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INVITED REVIEW

Putting primary metabolism into perspective to obtain better fruits

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• **Background** One of the key goals of fruit biology is to understand the factors that influence fruit growth and quality, ultimately with a view to manipulating them for improvement of fruit traits.
• **Scope** Primary metabolism, which is not only essential for growth but is also a major component of fruit quality, is an obvious target for improvement. However, metabolism is a moving target that undergoes marked changes throughout fruit growth and ripening.
• **Conclusions** Agricultural practice and breeding have successfully improved fruit metabolic traits, but both face the complexity of the interplay between development, metabolism and the environment. Thus, more fundamental knowledge is needed to identify further strategies for the manipulation of fruit metabolism. Nearly two decades of post-genomics approaches involving transcriptomics, proteomics and/or metabolomics have generated a lot of information about the behaviour of fruit metabolic networks. Today, the emergence of modelling tools is providing the opportunity to turn this information into a mechanistic understanding of fruits, and ultimately to design better fruits. Since high-quality data are a key requirement in modelling, a range of must-have parameters and variables is proposed.

Key words: Fruits, metabolism, agricultural practice, post-genomics, kinetic modelling, stoichiometric modelling, process-based modelling.

CONTEXT

Fruits have proven to be a huge success in the evolution of plants. Over 150 million years, the organ of angiosperms dedicated to seed dispersal has evolved in a myriad of forms, tastes and properties, sometimes to protect the seeds by becoming impenetrable or toxic, and sometimes to help their spread by becoming winged, floatable, explosive or even desirable. Humans have long enjoyed this profusion, first as consumers, then as farmers and eventually as breeders.

Today, production of fruit, which is essential in human nutrition, is under significant pressure not only from environmental stresses but also from changes in consumer preference for taste and nutritional value, resulting in a constantly renewed need for improved varieties to meet this demand. Metabolism is an obvious target for improving fruit production, especially in fleshy fruits, our main source of vitamins and antioxidants, and understanding the mechanisms linking it to fruit phenotypes will help to improve breeding strategies (Giovannoni, 2006). Indeed, traits such as pathogen and abiotic stress resistance during growth, as well as flavour, nutritional value and health benefits are all affected by the composition of metabolites in fruit tissues. A key goal is therefore to understand the factors that affect metabolite concentrations in cells and tissues and how they are balanced with growth, ultimately for manipulating their levels to improve crop traits.

The concept of metabolism can be sub-divided into primary and specialized metabolism, depending on absolute requirements for cell survival and growth (Verpoorte, 2000). Importantly, reactions involved in primary metabolism are highly conserved, whereas those involved in specialized pathways show much greater diversity between fruit species. It is nevertheless striking that a large part of fruit diversity involves primary metabolism. Furthermore, primary metabolism undergoes important changes throughout fruit development, as evidenced by large changes in acidity and/or sweetness that occur at ripening. Thus, while the reaction network remains the same, a set of regulations can direct metabolic fluxes to very different destinations. Moreover, while primary metabolism is probably one of the best known biological networks with regard to its different stages, the way in which metabolic fluxes are reoriented remains largely unknown.

The present review focuses on the contribution of primary metabolism to fruit growth and quality, and how it may be influenced to improve fruit quality and increase yield. After a brief description of fruit primary metabolism and the way in which it is programmed throughout fruit growth and ripening, different approaches that have been adopted to manipulate fruit quality and production are discussed: agricultural practice, breeding and the search for metabolic targets. The modelling of fruit development and metabolism is then presented as an ensemble

of emerging tools that could be used in any species, leading to a better understanding of fruits and ultimately to better fruits.

FRUIT PRIMARY METABOLISM

From a topological point of view, primary metabolism (Fig. 1) is not very different between organs, stages of development or cell types, and, as mentioned above, it is highly conserved between species. What makes it different is the way in which it functions (Peregrín-Alvarez *et al.*, 2009). Our focus is therefore on the pathways that are particularly important for both the growth and quality of most fleshy fruits.

Primary pathways that contribute to fruit growth and quality

While most developing fleshy fruits are photosynthetic, they are not self-sufficient regarding carbon supply (Lytovchenko *et al.*, 2011; for a review, see Cocaliadis *et al.*, 2014). The major source of carbon for fruit is usually sucrose, which is imported from leaves via the phloem. In some species, carbon traffic is enhanced by the transport of additional sugars such as stachyose and raffinose in Cucurbitaceae (Haritatos *et al.*, 1996) or sorbitol in Rosaceae (Noiraud *et al.*, 2001). Sugar import is mainly symplastic in the initial stages of fruit development and becomes mainly apoplastic at later stages (Ruan and Patrick, 1995; Zhang *et al.*, 2006). Breaking the symplastic continuum enables the accumulation of metabolites at very high concentrations within the fruit (e.g. molar sugar concentrations in grape berries), as energy-consuming apoplastic transport does not require a favourable water potential difference between fruit and phloem (Patrick, 1997). In contrast, symplastic transport enables a high flux of incoming carbon. Accordingly, the carbon demand of young tomato fruits is the highest in relation to fresh weight (Colombié *et al.*, 2015), which corroborates the massive abortion of young fruits when carbon supply drops (Jean and Lapointe, 2001). Furthermore, the flux capacity of the petiole and pedicel (expressed as the proportion of phloem vessels) correlates with fruit growth rates and size (Savage *et al.*, 2015).

Central carbon metabolism, which in fruits involves the pathways of sucrose, starch, major organic acids and respiration, provides energy and biosynthetic precursors to support fruit growth and maturation (Fig. 1). It is also essential for fruit quality, as sweetness and sourness are conditioned by sugars and organic acids, respectively, which are major components in most fruits. For example, sugars represent about 8 % of the fruit fresh matter weight at maturity in peach (Desnoues *et al.*, 2014) and 15 % in grapevine (Davies and Robinson, 1996). Organic acids, especially citrate and malate, also represent large metabolic pools, with citrate reaching 5 % of the fresh pulp in lemon

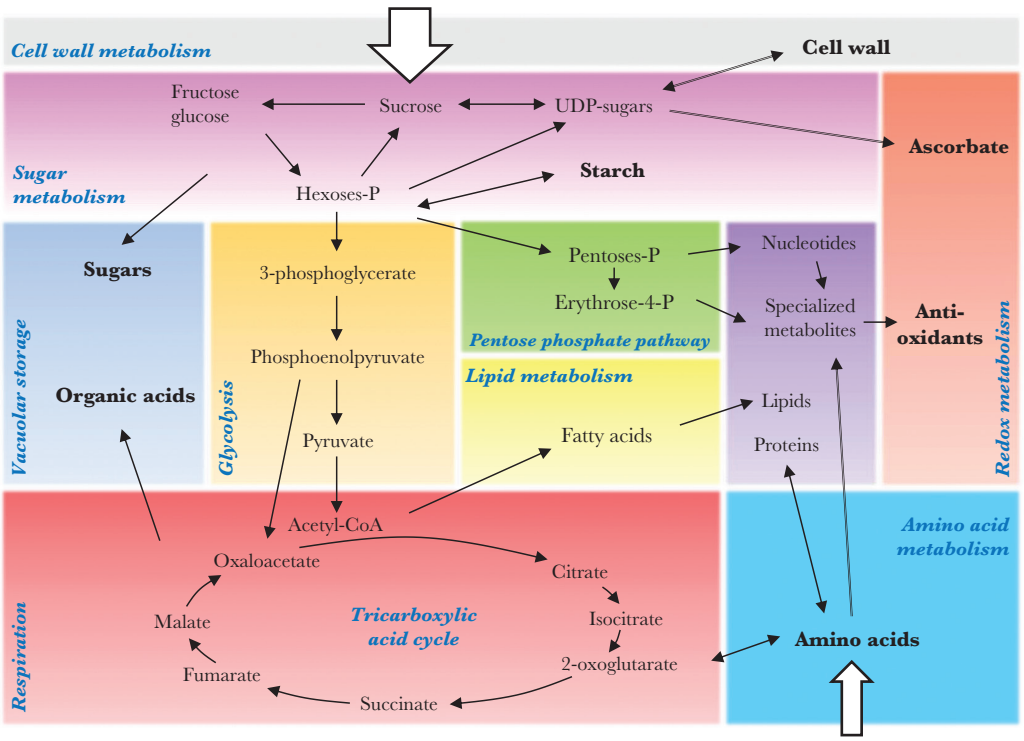

FIG. 1. Simplified representation of fruit primary metabolism. Major primary pathways and compounds involved in fruit growth and quality are represented. Sucrose and amino acids represent the major imported pools. Sucrose is first metabolized via sugar metabolism, which produces starch and precursors of cell wall components, and the major antioxidant ascorbate. A large fraction of sucrose and/or hexoses is stored in the vacuole. Hexose phosphates, which are intermediates of sucrose metabolism, are degraded via glycolysis to release energy and yield precursors of the TCA cycle and fatty acid metabolism. Alternatively, hexose phosphates are metabolized via the pentose phosphate pathway, which yields precursors of nucleotides and specialized metabolites. The TCA cycle coupled to respiration releases energy and provides precursors for amino acid synthesis. Imported amino acids, of which glutamine, glutamate, aspartate and asparagine are often the dominant forms, provide nitrogen and carbon skeletons for the synthesis of further amino acids. The major compounds that mainly influence fruit quality are in bold. Abbreviations: P, phosphate; UDP, uridine diphosphate; acetyl-CoA, acetyl-coenzyme A.

(Albertini *et al.*, 2006). The ratio between sugars and acids is also very important for taste. Remarkably, lemon (Albertini *et al.*, 2006) and tomato fruits (Causse *et al.*, 2004) do not taste sweet although they both have a relatively high sugar content of about 4 %. In most fruits, taste development occurring at ripening is due to increased sweetness, which is the result of a range of large metabolic changes leading to sugar accumulation (Bonghi and Manganaris, 2012). For example, the degradation of starch at the beginning of ripening is a major source of sugars and energy (e.g. McRae *et al.*, 1992; Hill and Ap Rees, 1994; Jourda *et al.*, 2017). Starch, which accumulates in many species at high levels during fruit development, also contributes considerably to the respiration climacteric (Colombié *et al.*, 2017). Nevertheless, some climacteric fruit species such as aubergine, melon and papaya do not store starch during fruit growth, thus raising the question of what are the other carbon sources that may potentially fuel the respiratory burst.

