AN INVESTIGATION ON THE SOFTENING AND RIPENING PROCESS OF TROPICAL MANGO (*Mangifera indica* L.) WITH A PARTICULAR FOCUS ON RAB GTPASES

TAMUNONENGIYEOFORI LAWSON (MSc.)

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School of Biosciences, Faculty of Science, the University of Nottingham, Malaysia Campus

ABSTRACT

Mango (*Mangifera indica* L.) is an economically important fruit crop grown in the tropics. This crop species is popularly consumed as fruit in Malaysia. Once ripening is initiated, the process proceeds at a fast rate making postharvest life short. Excessive softening during ripening is a major challenge in the postharvest storage of fruits as it renders the fruits unfit for long term storage leading to heavy postharvest losses. The improved storage of mango fruit would greatly enhance the economic potential of this crop. Hence, an in-depth understanding of the ripening-related events is essential to facilitate the development of strategies to improve fruit quality and reduce post-harvest losses. Fruit softening during ripening involves the trafficking of cell wall polymers and enzymes. The Rab (Ras related proteins in brain) GTPase family are key players in vesicle trafficking. Therefore it is important to understand the linkage between the Rab (Ras related proteins in brain) GTPases and the differential softening rate in fruit varieties for effective postharvest management, hence this research.

The first part of this research was conducted to characterize the ripening process of the mango varieties according to their postharvest quality attributes. The study was carried out on 'Chokanan' (CK), 'Golden phoenix'(GP) and 'Water lily'(WL) as exemplar mango varieties for which there are limited number of studies at the postharvest and molecular level respectively. Significant increase ($P < 0.05$) in the soluble solid concentration (SSC), ethylene production, respiration rate and weight loss coupled with significant decline in titratable acidity (TA) and fruit firmness occurred as ripening progressed. Significant differences were also found among the mango varieties. The analysis revealed that 'Chokanan' (CK) had greater fruit firmness ($P < 0.05$) than 'Golden phoenix' (GP) and 'Water lily'(WL) mango varieties. Multivariate analysis separated the unripe and ripe fruits according to their physicochemical

attributes. The ripening stages (unripe and ripe) as defined based on the measured postharvest parameters were selected for further studies.

Characterization of the mango Rab (Ras related proteins in brain) GTPase family by comparative analysis was performed. The study took advantage of the publicly available databases and transcriptome datasets to identify and conduct comprehensive comparison of the mango Rab (Ras related proteins in brain) GTPase family. A total of twenty-three members of the mango Rab (Ras related proteins in brain) GTPase family with similarity to those obtained from *Arabidopsis thaliana* and tomato (*Solanum lycopersicum*) were identified. Sequence similarity analyses and identification of conserved motifs, diagnostic of specific Rab family and subfamilies enabled the *bona fide* assignment of the deduced mango proteins.

A transcriptomic approach by RNA-sequencing (RNA-seq) was performed to investigate the molecular basis of mango ripening using two ripening stages (unripe and ripe) and two mango groups with contrasting firmness ($P < 0.05$). It is worth pointing out that this is the first experiment reported on Southeast Asian mango varieties employing the RNA-sequencing (RNA-seq) approach. Pairwise comparison between the ripening stages (unripe and ripe) of Chokanan ('CK') and Pool ('P') mango groups identified 9,765 and 13,651 differentially expressed genes (DEGs) (adjusted P value < 0.05; $\log_2$ fold change (FC) > 1 or $\log_2$ fold change (FC) < -1) respectively. On the other hand, comparison between mango groups at the same ripening stage identified 18,258 and 10,521 DEGs at the unripe and ripe stage respectively. Genes involved in the metabolism of energy, sugars, hormones and cell wall were differentially expressed. Notably, the *Rab* (Ras related proteins in brain) genes were also detected as differentially expressed 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 (Ras related proteins in brain) GTPases in fruit softening. The expression of ten genes evaluated by

both RNA-sequencing (RNA-seq) and reverse transcription quantitative polymerase chain reaction (RT-qPCR) showed a good correlation ($R^2 = 0.769$) indicating a good consistency between both techniques and the reliability of the RNA-sequencing data. Correlation analyses showed a significant relationship ($P < 0.05$) between the expression level of the *RabA3* and *RabA4* genes and fruit firmness at the unripe stage of 'Chokanan' (CK), 'Golden phoenix' (GP) and 'Water lily' (WL) suggesting that the differences in the *Rab* gene expression level may play an important role in the contrasting firmness of these varieties. The expression levels of these genes in other mango varieties were also analysed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) but no consistent regularity was found suggesting the contribution of other factors to bring about softening in the varieties.

In summary, these findings have provided insights into the molecular mechanisms underlying mango ripening and lay a foundation for the exploration of novel genes towards future development of strategies to improve fruit quality of mango and other non-model fleshy fruits.

LIST OF PUBLICATIONS

1. Published Papers

i. **Lawson, T**., Lycett, G.W., Ali, A., Chin, C.F., 2019. Characterization of Southeast Asia mangoes (*Mangifera indica* L.) according to their physicochemical attributes. Scientia Horticulturae 243, 189-196. doi:10.1016/j.scienta.2018.08.014

ii. **Lawson, T**., Mayes, S., Lycett, G.W., Chin, C.F., 2018. Plant Rabs and the role in fruit ripening. Biotechnology and Genetic Engineering Reviews 34, 181-197. doi:10.1080/02648725.2018.1482092

2. Conference Presentations

i. **Lawson, T**., Lycett, G.W., Mayes, S., Ali, A., Chin, C.F., 2018. A comparative transcriptomic approach toward understanding fruit softening in mango in: The International Conference and Workshop on Comparative Genomics and Interatomics for Agriculture (ICCGIA), 1-2 November 2018, Universiti Putra Malaysia.

ii. **Lawson, T**., Lycett, G.W., Mayes, S., Ali, A., Chin, C.F., 2018. The involvement of the Rab GTPases in mango ripening in: Nottingham-Adelaide Doctoral Training Partnership Symposium (NAPS), 10-12 October 2018, The University of Nottingham, Semenyih, Malaysia.

iii. **Lawson, T**., Lycett, G.W., Mayes, S., Ali, A., Chin, C.F., 2018. A Transcriptomic analysis on fruit ripening in mango (*Mangifera indica* var. 'Chokanan') in: LINK'18 Postgraduate Conference, 26 July 2018, The University of Nottingham, Semenyih, Malaysia.

iv. **Lawson, T**., Lycett, G.W., Mayes, S., Ali, A., Chin, C.F., 2018. The Rab GTPases to regulate rapid softening and spoilage of fruits in: Faculty of Science (FOS) Postgraduate Symposium Competition, 20 June 2018, The University of Nottingham, Semenyih, Malaysia.

v. **Lawson, T**., Lycett, G.W., Mayes, S., Ali, A., Chin, C.F., 2017. The use of Rab GTPases in mango ripening in: Crops for the future (CFF) Doctoral Training Partnership Symposium, 13-15 November, 2017, Semenyih, Malaysia.

vi. **Lawson, T**., Chin, C.F., Mayes, S., Ali, A., Lycett, G.W., 2015. The use of Rab GTPases to regulate rapid softening and spoilage in mango in: in: European Network for Plant Endomembrane Research (ENPER) 17-20 November, 2015. Leeds, United Kingdom.

vii. **Lawson, T**., Chin, C.F., Mayes, S., Ali, A., Lycett, G.W., 2015. The use of Rab GTPases to regulate rapid softening and spoilage in underutilized tropical fruits in: Plant Science Postgraduate Symposium, May 2015, The University of Nottingham, Sutton Bonington Campus, United Kingdom.

viii. **Lawson, T**., Chin, C.F., Mayes, S., Ali, A., Lycett, G.W., 2014. Amplification of the partial *Rab* gene fragments in Malaysia mango in: International Postgraduate Research Awards Seminar, 10–12 December, 2014, Kuala Lumpur, Malaysia.

ix. **Lawson, T**., Lycett, G.W., Mayes, S., Ali, A., Chin, C.F., 2014. The isolation and identification of the *Rab* gene fragments in Malaysian mango in: Crops for the future (CFF) Doctoral Training Partnership Symposium, 18-21 November, 2014. The University of Malaya, Kuala Lumpur, Malaysia.

x. **Lawson, T**., Chin, C.F., Mayes, S., Ali, A., Lycett, G.W., 2014. Identification and isolation of the *Rab* genes in Malaysia mango in: 21st MSMBB Annual Scientific Meeting on Biotecnology for global sustainability and prosperity, 1-3 October 2014, Monash University, Subang Jaya, Selangor, Malaysia.

DEDICATION

To God Almighty, with Whom nothing is impossible

&

To the memory of my father, the late Engr. Benoni Lawson

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My utmost gratitude to God Almighty, the Great Ordain Divine, my dearest Father for making it possible for me to undertake and complete this journey of life with perseverance. Thank you, Lord, for your endless grace, strength, peace and blessings showered on me through it all.

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LIST OF ABBREVIATIONS

°C	degrees Celsius
ABA	Abscisic acid
ACO	1-Aminocyclopropane-1-carboxylic acid oxidase
ACS	1-Aminocyclopropane-1-carboxylic acid synthase
ACT	Actin
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
bp	base pair
BP	Biological process
CC	Cellular component
cDNA	complementary DNA
CIA	Chloroform isoamyl alcohol
CO_2	Carbon dioxide
Cq	Cycle of quantification
CTAB	Cetyltrimethyl ammonium bromide
DEG	Differentially expressed genes
DNA	Deoxyribonucleic acid
DOA	Department of Agriculture, Malaysia

EDTA	Ethylenediaminetetraacetic acid
EE	Early endosome
ER	Endoplasmic reticulum
FAMA	Federal Agricultural Marketing Authority
FPKM	Fragments Per Kilo base of gene per Million mapped reads
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GAP	GTPase activating protein
GDI	Guanosine nucleotide dissociation inhibitor
GDF	GDI-displacement factor
GDP	Guanine diphosphate
GEF	Guanine nucleotide exchange factor
GO	Gene ontology
GTP	Guanine triphosphate
h°	Hue angle
KEGG	Kyoto Encyclopaedia of Genes and Genomes
KOG	Eukaryotic Orthologous Groups
L*	Lightness
LE	Late endosome
MF	Molecular function
MOA	Ministry of Agriculture and Agro based Industry, Malaysia

mRNA	messenger RNA
MT	Million tonnes
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
ng	nanogram
NRT	No reverse transcriptase
P	Pool
PCR	Polymerase chain reaction
PE	Pectinesterase
PG	Polygalacturonase
PM	Plasma membrane
PPI	Protein-protein interaction
PVP	Polyvinylpyrrolidone
R	Ripe
RAB	Ras-related proteins in brain
REP	Rab escort protein
RIN	RNA integrity number
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
ROS	Reactive oxygen species

RT-PCR	Reverse transcription PCR
RT-qPCR	Reverse transcription quantitative PCR
rRNA	ribosomal RNA
SEM	Standard error of the mean
SSC	Soluble solid concentration
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TA	Titratable acidity
TAIR	The *Arabidopsis* Information Resource
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TGN	*trans*-Golgi network
Tris-Cl	Tris-chloride
UBI	Ubiquitin
vs	Versus
w/v	Weight per volume
µg	Microgram
µl	Microliter
µM	Micromolar
UR	Unripe

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1 INTRODUCTION

1.1 BACKGROUND

Mango (*Mangifera indica* L.) is one of the most important tropical fruit with significant commercial value (Yahia, 2011). The consumption of mango is well appreciated worldwide due to its delicious taste, exotic flavour and nutritional value (Singh *et al*., 2013). Mango fruit is a rich source of health promoting compounds such as carotenoids, ascorbic acid and phenolic compounds (Lauricella *et al*., 2017). Currently, Asia is the largest mango-producing region with a production of approximately 30 million tonnes which accounts for 75 % of the global mango production in 2016 (FAOSTAT, 2017). Of the available varieties, most are grown for local consumption while a few are traded internationally (Kuhn *et al*., 2017). Mango production plays an important role in the economy of several tropical countries including Malaysia (DOA, 2016). However, several factors such as the fruit storage life, postharvest disease and lack of adequate postharvest infrastructures adversely affect the fruit supply chain (Yahia, 2011). This leads to postharvest losses from farm gate to the consumer and in Malaysia, it is estimated to be around 20 % (Mohammed, 2017).

Fruit ripening involves a spectrum of significant physiological, biochemical and molecular changes that give rise to an edible fruit of desired quality (Osorio and Fernie, 2013). An increased rate of respiration and a high level of ethylene production during ripening have been reaffirmed in climacteric fruit (Alexander and Grierson, 2002; Ong *et al*., 2014). This applies in the mango ripening process (Khaliq *et al*., 2015; Palafox-Carlos *et al*., 2012; Reddy and Srivastava, 1999; Zerbini *et al*., 2015). Most mango varieties ripen within 4-9 days (Singh *et al*., 2013) although there have been reports on ‘Alphonso’ and ‘Banganapalli’ mangoes with a ripening duration of 12-18 days from harvest (Deshpande *et al*., 2017; Nambi *et al*., 2015).

Rapid softening is the main factor limiting fruit's postharvest storage life (Brummell and Harper, 2001; Goulao and Oliveira, 2008; Wang *et al*., 2018a). As such, reducing fruit softening has been the subject of research groups for decades using molecular biology strategies (Hamilton *et al*., 1990; Sheehy *et al*., 1988; Smith *et al*., 1988). Cell wall degradation has been considered to be the main contributor to this aspect of ripening in fruits (Wang *et al*., 2018a). In tomato fruit, gene silencing has been used to inhibit the synthesis of polygalacturonase (PG; EC 3.2.1.15). The polygalacturonase (PG) levels were substantially reduced to as low as 1 % thereby inhibiting pectin breakdown (Sheehy *et al*., 1988; Smith *et al*., 1988). However, this inhibition has been found to have only a relatively small effect upon fruit firmness (Sheehy *et al*., 1988; Smith *et al*., 1988). Even though PG activity was not the sole determinant of fruit softening, it has been shown to lead to an extended shelf life (Giovannoni *et al*., 1989). This unique characteristic made the PG antisense plants sufficiently different and a commercial success. Similar studies have been carried out to elucidate the roles of other single enzymes including pectinesterase (PE; EC 3.1.1.11) (Phan *et al*., 2007) and endoglucanase (EC 3.2.1.4) (Brummell *et al*., 1999a) as well as non-enzymatic proteins such as expansin (Brummell *et al*., 1999b; Zhang *et al*., 2012). However, these resulted in little or no softening. An effect that is more significant has been achieved through the inhibition of pectate lyase (PL; EC 4.2.2.2) (Uluisik *et al*., 2016) and beta-galactosidase (EC 3.2.1.23) (Paniagua *et al*., 2016) gene expression. A study of tomato plants in which both polygalacturonase (PG) and expansin were inhibited showed a synergistic effect on fruit softening (Powell *et al*., 2003). Taken together, the experimental evidence provided by these studies has revealed that cell wall modification is a complex process involving several enzymes working in concert. In this regard, the alteration of softening may be dependent on the simultaneous reduction of several enzymes (Lycett, 2008). Hamilton *et al*., (1990) support this postulation from their observation that inhibition of ethylene biosynthesis altered most aspects

of ripening and had a significant effect on softening. This has spurred efforts to study the trafficking route which as suggested by Lu *et al*., (2001) is a promising strategy that could prevent over-softening as the synergistic actions of multiple enzymes would have been simultaneously reduced.

The plant endomembrane system consisting of the Golgi apparatus, endoplasmic reticulum, endosome, *trans*-Golgi network (TGN) and plasma membrane (PM) work together to synthesize, modify and ship proteins and other cellular materials. An appropriate delivery to the correct destination is maintained within the cell despite the influx of a vast array of gene protein products into the endomembrane system (Müntz, 1998; Zerial and McBride, 2001). The Rab (Ras related proteins in brain) GTPases have been found to be primary determinants of the steps of directing traffic within the endomembrane system (Fujimoto and Ueda, 2012; Hutagalung and Novick, 2011; Stenmark, 2009). The Rab (Ras related proteins in brain) GTPases oscillate between the 'active' guanine triphosphate (GTP) form and the 'inactive' guanine diphosphate (GDP) forms in the membrane and cytosol respectively (Stenmark, 2009; Zerial and McBride, 2001). This conformational change accounts for their roles as 'molecular switches' and the ability to perform several tasks in a coordinated manner (Stenmark, 2009). The Rab (Ras related proteins in brain) GTPase family which constitutes the largest group of the Ras (Rat sarcoma) (Cox and Der, 2010) superfamily has been found to exist in all eukaryotes studied (Stenmark, 2009). Thus far, members of the Rab (Ras related proteins in brain) GTPase family have been discovered in various plant species, including *Arabidopsis thaliana*, tomato (*Solanum lycopersicum*) and peach (*Prunus persica*) (Falchi *et al*., 2010; Lycett, 2008; Rutherford and Moore, 2002).

Fruit is one of several plant systems where the key role of vesicle trafficking in cell-wall related events has been well characterized (Lycett, 2008). The preferential expression of the *Rab* genes during fruit ripening has been reported (Abbal *et al*., 2008; Falchi *et al*., 2010; Liu *et al*., 2014;

Loraine *et al*., 1996; Lu *et al*., 2001; Zainal *et al*., 1996). Thus implicating the role of trafficking in fruit ripening. The *RabA* subclade provides a good illustration of how reduced expression could be used to modulate cell wall events in tomato (Lu *et al*., 2001). This study led to the postulation that difference in *RabA* gene expression level might be associated with fruit softening variants. Tomato as a model system has been useful in understanding and providing a research direction in fruit ripening (Alexander and Grierson, 2002; Brummell and Harpster, 2001; Seymour *et al*., 2013). It must be noted that although evidence is accumulating that gene family members involved in softening are conserved in fleshy fruits (Seymour *et al*., 2013), there is a need to confirm the results from tomato in other fruits (Goulao and Oliveira, 2008). This is because fruits species may have evolved diverse ways of cell wall alterations to bring about softening (Brummell, 2006; Goulao and Oliveira, 2008).

Different ripening and softening behaviours exist among mango varieties (Jha *et al*., 2013; Lawson *et al*., 2019; Mitcham and McDonald, 1992). Presumably, the different softening behaviour may be due to quantitative (gene expression level) changes as observed in apple (*Malus domestica*) (Wakasa *et al*., 2006), papaya (*Carica papaya*) (Chen and Paull, 2003), peach (Qian *et al*., 2016; Yang *et al*., 2016) and strawberry (*Fragaria ananassa*) (Salentijn *et al*., 2003). As such, taking advantage of the natural variation in a non-model crop such as mango constitutes a resource for dissecting the role of Rab GTPases in mango fruit softening. To date, no attempt has been made to linking the RabA GTPases and differential softening in fruit varieties, which makes this study, the first of its kind.

At the beginning of this study, unlike the situation prevailing in model plants such as *Arabidopsis* and tomato where members of the *Rab* gene family had been identified so far, there were only two published Rab sequences for mango (Zainal e*t al*., 1996; Liu *et al*., 2014). A wider characterization of these genes will not only lead to an integrated picture of their potential functionality during ripening but also provide new information and tools to support

crop improvement strategies involving the RabA GTPases. It is also hugely important to provide information on local mango varieties for which no study has been undertaken.

1.2 HYPOTHESIS

Antisense technology revealed that the *Rab* genes influence the ripening-associated fruit softening by controlling the secretion of cell wall components and hydrolytic enzymes. Notably, the inhibition of a *RabA* gene caused a reduction in softening and improved the fruit's shelf life. Therefore, it was hypothesised that the differential softening rate of mango varieties might be associated with the differences in the expression levels of the *RabA* genes.

1.3 AIM AND OBJECTIVES

This project aimed at investigating the involvement of the Rab GTPase family in the ripening and softening process of mango. The benefits of elucidating the mechanism of fruit ripening in this economically important fruit crop would be beneficial for both farmers and consumers since better post-harvest strategies for increasing and maintaining quality could be adopted. In addition, the knowledge gained from this less well-explored aspect of mango ripening will be an important contribution to the scientific community interested in fruit ripening research in *Mangifera* species and other tropical fruit crops.

The study was initiated with the following specific research objectives:

- Physiological and physicochemical characterization of the mango ripening process during postharvest storage. Among some of the ripening associated processes examined in this thesis were pulp firmness, soluble sugar concentration and ethylene production.
- Identification and characterization of the Rab GTPase family in mango fruit by comparative analysis.
- A comparative RNA-seq analysis of mango varieties with statistically different pulp firmness at two ripening stages (unripe and ripe).

- Using correlation analysis to examine the association between the RabA GTPases and the differential softening rate of mango varieties during postharvest storage.

The research methodology is outlined in Figure 1.1.

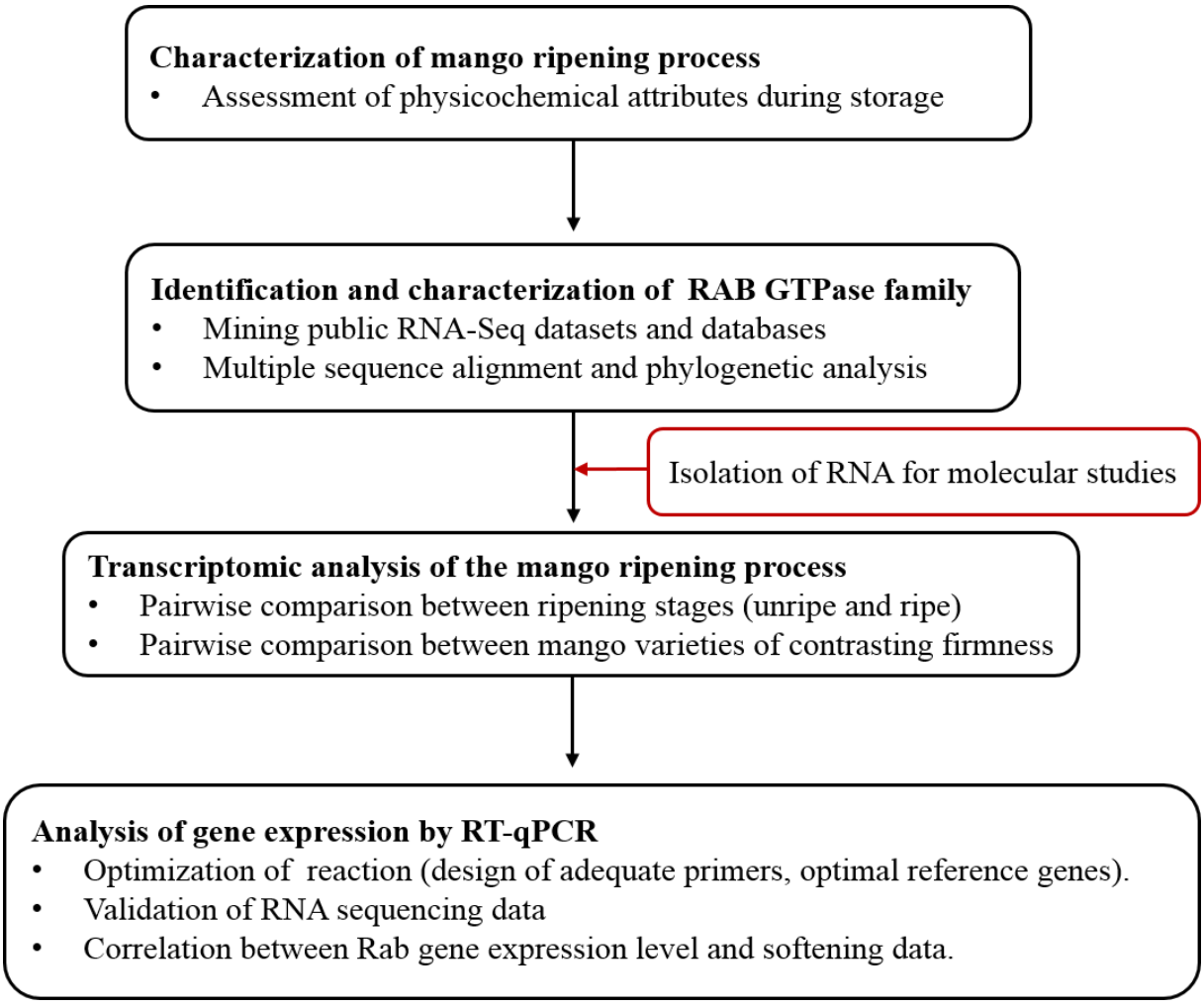


Figure 1.1: A flow chart of research methodology

1.4 THESIS OUTLINE

This dissertation includes the following chapters:

Introduction: An introduction to the mango ripening process and the importance of investigating mango fruit softening has been presented in this section.

Review of Literature: This section presents a review of literature on the physiology and molecular biology of fruit ripening. The Rab GTPases and their roles in ripening has been reviewed.

Material and Methods: This section provides details of plant materials used, protocols and techniques employed during this study.

Results: This section includes data obtained on the physiological and physicochemical changes in mango during ripening, identification and characterization of the Rab GTPase family, isolation of high quality RNA, transcriptomic analysis of mango during ripening and RT-qPCR gene assessment and correlation analysis between gene expression level and mango pulp firmness.

Discussion: This section contains inferences drawn from the results generated during the present study.

Conclusion and Future Perspectives: This section describes the conclusions derived from this project and further studies which could be carried out based on the results obtained in this study.

2 LITERATURE REVIEW

2.1 MANGO

2.1.1 Botanical description

Mango (*Mangifera sp*) of the family Anacardiaceae is one of the most economically important fruits around the globe (Mukherjee and Litz, 2009). Mango trees (Figure 2.1) are evergreen ranging from medium to large (10-40m) in height with foliage providing a good shade covering. The plant is supported by a long taproot and a dense mass of surface feeder root (Bally, 2006; Mukherjee and Litz, 2009). Young leaves appear brown and then gradually change to dark green as the leaves mature (Figure 2.1). Mango bears both hermaphrodite and male flowers on the same inflorescence (Ding and Darduri, 2013). Although flowers may be self-fertile, cross-pollination is necessary for high fruit set (Ding and Darduri, 2013; Huda *et al*., 2015). Mango fruit is a large, fleshy drupe, made up of the pericarp, mesocarp and an endocarp (Figure 2.1). It varies greatly in shape, size, colour and flavour; this can be a distinguishing feature among species and varieties (Yahia, 2011). Fruit peel colour changes from green to olive green, yellow, orange yellow sometimes reddish. These variations are dependent on the variety and ripening stage (Yahia, 2011). Depending on varieties and growing conditions, it takes three to six months from flowering to produce a harvest-ready mango fruit (Kader, 2003). In Malaysia, the early harvest season for mango varieties occurs in March through June and the late season in October. There are two groups of mango fruit based on the region and type of seed produced (Kusumo *et al*., 1984; Mukherjee and Litz, 2009). The Indochinese groups are polyembryonic (more than one seedling per seed) while the Indian groups are monoembryonic (one seedling per seed).

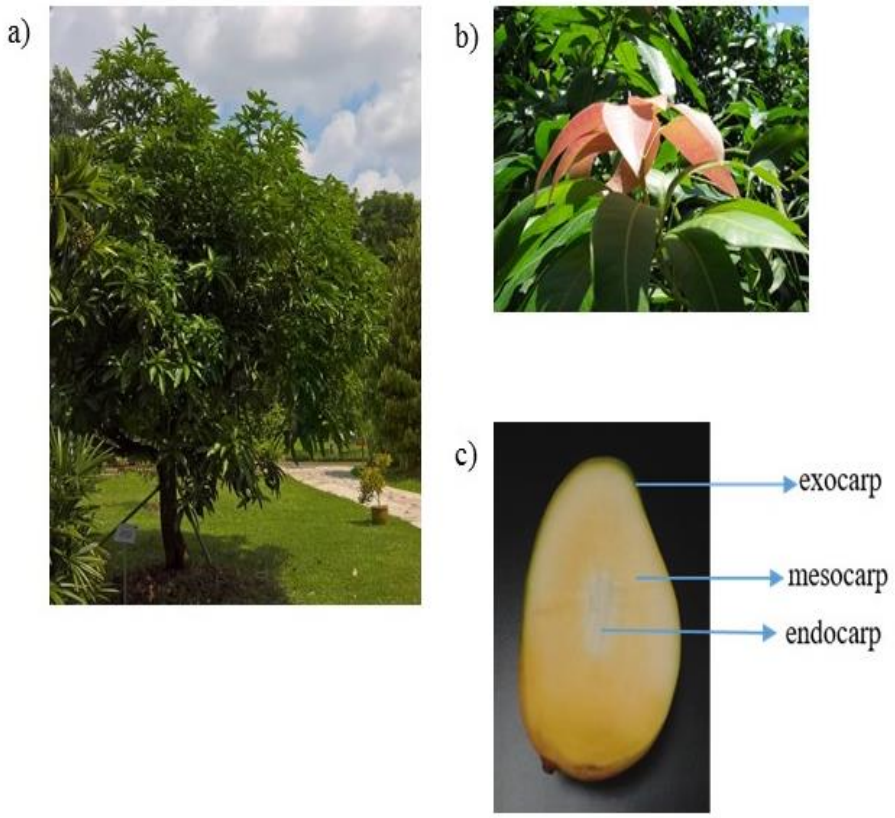


Figure 2.1: Mango tree (a) leaves (b) fruit (c)

(Original)

2.1.2 Distribution and diversity

Of the seventy two *Mangifera* species reported (Singh *et al*., 2016) at least thirty are indigenous to Malaysia (Salma *et al*., 2010). *Mangifera* species known to bear edible fruits include *Mangifera indica, Mangifera caesia, Mangifera foetida, Mangifera odoranta* and *Mangifera pajang* (Bally, 2006; Mukherjee and Litz, 2009; Ueda *et al*., 2016) (Figure 2.2). *Mangifera indica* is the most widely cultivated fruit crop tree for mango production (Singh *et al*., 2016). With the exception of *Mangifera indica*, these *Mangifera* species are often referred to as wild mangoes (Derese *et al*., 2017). These wild mangoes are found either in the forest or home gardens and sold in local markets (Ueda *et al*., 2016). *Mangifera pajang* locally known as 'Bambangan' is native to Malaysia states of Sabah and Sarawak, Brunei and Indonesia (Abu

Bakar and Fry, 2013). The fruit is ovoid with light brown peel and about three times larger than common mango (Abu Bakar and Fry, 2013). *Mangifera foetida* (‘Bachang’ or horse mango) is an oval, green coloured drupe with tiny dark spots. (Wong and Ong, 1993). *Mangifera odorata* (‘Kuini’ or fragrant mango) fruit remains green when fully ripe and it is known for its unique fragrance (Wong and Ong, 1993). *Mangifera caesia* (‘Binjai’ or white mango) is endemic to Borneo and peninsular Malaysia (Wong and Siew, 1994). This fruit possesses a thin yellowish pale-brown skin with an almost white flesh when fully ripe (Wong and Siew, 1994).

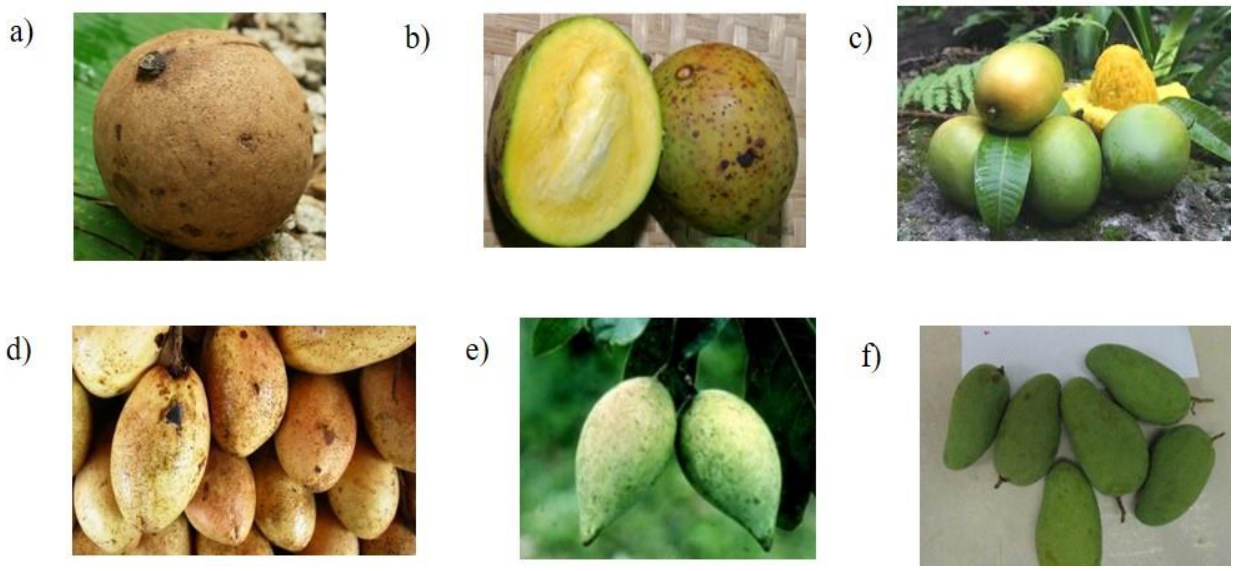


Figure 2.2: Fruit diversity within the *Mangifera* species

Note: (a) *M. pajang* (‘Bambagan’) (b) *M. foetida* (‘Bachang’); (c) *M. odoranta* (‘Kuini’); (d) *M. caesia* (‘Binjai’) (e) *M. sylvatica*; (f) *M. indica* (common mango; ‘Chokanan’ variety) Photo credit: wild mangoes (Parma, 2013) (with permission); common mango (original).

2.1.3 Production and trade

Mango is grown in approximately 94 countries (Singh *et al*., 2013). World production of mango has increased from 34 million tonnes (MT) in 2006 to 46.5 million tonnes (MT) in 2016 (FAOSTAT, 2017). Asia is the largest mango-producing region with an average production of 30MT (2006-2016) which accounts for 75 % of global mango production (FAOSTAT, 2017). This is followed by America (13 %; 5.20 MT), Africa (11 %; 4.70 MT) and Oceania (0.1 %; 0.05 MT) (FAOSTAT, 2017). The major mango-producing countries are India, China, Thailand, Indonesia, Mexico, Pakistan, Brazil, Bangladesh, Philippines and Nigeria (Figure 2.3). Hundreds of mango (*M. indica*) varieties exist worldwide of which Asia has over 500 fully characterized varieties (Singh *et al*., 2016). The vast majority of the mangoes produced are consumed locally with only a few mango varieties traded internationally (Kuhn *et al*., 2017). Commercial varieties that dominate the global export market include 'Tommy Atkins', 'Haden', 'Ataulfo', 'Kent', 'Keitt' and 'Alphonso' (Bally, 2011; Galán Saúco, 2015). Total mango production in Malaysia has increased over the years from 26,247 tonnes in 2006 to 102,046 tonnes in 2016 (Figure 2.4, FAOSTAT, 2017). The major mango producing states in Malaysia are Perak, Perlis, Kedah, Melaka and Kelantan (MOA, 2016). Mango varieties recommended for commercial planting include 'Chokanan', 'Harumanis', 'Masmuda' and 'Sala' (MOA, 2016). Mango production contributes to the rural economy of many developing countries (Bally, 2011). However, despite the buoyant growth of mango production within these regions, the high requirement concerning colour and storage affects its marketability and distribution in the global trade (Yahia, 2011; Bally, 2011).

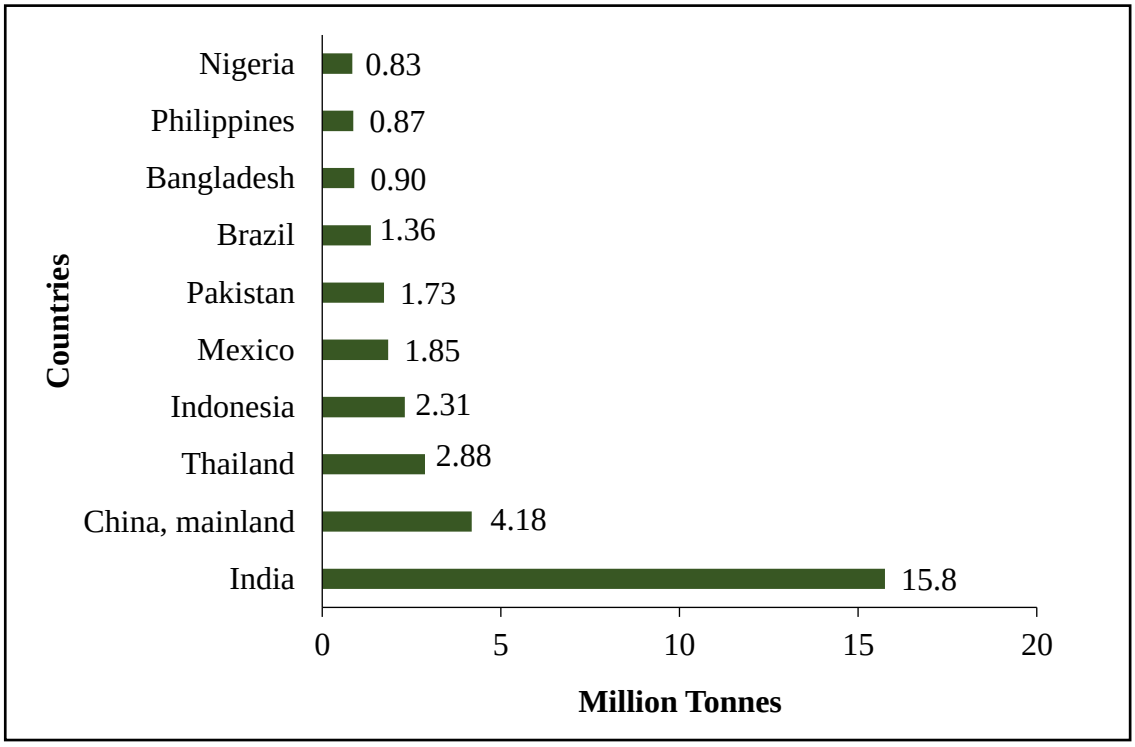


Figure 2.3: Top ten mango-producing countries in the world (FAOSTAT, 2017)

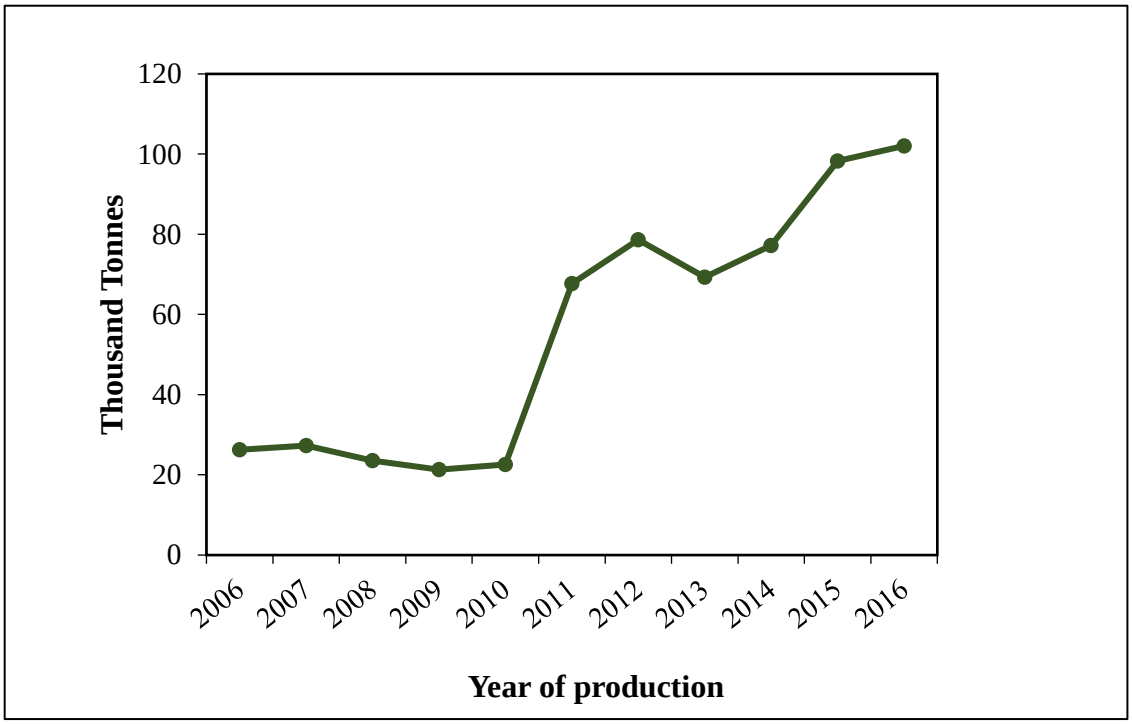


Figure 2.4: Production quantities in Malaysia (2006 - 2016) (FAOSTAT, 2017)

2.1.4 Economic importance

Mango fruit is utilised both for its food and non-food purposes. The fruits are consumed either fresh or as processed products including juices, jellies, chutney, pickles, jam etc. Mango fruit is a rich source of carbohydrates, amino acids, dietary fibres and vitamins. Mango fruit provide a range of health promoting compounds such as carotenoids, ascorbic acid and polyphenols (Lauricella *et al*., 2017). Studies have revealed that extracts from leaves, seeds, kernels, roots, fruits and stem barks have therapeutic properties (Abu Bakar and Fry, 2013; Ajila and Rao, 2008; Jahurul *et al*., 2015). These health benefits emphasise the need and importance of mango fruits in our daily diet.

2.2 FRUIT RIPENING

Ripening involves a series of physicochemical and physiological changes in the quality attributes of the fruits ultimately enhancing the fruit's edibility (Tucker *et al*., 2017). While ripening improves the eating quality of fruit, the postharvest life is reduced, thus it can be said that ripening-related changes are both beneficial and detrimental (Goulao and Oliveira, 2008; Seymour *et al*., 2013). Fruits are classified into two groups based on their ripening behaviour. Climacteric fruits that include bananas, mangoes, tomatoes etc. are associated with a rise in respiration and an outburst of ethylene production (Alexander and Grierson, 2002; Liu *et al*., 2015). On the other hand, non-climacteric fruits such as grapes and strawberries do not exhibit remarkable increase in respiration and ethylene production (Liu *et al*., 2015). The ripening process enhances the perishability of fresh produce after harvest (Mishra and Gamage, 2007; Tucker *et al*., 2017). While ripening enhances the physicochemical and nutritional quality of fruit, over ripening can lead to decreased fruit quality and postharvest losses. In this regard, fruit ripening has implications for human diet and agriculture with respect to food and

nutritional security (Giovannoni *et al*., 2017; Giovannoni and El-Rakshy, 2005; Seymour *et al*., 2013).

2.2.1 Mango ripening physiology and quality changes

Mango, as other climacteric fruit, is generally harvested at the mature-green stage (FAMA, 2014) and the ripening process completed in the postharvest phase (Yahia, 2011). Mango ripening process occurs within 4-9 days (Dautt-Castro *et al*., 2015; Srivastava *et al*., 2016; Yahia, 2011) although 'Alphonso' and 'Banganapalli' mangoes have been reported to ripen between 12-18 days from harvest (Desphande *et al*., 2017; Nambi *et al*., 2015). At cold storage (13° C), mango can be stored for 2-3 weeks (Carrillo-Lopez *et al*., 2000). Respiration, ethylene production, pulp firmness, acidity and soluble solid concentration are major changes that occur during mango ripening (Yahia, 2011). These parameters constitute a resource to explore either alone or in combination as the principal index for ripening and should be taken into consideration in studies aimed at improving mango fruit quality (Yahia, 2011).

2.2.1.1 Respiration and ethylene production

Respiration and ethylene production are two important metabolic processes that occur during ripening (Saquet and Streif, 2017). Respiration (i.e., oxygen consumption and carbon dioxide release) is a continuing process that occurs throughout the life of a fruit after harvest. Climacteric and non-climacteric fruit are distinguished according to their respiration rate and pattern (Tucker *et al.,* 2017). The start of ripening in a climacteric fruit such as mango is accompanied by a dramatic increase in respiration rate, also called the respiratory climacteric (Gapper *et al*., 2013). In contrast, non-climacteric fruit displays a gradual decline in their respiration during ripening (Tucker *et al*., 2017). An increase in respiration has been observed to be associated with an increase in soluble solid concentration (SSC) and a decrease in pulp firmness in several fruits (Asiche *et al*., 2017; Ong *et al*., 2011) including mango (Lawson *et*

al., 2019; Padda *et al*., 2011). The rate of respiration during ripening in mangoes is influenced by the variety (Lawson *et al*., 2019; Nordey *et al*., 2016), maturity stages at harvest (Lalel *et al*., 2003), storage condition (Patel *et al*., 2016; Noiwan *et al*., 2017) and postharvest treatment (Khaliq *et al*., 2015).

Ethylene plays a key role in regulating ripening by coordinating several aspects of the ripening process including cell wall modulation and colour development (Tucker *et al*., 2017). Exogenous application of ethylene has been found to trigger the ripening process in several mango varieties with increased levels of ethylene production (Schouten *et al*., 2018; Razzaq *et al*., 2015). On the other hand, the application of ethylene inhibitors such as 1-methylcyclopropene (1-MCP) or nitric oxide (NO) have impaired its production and affected the course of ripening in mango (Razzaq *et al*., 2015; Sakimin Siti and Singh, 2011). The ethylene induced softening is associated with the storage temperature, as low temperature scenarios show decreased softening (Schouten *et al*., 2018). The outburst of ethylene during ripening has been reported to either precede or coincide or lag behind the respiratory climacteric (Burg and Burg, 1962; Cua and Lizada, 1990; Lalel *et al*., 2003; Nordey *et al*., 2016; Palafox-Carlos *et al*., 2012; Reddy and Srivastava, 1999).These differences might be due to several factors including varietal differences in gas composition, maturity stages and postharvest storage conditions (Brecht and Yahia, 2009; Nordey *et al*., 2016). Additionally, the high ethylene production observed during mango fruit development has been attributed to the active growth of the fruit (Wongmetha *et al*., 2015).

The biochemical pathway for ethylene synthesis involves a series of reactions where *S*-adenosyl methionine is converted by ACC synthase (ACS) into 1-amino cyclopropane-1-carboxylic acid synthase (ACS), which in turn is converted to ethylene by ACC oxidase (ACO) (Grierson, 2013). Downstream of ethylene is a signalling pathway that involves several

components, including ethylene receptors (ETRs) that modulate different ripening responses in fruit (Liu *et al.*, 2015). Ethylene-related genes have been identified in a wide array of fruit crops including mango (Dautt-Castro *et al*., 2015; Srivastava *et al*., 2016), papaya (*Carica papaya*) (Shen *et al*., 2017) and banana (*Musa acuminata*) (Xiao *et al*., 2013).

2.2.1.2 Peel colour

Peel colour is an important visual attribute of mango (González-Aguilar *et al*., 2001; Karanjalker *et al*., 2018) which can be used for determining the appropriate harvesting stage (Cocozza *e*t *al*., 2004; Jha *et al*., 2007; Nambi *et al*., 2015) and consumption (Cocozza *et al*., 2004; Jha *et al*., 2007). When mango ripens, the peel can remain green or changes to greenish yellow, orange-yellow or yellow depending on the variety (Ketsa *et al*., 1999; Yahia, 2011). Colour change is due to the degradation of chlorophyll with a simultaneous pigment accumulation (Klee and Giovannoni, 2011). Carotenoids are the predominant pigments in yellow-coloured varieties whereas red-coloured mango varieties has been attributed to anthocyanins (Berardini *et al*., 2005; Karanjalker *et al*., 2018; Ma *et al*., 2018). The accumulation of carotenoids occurs during ripening as observed in 'Tainong 1' and 'Hongyu' mangoes (Ma *et al*., 2018). In the aforementioned varieties, β-carotene was found to be the most abundant carotenoid in the fruit pulp. Peel colour alone is not a standalone ripening index since the fruit colour change occurs when the fruit is already soft in some varieties (Vásquez-Caicedo *et al*., 2002; Lawson *et al*., 2019). Lawson *et al*., (2019) reported that 'Chokanan' developed more yellow colouration upon ripening compared to 'Golden phoenix' and 'Water lily' mangoes. In addition to varietal effect, temperature and postharvest treatment also have an impact on colour development (Silva *et al*., 2017; Khaliq *et al*., 2016).

2.2.1.3 Soluble sugars and organic acids

Carbohydrates are well known as the major components which play an important role in fruit physiology. Starch is the main carbohydrate present in mango fruits (Yashoda *et al*., 2006) and its accumulation during fruit development has been reported (Nakkanong *et al.,* 2012). Starch hydrolysis by amylase contributes to an increase in soluble sugars as ripening progresses (Silva *et al*., 2017). The conversion of complex carbohydrates to simple sugars has been reported in various mango varieties including 'Irwin' and 'Jinhwang' mangoes (Wongmetha *et al*., 2015; 2012). The starch content of mango pulp was found to decrease significantly ($P < 0.01$) from 12% to 4.3% in 'Ashwina' mango variety (Hossain *et al*., 2014).The development of sweetness in ripe fruit is due to the accumulation of soluble sugars as the result of the degradation of polysaccharides. The total soluble solids (TSS) increase with the ripening process (Lawson *et al*., 2019; Padda *et al*., 2011; Yahia, 2011) and are higher with increase in storage temperature, irrespective of sample/variety of the fruit (Baloch and Bibi, 2012). Ripe mango fruit contains 10-20 % sugars depending on the variety and ripeness stage (Yahia. 2011). For instance, SSC increased from 9 % to 17 % in 'Keitt' (Padda *et al*., 2011), 7 % to 21 % in 'Ataulfo' (Palafox-Carlos *et al*., 2012) and 11 % to 26 % in 'Chaunsa' (Rajwana *et al*., 2010) mangoes as ripening progressed. Sucrose synthesis is another mechanism for the increase in total sugars during fruit ripening (Wongmetha *et al*., 2015). Several enzymes are involved in sucrose metabolism in fruit growth and development. Sucrose phosphatase synthase (SPS) plays akey role in sucrose biosynthesis (Maloney *et al*., 2015). Sucrose synthase (SS; EC 2.4.1.13) is a sucrose-cleaving enzyme that provides UDP-glucose for the cellulose synthase complex (Moscatello *et al*., 2011). On the other hand, fruit invertases are involved in the degradation of sucrose which catalyse the irreversible hydrolysis of sucrose to glucose and fructose (Yahia, 2011). The study of the enzyme activities in sucrose metabolism of 'Jinhwang' mango variety during fruit growth and development (Wongmetha *et al*., 2015). These authors reported that starch

accumulation was associated with a decrease in sucrose phosphate synthase (SPS) and fruit invertase activities.

Organic acids are used as substrates in fruit respiration during ripening leading to a reduction in the organic acid content (Etienne *et al*., 2013). Citric and malic acid are the two most abundant organic acids in mango fruits including 'Palmer', 'Irwin', 'Jinhwang' and 'Tainong No1' mango varieties (Liu *et al*., 2013). Other organic acids present in lower concentrations in mango include tartaric, oxalic and succinic acids (Yahia, 2011). The reduction of fruit acidity in mango is accompanied by a large decrease in citric acid during postharvest ripening (Silva *et al*., 2017).In different studies it have been reported that acidity diminish through the ripening stages in 'Palmer' (Silva *et al*., 2017), 'Keitt*'* (Ibarra-Garza *et al*., 2015), 'Alphonso*'* (*Palafox*-Carlos *et al*., 2012) and 'Chokanan' (Lawson *et al*., 2019). Factors influencing the sugar and acid concentration in mango include variety (Nambi *et al*., 2015; Nassur *et al*., 2015; Vásquez-Caicedo *et al*., 2002), stage of ripening (Nambi *et al*., 2015), postharvest treatments (Gupta and Jain, 2014; Silva *et al*., 2017) and storage conditions (Medlicott *et al*., 1990). The changes in the concentration of soluble sugars and organic acids have an impact on the sensory quality of mango fruit (Malundo *et al*., 2001; Nassur *et al*., 2015). These two classes of metabolites are associated with the biosynthetic route of diverse compounds such as amino acids, vitamins, and terpenoids, which impact fruit aroma (Beauvoit *et al*., 2018).

2.2.1.4 Softening

Fruit softening is an important aspect of fruit ripening that has an impact on the storability, transportability and consumer acceptability (Tucker *et al*., 2017; Wang *et al*., 2018a). Fruit softening occurs as a result of the production of new cell wall polymers and enzymes (Lycett, 2008) as well as the modification in the structure and composition of the cell wall components (Tucker *et al*., 2017; Wang *et al*., 2018a). Mango fruit ripening involves changes in the

structure of the cell wall, which are mainly composed by pectin, cellulose and hemicelluloses, as well as loss of starch, which result in loss of firmness (Cárdenas-Coronel *et al*., 2012). Cell wall hydrolases implicated in mango fruit softening include endoglucanase (EC 3.2.1.4), beta-galactosidase (EC 3.2.1.23), polygalacturonase (PG; EC 3.2.1.15), pectinesterase (PE; 3.1.1.11), α-glucosidase (EC 3.2.1.20), α-mannosidase (EC 3.2.1.24), β-hexosaminidase (EC 3.2.1.52) amylase (EC 3.2.1.1) and pectate lyase (PL; EC 4.2.2.2) (Abu-Sarra and Abu-Goukh, 1992; Ali *et al*., 2004; Chourasia *et al*., 2008; Hossain *et al*., 2014; Mitcham and McDonald, 1992; Oaks *et al*., 2019; Prasanna *et al*., 2005; Prasanna *et al*., 2006; Rahman *et al*., 2011). Most cell wall modifying enzymes encoded by multigene families have specific functions in cell wall metabolism (Wang *et al*., 2018a) and have been isolated from various plants including mango (Dautt-Castro *et al*., 2015; Deshpande *et al*., 2017; Srivastava *et al*., 2016), papaya (Fabi et al., 2012), apple (Yang *et al*., 2018) and banana (Asif *et al*., 2014). Their involvement in mango ripening is further discussed below;

a. Endoglucanase

Endoglucanases are involved with the degradation of polymeric hemicellulose and cellulose in the cell wall (Yennamalli *et al*., 2013; Tucker *et al*., 2017). Previous studies have indicated a decrease in cellulose content and an increase in EGase activity in some mango varieties during ripening (Lazan *et al*., 2004). In the transcriptome of 'Dashehari' (Srivastava *et al*., 2016) and 'Kent' (Dautt-Castro *et al*., 2015) mango varieties, 11 and 2 genes encoding endoglucanases were identified respectively, and these were found to be expressed at higher levels in ripe samples. Additionally, Chourasia *et al.*, (2008) isolated an EGase gene from a ripe mango sample. Its expression correlated with an increase in EGase activity and degradation of cellulose.

b. Beta-galactosidase

Beta-galactosidase enzymes have been implicated in fruit softening by increasing the porosity of the cell wall and enhancing the access of other cell wall degrading enzymes (Ng *et al*., 2015; Tucker *et al*., 2017). Beta-galactosidase enzyme has been purified from several mango varieties including 'Harumanis' (Ali *et al.*, 1995), 'Ashwina' (Hossain *et al*., 2014) and 'Fazli' (Rahman *et al.*, 2010) and these have been reported to exhibit an increased activity upon ripening. Furthermore, some authors (Ali *et al*. 2004; Prasanna *et al.*, 2005) have observed a concordance between enzyme activity and a reduction in fruit. In 'Alphonso' mango, three isoforms of beta-galactosidase were purified and these were involved in pectin dissolution (Prasanna *et al.* 2005). However, Kermani *et al*., (2015) mentioned that although a high activity of beta-galactosidase was observed, it had no effect on the viscosity of the mango puree.

c. Polygalacturonase (PG)

PG is an important pectin degrading enzyme involved with pectin depolymerisation during fruit ripening (Tucker *et al*., 2017). The increase in the activity of *exo*-PG was more pronounced than the activity of *endo*-PG in 'Nam Dok Mai' mango during ripening (Chaimanee *et al*., 2000). Although studies have reported a significant correlation between firmness loss and PG activity in mango (Ali *et al*. 2004), there are some contradictory reports which suggested weak correlation (Abu-Sarra & Abu-Goukh 1992). Chaurasia *et al.*, (2010) reported that 'Dasheri' mango had very little PG enzyme activity and suggested that other pectin degrading enzymes might be playing more important roles in mango ripening. In 'Kent' (Dautt-Castro *et al*., 2015) and 'Dasheri' (Srivastava *et al*., 2016) mango transcriptomes *PG* genes were found to be up-regulated during the progression of ripening. However, in 'Alphonso' mango (Deshpande *et al*., 2017) most genes had a stable expression and the authors attributed these results to the longer shelf life of this mango variety.

d. Pectinesterase (PE)

PE is an important pectin degrading enzyme that catalyses the de-esterification of pectin makingthem susceptible to the action of PG and PL during ripening (Tucker *et al*., 2017). According to Sirijariyawat *et al*., (2012), pectinesterase improved the fruit texture of both fresh and frozen thawed mangoes. Duvetter *et al*., (2009) and Kermani *et al*., (2015) reported a lower PE activity in mango compared to other fruits. The enzyme activity exhibited a declining or constant trend during mango ripening (Kermani *et al*., 2015; Ali *et al*., 2004). In addition, genes encoding PE showed a decreasing expression during ripening in 'Dasheri' mango (Srivastava *et al*., 2016). Since the activity of PE precedes the activity of PL and PG, it might be required during the early phase of ripening and declined in the ripe fruit when PL and PG become active (Asif et al., 2014).

e. Pectate lyase (PL)

PL enzymes have been reported to be involved in the depolymerisation of pectin (Tucker *et al*., 2017). Chaurasia *et al.* (2010) suggested that the pectate lyases could be the most important pectin degrading enzymes in mango. These authors observed that the ripening-related *PL* genes showed increased expression levels during ripening. Similar expression profiles were observed in "Dashehari," and 'Kent' mangoes respectively (Dautt-Castro *et al*., 2015; Srivastava *et al*., 2016).

f. α-mannosidase and β-hexosaminidase

It has been reported that the activities of β-hexosaminidase and α-mannosidase enzymes enhanced the fruit shelf life of tomato (Meli *et al*., 2010). Ripening enzyme analysis in 'Ashwina' mango variety showed that β-hexosaminidase and α-mannosidase gradually increased significantly from the onset of ripening to later stage of ripening (Hossain *et al*., 2014). Similar results for α-mannosidase and β-glucosidase have been observed in 'Alphonso' mango (Oak *et al*., 2019).

g. Amylases

The loss of starch during ripening has been implicated in major loosening of the cell wall structure leading to fruit softening (Cárdenas-Coronel *et al*., 2012; Yashoda *et al*., 2006). In 'Kent' mango variety, Cárdenas-Coronel *et al*., (2012), observed that 90% of starch is broken down in the early stages of ripening and this is concordant with decrease of the fruit firmness. Furthermore, Dautt-Castro *et al*., (2015) identified three α-amylase and four β-amylase coding genes, out of which only two *β-amylase* genes exhibited an up-regulation during the progression of mango ripening.

h. Expansin

These are referred to as non-enzymatic proteins because they do not catalyse a chemical reaction (Tucker *et al*., 2017). Expansins have been reported to facilitate the disruption of hydrogen bonds between the cellulose microfibrils and xyloglucans which in turn enhances fruit softening (Zhang *et al*., 2012; Tucker *et al*., 2017). Genes encoding the expansins have been identified in several mango varieties including 'Zill' (Wu *et al*., 2014), Kent' (Dautt-Castro *et al*., 2015) and 'Dasheri' (Srivastava *et al*., 2016). In most cases, these gene exhibited an up-regulated trend as ripening occurred.

2.2.2 Modifying softening

Postharvest losses of fruits remains one of the biggest challenges of our world today (FAO, 2011). Once ripening is initiated, it cannot be reversed. Excessive softening adversely affect the fruit quality leading to the fruits being wasted. This wastage is estimated to be about 20 – 25 % from the farm-gate to the consumers (Kader, 2002). Effort to reduce post-harvest losses can be achieved through a better understanding of the ripening process (Seymour *et al*., 2013). Knowledge gained will enhance the ability to devise strategies to reduce postharvest losses.

Several postharvest methods have been employed to prolong fruit storage life such as chemicals, controlled atmosphere, coating and low temperature (Abbasi *et al*., 2009; Ali *et al*., 2016; Khaliq *et al*., 2016; Liu *et al*., 2017; Mustafa *et al.*, 2018; Payasi and Sanwal, 2009; Rojas-Graü *et al.*, 2009). However reports have shown that these technologies can be unsafe for human consumption, expensive and confer undesirable fruit qualities (Bibi and Baloch, 2014; Dhall, 2013; Gol *et al*., 2013; Mari *et al*., 2014; Theologis *et al*., 1992).

Great strides have been made in understanding ripening over the years using molecular biology approaches. Ethylene has been revealed to play a critical role in the ripening of climacteric fruits (Bapat *et al*., 2010; Barry and Giovanonni, 2007; Hamilton *et al.*, 1990). Inhibition of ethylene production using reverse genetics delayed the ripening process in tomato (Hamilton *et al*., 1990). The drawback of this approach is that the ripening process is triggered as normal when transgenic fruits are exposed to ethylene (Brummell and Harpster, 2001). A different approach to prolonging the storage life may be the modification of the softening process more directly (Brummell and Harpster, 2001; Tucker and Seymour, 1991). This is beneficial as the entire ripening process proceeds as normal but with softening controlled (Brummell and Harpster, 2001; Wang *et al*., 2018a). Fruit softening is a complex process as shown by attempts to reduce fruit softening using reverse genetics to inhibit the action of single enzymes.

In tomato fruit, gene silencing has been used to inhibit the synthesis of polygalacturonase (PG; EC 3.2.1.15). The PG levels were substantially reduced to as low as 1 % thereby inhibiting pectin breakdown (Sheehy *et al*., 1988; Smith *et al*., 1988). However, this inhibition has been found to have only a relatively small effect upon fruit firmness (Sheehy *et al*., 1988; Smith *et al*., 1988). Even though PG activity was not the sole determinant of fruit softening, it has been shown to lead to an extended shelf life (Giovannoni *et al*., 1989). This distinctive feature made the PG transgenic plants sufficiently different and a success on the commercial scale. Similar studies have been carried out to elucidate the roles of other single enzymes including

pectinesterase (PE; EC 3.1.1.11) and endoglucanase (EC 3.2.1.4) (Brummell *et al*., 1999a; Phan *et al*., 2007). However, these resulted in little or no softening. Altogether, the evidences provided by these studies revealed that fruit softening is a complex process involving several enzymes working in concert. As such, softening cannot be explained solely on the genes that have been identified so far and the role of other unidentified genes cannot be ruled out. Hence, there is a constant need to identify more genes in order to improve our current understanding of fruit softening. In this regard, Lycett (2008) suggested that the trafficking route is a promising strategy that might have a more significant effect on softening as the activities of multiple enzymes would have been reduced at the same time.

2.2.3 Role of trafficking in plant cell wall dynamics

The production and trafficking of new cell wall polymers and enzymes is a prerequisite for the synthesis and modification of the cell wall (Lycett, 2008; Ebine and Ueda, 2015). The plant endomembrane system consists of the nuclear envelope, Golgi apparatus, endoplasmic reticulum, plasma membrane, vacuole and vesicles working together to synthesize, modify and ship proteins and other cellular materials. This transport involves budding of the vesicles from the donor membranes, vesicle movement to its specific membrane, tethering and fusion with the target membrane. An appropriate delivery to the correct destination is strictly maintained within the cell despite the influx of a vast array of gene products into the endomembrane system (Stenmark, 2009). Although the molecular framework of trafficking is generally conserved among eukaryotes, a difference in the endomembrane system exist between plants and animals (Saito and Ueda, 2009). Due to the distinctive features of the plant cells such as the cell wall, plants have evolved a unique mechanism of trafficking to fulfil plant-specific functions (Fujimoto and Ueda, 2012; Uemura, 2016). The plant cell wall components and proteins involved in cell wall-altering events are synthesized in several parts of the cell. Cell wall-modifying enzymes and cellulose synthases are produced on the endoplasmic reticulum while

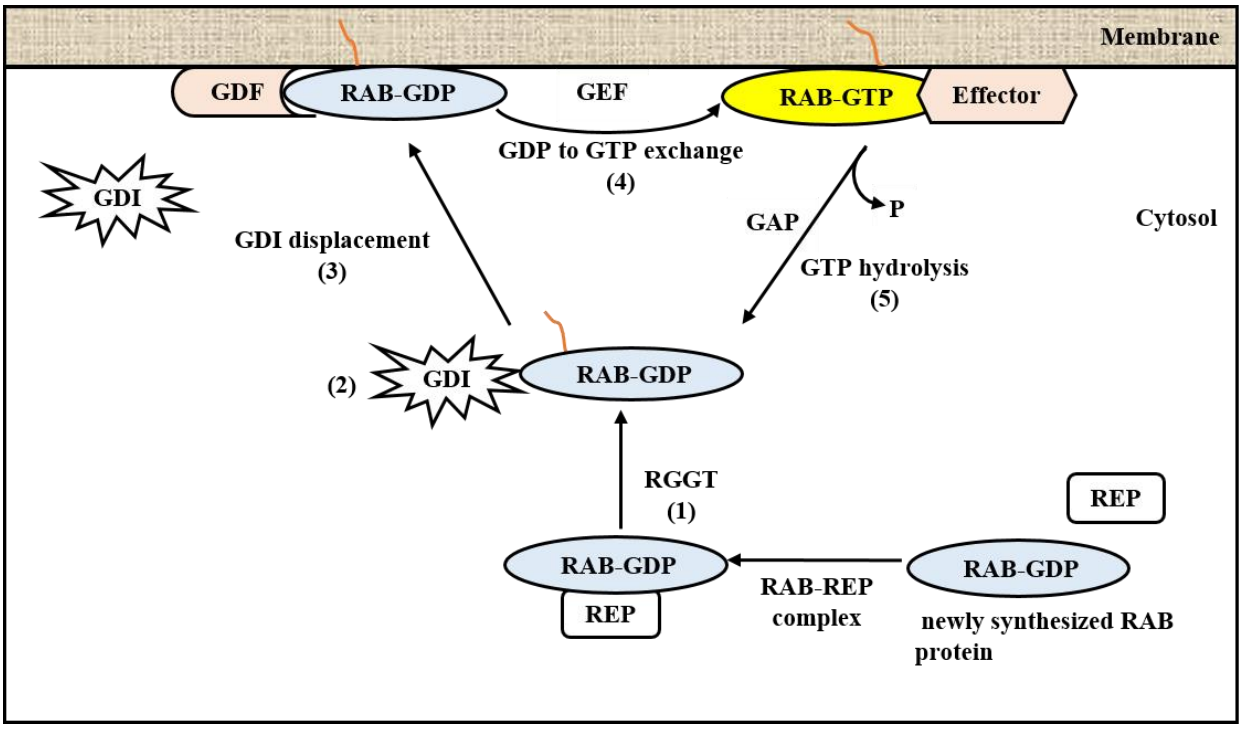
several major cell wall polysaccharides are made in the Golgi apparatus (Pauly *et al*., 2013; Harholt *et al*., 2010). Following this, these cargoes presumably are trafficked through the endomembrane system of the cell and secreted to the apoplast. That the transport system is maintained in a specific and coordinated manner raised questions; what factors ensure the correct fusion to its target membrane? The Rab GTPases have been implicated as key players of the above step (Stenmark, 2009).

2.3 THE RAB GTPASE FAMILY

2.3.1 Rab GTPases as molecular switches in membrane trafficking

The first members of this protein family were originally discovered in yeasts, where they are commonly referred to as YPTs (yeast protein transport) (Gallwitz *et al*., 1983; Salminen and Novick, 1987; Segev and Botstein, 1987). Following this discovery, the use of oligonucleotide probes to screen a rat brain cDNA library identified the first homologs in mammals (Touchot *et al*., 1987). Hence, the acronym 'Rab' (Ras-related proteins in brain) was adopted (Touchot *et al*., 1987). The Rab GTPases oscillate between the 'active' GTP-bound and the 'inactive' GDP-bound forms in the membrane and cytosol respectively (Stenmark and Olkkonen, 2001; Zerial and McBride, 2001) (Figure 2.5). This conformational change accounts for their roles as 'molecular switches' and their ability to perform several tasks in a coordinated manner (Stenmark and Olkkonen, 2001; Zerial and McBride, 2001). Rab GTPases are initially synthesized as soluble proteins in the cytosol (Ali and Seabra, 2005). Rab escort proteins (REP) recognizes and associates with the newly synthesized GDP-bound Rab proteins to form a Rab-REP complex (Andres *et al*., 1993). This interaction facilitates the prenylation of the Rab protein catalysed by the Rab geranylgeranyl transferase (RGGT) enzyme (Alexandrov *et al*., 1999). Prenylation in Rab proteins is a posttranslational modification that involves the addition of geranylgeranyl groups to the cysteine residues at the C-terminus (Glomset and Farnsworth,

1994; Seabra, 1998). This process is essential for Rab membrane targeting and attachment



(Casey and Seabra, 1996). Following prenylation, REP is released from the Rab-REP complex

Figure 2.5: The Rab GTPase cycle

(Rak *et al*., 2004). Another protein known as the Rab GDP dissociation inhibitor (GDI) protein

binds to the modified Rab to maintain its stability and solubility in the cytosol (Alexandrov *et*

Note: 1) Rab escort protein (REP) interacts with newly synthesized GDP-bound Rab *a*proteins and presents them to the Rab geranylgeranyl transferase (RGGT) enzyme for the posttranslational lipid modification. This is essential for membrane targeting and attachment. 2) Membrane cycling of the prenylated GDP-bound Rab is facilitated with the binding of GDP dissociation inhibitor (GDI) and release of REP. 3) GDI displacement factor (GDF) facilitates the release of GDI from the GDP-bound Rab-GDI complex. 4) Guanine exchange factor (GEF) aids the conversion from the 'inactive' GDP-bound Rab to its active GTP-bound state by the guanine nucleotide exchange factor (GEF) (Figure 2.5).

al., 2007). This is followed by the conversion of the Rab protein to its active GTP-bound state by the guanine nucleotide exchange factor (GEF) (Figure 2.5).

2.3.2 The Rab family in plants

The Rab GTPase family which constitutes the largest group of the 'Ras' (rat sarcoma) (Cox and Der, 2010) superfamily has been found to exist in all eukaryotes studied (Stenmark, 2009). This subfamily has been extensively studied in yeasts and humans with at least 11 and 60 members respectively (Pereira-Leal and Seabra, 2001). Members of the Rab GTPases have been identified in several plants (Table 2.1).

Table 2.1: Distribution of the Rab GTPase identified so far in selected plant species.

Plant species	**Total**	**Rab GTPase subfamily**							
		A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Arabidopsis thaliana[1]	57	26	3	3	4	5	3	8	5
Gossypium raimondii[2]	87	34	6	8	7	8	9	9	6
Glycine max[3]	94	41	4	11	7	8	7	8	8
Lotus japonicus[3, 4]	30	12	2	4	1	3	3	3	2
Medicago truncatula[3]	64	23	7	6	4	6	5	9	4
Oryza sativa[3, 5]	52	17	4	3	7	6	7	5	3
Prunus persica[6]	14	6	1	2	2	1	1	1	-
Solanum lycopersicum[3,7]	56	26	5	4	5	5	4	4	3
Triticum aestivum[8]	29	13	2	2	4	3	2	3	-
Vitis vinifera[9]	26	14	3	1	1	2	3	1	1
Zea mays[5]	41	15	3	3	8	5	3	3	1

Note: Classification is according to Pereira and Seabra, (2001) and Rutherford and Moore, (2002). Superscript numbers indicate articles where the plant Rab GTPase data can be found: 1, Rutherford and Moore, (2002); 2, Li and Guo, (2017); 3, Flores *et al*., (2018); 4, Borg *et al*., (1997); 5, Zhang *et al*., (2007); 6, Falchi *et al*., (2010); 7, Lycett, (2008); 8, Tyler *et al*., (2015); 9, Abbal *et al*., (2008). Published in Lawson *et al*., (2018).

