REVIEW

Understanding development and ripening of fruit crops in an 'omics' era

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Next generation sequencing has revolutionized plant biology. Not only has our understanding of plant metabolism advanced using model systems and modern chromatography, but application of 'omics'-based technology has been widely extended to non-model systems as costs have plummeted and efficiency increased. As a result, important fundamental questions relating to important horticultural crops are being answered, and novel approaches with application to industry are in progress. Here we review recent research advances on development and ripening of fruit crops, how next generation sequencing approaches are driving this advance and the emerging future landscape.

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IMPACT OF NEXT-GENERATION SEQUENCING (NGS)

NGS has revolutionized biological research. Since the publication of the *Arabidopsis thaliana* genome sequence in 2000,¹ a deeper understanding of plant biology of both model and crop species has developed by the generation of genome, transcriptome, and epigenome data sets using NGS technology and new bioinformatics algorithms enabling the analysis of resulting data. The rate at which new genomes are being sequenced correlates with the rate at which the cost of the technology has declined (Figure 1). However, software and algorithm development has to some extent lagged behind and shifted limitations from data generation to analysis.² The plant genomes that have been sequenced to date are shown in Supplementary Table 1. Interestingly, the genomes sequenced in the last five or six years are mainly from species regarded as crops or non-model plants, in contrast with earlier sequencing efforts that concentrated on model plant systems. This change reflects the availability of new technologies and reduced costs as noted above allowing increased applied studies in crops of interest, in addition to more effective use of model systems. Excellent recent reviews focus on advances in genomics related to postharvest physiology and fruit ripening^{3,4} and impacts of genome sequencing on the research of crops and plants.^{2,5,6} Here, we focus on NGS technology as a driving force in the advancement of the fields of -omics scale research in fruit crops.

GENOME SEQUENCING

A direct result of NGS technologies is that sequencing approaches employing BAC-by-BAC-based sequencing methodologies have been replaced by whole-genome shotgun, especially in the last few years with longer read lengths and improved assembly algorithms. It is important to note that the quality of genome sequences can vary depending on the depth of sequence coverage, combinations of sequence methods employed, heterozygosity and complexity of the genome in addition to annotation inputs and methodology, are among the most influential factors. Eighteen fruit genome sequences have been published to date, including grape,^{7,8} papaya,⁹ cucumber,^{10,11} apple,¹² strawberry,¹³ date

palm,¹⁴ tomato,¹⁵ melon,¹⁶ banana,¹⁷ Chinese plum,¹⁸ pear,^{19,20} watermelon,²¹ peach,²² kiwifruit²³ and pepper.²⁴ However, further progress on genome assembly as well as both physical and functional annotation, which greatly facilitates the use of the information, is needed. The tomato genome assembly is the most complete of all fruit species sequenced so far, but functional annotation of the gene models for this species remains incomplete. The assembly of the apple genome, which was sequenced using a similar strategy to tomato, initially BAC-by-BAC followed by whole-genome shotgun and NGS approaches, has been hampered by heterozygosity and recent gene duplications.¹² The assembly of this genome consists of relatively small contigs (averaging ~7 kb for apple, vs. ~28 kb for tomato), so building these into a scaffold which is routinely carried out during genome assembly has been difficult, and has led to a high level of redundancy. The estimated number of gene models for apple is relatively high, with 57 386 predicted,¹² but recent transcriptome analysis of apple using NGS has revealed an additional 17 524 genes that were not predicted from the original genome assembly.²⁵ Further sequencing and genome assembly is required to better understand the real number of genes encoded in the apple and many plant genomes.

MAP-BASED CLONING

The availability of genome sequence enables the possibility for accelerated cloning of genes that characterize a mapped locus. Traditionally, map-based cloning utilizes DNA-based markers that have been mapped to fairly precise areas of plant genomes. Mutants are crossed into other backgrounds over many generations until a small area is fine mapped and can be sequenced using more traditional means, including Sanger sequencing. This approach has been successful for isolation of many ripening mutant loci, especially in tomato,²⁶ as is covered in following sections of this review. In other fruit species, less progress has been made and such studies have focused on candidate genes responsible for ripening-related phenotypes based on their change in expression. The ACC synthase (ACS) alleles in apple have been extensively studied and appear to be involved in shelf-life periods.^{27–29} However, polygenic

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Figure 1. Sequencing cost and sequenced plant genomes this millennia. **(a)** The cost of sequencing a human genome during the twenty-first century, data uploaded from the National Human Genome Research Initiative (NIH, <http://genome.gov/sequencingcosts>). **(b)** Increase in the number of sequenced plant genomes.