Although fruits may contain nitrate and ammonium (Horchani *et al.*, 2008; Sanchez *et al.*, 2017), they do not assimilate nitrogen themselves but import amino acids from the phloem and, to a lesser extent, the xylem (Gourieroux *et al.*, 2016). Like the import of sugars, amino acids can take both the symplastic and apoplastic routes, depending upon the stage of fruit development (Zhang *et al.*, 2015). Amino acid metabolism provides precursors not only for protein synthesis but also for a range of specialized metabolites (Gonda *et al.*, 2010). Major amino acids and their derivatives can have an important influence on fruit taste and quality. In tomato, the accumulation of large amounts of glutamate and aspartate during ripening determines the umami taste. In cultivated tomato, which accumulates much higher levels than wild relatives, the accumulation of glutamate has been attributed to increased NAD-glutamate dehydrogenase activity (Ferraro *et al.*, 2015). γ-Aminobutyric acid (GABA), which results from the reaction catalysed by glutamate decarboxylase, also accumulates at high levels in growing tomato fruits, thus providing valuable nutritional properties (Takayama and Ezura, 2015). In grapevine berries, the accumulation of amino acids that occurs between veraison and ripeness is essential for winemaking as it represents the only natural source of amino acids for yeast growth (Bell and Henschke, 2005). More generally, the increase in amino acids occurring at ripening in many fruit species is often attributed to protein degradation (Sorrequieta *et al.*, 2010). However, increased protein synthesis at the beginning of ripening takes place in various climacteric fruit species (Brady, 1987), suggesting that the accumulation of amino acids might also result from a changed balance between the supply from the mother plant and the use for fruit growth.

Fruits are considered to be our major source of antioxidants, but domestication tended to reduce their concentrations, suggesting that there is a trade-off with growth and thus productivity (Gest *et al.*, 2013). For example, in cultivated kiwifruit, the ascorbate content is 20 times lower than in wild relatives. Although the reason for such trade-offs remains unknown, the lower ascorbate content decreases stress resistance in fruits (Gest *et al.*, 2013). Tartaric acid, which is a degradation product of ascorbate, is a major organic acid in several fruits including citrus (Albertini *et al.*, 2006) and grape berries, where it plays a major role in winemaking by controlling the acidity of the

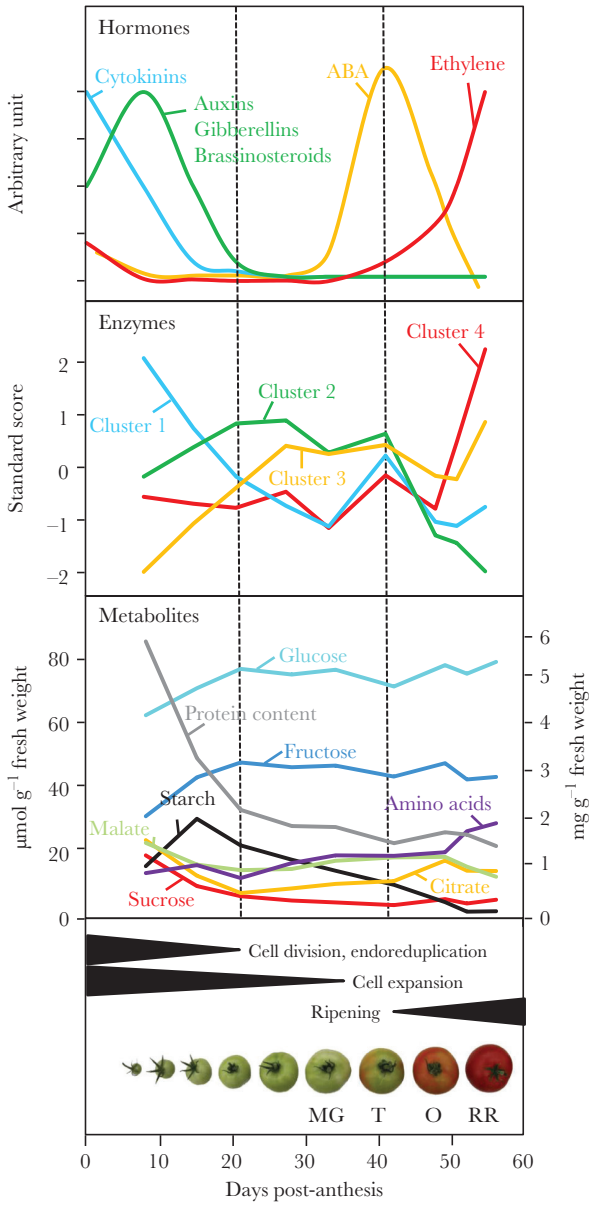

FIG. 2. Hormonal, enzymatic and metabolic changes occurring in tomato fruit pericarp during development and ripening. Hormone levels are expressed in arbitrary units, metabolite levels in µmol g^{-1} fresh weight and protein content in mg g^{-1} fresh weight. Enzyme capacities expressed in units mg^{-1} protein have been normalized, grouped into four clusters and averaged. Cluster 1:fructokinase, glucokinase, pyruvate kinase, aconitase, NAD-isocitrate dehydrogenase, fumarase, NAD-glutamate dehydrogenase and aspartate aminotransferase. Cluster 2:phosphoglucose isomerase, phosphoglucomutase, ADP-glucose pyrophosphorylase, ATP-phosphofructokinase, PPi-phosphofructokinase, plastidial fructose bisphosphatase, triose phosphate isomerase, NAD-glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, enolase, phosphoenolpyruvate carboxylase, NAD-malate dehydrogenase, NAD-malic enzyme and NADP-malic enzyme. Cluster 3:sucrose synthase, UDP-glucose pyrophosphorylase, cytosolic fructose bisphosphatase, NADP-glyceraldehyde-3-phosphate dehydrogenase, NADP-glutamate dehydrogenase and alanine aminotransferase. Cluster 4:acid invertase, neutral invertase, sucrose phosphate synthase, aldolase, glucose-6-phosphate dehydrogenase, citrate synthase, NADP-isocitrate dehydrogenase and succinyl-coenzyme A ligase. Adapted from Zhang *et al.* (2009) and McAtee *et al.* (2013) for changes in hormone levels and from Biais *et al.* (2014) for changes in enzyme activities and metabolite concentrations.

wine (de Bolt, 2006). There is also a cross-talk between fruit firmness and ascorbate content that comes from the sharing of GDP-D-mannose epimerase between the cell wall and ascorbate biosynthetic pathways (Mounet-Gilbert *et al.*, 2016).

Primary cell wall metabolism can be considered as part of primary metabolism since plant cells cannot grow or even survive in nature without a wall. The composition of the cell wall, the primary carbon sink in plants, is highly diverse among plant species, but the major components (cellulose, three matrix glycans composed of neutral sugars and three pectins rich in D-galacturonic acid) are usually the same (Brummell and Harpster, 2001). Cell walls are particularly important in fruits. During growth, they play a major role in shaping and protecting the fruit, and at ripening their partial degradation via a large solubilization of pectic polysaccharides has strong implications not only for fruit quality but also for shelf-life (Brummell and Hapster, 2001). In strawberry, cell wall degradation at ripening contributes substantially to the ascorbate pool via the recycling of D-galacturonic acid, which is a component of pectins (Agius *et al.*, 2003). Partial cell wall degradation at ripening also leads to a massive release of sugars including uronic acids (Cutillasiturralde *et al.*, 1993; Sakurai *et al.*, 1997), which in plants are recycled following the reactivation by phosphorylation and via the reaction catalysed by UDP-sugar pyrophosphorylase (Geserick and Tenhaken, 2013), thus providing energy and building blocks for a range of processes such as protein synthesis and sugar accumulation.

Sub-cellular compartmentalization should also be considered when studying metabolism, especially the vacuole. Indeed, most of the cell volume in fleshy fruits is occupied by a large central vacuole that participates in fruit growth via its enlargement driven by the accumulation of osmolytes such as organic acids and sugars (Ho, 1996). It is therefore of great importance for fruit quality. Although the transport of sugars and organic acids into the vacuole is active, which implies ATP or PPi consumption by proton pumps and thus significant energy expenditure (Shiratake and Martinoia, 2007), very little is known about the properties of fruit tonoplast transporters. *In vitro* experiments to investigate the metabolic changes that underlie fruit development are very difficult, mainly because it is impossible to measure the capacities of such transporters.

Briefly, characters such as sweetness, sourness, nitrogen content and the antioxidant properties of fruits, as well as fruit yield, are the result of a complex interplay between transport, compartmentalization and the transformation of metabolites. As we will see in the next paragraph, it is becoming clear that this interplay involves gene regulation affecting pathway enzymes.

Metabolism undergoes profound reprogramming during fruit development

The development of fleshy fruits is characterized by three partly overlapping phases: cell division, cell expansion and maturation, which all involve a profound reprogramming orchestrated mostly by hormones (Fig. 2). The involvement of hormones in fruit growth and development has been known for a long time, and hormonal treatments are common in fruit production (Ginzberg and Stern, 2016). Briefly, cytokinins (reviewed in Jameson and Song, 2016), auxins (reviewed in Pattison *et al.*, 2014) and gibberellins (Serrani *et al.*, 2007) are involved in the early events following pollination (Fig. 2). Cytokinin levels are high in ovaries and promote auxin synthesis, whereas pollination results in increased levels of gibberellins (Olimpieri *et al.*, 2007). Auxins and gibberellins promote cell division and/or cell expansion, so they influence fruit size (Pattison *et al.*, 2014) and are able to induce parthenocarpy (Ding *et al.*, 2013; Shinozaki *et al.*, 2015). Brassinosteroids, which have also been found to be involved in early cucumber fruit growth, induce cell cycle-related genes (Fu *et al.*, 2008). Ethylene and abscisic acid (Leng *et al.*, 2014) are considered to be major factors controlling fruit ripening, which occurs after fruit growth stops. Whereas the role of abscisic acid in ripening remains elusive (Jia *et al.*, 2016), the role of ethylene is increasingly understood (Giovannoni, 2004; Giovannoni *et al.*, 2017). Climacteric fruits such as tomato, banana and mango show a respiratory peak and a concomitant rise in ethylene, which initiates a range of ripening processes. Ethylene is first perceived by the ethylene receptor protein (ETR) and its action is mediated by ethylene response factors (ERFs), one of the largest families of plant transcription factors (Liu *et al.*, 2015). In non-climacteric fruits such as strawberry, grape and citrus, there is no respiratory peak and ethylene content remains relatively low. Interestingly, transcription factors acting upstream of ethylene signalling have been found in both climacteric and non-climacteric fruits (Giovannoni *et al.*, 2017). However, the nature of the prime signals initiating ripening remains elusive. It is still unknown whether the completion of fruit or seed growth is sensed or whether decreased sink demand leads to metabolic signals. While some data indicate that hormones can trigger metabolic changes, there is also emerging evidence that metabolic signals are involved in the control of fruit development and ripening. Thus, suppressing or enhancing the expression of a vacuolar invertase inhibitor revealed a link between sucrose metabolism, ethylene biosynthesis and ripening (Qin *et al.*, 2016).

