Model-Assisted Analysis of Sugar Metabolism throughout Tomato Fruit Development Reveals Enzyme and Carrier Properties in Relation to Vacuole Expansion

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INTRODUCTION

The development of fleshy fruits such as tomato (Solanum lycopersicum) occurs in three distinct but overlapping phases: cell division (in the very early days following anthesis), cell expansion, and maturation. Because most of the cell volume is occupied by a large central vacuole, it has been postulated that fruit growth mainly depends on osmotic-driven enlargement of vacuoles, thereby giving the fruit its fleshy characteristics (Ho, 1996). However, the stage-dependent changes in vacuole volume occurring during growth have remained elusive to date. This issue is particularly relevant since other evidence links fruit expansion to the ring during growth have remained elusive to date. This issue is particularly relevant since other evidence links fruit expansion to the ring during growth.

Because compounds responsible for the taste and flavors of fruits, such as sugars, organic acids, and secondary metabolites, are stored within this organelle (reviewed in Martinoia et al., 2007), highly active sugar transport into fruit vacuoles must therefore be assumed (Shiratake and Martinoia, 2007). However, knowledge of sugar transporters of the tomato fruit tonoplast in terms of capacity, specificity, and energy requirement is still limited (Milner et al., 1995), and in vitro experiments can hardly be extrapolated within the framework of metabolic changes that underlie fruit development.

For decades, targeted studies based on reverse genetics and enzyme purification have focused on the functional and biochemical characterization of small sets of enzymes in sucrose and starch metabolism. Accordingly, up- and downregulation of sucrose synthase, which catalyzes a potentially reversible reaction in tomato (Sun et al., 1992), provided evidence that it could be rate-limiting for sucrose cleavage and might thus regulate sink strength during early fruit development, ultimately establishing the final fruit size (Sun et al., 1992; Wang et al., 1993; D’Aoust et al., 1999; N’chombo et al., 1999). However, downregulation of the vacuolar acid invertase (Klann et al., 1996) and of fructose-1,6-bisphosphate aldolase is more critical (Ho, 1996). The high activity of the vacuole is important for fruit quality because compounds responsible for the taste and flavors of fruits, such as sugars, organic acids, and secondary metabolites, are stored within this organelle (reviewed in Martinoia et al., 2007). Highly active sugar transport into fruit vacuoles must therefore be assumed (Shiratake and Martinoia, 2007). However, knowledge of sugar transporters of the tomato fruit tonoplast in terms of capacity, specificity, and energy requirement is still limited (Milner et al., 1995), and in vitro experiments can hardly be extrapolated within the framework of metabolic changes that underlie fruit development.

A kinetic model combining enzyme activity measurements and subcellular compartmentation was parameterized to fit the sucrose, hexose, and glucose-6-P contents of pericarp throughout tomato (Solanum lycopersicum) fruit development. The model was further validated using independent data obtained from domesticated and wild tomato species and on transgenic lines. A hierarchical clustering analysis of the calculated fluxes and enzyme capacities together revealed stage-dependent features. Cell division was characterized by a high sucrolytic activity of the vacuole, whereas sucrose cleavage during expansion was sustained by both sucrose synthase and neutral invertase, associated with minimal futile cycling. Most importantly, a tight correlation between flux rate and enzyme capacity was found for fructokinase and PPI-dependent phosphofructokinase during cell division and for sucrose synthase, UDP-glucopyrophosphorylase, and phosphoglucomutase during expansion, thus suggesting an adaptation of enzyme abundance to metabolic needs. In contrast, for most enzymes, flux rates varied irrespectively of enzyme capacities, and most enzymes functioned at <5% of their maximal catalytic capacity. One of the major findings with the model was the high accumulation of soluble sugars within the vacuole together with organic acids, thus enabling the osmotic-driven vacuole expansion that was found during cell division.

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shown to increase the sucrose-to-hexose ratio and to decrease the fruit growth rate and size. Conversely, sucrose phosphate synthase (SPS) and neutral invertase are thought to play a minor role because of their very low abundance in tomato fruit. For instance, overexpression of SPS increases the sucrose turnover, suggesting that this activity may be a limiting step in sucrose synthesis in domesticated tomato fruit (Nguyen-Quoc et al., 1999). On the other hand, intensive investigations of related tomato species further emphasized the role played by acid invertase and SPS in the sucrose-accumulating trait of mature wild-type tomato (Miron and Schaffer, 1991; Dali et al., 1992), thus suggesting the existence of the so-called sucrose cycling during the maturation phase (Dali et al., 1992; reviewed in Nguyen-Quoc and Foyer, 2001). Meanwhile, pioneering applications of metabolic control analysis (MCA) in plant biology demonstrated that the influence of a particular enzyme cannot be solely inferred from its over- or under-expression and that all enzymes and metabolites in the system must be considered (Thomas et al., 1997).

Given the recent development of "omics" approaches, correlation network analysis of fruit metabolism during growth and ripening has been performed at the level of transcripts and metabolites (Carrari et al., 2006; Mounet et al., 2009), transcripts and enzymes (Steinhauser et al., 2010), transcripts, proteins, and metabolites (Osorio et al., 2011), and, more recently, metabolites and enzymes under challenging environmental conditions (Biais et al., 2014). These integrated approaches revealed the existence of stage-dependent metabolic shifts and, more specifically, led to the identification of gene and enzyme subsets that may be involved in metabolic reprogramming. For instance, hierarchical clustering showed that cell division is characterized by a relative abundance of enzymes involved in energy metabolism, whereas anaplerotic enzymes were rather abundant during cell expansion (Biais et al., 2014). Thus, large-scale enzyme profiling provides critical information about the metabolic capacity of cells in a given genetic, developmental, or environmental context. However, to what extent metabolic fluxes readily respond to enzyme reprogramming remains to be elucidated. In the absence of a validated protocol of \(^{13}\)C metabolic flux analysis within a developmental series, one strategy of integrating enzyme capacities and metabolic traits is kinetic modeling. This approach has been successfully applied to the Calvin cycle (Pettersson and Ryde-Pettersson, 1988; Poolman et al., 2000) and to sucrose metabolism in sugarcane (Saccharum officinarum; Rohwer and Botha, 2001; Uys et al., 2007). More recently, by taking the properties of tonoplastic proton pumps and carriers into account, a kinetic model satisfactorily fitted the evolution pattern of malate in developing peach (Prunus persica) fruit (Lobit et al., 2006).

The objective of this study was to obtain new insights into the control of sucrose metabolism throughout tomato fruit development. For this, we built a kinetic model of the sugar metabolism of pericarp by combining the subcellular compartmentation and enzyme properties. Simulations were performed throughout fruit development to fit the measured variations of soluble sugar contents. A sensitivity analysis of the calculated fluxes and concentrations was performed to quantify the control exerted by enzymes within the network. Finally, hierarchical clustering was used to unravel hidden properties of the network by linking fruit growth, vacuole expansion, sugar storage, and enzyme properties.

