

# **Understanding the Physiological Mechanisms of Ripening in Capsicum**

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## Abstract

*Capsicum* (*Capsicum annuum* L.) is considered a non-climacteric fruit, exhibiting limited respiration and ethylene levels. The physiological mechanisms of ripening in capsicum have not been fully understood to date, especially the probable reason behind the non-climacteric behaviour. In this thesis, the protein (Chapter 3) and metabolite (Chapter 4) profiles of capsicum at different ripening stages have been reported. Several proteomic and metabolic candidates, for example ACC oxidase (ACO) enzyme, sugars (glucose, fructose, sucrose) and malate were chosen and analysed in different tissue types (peel, pulp and seeds/placenta) and cultivars with different ripening times (Chapter 5). The results suggested that some of these candidates were differentially present in different tissues and cultivars which implied that ripening could be regulated spatially and temporally.

Furthermore, proteomic analysis also identified an ACO isoform 4 (CaACO4) which was found during capsicum ripening onset and corresponded to the increase in the overall ACO activity (Chapter 3). The expression of several ACO isoforms including CaACO4, and other identified ACC synthase (ACS) and Ethylene receptor (ETR) isoforms were therefore characterised to shed some light on their roles in this non-climacteric fruit (Chapter 6). CaACO4 was the only ACO isoform expressed significantly higher during capsicum ripening onset, confirming the earlier proteomic results. The expression of several ACS and ETR isoforms, normally associated with the climacteric increase of ethylene in tomato (a close relative of capsicum), was also limited as was ACS activity and ACC content. The production of ACC, as an ethylene precursor, may therefore be the rate-limiting step for ethylene production in non-climacteric capsicum. The postharvest application of ethylene did not promote capsicum ripening or induce the expression of most ACO, ACS and ETR isoforms, suggesting they are not regulated by ethylene as usually observed in climacteric fruit such as tomato. However, 1-methylcyclopropene

treatment significantly delayed capsicum ripening postharvest, particularly when applied at Breaker stage (the onset of ripening), suggesting that blocking ethylene perception could affect ripening and that the basal level of ethylene normally produced in non-climacteric fruit may be (partially) required for ripening (Chapter 6).

Other proteomic candidates such as *Copper chaperone*, *TCP chaperone*, *Cysteine synthase* and *Spermidine synthase* were also isolated and investigated due to their possible roles in capsicum ripening. However, unlike *CaACO4*, the RNA expression of these candidates did not follow their respective proteomic trends, suggesting a regulation at the post-translational level (Chapter 7). The identified candidates including *CaACO4* are now a resource for further investigation to identify factors that may be involved in capsicum ripening.

## **Thesis Declaration**

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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## Abbreviations

1-MCP	1-methylcyclopropene
2DGE	2D-gel electrophoresis
ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylate
ACO	ACC oxidase enzyme
ACS	ACC synthase enzyme
ADK	adenosine kinase
ANOVA	analysis of variance
ATP	adenosine triphosphate
βME	β-mercaptoethanol
B	Breaker capsicum fruit
BR1	Breaker Red 1 capsicum fruit
BR2	Breaker Red 2 capsicum fruit
<i>CaGAPdH</i>	<i>Capsicum annuum</i> glyceraldehyde 3-phosphate dehydrogenase
CCH	copper chaperone
cv.	cultivar
CYS	cysteine synthase
DAA	days after anthesis
DR	Deep Red capsicum fruit
ETR	ethylene receptor
G	Green capsicum fruit
GC-MS	gas chromatography-mass spectrometry
IPG	immobilised pH gradient
LC-MS	liquid chromatography-mass spectrometry
LR	Light Red capsicum fruit
l.s.d.	least significant difference
Met	methionine
MW	molecular weight
PCR	polymerase chain reaction
pI	isoelectric point
qPCR	quantitative real-time polymerase chain reaction
<i>rin</i>	<i>ripening inhibitor</i>
RT	room temperature
SAM	S-adenosyl-methionine
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SPS	spermidine synthase
sqRT-PCR	semi-quantitative Reverse Transcriptase-polymerase chain reaction
TCA/acetone	trichloroacetic acid/acetone
TCA cycle	tricarboxylic acid cycle
TF	transcription factor
Tris	tris(hydroxymethyl)amino methane

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# **Chapter 1: Literature Review**

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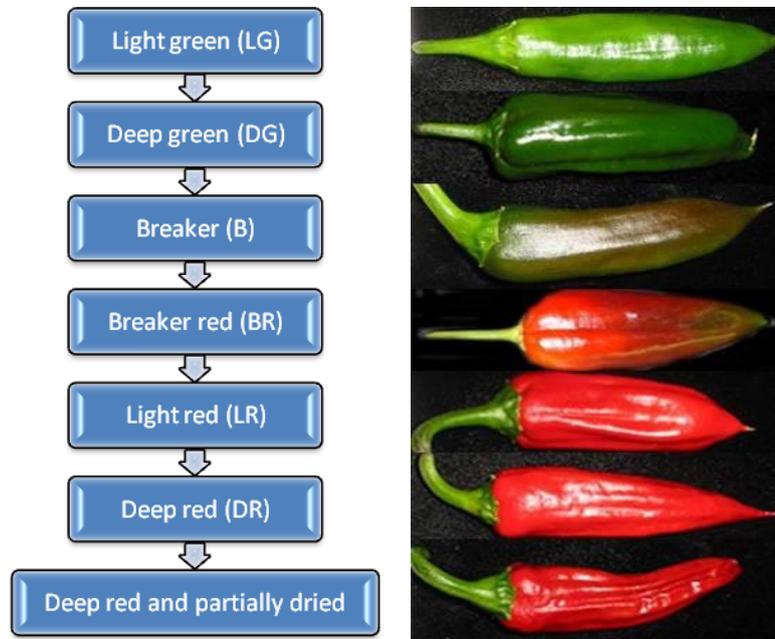
## 1.1 Background

One of the most important vegetables in the world is the capsicum (*Capsicum annuum* L.) which is predominantly used as a food colourant and spice. The two types of capsicum commonly used for these purposes are paprika and cayenne. Another common capsicum type, bell pepper, is mainly utilised fresh. The world production of fresh and dry capsicum reached 29 and 34 million tonnes in 2011, respectively, an increase of approximately nine million tonnes in the last decade [United Nations Food and Agricultural Organization (FAO, 2013)]. Australian production in 2011 was only around 51 thousand tonnes for fresh capsicum but the trend for the last 12 years has been remarkable with a production surge of nearly 40% (FAO, 2013). This data suggests that capsicum or pepper is becoming increasingly popular, not only for the food processing industry but also as a consumer choice as a raw vegetable for cooking.

Preliminary research by Pham (2007) has categorised three capsicum cultivars based on their ripening profiles. Capsicum cv. Papri Queen (paprika type) and cv. Aries (bell pepper type) exhibited a non-climacteric nature and hence did not have a peak of ethylene production during their ripening course. However, the cv. Caysan (cayenne type) produced some ethylene suggesting it behaved as a climacteric fruit. The physiological study also showed that there was no change in fruit diameter and length of all cultivars examined once they reached the Light Green (LG) stage, and that both parameters seemed greater when grown in summer than in winter. Furthermore, there was approximately a lag of seven days for fruit to reach the final ripening stage when grown in winter than in summer.

Further measurement by Pham (2007) also characterised fruit harvested in early ripening stages compared to fruit left to ripen on the plant (Figure 1.1). One notable observation was that fruit harvested at the Breaker stage (B) or later were able to ripen normally but not fruit harvested at earlier stages. Furthermore, Pham (2007) also observed

that ripening of fruit cinctured at the LG stage (on the plant) was delayed by five days when compared to control fruit. Hence, stage-specific molecules were postulated to control ripening at the B stage, in the form of a signal sent from the mother plant via the phloem.



**Figure 1.1** Ripening course of a capsicum (cv. Papri Queen) [adapted from Pham (2007)].

Pham (2007) also investigated the role of ethylene in promoting ripening in a non-climacteric capsicum, cv. Papri Queen. Ethephon (2-chloroethylphosphonic acid; a solution-based ethylene source) was shown to promote the ripening of harvested LG fruit but only at a very high concentration ( $144,000 \mu\text{L L}^{-1}$ ). The effects included an increase in extractable colour [known to be caused by carotenoid production (Vega-Gálvez et al., 2009)], as well as markedly reduced chlorophyll content which could only be seen 14 days after treatment. Furthermore, there was differential expression of two enzymes in carotenoid biosynthesis, *Lycopene- $\beta$ -cyclase* (*Lcy*) and *Capsanthin capsorubin synthase* (*Ccs*) during ripening. No *Lcy* transcripts were found using Northern blot analysis in all ethylene treatments and ripening stages. In contrast, *Ccs* was highly up-regulated in all cases but more prominent when treated with a high ethephon concentration. This evidence

suggests that ethylene may play a minor role in ripening induction of a non-climacteric fruit and affect the carotenoid biosynthesis pathway.

Further experiments are needed to verify these findings. For instance, comparison-based approaches such as proteomics and metabolomics can be employed to identify and characterise ripening factor candidates differentially expressed in different ripening stages. Moreover, the role of ethylene in inducing these identified factors also requires further investigation. The outcomes from these experiments will develop better understanding in the ripening of non-climacteric fruit such as capsicum.

In this literature review, the molecular mechanisms that lead to several ripening characteristics are detailed and gaps are highlighted. Comparison-based approaches are then discussed with a special focus on current studies of fruit ripening; especially the use of these methods in identifying differentially expressed molecules.

## **1.2 Fruit ripening physiology and biochemistry**

Ripening is an important process for plant survival where mature green fruit gradually become edible and attractive to animals (Rodríguez et al., 2013). Many physiological changes occur during ripening including colour alteration, sugar changes, texture softening and aroma build-up (Bapat et al., 2010; Seymour et al., 2013). Moreover, ripening fruit can also be either dependent on ethylene production to fully ripen (known as climacteric fruit) or ripen independently of ethylene production (non-climacteric) (Barry and Giovannoni, 2007; Gapper et al., 2013; Lin et al., 2009).

### *1.2.1 A comparison of the physiology of climacteric and non-climacteric fruit ripening*

Fruit are generally classified based on physiological changes during ripening. The two widely used benchmarks for climacteric or non-climacteric categorisation are biosynthesis

of the gaseous plant hormone ethylene and increases in respiration. Previous studies have shown that climacteric fruit such as tomato, banana and apples exhibit higher ethylene and CO<sub>2</sub> levels and are highly sensitive to ethylene treatment (Bapat et al., 2010; Matas et al., 2009). On the other hand, non-climacteric fruit like capsicum, strawberry and citrus do not ripen naturally when harvested at the mature green stage, exhibit minimal level of ethylene production and gradually declining CO<sub>2</sub> levels during ripening (Bapat et al., 2010).

The effects of ethylene treatment on climacteric fruit ripening have been extensively researched, using not only ethylene in its gaseous form, but also ethylene precursors such as propylene and ethephon. This has been presented in several reviews (Bapat et al., 2010; Lelièvre et al., 1998; Matas et al., 2009; Pech et al., 2008; Prasanna et al., 2007). Furthermore, a competitive ethylene inhibitor, 1-methylcyclopropene (1-MCP) has also been used as a tool for fruit classification (Watkins, 2006). The effect of 1-MCP inhibition towards climacteric fruit is also widely known, having been reviewed by Huber (2008) and Martínez-Romero et al. (2007). A recent study by Moradinezhad et al. (2010) showed that 1-MCP application on a climacteric fruit, banana, delayed its ripening process. Meanwhile, tomato treated with 1-MCP also exhibited the same effect where some characteristics of fruit ripening such as colour changes and textural softening were retarded (Guillén et al., 2007).

The effects of exogenous ethylene application and 1-methylcyclopropene on non-climacteric fruit ripening have been debatable (Huber, 2008; Watkins, 2006). One would expect that non-climacteric fruit does not respond to ethylene application and inhibitor treatment as they seem to be nearly independent of ethylene during ripening. In fact, non-climacteric fruit does have some sensitivity, to a certain extent, towards ethylene treatment and inhibition. For example, Villarreal et al. (2010) found that ethephon treatment accelerated some ripening aspects during strawberry ripening such as chlorophyll degradation, sugar changes and carotenoid accumulation while 1-MCP had an inverse

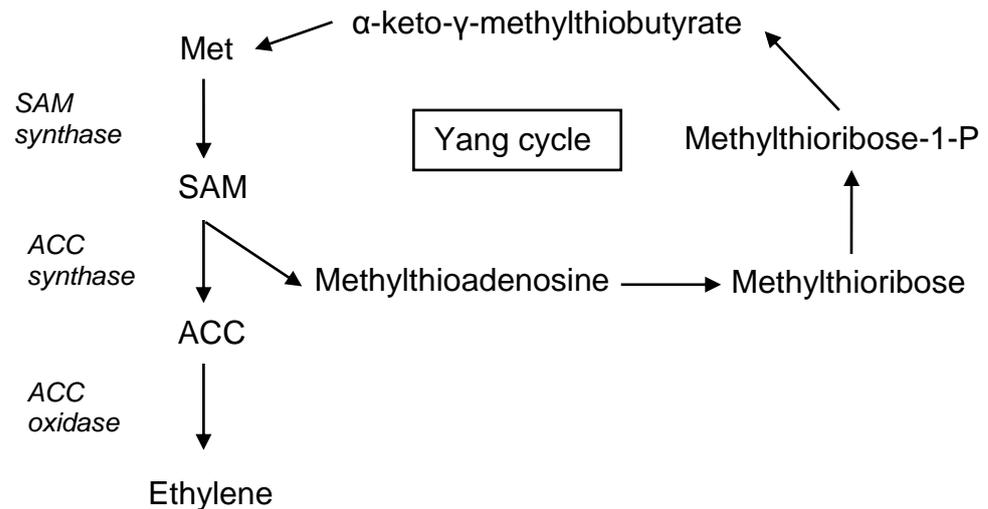
effect. Furthermore, 1-MCP has also been shown to reduce cell wall breakdown of ripening loquat (Wang et al., 2010), while the treatment of citrus fruit with propylene reduced the endogenous production of ethylene (Katz et al., 2004).

There are two systems controlling endogenous ethylene production in plants, System 1 and System 2 (Barry et al., 2000; Lelièvre et al., 1998; McMurchie et al., 1972). System 1 is responsible for minimal ethylene production in all plant tissues during growth including non-climacteric fruit. Furthermore, exogenous application of ethylene seems to negatively regulate this system (Katz et al., 2004). Meanwhile, System 2 appears to be mainly active during the ripening process of climacteric fruit and hence the climacteric increase of ethylene. Unlike System 1, System 2 is an autocatalytic system where ethylene existence increases biosynthesis of ethylene and respiration. In climacteric fruit, System 1 occurs before climacteric patterns emerge in System 2. Non-climacteric fruit generally only exhibit System 1 throughout ripening but System 2 may exist even earlier before ripening onset in fruit like citrus (Katz et al., 2004), System 2 may actually exist even earlier before ripening onset. One probable explanation for this finding is that System 2 may be necessary for preparing the non-climacteric fruit to ripen with minimal ethylene production (in System 1). Undeniably, more research is required to rationalise this deduction and unravel the true role of ethylene in ripening promotion of non-climacteric fruit. Regardless of the system, the ethylene biosynthesis pathway is still the same and this is detailed in section 1.2.2.

### *1.2.2 Ethylene biosynthesis and the role of the ripening transcription factor (RIN)*

The biosynthesis of ethylene and the methionine recycling pathway called the Yang cycle are shown in Figure 1.2. In the biosynthesis pathway, methionine (Met) is converted to S-Adenosyl-Methionine (SAM) by SAM synthase before conversion to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS). The last step of

ethylene biosynthesis is ACC conversion to ethylene (C<sub>2</sub>H<sub>4</sub>) by ACC oxidase (ACO). Meanwhile, the Yang cycle recycles back methylthioadenosine molecules which are the by-product of ACC synthase activity. This cycle is important because it keeps sufficient Met for subsequent ethylene synthesis (Génard and Gouble, 2005; Taiz and Zeiger, 2010).



**Figure 1.2** Biosynthesis of ethylene from methionine (Met). The by-product of S-Adenosyl-methionine (SAM) conversion to 1-aminocyclopropane-1-carboxylic acid (ACC), methylthioadenosine is recycled back to Met in the Yang cycle. Figure modified from Taiz and Zeiger (2010) and Bapat et al. (2010). Enzymes are shown in italics.

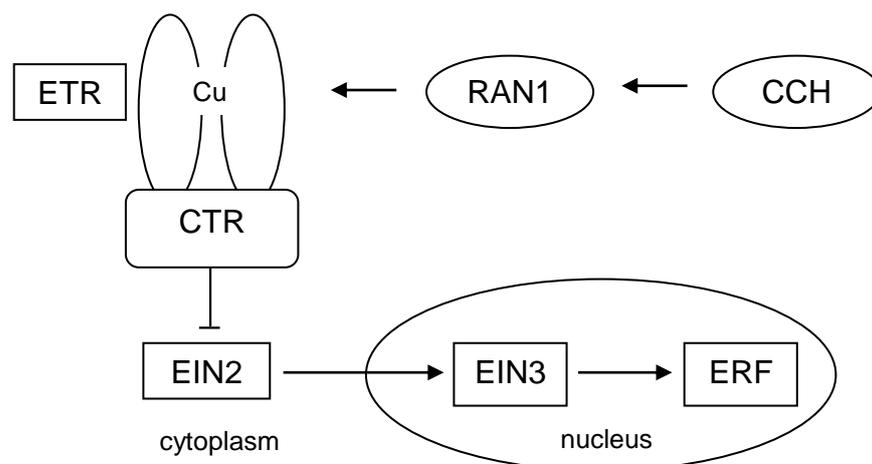
The main enzymes that regulate ethylene biosynthesis (ACS and ACO) seem to be up-regulated on several occasions including during the onset of ripening. There are at least nine gene homologues for ACS and six ACO homologues in tomato (Barry et al., 2000; Lin et al., 2009) and interestingly, these homologues are differentially regulated to control System 1 and System 2 ethylene production in tomato ripening (Figure 1.3) (Barry et al., 2000). The minimal production of ethylene during ripening of non-climacteric fruit may be due to a lack of some of these homologues as seen in non-climacteric melon (Zheng et al., 2002), pear (Yamane et al., 2007) and plum (El-Sharkawy et al., 2008). However, this needs further investigation as does the reason behind the differential expression of some of these homologues in tomato. Even so, a transcription factor mutant in tomato, *ripening-*

*inhibitor (rin)* down-regulated certain *ACS/ACO* homologue expression (Barry et al., 2000; Yokotani et al., 2004) and ceased all ripening aspects (Vrebalov et al., 2002), thus suggesting that the transcription factor plays a role in the ripening of climacteric fruit.

The *rin* mutant still retains an intact ethylene biosynthetic pathway (Vrebalov et al., 2002) suggesting that the transcription factor is one of a group of ripening regulators which acts upstream of ethylene induction (Giovannoni, 2007). Furthermore, these ripening regulators may actually be conserved among fruit types including non-climacteric fruit; and indeed strawberry *RIN* expression has been shown to be closely related to its ripening onset (Vrebalov et al., 2002). However, the central role of *RIN* in ripening of non-climacteric fruit especially in capsicum is still inconclusive. Vrebalov et al. (2002) found that an expressed sequence tag (EST) sharing homology with *RIN* was only isolated in pathogen-infected pepper while Moore et al. (2005) showed that there was limited expression of MADS box genes (which may include *RIN*) in different stages of pepper ripening, unlike tomato. Conversely, a microarray analysis by Lee et al. (2010) showed that *RIN* was up-regulated during capsicum ripening but surprisingly not during tomato ripening. This implies that more research is required to decipher the global regulatory control of fruit ripening prior to the ethylene regulatory pathway. Moreover, ethylene perception may also play a role in regulating System 1 and System 2 ethylene production during fruit ripening and this is detailed in the next section.



presence of ethylene, the receptors are bound and CTR is inactivated. EIN2 is switched on and the signalling is then relayed into the nucleus to activate the EIN3 transcription factor (TF) as well as other EIN3-like (EIL) TFs. Subsequently, EIN3 and EILs modulate gene expression corresponding to ethylene response. EIL proteins in climacteric melon (CmEIL) were shown to positively up-regulate *ACO* expression in a yeast-one hybrid experiment suggesting direct regulation of ethylene synthesis by EIL (Huang et al., 2010). Another TF which acted downstream of EIN3, Ethylene Response Factor (ERF) was also shown to regulate ethylene responsive genes, *ACS* and *ACO* expression in tomato (Zhang et al., 2009b). Furthermore, the role of these TFs (EIN3, EIL, ERF) have been implicated in various ripening processes such as sugar metabolism, cell wall breakdown and biosynthesis of pigments (Bapat et al., 2010; Seymour et al., 2013).



**Figure 1.4** Ethylene signalling pathway in plants [figure adapted from (Lin et al., 2009)]. EThylene Receptors (ETR) require Cu (copper) as a cofactor which is delivered by Copper CHaperone (CCH) and Responsive to ANtagonist1 (RAN1) proteins, sequentially (Woeste and Kieber, 2000). Without ethylene stimulation, the ETR are active to stimulate the Constitutive Triple Response (CTR) protein which suppresses Ethylene Insensitive2 (EIN2) and the subsequent downstream ethylene signalling pathway. When ethylene binds to the receptors, CTR is no longer activated and this will initiate the downstream pathway by removing the inhibition of EIN2. EIN2 activates transcription factor (TF) EIN3 in the nucleus which then activates another TF, Ethylene Response Factor (ERF). The activation of these transcription factors will regulate other downstream ethylene response genes.

In non-climacteric fruit, most studies have focused on isolating components of ethylene perception and signalling to verify its nature with some surprising results. For instance, three different ethylene receptor transcripts were isolated from non-climacteric loquat and were highly expressed during ripening (Wang et al., 2010), a piece of evidence that shows non-climacteric fruit also may have basal responses to ethylene. EIL-like protein was also found to be up-regulated at the onset of ripening in a non-climacteric pepper suggesting that ethylene or at least part of its pathway are needed for the ripening process in non-climacteric fruit (Lee et al., 2010). Growing evidence of ethylene response in non-climacteric fruit led to a challenge of the usual classification of fruit based on ethylene production by Lin et al. (2009). This notion however, still needs to be clarified by future experiments.

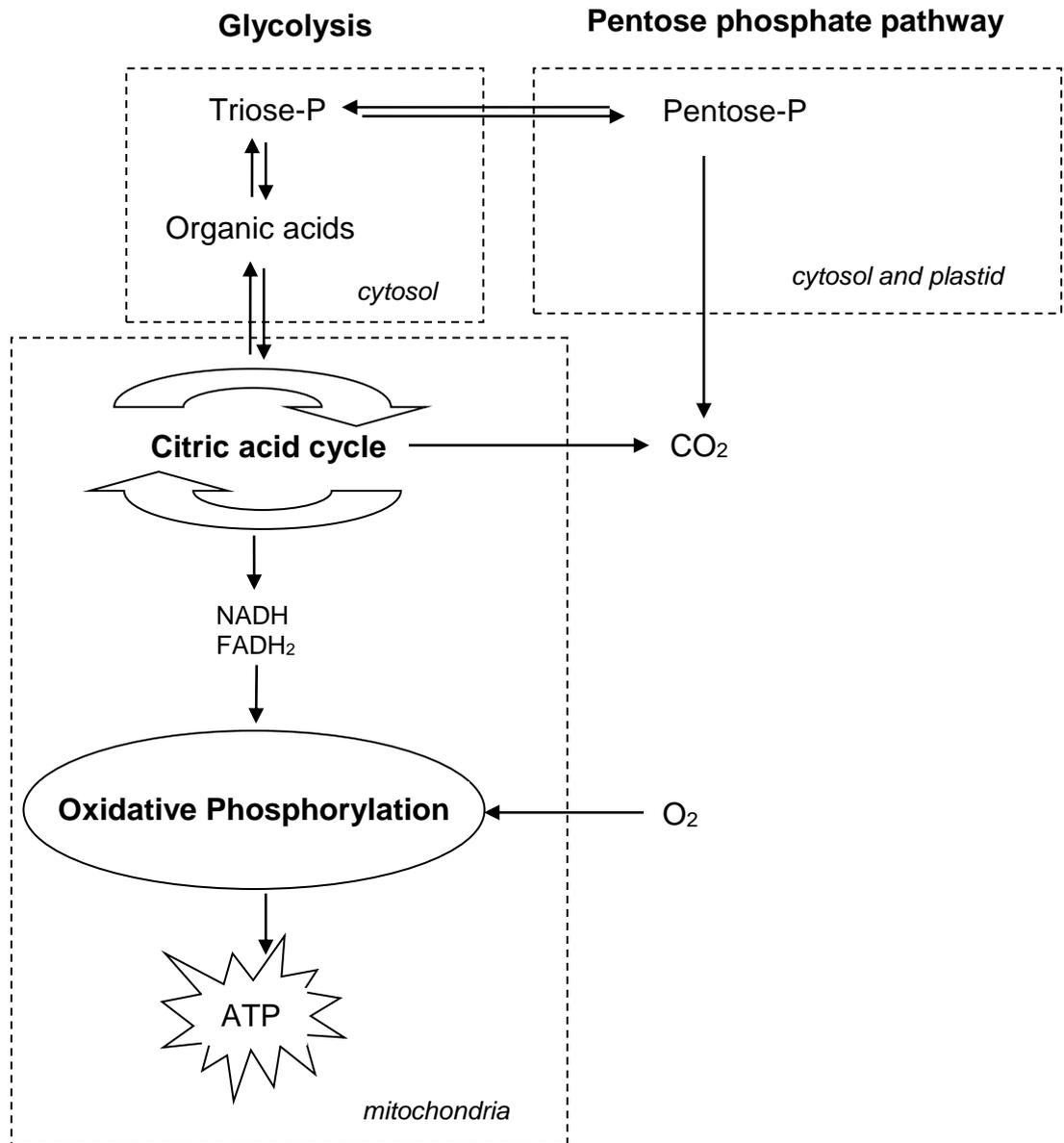
Research in fruit such as melon (Huang et al., 2010), guava (Liu et al., 2012b) and capsicum (Klieber, 2000) that have cultivars of either climacteric or non-climacteric behaviour may also assist in research of fruit ripening promotion. This is because these fruit cultivars may have several biochemical pathways but only differ in the most necessary pathway. Surprisingly, the ripening of these fruit has not been well studied and this will provide an interesting area to be explored. In climacteric fruit, the ethylene surge is usually followed by a respiration increase whereas non-climacteric fruit exhibit respiration levels that gradually decline during ripening with minimal ethylene production.

#### *1.2.4 Respiration of fruit during ripening*

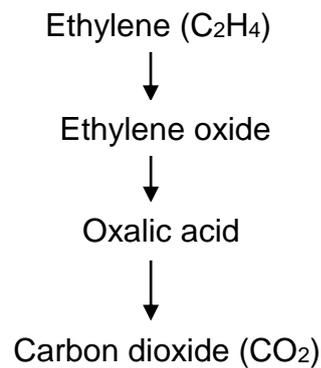
Though respiratory increase during the climacteric stage is well documented, the motive behind the process is poorly understood (Giovannoni, 2004; Lelièvre et al., 1998; Seymour et al., 1993). There are probably three plausible reasons for climacteric respiration. First, energy in the form of ATP is used to drive basic changes of ripening, for example synthesis of carotenoids and new amino acids (Seymour et al., 1993). The energy processing cycle,

called the respiratory cycle, is therefore up-regulated and produces CO<sub>2</sub> as a by-product (Figure 1.5 A) (Seymour et al., 1993). Since non-climacteric fruit are still able to ripen while inducing mostly the same ripening changes as in climacteric fruit independent of respiration increase (Bapat et al., 2010; Pham, 2007), this may not be accurate. However, energy may also contribute to the maintenance of homeostasis especially during the degradation of chloroplasts to chromoplasts (Romani 1984). Homeostasis may therefore be more easily maintained in non-climacteric tissue such that an increase in respiration is not required to the same extent as in climacteric tissue. Alternatively, in non-climacteric fruit, the membrane changes might proceed more slowly than climacteric fruit limiting respiration. The second reason is that the ATP is also required during the ethylene synthesis especially for feeding the Yang cycle (Figure 1.2) (Génard and Gouble, 2005) and hence CO<sub>2</sub> will be generated from the respiratory pathway. Lastly, ethylene can be readily oxidised to CO<sub>2</sub> in plant cells (Taiz and Zeiger, 2010) and thus the rate of the oxidation pathway (Figure 1.5 B) can be increased during the climacteric peak of ethylene. Since ethylene production is minimal during non-climacteric fruit ripening, there is no need to either increase ATP production to maintain the ethylene level or to induce the ethylene oxidation pathway, and hence reduce the respiration level.

A)



B)



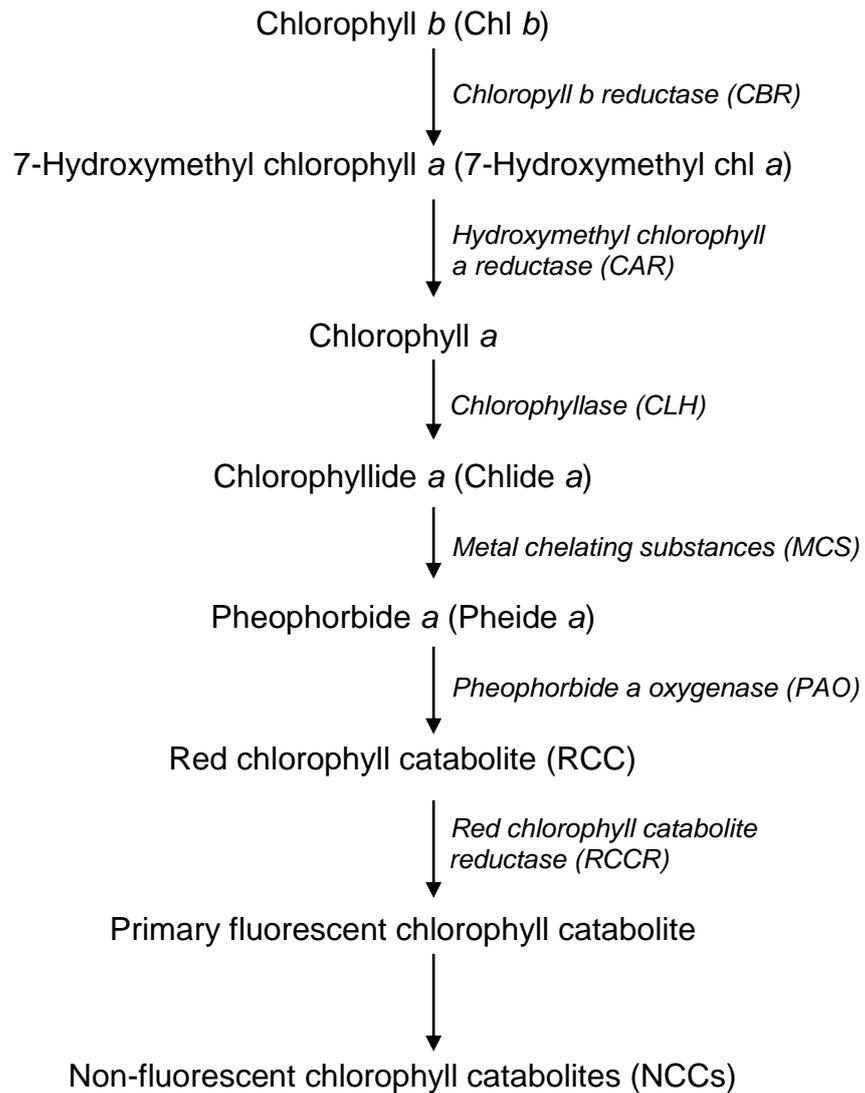
**Figure 1.5** A) The respiratory cycle in a plant cell. The major processes are bolded and organelles where the reaction takes place are in italics. Only major products and substrates are shown. The citric acid cycle is also known as the tricarboxylic acid (TCA) cycle or Krebs cycle. NADP, Nicotinamide adenine dinucleotide phosphate; FADH<sub>2</sub>, Flavin adenine dinucleotide-reduced form; ATP, Adenosine triphosphate [adapted from Taiz and Zeiger (2010)]. B) The oxidation pathway of ethylene [adapted from Taiz and Zeiger (2010)].

### *1.2.5 Colour-related processes that occur during ripening*

The final product of ripening is generally a different colour than the initial mature fruit prior to ripening. Usually, fruit ripens from green to other colours such as red and this phenomenon is caused by chlorophyll breakdown and carotenoid accumulation events. These events are detailed in the next subsections.

#### *1.2.5.1 Chlorophyll degradation*

At the onset of ripening, one of the earliest biochemical changes is chlorophyll breakdown which causes loss of green colour in fruit. The colour loss is mainly attributed to the conversion of fluorescent components of chlorophylls to non-fluorescent components of catabolites (details in Figure 1.6). More specifically, the enzyme Pheophorbide *a* oxygenase (PAO) which catalyses Pheophorbide *a* (Pheide *a*) to Red chlorophyll catabolite (RCC) is the critical enzyme for disappearance of the green colour. Hence, several studies of stay-green phenotype mutants in ripening fruit have shown that these mutants are deficient in the RCC compounds (Barry, 2009; Barry et al., 2008; Borovsky and Paran, 2008; Park et al., 2007; Roca and Minguéz-Mosquera, 2006). Furthermore, the gene expression of another important chlorophyll degrading enzyme, *Chlorophyllase (CLH)* was shown to be significantly up-regulated during ethylene treatment of mature mandarin, further suggesting the role of ethylene during ripening induction (Barry, 2009; Fujii et al., 2007). Moreover, during the ripening process, chloroplasts are degraded to much less organised organelles called chromoplasts which accumulate carotenoids (Roca and Minguéz-Mosquera, 2006).

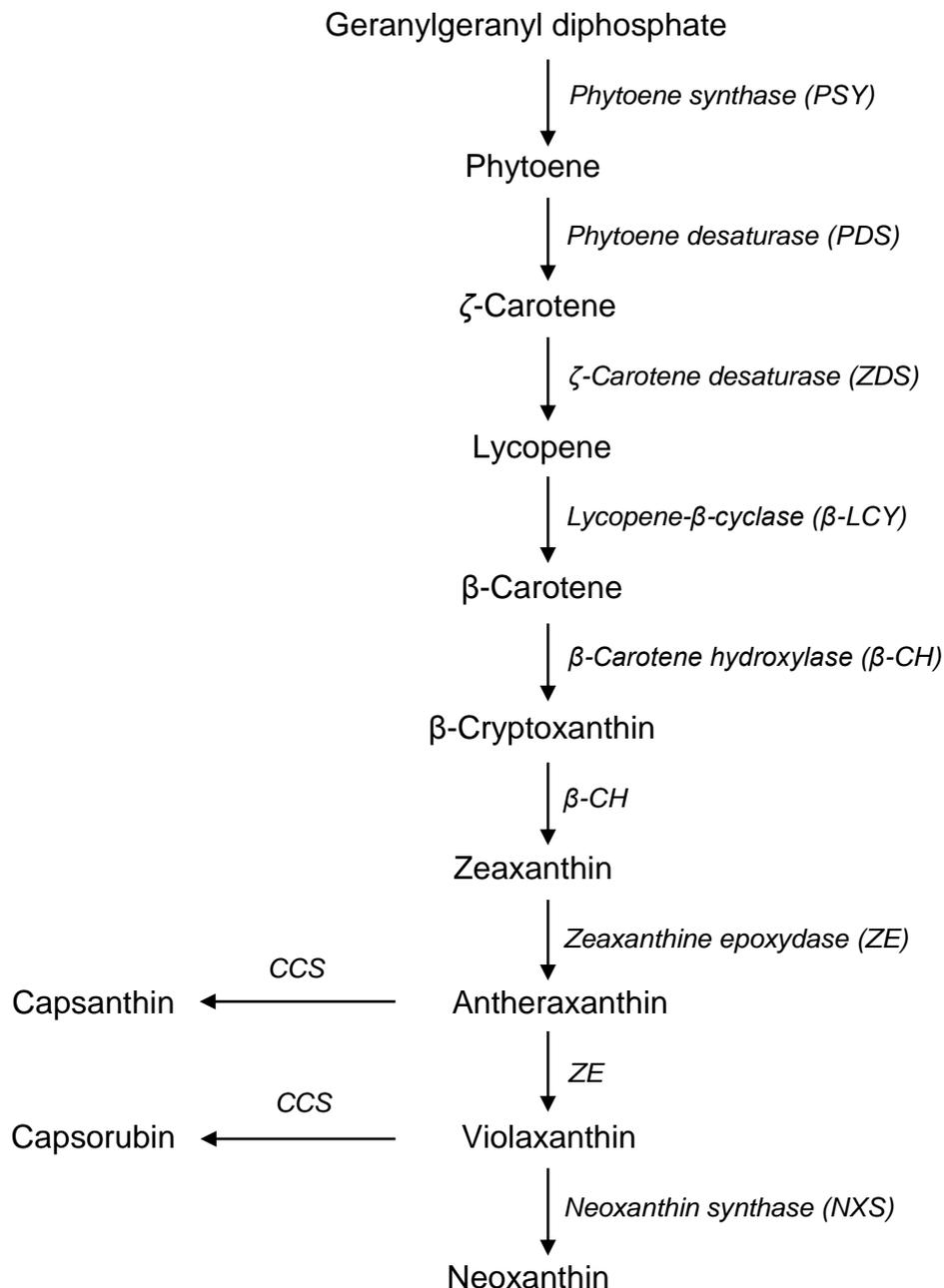


**Figure 1.6** The chlorophyll degradation pathway in plants [modified from (Barry, 2009)]. Enzymes are shown in italics.

#### 1.2.5.2 Carotenoid biosynthesis and accumulation

Carotenoids are essentially metabolites that can contribute to pigmented colours. Studies have shown that the composition of different carotenoids will determine the end-product colour of a ripening fruit when coupled with the previously described chlorophyll degradation (Giovannoni, 2004). For example, capsanthin and capsorubin are the carotenoids responsible for red coloured capsicum while different coloured capsicums such as white, orange and yellow capsicums accumulate different carotenoids (Guzman et al., 2010; Ha et al., 2007). Figure 1.7 shows the biosynthetic pathway of carotenoids in red

capsicums. Several carotenoid biosynthesis genes [*Phytoene synthase (PSY)*, *Phytoene desaturase (PDS)* and *ζ-Carotene desaturase (ZDS)*] have been shown to respond to ethylene treatment in varieties of apricots (Marty et al., 2005), implying that ethylene may also responsible for colour related changes in this fruit, at least, during ripening.



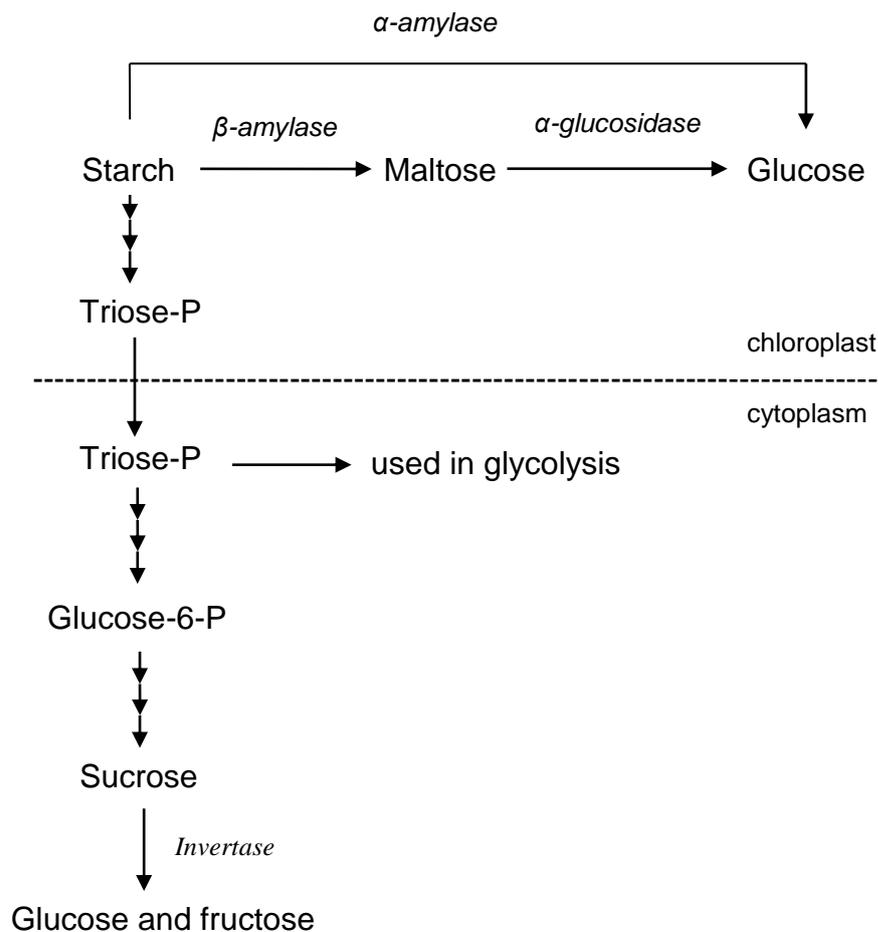
**Figure 1.7** Biosynthesis of carotenoids in capsicum (Guzman et al., 2010; Ha et al., 2007). CCS, Capsanthin-capsurobin synthase enzyme. Enzymes are shown in italics.

## *1.2.6 Other processes that occur during ripening*

### *1.2.6.1 Sugar changes*

Sweetness of fruit is one of the important aspects in edibility as well as marketability. Sugar can be obtained from assimilates of other photosynthetic organs, translocated actively into fruit during its development (Yamaki, 2010) or be derived from starch. Starch metabolism to less complex molecules like sucrose, glucose and fructose (Figure 1.8) is the main contributor to sweetness and this process is enhanced during fruit ripening (Nielsen et al., 1991; Seymour et al., 1993) and ethylene treatment (Bapat et al., 2010). These simple sugars were shown to be markedly increased in ripening fruit as seen in tomato, capsicum and others (Gautier et al., 2008; Matsufuji et al., 2007; Serrano et al., 2010).

Starch is mainly stored in the chloroplast as it is synthesised locally during photosynthesis (Seymour et al., 1993). Starch can be converted into simpler molecules like glucose inside the chloroplast itself by the enzymatic action of  $\alpha$ -amylase as well as through a maltose intermediate catalysed by  $\beta$ -amylase and  $\alpha$ -glucosidase, sequentially. Nevertheless, most of the sugar conversion takes place in the cytoplasm where glycolysis (Figure 1.5 A) of sugar is active (Seymour et al., 1993; Taiz and Zeiger, 2010). Therefore, during fruit ripening when chloroplasts are disintegrating, increased starch is likely to be free in the cytoplasm for sugar conversion.

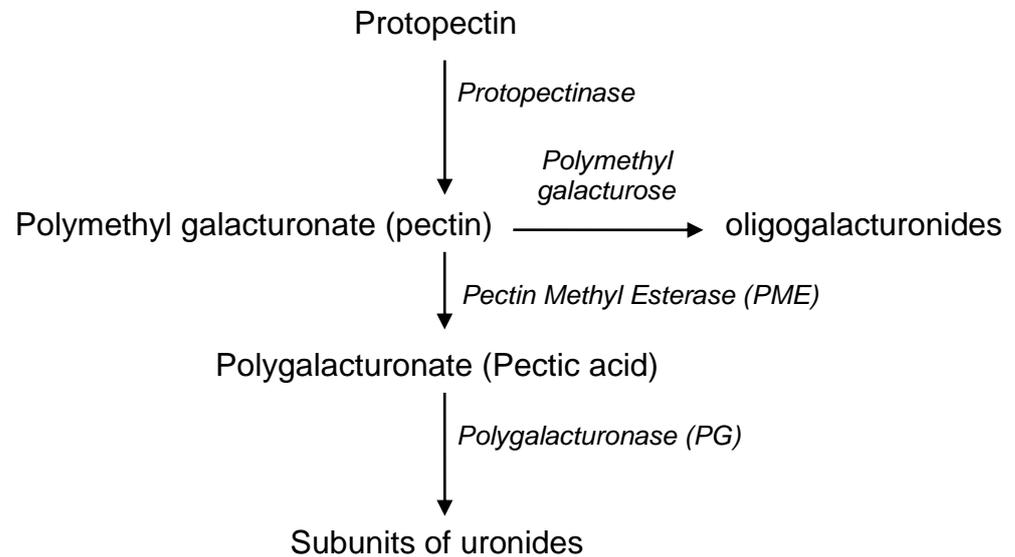


**Figure 1.8** The starch metabolism pathway in plant cells [figure modified from Seymour et al. (1993)]. Only key enzymes (in italics) and products are shown. The triose-P is used in the glycolysis pathway, as shown in Figure 1.5 A.

