



## Molecular approaches for regulation of fruit ripening-A review

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**Abstract:** Fruit ripening is a complex phenomenon with drastic changes in chemical composition, which enhance an important quality attributes such as flavour, softness, sweetness and color. These changes are very coordinated and involve many biochemical steps, which are upon a stringent and integrated control played by plant hormones and gene expression, including epigenetic mechanism. The expression of specific genes is essential to normal ripening and the synthesis of enzymes involved in the autocatalytic ethylene burst during climacteric fruit ripening. However, several other metabolic pathway are dependent on the modulation by gene expression, and some genes of enzymes putatively controlling the most important physiological changes associated to fruit ripening have been pointed. Based on the studies on tomatoes, there is already some understanding about the genes involved in the most important physiological changes, such as the accumulation of soluble sugars, cell wall disassembly, and synthesis of pigments during fruit ripening. In this way, the identification of genes induced during ripening could provide important clues about the activation of biochemical steps and its relationship to fruit quality.

**Keywords:** Fruit ripening, Gene expression, Molecular and quality

### Introduction

The ripe phenotype is the summation of biochemical and physiological changes that occur at the terminal stage of fruit development and render the organ edible and desirable to seed dispersing animals. Ripening also imparts value to fruit as agricultural commodities. These changes, although variable among species, generally include modification of cell wall ultrastructure and texture, conversion of starch to sugars, increased susceptibility to post-harvest pathogens, alterations in pigment biosynthesis and accumulation, and heightened levels of flavour and aromatic volatiles (Seymour *et al.*, 1993).

### Ethylene Biosynthesis and Transduction

Ethylene biosynthesis is induced in response to a variety of endogenous and environmental cues. One of the early responses after the sensing of these cues is the induction of ethylene, which is achieved via upregulation of ACS activity, the rate-limiting enzyme that catalyzes the first committed step of ethylene biosynthesis. Members in the two major subgroups of the ACS family, represented by ACS5 and ACS6, are regulated by different kinase pathways. Phosphorylation stabilizes ACS6 (and possibly ACS5), and interaction of ACS5 with ETO1 targets ACS5 for degradation by the proteasome. Fine-tuning of ethylene induction is achieved by tissue-specific expression, gene activation, and the formation of heterodimers between different ACS members. ACC oxidase (ACO) activity can also affect levels of ethylene induction. Ethylene gas is perceived by a family of ER-associated receptors (ETR1, ETR2, ERS1, ERS2, and EIN4).

CTR1, a negative regulator of downstream responses, is proposed to be activated by the unoccupied receptors via physical

interaction and is inhibited upon binding of ethylene by the receptor. CTR1 may function through unidentified MAPKK(s) and MAPK(s). Downstream components in the ethylene pathway include several positive regulators (EIN2, EIN5, EIN6, and EIN3). In the absence of ethylene, EIN3 protein is targeted for degradation by an SCF complex containing one of the two F-box proteins, EBF1 and EBF2. In the presence of ethylene, EIN3 accumulates in the nucleus and activates gene expression, which eventually leads to ethylene-induced responses. Liu and Zhang (2004) present strong evidence that lands the MPK6 MAPK module in the pathway regulating ACS6 activity—upstream of ethylene biosynthesis in response to stress.

