Reproducibility of tomato fruit enzyme activities

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Remarkable reproducibility of enzyme activity profiles in tomato fruits grown under contrasting environments provides a roadmap for studies of fruit metabolism

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Summary

In tomato fruits, the profiles of enzyme capacities involved in central metabolism are strongly influenced by developmental stage, but only weakly by environment.

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Abstract

To assess the influence of the environment on fruit metabolism, tomato (*Solanum lycopersicum* L.) cv. Moneymaker plants were grown under contrasting conditions (optimal for commercial production, water limited or shaded) and locations. Samples were harvested at nine stages of development and 36 enzymes activities of central metabolism were measured, as well as protein, starch and major metabolites such as hexoses, sucrose, organic acids and amino acids. The most remarkable result was the high reproducibility of enzyme activities throughout development irrespective of conditions or location. Hierarchical clustering of enzyme activities also revealed tight relationships between metabolic pathways and phases of development. Thus, cell division was characterized by high activities of fructokinase, glucokinase, pyruvate kinase and TCA-cycle enzymes, indicating ATP-production as a priority, whereas cell expansion was characterized by enzymes involved in the lower part of glycolysis, suggesting a metabolic reprogramming towards anaplerosis. As expected enzymes involved in the accumulation of sugars, citrate and glutamate were strongly increased during ripening. However, a group of enzymes involved in ATP-production, which is probably fueled by starch degradation, were also increased. Metabolites levels appeared more sensitive than enzymes to the environment, although such differences tended to decrease at ripening. The integration of enzyme and metabolite data obtained under contrasting growth conditions using principal component analysis suggests that with exception of alanine amino-transferase, and glutamate- and malate dehydrogenase and malate, there are no links between single enzyme activities and metabolite time-courses or levels.
Introduction

Tomato (*Solanum lycopersicum* L.) is ranked number 1 among fruits and vegetables with 14% of the total production worldwide. Some 4 million hectares are in production yielding 18 kg per habitant (Food & Agriculture Organization of the United Nations, http://faostat.fao.org). Tomato is also the most studied fleshy fruit (Giovannoni, 2001; Klee and Giovannoni, 2011), thanks to ease of cultivation, short generation times, a relatively small sized diploid genome and good tolerance of inter-specific crosses, inbreeding, high density mutagenesis and transformation. Numerous introgression lines (e.g. Eshed and Zamir, 1995; Tanksley et al., 1996), mutants (Saito et al., 2011) and transformants (e.g. Smith et al., 1988; Klee et al., 1991; Carrari et al., 2003) have been generated. Its genome has been sequenced and annotated recently (The Tomato Genome Consortium, 2012) and a number of post genomic approaches have been used to gain insights into molecular networks controlling fruit development and ripening. They include analyses of fruit transcriptomes (Alba et al., 2005; Lemaire-Chamley et al., 2005), proteomes (Rocco et al., 2006; Faurobert et al., 2007), and metabolomes (Roessner-Tunali et al., 2003; Schauer et al., 2006) as well as multilevel studies integrating transcriptomics and metabolomics (Carrari et al., 2006; Mounet et al., 2009), transcriptomics and enzyme profiles (Steinhauser et al., 2010), or transcriptomics, proteomics and metabolomics (Osorio et al., 2011). These efforts, mostly focused on the cellular level, are leading to an increasingly broad understanding of organogenesis, development and maturation of fleshy fruits. Yet, such approaches are usually conducted under standard growth conditions and do not take into account environmental factors known to affect yield quality and quantity.

Metabolite composition, which is an important component of fruit quality, results from numerous metabolic pathways that undergo profound reprogramming throughout fruit growth and ripening (Carrari et al., 2006). Although such reprogramming strongly depends on the genotype (Sinesio et al., 2010), producers and gardeners know very well that it is also modulated by the environment (Beckles, 2012). The main limiting resources for fruit growth and quality are water and carbon coming from the plant (Prudent et al., 2010). The main abiotic variables affecting these resources are light, water and temperature. Thus, shading leads to a reduction in fruit fresh weight, size and quality (Cockshull et al., 1992). Conversely, high temperature and light intensity have been found to increase hexoses in cherry tomato
fruits (Rosales et al., 2007). It is also well known that salinity (Cuartero and Fernández-Muñoz, 1998) and increased electrical conductivity of the growth medium (Fanasca et al., 2007) both lead to various metabolic responses in fruits, in particular to increased sugar and organic acid contents. More generally, it has been shown that interactions between genotype and the environment result in high variability for a wide range of primary and secondary metabolites (Schauer et al., 2008). Finally, fruit load (Do et al., 2010) and fruit position also influence quality. Tomato fruits are known to compete within a truss (Gautier et al., 2005) and greenhouse tomato producers usually prune trusses to maximize fruit growth and quality. The position of the truss on the plant has also been found to influence various metabolites in the fruits (Bénard et al., 2009) including lycopene and beta-carotene (Fanasca et al., 2007) as well as seed quality (Dias et al., 2006). Such variations are probably due to a combination of factors, especially in indeterminate varieties where new trusses are initiated while the plant continues to grow, which implies different micro or macro climatic conditions for each truss.

The study of metabolic fluxes and enzyme activities provides critical information towards a better understanding of metabolic control in fruits (Beckles et al., 2012). Accordingly, several reports show that modifications of enzyme properties can impact fruit metabolite composition. For example, a 30% decrease in aconitase activity in transgenic tomato fruits expressing an antisense gene construct led to a strong increase in organic acids at maturity (Morgan et al., 2013). Similarly introgression of the gene encoding the regulatory subunit of ADP-glucose pyrophosphorylase from Solanum hirsutum into the cultivated tomato resulted in higher starch levels in the developing fruit and then higher Brix in mature fruits (Schaffer et al., 2000). Finally introgression of a gene encoding cell wall invertase from Solanum pennellii, with higher affinity for sucrose, also resulted in fruits with higher Brix (Fridman et al., 2004). On the other hand, intensive investigation of a small set of enzymes from sucrose and starch metabolism pinpointed significant differences between cultivars and related species and eventually brought about better understanding of carbon source-sink relationships in fruits (e.g., Miron and Schaffer, 1991; Wang et al., 1993; Demnitz-King et al., 1997; Schaffer and Petreikov, 1997). For example, it was demonstrated that a strong increase in acidic invertase at ripening is responsible for the accumulation of hexoses at the expense of sucrose in the cultivated tomato (Yelle et al., 1991). More recently, the profiling of 22 enzymes revealed subtle but significant differences between two tomato varieties (Steinhauser et al., 2010), confirming that the genetic component of metabolite composition includes programming of pathway enzymes.
Conversely, very little is known about the influence of the environment on fruit metabolic pathways. In particular, it is still unclear to what extent metabolic responses to factors such as light intensity or water supply are due to a reprogramming of fruit central metabolism or simply to alterations in assimilate supply from the mother plant. Profiling enzyme activities from various pathways in fruits harvested at various developmental stages and grown under contrasting growth conditions therefore seems a rational step. Indeed, even when measured in vitro, enzymes provide integrated information about gene expression and post-translational regulation (Gibon et al., 2004). Furthermore, the recent development of robotized enzyme profiling now enables the profiling of tens of activities in large numbers of samples and at relatively moderate costs (Rogers and Gibon, 2009), which enables the performance of detailed experiments.