mutations are the likely cause of these phenotypes, as the allelic diversity of ACS could not fully explain the full range of shelf-life attributes.³⁰

As well as naturally occurring mutants and variants, collections of insertional, ethylmethane sulfonate (EMS) and fast neutron mutants are available, particularly for tomato.³¹ The availability of new genomics resources enables identification of the genes responsible for mutant phenotypes at a much faster rate, with a sequenced genome, taking more advantage of fine mapping with fewer inputs due to increased marker diversity. One recent example was the cloning of a *GOLDEN-LIKE2* (*GLK2*) Myb super family transcription factor regulating plastid development and underlying the *uniform* ripening (*u*) mutant in tomato.^{32,33} When a normal *U* gene is expressed in tomato, dark green areas at the pedicel end of the fruit (shoulders), attributed to higher levels of chlorophyll and numbers of chloroplasts, are prevalent. This mutant has been bred into most commercial cultivars as it results in more even, or 'uniform', ripening. Interestingly, *U* gene expression manifests in a latitudinal gradient in the fruit (higher at the pedicel end compared with the stylar end) opposite to the gradient observed during ripening (from bottom to top) yet consistent with the green shoulder phenotype.³³ Transcriptome analysis using RNAseq of these tissues has revealed ripening-related genes that follow a gradient pattern and some novel candidate genes for gradient control were identified, particularly in the more mature stages of ripening.

FUNCTIONAL GENOMICS OF FRUIT RIPENING

Transcriptional control of ripening

The availability of mutants has been critical to elucidating gene function in plants, and tomato has one of the best collections of characterized fruit ripening-related mutants (maintained and accessible through the Tomato Genetics Resource Center at the University of Davis (<http://tgrc.ucdavis.edu/>)). Recent reviews describing some of these and the transcriptional control of ripening are available.^{34–36} In brief, several single locus mutations, resulting in ripening inhibition phenotypes have facilitated understanding of transcriptional control upstream of the required ethylene response for fruit ripening in tomato. Most notable are three ripening mutants, *ripening-inhibitor* (*rin*), *non-ripening* (*nor*) and *colourless non-ripening* (*Cnr*), which show severely inhibited ripening, do not produce elevated endogenous ethylene, or ripen in response to exogenous ethylene, and produce fruit that remain firm and green for extended periods.^{37,38} However, these mutants show ethylene responsiveness at the level of gene expression indicating they normally control aspects of ripening control in addition to those mediated by ethylene.²⁶ The *rin* gene was the first cloned,³⁹ and is important not only as a research tool but also commercially. *RIN/rin* hybrid tomato cultivars delay ripening and extend storage and shelf-life. *RIN* encodes a MADS-box transcription factor protein of the SEPALATTA clade.⁴⁰ *Cnr* is a rare epigenetic mutation, resulting

from heritable hyper methylation in the promoter of the *Cnr* locus SQUAMOSA binding protein (SBP/SPL) causing greatly reduced transcription.³⁸ The expression of *CNR* is reduced in the *rin* mutant indicating that it may act downstream of RIN-MADS³⁸ and *CNR* is also required for RIN binding to target promoters.⁴¹

Global effects on ripening pathways, including ethylene independent metabolism, suggest that both RIN and *CNR* proteins are central to ripening regulation, and conserved through evolution in both climacteric and non-climacteric fruit species. Strawberry is probably the most studied non-climacteric fruit, and a ripening-related MADS-box sequence (*FaMADS9*)³⁹ was silenced resulting in inhibition of fruit development and ripening.⁴² This finding provides evidence that transcriptional regulators such as RIN are conserved in both climacteric and non-climacteric species. Furthermore, identification of ripening-related MADS-box genes in maturing banana peel and pulp tissue indicates that RIN function may also be conserved between fleshy fruited monocot and dicot species.⁴³

