9 Systems Biology Approaches Reveal New Insights into Mechanisms Regulating Fresh Fruit Quality

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9.1 FRUIT RIPENING AND QUALITY

Fruit quality is defined by traits such as fruit size and composition and is the result of a complex network of biological processes, involving exchanges (transpiration, respiration, photosynthesis, phloem and xylem fluxes, and ethylene emission) between the fruit and its environment, tissue differentiation, and cell functioning (Genard et al. 2007). The quality of horticultural products can be defined using a number of descriptors and is measured by several physicochemical parameters; a further quality evaluation occurs during or following consumption, when properties such as

flavor and texture are considered (Struik et al. 2005). Although the genetic regulation of fruit development and ripening is well documented in some cases (Giovannoni 2004), fruit should also be examined through a system and a process-based modeling approach (Genard et al. 2007).

Fruit ripening is a peculiar phase of development with direct implications for a large component of the food supply and related areas of human health and nutrition, in which a coordinated series (or syndrome) of developmental and biochemical events lead to changes in color, texture, aroma, and nutritional quality (reviewed in Alexander and Grierson 2002, Barry and Giovannoni 2007) (Figure 9.1). The color change during fruit ripening is mainly due to the degradation of chlorophyll and dismantling of the photosynthetic apparatus that allows the unmasking of pigments, and the neosynthesis of different types of anthocyanins as well as of carotenoids such as β-carotene, xanthophyll esters, xanthophylls, and lycopene. The increase in flavor and aroma during fruit ripening is attributed to the production of a complex mixture of volatile compounds and degradation of bitter principles, flavanoids, tannins, and related compounds (reviewed in Defilippi et al. 2009, Prasanna et al. 2007). The taste development is due to a general increase in sweetness, which is the result of increased gluconeogenesis, hydrolysis of polysaccharides, especially starch, decreased acidity, and accumulation of sugars and organic acids, resulting in

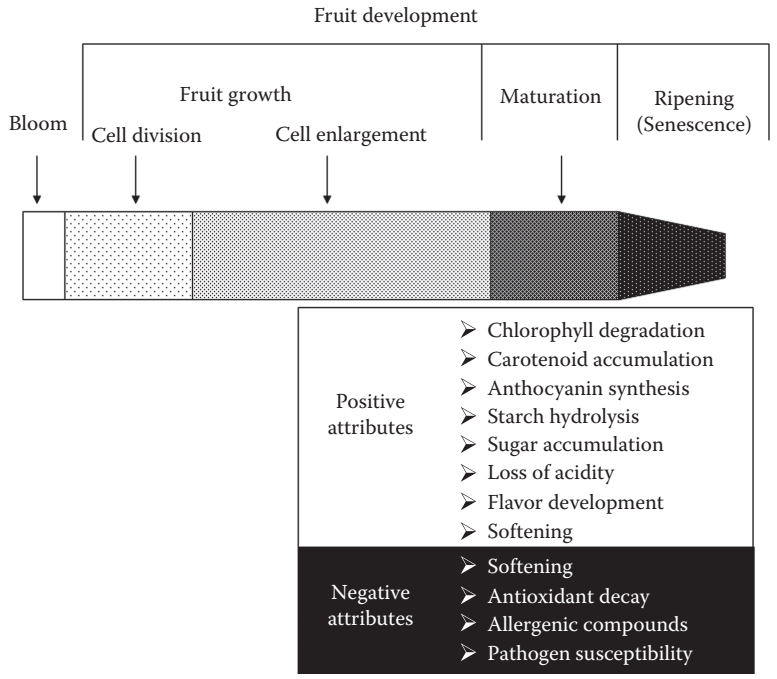

FIGURE 9.1 Main stages of fleshy fruit development. Maturity is a state achieved by the fruit at the end of maturation. Only mature fruits, when detached from the tree, can complete the ripening phase. The latter involves changes that transform the mature fruit into one that is ready to eat (positive attributes). However, these changes can also negatively affect some quality traits (negative attributes).

an excellent sugar/acid blend. The major textural change is the softening of fruit that implies an increase of susceptibility to pathogens and a lower tolerance to mechanical damage that may quickly render the fruit unmarketable.

Tissue softening of fleshy fruits is a developmentally programed process that involves cell wall disassembly and the coordinated and interdependent action of an array of hydrolytic enzymes (Brummell et al. 2004b). The role of individual cell wall–modifying enzymes in fruit softening and the composition of polymers in the fruit cell wall may differ between fruit species, leading to many different textures associated with ripe fruit even within cultivars of the same species (e.g., melting, nonmelting, and stony hard peaches). Modifications of the cell wall are believed to underlie changes in firmness and texture, but the type and magnitude of the alterations carried out during ripening may vary considerably. Pectin solubilization, depolymerization and demethylesterification, hemicellulose depolymerization, and neutral sugar loss are some common ripening-related cell wall modifications; however, other cell wall processes occur to very different extents or are absent in certain species (Vicente et al. 2007). Indicatively, the depolymerization of ionically bound pectin during ripening is almost undetectable in strawberry, banana, and apple; relatively slight in melon; moderate in tomato; and dramatic in avocado and watermelon, while the extent of pectin solubilization is high in kiwifruit, tomato, and plum and almost absent in apple and watermelon (reviewed in Brummell et al. 2004a).

Based on ripening mechanisms, fruits are characterized as climacteric and nonclimacteric. In the former, ripening is accompanied by a peak in respiration rate and a concomitant burst of ethylene, while in the latter, respiration shows no dramatic change and ethylene production remains at a very low level. As confirmed by biotechnology approaches, such as antisense (reverse genetics) technology, specific isoforms of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase, the key enzymes of ethylene biosynthesis, control ethylene production during the initiation and subsequent autocatalytic phase of ethylene production at fruit ripening (Lin et al. 2009). The complexity of ethylene action during ripening is confirmed by the activation of multiple receptors and signal transduction components. At the present time, the block of receptors, obtained by using substances such as 1 methylcyclopropene (1-MCP), proved to be a powerful tool in both basic (elucidation of fruit ripening syndrome) and applied aspects (extension of postharvest life) of fresh produce quality (reviewed in Huber 2008). However, although ethylene is the dominant trigger for ripening in climacteric fruit, both ethylene-dependent and ethylene-independent gene regulation pathways coexist to coordinate the process. For example, the suppression of ethylene production by antisense ACC oxidase RNA in "Charentais" melon has shown that, while many ripening pathways were ethylene regulated (e.g., aroma synthesis, climacteric rise of respiration rate and degreening of the rind), some were ethylene-independent (e.g., initiation of climacteric, sugar accumulation, loss of acidity, and pulp coloration) (Pech et al. 2008). These facts stimulated a reevaluation of the role of other hormones, besides ethylene, or their interactions in the ripening of climacteric fruit. Within this context, the ethylene/auxin cross-talk is becoming a critical point in the ripening regulatory network (Trainotti et al. 2007). Similarly, for nonclimacteric fruit, ethylene action has been deeply revised (Chervin et al. 2008, Maihlac and Chervin 2006), and its regulatory function is dependent on

