Shotgun proteomics analysis of fruit sweetness and ripening in Nam-Dokmai mango (*Mangifera indica* L.) peel

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ABSTRACT

Nam-Dokmai mango (*Mangifera indica*) is an important economic crop in Thailand because of its high sweetness and good smell. In order to get the suitable time for fruit harvesting, sugar content in mango flesh and sweetness related proteome in mango peel during ripening were investigated. Mango fruits were collected and divided into 8 developmental stages (15, 30, 45, 60, 75, 90, 105 and 120 days after flowering (DAF)). The sugar content of mango flesh was monitored by HPLC. Glucose and fructose were found in the initial stages (15 DAF), while sucrose began to appear since 30 DAF and obviously increased from 105 DAF to 120 DAF (31.20±0.05 µg/mg to 141.52±0.11 µg/mg). Protein profiles in mango peel determined by shotgun proteomics showed that several proteins were related to sugar content in flesh, including proteins playing role in cell metabolic process, cellular component organization or biogenesis, localization, response to stimulus, and developmental process. Association between sweetness related sugars, fruit ripening related hormones and proteins obtained from mango peel including acetyl-CoA carboxylase, chalcone synthase, NADP-specific isocitrate dehydrogenase and flavin-containing monooxygenase were observed. These proteins might be used to develop as biomarker for mango fruit harvesting.

Keywords: Sugar content; HPLC; GeLC-MS; mango

INTRODUCTION

Mangoes, *Mangifera indica* L., originated from Burma and India, are commonly grown in most tropical countries. Thailand ranks fourth in the world for their production after India, China, and Mexico (Tharanathan *et al.*, 2006). To date, there are over 200 vernacular names of mango cultivars in Thailand, i.e., Nam-Dokmai, Raed, Chok-Anan, Chao-Khoon-Thip, Falan, Ok-Rong, Thong-Dam, and etc (Eiadthong *et al.*, 1999). 'Nam-Dokmai' is the most popular one, this cultivar is therefore chosen as a mango representative to be studied. It has an exceptional appearance and taste. The fruits are long, slender and sigmoid in shape. The young fruit has a creamy-green skin and its flesh is crispy with sour taste, while the ripe ones change to a soft yellow. The flesh of the ripe mango is soft and juicy, with an extreme sweetness and aromatic flavor (Ketsa *et al.*, 1999). The Office of Agriculture Economics, Ministry of Agriculture and Cooperatives, Thailand (2016) had recently reported that there was an increasing tendency of exporting mango in three consecutive years from 2013 to 2015, where the costs of export were around two thousand million baht in 2013, and three thousand million baht in both 2014 and 2015 (equivalent to 80 million US Dollars).

Proteomics is an approach that the comprehensive survey of all proteins expressed at an interesting time, at any conditions, in any organism. Studies on proteomics concerning many types of plant tissue have been carried out. This technology may help to understand the relationship between the mechanisms of sugar production and the changes of peel morphology during ripening stages which are of interest and imperative for fruit harvest and export. In the previous works, 4 proteins in Jatropha kernel were changed in their abundance during seed development similar to fatty acids level (Booranasrisak *et al.*, 2013b). Many proteins related to fruit quality, color development and pulp softening were reported by Andrade *et al.*, (2012). In addition, expression level of 128 proteins in mango fruit pericarp were altered during ripening stage (Wu *et al.*, 2014).

In this work, shotgun proteomics approach consisting of SDS-PAGE analysis, in-gel digestion and LC-MS/MS was applied to understand the protein expression pattern in mango peel associated with sugar content in mango flesh during fruit ripening.

MATERIALS AND METHODS

Plant material

Nam-dokmai mango fruits were sampled from mango orchard in Bangkhla, Chachoengsao, Thailand on different days after flowering (DAF). All fruits were separated into 8 ripening stages from 15 to 120 DAF. The characteristics of the fruits were summarized as shown in Table 1 and Figure 1. The peel was sliced from the fruit for proteomics analysis while flesh was prepared for sugar analysis by HPLC. All samples were stored at -80 ºC prior to use.