### 1.2.6.2 Fruit softening

Textural softening is one of the most notable changes during fruit ripening. This change can be attributed to starch breakdown as well as loss of cell turgor and cell wall integrity (Prasanna et al., 2007; Seymour et al., 1993). Many polysaccharides are localised in the plant cell wall, contributing to the mechanical strength of the cell wall and at large, the plant and the fruit itself. Amongst these cell wall polysaccharides such as cellulose and hemicellulose, pectins may be regarded as the backbone of the cell wall maintaining its rigidity (Prasanna et al., 2007; Seymour et al., 1993). Hence, many studies have shown that the breakdown of pectin (Figure 1.9) is responsible for fruit softening during ripening (Ali

et al., 2004; Cheng et al., 2008; Sethu et al., 1996). Ethylene has also been implicated in increasing the expression of the *Polygalacturonase* gene in papaya (Fabi et al., 2009), another instance of where ethylene regulates ripening in fleshy fruit.



**Figure 1.9** The metabolism pathway of pectins [figure modified from Prasanna et al. (2007)]. Enzymes are shown in italics.

### *1.2.7 Ripening changes in capsicum*

Ripening in capsicum generally exhibit characteristics as detailed above. Furthermore, capsicum cultivars can be either climacteric or non-climacteric as described by Pham (2007). The non-climacteric capsicum has been shown to ripen when harvested at Breaker stage but not earlier (Krajayklang et al., 2000; Pham, 2007). The reason for this behaviour is not well understood and thus comparison-based approaches can be utilised to investigate the presence of differentially expressed molecules in different stages of capsicum ripening that may induce the process.

### **1.3 Comparison-based approaches in ripening research**

The biochemical networks presented earlier are based on well established concepts and research. However, most of these studies relied on experiments that analyse individual components of a biochemical pathway and not comparisons of multiple components at the protein and metabolite levels. Hence, comparison-based approaches such as proteomics and metabolomics are increasingly important to assist researchers in identifying and characterising the molecules responsible for certain biochemical processes. Here, the use of these two approaches in respect to ripening research is discussed and compared.

Proteomics is a study of differences at protein level and can be both global and targeted (Palma et al., 2011). In the global approach, total protein composition is analysed while the targeted approach only considers a specific type of protein. The global approach seems to be the best method for early analysis and comparison because the resulting data is broader and covering nearly the whole proteome (Rose et al., 2004). One example of a global approach in proteomics is using 2D-gel electrophoresis (2DGE) coupled with tandem mass-spectrometry (MS/MS). The 2DGE separates proteins based on polypeptide charges and their molecular weight. By using total protein extracts at different ripening stages, differences in protein profiles can be investigated. Selected protein spots that are different on the gel can then be isolated and their peptide sequences determined using MS/MS. Several studies have used this strategy to compare protein characteristics during ripening, for example in banana, apple, peach, strawberry and tomato (Bianco et al., 2009; Faurobert et al., 2007; Nilo et al., 2010; Song et al., 2006).

After identifying several differentially expressed proteins, a more targeted approach is needed to thoroughly examine the expression of these proteins in different ripening stages. Two examples of a directed proteomics method are the use of enzymatic activity assays and specific antibodies to detect and confirm the expression of selected proteins. Interestingly, there are still not many studies in ripening research that go beyond the global

approach in order to examine all identified ripening-specific proteins. This is probably because of the massive protein variation between ripening stages and targeting each of these proteins may not seem feasible. However, targeting specific proteins that are known to regulate certain components of ripening biochemistry is possible. For example, enzymes responsible for fruit softening in papaya and grapes (endoxy lanase and  $\beta$ -glucanase, respectively) have been targeted using antibodies to examine their expression over ripening stages (Manenoi and Paull, 2007; Wang et al., 2009).

In addition, metabolomics examines the products of biochemical pathways (metabolites) that have taken place in plant cells (Baginsky et al., 2010). Though metabolomics is a relatively new area of study (Villas-Boas et al., 2005), its usefulness cannot be denied as it covers more aspects than proteomics research, including the release of volatiles such as ethylene (Tikunov et al., 2005). Other metabolites such as lipids, sugars and amino acids are also detectable using metabolomics, although a method where these metabolites are converted into their volatile derivatives first, using certain chemical reactions, is required (Fiehn, 2002). Separating techniques such as gas chromatography (GC) are then used and coupled with MS for molecule identification (Liu et al., 2009). Though this approach is a very useful method, research comparing different fruit ripening stages has been limited except in climacteric tomato (Moco et al., 2007; Tikunov et al., 2005). Studies in other fruit types such as pepper are therefore necessary to elucidate more information about fruit ripening, particularly in non-climacteric fruit.

A combination of the -omics technologies can be a powerful tool for comparison and discovering novel cellular networks. However, this is rarely used as the data generated is massive and requires an expert level of bioinformatics analysis. Recently, Barros et al. (2010) reported the comparison of genetically modified (GM) maize when grown in different environmental factors using three different approaches (transcriptomics, proteomics and metabolomics). The study successfully isolated differentially expressed

transcripts, proteins and metabolites but no correlation between these molecules could be made (Barros et al., 2010). This shows that integrating the data and information between different profiling can be quite challenging and sometimes gives unexpected results especially when dealing with both endogenous and external variables. In terms of fruit ripening, the combination of these approaches to compare different stages is still narrowly documented. Therefore, using different approaches to discover the regulation of ripening is promising as the variable factors may only be present endogenously and may not involve changing environmental factors as in Barros et al. (2010).

A comprehensive analysis from the current literature using different comparison-based approaches to isolate stage-specific molecules is presented in Table 1.1. Two closely related Solanaceae family members, tomato and pepper are contrasted to give an overview of the differentially expressed molecules between the different ripening nature (climacteric and non-climacteric, respectively) of these fruit. Only Breaker and the earlier ripening stage (Green) are compared because Breaker is always considered to be the first stage where ripening has been initiated by an unknown signal (Pham, 2007). Thus these two stages are compared in order to list the ripening-related molecules that may assist further development in fruit ripening research.

**Table 1.1.** Ripening related proteins/transcripts in Solanaceae identified during Breaker (B) but not earlier (↑ at B) or vice versa (↓ at B). More specifically, as the generated data is substantial, molecules which are present or absent between these stages as well as being discussed in the cited manuscript with particular interest to fruit ripening, are listed. ACC, 1-aminocyclopropane-1-carboxylate; 2DGE, 2D gel electrophoresis; GC-MS, gas chromatography-mass spectrometry; E4 protein, peptide methionine sulphoxide reductase; EREBP, ethylene-responsive element binding protein transcription factor; HSP, Heat shock protein; LC-MS, liquid chromatography-mass spectrometry; MALDI-TOF/MS, matrix-assisted laser desorption ionisation-time-of-flight mass spectrometry; MS/MS, tandem mass-spectrometry; PG, polygalacturonase; PSY, phytoene synthase; qPCR, quantitative real-time polymerase chain reaction; TF, transcription factor; <sup>a</sup> soluble sugars; <sup>b</sup> sugar alcohols; <sup>c</sup> organic acids; <sup>d</sup> TCA cycle intermediate; <sup>e</sup> isoprenoids; <sup>f</sup> flavonoids; \* fruit ripening protein (accession number O82575) named as such by Iusem et al. (1993) because of its abundance (5% of total mRNA) in ripe tomato fruit. It is also known as an ASR [abscisic acid (ABA), stress and ripening] protein but its proposed function in sugar trafficking is still unclear (Srivastava and Handa, 2005).

Fruit	Techniques	Differentially-expressed molecules		References
		↑ at B	↓ at B	
<i>climacteric</i> <b>Tomato</b>	2DGE, MS/MS and MALDI-TOF/MS	HSP 21 and 70, acid invertase, E8 and E4 proteins, lipocalin, $\alpha$ and $\beta$ -galactosidase, $\beta$ -1,3-endoglucanases, ER6-like stress protein, NADP <sup>+</sup> -specific isocitrate dehydrogenase	3- Phosphoglycerate dehydrogenase (PGDH), Thiamin biosynthetic enzyme	(Faurobert et al., 2007)
	2DGE, MS/MS, qPCR and microarray	ACC synthase and oxidase, PSY, glucuronosyl transferase homolog, PG-2 $\alpha$ , E8 protein, putative pectate lyase, $\beta$ -galactosidase, arginine decarboxylase, invertase, Glutathione S-transferase (GST)	Pectinase, phenylalanine ammonia-lyase, alcohol dehydrogenase	(Kok et al., 2008)
	2DGE, MS/MS and MALDI-TOF	E4 protein, embryo-abundant protein (EMB) protein, fruit ripening protein*, lipocalin, protein phosphatase-2C (PP2C)	ACC oxidase	(Rocco et al., 2006)
	GC-MS	Arabinose <sup>a</sup> , mannitol <sup>b</sup> , glycerol <sup>b</sup> , glycerate <sup>c</sup> , malate <sup>d</sup> , fumarate <sup>d</sup> , succinate <sup>d</sup> , $\alpha$ -ketoglutarate <sup>d</sup> , citrate <sup>d</sup>	rhamnose <sup>a</sup>	(Carrari et al., 2006)
	LC-MS	All- <i>trans</i> -lycopene, violaxantine <sup>e</sup> , ascorbic acid, Rutin and naringenin chalcone <sup>f</sup>	Chlorophyll <i>a</i> and <i>b</i> , tocopherol, kaempferol-rutinoside <sup>f</sup>	(Moco et al., 2007)
	EST digital expression analysis	ACC oxidase1, E8 protein, NR receptor, lipoxygenase, PG 2S precursor, histidine carboxylase, PSY, putative acyltransferase, pectate lyase, MADS box genes	B-subunit PG1, EREBP (TC116320)	(Fei et al., 2004)

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<i>non-climacteric</i> <b>Pepper</b>	microarray	<i>EIL</i> -like, $\beta$ -hydroxylase like, <i>Cystathionine gamma synthase (CGS)</i> -like, <i>MADS-RIN</i> genes, <i>ASR</i> gene*	<i>ACC synthase</i> and <i>oxidase</i>	(Lee et al., 2010)
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#### 1.4 Research rationale

The molecular mechanisms of ripening in non-climacteric fruit are still not well characterised. Moreover, Breaker-harvested capsicum is able to ripen but not Green-harvested capsicum. Hence, this project aimed to address the following questions:

- i. Are there any stage-specific molecules as determined by proteomics (Chapters 2 and 3) and metabolomics (Chapter 4) in non-climacteric capsicum fruit that may play a role in ripening?
- ii. Are there any selected stage-specific molecules differentially distributed in different capsicum tissues and cultivars (Chapter 5)?
- iii. Do ethylene or 1-methylcyclopropene treatments affect a selected stage-specific candidate and components of ethylene pathway in capsicum (Chapter 6)?
- iv. Are there any other selected candidates particularly from the proteomic study (Chapter 3) which can be further studied to confirm their expression levels during capsicum ripening (Chapter 7)?

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**Chapter 2: Preliminary studies for the proteomic  
analysis of capsicum and the characterisation of  
capsicum ripening behaviour (cv. Aries)**

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## 2.1 Introduction

Fruit ripening involves changes in colour, texture and taste to ensure the fruit becomes more palatable for seed dispersal agents such as animals (Rodríguez et al., 2013). Fruit are also divided largely into two different groups, climacteric and non-climacteric fruit, based on their ripening physiology. Climacteric fruit such as banana, apple and tomato increase their respiration and ethylene production during ripening. Non-climacteric fruit, such as strawberry, grapes and capsicum, have relatively limited increases in respiration and ethylene production during ripening and generally do not respond to ethylene exposure (Barry and Giovannoni, 2007; Seymour et al., 2013).

Comparison-based approaches have been utilised to give an overview of the ripening process of various fruit including both climacteric and non-climacteric fruit. These approaches have used techniques such as microarray (transcriptomics), 2D gel electrophoresis (2DGE, proteomics) and Gas Chromatography-Mass Spectrometry (GC-MS, metabolomics). However, gene expression differences, measured by transcriptomics, do not always correspond to the downstream biochemical processes. This is due to possible post-translational modifications which affect protein abundance but not the gene expression (Hertog et al., 2011). Therefore, the utilisation of proteomics and metabolomics which investigate samples at the level of proteins and metabolites will give a better indication of biochemical activities, which in this case, leads to the changes in ripening.

Tomato has been the primary model fruit used to study ripening (Giovannoni, 2004) and a number of studies utilising tomato in these comparison-based approaches have already been reported (Alba et al., 2005; Carrari et al., 2006; Faurobert et al., 2007; Fei et al., 2004; Kok et al., 2008; Moco et al., 2007; Rocco et al., 2006; Roessner-Tunali et al., 2003). Some non-climacteric fruit have also been investigated including strawberry (Aharoni and O'Connell, 2002; Bianco et al., 2009; Fait et al., 2008; Ponce-Valadez et al., 2009) and grapes (Giribaldi et al., 2007; Grimplet et al., 2007). From these studies, several

enzymes and metabolites related to the process of ripening such as carbohydrate modification and lipid metabolism are known to be differentially expressed in both ripening types (Palma et al., 2011). However, what regulates the two different types of ripening is still largely unanswered, possibly due to significant differences in genetic background (Pech et al., 2012). The use of capsicum which has a very similar genetic content (in the same Solanaceae family) as the model fruit tomato (Livingstone et al., 1999) may help better understanding between climacteric and non-climacteric ripening. However, there are still not many reported studies in capsicum especially utilising the comparison-based approaches such as proteomics and metabolomics.

The primary aim of the work presented in this chapter was to optimise 2D gel electrophoresis (2DGE) for the proteomic study of capsicum ripening. Most of the 2DGE techniques had been optimised previously in our laboratory by Khoo et al. (2012) but using bread wheat anthers. Therefore pilot studies were needed to confirm the optimised running conditions for capsicum fruit. Different protein extraction methods were also investigated for choosing the most consistent and high protein yield method to be used in the downstream 2DGE. Different pH ranges of 2DGE were also tested for better visualisation and separation of the capsicum proteome. Different RNA extraction methods were also studied for gene expression analysis in the following chapters (Chapters 3, 6 and 7). Furthermore, capsicum plants were grown in the glasshouse to characterise the ripening behaviour of the fruit for corresponding studies in the proteomics (Chapter 3) and metabolomics (Chapter 4) of capsicum ripening.

## **2.2 Materials and Methods**

### *2.2.1 Capsicum tissue samples*

Green (G) and Red (R) bell pepper capsicum were obtained commercially and only the capsicum flesh without seeds and placental tissues was used for pilot experimentation. The

flesh was cut into ~2 cm cubes, incubated immediately in liquid N<sub>2</sub> until frozen solid and ground in a laboratory blender (Multiblender, Sunbeam) until a fine powder was obtained. The powder was stored at -80°C until required.

### *2.2.2 Protein extraction*

Both trichloroacetic acid (TCA)/acetone and phenol extraction methods were adapted from Saravanan and Rose (2004) which extracted protein from tomato tissues for 2DGE. Both methods were repeated twice in order to assess their consistency.

#### *2.2.2.1 TCA/acetone method*

Ground tissues (1.5 g) were weighed in chilled 50 mL tubes and four volumes of ice-cold acetone mix [10% TCA, 1% polyvinylpolypyrrolidone (PVPP), 2% β-mercaptoethanol (βME)] were added before vortexing. The samples were rotated at 4°C for 30 min and placed at -20°C overnight. The samples were then centrifuged (4°C, 6000 g for 30 min) and the final supernatants were removed leaving intact pellets for protein solubilisation (see section 2.2.2.3).

#### *2.2.2.2 Phenol method*

Ground capsicum (1.5 g) was weighed in chilled 50 mL tubes and mixed with 15 mL extraction buffer 1 [1% PVPP, 0.7M sucrose, 0.1 M potassium chloride (KCl), 0.5 M tris(hydroxymethyl)amino methane (Tris)-Cl pH 7.5, 500 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethanesulfonyl fluoride (PMSF), 2% βME]. A similar volume of ice-cold phenol saturated in 0.5 M Tris-Cl pH 7.5 [prepared as per Sambrook and Russell (2001)] was added, the sample rotated at 4°C for 30 min and then centrifuged (4°C, 6000 g for 30 min). The upper phenol phase was collected into a new tube and re-extracted twice with extraction buffer 1 as described earlier. The final phenol phase was

resuspended in 5 volumes of 100 mM acetate in methanol and placed at -20°C overnight. The samples were pelleted using centrifugation (4°C, 6000 g for 30 min) and the supernatants were then discarded.

#### *2.2.2.3 Protein solubilisation and quantification*

Regardless of extraction method, protein pellets were washed once with 10 mL absolute methanol (ice-cold) and two to three times (until no colour was visible particularly for R samples) with 10 mL absolute acetone (ice-cold). Each wash comprised of vortexing for 1-2 min, up to 30 min rotation at 4°C and centrifugation at 4°C, 6000 g for 30 min. The final pellets were air-dried at room temperature (RT, 22-23°C) and solubilised in 1 mL resuspension buffer [7.0 M urea, 2.0 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 10 mM dithiothreitol (DTT)] for 1 h on an orbital shaker at RT. The samples were then centrifuged (10000 g, 4°C, 10 min) and only the clear protein extracts were transferred into 1.5 mL tubes for quantification and storage at -80°C.

Protein extracts were quantified as per Bradford (1976). Samples were diluted 1:100 and analysed in triplicate. Samples (800 µL) were mixed with 200 µL Bradford dye reagent (BioRad, CA, USA) in cuvettes and left at RT for 7 min. The absorbance was read at 595 nm wavelength using a UV/VIS Spectrophotometer SP 8001 (AdeLab Scientific, Thebarton, Australia). A standard curve of Bovine Serum Albumin (BSA, BioRad) was also generated using 12.7, 9.5, 6.4, 3.2 and 1.3 µg mL<sup>-1</sup>. The concentration of protein samples was estimated from the standard curve using Genstat Release 10.2 (VSN International, Hemel Hempstead, England).

### *2.2.3 Protein gels*

#### *2.2.3.1 Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)*

SDS-PAGE was run as per Sambrook and Russell (2001). Separating (12%) and stacking (4%) gels were prepared fresh in 1.0 mm cassettes (Invitrogen, CA, USA) as per manufacturer's instructions. After the gel polymerised, the cassettes were slotted into the XCell SureLock™ Mini-Cell (Invitrogen, CA, USA) and the cell was filled with 1x Novex® Tris-glycine SDS running buffer. Samples (5 µg) in 1x NuPAGE® lithium dodecyl sulfate (LDS) Sample Buffer (Invitrogen, CA, USA) were loaded and electrophoresed for 101 min (125 V, 30 mA). A Precision Plus Protein Standard (Bio-Rad) was also run as a marker in the same gel to estimate protein size.

#### *2.2.3.2 2D gel electrophoresis (2DGE)*

Protein samples (100 µg in 199 µL) were mixed with 1 µL 1% carrier ampholyte according to the immobilised pH gradient (IPG) strip used (for example, pH 3-10 ampholyte for running sample in pH 3-10 IPG strips). Bromophenol blue (0.1%) was also added to the sample before running on an 11 cm IPG strip (either pH 3-10, pH 4-7 or pH 7-10) (BioRad, Hercules, CA, USA) in the first dimension electrophoresis using an Ettan™ IPGphor™ II IEF System (Amersham Bioscience, USA). The focusing steps used were: 30 V for 14 h, 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 6000 V for 30 min (gradient) and 6000 V for 45 000 Vh. The strips were then equilibrated for 15 min in a 5 mL reducing equilibration buffer (6 M urea, 30% w/v glycerol, 2% w/v SDS, 50 mM Tris-Cl, 1% w/v DTT, pH 8.8) before another 15 min in a 5 mL alkylating equilibration buffer (6 M urea, 30% w/v glycerol, 2% w/v SDS, 50 mM Tris-Cl, 2.5 % w/v iodoacetamide, pH 8.8). The second dimension was run using 10% BioRad Criterion™ Xt Bis-Tris precast gels (11 cm, IPG + 1 well) slotted in a BioRad Criterion™ Cell. The gels with inserted strips were run in a 1x

MES [2-(*N*-morpholino) ethanesulfonic acid] SDS running buffer (NuPAGE® Invitrogen™, USA) for 45 min at 200 V. For protein size estimation, 5 µL of a Precision Plus Protein Standards (Bio-Rad) was also run in all 2D gels.

### 2.2.3.3 Staining

SDS-PAGE and 2D gels were stained using Coomassie Brilliant Blue R250 followed by eriochrome black T (EBT) silver staining according to Khoo et al. (2012). Briefly, the gels were first fixed by immersing the gel in fixative solution (10% acetic acid, 10% methanol, 40% ethanol) for 1 h. The gels were then sensitised for 2 h in sensitisation solution (1% acetic acid, 10% ammonium sulphate) before being soaked in staining solution (5% acetic acid, 45% ethanol, 0.125% Coomassie Brilliant Blue R250) overnight with gentle shaking. The gels were then destained with destaining solution 1 (5% acetic acid, 40% ethanol) for 1 h followed by destaining solution 2 (3% acetic acid, 30% ethanol) until no significant background colour was observed. Gels were imaged using an Epson Perfection Photo Scanner (Epson Australia Pty Ltd, Adelaide, SA). The gels were then either preserved in preservation solution (5% acetic acid) or continued to silver staining.

For EBT silver staining, the Coomassie-stained gels were fixed in fixative solution (30% ethanol, 10% acetic acid) for 20 min, twice. The fixed gels were then sensitised in sensitizer solution (0.006% EBT, 30% ethanol), destained in destain solution (30% ethanol) and washed twice with nanopure water (2 min for each step) before being immersed in freshly prepared silver solution (0.25% silver nitrate, 0.037% formaldehyde) for 5 min and washed with nanopure water for 20 s, twice. Gels were then soaked in freshly prepared developer solution (2% potassium carbonate, 0.04% sodium hydroxide, 0.002% sodium thiosulphate, 0.007% formaldehyde) until complete (black protein spots appearing) and the reaction was ceased in the stop solution (30% ethanol, 10% acetic acid) before significant background developed. The gel images were captured as mentioned

earlier for Coomassie stained gels and preserved in preservation solution (20% methanol, 7% acetic acid).

#### *2.2.4 RNA extraction*

Pilot experiments were performed using TRIzol and Phenol:chloroform methods to extract RNA from G and R capsicum samples.

##### *2.2.4.1 TRIzol*

This protocol was adapted from Pham (2007). Ground capsicum tissues (1 g) were transferred into chilled tubes and 10 mL TRIzol (Roche, Mannheim, Germany) added. The samples were vortexed for 1 min and incubated on ice for 1 min. The samples were centrifuged for 6000 g, 20 min at 4°C. The aqueous phase was transferred into a new tube and 5 mL of chloroform was added, thoroughly mixed and centrifuged as described earlier. This step was repeated once more before the upper aqueous phase was mixed with 5 mL isopropanol and incubated on ice for 5 min. The samples were centrifuged (6000 g, 20 min, 4°C) and the supernatants were removed. Pellets were washed with 2 mL 70% ethanol with a brief vortexing followed by a centrifugation (6000 g, 20 min, 4°C). The pellets were air-dried and resuspended in double sterilised nanopure water.

##### *2.2.4.2 Phenol:chloroform*

This method was based on Moore et al. (2005). Ground tissues (2 g) were added to 5 mL RNA extraction buffer [1 M Tris-Cl (pH 9.0), 1 M sodium chloride (NaCl), 0.2 M EDTA (pH8.0)] and 5 mL phenol:chloroform:isoamyl alcohol (25:24:1). Samples were vortexed and incubated on ice for 20 min before centrifugation (4°C, 5800 g, 20 min). The aqueous phase was transferred into a new tube and 3 mL isopropanol was added, before precipitation at -20°C for 1 h and centrifugation at 4°C and 5800 g for 20 min. Pellets were

washed with 70% ethanol and resuspended in diethylpyrocarbonate (DEPC)-treated water before a second precipitation using 4M lithium chloride (LiCl) (1:1 volume to sample) at -20°C for 1 h. Samples were centrifuged (4°C, 16000 g, 20 min) and pellets rinsed with 70% ethanol before resuspension in double sterilised nanopure water. To maximise solubilisation, samples were incubated at 60°C for 10 min (Chomczynski and Sacchi, 2006). Insoluble materials were removed by centrifugation (4°C, 16000 g, 15 min).

#### *2.2.4.3 RNA quantification and RNA visualisation*

Samples were quantified using a NanoDrop 1000 (Thermo Scientific, USA). Total RNA (5 µg) was then loaded into 1% agarose gel stained with ethidium bromide and electrophoresed for 15-20 min at 200 V. The gels were viewed and imaged using a FirstLight UV transilluminator (UVP, Upland, CA, USA). A 2-log DNA ladder (New England Biolabs, USA) was used for size estimation.

#### *2.2.5 Plant growth*

In order to check the best condition to grow capsicum, plants were grown as per Pham (2007) at the Waite Campus (latitude: -34° 58' 6.7", longitude: 138° 38' 28.47") under glasshouse conditions (24-25°C with natural sunlight). Briefly, capsicum (cv. Aries) seeds were germinated in petri dishes with moist Whatman paper for five to seven days. Germinated seeds were then transplanted into 6-cell punnets with University of California (UC)-Davis soil mix [comprising of one third peat moss with 1 kg m<sup>-3</sup> Ag-lime, 0.7 kg m<sup>-3</sup> hydrated lime and 2.25 kg m<sup>-3</sup> Osmocote® fertiliser (16 N: 3.9 P: 10 K: 1.5 Mg and trace elements, Scotts®)] and the other two thirds of washed Waikerie sand. When the fourth leaves had appeared, the seedlings were transplanted into 15 cm pots filled with UC-Davis soil and watered with reverse osmosis (RO) water every two-three days. The RO water was then supplied daily when capsicum plants developed more leaves (approximately four

weeks after transplanting). Seven weeks after transplanting, developing flowers at anthesis were tagged which later developed into fruit at approximately seven days after anthesis (DAA). At this stage, only one fruit per plant was chosen based on consistent size and shape before being allowed to mature. Osmocote® fertiliser was supplied once again when fruit reached maturity to maintain full nutrition to the plants. The length of time (or DAA) for fruit to reach various ripening stages may change according to the season (longer ripening time in winter due to less daylight time) as described by Pham (2007). Therefore, in all experiments, the size of fruit was carefully recorded as was the DAA for each stage. Furthermore, due to sporadic mite and aphid infection, appropriate treatments (Omite spray for mite prevention and Dichlorvos fumigation for aphid control) were also scheduled every 3-4 weeks starting from transplanting until the end of experiment.

#### *2.2.6 Internal ethylene concentration in fruit*

In later experiments, the internal ethylene concentration of capsicum was measured using Gas Chromatography (GC). Fruit at six stages of ripening, G, Breaker (B), BR1, Breaker Red 2 (BR2), Light Red (LR) and Deep Red (DR) (approximately 50 to 90 DAA from G to DR) were harvested in February-May 2012 (autumn season) and transported into the laboratory for gas measurement. A 1 mL syringe fitted with a 25-inch gauge needle was used to draw the internal gas from the cavity of each fruit and run in a GC (Shimadzu, Kyoto, Japan) as per Moradinezhad et al. (2008) and the standard used was 1.93  $\mu\text{L L}^{-1}$  ethylene (nitrogen balanced, BOC Gases Australia Ltd., NSW, Australia)

## 2.3 Results

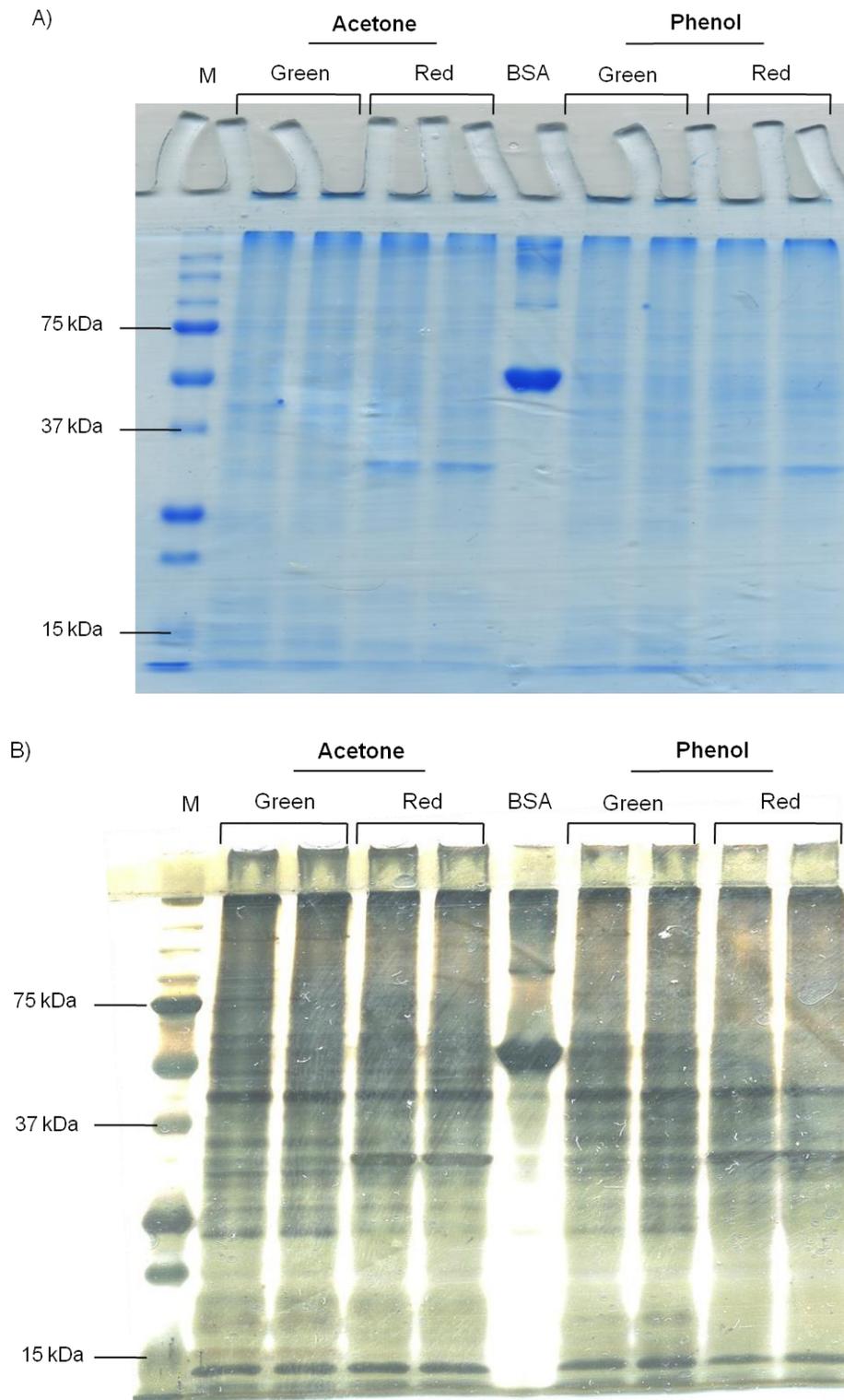
### 2.3.1 Protein extraction and 2DGE

Two protein extraction methods (TCA/acetone and Phenol) were compared. Protein extraction of G and R samples suggested a better and more consistent protein yield in the TCA/acetone method compared to the Phenol method (Table 2.1).

**Table 2.1** The comparison of protein yield between two protein extraction methods, TCA/acetone and Phenol methods from Green and Red capsicum. Both fruit were extracted twice (reps 1 and 2) and the protein concentration ( $\mu\text{g mL}^{-1}$ ) was estimated using the Bradford assay (Bradford, 1976).

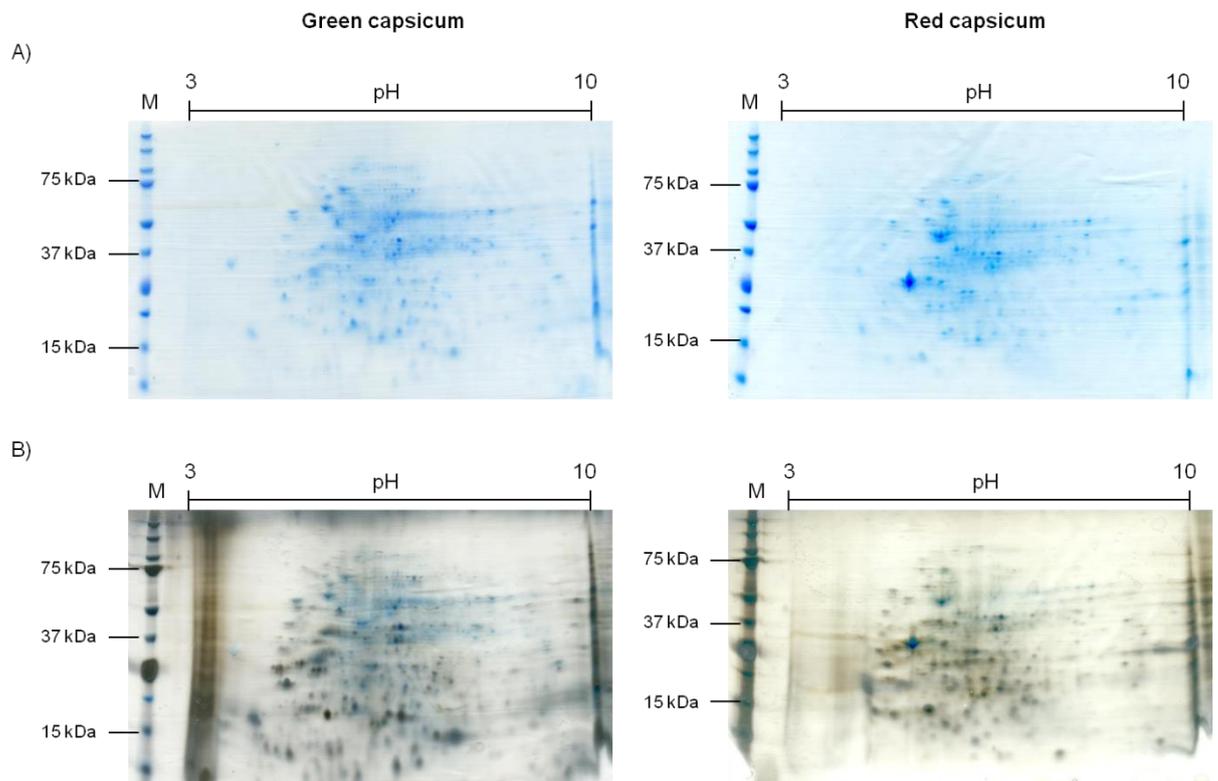
Extraction method	Fruit	Reps #	[protein] ( $\mu\text{g mL}^{-1}$ )
TCA/acetone	Green	1	557.8
		2	617.6
	Red	1	530.4
		2	448.1
Phenol	Green	1	435.7
		2	547.8
	Red	1	373.3
		2	308.5

SDS-PAGE of these protein samples (both G and R capsicum) suggested that protein samples from either extraction method produced distinct and sharp protein bands (Figure 2.1 A and B).



**Figure 2.1** SDS-PAGE of the protein extracts (5  $\mu$ g) from the two extraction methods, TCA/acetone and Phenol methods. Both Green and Red capsicum protein samples (reps 1 and 2, consecutively) listed in Table 2.1 were run to check for the protein bands from both extraction methods. A positive control Bovine Serum Albumin (BSA, 1  $\mu$ g) and a Precision Plus Protein marker (M, 5  $\mu$ L) were also run in the gel. The approximate size of the marker bands are shown in the left side of the Coomassie stained gel (A). The same gel was also stained with the silver staining method (B).

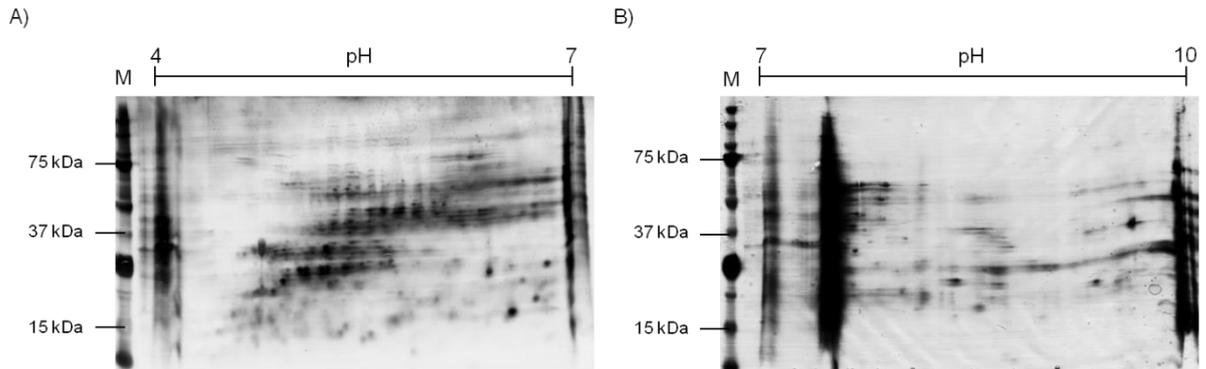
The 2DGE of both G and R capsicum produced considerably good focusing for protein spots and minimal streaking (Figure 2.2 A). This suggests that the running conditions used by Khoo et al. (2012) were also applicable for capsicum proteins. More protein spots were also observed when the gels were further stained with silver staining compared to only Coomassie staining (Figure 2.2 B). Hence all 2DGE were run and stained with silver staining to visualise more protein spots.



**Figure 2.2** 2D gel electrophoresis of 100 µg protein extracted with TCA/acetone method. One replicate of both Green (right) and Red (left) capsicum were run in pH 3-10 IPG strip as described in the Materials and Methods (pH range shown on top of each gel). The gels were first stained with Coomassie (A) before silver staining (B). M, 5 µL Precision Plus Protein marker with approximate protein sizes (kDa).

Since most protein spots resided approximately in the middle of the pH 3-10 gels (Figure 2.2), the 2DGE was further optimised by using smaller and different pH ranges to separate proteins (IPG strips of pH 4-7 and 7-10). Most proteins from the R capsicum were

present at pH 4-7 compared to pH 7-10 (Figure 2.3). Therefore, pH 4-7 IPG strips were used for all subsequent 2DGE experiments.



**Figure 2.3** Silver stained 2D gel electrophoresis of 100 µg Red capsicum protein extracted with TCA/acetone method using 4-7 (A) and 7-10 (B) pH range. M, 5 µL Precision Plus Protein marker with approximate protein sizes (kDa).

### 2.3.2 RNA extraction

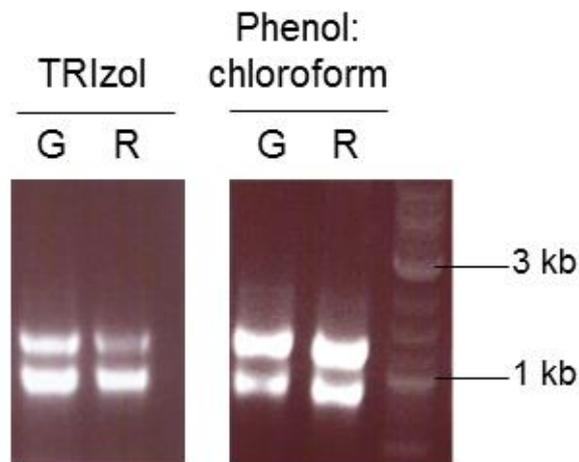
For pilot RNA extraction, two methods (TRIzol and Phenol:chloroform) were compared. One replicate of both G and R stages were extracted and the results suggested a better and more consistent yield in the Phenol:chloroform method compared to the TRIzol method, particularly for the R capsicum (Table 2.2).

**Table 2.2** The comparison of RNA yield ( $\mu\text{g } \mu\text{L}^{-1}$ ) from two RNA extraction methods, TRIzol and Phenol:chloroform methods.

Extraction methods	Fruit	[RNA] ( $\mu\text{g } \mu\text{L}^{-1}$ )
TRIzol	Green	2.543
	Red	0.7
Phenol:chloroform	Green	4.652
	Red	4.665

RNA gels also show that Phenol:chloroform extracted RNA may have better defined RNA bands (especially 28S RNA from Red capsicum) compared to the TRIzol method (Figure

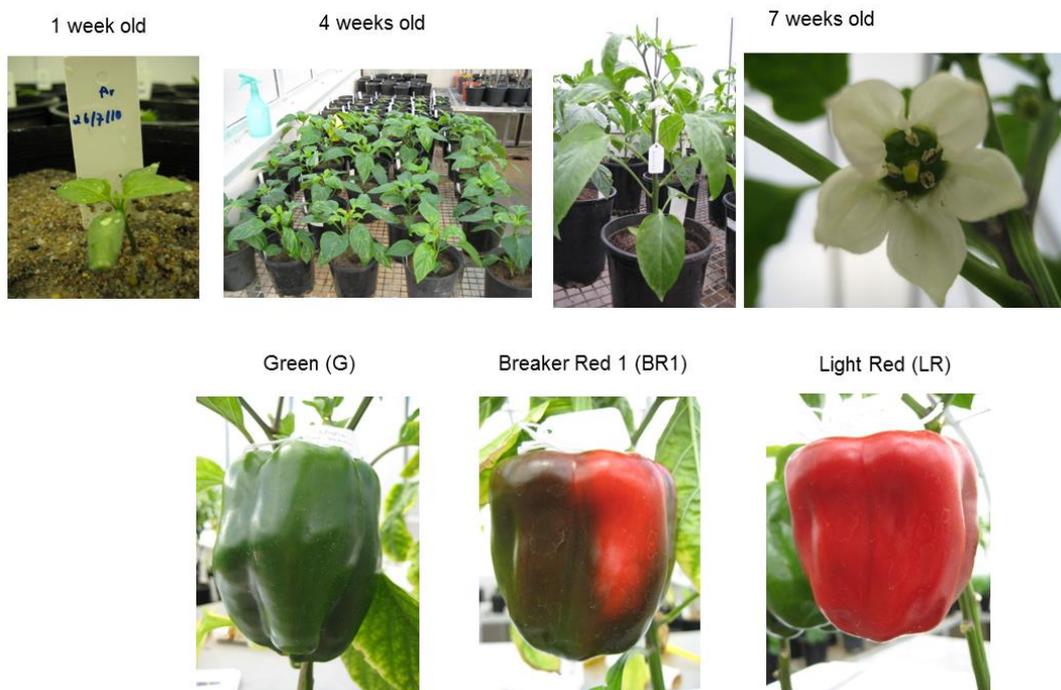
2.4). Hence, the Phenol:chloroform method was the method of choice for RNA extraction of capsicum.



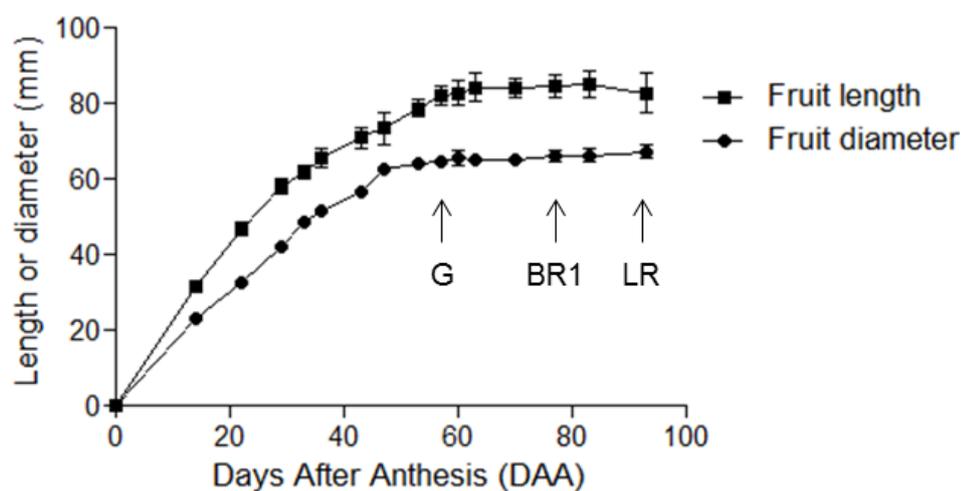
**Figure 2.4** Ethidium bromide gel (1% agarose) of the RNA extracts (5  $\mu$ g) from two extraction methods, TRIzol and Phenol:chloroform methods. One replicate of both Green (G) and Red (R) capsicum as listed in Table 2.2 were run together with a DNA ladder (2-log ladder) (not all band sizes are shown).

