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Abstract

Mango (*Mangifera indica* L.) is an economically important fruit. However, the marketability of mango is affected by the perishable nature and short shelf-life of the fruit. Therefore, a better understanding of the mango ripening process is of great importance towards extending its postharvest shelf life. Proteomics is a powerful tool that can be used to elucidate the complex ripening process at the cellular and molecular levels. This study utilized 2-dimensional gel electrophoresis (2D-GE) coupled with MALDI-TOF/TOF to identify differentially abundant proteins during the ripening process of the two varieties of tropical mango, *Mangifera indica* cv. 'Chokanan' and *Mangifera indica* cv 'Golden Phoenix'. The comparative analysis between the ripe and unripe stages of mango fruit mesocarp revealed that the differentially abundant proteins identified could be grouped into the three categories namely, ethylene synthesis and aromatic volatiles, cell wall degradation and stress–response proteins. There was an additional category for differential proteins identified from the 'Chokanan' variety namely, energy and carbohydrate metabolism. However, of all the differential proteins identified, only methionine gamma-lyase was found in both 'Chokanan' and 'Golden Phoenix' varieties. Six differential proteins were selected from each variety for validation by analysing their respective transcript expression using reverse transcription-quantitative PCR (RT-qPCR). The results revealed that two genes namely, glutathione S-transferase (GST) and alpha-1,4 glucan phosphorylase (AGP) were found to express in concordant with protein abundant. The findings will provide an insight into the fruit ripening process of different varieties of mango fruits, which is important for postharvest management.

Keywords (separated by '-')

Mangifera indica - Comparative proteomics - 2DE - MALDI-TOF/TOF - Fruit ripening - Methionine gamma-lyase

Footnote Information

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Comparative Proteomic Analysis on Fruit Ripening Processes in Two Varieties of Tropical Mango (*Mangifera indica*)

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Abstract

Mango (*Mangifera indica* L.) is an economically important fruit. However, the marketability of mango is affected by the perishable nature and short shelf-life of the fruit. Therefore, a better understanding of the mango ripening process is of great importance towards extending its postharvest shelf life. Proteomics is a powerful tool that can be used to elucidate the complex ripening process at the cellular and molecular levels. This study utilized 2-dimensional gel electrophoresis (2D-GE) coupled with MALDI-TOF/TOF to identify differentially abundant proteins during the ripening process of the two varieties of tropical mango, *Mangifera indica* cv. 'Chokanan' and *Mangifera indica* cv 'Golden Phoenix'. The comparative analysis between the ripe and unripe stages of mango fruit mesocarp revealed that the differentially abundant proteins identified could be grouped into the three categories namely, ethylene synthesis and aromatic volatiles, cell wall degradation and stress–response proteins. There was an additional category for differential proteins identified from the 'Chokanan' variety namely, energy and carbohydrate metabolism. However, of all the differential proteins identified, only methionine gamma-lyase was found in both 'Chokanan' and 'Golden Phoenix' varieties. Six differential proteins were selected from each variety for validation by analysing their respective transcript expression using reverse transcription-quantitative PCR (RT-qPCR). The results revealed that two genes namely, glutathione S-transferase (GST) and alpha-1,4 glucan phosphorylase (AGP) were found to express in concordant with protein abundant. The findings will provide an insight into the fruit ripening process of different varieties of mango fruits, which is important for postharvest management.

Keywords *Mangifera indica* · Comparative proteomics · 2DE · MALDI-TOF/TOF · Fruit ripening · Methionine gamma-lyase

1 Introduction

Mango (*Mangifera indica* L.) is a popular fruit characterised by its sweet taste, aromatic scent, and low fibre flesh. Being an economically important fruit, mango has an annual production of 46.5 million tonnes in tropical regions [1]. However, the international trade and export potential of mango are restricted due to several factors, such as short shelf life and risk of postharvest diseases, which are mainly associated with fruit ripening processes [2].

As a climacteric fruit, the ripening process of mango is associated with increased ethylene production and burst in respiration, leading to physiochemical changes in colour texture, firmness, flavour and aroma of the mango fruit [2]. These changes serve as critical parameters to evaluate fruit quality [3], which is often associated with cell wall softening, degradation of starch, sucrose accumulation, synthesis of colour pigments and production of aromatic volatiles. In-depth knowledge of the mango fruit ripening processes at the cellular and molecular levels are crucial in order to develop effective storage strategies, which could help reduce postharvest losses and mitigate global food insecurity.

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Proteomics has been recognized as a powerful tool to unravel fundamental biological pathways and processes. Differential protein analysis has been performed on many commercialized fruits to elucidate protein variations that occur during ripening and response towards ethylene [4]. For instance, in the peach protein profiling, a variation of key proteins under ethylene regulation, oxidative stresses, and carbon translocation were found to influence fruit tissue integrity [5]. While in tomato, 74 differential proteins were identified, allowing the establishment of the first proteomic reference map [6].

In addition, the effects of abscisic acid treatments on the ripening process of common grape on the proteomic level have been investigated [7]. Banana is another example of a fruit with economic importance that was investigated for change in proteins during ripening processes [8]. Recently, the roles of some proteins associated with fruit ripening in the tropical wax apple were discussed [9].

Despite its economic relevance, only a few proteomic studies have been conducted on mango ripening. For example, Andrande et al. [10] discovered significant ripening-related biochemical pathways in the 'Keitt' mango variety. However, as the variety originated from Florida, the 'Keitt' mango was found to be genetically divergent from the landrace cultivars in India [11], providing little insight into the mango ripening processes of Indian mango cultivars. Therefore, proteomic investigation of other cultivars is needed to provide novel insights into protein activities and their changes in mango during ripening.