RESULTS

Fruit Growth, Cell, and Vacuole Expansion throughout Fruit Development

Tomato (S. lycopersicum var Moneymaker) fruit growth follows a sigmoidal curve (Figure 1A), which is classically divided into a period of rapid cell division (0 to 10 d postanthesis [DPA]), followed by cell expansion (10 to 44 DPA) and then ripening (Mounet et al., 2009). The volume of the parenchymal cells of pericarp and of their subcellular compartments was estimated by a morphometric analysis. The mean cell volume was rather small during the division phase and increased largely during the expansion phase (Figure 1B). Meanwhile, the vacuolar and cytoplasmic volume fractions within cells changed with time, following a mirror-shaped pattern, i.e., between 2 and 15 DPA, the vacuole expanded from ~20 to 75%, whereas the cytoplasm shrunk from ~65 to 10% of the cell volume. Finally, the residual space, which is mainly occupied by the cell wall, remained constant (Figure 1C). Moreover, the plastid-to-cytoplasm volume ratio did not significantly change with time and was equal to 0.13 ± 0.04. In pericarp, the division phase is therefore characterized by an expansion of the vacuole within the dividing cell, whereas the fruit expansion phase results in a concomitant increase in all subcellular volumes. Therefore, water movements in the vacuole originate from two phenomena: (1) non-growth-associated vacuole expansion (i.e., volume readjustment between cytoplasm and vacuole) and (2) growth-associated cell expansion (i.e., water flow from phloem to pericarp cells). Consistent with this, the net water flow across the tonoplast, expressed in \(\mu\)mol of water min\(^{-1}\) g\(^{-1}\) fresh weight (FW), corresponding to each phenomenon was estimated by using the vacuole expansion rate derived from Figure 1C (see Equation 1) and the fruit growth rate derived from Figure 1A, multiplied by the vacuole volume fraction (see Equation 2), as follows:

\[
\frac{d}{dt} H_2O_{vacuole\ expansion} = \frac{d V_{vacuole}}{dt} \times \frac{1}{\rho_{tissue}} \times \frac{\rho_{water}}{MW_{water}}
\]

\[
\frac{d}{dt} H_2O_{cell\ expansion} = \frac{d (FW)}{dt} \times \frac{V_{vacuole}}{FW} \times \frac{\rho_{water}}{MW_{water}}
\]

where \(\rho_{tissue}\) and \(\rho_{water}\) are the tissue (in g FW mL\(^{-1}\)) and water densities, respectively; \(MW_{water}\) is the water molecular weight; \(V_{vacuole}\) is the vacuole volume fraction; and FW is the fruit fresh weight.

Figure 1D shows that the water inflow linked to vacuole expansion was the highest in the early stage and constantly decreased during cell division. Meanwhile, the water inflow associated with cell expansion followed a bell-shaped pattern with a maximal value for 15-DPA-aged fruit. Briefly, the majority of the water flow into the vacuole was associated with cell expansion, even though vacuole expansion per se accounted for at most 40 to 25% of the total water movement across the tonoplast during cell division (i.e., 4 to 10 DPA) (Figure 1D). The question is therefore to understand the relationships between the stage-dependent water movements in the vacuole and the concentration of sugars and organic acids within it. According to the general principles of kinetic modeling
applied to plant metabolic networks (reviewed in Schallau and Junker, 2010; Rohwer, 2012), these cytological data are combined below with enzyme data sets to parameterize mechanism-based models. Ultimately, this gives access to the carbon import, interconversion rates, and metabolite concentrations within cell compartments.

**Sugar Model Construction and Parameterization**

A kinetic model of the carbohydrate metabolism of pericarp cells was built according to Figure 2. The core of the network was based on the sugarcane model of sucrose metabolism developed by Rohwer and Botha (2001) and modified by Uys et al. (2007). The main implementation was the addition of the vacuole, containing acid invertase (AI) as well as tonoplastic carriers (the mono- and disaccharide carriers), and the plastids where starch synthesis occurs, containing the glucose-6-P/Pi translocator (Flügge et al., 2011). In addition, the impact of mass increase during growth was taken into account by adding output fluxes at the level of the most abundant cell constituents, namely, Glc, Fru, Suc, starch, and polysaccharides of the cell wall. These fluxes were calculated by multiplying the concentration of these compounds by the relative growth rate derived from Figure 1A. Note that the starch turnover, the cell wall invertase activity, and the apoplastic hexose import, which are likely to occur at the end of fruit expansion (Nguyen-Quoc and Foyer, 2001), were not included in the model because knowledge of their kinetic properties

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![Figure 1](image-url)  
**Figure 1.** Time-Course Evolution of Fresh Weight and Cellular and Subcellular Volumes throughout Fruit Development.  
(A) Growth curve of Moneymaker tomato fruit. Continuous line represents regression analysis using a three-parameter logistic function.  
(B) Mean volume of parenchyma cells of Moneymaker (closed circles) and Ailsa Craig (open circles) pericarp (mean ± so, n = 3 fruits).  
(C) Fractional volume of vacuole (circles), cytoplasm (triangles), and cell wall (squares) within cells of Moneymaker (closed symbols) and Ailsa Craig (open symbols) tomato pericarp (mean ± so, n = 3 fruits). Continuous lines represent nonlinear regression of vacuole and cytoplasm volume fractions and dashed line, that of the cell wall (see Supplemental Table 4 for details).  
(D) Water flow across tonoplast resulting from vacuole expansion within cell (dashed line), cell expansion within fruit (dotted line), or both (continuous line).
was lacking. Finally, glycolysis was the last output flux represented by the aldolase (ALD) reaction (Figure 2).

The model consists of 13 differential equations (Supplemental Table 1) describing the variations of hexoses, hexoses-phosphates, and Suc as a function of the 24 enzyme reactions of the network (Figure 2; Supplemental Table 2). The parameterization of the model was based on our measurements and on the literature. In general, half saturation constants or Michaelis constants ($K_m$) from the literature are more reproducible than maximal velocities ($V_{max}$), as they do not depend on enzyme concentration. $K_m$ values also tend to remain in the same order of magnitude across related species (see, e.g., the Brenda database, http://www.brenda-enzymes.org). For these reasons and after extensive reviewing, we used $K_m$ from various sources though preferably from studies in tomato or from other plants (Supplemental Table 3). Their values were assumed to be constant throughout fruit development. Moreover, time-dependent functions of the fruit relative growth rate and subcellular volumes (Figures 1A and 1C) were fitted by a logistic equation. Enzyme capacities ($V_{max}$) and metabolite contents of pericarp were determined by fitting data obtained with Money-maker (Biais et al., 2014) using cubic polynomials (Supplemental Figure 1). Vacular $\Delta$pH estimated from the malate plus citrate content of the pericarp was kept constant throughout the growth period (Supplemental Figure 2). ATP and ADP contents were assayed on the same samples and the cytoplasmic concentrations were subsequently calculated (Supplemental Figures 3A and 3B). Total acid extractable Pi was almost constant during development (Supplemental Figure 3C), and Pi concentration was assumed to be the same in cytoplasm and vacuole, as shown in vivo by $^{31}$P NMR (Rolin et al., 2000).

Biochemical reactions, rate equations, parameter settings, and corresponding references are listed in Supplemental Tables 2 to 5. Glucokinase (GK) and fructokinase (FK) were parameterized as irreversible Henri-Michaelis-Menten (HMM) (Deichmann et al., 2014) mechanisms, with a competitive Fru, glucose-6-P (Glc-6P), and fructose-6-P (Fru-6P) inhibition of GK (Claeyssen and Rivoal, 2007). Neutral and acid invertases (NI and AI, respectively) were parameterized as irreversible HMM processes, with competitive and noncompetitive inhibition by Fru and Glc, respectively (Sturm, 1999). The vacular sucrose- and hexose-$H^+$ antiporters were parameterized as reversible and symmetrical HMM processes driven by a proton motive force, with a competition of equal strength between Fru and Glc for the hexose carrier. Considering the pH difference across the tonoplast ($\Delta$pH) as the main component of the proton motive force (Shiratake and Martinoia, 2007), a driving force (i.e., $10^{\Delta$pH}) was introduced into the Haldane relationships, which express the kinetic parameters of the enzymes to the equilibrium constant of the reactions.