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 with mammalian Rab classes 11, 2, 18, 1, 8, 5, 7 and 6 respectively (Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002; Vernoud *et al*., 2003). The RabA clade is the largest of the plant Rabs (Table 2.1). Furthermore, the RabA clade is divided into six subgroups (RabA1 to RabA6) compared with only two Rab11 GTPases in mammals. The remarkably high number of Rab GTPases and their distribution across distinct membrane-bound compartments indicates their importance in plants-specific functions (Rutherford and Moore, 2002). Multiple sequence alignment analysis revealed 55 % sequence homology between various subfamilies of the Rab GTPase members (Agarwal *et al*., 2009) suggesting the occurrence of gene duplication events (Zhang *et al*., 2007). The conserved and non-conserved regions have been shown to contribute to the localization and specific function of the Rab proteins (Pfeffer, 2005). 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 (Pereira-Leal and Seabra, 2001. The Rab family regions (F1-F5) distinguish a Rab protein from other members of the Ras superfamily while the Rab subfamily regions SF1-3 facilitate the grouping of Rabs into subfamilies (Pereira-Leal and Seabra, 2001; Moore *et al*., 1995). Rab family and subfamily regions have also been shown to play essential roles in specific effector and membrane interaction (Ali and Seabra, 2005). 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 (Pfeffer, 2005). For instance, Rab5a mutants without a di-cysteine motif (replaced with mono-cysteine motif) led to their mistargeting to the endoplasmic reticulum/Golgi region rather than their designated cellular compartment (Shinde and Maddika, 2018).

2.3.3 Rab GTPases as directors of vesicle trafficking

Previous studies have revealed that there are many related Rab groups across many species to regulate protein trafficking in different parts of the endomembrane system (Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002) (Table 2.2; Figure 2.6). Rab GTPases carry out distinct membrane trafficking events, which span from vesicle formation, vesicle motility to vesicle tethering and fusion to the acceptor membranes (Gillingham *et al*., 2014). The roles of the Rab GTPases have been revealed in the exocytic (Hutagalung and Novick, 2011) and endocytic (Wandinger-Ness and Zerial, 2014) pathways (Table 2.2; Figure 2.6). Members of the RabA subclass have been localized in the *trans*-Golgi network (TGN) and mediate transport to the plasma membrane (PM) (Chow *et al*., 2008). The huge diversification of the plant RabA clade led to the hypothesis that distinct functions unique to plants may have evolved amongst them (Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002). This is supported by the observation of Choi *et al*., (2013) that in the leaf epidermal cells of *Nicotiana benthamiana*, RabA1b and RabA4c are involved in anterograde and retrograde trafficking between the TGN and PM respectively. Plant studies have shown that the RabB and RabD members are associated with ER to Golgi transport (Batoko *et al*., 2000). Mutations in a maize *Rab2* (*ZmRab2A1*) were shown to induce wart-like structures on leaf surfaces suggesting a role in cell wall secretion during expansion (Zhang *et al*., 2007). RabE is involved with Golgi to PM transport (Speth *et al*., 2009) whereas RabH is reported to function in the Golgi to the ER pathway (Bednarek *et al*., 1994). RabF and RabG are associated with endosomal trafficking (Ebine *et al*., 2014).

Table 2.2: Localization and functions of plant Rab GTPases

Arabidopsis names	Equivalent mammalian groups	Localization	Function in plants
RabA	Rab11	ER,TGN	TGN to PM traffic [1, 2, 3]
RabB	Rab2	ER, Golgi	ER to Golgi traffic[4]
RabC	Rab18	Golgi, PM	stress [5], abscission [6, 7]
RabD	Rab1	ER, Golgi	ER to Golgi traffic[8, 9]
RabE	Rab8/10/12	TGN, PM	Golgi to PM traffic[10, 11]
RabF	Rab5/22	EE	vacuole transport[12]
RabG	Rab7	LE	vacuole transport[12]
RabH	Rab6	Golgi	Golgi to ER traffic[13]

Note: Nomenclature and classification are according to Pereira-Leal and Seabra (2001) and Rutherford and Moore (2002). Superscript numbers indicate references where the details can be found: 1, Choi *et al*., (2013); 2, Inaba *et al*., 2002; 3, Lunn *et al*., (2013a); 4, Cheung *et al*., (2002); 5, Jiang *et al.,* (2017); 6, Corbacho *et al*., 2013; 7, Gil-Amado and Gomez-Jimenez, (2013); 8, Batoko *et al*., (2000); 9, Tyler *et al*., (2015); 10, Speth *et al*., (2009) ; 11, Inada and Ueda, (2014); 12, Ebine *et al.,* (2014); 13, Bednarek *et al*., (1994). EE, early endosome; ER, endoplasmic reticulum; LE, late endosome; TGN, trans-Golgi network; PM, plasma membrane. Published in Lawson *et al*., (2018).

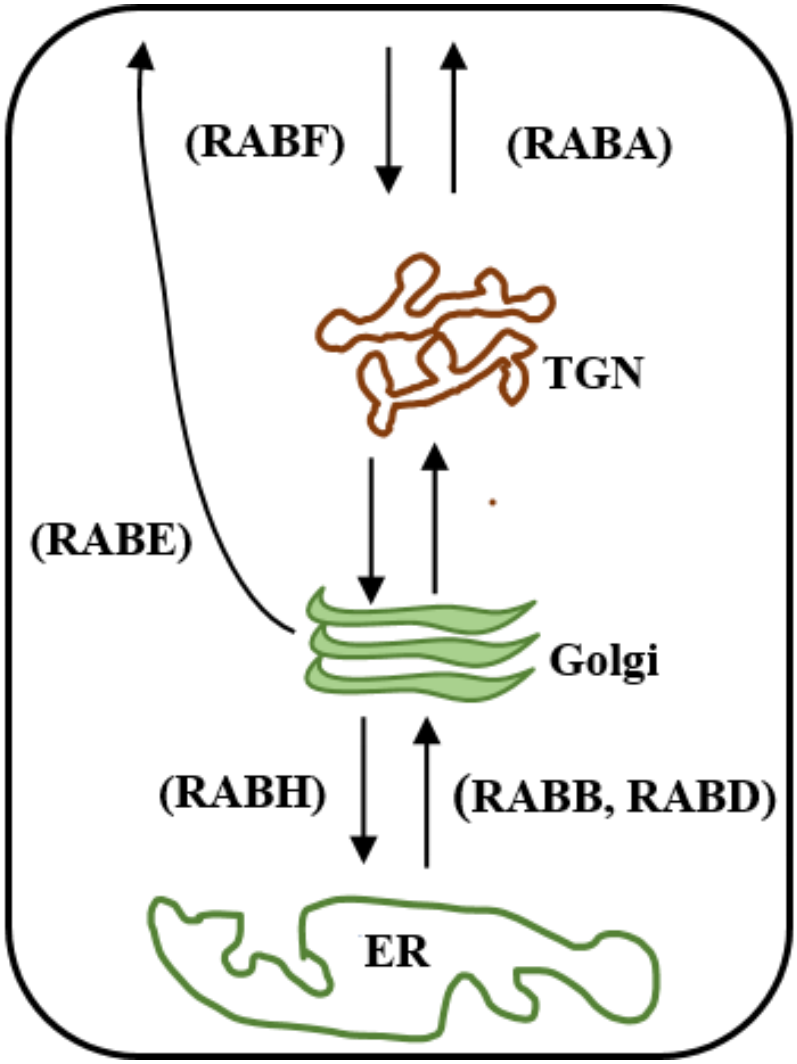


Figure 2.6: Simplified illustration of the Rab trafficking pathways involved with cell wall softening.

Note: The Rab family members involved at each step are indicated in parentheses. Arrows indicate pathways to and from the cell wall respectively. Nomenclature and classification is according to Pereira-Leal and Seabra (2001) and Rutherford and Moore (2002). TGN, *trans*-Golgi network; PM, plasma membrane; ER, endoplasmic reticulum. Published in Lawson *et al*., (2018).

2.3.4 The involvement of Rab GTPases in fruit ripening

Most research efforts to alter fruit softening have been focused on cell wall degradation. However, the process of ripening is far more complicated and cannot be explained only based on the few genes that have so far been identified. It is therefore advantageous to identify additional genes involved in other identified functions such as membrane trafficking. The identification of these genes is important not only to understand how a complex process such as ripening is governed but also because of the tremendous potential this study has for biotechnological application in terms of modulating fruit softening to mitigate post-harvest losses.

Fruit is one of several plant systems where the key role of vesicle trafficking in cell-wall related events has been well characterized (Lycett, 2008). It is clear that the process of cell wall disassembly requires cooperative action of multiple enzymes (Brummell, 2006). As such, it has been suggested that regulating the trafficking route is a promising strategy that might drastically reduce softening as the effect of a variety of enzyme activities would have been reduced at the same time (Lycett, 2008). Gene expression pattern of the Rab GTPases associated with fruit ripening offers insights into understanding the possible roles of Rab GTPases in this complex process. The preferential expression of the Rab GTPases during fruit ripening has been reported (Abbal *et al*., 2008; Falchi *et al*., 2010; Liu *et al*., 2014). Thus establishing their possible role in fruit ripening. More notably, the RabA subclade provides a good illustration of how altered *Rab* expression can affect the modification of the cell wall during ripening. For example, Lu *et al*., (2001) showed that an altered expression of the *RabA* gene member in tomato affected the trafficking of cell wall modifying enzymes by downregulating the trafficking step from the ER to the apoplast. The result was a decrease in

the levels of cell wall modifying enzymes and a marked reduction in fruit softening of the transgenic tomato compared to the wild type. From this study, it can be hypothesized that a reduced expression of the *RabA* in mango could decrease the rate of mango softening thereby improving storage potential of this economically important fruit. However, the situation in mango is less well understood and as such an in-depth study is required to ascertain if this phenomenon holds true. Studies performed on *Arabidopsis* stem (Lunn *et al*., 2013a) and green expanding tomato fruit (Lunn *et al*., 2013b) showed that the Rab GTPases are important in determining the proportion of the different cell wall polymers when the cell wall is made. Lunn and colleagues (2015) went further to assess the impact of altered cell wall composition on enzymatic breakdown in the stem tissue of *Arabidopsis RABA* gene knockout mutants. From this study, it emerged that the changes in the cell wall composition influences the susceptibility of the cell wall to enzymatic breakdown. Based on this evidence, it is possible to speculate that the implications may be applicable to ripening fruit too.

To our knowledge, no report has a) carried out a comprehensive study on the expression of Rab GTPase family in mango fruit and b) established a link between the RabA GTPases and the different softening characteristics that exist among mango varieties. This approach is particularly beneficial especially for a non-model crop such as *Mangifera indica* for which there are limited genomic resources.

2.4 ADVANCES IN TECHNOLOGIES TO STUDY FRUIT RIPENING

Recent years have witnessed huge developments in the ‘omics’ era and the world of omics has become a vast field (Gapper *et al*., 2014; Van Emon, 2015). These new technologies including genomics, proteomics, metabolomics, epigenomics and transcriptomics are paving new avenues for understanding the complexity of plant physiological processes including fruit

ripening (Gapper *et al*., 2014; Giovannoni *et al*., 2017; Kumar *et al*., 2017; Osorio *et al*., 2013; Van Emon, 2015; Zhuang *et al*., 2014). These approaches are discussed below;

2.4.1 Genomics study in fruit science

Genomics is defined as the study of an organism's genome. The genome refers to the complete set of genetic material present in an organism and provides all the information the organism requires to function. In living organisms, the genome is stored in long molecules of DNA called chromosomes Gapper *et al*., 2014). Small portions of the DNA, known as genes code for an RNA or protein molecules required by an organism. The history of a plant's domestication and breeding are recorded in its genome (Lin *et al*., 2014). Genomics techniques include DNA sequencing, genome editing and phylogenomics (Gapper *et al*., 2014; McKain *et al*., 2018; Mohanta *et al*., 2017). DNA sequencing provides information about the number, nature, and organization of genes in a genome and elucidates the mutational events that alter both genes and gene products. These mutations such as SSRs (simple sequence repeats) and SNPs (single-nucleotide polymorphisms) have been identified and are accelerating genetic mapping, trait identification, breeding efficiency through marker-assisted selection (MAS). Several fruit genome sequences have been published to date including durian (*Durio zibethinus*) (Teh *et al*., 2017), peach (Verde *et al*., 2013) and tomato (Tomato Genome Consortium, 2012). However, mango reference genome is not yet available. Genome editing is the addition, removal or alteration of an organism's selected DNA sequence at precise locations, with desired accuracy (Martín-Pizarro and Posé, 2018; Mohanta *et al*., 2017). Some of the genome editing tools that have been developed to aid precise changes into plant genomes include the CRISPR/Cas9 system, homologous recombination (HR), zinc finger nucleases (ZFNs) and RNA interference (RNAi) (Martín-Pizarro and Posé, 2018 ; Mohanta *et al*., 2017). Currently, CRISPR/Cas9 is now being widely used for targeted and stable editing of DNA to understand complex traits such as ripening (Martín-Pizarro and Posé, 2018). For instance, in ripening tomato,

CRISPR/Cas9-induced *pl* mutants has shown an effect on modulating pectin degradation and improved fruit shelf life (Uluisik et al., 2016; Wang *et al*., 2018b). The long juvenility of mango and limited genomic resources makes this technology unfeasible for mango fruit crop.

2.4.2 Proteomics applied in fruit science

The word "proteome" is derived from PROTEins expressed by a genOME (Chin and Tan, 2018). Proteomics is defined as the identification and quantification of overall proteins found in a cell, tissue or an organism at a given time (Bilal *et al*., 2017). To some degree, the proteome complements other omics approaches such as transcriptomics (Bilal *et al*., 2017). Proteomics can be subdivided into different areas, including, differential expression proteomics, descriptive proteomics posttranslational modification and interactomics (Chin and Tan, 2018). Commonly used proteomics-based technologies include Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional gel electrophoresis (2-DE), mass spectrometry (MS), Isobaric tag for relative and absolute quantization of tryptic peptides (iTRAQ), matrix assisted laser desorption ionization–time of flight (MALDI TOF), yeast two-hybrids screens and western blots complemented in varying degrees with computational prediction programs. Until recently, most proteomic studies of fruit have employed 2-DE electrophoresis to separate and identify proteins of interest. MS-based techniques are then used to analyse the sequence of the peptides associated with the desired protein spot (Gapper *et al*., 2014). The diverse proteomics approaches including mass spectrometry (MS) have developed to analyse the complex protein mixtures with higher sensitivity. Proteomic analysis have provided initial proteome insights into fruit ripening and softening, and generally validate prior transcriptomic studies. Comparison of the unripe and ripe stages of mango revealed altered abundance of 47 proteins, among these, proteins involved in carbon fixation, hormone biosynthesis, stress response and pathogen defence (Andrade *et al*., 2012). Ripening-induced changes in papaya (*Carica papaya*) revealed 27 differentially expressed proteins, among these

were proteins involved in cell wall metabolism, stress response, ethylene and carotenoid biosynthesis (Nogueira *et al.,* 2012). An analysis of the apple (*Malus x domestica*) proteome revealed 53 differentially expressed proteins associated with stress, energy metabolism, cell and protein synthesis (Shi *et al*., 2014). Despite the great promise of this technology, it is not widely adopted and this is due to the dynamic and unstable nature of proteins which makes handling in bulk challenging (Chin and Tan, 2018).

2.4.3 Metabolomics applied in fruit science

The metabolic networks in higher plants are highly complex and involves several multiplex biochemical steps (Kumar *et al*., 2017; Zhang *et al*., 2014a). Metabolomics is the detection of the metabolome from a biological sample (Gapper *et al*., 2014). The metabolome is the total number of metabolites (small organic compounds) present within a cell, tissue or organism. Metabolomics technology includes nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) coupled with gas chromatography (GC-MS) or liquid chromatography (LC-MS) (Gapper *et al*., 2014; Kumar *et al*., 2017; Zhang *et al*., 2014a). There are numerous reports employing metabolomics to dissect ripening in fruits such as tomato (*Solanum lycopersicum*) (Oms-Oliu *et al*., 2011; Osorio *et al*., 2012), avocado (*Persea americana*) (Pedreschi *et al*., 2014) and peach (*Prunus persica*) (Lombardo *et al*., 2011). Examples of findings include the distribution of metabolites obtained from different sampling positions of the apple fruit (Cebulj *et al*., 2017). Additionally, metabolite profiling of haskap berry (*Lonicera caerulae*) fruit revealed a relationship with colour and antioxidant activity (Lee *et al*., 2015).

2.4.4 Epigenomics applied in fruit science

Epigenomics/epigenetics refers to the study of the epigenome, which are heritable modifications of the genome not caused by alteration of the underlying nucleotide sequence (Giovannoni *et al*., 2017). Examples of mechanisms that lead to such changes include DNA

methylation, acetylation and histone modification, each of which can influence gene expression singularly and together (Gapper *et al*., 2014). There is increasing evidence of epigenome events during fruit development and impact ripening. The first evidence was reported in the tomato colourless ripening (*Cnr*) mutant (Manning *et al*., 2006). These authors revealed that the non-ripening phenotype of the *Cnr* epigenetic mutant was due to the hypermethylation of cytosine residues upstream the predicted ATG start site. In addition, the promoter of *Cnr* in wild type tomato fruits either contains a few methylated bases or appears to be demethylated prior to the onset of ripening (Manning *et al*., 2006). Furthermore, study by Zhong *et al*. (2013) has revealed epigenome modifications associated with fruit ripening. More recent studies has suggested a possible role of DNA methylation in the expression regulation of duplicated transcription factors (TF) in tomato fruit ripening (Wang *et al.*, 2018c). The emerging field of epigenetics offers innovative options for breeding applications (Gallusci *et al*., 2017; Pech *et al*., 2013).

2.4.5 Transcriptome and gene expression studies in fruit science

Identifying genes whose patterns of expression differ according to phenotype or experimental condition is an integral part of understanding the molecular basis of phenotypic variation (Alberts *et al*., 2002). Transcriptomics deals with the analysis of gene expression patterns under several conditions. Unlike the genome which is roughly fixed (excluding mutations), the transcriptome is highly dynamic and reflects the activity of genes at any given time across a wide array of phenotypes and conditions (Adam, 2008). On the other hand, the study of proteins is highly challenging due to their unstable nature and inability to be amplified (Chin and Tan, 2017). Thus by studying the transcriptome, it connects the genome to gene function (Adam, 2008). Transcriptome profilling involves detecting the expression level of one or more specific RNAs out of thousands of other RNAs. Several options are available for studying gene expression (Bustin, 2002; Fryer, 2002; Lockhart and Winzeler, 2000). However, the most

popularly used methods in recent times include reverse transcription quantitative PCR (RT-qPCR) and RNA-sequencing (RNA-seq) (Costa *et al*., 2013; Szabo, 2014). These methods can be further subdivided into targeted or untargeted approaches, each with its own pros and cons (Szabo, 2014). However, the utilization of a specific method mostly depends on the goals of the project, the budget and the study organism (Pavey *et al*., 2010).

The RT-qPCR is a targeted method that uses short DNA sequences called primers to amplify targeted set of genes in a biological sample. It is a method of choice particularly when only a selected number of genes are to be studied (Kozera and Rapacz, 2013). This technique has come a long way to becoming the "gold standard" for validating RNA-seq data due to its specificity, real time detection of reaction progress and broad range of quantification (Pfaffl, 2010; Kozera and Rapacz, 2013). In spite of being a robust technique for gene expression level assay, the reliability of RT-qPCR is dependent on appropriate normalization strategies to correct for the unavoidable differences introduced during the multistage process of RNA isolation and/or complementary DNA (cDNA) synthesis (Huggett *et al*., 2005; Sanders *et al*., 2014). RT-qPCR is limited by previous knowledge of gene sequences as well as challenges associated with reaction optimization (Bustin and Nolan, 2004; Szabo, 2014). Notwithstanding these limitations, this technique requires little bioinformatics expertise and offers an inexpensive means of assessing gene expression (Costa *et al*., 2013; Szabo *et al.,* 2014).

RNA-seq on its turn is the technology where the cDNAs are sequenced using next-generation sequencing (Wang *et al*., 2009). RNA-seq has been applied successfully in gene expression profiling of both model and non-model plants (Stricker *et al*., 2012). This experimental technique is rapidly advancing our knowledge and shedding light on how individual or groups of genes may regulate biological processes at the global scale (Jain, 2011). To date, RNA-seq is widely used mainly because: (1) of its ability to assess gene expression at a much broader range under defined experimental conditions (2) it requires no prior knowledge of gene

sequences, (3) the ability to detect low expressed transcripts and also novel transcripts (Everaert *et al*., 2017;Wang *et al.*, 2009). Currently, RNA-seq technology is considered the most powerful technique for whole transcriptome profiling in fruit science (Simsek *et al*., 2017). For instance, RNA-seq analysis of sweet orange red-fleshed mutant and its wild type revealed that genes associated with cell wall biosynthesis, carbohydrate and citric acid metabolism, carotenoid metabolism and the response to stress were the most differentially expressed (Yu *et al*., 2012). Transcriptome profiling of the unripe and ripe fruit tissues of banana revealed fruit softening as the most differentially regulated process (Asif *et al*., 2014). Comparative transcriptome analysis of two contrasting watermelon varieties during fruit development and ripening showed that a large number of the differentially expressed genes (DEGs) during fruit ripening were related to hormone, sugar and cell wall metabolism respectively (Zhu *et al*., 2017). RNA-seq analysis has also been employed to address questions relating to flower development (Liu *et al*., 2016), fruit abscission (Corbacho *et al*., 2013) and stress (Luria *et al*., 2014). In addition, RNA-seq analysis has also been used for molecular marker development in fruits such as pummelo (Liang *et al*., 2015), pomegranate (Ophir *et al*., 2014) and mango (Sherman *et al*., 2015; Kuhn *et al*., 2016). Some recent examples of RNA-seq analysis on fruit tree crops is provided in Table 2.3. RNA-seq technology, however, is not without challenges. It is expensive, more complicated and requires significant bioinformatics skill in data handling and analysis to extract biologically relevant information (Costa *et al*., 2013; Everaert *et al*., 2017). In addition, the absence of a suitable reference genome in a non-model fruit crop such as mango presents the *de novo* strategy as the only option for sequence assembly (Martin and Wang, 2011). Current comparative studies have demonstrated the advantage of applying multiple gene expression methods to reveal a more comprehensive picture of a transcriptome rather than relying solely on one method (Everaert *et al*., 2017; Kogenaru *et al*., 2012). As such, the RNA-seq technique will not likely replace current RT-

qPCR methods but will be a complementary approach depending on the need and the resources available (Costa *et al*., 2013). In fact, several reports have shown very good correlation between RNA-seq and RT-qPCR data (Everaert *et al*., 2017; Shi and He, 2014). The application of these two complementary technologies would be useful to better elucidate the molecular basis of mango softening process. Looking forward, it is clear that the advances in our fundamental understanding on fruit softening will have an impact to produce mango fruits more suitable for our needs.

Table 2.3: List of some studies of RNA-seq analysis in fruit trees.

Common name	**Scientific name**	**Mechanism**	**sample**	**References**
apple	*Malus domestica*	pathogen infection	root	Shin *et al*., 2016
blackberry	*Rubus spp*	fruit ripening	pulp	Garcia-Seco *et al*., 2015
Chinese bayberry	*Myrica rubra*	fruit ripening	pulp	Feng *et al*., 2012
grape	*Vitis vinifera*	fruit ripening	pulp	Balic *et al*., 2018
kiwi	*Actinidia arguta*	fruit ripening	pulp	Huang *et al*., 2018
litchi	*Litchi chinensis*	bud development	bud	Zhang *et al*., 2016a
mango	*Mangifera indica*	fruit ripening	pulp	Dautt-Castro *et al*., 2015; Srivastava *et al*., 2016
mango	*Mangifera indica*	fruit development and ripening	pericarp and pulp	Wu *et al*., 2014
mango	*Mangifera indica*	fruit development and ripening	flower, peel and pulp	Deshpande *et al*., 2017
mango	*Mangifera indica*	cuticle metabolism	peel	Tafolla-Arellano *et al*., 2017
mango	*Mangifera indica*	heat treatment	peel	Luria *et al*., 2014
melon	*Cucumis melo*	fruit abscission	abscission zone	Corbacho *et al*., 2013
melon	*Cucumis melo*	fruit development and ripening	pulp and flowers	Zhang *et al*., 2016b

nectarine	*Prunus persica*	cold stress	pulp	Sanhueza *et al*., 2015
persimmon	*Diospyros kaki*	fruit softening	peel	Jung *et al*., 2017
plum	*Prunus salicina Lindl.*	fruit ripening	pulp	Fang *et al*., 2016
tomato	*Solanum lycopersicum*	fruit ripening	pericarp	Li *et al*., 2016; Ye *et al*., 2015
sugar apple	*Annona squamosa*	flower development	flowers	Liu *et al*., 2016

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Laboratory chemicals and reagents

A list of the chemicals and reagents used in this thesis and their respective suppliers are provided in Appendix I. Recipes for buffers and solution are detailed in Appendix II.

3.1.2 Primers

Primers were commercially synthesised by Integrated DNA Technologies (Singapore). Primers were made up to 100 µM stock and diluted to 10 µM working concentrations followed by storage at -20 °C. All primers used in this are listed in Appendix III.

3.1.3 Plant materials

Mango (*Mangifera indica* L) varieties namely 'Chokanan', 'Golden phoenix' and 'Water lily' of maturity index 2 as recommended by FAMA (2014) were purchased from a commercial supplier at Melaka State of Malaysia. In order to avoid sample variability, mango fruit were selected for uniformity in size, shape and absence of external injury. After sorting, fruits were washed in running tap water, air dried and allowed to ripen at ambient temperature (25 ± 1 °C). Postharvest quality parameters were analysed on arrival (0th day) and every 2 days for a 9-day storage period.

Although a colour chart of the entire ripening period of the commercial variety 'Chokanan' is available (FAMA, 2014; Figure 3.1), none had been reported for the other mango varieties used this experiment. For this reason, the ripening stages of 'Golden phoenix' and 'Water lily' were defined based on the changes of the measured postharvest parameters such as pulp firmness (Jha *et al*., 2013; Nassur *et al*., 2015; Srivastava *et al*., 2016), titratable acidity (Vélez-Rivera

et al., 2014) and soluble solid concentration (Mitcham, 2012; Yahia, 2011). The completion of the ripening period (7 days for 'Golden phoenix' and 'Water lily') was considered as the time point when the decline in pulp firmness or titratable acidity remained unchanged (Jha *et al*., 2013; Vélez-Rivera *et al*., 2014).

A second batch of mangoes including 'Chokanan', 'Golden phoenix','Water lily','Apple mango', 'Black gold', 'Kemling' and 'Siku jaya' were obtained from the same supplier at a different fruit season to estimate the connection of the gene expression profile and fruit softening further (refer to section 4.5.6).

Index	Description
Index 1 	Immature fruit: The peel colour is dark green.
Index 2 	Mature green: The peel color is light green and ready for harvest
Index 3 	Quarter ripe, the peel is green with patches of yellow
Index 4 	Half ripe, greenish yellow peel colour
Index 5 	Ripe fruit; full yellow
Index 6 	Overripe fruit; orange-yellow

Figure 3.1: Maturity indices of 'Chokanan' mango (FAMA, 2014)

3.2 METHODS

The investigation of the ripening and softening process of mango was carried out at the postharvest and molecular levels respectively. As such, the following section has been divided into two subsections;

- Postharvest methods (section 3.2.1)
- Molecular methods (section 3.2.2)

3.2.1 Postharvest Methods

3.2.1.1 Weight loss

Weight loss was determined using a digital balance (EK-600H, Japan) at day 0 and on each sampling day. The same fruits were used for weight loss until the end of storage period. The percentage weight loss was calculated relative to the initial weight (Ali *et al*., 2016).

$$\text{weight loss} = \left[\frac{\text{initial weight} - \text{final weight}}{\text{initial weight}} \right] \text{X } 100\ \%$$

3.2.1.2 Colour

Colour was assessed on the basis of the Hunter Lab System using a MiniScan XE Plus colorimeter which was first standardized using a black tile and a white tile (X = 79.0, Y = 83.9, Z = 87.9). The values of L*, a*, b* were recorded. Coordinates, a* and b*, indicate colour directions: +a* is the red direction, –a* is the green direction, +b* is the yellow direction, and –b* is the blue direction. From these values, hue angle (h°) was calculated as h° = tangent^{-1}

b*/a*) where 0° = red purple, 90° = yellow, 180° = blue-green and 270° = blue. The readings were measured on three specified points along the equator of the fruit.

3.2.1.3 Firmness

Fruit firmness was assessed using an Instron Universal Testing Machine (Instron 2519-104, Norwood, MA) equipped with an 8 mm plunger tip (Figure 3.2). Firmness was assessed by subjecting fruits to puncture test at a constant speed of 20 mm/min (Ali *et al*., 2016). 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).

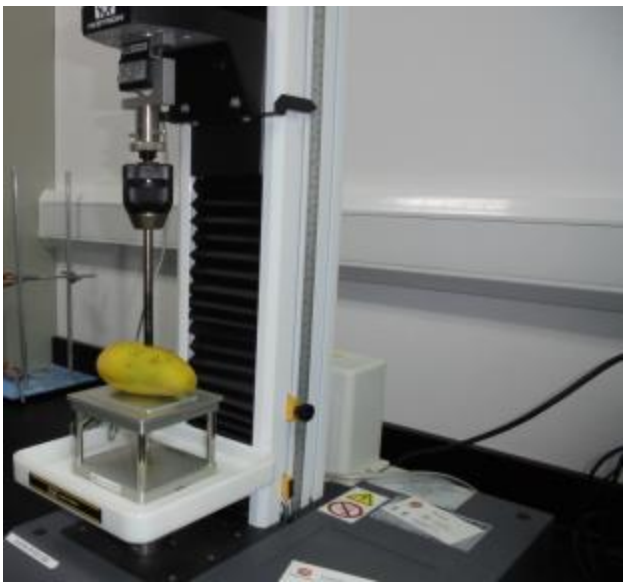


Figure 3.2: A bench mounted texture analyser (original)

3.2.1.4 Soluble solid contents (SSC) and Titratable acidity (TA)

SSC was determined as described by Ali *et al*., (2016). SSC was determined using a hand held digital refractometer (Model: PR-32α, Atago Co Ltd., Japan) (Figure 3.3). Fruit pulp samples (10 g) were homogenized in a blender with 40 ml of distilled water and filtered through a

double layer of muslin cloth to extract juice. 100 µl of the fruit juice was placed on the prism glass of the refractometer to obtain SSC reading. The refractometer was standardised with distilled water before analysis. The results were then expressed in percentage terms.

TA of mango was determined as described by Ranggana (1977). 5 ml of fruit homogenate was titrated against 0.1N NaOH using 0.1 % phenolphthalein as an indicator. The results were expressed as a percentage of citric acid by using the formula as shown below:

$$TA = \frac{\text{Titre value x 64 x volume made up x 100}}{\text{aliquot of sample used x sample weight x 100}}$$

Figure 3.3: A hand held refractometer (original)

3.2.1.5 Respiration and ethylene production

The measurement of respiration and ethylene production was carried out as described by Ong *et al*., (2013). Fruit were placed in a plastic container tightly sealed with a lid. After 1 hour of incubation, 1 ml of gas sample was withdrawn from the headspace and analysed in the gas chromatograph (GC) (Clarus-500 Perkin-Elmer, USA) equipped with a column (Agilent J&W, DB-5MS column: 30.00 m in length, 0.25 mm in diameter and 0.25µm in film thickness) with two detectors connected in series; a thermal conductivity detector (TCD) and flame ionization

detector (FID) for the quantification of carbon dioxide (CO_2) and ethylene respectively. Helium was used as the carrier gas for thermal conductivity (TCD) and temperatures were 60 °C, 150 °C and 200 °C for the oven, injector and detector respectively. The injector, oven and detector temperatures were 200 °C, 120 °C and 250 °C respectively with nitrogen as the carrier gas for the flame ionization detector (FID). Concentration of the standards used was 1 % carbon dioxide (CO_2) and 1 ppm ethylene (C_2H_4). Respiration and ethylene production rate are expressed as nmol kg^{-1} s^{-1} (Banks *et al*., 1995).

3.2.1.6 Statistical analysis

The experiments were conducted according to a completely randomized design (CRD) in four biological replications. For each replicate, three individual fruits were randomly selected for analysis at each evaluation time. Data were subjected to analysis of variance (ANOVA) using the GENSTAT (18th edition) software. Means were separated using Duncan's Multiple Range Test (DMRT; $P < 0.05$). Multivariate analysis was carried out using JMP statistical software version 8.0 (SAS Institute Inc., Cary, NC, USA). PCA and cluster analysis were performed to assess the pattern of association between the variables and mango samples at different storage days. The Pearson's correlation coefficient was employed to explore the relationship between the postharvest parameters.

3.2.2 Molecular Biology Methods

3.2.2.1 Data Mining to Retrieve Mango *Rab* Gene Sequences

Firstly, the published *Rab* protein sequences (Accession Z71276.1, KF768563) (Liu *et al*., 2014; Zainal *et al*., 1996) were used as queries to search against the mango RNA-sequencing database (http://bioinfo.bti.cornell.edu/cgi-bin/mango/index.cgi) (Tafolla-Arellano *et al*., 2017) with an e-value threshold of 1e-5 to identify potential members of the Rab GTPase family. Rab sequences were studied individually by comparison with *Arabidopsis* sequences using the *Arabidopsis* information resource (TAIR; https://www.arabidopsis.org) to check if they represented full-length coding regions. Predicted amino acid sequences were generated using ExPASy tool (https://web.expasy.org/translate). The predicted mango Rab GTPase proteins were assigned to subfamilies on the basis of their similarity to the sequences of *Arabidopsis* (Rutherford and Moore, 2002) and were named according to their closest similarity to *Arabidopsis* proteins. Where more than one mango Rab GTPase was present in the same subclade, a nomenclature based on numbers was adopted (Falchi *et al*., 2010).

3.2.2.2 Sequence and Similarity 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 with the protein sequences annotated and classified in *Arabidopsis* (Rutherford and Moore, 2002) as queries. Amino acid sequences of these proteins 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) (Corpet, 1988). A similarity tree was produced using the MEGA software (version 6) (Tamura, 2013). The reliability of the trees were examined using bootstrap replicates (1000 replicates). Percentage confidence values are shown on branches (Felsenstein, 1985; Saitou and Nei, 1987).

3.2.2.3 Isolation of RNA from Mango Pulp

3.2.2.3.1 Working with RNA

The following procedures were conducted to maintain an RNase free environment: All working surfaces, micropipettes, tube holders, pipette tip boxes and gel electrophoresis tanks were wiped with RNaseZap® RNase Decontamination Solution (Thermo Fisher Scientific, USA) before and after the isolation procedure. Disposable tubes and tips were used to reduce contamination. UltraPureTM DNase/RNase-free distilled water (Invitrogen, Thermo Fisher Scientific, USA) was used as the universal diluent in all RNA preparations.

3.2.2.3.2 Procedure for RNA Extraction

Total RNA was extracted from mango pulp samples as described by Zamboni *et al*., (2008) with slight modification. Tris-$EDTA_{0.1}$ ($TE_{0.1}$) buffer (10mM Tris-HCl; 0.1mM EDTA) was used as the resuspension buffer and spermidine was excluded from the extraction buffer. Briefly, frozen pulp tissues were ground with a mortar and pestle in the presence of liquid nitrogen. Approximately 2 g of the resulting powder was transferred to sterile centrifuge tubes containing 20 ml of pre-warmed extraction buffer (2.5 % CTAB, 5 % PVP, 100 mM Tris–HCl pH 8.0, 25 mM EDTA, 2 M NaCl, and 3 % β-mercaptoethanol added just before use) and incubated at 65 °C for 30 minutes with constant inversion to aid complete homogenization. Following the incubation, an equal volume of chloroform:isoamyl alcohol (CIA) (24:1) was added, mixed thoroughly by inversion and centrifuged at 12,000 ×g for 15 minutes at 4 °C. The supernatant was transferred into a new tube and an equal volume of chloroform:isoamyl alcohol

(CIA) (24:1) was added. This mixture was vortexed and centrifuged again for 15 minutes at 12,000 ×g. After a second centrifugation, the supernatant (approximately 12 ml) was transferred to a sterile tube following the addition of 4 ml of 10 M LiCl (0.3 volume) to precipitate RNA out of solution. After an overnight incubation on ice, precipitated RNA was collected in a pellet using centrifugation at 15,500 ×g for 35 minutes and re-suspended in 2 ml of $TE_{0.1}$ buffer. Chloroform: isoamyl alcohol (24:1) was then added to the solution and centrifuged (10 minutes; 15,000 ×g; 4 °C) to precipitate out remaining proteins. The clear supernatant was transferred into a new sterile tube and two volumes of absolute ethanol was added to precipitate the RNA at -80 °C for 30 minutes. RNA pellet was obtained by centrifugation (20 minutes; 17,000 ×g; 4 °C), dried and re-suspended in 25 µl UltraPure™ DNase/RNase-free distilled water (Invitrogen, Thermo Fisher Scientific, USA). Samples were finger flicked, incubated at 55 °C for 2-3 minutes for complete dissolution and spun down briefly. The quantity and quality of each RNA sample was examined as described in sections 3.2.2.4 and section 3.2.2.5 respectively. Samples were subsequently purified by DNase treatment to eliminate contaminating genomic DNA. DNase-treated RNA samples were divided into three aliquots: The first aliquot was used for quantification using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA), the second aliquot was sent to assess RNA integrity using the Agilent Bioanalyser (Agilent Technologies, Inc., California, USA) and the third aliquot was used for sequencing and/or complementary DNA (cDNA) synthesis by reverse transcription.

3.2.2.3.3 DNase treatment and RNA clean-up

Total RNA samples were treated with Qiagen RNase-free DNase to remove traces of genomic DNA followed by cleaning with an RNeasy MinElute cleanup kit (Qiagen, Germany). The procedure was carried out according to the manufacturer's instructions.

3.2.2.3.4 Synthesis of complementary DNA (cDNA) by Reverse Transcription

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 converted into cDNA. A genomic DNA elimination reaction was first performed using the gDNA wipeout buffer provided in the kit. The volumes and concentrations of the components for the genomic DNA elimination reaction are described in Table 3.1. The sample was incubated for 2 minutes at 42 °C and the placed on ice immediately.

Table 3.1: The components in a genomic DNA elimination reaction

Component	Volume/reaction	Final concentration
gDNA Wipeout Buffer (7×)	2 µl	1×
Template RNA	Variable (up to 1 µg)	1 µg per reaction
RNase-free water	Variable	-
Total volume	14 µl	

Subsequently, the RT reaction was performed on the genomic DNA elimination reaction using the components as described in Table 3.2. Reverse transcription was carried out for 15 minutes at 42 °C, followed by the inactivation of the reverse transcriptase at 95 °C for 3 minutes. The resulting cDNA was stored at -20 °C until further use in endpoint-PCR and qPCR respectively.

Table 3.2: The components of the reverse transcription reaction

Component	Volume/reaction	Final concentration
Quantiscript Reverse Transcriptase	1 µl	-
Quantiscript RT Buffer (5x)	4 µl	1x
Reverse Transcription primer mix	1 µl	-
Entire gDNA elimination reaction from above (Table 3.1)	14 µl	1000 ng/20 µl = 50 ng/µl
Total volume	20 µl	

3.2.2.4 Qualitative Assessment of Nucleic Acids

The concentration and purity of purified PCR products and RNA samples was determined using a Nanodrop 1000 spectrophotometer (Thermo Scientific). Prior to the start of each evaluation session, the instrument was initialised with 1.5 µl of water according to the manufacturer's instructions. Following this, the instrument was blanked with 1.5 µl of the respective elution buffer of each sample type and the absorbance of the samples was measured at 280 nm, 260 nm and 230 nm ultraviolent wavelengths. Any absorbance at 280 nm indicated the presence of contaminants such as proteins. On the other hand, an absorbance at 230 nm indicated the presence of contaminants such as carbohydrates and phenol. Nucleic acids showed strong absorbance at 260 nm, which was used to determine the concentration of the samples. Samples with 260 nm/280 nm and 260 nm/230 nm ratios between 1.8 - 2.0 were accepted as pure (free from contaminants) (Sambrook *et al*., 1989; Manchester, 1996). An aliquot of purified RNA was sent to First Base Laboratories (Malaysia) for the analysis of the RNA integrity number (RIN) using an Agilent 2100 Bioanalyser (Agilent Technologies, Inc., California, USA). RIN $\geq$ 7.0 (from the scale of 1, degraded, to 10, intact) were considered as good quality (Schroeder *et al*., 2006).

3.2.2.5 Quantitative Assessment of Nucleic Acids

A stock of 50x TAE gel electrophoresis buffer was diluted to 1x working concentration (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) with ultrapure (18 MΩ) water. Electrophoresis gels were prepared as 1.5 – 2.0 % (dependent on the product size) agarose gel in 1x TAE buffer. The molten agarose gel was slightly cooled and 3.0 µl (0.03 %) of SYBR® Safe DNA gel stain was added per 100 ml before being poured into a gel cast and left to solidify for 30 minutes to 1 hour. The PCR products were mixed with 6x DNA gel loading dye (10 mM Tris-HCl at pH 7.6, 60 % glycerol, 60 mM EDTA, 0.03% bromophenol blue, 0.03 % xylene cyanol FF) to bring the dye to a final 1x working concentration prior to electrophoresis. On the other hand, RNA samples were mixed with 2x RNA loading dye (95 % formamide, 0.025 % SDS, 0.025 % bromophenol blue, 0.025 % xylene cyanol FF, 0.025 % ethidium bromide, 0.5 mM EDTA) to bring the loading dye to 1x working concentration prior to gel electrophoresis. DNA or RNA ladder where required was run alongside samples for band size comparisons. For electrophoresis of PCR products, the DNA ladder used was GeneRuler 100 bp Plus DNA ladder, ready-to-use) (Thermo Fisher Scientific, USA) and for each run, 2.5 µl of the DNA ladder was loaded. The RNA ladder used was RiboRuler High Range RNA Ladder, ready-to-use (Thermo Fisher Scientific, USA) and 2 µl of the RNA ladder was loaded per run. Once products and ladder had been loaded, gels were run at 90 V for 30 - 50 minutes. Bands were visualized and photographed using a gel-Doc XR+ (Bio-Rad) documentation system.

3.2.2.6 Amplification and Quantification of Nucleic Acids

3.2.2.6.1 Primer design

To demonstrate that the isolated RNA obtained was suitable for downstream applications, RT-PCR reactions were carried out using the gene-specific primers corresponding to regions of the *RabA* genes. Primers were designed using the Primer3 Plus software. The amplicon sizes ranged between 400-700 bp. 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 specific primers that uniquely identify (differentiate) each of the input gene sequences. This was necessary especially because the targets belonged to a multigene family. ProbeFinder would not generate primer sequences if a unique design was not identified. The length of all primers used in endpoint and qPCR reactions ranged between 18 – 27 bp and melting temperatures (Tm) between 57 – 61 °C respectively.

3.2.2.6.2 Reverse transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR was carried out to a) assess the quality of the isolated RNA for downstream analysis (Ma *et al*., 2015; Tong *et al*., 2012) and b) validate the custom designed RT-qPCR primers (Figueiredo *et al*., 2009). A standard endpoint-PCR reaction tube contained 1× PCR buffer, 1 unit of HotStarTaq Plus DNA polymerase (Qiagen, Germany), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM of each primer (Integrated DNA Technologies, Singapore), 100 ng of template cDNA and water (DNase/RNase-free; Invitrogen, Thermo Fisher Scientific, USA) to a final volume of 20 µl. Where required, a touchdown (Table 3.3) or gradient (Table 3.4) PCR cycling condition was employed. A touchdown PCR was needed when the goal to increase the specificity of the amplified product without actually determining the annealing temperature. On the otherhand, gradient PCR was used for PCR optimization along with the determination of an optimal annealing temeperature. After the reaction, 5 µl of each assay mixture was electrophoresed as described in section 3.2.2.5 and the remaining products stored at 4 °C for later use.

Table 3.3: Touchdown PCR cycling condition

Number of cycles	Thermal cycling step	Temperature (°C)	Holding time
1	Initial denaturation	95	5 minutes
9	Denaturation	95	45 seconds
	Anneal*	65-55	45 seconds
	Extension	72	1 minute
20	Denaturation	95	45 seconds
	Anneal	55	45 seconds
	Extension	72	1 minute
1	Final elongation	72	7 minutes
	Cool	4	indefinite

* Temperature is reduced by 1 °C each cycle

Table 3.4: Gradient PCR cycling condition

Number of cycles	Thermal cycling step	Temperature (°C)	Holding time
1	Initial denaturation	95	5 minutes
30	Denaturation	95	45 seconds
	Anneal	55-60	45 seconds
	Extension	72	1 minute
1	Final elongation	72	7 minutes
	Cool	4	indefinite

3.2.2.7 PCR Product Purification for Sequencing

The desired PCR products of the correct size were purified using the High Pure PCR product purification kit (Roche) according to the protocol handbook. In some cases where non-specific secondary bands were present in a gel lane, the fragment of the correct size was excised and subsequently purified. Samples were sequenced by First Base laboratories (Malaysia). Sequenced products were submitted as queries using the *Arabidopsis* information resource database (TAIR, https://www.arabidopsis.org).

3.2.2.8 Sampling design for RNA-sequencing

Pulp samples of 'Chokanan', 'Golden phoenix' and 'Water lily' mangoes at the unripe and ripe stages respectively were used for this experiment. Based on the postharvest physiology studies (Table 4.4) 'Chokanan' mango remained significantly firmer ($P < 0.05$) (138.18 – 12.67 N) than 'Golden phoenix' (109.22 – 9.53 N) and 'Water lily' (104.40 – 7.50 N) during storage. This provided a resource to assess the differences in gene expression related to fruit softening. In this regard, the 'Chokanan' variety was selected to represent the firm mango group. Furthermore, there were no significant differences in fruit softening between the 'Golden phoenix' and 'Water lily' varieties during the storage period. For this reason, RNA samples obtained from 'Golden phoenix' and 'Water lily' mangoes were pooled in equal amounts (Rohland and Reich, 2012) to represent the less-firm mango group within the context of this study. Although pooling of samples reduces individual variability (Peng *et al*., 2003; Rajkumar *et al*., 2015), it can be applied as an alternative approach when the interest is solely on the desired characteristics of the groups (Kendziorski *et al*., 2005; Karp and Lilley, 2009; Li *et al*., 2015) and to reduce per-sample cost (Wylie *et al*., 2012). Thus, the study comprised of two mango groups namely 'Chokanan' (firm-mango group) and Pool (less-firm mango)

respectively. Three biological replicates were prepared for each ripening stage (unripe and ripe) within a mango group (Figure 3.4). Each biological replicate per ripening stage comprised three different fruits from the same batch respectively.

Total RNA extraction for each ripening stage was carried out using the CTAB method as previously described in section 3.2.2.3.2. Assessment of RNA quantity, quality and integrity was performed using Nanodrop ND1000 spectrophotometer, gel electrophoresis and an Agilent 2100 Bioanalyser system (Agilent Technologies, California, USA) as described in sections 3.2.2.4 and 3.2.2.5 respectively. RNA samples with 260 nm/280 nm and 260 nm/230 nm ratios between 1.8 to 2.0 and RIN (RNA integrity number) $\geq$ 7.0 were used for RNA-sequencing.

For simplicity, the mango groups used in this experiment were designated as follows:

i. CKUR for ‘Chokanan’ at the unripe stage
ii. CKR for ‘Chokanan’ sample at the ripe stage
iii. PUR for Pool (‘Golden phoenix’ + ‘Water lily’) sample at the unripe stage
iv. PR for Pool (‘Golden phoenix’ + ‘Water lily’) sample at the ripe stage

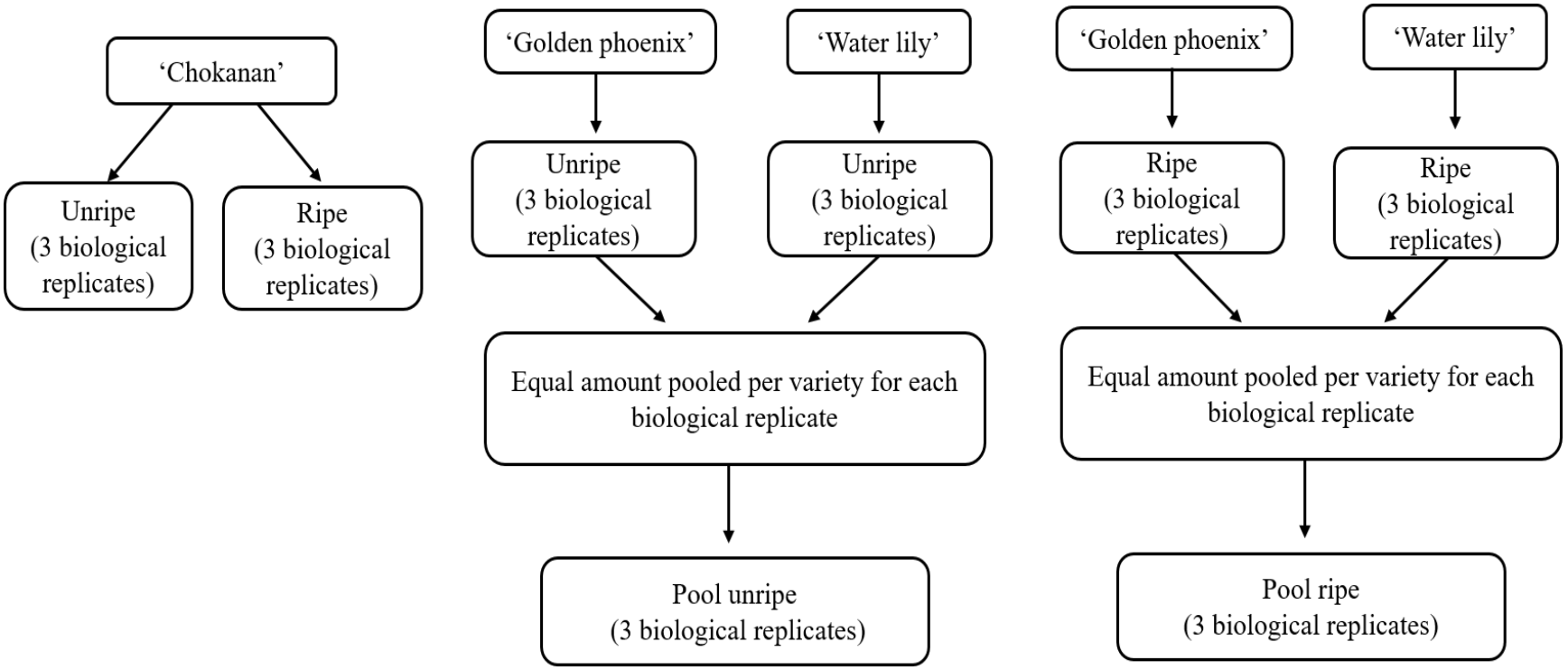


Figure 3.4: RNA sampling design for sequencing

3.2.2.9 Library preparation and sequencing

The extracted RNA samples were sent to Novogene Genome Sequencing Company (Singapore) library preparation and Illumina sequencing. The work flow is outlined in Figure 3.5.

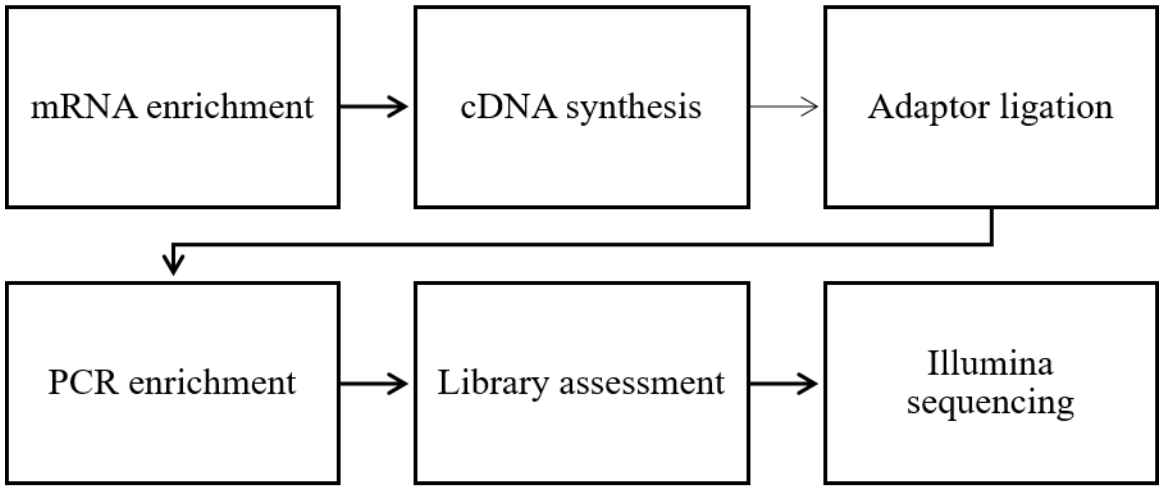


Figure 3.5: Flow diagram of library preparation and sequencing.

Briefly, a total amount of 1 μg total RNA per sample was used to prepare libraries using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's instructions. The mRNA was isolated using oligo(dT) magnetic beads. The purified mRNA was fragmented and reverse transcribed with random hexamer primers. Second-strand cDNA was synthesised and purified with Ampure XP SPRI beads (Beckman Coulter) followed by end repair and A-tailing. Finally, sequencing adaptors were ligated to the fragments and then amplified with PCR. Quality control of the library was carried out using a Qubit 2.0 fluorometer (Invitrogen, USA), qPCR and an Agilent 2100 Bioanalyser (Agilent Technologies, Inc., California, USA) followed by sequencing using an Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA) with 150 bp paired-end mode.

3.2.2.10 Bioinformatics analysis

Bioinformatics analysis was performed by Novogene Genome Sequencing Company (Singapore). The analysis workflow for RNA-sequencing is outlined in Figure 3.6.

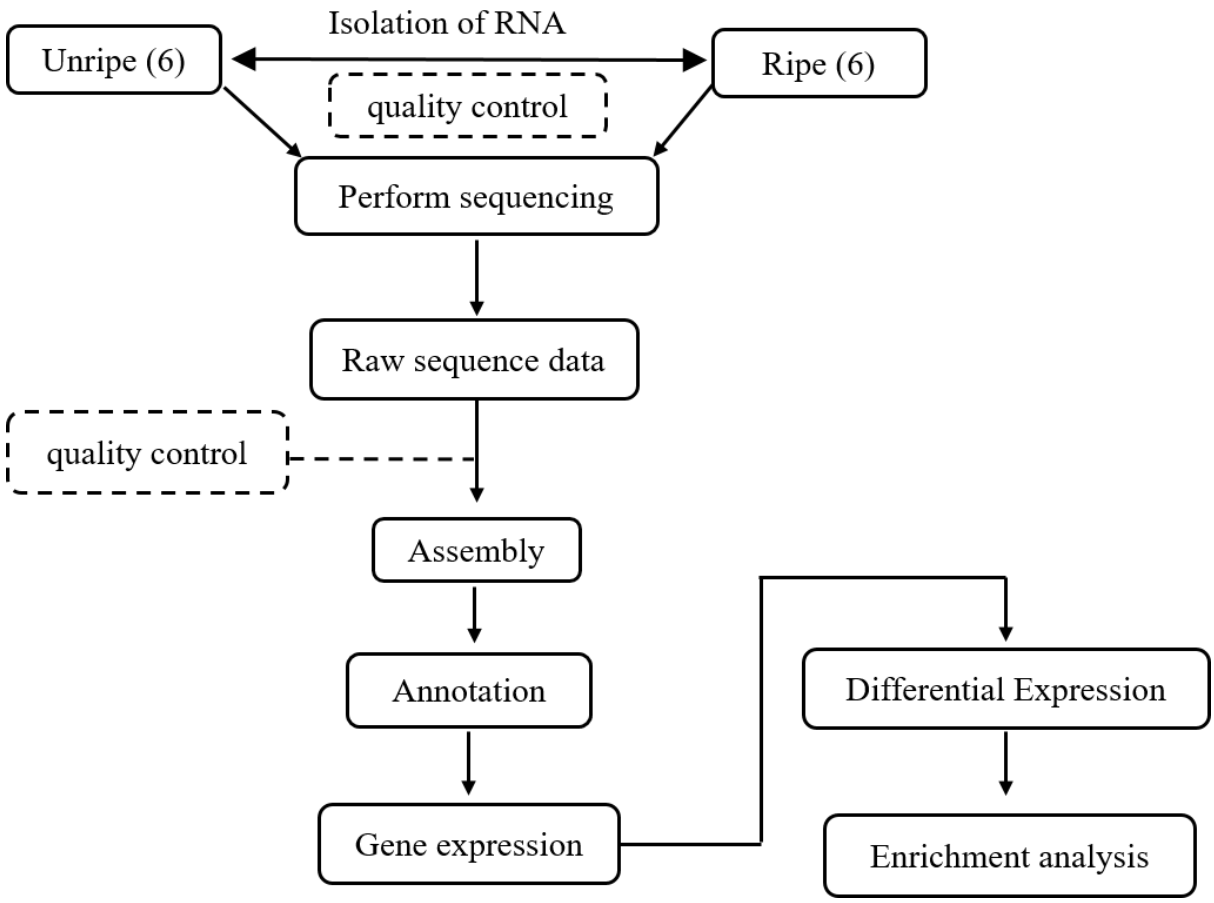


Figure 3.6: The work flow of the RNA-sequencing analysis

3.2.2.10.1 Quality Control and Transcriptome Assembly

The raw reads were initially processed through in-house perl scripts. In this step, adaptor sequences, low quality reads (i.e., those with unknown bases 'N' and reads having a quality score below 20 (Paszkiewicz and Studholme, 2010) were discarded to obtain high quality sequence data. The high quality clean reads were pooled and assembled using Trinity (Grabherr *et al*., 2011) with default settings. Trinity combined reads to form longer fragments called contigs. These contigs were subjected to further processing of sequence clustering using Corset software to remove redundancy (Davidson and Oshlac, 2014). The longest transcript of each cluster was taken as the unigene for further processing. Notably, all reads were combined into a single input for transcriptome assembly to facilitate differential gene analysis and also increase the chances of reconstructing lowly expressed transcripts (Asif *et al*., 2014; Dautt-Castro *et al*., 2015).

3.2.2.10.2 Transcriptome Annotation

To achieve comprehensive gene functional annotation, seven databases were applied: Unigenes were used to search against the NCBI NR database using BlastX (2.2.28+) with an E-value ≤ 10^{-5}. Annotation by the NT database, SwissProt database and KOG database was performed using DIAMOND (version 0.8.22) (Buchfink *et al*., 2015) with E-values ≤ 10^{-5}, ≤ 10^{-5} and ≤ 10^{-3} respectively. Furthermore, the sequences were compared against the Pfam database using HMMER (version 3.0) with an Hmmscan E-value ≤ 0.01. GO annotation of the unigenes were assigned using Blast2GO version 2.5 (Conesa and Götz, 2008) with an E-value ≤ 10^{-6}. Pathway assignments were performed according to the KEGG pathway database using KAAS (KEGG Automatic Annotation Server) (Moriya *et al*., 2007) with an E-value cut-off ≤ 10^{-10}. CDS (coding sequence) was obtained by blast search from the NR database and Swiss-Prot database respectively as well as by using the ESTScan software (Iseli *et al*., 1999).

3.2.2.10.3 Quantification of Gene Expression Levels and Differential Expression Analysis

RNA-seq by Expectation Maximization (RSEM) (Li and Dewey, 2011) was used to estimate the gene expression levels for each sample. RSEM quantifies gene abundances based on the mapping of the high quality RNA-seq clean reads to the assembled transcriptome. A read count for each gene in a sample was obtained from the mapping results. Following this, the normalized gene expression values were derived from the read counts of each gene using the FPKM (Fragments Per Kilo base of gene per Million mapped reads) method. In RNA-seq, FPKM is the most common method for the estimation of gene expression level which takes into account the total gene length and sequencing depth simultaneously (Conesa *et al*., 2016). Genes with FPKM values > 0.3 were considered as being expressed (Hart *et al*., 2013). Pearson's correlation coefficients were determined among the three biological replicates for each sample to assess the reliability of the experiment. Genes that were differentially expressed (adjusted P value < 0.05; $\log_2$ fold change (FC) > 1 or $\log_2$ fold change (FC) < -1) were identified using the DESeq package (Anders and Huber, 2010). The adjusted P value is an essential measure to control the number of false discoveries in the differential gene expression analysis (Benjamini and Hochberg, 1995).

3.2.2.10.4 Enrichment Analysis of Differentially Expressed Genes

GO terms enriched in the differentially expressed genes (DEGs) and altered KEGG pathways were performed using the GOseq package (Young *et al*., 2010) and KOBAS (KEGG Orthology-Based Annotation System) software (Mao *et al*., 2005) respectively.

3.2.2.10.5 Protein-Protein Interaction Analysis

To understand the functional associations between selected differentially expressed genes, a protein-protein interaction network analysis was performed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, version 10.5; (https://string-db.org/) (Szklarczyk *et al*., 2017). 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 (Jensen *et al*., 2009). Differentially expressed genes associated with softening and vesicle trafficking were used as inputs with default parameters. A BLAST search against the *Arabidopsis thaliana* proteins was lodged in the STRING database. Protein interactions with a confidence score ≥ 0.40 were retained in the network (Szklarczyk *et al.*, 2017). 'Nodes' in the network represent the proteins and each pairwise protein interaction, referred to as an 'edge'

3.2.2.11 Reverse-Transcription Quantitative PCR (RT-qPCR)

3.2.2.11.1 Target Gene Selection for RT-qPCR

The target genes analyzed in this study were chosen from the RNA-sequencing data based on their differential expression in pairwise comparisons (mango groups and ripening stages) and the experimental evidence provided in *Arabidopsis thaliana* (Lunn *et al.*, 2013a) and tomato (Lu *et al*., 2001; Lunn *et al.*, 2013b).

3.2.2.11.2 RT-qPCR Reaction

Target and reference genes were analysed with three biological replicates per ripening stage (unripe and ripe) for a single mango group. Each biological replicate per ripening stage for each variety was technically replicated three times. Prior to the quantification of the selected genes of interest, two reference genes (*Actin* and *Ubiquitin*) were selected from a total of four reference genes; *Ubiquitin* (*UBI*), β-*Actin* (*ACT*), α-*Tubulin* (*TUB*) and *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) based on their suitability. All RT-qPCR sample reactions were prepared using the master mix provided in the SENSIFAST^{TM} SYBR Kit (BIOLINE, UK) according to the protocol handbook. The volumes and concentrations of the components for RT-qPCR reaction are outlined in Table 3.5.

Table 3.5: Components of the RT-qPCR reaction

Component	Volume	Final concentration
2× SENSIFAST SYBR qPCR Master Mix	10 µl	1×
10 µM forward primer	0.8 µl	400 nM
10 µM reverse primer	0.8 µl	400 nM
DNase/RNase-free water	8.8 µl	-
Template from RT reaction	0.4 µl	20 ng/reaction
Total volume	20 µl	

Reactions were performed using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad, USA). Reaction conditions were as follows: initial denaturation at 95°C for 2 minutes (no acquisition, i.e. no fluorescence detection), 40 cycles of denaturation (95°C for 5 seconds, no acquisition) and annealing/extension (60°C for 30 seconds, acquisition at the end of step). Directly afterwards, each run was subjected to a melting-curve analysis (65 - 95°C with an increment of 0.2 °C per 10 seconds with fluorescence measured). Three biological replicates and three technical replicates for each biological replicate were analysed per gene for a ripening stage in a mango variety. To reveal the absence of contamination or primer dimers a non-template control (NTC) reaction with each primer pair was run.

3.2.2.11.3 Determination of RT-qPCR Amplification Efficiency

The qPCR amplification efficiency was validated using cDNA samples and the respective primers designed for each gene. Two methods were used to evaluate the amplification efficiencies:

a. Standard curve. Six dilution steps of two-fold (1:2) serial dilutions (Gallup and Ackermann, 2006) were prepared for each cDNA sample using DNase/RNase-free water. Each of the seven dilutions (the 0th to the 6th dilution) were amplified in triplicates. A standard curve was created for each target gene in Microsoft Excel ® and the amplification efficiency, *E*, was calculated from the slope of each concentration curve (Gallup and Ackermann, 2006) as follows:

$$E = (2^{-(1/slope)} - 1) \times 100\ \%$$

b. LinReg PCR software. The efficiency of each primer pair in each individual reaction (i.e. per well) was calculated from the fluorescence values of each amplification plot (Ramakers *et al*., 2003; Ruijter *et al*., 2009). The average amplification efficiency for each primer pair was calculated as the mean across all replicates.

3.2.2.11.4 Analysis of Gene Stability

Expression stability of the four potential reference genes was analyzed using a web based RefFinder tool (Xie *et al*., 2012) which integrates commonly used reference gene evaluation programs together including geNorm, NormFinder, Bestkeeper and the comparative ΔCq methods was used to generate the final overall stability ranking of the tested reference genes.

3.2.2.11.5 Relative Gene Expression Analysis

Gene expression level was determined relative to those of the reference genes to compensate for variations in individual reactions that were caused by initial quantities of cDNA templates. Therefore, the Cq values of the target genes in unripe and ripe samples were normalised with

the geometric Cq mean of two reference genes (*ACT and UBI*). The relative expression of each target gene per sample type was calculated using the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001). The delta Cq (ΔCq) of each sample of interest and control sample ($\Delta Cq_{\text{sample or control}}$) was first calculated by deducting the geometric Cq mean of the reference genes from the Cq mean of the respective sample for the target gene:

$$\Delta Cq \text{ sample} = (Cq \text{ (target, sample)} - CT \text{ (reference, sample)})$$

$$\Delta Cq \text{ control} = (Cq \text{ (target, control)} - CT \text{ (reference, control)})$$

Then, the delta delta Cq of each target gene in the samples of interest (ΔΔCq) was calculated by deducting the ΔCq control from the ΔCq sample:

$$\Delta\Delta Cq = \Delta Cq \text{ sample} - \Delta Cq \text{ control}$$

According to Livak and Schmittgen, (2001), the choice of a control (calibrator) could be any sample based on the user's discretion. In this regard, the comparison of relative expression between the mango varieties was performed with the 'Chokanan' mango variety chosen as the calibrator for all target genes in this study. With that, the relative expression level of the 'Chokanan'mango variety for a target gene was set to 1 and the expression level of other varieties were compared against 'Chokanan'. It is worth pointing out that the choice of 'Chokanan' sample was on the basis that it was a significantly firm variety as compared to other varieties investigated.

4 RESULTS

4.1 CHARACTERIZATION OF THE RIPENING PROCESS OF MANGO VARIETIES 'CHOKANAN', 'GOLDEN PHOENIX' AND 'WATER LILY'.

4.1.1 Weight loss during fruit ripening

A progressive weight loss was observed during the ripening for all the varieties under study (Figure 4.1). 'Chokanan' variety exhibited a 2.30 % weight loss after two days of storage. The highest rate of weight loss (6.98 %) was noticed on the 8th day of ripening for 'Chokanan' (a mean loss of 0.78 % per day). Furthermore, weight loss in 'Golden phoenix' variety significantly increased ($P < 0.05$) from the 2nd (2.76 %) to the 4th day (5.78 %). The percentage weight loss observed on the 4th day was not significantly different ($P < 0.05$) from that obtained on the 6th day of ripening. At the end of storage, 'Golden phoenix' had lost 7.76 % of initial weight with an average of 1.10 % per day. On the other hand, 'Water lily' lost 2.48 % of its initial weight after two days of storage and this was maintained with significant differences ($P < 0.05$) until the 6th day. At the end of storage, it attained an 8.44 % weight loss which averaged 1.40 % per day. Comparing among the varieties, there were no significant differences ($P > 0.05$) in weight loss between the varieties after 2 days of storage (Table 4.1; Appendix IV). However, as ripened progressed the rate of weight loss was greater in varieties 'Golden phoenix' and 'Water lily' compared to 'Chokanan'.

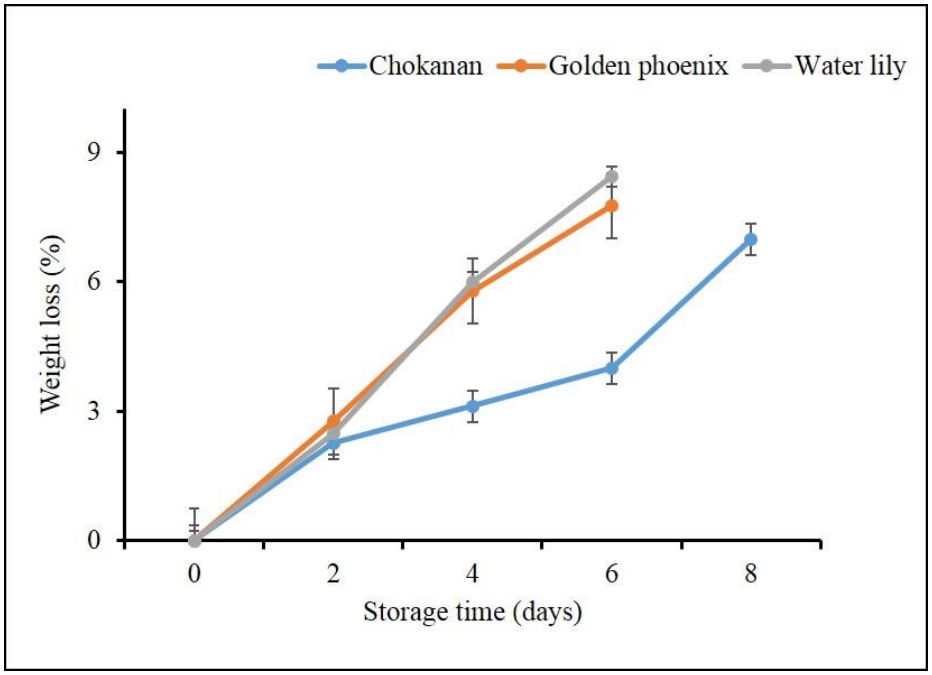


Figure 4.1: Weight loss of three mango varieties during postharvest storage.

Note: Values are means ± SE of four biological replicates.

Table 4.1: Variety effect on weight loss (%) after 2, 4 and 6 days of storage.

