

## **Mechanisms of Fruit Ripening: Retrospect and Prospects**

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### **Abstract**

**This paper aims at giving an overview of the progress made during the last decades on the mechanisms of fruit ripening and to present the most recent trends and prospects for the future. Important steps forward will be presented (respiratory climacteric, ethylene biosynthesis and action, isolation of genes involved in the ripening process, biotechnological control of fruit ripening....) by showing how the judicious exploitation of the data published previously, the strategies, methodologies and plant material adopted have been crucial for the advancement of knowledge. Opportunities of co-operation between geneticists and post-harvest physiologists as well as new possibilities offered by genomics, proteomics and metabolomics for the understanding of the fruit ripening process and the development of sensory quality will be developed.**

### **INTRODUCTION**

The mechanisms of fruit ripening have received great attention since many years for both scientific and societal reasons. For the scientist, fruit ripening is a process in which the biochemistry, physiology and structure of the organ are developmentally altered to influence appearance, texture, flavour and aroma (Giovanonni, 2004). For the consumer and for the partners of the distribution chain, fruit ripening corresponds to a series of chemical and structural modifications that allow fruit to become edible and attractive for consumption. There is nowadays growing emphasis not only on sensory properties but also on nutritive value and safety for human health. Since the majority of the quality attributes are elaborated during the ripening process it has always been considered as essential to better understand the mechanisms underlying fruit ripening. The fruit ripening process has been viewed over the last decades as being successively of physiological, biochemical and molecular nature. In this paper we will make a retrospect of the important discoveries made in the field of fruit ripening and present some perspectives for the near future. We will try to show that progress has generally been made through a good integration of earlier data into the newest concepts of plant science and through the choice of the best adapted methodologies and plant models.

### **THE RESPIRATORY CLIMACTERIC**

One of the earliest discoveries is the demonstration by Kidd and West (1925) that apple fruit undergo a rise in respiration associated with the ripening process that was termed ‘climacteric’. Subsequently it has been found that the respiration of many fruit undergoes a climacteric. The merit of Kidd and West to discover the climacteric respiration was high if one considers that the rise in respiration of the apple is very low as compared to other fruit. A review of the start of modern postharvest physiology by Kidd, West and Blackman has been published by Laties (1995). Further studies demonstrated that the climacteric rise of respiration was not universal and that some type of fruit named ‘non-climacteric’ showed no or relatively slow changes in respiration during ripening. This has led to a classification of fruit in two classes according to their respiratory pattern (Biale and Young, 1981). Many studies have been devoted to the determination of the nature of the climacteric respiration and the role of mitochondria. An increase in the rate of carbon flux through glycolysis has been demonstrated during the respiratory

climacteric associated with large increase in ATP levels (Barker and Solomos, 1962). The increase in glycolysis is suggested to be brought about by activation of phosphofructokinase and of pyrophosphate: fructose-6-phosphate phosphotransferase (Bennett et al., 1987). However, proof of their role in the climacteric respiration is still lacking. The respiratory climacteric has also been attributed to the increase in activity of the cyanide-insensitive alternative oxidase (AOX) associated with the uncoupling of the electron transport chain from oxidative phosphorylation (Solomos, 1977). Since then, genes encoding AOX (McIntosh, 1994) and uncoupler protein (Unc) (Laloi et al., 1997) have been isolated and the expression of some members of these families increase during the ripening process. Nevertheless the direct demonstration of a role of these genes (proteins) is lacking inasmuch as contradiction exists between the increase in ATP and the stimulation of the energy-dissipating systems, AOX and Unc. Furthermore, the relationship between the climacteric respiration and fruit ripening has been questioned few years ago when it was discovered that ripening on the vine of a number of fruit occur in the absence of any increase in respiration (see Lelièvre et al., 1997). Although the presence or absence of respiratory climacteric on the vine depends upon the environmental conditions (Bower et al., 2002), these observations indicate that the respiratory increase is probably not a trigger for the ripening process.

### **ORGANISATIONAL RESISTANCE VS DEVELOPMENTAL PROCESS**

One of the earliest theory on the regulation of fruit ripening was named 'organisational resistance' (Blackman and Parija, 1928) and considered the fruit ripening events as a senescence process mainly catabolic in nature and resulting from the loss of cell compartmentation (Sacher, 1973). However evidence emerged that fruit ripening corresponded to a developmental programme responsible for the changes in texture, flavour and aromas. Nevertheless, a legacy of the organisational resistance theory has been the evaluation of membrane deterioration through measuring solute leakage from fruit tissue as a marker for ripening and senescence. This is still being used nowadays, especially for non-climacteric organs where no such markers as the ethylene or respiratory peak can be used.

