Contribution of genomics to postharvest biology

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Abstract
Purpose of review: This review aims at presenting the actual and potential contribution of genomics to the understanding of the fruit ripening process and to the genetic improvement of fruit quality and storability.
Findings: The advent of high throughput technologies for the sequencing of mRNAs and genomic DNA has sped up the study of gene expression and the decoding of the genome of fruit species. Genomic resources are now available that facilitate the definition of molecular markers for marker-assisted breeding, the functional identification of genes involved in fruit quality traits, the understanding of the network of events underlying the fruit ripening process and of the impact of external factors such as postharvest treatments.
Directions for future research: Up to now, the development of genomic tools for studying the fruit ripening process have been carried out mostly using tomato as a model fruit. There is a need for applying genomic methods to the understanding of fruit ripening in other species, particularly non-climacteric fruit. Efforts should also be directed towards the elucidation of the function of the genes *in planta* and of the regulation of their expression. So far, among the several hundreds of genes whose expression is altered during ripening, very few have well characterized functions. The number of genes for which a picture of the regulatory events is available is extremely limited.

Keywords: molecular markers; QTLs; genome sequences; epigenome; transcription factors; ripening mutants

Abbreviations

EST	Expressed Sequence Tags
MAS	Marker-Assisted Selection
NGS	Next Generation Sequencing
QTLs	Quantitative Traits Loci
TILLING	Targeting Induced Local Lesions IN Genomes

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doi: 10.2212/spr.2013.4.2

Introduction
The sequencing of the *Arabidopsis* genome [1] can be considered as the birth of plant genomics. Since then, a number of plant genomes have been sequenced, particularly in recent years, largely due to the emergence of new technologies for sequencing and the development of bioinformatics tools. In parallel, high throughput mRNA sequencing methods have emerged providing extensive information on the expression of genes during various processes, including ripening. This information complements the Expressed Sequence Tags (EST) databases already available for many species including fruit. Among the plants of economic interest, fleshy fruit have received much attention. Current challenges in postharvest biology of fruit ripening have been recently reviewed [2]. The aims of the present paper are to describe the new opportunities offered in postharvest biology by the release of these sequences. Contribution of the knowledge of genome sequences and gene expression data to the basic understanding of the fruit ripening process will be considered as well as potential applications in improving fruit quality and storability.

Table 1: Inventory of the whole-genome sequences available for fruit species.

Species	Varieties Genotype	Chromosome number	Genome size (MB)	Assembly and annotation	References
Grapevine (*Vitis vinifera)*	PN40024	19	487	Draft	[3]
Grapevine (*Vitis vinifera)*	Pinot noir	19	487	Draft	[4]
Papaya (*Carica papaya*)	SunUp	9	372	Draft	[5]
Apple (*Malus domestica*)	Golden Delicious	17	742	Yes	[15]
Strawberry (*Fragaria vesca*)	Hawaii 4	7	240	Yes	[8]
Tomato (*Solanum lycopersicum*)	Heinz 1706	12	900	Yes	[16]
Banana (*Musa acuminata*)	DH-Pahang	11	523	Draft	[9]
Melon (*Cucumis melo*)	DHL92	12	450	Yes	[10]
Sweet orange (*Citrus sinensis*)	Valencia	9	367	Yes	[11]
Peach (*Prunus persica*)	Lovell	8	265	Draft	[13]
Pear (*Pyrus bretschneideri*)	Suli	17	527	Draft	[14]
Water melon (*Citrullus lanatus)*	97013	11	425	Draft	[12]

Sequencing of fruit genomes and EST resources

The earlier sequencing methods have used a BAC-based approach and applied this to the sequencing of the Arabidopsis genome. This method has been replaced by the less expensive and faster whole genome shotgun strategy which has been typically used for grapevine [3, 4] and papaya [5] sequencing. Recently, the Next Generation Sequencing (NGS) technique has greatly improved the output/cost ratio of genome sequencing [6, 7**]. The first fruit that has been totally sequenced using NGS alone is strawberry [8]. It has been followed by banana [9], melon [10], orange [11], watermelon [12], peach [13] and pear [14]. The sequences of the apple [15] and tomato genomes [16] were initially performed using a BAC-based approach, then NGS. So far, the full sequence of the genome of twelve fruit species has been released (Table 1). Nevertheless, progress remains to be made in the assembly of the gene sequences on the chromosomes. Genome assembly and annotation greatly facilitate the use of information. When too many unassembled scaffolds are obtained, they are not anchored to the genetic map, which renders their exploitation difficult. For some species (tomato), the assembly is quite in an advanced stage, which is not the case for several other species. In addition, the annotation, in terms of gene functions, is far from being completed.