The study by Andrande et al. [10] used a single variety of mango i.e. 'Keitt' to investigate protein changes over the pre-climacteric and climacteric stages. In this study, we aimed to investigate and compare the differential proteins expression associated with fruit ripening process of pre-climacteric and climacteric stages of two tropical mango varieties namely, *Mangifera indica* cv. 'Chokanan' and *Mangifera indica* cv. 'Golden Phoenix'. These two mango varieties were selected due to their economic importance and popularity in South East Asian and world market [12, 13]. Moreover, our previous findings revealed that each of the two varieties was found to have distinctive ripening characteristics such that the peel colour of 'Chokanan' turn yellow and the pulp is significantly firmer than Golden Phoenix when ripe [14].

2 Materials and Methods

2.1 Plant Material

Mature green mangoes (*Mangifera indica* vars. 'Chokanan' and 'Golden Phoenix') with maturity index 2, graded according to Grading Standards and Specification of Fruits and Vegetables (2017) [15], were purchased from a FAMA registered commercial supplier in Malacca, Malaysia (Juriano Enterprise, GBBS).

The fruit was stored at 25 ± 1 °C, 80 ± 5% relative humidity to simulate storage conditions adopted by farmers and retail fruit merchants. The firmness of the fruit was considered as a determining factor for selecting the end of the storage period for the fruits in this experiment [16].

As stated by Yahia [17], firmness and color change are principal indices for assessing the ripening process of mango. The ripening indices were determined as follows;

(a) Mango fruit firmness was analysed on arrival (0th day) and every 2 days for a 9-day storage period. This was assessed using an Instron Universal Testing Machine (Instron 2519-104, Norwood, MA) equipped with an 8 mm plunger tip at a constant speed of 20 mm/min [18]. The maximum amount of force (N) required to penetrate the fruits was recorded. Measurements were taken from three points of the equatorial region for each sampled fruit. An average of three readings was obtained and expressed in Newtons (N).

(b) Colour was assessed on the basis of the Hunter Lab System using a MiniScan XE Plus colorimeter which was first standardized using a black tile and a white tile (X = 79.0, Y = 83.9, Z = 87.9). The values of L*, a*, b* were recorded. Coordinates, a* and b*, indicate colour directions: + a* is the red direction, − a* is the green direction, + b* is the yellow direction, and − b* is the blue direction. From these values, hue angle (h°) was calculated as h° = tangent-1 b*/a* where 0° = red purple, 90° = yellow, 180° = blue-green and 270° = blue. The readings were measured on three specified points along the equator of the fruit.

Subsequently, the unripe and ripe fruits were peeled, sliced and ground under liquid nitrogen. Fruit fine powder was then stored in − 80 °C until further use. The experiments were conducted with analysis of three fruits (replicates) at each ripening stage.

2.2 Protein Extraction and Quantification

Total proteins were extracted from the pulp of mango using Phenol method modified from Carpentier et al. [19]. Briefly, one gram of pulp powder was suspended in 5.0 mL of cold extraction buffer (5 mM EDTA, 1% (w/v) DTT, 50 mM Tris–HCl (pH 8.8), 100 mM KCl and 30% (w/v) sucrose) containing protease and nuclease inhibitor cocktails (Sigma-Aldrich, USA). Subsequently, an equal volume of ice-cold phenol solution was added and the solutions were mixed by vortexing. After centrifugation (12,000×*g*, 30 min, 4 °C), the phenol phase was collected and precipitated with 5 volumes of 100 mM ammonium acetate in methanol overnight

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at 4 °C. The phenol phase was then recovered by centrifugation (12,000×*g*, 45 min, 4 °C). After precipitation, the protein pellet was rinsed with 1% DTT/acetone and air-dried. The protein pellet was re-suspended in 100 uL lysis buffer (4% (w/v) CHAPS, 2 M Thiourea, 7 M Urea, and 1% (w/v) DTT) and kept in − 80 °C until further use. The protein concentration was determined using Bradford protein method [20] with bovine serum albumin as the reference standard.

2.3 Two-Dimensional Gel Electrophoresis (2D-GE) and Gel Image Analysis

Isoelectric focusing (IEF) was conducted using 7 cm Immobilized pH gradient (IPG) strips (GE Healthcare Life Sciences, Sweden) with a linear pH gradient of 3–10 on PROTEAN® IEF cell (Bio-Rad Ltd, USA). The protein samples (100 µg) were loaded at the cathodic side of the IEF cell. The following running conditions were used: current limit at 50 µA per strip; 200 V for 1 h; 1000 V for 1 h; 4000 V for 1 h and then a rapid gradient to 4000 V until 20,000 Volt hours (Vh). The IPG strips were then incubated for 15 min in an equilibration buffer (30% (v/v) Glycerol, 50 mM Tris–HCl (pH 8.8), 6 M Urea and 2% (w/v) SDS) that contains 2% (w/v) DTT, followed by an incubation for another 15 min in the same buffer containing 135 mM iodoacetamide instead of DTT. For second dimension analysis, the equilibrated strips were transferred to 12% Acrylamide SDS-PAGE gels for electrophoresis fractionation at 150 V for 1 h. Three biological replicates were prepared for each ripening stage of the fruits.

The gels were stained with Coomassie® G-250 stain (Thermo Fisher Scientific, USA). The gel images were digitized using a GS-800TM calibrated densitometer (BioRad Ltd, USA) and analysed using the Progenesis SameSpot software (Totallab Ltd, UK). Spots were detected, matched and normalized according to the manufacturer's instruction. Differential protein spots between the ripe and unripe samples were ascertained using normalised spots and compared with the reference gel. One way ANOVA was used to calculate the fold difference and p values. The threshold value for fold change was set at 1.6 for up and down-regulation at $p \leq 0.05$.

