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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 **Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s10930-019-09868-x>) contains supplementary material, which is available to authorized users.



1 Comparative Proteomic Analysis on Fruit Ripening Processes in Two 2 Varieties of Tropical Mango (*Mangifera indica*)

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4 Tamunonengiyeofori Lawson¹

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7 Abstract

8 **ABSTRACT** Mango (*Mangifera indica* L.) is an economically important fruit. However, the marketability of mango is affected by the
9 perishable nature and short shelf-life of the fruit. Therefore, a better understanding of the mango ripening process is of great
10 importance towards extending its postharvest shelf life. Proteomics is a powerful tool that can be used to elucidate the com-
11 plex ripening process at the cellular and molecular levels. This study utilized 2-dimensional gel electrophoresis (2D-GE)
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13 of tropical mango, *Mangifera indica* cv. ‘Chokanan’ and *Mangifera indica* cv. ‘Golden Phoenix’. The comparative analysis
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16 response proteins. There was an additional category for differential proteins identified from the ‘Chokanan’ variety namely,
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18 was found in both ‘Chokanan’ and ‘Golden Phoenix’ varieties. Six differential proteins were selected from each variety for
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21 found to express in concordant with protein abundant. The findings will provide an insight into the fruit ripening process of
22 different varieties of mango fruits, which is important for postharvest management.

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

25 1 Introduction

26 Mango (*Mangifera indica* L.) is a popular fruit character-
27 ized by its sweet taste, aromatic scent, and low fibre flesh.
28 Being an economically important fruit, mango has an annual
29 production of 46.5 million tonnes in tropical regions [1].

30 However, the international trade and export potential of
31 mango are restricted due to several factors, such as short
32 shelf life and risk of postharvest diseases, which are mainly
33 associated with fruit ripening processes [2].

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

A1 **Electronic supplementary material** The online version of this
A2 article (<https://doi.org/10.1007/s10930-019-09868-x>) contains
A3 supplementary material, which is available to authorized users.

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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, Andrade 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 Andrade 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, $80 \pm 5\%$ 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 $h^\circ = \tan^{-1} b^*/a^*$ where $0^\circ =$ red purple, $90^\circ =$ yellow, $180^\circ =$ blue-green and $270^\circ =$ 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 ($12,000 \times g$, 30 min, 4 °C), the phenol phase was collected and precipitated with 5 volumes of 100 mM ammonium acetate in methanol overnight

144 at 4 °C. The phenol phase was then recovered by centrifuga-
145 tion (12,000×g, 45 min, 4 °C). After precipitation, the
146 protein pellet was rinsed with 1% DTT/acetone and air-dried.
147 The protein pellet was re-suspended in 100 uL lysis buffer
148 (4% (w/v) CHAPS, 2 M Thiourea, 7 M Urea, and 1% (w/v)
149 DTT) and kept in –80 °C until further use. The protein con-
150 centration was determined using Bradford protein method
151 [20] with bovine serum albumin as the reference standard.

152 2.3 Two-Dimensional Gel Electrophoresis (2D-GE) 153 and Gel Image Analysis

154 Isoelectric focusing (IEF) was conducted using 7 cm Immo-
155 bilized pH gradient (IPG) strips (GE Healthcare Life Sci-
156 ences, Sweden) with a linear pH gradient of 3–10 on PRO-
157 TEAN® IEF cell (Bio-Rad Ltd, USA). The protein samples
158 (100 µg) were loaded at the cathodic side of the IEF cell.
159 The following running conditions were used: current limit at
160 50 µA per strip; 200 V for 1 h; 1000 V for 1 h; 4000 V for 1 h
161 and then a rapid gradient to 4000 V until 20,000 Volt hours
162 (Vh). The IPG strips were then incubated for 15 min in an
163 equilibration buffer (30% (v/v) Glycerol, 50 mM Tris–HCl
164 (pH 8.8), 6 M Urea and 2% (w/v) SDS) that contains 2%
165 (w/v) DTT, followed by an incubation for another 15 min in
166 the same buffer containing 135 mM iodoacetamide instead
167 of DTT. For second dimension analysis, the equilibrated
168 strips were transferred to 12% Acrylamide SDS-PAGE gels
169 for electrophoresis fractionation at 150 V for 1 h. Three bio-
170 logical replicates were prepared for each ripening stage of
171 the fruits.

172 The gels were stained with Coomassie® G-250 stain
173 (Thermo Fisher Scientific, USA). The gel images were digi-
174 tized using a GS-800TM calibrated densitometer (BioRad
175 Ltd, USA) and analysed using the Progenesis SameSpot soft-
176 ware (Totalab Ltd, UK). Spots were detected, matched and
177 normalized according to the manufacturer's instruction. Dif-
178 ferential protein spots between the ripe and unripe samples
179 were ascertained using normalised spots and compared with
180 the reference gel. One way ANOVA was used to calculate
181 the fold difference and p values. The threshold value for fold
182 change was set at 1.6 for up and down-regulation at $p \leq 0.05$.

183 2.4 Protein Digestion and MALDI ToF–ToF analysis

184 Four gel samples of selected individual protein spots were
185 excised manually and the protein spots were sent for mass
186 spectrometry analysis for protein identification by a com-
187 mercial service provider (FirstBase© Sdn Bhd, Malaysia).
188 The protein samples were digested using trypsin and anal-
189 ysed by MALDI-TOF/TOF tandem mass spectrometer
190 5800 Proteomic Analyser (AB Sciex, Singapore). This was
191 then followed by spectral analysis for the identification of
192 the desired protein using the Mascot sequence matching

software (Matrix Science, USA). The parameters used for
193 database searches are: peptide mass tolerance of 200 ppm
194 and fragment mass tolerance of 0.5 Da, trypsin with one
195 missed cleavage allowed, carbamidomethylation was
196 selected as the fixed modification while methionine oxida-
197 tion was selected as the variable modification. Mass values
198 were monoisotopic. The precursor was selected automati-
199 cally to proceed for the MS/MS mode. The selection was the
200 mass from 800 to 3000 Da with the intensity of more than
201 800 in the run. The Mascot probability-based score with
202 confidence value greater than 99% ($p < 0.01$), at least two
203 peptide hits, bolded in red, and Mascot score over the mini-
204 mum, were prerequisites of accurate protein identification.
205 The databases used are NCBIInr and MSPnr100 Database
206 (Matrix Science, USA) with MASCOT as a search engine.
207 The protein functional classification was then searched
208 against established databases (NCBI and UniProt/PROSITE)
209 and the available literature.
210

211 2.5 RNA Extraction

212 Total RNA was extracted from pulp samples of 'Chokanan'
213 and 'Golden Phoenix' at the unripe and ripe stages using
214 the CTAB method [21]. Assessment of RNA quantity, qual-
215 ity and integrity were performed using Nanodrop ND1000
216 spectrophotometer, gel electrophoresis and 2100 Bioana-
217 lyzer system (Agilent Technologies, Santa Clara, CA, USA)
218 respectively. RNA samples with a 260/280 ratio between
219 1.8 and 2.0, 260/230 ratio between 2.0 and 2.5 and RIN
220 (RNA integrity number) ≥ 7.0 were used for real-time qPCR
221 analysis.