Variety	Day 2	Day 4	Day 6
'Chokanan'	2.26a	3.11b	4.00b
'Golden phoenix'	2.76a	5.78a	7.76a
'Water lily'	2.48a	5.99a	8.44a
SEM	0.525	0.677	0.509

4.1.2 Peel colour change during fruit ripening

The external appearance of each mango variety at the beginning and end of storage is presented in Figure 4.2. 'Chokanan' peel colour changed noticeably to yellow as ripening advanced compared to the other two varieties. From Table 4.2, it can be observed that while hue angle decreased, all three colour coordinates (L* a* b*) were increasing during the ripening of mango. The L* value (lightness) of 'Chokanan' was 53.63 on the 0th day of storage and gradually increased as the fruit ripening advanced (Table 4.2). When 'Chokanan' was fully ripened after eight days, there was a significant ($P < 0.05$) increase in lightness (L*) value to 63.78. 'Chokanan' peel colour exhibited a decline in hue angle which started at 118.20 and was maintained with significant differences from the 2nd to 8th day of storage. The lightness (L*) value of the 'Golden phoenix' mango peel increased beginning on the 2nd day and presented no significant changes until the end of storage. Similarly, there was a gradual increase in peel a* value beginning on the 2nd day and higher b* values on day four (Table 4.2). Meanwhile, hue angle dropped progressively from 119.03 to 108.61 during the ripening period. In 'Water lily' variety, hue angle decreased from 120.40 to 103.30 with significant differences ($P < 0.05$) between the storage days (Table 4.2). A progressive increase in peel a* value beginning on day two and higher L* value on day four was observed. Similarly, an increasing trend was observed for b* values with significant differences ($P < 0.05$) between storage time. Altogther, ripening had a significant effect ($P < 0.05$) (Appendix IV) on the postharvest quality attributes on the varieties. Furthermore, variety also had a significant effect ($P < 0.05$) on the colour of mangoes (Table 4.3; Appendix IV). With respect to peel colour, 'Golden phoenix' and 'Water lily' were similar in L* value and remained significantly lower than 'Chokanan'. The b* values significantly increased in the order of 'Water lily', 'Chokanan' and 'Golden phoenix' after 6 days. Although a decrease in hue angle was observed throughout the storage period, no

significant difference was observed between varieties from the 0th to 4th day. However, by the 6th day, the hue value of 'Chokanan' was significantly lower which is consistent with its increased yellow peel coloration. Overall, the peel colour of the mango varieties under study became lighter (higher L* values), less green (increased a* values) and tended to be more yellow (increased b* values) as ripening time progressed.

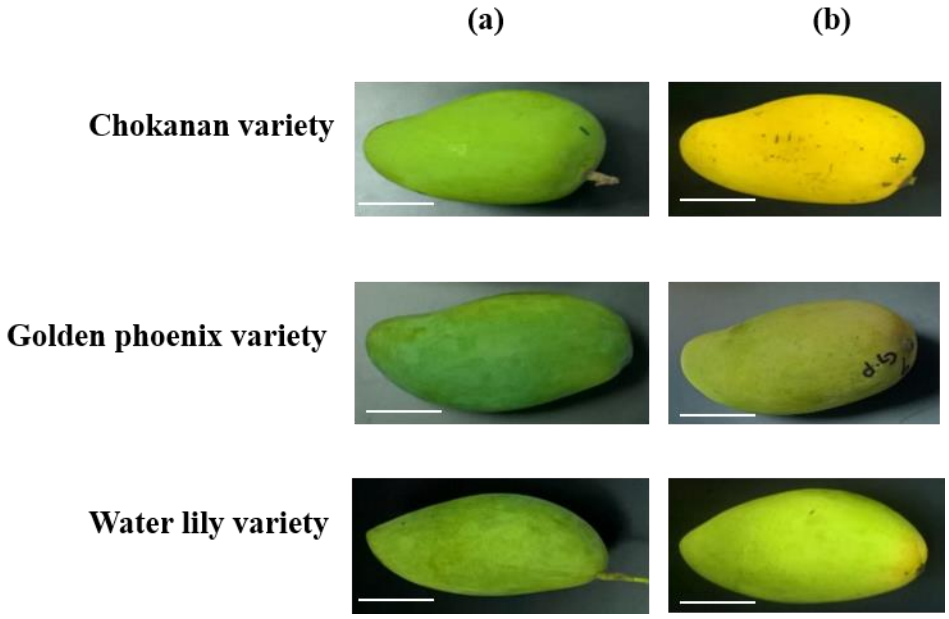


Figure 4.2: External peel colour appearance of mango varieties.

Note: (a) Fruit samples on arrival (day 0) and (b) samples at the end of storage (8th day for 'Chokanan' and 6th day for 'Golden phoenix' and 'Water lily' respectively.
(Scale bar = 2 cm) Published in Lawson *et al*., (2019).

Table 4.2: Peel colour changes in mango (*Mangifera indica*) varieties ('Chokanan', 'Golden phoenix' and 'Water lily')

Variety/ storage time	Hue	a* value	L* value	b* value
‘Chokanan’				
0	118.20a	-16.33d	53.63d	30.75d
2	116.61a	-15.72d	58.46c	34.53c
4	107.38b	-12.76c	60.22bc	41.38b
6	101.45c	-8.96b	62.53ab	43.51b
8	89.63d	-1.31a	63.78a	53.27a
SEM	1.133	0.492	1.086	1.204
‘Golden phoenix’				
0	119.03a	-15.71c	49.38b	28.70b
2	116.10b	-14.74bc	54.80a	30.40b
4	110.49c	-13.68ab	54.83a	36.94a
6	108.61c	-12.64a	57.59a	37.96a
SEM	1.005	0.482	0.946	1.471
‘Water lily’				
0	120.40a	-17.53c	49.00b	29.29d
2	117.00b	-17.13bc	52.65b	33.40c
4	110.50c	-15.38b	57.85a	41.20b
6	103.30d	-11.31a	57.97a	47.84a
SEM	0.844	0.611	1.228	0.763

Note**:** L*, a* and b* indicate lightness, indexes of red/green and yellow/blue colour of fruit respectively. Hue describes the visual colour of the fruit. Values are means of four replicates. Different letters within each column for each mango variety are significantly different across the storage time ($P < 0.05$) (Appendix IV).

Table 4.3: Effect of mango variety on peel coloration after 6 days of postharvest storage.

Day 0				
Variety	Hue	L*	a*	b*
'Chokanan'	118.22a	53.63a	-16.33ab	30.75a
'Golden phoenix'	119.03a	49.38b	-15.71a	28.70a
'Water lily'	120.42a	49.00b	-17.53b	29.90a
SE	0.935	0.832	0.474	1.463
Day 2				
Variety	Hue	L*	a*	b*
'Chokanan'	116.61a	58.46a	-15.72a	34.53a
'Golden phoenix'	116.10a	54.80b	-14.74a	30.40b
'Water lily'	117.00a	52.65b	-17.13b	33.89a
SE	1.034	1.004	0.340	0.894
Day 4				
Variety	Hue	L*	a*	b*
'Chokanan'	107.38a	60.22a	-12.76a	41.38a
'Golden phoenix'	110.49a	54.83b	-13.68ab	36.94b
'Water lily'	110.54a	57.85ab	-15.38b	41.10ab
SE	1.178	1.184	0.667	1.314
Day 6				
Variety	Hue	L*	a*	b*
'Chokanan'	98.95c	62.53a	-8.56a	43.51b
'Golden phoenix'	108.61a	57.59b	-12.64b	37.96c
'Water lily'	103.29b	57.92b	-11.31b	48.20a
SEM	1.288	1.008	0.707	1.106

Note**:** L*, a* and b* indicate lightness, indexes of red/green and yellow/blue colour of fruit respectively. Hue describes the visual colour of the fruit. Values are means of four replicates per variety. SEM: standard error of the mean. Mean values in the same column for each colour attribute per storage day followed by different letters are significantly different among the mango varieties ($P < 0.05$) (Appendix IV).

4.1.3 Changes in pulp firmness during fruit ripening

With storage, a loss of pulp firmness was observed in all mango varieties under study (Figure 4.3). Firmness of 'Chokanan' decreased significantly ($P < 0.05$) during storage from 138.18 N to 12.67 N after eight days. There were no significant changes in firmness during the first two days. 'Chokanan' presented a rapid loss of firmness (82.86 %) between the 2nd and 6th day of storage, with slow changes thereafter. In 'Golden phoenix', decline in firmness which started at 109.22 N was maintained with significant differences ($P < 0.05$) between sampling points. A significant decrease had begun on the second day by up to 36.02 % for 'Golden phoenix'. Firmness values at the end of storage (9.53 N) resulted in the loss of 91.27 % of the firmness recorded at the beginning of the study. Firmness of 'Water lily' decreased significantly during storage from 104.47 to 7.50 N after six days. A sharp decline was observed until the 4th day of ripening (16.61 N, 84.11 % loss), whereas from the 4th to 6th day firmness remained negligible. At the end of the ripening period, 'Water lily' had lost 92.82 % of its initial firmness. Furthermore, variety had an impact on the firmness, it was recorded that 'Chokanan' variety showed a higher pulp firmness than the other two varieties through the storage period. 'Golden phoenix' and 'Water lily' were similar in their firmness values (Table 4.4; Appendix IV).

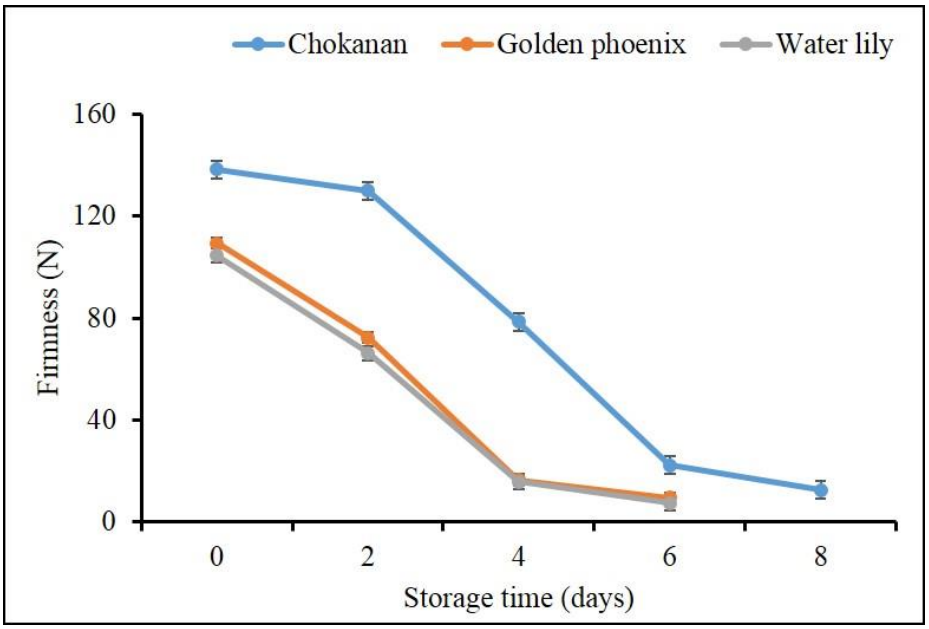


Figure 4.3: Changes in pulp firmness of three mango varieties during ripening

Note: Values are means ± SE of four biological replicates.

4.1.4 Changes in Soluble Solids Concentration (SSC) and Titratable Acidity (TA) during fruit ripening

As observed, the SSC value increased (Figure 4.4) while TA declined (Figure 4.5) during storage regardless of the variety. The initial SSC value for 'Chokanan' was 6.83 % and it peaked at 16.80 % on the 8th day of storage when the fruit was ripe. SSC did not present much variation between storage days. TA decreased from 1.05 % on day zero to 0.26 % on the 8th day of ripening. SSC value in 'Golden phoenix', which started at 7.18 % was maintained with significant differences between the days of ripening. However, on the 6th day of storage the highest SSC value (20.30 %) was observed. A decrease in TA was recorded for 'Golden phoenix' from 0.69 % to 0.25 % which was not statistically significant during the ripening period. Furthermore, 'Water lily' presented an increase in SSC value beginning on day four until the end of the storage. However, changes in SSC were negligible between the days four

and six. While SSC increased, TA decreased from 0.34 % to 0.12 % after six days of ripening. Taken together, a significant effect of ripening on the SSC and TA was observed (Appendix IV). In varietal comparison, 'Water lily' had given significantly lower values for TA compared to varieties 'Golden phoenix' and 'Chokanan' over the four day storage period. However, all varieties were similar for SSC values on days 0 and 2 respectively (Table 4.4; Appendix IV).

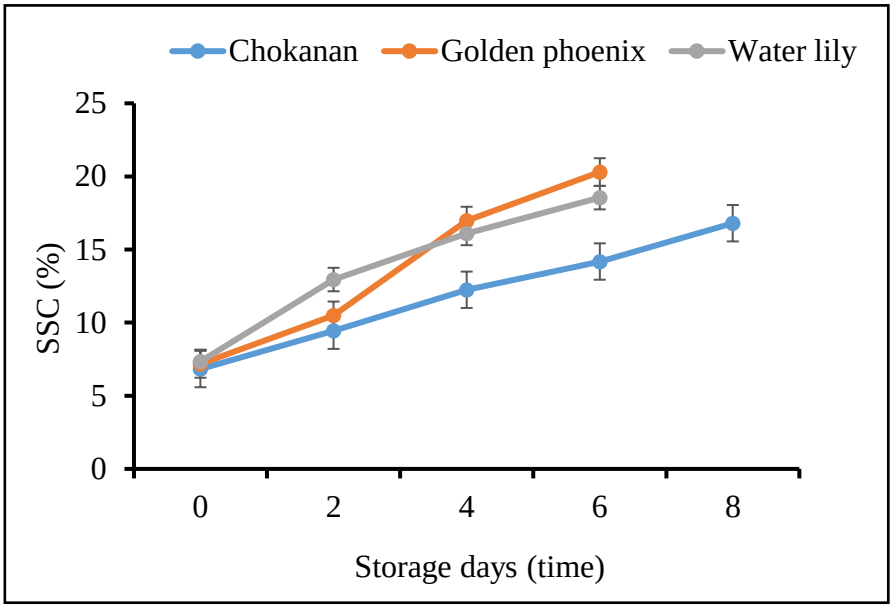


Figure 4.4: Changes in soluble solid concentration (SSC) of three mango varieties during ripening.

Note: Values are means ± SE of four biological replicates.

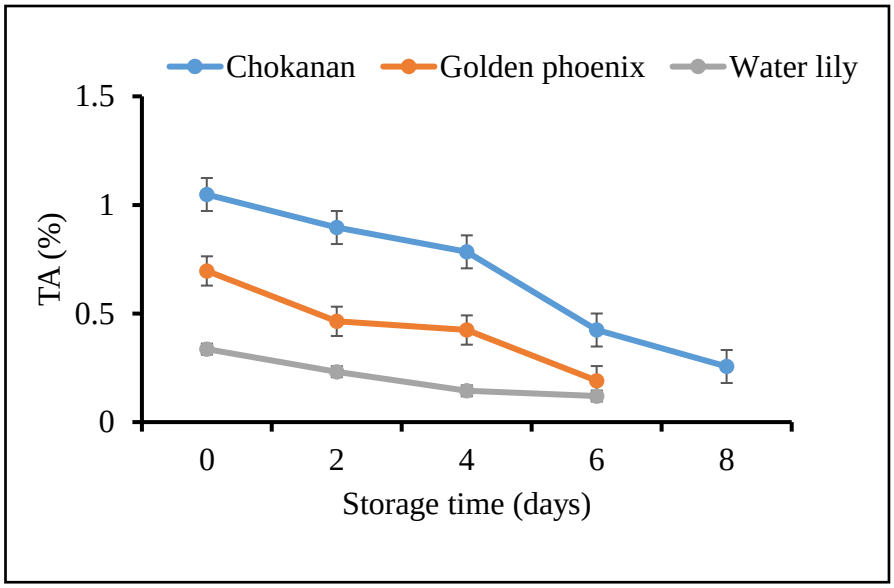


Figure 4.5: Changes in titratable acidity (TA) of three mango varieties during ripening.

Note: Values are means ± SE of four biological replicates.

4.1.5 Respiration rate and ethylene production during fruit ripening

A typical climacteric pattern of respiration (Figure 4.6) and ethylene production (Figure 4.7) was observed in all mango varieties during ripening. In 'Chokanan', the highest production of CO_2 was observed on the 6th day of storage at 590.41 nmol kg^{-1} s^{-1} when the fruit exhibited a more yellow peel colour. Ethylene production also peaked on the 6th day with a maximum value of 0.01 nmol kg^{-1} s^{-1} and decreased afterwards. On the other hand, CO_2 production in 'Golden phoenix' was 287.50 nmol kg^{-1} s^{-1} on day zero reaching a maximum of 940.20 nmol kg^{-1} s^{-1} on the 4th day. This was followed by a decrease to 778.80 nmol kg^{-1} s^{-1} on the sixth day. Maximum production of ethylene was observed in fruit from the 4th day (0.01 nmol kg^{-1} s^{-1}). In 'Water lily', maximum CO_2 and ethylene production were observed on the 4th day at 1118.01 nmol kg^{-1} s^{-1} and 0.01 nmol kg^{-1} s^{-1} respectively. Altogether, ripening had a significant effect (Appendix IV) on the respiration and ethylene production during postharvest storage. Comparing among the varieties, 'Chokanan' exhibited a significantly lower respiration

rate than the other two varieties after 6 days (Table 4.4; Appendix IV). 'Water lily' had the highest ethylene production among the tested varieties on day 0. However, this variety became similar with 'Golden phoenix' on the 2nd day while 'Chokanan' remained low. All varieties had similar rates of ethylene production on the 4th and 6th day respectively.

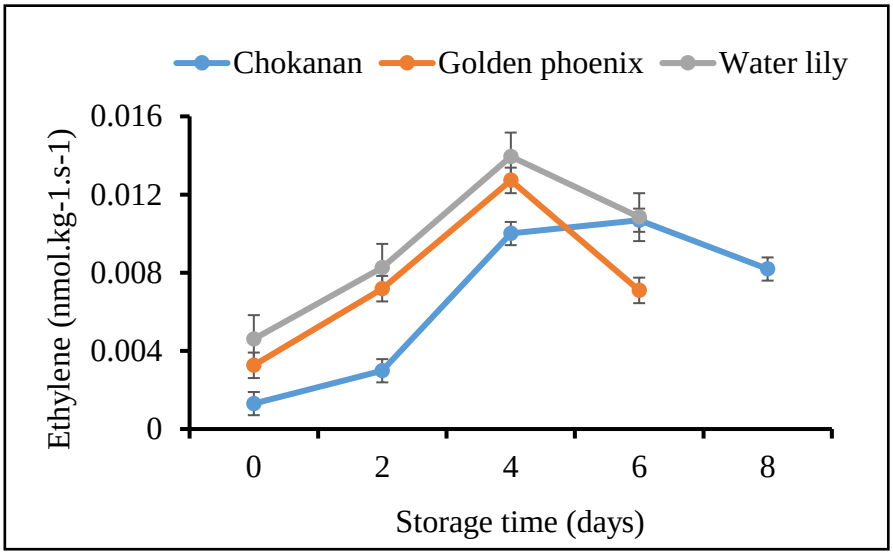


Figure 4.6: Respiratory pattern of three mango varieties during ripening.

Note: Values are means ± SE of four biological replicates.

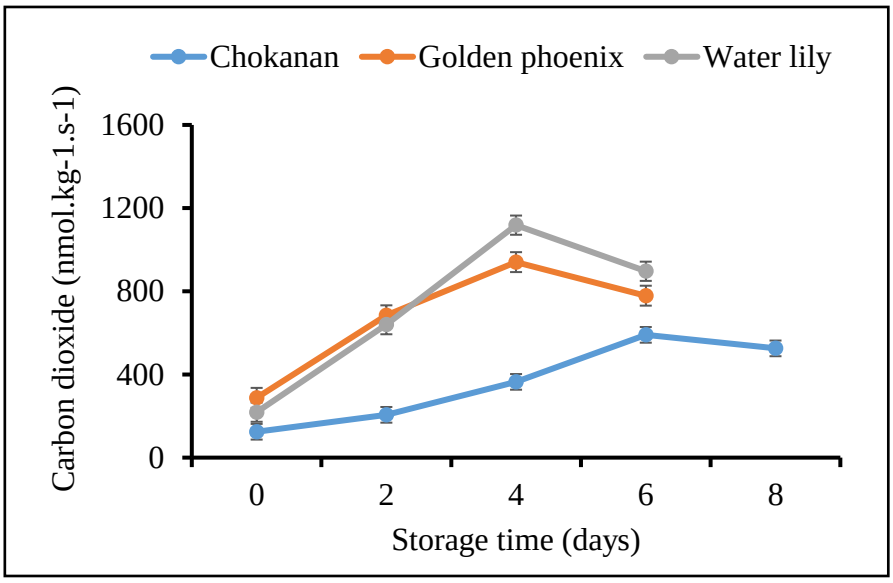


Figure 4.7: Ethylene production of three mango varieties during ripening.

Note: Values are means ± SE of four biological replicates.

Table 4.4: Effect of mango variety on the firmness, SSC, TA, respiration and ethylene production during storage.

Day 0					
Variety	Firmness (N)	SSC (%)	TA (%)	CO_2 (nmol kg/h)	Ethylene (nmol kg/h)
'Chokanan'	138.21a	6.83a	1.05a	124.40c	0.001c
'Golden phoenix'	109.20b	7.18a	0.70b	287.50a	0.003b
'Water lily'	104.53b	7.35a	0.34c	218.60b	0.005a
SEM	2.920	0.502	0.086	18.11	0.0003
Day 2					
Variety	Firmness (N)	SSC (%)	TA (%)	CO_2 (nmol kg/h)	Ethylene (nmol kg/h)
'Chokanan'	128.55a	9.45a	0.90a	205.80b	0.003b
'Golden phoenix'	72.30b	10.50a	0.52b	684.51a	0.007a
'Water lily'	66.33b	12.95a	0.23c	639.42a	0.008a
SEM	3.110	1.178	0.043	35.40	0.001
Day 4					
Variety	Firmness (N)	SSC (%)	TA (%)	CO_2 (nmol kg/h)	Ethylene (nmol kg/h)
'Chokanan'	78.41a	12.25b	0.78a	364.30c	0.010a
'Golden phoenix'	16.61b	16.98a	0.36b	940.20b	0.013a
'Water lily'	15.91b	16.10a	0.14c	1118.01a	0.014a
SEM	3.420	1.015	0.067	45.20	0.001
Day 6					
Variety	Firmness (N)	SSC (%)	TA (%)	CO_2 (nmol kg/h)	Ethylene (nmol kg/h)
'Chokanan'	22.25a	14.18b	0.42a	590.41c	0.011a
'Golden phoenix'	9.53b	20.30a	0.25b	778.80b	0.011a
'Water lily'	7.51b	18.55a	0.12b	896.21a	0.011a
SEM	1.990	1.055	0.036	35.70	0.002

Note**:** SSC, soluble solid concentration; TA, titratable acidity; CO_2, carbon dioxide. Values are means of four replicates per variety. SEM: standard error of the mean. Mean values in the same column for each attribute followed by different letters are significantly different among the mango varieties ($P < 0.05$) (Appendix IV).

4.1.6 Multivariate Studies

An investigation of all variables simultaneously was essential to fully explore the evolution of postharvest quality parameters during ripening (Table 4.5). That SSC was negatively correlated with firmness ($r = -0.868$, $P < 0.001$) and TA ($r = -0.637$, $P < 0.05$) can be explained by the observation that as ripening progresses the fruit becomes less firm and acidic respectively. Respiration showed a significant positive correlation with weight loss ($r = 0.699$, $P < 0.05$). This is expected because as respiration rate increases, water loss increases causing a loss of weight. Firmness showed a significant negative correlation with both ethylene production ($r = -0.851$, $P < 0.001$) and respiration ($r = -0.827$, $P < 0.001$). Thus, a respiratory climacteric and ethylene outburst will correspond to a lower firmness. A significant negative correlation between hue angle and the colour coordinates (a*, b* and L* values) would be expected because as a mango fruit ripens, it tends to be brighter (increased L* values), less green (increased a* values) and more yellow (increased b* values). On the other hand, there were weak correlations between physiological parameters (respiration and ethylene) and colour attributes (hue, a* and L* values).

Furthermore, to allow for a global study of the parameters that describe the ripening process, a principal component analysis was carried out. The results are presented as a biplot which represents information of the observations and the variables on the same plane (Figure 8). The observations were well separated on the biplot. Component 1 and 2 captured 66.50 % and 19.10 % of the variance respectively (Appendix IV). The first component separated them according to their ripening stages, with samples from early storage time located at the left hand side and late storage time on the right hand side. As ripening advanced, there was a shift from left to right along component 1 with increase in SSC, weight loss, ethylene and respiration rate. The second component showed separation related to the variety effect, with ‘Chokanan’ samples at the top (increased L* and a*) and the other varieties on the lower region (high hue

values). However, no clear demarcation was achieved for 'Waterlily' and' Golden phoenix' varieties along the second component. Further confirmation was carried out using cluster analysis (not shown). The dendrogram also demonstrated the same clustering of samples as observed in biplot. The similar grouping of samples from the biplot and cluster analysis provides further confidence in these findings.

Table 4.5: Correlation matrix among the postharvest parameters of the three mango varieties during ripening.

	CO_2	Ethylene	Firmness	Hue	a* value	L value	b* value	TA	SSC	WL
CO_2	**1**	**0.844**	**-0.827**	-0.255	0.156	0.178	0.477	**-0.604**	**0.691**	**0.699**
Ethylene		**1**	**-0.851**	-0.470	0.308	0.388	**0.727**	**-0.592**	**0.686**	**0.765**
Firmness			**1**	**0.667**	**-0.570**	**-0.485**	**-0.808**	**0.785**	**-0.868**	**-0.893**
Hue				**1**	**-0.957**	**-0.910**	**-0.728**	0.336	**-0.618**	**-0.673**
a* value					**1**	**0.845**	**0.559**	-0.246	0.474	0.499
L value						**1**	**0.583**	-0.099	0.513	**0.571**
b* value							**1**	**-0.637**	**0.756**	**0.891**
TA								**1**	**-0.637**	**-0.652**
SSC									**1**	**0.928**
WL										**1**

Note: Significant correlation ($P < 0.05$) are in bold. CO_2, carbon dioxide (respiration); SSC, soluble solid concentration; TA, titratable acidity; WL, weight loss.

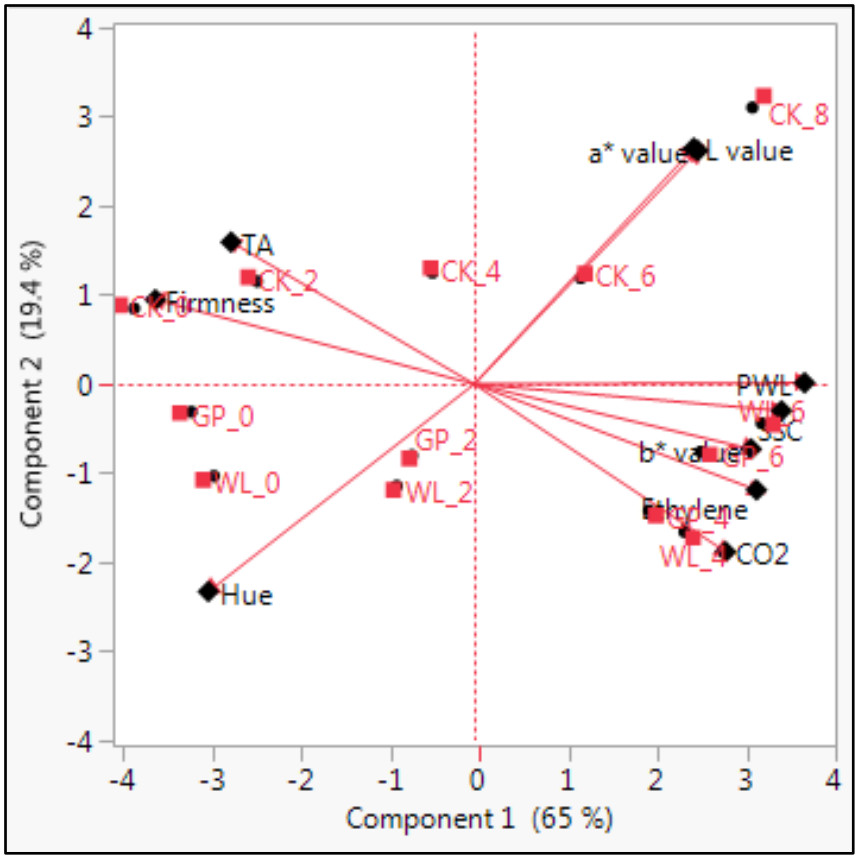


Figure 4.8: PCA biplot showing the relationship between the mango samples and the postharvest quality parameters during ripening

Note: The mango samples at different storage time (observations) are represented by blue square boxes while lines with diamond (black) represent the postharvest quality parameters (variables). CK, 'Chokanan'; GP, 'Golden phoenix'; WL, 'Water lily'. 0, (day 0); 2, (day 2); 4, (day 4); 6, (day 6); 8, (day 8). CO_2, carbon dioxide (respiration); SSC, soluble solid concentration; TA, titratable acidity.

4.2 CHARACTERIZATION OF THE RAB GTPASE FAMILY IN MANGO BY COMPARATIVE ANALYSIS

4.2.1 Identification of the Rab GTPase Family

To identify the genes encoding the Rab family in mango, the published *Rab* gene sequences (Accession Z71276.1, KF768563) (Liu *et al*., 2014; Zainal *et al*., 1996) were used as queries to BLAST the mango RNA-sequencing database (Tafolla-Arellano *et al*., 2017). More than fifty sequences were identified that displayed similarity to the query sequences. Each sequence was used to carry out a BLASTX search against the *Arabidopsis* information resource (TAIR; https://www.arabidopsis.org) to further confirm their 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 work is presented together with the closest corresponding *Arabidopsis* genes (Table 4.6) and the corresponding mango Rab cDNA sequences can be found in Appendix V. It is worth pointing out that 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 showed similarity to the same *Arabidopsis RabA* gene.

Table 4.6: Putative Rab GTPases identified in this study from mango (*Mangifera indica* L.) and their closest *Arabidopsis* homologues

Mango Rabs	*Arabidopsis* closest member	*Arabidopsis* AGI no
RabA1-1	*AtRABA1a*	At1g06400
RabA1-2	*AtRABA1f*	At5g60860
RabA1-3	*AtRABA1c*	At5g45750
RabA1-4	*AtRABA1f*	At5g60860
RabA2-1	*AtRABA2a*	At1g09630
RabA2-2	*AtRABA2b*	At1g07410
RabA3	*AtRABA3*	At1g01200
RabA4-1	*AtRABA4a*	At5g65270
RabA4-2	*AtRABA4c*	At5g47960
RabA5-1	*AtRABA5a*	At5g47520
RabA5-2	*AtRABA5c*	At2g43130
RabA6	*AtRABA6b*	At1g18200
RabB	*AtRABB1B*	At4g35860
RabC	*AtRABC1*	At1g43890
RabD-1	*AtRABD2C*	At4g17530
RabD-2	*AtRABD2A*	At1g02130
RabE-1	*AtRABE1A*	At3g46060
RabE-2	*AtRABE1E*	At3g09900
RabF-1	*AtRABF1*	At3g54840
RabF-2	*AtRABF2A*	At5g45130
RabF-3	*AtRABF2A*	At5g45130
RabG	*AtRABG3A*	At4g09720
RabH	*AtRABH1B*	At2g44610

Note: AGI, *Arabidopsis* Genome Initiative number

4.2.2 Similarity Analysis of Rab GTPases from Mango, Tomato and *Arabidopsis*

Examination of the resulting tree (Figure 4.9) indicated that the mango Rabs can be grouped into eight subgroups as reported in *Arabidopsis* (Vernoud *et al.*, 2003; Rutherford and Moore, 2002). 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. The Rab sequences as shown in Figure 4.9 do not cluster in a species specific manner but rather within clades supporting the findings of previous authors (Tyler, 2016; Zhang *et al*., 2007). According to the grouping of the members in the tree combined with the function of reported genes in the same group, it can be speculated that the members of the same cluster display similar functions (Zhang *et al*., 2007).

4.2.3 Rab Sequence Comparison

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. Sequences of *Arabidopsis* members that are representative of each subfamily were included for reference purposes. The mango Rab sequences contained conserved GTPase regions (G1-G5) present in all members of the Ras superfamily (Figure 4.10). The presence of the amino acid stretches (termed F1–F5) which are diagnostic for Rab family members as described by Pereira-Leal and Seabra (2000) was also observed. In addition, the C-terminal region containing the hypervariable region and the cysteine motif could be seen in the Rab GTPases.

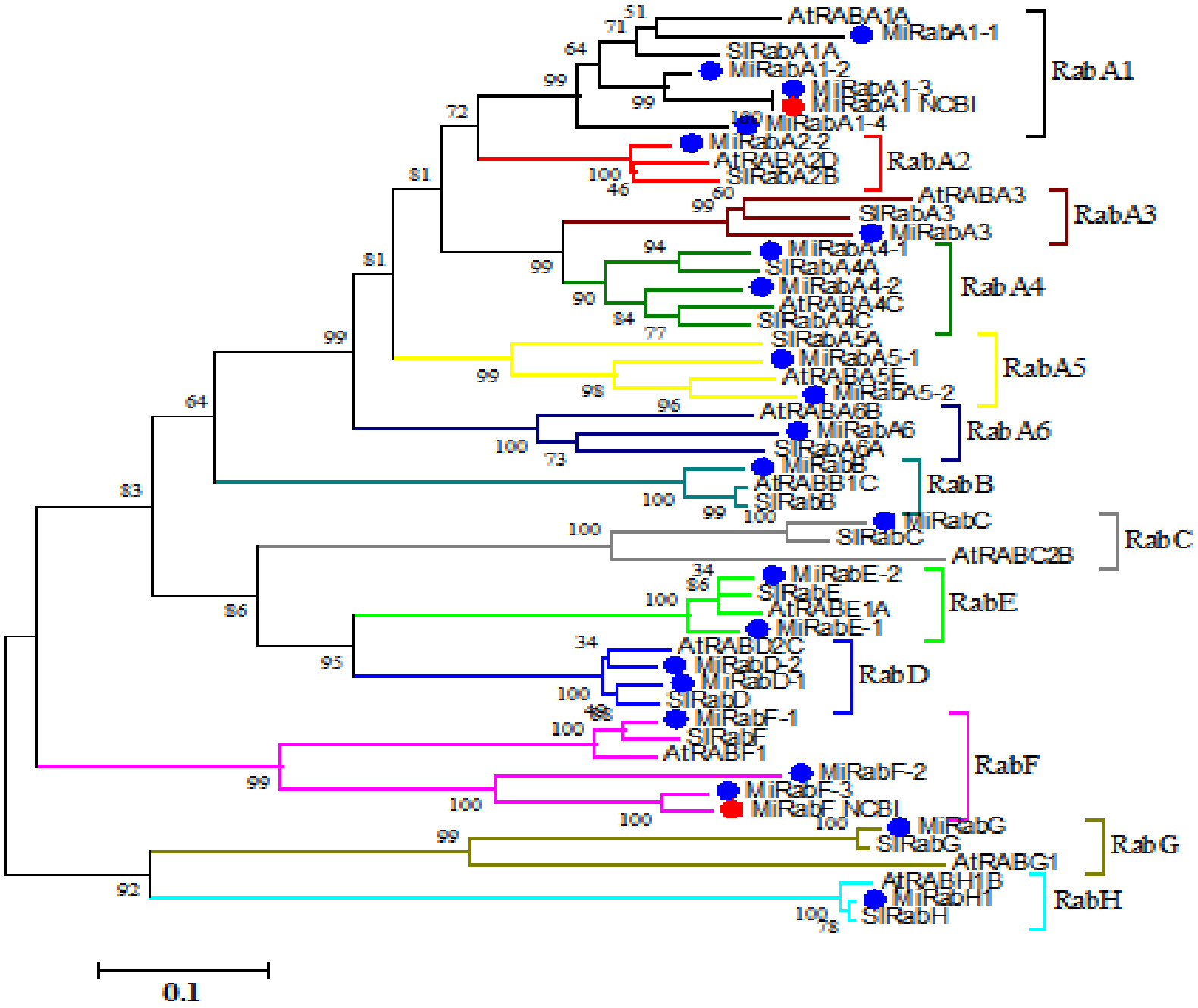


Figure 4.9: Similarity tree of Rab protein sequences from mango, tomato and *Arabidopsis thaliana*.

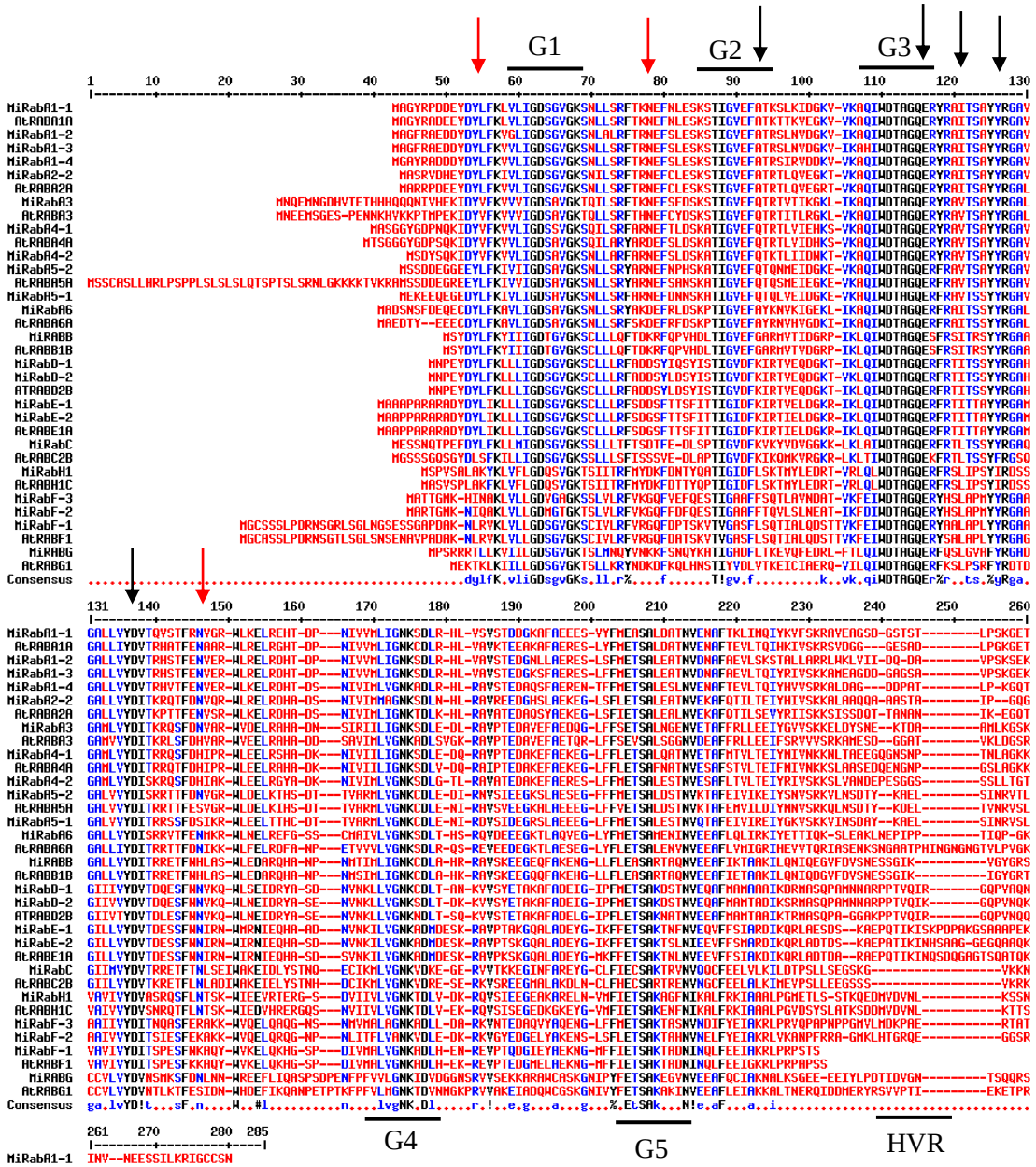


Figure 4.10: Multiple sequence alignment of Rab protein sequences from mango and *Arabidopsis*.

Note: Conserved motifs named 'G box' sequences are identified and indicated with black lines (G1, G2, G3, G4, and G5) (according to Jiang and Ramachandran, 2006. Residues highlighted in black indicate 100 % similarity; blue indicates 50 % similarity; red indicates < 50 % similarity. HVR, hypervariable region. Rab family (F) and Rab subfamily (SF) regions (defined according to Pereira-Leal and Seabra, 2000) are indicated by black and red arrows respectively. Sequence alignment was generated by MultiAlin software (Corpet, 1988).

4.3 ISOLATION OF TOTAL RNA FOR MOLECULAR STUDIES

4.3.1 Assessment of Quality and Quantity of Extracted RNA from Mango Pulp

The aim of this study was to find the most suitable RNA isolation method to obtain high quality RNA from mango pulp tissues. Prior to the use of the traditional cetyl trimethylammonium bromide (CTAB) method, commercially available TRIzol reagent (Invitrogen, USA) and RNeasy® mini kit (Qiagen, Germany) were tested individually and as a hybrid of the two but none of these worked well with mango fruit tissues. With TRIzol reagent, the yield was high (~1000 ng) but the purity of the extracted total RNA was insufficient. The A260/230 ratios were low (all below 1.0) and did not improve with multiple ethanol precipitations. With RNeasy® mini kit or TRIzol+ RNeasy® mini kit methods, the yield was very low (~200 ng) or absorbance ratios A260/280 and A260/230 consistently ranged between 0.22 – 1.32 and 0.22 – 0.95 respectively. The integrity of the RNA from these extraction methods could not be assessed by the Bioanalyser instrument due to the low sample concentration and/or low purity. These methods were discontinued as total RNA quality and quantity obtained did not meet the minimum requirement. A high quality RNA was obtained using the traditional CTAB method and this was confirmed in several ways. Agarose gel electrophoresis showed that ribosomal bands of 25S and 18S were intact and bright, with no visible signs of degradation (Figure 4.11). Genomic DNA contamination may appear as high molecular weight bands in agarose gels but as observed in Figure 4.11, there were no visible bands. Nanodrop results showed that the A260/A280 and A260/A230 ratios were ~2.0 for all samples tested (Table 4.7). This indicated that the isolated RNA was of high purity and free of contaminants such as proteins and polysaccharides (Ma *et al*., 2015; Sambrook *et al*., 1989). Analysis using the Agilent 2100 Bioanalyser confirmed high quality RNA (RIN > 7.0) (Figure 4.19). The electropherogram of the total RNA and the generated gel images showed clear peaks of ribosomal RNAs bands (Figure 4.12). As the performance of CTAB procedure was consistent and producing RNAs

with quality that met the requirements for downstream molecular experiments, this method was chosen to extract total RNA from mango pulp throughout the study.

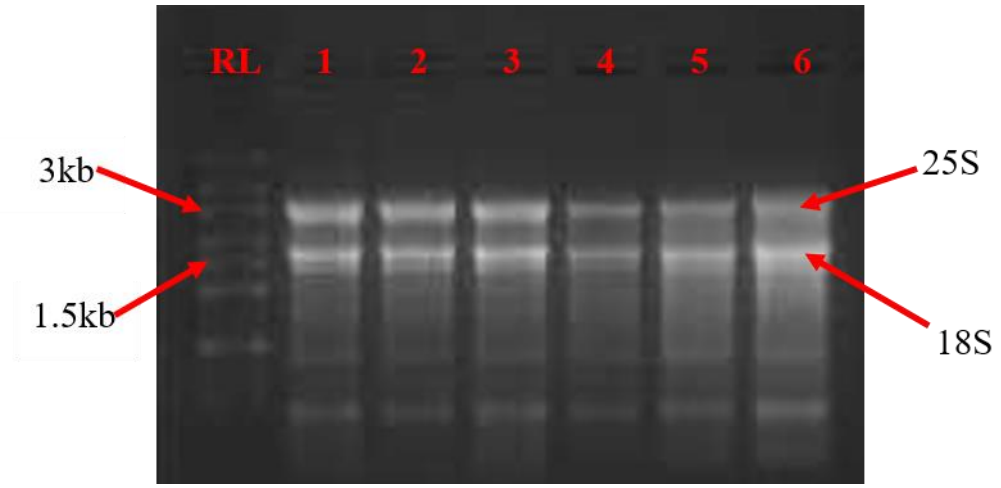


Figure 4.11: Representative total RNA samples separated on agarose gel.

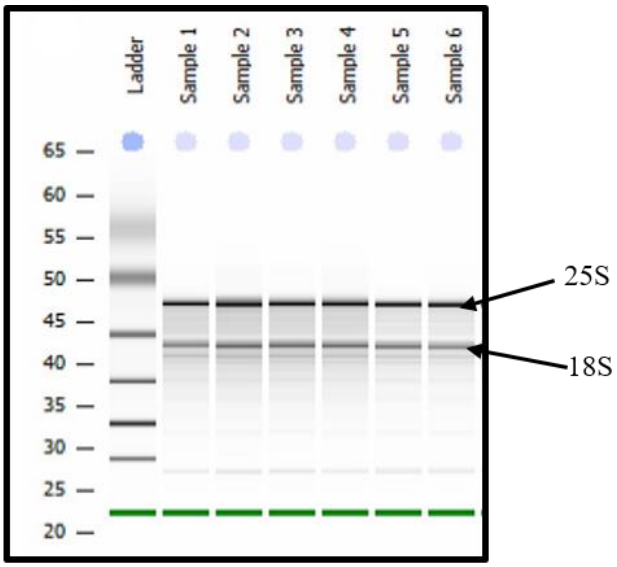
Note: RL, RiboRuler high range RNA ladder (Thermo Scientific, USA); Lanes 1-3, unripe samples and Lanes 4-6, ripe samples from mango ('*Mangifera indica*' variety 'Chokanan') fruit.

Table 4.7: Concentration and purity of isolated RNA samples analysed.

Sample	Concentration (ng/ µl)	A260/280 ratio	A260/230 ratio
1	128.80	2.01	2.39
2	241.44	2.02	2.37
3	319.63	2.04	2.38
4	270.21	2.05	2.14
5	421.98	2.09	2.21
6	231.26	2.03	2.24

*Samples labelled 1-3 represent isolated RNA from unripe samples; 4-6 represent isolated RNA from ripe samples of 'Chokanan' mango variety

(a)



(b)

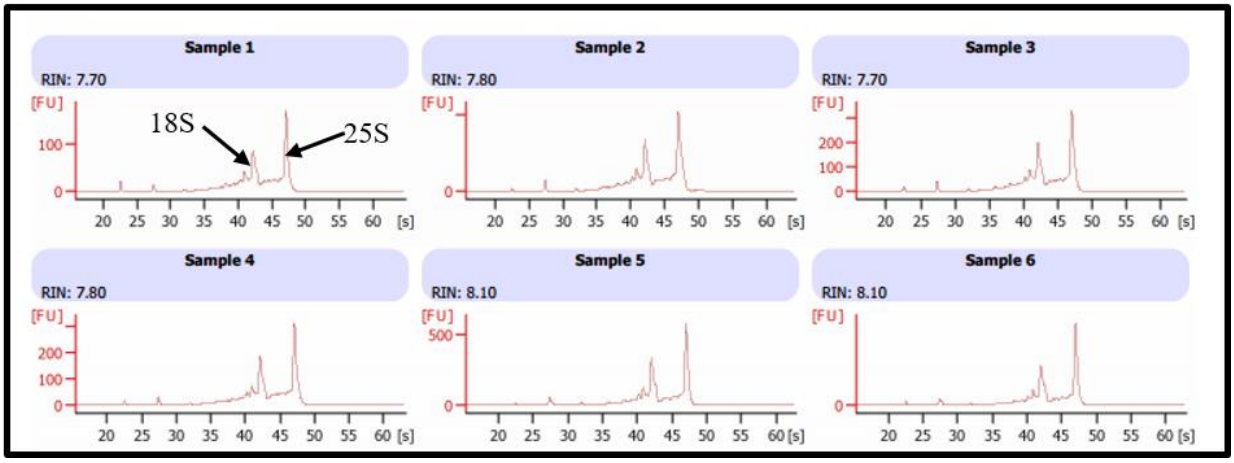


Figure 4.12: Gel like images (a) and electropherogram (b) generated from the isolated RNA samples using the Agilent 2100 Bioanalyzer.

Note: RIN values are indicated at the top left corner.

4.3.2 RNA Quality Assessment by RT-PCR Amplification

The quality of the extracted total RNA was further assessed by RT-PCR. If total RNA is a poor template for reverse transcription, the amplification process can be negatively affected (Tong *et al*., 2011). To determine its suitability for this purpose, the isolated RNA was used as a template for reverse transcription followed by PCR amplification of RabA GTPase cDNA fragments. *Actin* gene was included as a positive control. In addition, genomic DNA contamination of RNA samples was further assessed in RT-PCR reactions where reverse transcriptase was omitted. All tested genes were amplified using gene specific primers designed in this study (Appendix III). Amplicon sizes: 520 bp for *RabA1*, 700 bp for *RabA2*, 650 bp for *RabA3*, 400 bp for *RabA4*, 495 bp for *RabA5*, 550 bp for *RabA6* and 120 bp for *Actin* were observed (Figure 4.13a). Amplification did not occur in samples without reverse transcriptase (Figure 4.13b) indicating the absence of significant genomic DNA contamination. Furthermore, these PCR products were purified and then sequenced. Sequence alignment using the *Arabidopsis* information resource database (TAIR, https://www.arabidopsis.org) confirmed the identity of the RT-PCR products as genes coding for the Rab GTPase family.

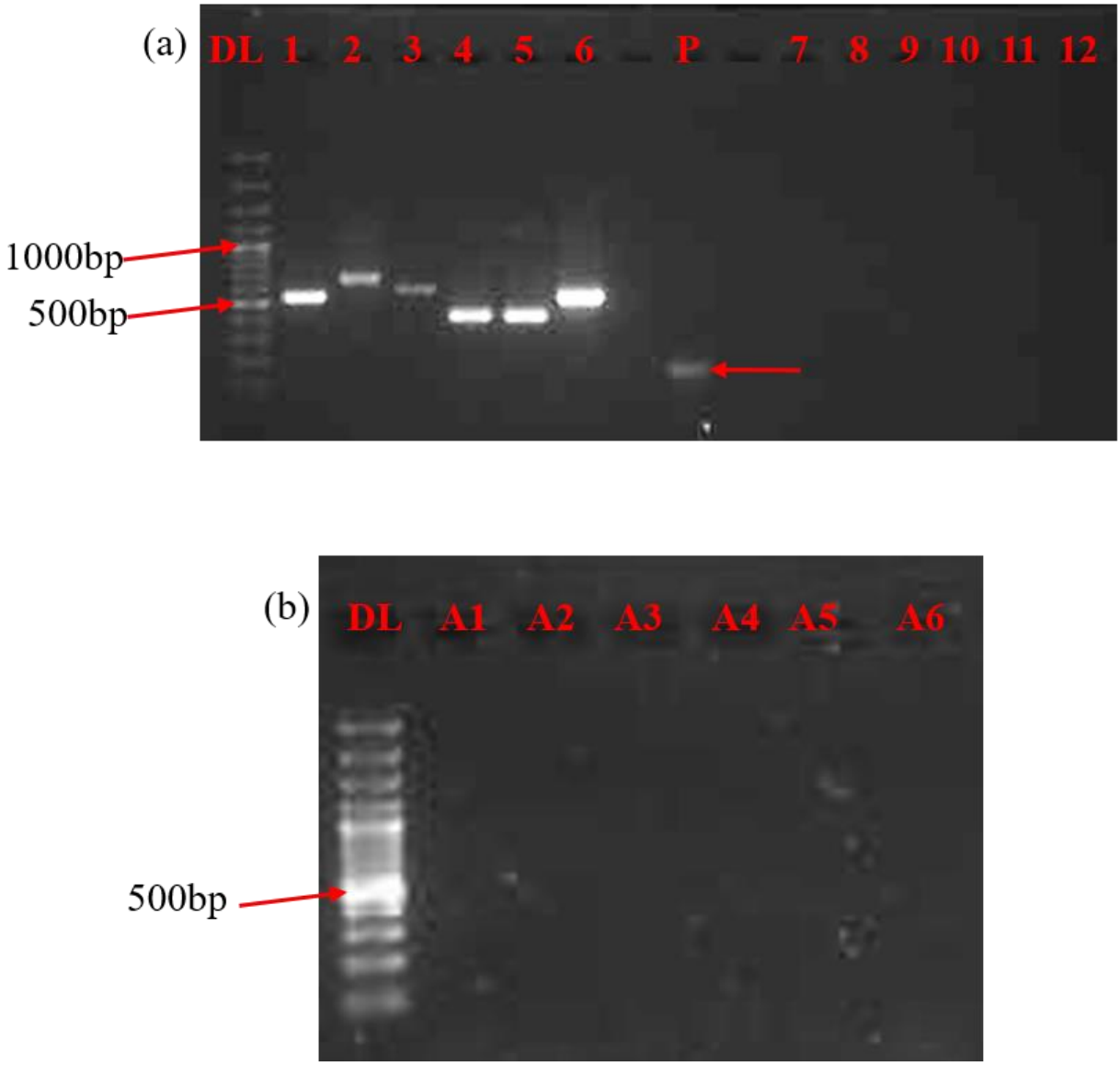


Figure 4.13: Agarose gel electrophoresis analysis of the PCR products from total RNA extracted.

Note: DL, Gene Ruler 100bp plus DNA Ladder (Thermo Scientific, USA). P, positive control (110bp *Actin* gene fragment). a) Lanes 1-6 represent gene fragments for *RabA1*, *RabA2*, *RabA3*, *RabA4*, *RabA5* and *RabA6* respectively. Lanes 7-12 represent negative controls *RabA1*, *RabA2*, *RabA3*, *RabA4*, *RabA5* and *RabA6* respectively. b) No reverse transcriptase controls for *RabA1*, *RabA2*, *RabA3*, *RabA4*, *RabA5* and *RabA6* respectively.

4.4 TRANSCRIPTOME ANALYSIS OF FRUIT RIPENING IN MANGO USING RNA SEQUENCING TECHNOLOGY

4.4.1 Summary of Transcriptome Sequencing Data

Total RNA samples from the unripe and ripe samples of the mango groups were successfully extracted using the CTAB method. Details of the quantity and quality of each RNA sample used in this study can be found in Appendix VI. Following the extraction of total RNA, twelve cDNA libraries were generated from different ripening stages (unripe and unripe). These included: three libraries from firm mango ('Chokanan') (CK) at the unripe stage (CKUR) or ripe stage (CKR) and three libraries from pooled (P) less-firm mango ('Golden phoenix' + 'Water lily') at the unripe stage (PUR) or ripe stage (PR) (Figure 3.4). A total of 1,125,567,452 raw reads were generated from all libraries. After trimming and discarding low quality reads, a total of 1,080,634,938 clean reads were obtained. As shown in Table 4.8, the Q30 and Q20 percentages (i.e. percentage of sequences with sequencing error rate of 0.001 % and 0.01 % respectively) for each sample was over 93 %. With the purpose of detecting differentially expressed genes, a mixed assembly was generated by pooling all reads into a single input in order to obtain a set of contigs that reflect the overall mango fruit transcriptome. A total of 165,140 unigenes with a mean length of 1,140 bp was obtained in this study. A comparison between the transcriptome assembly results from this study with previous studies on mango is presented in Table 4.9.

Table 4.8: Summary of transcriptome sequencing data

Category	CKUR	CKR	PUR	PR
Total raw reads	264,379,480	258,227,450	295,060,648	307,899,874
Total clean reads	253,524,510	247,492,900	284,711,604	294,905,924
Mapped reads	197,577,940	196,967,152	211,876,362	222,984,060
Q20 (%)	97.34	97.3	97.38	97.24
Q30 (%)	93.27	93.17	93.44	93.11
GC content (%)	44.51	44.03	44.64	45.43

Note: CKUR and CKR refer to 'Chokanan' mango at the unripe and ripe stage respectively; PUR and PR refer to the pool group ('Golden phoenix' + 'Water lily') at the unripe and ripe stage respectively; Raw reads indicate the original sequencing reads; Clean reads indicate the reads after filtering; Q30: represents an error rate of 1 in 1,000; Q20: represents an error rate of 1 in 100; Mapped reads: reads mapped back to the reference transcriptome.

Table 4.9: A comparative representation of the transcriptomic analysis from this study and previous reports in mango.

Category	This study	1	2	3	4	5	6	7
Tissue	mesocarp	peel	mesocarp	mesocarp	pericarp and pulp	mesocarp and exocarp	peel	leaf
Variety	'Chokanan', 'Golden phoenix' and 'Water lily'	'Keitt'	'Dasheri'	'Kent'	'Zill'	'Alphonso'	'Shelly'	'Langra'
Total unigenes	165,140	107,744	74,312	52,948	54,207	76,043	57,544	30,509
Average length of unigenes (bp)	1,140	1,717	942	836	838	1,326	863	536
N50 (bp)	1,722	2,235	-*	1,456	1,328	1,835	1,598	687
Sequencing system	HiSeq 2500	HiSeq 2500	Illumina Hi-Seq and 454 Titanium GS-FLX	Genome Analyzer GAIIx II	HiSeq 2000	HiSeq 1000	HiSeq 2000	HiSeq 2000

Note: * Not provided in the reference. The numbers represent the references where the data can be found.(1), Tafolla-Arellano *et al*., 2017; (2) Srivastava *et al*., 2016; (3) Dautt-Castro *et al*., 2015; (4) Wu *et al*., 2014; (5) Deshpande *et al*., 2017; (6) Luria *et al*., 2014; (7) Azim *et al*., 2014.

4.4.2 Functional Annotation of the Transcriptome

As shown in Figure 4.14, the unigenes were successfully annotated with the following databases: National Centre for Biotechnology Information (NCBI) non-redundant protein (NR, 102,072; 61.80 %), NCBI non-redundant nucleotide sequence (NT, 91,808; 55.59 %), Kyoto Encyclopaedia of Genes and Genomes (KEGG) Orthology (KO, 42,914; 25.98 %), Swiss-Prot (84,613; 51.23 %), PFAM (74,324; 45.01 %), Gene Ontology (GO, 75,196; 45.53 %) and Eukaryotic Orthologous Groups (KOG, 38,410; 23.25 %). In total, 120,104 (72.72 %) unigenes were found in at least one of these databases. Going further with the results, *Citrus* protein sequences were the most commonly matched with *Citrus sinensis* and *Citrus clementina* being the top two hits (Figure 4.15). Approximately 53,012 (52.00 %) of the total 102,072 annotated unigenes showed matches with *Citrus* sp. *Citrus sinensis* accounted for 34,680 sequences (34.00 %) followed by *Citrus clementina* with 18,332 sequences (18.00 %). Meanwhile only 269 sequences (0.30 %) presented blast hits from mango. This little portion could be due to the little publicly available sequence information for this species. So far, mango has 28,260 protein and 94,972 nucleotide sequences respectively (as of October 2018) deposited in the National Centre for Biotechnology Information (NCBI) GenBank compared with *Citrus* species (628,179 protein and 468, 721 nucleotide sequences respectively).

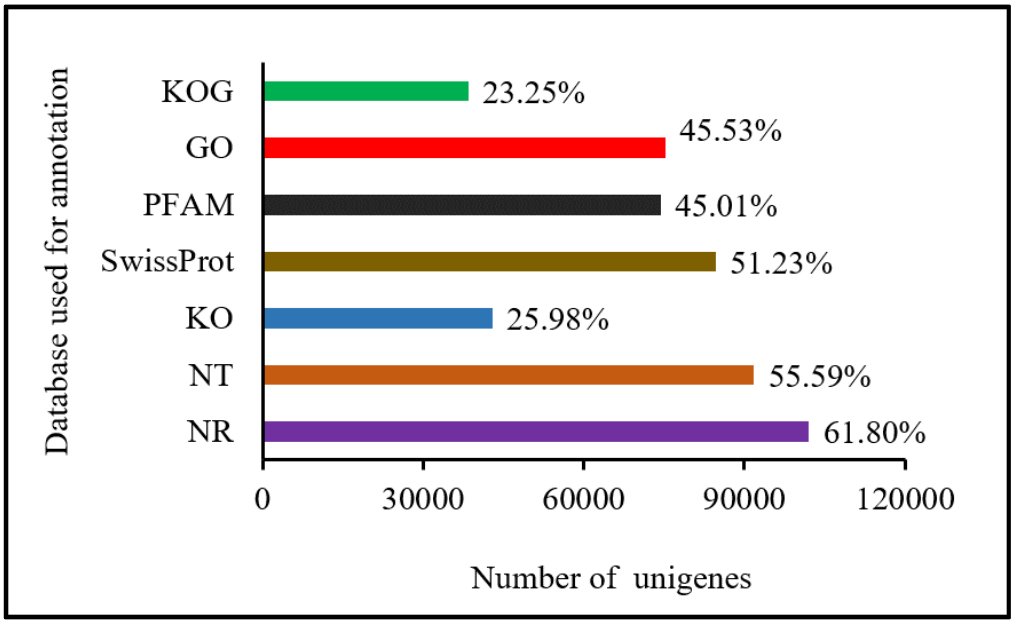


Figure 4.15: Unigenes annotated by seven public databases

Note: National Centre for Biotechnology Information (NCBI) non-redundant protein (NR); NCBI non-redundant nucleotide sequence (NT); Kyoto Encyclopaedia of Genes and Genomes (KEGG) Orthology (KO); Gene Ontology (GO) and Eukaryotic Orthologous Groups (KOG)

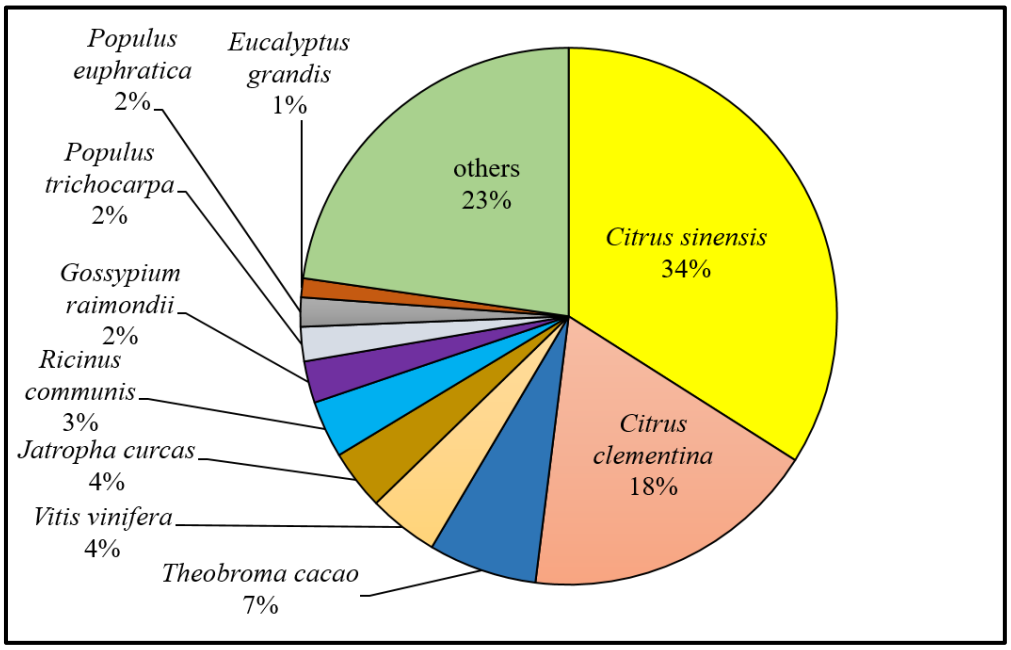


Figure 4.14: Top hit species distribution of the mango unigenes using BLASTX against NCBI NR database.

GO terms related to various biological processes (BP), molecular functions (MF) and cellular components (CC) were assigned to 75,196 annotated unigenes. The most enriched terms were cellular process (42,703) and metabolic process (39,425) in the biological process category (Figure 4.16). Under molecular functions, binding (42,235) was most abundant followed by catalytic activity (33,954) (Figure 4.17). Meanwhile, cell (22,668) and cell part (22,659) were the most represented in the cellular component category (Figure 4.18). The KEGG (Kyoto Encyclopedia of Genes and Genomes) database was also used as an alternative approach to categorize gene functions with emphasis on biological pathways. In total, 42,914 unigenes were assigned to KEGG pathways (Figure 4.19). The pathways with the most representation among the sequences were translation (3,697) followed by carbohydrate metabolic pathways (3,684) and folding, sorting and degradation (3,307) pathways. The annotated unigenes were also aligned to the Eukaryotic Orthologous Groups (KOG) database for functional prediction and classification. In total, 38,410 sequences were assigned into 26 different KOG categories. The cluster for general function prediction only (6,057) represented the largest group followed by post-translational modification, protein turnover, and chaperones (5,149), signal transduction mechanisms (3,939) and intracellular trafficking, secretion and vesicle transport (2,732) (Figure 4.20).

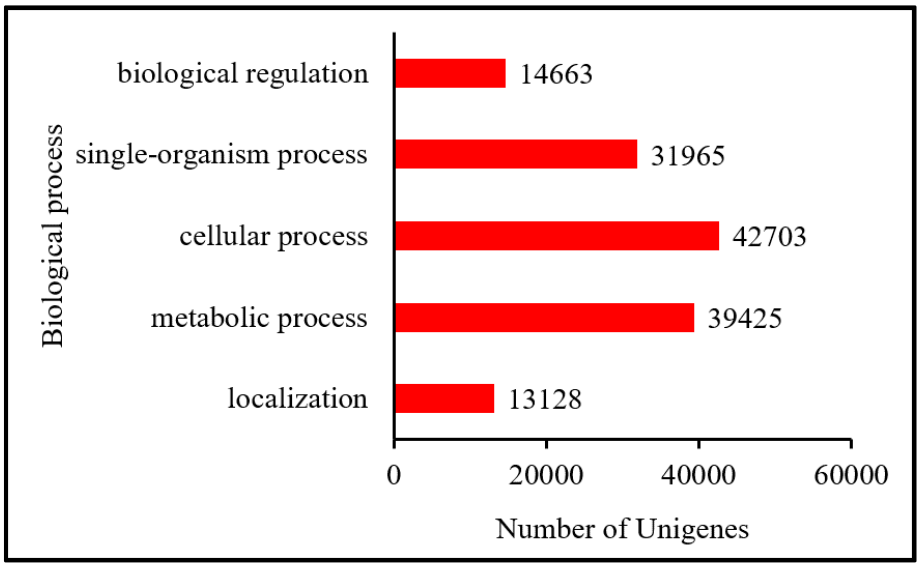


Figure 4.16: Top five GO annotation terms in the biological process category

Note: The number of unigenes annotated per GO term is shown on each bar.

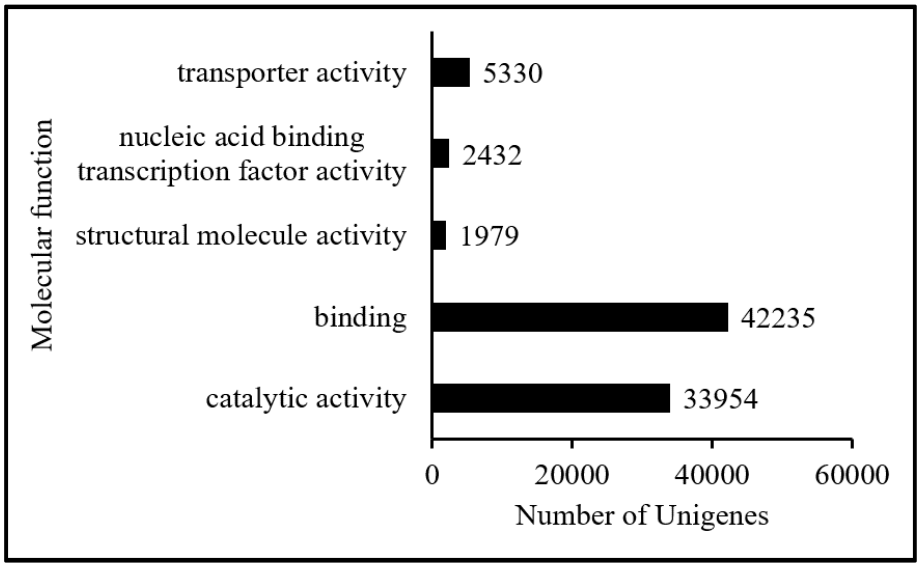


Figure 4.17: Top five GO annotation terms in the molecular function category

Note: The number of unigenes annotated per GO term is shown on each bar.

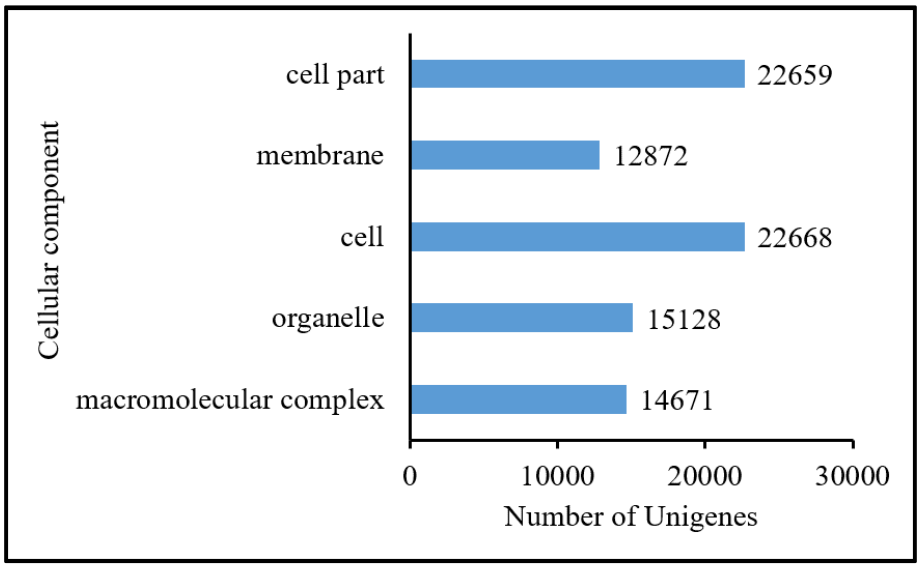


Figure 4.18: Top five GO annotation in the cellular component category

Note: The number of unigenes annotated per term is shown on each bar.