2.4 Protein Digestion and MALDI ToF–ToF analysis

Four gel samples of selected individual protein spots were excised manually and the protein spots were sent for mass spectrometry analysis for protein identification by a commercial service provider (FirstBase© Sdn Bhd, Malaysia). The protein samples were digested using trypsin and analysed by MALDI-TOF/TOF tandem mass spectrometer 5800 Proteomic Analyser (AB Sciex, Singapore). This was then followed by spectral analysis for the identification of the desired protein using the Mascot sequence matching software (Matrix Science, USA). The parameters used for database searches are: peptide mass tolerance of 200 ppm and fragment mass tolerance of 0.5 Da, trypsin with one missed cleavage allowed, carbamidomethylation was selected as the fixed modification while methionine oxidation was selected as the variable modification. Mass values were monoisotopic. The precursor was selected automatically to proceed for the MS/MS mode. The selection was the mass from 800 to 3000 Da with the intensity of more than 800 in the run. The Mascot probability-based score with confidence value greater than 99% ($p < 0.01$), at least two peptide hits, bolded in red, and Mascot score over the minimum, were prerequisites of accurate protein identification. The databases used are NCBInr and MSPnr100 Database (Matrix Science, USA) with MASCOT as a search engine. The protein functional classification was then searched against established databases (NCBI and UniProt/PROSITE) and the available literature.

2.5 RNA Extraction

Total RNA was extracted from pulp samples of 'Chokanan' and 'Golden Phoenix' at the unripe and ripe stages using the CTAB method [21]. Assessment of RNA quantity, quality and integrity were performed using Nanodrop ND1000 spectrophotometer, gel electrophoresis and 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA) respectively. RNA samples with a 260/280 ratio between 1.8 and 2.0, 260/230 ratio between 2.0 and 2.5 and RIN (RNA integrity number) ≥ 7.0 were used for real-time qPCR analysis.

2.6 RT-qPCR

The QuantiTect Reverse Transcription kit (Qiagen, Germany) was used to convert the RNA to cDNA following the manufacturer's protocol. For each qPCR reaction, the master mix from SensiFAST SYBR No-ROX kit (2x) (Bioline, London, UK), primers and distilled water were added to the cDNA, which made up to the final volume of 20 uL per tube. A total of 4 uL or 20 ng cDNA was used in the reaction. The qPCR amplification efficiency was validated via standard curve using cDNA samples and the respective primers designed for the reference gene only. The reference gene was chosen based on a stability test using the geNorm tool [22]. The qPCR reaction conditions were as follows: initial denaturation at 95 °C for 2 min, 39 cycles of denaturation at 95 °C for 5 s, annealing and extension at 60 °C for 30 s. The expression of the Actin gene [23] was used for normalization of every target gene studied. Relative expression level of the target genes was analysed using the $2^{-\Delta\Delta CT}$ method [24]. For each mango variety, three biological replicates (fruit) were taken and three technical

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replicates per biological replicate were analyzed per gene. The primers used are listed in Supplementary Table 1.

3 Results

3.1 Ripening Process of Mango

The storage life of mango has shown to be dependent on variety [25] based on physiochemical measurements. Our study has indicated that the colour and firmness of fruits varies between 'Chokanan' and 'Golden Phoenix' varieties [14] during ripening such that the colour of 'Chokanan' fruit turned from green to yellow within 8 days but remained firm while the fruit of 'Golden Phoenix' turned pale green, but the fruit was soft when it ripened. It is therefore interesting to investigate further to find out more about differential proteins involved in the ripening process of the two varieties of tropical mango.

A typical 2DE-gel of the ripe sample of 'Chokanan' and 'Golden Phoenix' mango are shown in Figs. 1 and 2 respectively.

3.2 Differential Proteins Involved in the Ripening Process of 'Chokanan'

The differential proteins with at least a 1.5-fold change in abundance between the ripe and unripe 'Chokanan' mango are presented in Table 1. The proteins can broadly be categorised into 4 groups namely, energy and carbohydrate metabolism, ethylene synthesis and aromatic volatiles, cell wall degradation and stress–response proteins.

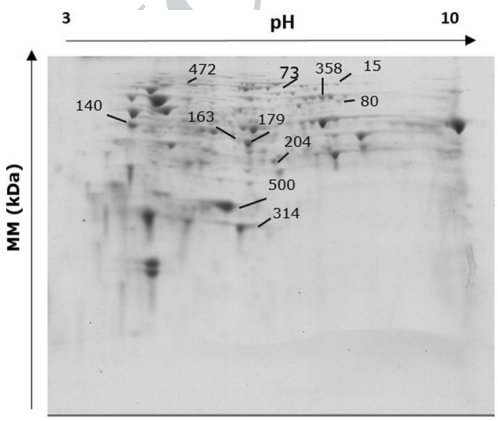

Fig. 1 2DE gel indicating the differential protein spots in a ripe 'Chokanan' mango sample

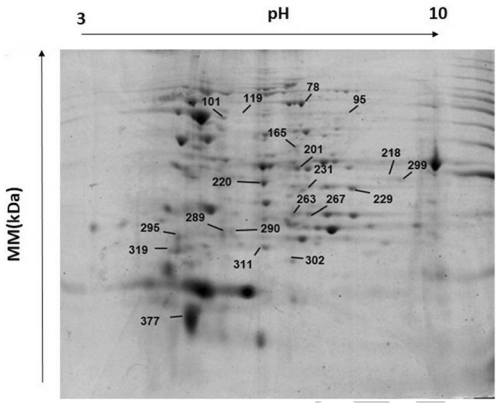

Fig. 2 2DE gel indicating the differential protein spots in a ripe Golden Phoenix mango sample

3.3 Differential Proteins Involved in the Ripening Process of 'Golden Phoenix'

The differential proteins between the ripe and unripe Golden Phoenix can be broadly categorised into three groups namely, ethylene synthesis and aromatic volatiles, cell wall synthesis and degradation and stress–response proteins (Table 2).

3.4 Comparison of Differential Proteins Related to Fruit Ripening Between 'Chokanan' and 'Golden Phoenix' Varieties

There were 11 differential proteins identified in 'Chokanan' and 13 in 'Golden Phoenix' (Tables 1 and 2). Out of these, only 1 common protein i.e. methionine gamma-lyase was found in both the ripe and unripe fruits of the 2 varieties of mango. Methionine gamma-lyase is involved in generating aromatic volatile via methionine degradation which is an important process to indicate the ripening status of the fruit. Another protein, 3-ketoacyl-CoA thiolase B, which was only found in the ripe fruit of 'Golden Phoenix', is also a precursor in the catabolic pathway for the production of aromatic volatiles. This protein was also identified in 3 spots (218, 229 and 299) indicating the presence of protein isoforms.