### 2.3.3 Time to maturity and ethylene production by capsicum

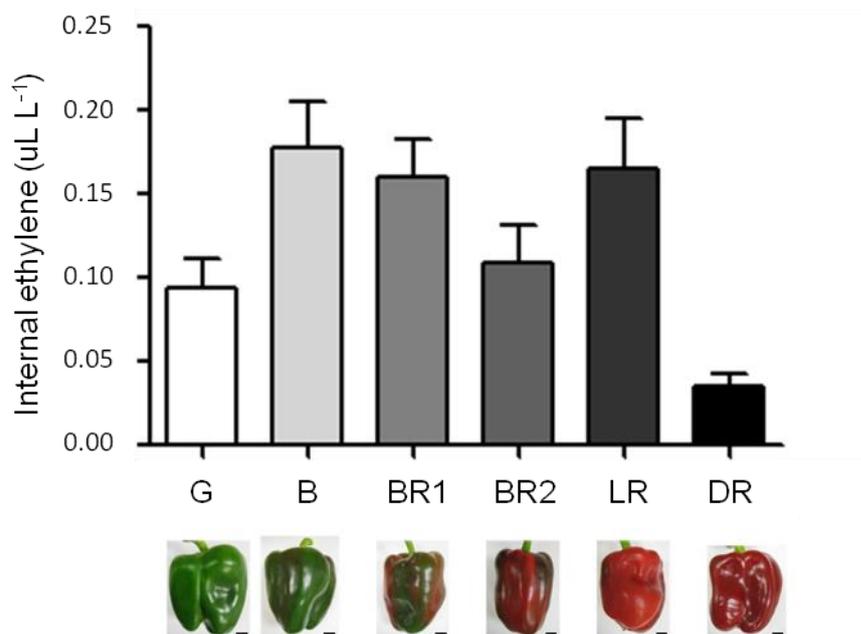
Capsicum plants (cv. Aries) were grown in glasshouse conditions until fruit harvest. This cultivar is a bell-shaped capsicum which ripens from green to red (Figure 2.5). Full Green (G), Breaker Red 1 (BR1) (30% red) and Light Red (LR, 100% red) stages were achieved at approximately 57, 78 and 93 DAA, respectively, when fruit ripened during winter to spring (August-October 2010). During the ripening period, capsicum fruit generally have a constant size after they reach the mature green stage (Figure 2.6). The information from this pilot experiment was used to help guide when fruit would be ready in subsequent experiments. Furthermore, the internal ethylene concentration in this capsicum throughout ripening ranged from 0.03 to 0.2  $\mu$ L L<sup>-1</sup> (Figure 2.7).



**Figure 2.5** Growing capsicum (cv. Aries) in glasshouse conditions (22°C with natural sunlight) during winter to spring. Seedlings germinated in petri dishes were transplanted into UC-Davis soil as per the Materials and Methods (see section 2.2.5) and reached anthesis after 7 weeks. The flowers were tagged at anthesis and only one fruit per plant was allowed to develop to maturity. The capsicum ripens from Green (G) to Breaker Red 1 (BR1) and Light Red (LR) at approximately 57-93 days after anthesis (DAA).



**Figure 2.6** The size of capsicum (cv. Aries) during development and ripening. Length and diameter (mm) of fruit reached a constant size during mature Green (G) stage followed by Breaker Red 1 (BR1) and Light Red (LR) stages.  $n = 5$  to 10 biological replicates  $\pm$ SE.



**Figure 2.7** The internal ethylene concentration ( $\mu\text{L L}^{-1}$ ) from the cavity of capsicum fruit ( $n = 3$  biological replicates  $\pm\text{SE}$ ). Six capsicum stages (G, Green; Breaker, B, Breaker Red 1, BR1; Breaker Red 2, BR2; Light Red, LR; Deep Red, DR) were analysed and the photos of corresponding stages are shown at the bottom. The scale bar at the bottom right hand corner of each photo is 1 cm.

## 2.4 Discussion

The ethylene level in non-climacteric fruit is known to be limited during ripening compared to climacteric fruit. The endogenous ethylene level in non-climacteric fruit such as orange, pineapple and lemon usually only reaches  $0.4 \mu\text{L L}^{-1}$  [reviewed in Burg and Burg (1965) and Paul et al. (2012)] while climacteric fruit such as tomato can produce as high as  $23 \mu\text{L L}^{-1}$  ethylene (Knee, 1995). Capsicum (cv. Aries) can be classified as non-climacteric fruit as its internal ethylene level remained limited during ripening (only up to  $0.2 \mu\text{L L}^{-1}$ , Figure 2.7) similarly as reported by Pham (2007). The levels of internal ethylene measured in other capsicum cultivars including bell peppers, chillies and paprika were also very low, approximately  $0.05\text{-}0.3 \mu\text{L L}^{-1}$  (Pham, 2007; Saltveit, 1977; Villavicencio et al., 1999), further suggesting that capsicum are largely non-climacteric fruit.

The use of capsicum in non-climacteric research may have a few advantages compared to using other non-climacteric fruit. For example, capsicum is in the same family as the well characterised model fruit, tomato (Solanaceae). The genetics and molecular regulation of capsicum is also very similar to tomato as reviewed by Paran and van der Knaap (2007). For instance, molecular markers for both species seem to be highly conserved within each chromosome, despite several genetic rearrangements that have been identified in capsicum (Livingstone et al., 1999). The promoter activities of capsicum's capsanthin/capsorubin synthase and fibrillin genes have also been shown to be up-regulated upon ripening even when transformed into tomato (Kuntz et al., 1998). This implies that tomato and capsicum may have similar molecular regulation especially during ripening (although both have different ripening behaviour, climacteric and non-climacteric ripening, respectively). Another unique behaviour of capsicum is that fruit are only able to ripen off the plant when harvested during Breaker stages but not the Green stage (Krajayklang et al., 2000; Pham, 2007). Hence, more studies in capsicum ripening are needed to compare its ripening stages especially utilising comparison-based approaches such as proteomics.

In the research presented in this chapter, 2DGE was run in a preliminary study to visualise protein spots for both Green and Red capsicum (obtained commercially). Most of the optimisation techniques including the 2DGE focusing steps, critical level for protein detection (100 µg protein samples) as well as gel staining with R250 Coomassie Brilliant Blue followed by EBT silver staining were documented by Khoo et al. (2012) for the proteomics of bread wheat, and as reported here, also appear suitable for capsicum (Figure 2.2). This suggests the applicability of 2DGE to analyse protein profiles of different organisms especially where their pI is similar. We also found that the capsicum proteome may be best visualised with a 4-7 pH range to better separate the proteins. Several other proteomic studies in fruit have utilised the 4-7 pH range as well, such as tomato (Qin et al.,

2012; Rocco et al., 2006; Saravanan and Rose, 2004), avocado (Saravanan and Rose, 2004), grape berry (Wang et al., 2009), citrus (Yun et al., 2010) and olive (Wu et al., 2011), suggesting this is an appropriate pH range to analyse the proteomics of capsicum fruit. Furthermore, both Green and Red capsicum have unique protein profiles as expected from different stages of ripening (Figure 2.2), suggesting the viability of 2DGE for comparing the capsicum proteome between ripening stages. However, the usefulness of 2DGE also depends on the protein extraction methods [reviewed by Rose et al. (2004)]. In this research, the TCA/acetone method was chosen based on the consistency and better protein yield compared to the Phenol method (Table 2.1). Further downstream analysis of gene expression for candidates identified via proteomics requires good RNA quality. The Phenol:chloroform method produced slightly better quality and higher yields than the TRIzol method and was therefore chosen for all further research. The mRNA of a few fruit rich in secondary metabolites such as tomato, eggplant (Moore et al., 2005) and citrus (Yun et al., 2012) have also been extracted with the Phenol:chloroform methods or similar. This suggests the applicability of the Phenol:chloroform-based method in extracting RNA from a variety of fruit rich in secondary metabolites including capsicum.

In conclusion, capsicum may be one of the best fruit for improving our knowledge about the non-climacteric model. The similarity with the climacteric tomato will definitely enhance our ability to further understand the regulation behind non-climacteric ripening. Furthermore, the use of comparison-based approaches such as proteomics (and metabolomics) to compare the ripening stages of capsicum will assist in identifying differentially expressed molecules in capsicum ripening (detailed in Chapters 3 and 4, respectively).

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**Chapter 3. Manuscript 1: Proteomic analysis  
during capsicum ripening reveals differential  
expression of ACC oxidase isoform 4 and other  
candidates**

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Accepted for publication and is available online:

Aizat, W.M., Able, J.A., Stangoulis, J.C.R. & Able, A.J.,  
(2013)

Functional Plant Biology, 40 (11), 1115-1128.

<http://www.publish.csiro.au/paper/FP12330.htm>

Statement of Authorship is located in Appendix A.

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NOTE:

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It is also available online to authorised users at:

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**Chapter 4. Manuscript 2: Metabolomics of  
capsicum ripening reveals modification of the  
ethylene related-pathway and carbon metabolism**

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Aizat, W.M., Dias D.A., Stangoulis, J.C.R., Able, J.A.,  
Roessner U. & Able, A.J. (2014)

Postharvest Biology and Technology, 89, 19-31.

[http://www.sciencedirect.com/science/article/pii/S09255214  
13003347](http://www.sciencedirect.com/science/article/pii/S0925521413003347)

Statement of Authorship is located in Appendix B.

Aizat, W.M., Dias, D.A. Stangoulis, J.C.R., Able, J.A., Roessner, U. & Able, A.J. (2014)  
Metabolomics of capsicum ripening reveals modification of the ethylene related-pathway and carbon metabolism.

*Postharvest Biology and Technology*, v. 89(March), pp. 19-31

NOTE:

This publication is included after page 47 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

<http://doi.org/10.1016/j.postharvbio.2013.11.004>

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**Chapter 5: Analysis of ACC oxidase and selected  
metabolic candidates in different capsicum tissues  
and cultivars**

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## 5.1 Introduction

The use of proteomics (Chapter 3) and metabolomics (Chapter 4) have identified several candidates of interest which were differentially expressed during capsicum ripening. Proteomic analysis utilising 2D gel electrophoresis (2DGE) identified several proteins including a protein which is involved in the ethylene pathway, 1-aminocyclopropane 1-carboxylate (ACC) oxidase (ACO) 4. The overall activity of ACO and its *CaACO4* transcript was shown to be highly up-regulated during B-BR stages, but not earlier (G) or later (LR and DR) stages (Aizat et al., 2013) (Chapter 3). Given that ACO is the final enzyme in ethylene biosynthesis, its up-regulation in non-climacteric capsicum may suggest a conserved ethylene pathway between climacteric and non-climacteric fruit. However, ethylene production could also be influenced by several factors such as plant cultivar, fruit maturity and tissue (Fernández-Otero et al., 2006; Zhang et al., 2009a). Therefore the ACO activity differences between capsicum fruit tissues and varieties with different ripening times may be important to understand the factors that regulate the proteomic candidate *CaACO4*.

The metabolomics approach also revealed several metabolites of interest but mostly in other pathways such as glycolysis (sugars) and the tricarboxylic acid (TCA) cycle (malate) (Chapter 4). Sugars (glucose, fructose and sucrose) were significantly increased during capsicum ripening, probably as a result of starch breakdown while malate decreased. This may suggest that glycolysis has taken place in capsicum during ripening to provide for various biochemical pathways including the TCA cycle. One of the TCA cycle intermediates, malate has been previously associated with the regulation of redox activity, starch levels (Osorio et al., 2012; Scheibe, 2004) and carotenoid content during ripening (Centeno et al., 2011). Malate down-regulation during capsicum ripening onset may therefore be important for the regulation of non-climacteric capsicum ripening but requires further investigation.

The ripening process in climacteric fruit has been shown to be regulated spatially and temporally. Banana for example, ripens from the pulp towards the peel (Domínguez and Vendrell, 1994) and both tissues have different expression of *MaMADS*-box transcription factors which may regulate their ripening induction (Elitzur et al., 2010). Furthermore, several metabolites such as chlorophyll, carotenoids and tocopherols were accumulated differently in multiple tissue types of tomato throughout ripening, suggesting that the ripening process may be determined not only by specific tissues but also by the ripening stage of the fruit (Moco et al., 2007). Several studies in different cultivars of tomato also showed that the expression of several ripening related genes were not similar (Anjanasree et al., 2005; Jafari et al., 2013), indicating that the ripening process may be differentially regulated even within the same species.

In an effort to further understand the behaviour of capsicum ripening, several proteomic and metabolic candidates were investigated in different capsicum tissues (peel, pulp and seeds/placenta) as well as cultivars with different ripening rates (cv. Aries and cv. Plato). Due to time limitations, only a select number of candidates were chosen to be analysed including ACO, sugars (glucose, fructose and sucrose) and malate.

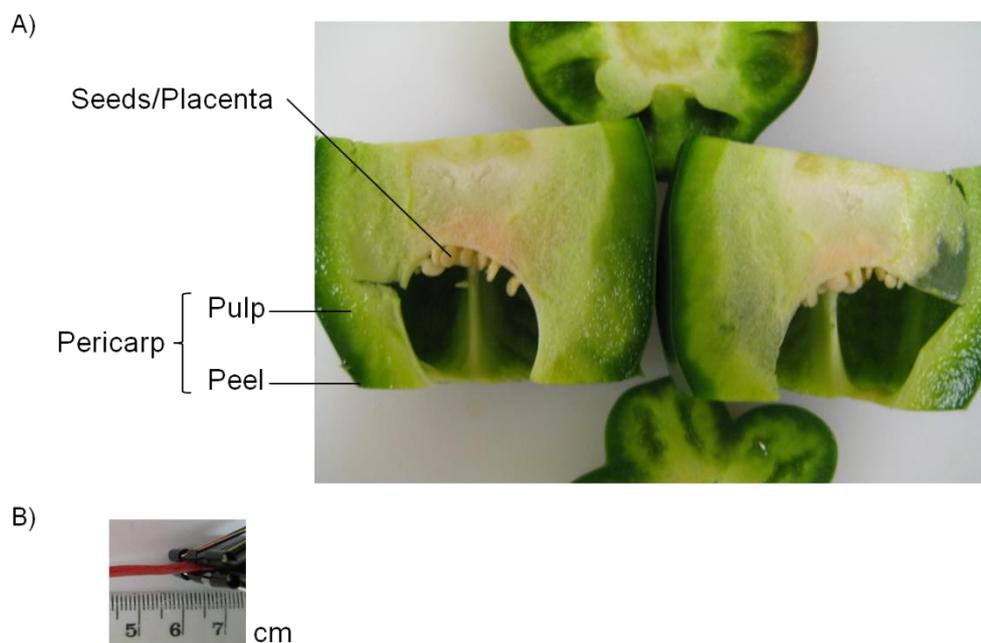
## **5.2 Materials and Methods**

### *5.2.1 Capsicum planting and sampling*

All capsicum plants were grown in a glasshouse and harvested according to Chapter 2 (section 2.2.5) unless otherwise stated.

For experimentation examining the different tissues, capsicum (cv. Aries) was grown during January-May 2012 (summer-autumn; referred to as the first set) or during September 2012-January 2013 (spring-summer season; referred to as the second set). Fruit were harvested at six different capsicum ripening stages (G, B, BR1, BR2, LR, DR) as per Aizat et al. (2013) and Chapter 3: Materials and Methods. Three different tissues (peel,

pulp, seeds/placenta) (Figure 5.1) were separated from three biological replicates of each stage. The top and bottom of the fruit were first discarded. Capsicum were then cut in half before the seeds/placental tissue was isolated using a knife and frozen in liquid N<sub>2</sub> while other tissues were sampled. The fruit pericarp was cut into smaller pieces (~2cm) and the peel was carefully separated (< 1.0 mm thickness) from the pulp using a kitchen peeler. Any peel which had too much pulp tissue was discarded. Both peel and pulp tissues were frozen immediately in liquid N<sub>2</sub>. The three tissues were ground to powder using a chilled mortar and pestle and stored in a -80°C freezer until required.



**Figure 5.1** Three different tissues of capsicum (peel, pulp and seeds/placental tissues) were sampled during capsicum ripening. A) Capsicum cut in sections with arrows pointing to the respective tissues (this sample is an example from the Breaker stage), and B) tissue regarded as peel (from a Light Red capsicum). cm, centimetre.

For experimentation investigating different cultivars, a pilot experiment was done to characterise three different capsicum cultivars (cv. Plato, cv. Tycoon and cv. Raptor) in terms of their ripening times (ripened in March-May 2012, autumn season). Capsicum (cv. Plato) ripened the earliest compared to the other cultivars (Appendix D; mean days to ripen

from mature G to LR stages were 25.8, 32.4 and 34.6 for cv. Plato, cv. Tycoon and cv. Raptor, respectively). Hence, cv. Plato was grown in another experiment with cv. Aries which was known to take longer to ripen. The fruit length as well as ripening times (days after anthesis, DAA) were recorded (both ripened during the October 2012-January 2013, spring-summer season) and were sampled at six time points [40 (G stage), 50 (B+0; Breaker at the first sighting of brown tissues), 56, 60, 65 and 78 DAA].

### *5.2.2 ACO activity assay and spectrophotometric analyses*

The ACO activity of all samples was measured as per Aizat et al. (2013) and Chapter 3: Materials and Methods. For spectrophotometric analysis, glucose, fructose, sucrose and malate were prepared as detailed in Chapter 4: Materials and Methods, with the exception of peel and seeds/placenta which were too dry and hence their juices were extracted by adding 1 mL nanopure water to the 0.5 g tissues (50% w/v). All fruit juices were then measured according to Anonymous (1984) as per Chapter 4: Materials and Methods and the dilution factor for each sample was taken into consideration when calculating respective metabolite concentration using the standard formula. Only the first set of different tissues was analysed in the spectrophotometric analyses.

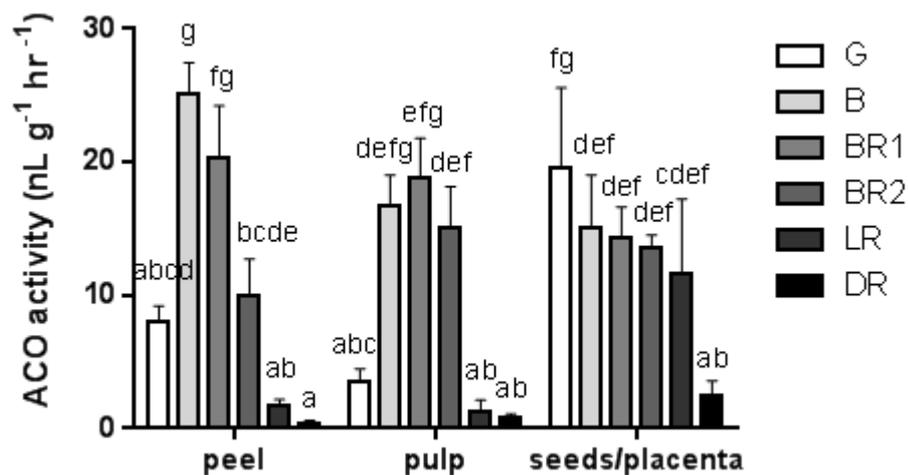
### *5.2.3 Statistical analysis*

All statistics were performed using Genstat 14 (Hemel Hempstead, UK). The statistical significance was determined using a Duncan's Multiple Range Test at  $P < 0.05$ . Where data from the two sets of harvest were combined, a two-way ANOVA ( $P < 0.05$ ) was first used to ensure both sets were not significantly different.

## 5.3 Results

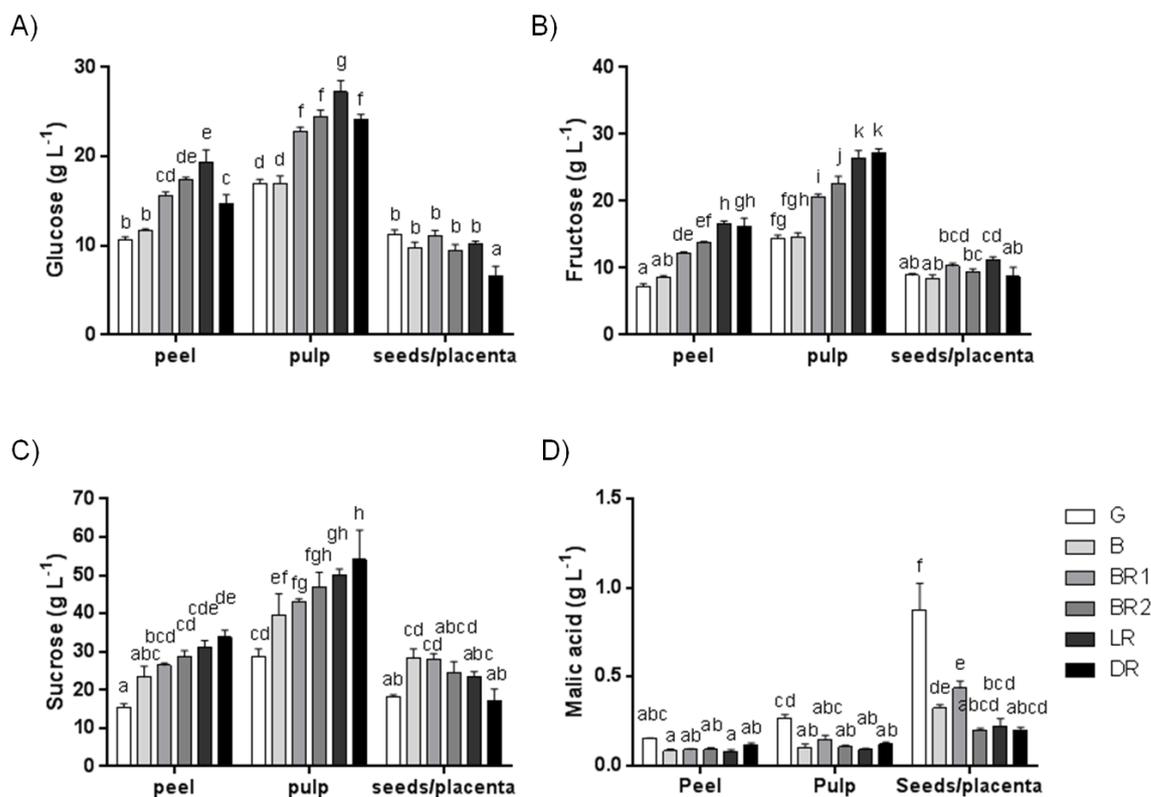
### 5.3.1 Different tissues (peel, pulp and seeds/placenta)

The ACO activity patterns during the six capsicum ripening stages were relatively similar for peel and pulp but differed for seeds/placenta (Figure 5.2). The ACO activity of both peel and pulp increased significantly during ripening onset (B-BR stages) whereas the ACO activity of seeds/placenta was constant during most ripening stages. During the G stage, the ACO activity of seeds/placenta was significantly higher than peel and pulp tissues. During the B stage, the ACO activity of peel was significantly higher than seeds/placenta but the ACO activity of pulp was not significantly different compared to the other tissues. Furthermore, during BR1 and BR2 stages, the ACO activity of all tissues was not statistically different. However, during the LR stage the ACO activity of seeds/placenta was the highest compared to the peel and pulp. During the DR stage, the ACO activity was limited and not significantly different across all tissues.



**Figure 5.2** ACC oxidase (ACO) activity of different tissues (peel, pulp and seeds/placental tissues) in six capsicum ripening stages (G, Green; B, Breaker; Breaker Red 1, BR1; Breaker Red 2, BR2; Light Red, LR; Deep Red, DR).  $n = 6$  biological replicates from two sets of harvest ( $\pm$ SE) and the same letter on each bar indicates no difference between means of the three tissues (peel, pulp, seeds/placenta) across six ripening stages (G, Green; B, Breaker; Breaker Red 1, BR1; Breaker Red 2, BR2; Light Red, LR; Deep Red, DR). Statistical significance was determined using a Duncan's Multiple Range Test ( $P < 0.05$ ).

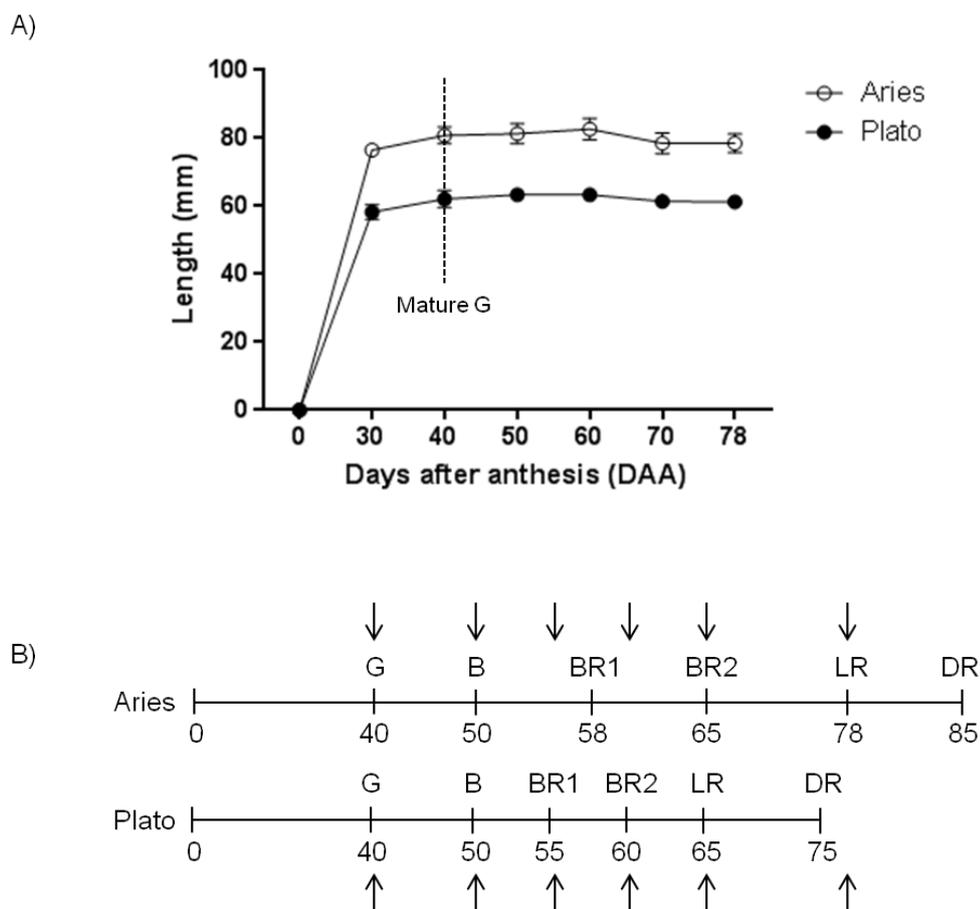
The spectrophotometric analysis of sugars (glucose, fructose and sucrose) and malate also revealed differences between the three tissues (Figure 5.3). Pulp contained the highest levels of sugars compared to the peel and seeds/placental tissues at all ripening stages while the sugars were significantly higher in peel than that observed in the seeds/placental tissue at the later ripening stages (Figure 5.3 A, B and C). Glucose in seeds/placenta was also constant throughout ripening except a significant reduction at the DR stage (Figure 5.3 A) whereas fructose in this same tissue (seeds/placenta) was significantly higher in LR compared to G, B and DR stages (Figure 5.3 B). Moreover, sucrose was significantly higher in seeds/placenta of B and BR1 stages compared to G and DR (Figure 5.3 C). The three sugars in peel and pulp were significantly greater during BR1 onwards compared to earlier stages (Figure 5.3 A, B and C). Furthermore, the malate level (Figure 5.3 D) in seeds/placenta was significantly higher than the level in peel and pulp at G to BR1 stages. Malate was relatively similar between tissue types after BR1. The malate level in peel was also constant throughout ripening stages but a significant reduction of malate from G to later stages was observed for pulp (except BR1) and seeds/placental tissues.



**Figure 5.3** Sugars [glucose (A), fructose (B) and sucrose (C)] and malate (D) of three different capsicum tissues (peel, pulp and seeds/placenta) at six different ripening stages (G, Green; B, Breaker; Breaker Red 1, BR1; Breaker Red 2, BR2; Light Red, LR; Deep Red, DR).  $n = 3$  biological replicates from the first set of harvest ( $\pm$ SE) and the same letter on each bar indicates no difference between means of the three tissues (peel, pulp, seeds/placenta) across six ripening stages (G, Green; B, Breaker; Breaker Red 1, BR1; Breaker Red 2, BR2; Light Red, LR; Deep Red, DR). The statistical analysis was determined using a Duncan's Multiple Range Test ( $P < 0.05$ ) for respective metabolites.

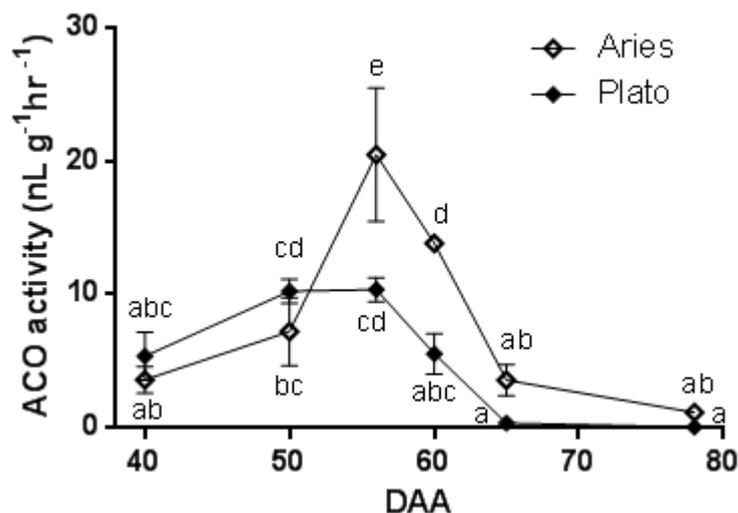
### 5.3.2 Different cultivars (*cv. Aries* and *cv. Plato*)

The length of fruit from *cv. Aries* was greater than the length of fruit from *cv. Plato* during ripening (Figure 5.4 A). Both cultivars also reached mature G and B+0 (Breaker at the first sighting of brown tissues) stages at similar DAA (40 DAA for G and 50 DAA for B+0) (Figure 5.4 A and B). For BR1, BR2, LR and DR stages, the DAA for *cv. Plato* were 55, 60, 65 and 70 DAA, respectively. For *cv. Aries*, the four ripening stages (BR1, BR2, LR and DR) were at 58, 65, 78 and 85 DAA, respectively. As a result, the two cultivars were sampled at 40 DAA, 50 DAA, 56 DAA, 60 DAA, 65 DAA and 78 DAA (Figure 5.4 B).



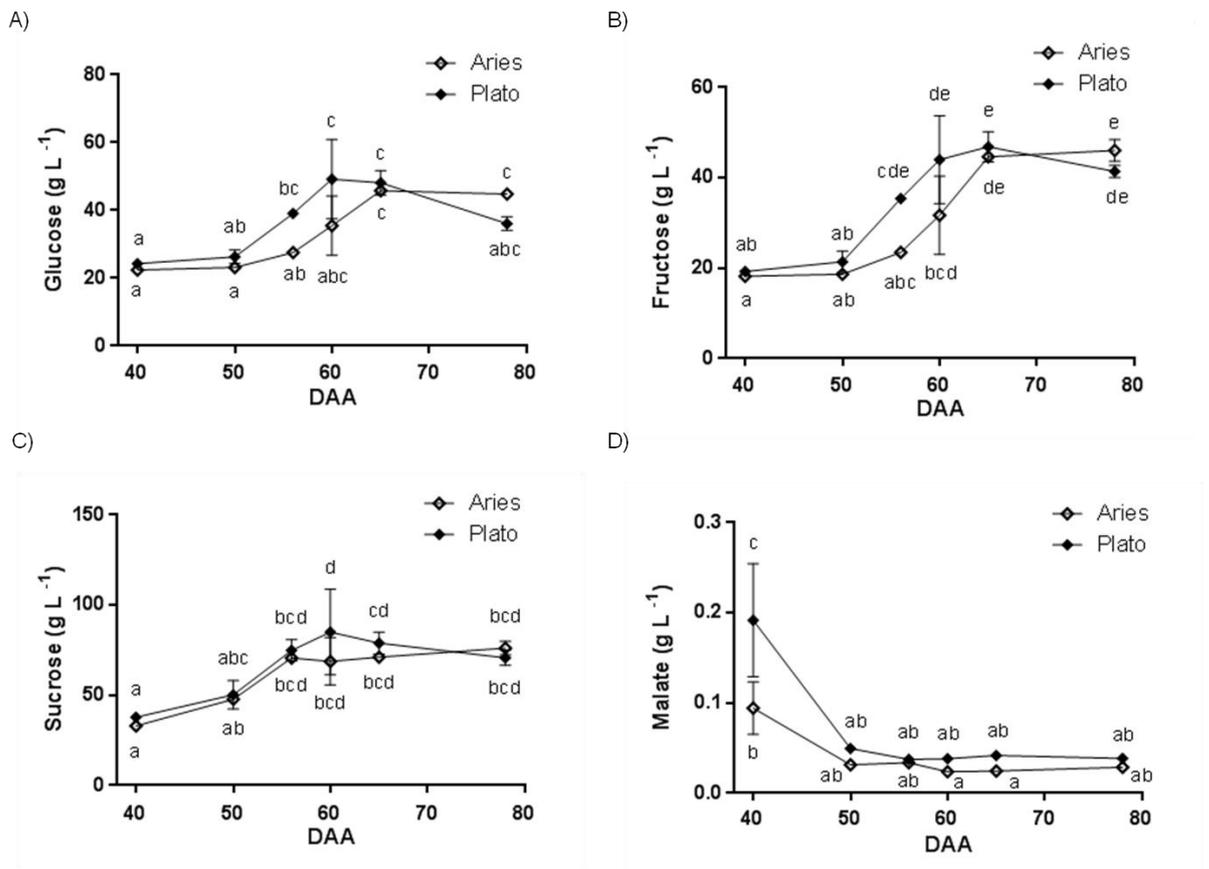
**Figure 5.4** Fruit from two different capsicum cultivars (cv. Aries and cv. Plato) ripened at different rates. A) The fruit length (mm) of both capsicum cultivars during ripening.  $n = 3$  to 6 biological replicates ( $\pm$ SE) and the dashed line indicates mature Green (G) stage. B) Conceptual figure of the six capsicum ripening stages (G; B+0, Breaker at the first sighting of brown tissues; Breaker Red 1, BR1; Breaker Red 2, BR2; Light Red, LR; Deep Red, DR) at different Days After Anthesis (DAA) of both cv. Aries and cv. Plato (observation from at least three fruit at each stage). Six sampling time points (arrows) for the two cultivars were at 40 (G), 50 (B+0), 56, 60, 65 and 78 DAA. Capsicum photos for each time point are shown in Appendix E.

The ACO activity of both cv. Aries and cv. Plato was not significantly different at most sampling times except at 56 and 60 DAA (Figure 5.5) when cv. Aries had significantly greater ACO activity than cv. Plato. Moreover, the ACO activity of cv. Aries was significantly higher at 56 and 60 DAA compared to earlier (40 and 50 DAA) or later (65 and 70 DAA) sampling times. In contrast, cv. Plato remained relatively constant except for a significantly lower ACO activity during later sampling times (65 and 78 DAA) compared to 50 and 56 DAA.



**Figure 5.5** ACC oxidase (ACO) activities of two capsicum cultivars (cv. Aries and cv. Plato) harvested at six different days after anthesis (DAA). The sampling times were 40, 50, 56, 60, 65 and 78 DAA. Plato ripened earlier than cv. Aries and means are  $n = 3$  biological replicates ( $\pm$ SE). Only pericarp tissues were analysed for both cultivars. The same letter on bars indicates no difference between means as determined using a Duncan's Multiple Range Test ( $P < 0.05$ ).

Glucose (Figure 5.6 A) and fructose (Figure 5.6 B) were not significantly different between cv. Aries and cv. Plato when compared at each time point. However, both sugars were significantly higher than the first sampling time (40 DAA) at 56 DAA for cv. Plato and cv. Aries only reached that significant level at 65 DAA (glucose) and 60 DAA (fructose). Furthermore, both cultivars had similar sucrose levels when compared at each sampling time (Figure 5.6 C). The sucrose level for both cultivars was significantly greater at 56 DAA compared to earlier sampling times before remaining constant thereafter. Malate (Figure 5.6 D) was only significantly greater in cv. Plato than cv. Aries at 40 DAA and not at other sampling times. However, cv. Plato seems to reach a significantly low level from 50 DAA onwards while cv. Aries was much later at 60 and 68 DAA, compared to the malate level at their first sampling time (40 DAA).



**Figure 5.6** Sugars [glucose (A), fructose (B) and sucrose (C)] and malate (D) of two capsicum cultivars (cv. Aries and cv. Plato) harvested at six different Days After Anthesis (DAA). The sampling times were 40, 50, 56, 60, 65 and 78 DAA. Cv. Plato ripened earlier than cv. Aries and n = 3 biological replicates ( $\pm$ SE). Only pericarp tissues were analysed for both cultivars. The same letter on bars indicates no difference between means as determined using a Duncan's Multiple Range Test ( $P < 0.05$ ).

## 5.4 Discussion

Further investigation of the selected proteomic and metabolic candidates such as ACO, sugars and malate revealed that these candidates were differentially expressed in different tissues (particularly seeds/placenta in comparison with the peel and pulp) as well as different cultivars with different ripening abilities.

*Seeds/placental tissue may be regulated differently than the peel and pulp during capsicum ripening*

In various fruit, the accumulation of metabolites has been associated with tissue specialisation (Arora et al., 2008; Moco et al., 2007). For example, total sugar and starch were mainly accumulated in the peel of several banana cultivars when compared to their pulp (Arora et al., 2008). In G stage tomato, metabolites such as starch and fructose were highly abundant in mesocarp compared to the locular tissues and *vice versa* for malate, glucose and sucrose (higher levels in locular tissues than mesocarp) (Lemaire-Chamley et al., 2005). Furthermore, Moco et al. (2007) showed that several metabolites were distributed differently in different tissues and ripening stages of tomato. For example,  $\gamma$ -tocopherol level in tomato was the highest in locular tissues during the G stage but significantly lower during B stage onwards. Other outer layer tissues such as the pericarp and epidermis had lower  $\gamma$ -tocopherol during the G stage but significantly increased, higher than the locular tissues as ripening progressed (Moco et al., 2007). Several other metabolites such as amino acids in melon were also differentially accumulated in various tissue types over different ripening stages (Moing et al., 2011). For instance, metabolites such as sucrose and  $\beta$ -carotene not only increased significantly when the melon ripened but sucrose was also significantly higher in the inner flesh tissue compared with the outer mesocarp (Moing et al., 2011). Metabolite accumulation and biosynthesis may therefore be regulated spatially (different tissues) and temporally (ripening stages) during fruit ripening.

However, the metabolite levels of different capsicum ripening tissues, particularly peel and pulp are still poorly investigated. To the best of my knowledge, the only study that differentiated peel and pulp of a capsicum was documented by Daood et al. (1989) which found that lipid and fatty acid levels were similar in both tissues during ripening. Other capsicum studies have generally sampled both peel and pulp together (Liu et al., 2012a; Moreno et al., 2012; Wahyuni et al., 2011; Wahyuni et al., 2013) as done previously in this research (Chapters 3 and 4). A few studies have also compared seeds/placental tissue with the pericarp (both peel and pulp) in capsicum. For instance,

seeds/placenta was shown to have higher capsaicinoid, the main contributor to pungency in capsicum, than the pericarp (Liu et al., 2012a; Wahyuni et al., 2011; Wahyuni et al., 2013). This suggests that the metabolic activity of seeds/placenta might be different than the pericarp.

Indeed, this study revealed that the patterns and levels of sugars, malate (Figure 5.3) and ACO activity (Figure 5.2) across ripening stages were different between the tissues (peel, pulp, seeds/placenta). For example, all sugars (glucose, fructose, sucrose) in both peel and pulp were significantly higher during later ripening stages compared to the G and B stages but the trends for the sugars in seeds/placenta were mostly consistent without dramatic changes in their levels (Figure 5.3 A, B and C). The significantly higher sugars in peel and pulp tissues during ripening onset may be related to the degradation of starch (as discussed in Chapter 4) which suggests glycolysis may be active in these capsicum tissues. Seeds are considered as a storage organ particularly for starch accumulation (Tetlow, 2011) and therefore, starch breakdown and glycolysis may not be as active in this tissue compared to the peel and pulp.

Malate was also differentially accumulated in different capsicum tissues (Figure 5.3 D). While all tissues except peel had significantly reduced malate upon ripening onset (G to B stage), malate in seeds/placenta was much higher than the other two tissues. Malate reduction during capsicum ripening onset may be correlated with the up-regulation of malate dehydrogenase enzyme (Aizat et al., 2013) (Chapter 3: Table 1) during this period which converts malate to oxaloacetate, although this observation was only from the pericarp (peel and pulp) and not seeds/placental tissue. Furthermore, malate has been shown to be closely associated with starch metabolism (Centeno et al., 2011) and the starch breakdown in the pericarp tissue may be related to the malate reduction upon ripening (Chapter 4: Figure 3). Interestingly, since starch breakdown may not be as active in seeds/placental tissue, as discussed earlier, the reduction of malate during capsicum

ripening may be related to other plant cellular processes. For instance, malate is an intermediate in the TCA cycle and this cycle is involved in the production of CO<sub>2</sub> during cellular respiration (Taiz and Zeiger, 2010). A previous study also reported that the accumulation of internal CO<sub>2</sub> in capsicum was contributed through respiration of the seeds/placental tissue (Blanke and Holthe, 1997). Given that malate in the seeds/placental tissue was significantly lower upon ripening onset compared to at the G stage, the TCA cycle activity of this tissue may be down-regulated (as no regeneration of malate) and thus further impacts fruit respiration/internal CO<sub>2</sub>. Indeed, the respiration level of various non-climacteric capsicum (Ben-Yehoshua et al., 1983; Biles et al., 1993; Blanke and Holthe, 1997) including cv. Aries (Pham, 2007) also decreased significantly upon ripening onset.

ACO activity of different capsicum tissues during ripening (Figure 5.2) was determined to further our understanding on the regulation of ethylene production and hence ripening in capsicum. The ACO activity of peel and pulp was significantly increased upon ripening onset (G to B-BR stages) before being reduced at the LR and DR stages. This pattern was similar to the ACO activity documented earlier for the whole pericarp tissue (Aizat et al., 2013) (Chapter 3) and there was no significant difference between pulp and peel tissues. In contrast, the ACO activity in banana peel was approximately five times higher than in the pulp (Domínguez and Vendrell, 1994). The epicarp (equivalent to peel) of climacteric plums also had a higher level of ACO activity (approximately two to five times) compared to its mesocarp tissue (equivalent to pulp) during ripening (Fernández-Otero et al., 2006). ACO activity may therefore be regulated differently in the different tissues of different fruit that vary in ripening behaviour (climacteric banana and plum versus non-climacteric capsicum). However, more studies are needed to confirm this hypothesis.

In addition, the ACO activity of seeds/placental tissue generally remained constant from G to LR stage before becoming significantly lower at DR stage. Interestingly, the

ACO activity at early ripening (G stage) of this tissue was significantly higher than the peel and pulp (Figure 5.2). Given that seeds/placental tissue have important roles in determining the microenvironment of internal capsicum (Blanke and Holthe, 1997), the constantly high ACO activity in this tissue might contribute to the basal level of endogenous ethylene throughout capsicum ripening (Chapter 2: Figure 2.7), but the role of the pulp in contributing to some level of endogenous ethylene also cannot be ruled out. In other climacteric fruit such as plum, seeds had the highest ACO activity during fruit development and early ripening stages before the pericarp and mesocarp tissues increased their ACO activity levels during later ripening stages (Fernández-Otero et al., 2006). Furthermore, the ACO activity of tomato seeds was higher and preceded the increase in other tissues during ripening (Zhang et al., 2009a). Perhaps the early increase of ACO activity in seeds [and hence ethylene production (Fernández-Otero et al., 2006; Zhang et al., 2009a)] is important for determining the exact timing for these climacteric fruit to ripen. Indeed, ethylene only induces ripening when applied to mature fruit (when the seeds have matured) but not during developmental or immature stages (Burg and Burg, 1965; Klee and Giovannoni, 2011). Seeds matured approximately a week before the normal ripening process began (climacteric stage) in tomato (Zhong et al., 2013). This may be corroborated by the early induction of ACO activity in seeds of tomato (Zhang et al., 2009a) and possibly other fruit as discussed earlier. However, the significance of relatively high ACO activity in capsicum, particularly seeds/placental tissue, during the G stage still requires additional experiments due to capsicum being a non-climacteric fruit and hence not responding to ethylene (Saltveit, 1977).

*Different ripening rates of different cultivars determined the rate of sugar, malate and ACO activity changes upon ripening*

Two different capsicum cultivars with different ripening rates were investigated in terms of their sugar, malate and ACO activity levels (only pericarp without seeds/placental tissue). Cv. Plato ripened quicker and reached the DR stage 10 days earlier than cv. Aries (Figure 5.4 B). Both cultivars however, exhibited similar patterns and were not significantly different in sugar accumulation (glucose, fructose, sucrose) at each sampling time (Figure 5.6 A, B and C). Several capsicum cultivars exhibited an increase in sugars, particularly glucose and fructose, during ripening (Barrera et al., 2008; Navarro et al., 2006; Nielsen et al., 1991; Serrano et al., 2010). Different cultivars of capsicum were known to accumulate different amount of sugars especially when hot and sweet peppers were compared (Blasiak et al., 2006). However, given that both cv. Aries and cv. Plato are sweet pepper capsicum, both might have similar sugar levels during ripening. In addition, malate level was significantly higher in cv. Plato than in cv. Aries particularly during the G stage (40 DAA) but malate was significantly decreased in the two cultivars during later sampling times such that they both have a similarly low level (Figure 5.6 D). The malate reduction during ripening was also observed in other capsicum cultivars (Osorio et al., 2012) as well as in other fruit such as tomato (Carrari et al., 2006; Guillet et al., 2002) and grape (Dai et al., 2013), indicating a similar ripening event across fruit species and cultivars.