Sugar Analysis of Mango Flesh by HPLC

Sugar contents in mango flesh were extracted by the modified method of Karkacier *et al.*, (2003). Mango flesh (50 mg) was ground in liquid nitrogen with micropestle and dissolved in 1 ml of water. All samples were centrifuged at 10,000xg for 15 min, supernatant was collected and filtered through a 0.2 µm millipore filter (VertiCleanTM; NYLON Syringe, Vertical Chromatography Co., Ltd.,Thailand). Fructose, glucose and sucrose were analyzed by high performance liquid chromatography (HPLC) coupled with a 410 differential refractometer (RI) detector that consisted of a Waters 600 gradient controller pump (Water, Milford, MA, USA) and on-line detection monitored by a RI detector. Chromatography of sugar was performed by a Metacarb 87C (7.8×300 mm) (Varian Inc., USA) equipped with guard column. Nanopure water was used as the mobile phase. The injection volume was 40 µl and the flow rate was set at 0.3 ml min/min at 85°C. Quantification of sugar contents were performed by comparing the peak areas with the sugar standard.

Table 1 The 8 ripening stages of mango fruits defined by days after flowering and morphological appearance of the fruits (Booranasrisak *et al.*, 2013a)

Ripening stages	Days after flowering	Flesh texture	Peel color
1	15	hard	Green
2	30	hard	Green
3	45	hard	Green
4	60	hard	Green
5	75	hard	Green
6	90	soft	Brown spots at the upper pole, yellowish-green
7	105	soft	Brown spots at the upper pole, yellowish-green and orange at the lower pole
8	120	soft	Brown spot all over the fruit, yellow peel and orange at the lower pole

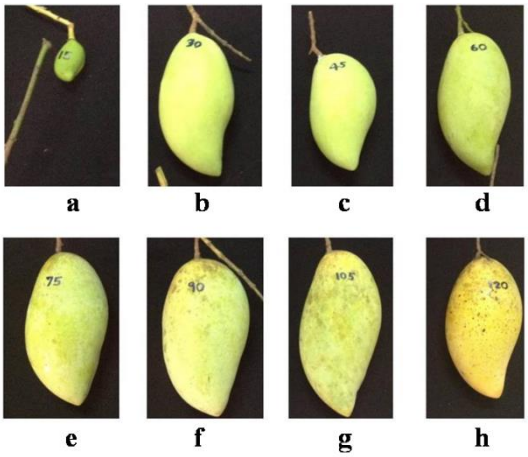

Figure 1 Photo of 8 ripening stage of Nam-dokmai mango fruits; (a-d) Young fruit at 15-60 DAF (e) Beginning of mature fruit at 75 DAF (f-g) Mature fruit at 90-105 DAF (h) Ripe fruit at 120 DAF (Booranasrisak *et al.*, 2013a).

Protein Analysis of Mango Peel by Proteomics

Protein Extraction

The mango flesh tissues from the individual stages were ground into fine powder in liquid nitrogen with pre-cooled mortars and pestles, and were subsequently dissolved in one ml of 0.1% (w/v) SDS. Each protein mixture was precipitated with cold acetone for overnight at -20ºC. The protein concentration of each sample was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as the protein standard.

SDS-PAGE Analysis

Each 50 µg protein sample was mixed with loading buffer, and subsequently heated at 95 ºC for 10 min before loading. The sample was resolved through 12.5% acrylamide resolving SDS-PAGE. Gel electrophoresis was carried out at 30 Volts for the stacking gel and 50 Volts for the separating gel. The gel was stained by Coomassie Brilliant Blue R250.