All the differential protein groups in 'Golden Phoenix' are of the same categories as 'Chokanan' namely, ethylene synthesis and aromatic volatiles, cell wall synthesis and degradation and stress–response proteins. However, there is one category of differential proteins only found in 'Chokanan', which is energy and carbohydrate metabolism. The higher abundance of these proteins namely, malic enzyme, ribulose-1,5-bisphosphate large subunit-binding protein subunit alpha and α-1,4 glucan phosphorylase present in the unripe fruit implicate the high energy required to maintain the firmness of the fruit during the ripening process in 'Chokanan'.

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Table 1 List of proteins with significant differential abundance ($P < 0.05$) between ripe and unripe 'Chokanan' mango samples

Spot number	Accession number (NCBInr)	Protein description	Function	PI	Mw (Da)	Sequence coverage (%)	Score	Organism
140	gi|703148942	RuBisCo large subunit-binding protein subunit alpha	Energy and carbohydrate metabolism	5.26	61,886	7	226	*Morus notabilis*
500	gi|658045224	Abscisic stress–ripening protein 3-like	Stress–response proteins	6.41	15,089	32	472	*Malus domestica*
472	gi|901807498	Chaperone protein ClpB 1	stress–response proteins	5.93	102,046	13	345	*Zostera marina*
15	gi|823259620	Alpha-1,4 glucan phosphorylase	Energy and carbohydrate metabolism	6.10	95,227	7	244	*Gossypium raimondii*
358	gi|1091514556	5-Methyltetrahydropteroyltriglutamate–homocysteine methyltransferase	Ethylene synthesis and aromatic volatiles	6.36	84,286	12	433	*Eucalyptus grandis*
80	gi|508723832	NADP-dependent malic enzyme	Energy and carbohydrate metabolism	6.46	65,072	19	587	*Theobroma cacao*
179	gi|590675246	Methionine gamma-lyase	Ethylene synthesis and aromatic volatiles	6.6	49,154	8	196	*Theobroma cacao*
204	gi|1044897083	Stearoyl-ACP desaturase	Ethylene synthesis and aromatic volatiles	5.63	45,035	11	308	*Paeonia lactiflora*
163	gi|1098730814	Homogentisate 1,2-dioxygenase-like	Ethylene synthesis and aromatic volatiles	5.89	51,540	10	317	*Juglans regia*
73	gi|848853991	Beta-galactosidase-like	Cell wall degradation	8.37	68,253	3	84	*Erythranthe guttata*
314	gi|926657597	Glutathione S-transferase-like protein	stress–response proteins	5.73	25,564	16	365	*Solanum chacoense*

Apart from methionine gamma-lyase, there were two other differential proteins identified in the ethylene synthesis and aromatic volatiles group of 'Chokanan'. The two proteins are 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (MET6), which was found abundant in unripe fruit and acyl-[acyl-carrier] protein desaturase, which was found only in ripe fruit. For 'Golden Phoenix', only one other protein namely ATP-sulfurylase was found in ripe fruit. These proteins are associated with either ethylene synthesis or aromatic volatiles production. It is interesting to take note that for the differential proteins in the cell wall synthesis and degradation category, β-galactosidase is found abundantly only in the ripe 'Chokanan' fruit.

3.5 RT-qPCR Analysis

To validate the results from MALDI ToF/ToF, RT-qPCR analysis was carried out. Six identified proteins from each mango variety were selected for RT-qPCR analysis to compare the protein abundance with the respective transcript expression. For 'Chokanan' variety, two proteins namely methionine gamma-lyase (MGL) and glutathione S-transferase (GST), were found abundant in ripe samples while four proteins namely chaperon protein ClpB 1 (ClpB1), alpha-1,4 glucan phosphorylase (AGP), 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (MET6) and malic enzyme (ME) were found to be abundant in unripe samples. The results of RT-qPCR analysis show that two genes, i.e. GST and AGP were expressed in concordance with protein abundance (Fig. 3a, c) while the other four genes tested were not in concordance with protein abundance (Fig. 3b, d, e, f). However, no significant difference was observed ($P < 0.05$) for all the comparisons of relative gene expressions between the ripe and unripe samples.

For the 'Golden Phoenix' variety, four proteins, 3-ketoacyl-CoA thiolase B (KCT), ATP-sulfurylase (AS), methionine gamma-lyase (MGL) and chitinase (Chiti) were found to be abundant in ripe samples while two proteins, xyloglucan endotransglucosylase/Hydrolase (XEG) and protein TSS (TSS) were found to be abundant in unripe samples. The RT-qPCR results revealed that three genes, i.e. KCT, TSS and Chiti were expressed in concordance with protein abundance (Fig. 4a, d, f) while the expression of the other three genes i.e. AS, MGL and XEG were not in concordance with their protein abundance (Fig. 4b, c, e). Even though the expression of MGL genes was not in concordance with the protein abundance, it is significantly ($P < 0.05$) downregulated in the ripe mango compared to the unripe mango samples.