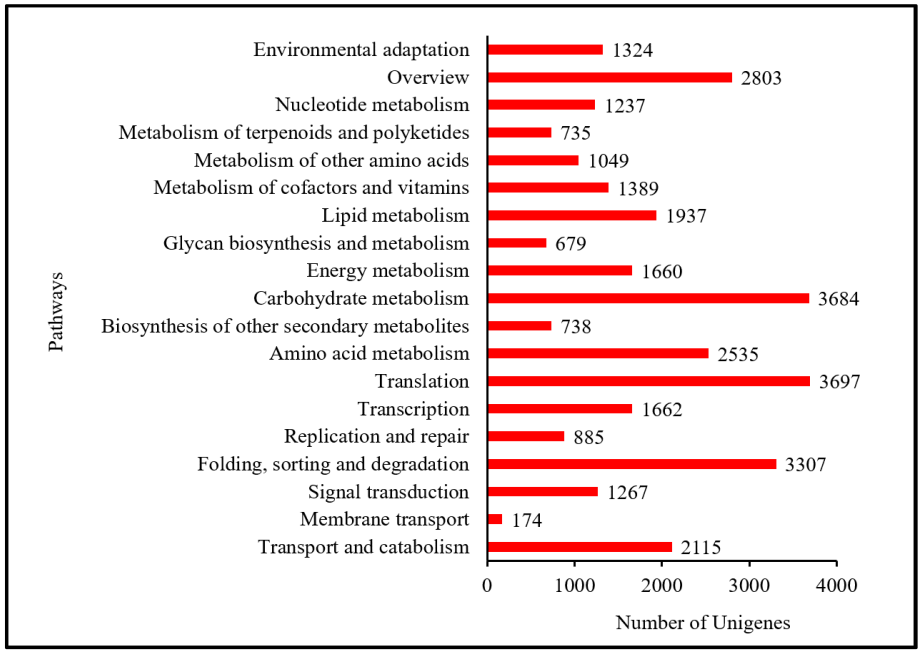


Figure 4.19: KEGG classification of annotated unigenes

Note: The number of unigenes annotated per term is shown on each bar.

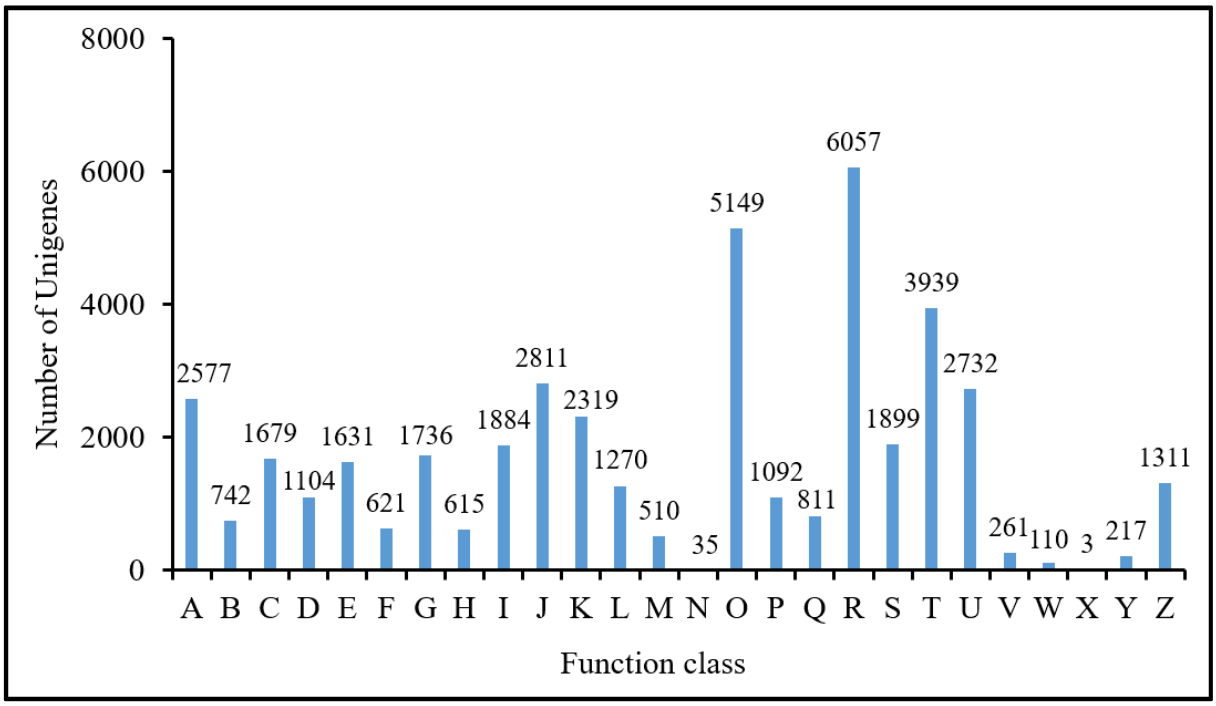


Figure 4.20: KOG classification of annotated unigenes

Note: The number of unigenes annotated per term is shown on each bar. The code descriptions for KOG categories are as follows: A, RNA processing and modification; B, Chromatin structure and dynamics; C, Energy production and conversion; D, Cell cycle control, cell division; E, Amino acid transport and metabolism; F, Nucleotide transport and metabolism; G, Carbohydrate transport and metabolism; H, Coenzyme transport and metabolism; I, Lipid transport and metabolism; J, Translation, ribosomal structure and biogenesis; K, Transcription; L, Replication, recombination and repair; M, Cell wall/membrane/envelope biogenesis; N, Cell motility; O, Posttranslational modification, protein turnover, chaperones; P, Inorganic ion transport and metabolism; Q, Secondary metabolites biosynthesis, transport and catabolism; R, General function prediction only; S, Function unknown; T, Signal transduction mechanisms; U, Intracellular trafficking, secretion, and vesicular transport; V, Defence mechanisms; W, Extracellular structures; X, Unnamed protein; Y, Nuclear structure; Z, Cytoskeleton

4.4.3 Analysis of Gene Expression

The correlation of gene expression between samples is an important indicator for reliability of experimental results. Correlation values among the samples were high (0.797 – 0.909) (Figure 4.21) indicating similarity of replicates and the reliability for further analysis (Wang *et al*., 2017b). The relative abundance of genes was determined using the FPKM (Fragments Per Kilo base of gene per Million mapped reads) method. Reads having an FPKM value lower than 0.3 were not considered for analysis, as they corresponded to genes too weakly expressed (Kang *et al*., 2013; Hart *et al*., 2013).

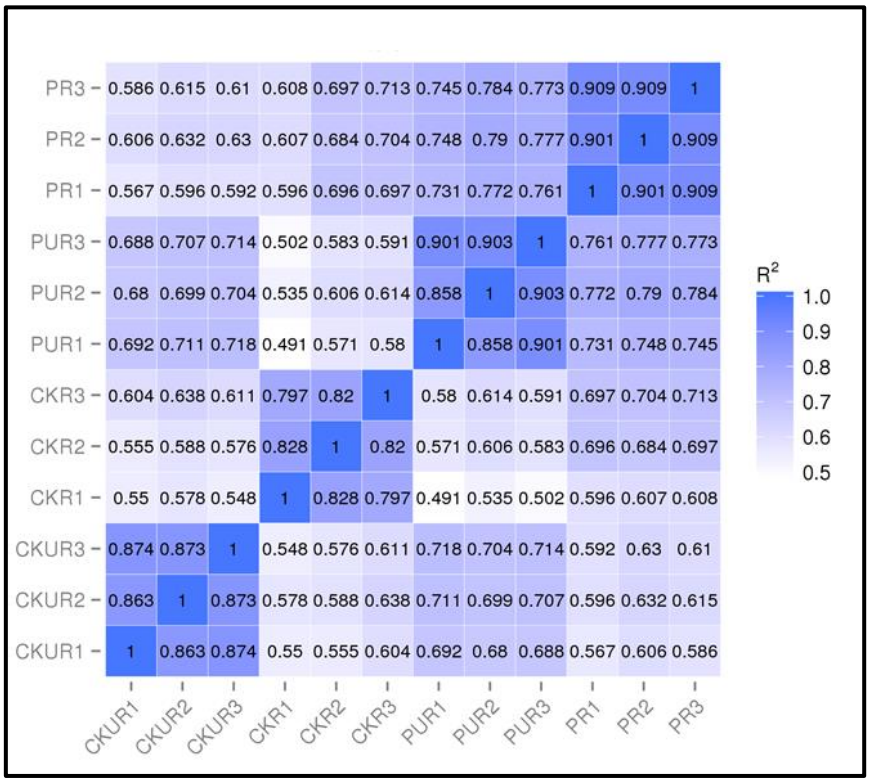


Figure 4.21: Correlation analysis of the unripe and ripe libraries of the mango groups.

Note: CKUR and CKR refer to 'Chokanan' mango at the unripe and ripe stage respectively; PUR and PR refer to the pool group ('Golden phoenix' + 'Water lily') at the unripe and ripe stage respectively; Labels 1, 2 and 3 represent three biological replicates respectively.

4.4.3.1 Genes Specific to a Ripening Stage

An overlap of genes expressed was observed between the two ripening stages and mango groups (Figure 4.22). Firstly, 35,159 genes were expressed in all ripening stages in all mango groups combined (CKUR, CKR, PUR and PR). Secondly, 28,800 genes were specifically expressed in the unripe samples (CKUR and PUR). Of these, 6,478 genes were shared between the CKUR and PUR groups while 8,550 and 13,772 genes were specific to the CKUR and PUR groups respectively. Furthermore, 17,274 genes were specifically expressed in the ripe stage. Of these, 1,740 genes overlapped between the mango groups with 9,959 and 5,575 genes being exclusively present in the CKR and PR group respectively. It was revealed that some genes associated with sugar transport, auxin, gibberellin and disease resistance were exclusively expressed in the unripe stage whereas some genes related to stress and cell wall degradation were exclusive to the ripe stage (Appendix VII).

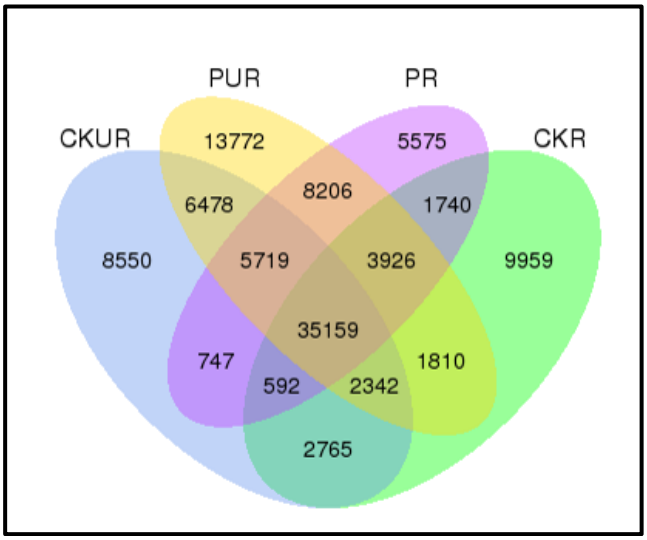


Figure 4.22: Venn diagram of expressed genes (FPKM > 0.3) for the mango samples.

Note: CKUR and CKR refer to 'Chokanan' mango at the unripe and ripe stage respectively; PUR and PR refer to the pool group at the unripe and ripe stage respectively.

4.4.3.2 Genes Expressed Differentially Between Ripening Stages

Differentially expressed genes (DEGs) were identified by comparisons of (a) different ripening stages in the same mango group and (b) the same ripening stage between different mango groups. Comparisons of the two ripening stages in the 'CK' group (CKUR vs CKR) identified 9,765 DEGs (4,435 up-regulated genes and 5,330 down-regulated genes) (Figure 4.23). On the other hand, comparisons of the two ripening stages in 'P' group (PUR vs PR) identified 13,651 DEGs (5,781 up-regulated genes and 7,870 down-regulated genes) (Figure 4.23). Comparisons of the different mango groups at the unripe (CKUR vs PUR) and ripe (CKR vs PR) stage identified 18,258 and 10,521 DEGs respectively (Figure 4.24). The aforementioned DEGs were found to be involved in major processes associated with ripening including energy metabolism, plant hormone metabolism, sugar metabolism and cell wall metabolism. It must be pointed out that for simplicity, the results below will be outlined based on;

a) DEGs identified by pairwise comparisons between the unripe and ripe stages in the 'CK' (CKUR vs CKR) (Table 4.10; Appendix VIII-a) and 'P' (PUR vs PR) (Tables 4.11; Appendix VIII-b) mango groups respectively.
b) DEGs identified by pairwise comparisons between the mango groups at the unripe (CKUR vs PUR) (Table 4.12; Appendix VIII-c) and ripe (CKR vs PR) (Table 4.13; Appendix VIII-d) respectively.

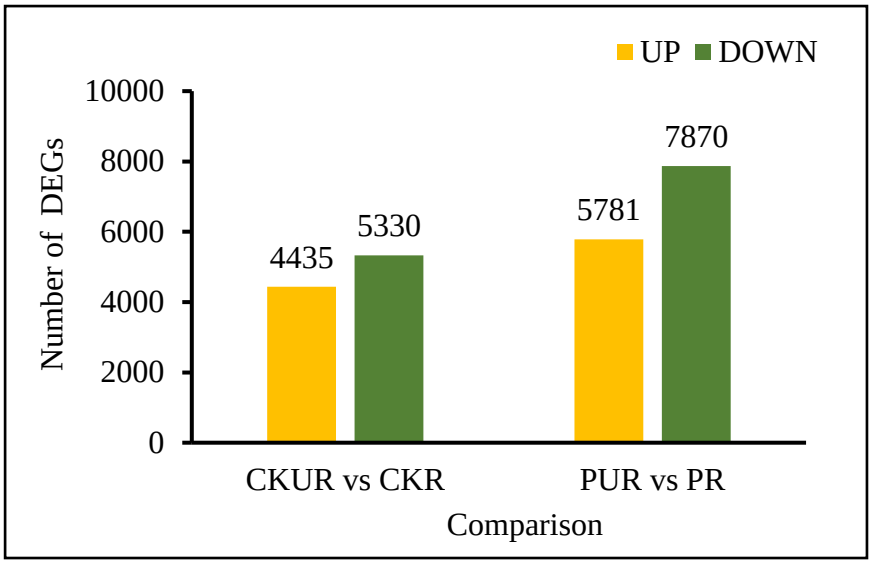


Figure 4.23: Differentially expressed genes between the ripening stages for each mango group.

Note: CKUR, 'Chokanan' group at the unripe stage; CKR, 'Chokanan' group at the ripe stage; PUR, Pool group at the unripe stage; PR, Pool group at the ripe stage. Up-regulated and down-regulated, respectively indicates that the expression level of the ripe stage (R) is higher and lower than that of the unripe stage (UR) respectively. The DEGs were defined by the criteria of adjusted P value < 0.05; $\log_2$ fold change (FC) > 1 or $\log_2$ fold change (FC) < -1).

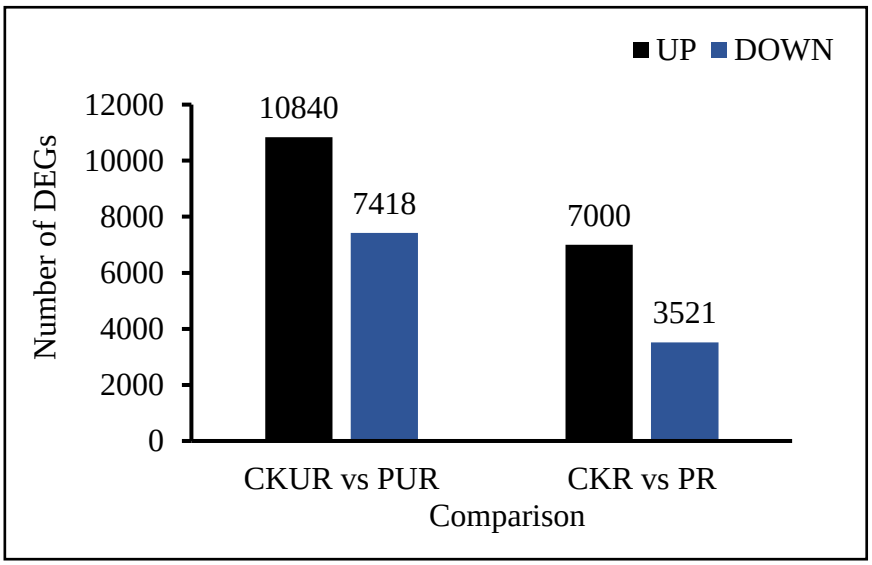


Figure 4.24: Differentially expressed genes between the mango groups for the same ripening stage.

Note: CKUR, 'Chokanan' group at the unripe stage; CKR, 'Chokanan' group at the ripe stage; PUR, Pool group at the unripe stage; PR, Pool group at the ripe stage. Up-regulated and down-regulated mean that the expression level in the P group is higher and lower than that in the CK group, respectively. The DEGs were defined by the criteria of adjusted P value < 0.05; $\log_2$ fold change (FC) > 1 or $\log_2$ fold change (FC) < -1).

4.4.3.2.1 Comparison between the Unripe and Ripe Stages in a Mango Group.

Based on the DEG analysis, the genes could be divided in the following categories:

4.4.3.2.1.1 Genes Associated With Energy Metabolism

Harvested fruits continue to respire throughout the postharvest life (Fagundes *et al*., 2013). As shown in Tables 4.10 and 4.11, genes encoding aconitase and citrate synthase of the citric acid cycle were found to be up-regulated in the ripe stage of both mango groups compared to their unripe stages. In addition, genes encoding malic enzyme showed a mixed (up or down) expression between the ripening stages of both mango groups.

4.4.3.2.1.2 Genes Associated With Hormone Metabolism and Signalling

Plant hormones are required for fruit development and ripening (McAtee *et al*., 2013). Comparative analysis revealed DEGs related to plant hormones such as ethylene, auxin and abscisic acid (ABA). Comparison between ripening stages in the 'CK' group (CKUR vs CKR) revealed that fourteen genes encoding 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) were expressed differentially (Table 4.10). Out of these, five showed an up-regulated trend in the ripe sample (CKR). On the other hand, a total of sixteen DEGs encoding ACO were present in the 'P' group (PUR vs PR), of which nine were up-regulated in the ripe sample (Table 4.11). In addition, the genes encoding 1-aminocyclopropane-1-carboxylate synthase (ACS) showed a mixed expression trend in the 'P' group. Genes encoding tryptophan aminotransferase (a key enzyme involved in auxin biosynthesis) displayed a decreasing trend during the ripening in both mango groups. In addition, genes coding the 9-cis-epoxycarotenoid dioxygenase (NCED), an important enzyme in ABA biosynthesis was detected. These genes were found to be down-regulated during the transition from the unripe to the ripe stage in the 'CK' and 'P' groups respectively. Genes encoding ethylene receptors (ETRs) were found to be differentially expressed during fruit ripening in the 'CK' and 'P' groups respectively. Most genes showed a

decreasing trend in both mango groups and presented a higher expression level in ‘P’ group compared with that in ‘CK’ group at the unripe.

4.4.3.2.1.3 Genes Associated With Sugar Metabolism

Soluble sugars are important components of mango fruit flavour and have an impact on the mango fruit quality (Malundo *et al*., 2001). Stage to stage comparison of the ‘CK’ group (CKUR vs CKR) revealed eight sucrose synthase (*Susy*) genes, of which five showed an increased expression during ripening (Table 4.10). On the other hand, the ‘P’ group (PUR vs PR) revealed eight sucrose synthase genes with six being up-regulated in the ripe sample (Table 4.11). Starch degradation contributes to the accumulation of sugar during ripening (Yashoda *et al*., 2006). Amylase encoding genes were found in both ‘CK’ (Table 4.10) and ‘P’ (Table 4.11) groups. In the ‘CK’ group (CKUR vs CKR), all genes were found to be up-regulated whereas a mixed expression (up and down) was observed in the ‘P’ group (PUR vs PR).

4.4.3.2.1.4 Genes Associated With the Cell Wall

One of the most important changes occurring during mango ripening is the loss of firmness (Yahia, 2011). Gene families encoding expansin, polygalacturonase (PG; EC 3.2.1.15), pectate lyase (PL; EC 4.2.2.2), pectinesterase (PE; EC 3.1.1.11) and endoglucanases (EC 3.2.1.4) involved in cell wall metabolism were expressed differentially in the ‘CK’ (Table 4.10) and ‘P’ (Table 4.11) groups respectively. For the ‘CK’ group, nine *PG* genes were obtained of which seven and two genes were up-regulated and down-regulated respectively. Similarly, more than half of the *PL* and *expansin* genes were up-regulated in the ripe stage of the ‘CK’ group (CKR). In addition, ten genes encoding endoglucanase were identified in the ‘CK’ group, of these, five genes were either down-regulated or up-regulated respectively. *PE beta-galactosidase* gene members also exhibited a mixed response during ripening.

Going further, comparison between the ripening stages of the ‘P’ group revealed ten *PG* genes, out of these eight were found to be up-regulated during ripening. Similar increased expression

were observed for genes encoding expansin, PL, PE, beta-galactosidase and endoglucanase respectively. Genes related to the cell wall softening were among the top up-regulated genes indicating that fruit softening is an important process during mango ripening.

4.4.3.2.1.5 Genes Associated With the Colour and Flavour Development

In this study, genes associated with carotenoid metabolism in mango mesocarp were found to be differentially expressed (Tables 4.10 and 4.11). Genes encoding Zeta carotene desaturase and lycopene beta-cyclase were upregulated trend in the ripe fruit of the 'P' group whereas no change was observed in the 'CK' group. An increase in β-carotene 3-hydroxylase gene expression was observed in the ripe fruit of the 'CK' group with no changes observed in the Pool group. In this study, genes encoding phytoene synthase showed a mixed expression (up regulation and down regulation) during the ripening of both groups.

Mango is an excellent system for the study of aroma due to the wide variety exhibited by the varieties (Srivastava *et al*., 2016). Several genes related to flavour showed significant differential regulation during ripening of the mango groups investigated. These included genes encoding alcohol dehydrogenase (ADH) and lipoxygenase (LOX). Most genes encoding the aforementioned genes exhibited a decreased expression trend during mango fruit ripening (Tables 4.10 and 4.11). Plants accumulate stress-related proteins during ripening (Huan *et al*., 2016). Data presented showed differential expression of the antioxidant genes such as *catalase* and *superoxide dismutase* during fruit ripening (Appendix VIII).

Table 4.10: Some of the genes differentially expressed between the unripe (UR) and ripe (R) stages of Chokanan (CK) mango group (CKUR vs CKR).

Gene_ID	log_2FC	Adjusted P value	Regulation	NR ID	Annotation*
ENERGY METABOLISM					
Citrate synthase					
Cluster-27569.67331	8.76	8.27E-22	up	XP_006448012.1	Citrate synthase
Cluster-27569.65031	8.47	4.29E-19	up	XP_006448012.1	Citrate synthase
Cluster-27569.63971	5.46	3.63E-12	up	XP_006448012.1	Citrate synthase
Cluster-27569.65540	3.88	1.87E-09	up	XP_010689947.1	citrate synthase
Cluster-27569.67597	4.38	1.74E-06	up	XP_006448012.1	citrate synthase
Cluster-27569.64957	6.96	0.0191	up	KDO58598.1	citrate synthase
Cluster-27569.58155	2.75	0.0343	up	AFB82642.1	citrate synthase
Aconitase					
Cluster-27569.67776	3.41	4.17E-07	up	KDO71345.1	Aconitase
Cluster-27569.67787	2.93	1.53E-05	up	KDO71349.1	Aconitase
Cluster-27569.64507	2.53	0.0004	up	XP_011022256.1	Aconitase
Cluster-27569.62673	2.87	0.0009	up	XP_012448314.1	Aconitase
Cluster-27569.43202	3.68	0.0053	up	XP_006452377.1	Aconitase
Cluster-27569.64093	4.98	0.0138	up	XP_012448314.1	Aconitase
Malic enzyme					
Cluster-27569.55138	4.87	1.29E-10	up	XP_007015739.1	Malic enzyme
Cluster-27569.78748	-3.85	1.16E-08	down	KDO66537.1	Malic enzyme
Cluster-27569.63006	3.13	5.13E-06	up	AFM08812.1	Malic enzyme 4
Cluster-27569.68188	-3.45	1.10E-07	down	AAF73006.1	Malic enzyme

Cluster-27569.70276	-2.77	1.90E-05	down	XP_003547744.1	Malic enzyme
Cluster-27569.68966	2.55	0.0001	up	KHN32646.1	Malic enzyme
Cluster-27569.67412	2.51	0.0005	up	AFM08812.1	Malic enzyme 4
Cluster-27569.70277	-2.31	0.0006	down	XP_002309534.1	Malic enzyme
Cluster-27569.67415	2.37	0.0142	up	KHM98720.1	Malic enzyme 4
Cluster-27569.61772	-1.69	0.0243	down	XP_011017312.1	Malic enzyme
Cluster-27569.45368	-1.55	0.0386	down	XP_003547744.1	Malic enzyme
HORMONE METABOLISM and SIGNALLING					
1-aminocyclopropane-1-carboxylate oxidase (ACO)					
Cluster-27569.43139	-6.58	1.35E-13	down	XP_006420181.1	1-aminocyclopropane-1-carboxylate oxidase homolog 1
Cluster-27569.72371	-4.63	3.18E-12	down	XP_006420188.1	1-aminocyclopropane-1-carboxylate oxidase homolog 3
Cluster-27569.72375	-7.66	9.84E-12	down	XP_006489571.1	1-aminocyclopropane-1-carboxylate oxidase homolog 2
Cluster-27569.85219	-4.28	8.81E-11	down	XP_007225869.1	1-aminocyclopropane-1-carboxylate oxidase homolog 7
Cluster-27569.55597	4.43	1.41E-10	up	XP_006489569.1	1-aminocyclopropane-1-carboxylate oxidase homolog
Cluster-27569.45316	-4.53	2.20E-10	down	CAB97173.1	1-aminocyclopropane-1-carboxylate oxidase
Cluster-27569.72372	-3.63	2.11E-07	down	XP_006420182.1	1-aminocyclopropane-1-carboxylate oxidase homolog 1
Cluster-27569.82597	-3.48	2.20E-06	down	XP_002320487.1	1-aminocyclopropane-1-carboxylate oxidase
Cluster-27569.72373	-4.82	6.91E-05	down	KDO38629.1	1-aminocyclopropane-1-carboxylate oxidase homolog
Cluster-27569.78714	3.72	8.99E-05	up	KDO61358.1	1-aminocyclopropane-1-carboxylate oxidase

Cluster-27569.46971	-4.90	0.0002	down	CAB97173.1	1-aminocyclopropane-1-carboxylate oxidase
Cluster-27569.56463	2.55	0.0002	up	XP_006477143.1	1-aminocyclopropane-1-carboxylate oxidase
Cluster-27569.52333	2.05	0.0031	up	XP_007050339.1	1-aminocyclopropane-1-carboxylate oxidase homolog 1
Cluster-27569.68916	3.92	0.0159	up	XP_006489569.1	1-aminocyclopropane-1-carboxylate oxidase homolog 7
Tryptophan aminotransferase					
Cluster-27569.105082	-7.30	3.92E-06	down	XP_006439890.1	Tryptophan aminotransferase-related protein 2
Cluster-27569.27507	-3.64	0.0001	down	XP_011042900.1	tryptophan aminotransferase-related protein 4-like
9-cis-epoxycarotenoid dioxygenase (**NCED**)					
Cluster-27569.91178	-5.33	5.22E-14	down	NP_001275864.1	9-cis-epoxycarotenoid dioxygenase NCED3
Cluster-27569.91181	-2.97	0.0001	down	NP_001275864.1	9-cis-epoxycarotenoid dioxygenase NCED3
Ethylene receptors (ETR)					
Cluster-27569.92251	-3.9664	2.90E-09	down	ACL81480.3	Ethylene receptor
Cluster-27569.98517	-4.9366	1.56E-06	down	AIT39449.1	Ethylene receptor 1
Cluster-27569.27348	-3.2265	5.48E-05	down	AIT39449.1	Ethylene receptor 1
Cluster-27569.20043	-3.0435	6.79E-05	down	NP_001275855.1	Ethylene response 3
Cluster-27569.27348	-3.2265	5.48E-05	down	AIT39449.1	Ethylene receptor 1
SUGAR METABOLISM					
Sucrose phosphatase synthase (**SPS**)					
Cluster-27569.64830	1.93	0.0133	up	BAM68535.1	Probable sucrose-phosphate synthase
Sucrose synthase (**Susy**)					
Cluster-27569.64909	-4.15	6.36E-08	down	XP_007050984.1	Sucrose synthase 2

Cluster-27569.74767	8.92	8.99E-08	up	XP_006481951.1	Sucrose synthase 5
Cluster-27569.28945	-7.84	2.54E-07	down	XP_006441344.1	Sucrose synthase 6
Cluster-27569.71227	8.84	4.24E-06	up	XP_006481951.1	Sucrose synthase 7
Cluster-27569.68858	6.99	9.08E-05	up	XP_006481951.1	Sucrose synthase 5
Cluster-27569.74768	5.62	0.0004	up	XP_006481951.1	Sucrose synthase 7
Cluster-27569.44187	2.92	0.0070	up	BAM68520.1	Sucrose synthase
Cluster-27569.86057	-4.58	2.87E-11	down	AJW82916.1	Sucrose synthase
Beta amylase					
Cluster-27569.64560	5.91	5.84E-18	up	XP_006385389.1	Beta-amylase 3
Cluster-27569.64814	5.13	1.10E-14	up	XP_006385389.1	Beta-amylase 3, 3
Cluster-27569.61143	4.56	3.93E-12	up	XP_006439286.1	Beta-amylase 1
Cluster-27569.64502	6.20	6.70E-10	up	XP_006385389.1	Beta-amylase 3
Cluster-27569.64488	3.87	2.32E-09	up	XP_007222488.1	beta-amylase 9
Cluster-27569.65014	3.21	6.99E-07	up	KDO75062.1	Beta-amylase 9
Cluster-27569.54769	4.00	2.59E-06	up	XP_006439285.1	Beta-amylase 1
Cluster-27569.61144	3.06	2.83E-05	up	XP_004503587.1	Beta-amylase 1
Cluster-27569.63332	2.12	0.0014	up	XP_006493994.1	Beta-amylase 1
Cluster-27569.63333	3.72	0.0188	up	XP_011091372.1	Beta-amylase 1

CELL WALL METABOLISM					
Polygalacturonase (PG)					
Cluster-27569.107909	-8.92	2.05E-13	down	XP_010652214.1	Probable polygalacturonase
Cluster-27569.65630	5.88	2.67E-12	up	XP_006448628.1	Polygalacturonase
Cluster-27569.59400	7.57	2.00E-09	up	XP_006427250.1	Probable polygalacturonase
Cluster-27569.9566	8.07	5.47E-08	up	XP_006493140.1	Polygalacturonase
Cluster-27569.103369	-3.94	1.33E-07	down	XP_012086833.1	Probable polygalacturonase
Cluster-27569.51988	2.51	0.0001	up	XP_006432212.1	Probable polygalacturonase
Cluster-27569.59398	4.09	0.0002	up	ACF16415.1	Probable polygalacturonase
Cluster-27569.101799	3.35	0.0063	up	KDO82433.1	Polygalacturonase 1
Cluster-27569.33497	5.60	0.0080	up	KDO51595.1	Polygalacturonase
Expansin					
Cluster-27569.47278	6.68	6.11E-06	up	XP_011070988.1	Expansin-A10
Cluster-27569.66259	8.59	1.67E-17	up	AHL25254.1	Expansin-A2
Cluster-27569.65111	5.49	3.39E-16	up	AAT11859.2	Expansin-A6
Cluster-27569.53261	5.25	1.49E-09	up	XP_010258310.1	Expansin-A1
Cluster-27569.9710	-3.16	0.0059	down	XP_006422480.1	Expansin-like A2
Pectinesterase (PE)					
Cluster-27569.103297	-4.61	2.99E-08	down	XP_007025998.1	Pectinesterase 2.1
Cluster-27569.103404	-4.63	3.05E-06	down	XP_007012505.1	Probable pectinesterase

Cluster-27569.64771	2.86	1.12E-05	up	XP_012068794.1	Pectinesterase 3
Cluster-27569.64338	2.70	3.60E-05	up	XP_002304256.1	Pectinesterase 1
Cluster-27569.103299	-5.18	0.0008	down	KDO75533.1	Pectinesterase 2.2
Endoglucanase					
Cluster-27569.109523	-3.99	0.0332	down	XP_006435157.1	Endoglucanase 10
Cluster-27569.100995	-7.11	4.38E-22	down	XP_012080633.1	Endoglucanase 11
Cluster-27569.30591	-6.58	2.69E-19	down	XP_006431347.1	Endoglucanase 25
Cluster-27569.64817	8.43	6.39E-14	up	XP_006423475.1	Endoglucanase 24
Cluster-27569.47150	8.37	3.80E-09	up	XP_006421697.1	Endoglucanase 8
Cluster-27569.71977	7.88	3.76E-08	up	XP_011047414.1	Endoglucanase 24
Cluster-27569.67542	4.19	0.0007	up	ABR10607.1	Endoglucanase 19
Cluster-27569.29620	-5.51	0.0022	down	XP_006447630.1	Endoglucanase 11
Cluster-27569.89592	4.78	0.0043	up	KDP26669.1	Endoglucanase
Cluster-27569.109523	-3.98	0.0332	down	XP_006435157.1	Endoglucanase 10
Pectate lyase (PL)					
Cluster-27569.64385	5.41	6.04E-14	up	AAX88800.1	Probable pectate lyase 8
Cluster-27569.65453	6.07	2.18E-07	up	XP_002322215.1	Probable pectate lyase 18
Cluster-27569.65305	3.53	0.0005	up	XP_010250930.1	Probable pectate lyase 18
Cluster-27569.18818	-5.63	0.0399	down	XP_006428993.1	Putative pectate lyase 21

Beta galactosidase					
Cluster-27569.62859	8.73	8.52E-13	up	CAJ09953.1	Beta-galactosidase
Cluster-27569.78361	8.51	2.25E-14	up	XP_006475097.1	Beta-galactosidase 7
Cluster-27569.69737	4.16	1.15E-10	up	XP_007012844.1	Beta-galactosidase
Cluster-27569.66680	3.94	1.27E-09	up	XP_007012844.1	Beta-galactosidase
Cluster-27569.66681	3.82	2.64E-09	up	XP_007012844.1	Beta-galactosidase
Cluster-27569.63507	7.40	3.65E-09	up	CAJ09953.1	Beta-galactosidase
Cluster-27569.66678	3.52	4.06E-07	up	XP_011045964.1	Beta-galactosidase-like isoform X1
Cluster-27569.108352	-3.30	2.42E-06	down	XP_012480371.1	Beta-galactosidase 5
Cluster-27569.63306	7.18	0.0001	up	CAJ09953.1	Beta-galactosidase
Cluster-27569.24702	-2.98	0.0010	down	XP_007024498.1	Beta-galactosidase 17
Cluster-27569.25707	-4.01	0.0023	down	XP_010274046.1	Beta-galactosidase 9
Cluster-27569.64539	6.81	0.0045	up	XP_007012844.1	Beta-galactosidase
Cluster-27569.112447	3.12	0.0078	up	AHC32021.1	Beta-galactosidase 3
Cluster-27569.60831	2.88	0.0165	up	XP_007012844.1	Beta-galactosidase
Cluster-27569.52583	-1.72	0.0499	up	XP_006493072.1	Beta-galactosidase 9
COLOR DEVELOPMENT					
Phytoene synthase					
Cluster-27569.28431	-7.83	3.19E-08	down	AFE85918.1	Phytoene synthase
Beta-carotene isomerase					
Cluster-27569.24323	-1.93	0.0112	down	XP_006492348.1	Beta-carotene isomerase D27
FLAVOUR DEVELOPEMENT					
Alcohol dehydrogenase (ADH)					
Cluster-27569.104639	-8.94	2.08E-22	down	XP_012073738.1	Alcohol dehydrogenase 9
Cluster-27569.93154	-6.59	1.97E-20	down	XP_012075882.1	Alcohol dehydrogenase 1
Cluster-27569.95147	-6.88	2.64E-17	down	XP_012075882.1	Alcohol dehydrogenase 1
Cluster-27569.106725	-7.64	2.25E-15	down	XP_012492075.1	Mannitol dehydrogenase

Cluster-27569.58720	-4.80	3.41E-12	down	XP_012073738.1	Alcohol dehydrogenase 6 isoform X1
Cluster-27569.60588	-3.84	2.28E-09	down	XP_006443700.1	Alcohol dehydrogenase class-3-like
Cluster-27569.104596	-6.67	4.21E-09	down	XP_003635280.1	Mannitol dehydrogenase
Cluster-27569.70050	3.91	5.33E-09	up	ADB43613.1	Alcohol dehydrogenase 1
Cluster-27569.61947	3.86	7.78E-09	up	ADB43613.1	Alcohol dehydrogenase 1
Cluster-27569.24221	-4.42	1.04E-08	down	XP_002529812.1	Alcohol dehydrogenase
Cluster-27569.62349	4.40	2.94E-08	up	ADB43614.1	Alcohol dehydrogenase 2
Cluster-27569.76545	-4.49	1.26E-06	down	XP_006473751.1	Alcohol dehydrogenase-like 7-like
Cluster-27569.70284	3.58	1.41E-06	up	ADB43613.1	Alcohol dehydrogenase 1
Cluster-27569.68183	-3.12	5.39E-06	down	XP_007039278.1	Alcohol dehydrogenase isoform 1
Cluster-27569.64951	-3.50	2.09E-05	down	NP_001238796.1	Alcohol dehydrogenase class III
Cluster-27569.23014	-2.88	0.000617	down	XP_006435310.1	Alcohol dehydrogenase-like 7
Cluster-27569.33734	-2.53	0.022566	down	XP_006426431.1	Alcohol dehydrogenase
Lipoxygenase (LOX)					
Cluster-27569.103807	-4.608	2.12E-10	down	XP_006494720.1	Linoleate 13S-lipoxygenase 2-1
Cluster-27569.103806	-4.4502	2.41E-10	down	XP_012444488.1	Linoleate 13S-lipoxygenase 2-1
Cluster-27569.103155	-9.0273	3.11E-09	down	XP_012444488.1	Linoleate 13S-lipoxygenase 2-1
Cluster-27569.66540	4.2906	5.23E-07	up	XP_006465905.1	Linoleate 13S-lipoxygenase 3-1
Cluster-27569.48014	6.0013	1.48E-05	up	XP_004141705.1	Linoleate 13S-lipoxygenase 3-1
Cluster-27569.67094	6.192	0.043197	up	AAK50778.4	Lipoxygenase

Note: *Based on the NCBI non redundant (NR) and Swissprot description. 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; $\log_2$FC > 1 or $\log_2$FC< -1.

Table 4.11: Some of the genes differentially expressed between the between the unripe (UR) and ripe (R) stages of Pool (P) mango group (PUR vs PR).

Gene_ID	log_2FC	Adjusted P value	Regulation	NR ID	Annotation*
ENERGY METABOLISM					
Citrate synthase					
Cluster-27569.63971	3.60	5.53E-11	up	XP_006448012.1	citrate synthase
Cluster-27569.65540	2.92	5.24E-09	up	XP_010689947.1	citrate synthase
Cluster-27569.64370	2.85	1.8E-08	up	CDP18733.1	citrate synthase
Cluster-27569.65031	3.59	0.0008	up	XP_006448012.1	citrate synthase
Cluster-27569.67331	4.51	0.0038	up	XP_006448012.1	citrate synthase
Cluster-27569.65541	4.22	0.0173	up	XP_012084988.1	citrate synthase
Cluster-27569.67597	2.33	0.0202	up	XP_006448012.1	citrate synthase
Cluster-27569.100666	1.70	0.0279	up	XP_012459840.1	citrate synthase
Aconitase					
Cluster-27569.43207	1.56	0.0208	up	XP_008455442.1	Aconitase
Cluster-27569.43202	1.97	0.0307	up	XP_006452377.1	Aconitase
Malic enzyme					
Cluster-27569.71023	-4.36	9.96E-14	down	AAF73006.1	Malic enzyme
Cluster-27569.70276	-3.20	4.32E-10	down	XP_003547744.1	Malic enzyme
Cluster-27569.78748	-3.15	5.54E-09	down	KDO66537.1	Malic enzyme
Cluster-27569.70277	-2.28	8.14E-06	down	XP_002309534.1	Malic enzyme
Cluster-27569.45368	-2.19	2.49E-05	down	XP_003547744.1	Malic enzyme
Cluster-27569.63008	5.95	0.0003	up	KEH39547.1	Malic enzyme
Cluster-27569.31106	-2.21	0.0005	down	XP_006476015.1	Malic enzyme

Cluster-27569.61885	1.90	0.0005	up	KDO86068.1	Malic enzyme
Cluster-27569.63009	-5.50	0.0030	down	KEH39547.1	Malic enzyme
Cluster-27569.62113	1.58	0.0062	up	KDO51777.1	Malic enzyme 4
Cluster-27569.18175	-2.21	0.0080	down	KDO79867.1	Malic enzyme
Cluster-27569.61772	-1.48	0.0108	down	XP_011017312.1	Malic enzyme
Cluster-27569.61307	1.45	0.0158	up	AFM08812.1	Malic enzyme 4
Cluster-27569.67415	1.57	0.0242	up	KHM98720.1	Malic enzyme 4
Cluster-27569.67412	1.35	0.0316	up	AFM08812.1	Malic enzyme 4
Cluster-27569.55138	1.34	0.0411	up	XP_007015739.1	Malic enzyme
Cluster-27569.105531	-2.30	0.0429	down	KDO79867.1	Malic enzyme
HORMONE METABOLISM AND SIGNALLING					
1-aminocyclopropane-1-carboxylate synthase 1 (ACS)					
Cluster-27569.2289	5.53	0.0042	up	AAA84895.1	1-aminocyclopropane-1-carboxylate synthase 1
Cluster-27569.99855	-2.92	0.0496	down	AJF36210.1	1-aminocyclopropane-1-carboxylate synthase 2
1 -aminocyclopropane-1-carboxylate oxidase (ACO)					
Cluster-27569.14298	5.89	4.61E-10	up	XP_011000640.1	1-aminocyclopropane-1-carboxylate oxidase
Cluster-27569.18002	7.78	1.51E-05	up	CDP04457.1	1-aminocyclopropane-1-carboxylate oxidase
Cluster-27569.76292	2.65	0.0182	up	XP_006477142.1	1-aminocyclopropane-1-carboxylate oxidase
Cluster-27569.43139	-3.02	0.0331	down	XP_006420181.1	1-aminocyclopropane-1-carboxylate oxidase homolog 1
Cluster-27569.65392	2.09	4.90E-05	up	XP_006489568.1	1-aminocyclopropane-

					1-carboxylate oxidase homolog 1
Cluster-27569.72371	-3.19	3.26E-10	down	XP_006420188.1	1-aminocyclopropane-1-carboxylate oxidase homolog 3
Cluster-27569.72375	-4.37	0.0047	down	XP_006489571.1	1-aminocyclopropane-1-carboxylate oxidase homolog 2
Cluster-27569.55597	1.95	0.0002	up	XP_006489569.1	1-aminocyclopropane-1-carboxylate oxidase homolog
Cluster-27569.104194	-4.09	0.0160	down	XP_010671061.1	1-aminocyclopropane-1-carboxylate oxidase homolog 1
Cluster-27569.72373	-5.43	0.0001	down	KDO38629.1	1-aminocyclopropane-1-carboxylate oxidase
Cluster-27569.52333	2.55	0.0030	up	XP_007050339.1	1-aminocyclopropane-1-carboxylate oxidase homolog 1
Cluster-27569.78289	-6.15	2.24E-21	down	XP_006420188.1	1-aminocyclopropane-1-carboxylate oxidase homolog 1
Cluster-27569.50278	-3.61	3.05E-07	down	XP_007225869.1	1-aminocyclopropane-1-carboxylate oxidase homolog 7
Cluster-27569.17960	2.03	0.0001	up	XP_002284638.2	1-aminocyclopropane-1-carboxylate oxidase homolog 1
Cluster-27569.17959	2.07	0.0004	up	XP_007035061.1	1-aminocyclopropane-1-carboxylate oxidase homolog
Cluster-27569.52332	3.16	0.0007	up	XP_007050339.1	1-aminocyclopropane-1-carboxylate oxidase homolog 1
Tryptophan aminotransferase					
Cluster-27569.54568	-5.68	3.77E-14	down	XP_006476853.1	tryptophan aminotransferase-

					related protein 2-like isoform X5
Cluster-27569.27507	-2.57	5.31E-07	down	XP_011042900.1	tryptophan aminotransferase-related protein 4-like
Cluster-27569.78968	-2.23	4.43E-05	down	XP_006439890.1	Tryptophan aminotransferase-related protein 2
9-cis-epoxycarotenoid dioxygenase (**NCED**)					
Cluster-27569.91182	-2.95	1.40E-06	down	KDO50508.1	9-cis-epoxycarotenoid dioxygenase NCED3
Cluster-27569.91178	-4.02	3.14E-05	down	NP_001275864.1	9-cis-epoxycarotenoid dioxygenase NCED3
Cluster-27569.91181	-3.94	0.0001	down	NP_001275864.1	9-cis-epoxycarotenoid dioxygenase NCED3
Cluster-27569.91180	-2.40	0.0088	down	NP_001275864.1	9-cis-epoxycarotenoid dioxygenase NCED3
Ethylene receptors (ETR)					
Cluster-27569.92251	-2.77	4.47E-08	down	ACL81480.3	ethylene receptor
Cluster-27569.38550	-3.59	6.32E-08	down	AEV21217.1	ethylene receptor
Cluster-27569.98517	-2.84	7.3E-07	down	AIT39449.1	ethylene receptor 1
Cluster-27569.27347	-3.18	8.18E-07	down	AIT39449.1	ethylene receptor 1
Cluster-27569.27348	-2.17	0.0016	down	AIT39449.1	ethylene receptor 1
Cluster-27569.85458	-2.57	0.0028	down	AIT39449.1	ethylene receptor 1
Cluster-27569.66807	1.38	0.019	up	XP_006420138.1	Ethylene receptor 2
Cluster-27569.20043	-1.38	0.0305	down	NP_001275855.1	ethylene response 3
Cluster-27569.92251	-2.77	4.47E-08	down	ACL81480.3	ethylene receptor
Cluster-27569.85458	-2.57	0.0028	down	AIT39449.1	ethylene receptor 1
SUGAR METABOLISM					

Sucrose synthase (Susy)					
Cluster-27569.71227	4.56	5.43E-16	up	XP_006481951.1	Sucrose synthase 7
Cluster-27569.86057	-3.54	6.24E-12	down	AJW82916.1	Sucrose synthase
Cluster-27569.79619	2.80	2.34E-08	up	AJW82916.1	Sucrose synthase
Cluster-27569.66803	2.76	2.11E-07	up	AJW82916.1	Sucrose synthase
Cluster-27569.68858	3.65	8.81E-05	up	XP_006481951.1	Sucrose synthase 5
Cluster-27569.79620	3.11	0.0001	up	AJW82916.1	Sucrose synthase
Cluster-27569.20356	-1.64	0.0048	down	AJW82918.1	Sucrose synthase 4
Cluster-27569.74767	2.26	0.0057	up	XP_006481951.1	Sucrose synthase 5
Beta amylase					
Cluster-27569.64814	2.42	1.51E-06	up	XP_006385389.1	Beta-amylase 3
Cluster-27569.64560	2.17	1.83E-05	up	XP_006385389.1	Beta-amylase 3
Cluster-27569.58506	-2.73	0.0001	down	XP_007035340.1	Beta-amylase 1
Cluster-27569.68148	2.03	0.0001	up	XP_009369103.1	Beta-amylase 9
Cluster-27569.60462	1.95	0.0003	up	XP_007035476.1	Beta-amylase 9
Cluster-27569.92208	-3.26	0.0003	down	XP_006493994.1	Beta-amylase 1
Cluster-27569.64502	1.77	0.0007	up	XP_006385389.1	Beta-amylase 3
Cluster-27569.63332	-1.49	0.0095	down	XP_006493994.1	Beta-amylase 1
Cluster-27569.71847	-2.02	0.0234	down	XP_011091372.1	Beta-amylase 1
Cluster-27569.69001	2.46	0.0343	up	XP_008390741.1	Beta-amylase 9
Cluster-27569.92206	-3.42	0.0454	down	XP_006493994.1	Beta-amylase 1
CELL WALL METABOLISM					
Polygalacturonase (PG)					
Cluster-27569.7350	-5.03	4.51E-15	down	XP_006439367.1	Probable polygalacturonase
Cluster-27569.17096	5.98	1.39E-12	up	XP_006489947.1	Polygalacturonase
Cluster-27569.65293	3.10	5.96E-10	up	CBI36307.3	Probable polygalacturonase

Cluster-27569.107909	-5.12	2.44E-09	down	XP_010652214.1	Probable polygalacturonase
Cluster-27569.59400	3.18	1.11E-07	up	XP_006427250.1	Probable polygalacturonase
Cluster-27569.59399	2.85	9.94E-07	up	ACF16415.1	Probable polygalacturonase
Cluster-27569.65630	3.96	3.18E-05	up	XP_006448628.1	Polygalacturonase
Cluster-27569.46817	3.19	0.0001	up	XP_007023628.1	Polygalacturonase
Cluster-27569.17095	5.86	0.0238	up	XP_006489947.1	Polygalacturonase
Cluster-27569.65629	1.29	0.0494	up	XP_007041025.1	Polygalacturonase
Expansin					
Cluster-27569.63411	7.43	1.51E-28	up	AHL25254.1	Expansin-A2
Cluster-27569.63815	4.60	8.27E-20	up	XP_011080662.1	Expansin-A2
Cluster-27569.66259	4.36	2.09E-17	up	AHL25254.1	Expansin-A2
Cluster-27569.17884	5.39	4.71E-09	up	XP_012091147.1	Expansin-A8
Cluster-27569.63861	3.13	1.07E-06	up	AHL25254.1	Expansin-A2
Cluster-27569.66261	3.69	7.71E-06	up	KHN04238.1	Expansin-A2
Cluster-27569.56489	-2.34	0.0010	down	XP_006430408.1	Expansin-A13
Cluster-27569.47278	2.04	0.0019	up	XP_011070988.1	Expansin-A10
Cluster-27569.33194	-1.47	0.0181	down	XP_006385562.1	Expansin-like B1
Pectinesterase (PE)					
Cluster-27569.17598	3.46	2.58E-12	up	XP_006479999.1	Probable pectinesterase
Cluster-27569.3912	-3.72	1.00E-06	down	XP_012084535.1	Probable pectinesterase
Cluster-27569.119546	-3.05	0.0008	down	XP_006427797.1	Probable pectinesterase
Cluster-27569.115149	1.61	0.0103	up	XP_007218171.1	Pectinesterase
Cluster-27569.103299	-2.72	0.0187	down	KDO75533.1	Pectinesterase
Cluster-27569.103404	-1.82	0.0014	down	XP_007012505.1	Probable pectinesterase
Pectate lyase (PL)					
Cluster-27569.65453	2.55	3.49E-07	up	XP_002322215.1	Pectate lyase 22

Cluster-27569.59684	3.27	3.51E-07	up	KHG22990.1	Pectate lyase-like isoform 1
Endoglucanase					
Cluster-27569.89592	4.39	3.80E-17	up	KDP26669.1	Endoglucanase
Cluster-27569.30591	-2.93	4.28E-09	down	XP_006431347.1	Endoglucanase 25
Cluster-27569.37213	5.97	4.24E-07	up	XP_006421697.1	Endoglucanase 8
Cluster-27569.70149	2.61	0.0001	up	ABR10607.1	Endoglucanase 6
Cluster-27569.100995	-4.59	0.0002	down	XP_012080633.1	Endoglucanase 11
Cluster-27569.26058	4.97	0.0002	up	XP_006435157.1	Endoglucanase 10
Cluster-27569.42916	-2.34	0.0010	down	XP_006483983.1	Endoglucanase E1
Cluster-27569.37212	5.72	0.0023	up	KDO65443.1	Endoglucanase 4
Cluster-27569.109523	-1.75	0.0046	down	XP_006435157.1	Endoglucanase 10
Cluster-27569.47150	8.01	0.0215	up	XP_006421697.1	Endoglucanase 8
Cluster-27569.29620	-4.08	0.0390	down	XP_006447630.1	Endoglucanase 11
Cluster-27569.66782	1.20	0.0423	up	ABR10607.1	Endoglucanase 19
Beta galactosidase					
Cluster-20281.3	4.95	1.65E-11	up	XP_006439048.1	Beta-D-glucosidase
Cluster-27569.114594	3.20	5.60E-06	up	XP_003538061.1	Beta-glucosidase 42
Cluster-27569.81308	3.53	6.04E-06	up	XP_007028105.1	Beta-glucosidase, putative isoform 2
Cluster-27569.52187	2.80	9.61E-05	up	XP_003538061.1	Beta-glucosidase 42
Cluster-27569.45224	3.88	0.0045	up	XP_010031675.1	Beta-glucosidase 11
Cluster-27569.111031	-2.15	0.0152	down	XP_006471035.1	Beta-glucosidase 44
Cluster-27569.48374	3.75	0.026	up	XP_006479944.1	Beta-glucosidase 11
Cluster-27569.73326	2.81	0.0364	up	XP_009346071.1	Beta-glucosidase 42
COLOR DEVELOPMENT					
Phytoene synthase					
Cluster-27569.61096	1.77	0.0050	up	AFE85918.1	Phytoene synthase
Cluster-27569.63499	1.22	0.0451	up	AFE85918.1	Phytoene synthase

Zeta carotene desaturase					
Cluster-27569.79928	1.46	0.0125		BAB68552.1	Zeta-carotene desaturase
Lycopene beta cyclase					
Cluster-27569.83348	-2.31	0.0095	down	ACT78995.1	Lycopene beta cyclase
Cluster-27569.122639	-3.68	0.0408	down	KDO54273.1	Lycopene beta cyclase
FLAVOUR DEVELOPEMENT					
Alcohol dehydrogenase					
Cluster-27569.95147	-3.88	3.62E-11	down	XP_012075882.1	Alcohol dehydrogenase 1
Cluster-27569.70284	2.95	6.42E-09	up	ADB43613.1	Alcohol dehydrogenase 1
Cluster-27569.61947	2.71	1.54E-07	up	ADB43613.1	Alcohol dehydrogenase 1
Cluster-27569.93154	-2.62	3.5E-07	down	XP_012075882.1	Alcohol dehydrogenase 1
Cluster-27569.70050	2.61	4.34E-07	up	ADB43613.1	Alcohol dehydrogenase 1
Cluster-27569.62349	2.44	4.25E-06	up	ADB43614.1	Alcohol dehydrogenase 2
Cluster-27569.106725	-4.11	2.65E-05	down	XP_012492075.1	Mannitol dehydrogenase
Cluster-27569.104639	-2.14	0.0001	down	XP_012073738.1	Alcohol dehydrogenase 6
Cluster-27569.58720	-1.85	0.0008	down	XP_012073738.1	Alcohol dehydrogenase 6
Cluster-27569.104596	-5.84	0.0062	down	XP_003635280.1	Mannitol dehydrogenase
Cluster-27569.64734	1.54	0.0069	up	XP_007048344.1	Alcohol dehydrogenase 1
Cluster-27569.76556	-5.35	0.0417	down	XP_006473751.1	Alcohol dehydrogenase-like 7-like
Lipoxygenase					
Cluster-27569.103806	-5.87	7.29E-24	down	XP_012444488.1	Linoleate 13S-lipoxygenase 2-1
Cluster-27569.67094	5.07	6.6E-22	up	AAK50778.4	Linoleate 9S-lipoxygenase 6
Cluster-27569.103807	-7.21	7.24E-15	down	XP_006494720.1	Linoleate 13S-lipoxygenase 2-1
Cluster-27569.119061	-4.55	5.28E-13	down	XP_011023610.1	Linoleate 9S-lipoxygenase 1
Cluster-27569.106032	-3.56	4.28E-06	down	XP_010025196.1	Linoleate 9S-lipoxygenase 5
Cluster-27569.20773	-3.48	7.86E-06	down	XP_006472029.1	Linoleate 9S-lipoxygenase 7

Cluster-27569.103155	-3.73	6.98E-05	down	XP_012444488.1	Linoleate 13S-lipoxygenase 2-1
Cluster-27569.120760	-5.17	0.0013	down	XP_011026291.1	Linoleate 9S-lipoxygenase 5
Cluster-27569.111681	-4.40	0.0013	down	XP_011026284.1	Linoleate 9S-lipoxygenase 5

Note: *Based on the NCBI non redundant (NR) and Swissprot description. 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; $\log_2$FC > 1 or $\log_2$FC< -1.

4.4.3.2.2 Comparison between Mango Groups ('CK' and 'P') at the Same Ripening Stage (Unripe and Ripe)

Comparison between mango groups at the unripe stage (CKUR vs PUR) (Table 4.12) revealed genes encoding citrate synthase (9), malic enzyme (10) and aconitase (6) and most of these genes were up-regulated in the 'P' group. Secondly, eleven genes encoding 1-aminocyclopropane-1-carboxylate oxidase (ACO) involved in ethylene biosynthesis were identified and out of these, six were strongly expressed in the unripe stage of 'P' (PUR) compared to the 'CK' group (CKUR). Four genes encoding 9-cis-epoxycarotenoid dioxygenase (NCED) associated with the biosynthesis of abscisic acid (ABA) were identified and all showed higher expression PUR compared to CKUR. Thirdly, genes encoding PG (11), PE (8), PL (2), expansin (8), endoglucanase (11) were also identified.The genes including *PG* and *PE endoglucanase* displayed a mixed expression (i.e. up-regulation or down-regulation) between mango groups. In contrast, *PL*, *expansin* and *GAUT* related genes were all up-regulated in PUR. Finally, the sugar-related genes including *Susy* and *amylase* were also found to be strongly expressed in PUR. Comparison at the ripe stage for the mango groups (CKR vs PR) (Table 4.13) showed that most genes outlined were highly expressed in PR. It is worth

pointing out that some of the genes which displayed differences in the expression level when mango groups were compared at the same ripening stage (i.e. the unripe [CKUR vs PUR] and/or ripe stage [CKR vs PR]) (Tables 4.12 and 4.13) were also observed to be expressed differentially during the unripe stage to ripe stage comparison in a mango group (Tables 4.10 and 4.11). For instance, a set of genes encoding malic enzyme (Cluster-27569.55138; Cluster-27569.67412), ACO (Cluster-27569.55597) and PG (Cluster-27569.65293) respectively which was strongly expressed in the unripe stage of the 'P' group (PUR) compared to the 'CK' group (CKUR) (Table 4.12) were found to be up-regulated in the ripe sample of the 'CK' and/or 'P' group (Tables 4.10 and 4.11**)**. The higher expression level of these genes in PUR than in CKUR might have contributed to the accelerated ripening programme in the 'P' group. Going further, a set of genes encoding PG (Cluster-27569.107909), malic enzyme (Cluster-27569.70276; Cluster-27569.78748) and ACO (Cluster-27569.72371; Cluster-27569.72375) respectively which displayed a down-regulated trend in both mango groups during fruit ripening, presented a higher expression level in CKUR compared to the PUR mango group (Table 4.12). This might have contributed to the delayed ripening in the 'CK' group. Pairwise comparison at either the unripe or ripe showed that the expression level of the colour-related gene β-carotene 3-hydroxylase was higher in the Pool group compared with the CK group. However, a mixed expression (up regulation and down regulation) was observed for *phytoene synthase* and *lycopene beta cyclase* when the same ripening stage for both mango groups were compared. Flavour-related genes were also found to be differentially expressed at either the unripe and ripe stages respectively.

Taken together, the different expression levels of these genes might have contributed to the differences observed in ripening associated characteristics of the mango groups investigated in this study.

Table 4.12: Some of the genes differentially expressed between the mango groups ('CK' and 'P') at the unripe stage (CKUR vs PUR).

Gene_ID	log_2FC	Adjusted P value	Regulation	NR ID	Annotation
ENERGY METABOLISM					
Citrate synthase					
Cluster-27569.74803	8.79	5.8E-44	up	KDO46052.1	Citrate synthase
Cluster-27569.58086	4.85	1.38E-19	up	XP_006480297.1	Citrate synthase
Cluster-27569.100667	4.11	6.37E-15	up	XP_012459840.1	Citrate synthase
Cluster-27569.97707	3.60	1.38E-08	up	XP_006488840.1	Citrate synthase
Cluster-27569.100664	3.06	1.54E-08	up	XP_002512567.1	Citrate synthase
Cluster-27569.100663	3.36	0.0005	up	XP_002512567.1	Citrate synthase
Cluster-27569.74094	1.52	0.0017	up	AFB82642.1	Citrate synthase
Cluster-27569.97708	5.70	0.0106	up	XP_011100760.1	Citrate synthase
Cluster-27569.100666	2.99	0.0110	up	XP_012459840.1	Citrate synthase
Aconitase					
Cluster-27569.43207	2.28	0.0023	up	XP_008455442.1	Aconitase
Cluster-27569.67787	1.41	0.0085	up	KDO71349.1	Aconitase
Cluster-27569.67777	1.15	0.0199	up	XP_007045642.1	Aconitase
Cluster-27569.58375	1.13	0.0206	up	XP_012448314.1	Aconitase
Cluster-27569.62674	-2.28	0.0216	down	XP_012448314.1	Aconitase
Cluster-27569.64507	1.25	0.0396	up	XP_011022256.1	Aconitase
Malic enzyme					
Cluster-27569.31106	6.45	3.29E-16	up	XP_006476015.1	Malic enzyme
Cluster-27569.67412	2.85	1.49E-09	up	AFM08812.1	Malic enzyme 4
Cluster-27569.63006	2.46	2.81E-07	up	AFM08812.1	Malic enzyme 4

Cluster-27569.18175	3.52	1.87E-05	up	KDO79867.1	Malic enzyme
Cluster-27569.105531	4.13	0.0001	up	KDO79867.1	Malic enzyme
Cluster-27569.55138	3.01	0.0001	up	XP_007015739.1	Malic enzyme
Cluster-27569.70276	-1.27	0.0083	down	XP_003547744.1	Malic enzyme
Cluster-27569.78748	-1.28	0.0098	down	KDO66537.1	Malic enzyme
Cluster-27569.61307	1.13	0.0240	up	AFM08812.1	Malic enzyme 4
Cluster-27569.26226	3.63	0.0439	up	XP_006450719.1	Malic enzyme
HORMONE METABOLISM AND SIGNALLING					
1-aminocyclopropane-1-carboxylate oxidase (ACO)					
Cluster-27569.17960	4.03	2.54E-15	up	XP_002284638.2	1-aminocyclopropane-1-carboxylate oxidase homolog 1
Cluster-27569.43139	-3.98	4.06E-14	down	XP_006420181.1	1-aminocyclopropane-1-carboxylate oxidase homolog 1
Cluster-27569.65392	2.43	7.85E-08	up	XP_006489568.1	1-aminocyclopropane-1-carboxylate oxidase homolog 1
Cluster-27569.55597	2.66	3.11E-07	up	XP_006489569.1	1-aminocyclopropane-1-carboxylate oxidase homolog
Cluster-27569.82597	2.48	8.34E-05	up	XP_002320487.1	1-aminocyclopropane-1-carboxylate oxidase
Cluster-27569.72372	-1.86	8.87E-05	down	XP_006420182.1	1-aminocyclopropane-1-carboxylate oxidase homolog 1
Cluster-27569.45316	2.38	0.0005	up	CAB97173.1	1-aminocyclopropane-1-carboxylate oxidase
Cluster-27569.72371	-1.42	0.0021	down	XP_006420188.1	1-aminocyclopropane-1-carboxylate oxidase homolog 3
Cluster-27569.72375	-1.49	0.0183	down	XP_006489571.1	1-aminocyclopropane-1-carboxylate oxidase homolog 2
Cluster-27569.104194	-1.89	0.0274	down	XP_010671061.1	1-aminocyclopropane-1-carboxylate oxidase homolog 1
Cluster-27569.68916	3.18	0.0358	up	XP_006489569.1	1-aminocyclopropane-1-carboxylate oxidase homolog 7
Tryptophan aminotransferase					

Cluster-27569.27507	3.32	2.63E-13	down	XP_011042900.1	tryptophan aminotransferase-related protein 4-like
9-cis-epoxycarotenoid dioxygenase (NCED)					
Cluster-27569.91182	2.38	1.22E-05	up	KDO50508.1	9-cis-epoxycarotenoid dioxygenase NCED3
Cluster-27569.91178	3.01	0.0004	up	NP_001275864.1	9-cis-epoxycarotenoid dioxygenase NCED3
Cluster-27569.91181	2.42	0.0077	up	NP_001275864.1	9-cis-epoxycarotenoid dioxygenase NCED3
Cluster-27569.91180	1.78	0.0255	up	NP_001275864.1	9-cis-epoxycarotenoid dioxygenase NCED3
Ethylene receptors (ETR)					
Cluster-27569.71327	4.89	3.89E-23	up	XP_006420138.1	Ethylene receptor 2
Cluster-27569.38550	2.69	1.64E-05	up	AEV21217.1	Ethylene receptor
Cluster-27569.94925	5.67	0.0088	up	NP_001275840.1	Ethylene receptor 2
Cluster-27569.71325	2.23	0.0437	up	XP_011029854.1	Ethylene receptor 2
Cluster-27569.71329	6.11	1.16E-33	up	XP_006420138.1	Ethylene receptor 2
SUGAR METABOLISM					
Sucrose synthase (Susy)					
Cluster-27569.79620	3.08	2.57E-07	up	AJW82916.1	Sucrose synthase
Cluster-27569.79619	2.11	3.71E-06	up	AJW82916.1	Sucrose synthase
Cluster-27569.71227	4.44	0.0003	up	XP_006481951.1	Sucrose synthase 7
Cluster-27569.20356	1.33	0.0091	up	AJW82918.1	Sucrose synthase 4
Polygalacturonase (PG)					
Cluster-27569.65293	4.72	1.59E-16	up	CBI36307.3	Probable polygalacturonase
Cluster-27569.46817	5.69	2.09E-16	up	XP_007023628.1	Polygalacturonase
Cluster-27569.59398	6.79	1.20E-12	up	ACF16415.1	Probable polygalacturonase
Cluster-27569.7350	2.62	2.40E-06	up	XP_006439367.1	Probable polygalacturonase
Cluster-27569.101799	6.83	7.00E-05	up	KDO82433.1	Polygalacturonase 1
Cluster-27569.59399	3.52	0.0026	up	ACF16415.1	Probable polygalacturonase

Cluster-27569.82565	5.46	0.0115	up	XP_007023876.1	Probable polygalacturonase
Cluster-27569.107909	-1.33	0.0313	down	XP_010652214.1	Probable polygalacturonase
Cluster-27569.80555	-1.15	0.0352	down	XP_010246894.1	Probable polygalacturonase
Cluster-27569.121795	-3.85	0.0411	down	KDO76318.1	Probable polygalacturonase
Cluster-27569.51988	1.08	0.0429	up	XP_006432212.1	Probable polygalacturonase
Pectinesterase (PE)					
Cluster-27569.14401	4.39	1.66E-15	up	NP_001275859.1	Pectinesterase 3
Cluster-27569.115149	3.90	7.25E-07	up	XP_007218171.1	Pectinesterase
Cluster-27569.64338	1.77	6.02E-05	up	XP_002304256.1	Pectinesterase 1
Cluster-27569.103298	1.88	0.0013	up	XP_007025998.1	Pectinesterase 2.1
Cluster-27569.17598	8.30	0.0030	up	XP_006479999.1	Probable pectinesterase
Cluster-27569.76572	-1.15	0.0314	down	KCW64453.1	Pectinesterase 31
Cluster-27569.11977	2.55	0.0444	up	XP_006474555.1	Pectinesterase
Cluster-27569.103404	1.20	0.0139	up	XP_007012505.1	Probable pectinesterase
Pectate lyase (PL)					
Cluster-27569.65453	1.68	0.0101	up	XP_002322215.1	Probable pectate lyase 18
Cluster-27569.82565	5.46	0.0115	up	XP_007023876.1	Probable polygalacturonase
Expansin					
Cluster-27569.53261	4.52	1.74E-19	up	XP_010258310.1	Expansin-A1
Cluster-27569.17884	6.38	6.13E-09	up	XP_012091147.1	Expansin-A8
Cluster-27569.15373	4.35	3.00E-07	up	KDO43109.1	Expansin-A1
Cluster-27569.65111	3.75	0.0014	up	AAT11859.2	Expansin-A6
Cluster-27569.47278	3.52	0.0015	up	XP_011070988.1	Expansin-A10
Cluster-27569.50951	3.88	0.0097	up	XP_010258311.1	Expansin-A1
Cluster-27569.33194	1.45	0.0168	up	XP_006385562.1	Expansin-like B1
Cluster-27569.9711	4.01	0.0443	up	XP_006422480.1	Expansin-like A1

Endoglucanase					
Cluster-27569.64817	5.48	4.75E-29	up	XP_006423475.1	Endoglucanase 24
Cluster-27569.71977	5.23	9.50E-21	up	XP_011047414.1	Endoglucanase 24
Cluster-27569.42916	7.21	4.80E-13	up	XP_006483983.1	Endoglucanase E1
Cluster-27569.64818	6.69	8.36E-10	up	XP_006423475.1	Endoglucanase 23
Cluster-27569.109523	3.07	7.28E-09	up	XP_006435157.1	Endoglucanase 10
Cluster-27569.89592	3.26	3.55E-06	up	KDP26669.1	Endoglucanase
Cluster-27569.64326	1.85	2.29E-05	up	ABR10607.1	Endoglucanase 6
Cluster-27569.66782	1.94	2.44E-05	up	ABR10607.1	Endoglucanase 19
Cluster-27569.100995	-1.92	0.0001	down	XP_012080633.1	Endoglucanase 11
Cluster-27569.67542	1.82	0.0021	up	ABR10607.1	Endoglucanase 19
Cluster-27569.93647	1.22	0.0430	up	XP_006484545.1	Endoglucanase 5
Beta amylase					
Cluster-27569.63333	4.36	8.51E-13	up	XP_011091372.1	Beta-amylase 1
Cluster-27569.64560	2.98	2.30E-10	up	XP_006385389.1	Beta-amylase 3
Cluster-27569.64814	2.54	2.84E-09	up	XP_006385389.1	Beta-amylase 3
Cluster-27569.63332	2.63	2.93E-09	up	XP_006493994.1	Beta-amylase 1
Cluster-27569.65896	3.56	1.44E-08	up	XP_010531694.1	Beta-amylase 3
Cluster-27569.64502	3.26	2.76E-07	up	XP_006385389.1	Beta-amylase 3
Cluster-27569.64488	2.18	4.37E-07	up	XP_007222488.1	Beta-amylase 9
Cluster-27569.65014	1.42	0.0022	up	KDO75062.1	Beta-amylase 9
Cluster-27569.58506	1.92	0.0030	up	XP_007035340.1	Beta-amylase 1
Beta galactosidase					
Cluster-27569.74286	-3.77	4.90E-06	down	XP_006487669.1	beta-galactosidase-like
Cluster-27569.119324	2.78	1.32E-05	up	XP_007048525.1	Beta-galactosidase 3
Cluster-27569.23429	4.55	1.00E-04	up	KJB32549.1	beta-galactosidase

Cluster-27569.66681	3.95	2.00E-04	up	XP_007012844.1	Beta-galactosidase
Cluster-27569.112447	2.96	4.00E-04	up	AHC32021.1	beta-galactosidase 3
Cluster-27569.108351	2.91	5.00E-04	up	XP_002263382.2	beta-galactosidase 3
Cluster-27569.66238	3.80	8.00E-04	up	AAB61470.1	beta-D-galactosidase
Cluster-27569.76723	-1.80	9.00E-04	down	XP_006487669.1	beta-galactosidase
Cluster-27569.54350	1.78	2.90E-03	up	XP_006466023.1	beta-galactosidase 17
Cluster-27569.56610	3.77	9.40E-03	up	AAB61470.1	beta-D-galactosidase
Cluster-27569.108352	3.22	1.05E-02	up	XP_012480371.1	beta-galactosidase 5
Cluster-27569.54352	-1.30	3.50E-02	down	XP_006466023.1	beta-galactosidase 17
COLOUR DEVELOPMENT					
Phytoene synthase					
Cluster-27569.63499	2.32	5.74E-06	up	AFE85918.1	phytoene synthase
Cluster-27569.28431	-2.28	6.66E-03	down	AFE85918.1	phytoene synthase
Cluster-27569.56796	3.24	4.34E-02	up	AFE85918.1	phytoene synthase
Beta-carotene 3-hydroxylase					
Cluster-27569.57556	6.21	2.81E-09	up	KDO56783.1	Beta-carotene hydroxylase 2
Cluster-27569.65769	2.76	7.09E-05	up	KDO56789.1	Beta-carotene hydroxylase 2
Cluster-27569.65043	2.22	7.44E-04	up	KDO56784.1	Beta-carotene hydroxylase 2
Cluster-27569.69891	1.61	1.92E-03	up	KDO56786.1	Beta-carotene hydroxylase 2
FLAVOUR DEVELOPEMENT					
Alcohol dehydrogenase					
Cluster-27569.102651	-8.78	2.97E-24	down	XP_002530071.1	Alcohol dehydrogenase,
Cluster-27569.47234	-3.93	2.75E-19	down	KDO65864.1	Alcohol dehydrogenase 1
Cluster-27569.95132	-5.71	5.15E-11	down	XP_006478148.1	Alcohol dehydrogenase-like 6
Cluster-27569.70050	3.18	1.56E-09	up	ADB43613.1	Alcohol dehydrogenase 1
Cluster-27569.70284	2.83	1.85E-09	up	ADB43613.1	Alcohol dehydrogenase 1
Cluster-27569.24221	-2.74	5.89E-08	down	XP_002529812.1	Alcohol dehydrogenase

Cluster-27569.61947	2.28	1.96E-06	up	ADB43613.1	Alcohol dehydrogenase 1
Cluster-27569.93154	-1.83	3.18E-05	down	XP_012075882.1	Alcohol dehydrogenase 1
Cluster-27569.24757	2.95	0.0001	up	KHG24369.1	Alcohol dehydrogenase 9 -like
Cluster-27569.95147	-1.33	0.01	down	XP_012075882.1	Alcohol dehydrogenase 1
Cluster-27569.23016	-1.25	0.047	down	KDO85026.1	Alcohol dehydrogenase-like 7
Lipoxygenase					
Cluster-27569.119061	4.79	3.86E-15	up	XP_011023610.1	Linoleate 9S-lipoxygenase 5
Cluster-27569.106032	3.98	6.64E-07	up	XP_010025196.1	Linoleate 9S-lipoxygenase 5
Cluster-27569.20773	3.16	4.33E-05	up	XP_006472029.1	Linoleate 9S-lipoxygenase 5-like isoform X2
Cluster-27569.120760	6.06	0.0013	up	XP_011026291.1	Linoleate 9S-lipoxygenase 5 isoform X2
Cluster-27569.66540	2.09	0.0105	up	XP_006465905.1	Linoleate 13S-lipoxygenase 3-1
Cluster-27569.111681	-1.83	0.0133	down	XP_011026284.1	Linoleate 9S-lipoxygenase 5 isoform X1
Cluster-27569.103155	-2.23	2.55E-02	down	XP_012444488.1	Linoleate 13S-lipoxygenase 2-1

Note: *Based on the NCBI non redundant (NR) and Swissprot description. 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; $\log_2$FC > 1 or $\log_2$FC< -1.

