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Quantitative proteomic analysis reveals the involvement of mitochondrial proteins in tomato fruit ripening

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ABSTRACT

Fruit ripening is characterized by dramatic changes in fruit quality and is considered to influence postharvest storage. The mitochondria are one of the most important organelles that play crucial roles in fruit quality formation, but the underlying mechanisms remain unclear. In this study, the expression profiles of mitochondrial proteins during tomato fruit ripening were investigated using quantitative proteomic analysis, leading to the identification of 27 proteins which showed altered abundance. We additionally evaluated changes in the mitochondrial proteome in the ripening-deficient mutant, *ripening-inhibitor* (*rin*), which carries a mutation in the transcription factor RIN. A total of 74 proteins were identified which changed abundance significantly in the *rin* mutant. Gene expression analysis combined with chromatin immunoprecipitation indicated that four genes, *DnaK*, *HSP*, *PYP*, and *ACC*, which encode chaperone DnaK, heat shock protein, pyruvate kinase, and acetyl-CoA carboxylase, respectively, are direct targets of RIN. These results suggest that mitochondria might participate in the regulation of fruit quality and ripening by specific proteins. Our findings provide new information to unravel of gene regulatory networks that control fruit quality and ripening.

1. Introduction

Fruit ripening is a major stage with multiple physiological and chemical changes in the primary and secondary metabolites in fruit development and plays a crucial role in fruit quality formation and shelf life (Klee and Giovannoni, 2011). Therefore, understanding the regulatory mechanism of fruit ripening is beneficial for improving fruit quality and reducing losses. Tomato serves as a model plant which helps to understand the fruit development and ripening. Over the past decades, many advances have been achieved in revealing the molecular mechanisms of fruit ripening in tomato (Alba et al., 2005; Giovannoni et al., 2017). Up to now, about 13 regulators which are necessary for tomato fruit ripening have been identified and characterized, including RIN (Ripening inhibitor) (Vrebalov et al., 2002), NOR (Non-ripening) (Giovannoni, 2007), CNR (Colorless non-ripening) (Manning et al., 2006), HB-1 (Lin et al., 2008), TAGL1 (Itkin et al., 2009), AP2a (Karlova et al., 2011), FUL1 and FUL2 (Bemer et al., 2012), NAC1 (Ma et al., 2014), ZFP2 (Weng et al., 2015), DML2 (Liu et al., 2015), MSI1(Liu et al., 2016a, 2016b) and ARF2 (Breitel et al., 2016). Among these regulators, RIN, NOR and CNR have been proved as critical regulators that control tomato fruit ripening (Giovannoni, 2007). *RIN* gene encoding MADS domain transcription factor which plays important roles in plant growth and development, is the most extensively studied regulator in tomato fruit (Vrebalov et al., 2002; Ito et al., 2008; Qin et al., 2012, 2016). RIN mutation led to non-ripening phenotypes (Vrebalov et al., 2002), and RIN regulates the expression of a large number of ripening-related genes *via* directly binding to the promoters. Numerous target genes of RIN have been identified by chromatin immunoprecipitation (ChIP) and these target genes were involved in cell wall metabolism, ethylene biosynthesis (Martel et al., 2011), aroma formation (Qin et al., 2012), carotenoid biosynthesis (Fujisawa et al., 2013), ubiquitin-mediated proteolysis (Wang et al., 2014) and sucrose metabolism (Qin et al., 2016). Moreover, Fujisawa et al. (2013) presented a comprehensive approach ChIP-chip to identify downstream targets of RIN and identified 241 direct targets genes. Although tremendous progress has been made to seek the direct target of RIN in regulation of fruit ripening, targets of RIN have not been completely identified.