For several fruit species sequence data of ESTs have been generated and deposited in databases. An inventory of EST resources of horticultural crops, including fruit species, has been made by Sonah *et al.* [17*]. The EST sequences have been used for the generation of micro-arrays. However, with the advent of RNAseq technologies [18] that has allowed the high throughput sequencing of mRNAs, the use of micro-arrays has become obsolete. Having a large scale picture of gene expression during the ripening process or evaluating the effect of environmental factors on gene expression has become easily accessible.

Facilitating breeding for quality traits by defining molecular markers

Traditional plant breeding has greatly contributed to the improvement of major agronomic traits related to yield, pest resistance and sometimes fruit quality and storability. The development of genomics offers new possibilities by defining molecular markers that allow the localization of genes responsible for agronomic traits in the genome. Following the heritability of the markers in the progenies it has been named marker-assisted selection (MAS). The utilization of MAS speeds up the time for selection by allowing the analysis of a great number of progenies at an early stage of development.

Now that it is possible to rapidly sequence multiple individuals within a species with limited technical expertise and at minimal cost, the mining of the sequence generated and identification of variants provides opportunities to define genetic makers. Several databases for molecular markers are available for tomato (reviewed in [19]). A Single Nucleotide Polymorphism (SNP, one of the most currently used DNA markers), high density map for fruit quality has been generated for peaches [20]. In melon a genetic map enriched for fruit quality traits has been constructed. It comprises 668 DNA markers of which 160 were newly developed from fruit ESTs and from mining in the melon genome sequence [21], thus demonstrating the interesting contribution of new genomic tools. Genome-wide discovery of SNPs can be achieved by re-sequencing related genotypes within each species. Re-sequencing efforts are currently launched for several fruit species including tomato and grapevine. Using the reference genomes as guides, the identification of alleles contributing to specific characters, particularly fruit quality, will become easier. The application of genomics tools in plant breeding has been reviewed by Perez-de-Castro *et al.* [22**].

Exploiting diversity

The comparison of the cultivated tomato genome with the genome of its wild relative, *Solanum pennellii*, has been used for elucidating the evolution of a complex locus responsible for the biosynthesis of terpenes in tomato [23]. It was demonstrated that a cluster of genes contains genes for terpene synthase that evolved by duplication and divergence of ancestral terpene synthase genes and alterations in substrate specificity. Similarly, the role of an esterase in volatile variation has been studied within various species of the tomato clade [24]. Red-fruited species accumulate low content of acetate esters in comparison with the green-fruited varieties. This was related to the insertion in the green-fruited species of a retrotransposon close to the most enzymatically active esterase causing higher expression of the esterase and lower production of esters. These two examples demonstrate the benefits of exploiting genetic diversity for understanding the generation of aroma volatiles in fruit, and, more generally, for understanding the development of fruit quality attributes.

Large-scale identification of target genes for transcription factors

Thanks to the analysis of ripening mutants, a number of transcription factors regulating the ripening process have been identified [25]. One of the most interesting is the *ripening inhibitor (rin)* gene which corresponds to a MADS-box, MADS-RIN, controlling the fruit ripening developmental process and which is present in both climacteric and non-climacteric fruit [26]. Efforts have been made in recent years to identify of the target genes for the MADS-RIN protein. A method has been employed consisting of a chromatin immune-precipitation (ChIP) analysis using an antibody against the transcription factor protein. The use of this method has revealed that RIN forms a stable homodimer that binds to MADS domain-specific DNA sites. Analysis of binding site selection experiments indicated that the consensus binding sites of RIN highly resemble those of the SEPALLATA (SEP) proteins, which are Arabidopsis MADS box proteins that control the identity of floral organs [27]. It was also demonstrated that direct RIN target genes are involved in ethylene synthesis and signaling, cell wall modification, carotenoid accumulation, aroma formation, and transcriptional regulation of ripening-related transcription factors genes in tomato, including NOR, CNR, TDR4 and HB-1 [28–30].