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Table 2 List of proteins with significant differential abundance ($P < 0.05$) between ripe and unripe 'Golden Phoenix' mango samples

Spot number	Accession number (NCBInr)	Protein description	Function	pI	Mw (Da)	Sequence Coverage (%)	Score	Organism
119	gil224064246	Protein TSS	Stress–response proteins	8.91	15,919	54	66	*Populus trichocarpa*
218	gil1129145	3-Ketoacyl-CoA thiolase B	Ethylene synthesis and aromatic volatiles	8.48	45,743	35	98	*Mangifera indica*
220	gil222846686	Methionine gamma-lyase	Ethylene synthesis and aromatic volatiles	5.97	49,684	17	63	*Populus trichocarpa*
229	gil1129145	3-Ketoacyl-CoA thiolase B	Ethylene synthesis and aromatic volatiles	8.48	45,743	33	212	*Mangifera indica*
231	gil18150421	ATP-sulfurylase	Ethylene synthesis and aromatic volatiles	8.7	51,499	31	124	*Allium cepa*
289	gil222840723	Rop guanine nucleotide exchange factor 12	Stress–response proteins	5.75	51,386	38	59	*Populus trichocarpa*
290	gil147770841	Uncharacterized protein	Unknown	4.93	23,588	52	58	*Vitis vinifera*
295	gil300162608	SNARE-interacting protein KEULE	Cell wall synthesis and degradation	8.49	75,170	33	65	*Selaginella moellendorffii*
299	gil1129145	3-Ketoacyl-CoA thiolase B	Ethylene synthesis and aromatic volatiles	8.48	45,743	35	152	*Mangifera indica*
302	gil189014948	Chitinase	Ethylene synthesis and aromatic volatiles/ stress–response proteins	5.77	26,352	9	96	*Mangifera indica*
311	gil226462277	Predicted protein	Unknown	6.04	97,639	26	58	*Micromonas pusilla*
319	gil356547573	Desiccation-related protein PCC13-62-like	Stress–response proteins	6.93	33,564	12	74	*Glycine max*
377	gil187373000	Xyloglucan endotransglucosylase/Hydrolase	Cell wall synthesis and degradation	7.16	38,735	40	56	*Malus domestica*

4 Discussion

4.1 Differential Proteins Involved in the Ripening Process of 'Chokanan'

4.1.1 Energy and Carbohydrate Metabolism

Three proteins, which are associated with carbohydrate and energy metabolism, malic enzyme, ribulose-1,5-bisphosphate large subunit-binding protein subunit alpha, and α-1,4 glucan phosphorylase were found to be abundant in unripe fruit.

Malic acid, or malate, is the second most abundant organic acid after citric acid, present in ripe mango [26]. In contrast to citric acid that undergoes rapid degradation, malate only undergoes minor degradation during ripening. In fruits, the malic enzyme is a NAD-dependent oxidoreductase that catalyses the reversible decarboxylation from malate to pyruvate [27]. The pyruvate product serves as an additional energy reserve by being converted into acetyl-CoA during the initial respiratory burst in mango [28]. Additionally, other proteomic studies in mango ripening have shown that the high abundance of malic dehydrogenase (MDH) in the unripe stage degrades malate into oxaloacetic acid (OAA), a constituent in the synthesis of citric acid [10, 16]. The high abundance of malic enzyme and MDH in pre-climacteric stages suggests the role of malate in early ripening is due to its respiratory and carbon fixation capacity, which is subsequently dismantled during ripening [28].

Fruits are able to photosynthesise due to the presence of chloroplast and mitochondria within the fruits [29]. The ribulose-1,5-bisphosphate (RuBisCo) is the main enzyme in Calvin's cycle that catalyses the conversion of carbon dioxide into organic materials during photosynthesis [30]. The enzyme activity is sensitive towards stress conditions, such as heat and oxidation [31, 32]. Previous studies suggested that the presence of extreme oxidative stress leads to the fragmentation of the RuBisCo large subunit [32]. Therefore, the assistance of a molecular chaperone, RuBisCo binding protein (RBP), is a requirement for the RuBisCo formation. Our study revealed the high abundance of RuBisCo large subunit-binding protein subunit alpha (Spot 140), a form

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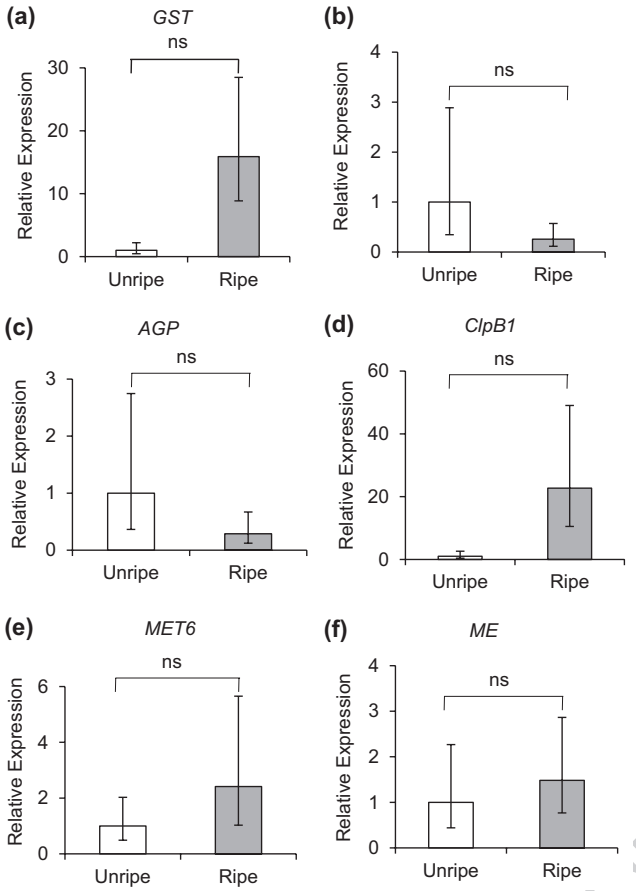

Fig. 3 RT-qPCR analysis of the transcript levels of differentially expressed proteins in ripe and unripe 'Chokanan' mango. **a** GST, **b** MGL, **c** AGP, **d** ClpB1, **e** MET6, **f** ME. Asterisks (*) indicate a statistically significant difference between the unripe and ripe samples at $P < 0.05$. The calculation for relative gene expression was made using the $2^{-\Delta\Delta CT}$ (Livak method). *ns* no significant difference. Actin gene was used as the reference gene. Error bars indicate ± SE of three biological replicates. *GST* glutathione S-transferase, *MGL* methionine gamma-lyase, *AGP* alpha-1,4 glucan phosphorylase, *ClpB1* chaperon protein ClpB 1, *MET6* 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase, *ME* malic enzyme