222 2.6 RT-qPCR

223 The QuantiTect Reverse Transcription kit (Qiagen, Ger-
224 many) was used to convert the RNA to cDNA following
225 the manufacturer's protocol. For each qPCR reaction, the
226 master mix from SensiFAST SYBR No-ROX kit (2x) (Bio-
227 line, London, UK), primers and distilled water were added
228 to the cDNA, which made up to the final volume of 20 uL
229 per tube. A total of 4 uL or 20 ng cDNA was used in the
230 reaction. The qPCR amplification efficiency was validated
231 via standard curve using cDNA samples and the respective
232 primers designed for the reference gene only. The refer-
233 ence gene was chosen based on a stability test using the
234 geNorm tool [22]. The qPCR reaction conditions were as
235 follows: initial denaturation at 95 °C for 2 min, 39 cycles
236 of denaturation at 95 °C for 5 s, annealing and extension at
237 60 °C for 30 s. The expression of the Actin gene [23] was
238 used for normalization of every target gene studied. Rela-
239 tive expression level of the target genes was analysed using
240 the $2^{-\Delta\Delta CT}$ method [24]. For each mango variety, three
241 biological replicates (fruit) were taken and three technical

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

244 3 Results

245 3.1 Ripening Process of Mango

246 The storage life of mango has shown to be dependent on
247 variety [25] based on physiochemical measurements. Our
248 study has indicated that the colour and firmness of fruits
249 varies between ‘Chokanan’ and ‘Golden Phoenix’ vari-
250 eties [14] during ripening such that the colour of ‘Cho-
251 kanan’ fruit turned from green to yellow within 8 days but
252 remained firm while the fruit of ‘Golden Phoenix’ turned
253 pale green, but the fruit was soft when it ripened. It is
254 therefore interesting to investigate further to find out more
255 about differential proteins involved in the ripening process
256 of the two varieties of tropical mango.

257 A typical 2DE-gel of the ripe sample of ‘Chokanan’
258 and ‘Golden Phoenix’ mango are shown in Figs. 1 and 2
259 respectively.

260 3.2 Differential Proteins Involved in the Ripening 261 Process of ‘Chokanan’

262 The differential proteins with at least a 1.5-fold change in
263 abundance between the ripe and unripe ‘Chokanan’ mango
264 are presented in Table 1. The proteins can broadly be cat-
265 egorised into 4 groups namely, energy and carbohydrate
266 metabolism, ethylene synthesis and aromatic volatiles, cell
267 wall degradation and stress–response proteins.

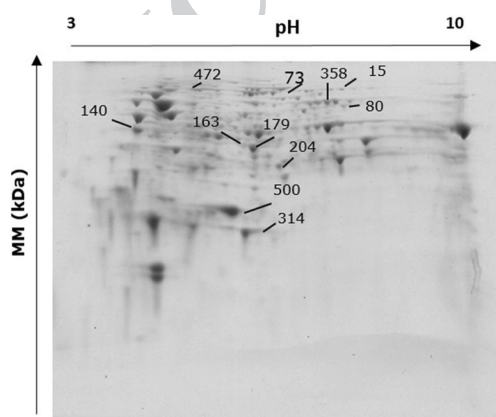


Fig. 1 2DE gel indicating the differential protein spots in a ripe ‘Chokanan’ mango sample

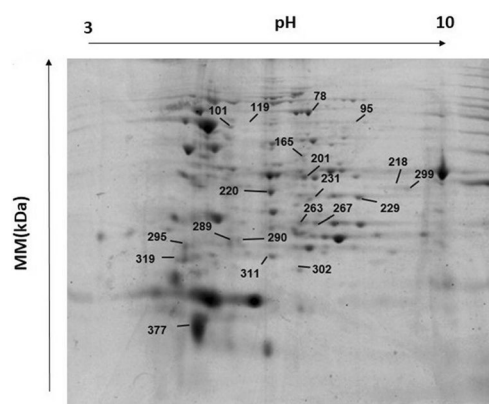


Fig. 2 2DE gel indicating the differential protein spots in a ripe Golden Phoenix mango sample

268 3.3 Differential Proteins Involved in the Ripening 269 Process of ‘Golden Phoenix’

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

275 3.4 Comparison of Differential Proteins Related 276 to Fruit Ripening Between ‘Chokanan’ 277 and ‘Golden Phoenix’ Varieties

278 There were 11 differential proteins identified in ‘Chokanan’
279 and 13 in ‘Golden Phoenix’ (Tables 1 and 2). Out of these,
280 only 1 common protein i.e. methionine gamma-lyase was
281 found in both the ripe and unripe fruits of the 2 varieties of
282 mango. Methionine gamma-lyase is involved in generating
283 aromatic volatile via methionine degradation which is an
284 important process to indicate the ripening status of the fruit.
285 Another protein, 3-ketoacyl-CoA thiolase B, which was only
286 found in the ripe fruit of ‘Golden Phoenix’, is also a pre-
287 cursor in the catabolic pathway for the production of aromatic
288 volatiles. This protein was also identified in 3 spots (218,
289 229 and 299) indicating the presence of protein isoforms.

290 All the differential protein groups in ‘Golden Phoenix’
291 are of the same categories as ‘Chokanan’ namely, ethylene
292 synthesis and aromatic volatiles, cell wall synthesis and deg-
293 radation and stress–response proteins. However, there is one
294 category of differential proteins only found in ‘Chokanan’,
295 which is energy and carbohydrate metabolism. The higher
296 abundance of these proteins namely, malic enzyme, ribulose-
297 1,5-bisphosphate large subunit-binding protein subunit alpha
298 and α -1,4 glucan phosphorylase present in the unripe fruit
299 implicate the high energy required to maintain the firmness
300 of the fruit during the ripening process in ‘Chokanan’.