Large-scale identification of direct RIN targets has been performed by chromatin immuno-precipitation coupled with DNA microarray analysis (ChIP-chip) targeting the predicted promoters of tomato genes [31**]. This allowed the identification of 241 direct RIN target genes that contain a RIN binding site and exhibit RIN-dependent positive or negative regulation during fruit ripening, suggesting that RIN has both activator and repressor roles. The predicted functions of RIN targets are numerous. They participate in the regulation of lycopene accumulation, ethylene production, chlorophyll degradation, and many other physiological processes. 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. Knowing the function of transcription factors in regulating the expression of ripening-related genes is of great interest not only for molecular breeding, but also for understanding the fruit ripening process.

Facilitating the characterization of quantitative trait loci and the cloning of candidate genes

The identification of genomic regions involved in quantitative traits, named as Quantitative Trait Loci (QTLs), has been achieved in several fruit species including fruit quality traits. The approaches used for identification of QTLs consist of the generation of introgression lines/advanced backcross populations by crossing inter- or intraspecific genotypes. Examples of QTLs for fruit quality include fruit texture of apple [32], pigment content of pepper [33], chilling injury in peaches [20], shape, soluble solids, and shelf-life of tomato [34], firmness of tomato [35], and the climacteric character in melons [36]. The search for QTLs most often exploits the genetic diversity by breeding varieties of the same species that differ in quality traits or by breeding cultivated genotypes with wild species. However, the QTL loci are generally very large and difficult to transfer genetically. In these conditions fine mapping of the QTL has proved necessary and in some occasions the candidate gene responsible for the trait has been isolated by positional cloning: eg, for tomato shape [37] and sugar content [38]. The recent advances in genomic resources will facilitate dissecting of the QTLs and cloning of the candidate gene(s).

The availability of the tomato genome sequence greatly facilitated the identification of candidate genes for fruit quality. As already mentioned, an esterase responsible for differences in volatile esters formation in different tomato species has been identified [24]. The Golden 2-like transcription factor was identified as responsible for the uniform ripening in tomato in great part thanks to the knowledge of the genomic sequence [39].

Accelerating the cloning of genes responsible for natural or induced mutants

The new methods of sequencing open the possibility for the direct sequencing of monogenic mutants and therefore have better accessibility than before to the genes responsible for the mutation. Several natural monogenic mutations have been characterized and the mutant gene identified, mostly in tomato [25]. However, several monogenic mutations of tomato still remain to be characterized. In other fruit, less progress has been made and studies have been only dedicated to the alteration of the expression of candidate genes suspected to be responsible for the ripening phenotype. For instance, extensive studies have been carried out on Fuji apples where mutations or alleles of ACC synthase genes appear to be involved in the long or short shelf-life behavior [40, 41]. However the phenotype is probably due to polygenic mutations

and the candidate genes identified cannot explain the full range of storage characteristics [42]. Many ripening mutants of other species have been characterized phenotypically and biochemically, but the gene actually mutated has not been identified (eg, peaches [43]; citrus [44, 45]; apple [46]. Besides natural mutants, collections of insertional, fast-neutron and EMS mutants are available, particularly for tomato [47]. The new genetic resources will facilitate the identification of the mutations.

The TILLING (Targeting Induced Local Lesions In Genomes) technology has been developed in the recent years for high throughput detection of point mutations. It consists, in the first steps, in generating a collection of ethyl methane sulphonate (EMS) mutants and in identifying interesting phenotypes. Then target genes are amplified by PCR using gene-specific primers in pools of mutants and mismatched heteroduplexes formed after annealing the PCR products from wild type and mutant plants are detected after cleavage by the Cel1 endonuclease, an enzyme capable of recognizing the mismatches and separation of cleavage products by electrophoresis. In these conditions the gene allele responsible for the phenotype can be identified.