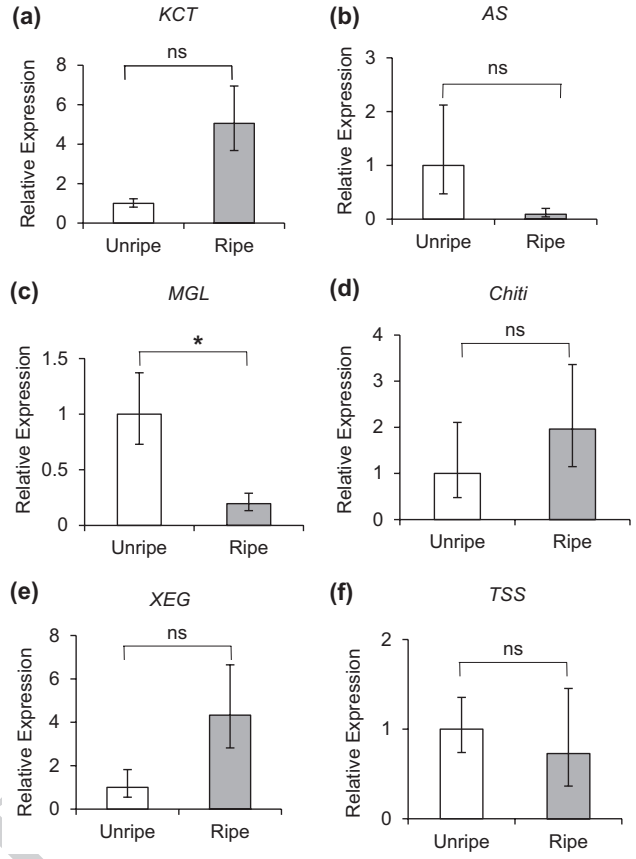

Fig. 4 RT-qPCR analysis of the transcript levels of differentially expressed proteins in ripe and unripe 'Golden Phoenix' mango. Note: **a** KCT, **b** AS, **c** MGL, **d** Chiti, **e** XEG, **f** TSS. Asterisks (*) indicate a statistically significant difference between the unripe and ripe samples at $P < 0.05$. The calculation for relative gene expression was made using the 2–ΔΔCt (Livak method). ns: no significant difference. Actin gene was used as the reference gene. Error bars indicate ± SE of three biological replicates. *KCT* 3-ketoacyl-CoA thiolase B, *AS* ATP-sulfurylase, *MGL* methionine gamma-lyase, *Chiti* chitinase, *XEG* xyloglucan endotransglucosylase/hydrolase, *TSS* protein TSS (TSS)

of RBP, from the Chaperonin 60 family in the unripe fruit, which is responsible for protein refolding under stress conditions. Previous studies have established a close relationship between fruit maturation and ripening to oxidative stress [33, 34]. Therefore, the absence of the RBP during ripening could lead to the exposure of the RuBisCo protein towards ripening-induced oxidative stress, resulting in the rapid dismantling of the photosynthetic pigment.

Starch is a polymer of glucose-1-phosphate (Glc-1-P), joined by an α-1,4 glycosidic bond, which is usually synthesized for energy storage or degraded as fuel. Starch phosphorylases, such as α-1,4 glucan phosphorylase, catalyses starch synthesis [35]. The high abundance of α-1,4 glucan phosphorylase in the unripe mango suggests the synthesis of starch during early ripening. The starch catabolism, catalysed by amylase, results in sucrose accumulation after the detachment from the mother plant [36]. To the best of our knowledge, the interchange mechanism between starch phosphorylases and amylase-related enzymes during ripening is unknown. However, the fact that there is a high correlation between starch degradation and sucrose accumulation in mango fruits is well-established [36], inferring that the starch phosphorylases can potentially serve as a fruit quality biomarker.

4.1.2 Ethylene Synthesis and Aromatic Volatiles

Ethylene acts as a trigger of the mango ripening process. This compound is formed from S-adenosyl methionine

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(AdoMet), a methionine derivative, and the ACC oxidase enzyme. Ethylene biosynthesis in mango is initiated by ethylene receptors, which are abundantly active from the flowering to ripe stage [37]. Upon initiation, the methionine is converted into AdoMet, which in turn forms the ethylene hormone.

Methionine can be synthesized through the de novo or the salvage pathways to maintain the methionine pool. One of the enzymes in the de novo pathway is the 5 methyltetrahydropteroyltriglutamate–homocysteine methyltransferase (MET6), which is responsible for the last stage of methionine formation through the methylation on homocysteine [38]. The enzyme MET6 was found to be abundant in the unripe sample, which indirectly implies the active participation of ethylene biosynthesis in the mango ripening process [38]. Additionally, the transcript increment of these proteins is correlated with the ethylene production. However, the reduced expression correlates to the reduced protein amount in both the mature and immature samples, suggesting that the ethylene acts only to initiate differential gene expression for ripening [39].

Methionine degradation is principally catalysed by 1-aminocyclopropane-1-carboxylic acid (ACC) synthase from AdoMet to produce ethylene. An alternative pathway, utilizing methionine gamma-lyase (MGL), was discovered in mature melon fruit to reduce excessive methionine and initiate the production of dominant aromatic precursor compounds [40]. The catabolic pathway produces methanethiol, α-ketobutyrate and ammonia gas, where the α-ketobutyrate is able to be converted into L-isoleucine, a precursor of propanoate ester [40], which contributes to the 270 aromatic volatile compounds of mango, including monoterpenes and esters [41].

Both observations on methionine synthesis and degradation suggest methionine plays a multifaceted role as an ethylene precursor and in profiling the aromatic volatiles.