Mitochondria, the vital organelles of eukaryotic cell, act as the powerhouses which generate energy for the cell and play a major role in a wide range of biological processes in plants, including photosynthesis (Araújo et al., 2014), programmed cell death (Lam et al., 2001), retrograde regulation (Rhoads and Subbaiah, 2007), cytoplasmic male sterility (Chase, 2007), senescence (Keech, 2011), defense response

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(Huang et al., 2013), and fruit ripening (Yang et al., 2017). Mitochondria also exert an enormous function on fruit quality formation since mitochondria are the major site for amino acid metabolism, biosynthesis of citrate, vitamins, γ-aminobutyric acid and other cofactors (Rébeillé et al., 2007; Schmidtmann et al., 2014; Hildebrandt et al., 2015; Li et al., 2017). Additionally, mitochondria are the major reactive oxygen species (ROS) generator in plant (Tripathy and Oelmüller, 2012). Thus, mitochondrial dysfunction can result in many adverse effects in fruit ripening and quality formation. Implementation of mitochondrial function requires the participation of a variety of proteins. So far, large-scale mitochondrial proteome has been broadly applied to study the global changes in protein abundance in various plants, such as rice (Huang et al., 2009), peach (Qin et al., 2009a), apple (Qin et al., 2009b), potato (Salvato et al., 2013), and *Arabidopsis* (Millar et al., 2001). Given the importance and multifunctional role of mitochondria in fruit, mitochondrial proteome studies have been conducted and provided valuable cues for understanding the function of mitochondria in fruit senescence. In previous study, we reported, through proteome analysis of mitochondria from different senescent apple fruit, ROS may influence fruit senescence through changing the abundance of mitochondrial proteins (Qin et al., 2009b). Oxidative damage of mitochondrial proteins participated in peach fruit senescence (Qin et al., 2009a). However, the global expression profiles of mitochondrial proteins during tomato fruit ripening and their regulation remain largely unknown.

In order to reveal the role of mitochondria in tomato fruit ripening, comparative proteome analysis of mitochondrial proteins from wild-type and *rin* mutant tomato was performed. Mitochondrial protein was isolated from fruits at various ripening stages and quantified by isobaric tags for relative and absolute quantification (iTRAQ) technology. A total of 74 proteins were identified which changed abundance significantly in the *rin* mutant compared with the wild-type. Further analysis demonstrated that four genes are novel direct targets of RIN. These findings are beneficial for unraveling gene regulatory networks that control fruit ripening.

2. Material and methods

2.1. Plant material

Wild-type tomato (*Solanum lycopersicum* cv. Ailsa Craig) and *rin* (*rin/rin*) mutant in the cv. Ailsa Craig background were planted in a climate-controlled greenhouse. Flowers were tagged at anthesis to determine fruit ripening stages. Fruit samples were harvested at 35, 38, 41 and 44 days post anthesis (dpa), respectively (Fig. S1 A). Collected fruit pericarp from wild-type and *rin* were immediately frozen in liquid nitrogen, and stored at −80 °C until used. For proteomic analysis, two biological replicates were performed and 10 tomato fruit from wild-type and *rin* were used for each replicate.