Protein Identification

Protein bands from the Coomassie Brilliant Blue R250 stained SDS-PAGE gel were excised manually according to the molecular mass range. Each protein band was subjected to in-gel digestion by trypsin. The peptides were extracted from the gel plugs after digestion, and then were analyzed using an HCTultra PTM Discovery System (Bruker Daltonics Ltd., Germany.) coupled to an UltiMate 3000 LC System (Dionex Ltd., U.K.). Peptides were separated on a nanocolumn (PepSwift monolithic column 100 µm i.d. x 50 mm). Eluent A was 0.1% formic acid and eluent B was 80% acetonitrile in water containing 0.1% formic acid. Peptide separation was achieved with a linear gradient from 10% to 70% B for 13 min at a flow rate of 300 nl/min, including a regeneration step at 90% B and an equilibration step at 10% B, one run took 20 min. Peptide fragment mass spectra were acquired in data-dependent AutoMS (2) mode with a scan range of 300−1500 *m/z*, 3 averages, and up to 5 precursor ions selected from the MS scan 50−3000 *m/z*.

The MS/MS spectra were analyzed with DeCyder MS 2.0 differential analysis software (GE Healthcare) and further submitted to a database search against the NCBI database using Mascot software (Matrix Science, London, UK, (Perkins *et al.*, 1999). All proteins were functionally identified by pantherdb (http://pantherdb.org). MultiExperiment Viewer (Mev) program version 4.6.1 (Howe *et al.*, 2010) was used to illustrate protein quantity as Heatmap for density comparison between each ripening stage. Analysis of time course log2 data was conducted with the software package Mev.

Bioinformatics using STITCH

Potential interaction of the identified proteins and biochemical molecules of interest was analyzed using the search tool for interacting chemicals using STITCH 5.0 software. Stronger associations are represented by the thicker lines. Protein-protein interactions are shown in blue, chemical-protein interactions in green and interaction between chemicals in red. High confident interaction was focused with a score higher than 0.7.

RESULTS

Sugar determination

During ripening of mango fruits, amount of some sugar increase and some remain high. These factors make mango flesh sweet when ripen. In this work, glucose and fructose were observed at the earliest stage when sucrose content increased in 30 DAF stage and existed thoroughly during ripening. Neither sorbitol nor mannitol was detected. The detected sugar profiles of mango flesh of individual 8 ripening stages are summarized in Figure 2. It was noticed that all of sugar contents gradually increased from 30 DAF stage to the last stage, except for sucrose that clearly increased from 105 DAF (31.20±0.05 µg/mg) and reached maximum at 120 DAF (141.52±0.11 µg/mg).

Protein analysis

SDS-PAGE and molecular mass distribution

The protein extracts from 8 developmental stages were resolved by 12.5% SDS-PAGE, and the gel was visualized by Coomassie Brilliant Blue R250 staining as shown in Figure 3. Proteins were expressed over a wide molecular weight range from 14.4 to 97 kDa. Compare with protein standard marker, the protein bands in range of 20 to 45 kDa were changed implying significant changes in abundance of many proteins. All gel bands in each lane were then excised according to size into 15 segments and tryptic in-gel digestion was subsequently performed. The extracted peptides of each mass range were individually injected to LC-MS/MS.

