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Current challenges in postharvest biology of fruit ripening

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ABSTRACT

This paper reviews the recent advances in the understanding of the fruit ripening process and describes future challenges. Fruit ripening is a complex developmental process which is orchestrated by the expression of ripening-related genes under the control of a network of signaling pathways. In climacteric fruit components responsible for the production of climacteric ethylene have been identified. Less progress has been made on non-climacteric fruit. Great advances have been made in the characterization of transcription factors (ERFs, RIN, etc...) that induce gene expression through the binding to their promoters. Genetic resources, genome sequencing and “omics” tools have been developed bringing a huge amount of data that will help to draw together an integrative network of regulatory and signaling pathways responsible for triggering and coordinating the ripening process. The discovery that some ripening events are controlled at the epigenetic level and, not in relation with the DNA sequences, opens novel perspectives.

Keywords: climacteric and non-climacteric fruit; ethylene; hormones; transcription factors; omics tools.

INTRODUCTION

Postharvest science is the study of the physiology of plant tissues and organs after their detachment from the plant. Because postharvest science is concerned with living organs, physiological and technological aspects are intimately linked. For instance, early studies have been carried out to understand the basic mechanisms of respiration of harvested organs. They have established that respiration is an essential component in the determination of postharvest shelf-life. These studies have served as a base for the definition of postharvest technologies devoted at reducing respiration such as cold storage, pre-refrigeration and, modified, controlled and dynamic atmospheres. In recent years postharvest science has integrated a number of modern methods such as molecular biology, biotechnology, and “omics” tools (metabolomics, proteomics, and transcriptomics) in order to elucidate the mechanisms of fruit ripening during which fruit develop their sensorial and nutritional quality. In this review we will concentrate on the most recent developments in the mechanisms of fruit ripening and development of quality. Challenges and perspectives offered by these new developments will be addressed.

1. ROLE OF ETHYLENE IN FRUIT RIPENING AND QUALITY

1.1 Climacteric and non-climacteric ripening

Climacteric fruit are characterized by a ripening-associated increase in respiration and in ethylene production whereas non-climacteric fruits undergo ripening without any ethylene-associated respiratory peak. Horticultural products derived from leaves, roots, and tubers are also non-climacteric. However, various studies on fruit attached to the plant have indicated that changes in the levels of CO₂ and ethylene occur at various stages of fruits development in non-climacteric fruit.

Nevertheless the timing of the climacteric syndrome in non-climacteric fruit is not related to the ripening period *per se*. In grapes it occurs at véraison (Chervin et al., 2004), and in citrus, in young immature fruit (Katz et al., 2004). In strawberries, ethylene production starts to increase once the fruit reaches the red ripe stage, but not before (Lannetta et al., 2006). It is now considered that some aspects of the ripening of non-climacteric fruit are regulated by ethylene and in climacteric fruit, some ripening pathways are independent of ethylene action. Differential cross-talks between ethylene and other phytohormones probably operate in each type of fruit (Pech et al., 2008; Paul et al., 2012). An extensive list of climacteric and non-climacteric fruit has been provided by Watkins (2002). However, there are still a great number of underexploited, non-traditional fruits in the world, especially in tropical areas (Rufino et al., 2009) which have not been characterized in terms of the presence or absence of climacteric behavior.

1.2 Ethylene biosynthesis

The transition from system 1 to system 2 in climacteric fruit it has long been considered that two distinct systems of ethylene biosynthesis take place during fruit development. System 1 is characterized by auto-inhibitory ethylene production and is

responsible for the basal production of ethylene levels in vegetative tissues, in non-climacteric fruit and at the pre-climacteric stages of climacteric fruit. System 2 operates during the climacteric burst and is autocatalytic. The two systems are distinguished by the expression of specific members of ACC synthase (ACS) genes (Barry et al., 2000). Yokotani et al. (2009) have investigated the mechanisms of the system 1 to system 2 transition in tomato fruit where the expression of an EIL transcription factor involved in the ethylene-transduction signal was suppressed. Despite ethylene insensitivity, the system 2-associated ACSs genes, *LeACS2* and *LeACS4*, exhibited significant expression that could not be inhibited by 1-MCP. These data indicate that system 2 is regulated at least partially by ethylene-independent developmental factor(s), but largely by an ethylene-dependent autocatalytic system. In winter varieties of pears, the system 1-to-system 2 transition of ethylene production cannot develop unless fruit are stored at low temperature. The cold treatment stimulates the ethylene-independent expression of a specific ACS gene that initiates ethylene production, and puts into motion the expression of system 2-associated ACSs (El-Sharkawy et al., 2004). A cold treatment, although not absolutely required, is also capable of hastening and synchronizing the onset of the climacteric rise of ethylene production and ripening of Bartlett and Conference pears, as well as in some apple (Pech et al., 2012) and peach varieties (Begheldo et al., 2008). The absence of climacteric behavior has been assigned to the absence of system 2 autocatalytic ethylene production in some guava varieties associated with a lack of ACC synthase activity (Liu et al., 2012a). The transition from system 1 to system 2 is also modulated at the level of the ethylene receptor(s). The ethylene receptor(s) negatively regulate ethylene signal transduction and suppress ethylene responses. It has been demonstrated that a reduction in the levels of either *LeETR4* or *LeETR6* ethylene receptors causes an early-ripening phenotype in tomato fruit (Kevany et al., 2008). It is concluded that the levels of receptor would act as a sensor for

memorizing cumulative ethylene exposure and would determine the timing of the onset of ripening. The low level of ethylene production of immature fruit by system 1 would be responsible for a reduction in the amount of receptor protein and therefore in an increase in ethylene sensitivity. How ethylene biosynthesis genes are regulated at the transcriptional level has been one of the challenges of the recent years.

