Transgenic Plant Journal ©2010 Global Science Books

Toward the Control of Ethylene Production in Papaya Fruit: A Model for Tropical Fruits

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ABSTRACT

Papaya (*Carica papaya*) is one of the healthiest and nutritious fruits in the world. This tropical plant contains diverse biologically active compounds of industrial and nutraceutical interest. As a climacteric fleshy fruit, papaya is highly vulnerable to the effects of the growth regulator ethylene, reducing its nutritional value and causing significant postharvest losses. To extend the shelf life of fruits several strategies are available to control the production of this gas during fruit ripening. Knowledge at the molecular level of the ethylene biosynthesis and action pathways permits to devise strategies to control ethylene metabolism in transgenic plants. The control of ethylene production is well documented in transgenic tomato plants and has been initiated in papaya. This paper reviews the molecular basis of ethylene metabolism in fruits and centers on papaya as an example of a climacteric fruit.

Keywords: antisense, climacteric, cosuppression, ripening, olefin

CONTENTS

INTRODUCTION

The fruit is an exclusive structure of angiosperms that develops from the ovary after fertilization. Fruit maturation and ripening are complex genetically regulated processes that undergo staggering changes in color, texture, flavor and aroma at the fruit flesh. These processes are the subject of permanent research at both the biochemical and genetic levels in several fruit crops with economic and/or nutritional values (Alexander *et al*. 2002). Papaya is a major fruit commodity reaching a production worldwide of 6708 million metric tons in 2004. The top ten papaya producers are Brazil, Mexico, Nigeria, Indonesia, India, Ethiopia, Congo, Peru, China and Philippines (Tecson-Mendoza *et al*. 2008). Papaya is a climacteric fruit with a short shelf life and substantial postharvest losses caused mainly by fruit overripening. It has been well established the central role of the gaseous plant hormone ethylene in fruit ripening as well as in the climacteric phenomenon. About 48% of overripe postharvest losses is assumed to be caused by ethylene (Paul *et al*. 1997). Papaya has the potential to be a model system for tropical fruits. A first draft of the papaya genome has been published, the genome is relatively small 372 Mbp, that is about three times the size of the genome of the model plant *Arabidopsis thaliana* (Ming *et al*. 2008). For instance, several genes involved in ethylene metabolism have been identified in this first draft of the genome (Paull *et al*. 2008). Papaya has also well-established transformation systems and the fruit generation time of about 12-16 months (Ascencio-Cabral *et al*. 2008).

PATHWAYS OF ETHYLENE BIOSYNTHESIS AND SIGNALING

The ethylene biosynthesis pathway in higher plants is understood in great detail (Yang *et al*. 1984; Bleecker *et al*. 2000). This olefin is synthesized in plants from methionine in three sequential steps (**Fig. 1**). Methionine is converted to S-adenosyl-L-methionine (SAM) by the SAM synthetase, then 1-aminocyclopropane-1-carboxylic acid (ACC) is generated from SAM via ACC synthase (ACS), and finally ethylene is generated from ACC by the action of the ACC oxidase (ACO). The genes encoding SAM synthetase, ACS and ACO are arranged in gene families. The papaya genome encodes fewer predicted genes for each one of these types of enzymes than the Arabidopsis and tomato genomes (**Fig. 1**). The role of the enzymes catalyzing the last two steps has been the focus of attention in model plants as well as in several crop species. Although the last two reactions are the limiting steps in the biosynthesis of ethylene, there is evidence that the genes involved in methionine synthesis and methionine salvage pathway are differentially expressed during ripening and in response to ethylene (Zegzouti *et al*. 1999; Alba *et al*. 2005).

Based on the respiration and ethylene biosynthesis rates during fruit ripening, fruits have been classified as climacteric or non-climacteric (Biale *et al*. 1981). Papaya as well as tomato, avocado, banana, apple are examples of climacteric fruits, they all show a burst in ethylene and CO_2 production during ripening. Ethylene is a key element in the ripening of climacteric fruits and two systems for ethylene production have been described. System 1 functions during normal growth and development and during stress respon-

Received: 1 August, 2009. Accepted: 1 May, 2010.