**Model Fitting and Validation**

The model covered the period of fruit growth starting at 4 DPA when cell proliferation was active and ending at 47 DPA where cell expansion ceased. The system of differential equations was solved in 10 stages describing pericarp growth as a succession of quasi-stationary states. The concentration of each intermediate is

![Figure 2. Schematic Network of Metabolism and Compartmentation of Carbohydrates in Tomato Fruit Pericarp.](image)

Network reactions: v1, sucrose import; v2, GK; v3, FK; v4, SPS; v5, sucrose-6-phosphatase (SPase); v6, Susy; v7, NI; v8 and v9, ATP- and PPi-dependent phosphofructokinase (PFK and PFP, respectively); v10, sucrose carrier; v11, AI; v12, sucrose storage; v13, glucose storage; v14, fructose storage; v15 and v16, hexose carrier; v17, PGI; v18, PGM; v19, UDP-glucopyrophosphorylase (UGPase); v20, ALD; v21, starch synthesis; v22, cell wall synthesis; v23, glucose-6-P/PI translocase; v24, FBPase. Output fluxes are italicized. Chemical reactions, rate equations, and kinetic parameters are detailed in Supplemental Tables 2 and 3.
constant at each stage but may vary from one stage at steady state to another. The initial parameterization of the model left three parameters unknown, i.e., sugar carrier capacities, namely, $V_{\text{max}10}$ and $V_{\text{max}15}$ (which is equal to $V_{\text{max}16}$), and Suc import ($V1$). The values of these three parameters were optimized at each stage by fitting the experimentally measured Glc, Fru, Suc, and Glc-6P contents. Briefly, parameter values were randomly searched by least square minimization. The whole iterative process was repeated using randomized initial conditions, and the 300 to 400 best scoring combinations of parameters were kept for each stage. Two means of comparing simulations and experiments were implemented. First, for each parameter combination, Glc, Fru, Suc, and Glc-6P contents were calculated. The resulting 300 to 400 values were averaged and superimposed with measured values. Figures 3A to 3C show that the averaged values of Glc, Fru, and Suc were fairly close to the measured ones, regardless of fruit stage. Second, as in Baker et al. (2010), a new set of parameters was built from the median values of the 300 to 400 combinations. The model was run with this new set of parameters and the calculated Glc, Fru, Suc, and Glc-6P contents were compared with measurements as above. Running the model with the median value of each optimized parameter resulted in a level of sugar contents fairly close to the corresponding mean values calculated above, on the one hand, and to the measured values, on the other hand (Figures 3A to 3C). Even though the general pattern of evolution of the calculated Glc-6P was consistent with the measurements, large discrepancies remained concerning the values. However, in view of the mechanism of the Glc-6P/Pi translocator, the Pi gradient between plastids and cytoplasm had to be considered (Pettersson and Ryde-Pettersson, 1988; Poolman et al., 2000, and references therein). By introducing this parameter into the rate equation, it became possible to accommodate the measured Glc-6P content of 8- to 40-d-aged fruits (Figure 3D). Overall, this optimization procedure provided suitable values of the three unknown parameters, thereby allowing values obtained with the model and the experiments to be matched throughout the entire growth period. Furthermore, for each of the 300 to 400 parameter combinations, fluxes were calculated and averaged, thus allowing the calculation of coefficients of variation (i.e., SD-to-mean ratio) as indicators of flux variability. For most fluxes, the coefficient

![Figure 3](image-url)
of variation was lower than 10% in the early and late stages and <25% in the middle and end of fruit growth (27 to 40 DPA), indicating that the mean of these fluxes was statistically reliable (Martin and Gendron, 2004). However, flux variability was noticeably high for NI and sucrose synthase (Susy) during the early and late growth stages (up to 50%) and was also high for the Glc carrier and phosphoglucomutase (PGM) in the intermediary growth stages, indicating that these fluxes were not precisely determined (Supplemental Figure 4).

The model was cross-validated using previously published enzyme profiles obtained with Moneymaker at three developmental stages (35, 42, and 49 DPA) (Carrari et al., 2006; Steinhauser et al., 2010). Figure 4A shows a good consistency between the measured and simulated Suc, Fru, and Glc contents, regardless of the stage (P value < 0.05). Likewise, the response of the model to changes in the sucrolytic enzyme activities was tested using published data obtained from various sucrose-accumulating tomatoes, i.e., on transgenic lines of Lycopersicon esculentum underexpressing Al (Klann et al., 1996), on the wild tomato Lycopersicon chmielewskii (Yelle et al., 1988), and on introgression lines obtained by breeding L. esculentum with L. chmielewskii (Yelle et al., 1991). Despite the presence of outliers, there was a consistency between measured and simulated Suc contents, regardless of the intra- or interspecies modulation of enzyme capacities (P value < 0.01) (Figure 4B). Interestingly, most of the outliers belong to a subgroup of Al underexpressing lines that are highly affected in final fruit size setting (Klann et al., 1996). This further emphasizes the importance of measuring the fruit growth rate to parameterize the model realistically.

Fitting of the three unknown parameters (i.e., Suc import and carrier’s $V_{\text{max}}$) and subsequent validation made it possible to investigate the control of the import, distribution, and accumulation of sugars at the system level.

**Retro-Inhibition of Acid Invertase and Glucokinase and Proton-Coupling of Tonoplast Carriers Are Essential for Model Fitness**

The response of the model to changes in parameter values was analyzed by applying MCA to calculate response coefficients (Kacser and Burns, 1973; Heinrich and Rapoport, 1974). Sugar contents were highly and positively sensitive to both the $\Delta \text{pH}$ and $K_{m}$ values of the hexose carrier. Conversely, Suc content was highly and negatively sensitive to the $K_{i}$ values of AI for Glc and Fru and to the $K_{m}$ value of the Suc carrier, as was the Glc content to the $K_{i}$ value of GK for Glc-6P (and not Fru-6P) (Supplemental Figure 5). To further address the respective influence of these parameters on data fitting, the model was optimized as above under conditions where AI and GK were not retroinhibited by their products. In both cases, a sum of weighted squared errors between calculations and measurements was calculated for each stage as a score of model fitness. Figure 5A shows that the score was very high at most growth stages. This was mainly due to differences in Suc or Glc contents in the case of AI or GK parameterization, respectively (Supplemental Figure 5). Similarly, changing the parameterization of carriers from an active to passive mechanism (no H+ coupling) drastically decreased the goodness of fit, regardless of the developmental stage (Figure 5A). In this case, it was mainly due to discrepancies between calculated and measured Glc, Fru, and Suc contents (Supplemental Figure 6). Overall, this approach tends to demonstrate that both the retroinhibition of AI and GK and the H+ -coupling of the tonoplast carriers are essential to accommodate the experimentally measured sugar content throughout fruit development.

Intriguingly, the only $K_{m}$ values ever published for the tonoplast of tomato fruit are 10- to 100-fold higher than in other species and the energy requirements of Suc and Glc transport remain unclear.

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**Figure 4.** Intra- and Interspecies Cross-Validation of Model.

(A) Model was parameterized using enzyme capacities measured with Moneymaker pericarp sampled at 35, 42, and 49 DPA (Steinhauser et al., 2010). Sucrose, glucose, and fructose contents (circles, squares, and triangles, respectively) are expressed relatively to 7-DPA-aged fruit as described by Carrari et al. (2006).

(B) Model was parameterized using enzyme capacities measured at breaker stage on ten transgenic lines of L. esculentum underexpressing acid invertase (Klann et al., 1996) (gray circles) and at seven developmental stages of L. chmielewskii wild tomato and its introgression lines (Yelle et al., 1988, 1991) (white and black circles, respectively). Suc content is expressed as percentage of total sugars, as described by Klann et al. (1996). Diagonals represent 100% match between simulations and experiments.
(Milner et al., 1995). Therefore, the capability of the model to respond to changes in carrier affinity was further tested by scanning the $K_m$ values from sub- to 100 mM ranges. Interestingly, a change in affinity from high to low and very low affinity only slightly decreased the goodness of fit (Figure 5B), thus making it possible to use all three parameterizations for further modeling.

**Stage-Dependent Changes in Tonoplast Carrier Capacities and in Sucrose Import into Pericarp Cells**

Figures 6A and 6B represent time courses of the carrier’s $V_{\text{max}}$ predicted by the model under conditions of high, low, and very low affinity parameterization. For both carriers and regardless of the growth stage, an increase in the $K_m$ values in the model was somehow compensated by an increase in the $V_{\text{max}}$ values, i.e., the higher the affinity of the carrier, the lower the predicted carrier capacity. Strikingly, $V_{\text{max}}$ reached extremely high values (up to 20 μmol min$^{-1}$ g$^{-1}$ FW) when the $K_m$ for hexose and Suc were set at 40 and 120 mM, respectively. Overall, the predicted $V_{\text{max}}$ values were high in the division phase for both carriers, decreased during expansion, and, in the case of the Suc carrier, increased again at the end of growth. This evolution pattern was about the same regardless of the carrier’s affinity (Figures 6A and 6B).

