Transcriptome-wide identification and characterization of the Rab GTPase family in mango

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Abstract

The Rab GTPase family plays a vital role in several plant physiological processes including fruit ripening. Fruit softening during ripening involves trafficking of cell wall polymers and enzymes between cellular compartments. Mango, an economically important fruit crop, is known for its delicious taste, exotic flavour and nutritional value. So far, there is a paucity of information on the mango Rab GTPase family. In this study, 23 genes encoding Rab proteins were identified in mango by a comprehensive *in silico* approach. Sequence alignment and similarity tree analysis with the model plant *Arabidopsis* as a reference enabled the *bona fide* assignment of the deduced mango proteins to classify into eight subfamilies. Expression analysis by RNA-Sequencing (RNA-Seq) showed that the *Rab* genes were differentially expressed in ripe and unripe mangoes suggesting the involvement of vesicle trafficking during ripening. Interaction analysis showed that the proteins involved in vesicle trafficking and cell wall softening were interconnected providing further evidence of the involvement of the Rab GTPases in fruit softening. Correlation analyses showed a significant relationship between the expression level of the *RabA3* and *RabA4* genes and fruit firmness at the unripe stage of the mango varieties suggesting that the differences in gene expression level might be associated with the contrasting firmness of these varieties. This study will not only provide new insights into the complexity of the ripening-regulated molecular mechanism but also facilitate the identification of potential Rab GTPases to address excessive fruit softening.

Keywords

Fruit ripening, Gene expression, Mango, Rab GTPase, Vesicle trafficking

1 Introduction

Mango (*Mangifera* sp) is one of the most economically important fruits around the globe. Mango fruit is highly appreciated for its delicious taste, exotic flavour and nutritional value [1]. Fruit softening is one of the most significant quality determination in mango for consumers as it reflects ripeness [2]. However, excessive softening adversely affects the fruit quality leading to the fruits being lost from the farm even before it has reached the consumers [3]. Effort to reduce post-harvest losses can be achieved through a better understanding of the ripening process [4]. Most research efforts to alter fruit softening have been focused on genes associated with cell wall degradation during ripening. However, the process of ripening is complex and cannot be explained based on only the genes that have so far been identified. It is therefore advantageous to identify and include more genes involved in other aspects such as membrane trafficking.

Fruit softening during ripening involves the production and trafficking of cell wall polymers and enzymes [5]. Using mostly reverse genetic approaches, the Rab guanosine triphosphatases (GTPases) have been found to be the primary regulator in directing traffic within endomembrane system [6].The Rab GTPase family has been found extant in all eukaryotes studied [7]. This subfamily has been extensively studied in yeasts and humans with at least 11 and 60 members respectively [8]. Thus far, members of the GTPase family have been discovered in several plant species, including *Arabidopsis thaliana*, tomato (*Solanum lycopersicum*), cotton (*Gossypium* sp), *Populus*, rice, maize, grape and peach (*Prunus persica*) [5, 9-12]. The plant Rab GTPase family has been grouped into eight clades, namely RabA, RabB, RabC, RabD, RabE, RabF, RabG, and RabH and these have been found to have a high degree of similarity to mammalian Rab classes 11, 2, 18, 1, 8, 5, 7 and 6 respectively [8,11,13]. Each type of Rab has a characteristic distribution on organelle membranes [14]. The RabA clade is the largest of the plant Rabs and is further divided into six subgroups (RabA1 to RabA6) compared with only two Rab11 GTPases in mammals. The conserved and non-conserved regions have been shown to contribute to the localization and specific function of the Rab proteins [15]. The Rabs share several common structural features, which include the guanine nucleotide-binding domains (termed G-boxes). Multiple sequence alignment analysis revealed Rab family specific regions (termed F1-F5) and Rab subfamily regions (termed SF1-3) respectively [8]. Despite the conserved nature of this gene family, great divergence exists at the hypervariable region which plays a crucial role in the specificity of membrane

association and targeting [15]. The preferential expression of the *Rab* genes during fruit ripening has been reported [9, 16-19]. Zainal *et al*. [19] carried out a pioneer investigation for the RabA sub-clade in mango fruit mesocarp and reported expression of a *RabA* gene in ripe fruit but not in green unripe fruit. A study by Liu *et al*. [16] demonstrated an increased expression of RabF during the later stages of mango fruit ripening. More notably, the RabA sub-clade provides a good illustration of how altered expression of a Rab GTPase could be used toeffect cell wall events necessary for expansion and loosening during ripening [18, 20]. For example, studies performed on green expanding tomato fruit showed that the Rab GTPases are important in determining the proportion of the different cell wall polymers when the cell wall is made [20].

To date, unlike the situation prevailing in model plants such as *Arabidopsis* and tomato where members of the Rab GTPase family had been elucidated, a detailed analysis of this gene family in mango remains to be conducted. Currently, there are only two published Rab sequences for mango [16, 19]. Although the mango genome is not yet available, transcriptome sequencing has a great potential to identify and characterize novel genes. In this study, we identified 23 *Rab* genes within the mango fruit transcriptome. Subsequently, multiple sequence alignments and similarity tree analysis were performed in addition to expression analysis of selected genes in different ripening stages (unripe and ripe).This study will not only present an integrated picture of the potential functionality of Rab GTPase during mango ripening but also provide new information to support mango improvement strategies involving the Rab GTPases.

2 Materials and Methods

2.1 Data Mining to Retrieve Mango Rab Gene Sequences

The known mango Rab *gene* sequences (Accession Z71276.1, KF768563) [16, 19] were used as queries to search against the mango RNA-sequencing database (http://bioinfo.bti.cornell.edu/cgi-bin/mango/index.cgi) [21] with an e-value threshold of 1e-2 to identify potential mango *Rab* genes. Each sequence was studied individually and using default parameters. All obtained mango Rab sequences were subjected to the BLASTP search of the *Arabidopsis* information resource (TAIR; https://www.arabidopsis.org). This was important in order to validate the protein conserved domains and check if they represented full-length coding regions. Predicted Rab protein sequences were generated using ExPASy tool (https://web.expasy.org/translate). The number of amino acids, molecular weight and

isoelectric point (pI) of the Rabs were predicted by EMBOSS Pepstats (https://www.ebi.ac.uk/Tools/seqstats/emboss_pepstats/). The predicted mango Rab proteins were assigned to subfamilies on the basis of their similarity to the sequences of *Arabidopsis* [11] and were named according to their closest similarity to *Arabidopsis* proteins. Where more than one mango Rab GTPase was present in the same sub-clade, a nomenclature based on numbers was adopted [9].

2.2 Sequence and Similarity Tree Analysis

Model plants *Arabidopsis thaliana* and tomato (*Solanum lycopersicum*) were used for comparative analysis. *Arabidopsis thaliana* Rab protein sequences were obtained from the *Arabidopsis* information resource (TAIR; https://www.arabidopsis.org). Members of the Rab GTPase family in tomato were identified from the Sol Genomics Network Browser (http://solgenomics.net) through BLASTP searches. Protein sequences were used for sequence and similarity analysis facilitating their classification in different families and subfamilies. The multiple sequence alignment was conducted using the software MultAlin (http://multalin.toulouse.inra.fr/multalin) [22]. A similarity tree was produced by the neighbour-joining (NJ) method [23] using the MEGA software (version 6.0) [24]. The reliability of the trees was examined using 1000 bootstrap replicates. Percentage confidence values were shown on branches.

2.3 Plant material and RNA preparation

Mango fruit varieties (‘Chokanan’, ‘Golden phoenix’ and ‘Water lily’) were obtained from a commercial supplier at the mature green stage. Following this, fruits were washed in running tap water, air dried and allowed to ripen at ambient temperature (25 ± 1 °C). Total RNA was isolated from the mango mesocarp tissue pulverized in liquid-nitrogen. Fruits were sampled at the unripe and ripe stages, using three individual fruits per ripening stage. The stages were defined based on firmness [25]. Total RNA extraction was performed as described by Zamboni *et al.* [26]. These samples were subsequently treated with Qiagen RNase-free DNase1 to remove traces of genomic DNA followed by purification with an RNeasy MinElute cleanup kit (Qiagen, Germany), according to the manufacturer's instructions. The concentration and purity of the RNA quantity was determined using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). RNA integrity was analyzed by 1.5 % agarose gel electrophoresis under denaturing conditions and with the Agilent 2100 Bioanalyser (Agilent Technologies, California, USA). Samples with 260/280, 260/230 ratios between 1.8 - 2.0 and RNA integrity number (RIN) ≥ 7.0

were used for further analysis. For RNA-Seq, equal amounts of RNA samples from the less-firm mango varieties 'Golden phoenix' and 'Water lily' [25] were combined to represent the less-firm mango group. Thus, the samples for RNA-Seq comprised of two mango groups namely the 'Chokanan' (abbreviated as 'CK'; firm mango group) and Pool (abbreviated as 'P'; less-firm mango group).