Table 4.13: Some of the genes differentially expressed between the mango groups ('CK' and 'P') at the ripe stage (CKR vs PR).

Gene_ID	log_2FC	Adjusted P value	Regulation	NR ID	Annotation
ENERGY METABOLISM					
Citrate synthase					
Cluster-27569.97707	5.37	7.55E-14	up	XP_006488840.1	citrate synthase
Cluster-27569.74803	3.58	9.63E-07	up	KDO46052.1	citrate synthase
Cluster-27569.100666	3.61	0.0001	up	XP_012459840.1	citrate synthase
Cluster-27569.70337	5.34	0.0003	up	XP_006480297.1	citrate synthase
Cluster-27569.58086	2.52	0.0011	up	XP_006480297.1	citrate synthase
Cluster-27569.100663	2.90	0.0035	up	XP_002512567.1	citrate synthase
Cluster-27569.100667	2.26	0.0041	up	XP_012459840.1	citrate synthase
Aconitase					
Cluster-27569.108062	3.93	0.0002	up	XP_006420372.1	Aconitase
Cluster-27569.36169	5.44	0.0049	up	XP_012077610.1	Aconitase
Cluster-27569.91550	2.78	0.0029	up	XP_006477157.1	Aconitase
Malic enzyme					
Cluster-27569.63009	-8.67	1.88E-25	down	KEH39547.1	Malic enzyme
Cluster-27569.68188	4.50	1.01E-10	up	AAF73006.1	Malic enzyme
Cluster-27569.18175	5.19	0.0002	up	KDO79867.1	Malic enzyme
Cluster-27569.72484	-6.53	0.0083	down	XP_003547744.1	Malic enzyme
Cluster-27569.31106	4.26	0.0187	up	XP_006476015.1	Malic enzyme

Cluster-27569.105531	4.27	0.0221	up	KDO79867.1	Malic enzyme
Cluster-27569.67412	1.67	0.0474	up	AFM08812.1	Malic enzyme 4
HORMONE METABOLISM AND SIGNALLING					
1-aminocyclopropane-1-carboxylate oxidase (ACO)					
Cluster-27569.17960	7.95	1.32E-21	up	XP_002284638.2	1-aminocyclopropane-1-carboxylate oxidase homolog 1
Cluster-27569.14298	8.52	2.67E-12	up	XP_011000640.1	1-aminocyclopropane-1-carboxylate oxidase
Cluster-27569.18002	6.25	0.0001	up	CDP04457.1	1-aminocyclopropane-1-carboxylate oxidase
Cluster-27569.46971	6.14	0.0034	up	CAB97173.1	1-aminocyclopropane-1-carboxylate oxidase
Tryptophan aminotransferase					
Cluster-27569.27507	4.33	0.004817	up	XP_011042900.1	tryptophan aminotransferase-related protein 4-like
9-cis-epoxycarotenoid dioxygenase (NCED)					
Cluster-27569.91178	4.35	4.49E-09	up	NP_001275864.1	9-cis-epoxycarotenoid dioxygenase NCED3
Sucrose synthase (Susy)					
Cluster-27569.64909	4.29	7.79E-10	up	XP_007050984.1	Sucrose synthase 2
Cluster-27569.79620	5.73	5.92E-09	up	AJW82916.1	Sucrose synthase
Cluster-27569.74768	-4.69	0.0061	down	XP_006481951.1	Sucrose synthase 7
Cluster-27569.86057	1.99	0.0210	up	AJW82916.1	Sucrose synthase
Cluster-27569.74767	-3.27	0.0316	down	XP_006481951.1	Sucrose synthase 5
Ethylene receptors (ETR)					
Cluster-27569.66899	-2.29	0.0023		KDO50509.1	ethylene receptor
Cluster-27569.71325	2.44	0.0139	up	XP_011029854.1	ethylene receptor 2-like isoform X2
Cluster-27569.92251	1.91	0.0246	up	ACL81480.3	ethylene receptor
CELL WALL METABOLISM					
Polygalacturonase (PG)					
Cluster-27569.46817	3.78	2.67E-06	up	XP_006493148.1	Polygalacturonase

Cluster-27569.42983	-4.16	1.43E-06	down	XP_007023628.1	Polygalacturonase
Cluster-27569.101799	3.32	3.27E-06	up	KDO82433.1	Polygalacturonase
Cluster-27569.82565	4.27	6.52E-06	up	XP_007023876.1	Probable polygalacturonase
Cluster-27569.59398	2.90	6.17E-05	up	ACF16415.1	Probable polygalacturonase
Cluster-27569.87912	-3.49	0.0002	down	XP_007203325.1	Polygalacturonase
Cluster-27569.113984	8.99	0.0005	up	XP_006489947.1	Polygalacturonase
Cluster-27569.103369	2.44	0.0090	up	XP_012086833.1	Probable polygalacturonase
Cluster-27569.33497	-4.23	0.0211	down	KDO51595.1	Polygalacturonase
Expansin					
Cluster-27569.66260	9.15	3.54E-29	up	XP_011080662.1	Expansin-A2
Cluster-27569.17884	6.15	1.14E-12	up	XP_012091147.1	Expansin
Cluster-27569.63861	-3.18	1.01E-05	down	AHL25254.1	Expansin-A2
Cluster-27569.66261	-4.08	0.0111	down	KHN04238.1	Expansin-A2
Cluster-27569.9710	3.15	0.0126	up	XP_006422480.1	Expansin-like A2
Pectinesterase (PE)					
Cluster-27569.17598	13.55	1.6E-34	up	XP_006479999.1	Probable pectinesterase
Cluster-27569.14401	5.36	2.71E-10	up	NP_001275859.1	Pectinesterase
Cluster-27569.115149	4.68	3.27E-08	up	XP_007218171.1	Pectinesterase
Cluster-27569.112257	7.63	0.0022	up	XP_006479999.1	Probable pectinesterase

Cluster-27569.103299	4.19	0.0316	up	KDO75533.1	Pectinesterase 2.2
Cluster-27569.103404	3.99	0.0005	up	XP_007012505.1	Probable pectinesterase
Endoglucanase					
Cluster-27569.30591	4.01	1.83E-06	up	XP_006431347.1	Endoglucanase 25
Cluster-27569.118046	5.02	3.31E-05	up	ACT54547.1	Endoglucanase 12
Cluster-27569.71976	-2.87	3.6E-05	down	XP_006423475.1	Endoglucanase 24
Cluster-27569.89592	2.90	4.08E-05	up	KDP26669.1	Endoglucanase
Cluster-27569.64817	-2.09	0.0060	down	XP_006423475.1	Endoglucanase 24
Cluster-19470.0	-5.34	0.0094	down	XP_009784759.1	Endoglucanase 1
Pectin lyase (PL)					
Cluster-27569.65453	-1.81	0.044152	down	XP_002322215.1	Probable pectate lyase 18
Beta galactosidase					
Cluster-27569.76723	-2.25	0.0056	down	XP_006487669.1	Beta-galactosidase
COLOUR DEVELOPMENT					
Phytoene synthase					
Cluster-27569.61096	6.31	2.28E-17	up	AFE85918.1	Phytoene synthase
Cluster-27569.56796	2.88	0.0172	up	AFE85918.1	phytoene synthase
Beta carotene 3-hydroxylase					
Cluster-27569.69891	2.15	0.0224	up	KDO56786.1	Beta-carotene 3-hydroxylase
FLAVOUR DEVELOPEMENT					
Alcohol dehydrogenase					
Cluster-27569.95132	-7.15	1.13E-12	down	XP_006478148.1	Alcohol dehydrogenase-like 6
Cluster-27569.102651	-6.75	2.06E-08	down	XP_002530071.1	Alcohol dehydrogenase
Cluster-27569.104639	5.99	7.92E-08	up	XP_012073738.1	Alcohol dehydrogenase 6 isoform X1
Cluster-27569.76545	3.64	0.0006	up	XP_006473751.1	Alcohol dehydrogenase-like 7

Cluster-27569.70284	2.17	0.0027	up	ADB43613.1	Alcohol dehydrogenase 1
Cluster-27569.68183	2.37	0.0038	up	XP_007039278.1	Alcohol dehydrogenase isoform 1
Cluster-27569.93154	2.14	0.0095	up	XP_012075882.1	Alcohol dehydrogenase 1
Cluster-27569.70050	1.85	0.0165	up	ADB43613.1	Alcohol dehydrogenase 1
Cluster-27569.58720	2.07	0.0208	up	XP_012073738.1	Alcohol dehydrogenase 6 isoform X1
Cluster-27569.24757	3.10	0.0274	up	KHG24369.1	Alcohol dehydrogenase 9 -like
Cluster-27569.27691	-2.67	0.0324	down	XP_002279832.1	Mannitol dehydrogenase
Cluster-27569.69367	2.72	0.0415	up	XP_004139644.1	Alcohol dehydrogenase
Cluster-27569.86861	-1.71	0.049	down	XP_012484144.1	Alcohol dehydrogenase class-3-like
Lipoxygenase					
Cluster-27569.66539	-3.11	0.0014	down	XP_006465905.1	Linoleate 13S-lipoxygenase 3-1
Cluster-27569.26957	2.00	0.0096	up	XP_007047990.1	Lipooxygenase
Cluster-27569.70107	1.69	0.0289	up	XP_006382594.1	Linoleate 9S-lipoxygenase 5
Cluster-27569.48014	-2.42	0.0386	down	XP_004141705.1	Linoleate 13S-lipoxygenase 3-1

Note: *Based on the NCBI NR and Swissprot description. 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.

4.4.3.2.3 The Involvement of the Rab GTPases in Mango Fruit Ripening

In order to investigate whether the Rab GTPases are important in mango softening, a pairwise comparison of the expression data was performed. Differentially expressed *Rab* genes were found between the ripening stages of the 'CK' (Table 4.14) and 'P' (Table 4.15) groups respectively. In addition, the *Rab* genes were also found to be expressed differentially between mango groups at the unripe (Table 4.16) and ripe (Table 4.17) stages respectively. This finding indicates that membrane trafficking is essential in mango ripening process. These genes belonged to the subclasses RabA, RabC, RabD, RabE and RabF indicating that at least some members of 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 (Table 4.14), which included *RabA* (4), *RabC* (1) and *RabE* (2) respectively. Out of these, two genes encoding RabA4 and RabE respectively were strongly expressed in the ripe sample while other genes showed an opposite trend. On the other hand, ten *Rab* genes were differentially expressed between the ripening stages of 'P' group (Table 4.15). This included *RabA* (7), *RabC* (2) and *RabF* (1) genes respectively. Out of these, one *RabC* and five *RabA* genes were strongly expressed in the unripe sample whereas other genes showed the opposite trend. A mixed gene expression (down-regulation and up-regulation) suggests that these genes play roles in fruit development and ripening (Tucker *et al*., 2017). Nevertheless, most of the *RabA* genes in both mango groups displayed higher expression levels at the unripe than at the ripe stage (Tables 4.14 and 4.15).

Going further with the results, pairwise comparison of the mango groups at either the unripe (Table 4.16) or ripe (Table 4.17) 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 (i.e. CKUR vs PUR) (Table 4.16),

eight encoded the RabA subfamily. Genes encoding RabA2, RabA3, RabC, RabD and RabE were 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’ group (Table 4.17). Together, the data presented here has reinforced the involvement of the Rab GTPase in mango fruit ripening. The RabA subfamily (RabA1, RabA2, RabA3 and RabA4) have been previously ascribed functions in plant cell wall dynamics (Lunn, 2013) which makes this subfamily a major focus of this study.

Table 4.14: *Rab* genes expressed differentially between the unripe (UR) and ripe (R) stages of the Chokanan ('CK') group (CKUR vs CKR).

Gene_ID	log_2FC	Adjusted P value	Regulation	NR ID	Annotation
Cluster-27569.63067	2.39	0.0011	up	XP_006426349.1	Ras-related protein RabA4a
Cluster-27569.55949	3.08	4.95E-05	up	KJB83797.1	Ras-related protein RabE
Cluster-27569.94833	-2.61	0.0002	down	XP_006426348.1	Ras-related protein RabA4a
Cluster-27569.53557	-3.17	0.0329	down	XP_011089516.1	Ras-related protein RabA2b
Cluster-27569.100367	-2.08	0.0374	down	XP_006438973.1	Ras-related protein RabA4d
Cluster-27569.106190	-3.67	1.42E-06	down	XP_006428467.1	Ras-related protein RabC2a
Cluster-27569.55074	-1.63	0.0236	down	KDO43940.1	Ras-related protein RabE1c

Note: *Based on the NCBI non redundant (NR) and Swissprot description. 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 4.15: *Rab* genes expressed differentially between the unripe (UR) and ripe (R) stages of the Pool ('P') group (PUR vs PR).

Gene_ID	log_2FC	Adjusted P value	Regulation	NR ID	Annotation
Cluster-27569.94833	-2.02	0.0001	down	XP_006426348.1	Ras-related protein RabA4a
Cluster-27569.39793	-1.91	0.0003	down	XP_012066252.1	Ras-related protein RabA5d
Cluster-27569.39791	-2.25	0.0002	down	XP_012066252.1	Ras-related protein RabA5d
Cluster-27569.69569	-2.00	0.0008	down	KDO63417.1	Ras-related protein RabA1a
Cluster-27569.53557	-2.04	0.0043	down	XP_011089516.1	Ras-related protein RabA2
Cluster-27569.22080	-4.55	0.0016	down	XP_006428467.1	Ras-related protein RabC2a
Cluster-27569.83684	1.51	0.0426	up	XP_006348894.1	Ras-related protein RabA1a
Cluster-27569.60513	1.42	0.0349	up	XP_007034146.1	Ras-related protein RabA5c
Cluster-27569.73649	1.68	0.0065	up	XP_007010044.1	Ras-related protein RabC2a
Cluster-27569.65261	1.49	0.0107	up	XP_007013197.1	Ras-related protein RabF

Note: *Based on the NCBI non redundant (NR) and Swissprot description. 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 4.16: *Rab* genes expressed differentially between the mango groups ('CK' and 'P') at the unripe stage (CKUR vs PUR).

Gene_ID	log_2FC	Adjusted P value	Regulation	NR ID	Annotation
Cluster-27569.69571	-3.89	2.34E-14	down	XP_006446826.1	Ras-related protein RabA1a
Cluster-27569.60513	-2.83	6.20E-10	down	XP_007034146.1	Ras-related protein RabA5c
Cluster-27569.69570	-1.46	0.0014	down	KDO63417.1	Ras-related protein RabA1a
Cluster-27569.50830	-1.29	0.0107	down	XP_009336164.1	Ras-related protein RabA1f
Cluster-27569.83684	-1.57	0.0127	down	XP_006348894.1	Ras-related protein RabA1a
Cluster-27569.24125	2.08	0.0007	up	XP_006444779.1	Ras-related protein RabA3
Cluster-27569.72659	1.18	0.0182	up	XP_002526431.1	Ras-related protein RabA2a
Cluster-27569.53557	1.45	0.0405	up	XP_011089516.1	Ras-related protein RabA2b
Cluster-27569.61122	4.39	1.47E-15	up	XP_003518654.1	Ras-related protein RabE1c
Cluster-27569.73649	5.45	2.38E-12	up	XP_007010044.1	Ras-related protein RabC2a
Cluster-27569.62477	5.67	1.11E-09	up	XP_006490023.1	Ras-related protein RabD2a
Cluster-27569.78912	1.61	0.0012	up	XP_007010044.1	Ras-related protein RabC2a

Note: *Based on the NCBI non redundant (NR) and Swissprot description. 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 4.17: *Rab* genes expressed differentially between the mango groups ('CK' and 'P') at the ripe stage (CKR vs PR).

Gene_ID	log_2FC	Adjusted P value	Regulation	NR ID	Annotation
Cluster-27569.69571	-3.46	4.35E-05	down	XP_006446826.1	Ras-related protein RabA1a
Cluster-27569.24125	1.87	0.0185	up	XP_006444779.1	Ras-related protein RabA3
Cluster-27569.73649	6.11	1.18E-12	up	XP_007010044.1	Ras-related protein RabC2a
Cluster-27569.50830	-1.68	0.0383	down	XP_009336164.1	Ras-related protein RabA1f
Cluster-27569.69569	-1.90	0.0407	down	KDO63417.1	Ras-related protein RabA1a
Cluster-27569.61122	4.15	3.72E-08	up	XP_003518654.1	Ras-related protein RabE1c
Cluster-27569.62477	Inf	9.87E-08	up	XP_006490023.1	Ras-related protein RabD2a

Note: *Based on the NCBI non redundant (NR) and Swissprot description. 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; $log_2FC > 1$ or $log_2FC < -1$. Inf, infinite (used when there is a zero expression in one group of sample).

4.4.4 Functional Categorization of Differentially Expressed Genes

Gene Ontology (GO) enrichment analysis was performed to analyse the functions of differentially expressed genes obtained from the pairwise comparison between the ripening stages of the mango groups (i.e. CKUR vs CKR [Figure 4.25] and PUR vs PR [Figure 4.26]) and between mango groups at the unripe (i.e. CKUR vs PUR [Figure 4.27]) and ripe (i.e. CKR vs PR [Figure 4.28]) stages respectively. Within the biological process category, most of the DEGs were classified into metabolic processes irrespective of the comparison being analysed.

A full list of enriched GO terms in the biological process category can be found in Appendix IX. Significantly enriched metabolic pathways in the DEGs were identified by Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analysis. To further identify the biological pathways in which the DEGs of fruit ripening are involved, the detected DEGs were mapped to the reference pathways in the KEGG database. The top 5 significant pathways found during the transition of the unripe to ripe stage of the 'CK' and 'P' group is presented in Table 4.18. The full list of significantly enriched pathways can be found in Appendix X.

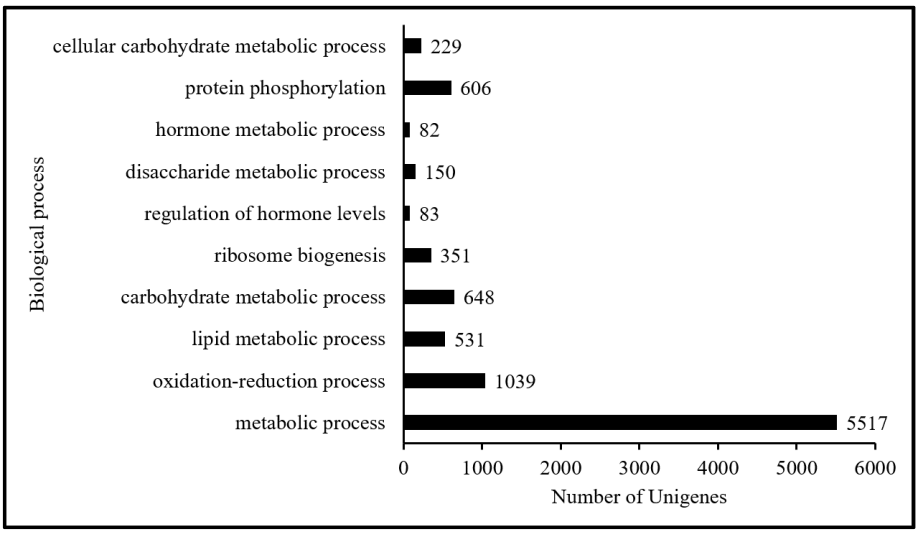


Figure 4.25: Gene ontology classification of differentially expressed genes during the ripening of the Chokanan ('CK') group under the biological process category.

Note: The top ten significant GO terms associated with the differentially expressed genes during the transition from unripe to ripe stage. The adjusted P value cut off was set at 0.05.

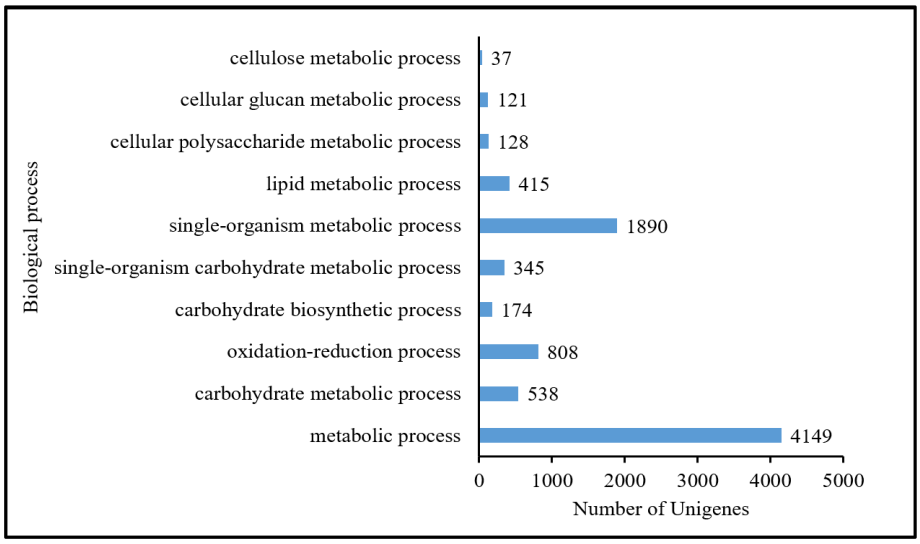


Figure 4.26: Gene ontology classification of differentially expressed genes during the ripening of the Pool ('P') group under the biological process category.

Note: The top ten significant GO terms associated with the differentially expressed genes during the transition from unripe to ripe stage. The adjusted P value cut off was set at 0.05.

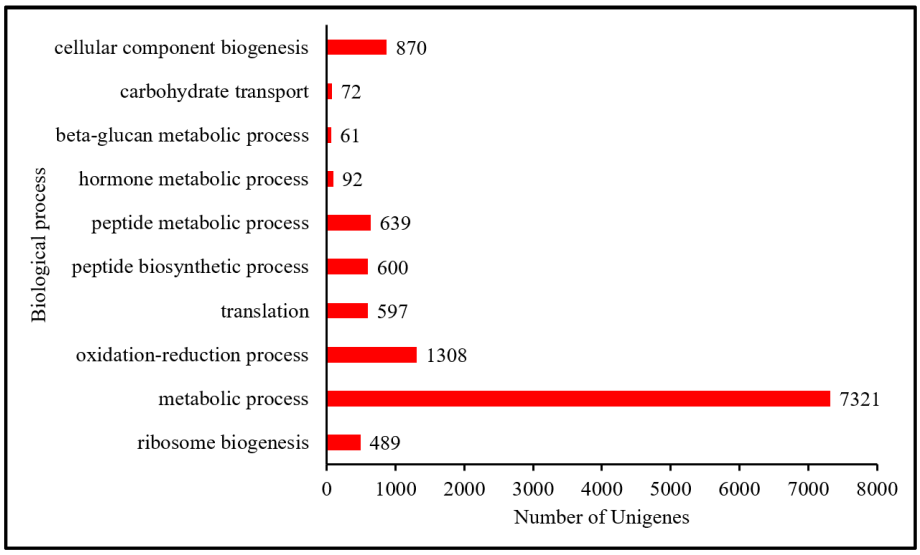


Figure 4.27: Gene ontology classification of differentially expressed genes between the unripe stages of Chokanan ('CK') and Pool ('P') group under the biological process category.

Note: The top ten significant GO terms associated with the differentially expressed genes during the transition from unripe to ripe stage. The adjusted P value cut off was set at 0.05.

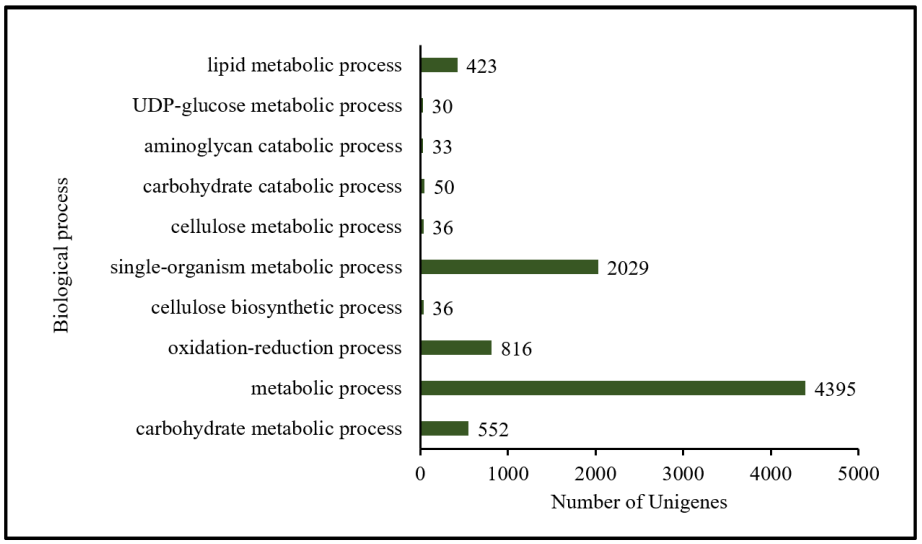


Figure 4.28: Gene ontology classification of differentially expressed genes between the ripe stages of Chokanan ('CK') and Pool ('P') group under the biological process category.

Note: The top ten significant GO terms associated with the differentially expressed genes during the transition from unripe to ripe stage. The adjusted P value cut off was set at 0.05.

Table 4.18: Top five significant enriched KEGG pathways during mango ripening

Term	ID	Count	Background	Adjusted P value
CKUR vs CKR				
Plant hormone metabolism	ko04075	247	850	3.2E-24
Ribosome	ko03010	265	1192	3.87E-13
Starch and sucrose metabolism	ko00500	183	806	1.14E-09
Fatty acid metabolism	ko01212	114	480	7.69E-07
Photosynthesis	ko00195	49	158	2.17E-05
PUR vs PR				
Plant hormone metabolism	ko04075	180	850	5.6E-19
Carbon metabolism	ko01200	231	1544	1.39E-09
Pyruvate metabolism	ko00620	106	605	4.07E-07
Fatty acid biosynthesis	ko00061	60	270	5.06E-07
Glycolysis	ko00010	110	682	4.14E-06

Note: KEGG, Kyoto Encyclopaedia of Genes and Genomes; Term refers to the name of the enriched KEGG pathway; Count refers to the number of differentially expressed genes related with a pathway. Background refers to the number of all genes related with the pathway. The adjusted P value cut off was set at 0.05.

4.4.5 Protein-Protein Interaction (PPI) Analysis

A Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database was used to reveal how differentially expressed genes interact with each other. This web-based database generates interaction networks based on known and predicted PPI (Szklarczyk *et al.*, 2017). 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 4.29. Besides the cell wall related proteins that were used as inputs (i.e. polygalacturonase, pectinesterase and endoglucanase) 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 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. GDI retrieves the GDP-bound Rab proteins from the target membranes after a vesicular transport event whereas syntaxins are involved in vesicle tethering and fusion with the target compartment. This indicates the combined efforts of various components within the secretory machinery to enable cargo transport during the softening process.

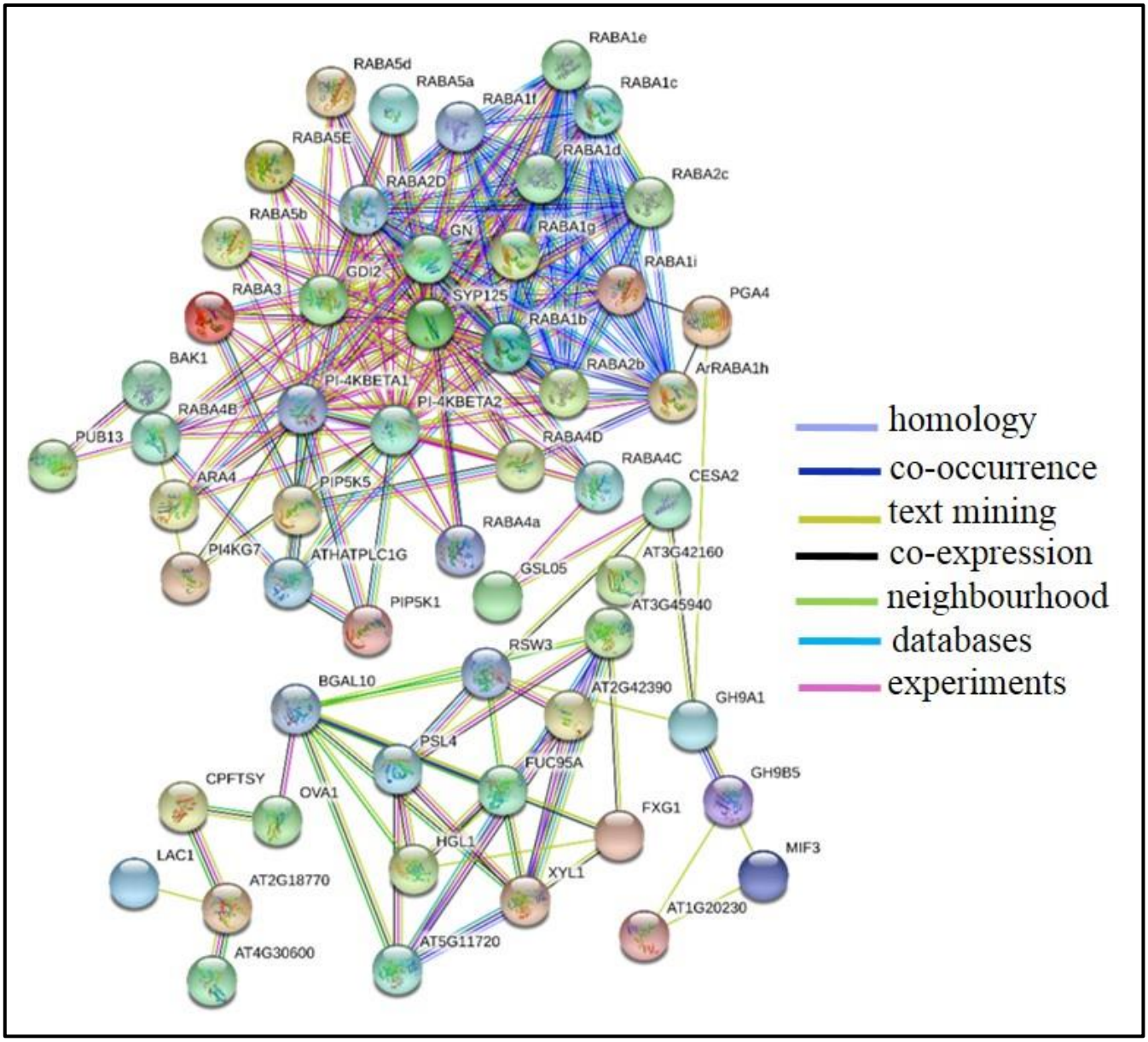


Figure 4.29: Protein network generated by STRING (v 10.0) for selected differentially expressed genes associated with fruit softening and vesicle trafficking

Note: Coloured nodes represent proteins whereas different colour of lines represents the types of evidence (depicted by the colour legend) for the association. Nodes are proteins and are labelled according to their corresponding gene symbols if present in TAIR, else labelled with their corresponding Gene ID. Input proteins include: PG4, polygalacturonase 4; AT1G09550, pectate lyase; pectinesterase; GH9B5, endoglucanase 2 and the RabA GTPase family. A complete list of the proteins within this network is provided in Appendix XI.

4.5 GENE EXPRESSION ANALYSIS BY REVERSE TRANSCRIPTION-QUANTITATIVE PCR (RT-qPCR)

Since a pooling design was employed in RNA-seq study (section 4.4), it was necessary to confirm the observation using a second method known as reverse transcription-quantitative PCR (RT-qPCR).

4.5.1 Primer Specificity

Primer specificity was verified by agarose gel electrophoresis of the amplified product and melt curve analysis. For each of tested genes, a PCR with gradient annealing temperatures (55.3, 57.3, 58.7 and 60.0 °C) was performed to verify the optimum annealing temperature and the amplicon size using 'Chokanan' ('CK') cDNA template. All tested genes were successfully amplified at temperatures as high as 60.0 °C and yielded single products of expected sizes (100 – 180 bp) respectively (Figures 4.30, 4.31 and 4.32). A no reverse transcriptase (NRT) control showed no amplification (Figure 4.33).

4.5.2 Expression Stability of Reference Genes

The availability and stable expression of reference genes for normalisation of RT-qPCR data is a prerequisite for obtaining reliable results. The RT-qPCR data were subjected to geNorm (Vandesompele *et al*., 2002) and NormFinder (Andersen *et al*., 2006) analysis. The geNorm program calculates the average expression stability (M-value) of a gene among all of the tested genes. Genes with an M-value below the threshold of 1.5 are stably expressed and the lower the M-value, the more stable the expression (Vandesompele *et al.*, 2002). Of the four references genes tested, *Ubiquitin* (*UBI*) and β-*Actin* (*ACT*) were ranked the most stable genes, both had an M-value of 0.020. *Glyceraldehyde 3-phosphate dehydrogenase* (*GADPH*, 0.087) was the least stable of the genes tested followed by α-*Tubulin* (*TUB*, 0.047). The results from geNorm

were further confirmed with NormFinder tool which measures gene expression stability taking into account intragroup and intergroup variations (Andersen *et al*., 2004). Genes with lowest stability values have the minimum variation and thus are top ranked. Similar results as observed in geNorm were obtained using NormFinder, which predicted a stability value of 0.122 and 0.310 for *ACT* and *UBI* respectively. Therefore, both geNorm and NormFinder outputs provide evidence for *ACT* and *UBI* as the most stable reference genes for the RT-qPCR assays. Finally, RefFinder tool (Xie *et al*., 2012) was used to generate a comprehensive ranking of the most stable reference genes (Figure 4.34). It is worth mentioning that this web based program takes into account various reference gene evaluation programs (the comparative ΔCt method, BestKeeper, NormFinder, and geNorm) to generate the ranking order of the tested genes. The reference genes were ranked from 1 (most stable) to 4 (least stable). The most stable genes from RefFinder were *ACT* followed by *UBI* thus validating the results obtained when a single method (either GeNorm or NormFinder) was used. Both *ACT* and *UBI* were found to be the most stable genes according to the ranking in at least two programs. As such, these genes were chosen for normalization in this study.

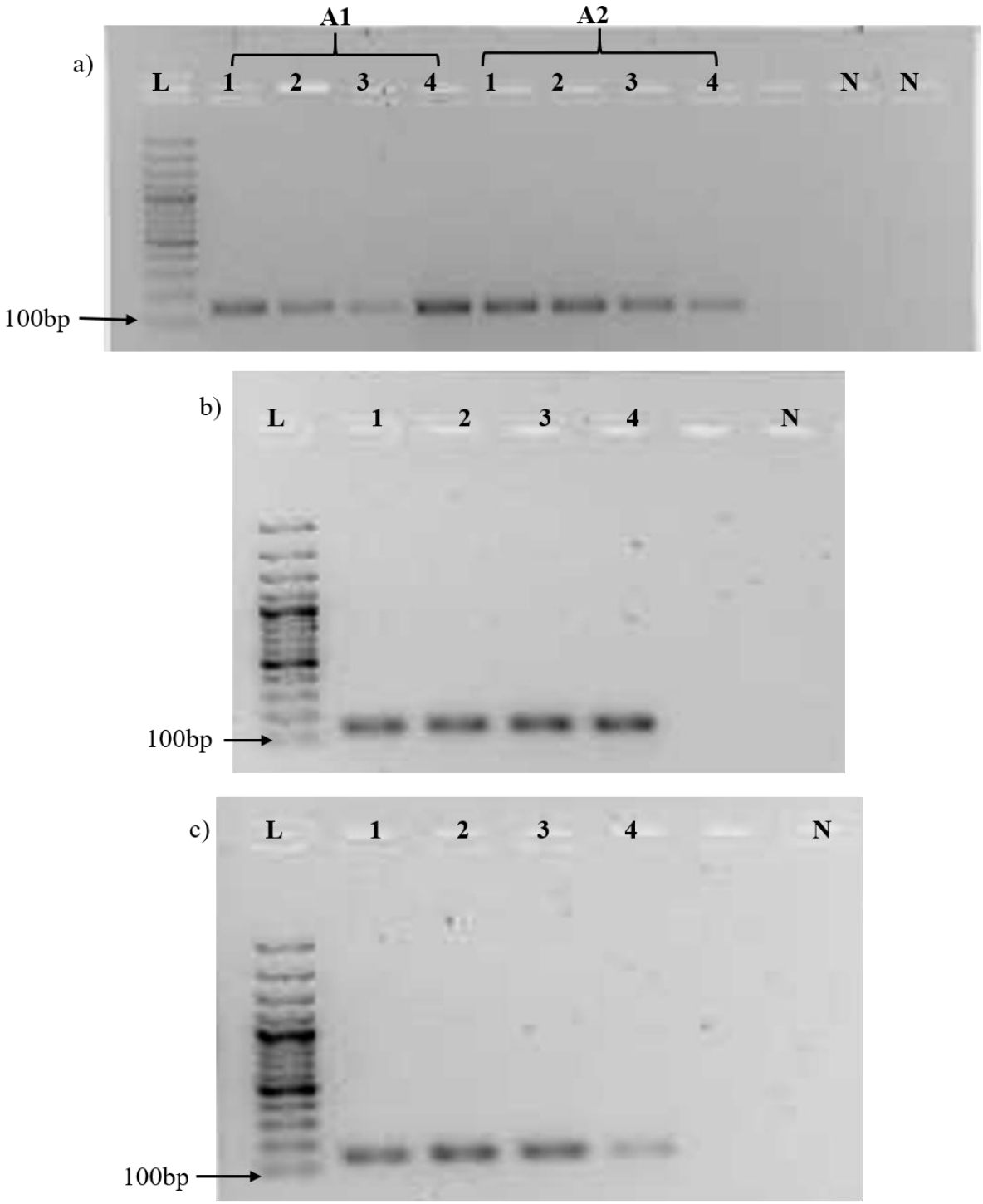


Figure 4.30: Gradient PCR profile of reference genes.

Note: L, GeneRuler 100bp Plus DNA ladder (Thermo Scientific, USA); a) A1, *ACT* ; A2, *GADPH* b) *UBI* c) *TUB*; N, negative control. Lanes 1 to 4 indicate 55.3 °C, 57.3 °C, 58.7 °C and 60.0 °C respectively.

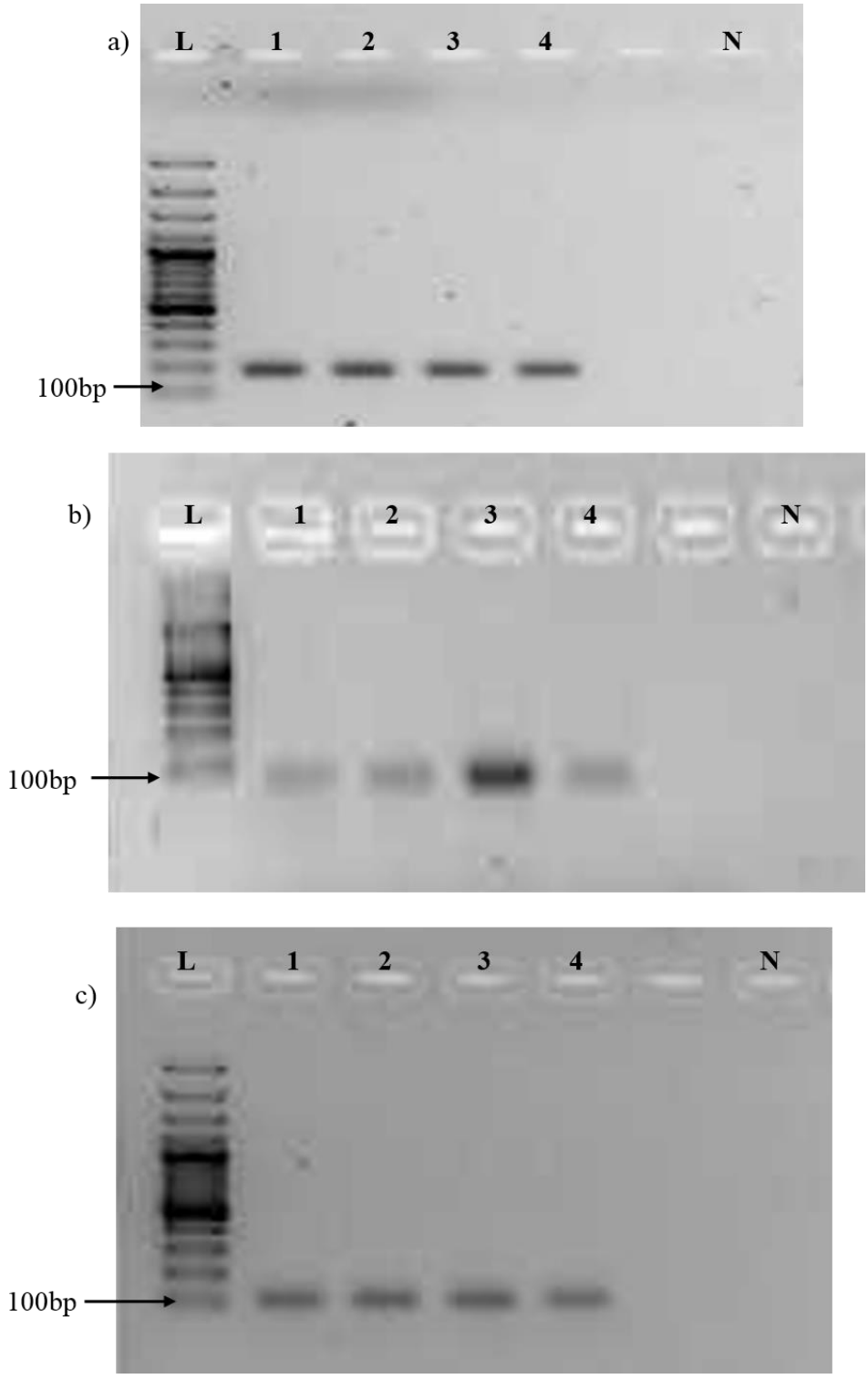


Figure 4.31: Gradient PCR profile of target genes a) *RabA1-1* b) *RabA1-2* and c) *RabA2*

Note: L, GeneRuler 100bp Plus DNA ladder (Thermo Scientific, USA); N, negative control. Lanes 1 to 4 indicate 55.3 °C, 57.3 °C, 58.7 °C and 60.0 °C respectively.

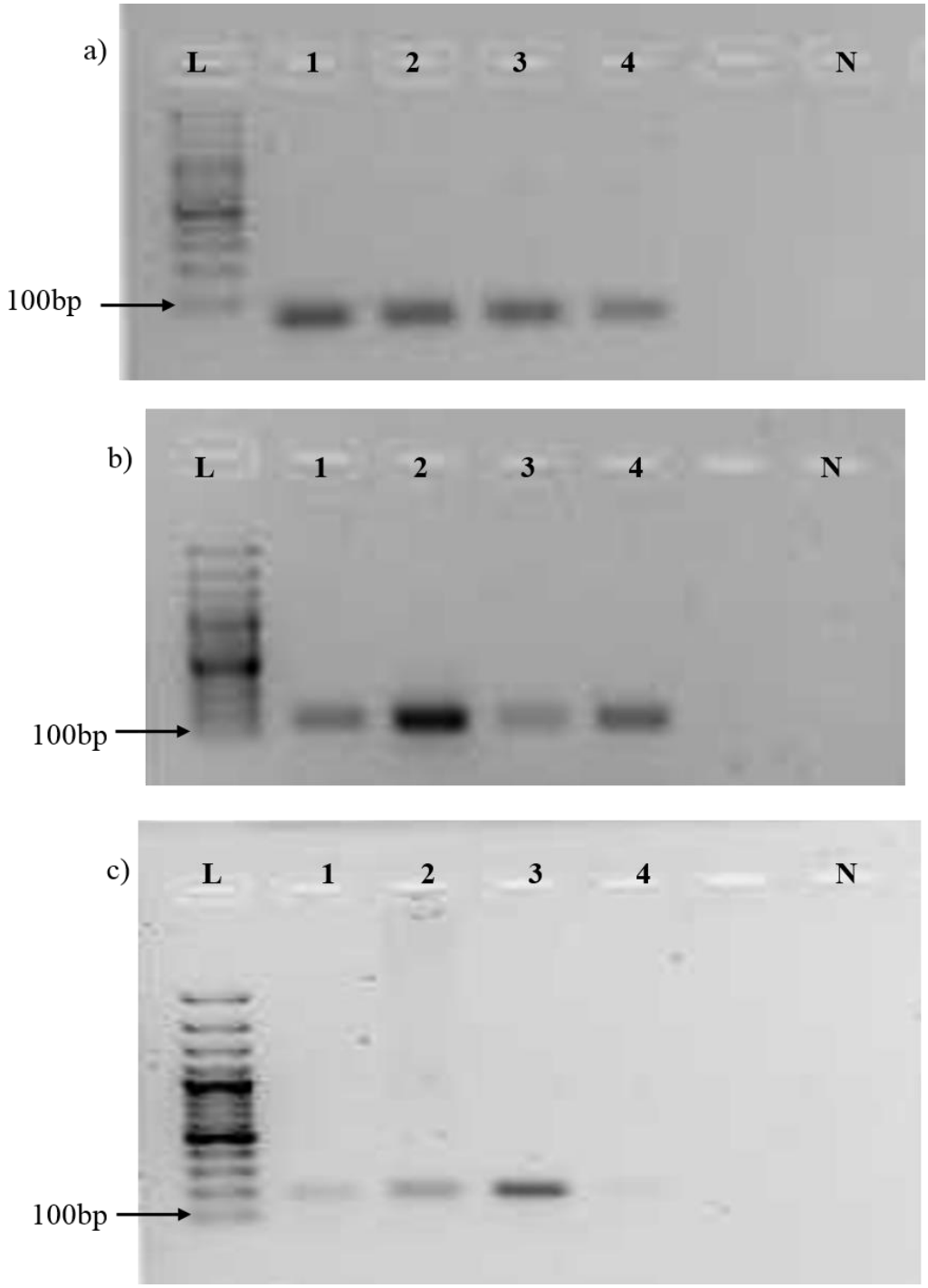


Figure 4.32: Gradient PCR profile of target genes a) *RabA3* b) *RabA4* and c) *PG*

Note: L, GeneRuler 100bp Plus DNA ladder (Thermo Scientific, USA); N, negative control. Lanes 1 to 4 indicate 55.3 °C, 57.3 °C, 58.7 °C and 60.0 °C respectively. *PG*, *polygalacturonase*

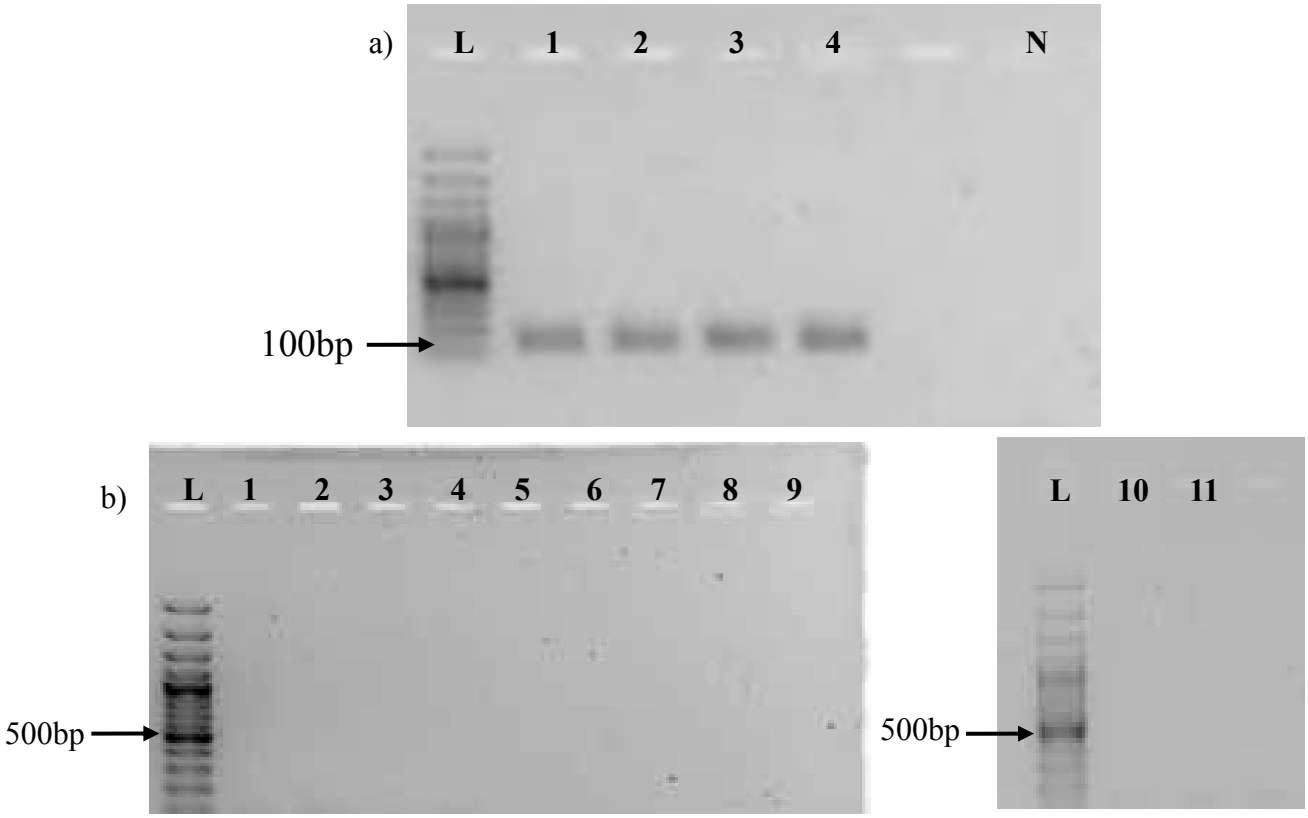


Figure 4.33: Gradient PCR profile of a) *PE* and b) no reverse transcription (NRT) control

Note: L, GeneRuler 100bp Plus DNA ladder (Thermo Scientific, USA); 1, *ACTIN*; 2, *GADPH*; 3, *UBI*; 4, *TUB*; 5, *Rab A1-1*; 6, *RabA1-2*; 7, *RabA2*; 8, *RabA3*; 9, *RabA4*; 10, *PG* (*polygalacturonase*); 11, *PE* (*pectinesterase*)

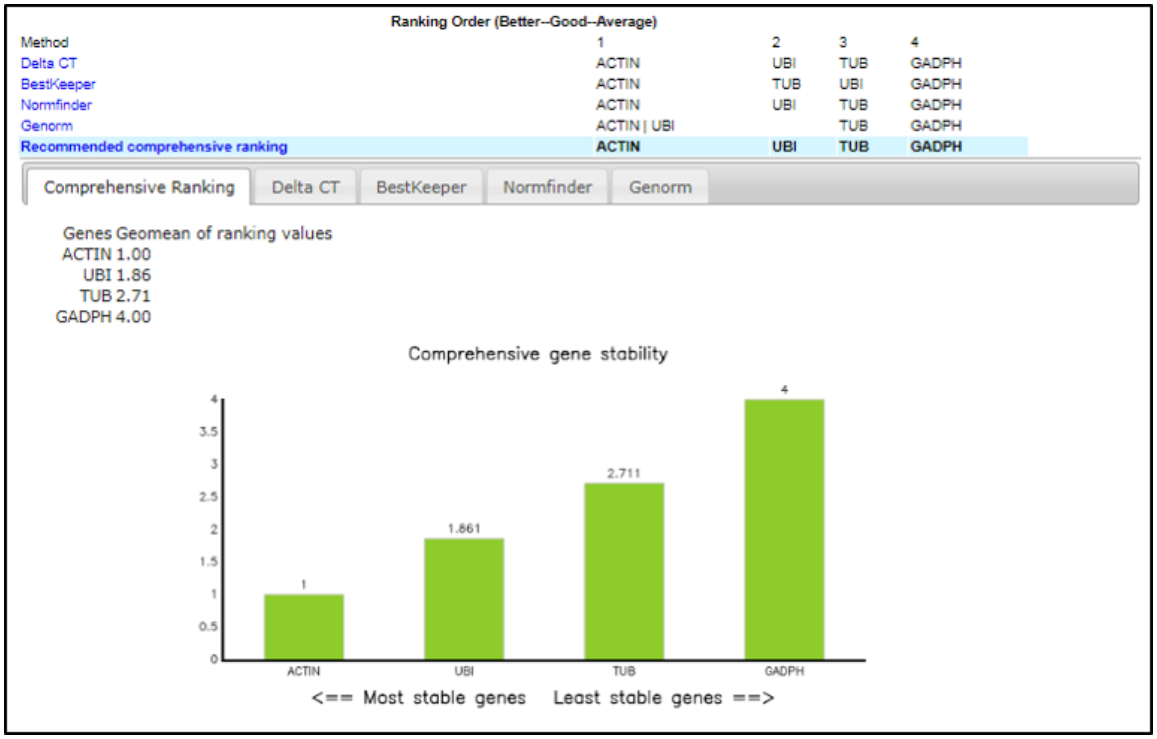


Figure 4.34: Gene expression stability ranked by RefFinder tool.

Note: Four reference genes were tested for gene stability using the unripe and ripe samples from Chokanan (CK), 'Golden phoenix' (GP) and 'Water lily' ('WL') mango varieties. *UBI, Ubiquitin*; *GADPH, Glyceraldehyde 3-phosphate dehydrogenase*; α-*Tubulin, TUB.*

4.5.3 Efficiency of the RT-qPCR Assay and Melt Curve Analysis

Prior to employing a relative quantification method it was important to demonstrate that the efficiencies of the target genes and the reference gene were similar. A standard curve based on a two-fold serial dilution (Gallup and Ackermann, 2006) was prepared from cDNA template obtained from a pool sample (unripe and ripe) of 'Chokanan' variety. Standard curves were generated using Microsoft Excel® (Figure 4.35). At least five magnitudes (Bustin *et al*., 2009) were included in the calculation of the amplification efficiency from the slope of each standard curve. The results from the standard curve were further confirmed by LinReg software

(Ramakers *et al*., 2003). LinRegPCR (v.11.0) calculates per-well amplification efficiencies of each individual sample and thereafter determines the mean efficiency of all samples of an amplicon group (set of samples with the same primer pair) (Ramakers *et al*., 2003). Based on these approaches adequate estimates of efficiencies ranging from 90 to 115 % were obtained (Table 4.19).

A melting curve analysis performed at the end of RT-qPCR reactions for the reference (Figure 4.36 and 4.37) and target (figure 4.38) genes was necessary to further confirm whether a single, specific product had been amplified. As observed, individual reactions revealed single melt peaks indicating the amplification of reference genes or target genes.

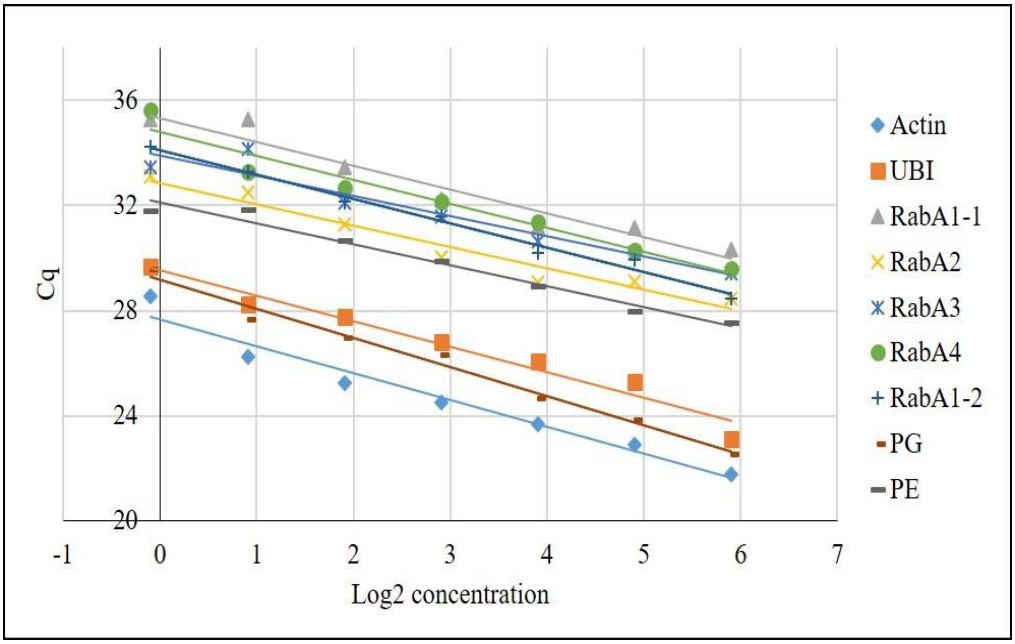


Figure 4.35: Standard curve for reference and target genes.

Note: Curves were plotted with Cq values against log starting quantities, and R^2 values ranged from 0.962 to 0.993. *UBI, Ubiquitin*; PG, *Polygalacturonase*; *PE, Pectinesterase*

Table 4.19: Amplification efficiency of reference and target genes.

Genes	Standard curve method		LinRegPCR method	
	Efficiency (%)	R^2	Efficiency (%)	R^2
β-*Actin* (*ACT*)	101.54	0.984	97.02	0.998
Ubiquitin (*UBI*)	104.32	0.962	101.00	0.997
RabA1-1	94.94	0.978	102.12	0.996
RabA1-2	110.49	0.971	104.73	0.995
RabA2	102.92	0.985	104.73	0.999
RabA3	106.81	0.992	103.72	0.997
RabA4	90.22	0.993	93.20	0.998
Polygalacturonase (*PG*)	104.59	0.986	96.09	0.998
Pectinesterase (*PE*)	104.97	0.984	98.77	0.998

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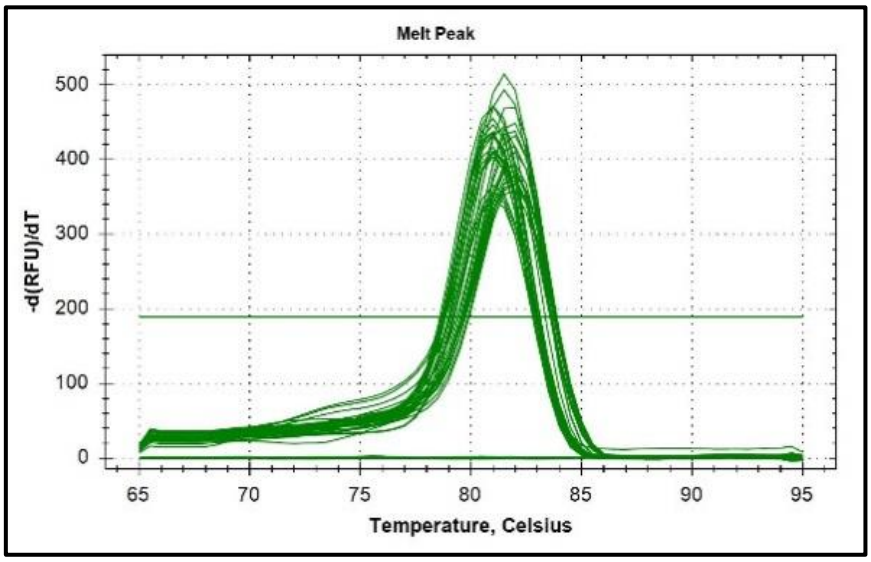

Figure 4.36: Melt peak for *Actin* gene.

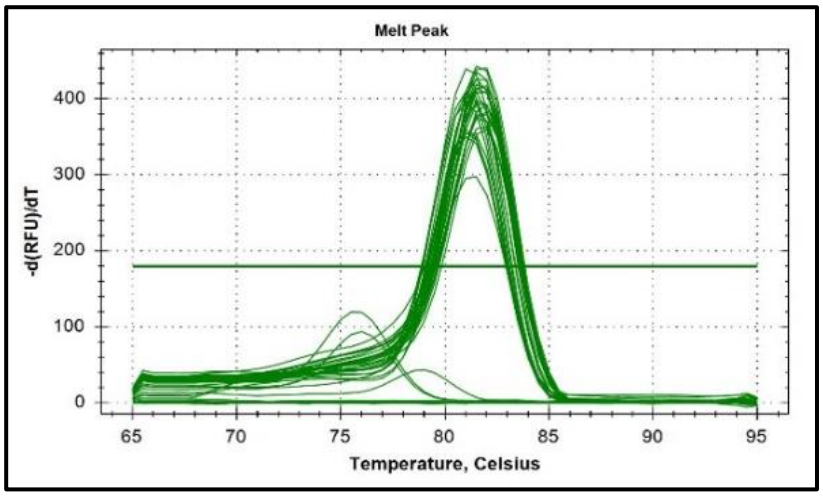

Figure 4.37: Melt peak for *Ubiquitin* gene.

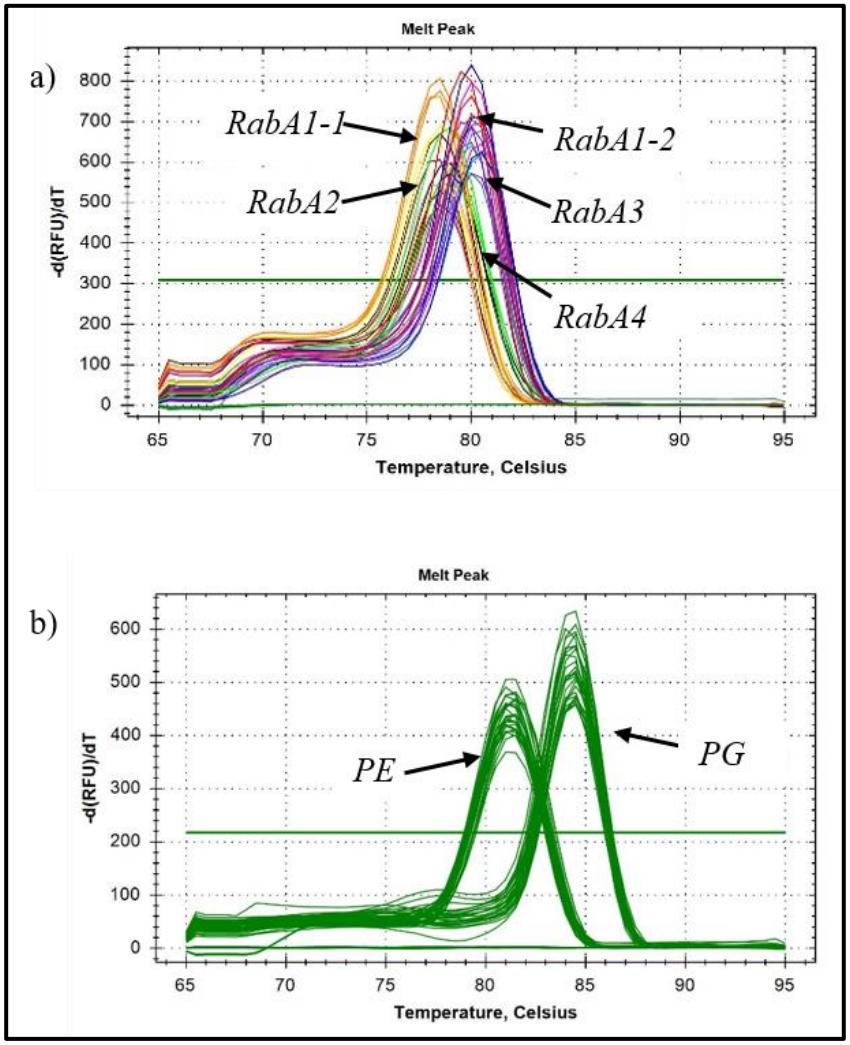

Figure 4.38: Melt peaks for all target genes.

Note: *PG*, *polygalacturonase*; *PE*, *pectinesterase*

4.5.4 Validation of RNA-seq Expression Data

To confirm the reliability and accuracy of the RNA results, ten genes displaying diverse expression profiles between the ripening stages of 'Chokanan' (CK) mango sample were selected from the RNA-seq data for RT-qPCR validation. It is worth noting out that some of the genes have a known involvement in fruit ripening. For each RT-qPCR assay, gene expression was normalized to the validated reference genes and the unripe sample was chosen as a calibrator for the calculation of the relative expression ratio of each target gene. The expression level of the calibrator sample was set at 1.00 and the relative expression, R of the target gene in the ripe sample was compared against it using the "$R = 2^{-\Delta\Delta Cq}$" method (Livak and Schmittgen, 2001). It was observed that the selected genes, except for *RabA5* showed the same trend for RNA-seq and RT-qPCR methods (Figure 4.39a). Although the fold changes estimated by the two methods (RNA-seq and RT-qPCR) varied, the expression patterns from both techniques were similar. Correlation between the RNA-seq and RT-qPCR data revealed a high similarity ($R^2 = 0.7698$) indicating the reliability of RNA-seq analysis (Figure 4.39b).

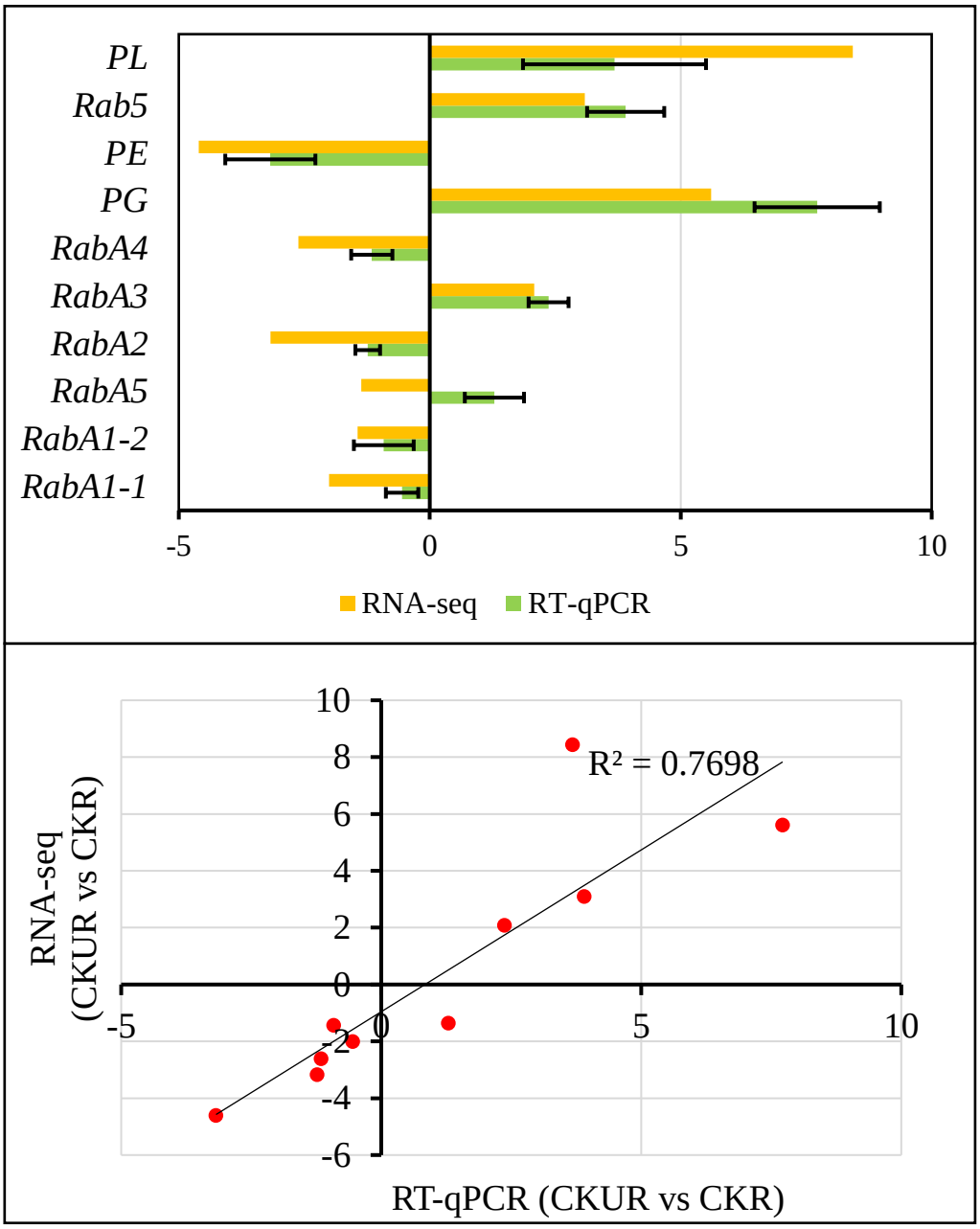

Figure 4.39: Comparison of gene expression results from RNA-seq and RT-qPCR.