For decades, the fatty acid metabolism of aromatic fruits has been widely studied due to its primary function as the precursor of aromatic volatile compounds and the capability to aid postharvest technology [41, 42]. During ripening, saturated fatty acids undergo oxidation to produce unsaturated fatty acids [43]. In this study, the acyl-[acyl-carrier] protein desaturase was found exclusively in the ripe stage that catalyses oxidation on the stearoyl acyl-carrier protein (ACP), forming oleoyl-ACP. The oleoyl-ACP catalyses the oleic acid production that accumulates in many ripe mango varieties, such as 'Alphonso' and 'Kensington Pride' [42, 43]. The unsaturated fatty acid then generates many aromatic volatile compounds through beta-oxidation, such as aldehyde, monoterpene, and esters [43, 44]. The significance of this knowledge has led to discoveries on the effect of postharvest factors such as harvest maturity and storage conditions in the expression of fruit aroma quality [44].

4.1.3 Cell Wall Degradation

The pectin cell wall of mango epidermis is the primary defence against microbial attacks and physical harm in the pre-climacteric stage, and its breakdown in the cell wall usually associated with the mango fruit ripening process [45]. The softening occurs through the enzymatic depolymerisation of the cell wall, where pectate lysate (PL), polygalacturonase (PG) and pectate esterase (PE) function as the main catalysts [39]. The β-galactosidase was present exclusively in the ripe stage, which is a pectin debranching enzyme that supplements the depolymerisation process [46]. Differential transcriptomic analysis on mango revealed that these three gene families, aided by β-galactosidase, had an exponential expression during ripening [37, 39] that was correlated to the overall decrease in hemicellulose, pectin and cellulose to less than 1% of the total fresh weight (FW) [47]. By losing their protective membrane, the mango is susceptible to physical damage and microbial attacks. Therefore, a postharvest technology that controls the cell-wall degrading enzymes, such as beta-galactosidase, should be further investigated.

4.1.4 Stress–Response Proteins

The oxidative burst during the ripening stage in both the epidermal layer and pulp generates reactive oxygen species (ROS) that must be negated to reduce the oxidative stress effect [48]. One of the mechanisms in response to oxidative stress utilizes superoxide dismutase protein during the ripening [10]. In this study, three differential stress–response proteins were identified throughout the mango ripening process. In which, two proteins, the abscisic stress ripening and chaperone protein, were abundant in the unripe mango, and one protein, the putative glutathione S-transferase protein, was present in the ripe samples.

The abscisic acid (ABA) in climacteric fruits reaches a peak during early ripening after abscission and accumulates as ethylene level increases in climacteric fruits [49]. The abscisic stress ripening (Asr) family is induced by ABA in response to a variety of abiotic stresses, including oxidative, osmotic and salt stress [50]. While a member of the gene family, Asr protein 3 (Asr3), abundant in the pre-climacteric stage are also found in tomato and banana species [51, 52]. Similar results were observed in grape skin, where the abundance of Asr3 had a significant decreased after ripening [53]. This suggests that Asr3 is abundantly produced to cope with the oxidative stress from the respiratory burst.

The chaperone ClpB 1 protein (Spot 472) is part of the heat shock protein (HSP) family that functions as a protection for plastid proteins, such as RuBisCo, against diverse environmental stresses [54]. Additionally, the chaperones ensure proper folding under stressful conditions to produce functional proteins [10]. As mentioned previously, the

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reduced expression of chaperones causes the dismantling of pigment proteins.

Alternatively, ROS can be regulated by the activity of cellular antioxidant systems. In mango, the ascorbate–glutathione cycle acts as an enzymatic antioxidant system that responds to ROS species [10]. The glutathione S-transferase (spot 314) in the cycle, found in ripe mango samples, performs ROS detoxification through the conjugation of a chemical group to glutathione [55]. The detoxification step would maintain the plant cell membrane integrity, protect the protein and DNA from denaturation during mango ripening [32]. Furthermore, high oxidation of mango fruit causes a decrease in aroma production and fruit quality [44]. Therefore, it is important to understand the control of oxidative stress in mango fruits, especially in the postharvest stage in order to maintain fruit quality.

4.2 Differential Proteins Involved in the Ripening Process of 'Golden Phoenix'

4.2.1 Ethylene Synthesis and Aromatic Volatiles

There are four proteins associated with ethylene and aromatic volatiles synthesis namely, methionine gamma lyase, 3-ketoacyl-CoA thiolase B, ATP sulfurylase and chitinase. The involvement of methionine gamma lyase in the production of aromatic volatiles has been discussed in Sect. 4.1.2.

3-ketoacyl-CoA thiolase B is a thiolase which is involved in fatty acids degradation in the final step of beta-oxidation by cleaving two carbon units from 3-ketoacyl-CoA to form shortened fatty acyl-CoA [56]. In higher plants, these fatty acids metabolism reactions occur mostly in the peroxisomes. The identified mango thiolase in this study is in close agreement with peroxisomal thiolase isolated by Bojorquez and Gomez-Lim [57] from ripening mango fruits with 430 amino acid residues and a molecular weight of 45,743 Da. The changes in the fatty acids may be associated with the production of aromatic volatile during mango ripening.

ATP sulfurylase (spot 231) was found abundantly in the ripe stage as it catalyses the reduction of sulphate to sulphite, which is assimilated into sulphur containing compounds such as methionine [58]. Methionine is subsequently degraded to produce ethylene and aromatic volatile in the ripe stage in mango.

The other protein which has been found to be abundant in the ripe sample was chitinase (spot 302). Even though chitinase has been known to be a pathogenesis-related (PR) protein which is involved in stress response [59], it was suggested to have a more prominent role in ethylene responsive induction in mango due to their hydrolytic action on *N*-acetylglucosamine-containing glycoproteins in the plant cell walls [37]. Therefore, chitinase may be involved in the fruit softening in the ripening process of mango.

4.2.2 Cell Wall Synthesis and Degradation

Two differential abundant proteins were found to be directly or indirectly involved in the cell wall synthesis or degradation in 'Golden Phoenix' mango. SNARE-interacting protein KEULE identified in this study was found to be involved in the regulation of vesicles transport and membrane fusion in cytokinesis [60]. The formation of cell plate could be an important process to prepare the ripe fruit for abscission.