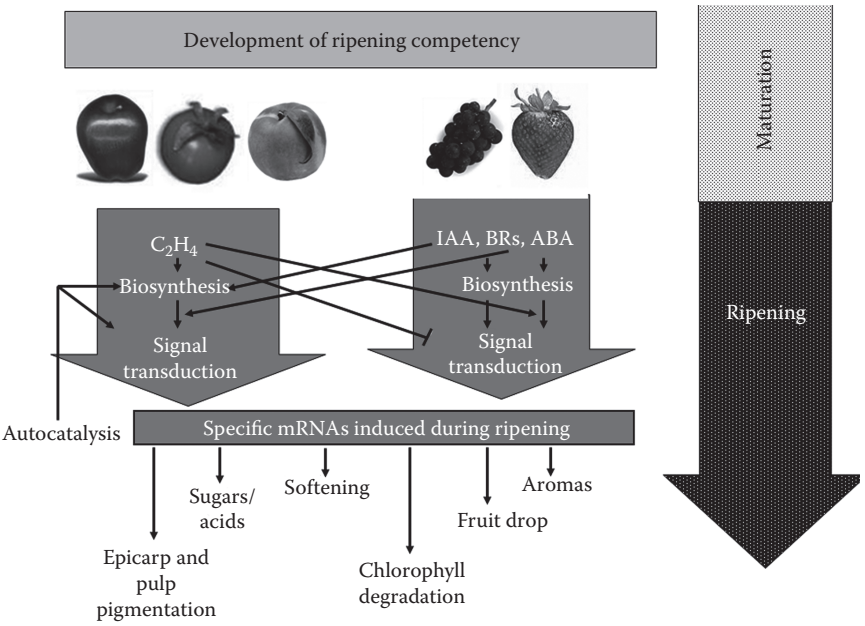

FIGURE 9.2 Model of interaction between factors involved in the ripening regulation of climacteric (apple, tomato, and peach) and nonclimacteric (grape and strawberry) fruits. Ripening competency is gained during the first phase of syndrome (maturation) that must be completed on the plant. Action of each hormone (ethylene, C_2H_4; auxin, IAA; brassinolides, BR; and ABA) and their interactions occur during the second phase (ripening) that can regularly proceed, also in the postharvest phase, as long as the fruit possesses the ripening competency.

the interaction with abscissic acid (ABA). In addition to ethylene, auxin, and ABA, significant involvement has also been proposed for jasmonates and brassinosteroids in climacteric (Ziosi et al. 2008) and nonclimacteric fruits (Symons et al. 2006), respectively. These results pointed out that all the hormone categories are directly or indirectly involved in the ripening of climacteric and nonclimacteric fruits, thus supporting the hypothesis of a model common for both fruit categories (Giovannoni 2001) (Figure 9.2). Furthermore, recent advances in ripening research have given insights regarding developmental signals responsible for the acquisition of the ripening competence (i.e., the state at which a mature fruit is capable of ripening in response to endogenous or exogenous hormones) mediated by transcription factors common to a number of fruit species (Martel and Giovannoni 2007).

In fruits, the transition from the immature to the mature stage is a crucial step involving the acquisition of edible traits and organoleptic quality. Due to the economic importance of fruit crop species, fruit ripening has received a substantial amount of attention from horticulturists, plant physiologists, biochemists, and molecularly oriented developmental biologists, having important practical and economical implications. Many fruits are harvested in the unripe (but about ready to ripe) state in order to assure good survival of storage and shipping conditions.

9.2 "OMICS" TECHNOLOGIES TO INVESTIGATE THE MOLECULAR AND ANALYTICAL BASIS OF FRESH FRUIT QUALITY

Quality traits are seldom subjected to modeling, since they are usually a result of poorly understood chain of processes, with only partly known, complex underlying mechanisms (Struik et al. 2005). The study of complex biological processes, such as fruit ripening, through comparative genomic studies is rapidly expanding, offering improved opportunities for gene identification and characterization (reviewed in Rose and Saladie 2005). The employment of state-of-the art techniques is particularly useful in defining processes that affect fruit quality.

Genomic technologies are currently being used in a range of crops coupled with other technologies such as genetic marker development and breeding lines in order to improve the quality of fruits and vegetables (Granell et al. 2007). Emerging genomic tools and approaches are rapidly providing new clues and candidate genes that are expanding the known regulatory circuitry of ripening (Adams-Phillips et al. 2004). Genomics are classically divided into two basic areas: (1) the characterization of the physical nature of whole genomes (structural genomics) and (2) the characterization of overall patterns of gene expression, usually indicated as transcriptomics. Besides RNA, targets of functional genomic studies are also proteins (proteomics) and metabolites (metabolomics). A multidisciplinary (systems biology) approach, including the coordinated approaches of transcriptomics, proteomics, and metabolomics, is instrumental for elucidating complex interplaying mechanisms that affect postharvest performance, taste, and flavor life of the horticultural commodities (Figure 9.3).

Transcriptome analysis represents an important approach that, in combination with other techniques, allows the elucidation and better understanding of complex physiological processes, as well as their genetic regulation (Blencowe et al. 2009, Forrest and Carninci 2009). The first works on transcriptome analysis were oriented to the alignments of expressed sequence tags (ESTs). *In silico* EST analyses have extensively been used to study fruit development and ripening and, to a less extent, the postharvest behavior and responses to different storage conditions. The *in silico* expression analysis is based on comparisons of tag frequencies in different libraries corresponding to an exact digital representation of the copy number of a transcript into the examined tissue. Large EST collections have been produced from many cDNA libraries of different tissues of several fruit species including apple, grape, melon, and tomato, where specific analyses of the sequence pool have been performed in relation to fruit development and, more specifically, to the ripening process. These analyses provide the primary tool for gene discovery especially for rapid screening of candidate genes related to interested quality traits. Usually candidate genes are isolated by studying associations between genes involved in relevant metabolic pathways and major genes or quantitative trait loci (QTLs) (Salvi and Tuberosa 2005). This strategy can be very time consuming and has been successful only infrequently. The comparison between ESTs obtained from mutant and wild individual with similar genetic background can make the identification of candidate genes easier (Eveland et al. 2008, Shi and Wang 2008).

Systems of high-throughput analysis of the transcriptome have appeared, thus providing more rapid and reproducible information on a large number of RNA sequences.