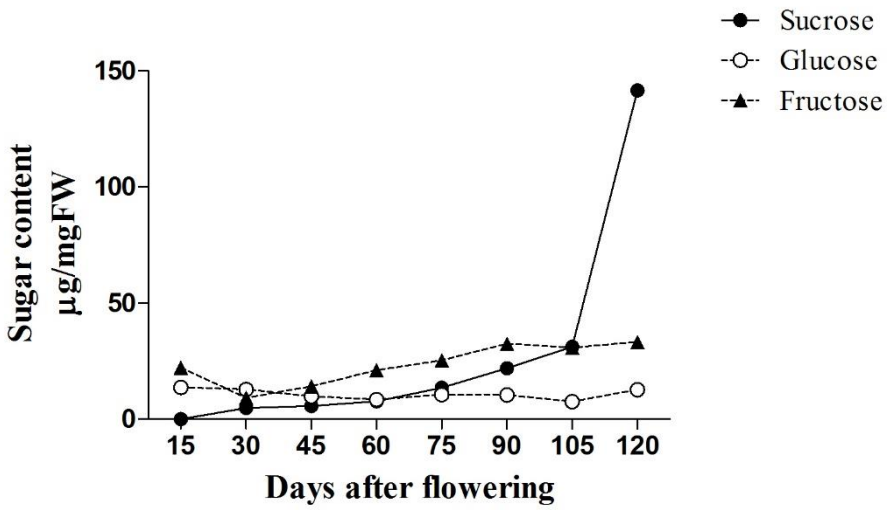

Figure 2 The quantity of sugar detected in all individual ripening stages (All data were the average values derived from triplicate experiments).

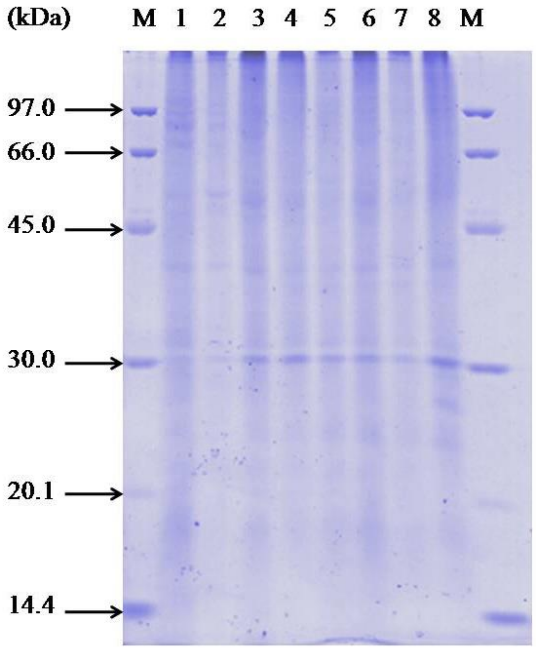

Figure 3 The Coomassie blue R-250 stained 12.5% SDS-PAGE of 50 µg protein isolated from 8 ripening stages of Nam-Dokmai mango peel (Lane M represents standard molecular weight marker; Lane 1-8 represent protein pattern of 8 ripening stages of Nam-Dokmai mango peel)

Protein identification

Raw LC-MS/MS data were analyzed by Decyder MS 2.0 differential analysis software (GE Healthcare) to glean peptides that change in expression during ripening. These identified 907 proteins were observed and shown in heatmap created by MultiExperiment Viewer (MEV) version 4.6.1 (Figure 4a). Their biological process, molecular function and cellular component were analyzed by Uniprot and Pantherdb. In the biological process classification, several metabolic processes (43%) and cellular process (20%) were found to alter during ripening (Figure 4b). While major proteins associated with catalytic activity and binding were differentially expressed during fruit development (Figure 4c). Figure 4d showed that the proteins most changes in their abundance were involved in cell part, organelle, macromolecular complex and membrane.

