lycopene accumulation, and yellow or orange fruit. Genes involved in carotenoid pathway flux and ethylene biosynthesis were consistently down-regulated. Silencing of *SNAC4/9* altered tomato ripening processing. In *SNAC4*-silenced fruit, softening-related factors were down-regulated and there was less ABA (abscisic acid) than the negative control, whereas *SNAC9*-slilenced fruit showed an opposite phenotype. Exogenous ABA accelerated softening of *SNAC4*-silenced fruit, while exogenous nordihydroguaiaretic acid (NDGA) slowed down the softening of *SNAC9*-silenced fruit. Our results suggest that pathways dependent on ethylene and ABA are regulated by *SNAC4* and *SNAC9,* respectively.

1. Introduction

Fruit ripening is an important developmental process in plants. This complex process is modulated by the genome, involving numerous metabolic pathways that result in changes in the texture, color, aroma, flavor, and nutrition of the fruit (Carrari and Fernie, 2006; Shen et al., 2014)· Fruit maturation is mediated by genetic regulators such as ethylene synthesis, pigment accumulation, and cell wall metabolism (Ergun et al., 2005; Eum et al., 2009). Tomatoes have the best genetic pattern in the asteroid clade and serve as a useful model in maturation and senescence research due to their high-resolution genetic maps and large mutant collections (Consortium, 2012).

NAC (NAM, ATF1/2 and CUC2) is one of the largest transcriptional factor families, the acronym of which derives from *NAM* (from Petunia), along with *ATAF1/2* and *CUC2* (from Arabidopsis). *NAC* proteins are highly conserved at the N-terminal region, and the NAC domain includes a DN A-B inding domain. The *NAC* C-terminus varies and includes a transcriptional activation domain (Ooka et al., 2003). *NAC* transcription factors with DN A-B inding activity are involved in several biological processes that modulate gene transcription in plants (Lindemose et al., 2013).

NAC families are related with most biological process, including lateral root formation (Xie et al., 2000), embryo development (Duval et al., 2002), flowering (Yoo et al., 2007), modulation of secondary cell wall accumulation, and cell division (Zhong et al., 2007). In chickpeas, *CarNAC5* is expressed at high levels during seed maturation as well as in the embryos during early germination, and *CaNACs* are involved in various tissues at different developmental stages (Ha et al., 2014). *EjNAC1* in loquat fruit is associated with lignin biosynthesis (Xu et al., 2015). *AtNAP* is related to the senescence of Arabidopsis siliques (Kou et al., 2012). Recently, several *MaNAC* transcription factors were found to function in banana ripening by interacting with component *MaEIL5*, a component of the ethylene signaling pathway (Shan et al., 2012). The *NAC*-domain transcription factor, *NOR* (non-ripening), can lead to a non-ripening phenotype, in which the production of ethylene is decreased (Giovannoni, 2007). Although tomato *SlNAC1* and *SlNAC4* have important roles in carotenoid pathway flux in tomatoes (Ma et al., 2014;Zhu et al., 2014; Meng et al., 2016), only a few tomato ripening-

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related genes associated with expression variation have been evaluated, and the mechanisms of numerous *NACs* related to fruit maturation are still undefined. SNAC4 (SGN Accession Number: SGN-U56342) was highly expressed in stems and leaves, SNAC9 (SGN Accession Number: SGN-U565186), and was highly expressed in old leaves (Kou et al., 2014).

Tomatoes are a major dietary source of lycopene. Lycopene is related to fruit reddening, which accompanies maturation. Overexpression of tomato *SlNAC1* leads to a decline in carotenoids by altering carotenoid flux and decreasing the synthesis of ethylene, which is basically regulated by interacting with the regulatory regions of *PSY1* (phytoene synthase 1), *ACS2* (1-aminocyclopropane-carboxylase synthase 2) and *ACO1* (1-aminocyclopropane-carboxylase oxidase 1) (Ma et al., 2014). Suppression of the *SlNAC1* transcription factor results in up-regulation of genes associated with the biosynthesis of lycopene and ethylene (Meng et al., 2016).

Ethylene is a critical phytohormone affecting the initiation of tomato ripening (Merchante et al., 2013). Two key enzymes take part in the biosynthetic pathway of ethylene: ACC synthase (ACS) and ACC oxidase (ACO), which have been studied at length (Eum et al., 2009). ACS and ACO activities are reduced, and the ripening process is impaired by inhibition of *ACS2, ACS4* and *ACO1* expression (Li et al., 2011). Ethylene Receptor 4 (*ETR4*) is responsible for the perception of ethylene (Kevany et al., 2007). *NR* (Never Ripening), which encoded an ethylene receptor, is important for the perception of ethylene (Bramley et al., 1992).

ABA, a phytohormone, plays a crucial role in fruit maturation. Galpaz et al. (2008) and other researchers have found that tomato plants lacking ABA have reduced growth, which suggests that ABA is important for fruit growth. 9-cis-epoxycarotenoid dehydrogenase (NCED) is a key enzyme involved in ABA synthesis (Sun et al., 2012); Overexpression of *NCED* accelerates the accumulation of ABA (Thompson et al., 2007), while *NCED*-suppressed tomato fruit are firmer than wild-type tomatoes (Sun et al., 2012). At the beginning of fruit ripening, ABA biosynthesis is initiated by the expression of *NCED*; the ABA concentration is reduced by biosynthetic inhibitors, effectively delaying softening (Zhang et al., 2009a, 2009b). Recent research has shown that *SlNAC1* increases ABA production, leading to early softening of fruit (Ma et al., 2014), while suppression of *SlNAC1* expression down-regulates genes associated with cell wall metabolism and ABA biosynthesis (Meng et al., 2016).