2.4 Gene expression analysis by RNA-Seq

RNA-Seq was performed at the Novogene Genome Sequencing Company using the HiSeq 2500 platform (Illumina, San Diego, CA, USA) according to the manufacturer's instructions with three biological replicates per sample. Briefly, 1 μg total RNA per sample was used to prepare libraries using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following the standard protocols. The raw sequence data of Fastq format were processed for various quality controls including the removal of adaptor sequences and low quality reads (i.e., those with unknown bases 'N' and reads having a quality score below 20). Following this, the clean reads obtained were pooled and assembled using Trinity [27] with default settings. RNA-seq by Expectation Maximization (RSEM) [28] was used to estimate the gene expression levels for each sample. The normalized gene expression values were derived using the Fragments Per Kilo base of gene per Million mapped reads (FPKM) method. In RNA-seq, FPKM is the most common method for normalization of gene expression as it eliminates the effect of gene length and sequencing depth [29]. Genes that were differentially expressed (adjusted P value < 0.05; log2 fold change (FC) > 1 or log2 fold change (FC) < -1) were identified using the DESeq package [30]. The adjusted P value is an essential measure to control the number of false discoveries in the differential gene expression analysis [31]. RNA-seq data generated from this study have been deposited in the NCBI Short Read Archive (SRA) database under the accession number PRJNA515564.

2.5 RT-qPCR Analysis

First strand cDNA synthesis was performed using QuantiTect® Reverse Transcription Kit (Qiagen, Germany) following the manufacturer's instructions. For each sample, 1μg of RNA was reverse transcribed. RT-qPCR was performed using the SENSIFAST™ SYBR Kit (Bioline, UK) on a CFX ConnectTM Real-Time PCR Detection System (BioRad, USA) according to the manufacturer's instructions. The Universal Probelibrary Assay Tool was used to design the RT-qPCR primers (amplicon length ranged from 70 to 180 bp). A differentiating assay mode was selected to design gene specific primers that uniquely identify (differentiate) each of the input gene sequences. This

was necessary especially because the targets belonged to a multigene family. This design tool would not generate primer sequences if a unique design was not identified. Primer pairs are listed in Supplementary Table S1. Furthermore, primer specificity was further confirmed by gel electrophoresis analysis of the amplicons and melting curve analysis and the amplification efficiency was carried out using the LinReg PCR software [33].The reaction mix (20 µl) contained 4 µl of diluted cDNA, 0.8 µl of each primer (10 µM), 10 µl of Master Mix, and 4.4 µl of RNase-free water. Reaction conditions were as follows: initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 5 sand 60°C for 30 s. All experiments were performed with three biological replicates for each sample and three technical replicates of each biological replicate. Average amplification efficiency for each amplicon was calculated using the LinRegPCR software [32]. Normalization was carried out with *Actin* and *Ubiquitin* genes based on the RefFinder tool [33], and the relative expression level was calculated using the $2^{-\Delta\Delta CT}$ method [34]. Statistical analysis was performed using the GENSTAT (18th edition) software. To compare the results for mean values of relative gene expression levels, one way analysis of variance (ANOVA) was used. Means were separated using Duncan's Multiple Range Test (DMRT; $P < 0.05$). Correlation analysis was carried out using JMP software version 8.0 (SAS Institute Inc., Cary, NC, USA). All data are presented as mean values ± standard error (SE).

2.6 Protein-Protein Interaction Analysis

A protein-protein interaction analysis was performed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, version 10.5; https://string-db.org/) [35]. This web-based tool identifies known and predicted protein associations based on the integrated information from numerous sources including high throughput experimental data, curated databases, co-expression data and public text mining [36]. Using default parameters, DEGs associated with softening and vesicle trafficking were used as inputs to understand the functional association of Rabs with fruit softening. A BLAST search against the *Arabidopsis thaliana* proteins was lodged in the STRING database. 'Nodes' in the network represent the proteins and each pairwise protein interaction, referred to as an 'edge'.

3 Results

3.1.1 Identification of the Rab GTPase Family

More than fifty sequences were identified that displayed similarity to the known mango *Rab* gene sequences. Each sequence was used to carry out a BLAST search against the *Arabidopsis* information resource

(https://www.arabidopsis.org) to further confirm its identity. After filtering for redundancy, a total of twenty-three genes with complete coding regions were retrieved. The most abundant were the RabA GTPases (12 in total) correlating with their relative abundance in other plant species such as *Arabidopsis*, tomato, wheat and grape amongst others. The mango Rabs were named according to their sequence similarity to the Rabs from *Arabidopsis*. A full list of the mango Rab GTPases identified in this study is presented together with the closest corresponding *Arabidopsis* genes (Table 1). A nomenclature based on numbers was adopted (RabA1-1, RabA1-2 etc.) to avoid misleading identification of putative mango genes orthologous to *Arabidopsis* Rab GTPases. This was important because while alignment of two RabA sequences showed differences at the amino acid level, they both showed greatest similarity to the same *Arabidopsis RabA* gene.

Table 1

Putative mango Rab GTPases (*Mangifera indica* L.) and their closest *Arabidopsis* homologues

Mango Rabs	No of aa residues	Mw (kDa)	pI	*Arabidopsis* closest member	*Arabidopsis* AGI no
RabA1-1	222	24.61	4.99	*AtRABA1a*	At1g06400
RabA1-2	217	24.15	5.38	*AtRABA1f*	At5g60860
RabA1-3	217	24.02	5.79	*AtRABA1c*	At5g45750
RabA1-4	217	24.08	5.73	*AtRABA1f*	At5g60860
RabA2-1	215	23.70	7.35	*AtRABA2a*	At1g09630
RabA2-2	220	24.15	6.25	*AtRABA2b*	At1g07410
RabA3	239	26.91	5.05	*AtRABA3*	At1g01200
RabA4-1	224	24.92	6.72	*AtRABA4a*	At5g65270
RabA4-2	227	25.07	5.14	*AtRABA4c*	At5g47960
RabA5-1	217	24.42	5.78	*AtRABA5a*	At5g47520
RabA5-2	218	24.28	4.87	*AtRABA5c*	At2g43130
RabA6	221	25.24	4.91	*AtRABA6b*	At1g18200
RabB	211	23.20	7.32	*AtRABB1b*	At4g35860
RabC	211	23.36	6.46	*AtRABC1*	At1g43890
RabD-1	202	22.60	5.95	*AtRABD2c*	At4g17530
RabD-2	203	22.57	4.86	*AtRABD2a*	At1g02130
RabE-1	216	23.90	8.23	*AtRABE1a*	At3g46060
RabE-2	203	22.77	9.71	*AtRABE1e*	At3g09900
RabF-1	200	21.84	7.13	*AtRABF1*	At3g54840
RabF-2	182	20.48	8.91	*AtRABF2a*	At5g45130
RabF-3	200	21.84	6.64	*AtRABF2a*	At5g45130
RabG	181	20.43	4.64	*AtRABG3a*	At4g09720
RabH	208	23.15	7.76	*AtRABH1b*	At2g44610

AGI, *Arabidopsis* Genome Initiative number; aa, amino acid; pI, isoelectric point; Mw, molecular weight

3.1.2 Sequence Comparison and Similarity Analysis

Multiple sequence alignments carried out with the deduced amino acid sequences of mango putative Rab GTPases aided the identification of family- and subfamily- specific regions, and further supported their assignment to a specific subgroup (Figure 1). Published mango Rab sequences were included for reference purposes. The Rab sequences obtained in this study contained the conserved GTPase regions (G1-G5) present in all members of the Ras superfamily. The presence of the amino acid stretches (termed F1–F5) which are diagnostic for Rab family members as described by Pereira-Leal and Seabra [8] was also observed. In addition, the C-terminal region containing the hypervariable region and the cysteine motif could be seen in the Rab GTPases. The Rab sequences obtained from the model plants *Arabidopsis* and tomato have been well characterized and thus were used as references. Rab sequences representing at least one member of each subclass from *Arabidopsis* and tomato were selected for analysis. This study allowed the investigation of the relationships between the Rab GTPases from different plant species. Examination of the resulting tree (Figure 2) indicated that the mango Rabs can be grouped into eight subgroups according to their homologs in *Arabidopsis* and tomato. The grouping of the Rab members suggests similar functions in these plant species. It was observed that half of the mango Rabs (12) belonged to the RabA group in six distinct subtypes (1-6). The other remaining eleven mango Rab members were distributed among seven other groups, with three RabF members, two RabD and E members, and one member in RabB, RabC RabG and RabH subfamilies.