**Figure 5.** Influence of Parameterization of Vacuolar Acid Invertase, Glucokinase, and Vacuolar Carriers on Model Fitness.

**(A)** Model was parameterized with (black bars) or without retroinhibition of glucokinase (dark-gray bars) and acid invertase (white bars) or without H$^+$-coupling of vacuolar carriers (light-gray bars).

**(B)** Model was parameterized with a $K_m$ value of the hexose carrier equal to either 40, 4, or 0.4 mM and that of the sucrose carrier, to either 120, 12, or 1.2 mM, corresponding to very low (white bars), low (gray bars), and high (black bars) affinity conditions, respectively. At each developmental stage and for each condition, parameter optimization ($V_1$, $V_{\text{max}}^{10}$ and $V_{\text{max}}^{15} = V_{\text{max}}^{16}$) was performed and the sum of squared residuals between measurements and calculations of Glc, Fru, and Suc (see Supplemental Figure 6 for raw results) weighted by the SD of each measurement was calculated to score the model fitness. Inserts show cumulative sum of squared residuals over all stages. Error bars were calculated from data of Supplemental Figure 6.
The calculated Suc import flux was high during early cell division but decreased sharply during expansion to reach a basal value at the beginning of maturation (Figure 6C). This trend was not significantly influenced by the parameterization of the carriers. Moreover, the Suc import calculated by the kinetic model was quite consistent with that estimated using the fruit construction cost model of Heuvelink (1995), which estimates the amount of carbon assimilated into biomass and that consumed by respiration throughout the growth period (Figure 6C).

Flux Partitioning Analysis Reveals Stage-Dependent Contributions of Sucrose Cleaving Enzymes

As already demonstrated for Suc import, fluxes within the network did not vary significantly with respect to the affinity of the vacuolar carriers. Consequently, in the following section, flux is presented as the average of values calculated under very low, low, and high affinity conditions. Figure 7A represents the splitting of the imported Suc into soluble sugars, polysaccharides (cell wall and starch), and glycolysis throughout fruit growth. First, fluxes toward soluble sugar and polysaccharides, which were of the same order of magnitude, decreased with time following a sigmoidal shape. Meanwhile, glycolytic flux, which was high during cell division (accounting for ~50% of the total imported Suc in 4-DPA-aged fruit), decreased sharply during cell expansion and accounted for ~80% of the total imported Suc in 47-DPA-aged fruit.

Most importantly, flux through the three cleaving enzymes, namely, AI, NI, and Susy, changed with time (Figure 7B). During early cell division, Suc was predominantly hydrolyzed in the vacuole by AI, with a flux representing ~80% of the total cleavage in 4-DPA-aged fruit. Then, as the vacuolar hydrolysis decreased with time, NI and Susy fluxes transiently increased with maximal values in the middle of cell expansion, each representing ~40 to 30% of the total Suc breakdown. Finally, flux through Susy and NI tended to zero at the end of growth, even though Susy never functioned in the reverse direction. Meanwhile, SPS flux slightly increased in the middle of cell expansion but always remained low compared with the total Suc breakdown (at most 10%) (Figure 7B). Overall, Suc synthesis was rather low compared with degradation, thus suggesting that the so-called Suc cleavage synthesis cycle (Nguyen-Quoc and Foyer, 2001) was almost inactive, regardless of the developmental stage.

The time courses of the three fluxes upstream of glycolysis are presented in Figure 7C. Phosphorylation of Fru-6P occurred mainly through PPI-dependent phosphofructokinase (PFK) during cell division and was relayed by a transient increase in the ATP-dependent phosphofructokinase (PFK) flux during expansion. In comparison, the fructose-1,6-bisPase (FBPase) flux was very low regardless of the stage, thus suggesting that the fructose-1,6-bis-P synthesis-hydrolysis cycle was almost inactive throughout the entire fruit growth period.

Sucrose Cleavage Is Mainly Controlled by Sucrose Synthase, Neutral Invertase, and the Tonoplast Sucrose Carrier

To unravel the kinetic control of sugar metabolism, MCA was performed by calculating the so-called flux and concentration control coefficients (Kacser and Burns, 1973; Heinrich and Rapoport, 1974; Reder, 1988). Unlike that in AI, fluxes through SPS, Susy, and NI were strongly controlled by their own activity. For instance, the control coefficient exerted by SPS on its own flux was close to 1 throughout fruit growth, i.e., any increase in SPS activity would produce a proportional effect on flux.
Transport of Sucrose into the Vacuole and Its Subsequent Hydrolysis Drive the Osmotic Potential of the Vacuole

The fate of the Suc transported into the vacuole is illustrated in Figure 8A. During early cell division (i.e., 4 DPA), only 25% of the transported carbon was stored in the vacuole, the remainder being exported to the cytosol via the hexose carrier. During cell division and expansion, the hexose efflux fell as the transport of Suc and its subsequent hydrolysis decreased within the vacuole (Figure 8A), thus increasing the proportion of carbon stored in the vacuole up to 100% for 27-, 30-, and 35-DPA-aged fruits. In sum, vacuolar sucrolysis in young fruit shunts the cytosolic Suc-cleaving enzymes and feeds the cytosolic metabolism with hexoses. The consequences of this shunt, which is particularly active during division and early maturation, were further analyzed in terms of osmotic strength and energy cost. Thus, the net flux of sugar transport across the tonoplast was calculated as the Suc in flux minus the efflux of hexoses (Figure 8B). Surprisingly, a net sugar efflux occurred during early division which, given the H+-coupling mechanism of the carriers, corresponded to a net H+ influx, thus participating in the generation of proton motive force. In contrast, during cell expansion, a net sugar influx occurred that corresponded to a net H+ efflux and to a dissipation of the energy in the tonoplast (Figure 8B). The question is therefore raised as to the consequences of these transport activities on the steady state distribution of sugars on both sides of the tonoplast.

Given the subcellular concentrations calculated by the model, the soluble sugars were predicted to be localized predominantly in the vacuole throughout fruit growth (Supplemental Figure 9), thus suggesting that their contribution to cytoplasmic osmotic strength was very small (<2 mOsm). Conversely, their contribution to the osmotic strength of the vacuole was rather high and almost constant throughout the growth period (~120 to 150 mOsm) (Figure 8C). Moreover, in view of the electrogenicity of malate transport across the tonoplast, it has been demonstrated that malate mainly accumulates in the vacuole in peach (Lobit et al., 2006). By applying the same approach that consists in taking into account the subcellular volumes, the pericarp content of malate and citrate, their acid-base dissociation constants and a thermodynamic equilibrium of the electrogenic anion channel of the tomato fruit tonoplast (Oleski et al., 1987), similar conclusions

Figure 7. Flux Partitioning within Network during Fruit Growth.

At each developmental stage, fluxes were calculated at steady state using the optimized parameters ($V_1$, $V_{\text{max}}^{10}$ and $V_{\text{max}}^{15} = V_{\text{max}}^{16}$) in Figure 6. Values are means ± so of $n = 3$ values calculated under conditions of very low, low, and high affinity vacuolar carriers.