The aim of the present study was to assess the effect of environment on fruit metabolism by comparing enzyme activity profiles obtained at various developmental stages in fruits from different trusses and/or from plants grown under contrasting growth conditions. The integration of this information with metabolite levels provides a better understanding about relationships between metabolism and fruit development and maturation.

**Results**

All experiments were performed with the Moneymaker cultivar, which is an indeterminate greenhouse variety that was bred in the early 20th century by F. Stonor and sons in Southampton (UK). This cultivar, which is still available on the market, essentially for gardeners and organic farmers, has been used in many studies focused on metabolism (Holtzapffel et al., 2002; Carrari et al., 2006; Luengwilai et al., 2010).

**Plant and fruit development under optimal conditions, shading and water limitation**

About 580 tomato plants were grown in a greenhouse in the south west of France (Sainte-Livrade sur Lot) during summer 2010 according to usual production practices. To avoid competition for assimilates between fruits of a given truss, fruit number was limited to six per truss by pruning.
To assess the effects of a decrease in light level, a mesh was installed above the plants to remove 60% of the photosynthetically active radiation. To assess the effect of water-limitation, the supply of the nutrient solution was decreased by 50%. This treatment provoked a global increase in nutrients in the draining solution, but only marginally affected their relative concentrations, indicating that there was no nutritive stress (not shown). Both treatments, which were started at 75 days after sowing, affected plant growth. Thus, in comparison to the control plants (average size at the end of the culture: 295 ± 15.2 cm, n=9), shaded plants were etiolated and taller (346 ± 15.4 cm, n=10) and plant under water shortage were slightly smaller (266 ± 11 cm, n=9). Trusses 1 to 4 were already growing on the plants at the onset of treatment. Whereas water shortage did not provoke any fruit abortion, shading resulted in the abortion of about 90% of the flowers and young fruits. Later on, the proportion of abortion decreased in shaded plants, resulting in 3 to 5 fruits per truss, indicating that source-sink relationships eventually improved. Fruits were harvested on three different trusses (5, 6 and 7) and at nine stages of development, i.e. 8, 15, 21, 28, 34 Days Post Anthesis (DPA), Mature Green (about 42 DPA), Turning (about 47 DPA), Orange (about 50 DPA) and Red Ripe (about 55 DPA). Figure 1A presents the fresh weight of fruits throughout development grown under the three conditions. At the Red Ripe stage, fruits were the largest under control conditions, with an average weight of 99.6 ± 3.5 g, while fruits weighed 86.7 ± 11.6 g under water shortage and only 60.3 ± 8.3 g under shaded conditions. Ripening of fruits of shaded plants was also delayed, as illustrated in figure 1B and 60 to 63 days were necessary to reach the red ripe stage instead of 55 for control and water shortage conditions.

Developmental changes in enzyme activities of fruits grown under optimal growth conditions

The activities of 36 enzymes involved in carbohydrate metabolism, glycolysis, the metabolism of organic acids and the Calvin-Benson cycle were measured at 9 stages of development. Figure 2 presents the results obtained for the seventh truss (see also supplemental Table I). To enable comparison with previous work (e.g., Steinhauser et al., 2009) we first expressed them on a fresh weight basis. Most enzyme activities were highest in the youngest stage and decreased sharply during the first 15 to 21 DPA, tending to a plateau until the end of fruit development and maturation. This trend was particularly marked for enzymes involved in carbohydrate metabolism, i.e. glucokinase (E.C. 2.7.1.2) and fructokinase (E.C. 2.7.1.4), which are involved in the phosphorylation of hexoses, ATP-
(PFK, E.C. 2.7.1.11) and PPI- (PFP, E.C. 2.7.1.90) phosphofructokinases, which are involved in the phosphorylation of fructose-6P, as well as phosphoglucoisomerase (PGI, E.C. 5.3.1.9) and phosphoglucomutase (PGM, E.C. 5.4.2.2), which catalyze interconversions of hexose-P. In contrast, enzymes involved in sucrose degradation (sucrose synthase, E.C. 2.4.1.13; invertases, E.C. 3.2.1.26) and synthesis (sucrose phosphate synthase, E.C. 2.4.1.14) showed less marked changes, with the exception of acid invertase, which dramatically increased at maturation, increasing 4-fold within a few days. UDP-glucose pyrophosphorylase (E.C. 2.7.7.9), which is involved in sucrose synthesis as well as in the synthesis of cell wall components, was particularly stable throughout fruit development. Stromal fructose-1,6-bisphosphatase (E.C. 3.1.3.11), which is exclusively located in the plastid, but also aldolase (E.C. 4.1.2.13), triose-P isomerase (E.C. 5.3.1.1), NADP-glyceraldehyde-3P dehydrogenase (E.C. 1.2.1.9) and phosphoglycerokinase (E.C. 2.7.2.3) that are also partly located in the plastids also showed a sharp decrease in activity between 8 and 20 DPA. NADP-malic enzyme (NADP-ME, E.C. 1.1.1.40) initially showed a similar pattern (sharp decrease between 8 and 21 DPA followed by stabilization), but then dropped significantly and abruptly at the beginning of ripening. Also NAD-malic enzyme (NAD-ME, E.C. 1.1.1.38), pyruvate kinase (PK, E.C. 2.7.1.40) as well as most enzymes involved in the TCA cycle, the main aminotransferases and both NAD⁺- and NADP⁺- glutamate dehydrogenase also showed an initial decrease followed by stabilization. Among them, NADP-isocitrate dehydrogenase (NADP-IDH, E.C. 1.1.1.42), citrate synthase (E.C. 2.3.3.1) and alanine aminotransferase (AlaAT, E.C. 2.6.1.2) increased quite dramatically during ripening. Finally, phosphoenolpyruvate carboxylase (PEPC, E.C. 4.1.1.31), enolase (E.C. 4.2.1.11) and NAD⁺ malate dehydrogenase (MDH, E.C. 1.1.1.37) showed a different pattern, as their activities decreased in a quasi-linear fashion throughout fruit development.