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

| Spot number | Accession number (NCBIInr) | Protein description | Function | PI | Mw (Da) | Sequence coverage (%) | Score | Organism |
|-------------|----------------------------|--|---|------|---------|-----------------------|-------|----------------------------|
| 140 | gil703148942 | RuBisCo large subunit-binding protein subunit alpha | Energy and carbohydrate metabolism | 5.26 | 61,886 | 7 | 226 | <i>Morus notabilis</i> |
| 500 | gil658045224 | Absciscic stress-ripening protein 3-like | Stress-response proteins | 6.41 | 15,089 | 32 | 472 | <i>Malus domestica</i> |
| 472 | gil901807498 | Chaperone protein ClpB 1 | stress-response proteins | 5.93 | 102,046 | 13 | 345 | <i>Zostera marina</i> |
| 15 | gil823259620 | Alpha-1,4 glucan phosphorylase | Energy and carbohydrate metabolism | 6.10 | 95,227 | 7 | 244 | <i>Gossypium raimondii</i> |
| 358 | gil1091514556 | 5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase | Ethylene synthesis and aromatic volatiles | 6.36 | 84,286 | 12 | 433 | <i>Eucalyptus grandis</i> |
| 80 | gil508723832 | NADP-dependent malic enzyme | Energy and carbohydrate metabolism | 6.46 | 65,072 | 19 | 587 | <i>Theobroma cacao</i> |
| 179 | gil590675246 | Methionine gamma-lyase | Ethylene synthesis and aromatic volatiles | 6.6 | 49,154 | 8 | 196 | <i>Theobroma cacao</i> |
| 204 | gil1044897083 | Stearoyl-ACP desaturase | Ethylene synthesis and aromatic volatiles | 5.63 | 45,035 | 11 | 308 | <i>Paeonia lactiflora</i> |
| 163 | gil1098730814 | Homogentisate 1,2-dioxygenase-like | Ethylene synthesis and aromatic volatiles | 5.89 | 51,540 | 10 | 317 | <i>Juglans regia</i> |
| 73 | gil848853991 | Beta-galactosidase-like | Cell wall degradation | 8.37 | 68,253 | 3 | 84 | <i>Erythranthe guttata</i> |
| 314 | gil926657597 | Glutathione S-transferase-like protein | stress-response proteins | 5.73 | 25,564 | 16 | 365 | <i>Solanum chacoense</i> |

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.

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

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

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

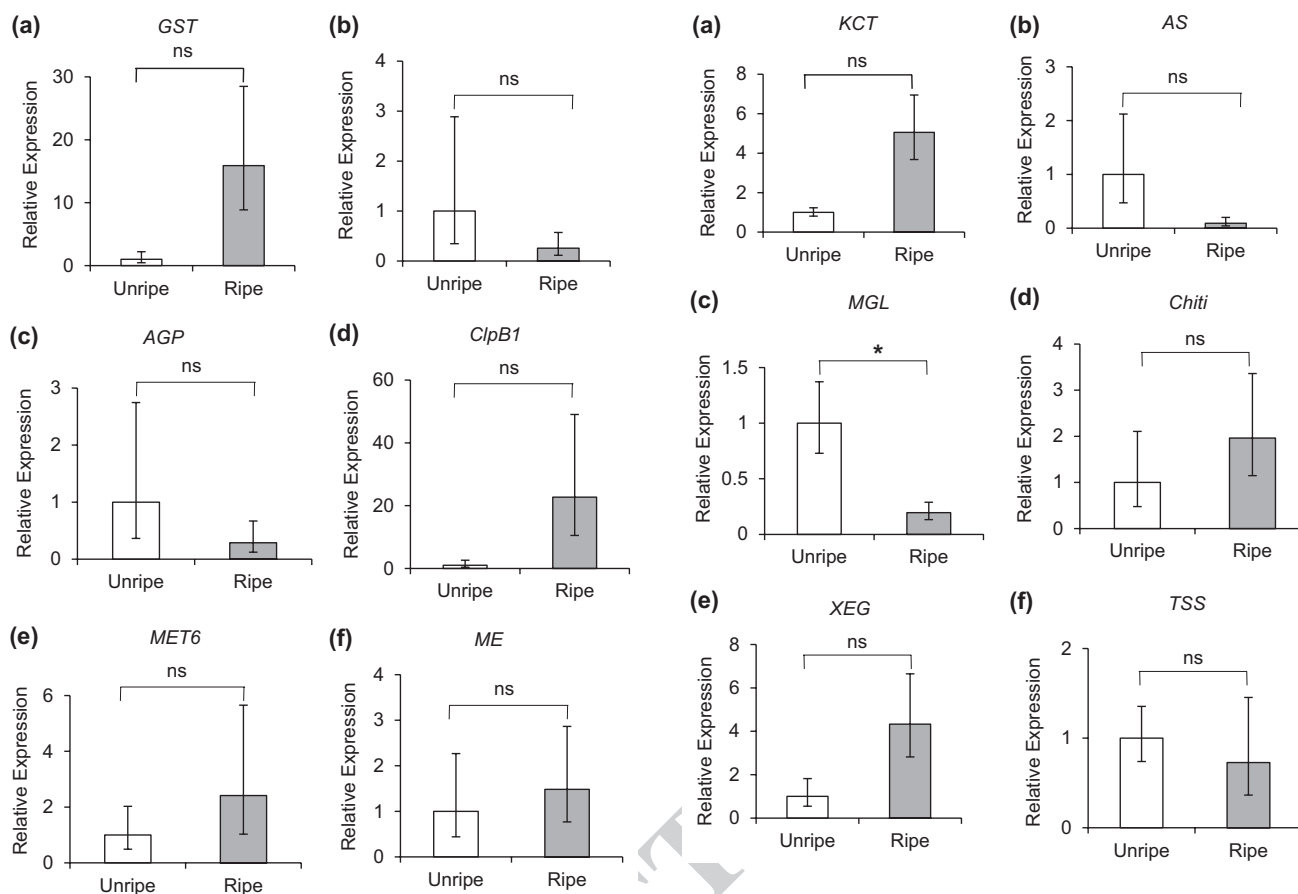


Fig. 3 RT-qPCR analysis of the transcript levels of differentially expressed proteins in ripe and unripe 'Chokanan' mango. **a** GST, **b** MGL, **c** AGP, **d** ClpB1, **e** MET6, **f** ME. Asterisks (*) indicate a statistically significant difference between the unripe and ripe samples at $P < 0.05$. 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 \pm 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