(A) Output fluxes of polysaccharide synthesis ($V_{21}+V_{22}$) (blue squares), sugar storage ($V_{12}+V_{13}+V_{14}$) (green triangles), and glycolysis ($V_{20}$) (magenta circles).
(B) Fluxes of sucrose cycle enzymes, i.e., acid ($V_{11}$) (magenta squares) and neutral invertase ($V_{7}$) (green circles), Susy ($V_{6}$) (blue triangles), and SPS ($V_{4}$) (black diamonds).
(C) Fluxes of Fru-1,6-bis-P cycle enzymes, i.e., PFP ($V_{9}$) (green squares), PFK ($V_{8}$) (blue circles), and FBPase ($V_{24}$) (magenta triangles). Note that flux values of SPS and FBPase are negative. Abbreviations are the same as in Figure 2.
could be drawn for the tomato pericarp (Supplemental Figure 9). The contribution of malate and citrate to the osmotic strength of the vacuole was thus calculated. Figure 8C shows that the contribution of these organic acids was high in the early division stages (100 mOsm) and constantly decreased during fruit growth to stabilize at around 25 mOsm during expansion. In sum, the contribution of sugars and organic acids to the osmotic strength of the vacuole started at a high value in the early division phase (260 mOsm) and constantly decreased to stabilize at around 150 mOsm during expansion.

**Sugar Accumulation Is Mainly Controlled by Sucrose Import and by the Tonoplastic Sucrose Carrier**

The control of the sum of vacuolar Glc, Fru, and Suc concentrations and therefore of vacuolar osmotic strength was further analyzed by calculating concentration control coefficients. The control exerted by Suc import into pericarp cells on the sugar accumulation within the vacuole was particularly high during cell division (4 and 8 DPA) and at the beginning of maturation (44 and 47 DPA) (at least +1.3) (Figure 8D). Conversely, the control exerted by the Suc tonoplastic transport was particularly high during cell expansion (15 to 44 DPA) (at least +0.6). AI did not noticeably control the vacuolar sugar concentration or, therefore, the osmotic strength of the vacuole, regardless of the growth stage (Figure 8D). In addition, the negative control of the vacuolar sugar concentration was shared between the relative growth rate and the tonoplastic hexose carrier (up to −0.8) throughout the growth period and, to a lesser extent, between GK, PFP, and Susy (up to −0.4) (Supplemental Figure 10).

---

**Figure 8. Role of Sugars and Organic Acids in Osmotic Strength of Vacuole during Fruit Growth.**

At each developmental stage, fluxes and concentrations were calculated at steady state using the optimized parameters (V1, Vmax10 and Vmax15 = Vmax16) of Figure 6. Values are means ± so of n = 3 values calculated under conditions of very low, low, and high affinity vacuolar carriers. (A) Partitioning of sucrose transported into vacuole between sugar storage (V12+V13+V14) and hexose efflux (V15+V16) (gray and white bars, respectively). (B) Net sugar influx across tonoplast (V10+V15+V16). (C) Contribution of sugars (open circles), organic acids (open triangles), and both (closed squares) to osmotic strength of vacuole. (D) Control of the sum of vacuolar Glc, Fru, and Suc concentrations by acid invertase (closed triangles), vacuolar sucrose carrier (closed circles), and cellular sucrose import (open circles).
DISCUSSION

This study investigated the interconversion and storage of soluble sugars throughout the growth of tomato fruit. A deterministic kinetic model describing the time course of sugars within pericarp tissue was built by integrating enzyme properties and the subcellular compartmentation of metabolites. In this model, only three parameters (V1, Vmax, 10, and Vmax 15 = Vmax 16) are unknown. Their optimization allowed us to accommodate the sugar contents measured at 10 developmental stages and to perform intra- and interspecies cross-validations using independent data sets.

Kinetic Modeling Reveals Unexpected Behavior of the Vascular Sugar Transport Systems

For decades, the kinetic properties of the tonoplastic transport systems have been extensively studied using a variety of techniques. While the specificity, affinity and maximum rate (Vmax) of transport have been measured only in isolated vacuoles or tonoplastic vesicles and frequently not from fruits (for tomato, Milner et al., 1995; reviewed in Shiratake and Martinioa, 2007), they differ widely between experiments and species (Martinioa et al., 2007). Therefore, the reported values expressed either as protein mass, tonoplast area, or volume are hardly convertible to rates per tissue FW (for peach, see Lobit et al., 2006). Given the lack of knowledge at present about sugar transport across the tonoplast, especially under in vivo conditions, doubts remain regarding the feasibility of quantitative modeling. Nevertheless, an optimization routine allowed us to find realistic kinetic parameters (e.g., Vmax and plausible mechanisms (e.g., H+-coupled antipor) for carriers that allow calculations to be made that match measurements at each developmental stage. One of the remarkable findings is that, like many enzymes of the carbohydrate metabolism (Blais et al., 2014), the predicted Vmax of the tonoplast carriers (expressed on an FW basis) undergoes a stage-dependent evolution (Figure 9). For instance, a high transport capacity of the Suc carrier characterizes the cell division phase and the beginning of maturation, thus positioning the Vmax of this carrier close to that of AI (Figure 9). In addition, a high transport capacity of the hexose carrier characterizes the cell division phase, thus making this carrier one of the members of the largest enzyme cluster that encompasses sugar kinases (FK, GK, PFK, and PFP), hexose-P interconverting enzymes (PGM and PGI), and cleaving enzymes (ALD and NI) (Figure 9). Overall, these data strongly suggest that tonoplast carriers are involved in the stage-dependent enzyme reprogramming that occurs during tomato fruit development (Steinhauser et al., 2010; Blais et al., 2014).

Fruit Growth Undergoes Stage-Dependent Changes in Cellular and Subcellular Volumes of Parenchyma Cells

The cytological analysis of pericarp cross sections confirmed that fruit growth is characterized by an increase in the volume of parenchymal cells during the expansion phase (Figure 1B). Importantly, the results show that the vacuole expands while the cytoplasm shrinks during cell division (Figure 1C). This expansion accounts for a significant part of the total water inflow across...
the tonoplast between 4 and 10 DPA. The fact that the vacuole expands faster than the cytoplasm in very young fruit implies that the osmotic strength of these two compartments varies accordingly (see below). Furthermore, this approach emphasizes the importance of knowing the dynamic of the subcellular volume changes when parameterizing a model and the need to interpret metabolomic data carefully. For instance, the ATP and ADP contents expressed as per unit of FW were shown to decrease with developmental time. However, when expressed as per unit of cytoplasmic volume, the ATP and ADP concentrations were shown to increase during cell expansion, with a nearly constant ATP-to-ADP ratio of 5.2 (Supplemental Figure 3).

Given the activity of H+/coupled sugar carriers, all soluble sugars accumulate mainly (up to 160 mM) in the vacuole throughout the entire growth period. The osmotic effect of sugar storage in the early division stages is further potentiated by the accumulation of malic and citric acids in the vacuole (up to 100 mM) (Figure 8C). In contrast, the contribution of free amino acids to the osmotic strength of the vacuole is likely to be weak throughout fruit development, i.e., at most 15 to 30 mOsm during division, assuming that amino acids are almost totally localized in the storage vacuoles (Tohge et al., 2011). Interestingly, the fact that organic acids result from the partial oxidation of sugars, which also provides ATP for the energization of the tonoplast, is in line with the occurrence of turbo metabolism (Biais et al., 2014) and probably compensates for
the predicted sugar efflux from the vacuole. Consequently, the osmotic potential of this organelle reaches high levels (the contribution of sugars and organic acids is about −0.6 MPa), corresponding to about two-thirds of the osmotic pressure of the whole fruit, thus triggering water inflow within dividing and expanding cells. There could be other physiological benefits of this H+-coupled accumulation of sugars in the vacuole. It might facilitate the inward transport of apoplastic hexoses into the pericarp cells down their concentration gradient during late development (Damon et al., 1988) and may be important for fruit growth when less Suc is available for uptake, especially under challenging environmental conditions. In this respect, the fact that sugar accumulation, and therefore the osmotic strength of the vacuole, is highly controlled by Suc import during cell division (Figure 8D) is in line with the reduced fruit size and low sugar content that are observed when Moneymaker tomato plants are grown under shaded conditions (Biais et al., 2014).