Invited Mini-Review

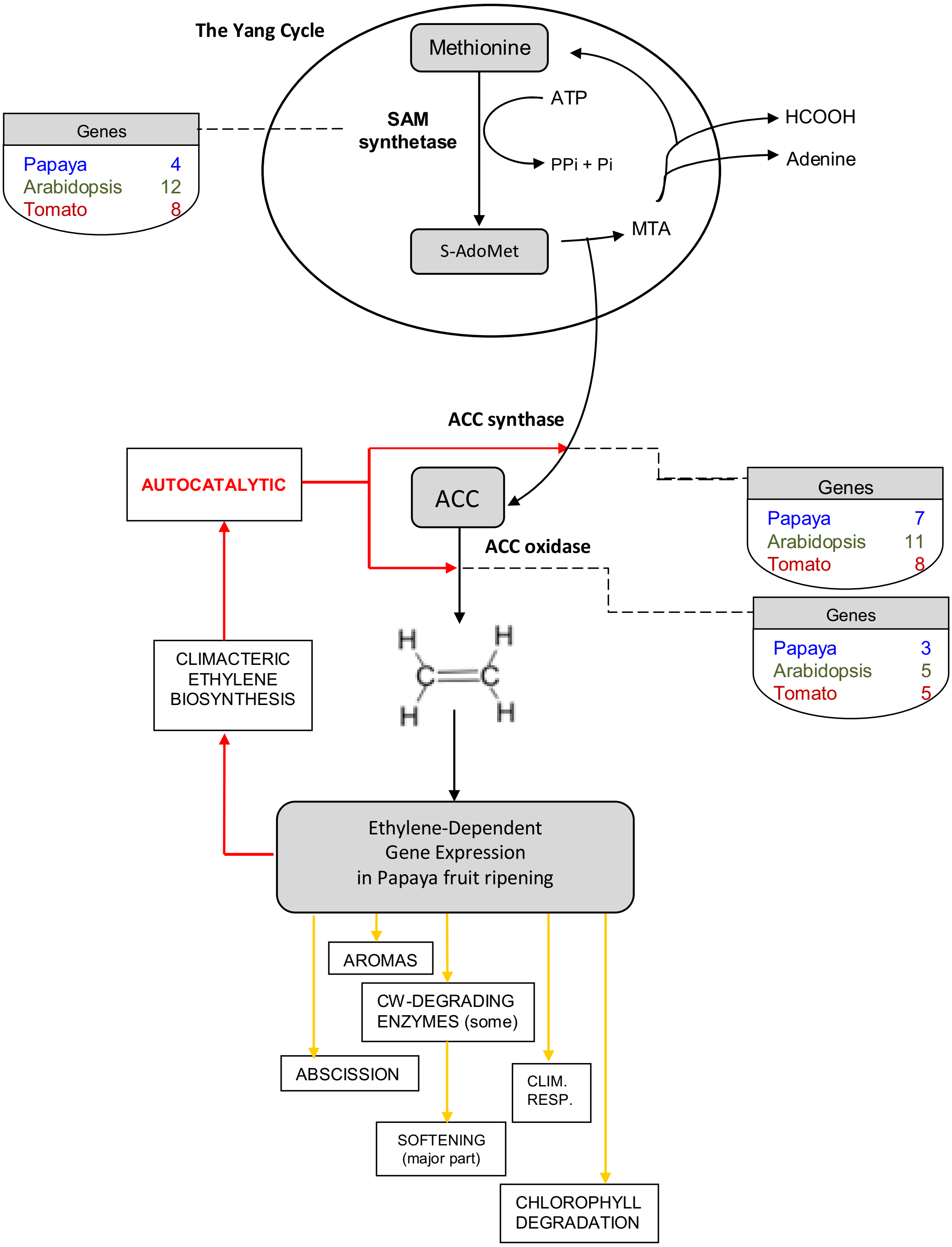

Fig. 1 Ethylene biosynthesis pathway adapted to papaya fruit ripening. The pathway is based on Pech *et al*. (2008) and Lin *et al*. (2009): see text for a brief description. Established points of the pathway are indicated by arrows. During fruit ripening ethylene induce the climacteric ethylene production, which induce the autocatalytic ethylene production by expression of ACC and ACO genes (red arrows). In a second step an enhanced ethylene production induce the expression of genes involve in papaya climacteric respiration, aroma, softening, chlorophyll degradation and abscission (yellow arrows). Round boxes include de number of genes of each class present in tomato, *Arabidopsis* and papaya (Paull *et al*. 2008).