These data are in agreement with previously published data (Steinhauser et al., 2010). For example, the maintenance of high SPS activity at ripening is confirmed. However, the present dataset covers earlier developmental stages (8, 15 and 21 DPA) corresponding to cell division and early cell expansion, at which most of the activities were much higher than at later stages.
Expressing enzyme activities per protein content minimizes the influence of vacuolar expansion

Expansion of the vacuole in fruit pericarp cells (Amemiya et al., 2006) probably explains a large proportion of the variations during development for enzyme activities expressed on a fresh weight basis (Steinhauser et al., 2010), especially during the first 20 days. Consistently, while fruit pericarp biomass increased (Figure 3A) protein content decreased sharply from 8 to 21 DPA, then tending to stabilize until ripening (Figure 3B). Figures 3C and 3D compare developmental changes expressed on fresh weight and protein basis, for fructokinase and PEPC, respectively. Whereas fructokinase decreased in both cases, PEPC showed very different patterns. Indeed, when expressed on fresh weight basis (nmol min⁻¹ g⁻¹ FW) it decreased throughout fruit development but when expressed on protein basis (nmol min⁻¹ mg⁻¹ protein), it increased during early stages, peaked at 28 DPA and then decreased until ripeness. Strikingly, SIPP2 transcripts, which encode a fruit-specific PEPC, have also been shown to peak during cell expansion (Guillet et al., 2002). Thus, assuming that the dilution effect due to vacuole expansion would be confounding for the interpretation of a large proportion of data, all values have been expressed per unit of protein from this point on.

Hierarchical clustering reveals tight associations between enzymes activities and developmental phase

Classically, fruit development is divided in three main phases: cell division, roughly corresponding to the 8- and 15-DPA samples, cell expansion corresponding to 21-, 27-, 34-DPA and Mature Green samples, and ripening, corresponding to Turning, Orange and Red Ripe samples. In order to investigate whether changes in enzyme activities could be assigned to these 3 phases, a hierarchical clustering analysis based on Pearson correlations was performed on mean-centered data obtained for trusses 5, 6 and 7 scaled to unit variance and displayed as a heat-map; white corresponds to the lowest activity (but not absence of activity) and dark red to the highest (Figure 4A). It first appeared that the unsupervised clustering analysis was always able to classify the nine developmental stages based on enzyme activities. Thus, 8 and 15 DPA (cell division), 21, 28, 34 DPA and Mature Green (cell expansion), Turning, Orange and Red Ripe (ripening) were found in 3 well separated clusters. The clustering of enzymes highlighted 4 main clusters.
The first cluster groups enzymes with high capacities during cell division (blue cluster) and to a lesser extent at the beginning of cell expansion (until 21 DPA), followed by a slow decrease, at the exception of, NAD-GluDH and PK, which show an increase at mature green stage and during ripening.

Enzymes of the second cluster presented a clearly different pattern (green cluster), with lower activity during cell division (8 and/or 15 DPA) followed by an increase to reach a plateau between 21 DPA and mature green. Their activities then decreased more or less gradually until the end of ripening. Thus, this cluster characterizes cell expansion.

The pattern of the third cluster (orange cluster) was similar to the second one with activities increasing during cell expansion with maxima reached close to the Mature Green and/or Turning stages, followed by a plateau or even a slight decrease during ripening.

The fourth cluster (red cluster) contained enzymes with large increases in activity during ripening between turning and orange and/or red ripe stage with mainly enzymes related to hexoses and sucrose (sucrose phosphate synthase, both acidic and neutral invertase) or citrate synthase (CS, E.C. 2.3.3.1).

As shown in figure 4B, the four clusters not only match developmental phases, they also correspond to pathways or sub-pathways in central metabolism. For instance, the first cluster associated to cell division contained glucokinase and fructokinase, and most enzymes of the TCA cycle. The cell expansion cluster could be clearly assigned to glycolysis and chloroplastic pathways (starch synthesis and Calvin-Benson cycle). The orange and red clusters comprised enzymes that are directly involved in reactions leading to metabolites accumulated during ripening, in particular invertase and citrate synthase.

**Principal component analysis of enzyme activities throughout fruit development**

Given the large number of variables, principal component analysis (PCA) was performed to facilitate the comparison between fruits obtained under various conditions. For this, a PCA was first performed on averaged data obtained from all fruits grown under optimal conditions. The score plot (Figure 5A) obtained for fruits grown under control conditions clearly distinguishes the nine developmental stages. The first principal component (PC1), which explains 43.8% of total variance, separates all green stages (from cell division to Mature Green, on the right part) from ripening stages (from Turning to Red Ripe, on the left
part). Because, for all stages except 8 DPA, the score on PC1 decreases when DPA increases, PC1 can be interpreted as expressing time. In contrast, PC2 which explains 24.4% of total variance clearly separates the cell expansion phase (from 21 DPA to Turning) from the 2 others. Thus, PC2 can be interpreted as expressing transitions between the three phases of fruit development.

The loading plot (Figure 5B) indicates that all enzymes participate in a balanced way to the separation of the developmental stages, as the loadings are relatively high for all of them. Positions of enzymes thus correspond to the stages at which they are highest.

PCA analysis was next used to compare developmental changes in enzyme activities of fruits grown on different trusses and under different conditions. To facilitate interpretation, loadings and scores of controls were maintained and data obtained under other growth conditions added as supplementary individuals (Lê et al., 2008). Thus, the closer to the control they are, the smaller the distance on the PCA plot.

**High Reproducibility of enzyme time courses from truss to truss**

As mentioned in the Introduction, a range of environmental and physiological variables influencing fruit development are likely to vary from truss to truss and one well known consequence is a variation in fruit metabolite composition (Winsor, 1979). To assess whether seasonal variations would be associated to changes at the level of the activome a PCA was performed for trusses 5, 6 and 7, which were obtained between July and the end of August (Figure 5C). It suggests that differences between the three trusses were very limited. This was confirmed by ANOVA and Tukey’s grouping test (supplemental Table II). Considering each enzyme activity throughout fruit development, no significant difference ($p$-value <0.05) was found between the three trusses (not shown). A few significant differences appeared when grouping time points according to the stage of development. Thus, during cell division (8 and 15 DPA), stromal fructose-1,6-bisphosphatase, PFP, and NAD-GAPDH were significantly different ($p$-value 0.04, 6E-3 and 0.03 respectively), although the coefficient of variation (CV) was below 15% for fructose-1,6-bisphosphatase and PFP, and below 21% for NAD-GAPDH. During cell expansion significant differences were found for aconitase ($p$-value 0.05, CV 20%) and NAD-GAPDH ($p$-value 2E-3, CV 22%). Finally, during ripening no significant difference was found for any of the 36 enzymes. The fact that differences observed between trusses were very limited might be explained by growth conditions being optimal.
Assuming that light intensity (Guan and Janes, 1991) and watering (Mitchell et al., 1991) are among the variables exerting the strongest influence on tomato fruit growth and quality, we next investigated their effects by profiling enzyme activities in fruits obtained under shading or water limitation.