However, the rate of sugar (particularly glucose and fructose) accumulation and malate reduction were different between the cultivars. For instance, cv. Plato accumulated glucose and fructose earlier (by approximately four to nine days) compared to cv. Aries (Figure 5.6 A and B). Malate level also decreased earlier (by approximately 10 days) for cv. Plato compared to cv. Aries (Figure 5.6 D). This may be linked to their different ripening rates of the cultivars; cv. Plato which ripens quicker might accumulate more sugars and reduce malate at an earlier DAA compared to cv. Aries which ripens later.

ACO activity of the two capsicum cultivars was also different particularly during the ripening onset (Figure 5.5). Both cultivars have similar ACO activity at the first two

time points before cv. Aries increased significantly during ripening onset (56 and 60 DAA) compared to cv. Plato. The reason behind this is still unclear but possibly different cultivars may have a different capacity to induce the ACO enzyme. Cv. Aries exhibited a peak-like pattern during ripening onset as documented earlier (Aizat et al., 2013) (Chapter 3: Figure 3) and cv. Plato seems to be mostly consistent during ripening before significantly decreasing during late ripening (in comparison to its ACO level during ripening onset). Given that capsicum's ACO activity reached maximum level during the ripening onset before being significantly limited during full red stages (Aizat et al., 2013) (Chapter 3), cv. Plato which ripens earlier might also have exhibited this pattern earlier compared to cv. Aries.

ACO activity in capsicum was shown to be correlated with the *CaACO4* and possibly be the major *ACO* isoform expressed in this non-climacteric fruit (Aizat et al., 2013) (Chapter 3: Figure 3). The differences in ACO activities of cv. Aries and cv. Plato may also be related to the differences in the expression of *CaACO4* but this requires further confirmation. Interestingly, in tomato, the expression of *LeACO1*, which is the major isoform in this climacteric fruit, was much higher during ripening of a cultivar that ripens quickly compared to cultivars that ripen later (Anjanasree et al., 2005). However, the ethylene production from all of these cultivars was not significantly different throughout ripening stages (Anjanasree et al., 2005). This could indicate: 1) the possibility of post-translational modification of the ACO enzyme such that all cultivars had similar ACO activity and hence similar ethylene production [however, the ACO activity or protein was not measured in the study by Anjanasree et al. (2005)], 2) other *ACO* isoforms might also contribute to the overall ACO activity, 3) the ACS enzyme was the rate limiting step in different cultivars which restricts the ethylene produced, and 4) ripening may be controlled at the transcriptional level (an upstream ethylene regulated ripening process) such that the transcription factors related to ripening would induce higher *LeACO1* transcript in the

cultivar that ripens quicker compared to the slower ripening cultivars. Additional studies are needed to verify these hypotheses and whether similar events are also conserved in capsicum cultivars with different ripening rates such as cv. Aries and cv. Plato.

### *Conclusion*

In conclusion, the results suggest that sugar and malate accumulation as well as ACO activity might be spatially and temporally regulated in different capsicum tissues. In particular, seeds/placental tissue had similar sugar levels during most of the ripening stages and significantly higher malate and ACO activity during early ripening stages compared to peel and pulp. Furthermore, cv. Plato ripens earlier than cv. Aries which might impact the rate of sugar accumulation, malate degradation and ACO activity level of both cultivars. In order to further confirm present results especially sugars and malate, a more sensitive technique such as High Pressure Liquid Chromatography (HPLC) could be utilised and perhaps seeds and placental tissues can be separated to determine the differences between these tissues. Furthermore, future studies on ACS activity as well as measuring the ACO transcripts between capsicum tissues and cultivars may be needed to develop a better understanding of their differences.

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**Chapter 6. Manuscript 3: Characterisation of  
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RESEARCH ARTICLE

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# Characterisation of ethylene pathway components in non-climacteric capsicum

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## Abstract

**Background:** Climacteric fruit exhibit high ethylene and respiration levels during ripening but these levels are limited in non-climacteric fruit. Even though capsicum is in the same family as the well-characterised climacteric tomato (*Solanaceae*), it is non-climacteric and does not ripen normally in response to ethylene or if harvested when mature green. However, ripening progresses normally in capsicum fruit when they are harvested during or after what is called the 'Breaker stage'. Whether ethylene, and components of the ethylene pathway such as 1-aminocyclopropane 1-carboxylate (ACC) oxidase (ACO), ACC synthase (ACS) and the ethylene receptor (ETR), contribute to non-climacteric ripening in capsicum has not been studied in detail. To elucidate the behaviour of ethylene pathway components in capsicum during ripening, further analysis is therefore needed. The effects of ethylene or inhibitors of ethylene perception, such as 1-methylcyclopropene, on capsicum fruit ripening and the ethylene pathway components may also shed some light on the role of ethylene in non-climacteric ripening.

**Results:** The expression of several isoforms of *ACO*, *ACS* and *ETR* were limited during capsicum ripening except one *ACO* isoform (*CaACO4*). *ACS* activity and *ACC* content were also low in capsicum despite the increase in *ACO* activity during the onset of ripening. Ethylene did not stimulate capsicum ripening but 1-methylcyclopropene treatment delayed the ripening of Breaker-harvested fruit. Some of the *ACO*, *ACS* and *ETR* isoforms were also differentially expressed upon treatment with ethylene or 1-methylcyclopropene.

**Conclusions:** *ACS* activity may be the rate limiting step in the ethylene pathway of capsicum which restricts *ACC* content. The differential expression of several ethylene pathway components during ripening and upon ethylene or 1-methylcyclopropene treatment suggests that the ethylene pathway may be regulated differently in non-climacteric capsicum compared to the climacteric tomato. Ethylene independent pathways may also exist in non-climacteric ripening as evidenced by the up-regulation of *CaACO4* during ripening onset despite being negatively regulated by ethylene exposure. However, some level of ethylene perception may still be needed to induce ripening especially during the Breaker stage. A model of capsicum ripening is also presented to illustrate the probable role of ethylene in this non-climacteric fruit.

**Keywords:** Capsicum, Ethylene pathway, Non-climacteric fruit, Ripening

## Background

Fruit can be divided into two different ripening behaviours, climacteric and non-climacteric types. Climacteric fruit such as banana and tomato generally exhibit ethylene and respiration surges during ripening but non-climacteric fruit such as grapes and capsicum do not [1-3]. The ethylene hormone also regulates the ripening rate of climacteric fruit but its function during non-climacteric ripening is still

inadequately understood [4,5]. Ethylene is produced from the 1-aminocyclopropane 1-carboxylate (ACC) precursor by the action of the ACC oxidase (ACO) enzyme [1]. ACC is synthesised by ACC synthase (ACS) from S-adenosyl methionine (SAM) which originates from the amino acid methionine [1]. Differences between the capability of climacteric and non-climacteric fruit to produce ethylene probably lie with the presence of two systems of ethylene production exclusively in climacteric fruit [4,6].

During fruit development, ethylene is produced at a basal level in climacteric and non-climacteric fruit alike, a process known as System 1 [4,6]. When climacteric fruit

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reach maturity, another process called System 2 is initiated to produce a burst in ethylene production to promote ripening while non-climacteric fruit are thought to remain in System 1 [4,6,7]. The regulation of these two systems has been associated with the differential expression of *ACO* and *ACS* isoforms, especially when first characterised during tomato ripening [8,9]. There are at least six *ACO* isoforms in tomato and nine known *ACS* isoforms but only some of them are expressed during ripening to regulate the two systems [2,10]. For example, *LeACS1A* and *LeACS6* were expressed during System 1 ethylene production and subsequently, *LeACS2* and *LeACS4* as well as *LeACO1* were highly induced during System 2 ethylene production. Furthermore, System 1 is also known to be an auto-inhibitory system whereas System 2 is an auto-stimulatory system [1,4]. In climacteric tomato, System 1-associated isoforms (such as *LeACS1A*) are negatively regulated by high ethylene whereas System 2-associated isoforms (such as *LeACO1* and *LeACS2*) are positively regulated [6,8]. Given that these *ACO* and *ACS* isoforms were regulated by the presence of ethylene, its perception also appears integral to climacteric ripening. Indeed, *ethylene receptors (ETRs)* have been shown to be differentially regulated during ripening and upon ethylene treatment [11]. The six tomato *ETR* isoforms can also be classified into two subfamilies, subfamily I (*LeETR1*, *LeETR2*, *LeETR3*) and subfamily II (*LeETR4*, *LeETR5*, *LeETR6*), with possible differences between these groups explained by their affinity towards the downstream protein, Constitutive Triple Response 1 (*CTR1*) [12,13]. However, the regulation of these isoforms and the two ethylene production systems during non-climacteric ripening is still inadequately documented and hence further research is needed.

Given that capsicum belongs to the Solanaceae family and shares genetic similarity with tomato, the characterisation of the same ethylene pathway in non-climacteric capsicum will enhance our understanding of differences in ethylene production in the two ripening types. Earlier microarray studies have reported that transcripts associated with ethylene signalling were up-regulated in both capsicum and tomato ripening [14,15]. Our recent proteomic analysis also revealed that during capsicum ripening, an *ACO* protein isoform 4 (*CaACO4*) was increased (which corresponded to the *ACO* activity and mRNA expression), suggesting a conserved ethylene pathway may be involved in the ripening of this non-climacteric fruit [16]. However, further components of this pathway such as other *ACO* isoforms, *ACS* and *ETR* isoforms and their regulation in capsicum are still not well described. Additionally, capsicum exhibits a unique ripening behaviour when harvested off the plant; only ripening properly when harvested at Breaker or later but not when harvested during the Green stage [17]. This suggests ripening regulators may be present exclusively during Breaker stage onwards

to induce ripening in non-climacteric capsicum, possibly in an ethylene independent pathway (as ripening can proceed without high levels of ethylene production). Therefore, further post-harvest studies employing ethylene or 1-methylcyclopropene (1-MCP) treatment of both Green and Breaker stages are necessary to characterise the ethylene pathway and/or the possible involvement of ethylene independent pathways in the non-climacteric ripening of capsicum.

In this study we have investigated the expression of *ACO*, *ACS* and *ETR* isoforms during capsicum (*Capsicum annuum* cv. Aries) ripening using quantitative real-time PCR (qPCR) at six different ripening stages (Green, G; Breaker, B; Breaker Red 1, BR1; Breaker Red 2, BR2; Light Red, LR; Deep Red, DR). *ACS* activity and ACC content during the ripening stages were also examined to contrast their levels with climacteric fruit. Furthermore, capsicum was treated with ethylene or 1-MCP at two different stages of ripening (G and B) and their effect on ripening, *ACO* and *ACS* activity, and ACC content was analysed during post-harvest storage. The expression of *CaACO*, *CaACS* and *CaETR* isoforms directly after treatment was also studied.

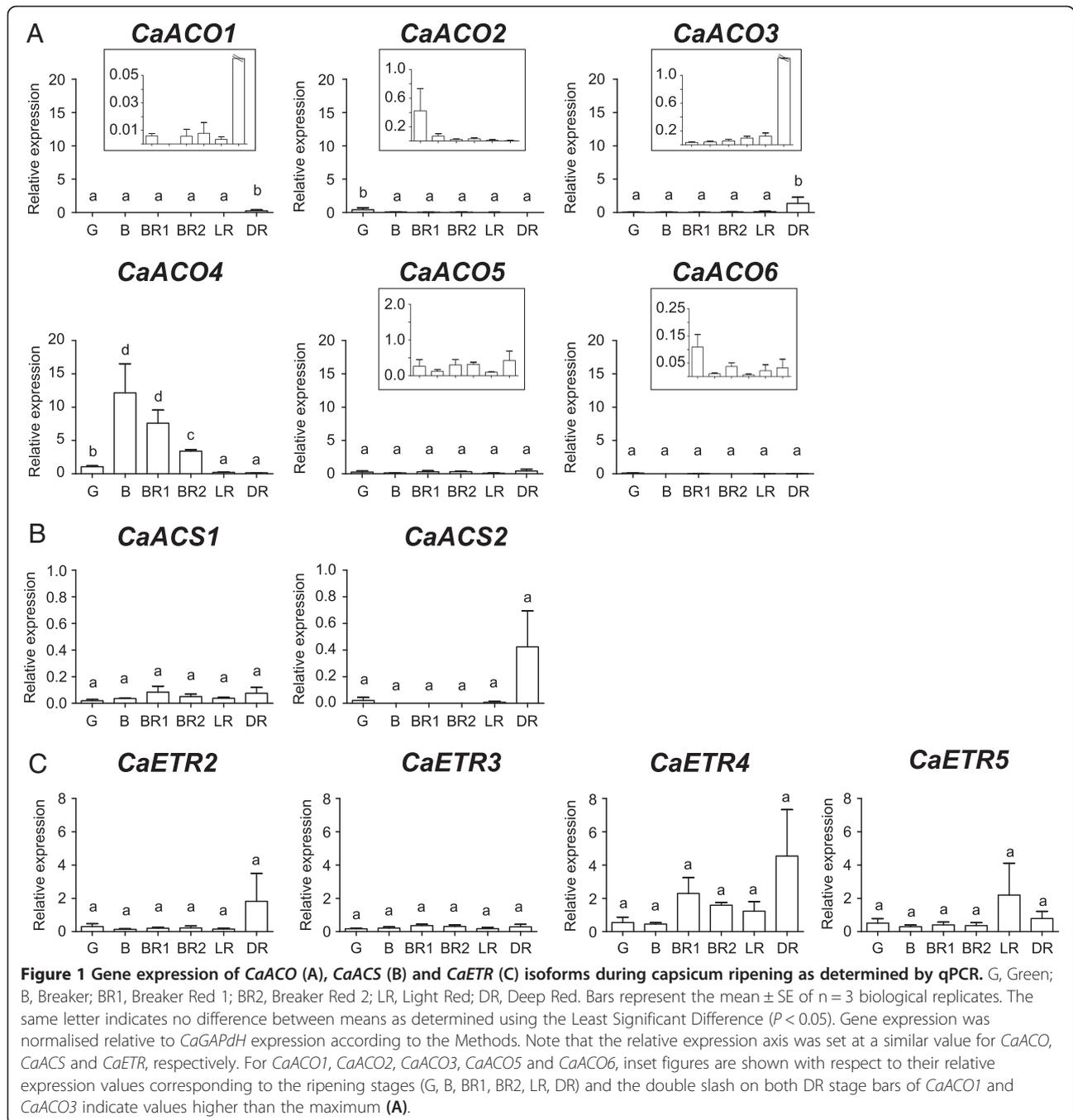
## Results

### **CaACO, CaACS and CaETR isoforms were differentially expressed during capsicum ripening**

Throughout capsicum ripening, the transcript expression of most *ACO* isoforms was limited except *CaACO4* (Figure 1A). *CaACO4* relative expression (normalised by *CaGAPdH*) was significantly greater during ripening onset (approximately seven to 12 times at B and BR1 compared to G) with minimal expression during the full red stages (LR and DR). Even though *CaACO1* and *CaACO3* transcripts were significantly increased at the DR stage and *CaACO2* was increased at the G stage, their relative expression levels throughout capsicum ripening stages were still very low compared to *CaACO4*. The relative transcript expression of *CaACO5* and *CaACO6* was also extremely low but constant during ripening.

Both *CaACS1* and *CaACS2* were not highly expressed during ripening relative to *CaGAPdH* (Figure 1B). The gene expression of both isoforms was also not significantly different during ripening but *CaACS1* was expressed more constantly throughout the six stages compared to *CaACS2*.

No significant change in the gene expression of any *ETR* isoforms was measured during ripening (Figure 1C). Comparing their levels, *CaETR4* was the main isoform expressed during capsicum ripening. *CaETR4* appeared to slightly increase from G (~0.5 relative expression) to the BR1 (~2.3 relative expression) and DR stages (~4.5 relative expression), but this was not statistically significant. In comparison, the expression of *CaETR2*, *CaETR3* and

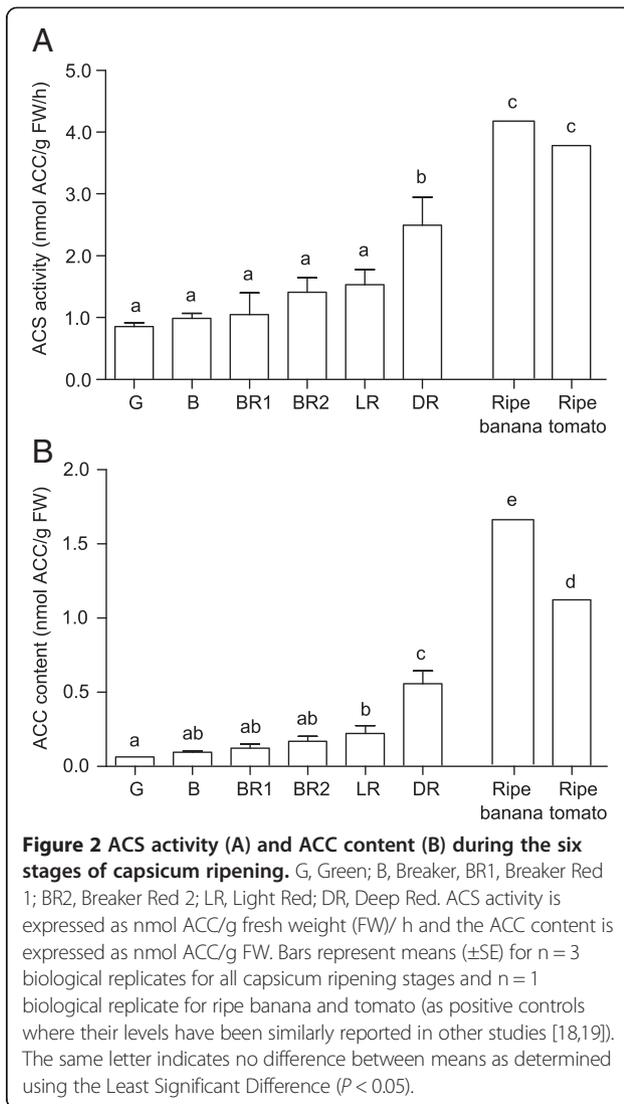


*CaETR5* was consistently low (mostly less than 2.0 mean relative expression) in all stages of ripening.

#### ACS activity and ACC content were limited during capsicum ripening

The mean level of ACS activity (Figure 2A) was not significantly different among any of the stages between G and LR but increased significantly at the DR stage by approximately two-fold. ACS activity in capsicum was approximately two

to four times lower than the two positive climacteric controls (ripe banana and tomato). The level of ACC content (Figure 2B) only increased significantly from the G stage to the LR and DR stages. The amount of ACC in ripe banana and tomato was approximately seven times higher than the average ACC level throughout capsicum ripening. Furthermore, the levels of ACS activity and ACC content reported here for banana and tomato as well as capsicum, correspond to other previous reports [18-20].



### The effects of ethylene or 1-MCP treatment

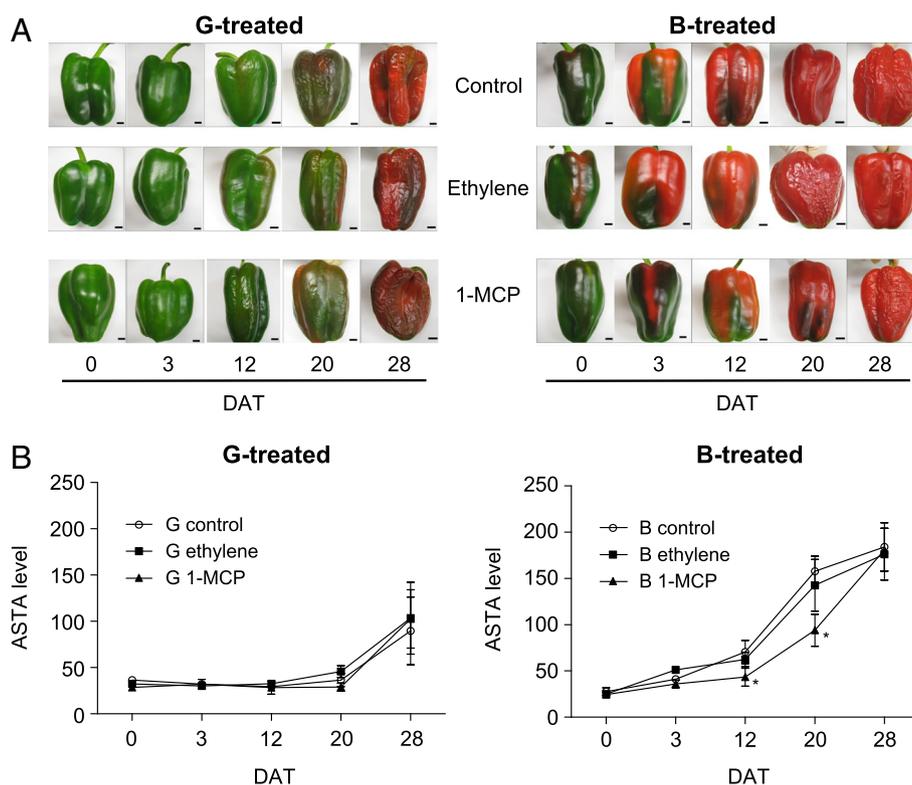
Untreated B-harvested fruit ripened normally and developed to DR after 28 days in storage but untreated G-harvested fruit ripened incompletely (Figure 3A). Regardless of sampling time during storage, ethylene or 1-MCP treatment did not significantly affect the ripening behaviour (Figure 3A, left) and colour development (Figure 3B, left) of G-harvested capsicum. The extractable colour of G-harvested capsicum in all treatments and the control was slightly increased at 28 days after treatment (DAT) compared to earlier sampling times (Figure 3B, left) but was still lower than the fully red coloured capsicum of untreated B-harvested control fruit (approximately 140 ASTA units at 20 DAT, Figure 3B, right). This amount of extractable colour was also achieved by others [17], confirming our present result. In contrast, the ripening behaviour of 1-MCP treated B-harvested fruit was shown to be

delayed compared to those treated with ethylene or the control especially from 3 to 20 DAT (obvious green/black tissues, Figure 3A, right). The extractable colour of both control and ethylene treated fruit reached 140 ASTA units at 20 DAT whereas the 1-MCP treated fruit did not do so until 28 DAT (Figure 3B, right). Regardless of sampling time, the ethylene treatment of B fruit did not significantly affect extractable colour but 1-MCP treatment significantly reduced it at 12 and 20 DAT (Figure 3B right). In terms of percentage weight loss, there were no significant differences regardless of treatment or when fruit was harvested but there was an obvious increase in the percentage weight loss throughout sampling (up to 30% weight loss at the 28 DAT for both G and B treated fruit) indicating that the fruit were dehydrated over time (data not shown).

In our previous report, the ACO activity of capsicum increased significantly at B-BR1 stages compared to the other ripening stages [16]. Indeed, the ACO activity reported here was also significantly higher in untreated B-harvested control fruit than untreated G-harvested control fruit at 0 DAT (Figure 4). Furthermore, at 0 DAT, the ACO activity of G fruit treated with ethylene was not significantly greater than the control while 1-MCP treated fruit had significantly lower ACO activity than the control (Figure 4A). On the other hand, the ACO activity of B-harvested fruit treated with ethylene or 1-MCP was significantly lower than the activity of the untreated B-harvested control fruit. Throughout storage, regardless of treatment, ACO activity peaked slightly at 20 DAT in G-harvested fruit while ACO activity in B-harvested fruit generally exhibited a downward trend (Figure 4).

The ACS activity of both G-harvested and B-harvested fruit (Figure 4B left and right, respectively) was not significantly different, regardless of sampling time and treatment, and significantly lower than the banana positive control (similar level as in Figure 2A, data not shown).

Regardless of sampling time during storage, the ACC content of both G-harvested and B-harvested fruit (Figure 4C left and right, respectively) was consistently lower than the banana positive control (similar level as in Figure 2B, data not shown). At 0 DAT, G-harvested fruit treated with 1-MCP had significantly greater ACC content compared to the control and ethylene-treated fruit. Furthermore, at 28 DAT, there was more ACC in G-harvested ethylene-treated fruit compared to the untreated control and *vice versa* for B-harvested fruit. However, these ACC levels (control and ethylene-treated fruit at 28 DAT) of both G and B harvested capsicum were not significantly different compared to 1-MCP-treated fruit. No other significant changes were observed between the control and treatments at any other DAT.



**Figure 3 Treatment of Green (G, left) and Breaker (B, right) harvested capsicum with 100  $\mu$ L/L ethylene or 500 nL/L 1-MCP (for 24 h).** Untreated control fruit were also prepared for both stages. **A**) Ripening of G and B treated fruit during storage after treatment. Representative images are shown. The scale at the bottom right of the pictures is 1 cm. **B**) The extractable colour (ASTA units) from each treatment and DAT (days after treatment). Asterisks adjacent to symbols indicate means were significantly different compared to the respective control at each DAT as determined by a Duncan's Multiple Range Test ( $P < 0.05$ ) (Additional file 4: Table S2 for details). Means are  $n = 3$  biological replicates ( $\pm$ SE).

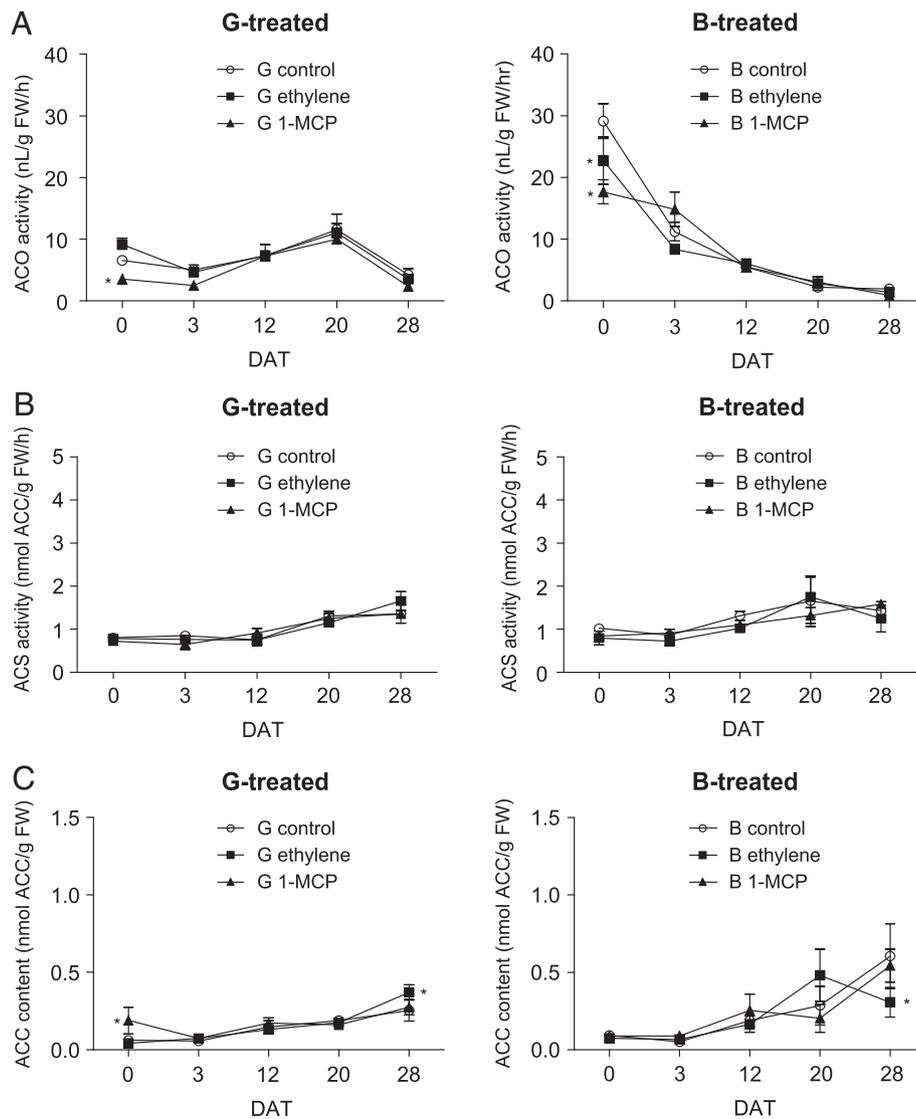
#### Differential expression of *CaACO*, *CaACS* and *CaETR* isoforms directly after treatment (0 DAT) with ethylene or 1-MCP

The six *CaACO* isoforms of capsicum exhibited differential expression upon ethylene or 1-MCP treatment (Figure 5A). When comparing control and ethylene-treated samples of G-harvested fruit, *CaACO1* was not statistically significant. However, the relative expression of *CaACO2* and *CaACO4* was significantly lower (approximately two-fold) in the ethylene-treated samples compared to the respective control. Moreover, the relative expression of other isoforms such as *CaACO3*, *CaACO5* and *CaACO6* was not significantly different between ethylene-treated and control G fruit samples. Conversely, G-harvested fruit treated with 1-MCP had significantly lower *CaACO2*, *CaACO3*, *CaACO4* and *CaACO5* expression compared to the control. Among these isoforms, *CaACO5* was affected the most by 1-MCP (approximately 52-fold lower expression than the control) while others had only approximately two to 11-fold differences. The 1-MCP treatment however did not significantly change *CaACO1* or *CaACO6* expression of G-harvested capsicum. For B-harvested capsicum, the relative expression of ethylene-treated samples was significantly higher for *CaACO1* but significantly lower for *CaACO4* compared to

the respective control. Ethylene had no effect on the expression of *CaACO2*, *CaACO3*, *CaACO5* and *CaACO6* while 1-MCP had no effect on *CaACO1*, *CaACO2*, *CaACO3*, and *CaACO6* in B-harvested fruit. In addition, 1-MCP treatment of B fruit caused *CaACO4* to be significantly lower than the control by three to five-fold and more so for *CaACO5* (approximately 33-fold less in treated samples). When compared across the different *ACO* isoforms within a particular treatment/control, *CaACO4* had the highest relative expression.

For the isolated *CaACS* isoforms (Figure 5B), both isoforms also showed differential expression upon treatment. *CaACS1* expression was significantly reduced in the ethylene-treated G fruit (approximately two-fold less relative expression compared to the untreated G fruit control) and in 1-MCP-treated samples of both G and B harvested fruit (approximately seven-fold less relative expression compared to the respective control). No significant relative expression differences were observed for *CaACS2* between treated samples and the control, where fruit was harvested at either ripening stage.

For the four *CaETR* isoforms (Figure 5C), different expression patterns were observed. Firstly and regardless

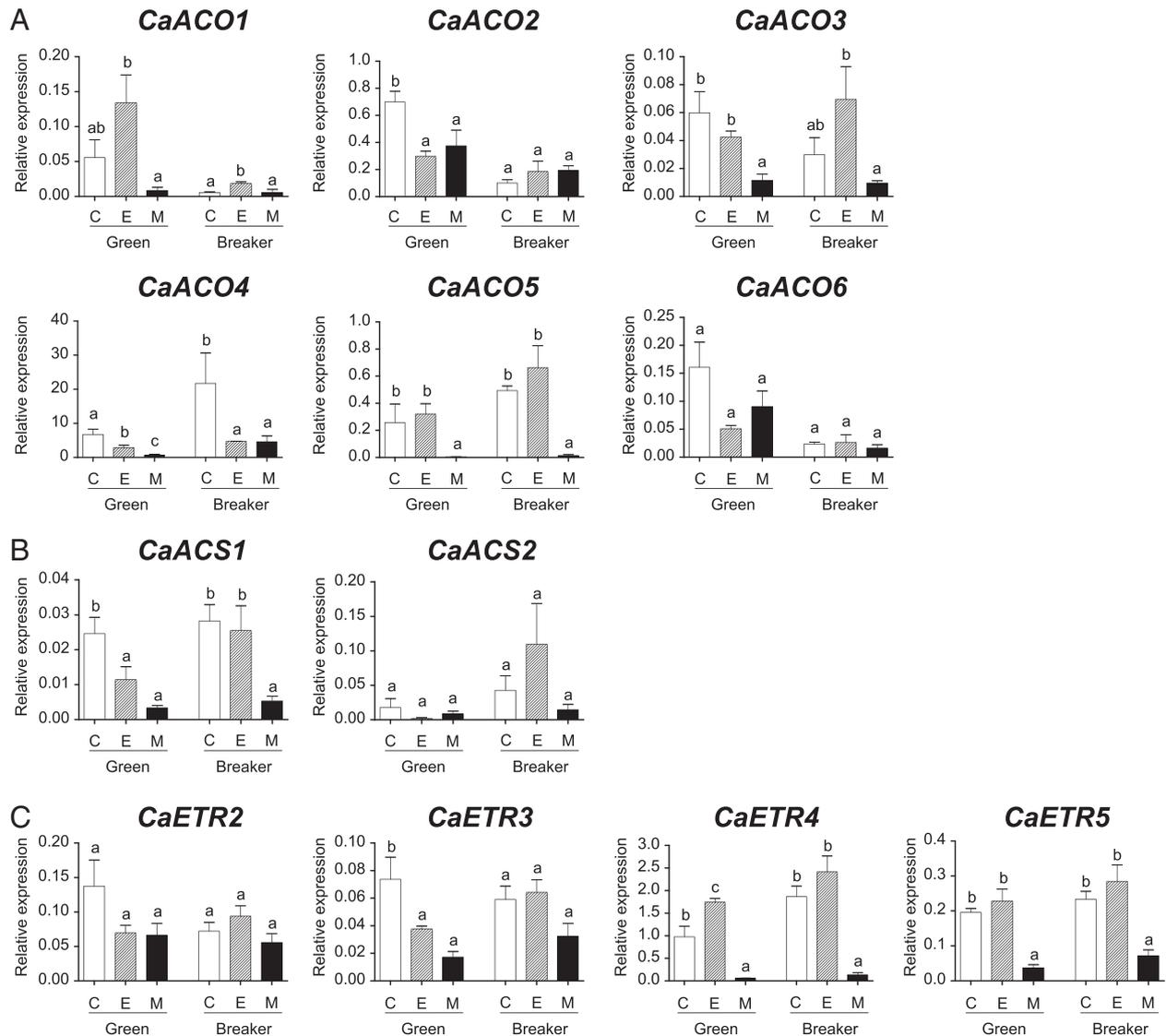


**Figure 4** ACO activity (A), ACS activity (B) and ACC content (C) of capsicum treated with 100  $\mu$ L/L ethylene or 500 nL/L 1-MCP (for 24 h) at two ripening stages, Green-harvested and treated (left) and Breaker-harvested and treated (right). Untreated control fruit

were also analysed for both stages. Asterisks adjacent to symbols indicate means were significantly different compared to the respective control at each DAT (days after treatment) as determined by Duncan's Multiple Range Test ( $P < 0.05$ ) (Additional file 4: Table S2 for details). Means are  $n = 3$  biological replicates ( $\pm$ SE).

of the ripening stage that fruit were harvested and the treatment, no significant changes were measured for *CaETR2*. However, ethylene or 1-MCP treatment of G-harvested fruit did result in significantly lower *CaETR3* relative expression (two to four-fold), while no significant differences were observed in B-harvested fruit for the two treatments. Moreover, the relative expression of *CaETR4* upon ethylene treatment at the G stage was significantly higher by approximately two-fold compared to the control while 1-MCP treatment caused *CaETR4* relative expression to be lower by approximately 17-fold when compared to the G-harvested control. However, no significant difference in

*CaETR4* expression between ethylene-treated and control fruit was observed when they had been treated at the B stage. Nonetheless in B-harvested fruit, *CaETR4* relative expression was significantly lower by approximately 14-fold when 1-MCP-treated fruit were compared with the control. For *CaETR5* expression, no significant difference was observed between the control and ethylene-treated samples in both G and B fruit. However, 1-MCP treatment caused the *CaACO5* transcript level to significantly drop by approximately three to five-fold in both ripening stages compared to the control. In summary and when comparing different *ETR* isoforms within a particular



**Figure 5** The qPCR analysis of *CaACO* (A), *CaACS* (B) and *CaETR* (C) isoforms of Green and Breaker treated capsicum at 0 DAT. C, Control (open bars); E, 100  $\mu$ L/L ethylene treatment for 24 h (striped bars); M, 500 nL/L 1-MCP treatment for 24 h (closed bars). Significantly different levels were determined using Least Significant Difference ( $P < 0.05$ ) of 1-way ANOVA at each stage (Green and Breaker, respectively) and are indicated by different letters on bars ( $\pm$ SE of  $n = 3$  biological replicates). Gene expression was normalised relative to *CaGAPdH* expression according to the Methods. Note that the relative expression axis was set according to respective isoforms.

treatment/control, *CaETR4* still had the highest relative expression (except G-harvested samples treated with 1-MCP which were similar for *CaETR4* and *CaETR2*).

## Discussion

The molecular mechanisms of capsicum ripening are inadequately understood, particularly for non-climacteric behaviour. Due to having genetic similarities with the model fruit tomato, capsicum may become a useful resource to elucidate the molecular regulation of non-climacteric ripening, especially with regards to the ethylene pathway. In this study we have demonstrated that ACS activity and ACC

content were limited in capsicum while several *ACO*, *ACS* and *ETR* isoforms were differentially regulated upon ripening and ethylene treatment. Furthermore, 1-MCP treatment during the onset of ripening (B stage) significantly delayed ripening and reduced the expression of several isoforms, indicating that ethylene perception may be required, to some extent, for non-climacteric fruit ripening to occur.

### Rate limiting ACS activity during capsicum ripening affects the level of its product, ACC

The presence of ACS protein appears to be the rate limiting step in the ethylene pathway of capsicum. Even

though ACO activity was greater in B fruit than other ripening stages (Figure 4A, [16]) and the level in capsicum seems to be comparable with the climacteric tomato during ripening [21,22]; capsicum ACS activity was approximately two to four-fold lower than that for the climacteric fruit used as positive controls (tomato and banana, Figure 2A). In addition, the pattern of *CaACS1* expression corresponded well with the basal level of ACS activity during G to LR, and the increase of ACS activity during DR (Figure 2A) may be due to both *CaACS1* and *CaACS2* expression at the same time (Figure 1B). An earlier study on *CaACS1* also showed that its expression was minimal but constant throughout capsicum ripening stages [23], thus corroborating our current findings. Furthermore, ACS activity in climacteric fruit has been shown to be increased during ripening onset [24,25] but its level in capsicum remained constant for most of the ripening stages (Figure 2A), suggesting that ACS was the rate limiting step in this non-climacteric fruit.

The level of ACC, the product formed from SAM by the action of ACS, was also very low in capsicum such that there was on average, seven-fold less ACC during capsicum ripening compared to the climacteric tomato and banana (Figure 2B). The level of ACC content in capsicum has previously been shown to be limited [26] in a similar manner as other non-climacteric fruit including strawberry [27] and grapes [28]. This is further corroborated by the significant increase in ACC level observed during the LR and DR stages (Figure 2B), probably in response to the limited ACO activity [16] preventing its conversion to ethylene. Furthermore, reducing the amount of ACO activity significantly by means of 1-MCP application during the G stage (at 0 DAT) also increased ACC content (Figure 4A and C, left). This confirms that not only ACC is limited in capsicum but that it might also be continually required for the basal level of ethylene production. However, no increase in ACC content was observed in B fruit directly after 1-MCP treatment despite the reduction in ACO activity, probably because the ACO activity level was still high in B-treated fruit compared to G-treated fruit (Figure 4). This suggests that the low levels of ethylene produced (approximately 0.1 nL/g fresh weight (FW)/h for our capsicum compared to 1.5-3 nL/g FW/h ethylene production for the banana and tomato used as controls in this study; data not shown) was probably due to the lack of precursors for ethylene production caused by the restricted ACS activity.

#### Differential expression of systems 1 and 2-associated isoforms in non-climacteric capsicum ripening

The ripening of climacteric tomato is generally accompanied by a burst in ethylene production which is caused by System 2. The transition from the negative feedback mechanism of System 1 during fruit development to the positive

feedback mechanism of System 2 during the ripening of mature fruit can be regulated by the differential expression of specific isoforms in the ethylene pathway [1]. For example, *LeACS1A* and *LeACS6* are considered to be System 1 components as they are expressed in immature fruit and are negatively regulated by ethylene treatment [8,9]. Once fruit reach maturity, *LeACS2* and *LeACS4* are highly expressed during ripening and upon ethylene treatment, suggesting that they are System 2-associated isoforms [8,22]. The climacteric increase in ethylene production during System 2 was also supported by the up-regulation of ACO isoforms particularly *LeACO1*, and to some extent *LeACO4* [9,29,30]. The application of ethylene to the mature G stage not only significantly increased these ACO isoforms [22,31] but also induced the expression of *ETR* isoforms, primarily *LeETR3*, *LeETR4* and *LeETR6* [11]. The regulation of the two ethylene production systems in non-climacteric fruit is however still not fully described but System 1 is generally considered to be operating throughout ripening [4,7].

Indeed, the expression and regulation of the ethylene pathway components in non-climacteric capsicum were somewhat different compared to the climacteric tomato. For instance, *CaACS2* was not highly expressed during ripening (Figure 1B) and upon ethylene treatment (Figure 5B), while *CaETR4* and *CaACO1* did respond to ethylene but only to some extent in either G or B-harvested fruit, respectively (Figure 5A and C). *CaACO1* expression was also limited throughout capsicum ripening (Figure 1A) which confirmed earlier reports [14,32]. The failure of *CaACO1* and *CaACS2* to be highly stimulated upon ripening and ethylene exposure as in climacteric tomato may suggest the absence of System 2, thus the burst in ethylene production associated with this system could not be induced in this non-climacteric fruit. Furthermore, *CaACO4* could be considered the major isoform expressed during capsicum ripening onset (B-BR stages) as no other ACO isoform exhibited similar levels of up-regulation (Figure 1A). This is in good agreement with our previous report on *CaACO4* expression using semi-quantitative RT-PCR and the overall ACO activity level during capsicum ripening [16]. Interestingly, *CaACO4* was negatively regulated by ethylene (Figure 5A) particularly in B-harvested fruit suggesting its negative regulation may result in the lower overall ACO activity observed after ethylene treatment when compared to the untreated control fruit (Figure 4A, right). Other isoforms such as *CaACO2*, *CaACS1* and *CaETR3* were also down-regulated upon ethylene treatment particularly at the G stage (Figure 5), which implies that System 1 may be predominantly operating in this non-climacteric fruit ripening rather than System 2.

The down-regulation of several transcripts upon ethylene treatment also suggests that other ripening regulators that might control their expression during capsicum

ripening. These ripening regulators might also be present exclusively during the B stage as G-harvested fruit did not ripen properly and the ACO activity of G-harvested capsicum cannot be induced to the level of B capsicum even with ethylene treatment (Figure 4A). *CaACO4*, which may be considered to be a System 1-associated isoform, was up-regulated during ripening onset (Figure 1A) which suggests that its up-regulation might be closely associated with these ripening regulators in an ethylene-independent manner. Two pathways, ethylene dependent- and independent-pathways, have been suggested to operate in climacteric fruit but only the latter pathway may be conserved in non-climacteric fruit to induce ripening [6,33]. The main regulators of the ethylene-independent pathways especially in non-climacteric fruit are still unknown but the *LeMADS-RIN* transcription factor (RIN) has been proposed to be one of the regulators [1,34]. RIN has been shown to control ripening even prior to the climacteric ethylene production in tomato, suggesting that this transcription factor may sit upstream of the ethylene pathway [33,35]. Indeed, RIN has been shown to regulate, directly or indirectly, the expression of several ethylene pathway components including *LeACS2* and *LeACO1* [1,36-38]. Interestingly, the expression of the two isoform homologues in capsicum, *CaACS2* and *CaACO1*, were limited (Figure 1). Furthermore, *LeACO4* may be considered to be a System 2-associated isoform [22] in contrast to the System 1-associated *CaACO4* (Figure 1A). This difference might be attributable to genetic rearrangement that heavily occurred in capsicum when compared with tomato [39], such that upstream promoter regulation is no longer similar between these (and possibly other) Solanaceae members. Further investigation is therefore needed to compare the isoform promoter regions of both fruit as well as the possible involvement of RIN or other ripening regulators in the ethylene-independent pathway(s) of capsicum ripening.