1.3 The role of transcription factors in the regulation of ethylene biosynthesis and action and in fruit ripening

In recent years, studies have been dedicated to the identification of transcription factors capable of binding to the promoters of ethylene biosynthesis genes and of regulating their expression. Lin et al. (2008), using *in silico* analysis observed that the *LeACO1* promoter harbors putative cis-elements capable of binding a class-I homeo-domain leucine zipper, HD-Zip. Using gel retardation assays they demonstrated that a tomato HD-Zip, LeHB-1, interacted with the promoter of *LeACO1*. The inhibition of LeHB-1 expression by virus-induced gene silencing (VIGS) greatly reduced *LeACO1* mRNA levels and fruit ripening. It is therefore concluded that LeHB1 stimulates ethylene synthesis by activating the transcription of *LeACO1*. Using the VIGS strategy, Itkin et al. (2009) performed a functional screen of tomato transcription factors that identified the TOMATO AGAMOUS-LIKE 1 (*TAGL1*) MADS box gene as altering tomato fruit pigmentation. In fact, transient promoter-binding assays indicated that *TAGL1* participates in the control of ethylene production by the activation of the *ACS2* gene.

A number of transcription factor mutants with altered ripening processes have been characterized (Giovannoni, 2007). Among these, the *ripening inhibitor (rin)* corresponded to a MADS-box gene, *non-ripening (nor)* to a member of the NAC domain transcription factor family and *Colorless non ripening (Cnr)* to a SBP-box gene. In a large-scale chromatin immunoprecipitation experiment Fujisawa et al. (2013)

found that *MADS-RIN* directly controls the expression of 241 target genes containing a RIN-binding site in their promoters. These genes are involved in the biosynthesis and perception of ethylene (as *LeACS2*, *LeACS4*, *NR*, and *E8*). Particularly interesting is that *MADS-RIN* is involved in switching on system-2 ethylene through the induction of *LeACS2* (Ito et al., 2008) and *LeACS4* (Martel et al., 2011), up-regulating *NOR* and *CNR* (Martel et al., 2011), and down-regulating *HB-1* (Lin et al., 2008). Ethylene production is negatively regulated by two Ethylene Response Factors, AP2a that binds to *CNR* (Karlova et al., 2011) and ERF6 whose binding target remains unknown (Lee et al., 2012). Other components of the RIN targeting system are two homologs of the Arabidopsis FRUITFULL transcription factor, *FUL 1* and *2* that form a complex with RIN and *CNR* to control several ethylene-independent aspects of the ripening process (Bemer et al., 2012). *MADS-RIN* also binds to its own promoter (Fujisawa et al., 2013) resulting in an auto-regulation process. It has also been demonstrated that *CNR* is required for RIN-binding activity (Martel et al., 2011). Overall these data indicate a complex mechanism for the regulation of ethylene biosynthesis in climacteric fruit including ethylene-dependent and developmentally regulated factors (Figure 1). Moreover, RIN also targets genes involved in other many ripening processes such as cell wall degradation and aroma biosynthesis (Qin et al., 2012; Fujisawa et al., 2013).

Homologs of the *MADS-RIN* accumulate during the ripening of non-climacteric fruit such as strawberry (Vrebalov et al., 2002) and pepper (Lee et al., 2010) indicating that MADS box-dependent ripening is probably conserved in non-climacteric fruit ripening. A comparative transcriptome analysis between tomato and pepper fruits using heterologous microarray hybridization indicated that divergent types of transcription factors were expressed in ripening tomato and pepper fruits, suggesting they may be key factors that differentiate these distinct ripening processes (Lee et al., 2010). The *MADS-RIN* is a member of the *SEPALLATA* family of MADS box transcription factors. Interestingly a

SEPALLATA (SEP) 1/2 like '(*FaMADS9*) gene is necessary for the development and ripening of strawberry (Seymour et al., 2011) indicating that

SEPALLATA genes play a role in the regulation of climacteric and non-climacteric ripening.