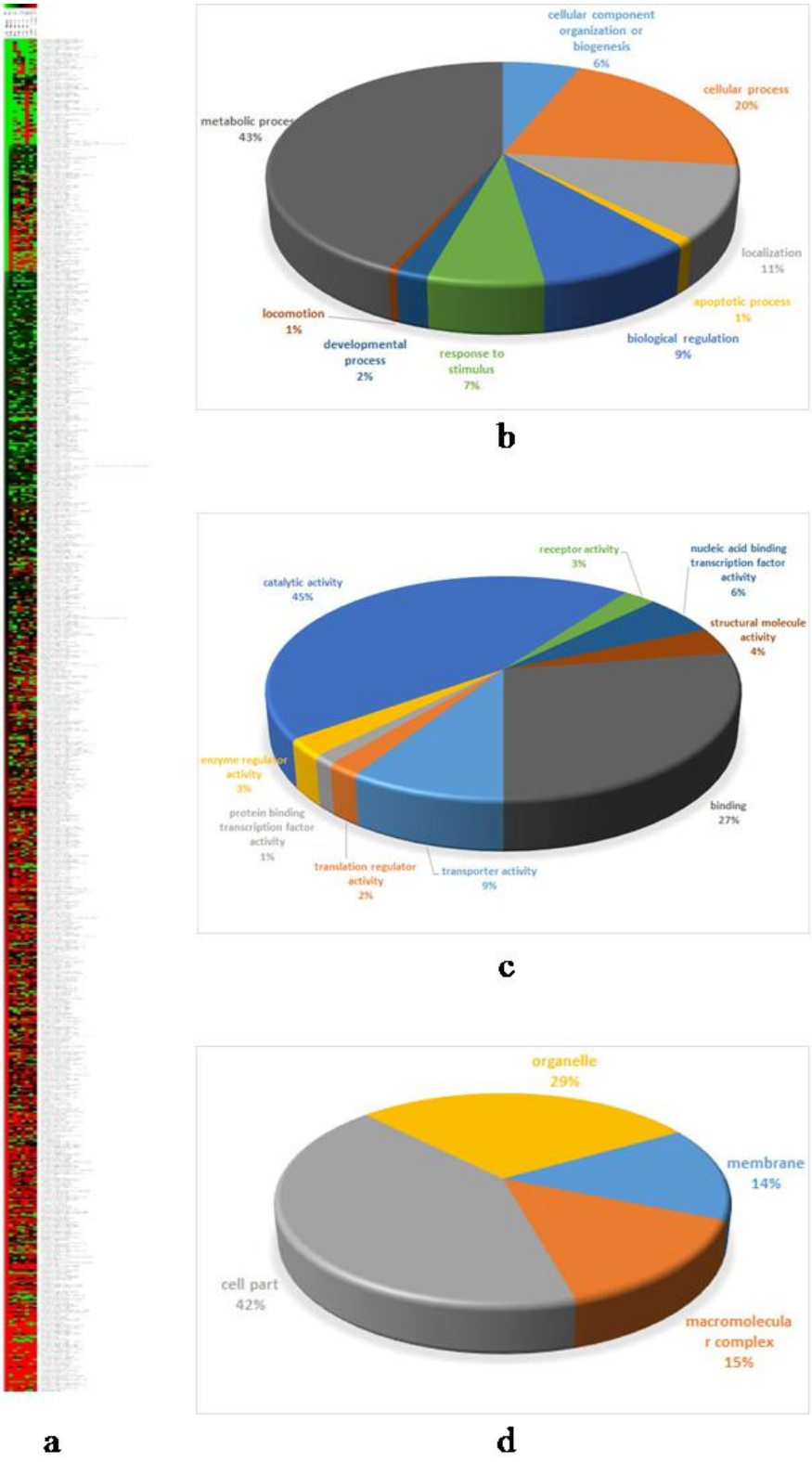

Figure 4 The heatmap of 907 proteins that changed in expression level during ripening were analyzed by MultiExperiment Viewer (MEV). Column 1-8 represent protein expression in Nam-Dokmai mango flesh at 15, 30, 45, 60, 75, 90, 105 and 120 days after flowering, respectively. Green, black and red colors represent proteins with low, average and high levels of expression, respectively. (a) The proteins were categorized based on (b) biological process, (c) molecular function and (d) cellular component by Uniprot and Pantherdb.