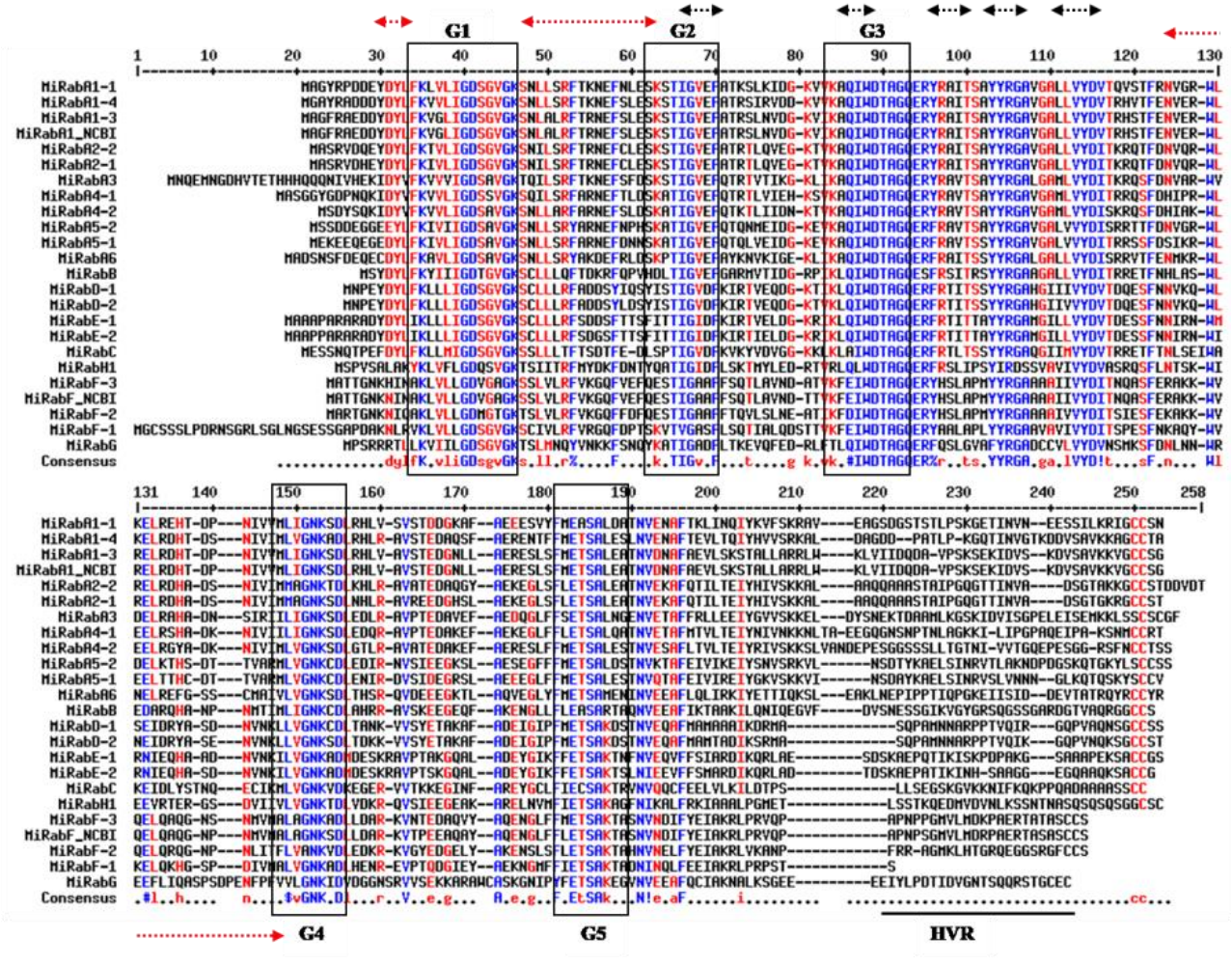

Fig. 1

Alignment of amino sequences from mango Rab sequences. Published Rabs on NCBI were included for reference. Conserved motifs named 'G box' sequences are identified and boxed with rectangles (G1, G2, G3, G4, and G5) (according to Jiang and Ramachandran [37]. Residues highlighted in black indicate 100% similarity in all sequences. HVR, hypervariable region. Rab -family (F) and -subfamily (SF) regions (defined according to Pereira-Leal and Seabra [8] are indicated by black and red arrows respectively. High and low consensus sequences are shown in blue and red colours respectively. Sequences in black colours are neutral.

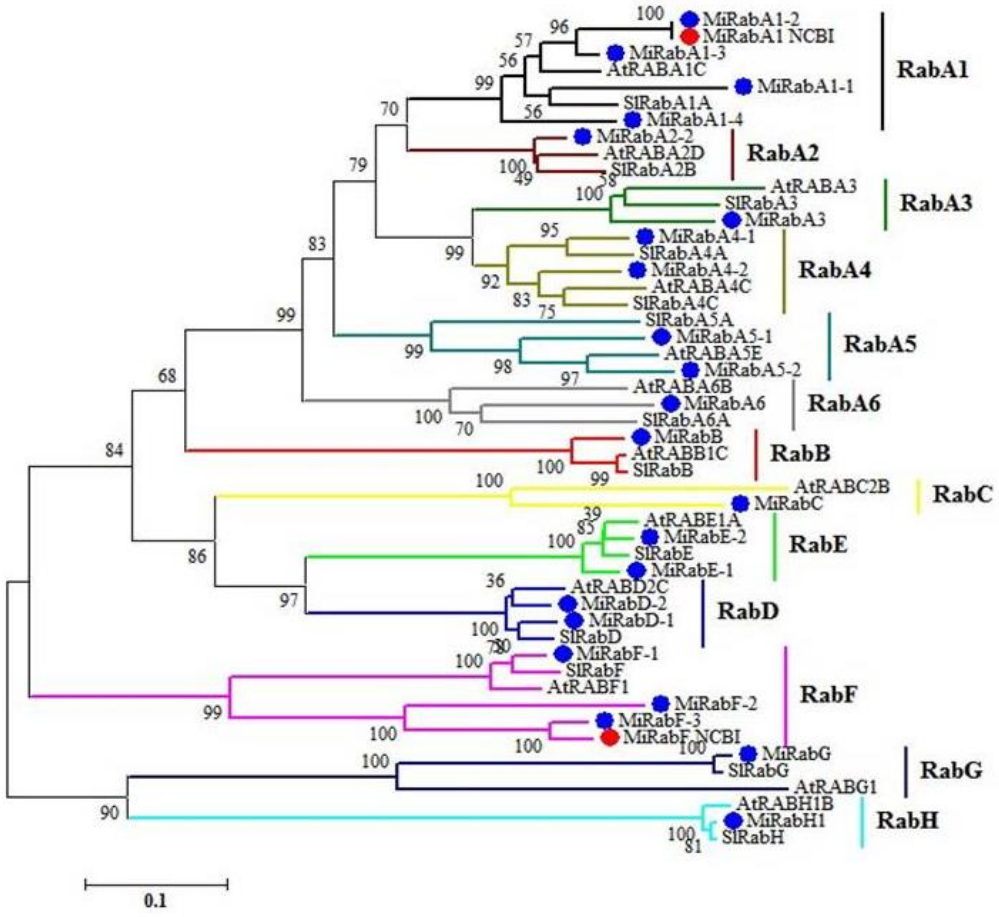

Fig. 2
Similarity tree of the mango Rabs and selected members from *Arabidopsis* and tomato.

3.1.3 Expression Profiling of the Rab GTPases during Ripening

The analysis of gene expression profiles provides useful insights towards gene function prediction [10]. To explore the potential roles of mango Rabs in ripening, the RNA-seq data generated from the mango groups (refer to Materials and Methods) was used to analyze their expression patterns. In this study, a stringent value of adjusted P value < 0.05; log2 fold change (FC) > 1 or < -1 as threshold was employed to identify significant differences in gene expression between the unripe and ripe stages. Thus, Tables 2-4 enlist the *Rab* genes that satisfied the criteria for differential expression in the pair-wise comparisons of samples. As observed, differentially expressed *Rab* genes were found in the 'CK' (Table 2) and 'P' (Table 3) groups respectively. In addition, the *Rab* genes were also found to be expressed differentially between mango groups at the unripe (Table 4) and ripe (Table 5) stages respectively. This finding indicates that membrane trafficking is essential in the mango ripening process. The genes identified

belonged to the subclasses *RabA*, *RabC*, *RabD*, *RabE* and *RabF* indicating that at least some members of the Rab GTPases play major roles in secretion and/or recycling events during mango ripening process. A total of seven differentially expressed *Rab* genes were obtained in the 'CK' group. Out of these, two genes encoding RabA4 and RabE respectively were strongly expressed in the ripe stage while other genes showed an opposite trend. On the other hand, ten *Rab* genes were differentially expressed between the ripening stages of 'P' group. Out of these, one *RabC* gene and five *RabA* genes were strongly expressed in the unripe sample whereas other genes showed the opposite trend. Nevertheless, most of the *RabA* genes in both mango groups displayed higher expression levels at the unripe than at the ripe stage.

Going further, pairwise comparison of the mango groups at either the unripe or ripe stage showed that most genes were up-regulated in the 'P' group as compared to the 'CK' group. Of the twelve *Rab* genes found to be expressed differentially between the mango groups at the unripe stage, eight encoded the RabA GTPase with *RabA2*, *RabA3* genes being strongly expressed in the 'P' group. At the ripe stage, a higher expression level of the *RabA3*, *RabC*, *RabD* and *RabE* genes was also observed in the 'P' group compared to the 'CK'. The RabA subfamily (RabA1, RabA2, RabA3 and RabA4) have been previously ascribed functions in plant cell wall dynamics [38] and as such, this subfamily was selected for further analysis.