#### **Ethylene perception may be partially required in capsicum ripening especially during ripening onset**

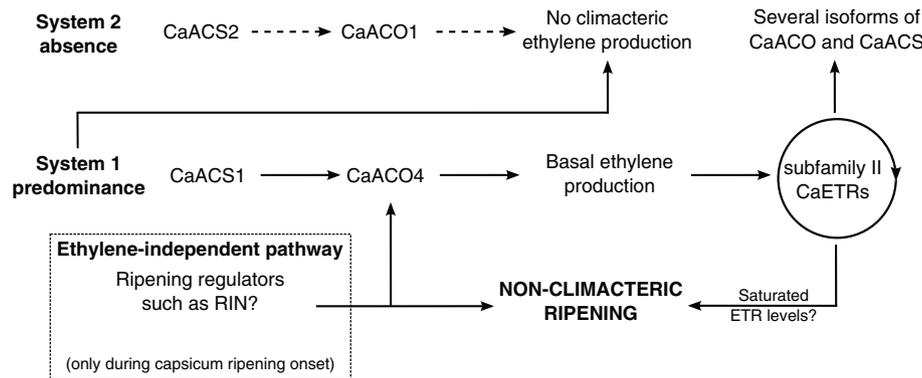
Although the ethylene independent pathway(s) may exist in non-climacteric ripening, our results also highlighted that ethylene perception may still be needed for capsicum ripening, to some extent. 1-MCP treatment, which blocks ethylene perception, delayed the ripening rate of B capsicum by approximately seven days (Figure 3A and B). The application of 1-MCP on other non-climacteric fruit such as grapes and strawberry also has been shown to slow some ripening aspects [28,40,41]. Furthermore, subfamily II receptors, *CaETR4* and *CaETR5* were significantly less in 1-MCP-treated samples compared to the control (Figure 5C), which may consequently influence the down-regulation of other downstream ethylene pathway components such as *CaACS1*, *CaACO4* and *CaACO5* (Figure 5A, B). Subfamily

II *CaETR4* may also be considered to be the major *ETR* isoform expressed in capsicum due to its higher overall relative expression during ripening (Figure 1C). Interestingly, in strawberry, *FaETR2* which is closely related to *LeETR4* was also the major isoform expressed [12]. Since both *CaETR4* and *FaETR2* belong to subfamily II ETRs which may have weaker binding with the CTR1 protein [13], the basal level of ethylene in the non-climacteric fruit may be sufficient to induce considerable changes to downstream ethylene responses and the inhibition of the ethylene perception may also severely impact ripening.

Ethylene binding has been associated with controlling the rate of ethylene receptor turnover in tomato [11], and in the case of non-climacteric fruit, the basal level of ethylene may be needed to sustain a certain receptor level and hence maintain ethylene perception for normal ripening to proceed. This was further corroborated by our findings in that ethylene treatment did not induce any significant changes towards ripening (Figure 4A, B), and the expression of all four measured *ETR* isoforms at the B stage in capsicum after ethylene exposure were also constant compared to the control (Figure 5C). This implies that there may be no more receptors available to accept ethylene (as they were possibly saturated) especially during ripening onset and thus there were no effects towards the ethylene perception and response for ripening. This further suggests that only a certain level of ethylene receptors and perception are required for the ripening process, together with the ethylene independent pathway(s) as described earlier. Therefore, a high ethylene level may not be needed and the energy saved can be utilised for other ripening related events such as colour and textural modification. However, any mRNA expression changes (especially for the *ETRs*) need to be confirmed with respective protein expression assays as post-translational regulation has been shown to strictly affect the receptor protein abundance [11]. In addition, given that the *Capsicum chinensis* gene *CcGH3*, which has been shown to influence fruit ripening, is regulated by both ethylene and auxin [42], whether a similar situation is possible for *CaETR* needs to be investigated.

#### **Conclusions**

Overall, the limited level of ethylene produced in non-climacteric capsicum may be contributed by the rate limiting ACS activity which restricts the ACC content. Furthermore, several isoforms of ethylene-related genes were differentially expressed in capsicum, suggesting alternative regulation and the likelihood that ethylene production in non-climacteric ripening is predominantly by System 1 with System 2 being absent (summarised in Figure 6). Ethylene independent pathway(s) may also be present during capsicum ripening onset but some level of ethylene perception may still be needed for the induction of non-climacteric fruit ripening (Figure 6).



**Figure 6 A proposed model for the ripening of non-climacteric capsicum.** The ethylene pathway generally involves different isoforms of ACO and ACS to produce ethylene before it is perceived by ETR for other downstream responses. In tomato, System 1 ethylene production during development will be followed by System 2 ethylene production to induce climacteric ripening [8]. However, in non-climacteric capsicum ripening, System 2 may be absent based on the limited expression (dotted arrows) of certain System 2-associated isoforms such as *CaACS2* and *CaACO1* during ripening and upon ethylene treatment (compared to their homologue expression in tomato). The expression of *CaACS1* and *CaACO4* during capsicum ripening was associated with System 1 due to their significant reduction upon ethylene treatment at either or both G and B stages. This is in contrast to the *CaACO4* homologue in tomato, *LeACO4*, which is a System 2-associated isoform [22]. Therefore System 1 may be predominantly operating in capsicum to produce the basal ethylene level (while inhibiting System 2 ethylene production). The basal ethylene level may be needed to maintain the rate of ETR turnover, particularly subfamily II CaETR4 and CaETR5, as ethylene perception removal (through 1-MCP treatment) severely affected the *CaETR* expression as well as other possible downstream *CaACO* and *CaACS* isoforms. These subfamily II CaETRs may become saturated, and perhaps together with ethylene independent pathways upon ripening onset, non-climacteric capsicum ripening could be initiated. The ethylene independent pathways may also involve some ripening regulators such as RIN transcription factors and their presence may induce the expression of *CaACO4* upon ripening onset.

## Methods

### Capsicum ripening tissues and the treatment of G and B harvested fruit with ethylene or 1-MCP

For different ripening stages, tissues from the six capsicum stages (G, B, BR1, BR2, LR and DR) were prepared and described in Aizat et al. [16]. For ethylene and 1-MCP treatments, capsicum plants were grown as per Aizat et al. [16], fruit length was measured every week beginning at 27 days after anthesis (DAA) onwards and harvested on Jan-Feb 2013 (summer season) when fruit reached either G (43 DAA) or B (50 DAA) stage. The average length of the matured stages was approximately 85 mm (data not shown). Harvested fruit at each stage were cleaned using nanopure water, dried at room temperature (RT, 22-23°C) for approximately 1 h, weighed and randomly allocated into nine 10 L plastic containers with a septum (five fruit each container). In each container, there was a heavy-duty towel at the bottom and approximately 100 g  $\text{Ca}(\text{OH})_2$  as a  $\text{CO}_2$  scrubber. Three containers were allocated for each treatment: control (no treatment), ethylene (100  $\mu\text{L}/\text{L}$  final concentration, Coregas) or 1-MCP (500 nL/L final concentration, prepared as in Moradinezhad et al. [43]). After a 24 h treatment in the dark at RT, fruit was aired in a laminar flow for 30 min, removed from the containers and placed into aluminium foil trays individually. The fruit were then stored at RT in the dark with potassium permanganate and  $\text{Ca}(\text{OH})_2$  to remove residual ethylene and carbon dioxide respectively [43,44]. At 0 (directly after treatment), 3, 12, 20 and 28 days after treatment (DAT),

three fruit for each treatment were weighed, photographed, sampled in liquid  $\text{N}_2$  according to Aizat et al. [16] and stored in  $-80^\circ\text{C}$  until further analysis.

### cDNA and genomic DNA stocks

For different ripening stages, all cDNA stocks were prepared and described as per Aizat et al. [16]. Genomic DNA was extracted from B fruit for genomic end-point PCR when no products were amplified from the cDNA stocks using a protocol adapted from Karakousis and Langridge [45]. Ground capsicum tissues (250 mg) were homogenised with 0.5 mL DNA extraction buffer pH 8.0 [100 mM Tris-HCl, 100 mM NaCl, 10 mM ethylenediamine tetraacetic acid (EDTA), 1% (w/v) N-Lauroyl Sarcosine (sarkosyl), 1% (w/v) poly(vinylpyrrolidone) (PVPP)]. Phenol: chloroform: isoamyl alcohol solution (25:24:1 v/v) (0.5 mL) was added, vortexed briefly and mixed on an orbital shaker for 15 min at 4°C. Samples were centrifuged (6000 rpm, 15 min, 4°C) and the upper aqueous solution was added to chloroform (0.5 volume for each 1.0 sample volume). Sample mixtures were then vortexed, centrifuged (13 000 rpm, 10 min, 4°C) and the chloroform extraction was repeated one more time. For every 1.0 mL of final aqueous sample, 90  $\mu\text{L}$  of 3 M sodium acetate and 900  $\mu\text{L}$  isopropanol were then added. The sample was then mixed on an orbital shaker for 10 min at 4°C and centrifuged to pellet (13 000 rpm, 10 min, 4°C). The DNA pellet was washed with 70% ethanol (500  $\mu\text{L}$ ) before being air-dried and resuspended in 30  $\mu\text{L}$  sterilised nanopure water. The extracted

genomic DNA was quantified using a NanoDrop 1000 (ThermoScientific, Wilmington, DE, USA) as per manufacturer's instructions.

For different treatments, RNA was extracted from the control and treated capsicum tissues at 0 DAT as per Aizat et al. [16] except that all centrifugation steps were done at 13 000 rpm for 20 min (4°C) and the capsicum materials, as well as buffers used, were scaled down to 1:10. The RNA was quantified, DNase-treated and synthesised to cDNA according to Aizat et al. [16].

#### End-point PCR

All end-point PCR was performed using primers shown in Additional file 1: Table S1, according to Aizat et al. [16] but with some modifications. The template used was cDNA mixture pooled from one biological replicate of all six stages of ripening (cDNA of G, B, BR1, BR2, LR and DR stages), while for all primer pairs the annealing was run at 55°C for 30 s and elongation was run at 72°C for 30 s for all primer pairs. PCR products were transformed into pGEM-T Easy (Promega, Madison, WI, USA) and sequenced from at least four independent colonies as per Aizat et al. [16]. Phylogenetic analysis was performed using Molecular Evolutionary Genetics Analysis (MEGA) program version 5.05 using the conditions stated in Khoo et al. [46].

For genomic end-point PCR the standard PCR procedure above was performed except, with the genomic DNA template, annealing temperatures were either at 55°C, 60°C or 65°C and the elongation step was for 1.5 min to take into account possible introns.

#### The identification of CaACO, CaACS and CaETR isoforms in capsicum

Full-length isoform sequences of tomato *ACO* (*LeACO1*, *LeACO2*, *LeACO3*, *LeACO4*, *LeACO5*, *LeACO6*), *ACS* (*LeACS1A*, *LeACS1B*, *LeACS2*, *LeACS3*, *LeACS4*, *LeACS5*, *LeACS6*, *LeACS7*, *LeACS8*) and *ETR* (*LeETR1*, *LeETR2*, *LeETR3*, *LeETR4*, *LeETR5*, *LeETR6*) were first obtained from the NCBI database ([47], accessed on 21 January 2013) before being used to identify any related capsicum accessions using the BLASTn search in the NCBI database as well as a capsicum EST database ([48], accessed on 21 January 2013) as per Aizat et al. [16]. The accession numbers are listed in Additional file 1: Table S1, and the corresponding phylogenetic analysis is presented in Additional file 2: Figure S1. Capsicum isoforms were named according to the corresponding homologues in tomato for easier reference and comparison. For the *ACO* isoforms, only a partial sequence of capsicum *CaACO1* from Garcia-Pineda and Lozoya-Gloria [32] and a full-length of *CaACO4* from Aizat et al. [16] were available in the NCBI database whereas all six possible capsicum isoforms were identified using the capsicum EST database which

encoded for full length proteins, except *CaACO5*. For the *ACS* isoforms, only *CaACS1* and *CaACS2* accessions were identified that encode full-length proteins and one EST transcript (partial) that closely matched *LeACS3*. For the capsicum *ETR* isoforms, no NCBI accession was found that closely related to any tomato *ETR* isoform. However using the capsicum EST database, one EST closely matched to *LeETR3* (named *CaETR3*) and two ESTs related to *LeETR4* and *LeETR5* (named *CaETR4* and *CaETR5*, respectively) were identified (all partial).

In order to further isolate any other possible *ACS* and *ETR* isoforms, degenerate primers were designed based on consensus sequences of highly conserved regions in *LeACS3*, *LeACS4*, *LeACS5*, *LeACS6*, *LeACS7* and *LeACS8* for *ACS* and subfamily I (*LeETR1*, *LeETR2*, *LeETR3*) as well as subfamily II (*LeETR4*, *LeETR5*, *LeETR6*) receptors for *ETR*. Available EST sequences from capsicum were also taken into consideration when designing these degenerate primers. However, no PCR product (using pooled cDNA from six ripening stages as templates) was detected for *ACS* degenerate primers but both sets of primers for *ETRs* produced a single band (Additional file 3: Figure S2) which after sequencing contained two different products. Phylogenetic analysis reveals that *ETR* subfamily I primers yielded products closely related to *LeETR2* and *LeETR3* while *ETR* subfamily II primers yielded products that matched *LeETR4* and *LeETR5* (Additional file 2: Figure S1). Three of these sequences matched to the three earlier EST sequences (*CaETR3*, *CaETR4* and *CaETR5*) but one sequence which is related to *LeETR2* (hence named *CaETR2*) did not match to any annotations in any databases searched.

Primers specific for each capsicum isoform were designed and run in end-point PCR. All primer pairs resulted in the amplification of a single PCR product using the pooled cDNA as templates (Additional file 3: Figure S2) and were specific to each isoform based on sequencing and a single qPCR melt curve (data not shown). However, *CaACS3* was not expressed as its primer was able to amplify genomic DNA but not its cDNA transcript (Additional file 3: Figure S2). Furthermore, *ACS4* primers from Osorio et al. [14] were not able to amplify any products even from the genomic PCR (Additional file 3: Figure S2), with no other information on *CaACS4* available in the NCBI and EST databases. *CaGAPdH* primers were obtained from Ogasawara et al. [49] and used in Aizat et al. [16] as well.

#### qPCR analysis

qPCR was performed as outlined by Schaarschmidt et al. [50]. Briefly, three biological replicates for each ripening stage and for different treatments at 0 DAT were analysed. A 1:10 dilution with double-sterilised nanopure water was made for all cDNA stocks and run in a qPCR

instrument (ViiA™ 7, Life Technologies, USA). In each 10 µL qPCR reaction (three technical replicates for each sample), the diluted cDNA template (1.5 ng reverse transcribed total RNA) and primers (5 pmol of each forward and reverse) were mixed with SYBR® Green reagent (iQ™ supermix, BioRad, USA). The qPCR running conditions were 95°C for 15 s, 40 cycles of 95°C for 15 s and 55°C for 30 s, followed by one step of 95°C for 15 s, 60°C for 1 min and 95°C for 15 s to generate the melt curve. A positive control (using 1:1000 dilution of purified plasmid transformed with *CaGAPdH* PCR product as the template and corresponding primers) and negative controls of no template were run in all qPCR plates. Ct values for each reaction were evaluated using in-built ViiA™ 7 version 1.2 software (Applied Biosystems) and imported into the Microsoft Excel program. The relative gene expression analysis was done according to the standard comparative Ct method ( $2^{-\Delta\Delta C_t}$ ) by correcting the Ct values of each gene to the positive control, before normalisation of the gene of interest to the *CaGAPdH* endogenous control. Furthermore, samples which did not possess any significant melt curve across all three technical replicates were considered to not be expressing that particular isoform and a value of 0 relative expression was set.

#### Enzymatic assays and colour (ASTA) measurement

The ACS activity assay was adapted from Kato et al. [51] with some modifications. Briefly, ground tissues [0.2 g FW] were homogenised with 1.8 mL ice-cold EPPS buffer A (0.1 M EPPS-KOH pH 8.5, 10 mM 2-mercaptoethanol, 10 µM pyridoxal phosphate). Samples were centrifuged at 13 000 rpm at 4°C for 20 min. A clear sample (0.5 mL) was added with an ice-cold EPPS buffer B (0.5 mL of 0.1 M EPPS-KOH pH 8.5, 0.2 mM SAM) in a test tube (12 × 75 mm) fitted with a rubber stopper (9.5 mm sub-seal). All reaction mixtures were incubated for 30 min at 30°C and the ACS activity was then measured as per Kato et al. [51]. Another reaction containing a spike solution (0.5 mL EPPS buffer B with 0.01 µM ACC) and 0.5 mL sample (extracted with the EPPS buffer A above) was also prepared similarly to calculate the efficiency of the assay as per Bulens et al. [52]. The ACC content was measured as per Tan et al. [20]. For both ACS activity and ACC content assays, ground tissues of one biological replicate of commercial ripe banana (Stage 5 according to CSIRO [53]) and red tomato were also run similarly as positive controls. The ACO activity assay was run as detailed in Aizat et al. [16].

Extractable colour was measured according to the standard method of the American Spice Trade Association, ASTA [54]. Ground tissues (1.5 g) were dried at 40°C for 24 h. Dried materials were weighed to approximately 43 mg, incubated in flasks containing 50 mL absolute

acetone and shaken in the dark for 18 h. The absorbance of the extracted colour was determined at a wavelength of 460 nm using a UV/VIS Spectrophotometer SP 8001 (Ade-Lab Scientific, Thebarton, Australia) and the ASTA units were calculated according to the standard formula [54].

#### Statistical analysis

All statistics were performed using Genstat 14 (Hemell Hempstead, UK). Least Significant Difference (LSD) at  $P < 0.05$  in the Analysis of Variance (ANOVA) was used to determine significantly different means, unless otherwise stated. The statistical significance for the different treatments at each of the sampling times was determined using Duncan's Multiple Range Test at  $P < 0.05$  (Additional file 4: Table S2).

#### Additional files

**Additional file 1: Table S1.** List of primers used in PCR and qPCR.

**Additional file 2: Figure S1. The phylogenetic analysis of ACO (A), ACS (B) and ETR (C and D) isoforms.** The tree was built based on full-length protein sequences of ACO isoforms (except CaACO5 which is partial), and the full length of ACS isoforms. The trees for ETRs were built based on mRNA sequences of tomato and the partial sequence of capsicum ETRs subfamily I (*CaETR2* and *CaETR3*, C) and subfamily II (*CaETR4* and *CaETR5*, D) obtained from an end point RT-PCR using degenerate primers for respective subfamilies (refer to Methods). Genbank accession numbers for the tomato are: LeACO1, P05116.2; LeACO2, CAA68538.1; LeACO3, CAA90904.1; LeACO4, NP\_001233867.1; LeACO5, NP\_001234037.1; LeACO6, ABP68407.1; LeACS1A, NP\_001233922.1; LeACS1B, AAB17279.1; LeACS2, NP\_001234178.1; LeACS3, NP\_001234026.1; LeACS4, NP\_001233875.1; LeACS5, NP\_001234156.1; LeACS6, BAA34923.1; LeACS7, NP\_001234346.1; LeACS8, NP\_001234160.1; *LeETR1*, NM\_001247220.1; *LeETR2*, NM\_001247224.1; *LeETR3*, NM\_001246965.1; *LeETR4*, NM\_001247276.1; *LeETR5*, NM\_001247283.1 and *LeETR6*, NM\_001247221.1. For capsicum, the available Genbank accession numbers are: CaACO4, AGG20315; CaACS1, BAG30909.1; and CaACS2, BAG30910.1. All other capsicum isoforms (from contigs of the EST database) are listed in Additional file 1: Table S1 translated *in silico* as per [16]. Le, tomato; Ca, Capsicum.

**Additional file 3: Figure S2.** End-point PCR for isolating capsicum ACO, ACS and ETR isoforms. Degenerate primers of ETR subfamily I (*ETRdeg1-3*) and subfamily II (*ETRdeg4-6*) as well as ACS isoforms (*ACSdeg*) were run in the end-point PCR (using cDNA pooled from six stages of ripening) to isolate other possible isoforms due to the lack of information in the databases (A). The primers for six *CaACO* isoforms (B), four *CaACS* isoforms (C) and four *CaETR* isoforms (D) were run in the end-point PCR using the pooled cDNA template. All bands are less than 0.26 kb using primers listed in Additional file 1: Table S1. The differences between band intensity (especially *CaACO1* and *CaETR4*) could be due to loading. The absence of *CaACS3* and *CaACS4* in the cDNA mix was also confirmed in independent RT-PCR and qPCR experiments (data not shown). (E) Genomic PCR using DNA template (annealing temperature 60°C) was also performed but only the *CaACS3* (and positive control *CaGAPdH* plus introns) produced specific products and not *CaACS4*. Experiments were repeated using two other annealing temperatures (55°C and 65°C), again without any amplification of *CaACS4* (data not shown). *CaACS4* primers were obtained from a previous qPCR study in capsicum and tomato [14] and no other information regarding its sequence was available in either the NCBI or capsicum EST databases.

**Additional file 4: Table S2.** Significant levels of colour (Figure 2B), ACO activity (Figure 3A), ACS activity (Figure 3B) and ACC content (Figure 3C) were determined using Duncan's Multiple Range Test ( $P < 0.05$ ). This analysis compares all data for control (C), ethylene (E) and 1-MCP (M) treated fruit at different days after treatment (DAT) within the respective ripening stage.

## Abbreviations

ACC: 1-aminocyclopropane 1-carboxylate; ACO: ACC oxidase; ACS: ACC synthase; ETR: Ethylene receptor; G: Green; B: Breaker; BR1: Breaker red 1; BR2: Breaker red 2; LR: Light red; DR: Deep red.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

WMA performed the experiments. WMA, JAA, JCRS and AJA participated in the design and interpretation of the study, all authors read and approved the final manuscript.

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## References

- Lin ZF, Zhong SL, Grierson D: Recent advances in ethylene research. *J Exp Bot* 2009, **60**:3311–3336.
- Cara B, Giovannoni JJ: Molecular biology of ethylene during tomato fruit development and maturation. *Plant Sci* 2008, **175**:106–113.
- Bapat VA, Trivedi PK, Ghosh A, Sane VA, Ganapathi TR, Nath P: Ripening of fleshy fruit: molecular insight and the role of ethylene. *Biotechnol Adv* 2010, **28**:94–107.
- Pech J-C, Purgatto E, Bouzayen M, Latché A: Ethylene and Fruit Ripening. In *Annual Plant Reviews: The Plant Hormone Ethylene*, Volume 44. 1st edition. Edited by McManus MT. Chichester: John Wiley & Sons Ltd; 2012:275–304.
- Gapper NE, McQuinn RP, Giovannoni JJ: Molecular and genetic regulation of fruit ripening. *Plant Mol Biol* 2013, **82**:575–591.
- Lelièvre J-M, Latché A, Jones B, Bouzayen M, Pech J-C: Ethylene and fruit ripening. *Physiol Plant* 1998, **101**:727–739.
- Katz E, Lagunes PM, Riov J, Weiss D, Goldschmidt EE: Molecular and physiological evidence suggests the existence of a system II-like pathway of ethylene production in non-climacteric citrus fruit. *Planta* 2004, **219**:243–252.
- Barry CS, Llop-Tous MI, Grierson D: The regulation of 1-aminocyclopropane-1-carboxylic acid synthase gene expression during the transition from System-1 to System-2 ethylene synthesis in tomato. *Plant Physiol* 2000, **123**:979–986.
- Nakatsuka A, Murachi S, Okunishi H, Shiomi S, Nakano R, Kubo Y, Inaba A: Differential expression and internal feedback regulation of 1-aminocyclopropane-1-carboxylate synthase, 1-aminocyclopropane-1-carboxylate oxidase, and ethylene receptor genes in tomato fruit during development and ripening. *Plant Physiol* 1998, **118**:1295–1305.
- Barry CS, Giovannoni JJ: Ethylene and fruit ripening. *J Plant Growth Regul* 2007, **26**:143–159.
- Kevany BM, Tieman DM, Taylor MG, Dal Cin V, Klee HJ: Ethylene receptor degradation controls the timing of ripening in tomato fruit. *Plant J* 2007, **51**:458–467.
- Trainotti L, Pavanello A, Casadoro G: Different ethylene receptors show an increased expression during the ripening of strawberries: Does such an increment imply a role for ethylene in the ripening of these non-climacteric fruits? *J Exp Bot* 2005, **56**:2037–2046.
- Cancel JD, Larsen PB: Loss-of-function mutations in the ethylene receptor ETR1 cause enhanced sensitivity and exaggerated response to ethylene in *Arabidopsis*. *Plant Physiol* 2002, **129**:1557–1567.
- Osorio S, Alba R, Nikoloski Z, Kochevenko A, Fernie AR, Giovannoni JJ: Integrative comparative analyses of transcript and metabolite profiles from pepper and tomato ripening and development stages uncovers species-specific patterns of network regulatory behavior. *Plant Physiol* 2012, **159**:1713–1729.
- Lee S, Chung EJ, Joung YH, Choi D: Non-climacteric fruit ripening in pepper: Increased transcription of EIL-like genes normally regulated by ethylene. *Funct Integr Genomic* 2010, **10**:135–146.
- Aizat WM, Able JA, Stangoulis JCR, Able AJ: Proteomic analysis during capsicum ripening reveals differential expression of ACC oxidase isoform 4 and other candidates. *Funct Plant Biol*. in press.
- Krajayklang M, Klieber A, Dry PR: Colour at harvest and post-harvest behaviour influence paprika and chilli spice quality. *Postharvest Biol Tec* 2000, **20**:269–278.
- Nakatsuka A, Shiomi S, Kubo Y, Inaba A: Expression and internal feedback regulation of ACC synthase and ACC oxidase genes in ripening tomato fruit. *Plant Cell Physiol* 1997, **38**:1103–1110.
- Inaba A, Liu XJ, Yokotani N, Yamane M, Lu WJ, Nakano R, Kubo Y: Differential feedback regulation of ethylene biosynthesis in pulp and peel tissues of banana fruit. *J Exp Bot* 2007, **58**:1047–1057.
- Tan CK, Ali ZM, Zainal Z: Changes in ethylene production, carbohydrate activity and antioxidant status in pepper fruits during ripening. *Sci Hortic* 2012, **142**:23–31.
- Zhang M, Yuan B, Leng P: The role of ABA in triggering ethylene biosynthesis and ripening of tomato fruit. *J Exp Bot* 2009, **60**:1579–1588.
- Li L, Zhu BZ, Yang PY, Fu DQ, Zhu Y, Luo YB: The regulation mode of RIN transcription factor involved in ethylene biosynthesis in tomato fruit. *J Sci Food Agr* 2011, **91**:1822–1828.
- Harpster M, Howie W, Dunsmuir P: Characterization of a PCR fragment encoding 1-aminocyclopropane-1-carboxylate synthase in pepper (*Capsicum annuum*). *J Plant Physiol* 1996, **147**:661–664.
- Sitrit Y, Riov J, Blumenfeld A: Regulation of ethylene biosynthesis in avocado fruit during ripening. *Plant Physiol* 1986, **81**:130–135.
- Vilaplana R, Soria Y, Valentines MC, Larrigaudière C: Specific response of apple skin and pulp tissues to cold stress and 1-MCP treatment. *Postharvest Biol Tec* 2007, **43**:215–220.
- Pretel MT, Serrano M, Amoros A, Riquelme F, Romojaro F: Non-involvement of ACC and ACC oxidase activity in pepper fruit ripening. *Postharvest Biol Tec* 1995, **5**:295–302.
- Atta-Aly MA, Brecht JK, Huber DJ: Ethylene feedback mechanisms in tomato and strawberry fruit tissues in relation to fruit ripening and climacteric patterns. *Postharvest Biol Tec* 2000, **20**:151–162.
- Chervin C, El-Kereamy A, Roustan JP, Latché A, Lamon J, Bouzayen M: Ethylene seems required for the berry development and ripening in grape, a non-climacteric fruit. *Plant Sci* 2004, **167**:1301–1305.
- Barry CS, Blume B, Bouzayen M, Cooper W, Hamilton AJ, Grierson D: Differential expression of the 1-aminocyclopropane-1-carboxylate oxidase gene family of tomato. *Plant J* 1996, **9**:525–535.
- Van de Poel B, Bulens I, Markoula A, Hertog M, Dreesen R, Wirtz M, Vandoninck S, Oppermann Y, Keulemans J, Hell R, Waelkens E, De Proft MP, Sauter M, Nicolai BM, Geeraerd AH: Targeted systems biology profiling of tomato fruit reveals coordination of the Yang cycle and a distinct regulation of ethylene biosynthesis during postclimacteric ripening. *Plant Physiol* 2012, **160**:1498–1514.
- Yokotani N, Tamura S, Nakano R, Inaba A, McGlasson WB, Kubo Y: Comparison of ethylene- and wound-induced responses in fruit of wild-type, rin and nor tomatoes. *Postharvest Biol Tec* 2004, **32**:247–252.
- García-Pineda E, Lozoya-Gloria E: Induced gene expression of 1-aminocyclopropane-1-carboxylic acid (ACC oxidase) in pepper (*Capsicum annuum* L.) by arachidonic acid. *Plant Sci* 1999, **145**:11–21.
- Giovannoni JJ: Fruit ripening mutants yield insights into ripening control. *Curr Opin Plant Biol* 2007, **10**:283–289.
- Giovannoni JJ: Genetic regulation of fruit development and ripening. *Plant Cell* 2004, **16**:S170–S180.
- Vrebalov J, Ruzinsky D, Padmanabhan V, White R, Medrano D, Drake R, Schuch W, Giovannoni J: A MAD5-box gene necessary for fruit ripening at the tomato ripening-inhibitor (Rin) locus. *Science* 2002, **296**:343–346.
- Ito Y, Kitagawa M, Ihashi N, Yabe K, Kimbara J, Yasuda J, Ito H, Inakuma T, Hiroi S, Kasumi T: DNA-binding specificity, transcriptional activation potential, and the rin mutation effect for the tomato fruit-ripening regulator RIN. *Plant J* 2008, **55**:212–223.
- Lin ZF, Hong YG, Yin MG, Li CY, Zhang K, Grierson D: A tomato HD-Zip homeobox protein, LeHB-1, plays an important role in floral organogenesis and ripening. *Plant J* 2008, **55**:301–310.

38. Fujisawa M, Nakano T, Ito Y: **Identification of potential target genes for the tomato fruit-ripening regulator RIN by chromatin immunoprecipitation.** *BMC Plant Biol* 2011, **11**:26.
39. Livingstone KD, Lackney VK, Blauth JR, van Wijk R, Jahn MK: **Genome mapping in capsicum and the evolution of genome structure in the Solanaceae.** *Genetics* 1999, **152**:1183–1202.
40. Villarreal NM, Bustamante CA, Civello PM, Martínez GA: **Effect of ethylene and 1-MCP treatments on strawberry fruit ripening.** *J Sci Food Agr* 2010, **90**:683–689.
41. Sun LA, Zhang M, Ren J, Qi JX, Zhang GJ, Leng P: **Reciprocity between abscisic acid and ethylene at the onset of berry ripening and after harvest.** *BMC Plant Biol* 2010, **10**:257.
42. Liu K, Kang B-C, Jiang H, Moore SL, Li H, Watkins CB, Setter TL, Jahn MM: **A GH3-like gene, CcGH3, isolated from Capsicum chinense L. fruit is regulated by auxin and ethylene.** *Plant Mol Biol* 2005, **58**(4):447–464.
43. Moradinezhad F, Sedgley M, Klieber A, Able AJ: **Variability of responses to 1-methylcyclopropene by banana: Influence of time of year at harvest and fruit position in the bunch.** *Ann Appl Biol* 2008, **152**:223–234.
44. Scott KJ, McGlasson WB, Roberts EA: **Potassium permanganate as an ethylene absorbent in polyethylene bags to delay ripening of bananas during storage.** *Aust J Exp Agric Anim Husb* 1970, **10**:237–240.
45. Karakousis A, Langridge P: **A high-throughput plant DNA extraction method for marker analysis.** *Plant Mol Biol Rep* 2003, **21**:95a–95f.
46. Khoo KHP, Able AJ, Able JA: **Poor homologous synapsis 1 (PHS1) interacts with chromatin but does not co-localise with Asynapsis 1 (ASY1) during early meiosis in bread wheat.** *Int J Plant Genomics* 2012. Article ID 514398, doi:10.1155/2012/514398.
47. *National Center for Biotechnology Information (NCBI) database.* [<http://www.ncbi.nlm.nih.gov/>]
48. *DFCI Pepper Gene Index.* [<http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=pepper>]
49. Ogasawara S, Abe K, Nakajima T: **Pepper  $\beta$ -galactosidase 1 (PBG1) plays a significant role in fruit ripening in bell pepper (*Capsicum annuum*).** *Biosci Biotechnol Biochem* 2007, **71**:309–322.
50. Schaarschmidt S, Roitsch T, Hause B: **Arbuscular mycorrhiza induces gene expression of the apoplastic invertase LIN6 in tomato (*Lycopersicon esculentum*) roots.** *J Exp Bot* 2006, **57**:4015–4023.
51. Kato M, Hayakawa Y, Hyodo H, Ikoma Y, Yano M: **Wound-induced ethylene synthesis and expression and formation of 1-aminocyclopropane-1-carboxylate (ACC) synthase, ACC oxidase, phenylalanine ammonia-lyase, and peroxidase in wounded mesocarp tissue of *Cucurbita maxima*.** *Plant Cell Physiol* 2000, **41**:440–447.
52. Bulens I, Van de Poel B, Hertog M, De Proft MP, Geeraerd AH, Nicolai BM: **Protocol: An updated integrated methodology for analysis of metabolites and enzyme activities of ethylene biosynthesis.** *Plant Methods* 2011, **7**:17.
53. Commonwealth Scientific and Industrial Research Organisation (CSIRO): *Banana Ripening Guide. Division of Food Research Circular 8.* Canberra, Australia: CSIRO; 1972.
54. American Spice Trade Association (ASTA): *Analytical Method 20.1.* Eaglewood Cliffs, USA: ASTA; 1986.

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**Additional file 1: Table S1. List of primers used in PCR and qPCR.**

Gene/ isoform names	NCBI accession numbers for tomato ( <i>Le</i> )	Contig accession numbers for capsicum ( <i>Ca</i> ) <sup>g</sup>	NCBI accession numbers for capsicum ( <i>Ca</i> )	Forward primer (5'-3')	Reverse primer (5'-3')	Product size
<i>GAPdH</i> <sup>a</sup>	-	-	AJ246011.1	GAAGAATTGGTCGATTGGTG	CCCCGTTGACTCTACGACAT	272
<i>ACO1</i>	NM_001247095.1	TC13435	GD053075.1 & GD054188.1	GCAAGTGCTTAAATTACAAGTGTG	TTGAGATGCAACCGTTACTCC	195
<i>ACO2</i>	XM_004251479.1	TC14180	GD052945.1, GD054373.1 & CA520300.1	GTTGAGAAAAGAGGCCAAAGGAT	AAATAATCTTGAGTTTGAATGTACG	188
<i>ACO3</i>	NM_001246999.1	TC12536	GD095125.1 & BM063409.1	ATCCAGCACCAGCTTTGATC	TGAATTGGAAATTAAGCACTTG	193
<i>ACO4</i>	AB013101.1	-	JX515597	GCAATGAAGGCTATGGAGACTA	TGACGCATCAAGAAACACAAG	217
<i>ACO5</i> <sup>b</sup>	AJ715790.1	TC15257	GD115323.1 & GD117950.1	GGACATTCCACCTTCCAAGA	AGAGCTTTGGTGCTGGAGAA	188
<i>ACO6</i>	EF501822.1	TC24188	GD089267.1, GD124776.1, GD101463.1 & GD099075.1	GGAGCCAAGGTTTGAAGCA	CATCACACTACCAGACAACAGTACA	259
<i>ACSI</i> <sup>c</sup>	NM_001246993.1 (A) and U72390.1 (B)	-	AB434926.1	AAGTTGTCGATGAAATGATTGG	CTAGCGATGTCCACTACTGTATCA	136
<i>ACS2</i> <sup>c</sup>	NM_001247249.1	-	AB434927.1	GTAGAAGACAACGAAATGAGTCAT	CGCAACTAACGACAGCATC	133
<i>ACS3</i> <sup>b</sup>	NM_001247097.1	KS23061E03	GD116714.1	CTTGAACCTCCTTACATTTCG	AACTCGTTCCCAAACATCTGT	156
<i>ACS4</i> <sup>d</sup>	NM_001246946.1	-	-	CCATCTTGTTTGCAGCAAATA	CGATGCTAACGAATTTTGGAGAA	-
<i>ACSdeg</i> <sup>f</sup>	accordingly	-	-	ATGGGTNTNGCNGAAAATCA	AANGCATCNCNGGNTNAG	287
<i>ETR2</i> <sup>e</sup>	NM_001247224.1	-	-	GGAACCAATGAGGGCTCG	CAGCCAGAAATCATTACGC	107
<i>ETR3</i> <sup>b</sup>	NM_001246965.1	KS16036C11	GD072810.1	AGAGTCCATGCGAGCCCA	CTTCTTGTCGAGCTACATCCAA	68
<i>ETR4</i> <sup>b</sup>	NM_001247276.1	KS20049F06 & TC15606	GD097854.1 & GD101436.1	TTGGGATGCAGCGTGCT	AAGGACGATTTGGAATGAGG	87
<i>ETR5</i> <sup>b</sup>	NM_001247283.1	TC12426	GD063530.1 & GD052924.1	CTTCATGTACTCGTTGCTGATG	CTAAAGCACTCAGGCATTGG	121
<i>ETR1-3deg</i> <sup>f</sup>	accordingly	-	-	AGATCAGGTNGCTGTNGCTC	GCATNGGNGTTCNCATTTCAT	179
<i>ETR4-6deg</i> <sup>f</sup>	accordingly	-	-	TGATGATGTNAATAGANNGGTAAC	TCNNGCANNTGAAGATCCAA	150

<sup>a</sup>*CaGAPdH* primers were obtained from other studies detailed in the manuscript.

<sup>b</sup>Contig sequences for *CaACO5*, *CaACS3*, *CaETR3*, *CaETR4* and *CaETR5* are partial and not encoded for full-length proteins.

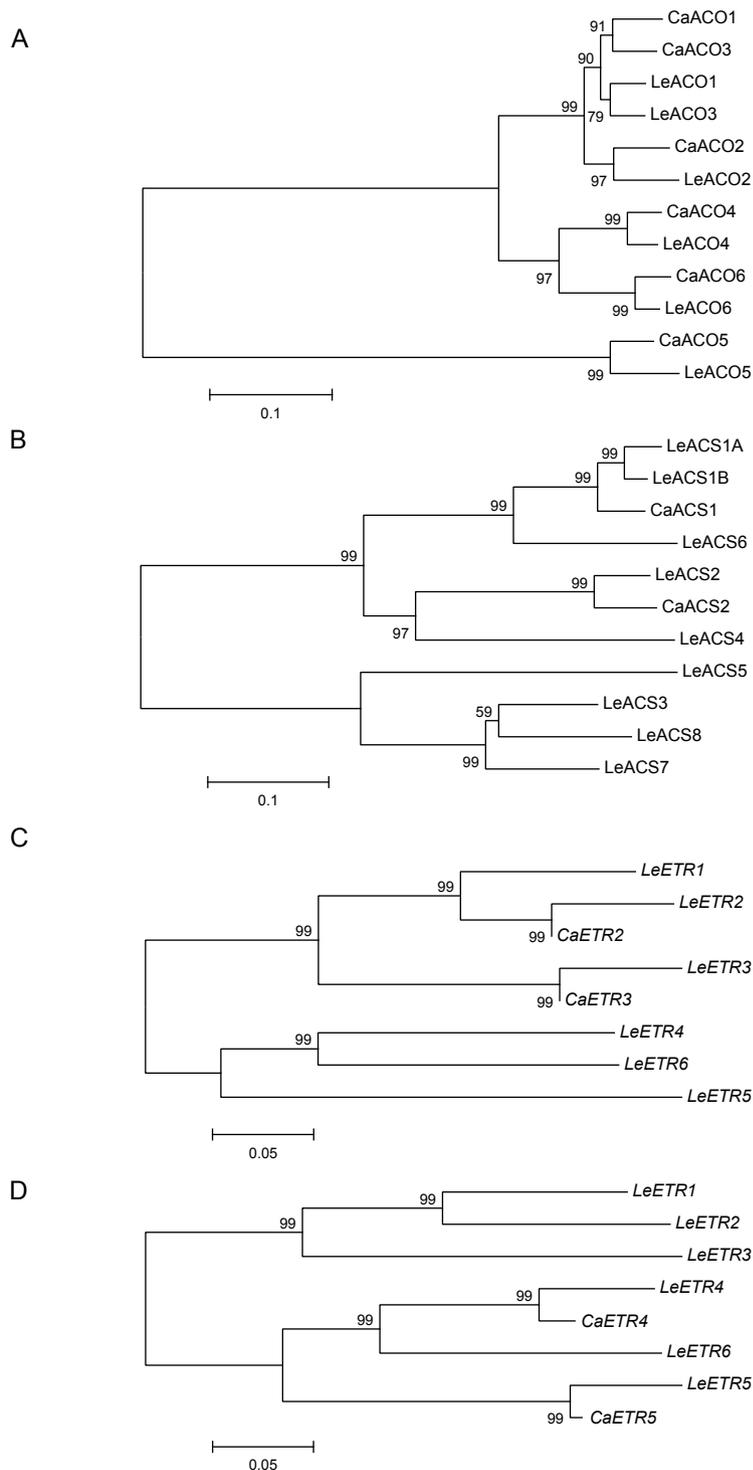
<sup>c</sup>*CaACS1* and *CaACS2* sequences are from *Capsicum chinense* obtained through the NCBI database (similar EST sequences were also obtained from the capsicum EST database). For the tomato *ACSI*, there are two different isoforms exist named *LeACSI A* and *LeACSI B*. However, only one isoform is available for capsicum which is closely related to both tomato *ACSI* isoforms (Additional file 2: Figure S1). Hence, the capsicum *ACSI* is named *CaACSI*.

<sup>d</sup>Primers for *CaACS4* were obtained from previous qPCR study in capsicum and no other information regarding its sequences are available in both NCBI and EST databases (detailed in manuscript).

<sup>e</sup>*CaETR2* was extracted from the degenerate primers (*ETR1-3deg*).

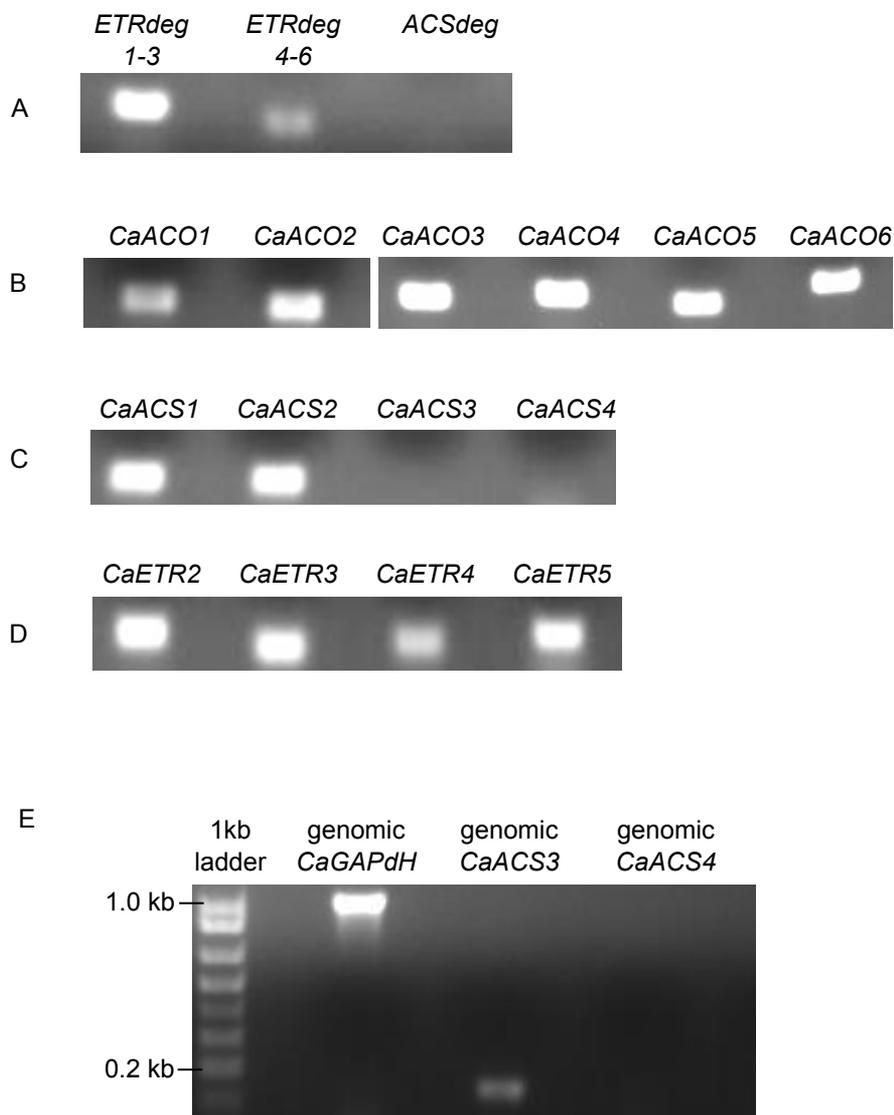
<sup>f</sup>Degenerate primers for *ACS* (*ACSdeg*) were designed based on consensus sequence of *LeACS3*, *LeACS4*, *LeACS5*, *LeACS6*, *LeACS7* and *LeACS8* and the degenerate primers for *ETR* (*ETR1-3deg* and *ETR4-6deg*) were designed based on the consensus sequences of tomato Type I (*LeETR1*, *LeETR2*, *LeETR3*) and Type II (*LeETR4*, *LeETR5*, *LeETR6*), respectively. Any available capsicum EST sequences were also taken into consideration when designing these primers.