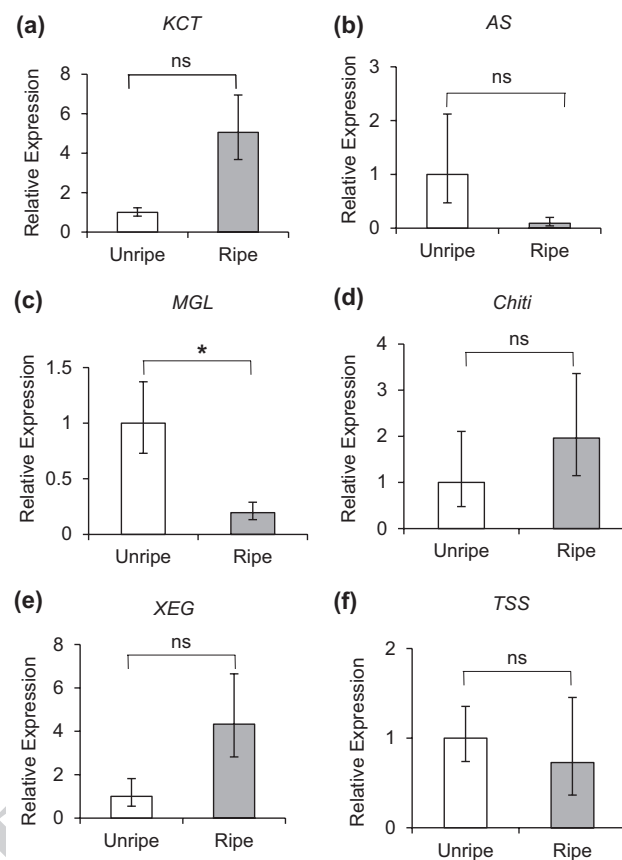


Fig. 4 RT-qPCR analysis of the transcript levels of differentially expressed proteins in ripe and unripe 'Golden Phoenix' mango. Note: **a** KCT, **b** AS, **c** MGL, **d** Chiti, **e** XEG, **f** TSS. Asterisks (*) indicate a statistically significant difference between the unripe and ripe samples at $P < 0.05$. The calculation for relative gene expression was made using the $2^{-\Delta\Delta Ct}$ (Livak method). *ns*: no significant difference. Actin gene was used as the reference gene. Error bars indicate \pm 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)

388 of RBP, from the Chaperonin 60 family in the unripe fruit,
 389 which is responsible for protein refolding under stress condi-
 390 tions. Previous studies have established a close relationship
 391 between fruit maturation and ripening to oxidative stress
 392 [33, 34]. Therefore, the absence of the RBP during ripening
 393 could lead to the exposure of the RuBisCo protein towards
 394 ripening-induced oxidative stress, resulting in the rapid dis-
 395 mantling of the photosynthetic pigment.

396 Starch is a polymer of glucose-1-phosphate (Glc-1-P),
 397 joined by an α -1,4 glycosidic bond, which is usually synthe-
 398 sized for energy storage or degraded as fuel. Starch phos-
 399 phorylases, such as α -1,4 glucan phosphorylase, catalyses
 400 starch synthesis [35]. The high abundance of α -1,4 glucan
 401 phosphorylase in the unripe mango suggests the synthesis

of starch during early ripening. The starch catabolism, cata- 402
 lysed by amylase, results in sucrose accumulation after the 403
 detachment from the mother plant [36]. To the best of our 404
 knowledge, the interchange mechanism between starch phos- 405
 phosphorylases and amylase-related enzymes during ripening is 406
 unknown. However, the fact that there is a high correla- 407
 tion between starch degradation and sucrose accumulation 408
 in mango fruits is well-established [36], inferring that the 409
 starch phosphorylases can potentially serve as a fruit quality 410
 biomarker. 411

4.1.2 Ethylene Synthesis and Aromatic Volatiles 412

Ethylene acts as a trigger of the mango ripening process. 413
 This compound is formed from S-adenosyl methionine 414

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

421 Methionine can be synthesized through the de novo or
422 the salvage pathways to maintain the methionine pool. One
423 of the enzymes in the de novo pathway is the 5 methyltetra-
424 rahydropteroyltryglutamate–homocysteine methyltransferase
425 (MET6), which is responsible for the last stage of methio-
426 nine formation through the methylation on homocysteine
427 [38]. The enzyme MET6 was found to be abundant in the
428 unripe sample, which indirectly implies the active participa-
429 tion of ethylene biosynthesis in the mango ripening process
430 [38]. Additionally, the transcript increment of these proteins
431 is correlated with the ethylene production. However, the
432 reduced expression correlates to the reduced protein amount
433 in both the mature and immature samples, suggesting that
434 the ethylene acts only to initiate differential gene expression
435 for ripening [39].

436 Methionine degradation is principally catalysed by
437 1-aminocyclopropane-1-carboxylic acid (ACC) synthase
438 from AdoMet to produce ethylene. An alternative pathway,
439 utilizing methionine gamma-lyase (MGL), was discovered
440 in mature melon fruit to reduce excessive methionine and
441 initiate the production of dominant aromatic precursor com-
442 pounds [40]. The catabolic pathway produces methanethiol,
443 α -ketobutyrate and ammonia gas, where the α -ketobutyrate
444 is able to be converted into L-isoleucine, a precursor of pro-
445 panoate ester [40], which contributes to the 270 aromatic
446 volatile compounds of mango, including monoterpenes and
447 esters [41].

448 Both observations on methionine synthesis and degra-
449 dation suggest methionine plays a multifaceted role as an
450 ethylene precursor and in profiling the aromatic volatiles.

451 For decades, the fatty acid metabolism of aromatic fruits
452 has been widely studied due to its primary function as the
453 precursor of aromatic volatile compounds and the capabil-
454 ity to aid postharvest technology [41, 42]. During ripening,
455 saturated fatty acids undergo oxidation to produce unsatur-
456 ated fatty acids [43]. In this study, the acyl-[acyl-carrier]
457 protein desaturase was found exclusively in the ripe stage
458 that catalyses oxidation on the stearoyl acyl-carrier protein
459 (ACP), forming oleoyl-ACP. The oleoyl-ACP catalyses the
460 oleic acid production that accumulates in many ripe mango
461 varieties, such as ‘Alphonso’ and ‘Kensington Pride’ [42,
462 43]. The unsaturated fatty acid then generates many aro-
463 matic volatile compounds through beta-oxidation, such as
464 aldehyde, monoterpene, and esters [43, 44]. The significance
465 of this knowledge has led to discoveries on the effect of post-
466 harvest factors such as harvest maturity and storage condi-
467 tions in the expression of fruit aroma quality [44].