Table 2

Rab genes expressed differentially between the unripe (UR) and ripe (R) stages of the Chokanan ('CK') group (CKUR vs CKR)

Gene_ID	Gene name	log_2FC	Adjusted P value	Regulation	*Arabidopsis* homolog
Cluster27569.63067	*RabA4-1*	2.39	0.0011	up	At5g65270
Cluster27569.55949	RabE-1	3.08	4.95E-05	up	At3g46060
Cluster27569.94833	RabA4-1	-2.62	0.0002	up	At5g65270
Cluster27569.53557	RabA2-2	-3.17	0.0329	down	At1g07410
Cluster27569.100367	RabA4-2	-2.08	0.0374	down	At5g47960
Cluster27569.106190	Rab C	-3.68	1.42E-06	down	At1g43890
Cluster27569.55074	RabE-1	-1.63	0.0236	down	At3g46060

FC, fold change; Up and down indicates that the expression level of the ripe stage is higher and lower than that of the unripe stage. The DEGs were defined by the criteria of adjusted P value < 0.05; $log_2FC > 1$ or $log_2FC < -1$

Table 3

Rab genes expressed differentially between the unripe (UR) and ripe (R) stages of the Pool ('P') group (PUR vs PR)

Gene_ID	Gene name	log_2FC	Adjusted P value	Regulation	*Arabidopsis* homolog
Cluster-27569.94833	*RabA4-1*	-2.02	0.0001	down	*AtRABA4a*
Cluster-27569.39793	*RabA5-2*	-1.91	0.0003	down	*AtRABA5c*
Cluster- 27569.39791	*RabA5-1*	-2.25	0.0002	down	*AtRABA5a*
Cluster-27569.69569	*RabA1-1*	-2.00	0.0008	down	*AtRABA1a*
Cluster-27569.53557	*RabA2-2*	-2.04	0.0043	down	*AtRABA2b*
Cluster-27569.22080	*RabC*	-4.55	0.0016	down	*AtRABC1*
Cluster- 27569.83684	*RabA1-1*	1.51	0.0426	up	*AtRABA1a*
Cluster-27569.60513	*RabA5-2*	1.42	0.0349	up	*AtRABA5c*
Cluster-27569.73649	*RabC*	1.68	0.0065	up	*AtRABC1*
Cluster-27569.65261	*RabF-3*	1.49	0.0107	up	*AtRABF2a*

FC, fold change; Up and down indicates that the expression level of the ripe stage is higher and lower than that of the unripe stage. The DEGs were defined by the criteria of adjusted P value < 0.05; $log_2FC > 1$ or $log_2FC < -1$

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Gene_ID	Gene name	log_2FC	Adjusted P value	Regulation	*Arabidopsis* homolog
Cluster-27569.69571	*RabA1-1*	-3.89	2.34E-14	down	*AtRABA1a*
Cluster-27569.60513	*RabA5-2*	-2.83	6.20E-10	down	*AtRABA5c*
Cluster-27569.69570	*RabA1-1*	-1.46	0.0014	down	*AtRABA1a*

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321 **Table 4**

Rab genes expressed differentially between the mango groups (‘CK’ and ‘P’) at the unripe stage (CKUR vs PUR)

Gene ID	Gene name	log_2FC	Adjusted P value	Regulation	*Arabidopsis* homolog
Cluster-27569.50830	*RabA1-4*	-1.29	0.0107	down	*AtRABA1f*
Cluster-27569.83684	*RabA1-1*	-1.57	0.0127	down	*AtRABA1a*
Cluster-27569.24125	*RabA3*	2.08	0.0007	up	*AtRABA3*
Cluster-27569.72059	*RabA2-1*	1.18	0.0182	up	*AtRABA2a*
Cluster-27569.53557	*RabA2-2*	1.45	0.0405	up	*AtRABA2b*
Cluster-27569.69571	*RabA1-1*	-3.46	4.35E-05	down	*AtRABA1a*
Cluster-27569.61122	*RabE-1*	4.39	1.47E-15	up	*AtRABE1a*
Cluster-27569.24125	*RabA3*	1.87	0.0185	up	*AtRABA3*
Cluster-27569.73649	*RabC*	5.45	2.38E-12	up	*AtRABC1*
Cluster-27569.62477	*RabD-2*	5.67	1.11E-09	up	*AtRABD2a*
Cluster-27569.78912	*RabC*	1.61	0.0012	up	*AtRABC1*

FC, fold change; Up and down indicates that the expression level in the unripe stage of the Pool group (PUR) is higher and lower than that in CK (CKUR). ‘P’, Pool mango group; ‘CK’, Chokanan mango group. The DEGs were defined by the criteria of adjusted P value < 0.05; $log_2FC > 1$ or $log_2FC < -1$

Table 5

Rab genes expressed differentially between the mango groups (‘CK’ and ‘P’) at the ripe stage (CKR vs PR)

Cluster-27569.73649	*RabC*	6.11	1.18E-12	up	*AtRABC1*
Cluster-27569.50830	*RabA1-2*	-1.68	0.0383	down	*AtRABA1f*
Cluster-27569.69569	*RabA1-1*	-1.90	0.0407	down	*AtRABA1a*
Cluster- 27569.61122	*RabE-1*	4.15	3.72E-08	up	*AtRABE1a*
Cluster- 27569.62477	*RabD-2*	Inf	9.87E-08	up	*AtRABD2a*

FC, fold change; Up and down indicates that the expression level in the ripe stage of the Pool group (PR) is higher and lower than that in CK (CKR). 'P', Pool mango group; 'CK', 'Chokanan' mango group. The DEGs were defined by the criteria of adjusted P value < 0.05; $\log_2$FC > 1 or $\log_2$FC< -1. Inf, infinite (used when there is a zero expression in one group of sample)

3.1.4 The Relationship between Gene Expression Level and Fruit Firmness

Based on the transcriptome analysis, the *RabA* genes were selected for further verification through qRT-PCR analysis in the varieties, 'Chokanan' (CK), 'Golden phoenix' (GP) and 'Water lily' (WL). Comparative expression analysis by RT-qPCR was used to further confirm the contributions of the RabA GTPases to contrasting mango pulp firmness. It is worth pointing out that the firmness data of these varieties has been published elsewhere [25]. The study showed that the Chokanan 'CK' variety was firmer than 'Golden phoenix' (GP) and 'Water lily' (WL). The 'CK' sample was arbitrarily chosen as a calibrator for the calculation of the relative expression ratio of each *RabA* gene. The expression level of the calibrator was set at 1.00 and the expression levels of the target genes in 'GP' and 'WL' were compared against it respectively. At the unripe stage, the expression levels of *RabA1-2* and *RabA3* genes

were lower in 'CK' as compared to other varieties (Figure 3). On the other hand, the expression level of *RabA2* was found to be similar in 'CK' and 'WL' while *RabA1-1* and *RabA4* expression levels showed no significant differences among the varieties ($P > 0.05$). A comparison of the expression levels of the tested *RabA* genes in the ripe stage (Figure 4) revealed similar tendency as observed in the unripe stage. Correlation analysis between *Rab* gene expression and pulp firmness of the mango varieties was performed. Supplementary Table S2 shows the relationship between pulp firmness of all varieties at the unripe stage. A non-significant ($P > 0.05$) negative correlation with pulp firmness was observed for *RabA1-1* ($r = -0.074$), *RabA1-2* ($r = -0.962$) and *RabA2* ($r = -0.784$) respectively. Conversely, significant negative correlations of pulp firmness with expression levels of *RabA3* ($r = -0.998$, $P = 0.043$) and *RabA4* ($r = -0.999$, $P < 0.01$) genes was observed. Although negative correlations were found between *RabA1-2*, *RabA2* and *RabA3* gene expression levels and pulp firmness at the ripe stage (Supplementary Table S3) albeit to a lesser extent compared to the unripe stage, these were not significant ($P > 0.05$ in all cases). Taken together, the result suggests that *RabA3* and *RabA4* may play an important role in the contrasting firmness of the mango varieties at the early stages of ripening.

3.1.5 Protein-Protein Interaction (PPI) Analysis

A Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database was used to reveal how differentially expressed *Rab* proteins interact with each other. This web-based database generates interaction networks based on known and predicted PPI [35]. In this network, nodes represent proteins and the edges (lines with different colours) between the nodes indicate the types of evidence supporting the association. The protein network resulting from STRING analysis is provided in Figure 5. The inputs for this analysis were the Rab proteins and the well-characterized cell wall softening proteins (polygalacturonase, pectinesterase and endoglucanase). As observed, several other related enzymes such as xylanase, laccase, callose synthase (GSL05) and xylosidase (XYL1) involved in cell wall biosynthesis and degradation respectively were found to be interacting partners with polygalacturonase, pectinesterase and endoglucanase in the network. Furthermore, from the cluster of Rab proteins, RabA1 was observed to interact with polygalacturonase (PGA4) whereas RabA4 protein was associated with callose synthase (GSL05). In addition, Rab-GDP dissociation inhibitor (GDI2) and syntaxin (SYP125) were also observed to interact with Rab GTPases respectively. This indicates the combined efforts of various components within the secretory pathway to bring about the biosynthesis modification of the cell wall.