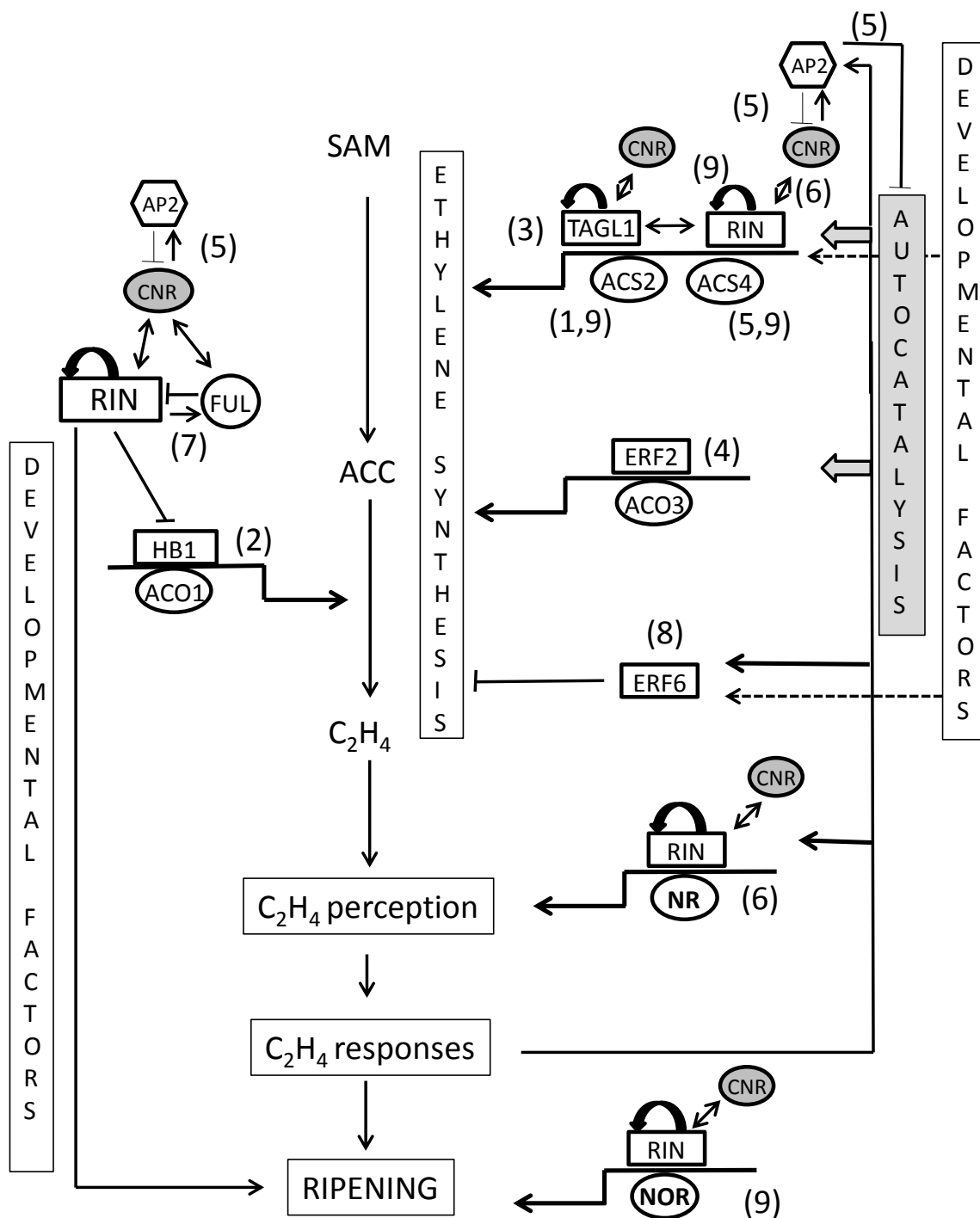


Figure 1. Schematic representation of the role of transcriptional regulators in climacteric fruit ripening. The numbers between brackets associated with the interactions correspond to the following publications: (1) Ito et al. (2008); (2) Lin et al. (2008); (3) Itkin et al. (2009); (4) Zhang et al. (2009); (5) Karlova et al. (2011); (6) Martel et al. (2011); (7) Bemer et al. (2012); (8) Lee et al. (2012); (9) Fujisawa et al. (2013). Arrows indicate positive regulation and blunt-ended lines indicate repression. Circle arrows in bold on RIN and TAGL1 indicate auto-regulation. Arrows with a star indicate the binding of the transcription factors to the promoter of the target genes.