4.1.3 Cell Wall Degradation 468

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

4.1.4 Stress–Response Proteins 488

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

500 The abscisic acid (ABA) in climacteric fruits reaches a
501 peak during early ripening after abscission and accumulates
502 as ethylene level increases in climacteric fruits [49]. The
503 abscisic stress ripening (Asr) family is induced by ABA in
504 response to a variety of abiotic stresses, including oxidative,
505 osmotic and salt stress [50]. While a member of the gene
506 family, Asr protein 3 (Asr3), abundant in the pre-climac-
507 teric stage are also found in tomato and banana species [51,
508 52]. Similar results were observed in grape skin, where the
509 abundance of Asr3 had a significant decreased after ripening
510 [53]. This suggests that Asr3 is abundantly produced to cope
511 with the oxidative stress from the respiratory burst.

512 The chaperone ClpB 1 protein (Spot 472) is part of the
513 heat shock protein (HSP) family that functions as a protec-
514 tion for plastid proteins, such as RuBisCo, against diverse
515 environmental stresses [54]. Additionally, the chaperones
516 ensure proper folding under stressful conditions to pro-
517 duce functional proteins [10]. As mentioned previously, the

518 reduced expression of chaperones causes the dismantling of
519 pigment proteins.

520 Alternatively, ROS can be regulated by the activity of
521 cellular antioxidant systems. In mango, the ascorbate–glu-
522 thatione cycle acts as an enzymatic antioxidant system that
523 responds to ROS species [10]. The glutathione S-transferase
524 (spot 314) in the cycle, found in ripe mango samples, per-
525 forms ROS detoxification through the conjugation of a
526 chemical group to glutathione [55]. The detoxification step
527 would maintain the plant cell membrane integrity, protect
528 the protein and DNA from denaturation during mango ripen-
529 ing [32]. Furthermore, high oxidation of mango fruit causes
530 a decrease in aroma production and fruit quality [44]. There-
531 fore, it is important to understand the control of oxidative
532 stress in mango fruits, especially in the postharvest stage in
533 order to maintain fruit quality.

534 4.2 Differential Proteins Involved in the Ripening 535 Process of 'Golden Phoenix'

536 4.2.1 Ethylene Synthesis and Aromatic Volatiles

537 There are four proteins associated with ethylene and aro-
538 matic volatiles synthesis namely, methionine gamma lyase,
539 3-ketoacyl-CoA thiolase B, ATP sulfurylase and chitinase.
540 The involvement of methionine gamma lyase in the produc-
541 tion of aromatic volatiles has been discussed in Sect. 4.1.2.

542 3-ketoacyl-CoA thiolase B is a thiolase which is involved
543 in fatty acids degradation in the final step of beta-oxidation
544 by cleaving two carbon units from 3-ketoacyl-CoA to form
545 shortened fatty acyl-CoA [56]. In higher plants, these fatty
546 acids metabolism reactions occur mostly in the peroxisomes.
547 The identified mango thiolase in this study is in close agree-
548 ment with peroxisomal thiolase isolated by Bojorquez and
549 Gomez-Lim [57] from ripening mango fruits with 430
550 amino acid residues and a molecular weight of 45,743 Da.
551 The changes in the fatty acids may be associated with the
552 production of aromatic volatile during mango ripening.

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

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

568 4.2.2 Cell Wall Synthesis and Degradation

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

576 As mentioned before, a plethora of enzymes were
577 involved in the depolymerisation of cell wall leading to fruit
578 softening during the ripening process. One of these cell wall
579 hydrolytic enzymes was identified as xyloglucan endotrans-
580 glucosylase/hydrolase, which was found to be abundant in
581 unripe 'Golden Phoenix' mango. This enzyme works as an
582 indicator for the start of fruit ripening process for the ethyl-
583 ene synthesis, that is why it could be high at the early stage
584 to switch on the production of ethylene. When the fruit reach
585 ripening stage the proteins expressed in the mature fruit is
586 less abundance to indicate sufficient amount of ethylene
587 produced. Similarly, the enzyme abundance has shown to
588 decrease in the softening stage in kiwifruit and apple [61,
589 62]. Even though the role of xyloglucan endotransgluco-
590 sylase/hydrolase has known to be an important enzyme in
591 xyloglucan metabolism [63], this enzyme has been found
592 to exist in many isozyme forms. Some of these isozymes,
593 however, were found to play a role in maintaining the cell
594 wall structural integrity [64, 65]. Therefore, it is evident that
595 xyloglucan endotransglucosylase/hydrolase may regulate the
596 fruit softening process during ripening by remodelling the
597 cell wall components.

598 4.2.3 Stress-Response Proteins

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

606 Our study found that the protein TSS was abundant in
607 unripe mango. Protein TSS has been found to be associ-
608 ated with the cell cycle and response to abscisic acid [66,
609 67]. The occurrence of such a protein suggests the adapta-
610 tion of the fruit tissues to cope with stress during ripening
611 processes.

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

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

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

635 4.3 Comparison of Differential Proteins Related 636 to Fruit Ripening Between 'Chokanan' 637 and 'Golden Phoenix' Varieties

638 β -galactosidase has been known as an enzyme that is able
639 to release galactosyl residues from various galactosyl-
640 containing cell wall substrates [71]. While in Golden
641 Phoenix, two differential abundant proteins were identified
642 namely SNARE-interacting protein KEULE and xyloglu-
643 can endotransglycosylase/hydrolase. SNARE-interacting
644 protein KEULE, which was found abundant in ripe fruit,
645 is related to fruit abscission while xyloglucan endotrans-
646 glycosylase/hydrolase was found abundant in unripe fruit,
647 which is mostly responsible for xyloglucan metabolism.
648 Since xyloglucan is a component of the cell wall, its deg-
649 radation in the unripe stage may be responsible for the sof-
650 tening process of 'Golden Phoenix' variety. The difference
651 in proteins isolated may account for the morphological
652 characteristics recording during the ripening stages of the
653 two different varieties.