**Comparison of developmental changes in enzyme activities of fruits grown under optimal conditions, water shortage and shading**

For fruits obtained under water shortage, the PCA plot indicates that there were very low differences with control fruits (Figure 5D, see also supplemental Table III) and most of the differences found were not significant, independently of the growth phase (supplemental Table IV). Exceptions were aconitase (p-value 0.02, CV 12%), stromal fructose-1,6-bisphosphatase (p-value 6E-5, CV 21%) and NAD-IDH (p-value 2E-5, CV 35%) during cell division, aconitase (p-value 0.04, CV 11%) during cell expansion and, finally, alanine aminotransferase (E.C. 2.6.1.2) (p-value 6E-4, CV 23%) and phosphoglycerokinase (p-value 1E-6, CV 11%) in red ripe fruits.

For fruits obtained under shading, the PCA plot (Figure 5D) indicates that at 8 and 15 DPA fruits were clearly divergent from the control fruits. Indeed, during cell division 7 of the measured activities were significantly different, i.e., FBP-aldolase (p-value 5E-3, CV 21%), stromal fructose-1,6-bisphosphatase (p-value 6E-5, CV 15%), NAD-GAPDH (p-value 5E-3, CV 22%), glucokinase (p-value 0.02, CV 23%), NAD-IDH (p-value 2E-5, CV 57 %), PFK (p-value 9E-3, CV 20%) and PFP (p-value 1E-3, CV 19 %). During cell expansion, there were fewer differences. Only PEPC showed a significantly lower activity in shaded fruits (p-value 9E-4, CV 15%) whereas glucokinase (p-value 0.03, CV 21%) and UGPase (p-value 2E-7, CV 11%) activities were higher. During ripening, most differences were not significant and only 11 enzymes (aconitase, AspAT, FBP-aldolase, cytosolic fructose-1,6-bisphosphatase, G6PDH, PEPC, PFP, PGK, pyruvate kinase, TPI and UGPase) were significantly different, but with an average coefficient of variation of less than 21%.
Comparison of developmental changes in enzyme activities of fruits grown at three locations

To increase heterogeneity in growth conditions, fruits obtained from two other batches of plants grown in glasshouses in Oxford (UK) during early spring and in Avignon (France) during late spring were analyzed and data compared to the previous ones (controls of Sainte-Livrade culture). It is worth mentioning that in addition to different light and temperature regimes, Oxford and Avignon used pots and soil whereas plants were grown in Sainte-Livrade hydroponically. Figure 5E presents the PCA score plot obtained for Oxford and Avignon samples (see also supplemental Table V). Contrary to the Sainte Livrade data, these fruit were staged according to DPA (see Materials and Methods), not to OECD standards (http://www.oecd.org). Globally, the PCA did not clearly separate the experiments, confirming that enzyme activities tend to follow a unique “trajectory” throughout fruit development. However, samples collected at 8 DPA in Oxford behaved differently, appearing very similar to those collected under shading at the same stage in Sainte-Livrade. At the latest harvested stages (55 days), Oxford and Avignon samples were very similar to samples from Sainte-Livrade collected at 42 DPA or mature green, which suggests that they were delayed in their development compared to Saint-Livrade. ANOVA and Tukey’s test performed for cell division, cell expansion and ripening stages, confirmed such differences between the experiments (Supplemental Table VI). Thus, during cell division, 30 enzymes were significantly different between the three locations, and the average of the variation coefficients calculated for each enzyme was 38 %. During cell expansion, variability decreased, with 22 enzymes being significantly different, but with an average of variation coefficients of less than 18%. During ripening, differences were again higher, with 27 enzymes statistically different and an average coefficient of variation of 27%. It was noticeable that enzymes responsible for metabolite accumulation (acid invertase and citrate synthase) remained much lower in Oxford and to a lesser extent in Avignon samples than in Sainte-Livrade experiment, even in shaded fruits, again suggesting that fruits of these experiments were delayed in their ripening. The fact that at young stages, Oxford samples resembled those obtained under shading suggests that the plants were carbon limited. Such limitation would also explain their slow development. This suggests that most differences found for activities were a consequence of differences in rates of fruit development. It is worth mentioning that differences were much lower when expressing enzyme activities on a fresh weight basis (not shown).
We conclude that throughout fruit development with only a few exceptions, activities of enzymes of central metabolism follow a pattern that is only weakly influenced by the environment.

**Metabolites are more sensitive to growth conditions than enzyme activities**

Major metabolites, such as carbohydrates (glucose, fructose, sucrose and starch), organic acids (citrate and malate) but also total amino acids, as well as protein content were measured in the pericarp during fruit development. Figure 6A presents the metabolite content depending on the truss position on the control plants (see also supplemental Table VII). The ANOVA and Tukey’s test showed that most metabolite concentrations were not statistically different from truss to truss (Supplemental Table VIII), or when significant, differences were relatively low (average coefficient of variation 12.3%). The largest changes in metabolites occurred during the first 15 to 20 days of development. Protein content (Figure 3) and sucrose (Figure 6A) decreased from 8 to 21 DPA, then reached a plateau until the end of ripening. In contrast, glucose and fructose concentrations increased between 8 and 15 DPA then remained constant. Starch increased from 8 to 15 DPA, was highest at 15 DPA and decreased until the red ripe stage. Citrate and malate presented a similar profile from 8 DPA to Mature Green stage (strong decrease during cell division followed by a plateau or a slight increase), then citrate was quickly accumulated while malate was decreasing. Total amino acids slightly increased until Mature Green and were accumulated much stronger during ripening. Most of the amino acids followed the same pattern and tended to increase or to reach a plateau during fruit development and ripening, at a few exceptions (Supplemental Table IX). Thus, GABA decreased slightly from 8 DPA to red ripe stage, proline decreased sharply from 8 to 21 DPA then remained constant until red ripe and tyrosine increased during cell division, remained constant during cell expansion and decreased during ripening. Finally, the most striking fact was that glutamate increased sharply during maturation, representing up to 50% of the total amino acids. Figure 6B compares metabolite profiles in fruits of control and stressed plants. The protein content was never significantly different between water shortage and control conditions (Supplemental Table X). The difference between shading and control conditions was always below 17%, even if significant at 15 DPA, Mature Green, Orange and Red Ripe stages. Sucrose content was not different between the treatments, except at the Red Ripe stage. Glucose and fructose contents were strongly affected by shading, but mainly during early fruit development. Differences were also found during ripening for glucose, which was
lower under shading and higher under water shortage. Starch content presented the biggest differences between growth conditions, with a coefficient of variation ranging from 27 to 45 %. Starch was always highest under water shortage and lowest under shading. Few differences were observed in organic acid contents between control and water shortage fruits where they were slightly higher. Under shading, malate was higher during the first fifteenth days of development. In contrast, citrate was not different during cell division, became lower during cell expansion and strikingly recovered at ripening. Similar to organic acids, amino acids were slightly higher under water shortage than under control conditions. Under shading, amino acids were not different during cell division but then decreased strongly compared to the two other treatments, during cell expansion and ripening.