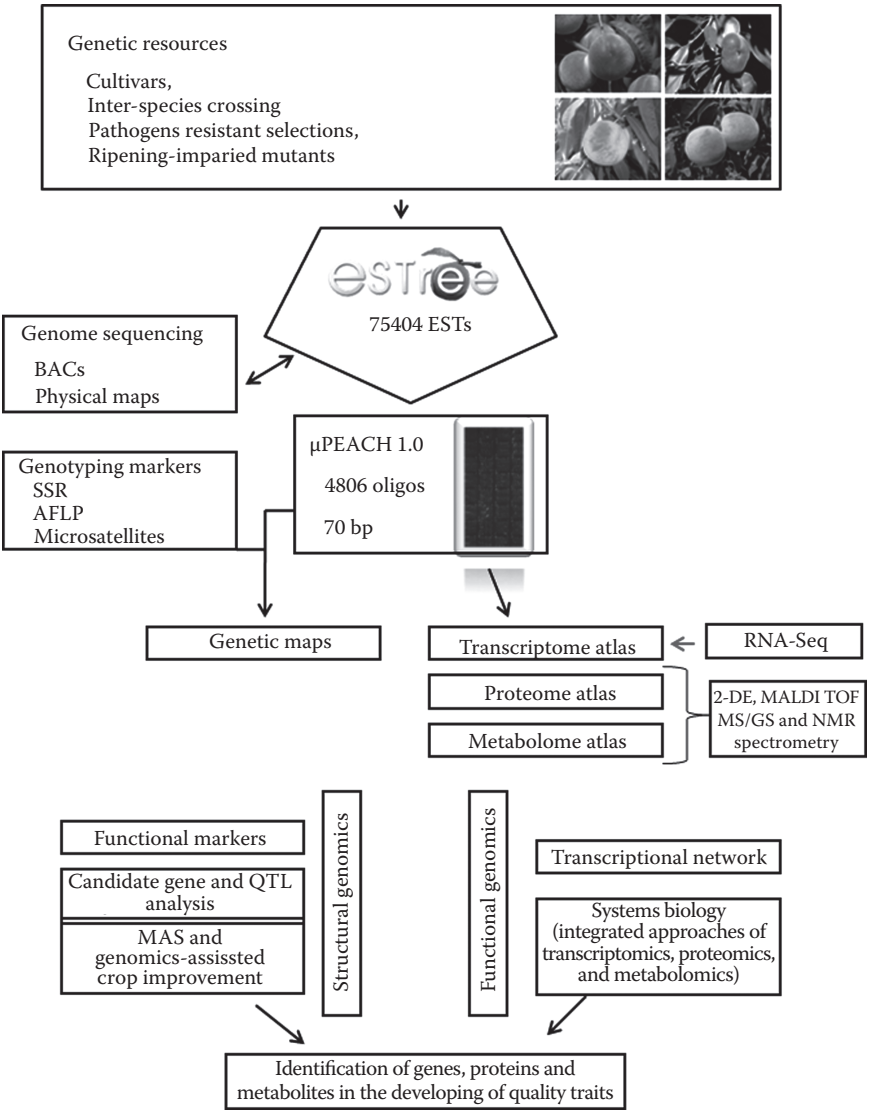

FIGURE 9.3 Diagram of genomic project for studying fruit development and ripening in fleshy fruits. The illustrated diagram refers to peach. In this species, genetics and genomic resources as well as tools, such as platform for transcriptome analysis, have been developed. These platforms can be employed to profile the transcriptome of other *Prunus* species taking into account the high levels of synteny present in this genus.

These studies can produce larger amounts of data in terms of gene expression and provide a good alternative for new transcriptomic studies in relation to candidate gene analysis and EST development. This is a crucial point for studying fruit physiology considering that most postharvest phenotypes are genetic traits associated with one or more genes. Therefore, the unraveling of the genetic determinants that confer

quality traits in fruits and other commodities is of prime importance for the development of new postharvest technologies. Among the tools developed for large-scale gene expression analysis, cDNA biochips (microarrays) are rapidly and successfully spreading because of their features and advantages mainly represented by the possibility of carrying out a massive gene analysis with a single experiment, thus avoiding the limits of the traditional single-gene approaches (Schena et al. 1995). Currently, most microarray profiling systems employ glass slides containing thousands of anchored sequences of interest (Bonghi and Trainotti 2006). The development of custom microarrays with probe sets designed to detect individual exons or using combinations of probes specific to exon and splice junction sequences was the strategy used by many groups whose research goal was the study of fruit ripening process (Bonghi and Trainotti 2006). Two different microarray hybridization strategies have been developed. The first, named two-channel microarrays (e.g., cDNA array), is performed by using a mixture of cDNAs prepared from two samples to be compared (e.g., treated tissue versus control tissue) and that are labeled with two different fluorophores. The second, named single-channel microarray (e.g., Affymetrix), provides intensity data for each probe or probe set indicating a relative level of hybridization with the labeled target. The comparison of two conditions for the same gene requires two separate single-dye hybridizations. Independently from the hybridization strategy adopted, the major drawback of microarray approach is that profiling coverage is strictly limited by the probe sets available for specific hybridization in each species. In some species, this limitation has been overcome thanks to the availability of the full genome sequence that allowed the development of new microarray platforms such as genomic tilling arrays based on a set of overlapping oligonucleotide probes representing the whole genome at very high resolution (Bertone et al. 2004). This approach could be very useful to investigate less known species at a molecular level belonging to a genus for which a full genome sequence is available and which is suitable as a reference. In addition, the sensitivity and specificity of this technology are limited by the fact that detection is indirect, generally measured by using a fluorescent signal, and is thus subject to a variety of confounding noise variables (Wechter et al. 2007). An alternative to microarrays approach is the massive (high-throughput) sequencing of transcriptome (RNA-Seq), allowed by a lowering of the costs thanks to the next-generation DNA sequencing platforms (Morozova and Marra 2008). Moreover, Wang et al. (2009) indicated that RNA-Seq, in contrast to tiling microarrays, has very low, if any, background signal because DNA sequences can be unambiguously mapped to unique regions of the genome.

The RNA-Seq approach yields a comprehensive view of both the transcriptional structure (e.g., identification of exons and introns and transcription start sites) and the expression levels of all the genes quantifying the number and density of reads corresponding to RNA from each exon (Nagalakshmi et al. 2008, Wang et al. 2009). With enough sequenced and mapped reads, it can detect and measure rare, yet physiologically relevant, species of transcripts (Mortazavi et al. 2008). In addition, RNA-Seq can be combined with other genomic and proteomic investigations to provide an integrated view of gene regulation (Fu et al. 2009, Hawkins et al. 2010). Furthermore, Werner (2010) indicated the great potential contribution of this technology to functional genomics with a special focus on gene regulation by

transcription factor–binding rates as well as other regulatory molecules such as small RNAs (sRNAs). The latter are small (19–24 nt) noncoding RNAs, organized into two classes named microRNAs (miRNAs) and short interfering RNAs (siRNAs), that play important roles in the regulation of various cellular processes by inhibiting gene expression at the posttranscriptional level (Jones-Rhoades et al. 2006). To date, large-scale analysis of expression of plant sRNAs and associated prediction of precursor sequences has been restricted to a small number of fruit species taking into account that extensive genomic information is required.