### **STUDY OF BIOCHEMICAL CHANGES OCCURRING DURING FRUIT RIPENING**

The identification of changes in the major components has been undertaken as an early step in the understanding of the ripening process. A good testimony of this period can be found in two major events managed by two scientific leaders of fruit biochemistry: (i) the publication by Hulme (1971) of two famous textbooks entitled "The biochemistry of fruit and their products" and (ii) the organisation of an international symposium entitled 'Facteurs et régulation de la maturation des fruits' (Ulrich, 1975). Among the most significant aspects of fruit biochemistry, some have received great attention due to their importance in fruit quality: texture changes, aroma volatiles, colour and pigments. These data have given an in-depth picture of the metabolic changes occurring during ripening and still represent an invaluable background for both the post-harvest physiologists and the molecular biologists.

### **MOLECULAR BIOLOGY AND BIOTECHNOLOGY OF FRUIT RIPENING**

Fruit ripening as a developmental event became considered as involving the synthesis of new proteins and the expression of specific genes. An increase in the incorporation of precursors into proteins or nucleic acids was demonstrated in the early stages of avocado fruit ripening suggesting the synthesis of new proteins or increased turnover (Richmond and Biale, 1967). Immunological methods have allowed to confirm the increase in the amount of several enzymes such as cellulase and polygalacturonase (Brady, 1987). One of the first studies of the isolation of RNA species was carried out by Romani (1975) but it was dedicated to transfer RNAs (tRNA) of tomato which undergo little changes during ripening and which are not thought to play a critical role in the

regulation of ripening genetic programme. The first studies aimed at studying gene expression at the mRNA level have been developed in Laties's laboratory using avocado fruit. In vitro translation of mRNA showed the appearance of new translation products (Christoffersen et al., 1982). This has led to the identification of the cDNA encoding cellulase and the determination of the full-length sequence of the gene and has given the first example of identification of a ripening-related gene (Bennett and Christoffersen, 1986). However the use of avocado fruit in these studies introduced a strong restriction in the use of reverse genetics since avocado is not amenable to genetic transformation. The merit of Grierson's group is to have undertaken molecular studies using tomato as a model. This model has proved to be highly convenient for biotechnology and genomics studies. Rattanapanone et al. (1978) have been the first to describe in vitro translation products from mRNA of tomatoes. This has been followed by the isolation of the polygalacturonase (PG) gene and by the use, for the first time in plants, of the antisense technology to knock-down the expression of a gene (Sheehy et al., 1988; Smith et al., 1988). These studies have led to the commercial release of the first transgenic edible fresh product in the USA in 1994, an antisense PG tomato. Since then, the ability to transfer genes in fruit and vegetables has been largely exploited for both a better understanding of the ripening process and also for potential applications for the improvement of agronomic and quality traits. The book edited by Valpuesta (2002) gives a good overview of the scientific advances in the area and also of the issues of consumers' attitudes and risk assessments.

### **ROLE OF ETHYLENE AND DISCOVERY OF THE ETHYLENE BIOSYNTHETIC PATHWAYS**

The role of ethylene in fruit ripening has a long history (Abeles et al., 1992). However one important step has been made when the climacteric rise in respiration has been associated with an increase in ethylene production (Biale et al., 1954) and when it was demonstrated that climacteric fruit had the specificity, contrary to non-climacteric to produced autocatalytic ethylene (McMurchie et al., 1972). The intimate mechanisms, at the molecular level, of autocatalytic ethylene production are still not fully understood. Ethylene has long been considered as deriving from lipids, particularly linolenic acid which was supposed to be released from membranes disintegration during the ripening/senescence process through a metal catalyzed peroxydation (Lieberman and Mapson, 1962; Gaillard et al., 1968). Methionine was initially introduced as a possible free radical quenching agent to inhibit ethylene formation derived from lipid peroxidation in apple tissues. Surprisingly, instead of suppression, ethylene production was increased (Lieberman et al., 1965), leading to the demonstration that methionine was the precursor of ethylene in vivo. This discovery has been the starting point for an exciting adventure towards the elucidation of the ethylene biosynthetic pathway led by Yang's group. S-adenosylmethionine (SAM) was discovered as an intermediate in the biosynthesis of ethylene from methionine through an ATP dependent step. The last step in the pathway between SAM and ethylene was discovered by remembering early experiments showing that ethylene synthesis could be inhibited by the absence of oxygen. The incubation of apple tissues under nitrogen in the presence of L-[U-<sup>14</sup>C]methionine resulted in the formation of an intermediate compound that was identified as 1-aminocyclopropane-1-carboxylic acid (ACC). Upon return to aerobic conditions, apple tissues released labelled ethylene from ACC (Adams and Yang, 1979). The discovery of ACC has been one of the major events of the early 1980's. Further, it was demonstrated that the 5'methyl group of MTA was recycled back to methionine. This recycling process has been referred as the Yang cycle.