Figure 6C presents the results obtained for the three growth locations. Overall, larger differences were observed between growth locations than between treatments. Fruits from Avignon and Sainte-Livrade cultures had similar profiles for proteins (Supplemental Table XII), hexoses, sucrose (excepted at 8 DPA) and organic acids. The main differences were the amino acids and starch, which were both higher in Avignon than in Sainte-Livrade. In fruits collected in Oxford each metabolite profile was different, especially hexoses at 8 DPA. Profiles of the latter were very similar to those found under shading in Saint-Livrade fruits, suggesting the occurrence of carbon limitation during the first days of development. Later on, starch content also became higher in Oxford fruits. In contrast, Oxford fruits contained lower amounts of organic acids. Malate content was nevertheless not significantly different for the firsts 20 days of fruit development while citrate content was always statistically lower than in Sainte-Livrade fruits.

To summarize these results, metabolite levels were more susceptible to environmental changes than enzyme activities, but the position of the fruit on the plant did not seem to be a major source of variability in metabolite content (see also results obtained with a PCA analysis in Supplemental Figures 1A-C). When comparing growth conditions, major differences were observed for starch, amino acids and organic acids. In contrast and as already observed in the cultivar Cervil (Gautier et al., 2008), hexoses and sucrose appeared to be very stable once cell division was achieved. Glucose and fructose concentrations measured during cell expansion were particularly reproducible between trusses, treatments and growth locations.
Metabolic responses to the environment tend to decrease at ripening

As shown above (Figure 5), most significant differences in enzyme activities were found for the youngest stages of fruit development. For major metabolites, the biggest differences were also observed at the youngest stages, *i.e.* during cell division and cell expansion until MG stage (Figure 6). However and as already mentioned, differences were bigger for metabolites than for enzymes and persisted for longer. But once fruits had attained their final size and underwent ripening, differences between growth conditions or locations tended to decrease. This trend is also visible on the PCA plots (supplemental Figures 1B and 1C) where the distance between fruits of the same age decreases towards ripening. A similar observation has been made with tomato fruits overexpressing hexokinase, where the influence of the genetic manipulation on metabolites decreased during ripening (Roessner-Tunali et al., 2003). Paradoxically, whereas during cell expansion enzyme profiles were very similar under the various growth conditions, metabolites showed strong differences. Furthermore, the opposite was observed at ripening, as enzymes tended to diverge while metabolites converged. Taken together these results suggest that the final reprogramming of metabolism occurring at ripening, which includes steps involved in sucrose and organic acid metabolism, results in a rather standard composition of fruit flesh.

Expressing metabolite content on fruit basis reveals hidden events

As previously shown, starch was more variable than most metabolites analyzed. When expressed on a fresh weight basis (figure 7A), the starch content increased from fruit set to 15 DPA and then decreased until ripeness. When expressed on whole fruit basis, (µmol glucose equivalents per fruit pericarp, figure 7B) starch accumulated until the Mature Green stage and degraded very quickly at the beginning of ripening (between Mature Green and Turning Stage, roughly corresponding to 5 days, see also Luengwilai and Beckles, 2009). Starch degradation operates via different pathways leading to maltose or glucose accumulation but also glucose-1-phosphate (G1P) as an intermediate, by the activity of α-glucan phosphorylase (Zeeman et al., 2007). The hexoses-P G1P, glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) were measured in the same samples and expressed on a fresh weight basis (Figures 7C, 7E and 7G). Although G1P was negatively correlated with starch, no relationship was apparent for G6P and F6P. When their amounts were calculated on a per fruit basis (Figures 7D, 7F and 7H), it was clear that all three hexoses-P, which probably tend to
equilibrate via the activities of PGI and PGM, were strongly increased at the very moment of starch degradation. Furthermore, this increase in hexoses-P, in particular G1P, seems to be roughly correlated with the amount of starch in the fruit. Indeed, fruits obtained under shading, which had the lowest starch content, also showed the smallest increase in hexoses-P. Taken together, these results suggest that in maturing fruits starch degradation results in a significant increase in hexoses-P that are then readily available for metabolic shifts associated with ripening.

When expressed on a fresh weight basis, hexose levels (Figures 7I and 7J) were almost constant during cell expansion and ripening and did not differ between the three treatments. In contrast, when expressed on fruit basis, differences were apparent. In particular and similarly to hexoses-P, the rate of increase in hexoses slowed just before the Mature Green stage but strongly accelerated until the Turning stage. However, the amount of hexoses accumulated during this period represented about 2500 µmol per fruit in the controls while the remobilization of starch corresponded to only 600 to 800 µmol in glucose equivalent per fruit. Thus, the strong increase in hexoses was probably due for the largest part to an increase in net sugar import, as already suggested (Balibrea et al., 2006). Indeed, starch degradation only represents 25 to 30% of hexoses accumulated during ripening and 10% of the hexose content in ripe fruits.

Integration of changes in enzyme activities and metabolite levels

The integration of multilevel metabolic data strongly depends on how the data are expressed. We assume that enzymes can control both metabolite concentrations and fluxes. Among all the possible ways of expressing enzyme activities and metabolite contents or net accumulation rates (per gFW, per protein, per fruit), results with the largest scattering of metabolites were obtained for enzyme activities per protein and net accumulation rates of metabolites per fruit (Figure 8A, see also supplemental Figures 2-5 for the complete set). Principal component analysis was used to integrate enzyme and metabolite data. Variables have been normalized over the three treatments (control, water limitation and shading) to enable comparison. Loadings have first been computed with the control dataset (Figure 8A), and data corresponding to water limitation (Figure 8B) and shading (Figure 8C) have then been added to the PCA as additional variables.
In control fruits, the loadings plot obtained with enzyme activities and net accumulation rates of metabolites was similar to that obtained with enzymes only (Figure 5). Thus, enzymes that have been associated to each developmental phase (Figures 4 and 5) were still clearly separated. Interestingly, the highest loadings (mainly on PC1) for metabolites and protein content, which correspond to their highest net accumulation rates, colocalised with enzymes peaking at early expansion. The only exception was amino acids, which were associated with enzymes rising at ripening. Strikingly, malate and citrate net accumulation rates were coincident with enzymes involved in interconversions between C3 and C4 organic acids. In contrast, sucrose and hexoses were not associated with enzymes involved in sucrose metabolism (Figure 8A). In fruits grown under water limitation most enzyme loadings were similar to those obtained with control fruits whereas metabolites appeared more scattered. For both groups of analytes, differences were more marked on PC2, which suggests the occurrence of shifts in transitions between developmental phases (Figure 8B). As expected, the loadings plot obtained with shaded fruits was the most divergent. For enzymes, this was mainly due to the cluster associated to cell division. For metabolites, differences in loadings were much more marked at both PC1 and PC2 (Figure 8C).