The study of complex biological processes, such as fruit ripening, through comparative proteomics is becoming increasingly attractive as the rapidly expanding plant genomic and EST sequence databases provide improved opportunities for protein identification (Rose and Saladié 2005). Numerous proteins involved in various metabolic pathways have already been reported for tomato, pepper, strawberry, grape, banana, apple, and pear. Better quantitative analyses and higher throughput proteomic technologies will further improve proteomic research in horticultural products, thus having significant impact on future breeding programs coupled with novel postharvest technologies in order to improve nutritional and eating quality (Song and Braun 2008).

As transcriptomics and proteomics aim to study the products of gene transcription and translation, metabolomic approaches aim at the quantification and the identification of all metabolites present within the cell under a given set of conditions; such approaches have emerged as a methodology that makes an important contribution to the understanding of complex molecular interaction in biological systems. Metabolites in plants function in many resistance and stress responses and contribute to color, taste, and aroma development. The employment of high-throughput techniques (gas [GC] or liquid [LC] chromatography coupled with mass spectrometry [MS] methods, nuclear magnetic resonance [NMR] spectroscopy, etc.) equipped with computer hardware and software that allow the interpretation of large datasets lead to the quantification molecules that govern quality attributes of horticultural products. Although only recently such high-throughput methods have been developed, metabolomic applications in plant biology and horticultural produce are rapidly increasing (Saito and Matsuda 2010).

Overall, genomic tools are commonly used in plant science to increasingly understand the biological basis of fruit ripening, both on- and off-tree. This chapter focuses on the molecular and analytical aspects of the main climacteric and nonclimacteric fruits in which systems biology approaches have been applied in the recent past.

9.3 MOLECULAR AND ANALYTICAL ASPECTS OF CLIMACTERIC FRUIT RIPENING

9.3.1 Tomato (*Solanum lycopersicum*)

Tomato is characterized as a model fruit for the ripening of climacteric fruits, where the most advanced functional genomic and proteomic tools for studying fruit ripening and quality traits have been applied. Currently, a combination of transcriptomic, proteomic, and metabolomic approaches has been applied in order to identify gene regions/QTLs relevant to tomato fruit quality (reviewed in Granell et al. 2007).

Positional cloning of genes defined by ripening defect mutations in tomato fruit system have led to the identification of novel ethylene signal transduction components, as well as to the elucidation of the function of unique transcription factors, affecting ripening-related ethylene production (reviewed in Barry and Giovannoni 2007). Tomato ripening mutations include (a) never ripe (*Nr*), (b) ripening inhibitor (*rin*), (c) colorless nonripening (*Cnr*), and (d) no ripening (*nor*). *Nr* phenotype, characterized by an ethylene insensitivity, is due to a dominant mutation in a member of the ethylene receptor gene family. The *rin* and *Cnr* mutations are recessive and dominant mutations, respectively, that effectively block the ripening process: *rin* encodes a partially deleted MADS-box protein of the SEPALLATA clade, whereas *Cnr* is an epigenetic change that alters the promoter methylation of a SQAMOSA promoter binding (SPB) protein. The *nor* locus harbors a gene with structural features suggestive of a transcription factor, although not a member of the MADS-box family. Fruit homozygous for either *rin* or *nor* or carrying a dominant *Cnr* allele undergoes complete fruit expansion and yields mature seed, yet fails to proceed in any significant way to ripening. However, both *rin* and *nor* are capable of ethylene synthesis in response to wounding, suggesting that the lack of ethylene-mediated ripening is attributed to the deficiency of appropriate developmental signals. These results, besides the fact that application of endogenous ethylene does not restore ripening to *rin*, *Nr*, or *Cnr* fruit but does result in the induction of ethylene-regulated genes, suggest that these mutants have a broader influence on aspects of climacteric ripening than those aspects controlled solely by ethylene (Giovannoni 2007).

Toward a better understanding of tomato gene expression in a plant system, a tomato functional genomics database (TFGD) has been recently generated (Fei et al. 2011). The TFGD includes all the tomato functional genomics resources organized in three major data components: gene expression, metabolite profiles, and sRNAs. Gene expression repertoire contains data obtained by using three microarray platforms (TOM1 cDNA array, TOM2 oligonucleotide array, and Affymetrix genome array) for a total of 1,308 hybridizations from 43 experiments. The employment of the TOM1 cDNA array platform showed that the *Nr*, which reduces ethylene sensitivity and inhibits ripening, altered the expression of 37% out of a total of 869 genes (Alba et al. 2005). The same research group also showed that the *Nr* affected fruit morphology, seed number, ascorbate accumulation, carotenoid biosynthesis, and ethylene evolution. Such data indicated the central role of ethylene on multiple aspects of development both prior to and during tomato fruit ripening, thus providing new insights into the molecular basis of ethylene-mediated ripening. The TOM1 cDNA array has also been employed for comparative transcriptomic studies with related Solanaceae species (pepper and eggplant fruit), and genes with central role in fruit ripening and development were identified (Moore et al. 2005).

A screening for genes, whose expression varied between lines genetically close but differing in fruit quality, showed that 39% of the unigenes corresponded to proteins that had never been isolated in fruit or with functions in fruit that were not clear or unknown. Since the BLAST comparison revealed that 41% of the unigenes were not included in this set, results revealed that some genes would not have been identified with commercially available microarrays and constitute new targets for fruit quality control (Page et al. 2008).

For metabolites, in addition to TFGD database (in which a total of more than 60 flavor and nutrition-related metabolites are collected), another database (MoToDB) was already available containing compounds that were detected in ripe tomato fruits of 96 cultivars using LC-MS (Grennan 2009, Moco et al. 2006). These databases appear to be of great help in studying the dynamics of metabolome to elucidate mutants and gene function based on differential metabolic profiles and to decipher the biological relevance of each metabolite. From this point of view, correlation analysis carried out between either the primary metabolites or the volatile organic compounds and organoleptic properties revealed a number of interesting associations such as aroma–guaiacol and sourness–alanine (Zanor et al. 2009). Furthermore, TFGD provides a central repository with tools for several large-scale tomato sRNA data sets (Fei et al. 2011, Itaya et al. 2008, Moxon et al. 2008, Pilcher et al. 2007). Among these, several conserved miRNAs showed fruit-specific expression, which, combined with target gene validation results, suggests that miRNAs may play a role in fleshy fruit development (Moxon et al. 2008).