Ethylene treatment induces *SNAC4* (*SlNAC48*) and *SNAC9* (*SlNAC19*) expression (Kou et al., 2014). We found that *SNAC4* and *SNAC9* transcription factors act in combination with ethylene synthesis genes, resulting in orange, mature tomato fruit (Kou et al., 2016). The important role of ethylene during the ripening and senescence of tomatoes is well established, but the interactions with ABA have not been fully determined. Thus, we explored the influence of *SNAC4* and *SNAC9* on tomato carotenoid biosynthesis and softening to elucidate the dependence of *NAC* on ethylene and ABA, leading to the proposal of an alternative way of understanding the maturation process regulated by *NAC*.

2. Materials and methods

2.1. Fruit materials and growth environments

Seeds of Micro-Tom tomatoes were immersed in distilled water for 24 h at 25 °C and then planted them in flowerpots containing soil and vermiculite at a ratio of 7:1. The tomatoes were then placed in a greenhouse under a 16 h/8 h light/dark cycle at 20 °C.

2.2. Agro-infiltration

The GV3101 strain of *Agrobacterium tumefaciens* carrying pTRV1, pTRV2 and pTRV2-*SNAC4/9* was preserved in the laboratory at −80 °C. Agrobacteria was cultured in LB (Luri A-B ertani) liquid medium containing kanamycin (3×10^{-5} g L^{-1}), rifampicin (5×10^{-5} g L^{-1}), and gentamicin (5×10^{-5} g L^{-1}) at OD_{600} = 2.0-3.0. The cells were centrifuged, and infiltration buffer containing $MgCl_2$ (10^{-5} kg mol^{-1}), MES (2-(N-Morpholino) ethanesulfonic acid) (pH 5.6), and acetosyringone (2×10^{-7} g mol^{-1}) were added to the supernatant. Then, pTRV2-*SNAC4/SNAC9* was mixed with pTRV1 at a ratio of 1:1 and retained with 200 × g centrifugal force for 3 h at 28 °C. We used the mixed suspension for surface infiltration of three-week-old seedlings with a needleless syringe. Four to six leaves were used for the seedling infection. In addition, a second injection was performed on the ovary at the anthesis stage. To measure ripening time, we considered flowers as the anthesis stage. The *PDS* (phytoene desaturase) gene was used as a marker to assess the efficiency of VIGS in the silencing of the *SNAC4/9* genes in tomatoes. For each VIGS plant, sixty seedlings were injected. We harvested 10 fruit at different ripening stages, including MG: 30 d after anthesis, and B stages: 35 d after anthesis, and 3, 7 and 10 d after the B stage (B + 3, B + 7, and B + 10, respectively).

2.3. Ethylene measurement

We exposed fruit to air for 3 h to remove the influence of wound-induced ethylene due to harvesting. We then placed the fruit in sealed jars at 20 °C for 3 h. Two mL of headspace gas was injected into a Ethylene analyzer (CNX-103, Tianjin, China). Ethylene concentrations of the samples were calculated using a formula (ethylene = concentration × V_{volumn}/time/kg). Three parallel experiments were carried. Each replicate involved than 10 fruit.

2.4. Measurement of respiration rate

Respiration rates of fruit at 20 °C and 80–85 % RH were determined according to Caleb et al. (2012), with slight adjustments. Fruit with uniform size from different treatments and ripening stages were divided into groups of 5 fruit and placed in chambers of Φ60*100 mm. The air flow through each chamber was 5×10^{-4} L min^{-1}. An infrared carbon dioxide analyzer (GXH-3051, Beijing, China) was used to analyze CO_2 concentrations..

2.5. Fruit firmness

Eight fruit from each ripening stage were evaluated with an sclerometer (FHM-1 Takemura, Japan) as described by Ecarnot et al. (2013), with slight modifications.

2.6. Analysis of fruit color and carotenoids

10 fruit surface color was assessed with a Chromameter (NR145, Sanenchi Co., ShenZhen, China) calibrated against a standard white tile. The L^*, a^* and b^* values were determined for fruit at each ripening stage. We then used the following equations to calculate hue angle values (in degrees): Hue = $\tan^{-1}(b^*/a^*)$ +180 if a < 0, and $\tan^{-1}(b^*/a^*)$ if a > 0[39]. Carotenoids were extracted from pericarp tissues, and then analyzed using a LC–MS (MIOROTOF-QII, NASDAQ, US), as described previously (Fantini et al., 2013). Chlorophyll was analyzed using the method of Wellburn (2004), with some modifications. We grinded 2×10^{-4} kg fruit into powder, used DMSO to accommodate to 10×10^{-3} L. The sample was in a constant temperature bath at 60 °C, then extracted 2 h, protected from light, collected supernatant. The equations for calculating the concentrations of Chla and Chlb were Chla g L^{-1} = (12.19 OD_{665}-3.45 OD_{649}) × V/1000 W and Chlb g L^{-1} = (21.99 OD_{665}-5.32 OD_{649}) × V/1000 W, where V and W are the volume of dimethyl sulfoxide and tissue weight (kg), respectively.