When considering all three treatments, only two statistical associations were found between a metabolite net flux and an enzyme activity. The closest was between total amino acids and Ala-AT, which suggests that the strong accumulation of glutamate at ripening (by far the largest pool among accumulating amino acids) involves this activity. One further interesting although looser interaction was found for malate and malate dehydrogenase. Other metabolites were rather associated to groups of enzymes or totally disconnected.

Discussion

The present work addresses the effect of the environment on the programming of metabolism in tomato fruits. Surprisingly, it appeared that within the range of conditions used, enzyme activities were only marginally affected by the environment whereas time courses of metabolites underwent larger changes in response to growth conditions, especially for starch and amino acids. These changes were attenuated during ripening and were not associated with specific reprogramming of enzyme activities.
Reprogramming of central metabolism during developmental transitions is mainly driven by de novo synthesis of enzyme clusters

While statistical analyses (ANOVA and Tukey’s grouping test) indicate that there were significant differences in given activities and stages of development, no “atypical” trajectories were found for individual enzymes. PCA score plots (Figure 5A) suggest that the environment was mainly affecting changes over time (on PC1), for example by slowing down the changes in enzyme activities, but without affecting the trajectories. Such shifts in rates of developmental changes could be explained by differences in e.g., temperature or carbon availability.

This analysis first reveals that the beginning of each of the three phases of fruit development was characterized by high activities for specific groups of enzymes. Thus, PC2 might be explained by a pronounced de novo synthesis of enzymes (Figure 5). Then, except for enzymes peaking at maturity, a gradual decrease in activity was observed (Figure 4), revealing that enzymes were decaying slower than they were made. Slow decay of enzymes might be explained in part by most enzymes having a slow turnover, as suggested for another plant model system (Piques et al., 2009). A further point is that there was obviously no induction of specific enzyme degradation that was associated with developmental shifts. The only exception was NADP-ME, which peaked at Turning stage and almost vanished at Orange stage, i.e. within a few days. We compared the present data with transcriptome data available from the literature and for comparable developmental series (Carrari et al., 2006; Osorio et al., 2011) but could not find any straightforward relationship between changes in transcripts and the activities of the encoded enzymes, as also concluded by (Steinhauser et al., 2010). In contrast, it is striking that the moments (20-28 DPA and 48-53 DPA in controls) at which changes occurred for enzyme activities almost perfectly match those found for more than 80 metabolites from various pathways in another study performed with Moneymaker fruits (Carrari et al., 2006). This suggests that coordinated changes in enzyme capacities, which integrate various levels of regulation, effectively impact the metabolome.

The analysis also reveals that the programming of metabolism throughout fruit development operates at a pathway level (Figure 4B). Each of the 4 clusters was associated with both a developmental phase and relatively well defined metabolic sectors. In particular, while cluster 1 contains enzymes involved in the upper part of glycolysis and the TCA cycle, cluster 2 is characterized by the lower part of glycolysis.
Cell division is characterized by a “turbo design”

Cluster 1 contains enzymes involved in glycolysis, especially glucokinase and fructokinase, which activities were particularly high during cell division. High levels of glucose-6P, were also observed during that phase. The occurrence of high ATP-consuming activities suggests that dividing fruit cells use a “turbo design” to drive glycolysis (Teusink et al., 1998). In brief, maintaining a low ATP to ADP ratio and high hexose-P levels probably results in high flux through glycolysis. This hypothesis is in agreement with the results of (Liu et al., 2007) indicating that the demand in sucrose is highest during cell division in tomato fruits. It is also in agreement with previous results obtained with discs of tomato pericarp collected at 21, 35 and 49 DPA indicating that the highest glycolytic flux is observed in the youngest fruits (Carrari et al., 2006). The fact that pyruvate kinase and TCA Cycle enzymes were also high (aconitase, succinyl CoA ligase and fumarase) reinforces the idea that a fine adjustment of the ATP production to the growth-linked ATP demand is a major issue during cell division (Takahashi et al., 2011).

Logically, fruits are particularly sensitive to carbon availability during cell division and carbon starvation leads to their abortion (Ruan et al., 2012). Fructokinase, which showed one of the highest variability at 8 DPA, has been shown to play a crucial role in floral initiation and abortion (Carrari and Fernie, 2006). The biggest differences found for sugars between treatments were at this developmental phase, especially for hexoses that were strongly decreased under shading.

Early cell expansion is characterized by anaplerosis

As shown in Figure 4, cell expansion can be subdivided in 2 steps, which have been assigned to early and late expansion. Cluster 2, which characterizes early cell expansion, includes a group of enzymes involved in the middle part of glycolysis, NAD-GAPDH, PGK and enolase, as well as PEPC. The strong correlation between these enzymes suggests that they operate together to adjust the level of oxaloacetate available for the TCA cycle, an anaplerotic process that has been proposed as a way to compensate for the loss of carbon induced by the numerous syntheses needed for cell expansion (O'Leary et al., 2011), in particular the accumulation of organic and amino acids to provide the osmotic driving force (Figure 6). The PK profile actually strengthens this hypothesis, as when PK was high, PEPC was low and vice versa. A further enzyme associated with cell expansion is AGPase, which
catalyzes the production of ADP-glucose for starch synthesis. Its activity was highest at the beginning of cell expansion, remained high until the Mature Green stage then decreased sharply between the Mature Green and Turning stages, thus mirroring starch accumulation and breakdown. SuSy, which reached its highest level during that phase, has been shown to control sink strength in growing tomato fruit (Wang et al., 1993) and could be involved in the synthesis of cellulose and other cell wall components, as suggested for other model systems (Winter et al., 1997). The activity of G6PDH peaked reproducibly at the beginning of cell expansion. This might reflect an activation of the pentose phosphate pathway for the biosynthesis of nucleotides. It is indeed striking that the G6PDH peak coincides with the strong increase in ploidy resulting from an increased rate of endoreduplication, which is observed in developing tomato fruit at the beginning of cell expansion (Nafati et al., 2011). NADPH produced by the oxidative pentose phosphate cycle may also be used for lipid synthesis that is required for cell expansion.