### Climacteric and Non-climacteric Ripening

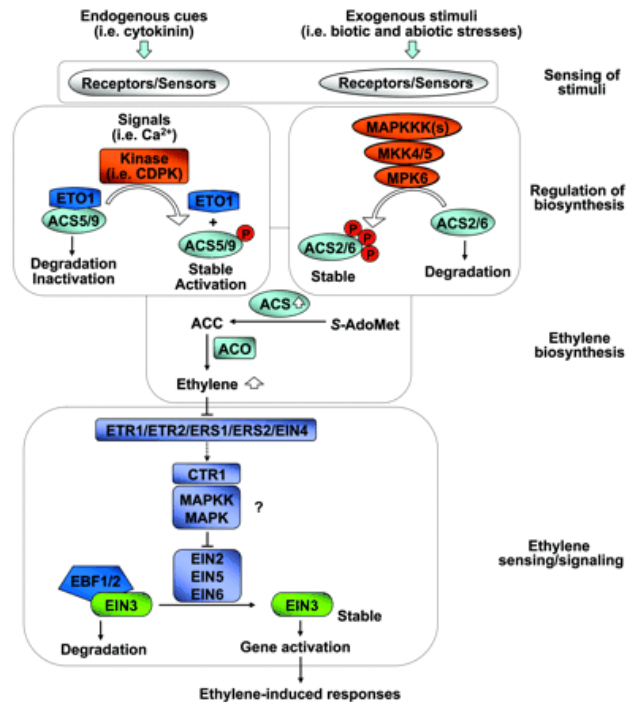
Although most fruit display modifications in color, texture, flavour, and pathogen susceptibility during maturation, two major classifications of ripening fruit, climacteric and non-climacteric have been utilized to distinguish fruit on the basis of respiration and ethylene biosynthesis rates. Climacteric fruit, such as avocado, banana, peaches, plums and apples are distinguished from non-climacteric fruits, such as strawberry, grape and citrus by their increased respiration and ethylene biosynthesis rates during ripening (Lelievre *et al.*, 1997). In non-climacteric fruits such as citrus may respond to ethylene induced mRNA and pigment accumulation in the flavo of orange (Alonso *et al.*, 1995), ethylene is not required for fruit ripening but necessary for the coordination and completion of ripening in climacteric fruit via analysis of inhibitors of ethylene biosynthesis and perception (Yang, 1985; Tucker and Brady, 1987). In Climacteric fruit ripening is triggered by the action of ethylene and results in activation of several cell wall hydrolases. Their action on cell wall results in wall disassembly leading to softening.

**Common Genetic Regulatory Mechanisms**

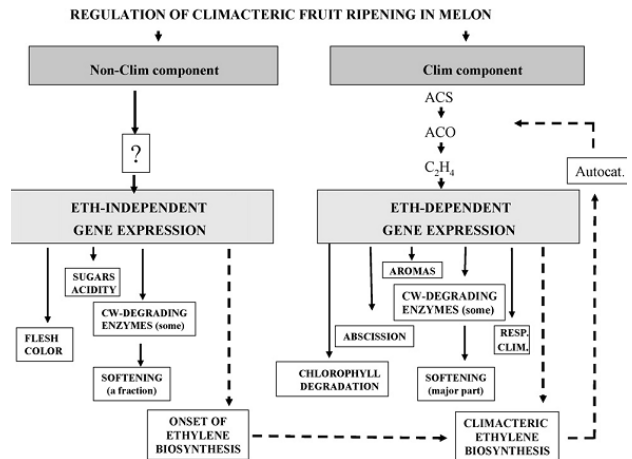
During ripening many important physico-chemical changes contribute to fruit quality, and they are precisely determined by gene expression. A clarification of the common genetic regulatory elements that are shared among climacteric and non-climacteric species is central to a full understanding of fruit ripening. Such primary regulators of fruit maturation might be shared by, or at least related to, those that regulate maturation of dehiscent fruit. Although such common regulatory elements remain elusive, *Arabidopsis* silique development genes, such as those from the MADS-box family of transcription factors (Doganlar *et al.*, 2000), may represent starting points in a search for common control mechanisms. Indeed, although antisense repression had no obvious effect on fruit ripening (Pnueli *et al.*, 1994), ectopic expression of the tomato AGAMOUS gene (TAG1) results in fleshy expansion, ripening-like cell wall metabolism and carotenoid accumulation in the sepals of transgenic tomatoes.

According to Gray *et al.* (1994) there is a hypothetical report regarding the TAG1 gene. TAG1 represents a redundant ripening control function. Alternatively, TAG1 may not regulate *in vivo* ripening, but the action may be related to or it mimics a similar regulatory gene when over-expressed in sepals. In addition to a further pursuit of candidate genes or gene families, investigators have identified a number of climacteric ripening mutants that fail to ripen in response to ethylene and represent an additional track toward identification of common ripening regulators. Ripening confers both positive and negative attributes to the resulting commodity. Although ripening imparts desirable flavour, color, and texture, considerable expense and crop loss result from negative ripening characteristics. For example, ripening-related increase in fruit pathogen susceptibility is a major contributor to fruit loss both before and after harvest. This genetically regulated change in fruit physiology currently necessitates the use of pesticides, post-harvest fumigants, and controlled atmosphere storage and shipping mechanisms in attempts to minimize loss. In addition to being wasteful of energy and potentially harmful to the environment, such practices represent major expenses in fruit production. Finally, it is important to reiterate that ripening imparts numerous quality and nutritional characteristics upon a significant component of the human diet fruit.