The discovery of the enzymes and genes involved in the ethylene biosynthetic pathway corresponded with the development of molecular biology and biotechnology. Until the early 1990s, all attempts to obtain genuine ACC oxidase (ACO) activity into a cellular fraction have failed. Activity was only measured on entire tissues in the presence of exogenous ACC and was referred to as ethylene-forming enzyme (EFE). This feature

led to the assumption that EFE was membrane bound (Lieberman, 1979; Yang and Hoffmann, 1984). The mystery of this enzyme was only fathomed once the gene encoding EFE protein has been isolated by Grierson and co-workers, giving a brilliant example of "reverse biochemistry". Among the ripening related cDNAs isolated from tomato fruit, the pTOM13 clone was selected as a putative ethylene biosynthetic gene based on its expression during ripening and upon wounding. The expression in tomato plants of an antisense construct of the pTOM13 cDNA resulted in reduced capacity to produce ethylene and significant delay in fruit ripening suggesting that this clone might encode the EFE protein (Hamilton et al., 1990). The ultimate identification of the gene was given by functional expression in *Saccharomyces cerevisiae* (Hamilton et al., 1991). Based on the sequence homology between pTOM13 and flavanone-3-hydroxylase genes and the demonstration that iron is an essential co-factor of EFE in vivo, a soluble EFE (now named ACO) activity could be obtained for the first time from melon fruit and measured in the presence of iron and ascorbate (Ververidis and John, 1991). Another major gene of the ethylene biosynthetic pathway, ACC synthase (ACS), has been isolated after total purification of the protein from wounded zucchini (Sato and Theologis, 1989). The protein being present at low levels, the authors enhanced the amount of ACS by wounding of the tissue, a procedure known to stimulate ethylene synthesis. This gives another example of a judicious exploitation in molecular biology of physiological data published long ago.

### **DISCOVERY OF THE ETHYLENE PERCEPTION AND TRANSDUCTION PATHWAY**

In an early work on the mode of action of ethylene, Burg and Burg (1967) concluded that biological activity of ethylene or other olefins such as propylene requires a double bond adjacent to the terminal carbon and hypothesised that ethylene binds to a receptor through a transition metal such as Cu(I). Saturable binding sites for ethylene have been detected in plant tissues using a method based on the competitive binding of <sup>14</sup>C- vs unlabelled ethylene (Sisler, 1979). However the biological relevance of these sites and their nature has never been discovered using biochemical and cell fractionation techniques (Sanders et al., 1991). Advances in the field have been made possible through the use of *Arabidopsis* ethylene insensitive mutants. By remembering the triple response effect of ethylene described long ago by Neljubow (1901) on pea epicotyls, Bleecker et al (1988) isolated the first ethylene-insensitive mutant. Thanks to a long "chromosome walk" the corresponding protein was isolated (Chang et al., 1993). Heterologous expression of the protein allowed to show that it could bind ethylene as predicted by Sisler (1967) and that it involved copper as predicted by Burg and Burg (1967). These very exciting data allowed the discovery of the ethylene receptors of fruit and of a number of elements of the ethylene transduction pathway (Klee and Clark, 2004). At least three lessons can be drawn from these experiments: (i) the *Arabidopsis* strategy was better adapted than the biochemical methods for the isolation of the receptor, (ii) former data have been very useful for the screening procedures of the mutants and characterisation of the protein and (iii) the *Arabidopsis* model has been critical for the understanding of the fruit ripening process by allowing the isolation of the fruit receptor(s).