Ripening control by ethylene

Ethylene is gaseous plant hormone playing roles in many response and developmental processes within plants. The role of ethylene in regulating fruit ripening in climacteric species is well documented.^{34,36,44} The biosynthetic pathway, perception and signal transduction of ethylene has been described, and the associated genes cloned and characterized in many plant species. Most studies have relied on homology to *Arabidopsis* sequences in identifying orthologs. This has been very successful, particularly in tomato. The biosynthesis of ethylene starts with the first dedicated and rate-limiting step of the pathway, the conversion of S-adenosyl-L-methionine to ACC by activity of ACS.⁴⁵ Subsequently ACC is converted to ethylene by activity of ACC oxidase (ACO). The transcriptional regulation of ACS is a major control point of ethylene synthesis,^{46–48} as it is encoded by a differentially expressed gene family of at least eight in tomato, two of which are upregulated during ripening (*LeACS2* and *LeACS4*).⁴⁸ At least four family members have been isolated from apple,^{27,49} of which *MdACS1* and *MdACS3* have received most attention as they are expressed in fruit.^{29,50,51} Suppression of ACS using an antisense construct for *LeACS2* was successful in tomato.⁵² ACO is also encoded by a differentially expressed gene family in both tomato and apple: at least four genes exist in tomato, two of which are expressed to high levels during ripening (*LeACO1* and *LeACO3*);⁵³ and at least four genes exist in apple, two of which are expressed in fruit (*MdACO1* and *MdACO2*).^{50,54,55} Despite ACO genes being transcriptionally upregulated by ethylene and ripening, ACO activity is generally not rate limiting in ripening fruit. Although this is the case, suppression of ACO results in reduced ethylene and delayed ripening in a number of fruit species including tomato, melon, apple, papaya and kiwifruit.^{56–60} Availability of the tomato genome sequence has led to *in silico* discovery of a further three putative ACS genes and another three putative ACO genes.¹⁵ Functional attributes of these genes remain to be elucidated.

Ethylene signaling starts with its perception by receptors; a multi-gene family, of seven in tomato.^{61,62} These receptors are differentially expressed,^{63,64} three are induced during ripening (*LeETR3/Nr*, *LeETR4* and *LeETR6*), and are negative regulators of ethylene. In the absence of ethylene, responses are repressed, but in the presence of the hormone, binding to receptors occurs and suppression of the response via a kinase cascade is reversed. Suppression of both *LeETR3/Nr* and *LeETR4* using a fruit-specific RNAi construct resulted in early ripening in transgenic tomato.⁶⁵ It has also been shown that the receptors are targets of protein degradation via the 26S proteasome pathway⁶⁴ and that *LeETR4* protein is phosphorylated during ripening and exogenous ethylene application.⁶⁶ Although the exact mechanisms of this protein turnover is not fully understood, it is

tempting to speculate that phosphorylation of the receptor labels them for ubiquitination and degradation. Further, it has been observed that both *ETR3/Nr* and *LeETR1* proteins interact with a tetratricopeptide repeat protein (*SITPR1*) in a yeast two-hybrid assay.⁶⁷ These classes of proteins are involved with ubiquitination of targets during 26S proteasome mediated degradation. However, when overexpressed, increased levels of *SITPR1* protein did not cause any ripening-related changes, indicating the requirement of additional regulators in ethylene receptor turnover.⁶⁴

The identification of the gene underlying the GREEN-RIPE (GR), a fruit-specific and ethylene-insensitive mutation of tomato was the first example of a novel ethylene signaling component discovered in a species other than *Arabidopsis*.⁶⁸ Its homolog was also described in *Arabidopsis*.⁶⁹ The function of GR is unknown, although its sequence suggests that it is membrane localized and has potential copper binding activities. It has been hypothesized that since the ethylene receptors are bound to the membranes of the endoplasmic reticulum,^{70,71} and that copper ions are required for their activity, GR may influence copper binding to the receptors.

Following perception of ethylene, downstream signaling takes place via a *CONSTITUTIVE TRIPLE RESPONSE1* (*CTR*) map kinase kinase family of three genes in tomato.⁷² It has been shown that *ETR3/Nr* in tomato interacts directly with multiple tomato *CTR* proteins,⁷³ supporting the hypothesis that ethylene receptors directly transmit a signal to the downstream *CTRs*. Later steps of ethylene signaling are mediated by the positive regulators *ETHYLENE INSENSITIVE 2* (*EIN2*) and *ETHYLENE INSENSITIVE 3* (*EIN3*). *CTR* negatively regulates *EIN2* and there is just one *EIN2* gene in both *Arabidopsis* and tomato through which all ethylene processes are transmitted. In tomato, *EIN2* expression increases at the onset of ripening.⁷⁴ *EIN2* then activates *EIN3* encoded by a family of four *EIN3-like* genes in tomato. Antisense suppression of tomato *EIL1*, *EIL2* and *EIL3* reduces ethylene sensitivity. All of these genes are expressed throughout the plant suggesting there is some functional redundancy in tomato.⁷⁵ A family of two *EIN3* BINDING FACTOR (*SIEBF1* and *SIEBF2*) *FBOX* proteins target the *EIN3* protein for degradation via the 26S proteasome and are functionally redundant in tomato. Repression of *SIEBF1/SIEBF2* resulted in constitutive ethylene responses and early ripening, indicating that ethylene signaling was activated by the stabilization of the *EIL* proteins.⁷⁶ The *EILs* transcriptionally activate a large family of *ETHYLENE RESPONSE FACTORS* (*ERFs*), due to the large number of family members some degree of functional redundancy is likely.³⁴