654 There were three differential stress-response proteins
655 identified in 'Chokanan', the abscisic stress ripening and
656 chaperone proteins, which was abundant in the unripe
657 mango, and the putative glutathione S-transferase protein,
658 which was present in the ripe samples. For 'Golden Pho-
659 enix', there were also three differential proteins identified.
660 The proteins are protein TSS, which are abundant in unripe
661 samples while ROP guanine nucleotide exchange factor 12
662 and desiccation-related protein PCC13-62-like were found
663 to be abundant in ripe samples. All the three differential
664 proteins identified in 'Golden Phoenix' are related to ABA
665 responses. The other stress-associated protein which has
666 been found abundantly in the ripe stage of 'Golden Pho-
667 enix' is chitinase.

668 4.4 RT-qPCR Analysis

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

687 5 Conclusions

688 The ripening of mango fruit is a complex process. In order to
689 successfully market mango fruit, the trajectory of the ripen-
690 ing process for each variety of mango needs to be revealed.
691 Our findings have shown that at the molecular levels, the
692 two varieties of mango namely, 'Chokanan' and 'Golden
693 Phoenix', produced diverse types of differentially expressed
694 proteins in both the ripe and unripe stages. However, these
695 proteins were mainly found to be involved in ethylene syn-
696 thesis process and production of aromatic volatiles, cell wall
697 degradation and stress-response proteins with an additional
698 group of proteins related to energy and carbohydrate metab-
699 olism found in 'Chokanan' variety. Further investigation on
700 these proteins will shed light into postharvest mango man-
701 agement and suggest directions for the mango improvement
702 programme.

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709 TL performed all the experimental work and data analysis. JAO and
710 NR contributed to the protein identification and quantification. CCF
711 performed the data interpretation. CCF and EYT drafted the manu-
712 script which was critically revised and approved for submission by all
713 the other authors.

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715 **Compliance with Ethical Standards**716 **Conflict of interest** The authors declare no conflict of interest.717 **References**

- 718 1. FAOSTAT (2018) Production of Mango (including Mangosteen and Guava). <http://www.fao.org/faostat/en/#home>. Accessed 16
719 Apr 2018
- 720 2. Singh Z, Singh RK, Sane VA, Nath P (2013) Mango—postharvest
721 biology and biotechnology. *Crit Rev Plant Sci* 32:217–236
- 722 3. Jha SN, Narsaiah K, Sharma AD, Singh M, Bansal S, Kumar
723 R (2010) Quality parameters of mango and potential of non-
724 destructive techniques for their measurement—a review. *J Food
725 Sci Technol* 47:1–14
- 726 4. Alexander L, Grierson D (2002) Ethylene biosynthesis and
727 action in tomato: a model for climacteric fruit ripening. *J Exp
728 Bot* 53:2039–2055
- 729 5. Prinsi B, Negri AS, Fedeli C, Morgutti S, Negrini N, Cocucci
730 M, Espen L (2011) Peach fruit ripening: a proteomic compara-
731 tive analysis of the mesocarp of two cultivars with different flesh
732 firmness at two ripening stages. *Phytochemistry* 72:1251–1262
- 733 6. Rocco M, D'Ambrosio C, Arena S, Faurobert M, Scaloni A, Marra
734 M (2006) Proteomic analysis of tomato fruits from two ecotypes
735 during ripening. *Proteomics* 6:3781–3791
- 736 7. Giribaldi M, Gény L, Delrot S, Schubert A (2010) Proteomic
737 analysis of the effects of ABA treatments on ripening *Vitis vinifera*
738 berries. *J Exp Bot* 61:2447–2458
- 739 8. Toledo TT, Nogueira SB, Cordenunsi BR, Gozzo FC, Pilau EJ,
740 Lajolo FM, Nascimento J (2012) Proteomic analysis of banana
741 fruit reveals proteins that are differentially accumulated during
742 ripening. *Postharvest Biol Technol* 70:51–58
- 743 9. Al-Obaidi JR, Jamil NA, Rahmad N, Rosli NH (2018) Proteomic
744 and metabolomic study of wax apple (*Syzygium samarangense*)
745 fruit during ripening process. *Electrophoresis* 39:2954–2964
- 746 10. Andrade J, Toledo T, Nogueira S, Cordenunsi B, Lajolo F, Nas-
747 cimento J (2012) 2-DIGE analysis of mango (*Mangifera indica*
748 L.) fruit reveals major proteomic changes associate with ripening.
749 *J Proteom* 75:3331–3341
- 750 11. Sherman A, Rubinstein M, Eshed R, Benita M, Ish-Shalom M,
751 Sharabi-Schwager M, Rozen A, Saada D, Cohen Y, Ophir R
752 (2015) Mango (*Mangifera indica* L.) germplasm diversity based
753 on single nucleotide polymorphisms derived from the transcrip-
754 tome. *BMC Plant Biol* 15:277–288
- 755 12. Jedele, Stefan, Hau AM, von Oppen M (2003) An analysis of
756 the world market for mangos and its importance for developing
757 countries. In: Conference on International Agricultural Research
758 for Development, Göttingen, October 8–10