Ripening impacts various critical aspects of mature fruit, including fiber content and composition, lipid metabolism, and the levels of vitamins and various antioxidants (Ronen *et al.*, 1999). The ability to understand key control points in global ripening regulation or within specific ripening processes, such as carotenoid, flavonoid, vitamin, and flavour volatiles will allow for manipulation of nutrition and quality characteristics associated with ripening. The most convincing argument for the promotion of safe plant genetic engineering will be the development of products with direct consumer impact and appeal, such as quality and nutritionally enhanced fruits. Regulation of climacteric and non-climacteric fruit ripening (Pech *et al.*, 2008). It has been observed that pulp coloration, accumulation of sugars and loss of acidity were ethylene independent processes, whereas yellowing of the rind, softening of the flesh, development of the peduncular abscission zone, aroma formation and climacteric respiration were totally or partially ethylene-dependent (Bower *et al.*, 1998).

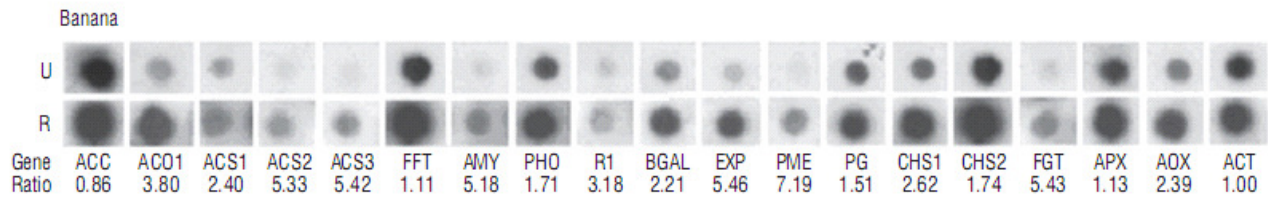


**Fig. 1:** A Simplified Model Depicting the Induction of Ethylene Biosynthesis and the Downstream Ethylene Sensing/Signaling Pathway

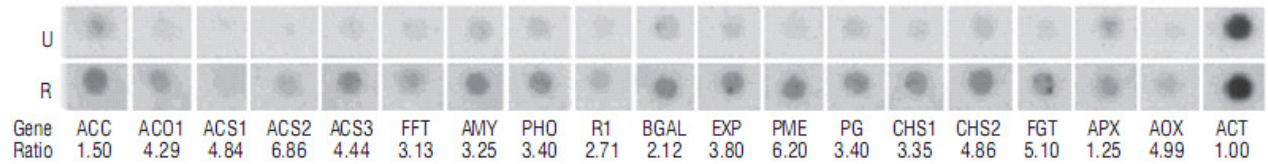


**Fig. 2:** Regulation of climacteric fruit ripening in melon

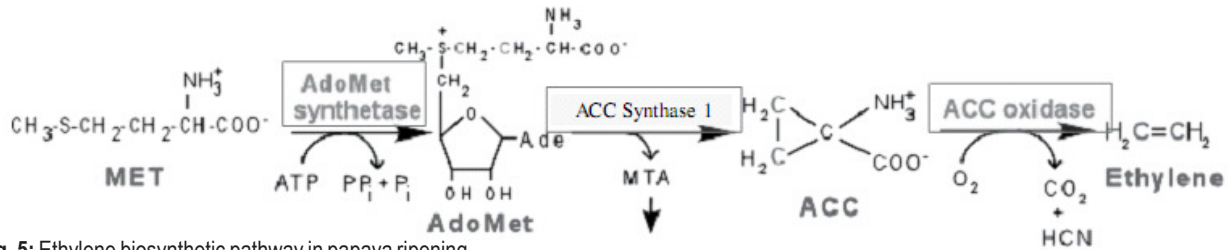
In banana fruit, MCP treatments led to the observation that peel degreening and aroma volatiles production were two ethylene dependent processes whereas sugar accumulation was ethylene independent (Golding *et al.*, 1993). Regulation of the softening process and of the biosynthesis of aromas will be studied in details below. Concerning gene expression, Hadfield *et al.* (1998) have isolated sixteen cDNAs whose abundance is regulated during ripening of Charentais melons, the majority of them being fruit specific. When examined in antisense ACO fruit, three distinct patterns of mRNA accumulation were observed. One group of cDNA corresponded to mRNAs whose abundance was reduced in transgenic fruit but inducible by ethylene treatment. A second group was not significantly altered in the transgenic fruit and was unaffected by treatment with ethylene, indicating that these genes are regulated