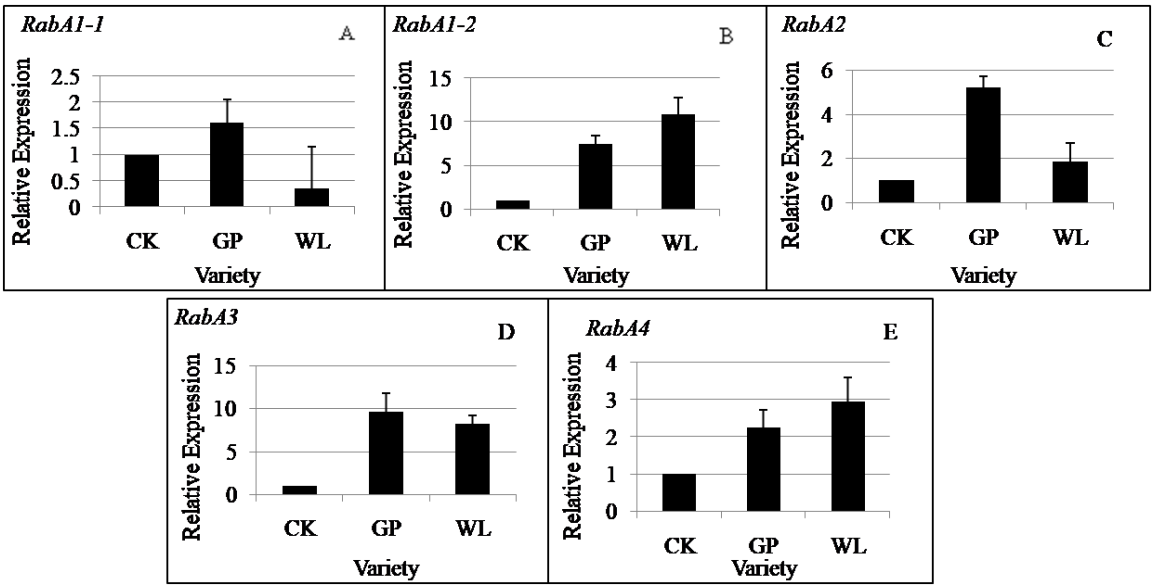

Fig. 3
Comparison of gene expression level between 'CK', 'GP' and 'WL' mango varieties at the unripe stage

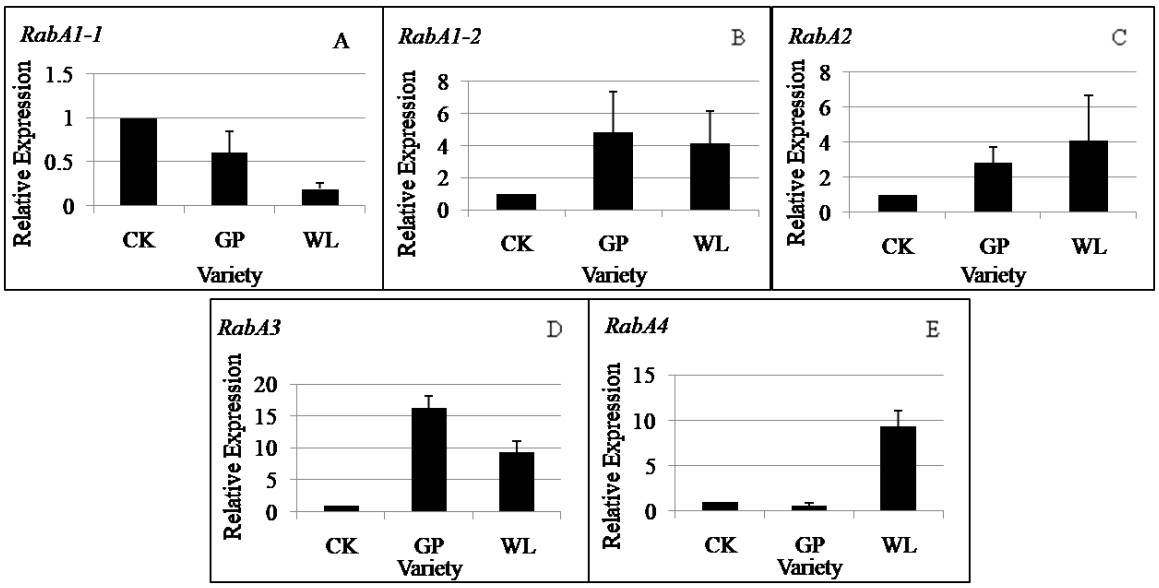

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Fig. 4
Comparison of gene expression level between 'CK', 'GP' and 'WL' mango varieties at the ripe stage

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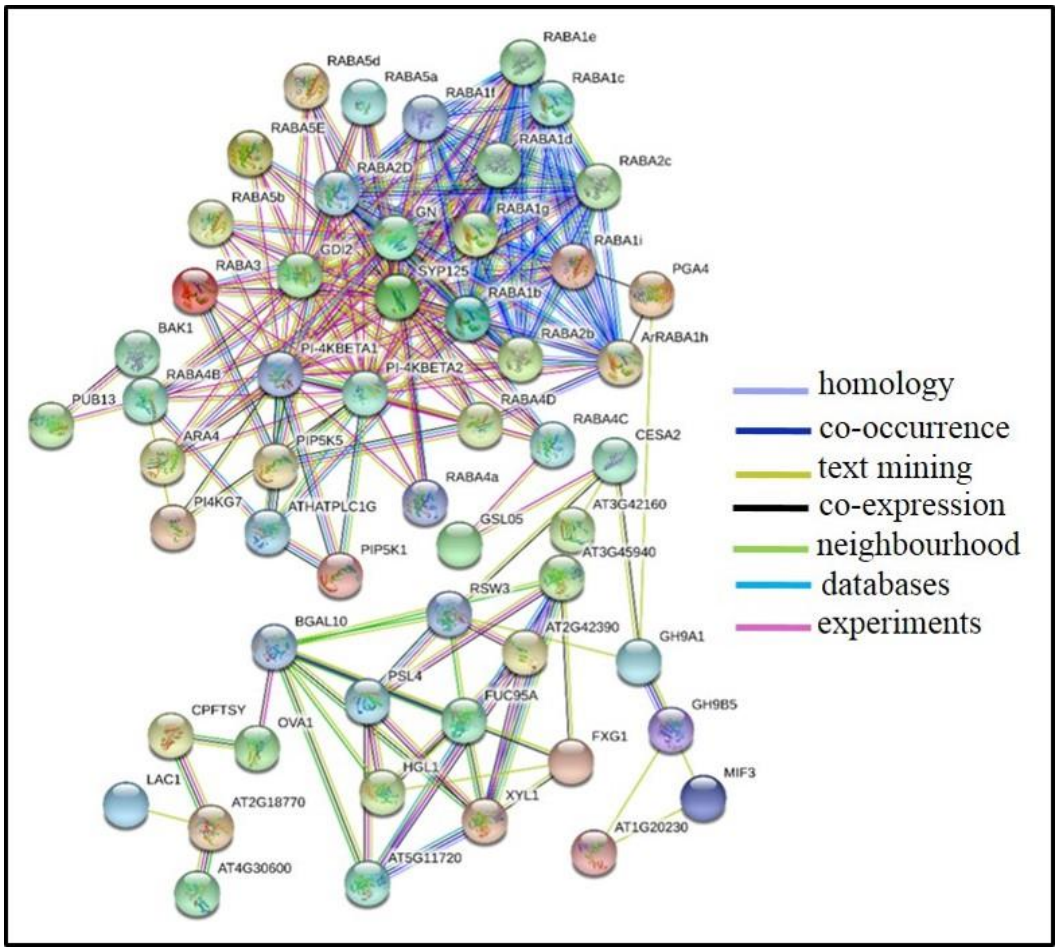

Fig. 5

Protein network generated by STRING (v 10.0) for selected differentially expressed genes associated with fruit softening and vesicle trafficking

Coloured nodes represent proteins whereas different colour of lines represents the types of evidence (depicted by the colour legend) for the association. Nodes represent proteins and are labelled according to their corresponding gene ID or gene symbols if present in TAIR. A complete list of the proteins within this network is provided in Supplementary Table S4.

4 Discussion

The Rab GTPase family has been studied extensively in various plants. The advent of next generation sequencing in recent years has provided a gateway to identify several genes for non-model plants such as mango. Comparative analysis is a powerful technique because information from well-studied groups can help guide less well-studied groups [39]. Rabs have been a model for this approach due to the high sequence conservation [40, 41]. The nomenclature used in this work is that defined in Pereira-Leal and Seabra [8]. The 23 Rabs found in this study might represent the total number expressed in fruit, though it is likely that this number only represents a portion of the total number of actual genes in mango. It is however smaller than the 57 Rab GTPases found in *Arabidopsis* [11], 87 in cotton [10] and 94 in soybean [40] but comparable to the 24 Rab GTPases in peach [9] and 26 in grape [9]. The higher number of genes might be due to additional duplication events in some plant species [42]. For instance, the comparative analysis of cotton and grape genomes revealed duplication events that were specific to the cotton lineage [43]. On the other hand, it is possible that the reduced number of genes found in grape and/or peach might have been due to gene loss during evolution [44, 45].

Nevertheless, the number of Rabs found in mango might also be due to the fact that the Rab sequences were retrieved from a fruit transcriptome dataset only. Future sequencing projects from other plant tissues will likely permit the identification of more Rab GTPases.