TRANSCRIPTOMIC STUDIES REVEAL NOVEL REGULATORS OF FRUIT DEVELOPMENT

There are numerous databases for fruit species where EST sequences have been deposited, and an inventory of these resources for horticultural crops is available.⁷⁷ These EST sequences have been used for electronic gene expression data sets and for establishing microarrays for analyzing the expression of large numbers of genes in any given sample. However, the target sequence and hybridization limitations of microarrays, combined with the availability of NGS-based RNAseq technologies, has meant that this method has been largely replaced in most systems. Establishing a large-scale picture of gene expression during ripening, or in response to treatment or environmental stress, has become feasible. However, there are still relatively few reports in the ripening literature describing RNAseq, a situation that is likely to change rapidly, especially as no prior sequencing is required for a *de novo* build of a reference transcriptome, in the absence of an available genome sequence.^{25,78} In addition, open access tools that provides users a platform for interactive large scale genome analysis further enable genome scale analyses.⁷⁹ Examples of how transcriptomics has driven the discovery of novel ripening regulators has been especially

effective in tomato with numerous examples including *LeHB1*, *TAGL1*, *FUL1/FUL2*, *SIAP2* and *SIERF6*.^{80–84}

Forward genetics has been useful in characterizing non-lethal mutants, but reverse genetics, has enabled the identification of many novel regulators of fruit ripening as noted above. For example, an HD-zip homeobox protein (*LeHB1*) was identified that binds to the promoter of the ripening-related ACC oxidase, *LeACO1*.⁸⁰ Antisense suppression of *LeACO1* in tomato results in reduced ethylene production and inhibited ripening.^{56,85} Previous studies have failed to demonstrate strong RIN binding to the *LeACO1* promoter; however, RIN was observed to interact with *LeHB1* promoter sequences suggesting a RIN-*LeHB1*-*LeACO1* regulatory pathway in tomato during ripening.⁴¹ Putative *LeHB1* binding sites are also present on a number of other ripening-related genes including *LeACO2*, *LePG1*, *MADS-RIN* and *NOR*; therefore, it is possible that *LeHB1* might also regulate these ripening-related genes directly.⁸⁰

Like *LeHB1*, the *Tomato AGAMOUS-like 1 (TAGL1)* gene, a ripening-related MADS-box transcription factor of the AGAMOUS clade, was identified through exploration of the tomato genome and its fruit ripening-associated expression profile. *TAGL1* is expressed at high levels during early carpel development in addition to ripening associated increase. Suppression of *TAGL1* using RNAi led to both ripening inhibition and reduction of carpel thickness due to reduced cell layers,^{81,86,87} indicating roles in both early and late fruit development associated with both early and late peaks of expression. *TAGL1* shares the AGAMOUS clade in tomato with *TAG1*, the tomato ortholog to the *Arabidopsis* AGAMOUS gene. *TAG1* is expressed in ripening fruit and induced in the absence of *TAGL1* expression, indicating possible functional redundancy. *TAG1* was shown to have a similar function in carpel development to its *Arabidopsis* ortholog,⁸⁸ however, its suppression caused the homeotic conversion of carpels to petals and therefore analysis of fruit development was not possible. However, overexpression of *TAG1* caused accumulation of lycopene, and fleshy expansion of sepals⁸⁸ similar to the ectopic expression of *TAGL1* homologs of both tomato and peach.⁸⁹ The use of fruit specific promoters in the characterization of *TAG1* in tomato would be useful to repress its expression without pleiotropic effects on the other aspects of plant development.