- 759 13. Loeillet D (1994) The European mango market: a promising tropi-
760 cal fruit. *Fruits* 49:332–334
- 761 14. Lawson T, Lycett GW, Ali A, Chin CF (2019) Characterization of
762 Southeast Asia mangoes (*Mangifera indica* L.) according to their
763 physicochemical attributes. *Sci Hort* 243:189–196
- 764 15. Grading Standards and Specification of Fruits and Vegetables
765 (2017) [http://www.fama.gov.my/documents/10157/af9f2d40-
766 c116-4a3e-8c8ccc1c897bb039](http://www.fama.gov.my/documents/10157/af9f2d40-c116-4a3e-8c8ccc1c897bb039)
- 767 16. Nassur RC, González-Moscoso S, Crisosto GM, Lima LC, Vilas
768 Boas EV, Crisosto CH (2015) Describing quality and sensory
769 attributes of 3 mango (*Mangifera indica* L.) cultivars at 3 ripe-
770 ness stages based on firmness. *J Food Sci* 80:S2055–S2063
- 771 17. Yahia EM (2011) Mango (*Mangifera indica* L.). In: Yahia
772 EM (ed) Postharvest biology and technology of tropical and
773 subtropical fruit: cocona to mango. Woodhead Publishing Lim-
774 ited, Cambridge, pp 492–586
- 775 18. Ali A, Hei GK, Keat YW (2016) Efficacy of ginger oil and
776 extract combined with gum arabic on anthracnose and quality of
777 papaya fruit during cold storage. *J Food Sci Technol* 53:1435–
778 1444. <https://doi.org/10.1007/s13197-015-2124-5>
- 779 19. Carpentier SC, Witters E, Laukens K, Deckers P, Swennen R,
780 Panis B (2005) Preparation of protein extracts from recalcitrant
781 plant tissues: an evaluation of different methods for two-dimen-
782 sional gel electrophoresis analysis. *Proteomics* 5:2497–2507
- 783 20. Bradford MM (1976) A rapid and sensitive method for the quan-
784 titation of microgram quantities of protein utilizing the principle
785 of protein–dye binding. *Anal Biochem* 72:248–254
- 786 21. Zamboni A, Pierantoni L, de Franceschi P (2008) Total RNA
787 extraction from strawberry tree (*Arbutus unedo*) and several
788 other woody plants. *iForest* 1:122–125
- 789 22. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N,
790 De Paepe A, Speleman F (2002) Accurate normalization of
791 real-time quantitative RT-PCR data by geometric averaging of
792 multiple internal control genes. *Flavour Fragr J* 8:147–151
- 793 23. Liu Z, Luo C, Dong L, Van Toan C, Wei P, He X (2014) Molec-
794 ular characterization and expression analysis of a GTP-binding
795 protein (MiRab5) in *Mangifera indica*. *Gene* 540:86–91
- 796 24. Livak K, Schmittgen T (2001) Analysis of relative gene expres-
797 sion data using real-time quantitative PCR and the 2⁻ $\Delta\Delta$ CT
798 Method. *Methods* 25:402–408
- 799 25. Araizá E, Osuna T, Siller J, Contreras L, Sanchez E (2005)
800 Postharvest quality and shelf-life of mango cultivars grown at
801 Sinaloa, México. *Acta Hort* 682:1275–1279
- 802 26. Medlicott AP, Thompson AK (1985) Analysis of sugars and
803 organic acids in ripening mango fruits (*Mangifera indica* L. var
804 Keitt) by high performance liquid chromatography. *J Sci Food
805 Agric* 36:561–566
- 806 27. Li M, Li D, Feng F, Zhang S, Ma F, Cheng L (2016) Proteomic
807 analysis reveals dynamic regulation of fruit development and
808 sugar and acid accumulation in apple. *J Exp Bot* 67:5145–5157
- 809 28. Sweetman C, Deluc LG, Cramer GR, Ford CM, Soole KL
810 (2009) Regulation of malate metabolism in grape berry and
811 other developing fruits. *Phytochemistry* 70:1329–1344
- 812 29. Blanke MM, Lenz E (1989) Fruit photosynthesis. *Plant, Cell
813 Environ* 12:31–46
- 814 30. Hauser T, Popilka L, Hartl FU, Hayer-Hartl M (2015) Role of
815 auxiliary proteins in rubisco biogenesis and function. *Nat Plants*
816 1:1–11
- 817 31. Demirevska-Kepova K, Holzer R, Simova-Stoilova L, Feller U
818 (2005) Heat stress effects on ribulose-1, 5-bisphosphate carbox-
819 ylase/oxygenase, rubisco binding protein and rubisco activase
820 in wheat leaves. *Biol Plant* 49:521–525
- 821 32. Knopf JA, Shapira M (2005) Degradation of rubisco SSU dur-
822 ing oxidative stress triggers aggregation of rubisco particles in
823 *Chlamydomonas reinhardtii*. *Planta* 222:787–793
- 824 33. Suzuki M, Takahashi S, Kondo T, Dohra H, Ito Y, Kiriiwa
825 Y, Hayashi M, Kamiya S, Kato M, Fujiwara M, Fukao Y,
826 Kobayashi M, Nagata N, Motohashi R (2015) Plastid proteo-
827 mic analysis in tomato fruit development. *PLoS ONE*
828 10(9):e0137266
- 829 34. Tian S, Qin G, Li B (2013) Reactive oxygen species involved in
830 regulating fruit senescence and fungal pathogenicity. *Plant Mol
831 Biol* 82(6):593–602
- 832 35. Da Mota RV, Cordenunsi BR, Do Nascimento JR, Purgatto E,
833 Rosseto MR, Lajolo FM (2002) Activity and expression of banana
834 starch phosphorylases during fruit development and ripening.
835 *Planta* 216:325–333
- 836 36. Silva A, Nascimento J, Lajolo F, Cordenunsi B (2008) Starch
837 mobilization and sucrose accumulation in the pulp of Keitt man-
838 goes during postharvest ripening. *J Food Biochem* 32:384–395
839