Note: a) A bar plot showing the fold changes measured by RT-qPCR and RNA-seq of 10 selected genes. *PG*, *polygalacturonase*; *PE*, *pectinesterase*; *PL*, *pectate lyase*. RT-qPCR expression ratio of the ripe samples (R) are relative to unripe samples (UR) and normalized using the validated references genes *ACT* and *UBI*. Values: > 0 indicates increasing trend; = 0 indicates no change; < 0 indicates decreasing trend. b) Correlation of the fold change between RT-qPCR results (x-axis) and the corresponding data from RNA-seq analysis (y-axis). CK, 'Chokanan', UR, unripe; R, ripe.

4.5.5 Relative Gene Expression Levels in Mango Varieties

As a means to gain a further understanding of relevant contributions of the RabA GTPases to contrasting mango pulp firmness, 'Chokanan' ('CK'), 'Golden phoenix' ('GP') and 'Water lily' ('WL') mango varieties were used for comparative expression analysis of a subset of *RabA* genes. RNA-seq analysis successfully revealed that the RabA GTPases are involved in the ripening process. These genes were then selected to examine whether there is a correlation between their expression patterns and rate of firmness loss measured in these mango varieties. The relative expression of the target *RabA* gene in either unripe or ripe samples of the aforementioned varieties was calculated using the "$R = 2^{-\Delta\Delta Cq}$" method. 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. Additionally, *polygalacturonase* (*PG*) and *pectinesterase* (*PE*) which are well studied softening-related genes were included to serve as a baseline in this assay. At the unripe stage, the expression levels of *Rab A1-2* and *RabA3* genes were lower in 'CK' as compared to other varieties (Figure 4.40a). On the other hand, the expression level of *RabA2* was found to be similar in 'CK' and 'WL' while *Rab A1-1* 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 4.41a) revealed similar tendency as observed in the unripe stage. A difference in the expression level of the *PG* gene was observed with 'WL' being significantly higher (Figures 4.40b and 4.41b). There were no significant differences in *PE* gene expression level between varieties at the unripe stage (Figure 4.40c) but at the ripe stage (Figure 4.41c) 'GP' was significantly higher. A heat map for easy visualization of changes in gene expression in all varieties at either the unripe (Figure 4.40d) or ripe stage (Figure 4.41d) is also provided.

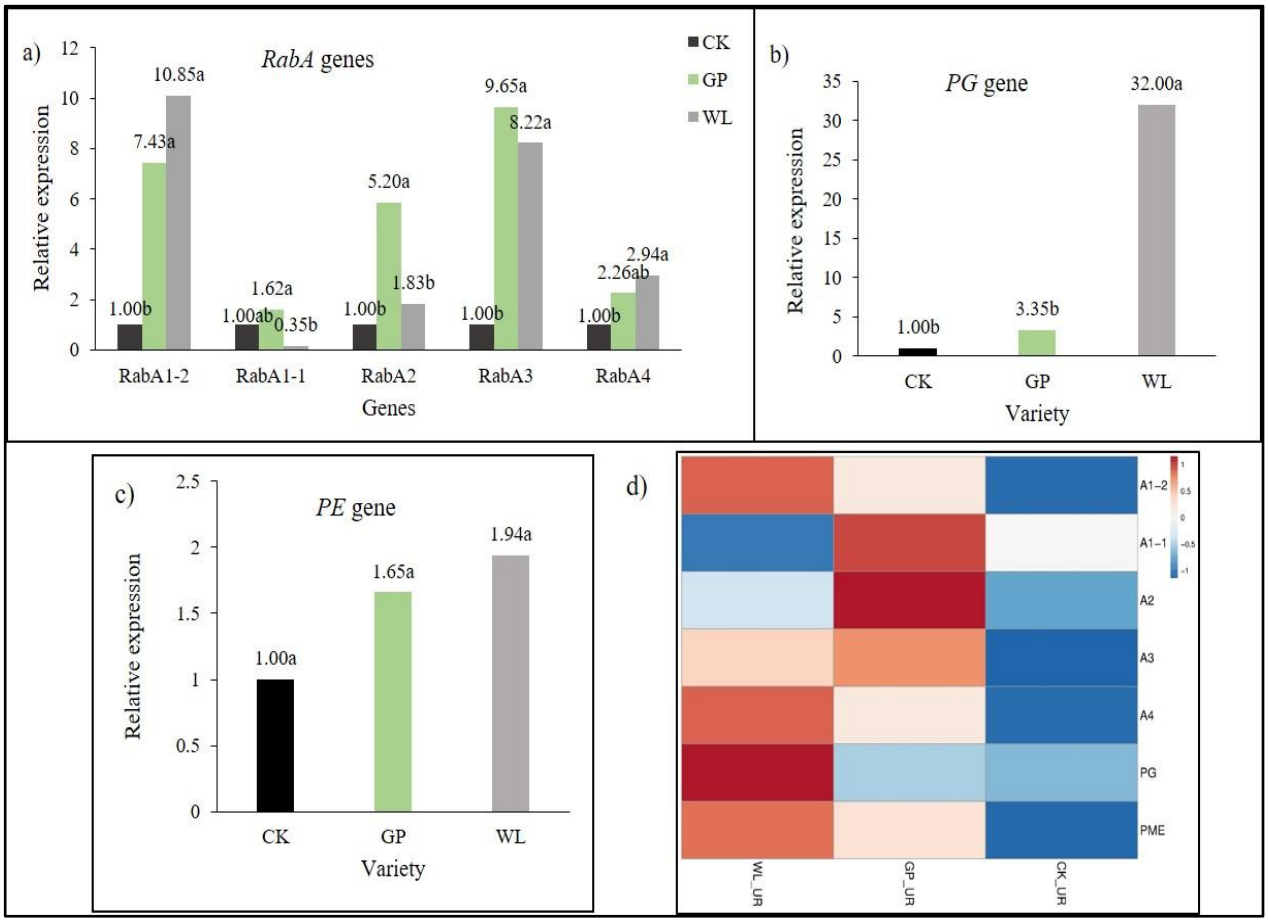

Figure 4.40: Comparison of gene expression level between 'CK', 'GP' and 'WL' mango varieties at the unripe stage.

Note: RT-qPCR analysis of a) *RabA*, b) *polygalacturonase* (*PG*) and c) *pectinesterase* (*PE*) gene expression. For each target gene, 'CK' ('Chokanan) sample (black bar) was used as a calibrator and the value was set at 1.00. Green and grey bars represent the relative gene expression of 'GP' ('Golden phoenix') and 'WL' ('Water lily') respectively. d) Heat map of the RT-qPCR data for all target genes in 'CK', 'GP' and 'WL' mango varieties respectively. Each row represents a gene and each column represents a variety. Different letters for each gene are significantly different ($P < 0.05$). The colour and intensity of the boxes represents changes of gene expression according to the key chart. Red and blue represents a high and low expression respectively. Heat map was generated by ClustVis tool (Metsalu and Vilo, 2015).

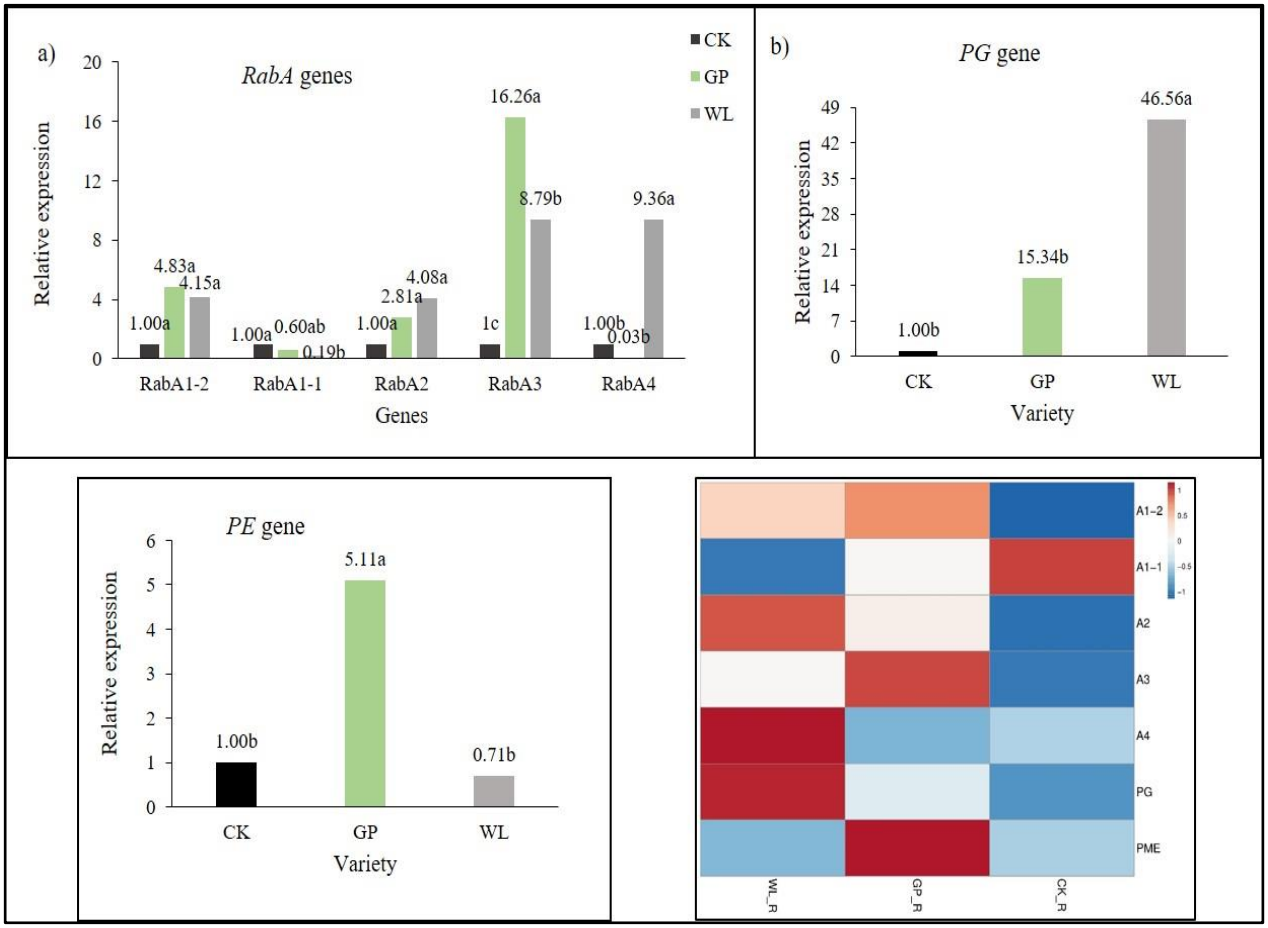

Figure 4.41: Comparison of gene expression level between ‘CK’, ‘GP’ and ‘WL’ mango varieties at the ripe stage.

Note: RT-qPCR analysis of a) *RabA*, b) *polygalacturonase* (*PG*) and c) *pectinesterase* (*PE*) gene expression. For each target gene, ‘CK’ (‘Chokanan) sample (black bar) was used as a calibrator and the value was set at 1.00. Green and grey bars represent the relative gene expression of ‘GP’ (‘Golden phoenix’) and ‘WL’ (‘Water lily’) respectively. d) Heat map of the RT-qPCR data for all target genes in ‘CK’, ‘GP’ and ‘WL’ mango varieties respectively. Each row represents a gene and each column represents a variety. Different letters for each gene are significantly different ($P < 0.05$). The colour and intensity of the boxes represents changes of gene expression according to the key chart. Red and blue represents a high and low expression respectively. Heat map was generated by ClustVis tool (Metsalu and Vilo, 2015).

4.5.6 The Relationship between Gene Expression Level and Fruit Firmness

In this study, a correlation analysis was performed to investigate how the changes in expression levels of the target genes were related to the pulp firmness variations observed in the mango varieties 'Chokanan' ('CK'), 'Golden phoenix' ('GP') and 'Water lily' ('WL') during ripening. The relationship between pulp firmness at the unripe stage (Table 4.20) and the expression of target genes was quite different from that observed at the ripe stage (Table 4.21). 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 (Table 4.20). 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. Furthermore, *PG* and *PE* expression levels were negative but not significantly correlated with pulp firmness ($r = -0.494$, $P = 0.671$; $r = -0.874$, $P = 0.323$) respectively. The cell wall softening genes *PG* and *PE* showed a non-significant positive relationship ($r = 0.854$; $P > 0.05$) (Table 4.20). Although negative correlations were found between *RabA1-2, RabA2 and RabA3* gene expression levels and pulp firmness at the ripe stage (Table 4.21) albeit to a lesser extent compared to the unripe stage, these were not significant ($P > 0.05$ in all cases).

Taken together, the result suggest that *RabA3* and *RabA4* may play an important role in the contrasting firmness of the mango varieties at the early stages of ripening. In addition, *PG* and *PE* genes investigated here may not be contributing significantly to the varietal differences in fruit softening. Based on these findings, an association can be inferred at the unripe stage for *RabA3* and *RabA4* gene expression level and the fruit softening variations observed in 'Chokanan', 'Golden phoenix' and 'Water lily' mango varieties.

Table 4.20: Pearson correlation of firmness loss, *Rab*A, *PG* and *PE* gene expression in 'Chokanan', 'Golden phoenix' and 'Water lily' mango varieties at the unripe stage.

RabA1-1	-							
RabA1-2	-0.073	-						
RabA2	0.675	0.686	-					
RabA3	0.139	0.978	0.824	-				
RabA4	0.072	0.990	0.784	0.997	-			
Firmness	-0.074	-0.962	-0.784	**-0.998**	**-0.999**	-		
PG	-0.832	0.615	-0.152	0.435	0.495	-0.494	-	
PE	-0.422	0.935	0.384	0.839	0.874	-0.874	0.854	-
	RabA1-1	*RabA1-2*	*RabA2*	*RabA3*	*RabA4*	Firmness	*PG*	*PE*

Note: Significant correlations ($P < 0.05$) of genes with firmness are indicated in bold

Table 4.21: Pearson correlation of firmness loss, *RabA, PG* and *PE* gene expression in 'Chokanan', 'Golden phoenix' and 'Water lily' mango varieties at the ripe stage.

RabA1-1	-							
RabA1-2	-0.766	-						
RabA2	-0.994	0.831	-					
RabA3	-0.504	0.941	0.595	-				
RabA4	-0.819	0.259	0.753	-0.082	-			
Firmness	0.632	-0.912	-0.712	-0.938	-0.073	-		
PG	-0.980	0.624	0.953	0.323	0.917	-0.466	-	
PE	0.066	0.590	0.042	0.828	-0.627	-0.732	-0.263	-
	RabA1-1	*RabA1-2*	*RabA2*	*RabA3*	*RabA4*	Firmness	*PG*	*PE*

Note: Significant correlations ($P < 0.05$) of genes with firmness are indicated in bold.

To estimate the connection between the *RabA* genes and pulp firmness further, gene expression level of 'Chokanan', 'Golden phoenix', 'Water lily' as well as other mango varieties ('Apple', 'Black gold', 'Siku Jaya', and 'Kemling') (Appendix XII) were analysed in the next fruit season. It is worth mentioning that the physiological measurements (firmness and soluble solid concentration) (Appendix XIII) and extraction of RNA samples (Appendix XIV) were performed at the same time to minimize batch variation on subsequent analysis. Notably, the study focused on the unripe stage for which a significant correlation pulp firmness and *RabA* gene expression had been earlier observed. The order of firmness of the test varieties from firm to less firm were 'Chokanan', 'Apple', 'Siku jaya', 'Golden phoenix', 'Water lily', 'Black gold' and 'Kemling' (Appendix XIII). 'Chokanan' was found to be the significantly ($P < 0.05$) firm variety once again. PE gene showed no significant differences between 'Chokanan', 'Golden phoenix', 'Water lily' (Figure 4.40) and thus was excluded. Results from the RT-qPCR analysis showed no clear differentiation of *RabA1-1* (Figure 4.42a), *RabA2* (Figure 4.42c), *RabA4* (Figure 4.43b) and *PG* (Figure 4.43c) gene expression level between the less-firm varieties and 'Chokanan' (the firm variety in this study). On the contrary, there were some consistency at least for *RabA1-2* (Figure 4.42b) and *RabA3* (Figure 4.43a) with low gene levels observed in 'Chokanan' ('CK') compared to all other varieties.

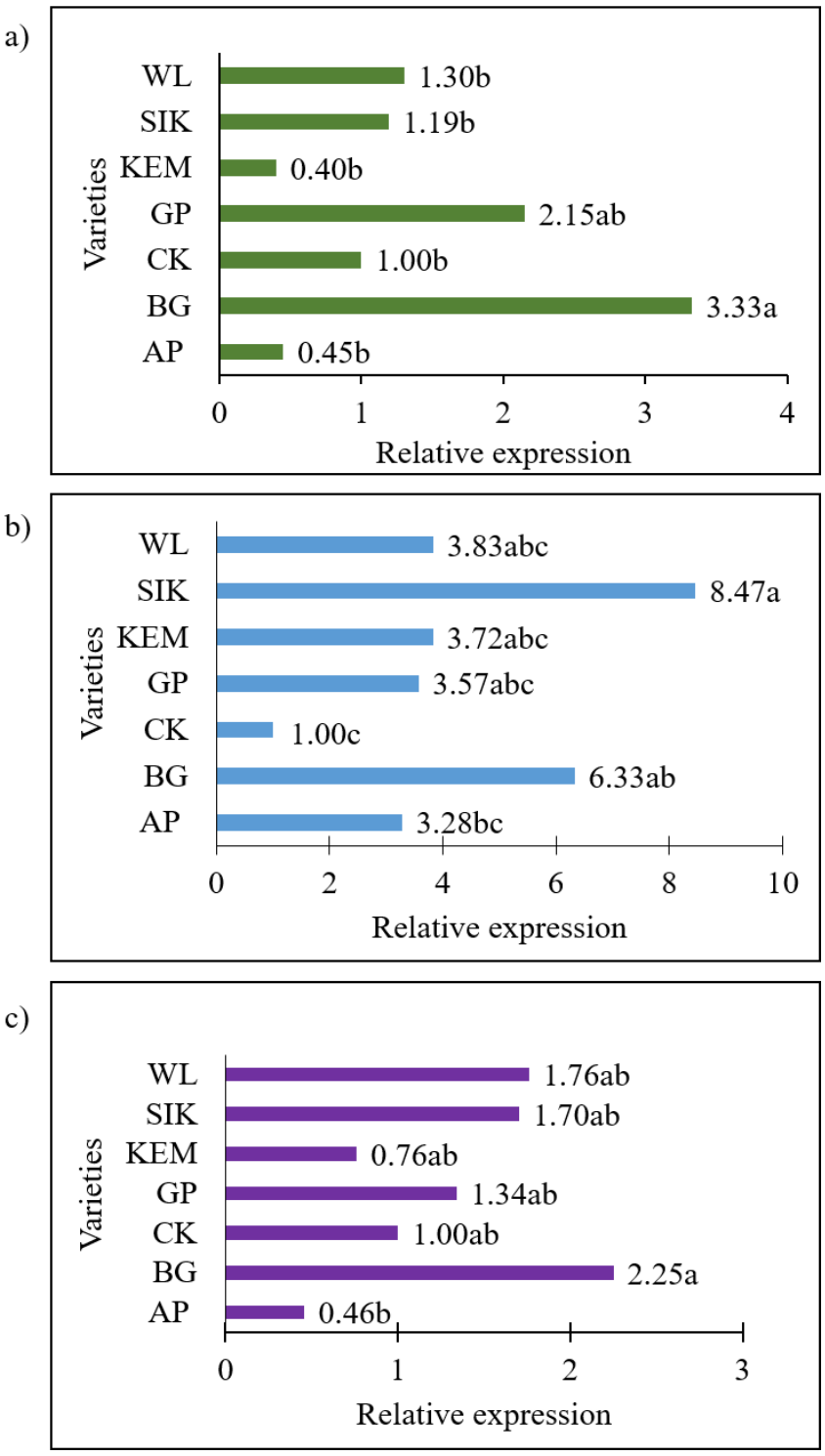

Figure 4.42: Comparison of a) *RabA1-1* b) *RabA1-2* c) *RabA2* gene expression level between the seven mango varieties at the unripe stage

Note: For each target gene, 'Chokanan' (CK) sample was used as a calibrator and the value was set at 1. Apple (AP); Black gold (BG); Golden phoenix (GP); Kemling (KEM); 'Siku jaya' (SIK) and Water lily (WL) mango varieties respectively. Different letters for each gene are significantly different ($P < 0.05$).

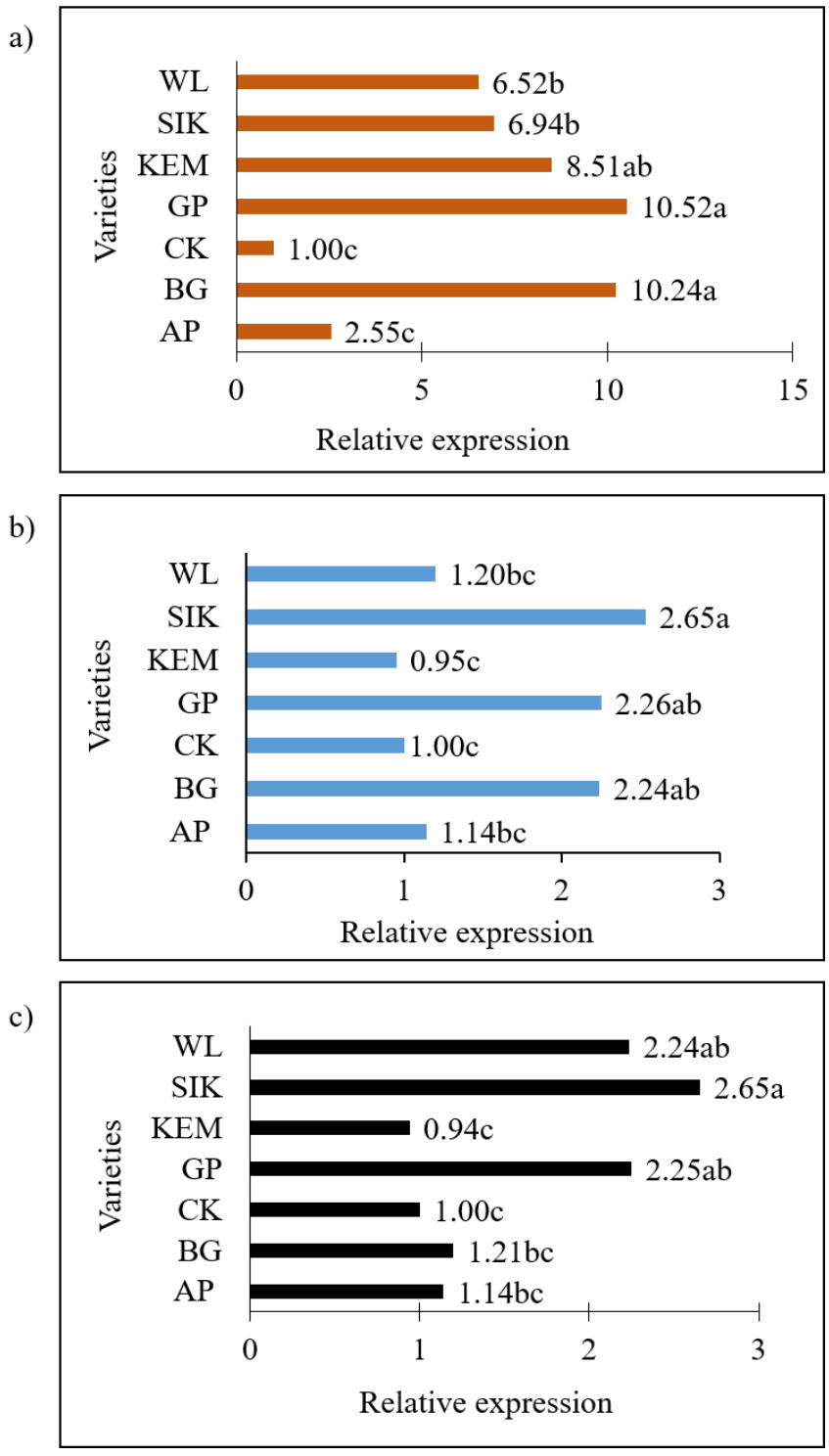

Figure 4.43: Comparison of a) *RabA3* b) *RabA4* and c) *polygalacturonase* (*PG*) gene expression level between the seven mango varieties at the unripe stage.

Note: For each target gene, 'Chokanan' (CK) sample was used as a calibrator and the value was set at 1. Apple (AP); Black gold (BG); Golden phoenix (GP); Kemling (KEM); 'Siku jaya' (SIK) and Water lily (WL) mango varieties respectively. Different letters for each gene are significantly different ($P < 0.05$).

5 DISCUSSION

5.1 CHARACTERIZATION OF THE RIPENING PROCESS OF MANGO VARIETIES ('CHOKANAN', 'GOLDEN PHOENIX' AND 'WATER LILY').

Mango fruit has been studied extensively but there is a paucity of published information on the postharvest profile of Southeast Asian mango varieties. Postharvest quality changes were observed to be influenced by variety and storage period in this study (section 4.1). The ripening period based on the evaluated postharvest parameters was 7, 7 and 10 days for 'Golden phoenix', 'Water lily' and 'Chokanan' respectively. The identification of the ripening stages for the mango varieties investigated in this study were successfully defined based on a combination of the measured postharvest parameters as previously reported (Jha *et al*., 2013; Mitcham, 2012; Vélez-Rivera *et al*., 2014; Yahia, 2011).

Weight loss is used as a quality index in the assessment of postharvest fruits (Khaliq *et al*., 2015; Ali *et al*., 2016; Ong *et al*., 2013). The increasing trend of weight loss observed for all varieties is in line with the findings obtained from 'Alphonso' mango (Yashoda *et al*., 2006), 'Dashehari' mango (Gupta and Jain, 2014) and other climacteric fruits (Del Angel-Coronel *et al*., 2010; Jan and Rab, 2012; Ong *et al*., 2013; Pongener *et al*., 2014) during postharvest storage. However, the different values recorded seem to be dependent on the species, variety or related to the storage environment employed for analysis (Khaliq *et al*., 2015; Baloch and Bibi, 2012). Gupta and Jain, (2014) showed that 'Dashehari' mango variety stored in cool storage showed less weight loss as compared to ambient condition during storage. The low temperature in cool storage might have brought about a reduction in the weight loss by decreasing the rate of respiration (McGlasson *et al*., 1979).

Colour is an important visible characteristic used to assess ripeness and it is a major factor in the consumer's purchase decision (Nassur *et al*., 2015). The loss of chlorophyll and the accumulation of carotenoids and other pigments improves the colour and attractiveness of mango fruit. (Ornelas-Paz *et al*., 2008). A reduction of the hue angle values and increase in L*, b* and a* values were observed in this study characterizing an increase in lightness, a more yellow coloration and a loss of green colour as ripening advanced. The trends of colour changes observed in this study are comparable with previous studies on other mango varieties (Ibarra-Garza *et al.*, 2015; Palafox-Carlos *et al*., 2012; Vásquez-Caicedo *et al*., 2002). Peel colour is observed after the fruit has started to soften and is usually inconsistent in several mango varieties (Yahia, 2011). Compared to 'Golden phoenix' and 'Water lily' mango varieties, 'Chokanan' showed visible changes from green to yellow which could make this variety more visually appealing to consumers (Vásquez-Caicedo *et al*., 2002). Varietal differences in peel colour changes observed in this study show the unreliability of peel colour as a stand-alone ripeness stage indicator for mango ripening. Nonetheless, in this study the peel colour attribute was useful in combination with other quality parameters.

Increase in SSC is associated with the breakdown of starch reserves into simple sugars during fruit respiration (Eskin *et al*., 2013). More notably, the SSC values for the 'Chokanan' variety were similar to those reported by Bejo and Kamarudin, (2014) from the same variety and geographical origin. Overall, the SSC values of the ripe mangoes obtained in the present study fitted well the 10 - 20 % SSC requirement for ripe mangoes (Mitcham, 2012; Yahia, 2011). Organic acids are energy reserves utilised as substrates during respiration and broken down, resulting in lower acidity of the fruit (Eskin *et al*., 2013). Decline in titratable acidity observed in this study has also been reported for other mango varieties such as 'Langra' (Baloch *et al*., 2012), 'Chaunsa' (Baloch *et al*., 2012) and 'Keitt' (Padda *et al*., 2011; Ibarra-Garza *et al*., 2015) and other climacteric fruits (Mahmood *et al*., 2012; Ong *et al*., 2013; Zhang *et al*., 2005).

The pattern of respiration and ethylene production in all varieties investigated in the present study was typical of a climacteric fruit. However, these rates varied with the different varieties and ripening stages. Decrease in the rate of respiration and ethylene production of a climacteric fruit following an outburst is likened to be the depletion of materials as the fruit progresses to the senescence stage (Yahaya, 2016). Respiratory activity is associated with the storage life and quality of fruits (Fagundes *et al*., 2013). As mentioned by Mitcham and McDonald (1992), the higher respiration rate of 'Tommy Atkins' mango compared to 'Keitt' might have contributed to its decreased storage life. It is well established that ethylene plays a pivotal role in regulating the ripening of climacteric fruits including mango (Barry and Giovannoni, 2007; Brecht and Yahia, 2009). Consistent with previous findings, the study showed respiration and ethylene production coincided in all varieties although it occurred on the 6th day for 'Chokanan' and 4th day for 'Golden phoenix' and 'Water lily' respectively. Similar patterns have been reported for other mango varieties such as 'Ataulfo' (Palafox-Carlos *et al*., 2012) and 'Cogshall' (Nordey *et al*., 2016). Conversely, 'Amrapali' and 'Dashehari' mangoes did not follow a climacteric pattern (Reddy and Srivastava, 1999). Previous studies have shown that an ethylene outburst may precede, coincide or lag behind the respiratory peak during mango ripening (Burg and Burg, 1962; Cua and Lizada, 1990; Lalel *et al*., 2003).

Firmness is one of the most significant quality aspects of mango for consumers as it reflects ripeness (Jha *et al*., 2010). The firmness test carried out on the mango varieties showed that 'Chokanan' mango was significantly firmer ($P < 0.05$) than 'Golden' 'phoenix' and 'Water lily' fruits on comparison (Table 4.4). Several authors have also found 'Chokanan' to be a firmer mango variety within the Southeast Asian region (Rimkeeree and Charoenrein, 2014; Vásquez-Caicedo *et al*., 2002). Nassur *et al*., (2015) mentioned that the end ripening point of 'Ataulfo', 'Haden', and 'Tommy Atkins' mangoes that meets consumer acceptance ranges between 4.5 and 26.7 N. This range correlates with the findings of this study despite the fact

that different varieties and geographical regions were used. Although fruit firmness is the preferred ripening index, other parameters such as colour, SSC and TA should not be disregarded as they also contribute to the appearance and eating quality of the fruit (Yahia, 2011).

ANOVA is commonly used to analyse the significance of differences between groups (such as ripening stages) for each parameter measured (Moreno and Moreno, 2008). However, ANOVA does not show how groups compare when all attributes are considered together or how these attributes may be inter-related (Bentham *et al*., 1992; Iezzoni and Pritts, 1991). Hence the need for multivariate analysis to allow a global study of the ripening process. The analysis did pick out features of the data in which the author had not noticed previously and the plots provided excellent summaries of the data. Correlation of some postharvest parameters observed in this study are in line with the studies in other mango varieties (Nambi *et al*., 2015) and tomato (Aoun *et al*., 2013). Hue (colour attribute) was not significantly associated with ethylene production in this study (Table 4.5) which was similarly observed by Ketsa *et al*., (1999) who found that 'Tongdum' mangoes, a ripe green fruit, had a high ethylene production compared with 'Nam Dok Mai' mangoes, which turn completely yellow upon ripening. Similar separation of fruit ripening stages observed in the multivariate analysis has been reported in other mango varieties (Padda *et al*., 2011; Nambi *et al*., 2015) as well as in banana (Valérie *et al*., 2014). The lack of separation between 'Golden phoenix' and 'Water lily' in the component 2 dimension of the biplot (Figure 4.14) could be due to a lesser variability of the peel colour changes between 'Golden phoenix' and 'Water lily' varieties as ripening progressed over the storage time. Principal component analysis and cluster analysis are 'unsupervised' techniques, meaning that a predicting variable was not specified in the analysis (Lynch *et al*., 2017). The benefit of such approaches is that the clustering of samples clearly shows intrinsic variance between the samples without being biased towards the desired outcomes (Thanaraj, 2010). The

clear separation of the unripe and ripe samples from multivariate study suggests a wide variation between their ripening characteristics irrespective of the mango variety. For this reason, these two ripening stages were considered to study the dynamics of the ripening process at the molecular level.

5.2 IDENTIFICATION OF THE RAB GTPASE FAMILY IN MANGO BY COMPARATIVE ANALYSIS

The Rab GTPase family have 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 (Mayes *et al*., 2011; Zhang *et al*., 2007). Rabs have been a model for this approach due to the high sequence conservation (Flores *et al*., 2018; Zhang *et al*., 2007). The nomenclature used in this work is that defined in Pereira-Leal and Seabra (2001). The 23 Rabs found in this study might represent the total number expressed, 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* (Rutherford and Moore, 2002), 87 in cotton (Li and Guo, 2017) and 94 in soybean (Flores *et al*., 2018) but comparable to the 24 Rab GTPases in peach (Falchi *et al*., 2010) and 26 in *Vitis vinifera* (Abbal *et al*., 2008). The higher number of genes might be due to additional duplication events in some plant species (Bowers *et al*., 2003; Rojas *et al*., 2012). For instance, additional rounds of genome duplication events has been reported in the model plant *Arabidopsis thaliana* (Bomblies and Maldlung, 2014; Bowers *et al*., 2003; Simillion, *et al*. 2002). In addition, comparative analysis of *Gossypium* and *Vitis* genomes revealed duplication events that were specific to the *Gossypium* lineage (Lin *et al*., 2011). On the other hand, it is possible that the reduced number of genes found in *Vitis vinifera* and/or *Prunus persica* might have been due to gene loss during evolution (Bowers *et al*., 2003; Tian *et al*.,

2014). 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 (Figure 4.16) on the basis of their localization and/or function in trafficking (Agarwal *et al*., 2009; Pereira-Leal and Seabra, 2001; Saito and Ueda, 2009). Analyses of sequence similarities and of the presence of specific family and subfamily conserved motifs in their sequence (Figure 4.17) allowed the identification of the closest homologues from *Arabidopsis* and the assignment of these sequences to the Rab family. 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 compared with their Rab11 counterparts in mammals (Vernoud *et al.*, 2003; Rutherford and Moore, 2002). All identified Rabs shared the typical conserved G-domains involved in binding and generally the double-cysteine motif in the C terminus (Pereira-Leal and Seabra, 2001). These conserved regions offer opportunities for designing degenerate primers to facilitate gene isolation in other plant species with less information. The hypervariable region (HVR) observed in the Rab proteins suggests specific membrane association and function (Pfeffer, 2013). Taking into account, these data confirm the definition of the 23 sequences as Rab GTPases presented in this study. An important feature of the Rab family is that Rab orthologues tend to perform similar functions even in divergent taxa (Diekmann *et al*., 2011). Thus, according to the function of the Rab GTPases reported in plants such as *Arabidopsis and tomato* (Chow *et al*., 2008; Lu *et al*., 2001; Qi and Zheng, 2013), 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.

5.3 ISOLATION OF RNA FROM MANGO PULP SAMPLES

To gain deeper insights into the possible roles of the Rab GTPase family during ripening, analysing the expression pattern of the *Rab* genes is required. However, the isolation of high quality RNA is a necessary step which is critical to the investigation of gene expression profiles using reverse transcriptase quantitative PCR (RT-qPCR) or RNA sequencing. Mango pulp tissues like those in many higher plant species contain high levels of polyphenols and polysaccharides which interfere with RNA isolation (Reddy *et al*., 2015). This is true even for identical tissues at different ripening stages in which the biosynthesis of these components is known to vary during ripening (Gasic *et al*., 2004). Additionally, pulp tissues contain high water concentrations leading to reduced RNA concentration, making isolation difficult (Davis *et al*., 2006). Hence the need for a suitable isolation procedure.

The success of an RNA isolation procedure is evaluated in terms of the quantity, quality and integrity of the recovered RNA (Ma *et al*. 2015). RNA extraction using TRIzol reagent (Life Technologies, USA) was unsuccessful on mango pulp based on the purity ratios. Moreover, even the commercially available Qiagen RNeasy Plant Mini Kit (Qiagen, Germany) could not efficiently extract RNA of high quality. While commercial kits may provide high sample throughput, these may have not been tailored to suit RNA isolation from plant tissues rich in polysaccharides and polyphenolic compounds (Tattersall *et al*., 2005). The CTAB method was found to be most effective for obtaining high quality (purity and integrity) and yield of total RNA from mango fruit. CTAB-based methods have been previously used successfully for RNA extraction from many plants rich in polyphenols and polysaccharides (Gudenschwager *et al*., 2012; Tong *et al*., 2012; Zamboni *et al*., 2008). Although the CTAB method is labour intensive and uses much time, the absence of phenol is an advantage leading to cost savings and less chemical toxicity. The suitability of the isolated total RNA in downstream molecular procedures was further evaluated using RT-PCR amplification. Since this technique is sensitive

to the presence of inhibitors in the extract or to RNA degradation (Ma *et al*., 2015; Nassuth, 2000; Tong *et al*., 2012), reverse transcription followed by PCR was performed to assess RNA quality. The genes encoding the Rab GTPases were successfully amplified (Figure 4.20). Sequence alignment using the *Arabidopsis* information resource database (TAIR, https://www.arabidopsis.org) confirmed the identity of the RT-PCR products as genes coding for the Rab GTPase family.

5.4 RNA-SEQUENCING ANALYSIS OF MANGO FRUIT RIPENING

5.4.1 Transcriptome Sequencing and Assembly

RNA-seq technology has allowed the characterization of the transcriptome dynamics during mango ripening. The ability of detecting expressed genes in an RNA sequencing experiment is dependent on the number of reads that can be mapped to the transcripts as well as the expression threshold (Marioni *et al*., 2008; Hart *et al*., 2013). Lowly expressed genes have less chance, so some may not be detected if insufficient sequencing depth is specified (Conesa *et al*., 2016). In the case of a model species with an available reference genome, a lower sequencing depth (as low as 10 million) may be sufficient (Garg and Jain, 2013). However, for a non-model species such as mango, a higher sequencing depth (>30 million reads) is needed to produce a meaningful assembled transcriptome (Garg and Jain, 2013; Tarazona *et al*., 2011). To obtain a global view of the mango transcriptome, approximately 1 billion reads (raw data) from the unripe and ripe samples of all mango groups (twelve libraries in total) was generated in accordance with the suggestion by Garg and Jain, (2013). This high-quality dataset provided a foundation for comparative transcriptome analysis in an attempt to identify key genes involved in the mango ripening and softening process.

Pooling samples in high throughput sequencing is a frequent practice among many researchers (Karp and Lilley, 2009; Kendziorski *et al*., 2005; Konczal *et al*., 2014; Williams *et al*., 2014).

In the context of detecting differential gene expression, divergent views on the wisdom of pooling samples have been reported. One of the arguments is that the biological variation can be reduced by pooling samples (Churchill and Oliver, 2001). However, as mentioned by Kendziorski *et al*., (2005) and Biswas *et al*., (2013), pooling designs were never found to perform significantly worse than non-pooled design in terms of identifying differentially expressed genes. Another argument in support of pooling samples is that it reduces financial cost (Zhang and Gant, 2005; Garg and Jain, 2013). Additionally, a pooling strategy has been suggested as a good option when the interest is on the characteristics of a group (Kendziorski *et al*., 2005; Karp and Lilley, 2009). In the present study, the trait of interest was the pulp firmness of the mango varieties. This characteristics was used as a basis for choosing the sample groups for sequencing. Based on the pulp firmness data obtained earlier (Table 4.4), 'Chokanan' ('CK') mango was found to be statistically firmer ($P < 0.05$) compared to the other mango varieties and as such was chosen to represent the 'firm' mango group whereas the Pool ('P') group comprising of 'Golden phoenix' and 'Water lily' represented the 'less-firm' mango group.

A mixed assembly of all reads was performed to faciliatate differential expression between ripening stages and mango groups. A total of 165,140 unigenes were obtained which is higher when compared to previous mango transcriptome studies (Table 4.9). This high number of unigenes suggests a redundancy in the contig assembly due to the polyploid nature and high degree of heterozygosity in mango (Singh *et al*., 2016). De novo transcriptome assembly of polyploid species is challenging due to the presence of several isoforms that originate from the different homoeologous chromosomes and alternative splicing of each of the gene families (Duan *et al*., 2012; Glover *et al*., 2016; Hoang *et al*., 2018; Schurch *et al*., 2014). Transcriptome assemblers differentiate between isoforms of the same gene and report each separately (Davidson and Oshlack, 2014). As a result, multiple contigs per gene are reported

even when they differ only by a single-nucleotide polymorphism (SNP) or insertion-deletion polymorphism (indel) (Davidson and Oshlack, 2014; Sánchez-Sevilla *et al*., 2017). Notably, the Trinity program used in this study has been reported to be the preferred transcriptome assembler for polyploidy species (Chopra *et al*., 2014). Furthermore, Hoang *et al*., (2018) mentioned that the number of isoforms obtained from an assembly can vary depending on the stage of maturity and the variety used in the respective study. In addition, Davidson and Oshlack, (2014) noted that a pooled design may also give rise to a higher number of contigs. It is possible that the high number of unigenes is a consequence of a combined transcriptome assembly approach (pooling of the unripe and ripe libraries from both mango groups) used in this study as homeologous genes from more than one mango variety will be present. On the basis of these findings, it indicates that the 'CK' and 'P' mango groups are highly heterozygous at many gene loci. It is worth pointing out that a combined transcriptome assembly was preferred to a) cover a wide range of genes that are expressed b) increase the chances of reconstructing low expressed genes and c) facilitate the comparative analysis between the mango groups and ripening stages (Asif *et al*., 2014; Dautt-Castro *et al*., 2015; Hoang *et al*., 2018).

Gene expression was analyzed by mapping reads from the individual samples on the assembled transcriptome for expression estimation. Fragments Per Kilo base of gene per Million mapped reads (FPKM) is a normalized estimation of gene expression. It is calculated from the number of reads that map to a particular gene sequence taking into account the gene length and the sequencing depth. The unigenes with an FPKM > 0.3 were considered to be expressed (Hart *et al*., 2013; Sánchez-Sevilla *et al*., 2017) and used for differential expression analyses. Differential gene expression analyses has been widely used in fruit transcriptomic studies (Table 2.3). A false discovery rate (FDR) approach was used as a criterion for identifying significant differentially expressed genes over the P value. FDR controls the number of false

positives within the significant results that was generated using P values in multiple comparisons. A way to look at the differences is that a P value of 0.05 means that 5 % of all tests will result in false positives while an FDR adjusted P value of 0.05 means that 5 % of the significant results will be false positives (Benjamin and Hochberg, 1995).

5.4.2 Functional Analysis of Differentially Expressed Genes (DEGs).

Functional analysis was carried out to investigate the biological processes associated with the differentially expressed genes. GO functional enrichment showed that metabolism (GO: 0008152) had the greatest number of DEGs for comparisons between ripening stages and mango groups respectively suggesting that the metabolic processes could be the key aspect differentiating the respective samples. These findings are consistent with the results from 'Alphonso', 'Kent' and 'Dashehari' mangoes (Dautt-Castro *et al*., 2015; Desphande *et al*., 2017; Srivastava *et al*., 2016) as well as other fruit species such as Chinese bayberry (Feng *et al*., 2013), grape (Balic *et al*., 2018), orange (Wang *et al*., 2017a), strawberry (Wang *et al*., 2017c) and watermelon (Guo *et al*., 2016; Zhu *et al*., 2017). The enrichment of the metabolic process suggests its contribution to fruit quality differences during mango ripening and also between the mango groups as reported in other fruits such as watermelon (Guo *et al*., 2016) and pear (Zhang *et al*., 2016b).

KEGG pathway analysis showed that the significantly enriched pathways in both mango groups during ripening included hormone metabolism and sucrose metabolism. It is worth pointing out that the order of the significantly enriched pathways of the 'CK' group varied from that of the 'P' group which might be due to varietal differences (Srivastava *et al*., 2016; Xiong *et al*., 2017). Nevertheless, these pathways have also been identified as being variable in other mango varieties such as 'Kent' (Dautt-Castro *et al*., 2015) and 'Dashehari' mangoes (Srivastava *et al*., 2016) as well as other fruit species such as persimmon (Jung *et al*., 2017),

Chinese bayberry (Feng *et al*., 2012) and watermelon (Zhu *et al*., 2017) during the ripening process. Taken together, the results were consistent with several mechanisms previously described and indicate that these pathways are essential to bring about the physicochemical changes that occur during fruit ripening process.

5.4.3 Transcriptome Dynamics during Fruit Ripening

Gene expression profiling provides a vast amount of information that may help to understand the molecular basis of fruit ripening (Osorio *et al*., 2013). There have been a few transcriptome studies by RNA-seq on mango varieties such as 'Zill', 'Kent', 'Dashehari' and 'Alphonso' which have provided insights into the mango fruit ripening. However, studies from the Chinese variety 'Zill' mango (Wu *et al*., 2014) provided information on transcriptomic characterization using pooled RNA from the pulp and peel tissues of four fruit developmental stages but not on the differentially expressed genes between the ripening stages (unripe and ripe). Secondly, Dautt-Castro *et al*., (2015) analysed the transcriptome changes between the unripe and ripe stages of 'Kent' mango, however only two replicates were used in this study. According to Manga *et al*., (2016) at least three biological replicates are recommended to increase the statistical power of detecting genes expressed differentially between investigated samples. Thirdly, Srivastava *et al*., (2016) provided information on the differentially expression genes utilising the unripe and mid-ripe stages of 'Dashehari' mango. Finally, the most recent report on the transcriptomic changes between the ripe and unripe stages was carried out on 'Alphonso' mango (Deshpande *et al*., 2017). The ripening duration of 'Alphonso' is 15-17 days from harvest (Nambi *et al*., 2015; Deshpande *et al*., 2017) compared to the duration (7-10 days) of the mango varieties investigated in this study. As mentioned by Hoang *et al*., (2018), the best transcriptome for expression profiling is that which is assembled directly from the samples due to varietal–specificity. For these reasons, it was necessary to sequence directly from the mango samples investigated in this study. To

the author's knowledge, there's been no published literature on the RNA-seq analysis of mangoes from the Southeast Asian region.

In the present study, genes associated with previously described pathways were found to be differentially expressed in comparisons between ripening stages and mango group respectively (Tables 4.10, 4.11, 4.12 and 4.13). In addition, this study revealed some findings highlighting the possible role of trafficking in the mango ripening process. This study has carried out comparative transcriptomic analysis in two mango groups [(Chokanan, 'CK') and (Pool 'P')] separated based on their statistically different pulp firmness in an attempt to identify potential RabA GTPases associated with contrasting pulp firmness.

5.4.3.1 Stage-Specific Gene Expression

Several genes were expressed in a stage-specific manner implicating their roles at a specific ripening stage (Appendix VII). Genes associated with plant hormones such as gibberellin, auxin as well as vacuolar activities were among the genes distinct to the unripe stages. Gibberellin (GA) is a plant hormone that plays a role in fruit set and development (Csukasi *et al*., 2011; Mariotti *et al*., 2011). GA is considered a ripening retardant because its exogenous application delays ripening associated events such as ethylene production, colour development and fruit softening (Khader, 1992). Notably, Mc Atee *et al*., (2013) mentioned that when a mature fruit becomes ready to undergo ripening, a shift in the hormone levels of the fruit occurs such as the decrease and increase of GA and ethylene levels respectively. This is in agreement with studies in tomato, fig and strawberry where the GA level decreased before the onset of ripening (Chen *et al*., 2016; Rosianski *et al*., 2016; Wang *et al.,* 2017c). Decrease in endogenous GA level has been attributed to gibberellin 2-oxidase (GA 2-oxidase) (Huang *et al*., 2010). In this regard, the *GA 2-oxidase* gene found to be exclusively present in the unripe mango sample might be involved in GA reduction and pave the way for ethylene biosynthesis

as ripening progresses. Furthermore, genes encoding sugar transporters known as SWEET were also found to be exclusively present in the unripe mango fruit. The vacuole is an important organelle for fruit quality because it stores components associated with fruit taste including sugars and organic acids (Shiratake and Martinoia, 2007). The accumulation of these components in the vacuole during fruit development and maturation requires transporters (Shiratake and Martinoia, 2007). The SWEET family have been implicated in the movement of sugars across the vacuolar membrane in plant cells (Doidy *et al*., 2012; Xuan *et al*., 2013). Expression analysis revealed that the *SWEET* genes were strongly expressed during fruit development in tomato (Reuscher *et al*., 2014), grape (Afoufa-Bastien *et al*., 2010) and apple (Wei *et al*., 2014) fruits, which was consistent with sugar accumulation. Thus, one might associate this phenomenon with the unripe-specific *SWEET* gene found in this study. Various genes related to auxin were also found to be uniquely expressed in the unripe stage but not at the ripe stages of the mango groups. The hormone auxin is known to play a critical role in many aspects of fruit development including fruit set and growth (Kumar *et al*., 2014; Pattison *et al*., 2014). Importantly, auxin levels increase at fruit set and the requirement of their higher levels at fruit set has been validated by exogenous application (Gustafson, 1936, Pandolfini *et al*., 2007). Several auxin-related genes were found to be expressed only in the developmental stages of 'Alphonso' mango (Deshpande *et al*., 2017) and apple (Devoghalaere *et al*., 2012) during ripening. Taking this into consideration, the auxin-related genes found to be specific in the unripe stage fits with their potential involvement in mango fruit development.

Various genes were exclusive to the ripe stage including the gene encoding the Universal Stress Protein (UPS). Fruit ripening has been described as an oxidative phenomenon (Thompson, 1984) that gives rise to an overproduction of reactive oxygen species (ROS) which can result in cell death and fruit damage (Jimenez *et al*., 2002; Pandey *et al*., 2013). UPS family endows tolerance and protects the cell from damage during prolonged exposure to oxidative stress

(Jung *et al*., 2015). The overexpression of the *USP* gene in *Arabidopsis thaliana* conferred a strong tolerance to oxidative stress (Jung *et al*., 2015). As such, it can be inferred that the exclusively present *USP* gene in the ripe stage appears to be responsible for preventing fruit damage during ripening. Furthermore, some genes encoding cell wall degrading enzymes such as polygalacturonase and endoglucanase were found to be expressed specifically in the ripe stage. These enzymes are involved in the degradation of the cell wall components (Crookes and Grierson, 1983; Smith *et al*., 1988; Brummell *et al*., 1994) and so this suggests their involvement in the loss of firmness observed in the ripe mango sample.

5.4.3.2 Differential Gene Expression

As shown in Figure 4.30, a greater number of genes were differentially expressed during the ripening of the 'P' group than in the 'CK' group. This finding suggest more active genes in the 'P' group which might have contributed to its increased rate of the ripening process compared to the 'CK' group. In support of this speculation, the modulation of a lesser number of differentially expressed genes has been considered an important characteristic of 'Alphonso' mango (a variety with a ripening duration of 15-17 days) compared to 'Kent' mango (a variety with 6 days duration) (Deshpande *et al*., 2017).

5.4.3.2.1 Genes Associated With Organic Acid and Sugar Metabolism

Fruit respiration is an important process through which energy is generated to facilitate the physicochemical changes that occur during ripening (Ambuko *et al*., 2017; Fagundes *et al*., 2013). The tricarboxylic acid (TCA) cycle is an essential component of respiratory metabolism in plants (Araújo et *al*., 2012). Organic acids are utilized as substrates in the TCA cycle during fruit ripening (Etienne *et al*., 2013). Fruit acidity as measured by titratable acidity (TA) is due to the presence of organic acids such as citric acid, malic acid and tartaric acid (Etienne *et al*., 2013; Yahia, 2011). Of these, citric acid has the highest concentration in several mango

varieties investigated (Malundo *et al*., 2001; Singh *et al*., 2013). Genes encoding the enzymes involved in the TCA cycle including citrate synthase (EC 2.3.3.1), aconitase (EC 4.2.1.3) and malic enzyme (EC 1.1.1.40) were identified in this study. The high expression of genes in the ripe stages of the 'CK' and 'P' group (Tables 4.10 and 4.11) suggests the contribution of TCA cycle in facilitating the climacteric burst in respiration during ripening (Araújo *et al*., 2012; Fabi *et al*., 2012). The genes associated with the TCA cycle showed an increased expression during the ripening process of banana (Asif *et al*., 2014) and papaya (Fabi *et al*., 2012), consistent with the results of this study. However, no significant change in expression during the ripening of Chinese bayberry was observed (Feng *et al*., 2012). These authors suggested that an alternative pathway for energy release was in play in Chinese bayberry since the genes associated with the Gamma-aminobutyric acid (GABA) shunt pathway were up-regulated during its ripening. Furthermore, functional studies on tomato (van der Merwe *et al*., 2010) and potato (Araújo *et al*., 2008) plants reported that mutants with reduced expression of genes associated with the TCA cycle showed a reduction in the rate of respiration compared to the wild type counterparts respectively. Based on these findings, the high level of energy-related genes found in the 'P' group compared to the 'CK' group (Tables 4.12 and 4.13) suggests an increased rate of respiration in the 'P' group which may have also contributed to its accelerated ripening rate.

The first step of the TCA cycle is catalysed by citrate synthase which is involved with citric acid biosynthesis (Etienne *et al*., 2013). Genes encoding citrate synthase were found to be up-regulated during the ripening of the 'CK' and 'P' mango groups respectively (Tables 4.10 and 4.11). This finding indicates the role of citrate synthase in citric acid biosynthesis during the ripening of the mango varieties investigated in this study similar to what has been reported in banana (Liu *et al*., 2013) and cherimoya (González-Agüero *et al*., 2016) fruits. Comparative study among the mango groups ('CK' and 'P' groups) revealed that the genes encoding citrate

synthase were highly expressed in the ‘P’ group at the unripe or ripe stage respectively (Tables 4.12 and 4.13). Previous studies on orange varieties showed that the expression of the *citrate synthase* genes were highly expressed in the low-acid variety ‘Hal’ compared to the high-acid variety ‘Al’ indicating that the low-acid variety had an increased rate of citric acid production compared to the high-acid variety (Guo *et al*., 2016). These authors concluded that the low acidity level may not have been due to the reduced expression of *citrate synthase* genes. In the present study, the ‘P’ group had a significantly low acidity level compared to the ‘CK’ group (refer to section 4.1.1.4) despite its higher expression level of citrate synthase genes. Taking the findings of the previous study into account, it implies that the low acidity level recorded in the ‘P’ group may have not been due to a reduced expression level of the *citrate synthase* genes. As suggested, another factor that may contribute to the differences in acidity level is the degradation of citric acid (Cercós *et al*., 2006; Etienne *et al*., 2013; Sadka *et al*., 2000). The involvement of aconitase enzyme which catalyzes the breakdown of citric acid in the TCA cycle has been described (Sadka *et al.* 2000; Morgan *et al*., 2013). In the present study, the pattern of *aconitase* gene expression mirrored the pattern of respiration in the ‘CK’ and ‘P’ group respectively. *Aconitase* genes were strongly expressed during the ripening process of the mango groups. A similar relationship has been reported during the ripening of apple (Shi *et al*., 2014), banana (Medina-Suárez *et al*., 1997), orange (Terol *et al*., 2010) and mandarin (Terol *et al*., 2010). Morgan *et al*., (2013) showed that the transgenic suppression of *aconitase* gene led to an increase in the citric acid level of the mutant ripe tomato. These authors suggested aconitase as a major determinant of the citric acid level. A study on lemon has also revealed that the inhibition of aconitase was associated with an increase in fruit acidity (Degu *et al*., 2011). Bearing this in mind, the increase in *aconitase* gene expression level could possibly explain the difference in the acidity level between the unripe and ripe stages of the mango groups respectively. Furthermore, the ‘CK’ group which had a significantly high acidity level

showed reduced expression of *aconitase* genes when compared with 'P' group. This findings imply that the high expression level in the 'P' group may have contributed to its reduced acidity level compared to the 'CK' group since a higher aconitase activity implies an increased citric acid degradation ability (González-Agüero *et al*., 2016; Saradhuldhat and Paull, 2007).

Malic enzyme catalyses the formation of pyruvate (a substrate for the citric acid cycle) and is potentially associated with both the synthesis and degradation of malic acid (Martinez-Esteso *et al*., 2011; Sweetlove *et al*., 2009). In this study, the genes encoding malic enzyme were found to be expressed differentially during the ripening of the 'CK' and 'P' groups. A similar observation during the ripening process of apple (Shi *et al*., 2014), cherimoya (González-Agüero *et al*., 2016) and papaya (Fabi *et al*., 2012) has been reported. These results support the premise that the genes encoding malic enzyme may be involved in sustaining the increased rate of respiration as the ripening process occurs. Additionally, it is also possible that the high expression level of the genes encoding malic enzyme found in 'P' group might partly explain its enhanced rate of respiration and accelerated ripening process compared to the 'CK' group. Nonetheless, the study has shown that malic acid metabolism may be playing an important role in the supply of energy in the mango groups during ripening. Furthermore, correlation analysis revealed that the difference in acidity between the varieties of Chinese dwarf cherry could be explained by the difference in the expression level of the genes encoding malic enzyme (Mu *et al*., 2018). In this regard, it is possible that the differences observed in the expression level of genes encoding malic enzyme between the 'CK' and 'P' group may have also contributed to the variation in acidity of these mango groups during storage. Although organic acids are utilized in the TCA cycle for the release of energy (Sweetlove *et al*., 2010), they can also be utilized in other metabolic pathways for sugar production or flavonoid synthesis (Fatland *et al*., 2005; Hu *et al*., 2015; Katz *et al*., 2007).

The most abundant soluble sugars found in the pulp of ripe mango fruits are sucrose, glucose and fructose (Castrillo *et al*., 1992; Wongmetha *et al*., 2015). Some enzymes associated with sugar metabolism in mango include sucrose phosphate synthase (SPS; EC 2.4.1.14) and sucrose synthase (Susy; EC 2.4.1.13) (Castrillo *et al*., 1992). The present study revealed a mixed expression pattern for genes encoding sucrose synthase consistent with the findings from 'Kent' mango and other fruits as *Citrus* and watermelon (Dautt-Castro *et al*., 2015; Gao *et al*., 2018; Islam *et al*., 2014) during ripening. As suggested by these authors, sucrose synthase may be playing different roles in fruit development and ripening. A gene encoding sucrose phosphate synthase showed an increasing trend in the 'CK' group during fruit ripening but none was found to be expressed differentially in the 'P' group. Analysis of the accumulation profiles of the *SPS* gene in 'Cavendish' and 'Kanthali' banana varieties during the different stages of ripening revealed that the *SPS* gene was strongly expressed during the early period of ripening in 'Cavendish' banana compared to 'Kanthali' banana (Choudhury *et al*., 2009a). Taking this into account, it is possible that the genes encoding SPS of the 'P' group may have been strongly expressed in a ripening stage which was not included in this study.

Starch degradation has been reported to be involved in sugar accumulation during mango ripening (Yashoda *et al*., 2006). The up-regulated genes encoding beta-amylase observed in the ripe stages of both mango groups ('CK' and 'P' group) suggests the occurrence of starch breakdown during ripening. These findings are in agreement with previous results on the ripening of other mango varieties (Dautt-Castro *et al*., 2015; Peroni *et al*., 2008) and other fruits such as tomato (Maria *et al*., 2016) and kiwi (Richardson *et al*., 2011). Furthermore, same stage comparison showed that most genes were up-regulated in 'P' group compared to the 'CK' group. A comparative study on low- and high-sugar content varieties of strawberry revealed that most genes involved in sugar accumulation were strongly expressed in the high-sugar content varieties (Lee *et al*., 2018). Based on this previous finding, it implies that these genes

may have contributed to the higher rate of sugar accumulation in the ‘P’ group compared to the ‘CK’ group as reflected by the soluble sugar concentration (SSC) (Cirilli *et al*., 2016). Soluble sugars and organic acids are important components of fruit taste and have an impact on organoleptic quality of mango fruit (Malundo *et al*. 2001; Yahia, 2011). In mango, taste is influenced by the varying concentration of sugars and organic acids which is the result of starch degradation, decreased acidity and the accumulation of sugars (Brecht and Yahia, 2009; Singh and Singh, 2012). A more recent study on watermelon varieties with differing fruit taste (Gao *et al*., 2018) revealed differences in the expression pattern of the genes involved in sugar and organic acid metabolism. Taking into account, the present and previous studies, it can be speculated that the differences in the expression profile of the aforementioned genes between the ‘CK’ and ‘P’ group might contribute to the variation in their taste quality. This variation in turn may have an impact on consumer preferences and the marketing potential of the mango varieties investigated here (Malundo *et al*., 2001). However, further analysis on sensory evaluation of the mango varieties will be needed to further support this notion.

5.4.3.2.2 Genes Associated with Hormone Metabolism

Plant hormones are very important for fruit development and ripening (McAtee *et al*., 2013). Ethylene is known to be the major hormone that governs climacteric ripening (Tucker *et al*., 2017). In this study, ethylene associated genes including 1-aminocyclopropane 1-carboxylate synthase (ACS) and 1-aminocyclopropane 1-carboxylate oxidase (ACO) were found to be differentially expressed in the ripening stages of the ‘CK’ and ‘P’ group respectively. These results are consistent with the findings from ‘Kent’ (Dautt-Castro *et al*., 2015) and ‘Dashehari’ (Srivastava *et al*., 2016) mangoes and other fruit species (Feng *et al*., 2012; Jung *et al*., 2017). Of note, these genes were also identified to be differentially expressed in same stage comparison between the ‘CK’ and ‘P’ group with most genes being highly expressed in the ‘P’ group. Although Singh *et al.,* (2017) attributed the increased loss of firmness in the apple

variety ‘Anna’ to the high rate of ethylene production associated with high ethylene-related genes. Ng *et al*., (2013) noted that this may not always be the case. These authors observed that the different softening rates of the apple varieties ‘Royal Gala’ and ‘Scifresh’ was due in part to the features of the cell wall as both varieties were high ethylene producers when compared to other apple varieties. In this regard, the difference in the fruit firmness of the mango groups investigated may have not been solely dependent on ethylene. Auxin plays a crucial role in plant growth and development (McAtee *et al*., 2013; Zhao *et al*., 2010). Regarding the effects on fruit ripening, auxin has been reported to play contradictory roles in different fruits. For instance, the application of exogenous auxin has been reported to delay the ripening process of fruits such as tomato and strawberry (Li *et al*., 2016; Su *et al*., 2015; Symons *et al*., 2012) whereas in peaches, auxin has been reported to promote the ripening process (Tatsuki *et al*., 2013; Trainotti *et al*., 2007). Tryptophan aminotransferase (TAA) is involved in auxin production (Woodward and Bartel, 2005; Shao *et al*., 2017). In the present study, genes encoding TAA were found to be strongly expressed in the unripe stage of the ‘CK’ and ‘P’ group respectively. The higher gene expression level observed in the unripe fruit support a role in the developmental phase as previously suggested for strawberry (Estrada-Johnson *et al*., 2017), tomato (Su *et al*., 2015) and papaya (Liu *et al*., 2017). Tatsuki *et al*., (2013) reported that the application of an anti-auxin (α-phenylethyl-2-one) to peaches decreased the expression level of genes encoding 1-aminocyclopropane-1-carboxylate synthase (ACS) (an enzyme associated with ethylene biosynthesis) and thus ethylene production. These authors suggested that the suppression of the *ACS* gene expression may have been caused by the low level of auxin. Furthermore, a higher expression level of auxin-related genes was detected in the ‘Fen Jiao’ banana variety (fast-ripening) compared to the ‘BaXi Jiao’ banana variety (slow-ripening) implying the potential involvement of auxin in promoting banana ripening (Hu *et al*., 2015). In this regard, the auxin-related gene found to be strongly expressed in the ‘P’ group

compared to the ‘CK’ group may have contributed to the accelerated ripening rate in the ‘P’ group during storage. In addition to ethylene and auxin related genes, the present study revealed the differential expression of genes encoding the plant hormone known as abscisic acid (ABA). ABA has been reported to participate in the ripening process of orange (Zhang *et al*., 2014), tomato (Sun *et al*., 2012) and strawberry (Jia *et al*., 2011). Genes encoding *9*-cis-*epoxycarotenoid dioxygenase* (*NCED*) (an enzyme involved in ABA synthesis) were differentially expressed in this study. Most genes encoding NCED were down-regulated in the ripe stages of the ‘CK’ and ‘P’ mango groups respectively. In peach, an *NCED* gene was observed to be strongly expressed at the unripe stage (Zhang *et al*., 2009) whereas in mandarin (Wang *et al*., 2017a) an opposite trend was observed. The results from the present study are in line with the observation from peach but contrast with that found in mandarin, a non-climacteric fruit where ABA seems to have a stronger role in promoting the ripening process (Jia *et al*., 2011; McAtee *et al*., 2011).

Taken together, the results has revealed the possible participation of other hormones in addition to ethylene during mango fruit ripening. Ethylene receptors (ETR) are involved in the transduction pathway that ultimately result in regulation of ethylene (Agarwal *et al*., 2012). In tomato, ETR is involved in the negative regulation of ethylene reactions (Chung *et al*., 2010). Thus, the decreasing trend of the genes in the ripe samples of the mango groups might have occurred to pave way for ethylene biosynthesis during the progression of ripening as previously described in ‘‘Kent’ mango (Dautt-Castro *et al*., 2015).

5.4.3.2.3 Genes Associated with Cell Wall Metabolism

Pulp softening is an obvious change that occurs in ripening mangoes and is denoted by the severe loss of firmness after just a few days (Yahia, 2011). Gene families encoding the softening-related enzymes such as PG and PE displayed a mixed gene expression (up-regulation and down-regulation) pattern during ripening. The down-regulated genes observed

suggests their involvement in fruit developmental processes rather than ripening (Brummell *et al*., 2006; Carrera *et al*., 2012; de Jong *et al*., 2010). The results of this study were similar to the observations from 'Kent' (Dautt-Castro *et al*., 2015) and 'Dasheri' (Srivastava *et al*., 2016) mangoes but contrary from what has been observed in 'Alphonso' mango (Deshpande *et al*., 2017) where most of the softening-related genes had a stable expression. The ripening duration for 'Kent' and 'Dashehari' mango is 10 and 6 days respectively similar to that observed in the present study. Conversely, the ripening duration of 'Alphonso' mango is 15 days from harvest (Deshpande *et al*., 2017). Keeping in view the varietal differences in ripening duration, the more stably expressed genes found in 'Alphonso' mango compared to the aforementioned varieties might be one of the reasons for its longer storage life as cell wall degradation during ripening may occur at a slower rate (Deshpande *et al*., 2017). Furthermore, it has been suggested that the differential softening characteristics in peach (Qian *et al*., 2016) and cotton (Li *et al*., 2015) varieties could be related to the amount of cell wall-related gene members. For example, more *PG* gene members were expressed in the fast-softening peach varieties compared to the slow-softening counterparts (Qian *et al*., 2016). In this regard, it can be inferred that the higher number of genes associated with cell wall degradation in the 'P' group compared to the 'CK' group may have also contributed to the increased rate of softening in the 'P' group. On the other hand, differences in the degree of mango softening during storage might also be due to the features of the cell wall (Tucker and Seymour, 1991). A study on 'Tommy Atkins' and 'Keitt' mangoes has shown some differences in cell wall composition despite a similarity in PG activity between the varieties (Mitcham and McDonald, 1992). Notwithstanding, these cell wall-related gene families have also been identified from other fruit species such as papaya, banana, peach and watermelon amongst others (Fabi *et al*., 2014; Asif *et al*., 2014; Qian *et al*., 2016; Zhu *et al*., 2017) suggesting a conserved gene action in the softening of a range of fleshy fruits species (Fabi *et al*., 2014). Albeit, their relative activities

may vary in fruit species (Brummell, 2006; Goulao and Oliveira, 2008). Taken together, the simultaneous expression of multiple genes of the same family during softening suggests the complexity of the softening process (Brummell, 2006).

5.4.3.2.4 Genes Associated with Colour and Flavour Development

Carotenoids such as beta-carotene, lycopene and lutein are pigments responsible for a wide range of fruit colouration in fruit (Cazzonelli and Pogson, 2010). During ripening, mango pulp colour changes from white or whitish yellow to yellow or orange-yellow due to the accumulation of carotenoids (Yahia, 2011). The relationship between carotenoid accumulation and gene expression has been investigated in various crop species. The relationship between carotenoid accumulation and gene expression has been investigated in various crop species. In sweet orange, beta-carotene accumulation was found to be directly related to the expression of *phytoene desaturase* rather than *LCYB* (Fanciullino *et al.*, 2008). The *PSY* knockout tomato mutant confirmed that a lack of phytoene led to the absence of carotenoid accumulation in ripe fruit of the mutant (Gady *et al*., 2012). *PSY* gene expression correlated positively with β-carotene accumulation in bitter melon (Tuan *et al*., 2011). Similarly, high phytoene synthase has also been associated with β-carotene accumulation in orange carrot roots (Maass *et al*., 2009). These findings indicate that carotenoid biosynthesis are regulated differently in different species. On the basis of these results, the different expression pattern of the carotenoid-related genes in the CK and Pool groups indicate that colour development during ripening might be achieved by different set of genes. Going further, a mutation in papaya *LCYB2* led to the accumulation of lycopene and was suggested to be responsible for the difference between

red- and yellow-fleshed papayas (Devitt *et al*., 2010). *Cucurbita moschata* showed lower expression levels of the *PSY1* gene compared with other squashes (Nakkanong *et al*., 2012). These authors suggested that the lack of phytoene (a requisite substrate for carotenoid synthesis) could explain the low levels of carotenoid accumulation in this species. Furthermore, a study in carrot suggested that the accumulation of major carotenoids in the red and yellow cultivars might partially be explained by the transcriptional level of genes directing the carotenoid biosynthesis pathway (Clotault *et al*., 2008). The carotenoid level in mango is variety dependent (Bretcht and Yahia, 2011). Keeping this in view, the differences in the coloration of the mango groups investigated might be explained by differences in colour-related genes.