At fruit set, auxins and gibberellins have a major impact on the genes involved in primary metabolism (Ruiu *et al.*, 2015). Unfortunately, changes at the level of the proteome and the metabolome have rarely been investigated in ovaries and very young fruits. This is probably a key point for future investigations. Indeed, in young tomato fruits, the catalytic capacities of enzymes involved in energy metabolism [i.e. enzymes involved in glycolysis and the tricarboxylic acid cycle (TCA)], including enzymes catalysing irreversible reactions (fructokinase, glucokinase and pyruvate kinase), are very high, indicating that fast growth in the early stages is supported by high glycolytic flux. Subsequently, the anaplerotic enzymes phosphoenolpyruvate carboxylase and NADP-malic enzyme become more abundant (Biais *et al.*, 2014). Their carboxylating activities can be used to replenish the pool of C_4 acids in order to produce biomass (Chollet *et al.*, 1996; Badia *et al.*, 2017). At ripening, the capacities of a number of enzymes involved in energy metabolism rise again, suggesting an increased demand for energy (Fig. 2). Strikingly, changes in the levels of enzymes (Biais *et al.*, 2014) and metabolites (Carrari *et al.*, 2006) occur concomitantly, indicating that co-ordinated changes in enzyme capacities impact the metabolome. Furthermore, an integrative study combining transcriptomics, proteomics and metabolomics conducted with tomato mutants impacting the production or the sensing of

ethylene showed that several metabolic events are mediated by ethylene (Osorio *et al.*, 2011). Although most integrative studies have been conducted in tomato, which is considered to be the model system for fleshy fruits, it will be important to consider other fruit species. Indeed, the profiles of protein, enzyme and/or metabolite abundance have been found to behave differently throughout fruit development in grape berries (Hawker, 1969), kiwifruit (Nardozza *et al.*, 2013), peach (Desnoues *et al.*, 2014) and apple (M. Li *et al.*, 2016), thus reinforcing the idea that changing the capacities and properties of enzymes and transporters would affect metabolite concentrations and fluxes.

STRATEGIES TO MANIPULATE FRUIT PRIMARY METABOLISM

In this section, growth conditions as well as forward and reverse genetic approaches that have been used to impact fruit primary metabolism and potentially fruit growth and quality are discussed.

Impact of crop management on fruit metabolism

Changes in agricultural practices have been driven mostly by their potential to increase yield or reduce pest attacks, and it is only recently that the use of agronomic levers has emerged to manipulate fruit quality, especially the levels and composition of antioxidant metabolites (Poiroux-Gonord *et al.*, 2010). The content of soluble sugars, acids, phenolic compounds, vitamins and carotenoids has been assessed under varying crop management conditions, for example in response to water deficit or salinity stress (Mills *et al.*, 1996; Yakushiji *et al.*, 1998), partial root drying (Zegbe *et al.*, 2006), temperature (Kliewer 1973; Gautier *et al.*, 2008), light intensity (Biais *et al.*, 2014), fertilizers (Bénard *et al.*, 2009; Spironello *et al.*, 2004) and grafting (Rouphael *et al.*, 2010).

Under high salinity or moderate water deficit, fruit size is inversely correlated to treatment intensity while the fruit contents in dry matter, soluble sugars and organic acids increase in a range depending on genotype (Berman and DeJong, 1996). For example, in tomato, fruit hexose content increases in response to high temperature and light intensity, in interaction with the plant source–sink ratio but also the genotype (Gautier *et al.*, 2005; Truffault *et al.*, 2015). The effects of crop management on fruit acidity are less clear. For instance, water deficit tends to increase the sugar:acid ratio, although again the response is genotype dependent (Ripoll *et al.*, 2016). During tomato fruit development under control, shaded or water-limited conditions, metabolite levels are more sensitive to the environment than enzyme capacities (Biais *et al.*, 2014). An increase in the activity of apoplastic invertase that facilitates sugar import into fruits under water deficit was suggested (Osorio *et al.*, 2014). Under high temperature and solar radiation, an increase in sucrose synthase activity resulted in higher fruit hexose and starch content in cherry tomatoes (Rosales *et al.*, 2007). In a high-density single truss tomato production system, supplying supplemental light to the lowest understorey leaves using light-emitting diodes (LEDs) with combined red and blue light increased tomato yield and fruit total soluble solids and ascorbic acid content (Tewolde *et al.*, 2016).

Concerning antioxidants, ascorbate is generally accumulated at higher levels at relatively low temperatures during the growth period, in contrast to carotenoids that decrease (Gautier *et al.*, 2008). Light also strongly affects the biosynthesis of antioxidants. Thus, ascorbate accumulation strongly depends on fruit irradiance, which may be increased by leaf pruning (Massot *et al.*, 2010). Furthermore, light and temperature interact to regulate the ascorbate pool size, not only at the level of ascorbate oxidation and recycling but also at the level of the expression of genes involved in ascorbate biosynthesis (Massot *et al.*, 2013). This probably explains large seasonal variations in fruit ascorbate content (Massot *et al.*, 2010). Carotenoid accumulation is also positively regulated by light exposure (Fanciullino *et al.*, 2014) or by an increase in the red to far-red ratio (Alba *et al.*, 2000). Regarding the effects of water and mineral supply, high salinity has a globally positive effect on the accumulation of ascorbate, lycopene and β-carotene (Frary *et al.*, 2010), with strong genotype by environment interactions (Gautier *et al.*, 2009). Ascorbate slightly increases under nitrogen depletion, possibly because more light reaches the fruits owing to the decreased canopy. While water deficit has a positive effect on ascorbate, the potential benefits of drought on fleshy fruit quality might be exacerbated or mitigated depending on genotype, seasonal factors or on the intensity and duration of treatment (Ripoll *et al.*, 2014). Crop management and in particular water deficit or high salinity may influence fruit metabolism, first through an effect on net photosynthesis and the supply of precursors for biosynthesis, and secondly through an oxidative stress signalling, which may trigger some biosynthetic pathways. In tomato and in grapevine, the synthesis of a range of metabolites is linked to oxidative stress (Deluc *et al.*, 2009). In contrast, carbohydrate availability does not limit the synthesis and accumulation of ascorbate in fruits (Poiroux-Gonord *et al.*, 2013), except under low light (Tewolde *et al.*, 2016).

The manipulation of plant fruit load via flower, fruit, leaf and/or shoot pruning, which is often used to regulate or increase fruit size, may induce a parallel increase in the content of individual metabolites expressed on a fresh weight basis (Kromdijk *et al.*, 2013). In peach and mango, Lechaudel *et al.* (2007) observed that an increase in the source–sink ratio decreased citrate concentrations and increased malate and sucrose concentrations near maturity. However, several exceptions have also been reported (e.g. Massot *et al.*, 2010; Fanwoua *et al.*, 2012). In tomato, most of the water entering the fruit comes from the phloem, together with assimilates, which explains why sugar and acid content hardly increase at low plant fruit load (Ho, 1996). In contrast, carotenoid and ascorbate contents can be significantly altered by fruit load and carbon availability (e.g. Gautier *et al.*, 2005; Massot *et al.*, 2010; Poiroux-Gonord *et al.*, 2013).

We have shown that various factors such as salinity, water stress, high light intensity, heat and sub- or supra-mineral nutrition can have a positive impact on fruit growth and/or quality. However, they can also result in oxidative stress and ultimately cell death. Blossom end rot is a type of necrosis appearing at the blossom end of the fruit (e.g. in tomato, pepper and apple) and is usually attributed to calcium deficiency, but it may result rather from complex interactions between environmental factors and involve secondary oxidative stress (Mestre, 2012; Saure, 2014). Solutions for preventing the appearance of such a

disease have so far been largely empirical, indicating that more mechanistic studies integrating metabolism and growth conditions are needed.

Forward genetic approaches for improving fruit metabolic traits

Plant domestication has resulted in considerable phenotypic modifications from wild species to modern varieties. Domestication modified expression levels for hundreds of genes, acting on entire gene networks, including genes involved in carbohydrate metabolism (Cao *et al.*, 2014; Sauvage *et al.*, 2017). Although genomic-assisted breeding (Kinkade *et al.*, 2013) is increasingly used, breeding using molecular markers and quantitative genetics is still relevant (Tomason *et al.*, 2013, Kumar *et al.*, 2014; Grandillo and Cammareri, 2016). Breeding continues to exploit the genetic diversity of wild relatives or ancestral varieties, as demonstrated for decades for various species (Burger *et al.*, 2006; Knapp and Peralta, 2016; Rasheed *et al.*, 2017). Diversity of genetic resources has been shared for tomato, for example through the Charles M. Rick Tomato Genetic Resource Centre (http://tgrc.ucdavis.edu/; Rick, 1986) as a central source of tomato wild species germplasm, various true-breeding populations and monogenic mutants (Giovannoni, 2016). Diversity has been induced by ethyl methane sulphonate (EMS) mutagenesis on Targeting Induced Local Lesions In Genomes (TILLING) platforms for tomato (Okabe and Ariizumi, 2016), melon (González *et al.*, 2011) and cucumber (Boualem *et al.*, 2014). Such collections can be used in forward genetics approaches, and rapid identification of causal mutations in tomato EMS populations is possible by using mapping by sequencing (Garcia *et al.*, 2016). However, the use of TILLING for the discovery of candidate gene function is presently being replaced by genome editing techniques, which are easily applied in several fruit species (Malnoy *et al.*, 2016).