In addition to *RIN*, *TAG*, and *TAGL1*, *FRUITFUL1 (FUL1*; formerly named *TDR4*) and *FUL2* (formerly named *MBP7*) are MADS-box transcription factors that play a role in regulating ripening.^{90,91} Silencing of *FUL1* results in impaired ripening in tomato, and a ripening-related *FUL* homolog exists in bilberry.⁹² Further, it was revealed that *FUL1* and *FUL2* interact with *RIN*.⁸² A transcriptome and ChIP-chip analysis in *FUL1/FUL2* suppressed lines identified *FUL1/FUL2* target genes that exhibited *FUL1/FUL2*-dependent expression during ripening. Further, *in vitro* protein binding assays revealed that *RIN*, *TAGL1*, *FUL1* and *FUL2* form DNA binding complexes suggesting a tetramer complex of these MADS-box proteins are responsible for transcriptional activation of ripening-related genes.⁸² A bZIP transcription factor from tomato, *AREB1*, is regulated by abscisic acid and was shown to be expressed during stress⁹³ and in seed and fruit tissues.⁹⁴ Overexpression of *AREB1* resulted in altered primary metabolism indicative of mature fruit, in immature fruit, implicating ripening regulation by abscisic acid.⁹⁴ A negative regulator of the ripening process was also identified based on its expression profile from a transcriptomic study, *SIAP2a*, a tomato *APETELA2 (AP2)* gene family member.^{83,95} *SIAP2a* is induced during ripening, however, silencing using RNAi caused higher levels of ethylene and carotenoids culminating in advanced ripening status. It was proposed that *SIAP2a* functions as a modulator of ripening, thereby providing some balance to the positive action of other ripening regulators such as *RIN*.⁸³

EPIGENETIC CONTROL OF FRUIT DEVELOPMENT AND RIPENING

Epigenetics is a relatively new area of research and can be simply defined as the study of changes in genomes and their activities, not caused by changes in the underlying DNA sequence. Unlike the effects of the DNA sequence (the genotype), changes in gene expression or cellular phenotype mediated by epigenetics are rooted in genome structure, packaging and non-sequence-based modifications. Examples of mechanisms that produce such changes are DNA methylation, acetylation and histone modifications, each of which can influence singularly and together, how genes are expressed without altering the underlying DNA sequence. Such changes may last through cell divisions for the duration of the cell's life, and may also last for multiple generations even though they do not involve changes in the underlying DNA sequence of the organism, instead, non-genetic factors cause the organism's genes to behave (or 'express themselves') differently. Recently, epigenome dynamics have been shown to occur during fruit development and impact ripening.

There is growing evidence that fruit ripening is influenced by the epigenome. The first evidence was reported by Manning *et al.*,³⁸ who showed the *Cnr* mutation in tomato is an epi-allele causing inhibition of ripening through heritable hypermethylation of cytosine residues within the *CNR-SPL* promoter. Epi-alleles are less stable and as such reversion to wild-type sometimes occurs which manifests as ripened sectors in otherwise non-ripened fruit. A number of whole genome/epigenome scale investigations have subsequently taken place in tomato indicating possible influences on development¹⁵ and sexual hybridization.^{96–98} More recently, epigenome dynamics has been associated with ethylene response and ripening.⁹⁹ Ripening in climacteric fruit occurs by a developmental change in ethylene response described as System 1 to System 2 ethylene.¹⁰⁰ The transition to System 2 ethylene promotes ripening. Mature Green fruit, full size fruit harboring mature seeds, but still a week or so prior to ripening, are able to respond to exogenous ethylene and ripen. However, early during fruit development, System 1 ethylene production is auto inhibitory; fruit of these stages are unable to ripen in response to exogenous ethylene. Treatment of immature fruit with an inhibitor of methyltransferases resulted in ripening of immature fruits well in advance of seed maturation and normal ripening.⁹⁹ These results suggested that DNA methylation influences the System 1 to System 2 ethylene transition. Whole-genome methylome analysis of tomato fruit at four stages of development from immature to ripe indicated that distinct regions of demethylation were often localized upstream of ripening-related genes.⁹⁹ In addition, it was observed that these same regions colocalized or were adjacent to the binding sites of the ripening associated transcription factor RIN-MADS protein, as determined by ChIP-seq analysis. In combination, demethylation of ripening gene promoters, RIN-MADS binding and the early ripening of fruit inhibited for methylation, suggests an important role of the epigenome and its dynamics in contributing to ripening control and in particular the control of the transition from System 1 to System 2 ethylene response.