1.4 Ethylene perception and signal transduction

Ethylene is perceived by membrane-associated receptor proteins. The general biochemical and molecular characteristics of ethylene perception and signal transduction in ripening fruit are well established (Pech et al., 2012). In tomato, ethylene receptors correspond to a family of genes comprising six members. Three of them, *NR*, *LeETR4* and *LeETR5* exhibit a significant increase during fruit ripening (Klee, 2002). Ethylene receptors have been identified not only in climacteric fruit but also in non-climacteric fruit (Trainotti et al., 2005). The receptors interact with a Raf kinase-like protein, CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) to release the transduction of the signal to the downstream elements (Ju et al., 2012). Such a mechanism has led to the definition of a negative regulatory model in which low amounts of receptor are required for stimulating ethylene responses. The model seems incompatible with the increased expression of the receptor during fruit ripening. However it has been clearly demonstrated that the levels of gene expression and the accumulation of the receptor protein are disconnected. When the levels of mRNA encoding the receptors increase in ripening fruit, the levels of the proteins decrease. Furthermore, ethylene stimulates the degradation of the receptor proteins 26S proteasome-dependent pathway (Kevany et al., 2007). Beside post-translational regulation, ethylene perception is also controlled by a Green-Ripe (GR) protein, a tomato homolog of the Arabidopsis Reversion To Ethylene Sensitivity (RTE1), which acts as negative regulator of ethylene response. The *Gr* mutant fails to ripen as a consequence of inhibition of ethylene responsiveness (Barry et al. 2005). The signaling process downstream of CTR involves the activation by *EIN2* of the ETHYLENE INSENSITIVE3 (*EIN3*) and EIN3-like (*EIL*) primary transcription factors that in turn activate secondary transcription

Components of ethylene signaling have been extensively studied mainly in the Arabidopsis model plant revealing a linear transduction pathway that leads to the activation of transcriptional regulators

belonging to the Ethylene Response Factor (ERF) type. These ERFs are members of the large AP2/ERF superfamily and they bind to a GCC box *cis*-element in the promoter of target genes in several plant species, regulate a number of biological processes including development, reproduction, responses to hormones, adaptation to biotic and abiotic stresses. They are supposed to regulate the expression of ethylene-responsive genes including ripening-related genes, although the function of ERFs in fruit ripening are still unclear. However, new data are emerging that may link them to the control of some aspects of fruit ripening (Li et al., 2007; Lee et al., 2012). Modulation of expression of individual ERFs in tomato has demonstrated their role in plant development and ripening. Mainly enabling ethylene to regulate a wide range of physiological processes in a highly specific and coordinated manner (Pirello et al., 2012). ERF proteins also have been characterized in the apple (Girardi et al., 2013) and, as reported by Xiao et al., (2013), in a number of other fruit, including banana, plum, persimmon, kiwi and longan, some of them related to expression of genes involved in ethylene biosynthesis, cell wall modification and senescence processes.

The control of ERFs regulation and of their target gene is a biotechnological challenge. Indeed, these new insights will give opportunity to improve fruit quality thanks to a better resistance to biotic and abiotic stresses (e.g., cold, heat, and drought). Recently, more and more evidence is appearing that suggests that probably ERFs and micro RNAs could be better utilized for this purpose which should lead to more specific changes in ripening parameters (ethylene production, color changes, softening, etc). Further, there is an urgent need to develop transformation and regeneration protocols for various other fruits that are economically important and for which post-harvest spoilage is high (Bapat et al., 2010).

In non-climacteric strawberries, correlated with the late increase in ethylene synthesis in red-ripe fruit, there is an increase in the expression of the *FaEtr2* ethylene receptor which is closely related to the tomato *LeETR4*)

(Trainotti et al., 2005). In pepper, *EIL-like* genes are induced during ripening (Lee et al., 2010). Grape traditionally has been classified as non-climacteric, but studies have shown that grape berry tissues have a fully functional pathway for ethylene synthesis and that this pathway is activated just before veraison (Chervin et al., 2004). Using a qRT-PCR platform encompassing the whole ERF/AP2 superfamily present in the grapevine, Licausi et al. (2010) show how *AP2/ERF-like* genes are expressed in both vegetative and reproductive tissues at different developmental stages. Skin and flesh tissues from grape berries differ greatly during the transition from *veraison* to full ripeness with respect to ERF-gene expression. In the skin, 31 genes were up-regulated at least four-fold and 18 were down-regulated. In the flesh tissues, only 18 *AP2/ERF* genes were up-regulated and 30 down-regulated. Two *AP2* genes, *VvAP2-5* and *VvAP2-16*, homologs to the Arabidopsis *PTL3 (AIL-6)* and *PTL4 (BBM)*, respectively were strongly up-regulated in skin tissues during ripening, whereas an *AIL-9* homolog, *VvAP2-9*, was repressed. The expression of ethylene receptor(s) together with the expression of downstream signaling elements in non-climacteric fruit reinforces the concept that climacteric and non-climacteric fruit share a minimum of common regulatory pathways. The observations that some aspects of non-climacteric fruit ripening are inhibited by the ethylene perception inhibitor 1-MCP also supports a role for ethylene in non-climacteric fruit ripening (Villarreal et al., 2010). Future experiments should be dedicated to confirm at the molecular level the role of ethylene perception and signaling in the regulation of some ripening-related genes in non-climacteric fruit, for instance, by knocking-down ethylene perception.