Sugar and protein results

HPLC results showed that sucrose, fructose and glucose were found in majority during ripening. The overall profiles of the protein log2 values at 15, 30, 45, 60, 75, 90, 105 and 120 days time points indicated changes in relative protein expression of mango peel during fruit ripening as shown as heatmap (Figure 4a). Then MEV software was used to cluster time course proteins profile with sugar content. Similar relative expression profiles of some proteins to each monosaccharide were listed in Table 2. A slight change in relative expression of Flavin-containing monooxygenase FMO, TATA-binding protein-associated factor MOT1 and NADP-specific isocitrate dehydrogenase was in the same trend as glucose content. The profile of peroxisomal 2,4-dienoyl-CoA reductase (DECR2) and pepsin A showed an initial decrease followed by an increase after 75 days, which was similar to changes of fructose in mango flesh during ripening. While a greater increase of sucrose in mango peel was found to fit to log2 time course profile of three proteins including chalcone synthase- like, proteasome component (PCI) domain- containing protein and acetyl-CoA carboxylase.

Table 2 The 8 peel proteins that considerably related to sugar content. The relative abundance level was expressed as log2 intensities.

Protein name (Uniprot number)	Accession number		15 day	30 day	45 day	60 day	75 day	90 day	105 day	120 day
flavin-containing monooxygenase FMO (AT1G12130)	gi|15221214	Glucose	18.7	18.1	19.2	17.7	19.1	19.7	16.7	20.6
pepsin A (D7LAV2)	gi|297834938	Fructose	17.1	16.1	14.0	15.8	16.9	16.5	13.8	15.2
NADP-specific isocitrate dehydrogenase (ICDH)	gi|31339158	Glucose	18.0	15.9	16.0	16.0	18.1	17.2	10.4	14.5
peroxisomal 2,4-dienoyl-CoA reductase (DECR2)	gi|195623738	Fructose	16.8	17.8	16.5	18.5	19.0	19.9	15.7	19.0
TATA-binding protein-associated factor (MOT1)	gi|255565952	Glucose	17.7	18.1	17.9	17.6	16.6	18.2	15.8	19.2
chalcone synthase-like (TT4)	gi|53793409	Sucrose	13.5	12.7	14.4	11.4	11.1	13.1	13.5	17.8
proteasome component (PCI) domain-containing protein (F4KEK8)	gi|42573586	Sucrose	16.7	16.8	17.8	16.4	15.2	15.3	15.3	19.2
acetyl-CoA carboxylase (CAC3),	gi|159477697	Sucrose	13.4	11.8	12.8	13.1	10.0	17.3	11.7	14.5

DISCUSSION

Here, proteomic approaches were selected for identifying proteins in the mango peel that are related to sweetness during fruit development. All mango fruit samples were divided into eight stages of development according to the time intervals or days after flowering and the morphological appearance of the fruits. This collecting step was different from a previous work that unripe fruits were collected and allowed to ripen naturally at 25ºC (Andrade *et al.,* 2012). However, the sugar contents in mango fruits were similar to our previous work (Booranasrisak *et al.,* 2013a). The fruit sweetness was gradually increased from 30 DAF (8.00 degree Brix) to 105 DAF (12.40 degree Brix), and finally reach maximum at 25.97 degree Brix of the last fruit stage. Sucrose was an absolute prevalent sugar in ripe mangoes but fructose had more quantity than the other from 45 to 90 DAF. Moreover, Krishnamurty and Subramanya (1973) reported high level of reducing sugar (glucose and fructose) at the beginning of ripening mango, while non-reducing sugar (sucrose) was the major sugar at the end of ripening.

Among the 907 peel proteins, expression level of acetyl-CoA carboxylase (CAC3), chalcone synthase (TT4), NADP-specific isocitrate dehydrogenase (ICDH), flavin-containing monooxygenase (AT1G12130) proteasome component (PCI) domain-containing protein (AT5G45620), and peroxisomal 2,4- dienoyl- CoA reductase (SDRB) was highly related to sweetness relative sugar content in mango flesh. However, the function of these 6 proteins is poorly understood in mango peel. The identified proteins and plant hormones as well as sugars were submitted to STITCH version 5.0 to search for understanding of cellular functions and annotate all functional interactions among proteins in the mango peel and sugar content in mango flesh (Kuhn *et al.,* 2014). The signaling pathway network connecting the identified proteins and sweetness relative sugars (Figure 5) imply the relatively specific role of AT1G12130, CAC3, ICDH, SDRB, and TT4 in the biosynthesis of sucrose, glucose and fructose in mango fruit. Interaction between these