<sup>g</sup>Contig accession numbers were obtained from the EST database which are linked to the NCBI dbEST database (some of them have more than one dbEST accessions as listed in Genbank accession numbers column of the table).



**Additional file 2:Figure S1. The phylogenetic analysis of ACO (A), ACS (B) and ETR (C and D) isoforms.**

The tree was built based on full-length protein sequences of ACO isoforms (except CaACO5 which is partial), and the full length of ACS isoforms. The tree for *ETRs* was built based on mRNA sequences of tomato and the partial sequence of capsicum *ETRs* Type I (*CaETR2* and *CaETR3*, C) and Type II (*CaETR4* and *CaETR5*, D) obtained from an end point RT-PCR using degenerate primers for respective types (refer to Methods). Genbank accession numbers for the tomato are: LeACO1, P05116.2; LeACO2, CAA68538.1; LeACO3, CAA90904.1; LeACO4, NP\_001233867.1; LeACO5, NP\_001234037.1; LeACO6, ABP68407.1; LeACS1A, NP\_001233922.1; LeACS1B, AAB17279.1; LeACS2, NP\_001234178.1; LeACS3, NP\_001234026.1; LeACS4, NP\_001233875.1; LeACS5, NP\_001234156.1; LeACS6, BAA34923.1; LeACS7, NP\_001234346.1; LeACS8, NP\_001234160.1; *LeETR1*, NM\_001247220.1; *LeETR2*, NM\_001247224.1; *LeETR3*, NM\_001246965.1; *LeETR4*, NM\_001247276.1; *LeETR5*, NM\_001247283.1 and *LeETR6*, NM\_001247221.1. For capsicum, the available Genbank accession numbers are: CaACO4, AGG20315; CaACS1, BAG30909.1 and CaACS2, BAG30910.1. All other capsicum isoforms (from contigs of the EST database) are listed in Additional file 1: Table S1 translated *in silico* as per [16]. Le, tomato; Ca, Capsicum.



**Additional file 3: Figure S2. End-point PCR for isolating capsicum *ACO*, *ACS* and *ETR* isoforms.** Degenerate primers of *ETR* Type I (*ETRdeg1-3*) and Type II (*ETRdeg4-6*) as well as *ACS* isoforms (*ACSdeg*) were run in the end-point PCR (using cDNA pooled from six stages of ripening) to isolate other possible isoforms due to the lack of information in the databases (A). The primers for six *CaACO* isoforms (B), four *CaACS* isoforms (C) and four *CaETR* isoforms (D) were run in the end-point PCR using the pooled cDNA template. All bands are less than 0.26 kb using primers listed in Additional file 1: Table S1. The differences between band intensity (especially *CaACO1* and *CaETR4*) could be due to loading. The absence of *CaACS3* and *CaACS4* in the cDNA mix was also confirmed in another independent RT-PCR and qPCR experiments (data not shown). (E) Genomic PCR using DNA template (annealing temperature 60°C) was also performed but only the *CaACS3* (and positive control *CaGAPdH* plus introns) produced specific products and not *CaACS4*. Experiments were repeated in two other annealing temperatures (55°C and 65°C), again without any amplification of *CaACS4* (data not shown). *CaACS4* primers were obtained from a previous qPCR study in capsicum and tomato [14] and no other information regarding its sequence was available in either the NCBI or capsicum EST databases.

**Additional file 4: Table S2. Significant levels of ASTA level (Figure 2B), ACO activity (Figure 3A), ACS activity (Figure 3B) and ACC content (Figure 3C) were determined using Duncan's Multiple Range Test ( $P<0.05$ ).** This analysis compares all data for control (C), ethylene (E) and 1-MCP (M) treated fruit at different days after treatment (DAT) within the respective ripening stage.

Stages	DAT	ASTA			ACO activity			ACS activity			ACC content		
		C	E	M	C	E	M	C	E	M	C	E	M
Green	0	a	a	a	bcde	ab	ef	a	a	a	ab	a	cde
	3	a	a	a	cdef	cdef	f	ab	a	a	ab	abc	abc
	12	a	a	a	bc	bc	bcd	a	a	ab	abcd	abcd	bcde
	20	a	ab	a	a	a	ab	c	bc	c	cde	bcde	abcde
	28	bc	c	c	cdef	def	f	cd	d	cd	de	f	ef
Breaker	0	ab	a	a	a	b	c	abcd	ab	ab	a	a	a
	3	cd	cde	abc	de	ef	cd	ab	a	abc	a	a	a
	12	ef	def	bcd	fg	fg	fg	abcd	abcd	abcd	ab	a	abc
	20	g	g	f	g	g	g	cd	d	abcd	abc	bcd	ab
	28	g	g	g	g	g	g	abcd	abcd	bcd	d	abc	cd

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**Chapter 7: The preliminary characterisation of  
several other proteomic ripening-related  
candidates**

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## 7.1 Introduction

Proteomic analysis using 2D gel electrophoresis (2DGE) has been used in several fruit studies including tomato, banana, apple, peach, strawberry, olive and grape (Bianco et al., 2009; Faurobert et al., 2007; Giribaldi et al., 2007; Nilo et al., 2010; Song et al., 2006; Wu et al., 2011). These studies have identified several classes of proteins unique to different ripening stages, postharvest treatments and/or different fruit tissues. However, further analyses of proteins identified by examining gene expression and protein activity has rarely been employed to confirm their findings.

In the proteomic study of capsicum ripening (Aizat et al., 2013) (Chapter 3), only one protein candidate, 1-aminocyclopropane 1-carboxylate (ACC) oxidase (ACO) was selected for additional experimentation to corroborate the proteomic results. *ACO isoform 4 (CaACO4)* mRNA expression and overall ACO activity closely reflected changes in proteomic analyses. However, several other proteomic candidates were also of interest. In particular, those peptides that shared similarity with a Copper CHaperone (CCH) from *Arabidopsis thaliana*, TCP (Teosinte branched1, Cycloidea, Proliferating cell factors) domain class transcription factors (TCP) from *Malus domestica*, CYsteine Synthase (CYS) from *Capsicum annuum*, SPermidine Synthase (SPS) from *Solanum lycopersicum* and ADenosine Kinase (ADK) from *Solanum tuberosum* (Aizat et al., 2013) (Chapter 3: Table 1) and they were chosen for further analysis. For instance, CCH may be associated with providing copper to the ethylene receptors (Woeste and Kieber, 2000) while CYS and SPS were involved closely with the ethylene pathway precursors, cysteine (Nikiforova et al., 2002) and S-adenosyl methionine (SAM) (Handa and Mattoo, 2010), respectively. TCP might be important due to its role as a putative transcription factor (Martín-Trillo and Cubas, 2010) while ADK may be vital for cytokinin metabolism and colour changes (Kobayashi et al., 2012; Spíchal, 2012) during capsicum ripening.

The aim of the research presented in this chapter was to isolate and confirm the presence of these candidates of interest (*CCH*, *TCP*, *CYS*, *SPS* and *ADK*). Full-length cDNA sequences were identified and their putative functional domains were determined. Gene expression was also investigated for most of these candidates using semi-quantitative Reverse Transcriptase-PCR (sqRT-PCR) as well as quantitative Real Time-PCR (qPCR) in six different capsicum ripening stages (G, Green; B, Breaker; BR1, Breaker Red 1; BR2, Breaker Red 2; LR, Light Red; DR, Deep Red). Their significance during capsicum ripening and in relation to their respective patterns in the earlier 2DGE is discussed.

## **7.2 Material and Methods**

### *7.2.1 cDNA stocks and primer design*

The cDNA of six capsicum ripening stages (G, B, BR1, BR2, LR and DR) were prepared and described as per Aizat et al. (2013) and Chapter 3: Materials and Methods. Primers for isolation of the full-length cDNA sequences of the proteomic candidates were designed according to the available Genbank NCBI accessions (*CaCYS*, X64874.1) or EST contig sequences (*CaCCH*, TC19828; *CaTCP*, TC19007 & TC22508; *CaSPS*, TC20247 and *CaADK*, TC13853) (<http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=pepper>, date of access: 27 June 2012). Primers were also designed for semi-quantitative RT-PCR (sqRT-PCR) and/or quantitative Real Time-PCR (qPCR) analyses using the isolated cDNA sequences (which were submitted to Genbank and have their own accession number as per Table 7.1).

**Table 7.1** The list of primers used in this study. These primers were used in isolating full-length cDNA product, sqRT-PCR and/or qPCR analyses as stated in the “Notes” column. *SPS* and *ADK* full-length sequences were extracted from two pairs of primers each (full contig 1 and full contig 2). Accession numbers for the full-length sequences were deposited in the Genbank NCBI database.

<b>Candidates &amp; accession numbers</b>	<b>Primer names</b>	<b>Sequence (5'-3') for either forward (pF) or reverse (pR) primers</b>	<b>PCR product size in base pairs</b>	<b>Notes</b>
<i>CCH candidate (CaCCH)</i> KF583452	CCHpF4	ATGTCTCAGACAGTTGTTCTCAAG	346 (CCHpF4-R4)	Full-length, sqRT-PCR qPCR
	CCHpR4	GTTAAGGTAACAATCATCATGCTG		
	CCHpF5	CGAAGCAAAGCCTGTAGAAG	115 (CCHpF5-pR4)	
<i>TCP candidate (CaTCP)</i> KF583453	TCPpF6	CTCTTCCGATACTCCGTCTTC	1789 (TCPpF6-pR5)	Full-length
	TCPpR5	ATCCATCTGTTCCGGTCTGTC		
	TCPpF5	CGAAGAGCGAAGAGGAGAA	245 (TCPpF5-pR4)	sqRT-PCR
	TCPpR4	AGTCCATCACGAACAGCATC		
	TCPpF7	ATGCGGAAACCAACTTAGC	144 (TCPpF7-pR5)	qPCR
<i>CYS candidate (CaCYS)</i> KF583454	CYSpF3	ATGGCATCTNTCATCAACAATCC	1254 (CYSpF3-pR3)	Full-length
	CYSpR3	TACACCAGCAGCCAACTAAATAAC		
	CYSpF4	ATTCAAGGGATTGGAGCAG	168 (CYSpF4-pR4)	sqRT-PCR & qPCR
	CYSpR4	AATGGCAGCAAGAGCAGC		
<i>SPS candidate (CaSPS)</i> KF583455	SPSpF5	GAAGCGACCTCAGATTTGTG	681 (SPSpF5-pR1)	Full contig 1 SPS
	SPSpR1	CCGTCACCAATGTGGAGAT		
	SPSpF1	AAGGTGCTGGTTATTGGAGG	824 (SPSpF1-pR4)	Full contig 2 SPS
	SPSpR4	GACAACTTTATCCCAGCACAA		
	SPSpR3	ATTATTTTCCTTTGGTTTCGATCA	643 (SPSpF1-pR3)	sqRT-PCR qPCR
	SPSpF6	TTCTACAACCTCTGAGATTCACCAA	263 (SPSpF6-pR4)	
<i>ADK candidate (CaADK)</i> KF583456	ADKpF3	AACAATGGAGTACGATGGGATTC	817 (ADKpF3-pR1)	Full contig 1 ADK
	ADKpR1	CTCAGCGACAACAACAGGAT		
	ADKpF2	TGGAAATGAGACAGAAGCAAG	399 (ADKpF2-pR3)	Full contig 2 ADK
	ADKpR3	CAAGAACTGAAGTAAGAATGAACTC		

### 7.2.2 Isolation of full-length sequences

All cDNA preparation, RT-PCR (including running conditions), PCR product identification, sequencing and phylogenetic analyses were as described in Aizat et al. (2013) and Chapter 3: Materials and Methods unless otherwise stated. For extracting full-length cDNA sequences, the primers of *CaCCH* (CCHpF4-pR4), *CaTCP* (TCPpF6-pR5), *CaCYS* (CYSpF3-pR3), *CaSPS* (SPSpF5-pR1, SPSpF1-pR4) and *CaADK* (ADKpF3-pR1, ADKpF2-pR3) were run in RT-PCR reactions using cDNA templates of either G stage (*CaCCH* and *CaTCP*) or BR1 stage (*CaCYS*, *CaSPS* and *CaADK*). The annealing temperature used in the PCR was either 55°C (*CaCCH*, *CaCYS* and *CaADK*), 60°C (*CaTCP*) or 62°C (*CaSPS*) and the elongation time was set up corresponding to the length of respective products (1 kb for 1 min). Putative conserved domains in the deduced residues of all candidates were identified from the NCBI database protein BLAST tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>; date of access: 1 September 2013).

### 7.2.3 sqRT-PCR analysis

All candidates identified earlier [except *CaADK* due to several mismatches with the proteomic result (Figure 7.1)] were run in sqRT-PCR according to Aizat et al. (2013) and Chapter 3: Materials and Methods. Briefly, all primer pairs for *CaCCH* (CCHpF4-pR4), *CaTCP* (TCPpF5-pR4), *CaCYS* (CYSpF4-pR4, specific to an additional region in *CaCYS1*) and *CaSPS* (SPSpF1-pR3) were first run in different PCR cycle numbers (24 to 36 cycles) using respective cDNA template for each candidate stated in 7.2.2. The cycle number just before the PCR products became saturated was chosen to be run in all ripening stages in the sqRT-PCR (all primer pairs were at 30 cycles, Appendix F). The annealing temperature was either 55°C

(*CaCYS*, *CaSPS*) or 60°C (*CaCCH*, *CaTCP*) and the elongation time was set up corresponding to the length of respective products (1 kb for 1 min). sqRT-PCR using a lower number of PCR cycles (27 cycles) was also performed in a biological replicate of all ripening stages but still no significant pattern across all ripening stages was observed (Appendix G). Hence, the more sensitive method of qPCR analysis was employed.

#### *7.2.4 qPCR analysis*

Primers for each candidate, *CaCCH* (CCHpF5-pR4), *CaTCP* (TCPpF7-pR5), *CaCYS* (CYSpF4-pR4, specific to additional region in *CaCYS1*) and *CaSPS* (SPSpF6-pR4) were first run in RT-PCR and pilot qPCR experiments using 1:10 diluted cDNA template of G stage for *CCH* and *TCP* while B stage for *CYS* and *SPS*. This was done to confirm the amplification of only specific product for each candidate (based on sequencing and a single qPCR melt curve, Appendix H). qPCR was then performed using cDNA from each of the six ripening stages (three biological replicates each) and with the same qPCR conditions as per Chapter 6: Methods.

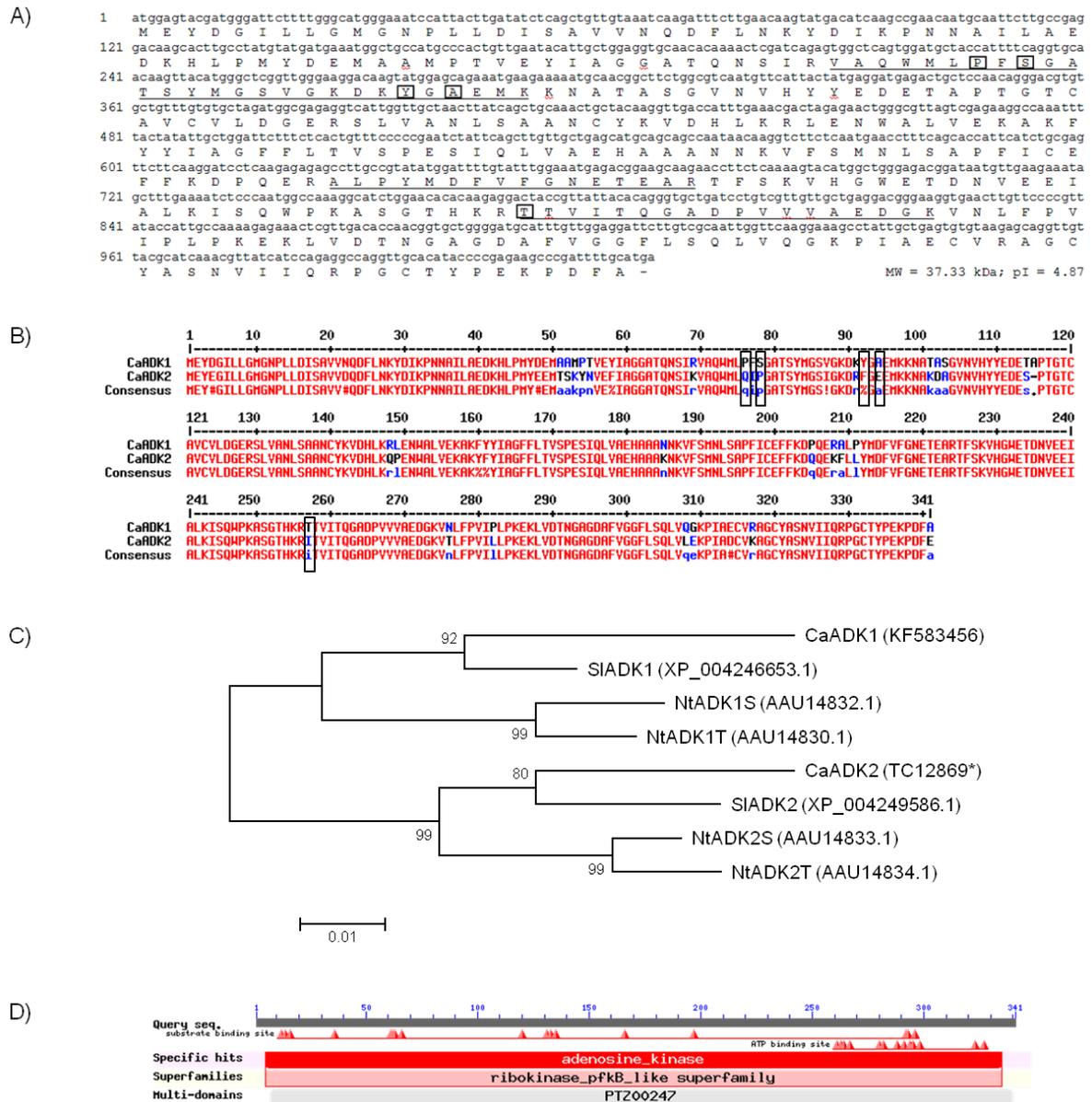
#### *7.2.5 Statistical analysis*

Data from the qPCR was analysed by One-way Analysis of Variance (ANOVA) using Genstat 14 (Hemel Hempstead, UK). Significantly different means were determined using least significant difference (l.s.d.) at  $P < 0.05$ .

## 7.3 Results

### 7.3.1 *CaADK*

The *ADK* isolated from capsicum (cv. Aries) encodes a 341 residue protein with a predicted molecular weight (MW) of 37.33 kDa and predicted isoelectric point (pI) of 4.87 (Figure 7.1 A). This *CaADK* is also closely related to isoform 1, and was therefore named *CaADK1*. Most peptide sequences identified by tandem mass spectrometry (MS/MS) in the earlier capsicum proteomic study (Aizat et al., 2013) (Chapter 3) matched to this isoform, except for five residues which matched to the other isoform, *CaADK2* (Figure 7.1 A and B). A phylogenetic analysis comparing *ADK* isoforms from the Solanaceae family; capsicum, tomato (*Solanum lycopersicum*) and tobacco (*Nicotiana tabacum*), revealed that isoform 1 and isoform 2 formed two different clades regardless of Solanaceae species (Figure 7.1 C) and the isolated *CaADK1* (KF583456) fell into the isoform 1 clade. *ADK* isoforms from other species such as wild strawberry (*Fragaria vesca*), *Arabidopsis thaliana* and grape (*Vitis vinifera*) were also compared in a phylogenetic analysis but no clear differentiation between these isoforms was observed (Appendix I). *CaADK1* putative protein sequence contains 17 substrate (adenosine) binding sites and 13 ATP binding sites. *CaADK1* also belongs to a larger superfamily of ribokinase/6-phosphofructokinase (PfkB)-like proteins (Figure 7.1 D) which are known to bind and phosphorylate a variety of substrates (in this case adenosine) at the expense of ATP (Kwade et al., 2005). Both *CaADK1* and *CaADK2* have the same conserved domains and a similar number of adenosine and ATP binding sites except that isoform 2 has one less amino acid (data not shown).

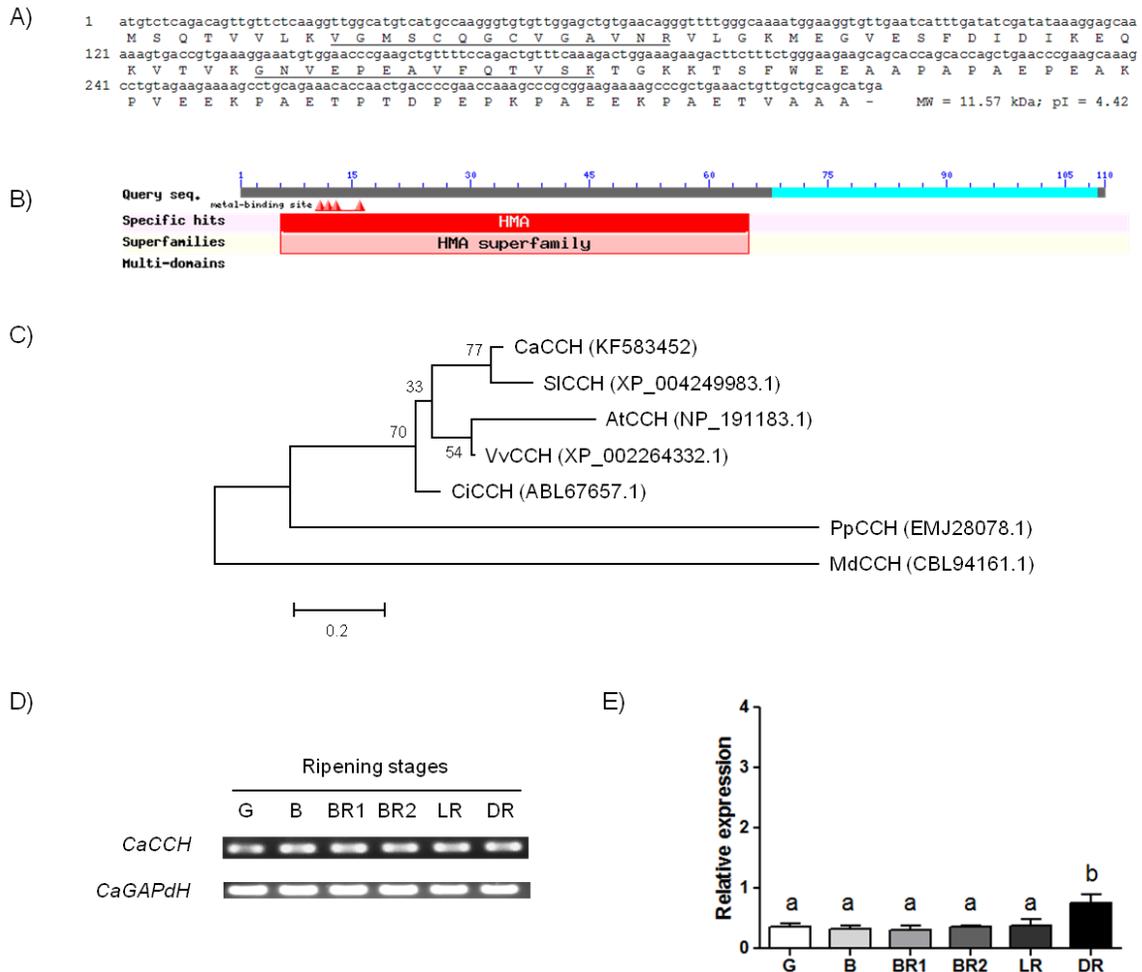


**Figure 7.1** *In-silico* analysis of *ADenosine Kinase (ADK)* isoform 1 from capsicum (*CaADK1*). A) cDNA sequences of *CaADK1* were determined and the deduced amino acids were compared with the peptide sequences (underlined residues) from Aizat et al. (2013) and Chapter 3: Table 1, spot 7. Mismatches between the peptide and deduced residues are boxed. B) Another isoform was identified from a capsicum EST database (<http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=pepper>, contig TC12869; date of access: 1 September 2013) which revealed that these mismatches (boxed residues) correspond to this other isoform (named *CaADK2*). C) A phylogenetic analysis comparing ADK protein isoforms from the Solanaceae family [*Capsicum annuum* (Ca), *Solanum lycopersicum* (Sl), *Nicotiana tabacum* (Nt)] revealed differentiation between isoform 1 and isoform 2 clades. Two isoforms (1 and 2) exist for Ca and Sl while four isoforms (1S, 1T, 2S and 2T) exist for Nt due to its allotetraploid nature (Kwade et al., 2005). Accession numbers are given in brackets beside respective proteins (\**CaADK2* was translated *in silico* from a contig accession mined from the EST database). The two ADK isoforms from Sl are originally named ADK2-

like genes with different accession numbers in the NCBI databases. Our phylogenetic analysis differentiated both isoforms into either isoform 1 or isoform 2 and hence renamed SIADK1 and SIADK2 accordingly in this chapter. Other isoforms from Nt are named as documented in the NCBI database. The branch length (bar) corresponds to an evolutionary distance at 0.01 amino acid substitutions per site. D) CaADK1 contains a putative adenosine kinase domain which has 17 substrate (adenosine) binding sites and 13 ATP binding sites. CaADK1 also contains a putative domain of ribokinase/6-phosphofructokinase (PfkB)-like superfamily and PTZ00247 multidomain which is a provisional domain yet to be characterised. Due to an inability to differentiate these two isoforms particularly during earlier proteomic analysis, this candidate was not further analysed in the gene expression analyses (sqRT-PCR and qPCR).

### 7.3.2 *CaCCH*

*CCH* isolated from capsicum (*CaCCH*) encodes a 110 amino acid chaperone which has a predicted MW and pI of 11.57 kDa and 4.42, respectively. The two peptides identified by MS/MS (Aizat et al., 2013) (Chapter 3: Table 1, spot 1) also matched to the deduced residues of *CaCCH* (Figure 7.2 A). Furthermore, *CaCCH* possesses four heavy metal (possibly copper) binding sites in its heavy-metal-associated (HMA) domain (Figure 7.2 B). No differentiation between CCH of different species [*Arabidopsis* (*Arabidopsis thaliana*), grape (*Vitis Vinifera*), citrus (*Citrus*), peach (*Prunus persica*) and apple (*Malus domestica*)] was observed but the Solanaceae, capsicum and tomato, grouped in one clade (Figure 7.2 C). The expression of *CaCCH* was constitutive throughout the ripening stages and appeared similar regardless of ripening stage when analysed using sqRT-PCR (Figure 7.2 D). However, *CaCCH* expression was significantly greater during the DR stage when analysed using qPCR (Figure 7.2 E).



**Figure 7.2** Analysis of *Copper CHaperone (CCH)* extracted from *Capsicum annuum* (*CaCCH*). A) cDNA sequences of *CaCCH* were determined and the deduced amino acids were compared with the peptide sequences (underlined residues) from Aizat et al. (2013) and Chapter 3: Table 1, spot 1. B) Putative conserved domains in the *CaCCH*. *CaCCH* contains a heavy-metal-associated (HMA) domain with four heavy metal binding sites. C) A phylogenetic analysis was performed comparing CCH proteins from a variety of fruit species including *Capsicum annuum* (Ca), *Solanum lycopersicum* (Sl), *Arabidopsis thaliana* (At), *Vitis vinifera* (Vv), *Citrus* (Ci), *Prunus persica* (Pp) and *Malus domestica* (Md). Accession numbers are given in brackets beside respective protein names. The branch length (bar) corresponds to an evolutionary distance at 0.2 amino acid substitutions per site. D) The semi-quantitative Reverse Transcriptase-PCR (sqRT-PCR) analysis of *CaCCH* and the positive internal control, *CaGAPdH* (Aizat et al., 2013) (Chapter 3). Gels shown are representatives of three biological replicates for the six ripening stages (G, Green; B, Breaker; BR1, Breaker Red 1; BR2, Breaker Red 2; LR, Light Red; DR, Deep Red). E) The same ripening stages were analysed in quantitative Real Time-PCR (qPCR) analysis of *CaCCH* which was normalised (relative expression) to *CaGAPdH* as per Chapter 6: Methods. n = 3 biological replicates ( $\pm$ SE) and different letters on bars are significantly different means between ripening stages (l.s.d. = 0.2461 relative expression at  $P < 0.05$ ).

### 7.3.3 *CaTCP*

*TCP* from capsicum (*CaTCP*) encodes a protein chaperone which consists of 536 residues corresponding to 58.96 kDa predicted MW and 6.02 predicted pI (Figure 7.2 A and B). A mismatch was identified between a MS/MS peptide sequence (Aizat et al., 2013) (Chapter 3: Table 1, spot 5) and a deduced residue of *CaTCP* (boxed in Figure 7.2 A). *CaTCP* putative domain is also associated with the zeta subunit of T-complex protein 1 (TCP1) chaperones and possesses several conserved residues for polypeptide binding, stacking interactions and ATP/Mg binding (Figure 7.3 B). A comparison between TCP chaperones from different species shows that the Solanaceae, capsicum and tomato formed a different clade than the others [apple, wild strawberry (*Fragaria vesca*), peach, grape and cucumber (*Cucumis sativus*)] and Arabidopsis acts as an outlier (Figure 7.3 C). The expression of *CaTCP* was constitutive during capsicum ripening (sqRT-PCR, Figure 7.3 D) and the qPCR analysis revealed that only the DR stage was significantly higher than the B-BR2 stages (Figure 7.3 E).



polypeptides can bind), six stacking interaction sites as well as 11 ATP/Mg binding sites for its putative function. C) A phylogenetic analysis was performed comparing TCP from a variety of fruit species including *Capsicum annuum* (Ca), *Solanum lycopersicum* (Sl), *Malus domestica* (Md), *Fragaria vesca* (Fv, wild strawberry), *Prunus persica* (Pp), *Vitis vinifera* (Vv), *Cucumis sativus* (Cs) and *Arabidopsis thaliana* (At). Accession numbers are given in brackets beside respective protein names. The branch length (bar) corresponds to an evolutionary distance at 0.01 amino acid substitutions per site. D) The semi-quantitative Reverse Transcriptase-PCR (sqRT-PCR) analysis of *CaTCP* and the positive internal control, *CaGAPdH* (Aizat et al., 2013) (Chapter 3). Gels shown are representatives of three biological replicates for the six ripening stages (G, Green; B, Breaker; BR1, Breaker Red 1; BR2, Breaker Red 2; LR, Light Red; DR, Deep Red). E) The same ripening stages were analysed in quantitative Real Time-PCR (qPCR) analysis of *CaTCP* which was normalised (relative expression) to *CaGAPdH* as per Chapter 6: Methods. n = 3 biological replicates ( $\pm$ SE) and different letters on bars are significantly different means between ripening stages (l.s.d. = 0.5506 relative expression at  $P < 0.05$ ).

### 7.3.4 *CaCYS1*

*CYS isoform 1* isolated from capsicum (cv. Aries) (*CaCYS1*) encodes a 385 residue protein with a predicted molecular weight (MW) of 40.94 kDa and predicted isoelectric point (pI) of 5.16 (Figure 7.4 A). All peptide sequences identified by MS/MS in the earlier capsicum proteomic study (Aizat et al., 2013) (Chapter 3) matched the deduced amino acids (Figure 7.4 A). Two isoforms of CYS from different plants/fruit species were also identified *in silico* (including *CaCYS2* which was not found in the cDNA isolation). There are 10 amino acids only present in isoform 1 but not in isoform 2 of capsicum, tomato, potato (*Solanum tuberosum*), peach, wild strawberry, cucumber and Arabidopsis. The deletion in *VvCYS2* was only nine residues and starts one amino acid earlier compared to the other species (Figure 7.4 B). The Solanaceae (capsicum, tomato and potato) grouped in one clade and there was no separation between isoform 1 and isoform 2. In contrast, the two isoforms of other species such as peach, wild strawberry, cucumber and Arabidopsis were differentiated into two different clades (both isoforms of grape are grouped in the isoform 1 clade) while apple  $\beta$ -cyanoalanine synthases [*MdCAS1* and *MdCAS2*, selected due to these enzymes being quite similar to the CYS in terms of their enzymatic activities (Han et al., 2007; Warrilow and

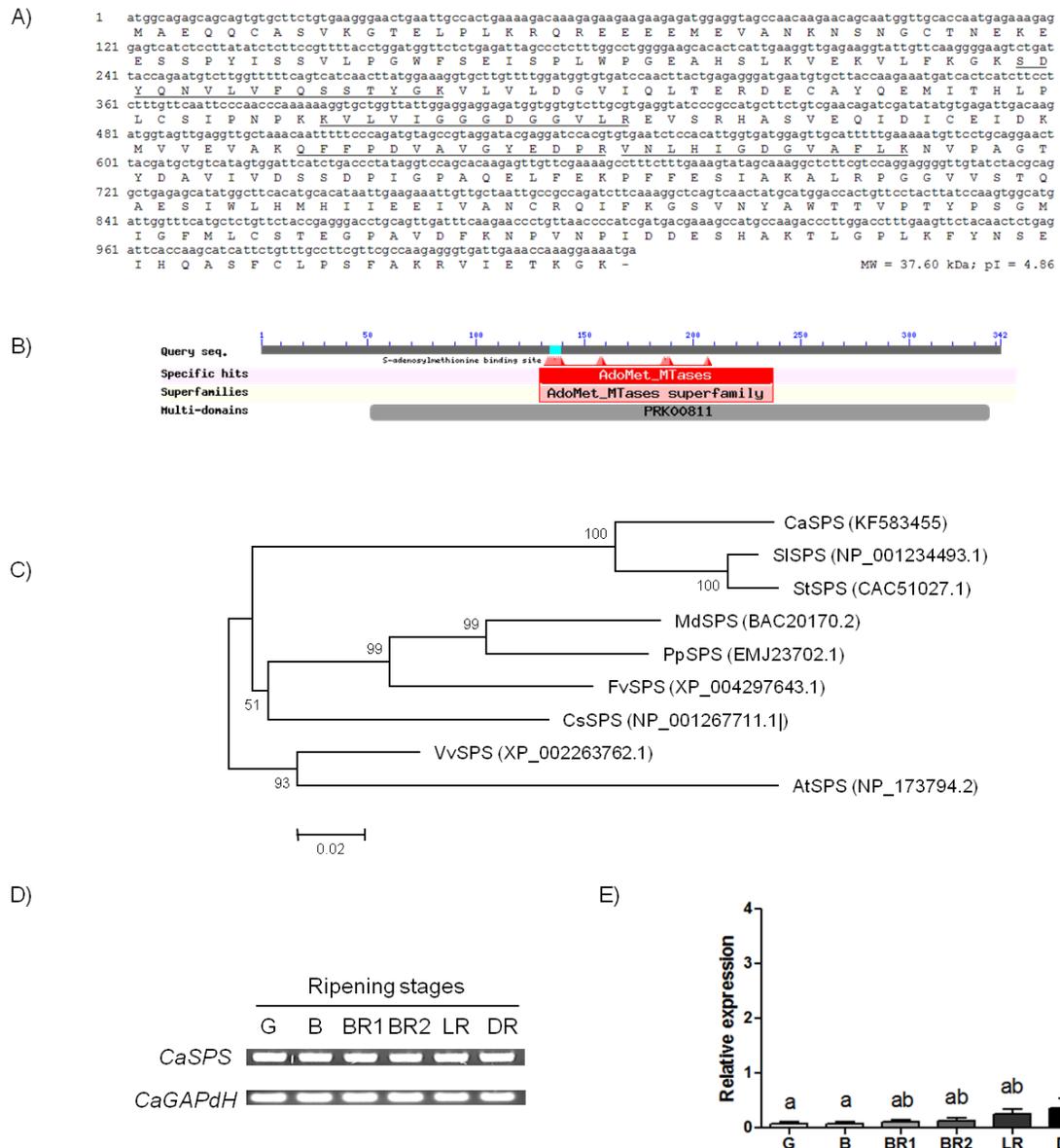
Hawkesford, 2000)] acted as an outgroup (Figure 7.4 C). There are also 12 pyridoxal 5' phosphate (PLP) binding sites in CaCYS1 but one less site for CaCYS2, but other conserved residues such as dimer interface of the cystathionine beta-synthase (CBS)-like domain are similar in both isoforms (Figure 7.4 D). The expression of *CaCYS1* (using primers designed in the deleted region) was constitutive throughout ripening stages (sqRT-PCR, Figure 7.4 E). qPCR analysis showed that *CaCYS1* increased during capsicum ripening such that there was approximately five times more expression in DR than G (Figure 7.4 F).



MdCAS2) are used as outgroup. Accession numbers are given in brackets beside respective protein names. The branch length (bar) corresponds to an evolutionary distance at 0.05 amino acid substitutions per site. D) Putative conserved domains in the CaCYS1. CaCYS1 contains cystathionine beta-synthase (CBS) domain which is also belongs to the pyridoxal 5' phosphate (PLP)-dependent enzyme family, Tryptophan synthase beta II superfamily. As such, CaCYS1 contains 12 sites of PLP binding, one catalytic site and 25 dimer interface. Boxed is the deleted region in the CaCYS2. E) The semi-quantitative Reverse Transcriptase-PCR (sqRT-PCR) analysis of *CaCYS1* and the positive internal control, *CaGAPdH* (Aizat et al., 2013) (Chapter 3). Gels shown are representatives of three biological replicates for the six ripening stages (G, Green; B, Breaker; BR1, Breaker Red 1; BR2, Breaker Red 2; LR, Light Red; DR, Deep Red). F) The same ripening stages were analysed in quantitative Real Time-PCR (qPCR) analysis of *CaCYS1* which was normalised (relative expression) to *CaGAPdH* as per Chapter 6: Methods. n = 3 biological replicates ( $\pm$ SE) and different letters on bars are significantly different means between ripening stages (l.s.d. = 0.883 relative expression at  $P < 0.05$ ).

### 7.3.5 *CaSPS*

*SPS* from capsicum (*CaSPS*) encodes 342 amino acids and has a predicted MW of 37.60 kDa and pI of 4.86 (Figure 7.5 A). *CaSPS* also possesses several *S*-adenosylmethionine (SAM) binding sites in the SAM-dependent methyltransferase (SAM-MTase or AdoMet-MTase) domain (Figure 7.5 B). A phylogenetic analysis of *SPS* also shows that the Solanaceae species (capsicum, tomato and potato) are grouped in one clade whereas apple, peach, wild strawberry and cucumber formed a different clade. Both grape and *Arabidopsis* act as an outgroup clade (Figure 7.5 C). The expression of *CaSPS* was constitutive throughout the ripening stages (sqRT-PCR, Figure 7.5 D) and was significantly greater during DR compared to G and B stages when analysed using qPCR (Figure 7.5 E).



**Figure 7.5** Analysis of *SPermidine Synthase* (*SPS*) extracted from *Capsicum annuum* (*CaSPS*). A) Translated cDNA sequences of *CaSPS* were compared with the peptide sequences (underlined residues) from Aizat et al. (2013) and Chapter 3: Table 1, spot 7. B) Putative conserved domains in the *CaSPS*. *CaSPS* contains S-adenosylmethionine-dependent methyltransferase (SAM-MTase or AdoMet-MTase) domain which is characterised by the SAM binding sites. *CaSPS* is also closely associated to a provisional uncharacterised domain, PRK00811. C) A phylogenetic analysis was performed comparing *SPS* from a variety of fruit species including *Capsicum annuum* (Ca), *Solanum lycopersicum* (Sl), *Solanum tuberosum* (St), *Malus domestica* (Md), *Prunus persica* (Pp), *Fragaria vesca* (Fv, wild strawberry), *Cucumis sativus* (Cs), *Vitis vinifera* (Vv) and *Arabidopsis thaliana* (At). Accession numbers are given in brackets beside respective protein names. The branch length (bar) corresponds to an evolutionary distance at 0.02 amino acid substitutions per site. D) The semi-quantitative Reverse Transcriptase-PCR (sqRT-PCR) analysis of *CaSPS* and the positive internal control, *CaGAPdH* (Aizat et al., 2013) (Chapter 3). Gels shown are representatives of three biological

replicates for the six ripening stages (G, Green; B, Breaker; BR1, Breaker Red 1; BR2, Breaker Red 2; LR, Light Red; DR, Deep Red). E) The same ripening stages were analysed in quantitative Real Time-PCR (qPCR) analysis of *CaSPS* which was normalised (relative expression) to *CaGAPdH* as per Chapter 6: Methods. n = 3 biological replicates ( $\pm$ SE) and different letters on bars are significantly different means between ripening stages (l.s.d. = 0.2635 relative expression at  $P < 0.05$ ).

## 7.4 Discussion

The full-length sequences of all five proteomic candidates (*CCH*, *TCP*, *CYS*, *SPS*, *ADK*) were successfully isolated from capsicum (cv. Aries) and sequenced [*ACO* was isolated in Aizat et al. (2013); Chapter 3]. All candidates encode full-length hypothetical proteins with the predicted MW and pI close to that observed for the protein spots identified in the 2DGE earlier (Aizat et al., 2013) (Chapter 3: Table 1). However, the ADK candidate contained the most mismatches (five in total) (Figure 7.1 A). This could be due to the presence of different ADK isoforms, particularly in the same 2DGE spot analysed by MS/MS. Indeed, the MS/MS peptide sequences were either matched to the translated *CaADK1* or *CaADK2* (Figure 7.1 A and B). A phylogenetic analysis on the Solanaceae family also suggests that there are two isoforms of ADK (isoforms 1 and 2) in capsicum, tomato and tobacco, differentiated into two different clades (Figure 7.1 C). These isoforms appear to have different affinity towards adenosine and cytokinin in tobacco and so they might have different functions (Kwade et al., 2005). Therefore, although both *CaADK1* and *CaADK2* share similar conserved domains, further characterisation, in terms of their mRNA expression and protein activities are needed. The capsicum ADK was only identified in BR1 and hence the enzyme may influence colour-related changes by regulating cytokinin metabolism (Kobayashi et al., 2012; Schoor et al., 2011) during ripening onset.

The predicted amino acid residues of other proteomic candidates such as *CCH* (Figure 7.2 A), *CYS* (Figure 7.4 A) and *SPS* (Figure 7.5 A) were identical to the peptide sequences

except one residue of CaTCP (Figure 7.3 A). The *CaTCP* cDNA was sequenced from at least four different transformed pGEMT-easy colonies as per Aizat et al. (2013) and Chapter 3: Materials and Methods with similar results, which indicates that the mismatch was probably not due to incorrect sequencing. Since there were no other isoforms identified from the NCBI and EST databases, the mismatch in the CaTCP (Gln in the deduced residue and Lys in the MS/MS peptide) (Figure 3 A) may be due to other reasons related to the MS/MS peptide such as post-translational modifications. Both Gln and Lys have a very similar molecular weight (approximately  $146.1 \text{ g mol}^{-1}$ ) and given that the mass-spectra of some residues could also be affected by post-translational modifications (Whitelegge, 2004), this might lead to the different (Lys instead of Gln) residue being identified during the proteomic study (Aizat et al., 2013) (Chapter 3).

Furthermore, the TCP candidate was originally selected because of the peptide hits to the TCP (Teosinte branched1, Cycloidea, Proliferating cell factors) transcription factor annotated in apple (Aizat et al., 2013) (Chapter 3). However, the functional domain in the full-length protein was not for a transcription factor but for a chaperone (Figure 7.3 B) sharing similarity with the TCP1 subunit zeta-like in tomato (Figure 7.3 C) and hence may be part of a larger protein complex comprising of different subunits. Indeed, another subunit of a TCP1 (subunit epsilon) was also found during G stage (Aizat et al., 2013) (Chapter 3). These subunits can be part of a chaperone complex called CCT (Chaperonin containing TCP1) which is responsible for cytoskeleton formation in plant secondary wall formation (Nick et al., 2000). The presence of CaTCP (and possibly CCT) during the early ripening (G stage) of capsicum (Aizat et al., 2013) (Chapter 3: Table 1, spot 5) may indicate its function in structural integrity of the fruit, especially during development. However, the *CaTCP* transcripts were constitutive throughout ripening (Figure 7.3 D and E) even though CaTCP was only present in G fruit.

CaTCP might therefore be degraded as ripening progresses, especially during fruit softening. Further analysis is needed to confirm whether and how CaTCP (and hence CCT) is regulated post-translationally including the use of specific antibodies to identify CaTCP and/or other subunits of CCT during ripening.