1.5 Inheritance and genetic diversity of the climacteric character

Studies on the inheritance of the climacteric character have been possible in fruit species where non-climacteric and climacteric genotypes were present. Such has been the case

for melon. Périn et al. (2002), using a segregating population of melons resulting from a cross between a climacteric and a non-climacteric melon found that the climacteric character was under the control of two duplicated independent loci. However, the potential genes involved were not identified. Crosses of Honeydew melons producing undetectable levels of ethylene with Cantaloupe melons producing high levels of climacteric ethylene gave F1 hybrids that generated ethylene in a climacteric manner (Ezura et al., 2002). Intriguingly, some introgression lines, but not all, generated from two non-climacteric melons exhibited a climacteric character (Obando et al., 2007). The absence of system 2 autocatalytic ethylene production has been suggested as responsible for the non-climacteric character of some guava varieties (Liu et al., 2012a). In some plum varieties, categorized as climacteric-suppressed, the level of ethylene is too low to allow proper ripening but treatment with propylene, a homolog of ethylene, was capable of inducing the whole ripening process (Abdi et al., 1997). Understanding the inheritance of the climacteric character is highly challenging. The number of genes and regulatory elements involved in ethylene production and perception is high so that different and complex genetic components probably exist for the control of climacteric ethylene. In addition, the number of genetically compatible fruit species comprising both climacteric and non-climacteric fruit is extremely limited so that studying the inheritance of the ripening character is not easy.

1.6 Ethylene synthesis and storage life

It has long been recognized that long storage life is associated with low ethylene production. Mutations of specific ACS genes appear to be responsible for the postharvest behavior of these genotypes. In apple fruit, the fruit-specific *MdACS1* gene is highly expressed during fruit ripening and is responsible for climacteric ethylene production. Low ethylene production is conferred by the presence in some cultivars of two allelic forms of the gene,

MdACS1-1 and *MdACS1-2* (Harada *et al.* 2000). Cultivars homozygous for the *MdACS1-2* allele showing low transcription due to the insertion of a retro-transposon-like element have low ethylene production and longer shelf life (Harada *et al.* 2000). Another ACS gene, *MdACS3a*, negatively regulated by ethylene is also pivotal in regulating the transition from system 1 to system 2 ethylene synthesis (Wang *et al.* 2009). In fact three *ACS3* genes are present in apple among which *MdACS3b* and *MdACS3c* are not expressed due to a transposon-like insertion in their 5' flanking region. *MdACS3a* has two null allelotypes, one affecting the enzyme activity of the encoded protein and one deficient in transcription activity (Wang *et al.*, 2009). However the *MdACS3a* null mutations affect the ripening phenotype of late harvest apple cultivars only, indicating that the *MdACS3a* genotype alone cannot explain the full spectrum of ethylene production and the storage characteristics of apples (Bai *et al.*, 2012). In Japanese pear fruit, where the shelf-life is also determined by the level of ethylene production, two types of *ACS* genes (*pPPACS1* and *pPPACS2*) were identified. Cultivars that produce high levels of ethylene possess at least two copies of *pPPACS1* and those producing moderate levels of ethylene have at least two copies *pPPACS2* (Itai *et al.*, 1999). In peaches, the stony hard phenotype associated with long shelf-life has been attributed to a low level of ethylene production. Among three *ACS* genes, *Pp-ACS1* is strongly expressed in melting-flesh peach varieties, but it is not expressed in stony hard peaches. Therefore the absence of expression of *Pp-ACS1* is considered as the responsible for the low ethylene production and suppression of softening of stony hard peaches (Tatsuki *et al.*, 2006). These data indicate that low ethylene production could be due to mutations in one or the other of ethylene synthesis gene alleles. Extending the storage life of fruit can be achieved case by case by using the low-ethylene producing and slow-ripening mutants (*e.g.*, *alcobaca*, *nor*) for genetic breeding.

2. OTHER HORMONES AND HORMONE CROSS-TALKS

The major emphasis of research on the regulatory mechanisms of fruit ripening has been on ethylene. However, functional characterization of several tomato (*Solanum lycopersicum*) mutants defective in ethylene response, such as *rin* and *Never Ripe*, the transgenic fruits with reduced expression of *ACS* and *ACO*, and the use of 1-MCP, have strengthened the evidence for the existence of several ethylene-independent ripening events in climacteric fruits. Furthermore, the application of 1-MCP in non-climacteric fruit has demonstrated the existence of several processes in these fruit types for which ethylene performs regulatory roles, such as loss of firmness in strawberries (Villarreal *et al.*, 2010), and the synthesis of anthocyanins in grapes (Chervin *et al.*, 2004).

The role of other hormones on fruit maturation has become apparent thanks to the development of new strategies, such as the use of a varied collection of tomato mutants defective both for signaling and for the synthesis of hormones such as indole-3-acetic acid (IAA), abscisic acid (ABA) and methyl jasmonate (MJ).

The identification of the elements of the signal transduction pathways of these hormones has revealed that some of its components may be targets in the signaling of more than one hormone. This fact points to a mechanism by which different hormones may act on the same pathways, sometimes exerting similar effects, sometimes the opposite. Among some examples is the synthesis of lycopene in tomato fruit; although often referred to as an event ethylene-dependent, it can be enhanced by exogenous ABA treatment (Zhang *et al.*, 2009). Likewise, several transcription factors of the ERF family have altered patterns of expression in IAA-treated peach fruit (Trainotti *et al.*, 2007), and some members of the ARF (auxin response factor) family are inhibited or stimulated in tomatoes after exogenous ethylene treatment (Jones *et al.*, 2002). The MJ promoted accumulation of lycopene in MJ-deficient

tomato spr2 and def1. Interestingly, the post-harvest treatment with MJ in NR tomato fruit, which notably does not accumulate lycopene, induced the expression of several genes encoding enzymes of the carotenoid biosynthetic pathway, resulting in the accumulation of lycopene in fruits, and suggesting the existence of a regulatory mechanism independent of NR (*LeETR3*) receptor signaling (Liu et al., 2012).