Fruit traits of interest can easily be detected and selected, even if the underlying mechanisms might be highly complex. Quantitative trait loci (QTLs) of fruit traits have been studied in a number of species as a result of the pioneering work by Paterson *et al.* (1988) who mapped QTLs controlling fruit mass, concentration of soluble solids and pH. More recently, the introgression of a regulatory subunit of ADP-glucose pyrophosphorylase from *Solanum hirsutum* into cultivated tomato (Schaffer *et al.*, 2000) resulted in stabilization of the activity of this enzyme during the early stages of fruit growth, thus increasing starch accumulation and ultimately the content in soluble compounds. Metabolite QTLs (mQTLs) are still used frequently, together with recombinant inbred lines (RILs). In melon, a map-based cloning strategy based on natural genetic variation for fruit acidity identified a gene family encoding membrane proteins responsible for acidity in fruit (Cohen *et al.*, 2014). QTLs controlling individual soluble sugars and organic acids have been mapped in tomato in relation to water deficit response (Albert *et al.*, 2016). In peach, co-locations between annotated genes, QTLs for enzyme activities and QTLs controlling major soluble sugar or organic acid concentrations (Desnoues *et al.*, 2016) revealed changing effects of alleles during fruit growth. In melon, single-gene resolution QTL mapping using 81 RILs has already been achieved (Katzir, 2015). Genotyping was conducted using almost 60 000 single nucleotide polymorphisms (SNPs) obtained by RNA sequencing of the flesh tissue of mature fruit. Phenotyping and metabolic profiling included >100 fruit traits affecting taste, aroma, colour and appearance. The details of this work were recently published (Galpaz *et al.*, 2018). Interestingly, a recent genetic study of sugar metabolism suggests that the maximal capacity of sucrose accumulation has been reached in melon (Argyris *et al.*, 2017).

Metabolite-based genome-wide association studies (mGWASs) have been undertaken (Luo, 2015). In tomato, a core collection of 163 tomato accessions was used to map loci controlling variation in fruit metabolites including amino acids, sugars and ascorbate. The accessions were genotyped with about 6000 SNP markers (Sauvage *et al.*, 2014). The mapping confirmed that cell wall invertase (Fridman *et al.*, 2000, 2004) is a candidate gene for the control of soluble sugar content and provided a list of other candidate loci including loci associated with malate and citrate levels in fruits.

Recently, an integration of mGWAS with analyses of gene expression patterns, genetic variations and T-DNA (CRISPR/Cas9)-derived mutants allowed the functional characterization of a major QTL for high malate content in tomato fruit as an InDel in the promoter of Al-ACTIVATED MALATE TRANSPORTER9 (Ye *et al.*, 2017). However, it is now acknowledged that classical breeding will inevitably reach a plateau in a given species so new strategies involving more fundamental knowledge will be needed (Vaughan *et al.*, 2007). Additionally, epialleles may determine the content of compounds of interest in fruits (Quadrana *et al.*, 2014), and epigenetic differences may provide new targets for breeding and crop improvement (Gallusci *et al.*, 2017).

Candidate genes for the manipulation of fruit metabolism

Knowledge of the topology of metabolic networks has generated a range of *a priori* approaches in which given enzymes are targeted to improve fruits. However, there are many examples indicating that manipulating enzymes does not necessarily lead to improvements of fruit biomass and/or quality. Several examples can be given. In tomato, the RNA interference (RNAi)-mediated inhibition of cell wall invertase led to a decrease in Brix as expected (see above), but also to other disturbances including decreased pollen viability, seed number and fruit size, as well as altered hormone and transcript profiles (Zanor *et al.*, 2009). The downregulation of vacuolar acid invertase increased sucrose but decreased hexoses and fruit growth rate and size (Klann *et al.*, 1996). Hexokinase overexpression resulted in lower sugar and starch, and impaired fruit growth (Menu *et al.*, 2004). Fruit-specific overexpression of a bacterial pyrophosphatase led to a significant increase in ascorbate content but also to a decrease in fruit size (Osorio *et al.*, 2013). The manipulation of malate concentrations via downregulation of fumarase or mitochondrial malate dehydrogenase resulted in major alterations of the metabolome, although fruit size was only marginally impaired (Centeno *et al.*, 2011). Regarding sub-cellular

compartmentalization, the overexpression of SICAT9, a tonoplastic amino acid exchanger, resulted in increased levels of GABA, aspartate and glutamate, paralleled by a decrease in citrate in tomato fruits (Snowden *et al.*, 2015). In tomato, the downregulation of the proton-pumping ATPase has been shown to increase the sucrose:hexose ratio but to decrease the fruit growth rate and size (Amemiya *et al.*, 2006). There are many more examples indicating that the manipulation of enzymes or transporters involved in primary metabolism rarely results in fruit and/or yield improvement. However, a rare success was the manipulation of sucrose sensing which led to tomato fruits with increased sweetness, without affecting plant or fruit growth (Sagor *et al.*, 2016).

Post-genomics, which can be defined as the shift in biology observed in the early 2000s once the first genomes had been sequenced, has opened the way for untargeted and multidisciplinary studies including transcriptomics, proteomics, metabolomics and bioinformatics. One aim of post-genomics is to search for 'better' candidate genes by performing large-scale correlational studies identifying 'suspects by association' (Usadel *et al.*, 2009; Toubiana *et al.*, 2013). Carrari and Fernie (2006) reviewed earlier works using targeted approaches, as well as pioneering studies in which metabolic or transcriptional profiling aimed at identifying candidate genes for modifying metabolite contents. They included primary metabolites and several specialized metabolites considered as important with respect to fruit quality. The focus here is on exemplary studies of the past few years.

The combination of at least two omics approaches has contributed to the characterization of metabolic shifts during development in a range of fruit species including tomato (Osorio *et al.*, 2011), grape berry (Dai *et al.*, 2013), apple (M. Li *et al.*, 2016; see also www.transcrapple.com), melon (Guo *et al.*, 2017) and mango (Wu *et al.*, 2014). Metabolic shifts during post-harvest storage have also been characterized, for example in litchi (Yun *et al.*, 2016) and citrus (Ding *et al.*, 2015). Moreover, omics approaches have been used to describe the effects of the environment on fruit metabolism in tomato (D'Esposito *et al.*, 2017) and of abiotic or biotic stresses such as water stress and botrytis infection in grape berry (Agudelo-Romero *et al.*, 2015; Ghan *et al.*, 2015). In addition, omics have been used to characterize cultivars and mutants. An example of the characterization of mutants is a study on low citrate accumulation in orange (Guo *et al.*, 2016). Omics will also be used to elucidate further the major biochemical and signal transduction pathways that are active for primary (Bastias *et al.*, 2014) and specialized metabolism (Wong and Matus, 2017), including the identification of transcription factors (Rohrmann *et al.*, 2011; Ye *et al.*, 2015) and their targets, as done recently for tomato (Fernandez-Moreno *et al.*, 2016) and citrus (S. J. Li *et al.*, 2016) fruit. In apple, a comprehensive 2-D gel-based proteomic analysis over five growth stages from young fruit to maturity, coupled with targeted metabolomic profiling of soluble sugars, organic acids and amino acids, provided insights into the metabolism and storage of fructose, sucrose and malate (M. Li *et al.*, 2016). Moreover, the decrease in amino acid concentrations during fruit development was found to be related to a reduction in the glycolytic enzyme abundance. In citrus, liquid chromatography–tandem mass spectrometry (LC-MS/MS)-based proteomic and metabolomic analyses showed that organic acid and amino acid accumulation shifted toward sugar synthesis during the later stage of citrus fruit development, and that an invertase inhibitor may be involved during maturation (Katz *et al.*, 2011). In grape exocarp, related trends between metabolites and proteins revealed clear links between primary and specialized metabolisms (Negri *et al.*, 2015). For instance, levels of several proteins involved in glycolysis, the TCA cycle and the metabolic intermediates of these pathways were correlated with anthocyanin content. In tomato, changes in protein abundance were measured in skin and flesh during development, including 61 differentially expressed transcription factors (Szymanski, 2017). The use of these data to estimate metabolic activity by employing the LycoCyc pathway annotations, the local topology of the pathways and protein expression values revealed a significant tissue-specific reprogramming of metabolism during fruit development.

The combination of transcriptomics, proteomics and metabolomics performed in five grapevine cultivars at maturity (Ghan *et al.*, 2015) revealed complex biochemical variations amongst the cultivars, including for amino acid metabolism. Mineral elements may be inhibitors or activators of enzymes or play a role in complex regulation cascades. However, the use of ionomics and metabolomics in fruit remains rare. Such an approach in melon (Moing *et al.*, 2011) enabled the identification of co-regulated hubs, including aspartic acid and 2-isopropylmalic acid in addition to several specialized metabolites, in metabolic association networks, and of links between primary and specialized metabolism and key mineral and volatile fruit complements. For example, potassium was highly correlated with pyruvic acid, and copper was associated with 14 amino compounds including proline. The development of 'hormonomics' in parallel with the analysis of primary metabolites and other omics is of special interest for studying the metabolic regulations linked with fruit set and maturation (Oikawa *et al.*, 2015).

While the candidate gene approach has proved until now to be complicated for central metabolism, it has been more successful for specialized metabolism (Lewinsohn *et al.*, 2001; Tohge *et al.*, 2015). For example, novel transcription factors and several microRNAs regulating different steps of the phenylpropanoid pathway and one long non-coding RNA compromising the expression of nine stilbene synthase genes have been found in grapevine (Wong and Matus, 2017). In peach, the analysis of volatile compounds and gene expression revealed a set of genes that are highly associated with fruit volatiles, which could prove useful in breeding and for biotechnological purposes (Sanchez *et al.*, 2013).

After less than two decades, it is probably too early to reach conclusions on the contribution of post-genomics to improving the performance of fruit crops. However, the complexity of metabolic networks has been exposed. Factors limiting the accumulation of metabolites in fruits have recently been reviewed (Morandini, 2013), revealing that the constraints shaping the responses of metabolic systems to manipulation are mass conservation, cellular resource allocation and, most prominently, energy supply, particularly in heterotrophic tissues. Modelling represents a promising way to link such factors with the complexity of metabolism, and is the topic of the third part of this review.