Another chaperone identified and selected was CCH due to its putative function in copper delivery to metalloproteins (Figure 7.2 A, B and C) which may include ethylene receptors (ETRs) (Woeste and Kieber, 2000). *CaCCH* expression was constitutive during ripening (Figure 7.2 D) but was significantly greater at the DR stage (Figure 7.2 E). This may be due to the induction of senescence at this final ripening stage, as suggested by the up-regulated expression of *AtCCH* during leaf senescence (Himmelblau et al., 1998). However, the CCH protein was only found during the G stage (Aizat et al., 2013) (Chapter 3: Table 1, spot 1) suggesting that it is degraded during the later ripening stages. If more ETRs are being made in capsicum during this period (G stage), then there may have been a greater requirement for CCH during the G stage. Interestingly, ETR proteins during tomato ripening were highly accumulated during the G stage and their abundance was also strictly controlled at the post-translational level, possibly involving 26S-proteasome protein degradation (Kevany et al., 2007). Further characterisation of CaETRs and the CaCCH candidate will be necessary to study their relationship and abundance during non-climacteric capsicum ripening.

CYS is involved in synthesising cysteine which is a precursor for methionine used in ethylene biosynthesis (Wirtz and Droux, 2005) and hence selected for further analysis. There are two isoforms of CYS in capsicum, CaCYS1 and CaCYS2 (Figure 7.4 B). The putative amino acid sequences of the two isoforms were similar except for a stretch of 10 residues only present in CaCYS1. At the nucleotide level, the open reading frames of both isoforms were also identical (Appendix J) which suggests that they were possibly splicing variants generated

during post-transcriptional modifications. The difference between both CYS isoform sequences (the additional region in isoform 1) was not unique to capsicum. Other species such as tomato, potato, plum, wild strawberry, cucumber and Arabidopsis also had the 10 amino acid deletion in their putative protein sequences of isoform 2 (Figure 7.4 B). A phylogenetic comparison however, only differentiated the isoform 1 and isoform 2 of plum, wild strawberry, cucumber and Arabidopsis into two different clades whereas other plants/fruit particularly the Solanaceae family members such as capsicum, tomato and potato were mainly grouped within their species (Figure 7.4 C). This suggests high sequence conservation within the Solanaceae family of CYS isoforms compared to other plants/fruit.

The 10 residue deletion of the CaCYS2 has reduced one site of PLP binding compared to CaCYS1 (Figure 7.4 D). PLP acts as the cofactor for CYS activity (Hesse et al., 1999) and the absence of one site might also reduce CaCYS2 activity. The study presented here has characterised *CaCYS1* expression during capsicum ripening. The *CaCYS1* transcripts increased gradually during ripening especially from G to BR2 to DR stages (Figure 7.4 F). In an earlier study by Römer et al. (1992), the capsicum (cv. Lamuyo) *CaCYS2* was characterised during G, BR and LR to reveal that the transcript level was highest during the LR stage. This suggests that both CaCYS1 and CaCYS2 may be required for chromoplast formation during the full-red stages. However, the CYS protein was found only during BR1 stage and not G or LR stages (Aizat et al., 2013) (Chapter 3: Table 1, spot 6). Due to the similarity of CaCYS1 and CaCYS2 sequences, the MS/MS peptides matched similarly to both isoforms and no peptides were identified in the 10 residue deleted region (Figure 7.4 A). We therefore cannot differentiate whether the CYS found during the proteomic analysis was isoform 1 or isoform 2. Thus, further analysis to discriminate both isoforms especially at the protein level is necessary to confirm their abundance during ripening and enzymatic activities in capsicum.

SPS is another candidate of interest which was chosen due to its close relationship with the ethylene pathway (Nambeesan et al., 2010). SPS sits in the polyamine pathway and is responsible for the generation of spermidine by metabolising putrescine and decarboxylated SAM (Mattoo et al., 2010). The protein spot associated with SPS was only found during the ripening onset of capsicum (Aizat et al., 2013) (Chapter 3: Table 1, spot 7) and concomitantly there was a significant reduction of putrescine during that ripening period (Chapter 4: Figure 4). Spermidine however was not increased during this period (Chapter 4: Figure 4), possibly due to its simultaneous use in other pathways such as spermine biosynthesis. The low level of ethylene during capsicum ripening may not only be caused by the rate-limiting ACS activity (Chapter 6: Figure 2) but also the increase in SPS which may also deprive one of the ethylene precursors, SAM. However, *CaSPS* transcripts were constitutive and relatively unchanged throughout ripening stages (Figure 7.5 D) except for a slightly higher expression during DR stage compared to G and B stages (Figure 7.5 E). This possibly indicates that the up-regulation of *CaSPS* during the BR1 stage (Aizat et al., 2013) (Chapter 3) might be due to its accumulation throughout early ripening stages before being degraded during the full red stages where putrescine becomes unavailable for *CaSPS* catalytic activity. Additional studies including the measurement of SPS activity during capsicum ripening as per Wang et al. (2005) will be necessary to confirm this notion.

### *Conclusion*

Several candidates of interest from the previous capsicum proteomic study were further investigated by isolating their full-length cDNA and characterising their gene expression during ripening. Two different isoforms of both *CaADK* and *CaCYS* (isoforms 1 and 2) were identified (either experimentally or *in silico*) but further experimentation is required to confirm

the specific role of each isoform during capsicum ripening and to corroborate their trends in the proteomic analysis. The mRNA expression levels of *CaCCH*, *CaTCP* and *CaSPS* however, did not conform to their respective proteomic trends which possibly highlights post-translational regulation of these candidates during capsicum ripening.

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## **Chapter 8: General Discussion**

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## 8.1 Unravelling complex ripening processes in capsicum

To unravel capsicum ripening processes, two comparison-based approaches, proteomics (Chapters 2 and 3) and metabolomics (Chapter 4) were chosen to identify and investigate differentially-expressed molecules during capsicum ripening. These ‘omic’-based approaches were selected because even though ripening had been investigated through transcriptomics (Lee et al., 2010), there have been no substantial study on the profiling of metabolites and proteins during the capsicum ripening phases. Furthermore, the integration of ‘omic’-based approaches could provide complementary information about the biochemical pathways in the target organism. Other fruit ripening studies such as in tomato (Alba et al., 2005; Carrari et al., 2006) and grape (Deluc et al., 2007) have also combined different ‘omic’-based approaches (but mostly transcriptomics and metabolomics), suggesting the applicability and usefulness of integrating various ‘omics’ platforms for elucidating complex processes such as ripening.

The proteomics study of capsicum ripening (Aizat et al., 2013) (Chapter 3) has identified several candidates that can be related to the metabolomics study (Chapter 4). For example, the increase of cysteine synthase, malate dehydrogenase and spermidine synthase enzymes (Chapter 3) may explain the up-regulation of cysteine and the degradation of malate and putrescine (Chapter 4), respectively during the onset of ripening. These approaches have also provided several other candidates which were further studied in different capsicum tissues and cultivars; for example sugars, malate and 1-aminocyclopropane-1-carboxylate (ACC) oxidase (ACO) activity (Chapter 5). The *ACO isoform 4 (CaACO4)* was identified in the proteomics study (Aizat et al., 2013) (Chapter 3) and was investigated relative to the other components of ethylene synthesis and perception such as *ACC synthase (ACS)*, *ETHylene Receptors (ETR)* and other *ACO isoform* expression (Chapter 6).

The components of ethylene synthesis and perception have primarily been investigated in climacteric fruit such as tomato, due to the importance of ethylene during climacteric ripening stimulation (Paul et al., 2012; Seymour et al., 2013). Interestingly, several studies characterising these components have also compared cultivars with different ripening behaviour including guava (Liu et al., 2012b), pear (Yamane et al., 2007), plum (El-Sharkawy et al., 2008) and melon (Périn et al., 2002). For instance, Liu et al. (2012b) reported that a guava cultivar which is non-climacteric had lower ACS activity but higher ACO activity than the climacteric cultivar. An ACC feeding experiment restored the climacteric ethylene increase in the non-climacteric guava (Liu et al., 2012b), suggesting that ACS is the rate limiting step in ethylene production by non-climacteric fruit. In non-climacteric capsicum, ACS activity and ACC content were limited (Chapter 6), perhaps also supporting the earlier hypothesis. Furthermore, Yamane et al. (2007) showed that *ACS* and *ACO* transcripts were highly up-regulated in climacteric pears yet *ACO* was transient and *ACS* was limited in non-climacteric pear. A climacteric plum cultivar has also been shown to have higher expression of *ACS* transcripts during ripening than a non-climacteric plum (El-Sharkawy et al., 2008). *ACS* transcripts were also limited in the non-climacteric capsicum but *CaACO4* was up-regulated during ripening (Chapters 3 and 6). The up-regulation of *CaACO4* is unexpected due to capsicum being a non-climacteric fruit and hence producing limited ethylene compared to climacteric fruit (Chapter 2). Phylogenetic comparison of some of these *ACO* and/or *ACS* transcripts was not able to differentiate these isoforms into either a climacteric or non-climacteric clade (Aizat et al., 2013; El-Sharkawy et al., 2008), perhaps due to high sequence divergence between species. Although the research presented in this thesis focused primarily on a non-climacteric cultivar (cv. Aries), some other capsicum cultivars (particularly hot peppers) have been reported to exhibit a climacteric behaviour (Biles et al., 1993; Gross et al.,

1986; Pham, 2007). Future studies should compare the ethylene perception and synthesis components between climacteric and non-climacteric capsicum cultivars, which might reveal some conserved ripening elements, such as *CaACO4* up-regulation. Interestingly, crossing climacteric melon with the non-climacteric type resulted in climacteric progenies, suggesting that climacteric characteristics are dominant (Périn et al., 2002). However, genetic analysis did not identify climacteric quantitative trait loci (QTL) that were possibly co-localised with known ethylene biosynthetic and transduction genes in melon (Pech et al., 2008; Périn et al., 2002), implying that other elements may be more important for ripening including transcriptional regulation (Gapper et al., 2013).

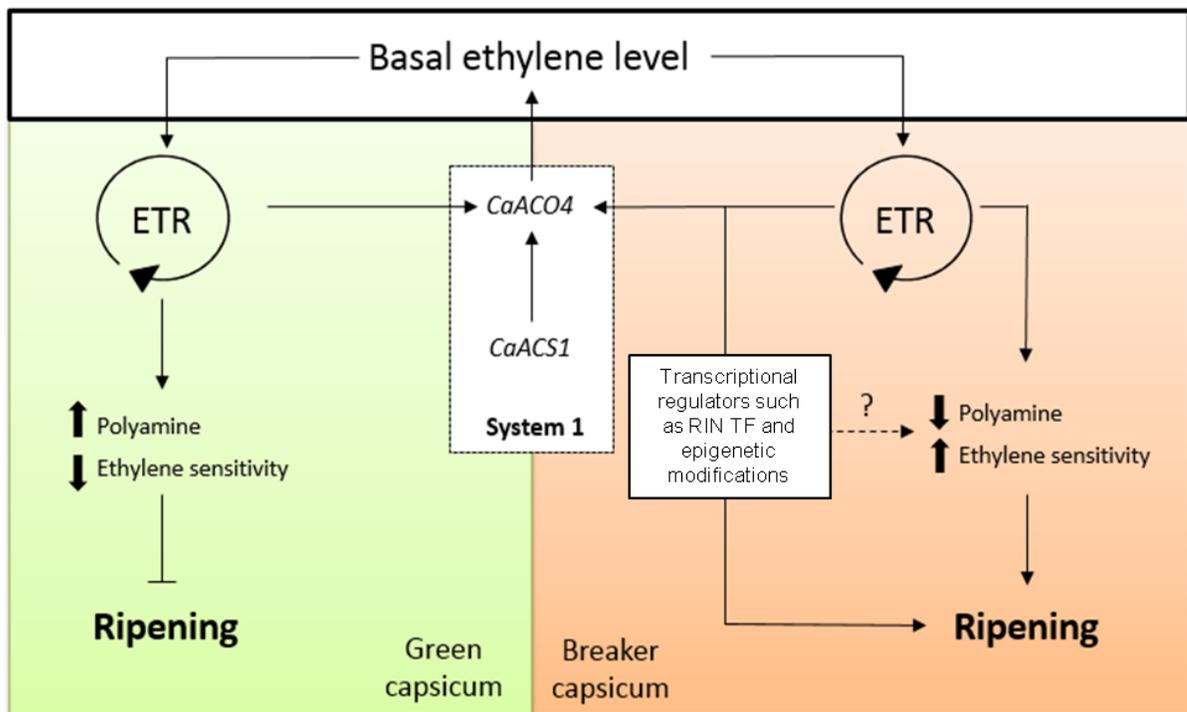
Transcription factors (TF) have been proposed to act not only upstream of the ethylene synthesis and perception components but also other major ripening processes (Giovannoni, 2007). Epigenetic modification (genome demethylation) has more recently been shown to regulate ripening in tomato (Zhong et al., 2013). Gene promoters including *Phytoene synthase1* and *Polygalacturonase-2a* were demethylated to allow TF binding [particularly Ripening INhibitor (RIN)] upon ripening onset, suggesting that these genes, which are important for colour and cell-wall metabolism respectively, could be controlled at the epigenetic level (Zhong et al., 2013). Perhaps these genes (and other related ripening genes) are also similarly induced in non-climacteric fruit, such that ripening can occur without the climacteric ethylene production (which stimulates and quickens the ripening of climacteric fruit). However, the reasons behind the limited expression of most ethylene synthesis and perception genes such as *ACS*, *ACO* and *ETR* in non-climacteric fruit including capsicum remain to be investigated.

In addition, the regulation of these *ACO*, *ACS* and *ETR* isoforms upon ripening and ethylene treatment was different compared to their homologue expression in climacteric

tomato. For example, the transient up-regulation of *CaACO4* (and not other ACO isoforms) during capsicum ripening is interesting because its homologue in tomato, *LeACO4* was not the major isoform expressed during climacteric tomato ripening (Nakatsuka et al., 1998). Moreover, *CaACO4* was considered a System 1 component due to its expression being negatively regulated by ethylene (Chapter 6) and yet *LeACO4* could be categorised as a System 2 component because of being auto-stimulated by ethylene (Li et al., 2011; Nakatsuka et al., 1998). The differences between *CaACO4* and *LeACO4* expression are still unknown but possibly relate to the differences in their upstream promoter sequences and/or binding with TF such as RIN (and therefore the methylation status of the promoter). The naturally occurring *rin* mutation in climacteric tomato yield fruit which cannot ripen even with ethylene application, suggesting that this TF is one of the transcriptional regulators in fruit ripening (Giovannoni, 2007; Vrebalov et al., 2002). However, the presence and involvement of RIN in non-climacteric fruit ripening has not been completely described. A *RIN* homologue was found in non-climacteric strawberry (*FaMADS9*) (Vrebalov et al., 2002) and its silencing resulted in severe inhibition of strawberry ripening (Seymour et al., 2011). Recently another *RIN* homologue from non-climacteric grape, *VviSEP4* was found to partially complement *rin* tomato mutants (Mellway and Lund, 2013), suggesting a possible conserved role of this *MADS*-box gene in both ripening types (climacteric and non-climacteric). Alternatively, other factors such as the phytohormones abscisic acid (ABA) and brassinosteroids have also been implicated in non-climacteric ripening (Jia et al., 2011; Symons et al., 2006) but the understanding behind their cross-talk is still in its infancy (Seymour et al., 2013).

## 8.2 Conclusion and future directions

Based on the results presented in this thesis, capsicum ripening appears to involve multiple factors in a stage-specific manner (Figure 8.1). During the Green (G) stage, due to high polyamine level (particularly putrescine; Chapter 4), ethylene sensitivity may be reduced (Yahia et al., 2001). The up-regulation of spermidine synthase (Chapter 3) may cause the degradation of putrescine during the Breaker (B) stage and concurrently increase the ethylene sensitivity. Basal ethylene production in capsicum (Chapter 2), possibly contributed by the System 1 components *CaACSI* and *CaACO4*, during ripening may then stimulate capsicum ripening from the B stage onwards, possibly through the subfamily II ETR isoforms (Chapter 6). The basal ethylene level may also be important to maintain the level of *ETR* as 1-methylcyclopropane (1-MCP) treatment severely impacted *ETR* isoform levels (Chapter 6). Whether this basal ethylene level also contributes and/or is initiated in other tissue types such as placenta/seeds remains to be investigated (Chapter 5). The up-regulation of *CaACO4* during the B stage was not ethylene-dependent (Chapter 6). How *CaACO4* expression is regulated and whether any TFs involved also regulate other proteomic candidates such as spermidine synthase (hence affecting polyamines) (Chapter 7) or other ripening processes in non-climacteric capsicum also requires further experimentation.



**Figure 8.1** A model of non-climacteric capsicum ripening. The basal level of ethylene production during non-climacteric capsicum ripening may be generated by *CaACS1* and *CaACO4* (System 1-associated isoforms) (Chapter 6). The basal ethylene level may be required to maintain a normal rate of turnover for the subfamily II ETR in both G and B stages, which in turn maintain the regulation of several ethylene pathway components including *CaACO4*. However, due to a high polyamine presence which may reduce the ethylene sensitivity (Chapter 4), the G-harvested capsicum do not ripen properly. In comparison with B-harvested fruit which do ripen, the ethylene sensitivity may be increased due to the degradation of polyamines and hence the basal level of ethylene may exert its effect on capsicum ripening. During this B stage, transcriptional regulators including RIN transcription factors (TF) and epigenetic modifications may also be important for the ripening process of capsicum and perhaps influence *CaACO4* up-regulation. The reduction of polyamines may be related to the transcriptional regulators too, but further confirmation is needed to verify this. ACS, 1-aminocyclopropane-1-carboxylate synthase; ACO, 1-aminocyclopropane-1-carboxylate oxidase; ETR, EThylene Receptors; RIN, Ripening INhibitor.

Future research should include:

1. The generation of *CaACO4* mutants to confirm its role during capsicum ripening. Whether *CaACO4* is directly or indirectly influenced by epigenetic modification or transcriptional regulators, such as *RIN*, will also be of interest in this experiment.
2. The identification and comparison of promoter regions from *ACO4* from both capsicum and tomato. Any elements for TF binding that might be different and can regulate their expression will be important for deciphering the regulation of ripening in both climacteric and non-climacteric fruit. Yeast-1-hybrid experiments could be used to confirm TF association with the promoters and hence identifying these transcriptional regulator components in capsicum. Furthermore, mutants of these identified TF can be generated to confirm their roles in capsicum ripening.
3. Examining the effects of polyamine and/or combined ethylene treatments on capsicum ripening to confirm a role(s) for polyamines in determining ethylene sensitivity of capsicum. The treatments can be done at different ripening stages to evaluate their ripening behaviours and the expression of some ethylene perception and synthesis components such as *CaETR* isoforms and *CaACO4* can be evaluated. Other related components of the ethylene pathway such as spermidine synthase and cysteine synthase can also be studied in relation to supporting a role for polyamines during ripening.
4. The identification of full-length *CaETR* to evaluate the differences between subfamily I and subfamily II ETR. Antibodies may be generated and protein expression assays used to assess the ETR presence at post-translational levels during ripening and ethylene/1-MCP/polyamine treatments. Copper chaperone involvement during

ethylene perception is also of interest and should be investigated further to elucidate its role.

5. An ACC (perhaps radioactively-labelled) feeding experiment on capsicum fruit (especially using the B-BR stages) to justify that the rate limiting step is ACS. The treated capsicum fruit may produce high ethylene levels as in climacteric fruit due to ACO enzyme up-regulation during ripening. Overexpression of one of the *CaACS* might be another option to clarify whether the lack of ethylene in capsicum is due to restricted ACS activity.

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## References

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- Aharoni, A. & O'Connell, A. P. 2002. Gene expression analysis of strawberry achene and receptacle maturation using DNA microarrays. *Journal of Experimental Botany*, 53, 2073-2087.
- Aizat, W. M., Able, J. A., Stangoulis, J. C. R. & Able, A. J. 2013. Proteomic analysis during capsicum ripening reveals differential expression of ACC oxidase isoform 4 and other candidates. *Functional Plant Biology*, 40, 1115-1128.
- Alba, R., Payton, P., Fei, Z. J., McQuinn, R., Debbie, P., Martin, G. B., Tanksley, S. D. & Giovannoni, J. J. 2005. Transcriptome and selected metabolite analyses reveal multiple points of ethylene control during tomato fruit development. *Plant Cell*, 17, 2954-2965.
- Ali, Z. M., Chin, L. H. & Lazan, H. 2004. A comparative study on wall degrading enzymes, pectin modifications and softening during ripening of selected tropical fruits. *Plant Science*, 167, 317-327.
- Anjanasree, K. N., Verma, P. K. & Bansal, K. C. 2005. Differential expression of tomato ACC oxidase gene family in relation to fruit ripening. *Current Science*, 89, 1394-1399.
- Anonymous 1984. *Methods of Enzymatic Food Analysis*, Boehringer Mannheim.
- Arora, A., Choudhary, D., Agarwal, G. & Singh, V. P. 2008. Compositional variation in  $\beta$ -carotene content, carbohydrate and antioxidant enzymes in selected banana cultivars. *International Journal of Food Science & Technology*, 43, 1913-1921.
- Baginsky, S., Hennig, L., Zimmermann, P. & Gruissem, W. 2010. Gene expression analysis, proteomics, and network discovery. *Plant Physiology*, 152, 402-410.
- Bapat, V. A., Trivedi, P. K., Ghosh, A., Sane, V. A., Ganapathi, T. R. & Nath, P. 2010. Ripening of fleshy fruit: Molecular insight and the role of ethylene. *Biotechnology Advances*, 28, 94-107.
- Barrera, J. A., Hernández, M. S., Melgarejo, L. M., Martínez, O. & Fernández-Trujillo, J. P. 2008. Physiological behavior and quality traits during fruit growth and ripening of four Amazonian hot pepper accessions. *Journal of the Science of Food and Agriculture*, 88, 847-857.
- Barros, E., Lezar, S., Anttonen, M. J., van Dijk, J. P., Röhlig, R. M., Kok, E. J. & Engel, K. H. 2010. Comparison of two GM maize varieties with a near-isogenic non-GM variety using transcriptomics, proteomics and metabolomics. *Plant Biotechnology Journal*, 8, 436-451.
- Barry, C. S. 2009. The stay-green revolution: Recent progress in deciphering the mechanisms of chlorophyll degradation in higher plants. *Plant Science*, 176, 325-333.
- Barry, C. S. & Giovannoni, J. J. 2007. Ethylene and fruit ripening. *Journal of Plant Growth Regulation*, 26, 143-159.
- Barry, C. S., Llop-Tous, M. I. & Grierson, D. 2000. The regulation of 1-aminocyclopropane-1-carboxylic acid synthase gene expression during the transition from System-1 to System-2 ethylene synthesis in tomato. *Plant Physiology*, 123, 979-986.
- Barry, C. S., McQuinn, R. P., Chung, M. Y., Besuden, A. & Giovannoni, J. J. 2008. Amino acid substitutions in homologs of the STAY-GREEN protein are responsible for the green-flesh and chlorophyll retainer mutations of tomato and pepper. *Plant Physiology*, 147, 179-187.
- Ben-Yehoshua, S., Shapiro, B., Chen, Z. E. & Lurie, S. 1983. Mode of action of plastic film in extending life of lemon and bell pepper fruits by alleviation of water stress. *Plant Physiology*, 73, 87-93.
- Bianco, L., Lopez, L., Scalone, A. G., Di Carli, M., Desiderio, A., Benvenuto, E. & Perrotta, G. 2009. Strawberry proteome characterization and its regulation during fruit ripening and in different genotypes. *Journal of Proteomics*, 72, 586-607.

- Biles, C. L., Wall, M. M. & Blackstone, K. 1993. Morphological and physiological changes during maturation of new Mexican type peppers. *Journal of the American Society for Horticultural Science*, 118, 476-480.
- Blanke, M. M. & Holthe, P. A. 1997. Bioenergetics, maintenance respiration and transpiration of pepper fruits. *Journal of Plant Physiology*, 150, 247-250.
- Blasiak, J., Kuang, A., Farhangi, C. S. & Musgrave, M. E. 2006. Roles of intra-fruit oxygen and carbon dioxide in controlling pepper (*Capsicum annuum* L.) seed development and storage reserve deposition. *Journal of the American Society for Horticultural Science*, 131, 164-173.
- Borovsky, Y. & Paran, I. 2008. Chlorophyll breakdown during pepper fruit ripening in the *chlorophyll retainer* mutation is impaired at the homolog of the senescence-inducible stay-green gene. *Theoretical and Applied Genetics*, 117, 235-240.
- Bradford, M. M. 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry*, 72, 248-254.
- Burg, S. P. & Burg, E. A. 1965. Ethylene action and ripening of fruits - Ethylene influences the growth and development of plants and is the hormone which initiates fruit ripening. *Science*, 148, 1190-1196.
- Carrari, F., Baxter, C., Usadel, B., Urbanczyk-Wochniak, E., Zanon, M.-I., Nunes-Nesi, A., Nikiforova, V., Centero, D., Ratzka, A., Pauly, M., Sweetlove, L. J. & Fernie, A. R. 2006. Integrated analysis of metabolite and transcript levels reveals the metabolic shifts that underlie tomato fruit development and highlight regulatory aspects of metabolic network behavior. *Plant Physiology*, 142, 1380-1396.
- Centeno, D. C., Osorio, S., Nunes-Nesi, A., Bertolo, A. L. F., Carneiro, R. T., Araújo, W. L., Steinhauser, M. C., Michalska, J., Rohrmann, J., Geigenberger, P., Oliver, S. N., Stitt, M., Carrari, F., Rose, J. K. C. & Fernie, A. R. 2011. Malate plays a crucial role in starch metabolism, ripening, and soluble solid content of tomato fruit and affects postharvest softening. *Plant Cell*, 23, 162-184.
- Cheng, J., Shen, H., Yang, X., Yu, S., Yuan, L., Sun, Z. & Sun, X. 2008. Changes in biochemical characteristics related to firmness during fruit development of pepper (*Capsicum annuum* L.). *European Journal of Horticultural Science*, 73, 155-161.
- Chomczynski, P. & Sacchi, N. 2006. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: Twenty-something years on. *Nature Protocols*, 1, 581-585.
- Dai, Z. W., Léon, C., Feil, R., Lunn, J. E., Delrot, S. & Gomès, E. 2013. Metabolic profiling reveals coordinated switches in primary carbohydrate metabolism in grape berry (*Vitis vinifera* L.), a non-climacteric fleshy fruit. *Journal of Experimental Botany*, 64, 1345-1355.
- Daood, H. G., Biacs, P. A., Kiss-Kutz, N., Hajdú, F. & Czinkotai, B. 1989. Lipid and antioxidant content of red pepper. In: Biacs, P., Gruiz, K. & Kremmer, T. (eds.) *Biological Role of Plant Lipids*. Springer, New York.
- Deluc, L., Grimplet, J., Wheatley, M., Tillett, R., Quilici, D., Osborne, C., Schooley, D., Schlauch, K., Cushman, J. & Cramer, G. 2007. Transcriptomic and metabolite analyses of Cabernet Sauvignon grape berry development. *BMC Genomics*, 8, 429.
- Domínguez, M. & Vendrell, M. 1994. Effect of ethylene treatment on ethylene production, EFE activity and ACC levels in peel and pulp of banana fruit. *Postharvest Biology and Technology*, 4, 167-177.

- El-Sharkawy, I., Kim, W. S., Jayasankar, S., Svircev, A. M. & Brown, D. C. W. 2008. Differential regulation of four members of the ACC synthase gene family in plum. *Journal of Experimental Botany*, 59, 2009-2027.
- Elitzur, T., Vrebalov, J., Giovannoni, J. J., Goldschmidt, E. E. & Friedman, H. 2010. The regulation of MADS-box gene expression during ripening of banana and their regulatory interaction with ethylene. *Journal of Experimental Botany*, 61, 1523-1535.
- Fabi, J. P., Cordenunsi, B. R., Seymour, G. B., Lajolo, F. M. & do Nascimento, J. R. O. 2009. Molecular cloning and characterization of a ripening-induced polygalacturonase related to papaya fruit softening. *Plant Physiology and Biochemistry*, 47, 1075-1081.
- Fait, A., Hanhineva, K., Beleggia, R., Dai, N., Rogachev, I., Nikiforova, V. J., Fernie, A. R. & Aharoni, A. 2008. Reconfiguration of the achene and receptacle metabolic networks during strawberry fruit development. *Plant Physiology*, 148, 730-750.
- Faurobert, M., Mihr, C., Bertin, N., Pawlowski, T., Negroni, L., Sommerer, N. & Causse, M. 2007. Major proteome variations associated with cherry tomato pericarp development and ripening. *Plant Physiology*, 143, 1327-1346.
- Fei, Z. J., Tang, X., Alba, R. M., White, J. A., Ronning, C. M., Martin, G. B., Tanksley, S. D. & Giovannoni, J. J. 2004. Comprehensive EST analysis of tomato and comparative genomics of fruit ripening. *Plant Journal*, 40, 47-59.
- Fernández-Otero, C., Matilla, A. J., Rasori, A., Ramina, A. & Bonghi, C. 2006. Regulation of ethylene biosynthesis in reproductive organs of damson plum (*Prunus domestica* L. subsp *Syriaca*). *Plant Science*, 171, 74-83.
- Fiehn, O. 2002. Metabolomics - the link between genotypes and phenotypes. *Plant Molecular Biology*, 48, 155-171.
- Food and Agriculture Organization (FAO) 2013. <http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#anchor> (date of access: 12th Oct 2013).
- Fujii, H., Shimada, T., Sugiyama, A., Nishikawa, F., Endo, T., Nakano, M., Ikoma, Y., Shimizu, T. & Omura, M. 2007. Profiling ethylene-responsive genes in mature mandarin fruit using a citrus 22K oligoarray. *Plant Science*, 173, 340-348.
- Gapper, N. E., McQuinn, R. P. & Giovannoni, J. J. 2013. Molecular and genetic regulation of fruit ripening. *Plant Molecular Biology*, 82, 575-591.
- Gautier, H., Diakou-Verdin, V., Bénard, C., Reich, M., Buret, M., Bourgaud, F., Poëssel, J. L., Caris-Veyrat, C. & Génard, M. 2008. How does tomato quality (sugar, acid, and nutritional quality) vary with ripening stage, temperature, and irradiance? *Journal of Agricultural and Food Chemistry*, 56, 1241-1250.
- Génard, M. & Gouble, B. 2005. ETHY. A theory of fruit climacteric ethylene emission. *Plant Physiology*, 139, 531-545.
- Giovannoni, J. J. 2004. Genetic regulation of fruit development and ripening. *Plant Cell*, 16, S170-S180.
- Giovannoni, J. J. 2007. Fruit ripening mutants yield insights into ripening control. *Current Opinion in Plant Biology*, 10, 283-289.
- Giribaldi, M., Perugini, L., Sauvage, F. X. & Schubert, A. 2007. Analysis of protein changes during grape berry ripening by 2-DE and MALDI-TOF. *Proteomics*, 7, 3154-3170.
- Grimplet, J., Deluc, L. G., Tillett, R. L., Wheatley, M. D., Schlauch, K. A., Cramer, G. R. & Cushman, J. C. 2007. Tissue-specific mRNA expression profiling in grape berry tissues. *BMC Genomics*, 8: 187.

- Gross, K. C., Watada, A. E., Kang, M. S., Kim, S. D., Kim, K. S. & Lee, S. W. 1986. Biochemical changes associated with the ripening of hot pepper fruit. *Physiologia Plantarum*, 66, 31-36.
- Guillén, F., Castillo, S., Zapata, P. J., Martínez-Romero, D., Serrano, M. & Valero, D. 2007. Efficacy of 1-MCP treatment in tomato fruit 1. Duration and concentration of 1-MCP treatment to gain an effective delay of postharvest ripening. *Postharvest Biology and Technology*, 43, 23-27.
- Guillet, C., Just, D., Bénard, N., Destrac-Irvine, A., Baldet, P., Hernould, M., Causse, M., Raymond, P. & Rothan, C. 2002. A fruit-specific phosphoenolpyruvate carboxylase is related to rapid growth of tomato fruit. *Planta*, 214, 717-726.
- Guzman, I., Hamby, S., Romero, J., Bosland, P. W. & O'Connell, M. A. 2010. Variability of carotenoid biosynthesis in orange colored *Capsicum* spp. *Plant Science*, 179, 49-59.
- Ha, S. H., Kim, J. B., Park, J. S., Lee, S. W. & Cho, K. J. 2007. A comparison of the carotenoid accumulation in *Capsicum* varieties that show different ripening colours: deletion of the capsanthin-capsorubin synthase gene is not a prerequisite for the formation of a yellow pepper. *Journal of Experimental Botany*, 58, 3135-3144.
- Han, S. E., Seo, Y. S., Kim, D., Sung, S. K. & Kim, W. T. 2007. Expression of *MdCAS1* and *MdCAS2*, encoding apple  $\beta$ -cyanoalanine synthase homologs, is concomitantly induced during ripening and implicates MdCASs in the possible role of the cyanide detoxification in Fuji apple (*Malus domestica* Borkh.) fruits. *Plant Cell Reports*, 26, 1321-1331.
- Handa, A. K. & Mattoo, A. K. 2010. Differential and functional interactions emphasize the multiple roles of polyamines in plants. *Plant Physiology and Biochemistry*, 48, 540-546.
- Hertog, M. L. A. T. M., Rudell, D. R., Pedreschi, R., Schaffer, R. J., Geeraerd, A. H., Nicolai, B. M. & Ferguson, I. 2011. Where systems biology meets postharvest. *Postharvest Biology and Technology*, 62, 223-237.
- Hesse, H., Lipke, J., Altmann, T. & Höfgen, R. 1999. Molecular cloning and expression analyses of mitochondrial and plastidic isoforms of cysteine synthase (O-acetylserine(thiol)lyase) from *Arabidopsis thaliana*. *Amino Acids*, 16, 113-131.
- Himelblau, E., Mira, H., Lin, S.-J., Culotta, V. C., Peñarrubia, L. & Amasino, R. M. 1998. Identification of a functional homolog of the yeast copper homeostasis gene *ATX1* from *Arabidopsis*. *Plant Physiology*, 117, 1227-1234.
- Huang, S. Z., Sawaki, T., Takahashi, A., Mizuno, S., Takezawa, K., Matsumura, A., Yokotsuka, M., Hirasawa, Y., Sonoda, M., Nakagawa, H. & Sato, T. 2010. Melon EIN3-like transcription factors (CmEIL1 and CmEIL2) are positive regulators of an ethylene- and ripening-induced 1-aminocyclopropane-1-carboxylic acid oxidase gene (*CM-ACO1*). *Plant Science*, 178, 251-257.
- Huber, D. J. 2008. Suppression of ethylene responses through application of 1-methylcyclopropene: A powerful tool for elucidating ripening and senescence mechanisms in climacteric and nonclimacteric fruits and vegetables. *Hortscience*, 43, 106-111.
- Iusem, N. D., Bartholomew, D. M., Hitz, W. D. & Scolnik, P. A. 1993. Tomato (*Lycopersicon esculentum*) transcript induced by water deficit and ripening. *Plant Physiology*, 102, 1353-1354.
- Jafari, Z., Haddad, R., Hosseini, R. & Garoosi, G. 2013. Cloning, identification and expression analysis of ACC oxidase gene involved in ethylene production pathway. *Molecular Biology Reports*, 40, 1341-1350.

- Jia, H.-F., Chai, Y.-M., Li, C.-L., Lu, D., Luo, J.-J., Qin, L. & Shen, Y.-Y. 2011. Abscisic acid plays an important role in the regulation of strawberry fruit ripening. *Plant Physiology*, 157, 188-199.
- Katz, E., Lagunes, P. M., Riov, J., Weiss, D. & Goldschmidt, E. E. 2004. Molecular and physiological evidence suggests the existence of a system II-like pathway of ethylene production in non-climacteric *Citrus* fruit. *Planta*, 219, 243-252.
- Kevany, B. M., Tieman, D. M., Taylor, M. G., Dal Cin, V. & Klee, H. J. 2007. Ethylene receptor degradation controls the timing of ripening in tomato fruit. *Plant Journal*, 51, 458-467.
- Khoo, K. H. P., Able, A. J., Chataway, T. K. & Able, J. A. 2012. Preliminary characterisation of two early meiotic wheat proteins after identification through 2D gel electrophoresis proteomics. *Functional Plant Biology*, 39, 222-235.
- Klee, H. J. 2004. Ethylene signal transduction. Moving beyond Arabidopsis. *Plant Physiology*, 135, 660-667.
- Klee, H. J. & Giovannoni, J. J. 2011. Genetics and control of tomato fruit ripening and quality attributes. *Annual Review of Genetics*, 45, 41-59.
- Klieber, A. 2000. *Chilli spice production in Australia : a report for the Rural Industries Research and Development Corporation Asian Foods Research and Development*. Rural Industries Research & Development Corporation, Barton.
- Knee, M. 1995. Do tomatoes on the plant behave as climacteric fruits? *Physiologia Plantarum*, 95, 211-216.
- Kobayashi, K., Baba, S., Obayashi, T., Sato, M., Toyooka, K., Keränen, M., Aro, E.-M., Fukaki, H., Ohta, H., Sugimoto, K. & Masuda, T. 2012. Regulation of root greening by light and auxin/cytokinin signaling in *Arabidopsis*. *Plant Cell*, 24, 1081-1095.
- Kok, E. J., Lehesranta, S. J., van Dijk, J. P., Helsdingen, J. R., Dijkema, W. T. P., Van Hoef, A. M. A., Koistinen, K. M., Kärenlampi, S. O., Kuiper, H. A. & Keijer, J. 2008. Changes in gene and protein expression during tomato ripening - Consequences for the safety assessment of new crop plant varieties. *Food Science and Technology International*, 14, 503-518.
- Krajayklang, M., Klieber, A. & Dry, P. R. 2000. Colour at harvest and post-harvest behaviour influence paprika and chilli spice quality. *Postharvest Biology and Technology*, 20, 269-278.
- Kuntz, M., Chen, H. C., Simkin, A. J., Römer, S., Shipton, C. A., Drake, R., Schuch, W. & Bramley, P. M. 1998. Upregulation of two ripening-related genes from a nonclimacteric plant (pepper) in a transgenic climacteric plant (tomato). *Plant Journal*, 13, 351-361.
- Kwade, Z., Świątek, A., Azmi, A., Goossens, A., Inzé, D., Van Onckelen, H. & Roef, L. 2005. Identification of four adenosine kinase isoforms in tobacco By-2 cells and their putative role in the cell cycle-regulated cytokinin metabolism. *Journal of Biological Chemistry*, 280, 17512-17519.
- Lee, S., Chung, E. J., Joung, Y. H. & Choi, D. 2010. Non-climacteric fruit ripening in pepper: increased transcription of *EIL*-like genes normally regulated by ethylene. *Functional and Integrative Genomics*, 10, 135-146.
- Lelièvre, J.-M., Latché, A., Jones, B., Bouzayen, M. & Pech, J.-C. 1998. Ethylene and fruit ripening. *Physiologia Plantarum*, 101, 727-739.
- Lemaire-Chamley, M., Petit, J., Garcia, V., Just, D., Baldet, P., Germain, V., Fagard, M., Mouassite, M., Cheniclet, C. & Rothan, C. 2005. Changes in transcriptional profiles

- are associated with early fruit tissue specialization in tomato. *Plant Physiology*, 139, 750-769.
- Li, L., Zhu, B. Z., Yang, P. Y., Fu, D. Q., Zhu, Y. & Luo, Y. B. 2011. The regulation mode of RIN transcription factor involved in ethylene biosynthesis in tomato fruit. *Journal of the Science of Food and Agriculture*, 91, 1822-1828.
- Lin, Z. F., Hong, Y. G., Yin, M. G., Li, C. Y., Zhang, K. & Grierson, D. 2008. A tomato HD-Zip homeobox protein, LeHB-1, plays an important role in floral organogenesis and ripening. *Plant Journal*, 55, 301-310.
- Lin, Z. F., Zhong, S. L. & Grierson, D. 2009. Recent advances in ethylene research. *Journal of Experimental Botany*, 60, 3311-3336.
- Liu, G. N., Zhu, Y. H. & Jiang, J. G. 2009. The metabolomics of carotenoids in engineered cell factory. *Applied Microbiology and Biotechnology*, 83, 989-999.
- Liu, S., Chen, C., Chen, G., Cao, B., Chen, Q. & Lei, J. 2012a. RNA-sequencing tag profiling of the placenta and pericarp of pungent pepper provides robust candidates contributing to capsaicinoid biosynthesis. *Plant Cell, Tissue and Organ Culture*, 110, 111-121.
- Liu, T.-C., Liu, Y.-C., Chen, K.-E., Chao, C.-W. & Wu, C.-T. 2012b. The nonclimacteric guava cultivar 'Jen-Ju Bar' is defective in System 2 1-aminocyclopropane-1-carboxylate synthase activity. *Postharvest Biology and Technology*, 67, 10-18.
- Livingstone, K. D., Lackney, V. K., Blauth, J. R., van Wijk, R. & Jahn, M. K. 1999. Genome mapping in capsicum and the evolution of genome structure in the Solanaceae. *Genetics*, 152, 1183-1202.
- Manenoi, A. & Paull, R. E. 2007. Papaya fruit softening, endoxylanase gene expression, protein and activity. *Physiologia Plantarum*, 131, 470-480.
- Martel, C., Vrebalov, J., Tafelmeyer, P. & Giovannoni, J. J. 2011. The Tomato MADS-Box Transcription Factor RIPENING INHIBITOR Interacts with Promoters Involved in Numerous Ripening Processes in a COLORLESS NONRIPENING-Dependent Manner. *Plant Physiology*, 157, 1568-1579.
- Martín-Trillo, M. & Cubas, P. 2010. TCP genes: A family snapshot ten years later. *Trends in Plant Science*, 15, 31-39.
- Martínez-Romero, D., Bailén, G., Serrano, M., Guillén, F., Valverde, J. M., Zapata, P., Castillo, S. & Valero, D. 2007. Tools to maintain postharvest fruit and vegetable quality through the inhibition of ethylene action: A review. *Critical Reviews in Food Science and Nutrition*, 47, 543-560.
- Marty, I., Bureau, S., Sarkissian, G., Gouble, B., Audergon, J. M. & Albagnac, G. 2005. Ethylene regulation of carotenoid accumulation and carotenogenic gene expression in colour-contrasted apricot varieties (*Prunus armeniaca*). *Journal of Experimental Botany*, 56, 1877-1886.
- Matas, A. J., Gapper, N. E., Chung, M. Y., Giovannoni, J. J. & Rose, J. K. C. 2009. Biology and genetic engineering of fruit maturation for enhanced quality and shelf-life. *Current Opinion in Biotechnology*, 20, 197-203.
- Matsufuji, H., Ishikawa, K., Nunomura, O., Chino, M. & Takeda, M. 2007. Anti-oxidant content of different coloured sweet peppers, white, green, yellow, orange and red (*Capsicum annuum* L.). *International Journal of Food Science and Technology*, 42, 1482-1488.
- Mattoo, A. K., Minocha, S. C., Minocha, R. & Handa, A. K. 2010. Polyamines and cellular metabolism in plants: transgenic approaches reveal different responses to diamine putrescine versus higher polyamines spermidine and spermine. *Amino Acids*, 38, 405-413.