**Fig. 3:** Sequencing of different ripening related genes in banana fruit



**Fig. 4:** Sequencing of genes related to mango ripening



**Fig. 5:** Ethylene biosynthetic pathway in papaya ripening

**Table-1:** Genes and their predicted function related to papaya ripening

Genes	Predicted gene	function
<i>AdeMet1</i>	S-adenosylmethionine synthetase 1	Ethylene biosynthesis and regulation
<i>AdeMet2</i>	S-adenosylmethionine synthetase2	Ethylene biosynthesis and regulation
<i>ACS1</i>	ACC synthase 1	Ethylene biosynthesis and regulation
<i>ACO1</i>	ACC oxidase	Ethylene biosynthesis and regulation
<i>ACO3</i>	ACC oxidase 3	Ethylene biosynthesis and regulation
<i>ACO4</i>	ACC oxidase 4	Ethylene biosynthesis and regulation
<i>ERF</i>	Ethylene-responsive transcription factor 7	Ethylene biosynthesis and regulation
<i>RAP2-4</i>	Ethylene-responsive transcription factor RAP2-4	Ethylene biosynthesis and regulation
<i>PG</i>	Polygalacturonase precursor	Fruit softening
<i>XYL</i>	Putative beta-D-xylosidase	Fruit softening
<i>FFT1</i>	Beta-fructofuranosidase1	Fruit softening
<i>FFT3</i>	Beta-fructofuranosidase 3	Fruit softening
	Probable xyloglucan endotransglucosylase	Fruit softening
<i>GLU</i>	Endo-1,4-beta-D-glucanase	Fruit softening
<i>EXPA8</i>	Expansin-A8 precursor	Fruit softening
<i>EXPB3</i>	Expansin-like B3 precursor	Fruit softening
<i>CHB4</i>	Basic endochitinase CHB4precursor	Fruit softening
<i>MT-3</i>	Metallothionein-like proteintype 3	Fruit softening
<i>LIP</i>	Putative lipoxygenase 5	Fruit aroma- Ester biosynthesis pathway
<i>ALD</i>	Alcohol dehydrogenase-like 5	Fruit aroma- Ester biosynthesis pathway
<i>CAD</i>	Cinnamic acid 4-hydroxylase	Fruit aroma- Phenyl propanoid biosynthesis pathway
<i>CMT</i>	Caffeic acid 3-O-methyltransferase	Fruit aroma- Phenyl propanoid biosynthesis pathway
-	Cytochrome P450 84A1	Fruit aroma- Phenyl propanoid biosynthesis pathway
-	Hydroxymethylglutaryl - CoA synthase	Fruit aroma- Terpene biosynthesis pathway

by ethylene-independent developmental cues. The third and largest group showed an unexpected pattern of expression, with mRNAs reduced in transgenic fruit and remaining low after exposure to ethylene. Regulation of this third group of genes thus appears to be ethylene-independent may be regulated by other developmental factors that require ethylene at a certain stage of development. Expression analysis of a set of genes related to the ripening of

bananas and mangoes (Godoy *et al.*, 2009) Specific genes are essential to normal ripening. The changes in gene expression, a small collection of genes related to ethylene biosynthesis, starch mobilization, cell wall disassembly and pigment synthesis and ascorbate metabolism were studied. In relation to starch metabolism, banana  $\alpha$ -amylase was induced during ripening while phosphorylase was more induced in mangoes. Similarly, expression