The tools for wide expression analyses ("omics" technologies) will provide an unprecedented increase in the information about the mechanisms by which auxin, abscisic acid and jasmonates affect the ripening of both climacteric and non-climacteric fruits, allowing detection of changes in thousands of genes, proteins and metabolites simultaneously. For now, the picture that emerges suggests that the biochemical changes during fruit ripening are the result of a multi-hormonal dialogue, more than the action of an isolated hormone (McAtee et al., 2013). The biggest challenge in this area will be to identify the points of crosstalk between these different hormonal classes. This challenge will require not only the adoption of new experimental designs (some examples are given in Allwood et al. 2011) or new means for the exogenous treatments with hormones and their respective inhibitors of action and/or biosynthesis (*e.g.*, hormone slow-release polymer films), but also a major effort to integrate the huge amount of information that will be generated by such experiments.

3 CONTRIBUTION OF HIGH THROUGHPUT TECHNOLOGIES TO THE UNDERSTANDING OF THE FRUIT RIPENING PROCESS

The main changes occurring during ripening and maturation derive from programmed, complex physiological processes further influenced by environmental conditions, biotic/abiotic stress and postharvest treatments. Given the multigenic factors affecting processes the study of single pathways and responses will provide a complete elucidation of the fruit ripening. In order to better understand how environmental factors,

stress and postharvest treatment and their inter-relationships affect fruit ripening, the most recent research strategies, including holistic studies in genomics, transcriptomics, proteomics and metabolomics will be needed (Seymour et al., 2013).

3.1. Genome sequencing

The genomes of twelve fleshy fruit species has been fully sequenced (Table 1). This will open a number of new opportunities: (i) making an inventory of the genes belonging to the same family, (ii) identifying regulatory elements and binding-site motifs, (iii) facilitating marker-assisted selection and (iv) allowing the cloning of candidate genes responsible for quantitative trait loci or responsible for mutations. Great progress can be expected in the near future from the sequencing of the genome of wild relative species which will further our understanding of the impact of domestication on genomes of cultivated crops. This will be facilitated by the use of novel high throughput sequencing technologies. Using the reference genomes as guides, the identification of alleles contributing to specific characters will become easier. Progress remains also to be made in the assembly of the gene sequences on the chromosomes. For some species (tomato), the assembly is quite an advanced stage, but is not the case for several other species. In addition, annotation in terms of gene functions is far from being completed.

3.2. Transcriptomics

The transcriptomics (simultaneous analysis of transcriptional levels of thousands of genes) is the "omics" technology that has evolved more quickly. By means of DNA microarrays, researchers have analyzed patterns of transcription in a number of fruits including grapes (Deluc et al., 2007), tomato (Alba et al., 2005) and citrus (Cercos et al., 2006). However, the technology of DNA microchips is dependent on the prior existence of a database of ESTs or whole genomes, and is limited to the set of selected genes that form the microarray. Such

limitations may now be circumvented by the Next-Generation Sequencing (NGS) technologies (Illumina Hi-Seq, SOLiD, and Roche FLX) that are capable of providing the sequencing of whole expressed genomes in a few days. Based on the counting frequency of the mRNAs sequences (RNA-Seq) in a sample, a virtually entire expressed genome can be assessed in a single run. This will push transcriptomic analysis to a new level. The increasing availability of whole genomes sequenced (Table I), as well as the rapid fall in the cost of the analysis (with many companies that outsource the service) will make RNA-Seq replace DNA chips over the next years.

Although RNA-Seq generates a large amount of data in a short time, the bioinformatic analyses of a single experiment may require months of intensive computer work. A major challenge will be the development of user-friendly pipeline tools, including statistical analyses that streamline the processing steps of data generated by sequencing machines. This is undoubtedly the biggest bottleneck to transcriptomics becoming a tool with easy access to postharvest scientists.