As mentioned before, a plethora of enzymes were involved in the depolymerisation of cell wall leading to fruit softening during the ripening process. One of these cell wall hydrolytic enzymes was identified as xyloglucan endotransglucosylase/hydrolase, which was found to be abundant in unripe 'Golden Phoenix' mango. This enzyme works as an indicator for the start of fruit ripening process for the ethylene synthesis, that is why it could be high at the early stage to switch on the production of ethylene. When the fruit reach ripening stage the proteins expressed in the mature fruit is less abundance to indicate sufficient amount of ethylene produced. Similarly, the enzyme abundance has shown to decrease in the softening stage in kiwifruit and apple [61, 62]. Even though the role of xyloglucan endotransglucosylase/hydrolase has known to be an important enzyme in xyloglucan metabolism [63], this enzyme has been found to exist in many isozyme forms. Some of these isozymes, however, were found to play a role in maintaining the cell wall structural integrity [64, 65]. Therefore, it is evident that xyloglucan endotransglucosylase/hydrolase may regulate the fruit softening process during ripening by remodelling the cell wall components.

4.2.3 Stress–Response Proteins

The association of abscisic acid (ABA) with fruit development and ripening as well as adaptive responses to biotic and abiotic stress have been well documented [49]. There were three differential abundant proteins identified in this study namely, TSS, ROP guanine nucleotide exchange factor 12 and desiccation-related protein PCC13-62-like, which are related to ABA responses.

Our study found that the protein TSS was abundant in unripe mango. Protein TSS has been found to be associated with the cell cycle and response to abscisic acid [66, 67]. The occurrence of such a protein suggests the adaptation of the fruit tissues to cope with stress during ripening processes.

ROPs are RHO-like small GTPases that act as molecular switches in a wide range of signalling pathways in plants. Guanine nucleotide exchange factors (GEFs) are activate ROPs by stimulating the exchange of GDP for GTP. In *Arabidopsis*, GEFs have been found to regulate ROP11 for a number of ABA-mediated processes such as

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seed germination and adaptation to drought stress [68]. The role of ROPGEF in fruit ripening is not well studied. However, since ROPGEFs regulate ABA responses, there is a strong likelihood that it is involved in the adaptation to cope with stress during the ripening processes.

The desiccation-related protein pcC13-62-like protein was isolated by Piatkowski and Co from resurrection plant *Craterostigma plantagineum* in 1990 [69]. This protein was found to be ABA-responsive related to desiccation and salt. Recently, pcC13-62 genes were reported to be highly expressed in desiccation-tolerant compared to the desiccation-sensitive species [70]. In addition, the gene promoters were found to contain ABA response elements. In this study, pcC13-62 protein was more abundant in ripe 'Golden Phoenix' mango samples compared to the unripe ones which indicates its role related to ABA response to stress during the fruit ripening stage.

4.3 Comparison of Differential Proteins Related to Fruit Ripening Between 'Chokanan' and 'Golden Phoenix' Varieties

β-galactosidase has been known as an enzyme that is able to release galactosyl residues from various galactosyl-containing cell wall substrates [71]. While in Golden Phoenix, two differential abundant proteins were identified namely SNARE-interacting protein KEULE and xyloglucan endotransglycosylase/hydrolase. SNARE-interacting protein KEULE, which was found abundant in ripe fruit, is related to fruit abscission while xyloglucan endotransglycosylase/hydrolase was found abundant in unripe fruit, which is mostly responsible for xyloglucan metabolism. Since xyloglucan is a component of the cell wall, its degradation in the unripe stage may be responsible for the softening process of 'Golden Phoenix' variety. The difference in proteins isolated may account for the morphological characteristics recording during the ripening stages of the two different varieties.

There were three differential stress–response proteins identified in 'Chokanan', the abscisic stress ripening and chaperone proteins, which was abundant in the unripe mango, and the putative glutathione S-transferase protein, which was present in the ripe samples. For 'Golden Phoenix', there were also three differential proteins identified. The proteins are protein TSS, which are abundant in unripe samples while ROP guanine nucleotide exchange factor 12 and desiccation-related protein PCC13-62-like were found to be abundant in ripe samples. All the three differential proteins identified in 'Golden Phoenix' are related to ABA responses. The other stress–associated protein which has been found abundantly in the ripe stage of 'Golden Phoenix' is chitinase.

4.4 RT-qPCR Analysis

The poor correlation between the mRNA expression and protein production has been well documented [72, 73]. This is often attributed to different levels of transcriptions, post-translational modifications and protein degradation. The results of our present study showed that most of the expression of our selected genes were not correlated with the corresponding protein abundance. It is interesting to note that MGL protein was identified in both the mango varieties in higher abundance in the ripe compared to the unripe samples. However, the corresponding genes were upregulated in the unripe instead of the ripe samples. As we have pointed out in Sect. 4.1.2, MGL has been known to be involved in the synthesis of precursors for aromatic volatiles. Since there are many aromatic volatiles (approximately 270 in mango) responsible to produce the fragrance aroma in a ripe mango fruit, it is possible that the complex pathways that involved MGL were initiated even at the mRNA level.

5 Conclusions

The ripening of mango fruit is a complex process. In order to successfully market mango fruit, the trajectory of the ripening process for each variety of mango needs to be revealed. Our findings have shown that at the molecular levels, the two varieties of mango namely, 'Chokanan' and 'Golden Phoenix', produced diverse types of differentially expressed proteins in both the ripe and unripe stages. However, these proteins were mainly found to be involved in ethylene synthesis process and production of aromatic volatiles, cell wall degradation and stress–response proteins with an additional group of proteins related to energy and carbohydrate metabolism found in 'Chokanan' variety. Further investigation on these proteins will shed light into postharvest mango management and suggest directions for the mango improvement programme.

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Author Contributions CCF designed the experiment. EYT, MC and TL performed all the experimental work and data analysis. JAO and NR contributed to the protein identification and quantification. CCF performed the data interpretation. CCF and EYT drafted the manuscript which was critically revised and approved for submission by all the other authors.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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