2.2. Mitochondria isolation and purification

The integrity and purity of mitochondria play a vital role in the quality of mitochondrial proteomics. Mitochondria from wild-type and *rin* fruit were isolated and purified using two-step percoll gradient centrifugation according to the method of Taylor et al. (2005) with some modifications. Briefly, 100 g of fruit pericarp from each sample was mixed with 300 mL ice-cold extraction buffer A (250 mM sucrose, 1 mM EDTA, 0.5% polyvinylpyrrolidone-40, 0.1% BSA, 10 mM β-mercaptoethanol, and 50 mM Tris-HCl, pH 7.5) and homogenized. KOH was added to maintain a stable pH between 7.0 and 7.1 during homogenization. Four layers of cheesecloth were applied to remove the debris and the resulting filtrate was centrifuged at 1200 × g for 15 min. After centrifugation, the supernatant was collected, centrifuged again at 17,000 × g for 20 min and then resuspended in 1 mL ice-cold wash buffer (250 mM sucrose, 0.1% (w/v) BSA, and 50 mM Tris-HCl, pH 7.5) using a soft brush. Next, the suspension was placed carefully onto the top of a step percoll gradient at 10, 17 and 23% (3:4:5) in wash buffer and centrifuged at 40,000 × g for 45 min. After centrifugation, bands were formed at the interface between the density gradient layers (Fig. S1B). To evaluate which band contains the crude mitochondria, the activities of marker enzymes of mitochondria (cytochrome c oxidase), peroxisomes (catalase), cytosol (alcohol dehydrogenase) were measured according to the method of Millar et al. (2001). Fractions of 0.5 mL each from top to bottom of the percoll step-gradient were collected and subjected to activity measurement. It was found that partially purified mitochondria were mainly enriched at 17%: 23%, contaminated by peroxisomes, but not by cytosol (Fig. S1C). The crude mitochondria fractions were collected, mixed with ten time volumes of ice-cold wash buffer and washed twice with ice-cold wash buffer at 25,000 × g for 15 min. To further purify mitochondria, the crude mitochondrial pellets were resuspended softly and placed on 35% (v/v) percoll gradient carefully. After centrifugation at 40,000 × g for 30 min, the mitochondria were enriched and formed a band at the upmiddle of the gradient (Fig. S1B), corresponding to a peak of cytochrome c oxidase activity (Fig. S1D). Activity of catalase and alcohol dehydrogenase could not be detected in this fraction, indicating there is no contamination by peroxisomes and cytosol (Fig. S1D). The mitochondria bands were collected and washed twice with ten time volumes of ice-cold wash buffer to remove percoll. Finally, purified mitochondria were carefully resuspended in 1 mL ice-cold wash buffer.

2.3. Protein extraction

Proteins from purified mitochondria were extracted according to method described by Saravanan and Rose (2004). Briefly, the purified mitochondria were resuspended in lysis buffer and sonicated at 35% amplitude for 3 min. After sonication, an equal volume of saturated phenol was added to each sample, vortexed for 10 min, and separated into layers by centrifugation at 13,000 × g for 30 min. The upper phenol containing proteins were collected, mixed with five volumes of saturated ammonium acetate in methanol and then precipitated overnight at −20 °C. The precipitated proteins were collected by centrifugation at 13,000 × g for 20 min and washed with ice-cold saturated ammonium acetate in methanol twice. Following, the proteins pellets were washed with ice-cold acetone twice. The resulting proteins were pelleted by centrifugation at 13,000 × g for 20 min and air dried. The dried proteins were dissolved and sonicated in protein buffer consisting of 500 mM triethylammonium bicarbonate (TEAB) and 1% SDS (w/v), pH 8.5. After that, the supernatant was collected by centrifugation and the concentration of mitochondrial proteins was quantified as described by Bradford (1976).

2.4. ITRAQ labeling

ITRAQ labeling was performed according to the method described by Wang et al. (2017). Briefly, one hundred micrograms of mitochondrial proteins were incubated with Tris-(2-carboxyethyle) phosphine (TCEP) reducing reagent at 37 °C for 4 h, and alkylated with methylmethanethiosulfonate (MMTS) Cysteine Blocking Reagent at 25 °C for 1 h. After alkylation, proteins were loaded onto the ultra-filtration column and washed three times with TEAB buffer to remove SDS. Proteins were digested by trypsin using the filter-aided sample preparation (FASP) method (Wiśniewski et al., 2009) for 16 h at 37 °C. After that, the tryptic peptides were eluted from ultra-filtration column, dried by vacuum centrifugation and then resuspended by iTRAQ dissolution buffer. Following, iTRAQ Reagents 4-plex Kit (Applied Biosystems, Framingham, USA) were used to labeled the tryptic peptides.

Mitochondrial proteins from wild-type and *rin* fruits at 38 dpa and 41 dpa were labeled with iTRAQ tags 114, 115, 116 and 117, respectively. The iTRAQ reagents dissolved in ethanol were mixed with each samples peptides, incubated at 25 °C for 2 h and then added ultrapure