The first characterization of the tomato fruit proteome and description of its variation during maturation included a comparative proteomic investigation on tomato fruits from a regional and commercial elite ecotype and specific proteins were recognized in each ecotype as differentially expressed during ripening (Rocco et al. 2006). A comparative analysis of the fruit pericarp proteome allowed 1,791 well-resolved spots to be selected, showing differential accumulation during cell division, cell expansion, and fruit-ripening stages (Faurobert et al. 2007). Ninety spots have been identified and most of these, showing an increasing accumulation at ripening, are related to carbohydrate metabolism or oxidative processes. A comparison between protein accumulation and expression profile of corresponding mRNA carried out on ripe tomatoes, using cDNA TOM1 microarray, indicated the presence of discrepancies between transcriptomic and proteomic data (Alba et al. 2005). Indicatively, 40% of the 90 identified varying spots corresponded to sequences present on TOM1 that had been classified as unchanged. These differences should be attributed to posttranscriptional and translational processes that modulate the quantity, temporal expression, and localization of proteins. As support to this fact, some results have shown that, even though a strong relationship between ripening-associated transcripts and specific metabolite groups (organic acids and sugar phosphates) was observed, posttranslational mechanisms dominate metabolic regulation during tomato fruit development (Carrari and Fernie 2006).

9.3.2 Apple (*Malus* × *domestica* Borkh.) and Related Species

Apple and pear fruit ripening involves complex biochemical and physiological changes and production of human health–promoting metabolites for which many molecular and genetic approaches have been undertaken to understand the associated cellular mechanisms.

The release of the apple ESTs into a public database has made possible large-scale expression studies, allowing the identification and characterization of genes with potential roles in fruit development, particularly those related to aroma production and protein degradation during ripening. Apple cDNA and oligonucleotide microarrays have been generated for more comprehensive examinations. Such tools

are powerful means for elucidating the molecular events involved in metabolite biosynthesis and physiological changes and may also enable researchers to understand how to control the ripening process (Seo and Kim 2009). For example, a gene expression analysis, using a microarray spotted with 6,253 cDNAs, showed that apple fruit development depends on the tight regulation of the expression of a number of genes, which are also expressed in other organs (Lee et al. 2007). Furthermore, an array designed from apple ESTs, representing approximately 13,000 genes, has been used to study gene expression during fruit development, and 1,955 genes showed significant changes in expression over this time course (Janssen et al. 2008). Intriguingly, the employment for comparative purposes of tomato microarrays during fruit development indicated that 16 genes showed similar pattern in apple and tomato; these genes may play fundamental roles in fruit development (Janssen et al. 2008). In addition, accumulating information about the involvement of some sRNAs in the apple fruit ripening process provides a better definition of the regulatory mechanism of expression operating in this process. In particular, sRNAs having target transcription factors such as AP2, TIR, SBP, and the ARF family, which are actively involved in the control of ripening, have been identified (Yu et al. 2011).

Taking into account that aroma governs apple fruit quality, the transcriptome approach has been used to analyze the expression of gene involved in (or related to) the aroma evolution. For this purpose, a transgenic line of "Royal Gala" apple was generated using an antisense ACC oxidase, harboring apples with no ethylene-induced ripening attributes including typical apple aroma volatile compounds such as terpenes. The comparison via microarray of untransformed and antisense ACC oxidase plants allowed the description of the expression profile of a repertoire of 179 candidate genes that might be involved in the production of aroma compounds. Among these, only 17 were typically affected by ethylene and most of them control the aroma biosynthesis, suggesting that only certain points (often the first and in all pathways the last steps) of this pathway are regulated by the hormone (Schaffer et al. 2007). The relation between ethylene and aroma was further investigated by Zhu et al. (2008). This research group examined the expression patterns of alcohol acyltransferase (*AAT*) and ACC synthase gene family members in two apple cultivars characterized by different levels of volatile ester production. The results pointed out that the climacteric expression of *ACS*1 greatly enhanced the expression levels of two alcohol acyltransferase (*AAT*1 and *AAT*2) genes that could be responsible for the emission of aromatic volatile esters. It was also suggested that the expression of *ACS*3 might play a role on induction of *AAT* gene expression during early fruit development as it is expressed prior to *ACS*1.

The complexity of the genetic control of important fruit quality traits has been confirmed by the analysis of apple genome (Velasco et al. 2010). In apple, as observed in other genomes, numerous events of duplication regarding genes involved in metabolism of anthocyanins and flavonoids, isoflavones and isoflavonones, terpenes, and carbohydrates occurred. For the latter, compared with other plant genomes, apple has considerably more copies of key genes related to sorbitol metabolism (71 in apple; while in other species, the number ranges between 9 and 43).

The availability of the full apple genome sequence with its synteny to the pear genome (Celton et al. 2009) has opened the way to efficient localization of a number of genes involved in pear ripening. This aspect is of paramount importance in pear

industry considering that fruits ripened on-tree generally do not develop the characteristic buttery and juicy texture required for marketing and consumption. Most European pears, unlike other climacteric fruits, possess varying degrees of resistance to ripening at harvest even when harvested at the appropriate maturity and require a period of chilling and/or ethylene exposure to ripen properly. This resistance to ripening poses a number of practical challenges for the pear industry in preparing the fruit for the market (Villalobos and Mitcham 2008).

The length of cold storage after harvest has a significant relationship with ethylene biosynthesis and the minimum chilling period required for normal ripening varies among pear cultivars. Pear ripening is associated with a burst of autocatalytic ethylene production. Some late pear cultivars such as Passe Crassane require a long (80 days) chilling treatment before the fruit will produce autocatalytic ethylene and ripen; therefore, cold requirement is linked to the capacity to respond to ethylene. Late ripening pears require several weeks at low temperatures and will then ripen rapidly at higher temperatures. In a few genotypes such as Passe Crassane, unchilled fruit will generally not ripen normally. In all cases, cold treatment stimulates ethylene biosynthesis that leads to ripening (Villalobos and Mitcham 2008).

Preclimacteric "Rocha" pears, stored under chilling conditions, had a larger increase of ACO activity and softened faster than those treated with ethylene. Nontreated fruit did not ripen or soften, acquired a rubbery texture, and showed barely detectable levels of ACO activity (Fonseca et al. 2005).

A collection of more than 25,000 ESTs from various tissues of the Japanese pear (*Pyrus pyrifolia* Nakai) cultivar "Housui," focusing on fruit tissues at several developmental stages have been put together from 11 different cDNA libraries. Several ESTs showing homology to genes involved in cell wall formation, phytohormone response and metabolism, transcriptional regulation, and other functions were also obtained (Nishitani et al. 2009).

Other "omics" approaches have been carried out in cultivars characterized by excellent organoleptic features in order to thoroughly investigate their genetic potential. An apple cultivar (cv Annurca), characterized by crispness, excellent taste, and long shelf life, was analyzed at the proteomic level. The results indicated 44 spots that were identified and associated to 28 different species. They were related to important physiological processes such as energy production, ripening, and stress response (Guarino et al. 2007). A metabolomic approach to characterize changes occur in an apple cultivar susceptible to superficial scald (cv. "Granny Smith") after 1-MCP or DPA treatment showed differentiation between treated and nontreated fruits within 1 week following storage initiation. α-farnese oxidation products with known associations to scald were associated with presymptomatic as well as scalded control fruit. The results demonstrate that extensive metabolomic changes associated with scald precede actual symptom development (Rudell et al. 2009).