ses, and system 2 functions in floral senescence and fruit ripening (**Fig. 1**). System 1 is autoinhibitory, exogenous ethylene inhibits ethylene biosynthesis, and inhibitors of ethylene action can stimulate its production. Conversely system 2 is autocatalytic as it is stimulated by ethylene. In this case, both ACS and ACO mRNAs are induced by ethylene (McMurchie *et al*. 1972; Barry *et al*. 2000). The effect of the reduction of ACS and ACO levels in transgenic plants is consistent with autocatalytic ethylene. Tomato, melon and apple carrying antisense constructs for ACS and ACO genes showed a decrease in ethylene production and a concomitant delay in fruit ripening (Oeller *et al*. 1991; Picton *et al*. 1993; Ayub *et al*. 1996; Shaffer *et al*. 2007). Afterward, autocatalytic ethylene synthesis triggers the ripening-associated genes expression that prompt fruit specific features as color, flavor, aroma and texture (**Fig. 1**) (Giovannoni 2004; Shaffer *et al*. 2007).

The climacteric process may also be controlled at the ethylene perception step since some transcripts encoding ethylene receptors in tomato are induced by this hormone

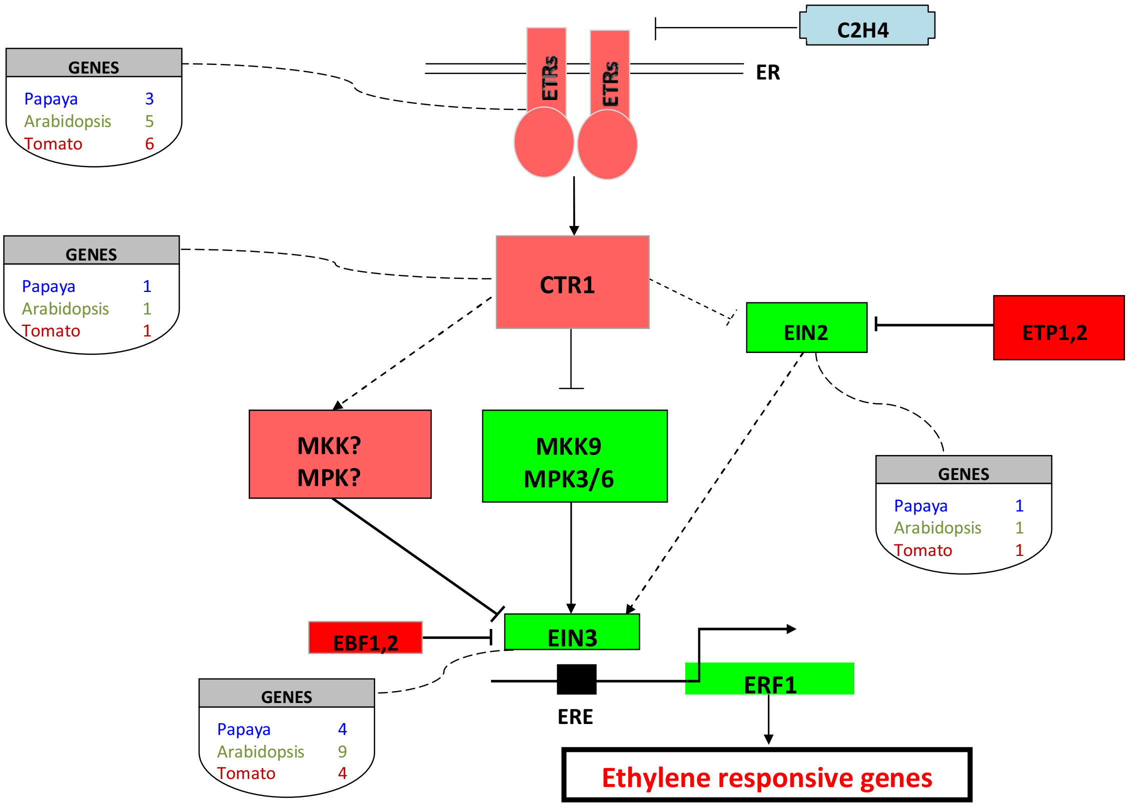

Fig. 2 Ethylene signal transduction pathway adapted to papaya fruit ripening. The pathway is based on Yoo *et al.* (2009): see text for a brief description. Established points of the pathway are indicated by continued arrows and presumed points by no continued arrows. Arrows indicate a positive effect on signaling. Blunted arrows indicate points of negative regulation. Round boxes include de number of genes of each class present in tomato, *Arabidopsis* and papaya (Paull *et al.* 2008).

and during fruit ripening (Gutiérrez-Martínez *et al.* 2001; Kevany *et al.* 2007; Lin *et al.* 2009). The five ethylene receptors genes in tomato display distinct pattern of expression during development and in response to environmental stimuli (Cara *et al.* 2008). The ethylene receptors show similarity to bacterial two-component regulators, they are integral membrane proteins dimers associated to the endoplasmic reticulum (Bleecker *et al.* 1998). The genetic dissection of the triple response of *Arabidopsis thaliana* seedlings has been the way to establish the ethylene perception and signaling pathway in plants (Stepanova *et al.* 2000). In papaya three ethylene receptors have been predicted which is less than *Arabidopsis* and tomato, each one of them with a putative orthologue in *Arabidopsis* (Paull *et al.* 2008) The binding of ethylene to the receptors has a negative effect on the ethylene signaling pathway. A constitutive ethylene response is generated by loss-of-function mutations in two or more of the ethylene receptor genes whereas hormone insensitivity results from a dominant gain-of-function allele (Hua *et al.* 1998; Wang 2003).