Another interesting adventure has been the discovery of antagonists of ethylene action. It was reported that some cyclic olefins are capable of counteracting the action of ethylene among which 2,5-norbornadiene (Sisler and Yang, 1984). However this latter compound was efficient at high levels only and had a disagreeable odour. Another compound diazocyclopentadiene (DACP) was examined (Sisler and Blankenship, 1993) that was active but required activation by light. An ingenious experiment showed that degradation of DACP resulted in the formation of cyclopropenes that were in fact the active compounds. This led to the synthesis of a compound, 1-methylcyclopropene (MCP), which proved to be a powerful antagonist of ethylene action. It is used as a research tool for the understanding of the ethylene-regulated developmental processes (Blankenship and Dole, 2003) and also at a commercial scale for extending the shelf-life of fruits and flowers. MCP probably represents the most remarkable innovation in post-

harvest horticulture of the past two decades.  
(<http://www.hort.cornell.edu/departement/faculty/watkins/ethylene/>).

### **CO-OPERATION BETWEEN FRUIT PHYSIOLOGISTS AND GENETICISTS**

Breeding for quality and storage life has long been a secondary goal as compared to yield and disease resistance. However in the case of apple for instance, the chance seedling Golden Delicious which has good agronomic characters has been crossed with old apple varieties having good sensory attributes to generate new apple cultivars that combine good agronomic and good sensory characters. Similarly, the poor keeping qualities of Delicious has been extended by crossing with long keeping apples (Rall's Janet) giving rise to the Fuji group of apples (Vaysse et al., 2000). In tomato, many long or mid-shelf-life modern varieties have been obtained by introgression of the *rin* and *nor* mutations. However, the long shelf-life character is often associated with poor sensory qualities. In Charentais type melons, long or mid-shelf life commercial genotypes are available. Some of them have been generated using a non-ripening melon named "Vauclusien". More basic work aimed at identifying genetic factors of fruit quality and storability has developed slowly in the recent years. For instance, in apple fruit, an allele of the ACC synthase gene (*MdACS1-2*) has been characterized as a good marker of storability. Cultivars that are homozygous for this allele have a long storage life (Sunako et al., 1999). In winter pears the presence of some ACS alleles is correlated with the chilling requirements for ripening and induction of autocatalytic ethylene production (El-Sharkawy et al., 2004). QTL analysis of fruit quality in fresh market tomato indicated that a few chromosome regions are capable of controlling the variations in sensory traits (Causse et al., 2002). In melon, RFLP polymorphisms linked to the ACC oxidase gene have been determined that give good markers for the intensity of ethylene production and shelf-life (Zheng and Wolff, 2000). The genetic analysis of a population of recombinant inbred lines resulting from the crossing of climacteric and non climacteric *Cucumis melo* indicated that the climacteric character is dominant and conferred by two independent loci only (Périn et al., 2002). One of the predictable future trends is a tighter co-operation between fruit physiologists, molecular biologists and geneticists

### **THE "OMICS" PERIOD**

The recent development of high throughput technology for analysing genome structure and functions is starting to have an important impact on fruit research. A number of national and multinational programmes are now attempting to combine genomics, proteomics metabolomics and reverse genetic approaches to unravel the molecular mechanisms of fruit development. The implementation of these genome-wide (Alba et al., 2004) and metabolomic technologies (Overy et al., 2005), together with bioinformatic tools is expected to provide new understanding of the fruit developmental program and reveal the networks of interactions between different pathways leading to the build up of fruit quality traits. The most important programs are implemented on the tomato taken as a model species for fruit research. A multinational consortium was recently set up which made available centralised facilities for tomato ESTs and derived DNA chips. This allows now to unravel global changes in gene expression during fruit development and ripening and to mine and analyse the expression profiling data in order to cluster the complete set of genes involved in specific metabolic and regulatory mechanisms. By comparing differences between natural variants, ripening mutants, or introgression lines, genes that are essential for specific aspects of fruit ripening and their corresponding impacts on fruit metabolism will be identified (Fei et al., 2004; Overy et al., 2005). In addition, reverse genetics for high throughput functional identification of target genes are being developed among which the emerging TILLING (Targeting Induced Local Lesions In Genomes) technology is the most promising. Finally, the launching of the tomato genome sequencing project and the availability of the tomato genome sequence in the near future represents major breakthroughs which will likely change fundamentally our way to address the fruit research topics.

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