TOWARDS INTEGRATIVE MODELLING OF FRUIT

Life sciences have reached a point where many aspects of the genotype–phenotype relationship can be quantified and used to construct mechanistic models of metabolism that allow for meaningful biological predictions (Bordbar *et al.*, 2014). In this section, we discuss three types of models that have been adopted in fruit research: enzyme-based (i.e. kinetic), reaction-based (i.e. stoichiometric) and process-based (i.e. biophysical) models, which may prove highly complementary and could be used to grasp the complexity of fruit metabolism.

Kinetic modelling of metabolic pathways

It has been frequently assumed that certain enzymes are rate limiting (Krebs, 1957), a concept that has been challenged in the light of results of metabolic control analysis (Kacser and Burns, 1973; Heinrich and Rapoport, 1974). Briefly, it is now accepted that the control of a metabolic flux is distributed between the different steps in the relevant pathway and that this distribution can vary with the physiological state. Consequently, it is almost impossible to predict the effect of an alteration of a given activity on the flux and metabolite concentrations of the corresponding pathway without implementing a kinetic model (Morandini, 2009). This probably explains why targeting enzymes has rarely resulted in improvement (see above). An enzyme-based kinetic model consists of sets of ordinary differential equations (ODEs) describing reactions of a metabolic network. When the reactions are adequately parameterized, ideally with experimental data, the computation of fluxes and concentrations becomes possible, as well as the estimation of so-called control coefficients for enzymes, which may allow the identification of candidate enzymes that could be manipulated to modify metabolism in a desired manner (Rohwer, 2012). High-quality experimental data on enzymes and metabolites are critical for building kinetic models, but they have hardly been available for modelling projects, mainly due to technical and organizational limitations (Kettner, 2007). Although such models were already used >60 years ago to describe biochemical processes, the number of validated available kinetic models remains astonishingly low, especially in plants (Rohwer, 2012; see also http://jjj.mib.ac.uk/ and http://www.ebi.ac.uk/biomodels-main/), despite their great potential for discovery. A model describing sucrose metabolism in sugarcane stems revealed that fructose and glucose uptake, vacuolar sucrose import and cytosolic neutral invertase are the most critical steps in determining the rate of sucrose accumulation (Rohwer and Botha, 2001; Uys *et al.*, 2007). Thereafter, the importance of neutral invertase in exerting a strong control over the hexose to sucrose ratio was demonstrated with transgenic sugarcane, in which this enzyme was downregulated (Rossouw *et al.*, 2010). The transfer of this model to the tomato fruit was made possible by adding the vacuolar compartment, indicating that transferring a model to another species is much more than a confirmatory procedure (Beauvoit *et al.*, 2014).

Building and parameterizing an enzyme-based compartmented kinetic model requires knowledge about three kinds of factor: (1) cellular reactions (i.e. network topology and enzymology); (2) cellular composition (i.e. biomass compounds, co-factors and accumulated metabolites); and (3) cell compartmentalization (i.e. sub-cellular volume fractions) (Fig. 3); for reviews, see Schallau and Junker (2010) and Rohwer *et al.* (2012). In this framework, fluxes are expressed as a function of reactant concentrations and kinetic properties using enzyme kinetic rate laws such as Michaelis–Menten and other *ad hoc* kinetics (Cornish-Bowden, 2004; Liebermeister and Klipp, 2006). Since enzyme capacities (i.e. maximal enzyme activities measured at substrate saturation) may vary during fruit development because of metabolic reprogramming (e.g. Biais *et al.*, 2014), they must be determined experimentally. However, kinetic constants can be taken from the literature or from experimental measurements (Fig. 3). The set of ODEs is solved by assuming that the growing fruit is at metabolic steady state, thus allowing modellers to perform a sensitivity analysis of the model which, in turn, pinpoints the most influential parameters whose values must be properly set. Ultimately, the parameters of a model are refined by comparing simulated and experimentally measured metabolites. Using optimization algorithms, the least-square fit of the data provides estimates for unknown parameters that are biologically relevant (for a review, see Tummler *et al.*, 2010), such as the carbon input flux or tonoplastic carrier capacities throughout tomato fruit development (Beauvoit *et al.*, 2014). Finally, independent data sets obtained, for example, with transgenic lines (Beauvoit *et al.*, 2014) can be used for validation, allowing the model to be established with a high level of confidence.

An advantage of kinetic modelling is that the model can be implemented with isoenzymes that catalyse the same reaction but display distinct kinetic properties and sub-cellular localization. An enzyme-based model of sucrose metabolism was able to discriminate the functioning of the various sucrose-degrading enzymes in developing tomato fruit (Beauvoit *et al.*, 2014). Sucrose cleavage was mainly sustained by acid invertase during cell division and was then relayed by neutral invertase and sucrose synthase during cell expansion. Meanwhile, the sucrose phosphate synthase activity remained at a low level. Altogether, these results indicated that each cleaving enzyme contributes to fruit sink strength, in contrast to previous findings, and that the sucrose synthesis–breakdown cycle was less active than previously hypothesized. Strikingly, the vacuolar sucrose carrier and acid invertase were found to exert a strong control over sugar composition, a prediction that has also been validated with data obtained from transgenic plants. Indeed, the transport of sucrose into the vacuole and its subsequent hydrolysis drive the osmotic potential of this organelle and, in turn, are likely to control vacuole expansion during early fruit growth.

Kinetic modelling can also be used to test the physiological relevance of regulatory features that have been previously biochemically characterized *in vitro*. For example, retro-inhibition of acid invertase and glucokinase, on the one hand, and the proton coupling mechanism of tonoplastic carriers, on the other, have been found to be essential to accommodate the experimentally measured sugar content through tomato fruit development (Beauvoit *et al.*, 2014).

Admittedly, kinetic analysis is usually restricted to small- and medium-scale networks not exceeding tens of reactions and transporters (Zhu *et al.*, 2007). A pioneering study attempted to account for the spatio-temporal specificity of sucrose metabolism, especially during the maturation of culm nodes of

sugarcane in close interactions with phloem (Rohwer, 2012). However, the detailed biochemical description of the network becomes difficult when kinetic models are scaled up, so the essential features captured by the model do not increase in proportion. One of the challenges in constructing realistic kinetic models is the scarcity of enzyme data, especially capacities within compartments and post-translational modifications, and the paucity of validation data sets. A further challenge will be to integrate information from transcriptomics, proteomics and metabolomics into mechanistic models. Such data sets are becoming more readily available for a growing number of fleshy fruits, but the data are usually expressed semi-quantitatively and are thus not readily usable.

Stoichiometric modelling of metabolism

Over the last 30 years, several hundreds of stoichiometric models, also called constraint-based models (CBMs), have been published (Bordbar *et al.*, 2014), including an increasing number of models describing plant metabolism. The reasons for such success are that stoichiometric models are amenable to the genome scale, do not necessitate massive computing resources and overcome experimental difficulties encountered with other modelling approaches (Shi and Schwender, 2016). Thus, unlike kinetic models, stoichiometric models do not require detailed knowledge about enzyme amounts and properties. However, while they do not enable predictions of metabolite concentrations, they offer the possibility of predicting fluxes, which is a valuable alternative when the use of isotopically labelled precursors is difficult. This is of great interest in fruits, which are very difficult to label (Sweetlove and Ratcliffe, 2011).

Stoichiometric modelling is based on a metabolic network description through stoichiometric equations of reactions and on the assumption of a pseudo-steady state. The network consists of coupled chemical conversions (reactions) that are mostly catalysed by enzymes. Nutrients are converted into building blocks such as nucleotides, fatty acids, lipids, amino acids and free-energy carriers that enable the synthesis of macromolecules such as DNA, proteins and cellulose. These macromolecules are required for the maintenance of cellular integrity and the formation of new cells. In a single reaction, substrates are converted into products, and the number of atoms of a given type such as C, H, O and N and the net charge should balance on each side of the equation. These balancing principles are followed in genome-scale metabolic reconstructions. Stoichiometric models have been widely used to estimate the metabolic flux distribution in the cell on the basis of an optimality hypothesis (flux balance analysis). Metabolic networks up to genome scale can be converted to stoichiometric matrices that enable constraint-based modelling when they are associated with input and/or output fluxes, and minimal and/or maximal reaction rates (Bordbar *et al.*, 2014). Once parameterized with such boundaries, these models can be used to generate a solution space for steady-state flux distributions. Objective functions can then be used to narrow the solution space. Commonly used objective functions include flux minimization, maximization of biomass production per unit substrate and maximized ATP yield. Stoichiometric models have proven very useful in the biochemical industry by enabling the optimization of the production of high-value molecules such as vanillin in yeast (Brochado *et al.*, 2010) and lycopene in *Escherichia coli* (Alper *et al.*, 2005). In plant research, stoichiometric models are still exploratory and face challenges such as tissue and cell metabolic specificities and sub-cellular compartmentalization. Thus,

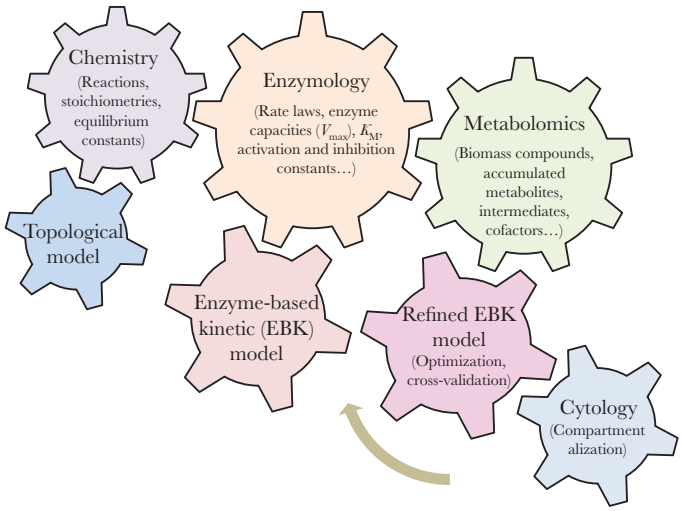

FIG. 3. Schematic representation of a data integration pipeline during construction and refinement of an enzyme-based kinetic model. Chemical information gives a structural framework describing the model topology. It is composed of chemical compounds connected through reactions and thermodynamic constants. The structural model is further enriched by enzyme data, which constrain the kinetic model. Enzyme rate laws are connected through a set of ordinary differential equations parameterized by numerous kinetic constants, e.g. enzyme capacity (V_{max}), Michaelis–Menten constants (K_M), as well as activation and inhibition constants. The enzyme-based model is further realistically constrained by metabolomics, first by giving access to co-factor concentrations and output fluxes, and secondly by enabling model parameterization via least-square fit of experimentally determined metabolites. Ultimately, independent data sets, when available, can be used to cross-validate the refined model. An additional layer of information is provided by cytological data. Thus, knowing the volume fraction of subcellular compartments and implementing the model with carriers and enzyme isoforms located in different compartments allows the model to calculate local concentrations and fluxes.

metabolic reconstructions will necessitate a more unified representation to make models comparable. In particular, co-factor specificity will need to be carefully addressed during reconstruction steps (Pfau at al. 2016).