Downstream of the receptors a MAP-kinase cascade initiating with the MAP-kinase kinase kinase CTR1 is likely to launch the signaling pathway, yet a direct connection between CTR1 and a MAP-kinase kinase has not been establish (Ouaked *et al.* 2003). CTR1 functions as a negative regulator of the response and interacts directly with ethylene receptors (Kieber *et al.* 1993; Clark *et al.* 1998; Gao *et al.* 2003). Downstream of CTR1 is EIN2, a positive regulator of the response which shows homology to the Nramp family metal transporter but whose biochemical function is unknown (Alonso *et al.* 1999). A family of transcription factors, EIN3/EIL1, that function downstream EIN2 drive the ethylene regulated gene expression. The papaya genome encodes possible homologues for all these genes, one *CTR1*, one *EIN2* and four *EIN3/EIL1* (**Fig. 2**).

EIN3/EIL1 transcription factors bind to the ERE elements present the transcription factor ERF1 that positively regulates ethylene-mediated gene expression (Fujimoto *et al.* 2000; Ohme *et al.* 2000). ERE elements are also frequently found in the promoter regions of senescence and ripening genes (Chao *et al.* 1997; Solano *et al.* 1998); several putative *ERF1* homologues genes have been predicted in the papaya genome (Paul *et al.* 2008). Additional important regulators of the ethylene biosynthesis and signaling pathways are components of the ubiquitin–26S proteasome system. The stability of several key elements in these pathways is controlled by protein turnover (Vierstra 2009). The specific components of the ubiquitin system regulating ethylene biosynthesis and signaling have not been searched in the papaya genome (EBF1,2 and ETP1,2; **Fig. 2**).

MANIPULATION OF ETHYLENE BIOSYNTHESIS IN PAPAYA

The last two reactions are the limiting steps in the ethylene biosynthesis pathway. ACS and ACO are encoded by multigene families in higher plants. In tomato nine ACS and five ACO have been described. Expression analysis has revealed that four ACS and three ACO genes are differentially expressed in the tomato fruit (Barry *et al.* 2007). The papaya genome encodes seven ACS and a minimum of three ACO genes (Paul *et al.* 2008). Based on early observations obtained with partial gene sequences, these genes may also display differential expression during fruit ripening. Two papaya ACS from cultivar 'Solo Kapoho' showed a differential expression during fruit ripening, one transcript was detectable during ripening whereas the other in green mature fruit (Mason *et al.* 1997). Likewise, partial sequences obtained from ACS and ACO genes from papaya cultivar Sunrise and Sunset showed expression during fruit ripening (Neupane *et al.* 1998).