**Table-2:** List of genes involved in fruit ripening and their regulatory activities

Genotype	Activity	Function	Crop	References
ACO	Amino carboxylic acid cyclopropane oxidase	Ethylene biosynthesis	Strawberry	Kess <i>et al.</i> , 2007
ACS	ACC synthase	Ethylene biosynthesis	Strawberry	Kess <i>et al.</i> , 2007
CTR1	Constitutive triple response gene controls kinase activity	Ethylene signaling	-	Gapper <i>et al.</i> , 2013
EIN	EIN binding protein	Reduces the ethylene sensitivity	<i>Citrus reticulata</i>	Gapper <i>et al.</i> , 2013
RAP2-4	ethylene-responsive transcription factor – fruit ripening via light and ethylene signaling pathway	Ethylene biosynthesis	Papaya cv. Eksotika	Shin <i>et al.</i> , 2011
AdoMet	S-adenosylmethionine synthetase	Ethylene biosynthesis	Papaya cv. Eksotika	Shin <i>et al.</i> , 2011
FFT	$\beta$ -fructofuranosidase – invertase enzyme breakdown the sucrose to fructose and glucose	Strach mobilization	Mango	Belfield <i>et al.</i> , 2005
R1	$\alpha$ -glucan-water-dikinase - phosphorylation of starch polymers both at developing and ripening stage	Strach mobilization	Mango	Belfield <i>et al.</i> , 2005
GH	Glycosyl hydrolase	Strach mobilization	Cherry	Lycoan <i>et al.</i> , 1996
TL	Thaumatococin like protein	Strach mobilization	Cherry	Lycoan <i>et al.</i> , 1996
PG	Polygalactouranase - Pectin depolymerization	Cell wall softening	Banana- Grand Naine	Godoy <i>et al.</i> , 2009
PME	Pectin methylation	Cell wall softening	-	-
PEL	Pectolytic activity	Cell wall softening	Banana- Grand Naine	Mbegule <i>et al.</i> , 2009
EXP	Non enzymatic protein – expansin action - Pectin methylation	Cell wall softening	-	Godoy <i>et al.</i> , 2009
XTH	Xyloglucan endo- transglycosylase	Cell wall softening	-	Mbegule <i>et al.</i> , 2009
XET	Xyloglucan endo hydrolases	Cell wall softening	Kiwi	Yin <i>et al.</i> , 2010
MT3	Metallothionein-like protein type 3	Fruit softening	-	Shin <i>et al.</i> , 2011
CHB4	Endochitinase precursor	Fruit softening	-	Shin <i>et al.</i> , 2011
APX	Peroxidase – oxidative action	Cell wall disassembly	Mango	Simao <i>et al.</i> , 2008
AOX	Ascorbate metabolism	Fruit softening and increase in Vit. C level	Mango	Belfield <i>et al.</i> , 2005
CHS	Catalyzation of flavonoids through Chalcone synthase activity	Pigment synthesis	Mango	Belfield <i>et al.</i> , 2005

of cell wall related genes for polygalacturonase and expansin were also different in those fruits. Fructan fructosyl transferase, chalcone synthase, and ascorbate oxidase genes were also induced in ripening mangoes, but not in bananas. In banana the genes related to ethylene biosynthesis presented a large increase in expression reaching more than threefold for ACO1 and fivefold for ACS2 and ACS3. Surprisingly, no significant change was noticed for one of the ACC sequence (ACC) was constant. In relation to the genes of enzymes related to the carbohydrate metabolism, no difference was noticed for the fructan metabolism (FFT) sequence, while those involved with starch degradation, such as  $\alpha$ -amylase (AMY), were clearly stimulated. Among the genes of cell wall metabolism, pectin methyl esterase was largely affected, while those of  $\beta$ -galacturonase (BGAL) and Polygalacturonase (PG) were almost invariable. The non enzymatic protein expansin (EXP) was also induced during ripening, with a fivefold increase.

An apparent increase in expression for the pigment related genes was noticed for the chalcone synthase 2 (CHS2), but not for the chalcone synthase 1 (CHS1). Clear differences were also observed between the ascorbate metabolism enzymes, since the oxidase (AOX) was induced while the peroxidase (APX) was almost invariable in banana. In contrast to banana fruit, all the three sequences involved in pigment synthesis were strongly stimulated during mango ripening. On the other hand, only the ascorbate oxidase was significantly induced. Figure 3 & 4. Presents the comparison between the expression patterns of the selected genes for both banana and mango fruits. Among the first group of sequences

there were ACO1, ACS2, ACS3, R1 and CHS, which presented similar ratios in bananas and mangoes. On the opposite, ACS1, FFT, AMY, PHO, EXP, PG, CHS2 and AOX were the sequences that changed differently between the two fruits. The characteristics of ethylene biosynthesis associated with ripening in banana (*Musa sp.* cv Grand Nain) fruit. MA-ACS1 encoding 1-aminocyclopropane-1-carboxylic acid (ACC) synthase in banana fruit was the gene related to the ripening process and was inducible by exogenous ethylene. At the onset of the climacteric period in naturally ripened fruit, ethylene production increased greatly, with a sharp peak concomitant with an increase in the accumulation of MA-ACS1 mRNA, and then decreased rapidly. At the onset of ripening, the in vivo ACC oxidase activity was enhanced greatly, followed by an immediate and rapid decrease.