37. Pandit S, Kulkarni R, Giri A, Koellner TG, Degenhardt J et al (2010) Expression profiling of various genes during the fruit development and ripening of mango. *Plant Physiol Biochem* 48:426–433
38. Amir R (2010) Current understanding of the factors regulating methionine content in vegetative tissues of higher plants. *Amino Acids* 39:917–931
39. Srivastava S, Singh RK, Pathak G, Goel R, Asif MH, Sane AP, Sane VA (2016) Comparative transcriptome analysis of unripe and mid-ripe fruit of *Mangifera indica* (var. “Dashehari”) unravels ripening associated genes. *Sci Rep* 6:1–13
40. Gonda I, Lev S, Bar E, Sikron N, Portnoy V, Davidovich-Rikanati R, Burger J, Schaffer AA, Tadmor Y, Giovannonni JJ, Huang M, Fei Z, Katzir N, Fait A, Lewinsohn E (2013) Catabolism of L-methionine in the formation of sulfur and other volatiles in melon (*Cucumis melo* L.) fruit. *Plant J* 74:458–472
41. Hadi M, Zhang F, Wu F, Zhou C, Tao J (2013) Advances in fruit aroma volatile researches. *Molecules* 18:8200–8299
42. Bandyopadhyay C, Gholap AS (1973) Relationship of aroma flavour characteristics of mango (*Mangifera indica* L.) to fatty acid composition. *J Sci Food Agric* 24:1497–1503
43. Lalel HJ, Singh Z, Tan SC (2003) Aroma volatiles production during fruit ripening of ‘Kensington Pride’ mango. *Postharvest Biol Technol* 27:323–336
44. Lalel HJD, Singh Z, Tan SC, Agustí M (2003) Maturity stage at harvest affects fruit ripening, quality and biosynthesis of aroma volatile compounds in ‘Kensington Pride’ Mango. *J Horticult Sci Biotechnol* 78:225–233
45. Han Y, Zhu Q, Zhang Z, Meng K, Hou Y, Ban Q, Suo J, Rao J (2015) Analysis of xyloglucan endotransglycosylase/hydrolase (XTH) genes and diverse roles of isoenzymes during persimmon fruit development and postharvest softening. *PLoS ONE* 10:e0123668
46. Ali ZM, Armugam S, Lazan H (1995) β -Galactosidase and its significance in ripening mango. *Phytochemistry* 38:1109–1114
47. Yashoda HM, Prabha TN, Tharanathan RN (2006) Mango ripening: changes in cell wall constituents in relation to textural softening. *J Sci Food Agric* 86:713–721
48. Tripathy BC, Oelmüller R (2012) Reactive oxygen species generation and signalling in plants. *Plant Signal Behav* 7:1621–1633
49. Leng P, Yuan B, Guo Y (2014) The role of abscisic acid in fruit ripening and responses to abiotic stress. *J Exp Bot* 65:4577–4588
50. Carrari F, Fernie AR, Iusem ND (2004) Heard it through the grapevine? ABA and sugar cross-talk: the ASR story. *Trends Plant Sci* 9:57–59
51. Maskin L, Gudesblat GE, Moreno JE, Carrari FO, Frankel N, Sambade A, Rossi M, Iusem ND (2001) Differential expression of the members of the *Asr* gene family in tomato (*Lycopersicon esculentum*). *Plant Sci* 161:739–746
52. Henry IM, Carpentier SC, Pampurova S, Van Hoylandt A, Panis B, Swennen R (2011) Structure and regulation of the *Asr* gene family in banana. *Planta* 234:785–798
53. Negri AS, Prinsi B, Rossoni M, Failla O, Scienza A, Cocucci M, Espen L (2008) Proteome changes in the skin of the grape cultivar ‘Barbera’ among different stages of ripening. *BMC Genom* 9:378
54. Lee U, Rioflorida I, Hong S, Larkindale J, Waters ER, Vierling E (2007) The *Arabidopsis* ClpB/Hsp100 family of proteins: chaperones for stress and chloroplast development. *Plant J* 49:115–127
55. Valenzuela-Chavira I, Contreras-Vergara CA, Arvizu-Flores AA, Serrano-Posada H, Lopez-Zavala AA, Garcia-Orozco KD, Hernandez-Paredes J, Rudiño-Piñera E, Stojanoff V, Sotelo-Mundo RR, Islas-Osuna MA (2017) Insights into ligand binding to a glutathione S-transferase from mango: Structure, thermodynamics and kinetics. *Biochimie* 135:35–45
56. Pye VE, Christensen CE, Dyer JH, Arent S, Henriksen A (2010) Peroxisomal plant 3-Ketoacyl-CoA Thiolase structure and activity are regulated by a sensitive redox switch. *J Biol Chem* 285:24078–24088
57. Bojorquez G, Gómez-Lim MA (1995) Peroxisomal thiolase mRNA is induced during mango fruit ripening. *Plant Mol Biol* 28:811–820
58. Prioretti L, Gontero B, Hell R, Mario Giordano M (2014) Diversity and regulation of ATP sulfurylase in photosynthetic organisms. *Front Plant Sci* 5:597
59. Grover A (2012) Plant chitinases: genetic diversity and physiological roles. *Crit Rev Plant Sci* 31:57–73
60. Wu J, Tan X, Wu C, Cao K, Li Y, Bao Y (2013) Regulation of cytokinesis by exocyst subunit SEC6 and KEULE in *Arabidopsis thaliana*. *Mol Plant* 6:1863–1876
61. Atkinson RG, Johnston SL, Yauk YK, Sharma NN, Schröder R (2009) Analysis of xyloglucan endotransglucosylase/hydrolase (XTH) gene families in kiwifruit and apple. *Postharvest Biol Technol* 51:149–157
62. Zhang Z, Wang N, Jiang S, Xu H, Wang Y, Wang C, Li M, Liu J, Qu C, Liu W, Wu S, Chen X, Chen X (2017) Analysis of the xyloglucan endotransglucosylase/hydrolase gene family during apple fruit ripening and softening. *J Agric Food Chem* 65(2):429–434
63. Han J, Tian S, Meng X, Ding Z (2006) Response of physiologic metabolism and cell structures in mango fruit to exogenous methyl salicylate under low-temperature stress. *Physiol Plant* 128:125–133
64. Hiwasa K, Nakano R, Hashimoto A, Matsuzaki M, Murayama H, Inaba A, Kubo Y (2004) European, Chinese and Japanese pear fruits exhibit differential softening characteristics during ripening. *J Exp Bot* 55:2281–2290
65. Lu W, Wang Y, Jiang Y, Li J, Liu H, Duan X, Song L (2006) Differential expression of litchi *XET* genes in relation to fruit growth. *Plant Physiol Biochem* 44:707–713
66. Skylar A, Sung F, Hong F, Chory J, Wu X (2011) Metabolic sugar signal promotes *Arabidopsis* meristematic proliferation via G2. *Dev Biol* 351:82–89
67. Kline KG, Barrett-Wilt GA, Sussman MR (2010) In planta changes in protein phosphorylation induced by the plant hormone abscisic acid. *Proc Natl Acad Sci USA* 107:15986–15991
68. Li Z, Liu D (2012) ROPGEF1 and ROPGEF4 are functional regulators of ROP11 GTPase in ABA-mediated stomatal closure in *Arabidopsis*. *FEBS Lett* 586:1253–1258
69. Piatkowski D, Schneider K, Salamini F, Bartels D (1990) Characterization of five abscisic acid-responsive cDNA clones isolated from the desiccation-tolerant plant *Craterostigma plantagineum* and their relationship to other water-stress genes. *Plant Physiol* 94:1682–1688
70. Giarola V, Jung NU, Singh A, Satpathy P, Bartels D (2018) Analysis of pcC13-62 promoters predicts a link between cis-element variations and desiccation tolerance in Linderniaceae. *J Exp Bot* 69:3773–3784
71. Smith DL, Gross KC (2000) A family of at least seven β -galactosidase genes is expressed during tomato fruit development. *Plant Physiol* 123:1173–1183
72. Vogel C, Marcotte EM (2012) Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Gen* 13:227–232
73. de Godoy LMF, Olsen JV, Cox J, Nielsen ML, Hubner NC, Fröhlich F, Walther TC, Mann M (2008) Comprehensive mass-spectrometry-based proteome quantification of haploid versus diploid yeast. *Nature* 455:1251–1255

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