With a medium-scale knowledge-based stoichiometric model describing central metabolism, fluxes have been determined throughout the development of the tomato fruit (Colombié *et al.*, 2015). This model has subsequently been implemented with a detailed description of the respiratory pathway including alternative oxidase and uncoupling proteins, which enabled the investigation of respiration and energy dissipation (Colombié *et al.*, 2017). With a large metabolic data set transformed into constraints, the model was then solved throughout the day by day development of the fruit. It detected a peak of CO_2 release as well as an excess of energy dissipation just before the onset of ripening, which coincided with the respiration climacteric. The unbalanced carbon allocation resulting from the simultaneous slowdown of biomass construction, on one hand, and the degradation of starch and cell wall polysaccharides, on the other, was found to explain the excess of energy that has to be dissipated. Additionally, constraint-based modelling is a promising tool for estimating fruit respiration, which is difficult to measure on fruits still attached to the mother plant. Therefore, it will be important to compare predicted and experimental data on respiration in fruits.

The most critical point regarding stoichiometric modelling is that flux predictions are highly dependent on the choice of the objective function used in the analysis. The function has to describe the metabolic 'purposes' appropriately even if cells are dedicated to several functions. While growth-based objective functions seem to be more appropriate for studying individual cells in culture, flux minimization is thought to be more adequate to describe complex metabolic networks of plant cells. The principle of flux minimization (Holzhutter, 2004) is based on the assumption that evolution selects cells able to fulfil vital functions such as growth and DNA repair by adjusting metabolic inputs. Stationary metabolic fluxes are considered to attain minimum values based on the availability of external substrates, i.e. substrates of the network under study. This principle has been shown to agree with the global behaviour of *in vivo* cellular systems and yields biological flux values (e.g. Grafahrend-Belau *et al.*, 2013).

While the 'enzyme cost', i.e. the amount of protein needed for a given metabolic flux, is crucial for the metabolic choices cells have to make, it has generally been ignored by constraint-based metabolic models, probably because information about protein amounts and/or enzyme activities was not available. A better description of the costs of protein synthesis and degradation (turnover) will be needed to define the energy (ATP) and carbon demand at the level of the whole metabolism. By developing a method for computing enzyme amounts needed to support a given metabolic flux at minimal protein costs, Noor *et al.* (2016) recently showed that the minimization of enzyme cost is a meaningful optimality principle for exponentially growing *E. coli* cells. In contrast, the modelling of fruit metabolism by using kinetic and stoichiometric approaches showed that most enzyme capacities always exceed the fluxes of the reactions they catalyse (Beauvoit *et al.*, 2014; Colombié *et al.*, 2015). This suggests that changing the capacities would have a limited effect on fluxes and their distributions. Paradoxically, all enzyme capacities measured throughout fruit development have been found to undergo major reproducible and stage-dependent changes, suggesting that the control of capacities still plays an important role during development (Biais *et al.*, 2014). Consequently, given the fact that highly conserved metabolic networks such as central and primary metabolism may operate very differently not only between species, organs, tissues and cell types but also between growing and steady cells or depending on the environment, stoichiometric modelling provides the opportunity to compare such diversity with relative ease. Thus, flux analysis and modelling of a range of plant systems have revealed the importance of the supply of metabolic inputs and demand for end-products as key drivers of metabolic behaviour (Sweetlove *et al.*, 2013). Thus, in fruits, the transposition of the heterotrophic model from tomato to other fruit species might prove very useful in improving our understanding of the links between metabolism and fruit phenotypes such as sweetness, acidity, growth rate and the occurrence of a respiration climacteric. Such models could then be used to describe metabolic diversity within species by exploiting the genetic diversity existing within species. For example, this could enable the identification of loci associated with fluxes (flux QTLs), which could lead to the identification of genes involved in flux control and ultimately to new breeding strategies. Given the fact that several species comprise cultivars exhibiting climacteric or non-climacteric behaviour (Barry and Giovannoni, 2007), it will also be interesting to compare flux maps obtained for climacteric and non-climacteric genotypes in order to gain further insights into the physiological meaning of the respiration climacteric.

Process-based modelling of fruit growth and quality

Fruit quality is *per se* the result of a complex chain of biological processes. As we have seen, sweetness results from hundreds of processes involved in sugar production in the leaves, loading and translocation in the phloem, unloading in the fruit cells, metabolism in the fruit cells and dilution by the water accumulated in the fruit. Agricultural practices influence these processes in a complex way. Clearly, not all the processes involved in fruit quality can be integrated in models. However, some degree of complexity is needed to be able to assess quality and the effect of agricultural practices.

Most plant simulation models were originally developed for agronomic applications (van Ittersum and Donatelli, 2003). Their success in such applications is largely due to their robust empirical description of the relationship between plant growth, environmental conditions and management practices. However, with the increase in knowledge, models with more processes and less empiricism have emerged during the last 20 years. Those process-based models offer a theory describing how the components of the system causally interact with one another to produce a given outcome. Simulations can be seen as the creation of a possible world that is constructed *in silico* using computer programs formally to represent relevant aspects of the real system under investigation. Process-based models decompose plant traits into various processes subjected to environmental variations, and enable the quantification of plant responses to genetic, environmental and management factors within a

mathematical framework that allows dynamic simulation of the physical, biophysical and physiological processes, with parameters independent of the environment and characteristic of a genotype or group of genotypes.

Prediction of fruit growth and composition requires an integrated view of plant functioning, with a formalization of interactions between resources, between organs and between processes. Such processes including organ emergence, growth and resource acquisition do not have the same sensitivity to the environment, thus resulting in large variations in source and/or sink phenotypes (T. Li *et al.*, 2015). As the plant is the main source of water, carbohydrates and minerals for the fruit, there is a need to link fruit growth with plant development and to take various organizational levels and the way they interact into account (Baldazzi *et al.*, 2012). For example, the contribution of fruit photosynthesis to the accumulation of carbohydrate in the fruit is marginal, whereas the position of a given fruit on the plant has a strong effect on the inflow of water and carbohydrates (Baldazzi *et al.*, 2013). To model fruit growth and its variability within the plant, some functional–structural plant models have been developed. They explicitly describe the architecture of the plant and its functioning by formalizing the processes of development, growth and acquisition of resources at the level of the organ. Such models allow the simulation of plant phenotypic plasticity with various environmental conditions (de Jong *et al.*, 2011) and agricultural practices (Louarn *et al.*, 2008; da Silva *et al.*, 2011) and hence are useful for investigating their effects on yield and fruit composition. A functional–structural plant model linking plant and fruit growth (the fruit growth model, after Fishman and Génard, 1998) has been developed for tomato (Baldazzi *et al.*, 2013). Estimations of resource acquisition (photosynthesis module), transpiration (radiative balance model), carbohydrate loading and leakage along the phloem pathway and transfer within the plant enable the simulation of water and carbohydrate availability at various locations within the plant. The water flow between the plant and the fruit is driven by the water potential gradient of the xylem and the phloem, and the carbohydrate import into the fruit is related to the phloem carbohydrate concentration through active uptake and mass flow. The model is able to simulate variations in leaf photosynthesis and transpiration with plant age and season, and hence can simulate carbohydrate concentration as well as water potential and their variability within the plant. Therefore, depending on plant age at anthesis and on the fruit position on the plant, variations in fresh and dry masses can be simulated. Thus, the model showed that fruits of the first truss are smaller because the leaf area is not fully developed, inducing lower carbohydrate availability. It also showed that within a given truss the distal fruits are smaller because of the progressive decrease of water potential along the truss rachis (Baldazzi *et al.*, 2013).

In the early 1980s, modelling of fruit growth was mainly limited to the accumulation of dry matter. Even to date, there are only a few models that simulate water accumulation. Models considering (1) water uptake and transpiration per unit of fruit area as a constant (Lee, 1990) or as a variable (Génard and Huguet, 1996); (2) the driving force resulting from the difference in water potential between the stem and the fruit; and (3) the role of fruit anatomy (Bussières, 1994) have been proposed. Thereafter, a model of fruit growth integrating both dry matter and water accumulation within the fruit was developed (Fishman and Génard, 1998; Liu *et al.*, 2007; de Swaef *et al.*, 2014). It is based on a biophysical representation of the fruit as one big cell in which sugars are transported from the plant phloem by mass flow, diffusion and active transport. Incoming water flows are regulated particularly by differences in water potential, and growth is effective only when the flow balance induces a sufficient turgor pressure on the cell walls. Fruit turgor pressure depends on carbon partitioning between soluble and insoluble solids. While soluble solids such as sugars and organic acids have rarely been the subject of modelling work, a sugar accumulation model (Génard and Souty, 1996) and two models for the accumulation of citrate (Lobit *et al.*, 2003; Etienne *et al.*, 2015) and malate (Lobit *et al.*, 2006; Etienne *et al.*, 2014) have been developed. The sugar accumulation model represents the transformation of phloemic sugars into different sugars accumulating in the fruit pulp (mainly sucrose, glucose and fructose), a part of which is used for synthesizing compounds other than sugars and for respiration. In this model, a simplified view of sugar metabolism relies on the 'rate law' of chemical kinetics, which states that the carbon flow between two compounds is proportional to the quantity of carbon in the source compound. Thermodynamic considerations of how cells function led to the inference that variations in mitochondrial metabolism explain citric acid concentrations, whereas vacuole storage would explain variations in malic acid (Etienne *et al.*, 2013). The citrate model is based on a simplified representation of the TCA cycle, in which pyruvate, malate and citrate are the only metabolites considered because they are at branch points between several reactions and are exchanged between the cytosol and the mitochondria. The model is able to simulate seasonal variations in both citric acid production and degradation. The malate model assumes that malate accumulation in fleshy fruits is mainly determined by the conditions of vacuolar storage in cells. The transport of malate is passive and occurs by facilitated diffusion of the di-anion form through specific ion channels and transporters. It follows the electrochemical potential gradient of the di-anion across the tonoplast, which is mainly controlled by the di-anion malate activity across the tonoplast and the electric potential gradient across the tonoplast.