9.3.3 Peach (*Prunus persica*) and Related Species

Among *Prunus* species, peach is becoming the model for molecular studies considering the huge amount of information regarding the ripening processes and especially the availability of molecular tools (microarray platforms) and,

recently, the release of genome sequence (IPGI consortium; see at http://services.appliedgenomics.org/projects/drupomics/).

To date, the largest collection of ESTs (79,580 out of 107,973 *Prunus* EST) has been generated in peach mainly starting from mesocarp at different development stages, and more than 28,000 putative unigenes (20,682 contigs and 7,709 singlets) have been detected. This information is compiled in the ESTree database (http://www.itb.cnr.it/estree), where ~200 ESTs have been selected for mapping on a physical framework map. A total of 33,189 single nucleotide polymorphisms (SNPs) have also been identified, and further analysis concentrated on a subset of different SNPs representing genes putatively involved in important aspects of secondary metabolism (Lazzari et al. 2008). Additional genes associated with other quality traits were identified with a candidate gene approach (Etienne et al. 2002, Ogundiwin et al. 2009) and comparative EST approaches (Trainotti et al. 2003, Vizoso et al. 2009). The EST repertoire was used for developing the first peach microarray (μPEACH1.0) and was initially used to identify genes differentially transcribed during the transition of nectarine fruit from preclimacteric to climacteric phase (Trainotti et al. 2006). A significant number (~30%) of these genes were not ethylene regulated (Trainotti et al. 2007) (Figure 9.4). The results indicated a dramatic upregulation of genes encoding transcription factors, belonging to several families including MADS-box, Aux/IAA, bZIP, bHLH, HD, and Myb, and enzymes involved in ethylene biosynthesis and action.

In climacteric peach and tomato fruits, it has been shown that, concomitant with ethylene production, increases in the amount of auxin can also be measured. A genomic approach has been used in order to understand if auxin affects peach climacteric ripening. The results showed that auxin plays a role of its own during peach ripening, having the ability to regulate the expression of a number of different genes, and the hypothesis that a cross talk between auxin and ethylene exist has been supported (Trainotti et al. 2007). A large-scale transcriptome analysis has been also conducted using μPEACH1.0 microarray on nectarine fruit treated with 1-MCP. This compound maintains flesh firmness but did not block ethylene biosynthesis. Microarray comparison of this sample with untreated fruit 24 h after harvest revealed that about 45% of the genes affected by 1-MCP at the end of the incubation period changed their expression during the following 48 h in air. Among these genes, an ethylene receptor (*ETR*2) and three ethylene-responsive factors (*ERFs*) were present, together with other transcription factors and ethylene-dependent genes involved in quality parameter changes (Ziliotto et al. 2008).

Peach has been also chosen as a model fruit to shed light on the physiological role of Jasmonates (JAs) during ripening. Results showed that exogenous JAs led to a ripening delay due to an interference with ripening- and stress/defense-related genes, as reflected in the transcriptome of treated fruit at harvest (Ziosi et al. 2008).

Peach fruit undergoes a rapid softening process that involves a number of metabolic changes. Storing fruits at low temperatures has been widely used to extend their postharvest life. However, this leads to undesired changes, such as mealiness (woolliness) and flesh browning, thus affecting the quality of the fruit (Manganaris et al. 2006). Transcriptomic studies have been extensively employed over the recent past in order to elucidate the molecular basis of the incidence of chilling injury (CI)

FIGURE 9.4 Among genes differentially expressed during the transition between preclimacteric (S3 stage) and climacteric stage (S4 stage) only a subset is controlled by ethylene. Data from Trainotti et al. (2007) were recomputed and visualized by using the Mapman platform. The overview pathway shows each category (listed on the right) of genes up- and downregulated during S4/S3 transition (above) and the effect of ethylene treatment, performed at S3 stage, on their transcription (below). The grayscale heat map shows changes in gene expression, calculated as $\log_2$ ratio in the S4/S3 and S3E (ethylene)/S3 air comparisons. Up- ($\log_2$ ratio >1) and downregulated ($\log_2$ ratio <–1) genes are displayed in light gray and dark gray, respectively, while those unchanged ($\log_2$ ratio between –1 and 1) in white. Only 50% of genes differentially expressed at S4/S3 transition are controlled by ethylene.

symptoms in peach fruit. The ChillPeach database was developed to facilitate the identification of genes controlling CI, a global-scale postharvest physiological disorder in peach. Microarray slides containing 4,261 ChillPeach unigenes were printed and used in a pilot experiment to identify differentially expressed genes in cold-treated compared to control mesocarp tissues, and in vegetative compared to mesocarp tissues. The microarray and qRT-PCR analyses indicated that ChillPeach is rich in putative fruit-specific and novel cold-induced genes and a web site (http://bioinfo.ibmcp.upv.es/genomics/ChillPeachDB) was created that has detailed information on the ChillPeach database (Ogundiwin et al. 2008). Other candidate genes for CI tolerance have been identified by using the μPEACH 1.0 platform. Falara et al. (2011) by comparing the transcriptome of peach fruit from "Morettini N°2" and "Royal Glory," two cultivars showing sensitivity and tolerance to CI, respectively, found that a β-D-xylosidase and PR-4B precursor could be related to the difference in tolerance to CI.

In addition, a comparative proteomic approach with 2-D DIGE allowed to point out differentially accumulated proteins in peach fruit during normal softening as well as under conditions that led to fruit CI proteins such as endopolygalacturonase, catalase, NADP-dependent isocitrate dehydrogenase, pectin methylesterase, and dehydrins, which were found to be very important for distinguishing between healthy and chilling-injured fruits (Nilo et al. 2009). A significant proportion of the proteins identified had not been associated with softening, cold storage, or CI–altered fruits before; thus, comparative proteomics has proven to be a valuable tool for understanding fruit softening and postharvest behavior.

The μPEACH1.0 was also used in comparative studies of fruit development of apricot and peach (Manganaris et al. 2011). When applied to μPEACH1.0, apricot target cDNAs showed significant hybridization with an average of 43% of spotted probes validating the use of μPEACH1.0 to profile the transcriptome of apricot fruit. Microarray analyses, carried out separately on peach and apricot fruits to profile transcriptome changes during fruit development, showed that 71% of genes had the same expression pattern in both species. Such data indicate that the transcriptome is quite similar in apricot and peach fruit, but also highlighted the presence of species-specific transcript changes. A similar comparative approach was used to dissect common and/or diverse mechanisms regulating plum (*Prunus salicina*) fruit ripening in genotypes characterized by different patterns of ethylene production (Manganaris et al. 2010).