Table 1. Inventory of references related to the genome sequences of fruit species and TILLING collections

| Species | References |
|--|---------------------------------|
| Sequence data | |
| Grapevine (<i>Vitis vinifera</i>) | Jaillon et al. (2007) |
| Papaya (<i>Carica papaya</i>) | Ming et al. (2008) |
| Cucumber (<i>Cucumis sativus</i>) | Huang et al. (2009) |
| Apple (<i>Malus domestica</i>) | Velasco et al. (2010) |
| Strawberry (<i>Fragaria vesca</i>) | Shulaev et al. (2011) |
| Tomato (<i>Solanum lycopersicum</i>) | Tomato Genome Consortium (2012) |
| Banana (<i>Musa acuminata</i>) | D'Hont et al.(2012) |
| Melon (<i>Cucumis melo</i>) | Garcia-Mas et al. (2012) |
| Sweet orange (<i>Citrus sinensis</i>) | Xu et al. (2012) |
| Peach (<i>Prunus persica</i>) | Arús et al. (2012) |
| Pear (<i>Pyrus bretschneideri</i>) | Wu et al. (2013) |
| Water melon (<i>Citrullus lanatus</i>) | Guo et al. (2013) |
| TILLING collection | |
| Tomato micro-tom (<i>Solanum lycopersicum</i>) | Saito et al. (2011) |
| Tomato (<i>Solanum lycopersicum</i>) | Minoia et al. (2010) |
| Melon (<i>Cucumis melo</i>) | Dahmani-Mardas et al. (2010) |
| Melon Piel de Sapo (<i>Cucumis melo</i>) | Gonzalez et al. (2011) |

3.3. Postranscriptional

Regulation by microRNAs. MicroRNAs represent a ubiquitous class of short RNAs that are crucial in mediating gene silencing at the post-transcriptional level. Zuo et al. (2013) and Karlova et al. (2013) have identified more than 100 conserved miRNAs in tomato. A number of them show differential expression during fruit ripening and in response to ethylene. Some of the targets for these micro RNAs are predicted

to be involved in several aspects of the fruit ripening process. We are still at the beginning of understanding of the role of microRNAs in fruit ripening.

3.4. Proteomics

The proteomic studies in fruits is particularly challenging, given the difficulty of extracting protein in these tissue types, generally rich in phenolic compounds, pigments,

sugars and complex carbohydrates. Some proteomic works on fruits after harvest have used gel-based approaches (usually 2D-electrophoresis, 2D-E) coupled to various mass spectrometry (MS) techniques (Hertog et al., 2011). Use of Fluorescence Difference Gel Electrophoresis (DIGE) substantially improves the gel reproducibility, one of the main problems in the application of 2D-E. Although it can resolve hundreds of proteins in a single gel, this approach is time consuming and tedious. However, many researchers have reported the identification of proteins associated with the ripening process, such as aldehyde dehydrogenase, Rubisco, chitinase, β -1,3-glucanase, in fruit samples at various stages of maturation (e.g., apple, tomato, grape, citrus and plum, Palma et al., 2011)

The use of prior separation by Nano-LC coupled MS/MS, in a gel-free approach along with a count the frequency of reading masses of the peptides (MS counting) has increased the resolution of proteomic analysis in fruits. MS technologies, such as the Orbitrap[®] mass analyzer, have evolved to get high sensitivity and accuracy in the determination of the masses of thousands of peptides in a single analysis. Thus, increasing even more the resolving power of mass spectrometers, improving the extraction of proteins from fruits and pre-preparation protocols for Nano-LC and the creation of bioinformatic tools that facilitates the proteome data analysis and its integration with transcriptomics and metabolomics data must be the next challenges to be met for the advancement of proteomics studies on postharvest.

3.5. Metabolomics of fruit ripening and quality

Metabolomics is based on the high throughput analysis of metabolites in order to define the global biochemical profiles of biological systems. In fruit physiology it has been used primarily for characterizing the metabolic changes occurring during fruit ripening (Hertog et al., 2011). As mentioned in Bernillon et al. (2013), it has also been used to assess metabolic diversity and genetic

variations, variations between growing seasons, the effect of biotic and abiotic stresses and of cultural practices. Metabolomics research generates a wide range of data matrices that often stay descriptive and generate correlation networks whose biological relevance is not always established. Nevertheless it provides an in-depth knowledge on fruit composition and nutritional value which may be helpful for guiding the improvement of fruit quality through plant breeding and cultural practices.

3.6. High throughput search for the target genes of transcriptional regulatory proteins

The binding of transcription factors to the promoter region of ripening related genes has been assessed recently by gel-retardation assays for studying the characteristics of the interaction between ERFs and the GCC box (Tournier et al., 2003) and the binding of the LeHB-1 tomato homeobox protein with the promoter of *LeACO1* (Lin et al., 2008). Another method that has been used successfully is a transient expression assay in tobacco protoplasts co-transformed with a transcriptional regulator construct driven by the 35S constitutive promoter and reporter constructs harbouring the gene coding the Green Fluorescent Protein driven by the target promoter. This method has been applied to functional analysis and binding affinity of tomato ERFs (Pirello et al., 2012). The same approach can be used for many other transcription factor families, such as ARFs and MYB.

In the recent years, a method has been established that allows identifying the target genes of transcriptional regulatory proteins by a chromatin immunoprecipitation (ChIP) analysis using an antibody against the transcription factor protein. This method has been applied to the identification of the target genes of the RIN transcription factor (Ito et al., 2008; Fujisawa et al., 2011; Martel et al., 2011; Qin et al., 2012). The method has been scaled-up by coupling ChIP with DNA microarray analysis leading to the identification of 241 target genes containing RIN binding sites in their promoter regions

(Fujisawa et al., 2013). In order to fully assess the biological function of the interaction, the binding of a given transcriptional protein to the promoter region of such an elevated number of genes probably requires confirmation one by one by other methods. This approach will be useful to identify targets of important regulators of gene transcription related to fruit ripening, such as NOR, TAGL1, HB and members of the ERF, ARF and MYB families.