**Cell expansion is driven by hexose content**

In plants experiencing mild water shortage, growth is usually affected earlier than photosynthesis, leading to carbon excess (Muller et al., 2011). It is likely that more carbon was available to fruits grown on water-limited plants, but they did not increase their concentration in soluble sugars during expansion. Instead, more starch was accumulated, perhaps in relation with the sugar-dependent post-translational activation of AGPase, as shown for potato tubers (Tiessen et al., 2002) and leaves of several species (Hendriks et al., 2003). Consistently, starch accumulation was weak in fruits grown under shading. Although, these observations confirm that net starch accumulation is triggered by the amount of available carbon (N’tchobo et al., 1999), there was no correlation between starch and soluble sugars during cell expansion, probably because soluble sugars are mainly located in the vacuole. Intriguingly, during cell expansion, concentrations in hexoses and sucrose were nearly identical across all growth conditions tested here. Hexoses are by far the most abundant metabolites in the pericarp of developing fruits. Their contribution to the osmotic potential can be estimated at -0.4 MPa (150 mOsmoles). Strikingly, a previous study reported an identical value in growing fruits of *S. lycopersicum* obtained under optimal conditions but also under salt stress (Bolarin et al., 2001). Such osmotic potential represents at least 50% of the fruit osmotic potential, which suggests that hexoses are key drivers of cell expansion. Then, the apparent stability of hexoses may simply result from an adjustment of cell
expansion, as fruits of shaded and water-limited plants were smaller. In the case of shaded plants, sugar supply to the fruits may limit cell expansion whereas under water limitation, at equal sugar concentrations, turgor potential would be less because of a decreased water potential. Thus, it can be concluded that in this model system, a mechanism determining vacuolar hexose concentrations plays a major role in cell expansion.

**Enzyme programming during late expansion anticipates ripening**

In tomato, the transition between the end of cell expansion and ripening takes place within a few days at the end of the Mature Green stage. It involves highly coordinated processes involving ethylene-sensitive and –insensitive pathways (Giovannoni, 2001). Interestingly, several enzymes, grouped in cluster 3 (Figure 4) increased gradually during cell expansion, to eventually peak just before the beginning of ripening, suggesting the anticipation of ripening. They include PGI, PFK and UGPase, which are probably involved in the recycling of the large amounts of hexoses-P released by starch degradation. This cluster also contains both malic enzymes. In tomato fruits and grape berries, NADP-ME has been shown to be involved in respiration during ripening, providing pyruvate and NADPH as substrates for respiration (Drincovich et al., 2001). Furthermore, it has recently been shown that fruits of transgenic tomato plants with reduced NADP-ME activity had lower starch levels at the breaker stage, in connection with a decrease in the redox activation of AGPase (Osorio et al., 2013). The fact that this enzyme shows a sharp peak at the Mature Green stage might be related to its involvement in the forthcoming climacteric crisis that will result in a strong increase in respiration (Hill and ap Rees, 1993) and initiate the beginning of ripening. The mitochondrial enzyme NAD-ME has been proposed to produce pyruvate from malate to maintain the TCA cycle when malate dehydrogenase is inhibited by high levels of NADH and oxaloacetate (Wedding, 1989). The cluster also contains NADP-GluDH, an enzyme whose role remains unknown, and which has been proposed to be located in plastids (Miyashita and Good, 2008). Our results confirm that it is not the same enzyme as NAD-GluDH, as their activity profiles were different. Like NAD-GluDH, NADP-GluDH did not respond to carbon limitation (Supplemental Table II, non-significant difference between shaded and control plants), but both activities increased strongly between expansion and ripening, when glutamate, an important component of tomato fruit quality (Carrari et al., 2007), started to accumulate. Whereas it is assumed that NAD-GluDH catalyses the oxidation of glutamate in the mitochondrion (Labboun et al., 2009), the opposite reaction could take place in the plastid
(or in the cytosol) via NADP-GluDH, suggesting that at ripening these activities could be involved in different processes, or participate in a glutamate shuttle between both compartments. Strikingly, the constitutive overexpression of a fungal NADP-GluDH in tomato has been shown to result in a 2 to 3 fold increase in glutamate content in fruits (Kisaka and Kida, 2003).

**Ripening involves both catabolism and accumulation of key metabolites**

Enzymes associated with ripening are essentially grouped in cluster 4 (red) although several enzymes found in cluster 1 (blue) also increased during that phase (Figure 4). All in all about one third of the enzymes studied here significantly increased during ripening, some dramatically.

Concomitantly, fruit biomass accumulation rate was increased while protein content was maintained or even slightly increased (Figure 2), which suggests a global rise in protein synthesis like in banana (Brady et al., 1970). Concomitantly, there was a peak in the accumulation rate of hexoses, which are likely to be stored in the vacuole. Clearly, both protein synthesis and hexose accumulation in the vacuole are processes that imply an increase in energy demand. This is in line with the fact that activities of enzymes involved in glycolysis (FBP aldolase, pyruvate kinase) and the TCA cycle (citrate synthase, succinyl CoA ligase) were strongly increased. However, glucokinase and fructokinase went on decreasing to reach very low activities. Starch degradation can therefore be seen as an alternative source of hexose-P as substrates for respiration that can be remobilized within a short period of time. The concomitant increases in G1P and to a lesser extent G6P and F6P, when expressed on a fruit basis, supports this view.

Metabolic intermediates were not only used for respiration, as ripening also resulted in the accumulation of citrate, which is consistent with high citrate synthase activity, and amino acids, in particular glutamate. The increases in NADP isocitrate dehydrogenase and alanine aminotransferase could be associated to the massive accumulation of glutamate, via the synthesis of 2-oxoglutarate in the cytosol (Sulpice et al., 2010) and its subsequent conversion into glutamate. This hypothesis is supported by the strong correlation found between amino acids (and thus glutamate) and alanine aminotransferase.

The strong increase in invertase activity has been proposed to account for the accumulation of hexoses in cultivated tomato (Yelle et al., 1991). The fact that the activity of
SPS also strongly increased at ripening seems contradictory at first sight, as the latter enzyme is involved in the synthesis of sucrose, which is a substrate of invertases. However, in other species related to tomato ripening fruits do not enhance their invertase activity and accumulate sucrose instead of hexoses (Yelle et al., 1991). Furthermore, it has been shown that SPS dramatically increases in maturing fruits of *S. pennellii* (Steinhauser et al., 2009). Also, increasing the capacity of sucrose synthesis at ripening can be associated to the increase in carbon import and remobilization of starch, both processes leading to hexoses and hexose-P.