proteins were strongly correlated with phytohormone related ripening including auxin, cytokinin and ethylene. How phytohormones regulate the identified proteins and how proteins in mango peel acts on sugar content in flesh should be explored in future. The major finding of this study is that AT1G12130, CAC3, ICDH, SDRB, and TT4 in peel are theoretically assumed to be involved in ripening of mango fruit via phytohormone. They may appear as suitable markers for quality, conservation technologies and storage time in mangoes. Gene expression at mRNA level by RT-qPCR or protein level by dot blot (or ELISA) may be an alternative for a better fruit characterization. In addition, the activation or inhibition of these proteins may be an alternative method for improving fruit harvesting, storage and transport in the future.

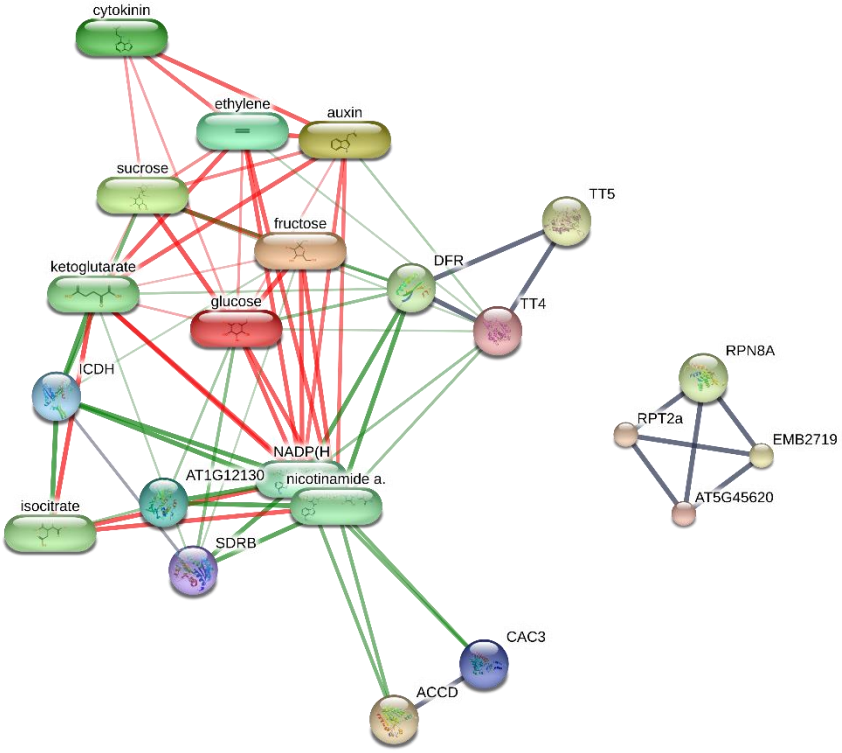

Figure 5 The involvement of 8 candidate proteins with sugar and phytohormones predicted by STITCH 5.0. Based on the online STITCH 5.0 database, an association of 8 proteins in mango flesh and sweetness related factors including sugars (sucrose, glucose, fructose) and phytohormones (auxin, cytokinin, gibberellin, ethylene) was analyzed. Abbreviations: acetyl-CoA carboxylase (CAC3), chalcone synthase (TT4), NADP-specific isocitrate dehydrogenase (ICDH), Flavin-containing monooxygenase FMO (AT1G12130), proteasome component (PCI) domain-containing protein (AT5G45620), and peroxisomal 2,4-dienoyl-CoA reductase (SDRB).

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