3.7. Systems biology

System biology aims at understanding complex biological systems such as fruit ripening using a combination of approaches (e.g. metabolomics, transcriptomics, proteomics) in order to establish a network of interactive regulatory events. A review has been published on how system biology can be used in postharvest research (Hertog et al., 2011). So far only one study has used system biology for unraveling regulatory networks during tomato fruit ripening (Osorio et al., 2011). It has been found that the correlation between the levels of a transcript and the abundance of the corresponding protein was lower during early ripening than at later stages, indicating changes in the importance of posttranscriptional regulation during the ripening process. On the contrary, a very strong correlation was found between gene transcription and specific metabolite groups. This study is a good example of the outputs of system biology in providing a network of key candidate regulators possibly involved in fruit ripening. However the network is based on mathematic correlations that require biological confirmation. This approach will be useful to reveal specific points of regulation of the different hormonal classes that influences the fruit ripening.

3.8. Functional genomics

The function of a gene *in planta* can be determined by different means. Knocking-down gene expression through the antisense and RNAi technologies has been largely used for elucidating the role of a single gene *in planta*.

Many examples exist in the area of postharvest physiology (Pech et al., 2005). The Virus Induced Gene Silencing (VIGS) strategy that uses a virus vector for transferring the antisense gene in a plant has often been used, but is restricted to few plant species due to the requirement of virus-host compatibility. It has been used in tomato for understanding the role of some transcription factors on the fruit ripening process (Zhou et al., 2012). In order to overcome functional redundancy in the case of multigene families, the Chimeric Repressor Silencing Technology consisting of the use of a SRDX repression domain is very efficient and has been employed in tomato (Itkin et al., 2009). When the function of the encoded protein can be easily evaluated *in vitro*, heterologous expression of the gene in yeast or bacteria is a method of choice.

TILLING (Targeting Induced Local Lesions IN Genomes) consists in generating a collection of ethyl methanesulphonate (EMS) mutants, identifying interesting phenotypes and detecting single-nucleotide mutations to identify the gene allele responsible for the phenotype. This technology has led to the isolation of two allelic gene mutants of tomato (Sletr1-1 and Sletr1-2) resulting in reduced ethylene responses (Okabe et al., 2011) and several alleles of tomato light signaling genes affecting phenylpropanoid and carotenoid composition (Jones et al., 2012). A TILLING platform of cantaloupe melons has allowed the characterization of a missense mutation in the *CmACO1* gene. As expected, the mutation inhibits fruit ripening and extends fruit storage life (Dahmani-Mardas et al., 2010). The functional characterization of new allele genes responsible for some ripening by TILLING is facilitated by the availability of genome sequences. TILLING strategies could be included in breeding programs. They have the advantage of not employing genetic engineering methods for which consumers are often reluctant, but they require the building-up and screening of a mutant collection for each individual elite line used for genetic crosses. All the methods of functional genomics mentioned above are complementary, but we are still far from

understanding the role *in planta* of several hundred of genes that are differentially expressed in ripening fruit.

3.9. Epigenetics

The possibility that epigenetics events (i.e. not related to DNA sequences) are involved in the fruit ripening process was first suggested by the study of the tomato colorless non-ripening (*Cnr*) mutant. The *Cnr* gene encodes a *SQUAMOSA* promoter binding protein–box transcription factor and the mutation is related to heritable cytosine hyper-methylation of the promoter (Manning et al., 2006). It was later demonstrated that the hyper-methylation of the *Cnr* promoter prevented the binding of RIN transcription and, as a consequence of *Cnr* gene expression and fruit ripening (Zhong et al., 2013). Whole-genome bisulfite sequencing indicated that DNA methylation in the 5' ends of genes (predicted to correspond to the promoters) gradually declines during fruit ripening. Interestingly promoter methylation remains high in the two ripening mutants *Cnr* and *rin*. So far, breeding for improving shelf-life and quality of fruit is relying on genetic variations only. Strategies exploiting epigenetic variations should now be considered.

CONCLUSIONS

In climacteric fruit the molecular basis for the transition from immature to mature fruit has made great progress with a better knowledge of the System1 to System2 ethylene production. The role of transcriptional regulation has received great development. The understanding of the role of other hormones and their interactions with ethylene is at the beginning. In contrast with climacteric fruit, the ripening mechanisms of non-climacteric fruit for, which ethylene does not play a major role, has made much less progress.

The sequencing of the genomes and the use of “omics” techniques is in full development. It has already provided a large amount of information on the metabolic, proteomic and transcriptomic changes occurring

during fruit ripening. However more work is needed to bring an integrative picture of the biological function of the ripening-related genes and transcriptional regulators. Epigenetics represents a new area which is opening novel perspectives.

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