Special attention should be paid to the role of Al. Our analysis suggests that it did not significantly control the rate of Suc cleavage in the vacuole or the extent of sugar accumulation into this organelle. This might explain why a 100-fold decrease in its expression level in tomato is required to detect any effect on fruit size and on the sucrose-to-hexose ratio (Klann et al., 1996). In this regard, our modeling approach illustrates that a prerequisite for high Suc storage within sucrolytic tissues is a feedback regulation of the Al by hexoses, on the one hand, and of the GK by hexoses-P, on the other hand. Such feedback inhibition has already been used to constrain a model fitting the day-night Suc fluctuation in leaves (Nägele et al., 2010). In fruit, such properties are essential to fit the Suc content of both

Figure 10. Fractional Velocity of Enzymes during Fruit Growth.
Fractional velocity, i.e., flux-to-V_{max} ratio, was calculated from flux and V_{max} values of the respective enzymes. Values are means ± sd of n = 3 values calculated under conditions of very low, low, and high affinity vacuolar carriers.
(A) Enzymes of sucrose cycle.
(B) Aldolase, glucokinase, and fructokinase.
(C) Enzymes of Fru-1,6BP cycle.
(D) Hexose-P interconverting enzymes. Abbreviations are the same as in Figure 2.
sucrose- and hexose-accumulating tomato lines (Figure 4), especially during cell division when Suc particularly accumulates (Figure 3C).

The occurrence of a high sucrolytic activity in the vacuole during cell division induces a hexose efflux coupled to a Suc influx that energizes the vacuole membrane and achieves osmolyte homeostasis. This phenomenon leads to a steady state of sugar transport across the tonoplast with a minimal impact on the overall water movement, since these transport rates remain negligible compared with the water influx (10 versus 2000 nmol min\(^{-1}\) g\(^{-1}\) FW in 4-DPA-aged fruit). In contrast, the impact of sugar transport on vacuole energization might be physiologically significant. Consistent with this, the division phase was characterized by the highest accumulation rates of organic acids (i.e., 5 to 2 nmol min\(^{-1}\) g\(^{-1}\) FW between 4 and 15 DPA), in agreement with previous estimations made for peach (Lobit et al., 2006). Interestingly, these values are of the order of magnitude of those calculated for the sugar-linked proton efflux (i.e., 9 to 2 nmol min\(^{-1}\) g\(^{-1}\) FW; Figure 8B). This indicates that sugar transport might be coupled with other forms of energy-dependent solute transport during cell division. This does not rule out the involvement of the tonoplastic H\(^+\)-ATPase, which is likely to be active during this phase (Milner et al., 1995; Amemiya et al., 2006). Taken together, these data suggest a functional interplay between sugar and organic acid stores to trigger osmotic-driven vacuole expansion during early growth.

**Cycle-Linked Energy Expenditure**

An interesting outcome of the present model is that Suc cleavage was mainly sustained by AI during cell division and then was relayed by NI and Susy during cell expansion, thus tending to demonstrate that each cleaving enzyme contributes to fruit sink strength, in contrast to previous findings (Sun et al., 1992; D’Aoust et al., 1999; N’Gonchobo et al., 1999). As discussed above, AI participates in the osmotic homeostasis of the vacuole during cell division, which confirms the key role that it plays in the osmotic-driven elongation of other sink organs such as Arabidopsis thaliana root and cotton (Gossypium hirsutum) fiber (Wang and Ruan, 2010). Our results also emphasize the potential role played in the tomato fruit by NI, which is usually considered as a “maintenance” enzyme involved in Suc degradation when the activity of other invertases and Susy are low (Winter and Huber, 2000; Nguyen-Quoc and Foyer, 2001).

Given the fact that Susy has a relatively low affinity for Suc (~20 to 65 mM in various fleshy fruits), its highest flux rate during cell expansion is associated with a concomitant increase in its catalytic capacity (see also Wang et al., 1993; Blais et al., 2014) and in the cytoplasmic Suc concentration (Supplemental Figure 9). The latter increase during expansion results from a lower vacuolar hydrolysis and, to a lesser extent, from a higher SPS rate. However, the extent of this resynthesis does not seem to exceed 10% of the cleavage, thus indicating that the Suc synthesis breakdown cycle is less active during growth than previously hypothesized (Nguyen-Quoc and Foyer, 2001). Nevertheless, given the high control exerted by SPS on Suc synthesis, the 3- to 4-fold induction of SPS during ripening (Supplemental Figure 1) can be expected to end with a proportional increase in Suc synthesis, in addition to the increase in carbon import and the remobilization of starch that occurs at ripening (Blais et al., 2014). Furthermore, the extent of the fructose-1,6BP cycle also seems to be very low. Overall, our results suggest that the metabolic shifts underlying fruit development somehow minimize energy expenditure both in the vacuole during cell division (Suc influx-hexose efflux cycle) and in the cytoplasm during fruit expansion (Suc and fructose-1,6BP cycles). This energy-saving priority of fruit growth is further supported by the fact that metabolic shifts occurred without any significant changes in energy status, as attested by the ATP-to-ADP ratio (Supplemental Figure 3C). At steady state, the maintenance of this ratio suggests that the ATP-consuming fluxes decline in parallel with the ATP-synthesizing fluxes (such as glycolysis; Figure 7A) throughout fruit development. Altogether, these data are in line with the decrease in the capacity of the tricarboxylic acid (TCA) cycle and the lower glycolysis (e.g., pyruvate kinase) that Blais et al. (2014) observed during expansion.

**Inherent (Enzyme-Based) versus Hierarchical (Network-Based) Control of Sugar Metabolism throughout Fruit Development**

To unravel the unexpected regulatory aspects of sugar metabolism, an integrated approach was based upon the calculation of flux control coefficients and fractional velocities and upon the comparison of flux and enzyme capacity patterns. Several case studies have been highlighted.

First of all and most interestingly, Susy, together with UGPase, PGM, PFP, and GK, belongs to a group of enzymes whose flux increases in parallel with capacity (Figure 9), thus minimizing the changes in fractional velocity with time (Figure 10) and in turn decreasing its control coefficient during cell expansion (Supplemental Figure 4). This behavior somehow illustrates an adaptation of the enzyme capacity to the metabolic needs at a given stage, such as glycolysis (PFP and GK) and cell wall synthesis (PGM and UGPase) during cell division and Susy during cell expansion. A hierarchical clustering of enzyme capacities expressed on a tissue protein basis previously showed that PFP, PGM, and GK are more abundant during the division phase, while Susy and UGPase are more abundant during expansion (Blais et al., 2014). Taken together, these data suggest that the close match of the catalytic capacity to flux needs may be due, at least in part, to protein neosynthesis. Nevertheless, in these correlation analyses, one cannot rule out that posttranslational modifications or protein-protein interactions modulate \(V_{\text{max}}\) while the enzyme content remains constant. For instance, a Susy isoform has been shown to be phosphorylated and subsequently translocated to the plasma membrane in developing tomato fruit (Anguenot et al., 2006), raising the possibility that UDP-glucose could somehow be channeled into cell wall constituents (Winter and Huber, 2000). Moreover, acid invertases can be inhibited by proteinaceous inhibitors in tomato fruit (Tazinede et al., 2014), thus interfering in vivo with its development and hexose content (Jin et al., 2009).

The remaining enzymes of sugar metabolism exhibit different evolution patterns of flux and capacity, so the velocity ratio...
varies with time. In this framework, NI, FK, and PFK belong to a subgroup of irreversible reaction enzymes whose velocity ratio drastically increases during cell expansion (up to 0.2). However, in this context, only NI was shown to exert a high kinetic control on its own rate. Conversely, PGI, FBPase, SPS, and, to some extent, AI belong to another subgroup of enzymes whose velocity ratio varies with time but remains very low (up to 0.05), thus reflecting an excess of catalytic capacity. Such evolution patterns resemble those noted in highly glycolytic animal tissues, where the fractional velocity of both hexokinase and PFK is subject to large variations (from 0.001 to 0.9) with increasing physiological loads, whereas that of PGI remains unchanged (Suarez et al., 1997).