INVESTIGATION OF THE PROTEOME DURING RIPENING

Commonly used techniques to identify proteins and map their interactions in a cellular context in plant-based proteomic research includes two-dimensional gel electrophoresis, mass spectrometry (MS), matrix-assisted laser desorption/ionization-time of flight (MALDI TOF), yeast two-hybrids screens, ELISA and western blots, complemented in varying degrees with computational prediction programs. Some of these methods have been used for years, but are now being adapted to high throughput screening techniques enabled for improved predictive power by annotated genome sequences and robust computational tools. It is the combined high-throughput screening and increased predictive ability that

defines modern proteomics. The accurate quantitation of proteins and peptides in complex biological systems is one of the most challenging areas of proteomics.¹⁰¹ MS-based methodologies have provided significant advances in accurate and sensitive quantitation and the ability to multiplex vastly complex samples through the application of robust bioinformatic tools.¹⁰¹ MS-based methodologies are relatively simple and address the issues of reproducibility¹⁰² and under representation of low abundance,¹⁰³ low mass and basic proteins.¹⁰⁴ Accordingly, MS-based methods have come into prominence compared with antibody-based methods due to their higher specificity, good reproducibility and precision, and ability to rapidly analyze hundreds of peptide transitions in a single assay.¹⁰⁵

Until recently, most proteomic studies of fruit had been carried out using two-dimensional electrophoresis to separate and identify proteins of interest. MS-based techniques are then used to analyze the sequence of the peptides associated with a 'picked' spot. The results of these studies highlight the need for improvements typically low numbers of proteins of interest have been identified and often with a high degree of contamination. These limited proteomes have provided initial proteome insights into fruit ripening and softening, and generally validate prior transcriptomic studies. Comparison of two ripening stages of peach revealed altered abundance of 53 proteins, among these, proteins involved in both primary and secondary metabolism, ethylene biosynthesis, and stress response.¹⁰⁶ In a fruit softening study of five peach and nectarine cultivars,¹⁰⁷ it was revealed that 164 proteins were differentially changed. Only 14 of these changed during softening in all of the cultivars tested. These proteins were mostly involved in carbohydrate and cell wall metabolism and fruit senescence. In ripening apricot fruit, 106 proteins involved in the biochemical processes influencing metabolic and structural changes during ripening were identified.¹⁰⁸ In papaya fruit, 27 ripening-related proteins involved in cell wall metabolism, stress response, ethylene and carotenoid biosynthesis were identified.¹⁰⁹ An analysis of the strawberry proteome defined 68 identifiable proteins that were differentially accumulated during ripening, among these, proteins involved with anthocyanin production, heat shock, storage and ripening.¹¹⁰ The proteome of grape revealed changes in photosynthesis, carbohydrate metabolism and stress response at the onset of ripening.¹¹¹ Further, it was observed that the most significant changes occurred during the first 2 weeks following the onset of ripening, the most notable changes corresponding to pathogen defense, oxidative stress, carbon and nitrogen metabolism.¹¹² Ripening-induced changes in mango revealed 47 differentially expressed proteins, among those involved with carbon fixation, hormone biosynthesis, stress response and pathogen defense.¹¹³ In apple, 97 proteins were identified in response to both ripening and ethylene treatment of the following classes: ethylene production, antioxidant and redox, carbohydrate metabolism, oxidative stress, energy and defense response.¹¹⁴ A similar study in apple revealed 53 differentially accumulating proteins involved in stress response and defense, energy and metabolism, fruit ripening and senescence, signal transduction, cell structure and protein synthesis during ripening and storage.¹¹⁵ Proteomic analysis of tomato during ripening in two ecotypes revealed 83 proteins that were differentially expressed involved with redox status, defense, stress, carbon metabolism, energy production and cellular signaling.¹¹⁶ In tomato pericarp, 424 proteins were identified as unique from 12 different genotypes¹¹⁷ and changed in abundance either in response to developmental age (333 proteins), according to genotype (321 proteins) and according to stage and genotype interactions (215 proteins). To isolate the proteome involved in the cuticle of tomato, fruit were dipped in an organic solvent and surface proteins extracted and identified.¹¹⁸ Approximately 200 proteins were identified, a subset of which are potentially involved in the transport, deposition, or modification of cuticle constituents. For a recent review on proteomic investigations on fruit development and ripening see Ref. 119.