9.4 MOLECULAR AND ANALYTICAL ASPECTS OF NONCLIMACTERIC FRUIT RIPENING

Notwithstanding the economic importance of nonclimacteric fruits like grape, citrus fruit, and strawberry, little is known about the mechanisms that regulate their ripening. Up to date, no growth regulator has emerged with a primary role similar to that played by ethylene in the ripening of the climacteric fruits. Strawberries can produce ethylene, although in limited amounts. Overall, similarities between nonclimacteric and climacteric fruit ripening exist and certain ethylene-dependent events in climacteric fruits are observed, apparently in the absence of or with extremely low levels of ethylene, in nonclimacteric fruits. Identification of additional components involved in ethylene signal transduction, the further characterization of ripening mutants, and additional studies on the biochemistry of ripening are essential for complete understanding of the ripening process. Therefore, understanding what controls these processes in nonclimacteric ripening may prove pertinent in gaining a full understanding of climacteric fruit ripening and vice versa (Alexander and Grierson 2002).

9.4.1 Citrus Fruit (*Citrus* spp.)

Citrus includes orange (*Citrus sinensis*), mandarin (*Citrus nobilis*), *Citrus aurantiumm*, *Citrus bergamina*, *Citrus grandis*, lemon (*Citrus limon*), *Citrus medica*, grapefruit (*Citrus paradisii*), as well as many interspecific hybrids. The major commercial traits in citrus include improved fruit quality, higher yield, and tolerance to environmental stresses (Terol et al. 2007). In terms of postharvest citrus fruit

performance, the existence of differential preformed mechanisms as well as inducible responses to cold temperature storage has been revealed with the employment of microarray analysis. These tools and analysis provide valuable information to design postharvest protocols and to introduce those candidate genes in the plant breeding programs (Granell et al. 2007).

The first functional genomic projects, initiated to approach the molecular characterization of the main biological and agronomical traits of citrus fruits, consisted in the generation of ESTs from different fruit tissues (Forment et al. 2005) or from cultivars with different ripening properties (Terol et al. 2007). The main positive effect of these projects was the development of cDNA platforms to profile changes in the transcriptome of fruit (whole or its tissues separately) during development and ripening or postharvest phase. A custom microarray platform, based on 366 genes of interest in peel pericarp and endocarp during three developmental stages of "Washington Navel" orange fruit, was used to profile peel-specific genes. Most highly differentially expressed genes were those involved in the modification of the cell wall architecture during growth and in the development of color and aroma (Goudeau et al. 2008).

In-house citrus microarrays have been developed to identify candidate for fruit quality genes and to study the impact of genetic background and environment on gene expression (reviewed in Granell et al. 2007). A Citrus GeneChip microarray, developed by using the Affymetrix technology, was employed to gain insight into the molecular mechanisms involved in the responses of citrus fruit to low-temperature storage (Maul et al. 2008). Exposure to chilling, besides the well-known gene dataset including those associated with cell wall turnover; pathogen defense; photosynthesis; respiration; and protein, nucleic acid, and secondary metabolism, enhanced the transcript levels of genes related to membranes; lipid, sterol, and carbohydrate metabolism; stress stimuli; hormone biosynthesis; and modifications in DNA binding and transcription factors as adaptive mechanism (Maul et al. 2008).

A RNA-Seq approach was used to identify and quantitatively profile small RNAs involved in the posttranscriptional mechanism responsible for the higher lycopene accumulation in the red-flesh sweet orange mutant (MT) in comparison to its wild type (WT). Comparative profiling revealed that 60 miRNAs exhibited significant expression differences between MT and WT. Among their target predictions, those implicated in carotenogenesis were present suggesting that this pathway is deeply altered in the red-flesh sweet orange mutant (Xu et al. 2010).

Proteomics can be used to study processes strictly related to fruit quality. Within this context, Katz et al. (2007) showed that in mature juice-sac cells, the decline in acidity is a consequence of the use of citric acid for the synthesis of amino acid and sugar. This process, together with the increase in invertase activity and sugar transporters, is part of a mechanism that maintains juice-sac cell sugar homeostasis. A combination of 2-DE and LC-MS/MS approaches was used to identify the differentially expressed proteome of a pigmented sweet orange (cv Moro) compared to a common cultivar (cv Cadenera) at the ripening stage. The comparison of the protein patterns of the 2 cultivars showed 64 differentially expressed protein spots. Most of the proteins related to sugar metabolism were overexpressed in "Moro" fruit, while those related to stress responses were overexpressed in "Cadenera" fruit. Proteomic results were compared with the known variations

of the same fruits at transcript level, outlining the existence of many discrepancies. Therefore, the necessity to associate both proteomic and transcriptomic approaches in order to achieve a more complete characterization of the biological system is essential (Muccilli et al. 2009).

9.4.2 Grape (*Vitis* spp.)

In the recent past, grape ripening was studied with holistic approaches based mainly on microarray chips obtained selecting suitable probes from the large EST collection present in the public databases (Deluc et al. 2007, Grimplet et al. 2007, Pilati et al. 2007). However, the availability of extended genome sequence (Jaillon et al. 2007) facilitated the analysis of transcriptomic data, rendering grape as a model fruit for studying the nonclimacteric ripening. Recently, the massively parallel sequencing technologies have been applied to dissect the wide range of transcriptional responses that are associated with berry development in *Vitis vinifera* cv "Corvina" (Zenoni et al. 2010). Through this approach, 17,324 genes (out of 30,434 protein-coding genes predicted in the genome) were expressed during berry development, and about 30% of these showed a stage-specific expression, suggesting a significant complexity in terms of transcriptional regulatory mechanisms. Among these mechanisms an important role is played by sRNA, thus an RNA-Seq approach, via 454 technology, was performed to identify sRNA actively transcribed during berry development (Carra et al. 2009). Stage-specific expression was observed for sRNA, 53 at green phase, 42 at veraison (the point where growth ends and ripening begins), and 40 at fully-ripe stage. Among miRNAs upregulated at the fully-ripe stage, an abundance of miR169 family members was observed. Li et al. (2008) showed that the transcription factor NFYA5 is targeted by miR169 and that overexpression of miR169 leads to excessive water loss through leaves and hypersensitivity to drought stress in *Arabidopsis thaliana*. In this light, the upregulation of miR169 family members might represent a system leading to a reduction in cell turgor, an event positively associated to the initiation of softening (Saladiè et al. 2007). In grape, the kinetic of water losses is also important in the postharvest phase when grape berries are subjected to withering, a technique used for the production of dessert and fortified wines. The molecular processes that occur during withering are poorly understood, so a detailed postharvest transcriptomic analysis of grape berries was carried out by AFLP-transcriptional profiling (AFLP-TP) analysis (Zamboni et al. 2008) as well as a microarray approach (Rizzini et al. 2009). These two approaches pointed out that genes involved in transcriptional regulation; hormone, carbohydrate, and secondary metabolism; transport; and stress responses are particularly affected by dehydration, demonstrating that grape berries are metabolically reactive to water stress even after harvest. Among the different secondary metabolic processes, those concerning the phenylpropanoid pathway are of paramount relevance in terms of quality properties in particular for red wines (Figure 9.5). Associated to withering, other postharvest treatments can be performed for modulating the metabolism of important molecules associated with grape berry quality traits. Among these, particular attention was paid to transcriptome changes induced by CO_2 (Becatti et al. 2010) or ethylene (Rizzini et al. 2010) application. In CO_2-treated berries, functional categorization and gene