A virtual fruit model (VFM) has been proposed (Lescourret and Génard, 2005; Martre *et al.*, 2011) that integrates the main processes involved in fruit quality development into one system. This model, which has been applied to species such as peach, mango, grape and tomato, has interesting complex features. For example, it predicts that the application of a water stress after a period of optimal irrigation results in a strong decrease in growth, whereas fruits grown on plants under continuous stress grow normally. This suggests that fruits can adapt to stressful situations. In real plants, this kind of adaptation has been called a memory effect (Trewavas, 2004). The model also predicts that enhanced unloading of sugars into the fruit leads to an increase in the amount of water accumulated in the fruit and, consequently, to an increase in fruit size. In turn, an increase in water supply leads to an increase in the amount of water accumulated in the fruit and, consequently, to an increase in fruit size, whereas the sugar concentration decreases. The quality traits are therefore affected differently according to the factor (carbon or water) considered, with either positive or negative correlations between fruit mass and sugar concentrations.

The VFM has been used to study intraspecific genetic variability of fruit growth, dry matter content and sugar concentration (Quilot *et al.*, 2005). Fruit species diversity, which is high regarding traits such as size, sweetness, acidity, starch accumulation, skin transpiration, xylem fluxes and growth rates, could be advantageously analysed with this modelling approach. The VFM could be improved by refining the coupling between cell division and cell expansion, and by integrating endoreduplication (Fanwoua *et al.*, 2013), for which an independent model is available (Apri *et al.*, 2014). Despite their importance, the interactions between cell growth processes (division, endoreduplication and expansion) during fruit development are still unclear and are subject to debate (Beemster *et al.*, 2003; Sugimoto-Shirasu and Roberts, 2003; John and Qi, 2008; Breuninger and Lenhard, 2012). To overcome this problem, *in silico* analyses of various coupling strategies could help to clarify the debate, providing insights into the control of organ development. In parallel, recent models describing cell growth and resource allocation developed for unicellular systems could also be used as a benchmark to better investigate the links among cell growth, metabolism and ploidy, in a general theory of cell economy (Molenaar *et al.*, 2009; Scott *et al.*, 2010; Weiße *et al.*, 2015).

Since most parameters are usually fitted in process-based models, the search for their genetic bases is possible only by forward genetics approaches such as QTL mapping, in which co-localizations between QTLs for traits and QTLs for model parameters are searched (e.g. Quilot *et al.*, 2005). Although the approach is very promising, it is relatively slow and work intensive, especially in species in which genetic resources and tools are limited. The integration of process-based models with more mechanistic models might represent an easier way to identify the parameters having the strongest control over a trait of interest.

Integrative modelling

While process-based modelling takes environmental variables into account, this is not the case with the mechanistic models of metabolism. Fruit growth and quality depend on an integrative system that functions at different levels of the plant and combines metabolic networks and biophysical processes. For example, fruit size is a function of cell number and cell expansion (Bertin *et al.*, 2007), where the former is tightly related to cell division and the latter largely depends on the biophysical properties of water transport that cannot be predicted solely from metabolic reactions. As discussed above, stoichiometric and enzyme-based kinetic models focused at sub-cellular or cellular levels can capture a clear picture of metabolic fluxes, but often overlook the dependencies and co-ordination between different compartments of a whole plant (Grafahrend-Belau *et al.*, 2013; Rennenberg and Herschbach, 2014). On the other hand, the process-based dynamic models are often too simplistic to account for biological processes. Linking process-based models (Fig. 4) to the genetic basis of metabolism could

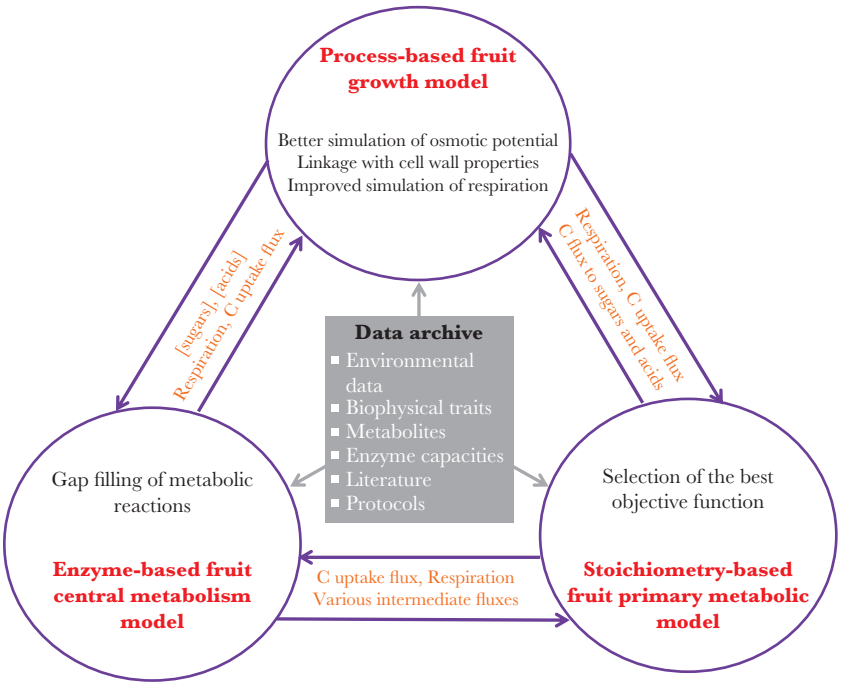

FIG. 4. Fruit model comparison and integration. The comparison of common variables enables cross-validation. The arrows indicate examples of potential benefits that will be obtained by comparing or coupling kinetic, stoichiometric and/or process-based models describing fruit growth and metabolism. Thus, enzyme-based kinetic models can be used to obtain data for osmolyte concentrations (i.e. sugars and organic acids), which can then be used to improve simulations of the osmotic potential and its link with cell wall properties in process-based models. Thereafter, both kinetic and stoichiometric models can be used to improve flux simulations for, for example, carbon influx or respiration within process-based models. In turn, process-based models can be used to constrain input and output variables, e.g. carbon influx and fluxes towards biomass components. Such constraining also makes it possible to study the effects of environmental variables such as temperature, light and watering on metabolic networks. Model integration necessitates the archiving of data in common or translatable formats. Common variables are summarized in the box between the models.

lead to powerful tools to manipulate fruit biomass and quality (Struik *et al.*, 2005). The interest in developing integrative models is 2-fold (Baldazzi *et al.*, 2012): from the point of view of molecular biology, an integrated multi-scale model would offer a useful framework to interpret omics data in relation to environmental factors, developmental stages and agricultural practices; from an ecophysiological perspective, the integration of cellular and molecular levels can help refine plant models, shedding light on the complex interplay between different spatial and temporal scales in the emerging system response (Chew *et al.*, 2014). In particular, the integration of an enzyme-based kinetic model (Beauvoit *et al.*, 2014) into a process-based model (Fishman and Génard, 1998) would enable the identification of those enzymes and/or transporters having the strongest control over a trait of interest (e.g. fruit size or sugar concentration), thus making it possible to manipulate this trait.

To our knowledge, however, there is still no integrated fruit model linking detailed fruit metabolism with biophysical fruit growth. Nevertheless, the crop research community is now attempting to create an integrative multi-level '*in silico* crop' platform (Marshall-Colon *et al.*, 2017). A model covering various levels of organization such as the sub-cellular, cellular, organ and whole plant would provide a holistic view of the system regulation and co-ordination that cannot be achieved with a model that is specific for a single level. Moreover, the advent of multi-scale models would pave the way for exploiting trade-offs in the configuration of metabolism between levels of organization (Sweetlove and Fernie, 2013). Multi-scale and combined metabolic models are required to be able to use flux balance models as a framework for metabolic engineering, especially to improve crop yield and quality (Baghalian *et al.*, 2014).

An integrated model could be achieved by various strategies from manual and loose integration to tight and automatic integration. This would also affect the efficiency and performance of the model (Borgdorff *et al.*, 2012; Zhu *et al.*, 2016). Several platforms have been developed to facilitate model integration with different frameworks, but they are still rarely used by plant modellers (see the detailed review in Marshall-Colon *et al.*, 2017). Process-based simulation models were successfully integrated into a so-called virtual peach fruit model by manually recoding and connecting several existing models (Lescourret and Génard, 2005), a process that turned out to be time consuming. Flux balance analysis (FBA) models have also been integrated with other types of models to provide an organ or even a whole-plant view. Multi-scale and combined metabolic models are required to be able to use flux-balance models as a framework for metabolic engineering, especially to improve crop yield and quality (Baghalian *et al.*, 2014). For example, the role of photorespiration during the evolution of C_4 photosynthesis was studied by coupling the genome-scale FBA model C4GEM (de Oliveira Dal'Molin *et al.*, 2010) with a mechanistic model of carbon fixation. The same authors also applied the FBA model of metabolism for leaf, stem and root systems across a day and night cycle to investigate how the metabolism of a given tissue is co-ordinated within the whole plant and to assess the effect of translocation costs on tissue metabolism (de Oliveira Dal'Molin *et al.*, 2015).

In addition to spatial integration, it is also possible to extend the static FBA into dynamic mode (dFBA) by integrating the simulated outputs at an earlier step to update the substrate and product amounts of the metabolic network, which are then used as inputs for the next time step (Mahadevan *et al.*, 2002). Grafahrend-Belau *et al.* (2013) developed FBA models for leaf, stem, ear and root of a barley plant and integrated each of them into a dynamic whole-plant function–structure model. The resulting model revealed source to sink shifts during plant development and provided a novel approach for *in silico* analysis of whole-plant metabolism. Chew *et al.* (2014, 2017*a*) created another elegant model in arabidopsis, from gene regulation via metabolism to whole-plant growth, by integrating several existing models in a modular way with minimal modifications of the original model. Recently, it has been proposed that epigenetic regulation, gene expression and metabolism could be integrated to simulate lycopene biosynthesis in growing tomato fruit (Gallusci *et al.*, 2017).