Expression of the MA-ACO1 gene encoding banana ACC oxidase was detectable at the preclimacteric stage, increased when ripening commenced, and then remained high throughout the later ripening stage despite of a rapid reduction in the ACC oxidase activity. This discrepancy between enzyme activity and gene expression of ACC oxidase could be at least in part, due to reduced contents of ascorbate and iron, cofactors for the enzyme, during ripening. Addition of these cofactors to the incubation medium greatly stimulated the in vivo ACC oxidase activity during late ripening stages. The results suggest that ethylene production in banana fruit is regulated by transcription of MA-ACS1 until climacteric rise and by reduction of ACC oxidase activity possibly through limited in situ availability of its cofactors once ripening has commenced, which in turn characterizes the sharp peak of ethylene production.

R1 has  $\alpha$ -glucan-water-dikinase activity which is thought to be essential in phosphorylation of starch polymers both at development and degradation of starch granules (Ritte *et al.*, 2006). This enzyme can potentially contribute to starch granule disassembly during fruit ripening. The relative change in  $\alpha$ -amylase expression would in agreement to the previous observation of enzymatic changes during ripening of both fruit (Simao *et al.*, 2008). Fruit softening is an important change observed during fruit ripening. However, its dependence on cell wall disassembly seems to be different for bananas and mangoes (Fioravante *et al.*, 2008). The texture of banana pulp seems to be more dependent on the starch, which is stored at amounts as high as 25% of fresh weight (Cordenunsi and Lajolo, 1995). This could explain why PG expression in mango fruit was almost twofold that seen for banana fruit. The observed differences could indicate that pectin depolymerization by PG would be a more relevant event for cell wall disassembly during mango ripening, while relaxation of the structure by changes in pectin methylation by PME and expansin action would predominate in starchy banana fruit.

Flavonoids are catalyzed by chalcone synthase and leads to biosynthesis of anthocyanin. But banana and mango are not recognized as source of anthocyanins, in the pulp, there were expressive changes in gene expression related to these genes. Both CHS1 and FGT increased similarly in bananas and mangoes, but mango had a fivefold increase in CHS2. It is possible that, in spite of the large stimulation on the expression of these enzymes, the availability of precursor compounds is limiting, precluding the accumulation of appreciable amounts of anthocyanins. APX and AOX, are potentially relevant to the levels of ascorbate in the pulp of the fruits, suggest that oxidative steps are favored during mango ripening. Since the ascorbate (component of the cell redox system) is implicated in some important changes during ripening, such as cell wall disassembly (Belfield *et al.*, 2005). AOX induction in mango fruit would not be only important in relation to the vitamin C levels in the pulp, but also for fruit softening. Isolation of fruit ripening genes from *Carica papaya* Var. Eksotika 1 cDNA libraries (Shin *et al.*, 2011). The objective of this study is to identify the genes and their regulatory pattern related to papaya ripening. They predicted 8 unigenes related to ethylene biosynthesis process, 8 unigenes and 6 unigenes for fruit softening and synthesis of fruit aroma respectively.

#### **Changes in Endogenous ABA Content and Levels of FaASR mRNA and Protein at Different Ripening Stages**

ABA content increases during fruit ripening in both climacteric and non-climacteric fruit. ABA is thought to control ripening through activation of ethylene biosynthesis. Biochemical and genetic evidence has also indicated that ABA biosynthesis and signaling appear to involve a complex network of both positively and negatively regulating components, including kinases pathway. ABA stress and ripening induced (ASR)-downstream component involved in ABA signal transduction. During strawberry fruit development, endogenous ABA content, levels of FaASR mRNA and protein.

Fruit ripening is a complex phenomenon with drastic changes in chemical composition, which enhance an important quality attributes such as flavour, softness, sweetness and color. These

changes are very coordinated and involve many biochemical steps, which are upon a stringent and integrated control played by plant hormones and gene expression, including epigenetic mechanism. The expression of specific genes is essential to normal ripening and the synthesis of enzymes involved in the autocatalytic ethylene burst during climacteric fruit ripening. However, several other metabolic pathway are dependent on the modulation by gene expression, and some genes of enzymes putatively controlling the most important physiological changes associated to fruit ripening have been pointed. Based on the studies on tomatoes, there is already some understanding about the genes involved in the most important physiological changes, such as the accumulation of soluble sugars, cell wall disassembly, and synthesis of pigments during fruit ripening. In this way, the identification of genes induced during ripening could provide important clues about the activation of biochemical steps and its relationship to fruit quality.

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