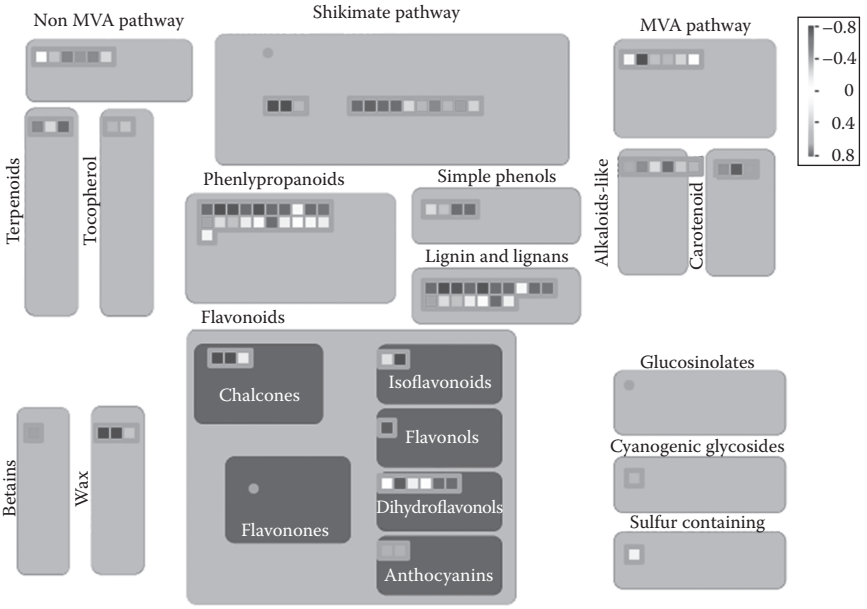

FIGURE 9.5 Expression of genes involved in secondary metabolism in the skin of dehydrated grape berries. Data adapted from Rizzini et al. (2009) were recomputed and visualized by using the Mapman platform. In the secondary metabolism pathway, genes involved in phenylpropanoids are significantly affected by dehydration (30% of water loss). The grayscale heat map shows changes in gene expression, calculated as $\log_2$ ratio in the dehydrated versus control berry skin comparison. Up-($\log_2$ ratio >0.8) and downregulated ($\log_2$ ratio <−0.8) genes are displayed in light gray and dark gray, respectively, while those unchanged ($\log_2$ ratio between −0.8 and 0.8) in white.

enrichment analyses pointed out that epicarp cells, in comparison to mesocarp ones, undergo more pronounced changes in transcript profiling at the end of the incubation period. In the skin, highly represented categories were fermentation, CHO metabolism, and redox regulation, while in both tissues the categories were those related to protein, stress, transcript, RNA, and hormone (ethylene, ABA) metabolism. Ethylene application affected the transcription of the majority of genes involved in phenylpropanoid biosynthetic pathway with the exception of the UDP-glucose:flavonoid 3-*O*-glucosyltransferase (*UFGT*) gene. This result is in contrast with the observation of El-Kereamy et al. (2003) who reported an upregulation induced at veraison by ethylene, suggesting that UFGT is differentially regulated during fruit development and ripening. Among upregulated targets by ethylene treatment, there are also some present that correspond to cell wall hydrolases. Considering their role in cell wall structure and architecture, the upregulation could be related to the increased extractability of polyphenols induced by ethylene and, consequently, to the different characteristics observed in the resulting wines.

Recently, grape berries have been studied throughout the integration of transcriptomic, proteomic, and metabolomic data achieved using a hierarchical clustering strategy based on the multivariate bidirectional orthogonal projections to latent

structures technique (Zamboni et al. 2010). This technique identified stage-specific functional networks of linked transcripts, proteins, and metabolites. In the case of ripening and withering, the characteristic accumulation of secondary metabolites such as acylated anthocyanins was confirmed. The accumulation pattern of this compound is strictly related to that of a BEACH transcript that encodes a protein that could facilitate the compartmentalization of anthocyanins through membrane trafficking. It was also pointed out that withering involves the activation of specific osmotic and oxidative stress response genes and the specific production of stilbenes and taxifolin.

To date, grape is probably the species in which "omics" technologies have been fully integrated in the dissection of genomic basis of fruit quality, thus providing a compendium of strategies for other species.

9.4.3 Strawberry (*Fragaria* × *ananassa*)

Following the pioneering work of Aharoni et al. (2000) who identified a novel alcohol acyltransferase (SAAT) gene responsible for flavor biogenesis in ripening strawberry using a cDNA microarray, some other cDNA-based arrays have been produced and used for transcript profiling during strawberry ripening. Using cDNA microarrays, containing 1,701 probes obtained mainly from red fruit, a comprehensive investigation of gene expression was carried out in strawberry fruit in order to understand the flow of events associated with its maturation (Aharoni et al. 2002, Aharoni and O'Connell 2002). In particular, the hormonal control of nonclimacteric fruit ripening and the differences in terms of transcripts between receptacle and achene tissues was dissected. The results emphasized the role of auxin and oxidative stress condition as regulatory elements of the ripening process. Gene sets specific for achene and the receptacle were different, for an enrichment of genes involved in the desiccation tolerance in the achene, while in receptacle tissue genes associated with the metabolism of ripening related compounds (pigments, cell wall components, fatty acids, volatile flavor, etc.) were largely represented. No further efforts have been reported for the improvement of microarray platform in strawberry. This could be important for further elucidation of the ripening biology of this nonclimacteric fruit since, as previously mentioned, the original 1,700 probes were all coming from ripe fruit. Furthermore, the microarray developed by Carbone et al. (2005) has the same bias toward ripening associated mRNAs, since it contains about 1,800 probes all derived from red fruit. New insights concerning valuable horticultural traits have been obtained after the completion of *Fragaria vesca* genome sequence (Shulaev et al. 2010). In particular, many structural genes responsible for the flavor production (acyltransferases, the terpene synthases, and small molecule *O*-methyltransferases) as well as transcription factors involved in their transcriptional regulatory mechanism have been identified.

9.5 FUTURE PERSPECTIVES

Taking into account the great variability that occurs within cultivars of the same species, the integration of genomic, metabolomic, and proteomic data will be indispensable for future molecular and analytical characterization and hence full

exploitation of the peculiar organoleptic, nutritional, and agronomic traits of produce from cultivars with diverse properties. Moreover, genomic approaches are expanding the gene pools available for crop improvement and increasing the precision and efficiency with which superior individuals can be identified and selected. Nevertheless, efforts are still required for full exploitation of existing bioinformatics platforms and for developing new ones that enable large-scale data sets to be meaningfully managed. In particular, methods for integrating large datasets from different biological systems or provided through different "omics" approaches need to be developed.

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