Overall, this study demonstrates that most enzyme capacities exceed the actual metabolic needs at each developmental stage, even though the amount of most of the enzymes of sugar metabolism dramatically declines during cell division. A similar conclusion was drawn for the TCA cycle of cultured Brassica napus embryos by comparing enzyme capacities and $^{13}$C measured fluxes (Junker et al., 2007). However, caution is required since the concept of “flux capacity provision” (Newsholme and Crabtree, 1986) does not automatically imply that the reversible reactions with low velocity ratio lie close to equilibrium and that they do not exert any kinetic control (e.g., Susy and PFP versus PGI, PGM, and UGPase) nor that the irreversible reactions with high velocity ratio exert a high kinetic control (e.g., GK and PFK versus neutral invertase). In fact, the flux, the fractional velocity, the proximity of the reaction to equilibrium and the control coefficients of individual enzymes are mutually dependent parameters that are differently related to the network kinetic properties (Kruckenberg et al., 1989; Rohwer and Hofmeyr, 2010). Kinetic modeling is a way to calculate all these parameters, thus allowing an exhaustive analysis of the functioning and regulation of the network to be performed.

In conclusion, the incorporation of enzyme capacities, sugar and organic acid concentrations, and subcellular compartmentation in a realistic kinetic model has increased our understanding of the metabolic reprogramming underlying tomato fruit development. One of the remarkable features highlighted is the high sugar transport capacity of the vacuole during early cell division and, to some extent, at the beginning of ripening, and the energy-saving priority of fruit growth. Further analysis of the respiratory chain components and subsequent model developments connecting the sugar metabolism to the mitochondrial TCA cycle will also be needed to extend knowledge of the interplay between energy metabolism and anaplerosis in the developing fruit. More work will also be needed to identify the genes underlying these tonoplastic transports, as already done for the H+-coupled symporters involved in the retrieval of apoplastic sugars at the end of expansion and at ripening (McCurdy et al., 2010). In this context, implementation of the model with these carriers and the cell wall invertase will help in deciphering the control exerted by these steps on the final sugar accumulation setting, as already suggested by genetic and biochemical experiments (Fridman et al., 2004; Baxter et al., 2005).

**METHODS**

**Plant Growth, Fruit Harvest, and Sample Processing**

Solanum lycopersicum var Moneymaker cultivar was grown as described by Biaya et al. (2014). Briefly, ~200 tomato plants were grown under optimal conditions in a greenhouse according to usual production practices. Flower anthesis was recorded and trusses were pruned at six developed fruits. Ten developmental stages from 4 to 53 DPA (red ripe) were harvested on three different trusses (truss 5, 6, and 7). For each sample, three biological replications were prepared with a minimum of four fruits per replication. Samples were prepared by cutting the fruits (after removing seeds, jelly, and placenta) in small pieces (~1 cm × 0.2 cm) of pericarp immediately frozen in liquid nitrogen. Frozen samples were then ground into fine powder with liquid nitrogen and stored at −80°C until analysis. Given the high reproducibility of the biochemical composition of fruit irrespective of the truss (Biaya et al., 2014), the analyses performed on the three trusses were averaged at each developmental stage.

**Perchloric Acid Extraction and Determination of ATP, ADP, and Pi**

Frozen tissues (50 to 100 mg FW) were ground in liquid nitrogen and extracted at 4°C using 500 μL of 7% (v/v) perchloric acid supplemented with 25 mM Na$_2$EDTA. The extract was centrifuged for 5 min at 13,000g. The supernatant was quickly adjusted to pH 5.6 to 6.0 using a 2 M KOH-0.3 M MOPS solution. KClO$_3$ precipitate was discarded by centrifugation (5 min, 13,000g). Adenine nucleotides of the supernatant were measured in a luminometer (Bio-Orbit) using the luciferine-luciferase assay (ATP-Lite Kit; Perkin-Elmer) according to the manufacturer’s instructions and using external ATP standardization. Total acid extractable Pi was measured on the same perchloric acid extracts using the colorimetric assay described by Cogan et al. (1999).

**Cytological Study**

Three Moneymaker fruits were collected at each stage (8, 15, 28, and 41 DPA) and fragments (1 to 2 mm thick) of equatorial pericarp were fixed for 4 h in glutaraldehyde (2.5% [v/v] in 0.1 M [pH 7.2] sodium phosphate buffer) at 4°C. During the first hour of fixation, an increasing vacuum (800 to 200 mbar) was applied. The samples were rinsed three times in phosphate buffer and treated for 2 h with osmium tetroxide (1% [w/v] in 0.1 M phosphate buffer) at 4°C, then with tannic acid (1% [w/v] in water) for 30 min. After three rinses, the samples were dehydrated by an ethanol series and embedded in Epon 812. Sections (1 μm) obtained with glass or diamond knives were stained with 0.04% (w/v) toluidine blue and photographed using a Zeiss Axioskop microscope coupled with a Spot RTKE digital camera. Cell and vacuole lengths and areas were measured using the Image-J software, using either automatic thresholding or manual or semiautomatic drawing. Subcellular volumes were calculated assuming that the cell, vacuole, and nucleus are prolate spheroids of radius $a$, $b$, and $c$ (with $a > b = c$). The cell wall was assumed to be delimited by two concentric prolate spheroids. Amyloplasts were assumed to be spherical and homogeneously distributed within the cytoplasm. Finally, the cytoplasmic space was determined by calculating the difference between the total cell volume and the above calculated volumes. Similar calculations were performed on three samples of Ailsa Craig fruits collected at 25 and 40 DPA and on previous electron microscopy observations of parenchymal cells of 1, 2, and 10 DPA Ailsa Craig tomato (Mohr and Stein, 1969).

**Computer Modeling**

The model was constructed from the mass balance equations of the compartmentalized biochemical network shown in Figure 2. The general form of the differential equations used is:
\[
\frac{dC_i}{dt} = \sum_{j=1}^n n_{ij} \cdot v_i \cdot \frac{\text{Vol}_j}{\rho}
\]

where \(C_i\) is the concentration of the \(i\)th species (in \(\mu\text{mol} \cdot \text{g}^{-1} \text{FW}\)), \(n_{ij}\) the stoichiometry of the \(i\)th species in the reaction \(j\), \(v_i\), the volume fraction of the compartment (in mL mL\(^{-1}\) tissue) where the \(j\)th reaction takes place, \(\rho\), the tissue density (in g FW mL\(^{-1}\) tissue), and \(v_i\), the rate of the \(j\)th reaction (mM min\(^{-1}\)) involved in the consumption and production of the \(i\)th species. The reaction rate equations, taking into account the measured maximal velocity (Vmax) of the enzymes, the volume space where they are located, and their kinetic parameters, are listed in Supplemental Tables 2 to 5. The set of differential equations listed in Supplemental Table 1 was solved by the Copasi 4.7 software (Hoops et al., 2006) to satisfy the steady state condition of metabolic intermediates, i.e., \(\frac{dC}{dt}\) close to zero. Ten Copasi files corresponding to the ten developmental stages are available as Supplemental Data Sets 1 to 10. Note that the compartment volumes (in mL g\(^{-1}\) FW) were implicitly implemented in Copasi as volume fractions divided by tissue density.

The tissue contents of Glc, Fru, Suc, and Glc-6P at steady state were calculated by taking into account the local concentrations of the metabolites given by the model, the compartment volume fractions, and the tissue density, according to the following equation:

\[
X_{\text{total}} = \frac{[X]_{\text{vac}} \cdot V_{\text{vac}} + [X]_{\text{cyt}} \cdot V_{\text{cyt}} + [X]_{\text{plast}} \cdot V_{\text{plast}}}{\rho}
\]

where \(X_{\text{total}}\) is the tissue content of the metabolite \(X\) (in \(\mu\text{mol} \cdot \text{g}^{-1} \text{FW}\)) and the subscripts vac, cyt, and plast symbolize the volume fraction (in mL mL\(^{-1}\) tissue) and the steady state concentrations (in mM) for the cytosolic, vacuolar, and plastidial compartment, respectively, and \(\rho\), the tissue density (in g FW mL\(^{-1}\) tissue). V value was calculated from a linear fit of the time course of the fresh-to-dry weight ratio of perecarp (Supplemental Table 5).