- McMurchie, E. J., McGlasson, W. B. & Eaks, I. L. 1972. Treatment of fruit with propylene gives information about biogenesis of ethylene. *Nature*, 237, 235-236.
- Mellway, R. D. & Lund, S. T. 2013. Interaction analysis of grapevine MIKC<sup>c</sup>-type MADS transcription factors and heterologous expression of putative *véraison* regulators in tomato. *Journal of Plant Physiology*, 170, 1424-1433.
- Moco, S., Capanoglu, E., Tikunov, Y., Bino, R. J., Boyacioglu, D., Hall, R. D., Vervoort, J. & De Vos, R. C. H. 2007. Tissue specialization at the metabolite level is perceived during the development of tomato fruit. *Journal of Experimental Botany*, 58, 4131-4146.
- Moing, A., Aharoni, A., Biais, B., Rogachev, I., Meir, S., Brodsky, L., Allwood, J. W., Erban, A., Dunn, W. B., Kay, L., de Koning, S., de Vos, R. C., Jonker, H., Mumm, R., Deborde, C., Maucourt, M., Bernillon, S., Gibon, Y., Hansen, T. H., Husted, S., Goodacre, R., Kopka, J., Schjoerring, J. K., Rolin, D. & Hall, R. D. 2011. Extensive metabolic cross-talk in melon fruit revealed by spatial and developmental combinatorial metabolomics. *New Phytologist*, 190, 683-696.
- Moore, S., Payton, P., Wright, M., Tanksley, S. & Giovannoni, J. 2005. Utilization of tomato microarrays for comparative gene expression analysis in the Solanaceae. *Journal of Experimental Botany*, 56, 2885-2895.
- Moradinezhad, F., Sedgley, M. & Able, A. J. 2010. Effect of pre-ripening chilling temperatures on ripening, shelf life and quality of bananas treated with 1-methylcyclopropene. *International Journal of Food Science and Technology*, 45, 312-318.
- Moradinezhad, F., Sedgley, M., Klieber, A. & Able, A. J. 2008. Variability of responses to 1-methylcyclopropene by banana: Influence of time of year at harvest and fruit position in the bunch. *Annals of Applied Biology*, 152, 223-234.
- Moreno, E., Fita, A., González-Mas, M. C. & Rodríguez-Burruezo, A. 2012. HS-SPME study of the volatile fraction of *Capsicum* accessions and hybrids in different parts of the fruit. *Scientia Horticulturae*, 135, 87-97.
- Nakatsuka, A., Murachi, S., Okunishi, H., Shiomi, S., Nakano, R., Kubo, Y. & Inaba, A. 1998. Differential expression and internal feedback regulation of 1-aminocyclopropane-1-carboxylate synthase, 1-aminocyclopropane-1-carboxylate oxidase, and ethylene receptor genes in tomato fruit during development and ripening. *Plant Physiology*, 118, 1295-1305.
- Nambeesan, S., Datsenka, T., Ferruzzi, M. G., Malladi, A., Mattoo, A. K. & Handa, A. K. 2010. Overexpression of yeast spermidine synthase impacts ripening, senescence and decay symptoms in tomato. *Plant Journal*, 63, 836-847.
- Navarro, J. M., Flores, P., Garrido, C. & Martinez, V. 2006. Changes in the contents of antioxidant compounds in pepper fruits at different ripening stages, as affected by salinity. *Food Chemistry*, 96, 66-73.
- Nick, P., Heuing, A. & Ehmann, B. 2000. Plant chaperonins: A role in microtubule-dependent wall formation? *Protoplasma*, 211, 234-244.
- Nielsen, T. H., Skjærbaek, H. C. & Karlsen, P. 1991. Carbohydrate metabolism during fruit development in sweet-pepper (*Capsicum annuum*) plants. *Physiologia Plantarum*, 82, 311-319.
- Nikiforova, V., Kempa, S., Zeh, M., Maimann, S., Kreft, O., Casazza, A. P., Riedel, K., Tauberger, E., Hoefgen, R. & Hesse, H. 2002. Engineering of cysteine and methionine biosynthesis in potato. *Amino Acids*, 22, 259-278.
- Nilo, R., Saffie, C., Lilley, K., Baeza-Yates, R., Cambiazo, V., Campos-Vargas, R., Gonzalez, M., Meisel, L. A., Retamales, J., Silva, H. & Orellana, A. 2010. Proteomic analysis of

- peach fruit mesocarp softening and chilling injury using difference gel electrophoresis (DIGE). *BMC Genomics*, 11, 43.
- Osorio, S., Alba, R., Nikoloski, Z., Kochevenko, A., Fernie, A. R. & Giovannoni, J. J. 2012. Integrative comparative analyses of transcript and metabolite profiles from pepper and tomato ripening and development stages uncovers species-specific patterns of network regulatory behavior. *Plant Physiology*, 159, 1713-1729.
- Palma, J. M., Corpas, F. J. & del Río, L. A. 2011. Proteomics as an approach to the understanding of the molecular physiology of fruit development and ripening. *Journal of Proteomics*, 74, 1230-1243.
- Paran, I. & van der Knaap, E. 2007. Genetic and molecular regulation of fruit and plant domestication traits in tomato and pepper. *Journal of Experimental Botany*, 58, 3841-3852.
- Park, S. Y., Yu, J. W., Park, J. S., Li, J., Yoo, S. C., Lee, N. Y., Lee, S. K., Jeong, S. W., Seo, H. S., Koh, H. J., Jeon, J. S., Park, Y. I. & Paek, N. C. 2007. The senescence-induced staygreen protein regulates chlorophyll degradation. *Plant Cell*, 19, 1649-1664.
- Paul, V., Pandey, R. & Srivastava, G. C. 2012. The fading distinctions between classical patterns of ripening in climacteric and non-climacteric fruit and the ubiquity of ethylene - An overview. *Journal of Food Science and Technology-Mysore*, 49, 1-21.
- Pech, J.-C., Purgatto, E., Bouzayen, M. & Latché, A. 2012. Ethylene and fruit ripening. In: McManus, M. T. (ed.) *Annual Plant Reviews: The Plant Hormone Ethylene*. John Wiley & Sons Ltd, Chichester.
- Pech, J.-C., Bouzayen, M. & Latché, A. 2008. Climacteric fruit ripening: Ethylene-dependent and independent regulation of ripening pathways in melon fruit. *Plant Science*, 175, 114-120.
- Périn, C., Gomez-Jimenez, M., Hagen, L., Dogimont, C., Pech, J.-C., Latché, A., Pitrat, M. & Lelièvre, J.-M. 2002. Molecular and genetic characterization of a non-climacteric phenotype in melon reveals two loci conferring altered ethylene response in fruit. *Plant Physiology*, 129, 300-309.
- Pham, T. N. T. 2007. *Ripening behaviour of capsicum (Capsicum annuum L.) fruit*. PhD thesis, University of Adelaide.
- Ponce-Valadez, M., Fellman, S. M., Giovannoni, J., Gan, S. S. & Watkins, C. B. 2009. Differential fruit gene expression in two strawberry cultivars in response to elevated CO<sub>2</sub> during storage revealed by a heterologous fruit microarray approach. *Postharvest Biology and Technology*, 51, 131-140.
- Prasanna, V., Prabha, T. N. & Tharanathan, R. N. 2007. Fruit ripening phenomena - An overview. *Critical Reviews in Food Science and Nutrition*, 47, 1-19.
- Qin, G. Z., Wang, Y. Y., Cao, B. H., Wang, W. H. & Tian, S. P. 2012. Unraveling the regulatory network of the MADS box transcription factor RIN in fruit ripening. *Plant Journal*, 70, 243-255.
- Roca, M. & Mínguez-Mosquera, M. I. 2006. Chlorophyll catabolism pathway in fruits of *Capsicum annuum* (L.): Stay-green versus red fruits. *Journal of Agricultural and Food Chemistry*, 54, 4035-4040.
- Rocco, M., D'Ambrosio, C., Arena, S., Faurobert, M., Scaloni, A. & Marra, M. 2006. Proteomic analysis of tomato fruits from two ecotypes during ripening. *Proteomics*, 6, 3781-3791.
- Rodríguez, A., Alquézar, B. & Peña, L. 2013. Fruit aromas in mature fleshy fruits as signals of readiness for predation and seed dispersal. *New Phytologist*, 197, 36-48.

- Roessner-Tunali, U., Hegemann, B., Lytovchenko, A., Carrari, F., Bruedigam, C., Granot, D. & Fernie, A. R. 2003. Metabolic profiling of transgenic tomato plants overexpressing hexokinase reveals that the influence of hexose phosphorylation diminishes during fruit development. *Plant Physiology*, 133, 84-99.
- Romani R.J., 1984. Respiration, ethylene, senescence, and homeostasis in an integrated view of postharvest life. *Canadian Journal of Botany*, 62, 2950-2955.
- Römer, S., d'Harlingue, A., Camara, B., Schantz, R. & Kuntz, M. 1992. Cysteine synthase from *Capsicum annuum* chromoplasts - Characterization and cDNA cloning of an up-regulated enzyme during fruit development. *Journal of Biological Chemistry*, 267, 17966-17970.
- Rose, J. K. C., Bashir, S., Giovannoni, J. J., Jahn, M. M. & Saravanan, R. S. 2004. Tackling the plant proteome: practical approaches, hurdles and experimental tools. *Plant Journal*, 39, 715-733.
- Saltveit, M. E. 1977. Carbon-dioxide, ethylene, and color development in ripening mature green bell peppers. *Journal of the American Society for Horticultural Science*, 102, 523-525.
- Sambrook, J. & Russell, D. W. 2001. *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory Press, New York.
- Saravanan, R. S. & Rose, J. K. C. 2004. A critical evaluation of sample extraction techniques for enhanced proteomic analysis of recalcitrant plant tissues. *Proteomics*, 4, 2522-2532.
- Scheibe, R. 2004. Malate valves to balance cellular energy supply. *Physiologia Plantarum*, 120, 21-26.
- Schoor, S., Farrow, S., Blaschke, H., Lee, S., Perry, G., von Schwartzberg, K., Emery, N. & Moffatt, B. 2011. Adenosine Kinase Contributes to Cytokinin Interconversion in Arabidopsis. *Plant Physiology*, 157, 659-672.
- Serrano, M., Zapata, P. J., Castillo, S., Guillén, F., Martínez-Romero, D. & Valero, D. 2010. Antioxidant and nutritive constituents during sweet pepper development and ripening are enhanced by nitrophenolate treatments. *Food Chemistry*, 118, 497-503.
- Sethu, K. M. P., Prabha, T. N. & Tharanathan, R. N. 1996. Post-harvest biochemical changes associated with the softening phenomenon in *Capsicum annuum* fruits. *Phytochemistry*, 42, 961-966.
- Seymour, G. B., Chapman, N. H., Chew, B. L. & Rose, J. K. C. 2013. Regulation of ripening and opportunities for control in tomato and other fruits. *Plant Biotechnology Journal*, 11, 269-278.
- Seymour, G. B., Ryder, C. D., Cevik, V., Hammond, J. P., Popovich, A., King, G. J., Vrebalov, J., Giovannoni, J. J. & Manning, K. 2011. A SEPALLATA gene is involved in the development and ripening of strawberry (*Fragaria×ananassa* Duch.) fruit, a non-climacteric tissue. *Journal of Experimental Botany*, 62, 1179-1188.
- Seymour, G. B., Taylor, J. E. & Tucker, G. A. 1993. *Biochemistry of fruit ripening*. Chapman & Hall, New York.
- Song, J., Braun, G., Bevis, E. & Doncaster, K. 2006. A simple protocol for protein extraction of recalcitrant fruit tissues suitable for 2-DE and MS analysis. *Electrophoresis*, 27, 3144-3151.
- Spíchal, L. 2012. Cytokinins - Recent news and views of evolutionally old molecules. *Functional Plant Biology*, 39, 267-284.
- Srivastava, A. & Handa, A. K. 2005. Hormonal regulation of tomato fruit development: A molecular perspective. *Journal of Plant Growth Regulation*, 24, 67-82.

- Symons, G. M., Davies, C., Shavrukov, Y., Dry, I. B., Reid, J. B. & Thomas, M. R. 2006. Grapes on steroids. Brassinosteroids are involved in grape berry ripening. *Plant Physiology*, 140, 150-158.
- Taiz, L. & Zeiger, E. 2010. *Plant Physiology*. Sinauer Associates Inc, Sunderland.
- Tetlow, I. J. 2011. Starch biosynthesis in developing seeds. *Seed Science Research*, 21, 5-32.
- Tikunov, Y., Lommen, A., de Vos, C. H. R., Verhoeven, H. A., Bino, R. J., Hall, R. D. & Bovy, A. G. 2005. A novel approach for nontargeted data analysis for metabolomics. Large-scale profiling of tomato fruit volatiles. *Plant Physiology*, 139, 1125-1137.
- Vega-Gálvez, A., Di Scala, K., Rodríguez, K., Lemus-Mondaca, R., Miranda, M., López, J. & Perez-Won, M. 2009. Effect of air-drying temperature on physico-chemical properties, antioxidant capacity, colour and total phenolic content of red pepper (*Capsicum annuum*, L. var. Hungarian). *Food Chemistry*, 117, 647-653.
- Villarreal, N. M., Bustamante, C. A., Civello, P. M. & Martínez, G. A. 2010. Effect of ethylene and 1-MCP treatments on strawberry fruit ripening. *Journal of the Science of Food and Agriculture*, 90, 683-689.
- Villas-Boas, S. G., Rasmussen, S. & Lane, G. A. 2005. Metabolomics or metabolite profiles? *Trends in Biotechnology*, 23, 385-386.
- Villavicencio, L., Blankenship, S. M., Sanders, D. C. & Swallow, W. H. 1999. Ethylene and carbon dioxide production in detached fruit of selected pepper cultivars. *Journal of the American Society for Horticultural Science*, 124, 402-406.
- Vrebalov, J., Ruezinsky, D., Padmanabhan, V., White, R., Medrano, D., Drake, R., Schuch, W. & Giovannoni, J. 2002. A MADS-box gene necessary for fruit ripening at the tomato *Ripening-Inhibitor (Rin)* locus. *Science*, 296, 343-346.
- Wahyuni, Y., Ballester, A. R., Sudarmonowati, E., Bino, R. J. & Bovy, A. G. 2011. Metabolite biodiversity in pepper (*Capsicum*) fruits of thirty-two diverse accessions: variation in health-related compounds and implications for breeding. *Phytochemistry*, 72, 1358-1370.
- Wahyuni, Y., Ballester, A. R., Tikunov, Y., de Vos, R. C. H., Pelgrom, K. T. B., Maharijaya, A., Sudarmonowati, E., Bino, R. J. & Bovy, A. G. 2013. Metabolomics and molecular marker analysis to explore pepper (*Capsicum* sp.) biodiversity. *Metabolomics*, 9, 130-144.
- Wang, P., Zhang, B., Li, X., Xu, C. J., Yin, X. R., Shan, L. L., Ferguson, I. & Chen, K. S. 2010. Ethylene signal transduction elements involved in chilling injury in non-climacteric loquat fruit. *Journal of Experimental Botany*, 61, 179-190.
- Wang, Q., Yuan, G., Sun, H., Zhao, P., Liu, Y. & Guo, D. 2005. Molecular cloning and expression analysis of spermidine synthase gene during sex reversal induced by Ethrel in cucumber (*Cucumis sativus* L.). *Plant Science*, 169, 768-775.
- Wang, W., Bianchi, L., Scali, M., Liu, L. W., Bini, L. & Cresti, M. 2009. Proteomic analysis of beta-1,3-glucanase in grape berry tissues. *Acta Physiologiae Plantarum*, 31, 597-604.
- Warrilow, A. G. & Hawkesford, M. J. 2000. Cysteine synthase (O-acetylserine (thiol) lyase) substrate specificities classify the mitochondrial isoform as a cyanoalanine synthase. *Journal of Experimental Botany*, 51, 985-993.
- Watkins, C. B. 2006. The use of 1-methylcyclopropene (1-MCP) on fruits and vegetables. *Biotechnology Advances*, 24, 389-409.
- Whitelegge, J. P. 2004. Mass spectrometry for high throughput quantitative proteomics in plant research: Lessons from thylakoid membranes. *Plant Physiology and Biochemistry*, 42, 919-927.

- Wirtz, M. & Droux, M. 2005. Synthesis of the sulfur amino acids: cysteine and methionine. *Photosynthesis Research*, 86, 345-362.
- Woeste, K. E. & Kieber, J. J. 2000. A strong loss-of-function mutation in *RAN1* results in constitutive activation of the ethylene response pathway as well as a rosette-lethal phenotype. *Plant Cell*, 12, 443-455.
- Wu, M.-C., Hu, H.-T., Yang, L. & Yang, L. 2011. Proteomic analysis of up-accumulated proteins associated with fruit quality during autumn olive (*Elaeagnus umbellata*) fruit ripening. *Journal of Agricultural and Food Chemistry*, 59, 577-583.
- Yahia, E. M., Contreras-Padilla, M. & Gonazalez-Aguilar, G. 2001. Ascorbic acid content in relation to ascorbic acid oxidase activity and polyamine content in tomato and bell pepper fruits during development, maturation and senescence. *Lebensmittel-Wissenschaft Und-Technologie-Food Science and Technology*, 34, 452-457.
- Yamaki, S. 2010. Metabolism and accumulation of sugars translocated to fruit and their regulation. *Journal of the Japanese Society for Horticultural Science*, 79, 1-15.
- Yamane, M., Abe, D., Yasui, S., Yokotani, N., Kimata, W., Ushijima, K., Nakano, R., Kubo, Y. & Inaba, A. 2007. Differential expression of ethylene biosynthetic genes in climacteric and non-climacteric Chinese pear fruit. *Postharvest Biology and Technology*, 44, 220-227.
- Yokotani, N., Tamura, S., Nakano, R., Inaba, A., McGlasson, W. B. & Kubo, Y. 2004. Comparison of ethylene- and wound-induced responses in fruit of wild-type, *rin* and *nor* tomatoes. *Postharvest Biology and Technology*, 32, 247-252.
- Yun, Z., Jin, S., Ding, Y., Wang, Z., Gao, H., Pan, Z., Xu, J., Cheng, Y. & Deng, X. 2012. Comparative transcriptomics and proteomics analysis of citrus fruit, to improve understanding of the effect of low temperature on maintaining fruit quality during lengthy post-harvest storage. *Journal of Experimental Botany*, 63, 2873-2893.
- Yun, Z., Li, W., Pan, Z., Xu, J., Cheng, Y. & Deng, X. 2010. Comparative proteomics analysis of differentially accumulated proteins in juice sacs of ponkan (*Citrus reticulata*) fruit during postharvest cold storage. *Postharvest Biology and Technology*, 56, 189-201.
- Zhang, M., Yuan, B. & Leng, P. 2009a. The role of ABA in triggering ethylene biosynthesis and ripening of tomato fruit. *Journal of Experimental Botany*, 60, 1579-1588.
- Zhang, Z. J., Zhang, H. W., Quan, R. D., Wang, X. C. & Huang, R. F. 2009b. Transcriptional regulation of the ethylene response factor LeERF2 in the expression of ethylene biosynthesis genes controls ethylene production in tomato and tobacco. *Plant Physiology*, 150, 365-377.
- Zheng, X. Y., Wolff, D. W. & Crosby, K. M. 2002. Genetics of ethylene biosynthesis and restriction fragment length polymorphisms (RFLPs) of ACC oxidase and synthase genes in melon (*Cucumis melo* L.). *Theoretical and Applied Genetics*, 105, 397-403.
- Zhong, S., Fei, Z., Chen, Y.-R., Zheng, Y., Huang, M., Vrebalov, J., McQuinn, R., Gapper, N., Liu, B., Xiang, J., Shao, Y. & Giovannoni, J. J. 2013. Single-base resolution methylomes of tomato fruit development reveal epigenome modifications associated with ripening. *Nature Biotechnology*, 31, 154-159.

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## **Appendices**

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## **Appendix A**

Statement of Authorship

Manuscript 1 Chapter 3

**Chapter 3**  
Statement of Authorship

Title of Paper	Proteomic analysis during capsicum ripening reveals differential expression of ACC oxidase isoform 4 and other candidates.
Publication Status	Accepted for Publication
Publication Details	Aizat, W.M., Able, J.A., Stangoulis, J.C.R. & Able, A.J., (2013). Proteomic analysis during capsicum ripening reveals differential expression of ACC oxidase isoform 4 and other candidates. Functional Plant Biology, doi.org/10.1071/FP12330.

**Author Contributions**

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Wan M. Aizat		
Contribution to the Paper	Performed all experimental work, data interpretation and wrote the manuscript.		
Signature		Date	17/08/2013

Name of Co-Author	Jason A. Able		
Contribution to the Paper	Supervised work development, helped in data interpretation and critical assessment of the manuscript.		
Signature		Date	17/10/13

Name of Co-Author	James C.R. Stangoulis		
Contribution to the Paper	Supervised work development, helped in data interpretation and critical assessment of the manuscript.		
Signature		Date	22/10/13

Name of Co-Author	Amanda J. Able		
Contribution to the Paper	Principal supervisor of the project, helped in data interpretation, critical assessment and editing of the manuscript. Acted as corresponding author.		
Signature		Date	23/10/13

## **Appendix B**

Statement of Authorship

Manuscript 2 Chapter 4

**Chapter 4**  
Statement of Authorship

Title of Paper	Metabolomics of capsicum ripening reveals modification of the ethylene pathway and carbon metabolism.
Publication Status	Submitted for Publication
Publication Details	Aizat, W.M., Dias D.A., Stangoulis, J.C.R., Able, J.A., Roessner U. & Able, A.J., (2013). Metabolomics of capsicum ripening reveals modification of the ethylene pathway and carbon metabolism. Postharvest Biology and Technology, submitted.

**Author Contributions**

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Wan M. Aizat	
Contribution to the Paper	Performed the majority of experimental work, data interpretation and wrote the manuscript.	
Signature		Date 17/08/2013

Name of Co-Author	Daniel A. Dias	
Contribution to the Paper	Performed the GC-MS and LC-MS analyses as part of collaboration with Metabolomics Australia, helped in data interpretation and critical assessment of the manuscript.	
Signature		Date 25/10/13.

Name of Co-Author	James C.R. Stangoulis	
Contribution to the Paper	Supervised work development, helped in data interpretation and critical assessment of the manuscript.	
Signature		Date 22/10/13

Name of Co-Author	Jason A. Able	
Contribution to the Paper	Supervised work development, helped in data interpretation and critical assessment of the manuscript.	
Signature		Date 17/10/13

Name of Co-Author	Ute Roessner	
Contribution to the Paper	Helped in data interpretation and critical assessment of the manuscript.	
Signature		Date 25/10/13

Name of Co-Author	Amanda J. Able	
Contribution to the Paper	Principal supervisor of the project, helped in data interpretation, critical assessment and editing of the manuscript. Acted as corresponding author.	
Signature		Date 23/10/13

## **Appendix C**

Statement of Authorship

Manuscript 3 Chapter 6

**Chapter 6**  
Statement of Authorship

Title of Paper	Characterisation of ethylene pathway components in non-climacteric capsicum.
Publication Status	Submitted for Publication
Publication Details	Aizat, W.M., Able, J.A., Stangoulis, J.C.R. & Able, A.J., (2013). Characterisation of ethylene pathway components in non-climacteric capsicum. BMC Plant Biology, submitted.

**Author Contributions**

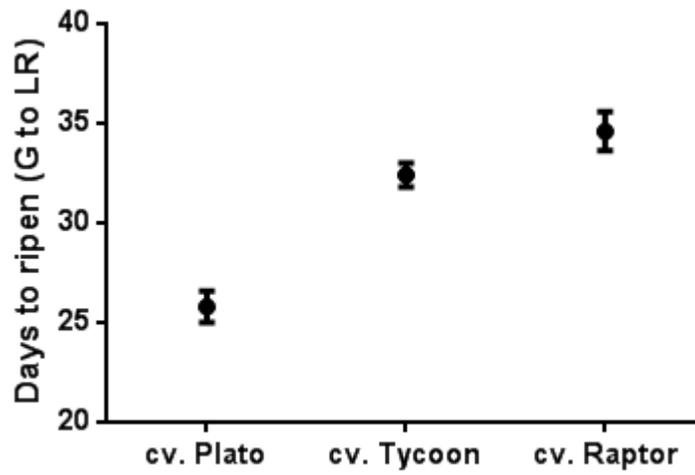
By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Wan M. Aizat		
Contribution to the Paper	Performed all experimental work, data interpretation and wrote the manuscript.		
Signature		Date	17/08/2013

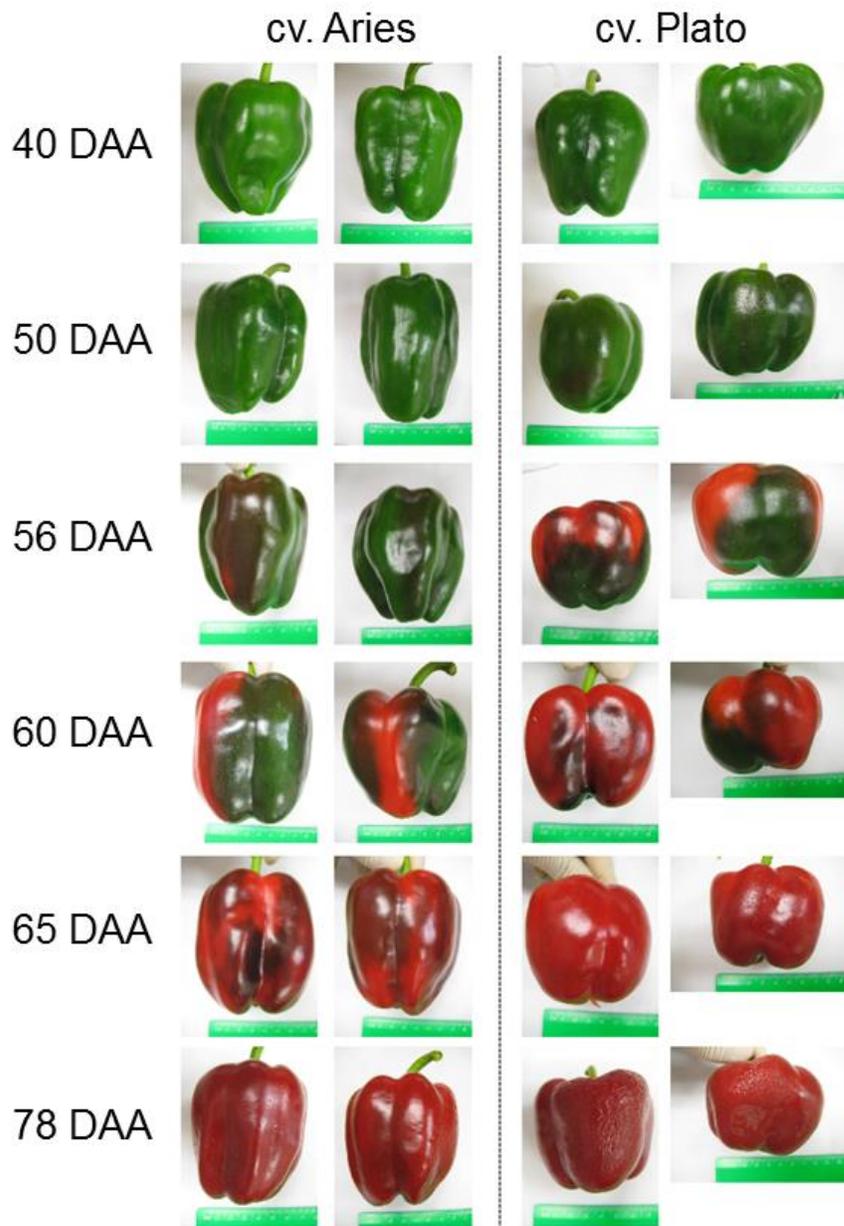
Name of Co-Author	Jason A. Able		
Contribution to the Paper	Supervised work development, helped in data interpretation and critical assessment of the manuscript.		
Signature		Date	17/10/13

Name of Co-Author	James C.R. Stangoulis		
Contribution to the Paper	Supervised work development.		
Signature		Date	22/10/13

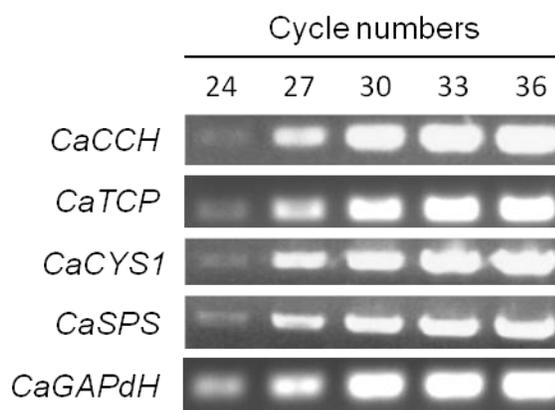
Name of Co-Author	Amanda J. Able		
Contribution to the Paper	Principal supervisor of the project, helped in data interpretation, critical assessment and editing of the manuscript. Acted as corresponding author.		
Signature		Date	23/10/13



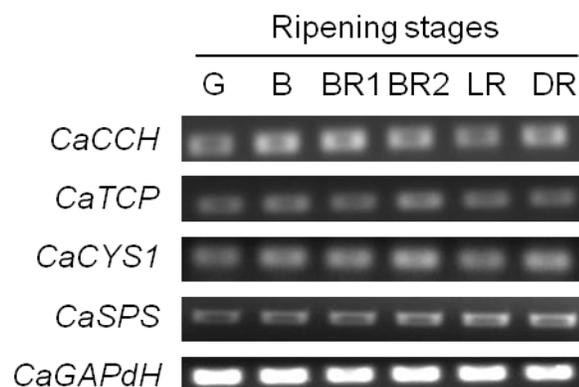
**Appendix D** Three cultivars of capsicum (cv. Plato, cv. Tycoon, cv. Raptor) have different ripening times measured as days to ripen from Green (G) to Light Red (LR) stages. Means are  $n = 5$  biological replicates ( $\pm$ SE).



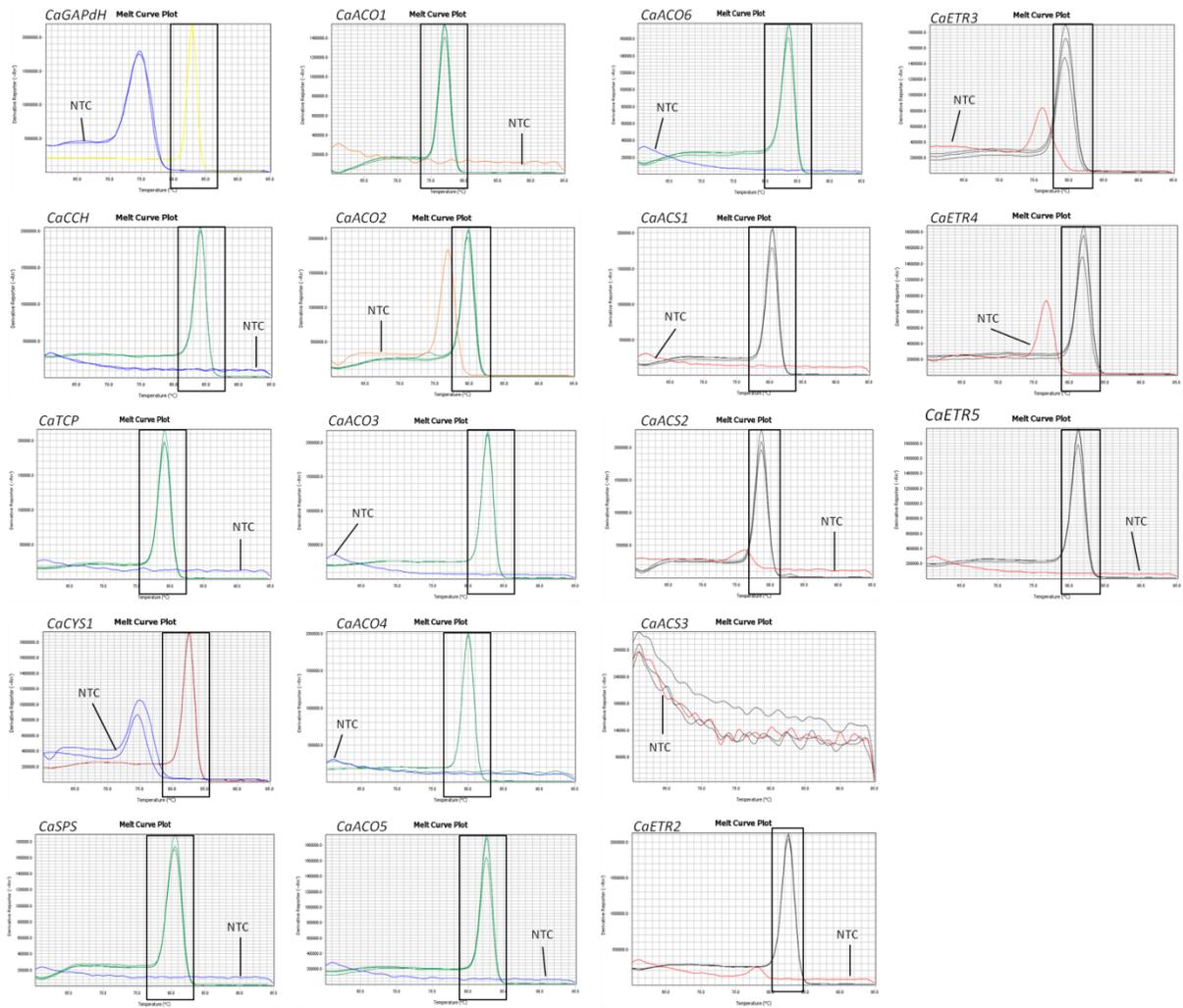
**Appendix E** Both capsicum cultivars (cv. Aries and cv. Plato) were harvested at six different time points as per 5.2.1. Only two out of three biological replicates are shown. DAA, Days After Anthesis.



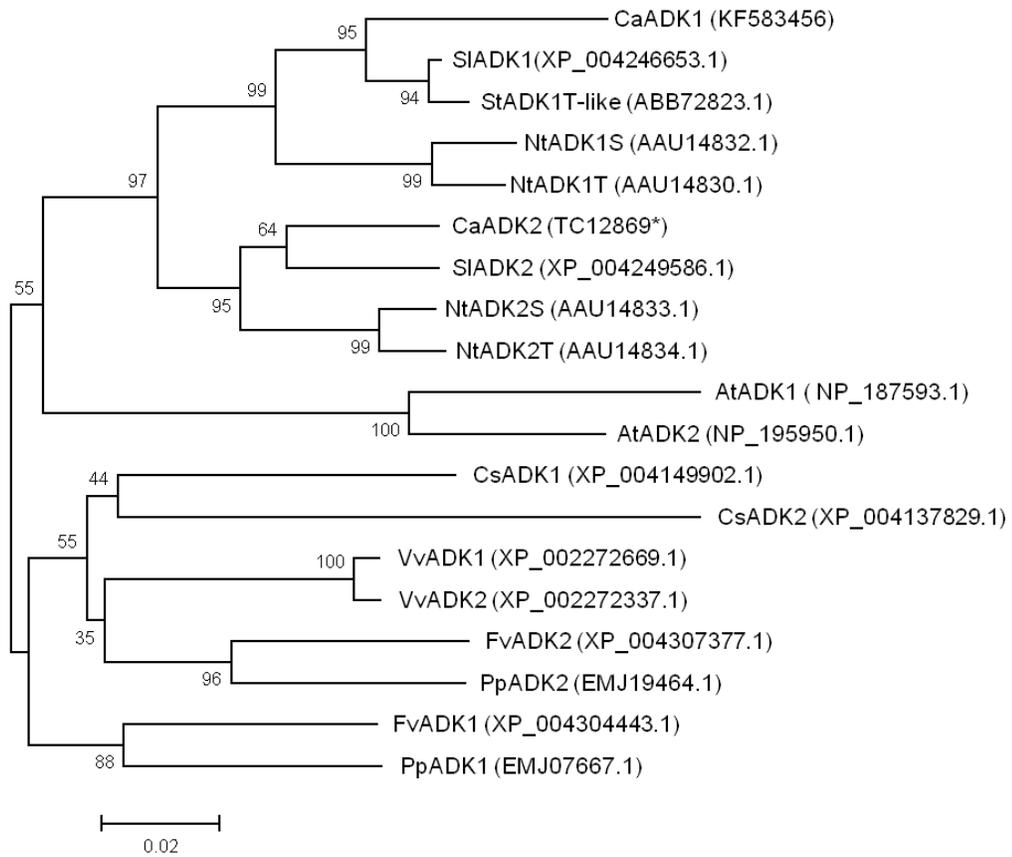
**Appendix F** The saturated cycle number of all primers for the four proteomic candidates (*CaCCH*, *CaTCP*, *CaCYS*, *CaSPS*) and the internal positive control, *CaGAPdH* (Aizat et al., 2013) (Chapter 3) were determined by running PCR at different cycle numbers (24 to 36 cycles) (refer Chapter 7: section 7.2). Each product size is listed in Table 7.1 for sqRT-PCR primers.



**Appendix G** Four proteomic candidates (*CaCCH*, *CaTCP*, *CaCYS*, *CaSPS*) were analysed in semi-quantitative RT-PCR using a lower number of PCR cycles (27 cycles). Six different ripening stages (G, Green; B, Breaker; BR1, Breaker Red 1; BR2, Breaker Red 2; LR, Light Red; DR, Deep Red) from a biological replicate was examined. Each product size is listed in Table 7.1 for sqRT-PCR primers (refer Chapter 7: section 7.2). *CaGAPdH* expression for internal positive control as per Aizat et al. (2013) and Chapter 3 was also determined at 27 PCR cycles.



**Appendix H** The quantitative Real Time-PCR (qPCR) melt curve plots of all candidates analysed in capsicum ripening. All candidate names are at the left-hand side top corner of each plot and the primers as well as templates used are detailed in Chapter 7 (section: 7.2.4 for *CaCCH*, *CaTCP*, *CaCYS1* and *CaSPS* whereas other candidates are detailed in Chapter 6: Methods). All peaks specific for the candidates are boxed and any peaks from the negative template control (NTC) are primer dimers. No product was obtained for *CaACS3*. y-axis: derivative reporter (-R') values; x-axis: melting temperatures (°C).



**Appendix I** Phylogenetic analysis of different adenosine kinase (ADK) isoform from various species. *Capsicum annuum* (Ca), *Solanum lycopersicum* (Sl), *Nicotiana tabacum* (Nt), *Solanum tuberosum* (St), *Vitis vinifera* (Vv), *Prunus persica* (Pp), *Fragaria vesca* (Fv, wild strawberry), *Cucumis sativus* (Cs) and *Arabidopsis thaliana* (At). The branch length (bar) corresponds to an evolutionary distance at 0.02 amino acid substitutions per site.

	1	10	20	30	40	50	60	70	80	90	100	110	120	130
CaCYS1	ATGGCATCATCATCAACATCCCTTCACTTCTCTGCTGCAACACCAACAAATGTGAACTAATAGAAATTTGCTCTTTAAGGCTCAGCAGTCATTTGGTGTGTCACAGCTCAGCAGAAAGTTGGT													
CaCYS2	ATGGCATCATCATCAACATCCCTTCACTTCTCTGCTGCAACACCAACAAATGTGAACTAATAGAAATTTGCTCTTTAAGGCTCAGCAGTCATTTGGTGTGTCACAGCTCAGCAGAAAGTTGGT													
Consensus	ATGGCATCATCATCAACATCCCTTCACTTCTCTGCTGCAACACCAACAAATGTGAACTAATAGAAATTTGCTCTTTAAGGCTCAGCAGTCATTTGGTGTGTCACAGCTCAGCAGAAAGTTGGT													
	131	140	150	160	170	180	190	200	210	220	230	240	250	260
CaCYS1	TCCCTTCTGTGTTTGCRAAGCAGTGTCTGTGCAACGAAATCACTACGAAATTTGAGGCTTAACTTGTGAGAGTGTACACAGCTTATTGGAAACACCAATGGTTACCTTACACCAATTTG													
CaCYS2	TCCCTTCTGTGTTTGCRAAGCAGTGTCTGTGCAACGAAATCACTACGAAATTTGAGGCTTAACTTGTGAGAGTGTACACAGCTTATTGGAAACACCAATGGTTACCTTACACCAATTTG													
Consensus	TCCCTTCTGTGTTTGCRAAGCAGTGTCTGTGCAACGAAATCACTACGAAATTTGAGGCTTAACTTGTGAGAGTGTACACAGCTTATTGGAAACACCAATGGTTACCTTACACCAATTTG													
	261	270	280	290	300	310	320	330	340	350	360	370	380	390
CaCYS1	TAGGGTTGTGTGCRAATATTGCTGCTAACTTGAAGATTATGGAGCATGTTGCAGTGTAAAGACAGGATAGGGTTCAAGTATGATATCTGATGCTGAGGAGAGGGACTTATATCCAGGAGAGCT													
CaCYS2	TAGGGTTGTGTGCRAATATTGCTGCTAACTTGAAGATTATGGAGCATGTTGCAGTGTAAAGACAGGATAGGGTTCAAGTATGATATCTGATGCTGAGGAGAGGGACTTATATCCAGGAGAGCT													
Consensus	TAGGGTTGTGTGCRAATATTGCTGCTAACTTGAAGATTATGGAGCATGTTGCAGTGTAAAGACAGGATAGGGTTCAAGTATGATATCTGATGCTGAGGAGAGGGACTTATATCCAGGAGAGCT													
	391	400	410	420	430	440	450	460	470	480	490	500	510	520
CaCYS1	GTTCTAGTTGAGCTACGAGTGGAAACACAGGCATTTGGCTTTCATTTGCTGCTCCAGAGGATATAGCTCATCTTACGATGCTGCTCAATGAGTCTTGAAGAGAGGGTTATCTAAAGCTT													
CaCYS2	GTTCTAGTTGAGCTACGAGTGGAAACACAGGCATTTGGCTTTCATTTGCTGCTCCAGAGGATATAGCTCATCTTACGATGCTGCTCAATGAGTCTTGAAGAGAGGGTTATCTAAAGCTT													
Consensus	GTTCTAGTTGAGCTACGAGTGGAAACACAGGCATTTGGCTTTCATTTGCTGCTCCAGAGGATATAGCTCATCTTACGATGCTGCTCAATGAGTCTTGAAGAGAGGGTTATCTAAAGCTT													
	521	530	540	550	560	570	580	590	600	610	620	630	640	650
CaCYS1	TTGGAGCTGAACTTGTTTTAACTGACCCAGCCAAAGGGATGAAAGGAGCTGTTCAAGGCTGAAAGAAATTTGATACACACCAAGATGCTATATCCCTTCAACATTTGACCAATCCCGCCACCCCA													
CaCYS2	TTGGAGCTGAACTTGTTTTAACTGACCCAGCCAAAGGGATGAAAGGAGCTGTTCAAGGCTGAAAGAAATTTGATACACACCAAGATGCTATATCCCTTCAACATTTGACCAATCCCGCCACCCCA													
Consensus	TTGGAGCTGAACTTGTTTTAACTGACCCAGCCAAAGGGATGAAAGGAGCTGTTCAAGGCTGAAAGAAATTTGATACACACCAAGATGCTATATCCCTTCAACATTTGACCAATCCCGCCACCCCA													
	651	660	670	680	690	700	710	720	730	740	750	760	770	780
CaCYS1	GATACACTATGAAACACGGGTCAGAAATCTGGGAGACACAAAGGCAAAATCGACATACCTTGTGCTGGCATTGGAACTGGCGAAACATTTCTGGAAACGGCCGATACCTTAAAGAGAAATCCA													
CaCYS2	GATACACTATGAAACACGGGTCAGAAATCTGGGAGACACAAAGGCAAAATCGACATACCTTGTGCTGGCATTGGAACTGGCGAAACATTTCTGGAAACGGCCGATACCTTAAAGAGAAATCCA													
Consensus	GATACACTATGAAACACGGGTCAGAAATCTGGGAGACACAAAGGCAAAATCGACATACCTTGTGCTGGCATTGGAACTGGCGAAACATTTCTGGAAACGGCCGATACCTTAAAGAGAAATCCA													
	781	790	800	810	820	830	840	850	860	870	880	890	900	910
CaCYS1	AHCATTAGATTATGGTGGAGCCACAGAAAGTACGTTCTATCAGGGGGCAGCCTGGCCCTACAAATTCAGGGGATTGGAGCAGGTTTATTCCAGGAACCTGGATCAGATGTCATGGATG													
CaCYS2	AHCATTAGATTATGGTGGAGCCACAGAAAGTACGTTCTATCAGGGGGCAGCCTGG-----TTTATTCCAGGAACCTGGATCAGATGTCATGGATG													
Consensus	AHCATTAGATTATGGTGGAGCCACAGAAAGTACGTTCTATCAGGGGGCAGCCTGG.....TTTATTCCAGGAACCTGGATCAGATGTCATGGATG													
	911	920	930	940	950	960	970	980	990	1000	1010	1020	1030	1040
CaCYS1	AAGTGTATGAGATCATAGTACGAGGCTGTTGAACTGCAAGCAATTTAGCCCTACAGAGAGGGTTGTTGGTTGGATTCTTCGGGGCAGCTGCTCTGCTGCCATTCAGGTTCCGAAAGGCTGGA													
CaCYS2	AAGTGTATGAGATCATAGTACGAGGCTGTTGAACTGCAAGCAATTTAGCCCTACAGAGAGGGTTGTTGGTTGGATTCTTCGGGGCAGCTGCTCTGCTGCCATTCAGGTTCCGAAAGGCTGGA													
Consensus	AAGTGTATGAGATCATAGTACGAGGCTGTTGAACTGCAAGCAATTTAGCCCTACAGAGAGGGTTGTTGGTTGGATTCTTCGGGGCAGCTGCTCTGCTGCCATTCAGGTTCCGAAAGGCTGGA													
	1041	1050	1060	1070	1080	1090	1100	1110	1120	1130	1140	1150	1155	
CaCYS1	GAATGCAGGGAACTTATTGCGGTTGATTTCCAGTTTGGGGACGGTACTCTCCTCTATCTTTCCAGTCAATACGAGAGGATGCGAGAAATGAGCCAGATTTGTGA													
CaCYS2	GAATGCAGGGAACTTATTGCGGTTGATTTCCAGTTTGGGGACGGTACTCTCCTCTATCTTTCCAGTCAATACGAGAGGATGCGAGAAATGAGCCAGATTTGTGA													
Consensus	GAATGCAGGGAACTTATTGCGGTTGATTTCCAGTTTGGGGACGGTACTCTCCTCTATCTTTCCAGTCAATACGAGAGGATGCGAGAAATGAGCCAGATTTGTGA													

**Appendix J** The alignment of *CaCYS1* and *CaCYS2* open reading frame (ORF) sequences reveals identical nucleotides except the deleted 30 base pair region in the *CaCYS2* (position 843-872).