TILLING collections have been generated for tomato [48, 49], for climacteric cantaloupe melons [50] and for non-climacteric "piel de sapo" melons [51]. Using the TILLING collection mutants described by Saito *et al.* [49], Okabe *et al*., [52] have isolated two allelic gene mutants of the tomato ethylene receptor (Sletr1-1 and Sletr1-2) resulting in reduced ethylene responses. Also, point mutations to the *DEETIOLATED1 (DET1)*gene*,* which is responsible for the high pigment2 (hp2) tomato mutant, have been detected in tomato by TILLING [53]. It resulted in elevated levels of both carotenoid and phenylpropanoid phytonutrients in ripe fruit, while immature fruit showed increased chlorophyll content and photosynthetic capacity and altered fruit morphology. Furthermore, genotypes with mutations to the UV-DAMAGED DNA BINDING PROTEIN 1 (DDB1), COP1 and COP1like were also characterized but they did not display phenotypes characteristic of mutation to light signaling components.

Gady *et al.* [53] through TILLING applied to an EMS-mutated collection have identified two point mutations in the phytoene synthase 1 gene (Psy1) gene corresponding to a knockout allele and an amino acid substitution. Plants carrying the Psy1 knockout allele show fruit with a yellow flesh color with no further change in color during ripening. In the line with amino acid substitution, fruit remain yellow until 3 days post-breaker and eventually turn red. A TILLING platform of Cantaloupe melons has allowed the characterization of a missense mutation in the ACC oxidase 1 gene (CmACO1) that inhibits fruit ripening and extends fruit storage life [50]. TILLING strategies could be included in breeding programs. They have the advantage of not employing genetic engineering methods for which consumers are often reluctant. The availability of genome sequences facilitates the characterization of new allele genes responsible for ripening characters. With the new sequencing technologies it is conceivable to directly sequence the TILLING mutants and have rapid access to the mutation.

Other functional genomics methods have been developed including insertional mutagenesis with the maize Ac/Ds transposable elements, fast-neutron mutagenesis, and chemical mutagenesis by EMS. Detailed information on the use and outputs of these methods in the case of tomato can be found in a paper by Eyal and Levy [47]. All the methods of functional genomics mentioned above have led to the identification of a limited number of genes conferring fruit quality traits. We are still far from understanding the role *in planta* of several hundred of genes that are differentially expressed in ripening fruit.

Epigenome

The first demonstration that epigenetics events (ie, not related to DNA sequences) participate in the control of fruit ripening has been provided by the characterization of the colorless non-ripening (*Cnr*) mutant [55**]. Cnr encodes a *SQUAMOSA* transcription factor. The mutation was not related to any genetic alteration but rather to an heritable hyper-methylation of *Cnr* promoter. It has recently been shown that the hyper-methylation of the promoter resulted in the inhibition of *Cnr* gene expression by preventing the binding of RIN transcription factor [56]. Interestingly, the infiltration of a methyltransferase inhibitor 5-azacytidine into tomato fruit resulted in premature ripening, indicating that epigenetic changes may occur during the fruit ripening process. A whole-genome bisulfite sequencing was performed on fruit in four stages of development, from immature to ripe [56] that demonstrated that the level of DNA methylation in the predicted promoter regions gradually declines during fruit ripening. In contrast, methylation remains high and unchanged not only in the *Cnr* mutant but also in the *rin* mutant. Furthermore, the binding sites for the RIN protein were often localized in demethylated regions indicating that the binding of RIN is dependent upon de-methylation for switching the expression of ripening-related genes.

These data on the epigenome offer novel possibilities in terms of breeding for improving shelf-life and quality of fruit by exploiting epigenetic variations.

Conclusion

Great advances have already been made in the understanding of the fruit ripening process at the molecular level. Genes participating in the development of fruit quality (eg, texture, aromas, etc) and in the hormonal regulation (ethylene biosynthesis and signal transduction, etc) have been isolated and functionally characterized. Progress has been also achieved in the identification of transcription factors that regulate the expression of fruit ripening-related genes although many interactions occur that are far from being elucidated. The advent of high throughput mRNA sequencing has increased

information in this area and will facilitate the inventory of regulatory factors.

The knowledge of the genome sequences of fruit species has made great progress in recent years. The identification of markers and candidates responsible for quality traits in genomic regions already identified will become faster and more efficient. Map-based cloning strategies will be greatly facilitated by using *in silico* analysis of the genomic sequences. The identification of natural or induced mutations (eg, generated by TILLING) for genes related to quality traits will also be much easier. Finally, data on the epigenomic regulation will be increasingly released that will offer novel possibilities for improving fruit quality.

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