**Model Parameter Optimization**

Parameter optimization was performed using the random search algorithm by and minimizing an Obj score, i.e., the sum of the squared residuals weighted by the sd of each measurement, according to the following equation:

\[
\text{Obj} = \sum_{i=1}^n \left( \frac{X_{\text{cal}} - X_{\text{exp}}}{\sigma_{\text{exp}}} \right)^2
\]

where Obj is the objective score, \(n\) the total number of species, \(X_{\text{cal}}\), the calculated value, and \(X_{\text{exp}}\), the experimental value of a particular species \(X_i\) and \(\sigma_{\text{exp}}\), its sd. Sets of initial parameter values were randomly generated to avoid finding only local minima. At each developmental stage, the whole iterative process was repeated and the 300 to 400 best-scoring parameter sets were kept for further analysis. As a matter of comparison, random-based optimization gave solutions within the range of values obtained using the evolutionary programming algorithm implemented in Copasi (Supplemental Figure 11).

**Calculation of Carbon Import into Fruit Using Construction Cost Model**

The phloem sucrose flux can be calculated as the sum of the assimilate needs for biomass synthesis and respiration, according to the following equations (Heuvelink, 1996):

\[
\frac{dC_{\text{sucrose}}}{dt} = \frac{dC_{\text{mets}}}{dt} + \frac{dC_{\text{respiration}}}{dt} = \left(C_{\text{DW}} + q_{\text{growth}}\right)\frac{dC_{\text{DW}}}{dt} + q_{\text{maintenance}} \cdot DW \cdot Q_{\text{DW}}(T - 20)/10
\]

where \(dC_{\text{sucrose}}/dt\) is the total sucrose import (g of C day\(^{-1}\) g\(^{-1}\) DW); \(C_{\text{DW}}\), the measured carbon content of the pericarp (\(C_{\text{DW}} = 0.413 \pm 0.005 \text{ g C g}^{-1} \text{ DW}\)); \(q_{\text{growth}}\) and \(q_{\text{maintenance}}\), the assimilate requirement for growth-linked and maintenance respiration of fruit, respectively (\(q_{\text{growth}} = 0.15 \text{ g C g}^{-1} \text{ DW}, q_{\text{maintenance}} = 0.004 \text{ g C g}^{-1} \text{ DW day}^{-1} \text{ at } 20 \text{ C}\)); \(Q_{\text{DW}}\), the temperature-dependent coefficient for maintenance respiration (\(Q_{\text{DW}} = 2\)); and \(T\), the growth temperature (averaged temperature of the culture was 25.4 C) (Heuvelink, 1995; Liu et al., 2007).

**MCA**

According to the formalism of MCA (Kacser and Burns, 1973; Heinrich and Rapoport, 1974; Reder, 1988), response coefficients are defined as the scaled partial derivative of the simulated values with respect to each parameter:

\[
R_i^j = \frac{\partial \ln X_i}{\partial \ln p_j}
\]

where \(R_i^j\) is the response of concentrations \(X_i\) with respect to parameter \(p_j\). Coefficients were calculated using the implemented function in Copasi 4.7 and a delta factor of 0.001 for fixed parameters. The same method was used to calculate the flux \(C_{i}^j\) and concentration \(C_{i}^j\) control coefficients of an enzyme, which are defined as:

\[
C_i^j = \frac{\partial \ln J_i}{\partial \ln p_j} \quad \text{and} \quad C_i^k = \frac{\partial \ln X_i}{\partial \ln p_k}
\]

where \(v_i\) is the activity of the targeted enzyme \(j\), \(J_i\), a given flux; and \(X_i\), a given metabolite concentration.

Then, the control exerted by any enzyme \(j\) on the vacuolar sugar concentration was calculated as follows:

\[
C^S_j = \frac{C^S_{\text{Suc vac}}(\text{Suc vac}) + C^S_{\text{Glc vac}}(\text{Glc vac}) + C^S_{\text{Fruc vac}}(\text{Fru vac})}{C^S_{\text{Suc vac}} + C^S_{\text{Glc vac}} + C^S_{\text{Fruc vac}}}
\]

**Statistics and Mathematical Regressions**

Hierarchical clustering analysis and heat map visualization were performed using the TMeV4 software (Saeed et al., 2003) with the correlation-based distance measure and the average linkage clustering method. Linear and nonlinear regressions of the experimental data were performed using the solver function in Microsoft Excel. Statistical tests were performed with Student’s t-test and deemed significant if \(P < 0.05\). Coefficient of variation was defined as the ratio between the sd and the mean values (Martin and Gendron, 2004).

**Supplemental Data**

The following materials are available in the online version of this article.

- **Supplemental Figure 1.** Time Courses of Enzyme Capacities and Polysaccharide Contents throughout Fruit Growth.
- **Supplemental Figure 2.** Estimation of Vacuolar pH throughout Fruit Growth.
- **Supplemental Figure 3.** Time Courses of Adenylate Nucleotides and Phosphate Content of Pericarp Tissue throughout Fruit Growth.
- **Supplemental Figure 4.** Flux Variability as a Function of Developmental Stage.
- **Supplemental Figure 5.** Sensitivity Coefficients of Calculated Glucose, Fructose, and Sucrose Content toward Parameterization of Glucokinase, Acid Invertase, and Vacuolar Carriers.
- **Supplemental Figure 6.** Influence of Parameterization of the Vacuolar Acid Invertase, Glucokinase, and Sugar Carriers on Model Fitness.
- **Supplemental Figure 7.** Box Plot Graphs of Optimized Values of Vacuolar Carrier Capacities and Sucrose Import.
- **Supplemental Figure 8.** Control of Sucrose Interconverting Fluxes by Enzymes.
- **Supplemental Figure 9.** Evolution of Concentrations of Sugars and Organic Acids within Vacuole and Cytoplasm.
- **Supplemental Figure 10.** Control of Sugar Accumulation by Enzymes and Fruit Growth.
Supplemental Figure 11. Comparison between Random Search and Evolutionary Programming Algorithms for Parameter Optimization.

Supplemental Table 1. Ordinary Differential Equations Corresponding to Network in Figure 2.

Supplemental Table 2. Enzymatic Steps, Reactions, and Equations Rates of Kinetic Model.

Supplemental Table 3. Enzymes, Reactions, and Parameters of Kinetic Model.

Supplemental Table 4. Nucleotide Concentrations, Vacuolar ΔpH, and Compartment Volumes of Kinetic Model.

Supplemental Table 5. Time-Dependent Function of Relative Growth Rate, Metabolites, and Enzyme Capacities.

The following materials have been deposited in the DRYAD repository under accession number http://dx.doi.org/10.5061/dryad.39vb6.

Supplemental Data Set 1. Kinetic Model of 4-DPA-Aged Fruit.

Supplemental Data Set 2. Kinetic Model of 8-DPA-Aged Fruit.


Supplemental Data Set 8. Kinetic Model of 40-DPA-Aged Fruit.


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AUTHOR CONTRIBUTIONS

B.P.B., C.C., M.D.-N., J.-P.M., and Y.G. designed the research. B.P.B., B.B., C.B., M.-H.A., and A.M. performed the research. B.P.B., S.C., C.N., and M.D.-N. analyzed the data. B.P.B., S.C., J.-P.M., and Y.G. wrote the article, which was later approved by all the other authors.

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Model-Assisted Analysis of Sugar Metabolism throughout Tomato Fruit Development Reveals Enzyme and Carrier Properties in Relation to Vacuole Expansion

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