Similarity tree analyses showed the grouping of the Rab GTPases into subfamilies on the basis of their localization and/or function in trafficking [8]. Analyses of sequence similarities and of the presence of specific family and subfamily conserved motifs in their sequence allowed the identification of the closest homologues from *Arabidopsis* and the assignment of these sequences to members of the Rab family. The Rab sequences do not cluster in a species specific manner but rather within subfamilies supporting the findings of previous authors [41, 46]. According to these authors, it can be speculated that the members of the same cluster display similar functions. Within the mango Rab GTPase family, the predominant subgroup was the RabA subclade which included twelve RabA members. This is consistent with the significant expansion of the RabA group in plants [13]. All identified Rabs shared the typical conserved G-domains involved in binding and generally the double-cysteine motif in the C terminus [8]. These conserved regions offer opportunities for designing degenerate primers to facilitate gene isolation in other plant species with less information. An important feature of the Rab family is that Rab orthologues tend to perform similar functions even in divergent taxa [47]. Thus, according to the function of the Rab GTPases reported in plants such as

Arabidopsis and tomato [18, 48, 49], the possible functions of the mango Rab GTPases can be inferred based on the grouping of the Rab GTPase members observed from the plant species. Reverse genetic approaches on tomato fruits [18] have established the importance of vesicle trafficking in fruit ripening. Vesicle trafficking or the flow of membrane material between the endomembrane compartments is essential for the transport of proteins and polysaccharides to various destinations inside and outside of the cell [5]. Changes in the deposition of cell wall material during ripening require transport reflected by the differentially expressed *Rab* genes. Several classes of the Rab GTPases (RabA, Rab C, RabD, Rab E and Rab F) were detected and therefore may participate in secretion and/or endocytosis [14, 50] during mango ripening.A mixed expression pattern (up-regulated or down-regulated) was observed during the ripening of the mango groups ('CK' and P group respectively) in agreement with previous studies [9, 18, 19]. A mixed gene expression (down-regulation and up-regulation) suggests that these genes play roles in fruit development and ripening [51]. Lu *et al*. [18] found that the rabA tomato mutant maintained a higher firmness than its wild type. This result contrasts with the findings of the present study as the firm mango group ('CK') exhibited a higher level of *RabA1* gene transcript than the less-firm mango group ('P') at either the unripe or ripe stage. Lunn *et al*. [20] has shown that in tomato, the *RabA1* gene is more highly expressed in the mature green fruit than in the ripe fruit and even more strongly in the developing fruit. Thus, it is possible that a peak expression of the *RabA* gene in the 'P' group compared to the 'CK' group might have occurred earlier in the developmental stages not included in the study. It is also possible that different fruits may have adapted different ways to bring about fruit softening [52] as variation in cell wall changes between mango and tomato has been reported [53]. A study on *Arabidopsis* has shown that the RabA1, RabA2, RabA3 and RabA4 GTPases impact the pectin, cellulose, lignin and hemicellulose content of the cell wall respectively [38, 54]. Lunn *et al*. [55] went further to assess the effect of the RabA GTPase-deficient *Arabidopsis* mutant lines on cell wall digestion. These authors found out that the cell wall of the RabA4 mutants with reduced hemicellulose levels displayed increased susceptibility to enzymatic breakdown. Meanwhile, *RabA3* mutant lines which had raised level of lignin exhibited a reduction in enzyme degradation compared to the wild type. Lignin is an important component of the plant cell wall and its biosynthesis has been studied in fruits such as mangosteen [56] and peach [57]. Notably, high levels of lignin have been reported to be associated with increased fruit firmness in mangosteen [56], loquat [58] and pear [57]. Moreover, Salentijn *et al*. [59] mentioned that the contrasting firmness of strawberry varieties could be related to the lignin level as well as its composition. In the present study, same stage comparison between the 'CK' and 'P' groups

revealed a higher expression level of the *RabA3* gene in the 'P' group. Based on the previous reports, it is possible that the high expression level of *RabA3* observed in the 'P' group may have led to a reduction in lignin level and/or altered its composition leading to an increased susceptibility to enzymatic degradation and consequently increased loss in pulp firmness. Taken together, the differences in the level of *RabA* gene expression observed in the mango groups investigated would support the notion that the differential softening rate could be related to the variation in cell wall composition.

The present study represents one of the few studies linking the RabC GTPase subfamily to fruit ripening. The genes encoding the RabC GTPase showed a mixed expression (up-regulation and down-regulation) during the ripening of the mango group suggesting their roles in the development and ripening. The expression of *RabC* gene has also been shown to be regulated by dehydration and salinity [60]. The *RabC* gene strongly expressed in the unripe fruit may have been due to pre-harvest stresses such as exposure to sun light and pesticides while on the tree [61]. Taking this into account, the *RabC* genes strongly expressed in the ripe fruit may be required to prevent cell damage in some way.

The Rab GTPases of the D subclade mediate ER to Golgi trafficking steps in plants [61]. Comparison between the 'CK' and the 'P' group at the same ripening stage (unripe or ripe stage) revealed significantly higher levels in the 'P' group. Evidence from wheat has shown that transgenic lines with down-regulated *RabD* gene resulted in grains with altered bread making quality [46]. These authors suggested that the reduced bread making quality observed in the transgenic grains might have been due to the altered trafficking of the gluten proteins. In addition, Loraine *et al*. [17] found that the *RabD* genes were expressed at the breaker stage of ripening progressed of tomato fruit which is a stage when polygalacturonase is secreted strongly. This is consistent with the role of the Rab GTPase in increased synthesis and trafficking of cell wall modifying enzymes. Taking this into consideration, the up regulated *RabD* gene observed may have contributed to an increased trafficking of cell wall cargos in the 'P' group which might have led to an increased pulp softening compared to the 'CK' group.

The Rab GTPases of the E subclade mediate trafficking from the Golgi to PM [62]. The *RabE* genes have been reported to be expressed in fruits such as peach [9], grape [9] and apple [63]. Comparing the ripening stages of the mango groups, the *RabE* genes displayed a mixed expression (up-regulation and down-regulation) in the 'CK' group. However, no *RabE* gene was found to be expressed differentially in the 'P' group. An explanation for this may be due to the differences in the timing of gene expression [64]. It is possible that expression level may have

been very high and/or low during the pre-harvest or mid-ripe stages of the ‘P’ group which was not included in this study.

Studies using the loss-of-function mutation in *Arabidopsis* have established the role of RabF GTPases in vacuolar trafficking [65, 66]. The *RabF* gene was found to be up-regulated during the ripening of the ‘P’ group consistent with the findings of ‘Siji’ mango [16]. However, none was expressed differentially during the ripening of the ‘CK’ group. One possible reason could be due to the timing of gene expression [64]. It is also possible that this gene may have been induced in the ‘P’ group due to the stress-related events [16] associated with its fast ripening [25].

Altogether, the comparative analysis of expression has revealed the differential gene expression profile between the ripening stages for a mango group or between the same ripening stages of the mango groups. This finding suggests that the variability of ripening-related quality of the mango groups [25] could be, at least in part, due to the differences in the level of the *Rab* gene expression. In support of this, differential expression analyses of ripening associated genes in fruit varieties of watermelon [67], orange [68] and strawberry [59] have also shown stage- and/or variety-dependent expression profiles.

Proteins do not act alone but in association with other proteins which is essential for the biological processes that occur in the cell [69]. Molecular interactions play a key role in predicting the function of a protein and the biological processes the protein is associated with [70]. This bioinformatics approach using STRING 10.0 allowed the identification and interaction of the proteins related to cell wall metabolism and vesicle trafficking. Clusters of proteins identified were observed to be linked suggesting that these proteins often act in cooperation with each other [35]. For instance, the Rab GDP dissociation inhibitor (GDI) retrieves the GDP-bound Rab GTPases from the target membranes after a vesicular transport from the donor to acceptor membrane. Also, the Rab GTPases depend on specific binding to the correct syntaxin to mediate the docking and fusion of vesicles with the correct target membranes. A RabA was discovered because of its strong expression in mango fruit [19] and its tomato orthologue, which was shown to be required for fruit softening [18], seems to interact specifically with the syntaxins SYP122 rather than SYP121 [71]. Meanwhile, when localized in the cell wall, the pectinesterase (PE) modifies the pectins to make them more accessible for degradation by polygalacturonase (PG) [51]. Taken together, the protein-protein interaction analysis has indicated the synergistic action between several cell wall-related enzymes and more importantly strengthens the involvement of Rab GTPases in cell wall biosynthesis and modification.

The expression levels of the *RabA3* and *RabA4* genes were found to be significantly negatively correlated with

differences in fruit firmness among the three mango varieties ‘Chokanan’, ‘Golden phoenix’ and ‘Water lily’ in the unripe stage. The significant correlation of gene expression and fruit firmness at the unripe stage suggests that changes in cell wall composition leading to varietal differences in softening may have occurred early during mango fruit development. In this respect, differences in gene expression level among mango varieties would correlate most strongly and significantly with differences in softening at the developmental phase. This is supported by Ng *et al*. [72] who demonstrated that the variable rates of softening in apple varieties manifested in the early stages of fruit development. Additionally, in a study by Lunn *et al*. [20] the really high level of expression of the *RabA1a* gene was during the expansion of immature fruit which is the stage at which pectin is being laid down in the cell wall and these authors showed that there were significant differences in pectin content when gene expression was inhibited.