Entire genomic and cDNA sequences from papaya ACO have been isolated and their expression analyzed (Lin *et al.* 1997; Chen *et al.* 2003; López-Gómez *et al.* 2004). Sequence comparison of two of the genes suggests that they correspond to the same gene from different cultivars, *Cp-*

Table 1 Comparison of different fruit phenotypes of transgenic lines altered in ethylene biosynthesis.

	Climacteric respiration	**Softening**	**Fruit color**	**Shelf life time**	**Aroma**	**Reverted by ethylene**	**Reference**
Tomato *ACS* Antisense	Inhibited	Inhibited	Partially altered	90-120 days	Inhibited	Yes	Oeller *et al.* 1991
Tomato *ACO* Antisense	Inhibited	No inhibited	Partially altered	28 days	Unpublished	Yes	Murray *et al.* 1993
Tomato *ACC* Deaminase	Inhibited	Inhibited	No altered	121 days	Unpublished	Unpublished	Klee *et al.* 1991
Melon *ACO* Antisense	Inhibited	Inhibited	No altered	Unpublished	Inhibited	Yes	Pech *et al.* 2008
Apple *ACO* Antisense	Inhibited	Inhibited	No altered	85 days	Inhibited	Yes	Schaffer *et al.* 2007
Papaya *ACS* Antisense	Unpublished	Inhibited	No altered	14 days	Unpublished	Unpublished	Tecson-Mendoza *et al.* 2008
Papaya *ACO* Cosuppression	Inhibited	Inhibited	Altered	14 days	Unpublished	No	López-Gómez *et al.* 2009
Papaya 1-MCP	Inhibited	Inhibited	Altered	Unpublished	Inhibited	No	Fabi *et al.* 2007

ACO2 from 'Tainong 2' and *Cp-ACO1* from 'Maradol' (Chen *et al.* 2003; López-Gómez *et al.* 2004). Expression analysis revealed that *Cp-ACO1* is ripening-associated while *Cp-ACO2* was specific for late stages of fruit ripening and leaf senescence. This difference in gene expression may uncover an intrinsic and specific feature on each papaya cultivar. Further expression analysis showed that *Cp-ACO1* transcript is detected in the pulp but not in peel at pre-clicamacteric stage, then there is a dramatic increment of the messenger at climacteric stage in both pulp and peel followed by a reduction at postclimacteric stage in both structures (López-Gómez *et al.* 2004). *Cp-ACO1* and *Cp-ACO2* are induced by ethylene in fruits also are also detected after wounding (Chen *et al.* 2003; López-Gómez *et al.* 2004). These results suggest that *Cp-ACO1* is developmentally regulated and that the ripening process may advance from within to outside the fruit. In addition, they imply the involvement of ACO in the system 2 of ethylene production in climacteric fruits.

Based on the results obtained for ethylene production inhibition in tomato, similar strategies have been developed in papaya (**Table 1**) (Oeller *et al.* 1991; Picton *et al.* 1993). Using antisense ACS constructs transgenic papaya plants were generated (Tecson- Mendoza *et al.* 2008). The fruits of transgenic and non-transgenic lines exhibited similar number of days from color break to full color. However, the number of days from full yellow to fully ripe stage was more pronounced and significant: 4-14 days for selected transgenic lines compared with 2 days for control non-transgenic papayas. Among the quality traits determined, softening was most significantly different between the transgenic and non-transgenic fruit. The papaya transgenic fruit was firm from 4 to 14 days after reaching full yellow stage at room temperature (28-30°C) while hardness on the non-transgenic fruit was lost 1 to 2 days after the full yellow stage.