Flavour is due to the blending of acidity, sweetness, acidity and the odour produced by the volatile compounds in the fruit (El Hadi *et al*., 2013). Mango varieties exhibit vast differences in aroma and this is dependent on the composition and concentrations of the volatiles including alcohols, aldehydes, esters, furanones and lactone (Pandit *et al*., 2009). Several genes related to flavour showed significant differential regulation during ripening of the mango groups investigated. These included genes encoding alcohol dehydrogenase (ADH) and lipoxygenase (LOX). *ADH* genes are expressed in a developmentally regulated manner particularly and have been shown to be associated with flavour development in mango (Singh *et al*., 2010) and peaches (Zhang *et al*., 2010). In the present study, *ADH* genes showed a mixed expression during the ripening of the mango groups suggesting their involvement in fruit development and ripening. Singh *et al*., (2010) reported a decreased trend of the *ADH* genes during the progression of mango ripening. In apple, LOX activity has been reported to be associated with aroma volatile production (Vogt *et al.,* 2013). Most genes encoding LOX were down-regulated during the ripening of the mango groups and most genes were highly expressed in the 'P' group compared to the 'CK' group. A decreasing expression trend was reported in 'Dashehari' mango

(Srivastava *et al*., 2016) whereas an up-regulation was observed 'Kent' mango (Dautt-Castro *et al*., 2015). As mentioned by Srivastava *et al*., (2016), the differences in the expression profiles of the *LOX* genes between 'Kent' and 'Dashehari' again suggest the involvement of different aroma pathways in different varieties.

5.4.3.2.5 The Role of Rab GTPases in Mango Fruit Ripening

Reverse genetic approaches on tomato fruits (Lu *et al*., 2001) have established the importance of vesicle trafficking in fruit ripening. Vesicle trafficking or the flow of membrane material between endomembrane compartments is essential for the transport of proteins and polysaccharides to various destinations inside and outside of the cell (Alice and Vries, 2008; Lycett, 2008). Elements of the secretory machinery, notably the Rab GTPases, have been shown to mediate communication between the plasma membrane, endoplasmic reticulum, Golgi and cell wall (Lycett 2008; Saito and Ueda, 2009). 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 (Saito and Ueda, 2009; Lawson *et al*., 2018) 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 (Abbal *et al*., 2008; Falchi *et al*., 2010; Lu *et al*., 2001; Lunn *et al*., 2013b; Zainal *et al*., 1996). These results suggests their involvement in fruit development and ripening. In particular, the plant RabA GTPase family is deserving of investigation for which evidence related to fruit softening has emerged using a reverse genetic approach (Lu *et al*., 2001). These authors 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 than the less-firm mango group ('P') at

either the unripe or ripe stage. Lunn *et al*., (2013b) has shown that in tomato, the *RabA1* is 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 (Brummell, 2006) as variation in cell wall changes between mango and tomato has been reported (Tucker and Seymour, 1991; Muda *et al*., 1995). 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 (Lunn, 2013; Lunn *et al*., 2013a). Authors on fruit studies have also observed significant differences in the cell wall components of apple (Kertesz *et al*., 1959; Yang *et al*., 2018), mango (Mitcham and McDonald, 1992) and strawberry (Rosli *et al*., 2004) varieties with contrasting firmness. Lunn *et al*., (2015) 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. Conversely, the *RabA1* and *RabA2* mutants in which pectin and cellulose appeared to have been reduced respectively showed no significant effect on enzymatic degradation compared to the wild type. 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 model plants and woody trees such as *Arabidopsis* (Zhao *et al*., 2011) and *Populus* (Lu *et al*., 2013; Wang *et al*., 2014) as well as in fruits such as loquat (Cai *et al*., 2006; Wang *et al*., 2016), mangosteen (Kamdee *et al*., 2014) and peach (Xue *et al*., 2018). Notably, high levels of lignin have been reported to be associated with increased fruit firmness in mangosteen (Kamdee *et al*., 2014; Ketsa and Atantee, 1998), loquat (Xu *et al*., 2015; Zeng *et al*., 2016) and

pear (Xue *et al*., 2018). On the other hand, Salentijn *et al*., (2003) 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 (Tables 4.16 and 4.17). Based on the previous findings, 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 the composition leading to an increased susceptibility to enzymatic degradation and consequently increased loss in fruit 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 RabC GTPases have been described as dehydrins (Hanin *et al*., 2011; Lang and Palva 1992). Dehydrins play an important protective role in plants exposed to abiotic stresses (Hanin *et al.*, 2011). Fruit ripening has been described as an oxidative process that leads to an excess of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) (Pandey *et al*., 2013; Mondal *et al*., 2009). ROS can cause damage to the membranes, proteins and DNA resulting in cell death (Mittler *et al*., 2004; Resende *et al*., 2012). Thus, the process of fruit ripening can be termed a stressful process (Resende *et al*., 2012). Oxidative stress during ripening has been reported in fruits such as peach (Camejo *et al*., 2010) and tomato (Mondal *et al*., 2004). 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 *RabC* gene strongly expressed in the unripe fruit may have been due to pre-harvest stresses such as exposure to sun light and pesticides (Bower *et al*., 1989; Pang *et al*., 1993) while on the tree. Lang and Palva, (1992) reported the accumulation of *RabC* gene in *Arabidopsis* exposed to cold stress. Campo *et al*., (2014) mentioned that the

up-regulation of the *RabC* gene in the roots of the rice might serve to prevent stress-induced oxidative damage in cellular membranes. Taking this into account, the *RabC* genes strongly expressed in the ripe fruit of the 'CK' and 'P' groups may be required to prevent cell damage as the rate of respiration leading to oxidative stress increases during the ripening process. Furthermore, the expression of a *RabC* gene was almost constant during the ripening of grape (Abbal *et al*., 2008) whereas an increased expression was observed in peach (Falchi *et al*., 2010). An explanation for these differences could be related to the different roles in different fruit species (Goulao and Oliveira, 2008) or the ripening stages and/or the type of isoform that was analysed in these studies since the RabC GTPases are encoded by a multigene family (Rutherford and Moore, 2002). Furthermore, comparison of the 'CK' and 'P' group at the same ripening stage (unripe or ripe) revealed that the *RabC* genes were strongly expressed in the 'P' group. Increase in the rate of respiration and/or water loss (measured as weight loss) result in an increase in the level of oxidative stress (Pandey *et al*., 2013). As such, the result indicates that the 'P' group with a higher rate of respiration and water loss showed a strong expression of the *RabC* gene to withstand the increased oxidative stress. The Rab GTPases of the D subclade mediate ER to Golgi trafficking steps in plants (Batoko *et al*., 2000; Cheung *et al*., 2002). 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 (Tyler *et al*., 2015). These authors suggested that the reduced bread making quality observed in the transgenic grains might have been due to the reduced trafficking of the gluten proteins. In addition, Loraine *et al*., (1996) found that the genes encoding the RabD GTPases accumulated during the ripening process in tomato. These authors suggested that the high expression level of *RabD* gene in the ripe stage may be linked to increased synthesis and trafficking of cell wall-related 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 (Speth *et al*., 2009). The genes encoding RabE GTPases have been reported to be expressed in fruits such as tomato (Zegzouti *et al.*, 1999), peach (Falchi *et al*., 2010), grape (Abbal *et al*., 2008) and apple (Park *et al.*, 2006). Comparing the ripening stages of the mango groups, the genes encoding the RabE GTPases 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 (Choudhury *et al*., 2009a, b). 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. Nonetheless, the results from the 'CK' group indicate a role for RabE GTPases during the development and ripening process of mango.

Studies using the loss-of-function mutation in *Arabidopsis* have established the role of RabF GTPases in vacuolar trafficking (Ebine *et al*. 2014; Sohn *et al*., 2003). The *RabF* gene was found to be up-regulated during the ripening of the 'P' group consistent with the findings of 'Siji' mango (Liu *et al*., 2014). However, no *RabF* gene was expressed differentially during the ripening of the 'CK' group. One possible reason could be that a maximum or minimum expression may have occurred earlier (Choudhury *et al*., 2009a, b). It is possible that this gene may have been induced in the 'P' group due to the stress-related events (Liu *et al*., 2014) such as a higher rate of respiration associated with its fast ripening.

Altogether, the comparative analyses 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 suggest that the variability of ripening-related quality of the mango groups 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 (Zhu *et al*., 2017), orange (Wang *et al*., 2017b), apple (Schaeffer *et al*., 2016), strawberry (Salentijn *et al*., 2003), banana (Choudhury *et al*., 2009a,b) and peach (Hiwasa *et al*., 2004) have also shown stage- and/or variety-dependent expression profiles. However, it is worth pointing out that since a pool group ('P') was used in this study, evidence of the specific variety(s) contributing to the high expression level is not presented. In order to address this, verification via RT-qPCR is needed to further support the implication of the RabA GTPases in mango softening.

5.4.3.3 Protein-Protein Interaction

Proteins do not act alone but in association with other proteins which is essential for the biological processes that occur in the cell (Launay *et al.,* 2017). Molecular interactions play a key role in predicting the function of a protein and the biological processes the protein is associated with (Rao *et al.,* 2014). 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 (Figure 4.36) suggesting that these proteins often act in cooperation with each other (Szklarczyk *et al*., 2017). For instance, the Rab GTPases depend on syntaxin to mediate the docking and fusion of vesicles with the target membranes (Rehman *et al*., 2008; Simonsen *et al*., 1999). Also, 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. Meanwhile, when localized in the cell wall, the pectinesterase (PE) modifies the pectins to make them more accessible for degradation by polygalacturonase (PG) (Tucker *et al*., 2017). Taken together, the PPI analysis has indicated the synergistic action between several cell wall-related enzymes and more

importantly strengthens the involvement of Rab GTPase in cell wall biosynthesis and degradation.

5.5 GENE EXPRESSION ANALYSIS BY REVERSE TRANSCRIPTION-QUANTITATIVE PCR (RT-qPCR)

5.5.1 Stability of Reference Genes and RT-qPCR Efficiency

RT-qPCR has become one of the most commonly used techniques for the quantification of gene expression and validation of data from RNA sequencing (RNA-seq) due to its rapidity, sensitivity and specificity (Chen *et al*., 2011). According to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al*. 2009), more than one reference gene is necessary for accurate data normalization. Thus, the accuracy of this approach is dependent on the correct choice of stable reference genes across the tested samples (Vandesompele *et al*., 2002). Unfortunately, there are no universally-suitable reference genes under every experimental condition (Bustin 2002; Vandesompele *et al*. 2002). Therefore, it was necessary to select and analyse a set of genes suitable under the desired experimental condition for gene expression analysis prior to RT-qPCR. Further, it has been recommended that more than one evaluation tool should be used for the assessment of reference gene stability (Xiao *et al*., 2015; Sarker *et al*., 2018). In this study, four genes were assessed for their suitability as reference genes using three computational programs GeNorm, NormFinder and RefFinder respectively. It is worth mentioning that in addition to ranking the tested genes, the geNorm also has the function of selecting the optimal number and combinations of reference genes for an experimental study (Vandesompele *et al*., 2002). The web-based tool RefFinder which integrates all four approaches (geNorm, NormFinder, BestKeeper and the ΔCt method) was used as a confirmatory tool to finally select the reference genes since geNorm and NormFinder programs produced slightly different ranking of the

stable genes. This discrepancy in gene ranking among the programs (geNorm, NormFinder, BestKeeper and the ΔCt method) has been noted in previous studies and as reported it might be as a result of the different principles employed by these programs in assessing gene stability (Qi *et al*., 2016; Sarker *et al*., 2018).

The standard curve method has been the gold standard for estimating amplification efficiencies (Svec *et al.* 2015). However, sample dilutions at high concentrations could lead to an overestimation of efficiency due to the presence of high amounts of PCR inhibitors whereas samples with very low concentrations may not be accurately quantified due to very few target molecules present (Rutledge and Côté, 2003; Wong and Medrano, 2005). Together, this could lead to a slope of the standard curve that is either higher or lower and thus affect the subsequent calculation. To overcome these limitations, an alternative method without the need of a standard curve is the LinRegPCR (Ranmakers *et al*., 2003; Ruijter, 2009). This software estimates the efficiency from individual amplification curves and provides a mean efficiency for an amplicon group (Ranmakers *et al*., 2003; Ruijter, 2009). Keeping this in mind, the calculated amplification efficiencies by standard curve were within the acceptable range of 90 –110 % (Bustin, 2009) and the results were comparable to those obtained from LinRegPCR (Table 4.19).

5.5.2 Comparison of RNA-Sequencing Results to RT-qPCR Analysis

Validation has been an important part in transcriptome expression literature. The differentially expressed genes (at least some) identified using RNA sequencing are often validated using RT-qPCR. For example, Dautt- Castro *et al*., (2015) validated ten ripening-related genes found to be differentially expressed genes from RNA-seq in 'Kent' mango. They showed that the trend from RNA-seq had a high correlation (r = 0.970) with that from the RT-qPCR. High consistency between RNA-seq and RT-qPCR results has also been observed in studies of other fruits including grape (Balic *et al*., 2018), tomato (Li *et al*., 2016) and pear (Huang *et al*., 2014).

A high correlation (r = 0.769) between the RT-qPCR and RNA-seq data was observed in this study which is in line with the aforementioned studies. However, the inconsistency between both platforms for *RabA5* gene could be due to one of several reasons. Firstly, this discrepancy may, in part, be explained by differences in the expression quantification and normalisation strategies between the two techniques (Everaert *et al*., 2017). In RNA-seq, an expressed gene is based on the total reads that map to a particular gene and normalised based on the gene length and sequencing depth whereas in RT-qPCR primer pairs are designed to amplify a little portion (< 200 bp) of the entire gene and normalisation is based on reference genes. It is also possible that other technical issues such as the library preparation and removal of low quality reads could be responsible (Merrick *et al*., 2013; van Dijk *et al*., 2014). Fundamental to RNA-seq analysis is the preparation of libraries which involves a coordinated series of enzymatic reactions to generate fragments fused with adapters followed by PCR amplification and sequencing. Biases in RNA-seq library preparation can be introduced from several steps such as adaptor ligation, reverse transcription and amplification steps (van Dijk *et al*., 2014). The attachment of adapter sequences at the ends of the fragments by ligases is a critical step in library preparation. These ligated products are subsequently reverse transcribed, amplified by PCR and sequenced. However, the differences in the efficiency of ligation reaction can alter the detection and quantification of individual templates (Baran-Gale *et al.*, 2015). Another important issue for library preparation is the formation of adapter dimers which can compete with the cDNA fragments thus reducing the sequencing depth (Baran-Gale *et al.*, 2015). On the other hand, the possibility of a false positive cannot be excluded as a source of inconsistency between the RNA-seq and RT-qPCR data (Fabi *et al*., 2012).

5.5.3 Comparison of Expression Levels in Mango Varieties

Fruit softening is a complex attribute (Seymour *et al*., 2013) and more information on the factors associated with this process are constantly needed. The present study has provided

evidence on the potential RabA GTPases that might be related to the difference in firmness among ‘Chokanan’, ‘Golden phoenix’ and ‘Water lily’ mango varieties investigated in this study. From RT-qPCR assays, it was demonstrated that the genes putatively involved in cell wall modification and vesicle trafficking were differential expressed as earlier observed in the RNA-seq data. This study investigated the expression of genes encoding two most intensively studied cell wall degrading enzymes involved in fruit softening: polygalacturonase (PG) and pectinesterase (PE). The genes were selected based on the RNA-seq data and their previous characterization in relation to fruit softening (Phan *et al*., 2007; Smith *et al*., 1988). The study showed that *PE* gene negatively correlated with the firmness of the mango varieties at the unripe stage. Based on previous study (Phan *et al*., 2007), a higher *PE* expression would result in the generation of more de-esterified pectin making them more accessible for the action of PG and thus setting the basis for increased loss of firmness. However, since the correlation for *PE* with the firmness of the mango varieties was not significant, it suggests that the *PE* gene investigated here might not be playing an essential role in the softening differences observed amongst the varieties. Furthermore, *PG* gene expression showed similar accumulation patterns in ‘Chokanan’ and ‘Golden phoenix’, even though these two varieties were significantly different in terms of pulp firmness. This result indicates that although this gene may be involved in softening, it is not sufficient alone to induce the contrasting firmness observed in the mango varieties. This finding contradicts with the observation from apple (Wakasa *et al*., 2006), peach (Qian *et al*., 2016) and strawberry (Salentijn *et al*., 2003) varieties. One possible reason for this discrepancy may be due to the marked diversity in cell wall modification that occur in different fruit species (Brummell, 2006). A positive relationship ($r = 0.8544$; $P > 0.05$) was observed for genes *PG* and *PE* suggesting a co-expression, consistent with what is already known about the relationships between these genes in other fruits raspberry (Simpson *et al*., 2017) and apple (Gwanpua *et al*., 2016). However, the relationship was not significant

suggesting that the concormitant action of these genes may not be required for pulp softening. This agrees with the study in papaya in which a stable amount of esterified pectin was observed with progression in ripening (Fabi *et al*., 2012).

Cell wall modification enzymes, such as PG and PE are synthesized on the endoplasmic reticulum (ER) (Ray *et al.*, 1977) and transported through the endomembrane system to the apoplast (Staehelin and Moore, 1995). Therefore, the dynamics of cell wall changes would require membrane trafficking which enable the delivery between compartments (Ebine and Ueda, 2015). Differences in *RabA* gene expression level among mango varieties suggest that alterations in the membrane trafficking system would have a potential role in fruit softening rates (Lycett, 2008). The expression level 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'. Transgenic *Arabidopsis* lines deficient of *RabA1, RabA2, RabA3 and RabA4* gene expression showed alteration in the proportion of the cell wall components: pectin, cellulose, lignin and hemicellulose respectively (Lunn *et al*., 2013a) suggesting a role in the trafficking of specific cell wall components. Several authors have also reported a close relationship between differences in cell wall composition and softening rate in other fruit species. For instance, differences in pectin content were detected for strawberry (Rosli *et al*., 2004) and mango (Mitcham and McDonald, 1992) varieties with contrasting fruit firmness. Besides, tomato plants with antisense expression of a *RabA1* gene (involved in pectin transport) produced fruit that were significantly firmer than the wild type (Lu *et al*., 2001).

Although 'Apple' and 'Kemling' varieties were significantly less-firm, these varieties showed similar expression pattern to 'Chokanan' (the firm mango) in most genes. This suggests that although differences in gene expression level could have contributed to softening, this may not have been the sole determinant of the differential softening rates observed in all mango

varieties and that other factors may be important. Cell turgidity (Tong *et al*., 1999; Vicente *et al*., 2007), transpirational water loss (Saladié *et al*. 2007) and cell shape (Harker *et al*., 2010) have been suggested to be integral in determining fruit firmness. Loss of turgidity during ripening is due to the accumulation of solutes (sugars and organic acids) in the apoplast and transpirational water loss (Vicente *et al*., 2007). Ripening tomato fruits have been reported to show a decline in turgidity that roughly corresponds with the ripening-associated softening (Shackel *et al*., 1991). In addition, Saladié *et al*. (2007) reported that the ripening tomato mutant [known as delayed fruit deterioration (DFD)] which displayed a substantially reduced softening showed minimal transpirational water loss and elevated cellular turgor compared to the wild type [Alisa Craig (AC)]. These authors concluded that cell turgidity is of critical importance as is cell wall degradation in the substantial variation of fruit firmness. Additionally, a study on apples showed that 'Delicious' and 'Honeycrisp' (firm varieties) maintained a higher turgor potential than 'Macoun' and 'Honeygold' (soft varieties) (Tong *et al*., 1999). Several investigators have also reported that differences in the structural features of the cell wall established during the stages of fruit development may also play a role in softening during postharvest storage. For instance, Ng *et al*., (2013) found that the firmer-fleshed 'Scifresh' apples had cells which were more angular compared to the softer-fleshed 'Royal Gala' apples. More angular cells in firmer-fleshed varieties have been attributed to lesser airspace and greater cell-to-cell contact compared to the rounder cells of softer-fleshed varieties (Vincent, 1989). Based on these findings, the results of the present study imply that a) mango varieties may have developed different ways to bring about differential softening rates during ripening and b) early stages of fruit development may have an influence on the differential softening rate of mango varieties during postharvest storage.

Going further, the positive correlation between the expression of softening related genes *PE*, *PG*, *RabA1-2*, *RabA3* and *RabA4* observed this study led to some parallels with the results

observed in tomato Lu *et al.* (2001) and mango (Zainal *et al*., 1996). Lu *et al*., (2001) stated that the inhibition of a tomato *RABA1a* orthologue resulted in reduced levels of cell wall-modifying enzymes in the antisense plant compared to the wild type. Considering this finding, an alteration in the *RabA* gene expression level might also have affected the secretion of cell wall modifying enzymes leading to the contrasting firmness among the mango varieties.

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*., (2013) 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*., (2013b) the really high level of expression of the *RabA1* 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.

6 CONCLUSION

Research regarding the regulation of fruit softening has mainly focused on the degradation of the cell wall at the molecular level. However, the knowledge of the involvement of vesicle trafficking during mango ripening is still rudimentary which made it exciting to explore the possible involvement of the Rab GTPases. To truly appreciate the context of the data presented in this study, it is in the first instant best to refresh the overall hypothesis of the project and individual objectives. The project aimed to investigate the ripening and softening process of tropical mango with a particular focus on the Rab GTPases. Towards achieving this aim, the objectives were (i) the physicochemical and physiological characterization of 'Chokanan', 'Golden phoenix' and 'Water lily' mango varieties during ripening, (ii) identification and analysis of the Rab GTPase family in mango fruit iii) global comparative transcriptomic profiling of mango varieties at two ripening stages, namely unripe and ripe iv) to examine the relationship between *RabA* gene expression level and fruit firmness.

Changes in the physiological and physicochemical characteristics of mango varieties namely 'Chokanan', 'Golden phoenix' and 'Water lily' were investigated to examine the influence of ripening and variety on postharvest quality during the storage. With the exception of 'Chokanan', it is worth mentioning that no literature has been found so far on the characterization of 'Golden phoenix' and 'Water lily' mango varieties according to their physicochemical attributes. The result of the study showed that the varieties along with storage time contributed to the differences in postharvest quality. It was found that the ripening characteristics of 'Chokanan' differed from other varieties in terms of reduced weight loss, higher pulp firmness, timing of the climacteric peak in respiration and ethylene outburst.

The identification of ripening stage from this study was successfully defined based on the measured quality parameters thus confirming their reliability as indicators of fruit ripening. It also emerged from this study that multivariate analysis can be successfully applied as a

grouping tool on the basis of fruit quality attributes. The biplot showed clear separation of the mango samples by the ripening stages and demonstrated that firmness and SSC attributes contributed most to the variability between the unripe and ripe fruits. In addition, the study showed that peel colour alone may not be a useful descriptor in the differentiation of 'Golden phoenix' and 'Water lily 'mango varieties. The data obtained from the physicochemical studies would be useful to local producers who have now a better knowledge of the different varieties and by industries wishing to use these as processed products.

Up to now, the identification and characterization of the Rab GTPase family has been carried out in several plant species. An investigation of the Rab GTPase family expressed in mango fruit has been successfully carried out for the first time in the current study using an *in silico* approach. This was 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. Identification and characterization of the Rab GTPases was established using the known Rab GTPase family of the model plants viz *Arabidopsis* and tomato. The study confirmed that the Rab GTPases are conserved within clades rather than within species which might indicate shared putative functions. Furthermore, similarity tree analysis for the grouping of the Rab GTPases into sub-families coupled with sequence comparison allowed the identification of the closest homologues from *Arabidopsis* and their assignment to specific subgroups. Together, this study has provided insights into the functional diversity of the mango Rab GTPase family.

The RNA extraction procedure described in this study allowed for successful isolation of high yield of pure, high-quality RNA from mango fruit as indicated by the Bioanalyzer results. The RNA samples were devoid of contaminating DNA and suitable for downstream applications such as RT-PCR, RNA sequencing and RT-qPCR analysis. The presented method combines the advantages of being efficient, phenol-free, highly reproducible and inexpensive. This

protocol could be applicable in other plants where higher polyphenol and polysaccharides problems exist, especially in fruit tissues.

Going further, the study employed RNA-sequencing technology and this has proven to be a robust method in addressing the questions of mango fruit ripening. The global picture of the dynamic changes in gene expression obtained enabled new biological insights which could not have been obtained using conventional single observation methodologies. Comparative analyses provided an invaluable resource to reveal several genes associated with plant hormone metabolism, cell wall metabolism, starch and sucrose metabolism amongst others consistent with published literature. Pairwise comparisons revealed differential gene expression patterns between ripening stages for a mango group or between the same ripening stages of the mango groups. This has shown that the expression of ripening-related genes, at least of those examined in this study, is stage- and variety-dependent, which might in turn contribute to different phenotypes of mango varieties during postharvest storage. In addition, this comprehensive study has provided evidence and increased knowledge on the involvement of key players of trafficking, notably the Rab GTPases during mango ripening. Given the complexity of fruit ripening, the data reported here has opened up a new perspective to study the underlying mango fruit softening and contributed to a better understanding of the molecular basis underlying fruit ripening. Besides enriching the mango genes in the database, this study has provided a platform for further research on this economically important fruit crop and a reference for other related fleshy fruits towards the goal of reducing postharvest losses.

The present study has demonstrated that a targeted approach such as RT-qPCR is needed in combination with untargeted approaches such as RNA-seq for understanding the molecular basis of mango fruit softening. Accordingly, *ACT* and *UBI* were found to be the appropriate reference genes for gene expression analysis in mango. The stable reference genes identified by a combinatorial use of gene evaluation programs will facilitate future work gene expression

studies on mango fruit ripening. In addition, the LinRegPCR program has been shown to be a suitable alternative to standard curve method in amplification efficiency determination. An overview of the relationship between changes in pulp firmness and the expression level of the *RabA* genes in selected mango varieties has been obtained thus providing evidence for the involvement of vesicle trafficking in fruit softening. The information obtained, although correlative in nature, indicates that cell wall composition as well as secretion of cell wall modifying enzymes might be associated with contrasting firmness in mango varieties. In particular, *RabA3* may be considered an interesting gene for improving the firmness of mango fruit although a larger sample size will be needed for further confirmation. Altogether, the results of this study would be of interest to the research community particularly those interested in fruit ripening research.

7 FUTURE DIRECTIONS

The present study carried out an in-depth investigation of the physiological and physicochemical characterization of three mango varieties during ripening. Since mango fruit is considered a good source of dietary antioxidants such as ascorbic acid and carotenoids which have demonstrated health-promoting benefits (Lauricella *et al*., 2017), future research may consider evaluating and comparing the antioxidant properties of the mango varieties investigated. Such a study could serve as a platform to accumulate evidence to support this edible fruit as a potential source for functional food. Consumer perception for the fruit is an important factor that influences the marketability of fruits (Jha *et al*., 2013; Nassur *et al*., 2015). As such, further investigation on these mango varieties aiming at the evaluation of their sensory properties will provide valuable information which could be used by growers, plant breeders, exporters and marketing agents to facilitate increased utilization and export of varieties that would be acceptable by consumers globally. As mango is a seasonal fruit, processing is

increasingly needed to be considered, as an additional alternative to reduce postharvest losses and add the value of finished products. Taken together, dissemination of the knowledge on antioxidant and sensory properties will add value to these varieties, provide information for the best use of the mango varieties and also open new perspectives to the farmers and to the local industry on their potential for technological and nutritional utilization.

With the exception of 'Chokanan' mango, there is currently no published information regarding the changes in cell wall components of 'Water lily' and 'Golden phoenix' mangoes during ripening, let alone how these components may differ between the varieties investigated in this study. Thus, it would be of great importance to perform a comparative study and examine the correlation with pulp firmness in 'Chokanan', 'Golden phoenix' and 'Water lily' in relation to changes in their cell wall constituents.

The study has identified Rab GTPase sequences expressed in mango fruit; however, this may not represent all members in mango, thus further identification may be considered including other organs such as leaves, roots and flowers. Furthermore, the Rab sequence information could be used as a reference for designing degenerate primers. Degenerate primer design will facilitate the amplification of homologous sequences even when the species in question is poorly characterized at the molecular level. Further gene characterisation can be performed using the gene-specific primers in RACE-PCR to obtain full length cDNA sequences. A comparative study between full length cDNA sequences and the corresponding gDNA sequences will would provide information on the intron-exon structure of the mango *Rab* genes.

Furthermore, a comparative analysis of the RNA-seq data generated from this study and that of previous reports will facilitate the identification and characterization of common as well as variety and/or tissue-specific genes associated with ripening. In addition to differences in gene expression level, nucleotide sequence variation may also contribute to the phenotypic

differences observed between the mango varieties (Dillon *et al*., 2014; Hoang *et al*., 2015). Thus, it would be worth exploring this aspect. The transcriptome datasets generated in this study constitutes a rich genetic resource which can be further exploited for the identification and characterization of nucleotide variations such as single nucleotide polymorphisms (SNPs) and simple sequence repeats (SSRs). This will provide valuable information that may contribute to (1) the genetic relationships of *Mangifera indica* and related *Mangifera* species, (2) association of molecular markers with important traits of *Mangifera species* and (3) facilitating marker-assisted selection in future research. Transcriptomic analysis performed in this study has proven to be valuable in the identification of putative ripening-related genes. However since mRNA and protein abundance are not always consistent, future investigations may consider characterizing the dynamic changes that occur at the protein level. This can aid the identification of proteins that are specifically relevant to the trait of interest (Chin and Tai, 2018). Integrating knowledge gained from physiological and transcriptomic data coupled with proteomic study could provide more detailed evidence to comprehensively understand the molecular events that occur during mango fruit ripening.

Finally, since the sampling of mango fruits began at the mature green (harvest) stage. Future investigations might consider including the developmental (immature) stages. It would also be interesting to compare the parameters measured in this study with more varieties since the species *Mangifera indica* is highly diverse with respect to many attributes, including firmness. In particular, 'Alphonso' mango which has been well documented as a variety with a long term storage potential (15-17 days) (Deshpande *et al*., 2017; Nambi *et al*., 2015).

Even though more studies are needed to elucidate mango fruit softening, the results of this PhD project obtained through a large range of activities and experimental tools can be considered as an appreciable contribution to a better understanding of the mango ripening process and strengthens the emerging role of the Rab GTPases as potential targets to address postharvest

losses not in this economically important fruit. In addition, knowledge gained could be transferred to minor/underutilised fruits since these molecular elements are heavily conserved in plant species.

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9 APPENDICES

9.1 Appendix I: Suppliers of Reagents and Chemicals

Material	**Supplier**
Chemicals	
Chloroform	Sigma-Aldrich
Ethanol, 99. 5 %	Nacalai Tesque
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Hexadecyltrimethylammonium bromide	Sigma-Aldrich
Isoamyl alchohol	Sigma-Aldrich
Lithium chloride (LiCl)	Sigma-Aldrich
Sodium chloride (NaCl)	Sigma-Aldrich
Sodium hydroxide (NaOH)	Fisher Scientific
Tris base	R&M Chemicals
PCR/RT-qPCR reagents	
Primers	Integrated DNA Technologies
Hot star taq plus master mix	Qiagen
qPCR master mix	Bioline
DNase/RNase-free distilled water	Invitrogen
Gel Electrophoresis	
100 bp plus DNA ladder	Thermo Scientific
6 x DNA loading dye	Thermo Scientific
2 x RNA loading dye	Thermo Scientific

50× Tris-acetate-EDTA (TAE) buffer	Thermo Scientific
Agarose	First base
High range RNA ladder	Thermo Scientific
SYBR® Safe DNA gel stain	Invitrogen
Commercial Kits	
PCR and gel purification kit	Roche
DNase treatment and RNA clean-up kit	Qiagen
Reverse transcription kit	Qiagen
Consumables	
1.5 ml microcentrifuge tubes	Eppendorf
2.0 ml microcentrifuge tubes	Eppendorf
15 ml and 50 ml centrifuge tubes	Labcon
PCR tubes	Labcon
Filter pipette tips	Labcon

9.2 Appendix II: Standard Reagents

All reagents were prepared in double distilled water (18 ohms) and were sterilised using a stem autoclave for 1 hour at 121 °C.

1 N NaOH: This was prepared by dissolving 4 g of sodium hydroxide (NaOH) pellets in 100 ml of water.

5 M NaCl: A 5 M stock of NaCl solution was prepared by dissolving 29.22 g of NaCl in 100 mL of water.

0.5 M EDTA (pH 8.0): A 0.5 M stock of EDTA solution was prepared by dissolving 18.61 g

of EDTA disodium salt in 50 ml of water, adjusting the pH to 8.0 using NaOH and bringing the final volume to 100 ml.

1 M Tris (pH 8.0): 12.11 g of Tris base was dissolved in 70 ml distilled water, adjusting the pH to 8.0 using concentrated HCl and then adding water to a final volume of 100 ml.

Tris-EDTA (TE) buffer with low EDTA (100 ml) ($TE_{0.1}$ buffer): 1 ml of 1M Tris-Cl (pH 8.0) and 0.02 ml of 0.5 M EDTA (pH 8.0) were mixed with 50 ml water and then adding water to bring the final volume to 100 ml. The final concentrations were 10 mM Tris-Cl and 0.1 mM EDTA

10 M LiCl: This was prepared by dissolving 42.4 g in 80 ml of water and then adjusting to a final volume of 100 ml.

Chloroform: Isoamyl alchohol (CIA) (24:1): A 100ml of CIA was prepared by mixing 96 ml chloroform with 4 ml isoamyl alchohol.

CTAB extraction buffer: 2.5 g of Cetyltrimethyl ammonium bromide (CTAB) powder, 40 ml of 5 M NaCl, 5 ml of 0.5 M EDTA and 10 ml of 1.0 M Tris-Cl were mixed to make 100 ml of CTAB extraction buffer. The final concentrations were 2.5 % CTAB, 2 M NaCl, 25 mM EDTA and 100 mM Tris

9.3 Appendix III: Primers used in this study

Primer name	Sequence (5'- 3')	Product (bp)	Reference
RT-PCR			
RbA1a_R	TGGGTACAGGCCTGATGATGA	520	This study
RbA1a_F	TTCAGAAAGGAAGCATCCGCA		
RabA2a_F	AGACCGGACGAGGAATACGA	700	
RabA2a_R	GCTCCTACATCGATAGTCTGACCTT		
RabA3_F	TAATCGGAGACTCGGCGGTG	650	
RabA3_R	AGCATGGCTGCGTCAGTTTT		
RabA4a_F	TGGCTTCTGGAGGCTATGGAGAT	400	
RabA4a_R	CTTGGCCTTCTTCTGCTGTTAG		
RabA5_F	ATCTCCTCTCTCGCTACGCTC	495	
RabA5_R	CCTGTTTGCTTCGATCCATCCG		
RabA6_F	GGCAGTGTTGATTGGGGACT	559	
RT-qPCR			
QA1-1F	AGTCAGATCTTCGGCACCTC	85	This study
QA1-1R	GACTCCTCCTCGGCAAATG		
QA1-2F	TCACTCAGATATATCATGTTGTCAGC	104	
QA1-2R	GCAAGGTTGCTGGGTCAT		
QA2-2F	GACTTAAATCATCTTAGAGCTGTTCG	113	
QA2-2R	GACCTTCCTTCTCAGCCAAG		
QA3-F	GGAAGAAATTTATGGCGTGGT	90	
QA3-R	GCGTCAGTTTTCTCATTGCTG		
QA4-1F	CAAGACACCAGAGCTTTGACC	100	
QA4-1R	TTCTTATCAGCATGGCTTCG		

MiACT1_F	CCCAAGGCTAACAGAGAGAAGATG	120	Liu *et al*., 2014
MiACT1_R	ATCACCAGAATCCAGCACAATACC		
MiGADPH_F	GTGGCTGTTAACGATCCCTT	124	Dautt-Castro *et al*., 2015
MiGADPH_R	GTGACTGGCTTCTCATCGAA		
PE_F	GCAGCTTAGGAGGTGGAACAATC	111	
PE_R	TTAACTGGCCGAGCCAAATTCG		
MiUBI_F	AGCCATGCAGATCTTCGTCA	127	This study
MiUBI_R	CTGTTGGTCCGGAGGGATAC		
MiTUB_F	CTGTGGGGACTCCGATCTTC	125	
MiTUB_R	TCCATAGTACCCGGCTCCAG		
QA5_F	CAGAGGGATTGTTCTTTATCGAG	105	
QA5_R	TCTCGAAAGCTGTTTCAACG		
QRAB5_F	TCGAGCCGGAATGAAGTTAC	101	
QRAB5_R	TGAAGAGCAACAAAACCCTCTA		
PG_F	GAAAGCTGGTGGAAGAATG	121	Srivastava *et al*., 2016
PG_R	CTGTTGACGTGATGCAATCC		

9.4 Appendix IV: Analysis of Variance (ANOVA) Table

a) Effect of ripening on the postharvest quality parameters

Weight loss					
'Chokanan'					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	4	107.3163	26.8291	71.54	<.001
Residual	15	5.6251	0.3750		
Total	19	112.9414			
'Golden phoenix'					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	3	139.189	46.396	20.16	<.001
Residual	12	27.613	2.301		
Total	15	166.802			
'Water lily'					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	3	167.1168	55.7056	255.09	<.001
Residual	12	2.6205	0.2184		
Total	15	169.7374			
Hue					
'Chokanan'					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	4	2195.075	548.769	100.88	<.001
Residual	15	81.598	5.440		
Total	19	2276.673			
'Golden phoenix'					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	3	281.278	93.759	26.22	<.001
Residual	12	42.918	3.576		
Total	15	324.196			
'Water lily'					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	3	684.691	228.230	80.19	<.001
Residual	12	34.155	2.846		
Total	15	718.846			
L* value					
'Chokanan'					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	4	253.416	63.354	25.36	<.001
Residual	15	37.469	2.498		
Total	19	290.884			
'Golden phoenix'					

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	3	141.960	47.320	11.71	<.001
Residual	12	48.501	4.042		
Total	15	190.461			
‘Water lily’					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	3	226.028	75.343	12.49	<.001
Residual	12	72.389	6.032		
Total	15	298.417			
b* value					
‘Chokanan’					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	4	1213.561	303.390	52.36	<.001
Residual	15	86.920	5.795		
Total	19	1300.481			
‘Golden phoenix’					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	3	257.484	85.828	9.92	0.001
Residual	12	103.825	8.652		
Total	15	361.309			
‘Water lily’					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	3	783.390	261.130	112.23	<.001
Residual	12	27.920	2.327		
Total	15	811.310			
a* value					
‘Chokanan’					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	4	614.444	153.611	120.77	<.001
Residual	15	19.078	1.272		
Total	19	633.522			
‘Golden phoenix’					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	3	21.0803	7.0268	7.56	0.004
Residual	12	11.1549	0.9296		
Total	15	32.2352			
‘Water lily’					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	3	96.987	32.329	21.65	<.001
Residual	12	17.921	1.493		
Total	15	114.908			
Soluble solid concentration (SSC)					
‘Chokanan’					
Source Of Variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	4	457.023	114.256	25.81	<.001
Residual	15	66.395	4.426		
Total	19	523.418			

‘Golden phoenix’					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	3	827.947	275.982	32.85	<.001
Residual	12	100.817	8.401		
Total	15	928.764			
‘Water lily’					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
DAY	3	314.702	104.901	56.46	<.001
Residual	12	22.295	1.858		
Total	15	336.998			
Titratable acidity (TA)					
‘Chokanan’					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	4	45807.34	11451.84	144.32	<.001
Residual	15	1190.23	79.35		
Total	19	46997.58			
‘Golden phoenix’					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	3	22153.27	7384.42	244.46	<.001
Residual	12	362.48	30.21		
Total	15	22515.76			
‘Water lily’					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	3	24305.76	8101.92	304.96	<.001
Residual	12	318.80	26.57		
Total	15	24624.56			
Firmness					
‘Chokanan’					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	4	1.478320	0.369580	37.53	<.001
Residual	15	0.147703	0.009847		
Total	19	1.626023			
‘Golden phoenix’					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	3	0.51426	0.17142	9.41	0.002
Residual	12	0.21857	0.01821		
Total	15	0.73283			
‘Water lily’					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Day	3	0.051968	0.017323	11.94	<.001
Residual	12	0.017408	0.001451		
Total	15	0.069376			
Ethylene					
‘Chokanan’					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Stage	4	2.624E-04	6.561E-05	64.13	<.001

Residual	15	1.535E-05	1.023E-06		
Total	19	2.778E-04			
‘Golden phoenix’					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Stage	3	1.571E-04	5.235E-05	61.30	<.001
Residual	12	1.025E-05	8.540E-07		
Total	15	1.673E-04			
‘Water lily’					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Stage	3	1829119.	609706.	123.59	<.001
Residual	12	59200.	4933.		
Total	15	1888319.			
		Carbon dioxide			
‘Chokanan’					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Stage	4	631417.	157854.	42.83	<.001
Residual	15	55281.	3685.		
Total	19	686698.			
‘Golden phoenix’					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Stage	3	1001123.	333708.	60.07	<.001
Residual	12	66669.	5556.		
Total	15	1067791.			
‘Water lily’					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Stage	3	1.516E-04	5.055E-05	13.03	<.001
Residual	12	4.655E-05	3.879E-06		
Total	15	1.982E-04			

Note: d.f, degree of freedom; s.s, sum of squares; m.s, mean sum of square; v.r; variance ratio; F.pr, F probability or P value.

b) Effect of variety on postharvest quality parameters

Weight loss					
Day 2					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	0.502	0.251	0.23	0.801
Residual	9	9.936	1.104		
Total	11	10.438			
Day 4					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	20.738	10.369	5.66	0.026
Residual	9	16.497	1.833		
Total	11	37.235			
Day 6					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	45.656	22.828	22.03	<.001
Residual	9	9.325	1.036		
Total	11	54.981			
Hue					
Day 0					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	10.396	5.198	1.49	0.277
residual	9	31.45	3.495		
total	11	41.854			
Day 2					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	1.605	0.802	0.19	0.832
residual	9	38.502	4.278		
total	11	40.107			
Day 4					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	26.189	13.095	2.36	0.150
residual	9	49.918	5.546		
total	11	76.108			
Day 6					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	187.309	93.655	14.11	0.002
Residual	9	59.736	6.637		
Total	11	247.045			
L* value					
Day 0					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	52.771	26.385	9.52	0.006
Residual	9	24.942	2.771		

Total	11	77.713			
Day 2					
Source of variation	d.f.	s.s.	m.s	v.r.	F pr.
Variety	2	68.995	34.497	8.55	0.008
Residual	9	36.299	4.033		
Total	11	105.294			
Day 4					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	58.364	29.182	5.21	0.031
Residual	9	50.428	5.603		
Total	11	108.792			
Day 6					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	60.867	30.433	7.48	0.012
Residual	9	36.609	4.068		
Total	11	97.476			
		a* value			
Day 0					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	6.8892	3.4446	3.83	0.063
Residual	9	8.1037	0.9004		
Total	11	14.9929			
Day 2					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	11.5972	5.7986	12.53	0.003
Residual	9	4.1640	0.4627		
Total	11	15.7612			
Day 4					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	14.111	7.056	3.97	0.058
Residual	9	15.984	1.776		
Total	11	30.095			
Day 6					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	34.692	17.346	8.68	0.008
Residual	9	17.984	1.998		
Total	11 52.677				
		b* value			
Day 0					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	8.479	4.239	0.50	0.625
Residual	9	77.005	8.556		
Total	11	85.484			
Day 2					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	39.444	19.722	6.17	0.021
Residual	9	28.761	3.196		
Total	11	68.205			

Day 4					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	49.566	24.783	3.59	0.071
Residual	9	62.166	6.907		
Total	11	111.732			
Day 6					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	209.908	104.954	21.46	<.001
Residual	9	44.026	4.892		
Total	11	253.934			
Firmness					
Day 0					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	2663.48	1331.74	39.01	<.001
Residual	9	307.22	34.14		
Total	11	2970.7			
Day 2					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	9433.5	4716.75	121.57	<.001
Residual	9	349.17	38.8		
Total	11	9782.67			
Day 4					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	10300.04	5150.02	110.19	<.001
Residual	9	420.65	46.74		
Total	11	10720.7			
Day 6					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	511.18	255.59	16.15	0.001
Residual	9	142.41	15.82		
Total	11	653.59			
Soluble solid concentration (**SSC**)					
Day 0					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	0.572	0.286	0.28	0.759
Residual	9	9.065	1.007		
Total	11	9.637			
Day 2					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	25.807	12.903	2.32	0.154
Residual	9	49.98	5.553		
Total	11	75.787			
Day 4					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	50.552	25.276	6.13	0.021

Residual	9	37.118	4.124		
Total	11	87.669			
Day 6					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	79.625	39.813	8.94	0.007
Residual	9	40.057	4.451		
Total	11	119.683			
Titratable acidity (TA)					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	1.01393	0.50697	17.1	<.001
Residual	9	0.26675	0.02964		
Total	11	1.28068			
Day 2					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	0.934059	0.467029	64.64	<.001
Residual	9	0.065024	0.007225		
Total	11	0.999083			
Day 4					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	0.8192	0.4096	23.23	<.001
Residual	9	0.15872	0.01764		
Total	11	0.97792			
Day 6					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	0.202545	0.101272	19.16	<.001
Residual	9	0.047563	0.005285		
Total	11	0.250108			
Carbon dioxide					
Day 0					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	53613	26806	20.36	<.001
Residual	9	11848	1316		
Total	11	65461			
Day 2					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	558944	279472	55.87	<.001
Residual	9	45019	5002		
Total	11	603962			
Day 4					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	1241575	620787	75.87	<.001
Residual	9	73642	8182		
Total	11	1315217			
Day 6					

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
VAR	2	190389	95194	18.69	<.001
Residual	9	45837	5093		
Total	11	236226			
Ethylene					
Day 0					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	2.307E-05	1.154E-05	19.49	<.001
Residual	9	5.326E-06	5.918E-07		
Total	11	2.840E-05			
Day 2					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	4.964E-05	2.482E-05	24.56	<.001
Residual	9	9.104E-06	1.012E-06		
Total	11	5.874E-05			
Day 4					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	1.300E-04	6.498E-05	7.35	0.013
Residual	9	7.959E-05	8.843E-06		
Total	11	2.095E-04			
Day 6					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	2	0.000025	0.000012	0.85	0.457
Residual	9	0.000135	0.000015		
Total	11	0.00016145			

Note: d.f, degree of freedom; s.s, sum of squares; m.s, mean sum of square; v.r; variance ratio; F.pr, F probability or P value.

c) Multivariate analysis

a. P values obtained from the correlation analysis of samples and postharvest quality parameters

	CO_2	Ethylene	Firmness	Hue	a* value	L value	b* value	TA	SSC	WL
CO_2	<.0001	0.0003	0.0005	0.4005	0.6097	0.5598	0.0991	0.029	0.0089	0.0078
Ethylene	0.0003	<.0001	0.0002	0.1046	0.3055	0.1895	0.0048	0.0331	0.0096	0.0023
Firmness	0.0005	0.0002	<.0001	0.0127	0.0418	0.0924	0.0008	0.0015	0.0001	<.0001
Hue	0.4005	0.1046	0.0127	<.0001	<.0001	<.0001	0.0048	0.2622	0.0244	0.0117
a* value	0.6097	0.3055	0.0418	<.0001	<.0001	0.0003	0.0468	0.4184	0.102	0.0824
L value	0.5598	0.1895	0.0924	<.0001	0.0003	<.0001	0.0362	0.7467	0.0731	0.0416
b* value	0.0991	0.0048	0.0008	0.0048	0.0468	0.0362	<.0001	0.0191	0.0028	<.0001
TA	0.029	0.0331	0.0015	0.2622	0.4184	0.7467	0.0191	<.0001	0.0192	0.0159
SSC	0.0089	0.0096	0.0001	0.0244	0.102	0.0731	0.0028	0.0192	<.0001	<.0001
WL	0.0078	0.0023	<.0001	0.0117	0.0824	0.0416	<.0001	0.0159	<.0001	<.0001

b. Eigen values and vectors generated from multivariate analysis

Eigenvalues

Number	Eigenvalue	Percent	20 40 60 80	Cum Percent
1	6.4999	64.999		64.999
2	1.9417	19.417		84.417
3	0.5497	5.497		89.913
4	0.5000	5.000		94.913
5	0.2885	2.885		97.798
6	0.1291	1.291		99.088
7	0.0795	0.795		99.883
8	0.0081	0.081		99.964
9	0.0031	0.031		99.995
10	0.0005	0.005		100.000

Eigenvectors

	Prin1	Prin2	Prin3	Prin4	Prin5	Prin6	Prin7	Prin8	Prin9	Prin10
CO2	0.28979	-0.35357	0.45910	-0.40479	0.09533	0.08801	0.33786	-0.11676	0.51656	-0.07025
Ethylene	0.32530	-0.22305	0.45856	0.03795	-0.51597	-0.15075	-0.48921	0.21473	-0.20654	0.12516
Firmness	-0.36760	0.17811	0.12758	0.25356	-0.00800	-0.32462	-0.18716	0.37015	0.68576	0.06958
Hue	-0.30658	-0.43726	0.07167	0.06636	0.16827	-0.05832	0.21724	0.08520	-0.17874	0.76626
a* value	0.25759	0.49258	-0.16301	-0.33349	-0.13445	0.40180	-0.10817	0.11497	0.20462	0.55400
L value	0.25288	0.49799	0.27322	0.09525	0.00937	-0.58875	0.38837	-0.22408	-0.15019	0.18445
b* value	0.31919	-0.13674	-0.13324	0.73190	-0.21406	0.25319	0.12738	-0.32677	0.27613	0.12966
TA	-0.28034	0.29974	0.66181	0.23203	0.21899	0.50471	-0.04891	-0.07721	-0.16236	-0.04822
SSC	0.35415	-0.05601	-0.01636	0.07324	0.72261	-0.14423	-0.52970	-0.16203	0.05167	0.11550

Note: Cum, cumulative; Prin, principal component; CO2, carbondioxide (respiration); TA, titratable acidity; SSC, soluble solid concentration

9.5 Appendix V: RabA cDNA sequences

These were generated using the Expansy tool (https://web.expasy.org/translate/). The output font type was retained to enable a good presentation of the nucleotide and amino acid sequences. The start and stop codons are highlighted.

- **RabA1-1**

```
atggctgggtacaggcctgatgatgagtatgattacttgttcaagctggtgttgattggt
 M  A  G  Y  R  P  D  D  E  Y  D  Y  L  F  K  L  V  L  I  G
gattcaggtgtgggtaagtccaacttgctttccaggtttaccaaaaacgagtttaatctc
 D  S  G  V  G  K  S  N  L  L  S  R  F  T  K  N  E  F  N  L
gagtccaagtctactattggtgttgagtttgctaccaaaagtttgaagattgatggcaag
 E  S  K  S  T  I  G  V  E  F  A  T  K  S  L  K  I  D  G  K
gtcgtcaaggctcagatttgggacactgctggccaagaaaggtaccgtgccattacaagt
 V  V  K  A  Q  I  W  D  T  A  G  Q  E  R  Y  R  A  I  T  S
gcttattatagaggtgctgttggtgctttgcttgtgtatgatgtcactcaagtttccacc
 A  Y  Y  R  G  A  V  G  A  L  L  V  Y  D  V  T  Q  V  S  T
tttaggaatgttgggaggtggttgaaggagttgagagagcacaccgaccccaacattgtt
 F  R  N  V  G  R  W  L  K  E  L  R  E  H  T  D  P  N  I  V
gtcatgctgataggcaacaagtcagatcttcggcacctcatagctgtctccactgaggat
 V  M  L  I  G  N  K  S  D  L  R  H  L  I  A  V  S  T  E  D
ggtaaagcatttgccgaggaggagtctgtatatttcatggaaacatcagcattagatgca
 G  K  A  F  A  E  E  E  S  V  Y  F  M  E  T  S  A  L  D  A
acaaacgtggaaaatgcttttacagaagttattaatcagatatacaaggttgttagcaag
 T  N  V  E  N  A  F  T  E  V  I  N  Q  I  Y  K  V  V  S  K
aggacagtcgaggcaggcgtcgatggatcttcttccacacttccatcaaaaggagagaca
 R  T  V  E  A  G  V  D  G  S  S  S  T  L  P  S  K  G  E  T
ataaatgtcaatgcggatgctcctttgaggaatcttccattttaaagagaattggatgct
 I  N  V  N  A  D  A  P  L  R  N  L  P  F  -  R  E  L  D  A
gctcaaactag
 A  Q  T  -
```

- **RabA1-2**

```
atgggagcatacagagctgatgatgactacgattacttgttcaaggtggtgttgattggc
 M  G  A  Y  R  A  D  D  D  Y  D  Y  L  F  K  V  V  L  I  G
gactctggagttggtaaatccaatctcttgtctcgattcactaaaaatgaattcagtcta
 D  S  G  V  G  K  S  N  L  L  S  R  F  T  K  N  E  F  S  L
gaatccaaatccaccatcggcgtcgaattcgccactcgcagtattcgcgtcgatgacaag
 E  S  K  S  T  I  G  V  E  F  A  T  R  S  I  R  V  D  D  K
gtcgtcaaggcccagatttgggatactgcgggccaagaacggtaccgtgcaatcactagt
 V  V  K  A  Q  I  W  D  T  A  G  Q  E  R  Y  R  A  I  T  S
gcatattacagaggagcagtaggcgcactgcttgtttacgatgttacacgacatgtcaca
 A  Y  Y  R  G  A  V  G  A  L  L  V  Y  D  V  T  R  H  V  T
tttgagaatgtggagagatggttaaaggagcttagagatcacaccgattccaacattgtg
 F  E  N  V  E  R  W  L  K  E  L  R  D  H  T  D  S  N  I  V
```

atcatgcttgtgggaaacaaggcagatttacgtcatttgcgtgcagtctccaccgaggat
 I M L V G N K A D L R H L R A V S T E D
gctcagtcgtttgctgagagagaaaatacctttttcatggaaacctctgccctagagtca
 A Q S F A E R E N T F F M E T S A L E S
ctaaacgttgaaaatgccttcactgaggtactcactcagatatatcatgttgtcagccgg
 L N V E N A F T E V L T Q I Y H V V S R
aaggctcttgatgctggcgatgacccagcaaccttgcccaagggacaaactatcaatgtt
 K A L D A G D D P A T L P K G Q T I N V
ggcactaaagatgatgtatctgcagtgaaaaaggctggctgctgcaccgcataa
 G T K D D V S A V K K A G C C T A -

- **RabA1-3**

atggctggcttcagagctgaagatgactatgattacctttcaaggtcgggttgattggt
 M A G F R A E D D Y D Y L F K V G L I G
gactctggtgtgggcaagtctaatcttgctctcaggttcactaggaacgagtttagtctc
 D S G V G K S N L A L R F T R N E F S L
gagtctaagtccaccataggtgttgagttcgctactcgtagtttgaatgttgatggcaag
 E S K S T I G V E F A T R S L N V D G K
gtcatcaaggctcagatttgggatactgctggccaagaaaggtaccgtgccataaccagt
 V I K A Q I W D T A G Q E R Y R A I T S
gcttactaccgtggagctgttggtgcccttctggtgtatgatgtaactcggcactccaca
 A Y Y R G A V G A L L V Y D V T R H S T
tttgagaatgtagagaggtggttaagggagctgagagatcacacagatcccaacatcgtg
 F E N V E R W L R E L R D H T D P N I V
gtcatgctcattggtaacaaatcagatctccgccatctcgttgcggtctcaaccgaggat
 V M L I G N K S D L R H L V A V S T E D
ggaaatcttttggctgaaagggaatctctctctttcatggaaacatctgcccttgaggct
 G N L L A E R E S L S F M E T S A L E A
actaatgtggacaatgcatttgccgaagttctctccaagtctacggcattgttagcaaga
 T N V D N A F A E V L S K S T A L L A R
aggctatggaagctggtgatcattgatcaggatgctgtaccctcaaaaagtgagaaaatt
 R L W K L V I I D Q D A V P S K S E K I
gatgtgagtaaagacgtttctgctgtgaagaaagtcggctgctgctcaggctag
 D V S K D V S A V K K V G C C S G -

- **RabA1-4**

atggcagcttacagagcggacgacgactacgattatctattcaaactggtgttgattggc
 M A A Y R A D D D Y D Y L F K L V L I G
gattcgggtgtgggcaaatccaatttgctatccagattcacccggaacgagttcagtcta
 D S G V G K S N L L S R F T R N E F S L
gagtccaaatcaactatcggagtcgaattcgccacccgaagcattcgggtcgacgacaag
 E S K S T I G V E F A T R S I R V D D K
attgttaaggctcagatttgggacaccgctggccaggaaagataccgagctatcacaagt
 I V K A Q I W D T A G Q E R Y R A I T S
gcatactacaggggagctgttggtgcattgcttgtctatgatgttacaagacatgtcaca

A Y Y R G A V G A L L V Y D V T R H V T
tttgagaatgttgaaagatggttaaaggaattgcggggtcacacagatgccaatattgtc
F E N V E R W L K E L R G H T D A N I V
attatgcttgtgggtaacaaggcagacttgcgtcatttgcgtgctgtttctgtggaggat
I M L V G N K A D L R H L R A V S V E D
gctacagcctttgctgagagggaaaacacattctttattgagacatcagctcttgagtct
A T A F A E R E N T F F I E T S A L E S
atgaatgttgacaaggcattcactgaagtgctaacccaaattcataatgtagtaagccgg
M N V D K A F T E V L T Q I H N V V S R
aaagcacttgatataggggatgaccctgcagccttgcctaaggggcaaactattaatatt
K A L D I G D D P A A L P K G Q T I N I
ggaggcaaggatgatgtctcggctgtaaagaaagaaggttgctgcagtgcttga
G G K D D V S A V K K E G C C S A -

- **RabA2-1**

atggcgagtagagttgatcacgagtacgattatttgttcaagatcgtgctgatcggcgac
M A S R V D H E Y D Y L F K I V L I G D
tctggtgtggggaaatcaaacattctttctaggtttacgaggaatgagttttgcttggaa
S G V G K S N I L S R F T R N E F C L E
tctaaatccactatcggagttgagttcgccaccagaactcttcaggtagaaggaaagaca
S K S T I G V E F A T R T L Q V E G K T
gttaaggcacagatctgggacacagcagggcaggagagataccgagctatcactagtgct
V K A Q I W D T A G Q E R Y R A I T S A
tattatagaggagctgttggtgccctccttgtgtatgacataaccaagaggcaaactttt
Y Y R G A V G A L L V Y D I T K R Q T F
gacaatgtccagaggtggctccgtgaattaagagatcatgctgattctaacattgtgata
D N V Q R W L R E L R D H A D S N I V I
atgatggctgggaataagtctgacttaaatcatcttagagctgttcgagaggaggatggt
M M A G N K S D L N H L R A V R E E D G
cattccttggctgagaaggaaggtctctcattccttgagacatctgcactggaagcgacc
H S L A E K E G L S F L E T S A L E A T
aatgttgagaaggcgtttcagacaattttgacggagatttaccatatcgtaagcaaaaaa
N V E K A F Q T I L T E I Y H I V S K K
gcattggcagcccagcaggcagctgcttctaccgctattcccggtcaaggaaccactatt
A L A A Q Q A A A S T A I P G Q G T T I
aatgttgctgattcagggactgggaaaagaggttgctgttctacttaa
N V A D S G T G K R G C C S T -

- **Rab A2-2**

atggcgagtagagttgatcaagagtacgattatttgttcaagaccgtgctgatcggcgac
M A S R V D Q E Y D Y L F K T V L I G D
tctggtgtggggaaatcaaacattctttctaggtttacgaggaatgagttttgcttggaa
S G V G K S N I L S R F T R N E F C L E
tctaaatccactatcggagttgagttcgccaccagaactcttcaggtagaaggaaagaca
S K S T I G V E F A T R T L Q V E G K T
gttaaggcacagatctgggacactgcagggcaggagcgataccgagctatcactagtgct

```
 V  K  A  Q  I  W  D  T  A  G  Q  E  R  Y  R  A  I  T  S  A
tattatagaggagctgtcggtgccctccttgtctatgacataaccaagaggcaaactttt
 Y  Y  R  G  A  V  G  A  L  L  V  Y  D  I  T  K  R  Q  T  F
gacaatgtccagaggtggctccgtgagttgagagatcatgctgattctaacattgtgata
 D  N  V  Q  R  W  L  R  E  L  R  D  H  A  D  S  N  I  V  I
atgatggctgggaataagactgatctgaagcatcttcgagcagttgccacagaggatgcc
 M  M  A  G  N  K  T  D  L  K  H  L  R  A  V  A  T  E  D  A
caaggttatgcagagaaagaaggtctctcattccttgagacatctgcactggaagcgacc
 Q  G  Y  A  E  K  E  G  L  S  F  L  E  T  S  A  L  E  A  T
aatgttgagaaggcgtttcagacaattttgacggagatttaccatatcgtaagcaaaaaa
 N  V  E  K  A  F  Q  T  I  L  T  E  I  Y  H  I  V  S  K  K
gcattggcagcccagcaggcagctgcttctaccgctattcctggtcaaggaaccactatt
 A  L  A  A  Q  Q  A  A  A  S  T  A  I  P  G  Q  G  T  T  I
aatgttgctgattcagggactgcgaaaaaaggttgctgttctacttaagatgatgtggat
 N  V  A  D  S  G  T  A  K  K  G  C  C  S  T  -  D  D  V  D
acttga
 T  -
```

- **RabA3**

```
atgaaccaagagatgaatggtgatcatgtcacggagactcatcatcatcaacaacaaaat
 M  N  Q  E  M  N  G  D  H  V  T  E  T  H  H  H  Q  Q  Q  N
attgttcatgagaaaatagactatgtgtttaaggtagtggtaatcggagactcggcggtg
 I  V  H  E  K  I  D  Y  V  F  K  V  V  V  I  G  D  S  A  V
ggaaagacgcagatactatcaaggtttaccaagaatgagtttagcttcgactctaaatca
 G  K  T  Q  I  L  S  R  F  T  K  N  E  F  S  F  D  S  K  S
acaattggtgttgagttccaaactaggactgtcaccattaaagggaagctcatcaaagct
 T  I  G  V  E  F  Q  T  R  T  V  T  I  K  G  K  L  I  K  A
cagatctgggacaccgctggccaagaaagataccgggctgtgacaagtgcatactacaga
 Q  I  W  D  T  A  G  Q  E  R  Y  R  A  V  T  S  A  Y  Y  R
ggtgcactaggagctatgctggtgtatgatataacaaaaaggcagtcatttgataatgtg
 G  A  L  G  A  M  L  V  Y  D  I  T  K  R  Q  S  F  D  N  V
gctcggtgggtggatgagctccgagcccatgctgataattccattagaattatactcatc
 A  R  W  V  D  E  L  R  A  H  A  D  N  S  I  R  I  I  L  I
ggaaacaaatcagatcttgaggatcttcgtgcagttccaacagaagatgcagttgagttt
 G  N  K  S  D  L  E  D  L  R  A  V  P  T  E  D  A  V  E  F
gctgaggatcaagggttgtttttctcggagacatctgcacttaatggtgaaaatgttgaa
 A  E  D  Q  G  L  F  F  S  E  T  S  A  L  N  G  E  N  V  E
actgcatttttaggctgctggaagaaatttatggcgtggtttcgaagaaggaactggac
 T  A  F  F  R  L  L  E  E  I  Y  G  V  V  S  K  K  E  L  D
tacagcaatgagaaaactgacgcggccatgcttaaaggttccaagattgatgttatatct
 Y  S  N  E  K  T  D  A  A  M  L  K  G  S  K  I  D  V  I  S
gggcctgaattagaaattagtgagatgaagaaattatcttcttgttcttgtggattttaa
 G  P  E  L  E  I  S  E  M  K  K  L  S  S  C  S  C  G  F  -
```

- **RabA4-1**

```
atggcttctggaggctatggagatcccaaccaaaagatagactatgtcttcaaagttgta
 M  A  S  G  G  Y  G  D  P  N  Q  K  I  D  Y  V  F  K  V  V
ttaatcggtgactcttccgttggcaagtctcagattctttcaaggtttgcaagaaatgaa
 L  I  G  D  S  S  V  G  K  S  Q  I  L  S  R  F  A  R  N  E
ttcactttggattccaaggccaccattggagttgagtttcaaactcgaactttagttatt
 F  T  L  D  S  K  A  T  I  G  V  E  F  Q  T  R  T  L  V  I
gaacataagagcgttaaggctcagatctgggacactgctggccaagaacgatacagagct
 E  H  K  S  V  K  A  Q  I  W  D  T  A  G  Q  E  R  Y  R  A
gttacaagtgcatattacagaggtgctgttggggcaatgctggtttatgacataacaaga
 V  T  S  A  Y  Y  R  G  A  V  G  A  M  L  V  Y  D  I  T  R
cgccagagctttgaccacataccccgttggctggaagagctgcgaagccatgctgataag
 R  Q  S  F  D  H  I  P  R  W  L  E  E  L  R  S  H  A  D  K
aatattgtcatcattctgataggaaacaagagcgatcttgaggaccagcgagctgttccc
 N  I  V  I  I  L  I  G  N  K  S  D  L  E  D  Q  R  A  V  P
acagaggatgccaaagaatttgcagagaaggaaggactatttttcttggagacgtcagca
 T  E  D  A  K  E  F  A  E  K  E  G  L  F  F  L  E  T  S  A
ttgcaagcaactaatgttgagactgccttcatgacagtgttgactgagatttacaatatt
 L  Q  A  T  N  V  E  T  A  F  M  T  V  L  T  E  I  Y  N  I
gtgaacaagaagaatctaacagcagaagaaggccaaggcaacagcaaccccacaaacctt
 V  N  K  K  N  L  T  A  E  E  G  Q  G  N  S  N  P  T  N  L
gctggaaagaagatccttattccaggacctgcacaggaaatcccagccaagagcaacatg
 A  G  K  K  I  L  I  P  G  P  A  Q  E  I  P  A  K  S  N  M
tgctgtagaacatga
C C R T -
```

- **RabA4-2**

```
atggcttataactaccaaggtaacttcaataataaaatcgattatgtgttcaaaatagtg
 M  A  Y  N  Y  Q  G  N  F  N  N  K  I  D  Y  V  F  K  I  V
ttgattggtgactccgccgtgggaaaatctaatctcctcgcacgttttgctagaaatgaa
 L  I  G  D  S  A  V  G  K  S  N  L  L  A  R  F  A  R  N  E
tttagcctcgattctaaagcaacaattggagtcgaatttcagaccaaaacgcttattatt
 F  S  L  D  S  K  A  T  I  G  V  E  F  Q  T  K  T  L  I  I
gataacaaaacggttaaagcacagcagatttgggacactgctggtcaagaacggtatcgt
 D  N  K  T  V  K  A  Q  Q  I  W  D  T  A  G  Q  E  R  Y  R
gctgttacaagtgcttactacagaggtgcagtgggggcaatgctggtctatgacataagt
 A  V  T  S  A  Y  Y  R  G  A  V  G  A  M  L  V  Y  D  I  S
aagcgtcagtcatttgatcatatagccaaatggttggaggaactgcggggatatgcagat
 K  R  Q  S  F  D  H  I  A  K  W  L  E  E  L  R  G  Y  A  D
aaaaatattgtgatcatgcttgtaggcaacaagtctgatctggggacccttcgagctgta
 K  N  I  V  I  M  L  V  G  N  K  S  D  L  G  T  L  R  A  V
gctactgaagatgcaaaagagtttgctgaaagggaaagcctgttctttatggagacatca
 A  T  E  D  A  K  E  F  A  E  R  E  S  L  F  F  M  E  T  S
gctcttgagtcaactaatgttgaatcagcttttctcactgtcttgactgagatttatcga
 A  L  E  S  T  N  V  E  S  A  F  L  T  V  L  T  E  I  Y  R
```

attgtttccaaaaagtctcttgttgccaatgatgaacctgaatctggtggcagttcatcg
 I V S K K S L V A N D E P E S G G S S S
cttctcaccggtaccaatattgttgtcactggtcaggagccagagtctgggggaaggagt
 L L T G T N I V V T G Q E P E S G G R S
ttcaactgttgcacatcatcatag
 F N C C T S S -

- **RabA5-1**

atggcttttattccgaagaggagaaaactgaggattatcttttaagattgttctaatt
 M A F Y S E E E K T E D Y L F K I V L I
ggtgattctgctgttgggaaatcaaatttgcttgcaagatttgctagagatgagttctac
 G D S A V G K S N L L A R F A R D E F Y
ccaaattcaaaatcgaccataggagtagagttccagacacaaaagatgcatattaatgga
 P N S K S T I G V E F Q T Q K M H I N G
aaggaaatcaaggcacagatctgggatacagctggtcaggagcgattcagggctgttacg
 K E I K A Q I W D T A G Q E R F R A V T
tctgcatattatcgaggtgcagttggagctcttctggtgtatgacattagtagacgaccg
 S A Y Y R G A V G A L L V Y D I S R R P
acatttgatagcattggcagatggcttaatgaacttcacacacactcagacatgaatgta
 T F D S I G R W L N E L H T H S D M N V
gtaaccatactagtaggcaacaagacagatctcagggatgcaagggaggtaaccaccgcc
 V T I L V G N K T D L R D A R E V T T A
gaaggcaaggccttggcagaggcacagggtttgttctttatcgagacatctgctctcgat
 E G K A L A E A Q G L F F I E T S A L D
tctactaacgttgaaacagctttcgagattgtgattcgagagatctataacaatgtaagc
 S T N V E T A F E I V I R E I Y N N V S
aggaaagtgctggattcagattcctacaaagctgatttgagcatcaacagggtaagcctt
 R K V L D S D S Y K A D L S I N R V S L
gtgaataataatggtttaaagcaaactcagagcaaatactcatgttgtgtttag
 V N N N G L K Q T Q S K Y S C C V -

- **RabA5-2**

atgtcctctgacgacgagggcggcgaggagtatctcttcaagatcgtcataattggcgat
 M S S D D E G G E E Y L F K I V I I G D
tccgcggtcggcaaatccaatctcctctctcgctacgctcgcaacgagttcaatcctcac
 S A V G K S N L L S R Y A R N E F N P H
tcgaaggccaccattggcgtcgagtttcagacgcagaacatggagatcgacggcaaagag
 S K A T I G V E F Q T Q N M E I D G K E
gtcaaagctcagatttgggacaccgccggtcaagaacggttccgtgctgtcacttctgct
 V K A Q I W D T A G Q E R F R A V T S A
tactaccgcggcgctgtgggtgctctcgttgtgtatgatatcagtaggagaactactttt
 Y Y R G A V G A L V V Y D I S R R T T F
gataatgtcggtcgatggcttgatgagctgaagactcactctgataccactgtggcaagg
 D N V G R W L D E L K T H S D T T V A R
atgcttgtggggaacaagtgtgatttggaggatataaggaacgtgagcatagaggaaggg
 M L V G N K C D L E D I R N V S I E E G
aaaagccttgctgaatcagaaggatttttcttcatggaaacttctgctttagattcaaca

K S L A E S E G F F F M E T S A L D S T
aacgttaaaacggcatttgagatagttatcaaagagatttacagcaacgtgagcaggaag
N V K T A F E I V I K E I Y S N V S R K
gtcttgaactcagatacttacaaagccgaattatccatcaaccgagtaacgctagcaaaa
V L N S D T Y K A E L S I N R V T L A K
aatgacccggatggatcgaagcaaacaggaaagtatctttcttgctgttcgagttga
N D P D G S K Q T G K Y L S C C S S –