The use of iTRAQ (isobaric tag for relative and absolute quantization of tryptic peptides) technology provides a deeper view of the proteome within a sample, and this prompted the development of a bioinformatics pipeline for processing EST data to enable grape peptide prediction.¹²⁰ Using this peptide database and iTRAQ MS/MS, 76 of 674 identified proteins were shown to be differentially expressed during development and véraison (ripening) of the grape berry, including proteins involved in abiotic and biotic stimuli and sucrose and hexose metabolism.¹²¹ A similar investigation was recently carried out in fruit of the Gr non-ripening tomato mutant. Again an iTRAQ MS/MS approach was used and revealed 43 proteins involved in pathways including cell wall metabolism, photosynthesis, oxidative phosphorylation, carbohydrate and fatty acid metabolism, protein synthesis and processing.¹²² What these, and the above studies suggest, is that fruit development and ripening is a very metabolically active process, involving changes in numerous, pathways related to color, texture, cell wall metabolism, stress response and hormones synthesis.

METABOLOMICS IN RIPENING

Comprehensive metabolomic studies aim to provide an efficient and accurate determination of the chemical constituents present in a tissue, and are being applied in a large number of biological disciplines.¹²³ Metabolomics technology includes use of nuclear magnetic resonance, and gas- or liquid-chromatography coupled to MS. Fruit is a very rich source of primary and especially secondary metabolites and so was one of the first plant organs to be subjected to investigation. Metabolic studies on ripening of fruit has been carried out in many species including peach, melon, tomato, apple, pear, avocado and pepper.^{124–130} Overall, these studies have provided new insights into metabolic pathways and sometimes unique chemistry leading to maturation associated changes in appearance, flavor and quality of ripe fruit.

Examples of findings include identification of metabolic networks that underpin each developmental stage during peach fruit development and ripening.¹²⁴ Early stages were characterized by decreases in protein abundance and elevated levels of polyphenols and amino acids, both substrates for lignin biosynthesis during stone hardening. Sucrose levels increased during development related to translocation from the leaf, and posttranscriptional mechanisms were important for metabolic regulation during early stages of development. Amino-acid levels decreased, coupled with the elevation of transcripts involved with amino acid and organic acid catabolism during ripening stage, consistent with the mobilization of amino acids to support respiration. Sucrose cycling also occurred during storage.

One of the more intriguing recent studies was a comparative investigation of three climacteric (peach and two tomato cultivars) and two non-climacteric species (strawberry and pepper).¹³¹ Using a principal component-based analysis, called STASIS, in combination with pathway over enrichment analysis, conserved dynamics of metabolic processes were observed using publically available metabolic profiles for the investigated species. Further, it was demonstrated that this novel computational approach could be used to identify similarities in metabolic processes during fruit development and ripening, thus providing insight into what pathways are essential during these processes across species.

SYSTEMS BIOLOGY

Systems biology is an increasingly important interdisciplinary field, focusing on complex interactions within biological systems, using a holistic rather than a reductionistic approach. Such analyses have been made feasible through the availability of large-scale comprehensive data sets and ever increasing computational power. Typically metabolic, proteomic, transcriptomic, genetic and/or cell signaling networks are generated, making use of mathematical and

computational models to find common themes such as correlation among large data sets. One of the major aims of systems biology is to use these models and networks to predict outcomes in the network when perturbed by an environmental cue, treatment, developmental transition or change in genotype. To date, much of this work in fruit has been done in tomato due to the genetic and genomic resources available for this system. As an example, targeted metabolic analysis, coupled with microarray transcriptome profiling, in the ethylene receptor mutant of tomato *Nr*, revealed multiple points of ethylene control during fruit development.¹³² It was observed that 869 genes were differentially expressed during normal fruit development, and that the *Nr* mutation (i.e., ethylene) influenced 37% of these genes. *Nr* also influenced fruit morphology, seed number, ascorbate accumulation, carotenoid biosynthesis and ethylene evolution, consistent with the 'well known' role of ethylene in ripening. Further, 72 of the differentially expressed genes are homologous to known transcription factors and genes involved in signal transduction, indicating complex regulatory control. In a subsequent study, the ripening mutants *rin* and *nor* were also compared (in addition to *Nr*) to wild-type tissues, at the transcriptomic, proteomic, and metabolomic levels during development and ripening.¹³³ Shifts in primary metabolism were revealed by untargeted metabolic profiling, leading to a decrease in metabolic activity during ripening. Combining metabolomics, transcriptomic and proteomic data highlighted several aspects of metabolic control during ripening. Although expression level of transcripts and their corresponding proteins may not always correlate during earlier stages of ripening, much higher correlation was

observed during the later stages of ripening, indicating complex posttranscriptional control at the onset and early stages of ripening. Strong correlations were also observed for ripening associated transcripts and specific groups of metabolites including sugars, cell wall-related metabolites and organic acids indicating their importance during the ripening process in tomato. Together these results confirm multiple ethylene-associated events during ripening in tomato. In a comparative systems biology-based study of a climacteric tomato and non-climacteric pepper fruits, both similarities and differences were observed in ripening regulation in these species.¹³⁰ While both species have similar ethylene-mediated signaling components, their regulation is different indicating possible ethylene insensitivity, or other non-ethylene-mediated regulators in pepper. Ethylene biosynthetic genes were not induced during ripening in pepper, yet genes downstream of ethylene signaling such as carotenoid biosynthesis, cell wall metabolism and the never-ripe receptor were induced in both pepper and tomato.