5 Conclusion

An investigation of the Rab GTPase family in mango has been successfully carried out for the first time in the current study. This is a starting point towards facilitating our understanding of the involvement of the Rab GTPases in mango fruit ripening. Mining of the publicly available mango RNA-seq database allowed for the retrieval of these sequences. Sequence comparison and similarity tree analysis of the mango Rab GTPases with *Arabidopsi*s and tomato as references allowed their identification and assignment into specific subgroups. The study indicated that the Rab GTPases are conserved within subgroups rather than within species which might indicate shared putative functions. This has provided insights into the possible functional diversity of the mango Rab GTPase family. The expression profile of the Rab GTPasefamily suggests their potential function in mango ripening, as previously documented in other mango varieties and fruits including grape, peach and tomato. The relationship between pulp firmness and the *RabA* gene expression in the mango varieties has been obtained thus providing evidence for the importance of trafficking in fruit softening. The information obtained, although correlative in nature, indicates that the trafficking of cell wall polymers and modifying enzymes might be associated with contrasting firmness in mango varieties. Nevertheless, these findings, together with the direct experimental evidence of some Rabs in plants, indicate that Rab GTPases are of great importance for crop improvement. In particular, *RabA3* may be considered an interesting gene for addressing the excessive softening of mango fruit although a larger sample size and more experimentation will be needed for further confirmation. This comprehensive study serves to facilitate our understanding of Rab GTPase in association with fruit ripening, provides a reference for Rab GTPase family

classification in fruit trees and lays out a foundation for future molecular breeding strategies involving Rab GTPases.

Declarations

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of Data

All RNA-Seq datasets generated during the current study are available in the NCBI Sequence Read Archive (SRA) database with the Bioproject accession number PRJNA515564.

Authors' contribution

CCF and GWL conceptualized and supervised the study. TL carried out RNA and cDNA preparation, *in silico* analysis and RT-qPCR experiments. All authors contributed to data interpretation. The draft of the manuscript was written by TL and all authors read, approved and contributed to the final version of the manuscript.

591 References

592 1. Singh Z, Singh R, Sane V, Nath P (2013) Mango - Postharvest Biology and Biotechnology. Crit Rev Plant
593 Sci 32:217-236. doi:10.1080/07352689.2012.743399
594 2. Jha S, Sethi S, Srivastav M, Dubey A, Sharma R, Samuel D, Singh, A (2010) Firmness characteristics of
595 mango hybrids under ambient storage. J Food Eng 97:208-212. doi:10.1016/j.jfoodeng.2009.10.011
596 3. Yahia EM (2011) Mango (*Mangifera indica* L.). In: Yahia EM (ed) Postharvest biology and technology of
597 tropical and subtropical fruit: cocona to mango, Woodhead Publishing Limited, pp 492B-586
598 4. Seymour G, Østergaard L, Chapman N, Knapp S, Martin C (2013) Fruit Development and Ripening. Annu
599 Rev Plant Biol 64:219-241. doi:10.1146/annurevarplant-050312-120057
600 5. Lycett GW (2008) The role of Rab GTPases in cell wall metabolism. J Exp Bot 59:4061-4074.
601 http://dx.doi.org/10.1093/jxb/ern255
602 6. Schimmoller F, Simon I, Pfeffer SR (1998) Rab GTPases, directors of vesicle docking. J Biol
603 Chem. 273**,** 22161–22164
604 7. Stenmark H (2009) Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol 10:513-525.
605 http://dx.doi.org/10.1038/nrm2728
606 8. Pereira-Leal J, Seabra M (2001) Evolution of the Rab family of small GTP-binding proteins. J Mol Biol
607 313:889-901. http://dx.doi.org/10.1006/jmbi.2001.5072
608 9. Abbal P, Pradal M, Muniz L, Sauvage F, Chatelet P, Ueda T, Tesniere, C (2008) Molecular
609 characterization and expression analysis of the Rab GTPase family in *Vitis vinifera* reveal the specific
610 expression of a VvRabA protein. J Exp Bot 59:2403-2416. http://dx.doi.org/10.1093/jxb/ern132
611 10. Li P, Guo W (2017) Genome-wide characterization of the Rab gene family in *Gossypium* by comparative
612 analysis. Bot Stud 58. doi:10.1186/s40529-017-0181-y
613 11. Rutherford S, Moore I (2002) The *Arabidopsis* Rab GTPase family: another enigma variation. Curr Opin
614 Plant Biol 5:518-528. http://dx.doi.org/10.1016/s13695266 (02)00307-2
615 12. Zhang J, Li Y, Liu B, Wang L, Zhang L et al (2018) Characterization of the Populus Rab family genes and
616 the function of PtRabE1b in salt tolerance. BMC Plant Biol 18:124. https://doi.org/10.1186/s12870-018-
617 1342-1
618 13. Vernoud, V., Horton, A., Yang, Z., Nielsen, E., 2003. Analysis of the small GTPase Gene Superfamily of
619 *Arabidopsis*. Plant Physiol 131:1191-1208. http://dx.doi.org/10.1104/pp.013052
620 14. Saito C, Ueda T (2009) Functions of RAB and SNARE proteins in plant life. Int Rev Cell Mol Biol
621 274:183-233
622 15. Pfeffer S (2005) Structural Clues to Rab GTPase functional diversity. J Biol Chem 280:15485-15488.
623 http://dx.doi.org/10.1074/jbc.r500003200
624 16. Liu Z, Luo C, Dong L, Van Toan C, Wei P, He X (2014) Molecular characterization and expression
625 analysis of a GTP-binding protein (MiRab5) in *Mangifera indica*. Gene 540:86-91.
626 http://dx.doi.org/10.1016/j.gene.2014.02.022
627 17. Loraine A, Yalovsky S, Fabry S, Gruissem W (1996) Tomato Rab1A homologs as molecular tools for
628 studying Rab geranylgeranyl transferase in plant cells. Plant Physiol 110:1337-1347.
629 http://dx.doi.org/10.1104/pp.110.4.1337
630 18. Lu C, Zainal Z, Tucker G, Lycett G (2001) Developmental abnormalities and reduced fruit softening in
631 tomato plants expressing an antisense Rab11 GTPase gene. The Plant Cell 13:1819-1833.
632 http://dx.doi.org/10.2307/3871321
633 19. Zainal Z, Tucker G, Lycett GW (1996) A Rab11-like gene is developmentally regulated in ripening mango
634 (*Mangifera indica* L.) fruit. Biochimica et Biophys Acta Mol Cell Res 1314:187-190.
635 http://dx.doi.org/10.1016/s0167-4889 (96)00133-4
636 20. Lunn D, Phan T, Tucker G, Lycett G (2013a) Cell wall composition of tomato fruit changes during
637 development and inhibition of vesicle trafficking is associated with reduced pectin levels and reduced
638 softening. Plant Physiol Biochem 66:9197. doi:10.1016/j.plaphy.2013.02.005

21. Tafolla-Arellano J, Zheng Y, Sun H, Jiao C, Ruiz-May E, Hernández-Oñate M et al (2017) Transcriptome analysis of mango (Mangifera indica L.) fruit epidermal peel to identify putative cuticle-associated genes. Sci Rep 7. doi:10.1038/srep46163
22. Corpet F (1988) Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res 16:10881-10890. doi:10.1093/nar/16.22.10881
23. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425. doi:10.1093/oxfordjournals.molbev.a040454
24. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:27252729. doi:10.1093/molbev/mst197
25. Lawson T, Lycett G, Ali A, Chin C (2019) Characterization of Southeast Asia mangoes (*Mangifera indica* L) according to their physicochemical attributes. Sci Hort 243:189-196. doi:10.1016/j.scienta.2018.08.014
26. Zamboni A, Pierantoni L, De Franceschi P (2008) Total RNA extraction from strawberry tree (*Arbutus unedo*) and several other woody-plants. iForest 1:122-125. doi:10.3832/ifor0465-0010122
27. Grabherr M, Haas B, Yassour M, Levin J, Thompson D, Amit I, et al (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotech 29:644-652. doi:10.1038/nbt.1883
28. Li B, Dewey C (2011) RSEM: accurate transcript quantification from RNA-seq data with or without a reference genome. BMC Bioinformatics 12:323. doi:10.1186/1471-2105-12323
29. Conesa A, Madrigal P, Tarazona S, Gomez-Cabrero D, Cervera A, McPherson A et al (2016) A survey of best practices for RNA-seq data analysis. Genome Biol 17:181 doi:10.1186/s13059-016-08818
30. Anders S, Huber W (2010) Differential expression analysis for sequence count data. Genome Biol 11:R106. doi:10.1038/npre.2010.4282.1
31. Benjamini Y, Hochberg Y (1995) Controlling the False Discovery Rate: a practical and powerful approach to multiple testing. J R Stat Soc Series B (Methodological) 57:289-300. doi:10.2307/2346101
32. Ruijter J, Ramakers C, Hoogaars W, Karlen Y, Bakker O, van den Hoff M, Moorman A (2009) Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res 37:e45. doi:10.1093/nar/gkp045
33. Xie F, Xiao P, Chen D, Xu L, Zhang B (2012) miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. Plant Mol. Biol. 80:75-84. doi:10.1007/s11103-012-9885-2
34. Livak K, Schmittgen T (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods 25:402-408. doi:10.1006/meth.2001.1262
35. Szklarczyk D, Morris J, Cook H, Kuhn M, Wyder S, Simonovic M et al (2016) The STRING database in 2017: quality-controlled protein–protein association networks, made broadly accessible. Nucleic Acids Res 45:D362-D368. doi:10.1093/nar/gkw937
36. Jensen L, Kuhn M, Stark M, Chaffron S, Creevey C, Muller J et al (2009) STRING 8--a global view on proteins and their functional interactions in 630 organisms. Nucleic Acids Res 37:D412-D416. doi:10.1093/nar/gkn760
37. Jiang S, Ramachandran S (2006) Comparative and evolutionary analysis of genes encoding small GTPases and their activating proteins in eukaryotic genomes. Physiol Genomics 24:235-251. http://dx.doi.org/10.1152/physiolgenomics.00210.2005
38. Lunn D, Gaddipati S, Tucker G, Lycett G (2013b) Null mutants of individual RABA genes impact the proportion of different cell wall components in stem tissue of Arabidopsis thaliana. Plos One 8:e75724. doi:10.1371/journal.pone.0075724
39. Mayes S, Massawe F, Alderson P, Roberts J, Azam-Ali S, Hermann M (2011) The potential for underutilized crops to improve security of food production. J Exp Bot 63:1075-1079. doi:10.1093/jxb/err396
40. Flores A, Via V, Savy V, Villagra U, Zanetti M, Blanco F (2018) Comparative phylogenetic and expression analysis of small GTPases families in legume and non-legume plants. Plant Signal Behav 13:e1432956. doi:10.1080/15592324.2018.1432956