Flux balance models often provide a range of solutions with equal goodness-of-fit for the objective function, while a unique solution is needed for the subsequent iterations when it is integrated into a dynamic growth model. This may result in large derivations of model simulation so novel algorithms will have to be developed to solve this problem (Martins Conde *et al.*, 2016). Integrating a detailed metabolic model with a process-based biophysical fruit growth model would considerably increase the number of parameters, thereby making it difficult to parameterize the integrated model. Thus, model reduction during model integration might be necessary to obtain combined models with a reasonable number of parameters (Baldazzi *et al.*, 2012). The challenge here will be to perform large numbers of simulations in which parameters would be merged and environmental factors removed or simplified. To this end, the following steps or components seem to be crucial for model integration (Fig. 4). (1) Standardizing data collection and organization to create a comprehensive data depository accessible to the public. It will be crucial to have a database with sufficient quality and scope covering the various organizational levels for model integration (Zhu *et al.*, 2016). (2) Performing model cross-validations by comparing common variables. This will also open up a range of possibilities regarding the analysis of metabolism. (3) Reducing model complexity. As mentioned above, integrating models might considerably increase the number of parameters to be estimated or determined experimentally, and thus strongly increase the need for phenotypic data. Therefore, a compromise between performance and complexity could be sought by excluding non-essential components, i.e. parameters that have little influence on the simulations. Finally, it can be anticipated that integrated models will enable *in silico* analyses of the interactions between fruit biophysical properties and the distribution of metabolic fluxes, and ultimately provide valuable clues for potential targets of metabolic engineering.

Overall, with the development of high-performance computing, progress in FBA and enzyme-based kinetic models and the expansion of process-based dynamic models, the time has come to integrate isolated models into a multi-scale model framework covering gene regulatory networks, activities and properties of enzymes, metabolic pathways and their compartmentalization, and plant growth. Thanks to a multi-disciplinary approach within the plant sciences (Zhu *et al.*, 2016; Chew *et al.*, 2017*b*; Marshall-Colon *et al.*, 2017), such multi-scale models for both crops and fruits could lead to ideotype design by picking the right parameters and could eventually allow breeding to be accelerated (Long *et al.*, 2015; Constantinescu *et al.*, 2016; Zhu *et al.*, 2016; Chenu *et al.*, 2017).

TABLE 1. *Experimental variables and parameters for kinetic, stoichiometric and process-based modelling measured in the field, greenhouse or growth chamber*

Variable/parameter	Type	Biological material	Methodology	Purpose
Fruit age	A	Flowers and fruits	Tagging flowers at fertilization	Determination of the time course of development (M. Li *et al.*, 2015)
Fruit size	A	Whole fruit	Metric scales	Plotting of the growth curve (M. Li *et al.*, 2015)
Fruit surface area	P			Calculatation of fruit transpiration and mass flow of phloem and xylem water
Fruit fresh mass	A	Ovaries, whole fruits or specific fruit tissues	Weighing scales	Plotting of the growth curve and calculation of relative growth rate
Stone or seed proportion	A	Fruit stone or seed		Estimation of flesh proportion
Air temperature	P, K	Ambient air around fruit	Thermometer	Calculation of transpiration, respiration, water and sugar flow (Both *et al.*, 2015)
Air relative humidity	P		Hygrometer	
Stem water potential	P	Plant stem	Pressure chamber	Calculation of water mass flow from xylem into fruit (Scholander *et al.*, 1965)
Fruit maintenance respiration	P	Whole fruit or specific fruit tissues	CO2 gas analyser	Calculation of maintenance respiration coefficient and Q10 temperature coefficient (Walton and Dejong, 1990)

Variables and parameters, as well as fruit samples, are preferably collected throughout fruit development, from very young to ripening fruits

Type refers to the modelling approach (A, all models; K, kinetic, P, process based; S, stoichiometric).

Note that parameters that are very difficult or impossible to measure can be fitted (model calibration).

CONCLUSION

Our knowledge of fruit metabolism is now extensive. So far, progress in manipulating fruit quality and biomass production has mainly resulted from forward approaches, i.e. the phenotype has been used to select the best genotypes and/or agricultural practices. The fact that reverse approaches have been less successful implies that the right targets for improvement remain to be found. Indeed, several examples show that increasing or decreasing the activity of enzymes or transporters does not necessarily lead to the desired phenotypes. Therefore, despite the considerable work that has been required to collect and interpret post-genomic data, our understanding of the functioning of central and primary metabolism remains patchy.

Trade-offs between metabolic pools, on one hand, and between quality and growth, on the other, are often invoked although rarely expected, so it is challenging to understand what determines the size and composition of fruits. Indeed, these traits result from a range of processes that are controlled at different levels of organization, with subtle interactions occurring within or between these levels. They are determined through successive phases of development including cell division, cell expansion with potential endoreduplication, carbon storage and accumulation of specialized metabolites, and finally maturation, which can be seen as sinks in competition. Thus, whereas metabolism and development are traditionally studied separately, it has now become important to integrate them. For example, the changes observed in levels of pathway enzymes during fruit growth and ripening (Fig. 2) call for studies integrating not only hormonal signalling, gene expression, protein synthesis and degradation but also dilution by growth. Furthermore, fruit traits are not only a matter of molecular and biochemical events; biophysical processes also need to be taken into account, in particular to understand what underpins the trade-offs mentioned above.

Whereas statistical approaches are now widely used in fruit research to model high-dimensional post-genomic data sets, mechanistic models of metabolism are just emerging. It is almost certain that once these mechanistic models have incorporated environmental factors, they will become extremely useful in advancing our knowledge of fruit metabolism. However, experimentation on fruit-producing crops is usually costly and time-consuming, especially when slow-growing fruits are studied. In consequence, anticipating as much as possible future needs in terms of modelling might prove very useful. Tables 1 and 2 propose a range of parameters and variables that are needed in the modelling approaches presented above. It is estimated that all analyses mentioned in Table 2 could be performed with samples of 2–3 g of fresh material. Sampling would be best performed under cryogenic conditions and throughout fruit growth and development. It can indeed be anticipated that fruit modelling will increasingly benefit from high quality data, especially data about biomass composition.

For many reasons including its relatively short life cycle and genome, the ease of transformation, and the large number of available genetic resources concerning varieties, mutant collections and crossable related species, tomato is by far the most studied fleshy fruit species. The time has probably come to study and to compare the way primary metabolism operates in many other fruit species in order to learn more about factors such as sweetness, acidity, growth rates and climacteric character that make fruits so diverse. To this end, mechanistic modelling is particularly propitious, especially stoichiometric modelling which can be implemented quite easily provided that the evolution of biomass composition over time is known. A further promising approach will be to develop simplified integrative models, i.e. with few parameters of major importance to measure or to fit. Such models could prove very useful to study intra- and inter-specific diversity, and ultimately to propose new targets for the improvement of fruit crops.

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TABLE 2. *Experimental variables and parameters for kinetic, stoichiometric and process-based modelling measured in the laboratory*

Variable/parameter	Type	Biological material	Methodology	Purpose
Fruit dry weight	A	Ovaries, whole fruits or specific fruit tissues	Lyophilzation or oven at 70 °C Weighing scale	Calculation of relative growth rate and fresh to dry weight ratio (Gary *et al.*, 1998)
Initial fruit hydrostatic pressure (turgor)	P	Whole fruit or specific tissue of fresh fruit	Pressure probe or calculated from fruit water potential and osmotic pressure	Model initialization (Lechaudel *et al.*, 2007)
Fruit water potential	P		Chilled mirror hygrometer	Calculation of hydrostatic pressure (turgor) in fruit initialization (Lechaudel *et al.*, 2007)
Fruit surface conductance to water	P	Whole fruit	Mass loss registered using weighing scales	Calculation of fruit transpiration (Gibert *et al.*, 2005)
Fruit hydrostatic pressure (turgor)	P		Pressure probe or calculated from water potential and osmotic pressure	Estimation of cell wall extensibility/elasticity and yield threshold (Lechaudel *et al.*, 2007)
Fruit osmotic pressure	P	Fruit juice	Freezing point (osmometer)	Calculation of hydrostatic pressure (turgor) in fruit (Galindo *et al.*, 2016)
Fruit pH	P, K		pH meter	Parameterization of vacuolar H+-coupled transport (Beauvoit *et al.*, 2014; Etienne *et al.*, 2015)
Fruit growth respiration	P	Whole fruit or specific tissue of fruit	Estimated from carbon and nitrogen content of fruit ashes	Calculation of growth respiration coefficient (Gary *et al.*, 1998)
Stem phloem sugar concentration	P	Stem apex, cut stem or petioles	Aphid stylectomy or phloem exudation Analysis of phloem metabolic composition	Calculation of sugar mass flow from phloem into fruit and active uptake of sugars (Grossman and DeJong, 1994; Palmer *et al.*, 2013)
Osmotic pressure of other solutes in stem phloem	P			
Fruit mineral concentrations	P	Fruit ash	Atomic absorption spectrophotometry	Calculation of contribution of minerals to fruit osmotic pressure and vacuolar transport of acids (Leterme *et al.*, 2006)
Intermediary metabolites	K	Fresh fruit frozen powder or lyophilized powder	Mass spectrometry (IC-Qtrap-MS)	Fitting unknown parameters and model validation (Dai *et al.*, 2013; Beauvoit *et al.*, 2014)
Accumulated metabolites	K, S		Spectrophotometry	Calculation of outfluxes towards accumulated metabolites and biomass compounds (Beauvoit *et al.*, 2014; Colombié *et al.*, 2017)
Total soluble proteins	K, S			
Starch	K, S			
Nucleic acids	K, S			
Lipids	K, S		GC-FID	
Cell wall proportion	K, S	Whole fruit or specific tissue of fruit	Calculated from fruit dry mass, total soluble carbohydrate content and starch content	
Cell wall polysaccharides	S	Dry fruit powder	GC-MS	
Enzymes capacities	K	Fresh fruit frozen powder	Spectrophotometry	Model parameterization (Beauvoit *et al.*, 2014)
Estimation of subcellular volumes	K	Fixed fruit tissue	Photonic microscopy	

Analyses are performed on whole fruits, phloem samples or fruit samples that have been shock-frozen in liquid nitrogen when collected, ideally at various stages throughout fruit development, from very young to ripe fruits.

Type refers to the modelling approach (A, all models; K, kinetic, P, process based; S, stoichiometric).

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