- **RabA6**

atggcggattcgaattcatttgatgaacaagagtgcgattatttgttcaaggcagtgttg
M A D S N S F D E Q E C D Y L F K A V L
attggggactctgcggttgggaaatcgaatttgttatcaagatatgcgaaagatgaattc
I G D S A V G K S N L L S R Y A K D E F
agattagattcaaagccaacaatcggtgttgaatttgcttacaagaatgttaagattgga
R L D S K P T I G V E F A Y K N V K I G
gaaaagctcatcaaggctcaaatttgggacactgctggacaagaaaggttcagagccatc
E K L I K A Q I W D T A G Q E R F R A I
acaagttcgtattatcgtggagccctaggagctttactagtttacgacatatcgagaaga
T S S Y Y R G A L G A L L V Y D I S R R
gtaacatttgaaaatatgaagagatggttaaatgaactcagagaatttggcagttcatgc
V T F E N M K R W L N E L R E F G S S C
atggcgattgttcttgttggcaacaaatctgatttaacacattccaggcaagttgatgaa
M A I V L V G N K S D L T H S R Q V D E
gaagaaggcaaaacccttgcacaggttgaaggtttgtatttcatggaaacttctgccatg
E E G K T L A Q V E G L Y F M E T S A M
gaaaatattaacgtggaggaggcttttctgcaacttattcgtaaaatttatgaaactact
E N I N V E E A F L Q L I R K I Y E T T
atccagaaaagtttagaggctaaattgaatgaacccatccccccaactattcagcctggg
I Q K S L E A K L N E P I P P T I Q P G
aaagaaattatcagtattgatgatgaagttactgcaactagacaatatagatgttgttat
K E I I S I D D E V T A T R Q Y R C C Y
aggtaa
R -

- **RabB**

atgtcctacgcttatctcttcaagtacatcatcatcggcgatactggagttggcaaatcg
M S Y A Y L F K Y I I I G D T G V G K S
tgtcttctcttgcagttcactgacaagcgctttcagcctgttcatgacctaaccattggt
C L L L Q F T D K R F Q P V H D L T I G
gttgaatttggggccagaatgatcaccattgataacaaacctattaagctccaaatttgg
V E F G A R M I T I D N K P I K L Q I W
gacacggcgggtcaagaatccttcagatctattaccaggtcttattatagaggggctgct

```
 D  T  A  G  Q  E  S  F  R  S  I  T  R  S  Y  Y  R  G  A  A
ggtgctttacttgtctatgatataaccaggagggaaacttttaatcacttggctagttgg
 G  A  L  L  V  Y  D  I  T  R  R  E  T  F  N  H  L  A  S  W
ctggaggatgcaaggcagcacgcaaatgccaacatgacaattatgcttattggtaacaag
 L  E  D  A  R  Q  H  A  N  A  N  M  T  I  M  L  I  G  N  K
tgtgatcttgctcatagaagggctgtcagtacagaggaaggtgagcaattcgccaaggag
 C  D  L  A  H  R  R  A  V  S  T  E  E  G  E  Q  F  A  K  E
catggattgatcttcatggaagcctctgcaaaaactgctcaaaacgtggaggaggctttt
 H  G  L  I  F  M  E  A  S  A  K  T  A  Q  N  V  E  E  A  F
ataaaaactgctgcaacaatttacaagaagattcaggatggagttttgatgtatcaaat
 I  K  T  A  A  T  I  Y  K  K  I  Q  D  G  V  F  D  V  S  N
gagtcatatggaataaaggttggatacggaggaatccctggaccatcaggagggagagat
 E  S  Y  G  I  K  V  G  Y  G  G  I  P  G  P  S  G  G  R  D
gggtcttcttctcaagctggaggatgttgcagttga
 G  S  S  S  Q  A  G  G  C  C  S  -
```

- **RabC**

```
atgggatcctcttcagctcaaagcaatagctatgatctttctttcaagatcttgttgatc
 M  G  S  S  S  A  Q  S  N  S  Y  D  L  S  F  K  I  L  L  I
ggtgattcaggtgttggtaaaagcagccttctagtcagcttcatatcaagctctgtagaa
 G  D  S  G  V  G  K  S  S  L  L  V  S  F  I  S  S  S  V  E
gatcttgctcccaccattggtgtcgattttaagatcaagcacctaaaattgggtggaaag
 D  L  A  P  T  I  G  V  D  F  K  I  K  H  L  K  L  G  G  K
agattgaagctgacgatttgggacacagctgggcaggaaaggttcagaacattaacaggc
 R  L  K  L  T  I  W  D  T  A  G  Q  E  R  F  R  T  L  T  G
tcttattatagaggtgcccaaggaatcattcttgtttatgatgtcacaaggagagaaact
 S  Y  Y  R  G  A  Q  G  I  I  L  V  Y  D  V  T  R  R  E  T
ttcacaaatttatcagatgtttgggctaaggaagtggacctttactctaccaatcaggat
 F  T  N  L  S  D  V  W  A  K  E  V  D  L  Y  S  T  N  Q  D
tgtgtcaagatgcttgttggaaataaagttgacagagattctgaaagggttgtaagcaga
 C  V  K  M  L  V  G  N  K  V  D  R  D  S  E  R  V  V  S  R
gaagaggggatgaatcttgcaaaagagcatgggtgtttgttccttgaatgtagtgctaaa
 E  E  G  M  N  L  A  K  E  H  G  C  L  F  L  E  C  S  A  K
actagagaaaatgtggagcaatgctttgaggagcttgcattgaagataatggaggttcct
 T  R  E  N  V  E  Q  C  F  E  E  L  A  L  K  I  M  E  V  P
agtcttttggaagaaggatccactgcagtcaagagaaacattttaaagcagaaaccagaa
 S  L  L  E  E  G  S  T  A  V  K  R  N  I  L  K  Q  K  P  E
tacccggcgcctcccagcggtggctgttgccaataa
 Y  P  A  P  P  S  G  G  C  C  Q  -
```

- **RabD-1**

```
atgaataacgaatatgactatttgtttaagcttttgctcattggagattctggtgtcggc
 M  N  N  E  Y  D  Y  L  F  K  L  L  L  I  G  D  S  G  V  G
aaatcttgtctgcttttgaggtttgctgatgattcatatctggagagctacattagcacc
 K  S  C  L  L  L  R  F  A  D  D  S  Y  L  E  S  Y  I  S  T
attggagttgacttcaaaatccgcactgtggaacaggatggaaaaaccattaagctccaa
 I  G  V  D  F  K  I  R  T  V  E  Q  D  G  K  T  I  K  L  Q
atttgggacactgctggccaagagcgttttaggacaatcactagtagctactatcgtggg
```

```
 I  W  D  T  A  G  Q  E  R  F  R  T  I  T  S  S  Y  Y  R  G
gctcatggcatcattgtcgtttatgatgtcactgaccaagagagcttcaacaatgttaaa
 A  H  G  I  I  V  V  Y  D  V  T  D  Q  E  S  F  N  N  V  K
caatggttgaatgagattgaccgctatgcaagtgaaaacgtgaacaagcttttagttggt
 Q  W  L  N  E  I  D  R  Y  A  S  E  N  V  N  K  L  L  V  G
aacaagtgcgatctcacagcaaacaaagttgtgtcctatgagacagctaaggcatttgct
 N  K  C  D  L  T  A  N  K  V  V  S  Y  E  T  A  K  A  F  A
gatgaaattgggatcccattcatggaaacaagtgcaaaaaatgccaccaatgttgaacag
 D  E  I  G  I  P  F  M  E  T  S  A  K  N  A  T  N  V  E  Q
gctttcatggccatggctgctgcaatcaagaacaggatggcaagccaacctatgaacaat
 A  F  M  A  M  A  A  A  I  K  N  R  M  A  S  Q  P  M  N  N
gctaggccaccaacggtgcagattcgaggacagcctgtcaaccaaaaatccggttgctgc
 A  R  P  P  T  V  Q  I  R  G  Q  P  V  N  Q  K  S  G  C  C
tcaacttga
 S  T  -
```

- **RabD-2**

```
atgaaccccgagtatgattatttgtttaagcttctgcttattggagattctggtgttgga
 M  N  P  E  Y  D  Y  L  F  K  L  L  L  I  G  D  S  G  V  G
aaatcatgtcttctattgagatttgctgatgattcatatattgagagttacatcagcaca
 K  S  C  L  L  L  R  F  A  D  D  S  Y  I  E  S  Y  I  S  T
ataggagtcgattttaaaatacgcactgtggagcaagatgggaagactattaaacttcaa
 I  G  V  D  F  K  I  R  T  V  E  Q  D  G  K  T  I  K  L  Q
atatgggatactgctgggcaggaacgatttaggacaatcaccagtagctactaccgtggg
 I  W  D  T  A  G  Q  E  R  F  R  T  I  T  S  S  Y  Y  R  G
gcacatggtatcataattgtttatgatgtaacagaccaagagagctttaataatgtcaag
 A  H  G  I  I  I  V  Y  D  V  T  D  Q  E  S  F  N  N  V  K
cagtggttgagtgaaattgatcgctatgctagtgataatgtcaacaaacttctggttgga
 Q  W  L  S  E  I  D  R  Y  A  S  D  N  V  N  K  L  L  V  G
aacaagtgtgatctcaccgctaataaagttgtgtcatatgaaacagctaaggcatttgca
 N  K  C  D  L  T  A  N  K  V  V  S  Y  E  T  A  K  A  F  A
gatgaaattggaatacctttcatggaaaccagtgctaaagattctacaaatgtggagcaa
 D  E  I  G  I  P  F  M  E  T  S  A  K  D  S  T  N  V  E  Q
gctttcatggctatggctgctgccatcaaggatagaatggcaagtcaaccagccatgaac
 A  F  M  A  M  A  A  A  I  K  D  R  M  A  S  Q  P  A  M  N
aatgcaaggcctccaacagtacagatccgaggtcagccagttgcacagaacagtggctgc
 N  A  R  P  P  T  V  Q  I  R  G  Q  P  V  A  Q  N  S  G  C
tgctcctcttag
 C  S  S  -
```

- **RabE-1**

```
atggcagctgcgccggctagggctcgggccgattacgattacctgatcaagctccttctc
 M  A  A  A  P  A  R  A  R  A  D  Y  D  Y  L  I  K  L  L  L
atcggggacagtggggtgggcaaaagttgcctgctattgcgtttctcagatgattctttt
 I  G  D  S  G  V  G  K  S  C  L  L  L  R  F  S  D  D  S  F
acaacaagtttcattaccacaattgggattgattttaagattaggaccgttgagctagat
 T  T  S  F  I  T  T  I  G  I  D  F  K  I  R  T  V  E  L  D
```

gggaagcgaattaaattacaaatatgggatactgctggacaagaacgttttcgaacaatt
 G K R I K L Q I W D T A G Q E R F R T I
acaacagcttattacaggggagccatgggcatattgctggtctatgacgtgacagatgaa
 T T A Y Y R G A M G I L L V Y D V T D E
tcatcttttaacaacatcaggaactggatgaggaacatagagcagcatgctgctgataat
 S S F N N I R N W M R N I E Q H A A D N
gtcaacaaaatattggtgggtaacaaagccgacatggatgagagcaaaagggctgtcccg
 V N K I L V G N K A D M D E S K R A V P
actgcaaagggccaagcattagctgatgaatatggtatcaaatttttgagacgagtgca
 T A K G Q A L A D E Y G I K F F E T S A
aaaacaaatttcaatgtggagcaagttttcttttcaattgcaagagatataaagcaaaga
 K T N F N V E Q V F F S I A R D I K Q R
ctcgcagaaagtgactcaaaagcagaacctcaaactatcaagatcagtaaaccagacccc
 L A E S D S K A E P Q T I K I S K P D P
gccaagggctcagcagctgctccagagaaatcagcatgctgtggttcttaa
 A K G S A A A P E K S A C C G S -

- **RabE-2**

atggccaaagcctacgaccacctcttcaagttgctgctgatcggggactcgggggtgggc
 M A K A Y D H L F K L L L I G D S G V G
aagacttgtctgatcattcgctttgcagaggacaacttcaacaacacttacatctccacc
 K T C L I I R F A E D N F N N T Y I S T
atcggaattgatttcaagatccgcactgtggatatagaggggaagaagatcaaactacaa
 I G I D F K I R T V D I E G K K I K L Q
gtctgggacacggctggccaagagcggttcaagacaataactactgcctactaccgtgga
 V W D T A G Q E R F K T I T T A Y Y R G
gccatgggcattatcctagtatacgacatcacggatgagaaatctttcgagaatattcag
 A M G I I L V Y D I T D E K S F E N I Q
aactggatgaaaagcatcaaggagaatgcctcggctggggtggagcgcctcttgctgggg
 N W M K S I K E N A S A G V E R L L L G
aacaaatgtgacatggaggccaagaggaaggtgcagaaggagcaggccgataagttggct
 N K C D M E A K R K V Q K E Q A D K L A
cgagagcatggaatccgatttttcgaaactagtgctaaatccagtatgaatgtggatgag
 R E H G I R F F E T S A K S S M N V D E
gcttttagttccctggcccgggacatcttgctcaagtcaggaggccggagatcaggaaac
 A F S S L A R D I L L K S G G R R S G N
ggcaacaagcctcccagtactgacctgaaaacttgtgacaagaagaacaccaacaagtgc
 G N K P P S T D L K T C D K K N T N K C
tccctgggctga
 S L G -

- **RabF-2**

atgggttgctcttcttcccttcctgataggaattctgggcggttgagtggacttaatggt
 M G C S S S L P D R N S G R L S G L N G
tcagagagcagtggagcacctgatgcgaaaaacctgcgtgttaagctggttttgttaggt
 S E S S G A P D A K N L R V K L V L L G
gattcaggtgttgggaaaagttgtatagttcttcgctttgttcgtggccagtttgaccca

D S G V G K S C I V L R F V R G Q F D P
acatccaaggttacagttggagcttcattcttgtcacaaacaatagctctgcaagattct
T S K V T V G A S F L S Q T I A L Q D S
acgacagttaaatttgaaatatgggacacagctggtcaagaaagatatgctgcactggca
T T V K F E I W D T A G Q E R Y A A L A
ccgctgtactacagaggtgctgcagttgcagttattgtgtatgatataacaagcccggag
P L Y Y R G A A V A V I V Y D I T S P E
tcattcaacaaagcacaatattgggttaaggagctacaaaaacatgggagtcctgatata
S F N K A Q Y W V K E L Q K H G S P D I
gtcatggctttagttggtaacaaagctgatcttcatgagaatcgagaagttccaacacag
V M A L V G N K A D L H E N R E V P T Q
gatggcattgagtatgcagagaagaacgggatgttctttattgagacatctgccaagact
D G I E Y A E K N G M F F I E T S A K T
gcagataatataaatcagctgtttgaggaaattgctaaacgacttccccgtccatcgacc
A D N I N Q L F E E I A K R L P R P S T
tcataa
S -

- **RabF-3**

atggccaccacggggaacaaacacatcaatgctaaattggtgttgcttggagatgttgga
M A T T G N K H I N A K L V L L G D V G
gctggaaagtctagtcttgtgttgcgcttcgttaaagggcaatttgttgaatttcaggaa
A G K S S L V L R F V K G Q F V E F Q E
tcaactataggtgctgccttttttcacaaacattggctgtaaatgatgccactgtaaag
S T I G A A F F S Q T L A V N D A T V K
tttgagatttgggatacagcaggtcaagagaggtaccatagtttggcaccaatgtactac
F E I W D T A G Q E R Y H S L A P M Y Y
agaggagctgcagccgccattattgtctatgatataacaaatcaggcctcatttgaacgt
R G A A A A I I V Y D I T N Q A S F E R
gctaagaaatgggtccaagagcttcaagcgcaaggcaattcaaacatggttatggcactt
A K K W V Q E L Q A Q G N S N M V M A L
gctgggaacaaagctgatttgctggatgctaggaaggtcaatacagaggatgctcaagtt
A G N K A D L L D A R K V N T E D A Q V
tatgctcaagagaatggacttttctttatggaaacctctgcaaaaacagcatctaatgtc
Y A Q E N G L F F M E T S A K T A S N V
aatgacattttctatgaaattgcaaagagactacctcgtgtgcagccggcacctaaccca
N D I F Y E I A K R L P R V Q P A P N P
ccaggaatggttctcatggacaaacctgctgagaggacagcaactgcatcatgttgctct
P G M V L M D K P A E R T A T A S C C S
tag
-

- **RabF2-2**

atgggaactgggaaaacaagtttggtactgagatttgtcaaaggccaatttttgatttc
M G T G K T S L V L R F V K G Q F F D F
caggaatcaacaattggagcagcattcttcacacaggttctgtcattaaatgaagccact
Q E S T I G A A F F T Q V L S L N E A T
ataaagtttgatatatgggatacagctgggcaggaacgataccatagtttggctccaatg

```
 I  K  F  D  I  W  D  T  A  G  Q  E  R  Y  H  S  L  A  P  M
tattaccgtggtgcagctgcagctgttgttgtttatgacgtcacgagcatggattcattt
 Y  Y  R  G  A  A  A  A  V  V  V  Y  D  V  T  S  M  D  S  F
gaaaaagctaagaaatgggttcaagaattgcaaagacagggaaatccaaatttgataatg
 E  K  A  K  K  W  V  Q  E  L  Q  R  Q  G  N  P  N  L  I  M
tttttggtggcgaataaggttgacttggaagataagagaaaagtgggatatgaggatggt
 F  L  V  A  N  K  V  D  L  E  D  K  R  K  V  G  Y  E  D  G
gagctatatgctaaagaaaatggcctatcttttcttgaaacatctgcaaagactgcacaa
 E  L  Y  A  K  E  N  G  L  S  F  L  E  T  S  A  K  T  A  Q
aatgtcaatgaactcttttatgaaatagcaaaaagattggtaaaagctaacccttttcgt
 N  V  N  E  L  F  Y  E  I  A  K  R  L  V  K  A  N  P  F  R
cgagccggaatgaagttacatactggacgccaagaaggtggaagtagagggttttgttgc
 R  A  G  M  K  L  H  T  G  R  Q  E  G  G  S  R  G  F  C  C
tcttcataa
 S  S  -
```

- **RabG**

```
atgaatcaatatgttcacaagaagtttagtcagcagtataaagctacaattggtgctgat
 M  N  Q  Y  V  H  K  K  F  S  Q  Q  Y  K  A  T  I  G  A  D
tttgttactaaagaactccaaattgatgaccgtcttgtcactctacagatatgggacaca
 F  V  T  K  E  L  Q  I  D  D  R  L  V  T  L  Q  I  W  D  T
gctgggcaagagagatttcagagtcttggggttgctttctatagaggggcagattgctgt
 A  G  Q  E  R  F  Q  S  L  G  V  A  F  Y  R  G  A  D  C  C
gtactagtttatgatgtcaatgtaatgaagtcatttgatacgcttgacaattggcatgag
 V  L  V  Y  D  V  N  V  M  K  S  F  D  T  L  D  N  W  H  E
gagtttcttaagcaggcaaacccagctgaccccaggatatttccatttatattacttggg
 E  F  L  K  Q  A  N  P  A  D  P  R  I  F  P  F  I  L  L  G
aacaagattgatattgatggtgggaacagccgagtggtgtctgagaagaaagcaaaggac
 N  K  I  D  I  D  G  G  N  S  R  V  V  S  E  K  K  A  K  D
tggtgcacctccaaagggaacattccttactttgagacctctgcaaaagaggatatcaat
 W  C  T  S  K  G  N  I  P  Y  F  E  T  S  A  K  E  D  I  N
gttgatgctgcattcttgagtatcgctagaactgctctagccaatgagcatgagcaggac
 V  D  A  A  F  L  S  I  A  R  T  A  L  A  N  E  H  E  Q  D
atatacttcgaagggattccagaggctgagcaaagaggtggttgtgcatgctga
 I  Y  F  E  G  I  P  E  A  E  Q  R  G  G  C  A  C  -
```

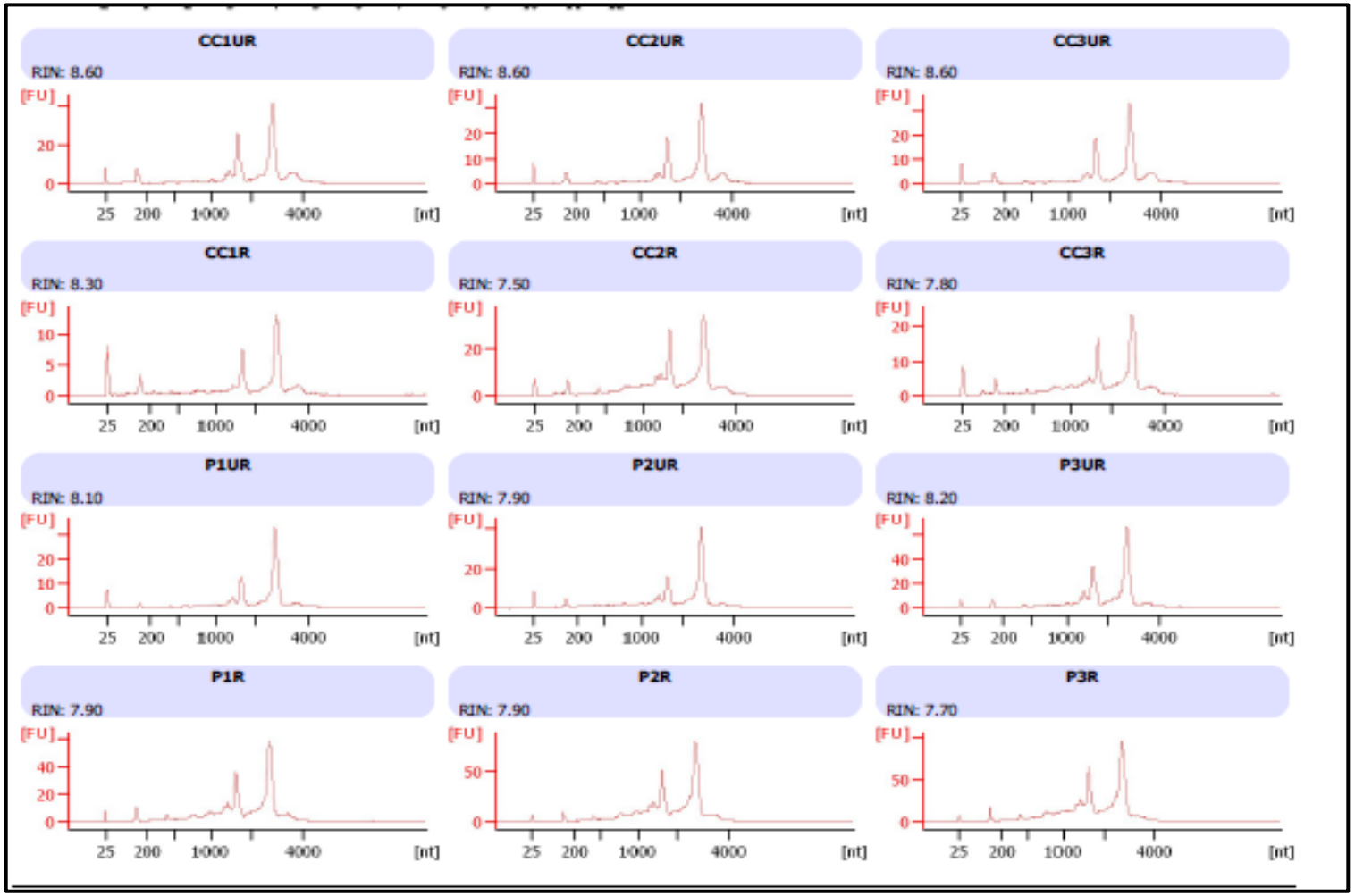
- **RabH**

```
atggcgccagtttccgctctcgctaagtacaagctcgttttcctgggagatcagtctgtt
 M  A  P  V  S  A  L  A  K  Y  K  L  V  F  L  G  D  Q  S  V
ggcaagaccagtattattactcgctttatgtacgataaattcgacaacacctatcaggct
 G  K  T  S  I  I  T  R  F  M  Y  D  K  F  D  N  T  Y  Q  A
actattggcatagatttttatcaaagacgatgtaccttgaagaccgaacagttcgactg
 T  I  G  I  D  F  L  S  K  T  M  Y  L  E  D  R  T  V  R  L
cagctttgggatactgcaggacaagaaaggttcaggagtctcattccgagttatatcagg
 Q  L  W  D  T  A  G  Q  E  R  F  R  S  L  I  P  S  Y  I  R
gattcttcagttgctgtgattgtatatgatgttgcaagcagacagtcattcctaaacact
 D  S  S  V  A  V  I  V  Y  D  V  A  S  R  Q  S  F  L  N  T
```

```
tccaagtggattgaagaggttcgcactgagcggggcagtgatgttatcattgtacttgtt
 S  K  W  I  E  E  V  R  T  E  R  G  S  D  V  I  I  V  L  V
gggaacaaaacagatctggtggacaaaaggcaagtttcaattgaggaaggagaagccaaa
 G  N  K  T  D  L  V  D  K  R  Q  V  S  I  E  E  G  E  A  K
gctcgtgaactaaatgttatgtttattgaaactagtgccaaggctggcttcaatataaag
 A  R  E  L  N  V  M  F  I  E  T  S  A  K  A  G  F  N  I  K
gcactcttccggaaaattgctgcagcgttaccagggatggaaacactttcttcaacaaag
 A  L  F  R  K  I  A  A  A  L  P  G  M  E  T  L  S  S  T  K
caagaagatatggttgatgttaatctgaagtcttccaacacaaatgcatctcagtcacaa
 Q  E  D  M  V  D  V  N  L  K  S  S  N  T  N  A  S  Q  S  Q
tctcagtctggaggatgttcttgttga
 S  Q  S  G  G  C  S  C  -
```

9.6 Appendix VI: Total RNA quality of mango samples used for RNA sequencing

a) RNA integrity by Agilent Bioanalyzer



b) Total RNA purity and concentration by NanoDrop spectrophotometer

Sample name	A260/280	A260/230	Concentration (ng/ µl)
CC1UR	2.06	2.31	357.80
CC2UR	2.03	2.37	195.44
CC3UR	2.03	2.32	289.40
CC1R	2.06	2.14	170.40
CC2R	2.09	2.21	460.54
CC3R	2.08	2.13	293.68
P1UR	2.16	1.95	435.24
P2UR	2.16	1.90	229.80
P3UR	2.12	1.98	289.36
P1R	2.12	2.28	410.80
P2R	2.11	2.43	454.44
P3R	2.17	2.35	519.36

9.7 Appendix VII: Some stage-specific genes identified in mango

Genes	NR ID	Annotation
Unripe stage		
Cluster-27569.105015	XP_006433440.1	Sugar transporter SWEET17-like
Cluster-27569.105116	XP_006427333.1	Probable disease resistance protein isoform X1
Cluster-27569.33800	XP_010051569.1	Putative receptor-like protein kinase
Cluster-27569.26025	AKE49463.1	NBS-LRR disease resistance protein NBS48
Cluster-27569.27503	XP_003635559.1	Ethylene-responsive transcription factor
Cluster-27569.27515	KDO73077.1	Putative disease resistance protein
Cluster-27569.2761	XP_002521714.1	Cytochrome P450 71D11-like
Cluster-27569.71872	KDP37976.1	Gibberellin 2-oxidase
Cluster-27569.27587	XP_002509479.1	Auxin-induced protein 22E
Cluster-27569.27721	KDO43940.1	Aux/IAA protein isoform 1
Cluster-27569.103038	XP_006442036.1	Cellulose synthase-like protein E6 isoform X1
Cluster-27569.18959	XP_009347232.1	40S ribosomal protein
Cluster-27569.18863	XP_002311070.1	Endoglucanase 9
Cluster-27569.18817	XP_012479374.1	Probable pectate lyase 4
Cluster-27569.28196	XP_011045108.1	Auxin transporter-like protein 2-like
Cluster-27569.27933	XP_002276380.1	Auxin-responsive protein IAA3
Cluster-27569.27812	KJB27547.1	Sugar transporter ERD6-like
Cluster-27569.28113	XP_007043809.1	Auxin-induced protein IAA4
Cluster-27569.28117	XP_006428995.1	Aux/IAA protein isoform 1
Cluster-27569.27995	XP_006441344.1	40S ribosomal protein
Cluster-27569.28200	XP_007018336.1	Auxin transporter-like protein 2-like
Cluster-27569.28693	XP_006477949.1	Polygalacturonase
Cluster-27569.28344	XP_006445915.1	Probable flavin-containing monooxygenase 1
Cluster-27569.29022	XP_007209641.1	Aux/IAA protein isoform 1
Cluster-27569.29274	XP_004310130.1	Auxin response factor 8-like isoform X3
Cluster-27569.30474	XP_007018336.1	S-adenosylmethionine
Cluster-27569.2902	XP_006467061.1	Isocitrate dehydrogenase
Cluster-27569.30204	XP_006471945.1	Auxin-responsive protein IAA8
Cluster-27569.28971	KDO56957.1	Vacuolar-processing enzyme
Cluster-27569.39123	KHG24041.1	Glucan endo-1,3-beta-glucosidase
Cluster-27569.39720	XP_006480932.1	Ras-related protein RABE1c
Cluster-27569.42152	XP_007018336.1	Probable disease resistance protein
Cluster-27569.35660	XP_007018336.1	Phosphofructokinase 7-like
Cluster-27569.41897	XP_010276540.1	Disease resistance protein
Cluster-27569.40833	XP_006419683.1	Sugar transporter
Cluster-27569.3757	XP_012486133.1	Malate dehydrogenase
Cluster-27569.44177	KDO50720.1	S-adenosylmethionine synthase 2
Ripe stage		
Cluster-27569.101874	XP_004952973.1	Isocitrate dehydrogenase

Cluster-15315.0	XP_011080662.1	Universal stress protein family
Cluster-27569.15735	AIX87533.1	Probable beta-1,3-galactosyltransferase 16
Cluster-27569.34074	XP_006430440.1	laccase-15-like
Cluster-27569.13890	XP_002323767.2	Polygalacturonase
Cluster-27569.17766	KDO44912.1	Glucan endo-1,3-beta-D-glucosidase
Cluster-27569.47150	XP_002535920.1	Endoglucanase 8
Cluster-27569.45224	XP_011007415.1	Beta-glucosidase 11-like
Cluster-27569.9566	XP_006421697.1	Polygalacturonase-like
Cluster-27569.97068	AFH77956.1	Putative beta-glucosidase 41-like
Cluster-9366.0	XP_006493140.1	Alpha-amylase
Cluster-27569.75387	XP_008245054.1	Sugar transporter ERD6-like 7
Cluster-27569.7992	KDO72075.1	Respiratory burst oxidase
Cluster-27569.66260	XP_006448012.1	Expansin-A4-like
Cluster-27569.76718	XP_006448536.1	Carotenoid cleavage dioxygenase 1A
Cluster-27569.79115	XP_006451738.1	Sucrose synthase family protein
Cluster-27569.81308	XP_006451738.1	Beta-glucosidase, putative isoform 2
Cluster-27569.8466	KDO77128.1	Vacuolar invertase
Cluster-27569.73326	XP_011044810.1	Beta-glucosidase 42-like
Cluster-27569.63411	KDO74905.1	Expansin
Cluster-27569.58020	XP_006376297.1	Stress-response A/B barrel domain-containing protein
Cluster-27569.75790	AFH77956.1	Beta-glucosidase
Cluster-27569.64892	KDO68594.1	Malate dehydrogenase
Cluster-27569.71930	XP_006443948.1	Malate dehydrogenase
Cluster-27569.73525	XP_006481951.1	Beta-glucosidase
Cluster-27569.68858	KHG22990.1	Sucrose synthase 6-like
Cluster-27569.59684	XP_006380428.1	Probable pectate lyase 18
Cluster-27569.59704	XP_011007518.1	Probable glucan 1,3-beta-glucosidase A
Cluster-27569.85212	XP_006448482.1	Protease 2
Cluster-27569.65630	XP_006475282.1	Polygalacturonase
Cluster-27569.55327	XP_012480180.1	Pyruvate kinase
Cluster-27569.87912	XP_012473964.1	Polygalacturonase
Cluster-27569.87908	XP_007050339.1	Vacuolar invertase
Cluster-27569.86913	XP_012068541.1	Beta-carotene isomerase

9.8 Appendix VIII: Additional genes expressed differentially between the ripening stages of a mango group and between mango groups at the same ripening stage.

a) Comparison between the unripe (UR) and ripe (R) stages of 'Chokanan' (CKUR vs CKR)

Gene_ID	Log_2FC	Adjusted P value	NR ID
CELL WALL-RELATED			
Beta glucosidase			
Cluster-27569.97072	6.32	0.0077	KHN28382.1
Cluster-27569.79913	-1.97	0.0089	XP_006445671.1
Cluster-27569.25347	-2.78	0.0205	XP_006445671.1
Cluster-27569.109596	-5.77	0.0249	XP_008385487.1
Xylosidase			
Cluster-27569.54248	5.51	2.05E-13	XP_007015579.1
Cluster-27569.111061	-5.25	4.80E-07	XP_006484460.1
Cluster-27569.120049	5.19	0.0006	XP_006444470.1
Cluster-27569.5309	-3.06	0.0353	XP_006472631.1
STRESS-RELATED			
Catalase			
Cluster-27569.63111	-5.11	2.31E-14	XP_012838970.1
Cluster-27569.65558	-4.43	1.01E-11	ACJ76836.3
Cluster-27569.63319	-2.91	0.0029	XP_012838970.1
Cluster-27569.66640	-1.75	0.0107	XP_011041225.1
Cluster-27569.65846	1.75	0.0147	XP_011041225.1
Cluster-27569.64244	2.25	0.0269	CAB56850.1
Cluster-27569.67612	3.24	0.0276	AET97564.1
Cluster-33812.1	Inf	0.0421	AFR42412.1
Universal stress protein			
Cluster-27569.49316	-4.95	8.25E-14	XP_006442550.1
Cluster-27569.111599	-6.41	7.69E-13	XP_007038300.1
Cluster-27569.39568	-4.31	7.53E-09	KDO75672.1
Cluster-27569.25479	-7.65	3.73E-08	XP_012066343.1
Cluster-27569.39967	-3.11	2.28E-06	KDO66689.1
Cluster-27569.39569	-3.18	2.75E-06	XP_011048491.1
Cluster-27569.119570	-3.70	5.61E-06	XP_007051086.1
Cluster-27569.59981	-2.89	1.46E-05	XP_010259285.1
Cluster-27569.74154	11.56	1.58E-05	XP_008458777.1
Cluster-27569.105508	-4.07	6.21E-05	XP_006486684.1
Cluster-27569.105514	-4.01	0.0001	XP_006486684.1
Cluster-27569.65671	-2.00	0.0029	NP_001238489.1
Cluster-27569.80326	2.98	0.0044	XP_006429235.1
Cluster-27569.62722	1.96	0.0074	XP_006468711.1
Cluster-27569.39564	-2.39	0.0118	XP_006467927.1

Cluster-27569.62721	1.83	0.0128	XP_012082109.1
Superoxide dismutase			
Cluster-27569.75308	-2.33	0.0002	ABY65355.1
Cluster-27569.77010	-1.98	0.0028	CAE54085.1
Cluster-27569.61950	2.02	0.0138	XP_007011340.1
Cluster-27569.69115	3.75	0.0208	ABR29644.1
Cluster-27569.78563	-1.62	0.0276	ADG26761.2
Cluster-27569.69587	1.60	0.0308	XP_006437873.1
Glutathione peroxidase			
Cluster-27569.92633	-4.32	2.48E-08	KJB52091.1
Cluster-27569.25233	-3.79	4.65E-07	XP_006476628.1
Cluster-27569.92634	-3.18	1.08E-05	XP_010660798.1
Cluster-27569.21895	-3.79	1.32E-05	XP_012077107.1
Cluster-27569.57249	2.43	0.0016	XP_006443084.1
Cluster-27569.82096	-2.09	0.0022	XP_006476628.1
Cluster-27569.57763	1.77	0.0225	NP_001292953.1
Cluster-27569.104902	-2.32	0.0315	AGT98544.1
Cluster-27569.66477	1.62	0.0361	XP_006584497.1

b) Comparison between the unripe (UR) and ripe (R) stages of 'Pool'(PUR vs PR)

Gene ID	log_2FC	Adjusted P value	NR ID
CELL WALL-RELATED			
Beta-glucosidase			
Cluster-20281.3	4.95	1.65E-11	XP_006439048.1
Cluster-27569.65957	3.16	7.65E-07	KDO77122.1
Cluster-27569.114594	3.20	5.60E-06	XP_003538061.1
Cluster-27569.81308	3.53	6.04E-06	XP_007028105.1
Cluster-27569.79913	-2.34	1.59E-05	XP_006445671.1
Cluster-27569.73526	2.08	6.84E-05	KDO77122.1
Cluster-27569.52187	2.80	9.61E-05	XP_003538061.1
Cluster-27569.45224	3.88	0.0045	XP_010031675.1
Cluster-27569.111031	-2.15	0.0152	XP_006471035.1
Cluster-27569.111032	-2.10	0.0235	XP_006431835.1
Cluster-27569.48374	3.75	0.0260	XP_006479944.1
Cluster-27569.88086	-1.29	0.0320	KDO72039.1
Cluster-27569.73326	2.81	0.0364	XP_009346071.1
Cluster-27569.49218	3.24	0.0403	XP_006486817.1
Cluster-27569.68293	-2.68	0.0422	XP_006445671.1
Xylosidase			
Cluster-18200.0	4.75	2.68E-06	XP_008236322.1
Cluster-27569.92544	-2.96	0.0043	XP_007035744.1

STRESS-RELATED			
Catalase			
Cluster-27569.58182	4.60	5.84E-17	CAB56850.1
Cluster-27569.65846	3.43	9.48E-12	XP_011041225.1
Cluster-27569.67612	2.69	1.71E-06	AET97564.1
Cluster-27569.67641	3.76	2.99E-06	CAB56850.1
Cluster-27569.64244	2.32	6.14E-06	CAB56850.1
Cluster-27569.111761	3.19	1.53E-05	XP_008237987.1
Cluster-27569.67419	-4.39	0.0022	XP_011041225.1
Cluster-27569.63111	-1.56	0.0041	XP_012838970.1
Cluster-27569.65558	-1.30	0.0274	ACJ76836.3
Universal stress protein			
Cluster-27569.111599	-5.01	4.90E-16	XP_007038300.1
Cluster-27569.74154	4.65	3.86E-13	XP_008458777.1
Cluster-27569.62721	3.35	6.38E-11	XP_012082109.1
Cluster-27569.66454	3.02	2.13E-07	XP_006448455.1
Cluster-27569.74459	2.36	5.16E-06	XP_006479977.1
Cluster-27569.74460	2.18	3.29E-05	KDO87197.1
Cluster-27569.62377	1.94	0.0002	XP_006487496.1
Cluster-27569.74461	1.88	0.0004	XP_006479977.1
Cluster-27569.56324	2.38	0.0010	XP_008389277.1
Cluster-27569.39967	-1.80	0.0014	KDO66689.1
Cluster-27569.48903	-2.53	0.0029	XP_008389277.1
Cluster-27569.49316	-1.49	0.0066	XP_006442550.1
Cluster-27569.59375	1.52	0.0086	XP_006487496.1
Cluster-27569.40151	-3.52	0.0269	XP_012846628.1
Superoxide dismutase			
Cluster-27569.56446	2.79	0.0001	KHG05610.1
Cluster-27569.63848	1.58	0.0059	XP_006440468.1
Cluster-27569.69886	1.94	0.0122	AIG52321.1
Cluster-35876.0	-5.52	0.0135	XP_012092832.1
Glutathione peroxidase			
Cluster-27569.63270	-4.52	1.55E-12	XP_007040204.1
Cluster-27569.21895	3.24	1.14E-10	XP_012077107.1
Cluster-27569.104902	-4.05	5.06E-10	AGT98544.1
Cluster-27569.25233	-3.17	1.07E-07	XP_006476628.1
Cluster-27569.70074	2.40	3.75E-06	NP_001292953.1
Cluster-27569.57249	1.86	0.0019	XP_006443084.1
Cluster-27569.92634	-2.01	0.0092	XP_010660798.1
Cluster-27569.92633	-2.48	0.0093	KJB52091.1

c) Comparison between 'Chokanan' (CK) and 'Pool' (P) at the unripe (UR) stage (CKUR vs PUR)

Gene_ID	log_2FC	Adjusted P value	NR ID
CELL WALL-RELATED			
Beta glucosidase			
Cluster-27569.91777	-5.86	5.59E-23	XP_009356956.1
Cluster-20281.0	-8.62	1.00E-16	XP_006439047.1
Cluster-27569.60595	3.04	9.36E-06	AAQ17461.1
Cluster-27569.54248	2.80	6.81E-05	XP_007015579.1
Cluster-27569.68293	-1.78	0.0008	XP_006445671.1
Cluster-27569.109596	-3.75	0.0106	XP_008385487.1
Xylosidase			
Cluster-27569.54248	2.80	6.81E-05	XP_007015579.1
Cluster-27569.96669	1.77	0.0205	XP_012084033.1
Cluster-27569.92544	2.20	0.0379	XP_007035744.1
STRESS-RELATED			
Catalase			
Cluster-27569.74200	3.39	3.67E-14	KDO76524.1
Cluster-27569.81304	5.88	0.0045	XP_011022505.1
Cluster-27569.64244	-1.23	0.0108	CAB56850.1
Cluster-27569.65846	-1.18	0.0136	XP_011041225.1
Cluster-27569.67612	3.17	0.0240	AET97564.1
Universal stress protein			
Cluster-27569.49316	-2.56	1.76E-09	XP_006442550.1
Cluster-27569.56324	-3.33	1.54E-08	XP_008389277.1
Cluster-27569.54952	3.70	3.45E-08	XP_012082104.1
Cluster-27569.105507	-7.29	1.44E-07	XP_006486684.1
Cluster-27569.62722	-2.19	3.03E-05	XP_006468711.1
Cluster-27569.84021	-2.24	3.68E-05	XP_006446520.1
Cluster-27569.25479	-4.53	0.0001	XP_012066343.1
Cluster-27569.119570	-2.22	0.0005	XP_007051086.1
Cluster-27569.105514	-2.40	0.0007	XP_006486684.1
Superoxide dismutase			
Cluster-27569.69115	5.91	4.38E-26	ABR29644.1
Cluster-27569.45042	-4.52	9.97E-08	XP_006484502.1
Cluster-27569.61949	-1.99	4.75E-06	XP_007011340.1
Cluster-27569.45041	-2.60	4.26E-05	XP_006484502.1
Cluster-27569.77010	-1.72	9.55E-05	CAE54085.1
Cluster-27569.68600	3.48	0.0003	XP_007011340.1
Cluster-27569.61950	-4.62	0.0006	XP_007011340.1
Cluster-27569.68978	-1.31	0.0118	CAE54085.1
Cluster-27569.68976	-1.56	0.0132	CAE54085.1
Cluster-27569.29024	-1.24	0.0159	XP_006437025.1
Cluster-27569.75308	-1.13	0.0195	ABY65355.1
Cluster-27569.99323	-5.66	0.0350	XP_001770912.1

Glutathione peroxidase			
Cluster-27569.92633	-2.96	6.08E-09	KJB52091.1
Cluster-27569.92634	-2.47	5.00E-07	XP_010660798.1
Cluster-27569.82096	-2.22	5.52E-07	XP_006476628.1
Cluster-27569.47567	-5.66	3.85E-06	XP_009778335.1
Cluster-27569.57763	-2.49	1.03E-05	NP_001292953.1
Cluster-27569.47569	-1.78	9.67E-05	XP_010038116.1
Cluster-27569.21895	2.63	0.0001	XP_012077107.1
Cluster-27569.109249	-4.61	0.0003	XP_007223774.1
Cluster-27569.92635	-4.10	0.0004	XP_002272936.1
Cluster-27569.66477	-1.31	0.0115	XP_006584497.1
Cluster-27569.64735	-1.23	0.0142	XP_006579376.1
Cluster-27569.64141	-1.11	0.0265	XP_012486281.1

a) Comparison between 'Chokanan' (CK) and 'Pool' (P) at the ripe (R) stage CKR vs PR)

a) CKR vs PR			
GENE_ID	log_2FC	Adjusted P value	NR ID
CELL WALL-RELATED			
Beta glucosidase			
Cluster-27569.97072	-4.04	0.0376	KHN28382.1
Xylosidase			
Cluster-27569.111061	4.99	2.73E-06	XP_006484460.1
Cluster-18200.0	3.01	0.0049	XP_008236322.1
Cluster-27569.5309	3.06	0.0225	XP_006472631.1
Cluster-27569.120049	-3.03	0.0327	XP_006444470.1
Cluster-27569.54248	-1.70	0.0441	XP_007015579.1
STRESS-RELATED			
Catalase			

Cluster-27569.58182	6.84	1.40E-15	CAB56850.1
Cluster-27569.67641	4.33	1.20E-05	CAB56850.1
Cluster-27569.63111	2.72	0.0001	XP_012838970.1
Cluster-27569.65558	2.57	0.0002	ACJ76836.3
Cluster-27569.67612	2.61	0.0016	AET97564.1
Glutathione Peroxidase			
Cluster-27569.21895	9.66	3.98E-30	XP_012077107.1
Cluster-27569.57763	-4.15	2.00E-08	NP_001292953.1
Cluster-27569.66477	-2.83	7.92E-05	XP_006584497.1
Cluster-27569.66477	-2.83	7.92E-05	XP_006584497.1
Cluster-27569.64141	-2.77	0.0001	XP_012486281.1
Cluster-27569.64735	-2.65	0.0002	XP_006579376.1
Cluster-27569.92635	-5.64	0.0005	XP_002272936.1
Cluster-27569.64737	-2.34	0.0045	XP_002310444.1
Superoxide dismutase			
Cluster-27569.61950	-6.09	3.69E-12	XP_007011340.1
Cluster-27569.68600	5.63	5.06E-11	XP_007011340.1
Cluster-27569.45042	-4.23	0.0002	XP_006484502.1
Cluster-27569.69115	3.04	0.0003	ABR29644.1
Cluster-27569.63163	-3.56	0.0004	CAN76051.1
Cluster-27569.68976	-2.01	0.0407	CAE54085.1
Universal stress protein			
Cluster-27569.62722	-4.85	9.05E-11	XP_006468711.1
Cluster-27569.54952	4.44	8.05E-06	XP_012082104.1
Cluster-27569.39568	3.37	3.98E-05	KDO75672.1
Cluster-27569.56439	3.82	0.0013	XP_002265489.2
Cluster-27569.66099	-3.11	0.0023	KDO62808.1
Cluster-27569.39569	2.25	0.0046	XP_011048491.1
Cluster-27569.93596	2.42	0.0068	XP_006432127.1
Cluster-27569.39563	2.15	0.0148	XP_006449182.1
Cluster-27569.39564	2.33	0.0176	XP_006467927.1
Cluster-27569.80326	-2.69	0.0313	XP_006429235.1

9.9 Appendix IX: Gene ontology (GO) functional enrichment analysis of genes differentially expressed between the ripening stages of each mango group and between mango groups for the same ripening stage.

[Unripe (UR) and ripe (R)] of a) 'CK' and b) 'P' groups respectively [Chokanan (CK) and Pool (P)].

CKUR vs CKR			
GO accession	Description	Adjusted P value	Number of DEGs
GO:0008152	metabolic process	1.45E-15	5517
GO:0055114	oxidation-reduction process	6.03E-11	1039
GO:0006629	lipid metabolic process	2.54E-07	531
GO:0005975	carbohydrate metabolic process	3.37E-07	648
GO:0042254	ribosome biogenesis	1.09E-06	351
GO:0010817	regulation of hormone levels	2.68E-06	83
GO:0005984	disaccharide metabolic process	2.81E-06	150
GO:0042445	hormone metabolic process	4.04E-06	82
GO:0022613	ribonucleoprotein complex biogenesis	4.19E-06	354
GO:0034754	cellular hormone metabolic process	1.02E-05	80
GO:0006468	protein phosphorylation	1.71E-05	606
GO:0044262	cellular carbohydrate metabolic process	1.73E-05	229
GO:0008202	steroid metabolic process	2.42E-05	100
GO:0034637	cellular carbohydrate biosynthetic process	3.09E-05	91
GO:0006694	steroid biosynthetic process	3.36E-05	99
GO:0044723	single-organism carbohydrate metabolic process	7.61E-05	409
GO:0009311	oligosaccharide metabolic process	0.0001	173
GO:0005985	sucrose metabolic process	0.0001	126
GO:0005982	starch metabolic process	0.0001	126
GO:0033015	tetrapyrrole catabolic process	0.0002	34
GO:0006073	cellular glucan metabolic process	0.0003	137
GO:0051187	cofactor catabolic process	0.0003	34
GO:0046149	pigment catabolic process	0.0003	31

GO:0044264	cellular polysaccharide metabolic process	0.0005	144
GO:0044710	single-organism metabolic process	0.0005	2437
GO:0030258	lipid modification	0.0005	42
GO:0009725	response to hormone	0.0008	51
GO:0030244	cellulose biosynthetic process	0.0009	34
GO:1901565	organonitrogen compound catabolic process	0.0009	136
GO:0071704	organic substance metabolic process	0.0024	4551
GO:0009719	response to endogenous stimulus	0.0026	52
GO:0010033	response to organic substance	0.0035	66
GO:0006012	galactose metabolic process	0.0046	46
GO:0030243	cellulose metabolic process	0.0052	34
GO:0006631	fatty acid metabolic process	0.0059	103
GO:0006026	aminoglycan catabolic process	0.0059	32
GO:0008610	lipid biosynthetic process	0.0062	272
GO:0005992	trehalose biosynthetic process	0.0063	30
GO:0044238	primary metabolic process	0.0066	4346
GO:0034219	carbohydrate transmembrane transport	0.0067	10
GO:0016051	carbohydrate biosynthetic process	0.0069	183
GO:0044255	cellular lipid metabolic process	0.0079	355
GO:0030259	lipid glycosylation	0.0093	20
GO:0009250	glucan biosynthetic process	0.0097	49
GO:0019318	hexose metabolic process	0.0110	129
GO:0006338	chromatin remodeling	0.0111	43
GO:0005976	polysaccharide metabolic process	0.0115	148
GO:0030682	evasion or tolerance of host defense response	0.0173	14
GO:0046184	aldehyde biosynthetic process	0.0196	9
GO:0047746	chlorophyllase activity	0.0208	23
GO:0042167	heme catabolic process	0.0208	8
GO:0006011	UDP-glucose metabolic process	0.0208	27
GO:0046351	disaccharide biosynthetic process	0.0221	30
GO:0055085	transmembrane transport	0.0253	586
GO:0033692	cellular polysaccharide biosynthetic process	0.0256	56

GO:0009225	nucleotide-sugar metabolic process	0.0275	35
GO:0005991	trehalose metabolic process	0.0337	30
GO:0016310	phosphorylation	0.0347	789
GO:0005996	monosaccharide metabolic process	0.0352	131
GO:0019538	protein metabolic process	0.0383	1825
GO:0051274	beta-glucan biosynthetic process	0.0411	41
PUR vs PR			
GO:0008152	metabolic process	1.72E-17	4149
GO:0005975	carbohydrate metabolic process	8.45E-13	538
GO:0055114	oxidation-reduction process	1.18E-12	808
GO:0016051	carbohydrate biosynthetic process	3.48E-09	174
GO:0044723	single-organism carbohydrate metabolic process	3.48E-09	345
GO:0044710	single-organism metabolic process	1.77E-08	1890
GO:0006629	lipid metabolic process	4.97E-08	415
GO:0044264	cellular polysaccharide metabolic process	2.09E-07	128
GO:0044042	glucan metabolic process	2.09E-07	121
GO:0030243	cellulose metabolic process	4.87E-07	37
GO:0030244	cellulose biosynthetic process	5.42E-07	35
GO:0051273	beta-glucan metabolic process	6.67E-07	48
GO:0051274	beta-glucan biosynthetic process	1.33E-06	46
GO:0009250	glucan biosynthetic process	1.69E-06	50
GO:0009311	oligosaccharide metabolic process	1.69E-06	144
GO:0044262	cellular carbohydrate metabolic process	1.93E-06	184
GO:0005985	sucrose metabolic process	3.21E-06	106
GO:0005982	starch metabolic process	3.21E-06	106
GO:0033692	cellular polysaccharide biosynthetic process	3.40E-06	57
GO:0044255	cellular lipid metabolic process	3.40E-06	295
GO:0006006	glucose metabolic process	4.10E-06	81
GO:0034637	cellular carbohydrate biosynthetic process	1.57E-05	74
GO:0005976	polysaccharide metabolic process	1.60E-05	129
GO:0005984	disaccharide metabolic process	1.65E-05	115

GO:0019318	hexose metabolic process	2.02E-05	113
GO:0005996	monosaccharide metabolic process	2.96E-05	117
GO:0008610	lipid biosynthetic process	0.0001	220
GO:0032787	monocarboxylic acid metabolic process	0.0002	227
GO:0006011	UDP-glucose metabolic process	0.0002	27
GO:0046364	monosaccharide biosynthetic process	0.0002	70
GO:0044699	single-organism process	0.0002	3116
GO:0000271	polysaccharide biosynthetic process	0.0002	57
GO:0015976	carbon utilization	0.0002	56
GO:0006793	phosphorus metabolic process	0.0003	891
GO:0006012	galactose metabolic process	0.0004	40
GO:0071704	organic substance metabolic process	0.0005	3401
GO:0009225	nucleotide-sugar metabolic process	0.0006	33
GO:0044711	single-organism biosynthetic process	0.0007	624
GO:0044238	primary metabolic process	0.0008	3256
GO:0016310	phosphorylation	0.0009	617
GO:0006979	response to oxidative stress	0.0010	53
GO:0009725	response to hormone	0.0020	40
GO:0051156	glucose 6-phosphate metabolic process	0.0022	76
GO:0055085	transmembrane transport	0.0024	456
GO:0044712	single-organism catabolic process	0.0035	179
GO:0006098	pentose-phosphate shunt	0.0040	74
GO:1901135	carbohydrate derivative metabolic process	0.0046	459
GO:0006108	malate metabolic process	0.0046	18
GO:0006631	fatty acid metabolic process	0.0076	80
GO:0009719	response to endogenous stimulus	0.0081	40
GO:0046939	nucleotide phosphorylation	0.0081	60
GO:0006081	cellular aldehyde metabolic process	0.0082	95
GO:0072350	tricarboxylic acid metabolic process	0.0139	36
GO:0006090	pyruvate metabolic process	0.0140	86
GO:0030258	lipid modification	0.0160	30
GO:0009733	response to auxin	0.0160	15

GO:0019682	glyceraldehyde-3-phosphate metabolic process	0.0163	74
GO:0019637	organophosphate metabolic process	0.0167	390
GO:0006099	tricarboxylic acid cycle	0.0167	35
GO:0034219	carbohydrate transmembrane transport	0.0196	8
GO:0006165	nucleoside diphosphate phosphorylation	0.0274	56
GO:0006757	ATP generation from ADP	0.0290	53
GO:0006638	neutral lipid metabolic process	0.0313	10
GO:0016042	lipid catabolic process	0.0314	46
GO:0006633	fatty acid biosynthetic process	0.0314	63
GO:0006732	coenzyme metabolic process	0.0320	230
GO:0006091	generation of precursor metabolites and energy	0.0346	199
GO:0009060	aerobic respiration	0.0351	36
GO:0046496	nicotinamide nucleotide metabolic process	0.0351	100
GO:1903509	liposaccharide metabolic process	0.0432	92
GO:0046149	pigment catabolic process	0.0473	20
CKUR vs PUR			
GO:0042254	ribosome biogenesis	2.75E-10	489
GO:0022613	ribonucleoprotein complex biogenesis	1.17E-09	495
GO:0008152	metabolic process	6.92E-06	7321
GO:0055114	oxidation-reduction process	1.58E-05	1308
GO:0006412	translation	5.45E-05	597
GO:0043043	peptide biosynthetic process	0.000806	600
GO:0006518	peptide metabolic process	0.001671	639
GO:0030244	cellulose biosynthetic process	0.001676	42
GO:0042445	hormone metabolic process	0.002607	92
GO:0044085	cellular component biogenesis	0.003134	870
GO:0030243	cellulose metabolic process	0.004835	43
GO:0051274	beta-glucan biosynthetic process	0.004835	60
GO:0071840	cellular component organization or biogenesis	0.006617	1425
GO:0051273	beta-glucan metabolic process	0.011575	61
GO:0009250	glucan biosynthetic process	0.013336	65
GO:0044710	single-organism metabolic process	0.02735	3287
GO:0044723	single-organism carbohydrate metabolic process	0.031776	524

GO:0044267	cellular protein metabolic process	0.037534	2009
CKR vs PR			
GO:0005975	carbohydrate metabolic process	1.59E-07	552
GO:0008152	metabolic process	1.59E-07	4395
GO:0055114	oxidation-reduction process	1.19E-06	816
GO:0030244	cellulose biosynthetic process	4.92E-06	36
GO:0044710	single-organism metabolic process	7.27E-06	2029
GO:0030243	cellulose metabolic process	3.79E-05	36
GO:1901136	carbohydrate derivative catabolic process	7.94E-05	50
GO:0006026	aminoglycan catabolic process	0.000113	33
GO:0006011	UDP-glucose metabolic process	0.000121	30
GO:0006629	lipid metabolic process	0.000156	423
GO:0006073	cellular glucan metabolic process	0.000246	119
GO:0044042	glucan metabolic process	0.000246	119
GO:0044723	single-organism carbohydrate metabolic process	0.000267	344
GO:0006032	chitin catabolic process	0.000339	15
GO:0046348	amino sugar catabolic process	0.000339	15
GO:1901072	glucosamine-containing compound catabolic process	0.000339	15
GO:0044264	cellular polysaccharide metabolic process	0.000565	124
GO:0044262	cellular carbohydrate metabolic process	0.000745	188
GO:0016998	cell wall macromolecule catabolic process	0.001271	14
GO:0005976	polysaccharide metabolic process	0.001319	134
GO:0009072	aromatic amino acid family metabolic process	0.001564	110
GO:0009225	nucleotide-sugar metabolic process	0.002321	35
GO:0051274	beta-glucan biosynthetic process	0.002831	42
GO:0042445	hormone metabolic process	0.003563	61
GO:0010817	regulation of hormone levels	0.003989	61
GO:0005984	disaccharide metabolic process	0.004952	117
GO:0032787	monocarboxylic acid metabolic process	0.007503	235
GO:0051273	beta-glucan metabolic process	0.007503	42

GO:0005985	sucrose metabolic process	0.007815	102
GO:0005982	starch metabolic process	0.007828	102
GO:0034754	cellular hormone metabolic process	0.007856	59
GO:0009250	glucan biosynthetic process	0.009508	44
GO:0034637	cellular carbohydrate biosynthetic process	0.009952	71
GO:0044699	single-organism process	0.014632	3372
GO:0006030	chitin metabolic process	0.015009	18
GO:0009410	response to xenobiotic stimulus	0.015009	42
GO:0071466	cellular response to xenobiotic stimulus	0.015009	42
GO:0006012	galactose metabolic process	0.029275	37
GO:0033692	cellular polysaccharide biosynthetic process	0.030288	49
GO:0006631	fatty acid metabolic process	0.033366	83
GO:0000271	polysaccharide biosynthetic process	0.034007	54
GO:0006558	L-phenylalanine metabolic process	0.036302	48
GO:1902221	phosphoenolpyruvate family amino acid metabolic process	0.036302	48
GO:0008610	lipid biosynthetic process	0.036636	219

9.10 Appendix X: Significantly enriched pathways between the ripening stages of 'CK' and 'P' mango groups.

Term	Count	Background	Adjusted P value
CKUR vs CKR			
Plant hormone metabolism	247	850	3.2E-24
Ribosome	265	1192	3.87E-13
Starch and sucrose metabolism	183	806	1.14E-09
Fatty acid metabolism	114	480	7.69E-07
Photosynthesis	49	158	2.17E-05
Biosynthesis of unsaturated fatty acids	50	188	0.0005
Amino sugar and nucleotide sugar metabolism	115	574	0.0005
alpha-Linolenic acid metabolism	45	164	0.0005
Cyanoamino acid metabolism	48	191	0.0017
Citrate cycle (TCA cycle)	79	379	0.0026
Fatty acid biosynthesis	60	270	0.0037
Glutathione metabolism	76	370	0.0043
Phenylpropanoid biosynthesis	71	343	0.0050
Glycine, serine and threonine metabolism	75	368	0.0050
Galactose metabolism	57	261	0.0058
Carbon metabolism	248	1544	0.0074
Isoquinoline alkaloid biosynthesis	30	112	0.0092
Glyoxylate and dicarboxylate metabolism	75	387	0.0137
Linoleic acid metabolism	15	40	0.0141
Carotenoid biosynthesis	33	135	0.0155
Carbon fixation	78	412	0.0158
Pyruvate metabolism	105	605	0.0315
Phenylalanine metabolism	56	284	0.0315
Pentose and glucuronate interconversions	53	271	0.0448
PUR vs PR			
Plant hormone metabolism	180	850	5.6E-19
Carbon metabolism	231	1544	1.39E-09
Pyruvate metabolism	106	605	4.07E-07
Carbon fixation in photosynthetic organisms	80	412	5.06E-07

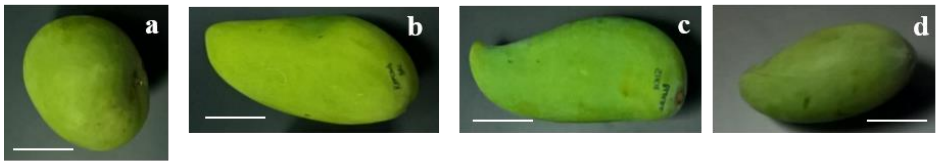
Fatty acid biosynthesis	60	270	5.06E-07
Fatty acid metabolism	88	480	6.73E-07
Glycolysis / Gluconeogenesis	110	682	4.14E-06
Starch and sucrose metabolism	122	806	1.51E-05
Photosynthesis	38	158	2.76E-05
Phenylpropanoid biosynthesis	63	343	4.05E-05
Methane metabolism	57	313	0.0001
Pentose phosphate pathway	52	275	0.0001
Linoleic acid metabolism	16	40	0.0002
Galactose metabolism	49	261	0.0002
Carotenoid biosynthesis	31	135	0.0003
Glutathione metabolism	62	370	0.0003
alpha-Linolenic acid metabolism	33	164	0.0014
Pentose and glucuronate interconversions	47	271	0.0014
Amino sugar and nucleotide sugar metabolism	83	574	0.0015
Ribosome	146	1192	0.0075
Phenylalanine metabolism	45	284	0.0095
Biosynthesis of unsaturated fatty acids	33	188	0.0100
Diterpenoid biosynthesis	11	40	0.031732
Glyoxylate and dicarboxylate metabolism	53	387	0.048917

9.11 Appendix XI: A list of the proteins in interaction network

Node	Annotation
PSL4	PRIORITY IN SWEET LIFE 4;
CPFTSY	Fused signal recognition particle receptor
LAC1	Laccase 1
ArRABA1h	RAB GTPase homolog A1H
RABA1g	RAB GTPase homolog A1G
RABA1i	RAB GTPase homolog A1I
AT4G30600	Signal recognition particle receptor subunit alpha
PI-4KBETA1	Phosphatidylinositol 4-kinase beta 1
BGAL10	Beta-galactosidase 10
RABA2D	RAB GTPase homolog A2D;
PIP5K1	Phosphatidylinositol-4-phosphate 5-kinase 1
PI-4KBETA2	Phosphatidylinositol 4-kinase 2
FXG1	Alpha-fucosidase 1
BAK1	BRI1-associated receptor kinase
GH9A1	Endoglucanase 25
RABA4D	RAB GTPase homolog A4D
AT2G42390	Protein kinase C
AT5G11720	Alpha-glucosidase
GDI2	RAB GDP dissociation inhibitor 2
AT3G45940	Alpha-glucosidase
RABA5d	RAB GTPase homolog A5D;
AT3G42160	Pectinesterase-related protein
RABA2c	RAB GTPase homolog A2C
RABA2b	RAB GTPase homolog A2B
OVA1	Ovule abortion 1
RABA1d	RAB GTPase homolog A1D
MIF3	Mini zinc finger

Node	Annotation
CESA2	Cellulose synthase A2
HGL1	Heteroglycan glucosidase 1
PIP5K5	Phosphatidylinositol-4-phosphate 5-kinase 5
RABA1e	RAB GTPase homolog A1E
PGA4	Polygalacturonase 4
ATHATPLC1G	Phospholipase C1
RABA4C	RAB GTPase homolog A4C
RABA5b	RAB GTPase homolog A5B
AT1G20230	Pentatricopeptide repeat-containing protein
RABA1c	RAB GTPase homolog A1C
ARA4	Ras-related protein ARA-4
XYL1	Alpha-xylosidase 1
RABA5E	RAB GTPase homolog A5E
PUB13	Plant U-box 13
AT2G18770	nucleoside triphosphate hydrolase-like protein
RABA4a	RAB GTPase homolog A4A
RABA1f	RAB GTPase homolog A1F
FUC95A	alpha-L-fucosidase 2
RABA1b	RAB GTPase homolog A1B
RSW3	Radial swelling
RABA3	Rab family protein
GH9B5	Endoglucanase 2
GSL05	Callose synthase 12
SYP125	Syntaxin-125
RABA5a	RAB GTPase homolog A5A
PI4KG7	Phosphoinositide 4-kinase gamma 7
GN	GNOM
RABA4B	RAB GTPase homolog A4B

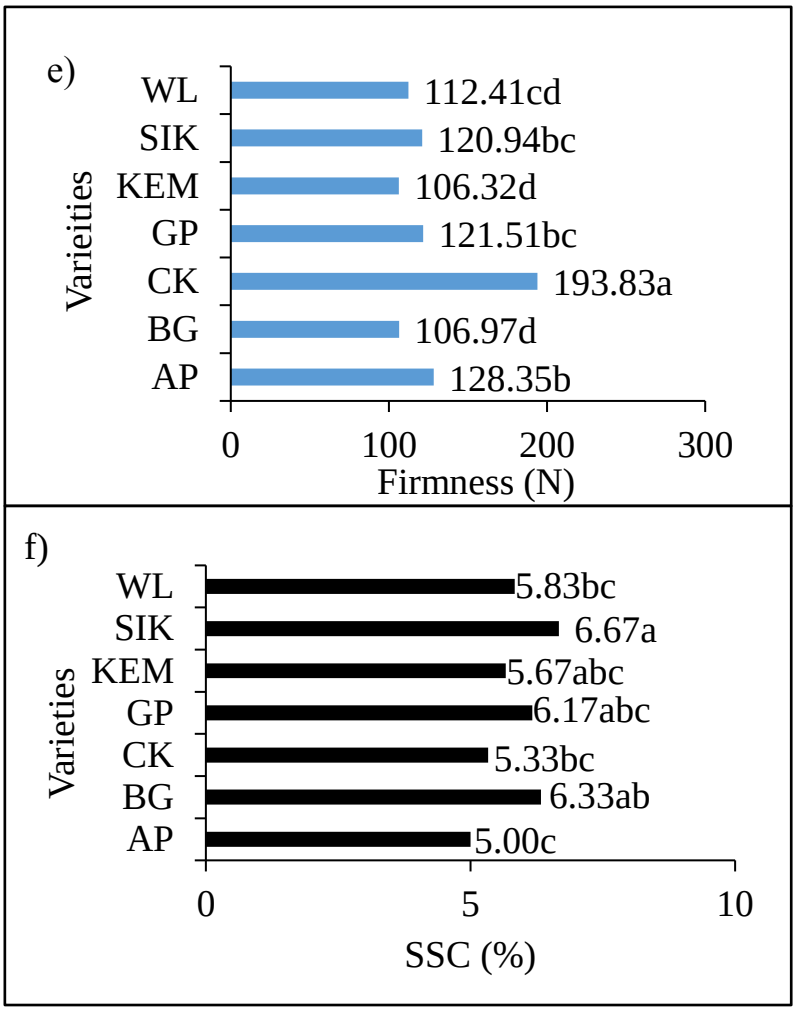
9.12 Appendix XII: Additional mango varieties used for RT-qPCR



a : 'Apple'
b : 'Black gold'
c : 'Kemling'
d : 'Siku jaya'

(Scale bar = 2 cm)

9.13 Appendix XIII: Firmness and SSC measurements of the seven mango varieties



Note: AP, 'Apple mango'; BG, 'Black gold'; CK,'Chokanan'; GP, 'Golden phoenix'; KEM, 'Kemling'; SIK, 'Siku jaya'; WL; 'Water lily'

9.14 Appendix XIV: RNA sample quality of mango varieties used in RT-qPCR

Sample	A260/A280	A260/A230	Concentration (ng/µl)	RIN
'Apple mango' 1	2.80	2.13	146.80	7.90
'Apple mango' 2	2.00	2.22	205.30	8.20
'Apple mango' 3	2.02	1.65	189.40	7.70
'Black gold' 1	1.96	1.88	353.10	7.90
'Black gold' 2	1.93	2.17	1777.60	8.20
'Black gold' 3	1.93	2.25	340.00	8.10
'Chokanan' 1	2.10	2.03	1253.10	7.60
'Chokanan' 2	2.06	1.80	170.20	7.10
'Chokanan' 3	2.00	1.76	120.50	8.00
'Golden phoenix' 1	2.00	2.24	1472.10	7.70
'Golden phoenix' 2	1.97	1.74	302.20	7.90
'Golden phoenix' 3	1.98	2.27	291.10	8.00
'Kemling' 1	2.05	2.26	145.70	8.10
'Kemling' 2	1.90	1.83	102.40	8.20
'Kemling' 3	2.03	1.73	209.20	8.30
'Siku jaya' 1	1.95	1.61	289.00	7.50
'Siku jaya' 2	2.01	2.11	913.00	7.20
'Siku jaya' 3	2.05	1.76	183.50	8.00
'Water lily' 1	1.89	1.85	334.50	8.40
'Water lily' 2	1.98	2.04	1962.60	7.30
'Water lily' 3	1.98	2.11	2526.00	7.50

Note: Biological replicates are represented as 1, 2 and 3 respectively

9.15 Appendix XV: Permission to Use Figure 2.2

Dr. Chiranjit Parmar <parmarch@gmail.com>

Dear Tamunonengiyeofori Lawson,

Material Requested:

Title: Online Encyclopaedia on edible fruits (Mango)

Author: Dr. Chiranjit Parmar

Website: http://www.fruitipedia.com/

DOI: 10.5897/JMPR2013.5299

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9.16 Appendix XVI: Permission to use Figures 2.5 and 2.6

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Publication: Biotechnology and Genetic Engineering Reviews
Publisher: Taylor & Francis
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Title: Characterization of Southeast Asia mangoes (Mangifera indica L) according to their physicochemical attributes
Author: Tamunonengiyeofori Lawson,Grantley W. Lycett,Asgar Ali,Chiew Foan Chin
Publication: Scientia Horticulturae
Publisher: Elsevier
Date: 3 January 2019
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