41. Zhang J, Hill D, Sylvester A (2007) Diversification of the Rab guanosine triphosphatase family in dicots and monocots. J Integr Plant Biol 48: 1129-1141. http://dx.doi.org/10.1111/j.1672-9072.2007.00520.x
42. Rojas A, Fuentes G, Rausell A, Valencia A (2012) The Ras protein superfamily: Evolutionary tree and role of conserved amino acids. J Cell Biol 196:189-201. doi:10.1083/jcb.2011030081964c
43. Lin L, Tang H, Compton R, Lemke C, Rainville L, Wang X, Rong J, Rana M, Paterson A (2011) Comparative analysis of *Gossypium* and *Vitis* genomes indicates genome duplication specific to the *Gossypium* lineage. Genomics 97:313-320. doi:10.1016/j.ygeno.2011.02.007
44. Bowers J, Chapman B, Rong J, Paterson A (2003) Unravelling angiosperm genome evolution by phylogenetic analysis of chromosomal duplication events. Nature 422:433438. doi:10.1038/nature01521
45. Tian X, Wang P, Hu W, Gao D, Li Q (2014) Gene loss in plants: Evidence from genome wide phylogenetic analysis of thiamine pyrophosphate-dependent enzymes. J Syst Evol 53:221-227. doi:10.1111/jse.1212
46. Tyler A, Bhandari D, Poole M, Napier J, Jones H, Lu C, Lycett GW (2015) Gluten quality of bread wheat is associated with activity of RabD GTPases. Plant Biotechnol J 13:163-176. http://dx.doi.org/10.1111/pbi.12231
47. Diekmann Y, Seixas E, Gouw M, Tavares-Cadete F, Seabra MC, Pereira-Leal JB (2011) Thousands of Rab GTPases for the Cell Biologist. Plos Comput Biol 7:e1002217. doi:10.1371/journal.pcbi.1002217
48. Chow C, Neto H, Foucart C, Moore I (2008) Rab-A2 and Rab-A3 GTPases define a trans Golgi endosomal membrane domain in *Arabidopsis* that contributes substantially to the cell plate. The Plant Cell 20:101-123. http://dx.doi.org/10.1105/tpc.107.052001
49. Qi X, Zheng H (2013) Rab-A1c GTPase defines a population of the trans-Golgi network that is sensitive to endosidin1 during cytokinesis in *Arabidopsis*. Mol Plant 6:847-859. doi:10.1093/mp/sss116
50. Lawson, T, Mayes S, Lycett G, Chin C (2018) Plant Rabs and the role in fruit ripening. Biotechnol Genet Eng Rev 1-17. doi:10.1080/02648725.2018.1482092
51. Tucker G, Yin X, Zhang A, Wang M, Zhu Q, Liu X et al (2017) Ethylene† and fruit softening. Food Qual Saf 1:253-267. doi:10.1093/fqsafe/fyx024
52. Brummell D (2006) Cell wall disassembly in ripening fruit. Funct Plant Biol 33:103. http://dx.doi.org/10.1071/fp05234
53. Muda P, Seymour G, Errington N, Tucker G (1995) Compositional changes in cell wall polymers during mango fruit ripening. Carbohydr 26:255-260. doi:10.1016/0144-8617(95)00028-6
54. Lunn D (2013) Role of Rab GTPase proteins in cell wall deposition and potential use of RabA mutants in bioenergy. Dissertation, University of Nottingham
55. Kamdee C, Imsabai W, Kirk R, Allan A, Ferguson I, Ketsa S (2014) Regulation of lignin biosynthesis in fruit pericarp hardening of mangosteen (*Garcinia mangostana* L.) after impact. Postharvest Biol Technol 97:68-76. doi:10.1016/j.postharvbio.2014.06.004
56. Xue C, Yao J, Qin M, Zhang M, Allan A, Wang D, Wu J (2018) PbrmiR397a regulates lignification during stone cell development in pear fruit. Plant Biotechnol J doi:10.1111/pbi.12950
57. Zeng J, Li X, Zhang J, Ge H, Yin X, Chen K (2016) Regulation of loquat fruit low temperature response and lignification involves interaction of heat shock factors and genes associated with lignin biosynthesis. Plant Cell Environ 39:1780-1789. doi:10.1111/pce.12741
58. Salentijn E, Aharoni A, Schaart J, Boone M, Krens F (2003) Differential gene expression analysis of strawberry cultivars that differ in fruit-firmness. Physiol Plant 118:571-578. doi:10.1034/j.1399-3054.2003.00138.x
59. Khassanova G, Kurishbayev A, Jatayev S, Zhubatkanov A, Zhumalin A, Turbekova A (2019) Intracellular vesicle trafficking genes, RabC-GTP, are highly expressed under salinity and rapid dehydration but down-regulated by drought in leaves of chickpea (*Cicer arietinum* L.). doi:10.3389/fgene.2019.00040
60. Bower J, Cutting J, Wolstenholme, B (1989) Effect of pre- and post-harvest water stress on the potential for fruit quality defects in avocado (*Persea Americana* Mill.). S Afr J Plant Soil 6:219-222. doi:10.1080/02571862.1989.10634516

61. Batoko H, Zheng H, Hawes C, Moore I (2000) A Rab1 GTPase is required for transport between the endoplasmic reticulum and Golgi apparatus and for normal Golgi movement in plants. The Plant Cell 12:2201. http://dx.doi.org/10.2307/3871115
62. Speth E, Imboden L, Hauck P, He S (2009) Subcellular localization and functional analysis of the *Arabidopsis* GTPase RabE. Plant Physiol 149:1824-1837. http://dx.doi.org/10.1104/pp.108.132092
63. Park S, Sugimoto N, Larson M, Beaudry R, van Nocker S (2006) Identification of genes with potential roles in apple fruit development and biochemistry through large-scale statistical analysis of expressed sequence tags. Plant Physiol 141:811-824. http://dx.doi.org/10.1104/pp.106.080994
64. Choudhury SR, Roy S, Sengupta D (2009) Characterization of cultivar differences in β 1,3 glucanase gene expression, glucanase activity and fruit pulp softening rates during fruit ripening in three naturally occurring banana cultivars. Plant Cell Rep 28:16411653. doi:10.1007/s00299-009-0764-5
65. Ebine K, Inoue T, Ito J, Ito E, Uemura T, Goh T et al (2014) Plant vacuolar trafficking occurs through distinctly regulated pathways. Curr Biol 24:1375-1382. doi:10.1016/j.cub.2014.05.004
66. Sohn E, Kim ES, Zhao M, Kim SJ, Kim H, Kim YW et al (2003) Rha1, an *Arabidopsis* Rab5 homolog, plays a critical role in the vacuolar trafficking of soluble cargo proteins. The Plant Cell 15:1057-1070. doi:10.1105/tpc.009779
67. Zhu Q, Gao P, Liu S, Zhu Z, Amanullah S, Davis A, Luan F (2017) Comparative transcriptome analysis of two contrasting watermelon genotypes during fruit development and ripening. BMC Genomics 18:3 doi:10.1186/s12864-016-3442-3
68. Wang J, Liu J, Chen K, Li H, He J, Guan B, He L (2017) Comparative transcriptome and proteome profiling of two *Citrus sinensis* cultivars during fruit development and ripening. BMC Genomics 18:984. doi:10.1186/s12864-017-4366-2
69. Launay G, Ceres N, Martin J (2017) Non-interacting proteins may resemble interacting proteins: prevalence and implications. Sci Rep 7. doi:10.1038/srep40419
70. Rao V, Srinivas K, Sujini G, Kumar G (2014) Protein-protein interaction detection: methods and analysis. Int J Proteomics 2014:147648. doi: 10.1155/2014/147648
71. Rehman R, Stigliano E, Lycett GW, Sticher L, Sbano F, Faraco M, Dalessandro G, Di Sansebastiano G (2008) Tomato Rab11a characterization evidenced a difference between SYP121-dependent and SYP122-dependent exocytosis. Plant Cell Physiol 49:751-766. doi:10.1093/pcp/pcn051
72. Ng J, Schröder R, Sutherland P, Hallett I, Hall M, Prakash R et al (2013) Cell wall structures leading to cultivar differences in softening rates develop early during apple (*Malus* x *domestica*) fruit growth. BMC Plant Biol 13:183. http://dx.doi.org/10.1186/1471-2229-13-183