The use of systems biology in correlation or co-expression-based approaches, have been utilized successfully by the metabolic engineering field to fill gaps in biosynthetic pathways, or to discover novel regulators of biosynthetic pathways. One such example was reported in tomato, where the genetic diversity of the *Solanum pennellii* introgression lines was exploited to find regulators of carotenoid biosynthesis.⁸⁴ Transcriptome analysis using the TOM2 microarray and targeted metabolic profiling revealed 953 carotenoid-correlated genes, a subnetwork revealed 38 candidate transcription factors. One of these, *SIERF6* was functionally characterized by RNAi and it was revealed that suppression of this

Figure 2. Phylogeny and expression of putative fruit MADS-box transcription factors. **(a)** Phylogenetic tree showing the relationship of kiwifruit MADS-box proteins and their homologs from characterized sequences from *Arabidopsis*, tomato and other fruit species. Bootstrap values are shown on branches, for further details, see Supplementary Data S1. **(b)** Expression profiles of 14 kiwifruit MADS-box transcription factors.

gene caused enhanced ethylene and carotenoid synthesis suggesting an important role for *SIERF6* in ripening connecting the ethylene and carotenoid pathways.

TRANSLATIONAL BIOLOGY OF RIPENING

As the number of sequenced genomes of fruit species grows, so do opportunities to exploit these genomics resources for translational experiments. The transcriptional regulation of ripening is well characterized in tomato as outlined in previous sections of this review providing opportunities for comparative genomics approaches to identify candidate genes from other fruit species taking advantage of leveraging functional studies performed in tomato. As an example, MADS-box transcription factors play a major role in the transition to and controlling ripening in tomato and other species. In a phylogenetic analysis of the kiwifruit MADS-box transcription factors, mined from the published genome,²³ a total of 14 kiwifruit sequences were discovered, and were aligned with other known fruit and *Arabidopsis*-predicted protein sequences as described previously.^{43,134} Their expression was confirmed by mining the RNAseq data that were released with the kiwifruit genome.²³ Figure 2 shows the phylogenetic relationship and the expression profiles of these 14 putative MADS-box transcription factors from kiwifruit. All were expressed in fruit tissues, however, half of these genes were expressed in low levels in maturing fruit, indicating a role more aligned with floral regulation and early fruit development rather than ripening. The other half of these genes increased during development, either at or following 120 days after pollination indicating a possible role in ripening control. Further, all of the main clades of the tree were represented with at least one kiwifruit gene that was expressed in mature fruit. This approach of mining regulators of ripening provides opportunities for biotechnological and marker-assisted breeding control of ripening.

FUTURE PROSPECTS

The pace at which sequencing technologies, computer infrastructure and computational biology has developed has been staggering in the last 20 years. We now have the blueprints of how plants grow and undergo complex developmental processes through the rapidly expanding collection of plant genome sequences. However, interpreting these blueprints remains a challenge. These new 'omics'-based research tools have already provided novel insights into the underlying biology of fruit development. Opportunities exist to expand this knowledge to postharvest systems, especially to responses of products to treatments such as temperature and modified atmospheres, as technology develops. Earlier this year, Illumina announced the release of their new generation of sequencing platforms, NextSeq 500 and HiSeq X Ten, both promising to lower sequencing costs and the time to generate sequence data. According to Illumina, HiSeq X Ten will yield whole human genome sequences for \$1000 each and will have the capability of generating up to 20 000 genomes per year. With these technological advances in mind, the Plant Science Decadal Vision has highlighted 'Increase the ability to predict plant traits from plant genomes in diverse environments' and 'Enhance the ability to find answers in a torrent of data' as two of its five goals to shape plant biology research in the decade 2015–2025.¹³⁵ These technical challenges faced will be overcome by the continuing advances in information technology. The prospects for greater understanding of fruit development and the ability to model and predict the effects of the environment during postharvest storage of these fruits look bright.

CONFLICT OF INTERESTS

The authors do not have any conflicts of interest.

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