Recently, ethylene biosynthesis inhibition in 'Maradol' papaya was reported by ACO cosuppression (López-Gómez *et al.* 2009). In transgenic fruits, ethylene was reduced to about 60%, CO_2 production was also found to be reduced, suggesting that the CO_2 is associated to the ethylene production. Furthermore, fruits do not present the climacteric behavior, providing evidence that ethylene causes the climacteric in papaya as in other fruits (Lin *et al.* 2009). Although the inhibition of ethylene was partial, some effects on the peel color and softening of the fruit were observed. In transgenic fruits, the color of the peel remained green longer than control fruits and the fruits stayed firm much longer; these features remained by 15 days post harvest. In addition, the usual brake stage of the fruit in the tree could not be observed. With time, a noticeable wrinkling of the peel of transgenic fruits was detected, probably related with a loss of turgor of the cells and after 15 days after harvest, even though the pulp still presented some firmness, the peel was very thin. This observation suggests that the process of ripening probably involves different mechanisms in pulp and peel and that ethylene could affect each tissue differently (López-Gómez *et al.* 2009). It could also be considered that there are processes in the papaya ripening program that are independent of ethylene as has been shown in tomato, melon and apple (Barry *et al.* 2007). The senescence of leaves of transgenic papaya plants was altered too. Leaf abscission on the ACO cosuppression plants was delayed compared to the non-transformed plants; this delay was also detected in transgenic tomato (Picton *et al.* 1993).

The overall growth and development of the transgenic ACO cosuppression plants grown on the field were similar as the non transgenic plants. Papaya is a fruit that normally ripens either on or off the tree. The transgenic fruits did not reach full maturity even after being 15 days off the tree; the fruits looked dehydrated and the experiment ended (López-Gómez *et al.* 2009). Leaving the fruits on the tree for up to 21 days had no apparent effect on fruit ripening, fruit did not ripe. When the expression of the ACO mRNA was assessed, a drastic reduction was detected by northern blot analysis. Thus, it is possible to infer that the expression of the enzymes such as those involved in peel color and pulp firmness may be regulated by ethylene in papaya (López-Gómez *et al.* 2009). Taken together, all the information suggests that papaya fruit is very sensitive to ethylene and that a reduction in ethylene levels could dramatically affect the progress of ripening.

The phenotypes of the ACO cosuppression transgenic lines resemble the observations of 1-methylcyclopropene (1-MCP) treatments. Papaya fruits of the cultivars 'Golden', 'Gold' and 'Rainbow' treated with 1-MCP showed a reduction in the ethylene production and a delayed fruit softness (Manenoi *et al.* 2006; Fabi *et al.* 2007). 1-MCP binds to ethylene receptors and strongly inhibits ethylene-mediated fruit ripening in climacteric fruit (Blankenship and Dole 2003). Papaya fruits of cultivar 'Golden' treated with 1-MCP were altered in the contents of the three main carotenoids; *all-trans*-lycopene, all-*trans*-β-cryptoxanthin and *all-trans*-β-carotene and the normal softening of the papaya fruit ripening was impaired. These alterations could not be reversed, so it seems that the deleterious effects of 1-MCP over the ethylene sensitivity are irreversible (Fabi *et al.* 2007). Similar results were obtained when 'Maradol' papaya fruit was treated with a combination of 1-MCP and the ethylene releasing compound etephon (2-chloroethylphosphonic acid), coincident solubilization and depolymerization of pectin polymerization associated to softening was dependent on ethylene perception (Señudo-Barajas *et al.* 2009). The reduction of ethylene levels in the ACO cosuppression transgenic lines may imply that less ethylene is binding to the receptors, having therefore an effect on fruit ripening. In the case of apple cultivars, the rate of ripening correlates with the abundance of ethylene receptors (Tatsuki and Endo 2006). The 1-MCP effect on papayas although ethylene is present, the number of receptors blocked prevent their degradation (Kanavy *et al.* 2007).

In spite of the reduction of ethylene production in transgenic papayas (López-Gómez *et al.* 2009), the fruit did not stay firm as long as the transgenic tomatoes (4 to 5 months after harvest) generated by similar approaches (Klee *et al.* 1991; Oeller *et al.* 1991; Picton *et al.* 1993; Xiong *et al.* 2005). This observation suggests that the ripening program in papaya may be different to that in tomato and other climacteric fruits (**Table 1**) and that role of ethylene in the ripening of these types of fruits may not be quite the same. Although the inhibition of fruit ripening in papaya have potential commercial application, more studies are necessary to be certain that the inhibition of ethylene evolution does not impair papaya fruit quality.

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