**Fruit quality depends for a large part on carbon import during ripening**

During ripening, concentrations in metabolites of tomato fruits grown under contrasted environments were converging thus tending to generate a reproducible metabolic phenotype, despite differences in fruit size and morphology. One interpretation is that final reprogramming of metabolism participates in generating a reproducible fruit quality as a guarantee to attract the consumer who will eventually disseminate the seeds. However, it is important to notice that sugar import during ripening might be essential to achieve such “standard” fruit quality. Indeed starch would represent only 10% of the final sugar content if totally converted into hexoses, whereas late carbon import would represent 20%. In other words, harvesting such fruits at the turning stage (followed by shelf maturing) would inevitably result in poorer quality, even when they are grown under optimal conditions (see also Beckles, 2012). It is worth mentioning that this probably explains for a large part why garden tomatoes are usually considered better than commercial fruits.

**Conclusion and Perspectives**

The surprisingly high reproducibility and consistency of enzyme time-courses monitored in fruits of *S. lycopersicum* var. Moneymaker throughout their development suggests that the environment exerts only a minor influence on the programming of fruit metabolism. Whereas enzyme profiles of young fruits are the most variable, they tend to converge –sooner or later- in expanding and maturing fruits. This is reflected by the rather reproducible composition in metabolites at maturity. One hypothesis is that fruit metabolism
would have evolved (or been selected) to guarantee reproducibility of the edible character, essential for seed dispersion. It will be important to investigate enzyme profiles of other genotypes, in particular wild relatives, in order to verify if this can be generalized. Then, knowing that each developmental phase is characterized by a reproducible and consistent enzyme profile provides new opportunities. A minimal set of enzymes could be selected to diagnose developmental stages in fruits. This could for instance be useful when studying mutants impaired in visual traits. This will also be useful to search for the molecular bases of the metabolic shifts that accompany developmental changes. Metabolic pathways could also be modeled for each developmental phase in order to get a better understanding of how metabolism participates in fruit growth and quality. Importantly, the parameterization of such models with enzyme data will be much easier than initially thought. It can be expected that such an approach would ultimately pinpoint future targets including maximal activities, kinetic properties or posttranslational mechanisms for fruit improvement.

**Material and Methods**

**Plant material and growth conditions**

Seeds of the Moneymaker variety were kindly provided by Alisdair Fernie (Max Planck Institute of Molecular Plant Physiology, Potsdam, Germany). They have been used in three distinct experiments, which took place in Sainte-Livrade (France), Avignon (France) and Oxford (UK). All seeds were coming from a common lot.

In the Sainte-Livrade experiment (south-west of France, 44° 23' 56" N and 0° 35' 25" E), plants were cultivated under realistic production conditions between June and October 2010. Seeds were germinated in a nursery on 30th April 2010. Seedlings were transferred to rockwool blocks on 4th June 2010 at a density of 2 plants m⁻² in a 350 m² greenhouse. The nutrient solutions were supplied with a drip system and the volume of water supply was adjusted to the climate. Three growing scenarios have been performed: Control with commercial production practices (276 plants), (2) water shortage by providing only half of the volume of nutritive solution as compared to the control plant (138 plants) and (3) low light treatment by hanging a shadow net in a part of the greenhouse. This shadow net stopped 60%
of the photosynthetically active radiation (138 plants). Stresses were applied at the flowering of the fourth truss. Environmental variables (Temperature, Relative Humidity, and PAR and Light intensity) were recorded on data loggers (Delta-T Devices, UK). Lateral stems were systematically removed. Each flower anthesis was recorded and trusses were pruned at 6 developed fruits to limit fruit size heterogeneity.

In the Avignon experiment (south of France, 43° 57' 00" N and 4° 49' 01" E), on 31th January 2011, seeds were sown in Petri dishes containing MS medium (Murashige and Skoog, 1962). Plants with 5 growing leaves were transplanted into 5 l pots containing potting soil (H21 Tref, Tref EGO Substrates B.V., Moerdijk, Netherlands) at a density of 1.8 plants m⁻² in a glasshouse. Plant nutrition and disease control were in accordance with the commercial practices. Water was supplied with a drip irrigation system to maintain 20 to 30% drainage. Flowers were mechanically pollinated three times a week. Each flower anthesis was recorded and trusses were pruned at 5 (rather than 6 given less light was available than in the other experiments) developed fruits to limit fruit size heterogeneity.

In the Oxford experiment (south-east of England, 51° 45' 07" N and 1° 15' 28" W), seeds were germinated on John Innes potting compost no. 3 (The John Innes Manufacturer Association, Reading, UK) and grown for 30 days before potting into 10 l pots in the same compost with the addition of slow-release fertilizer grains (Osmocote Exact Standard by Scotts with 5 months release time). Plants were grown in a temperature and light-controlled greenhouse (16 hours light from 6 am to 10 pm, 8 hours dark) under high pressure sodium and metal halide lamps at a density of 4 plants m⁻². Environmental conditions were recorded using a Hobo U12 data logger (onsetcomp.com) set to one recording per hour. On average, the light intensity was 200 μmoles m⁻² s⁻¹ during the illumination period with an average temperature of 25.7°C and 32 % relative humidity. During darkness, the average temperature was 19°C with 42 % relative humidity. Plants were watered daily from the top with addition of liquid fertilizer (Levington Tomorite) once per week when the plants started flowering. Fruit samples were collected between 2nd March and 4th May 2011 from plants sown 26th November 2010 and on 4th January 2011. Trusses were limited to six per plant, each flower anthesis was recorded and trusses were pruned at 6 developed fruits to limit fruit size heterogeneity.
Harvest and sample processing

In Sainte-Livrade experiment, nine developmental stages from 8 DPA to Red Ripe stage were harvested on three different trusses (Truss 5, 6 and 7). Six stages were based on the age of the fruit (8, 15, 21, 28 DPA, 34 DPA and 42 DPA (Mature Green)). Three stages were based on the fruit color according to OECD color gauge: Turning (grade 4), Orange (grade 8) and Red Ripe (grade 11-12) roughly corresponding to 47DPA, 50DPA and 55 DPA respectively. For each sample, three biological replications were prepared with a minimum of 4 fruits per replication. In Oxford experiment, seven developmental stages were harvested (7, 21, 35, Mature Green, Turning, Orange and Red Ripe) with a minimum of 4 biological replications. In Avignon experiment, fruits were harvested on truss 4 and truss 5 at 12, 15, 21, 28, 34, 42 (Mature Green), 47 (Turning), 50 (Orange) and 55 DPA (Red Ripe).

For the sample preparation, fruits were cut; seeds, jelly and placenta were removed and small pieces (approximately 1cm x 0.2cm) of pericarp were immediately deep frozen in liquid nitrogen in less than 1 min from the fruit harvest to the frozen piece of tissue. Frozen samples were then ground into fine powder with liquid nitrogen. Aliquots of about 20 mg were then weighted in 1.1 ml Micronic™ tubes (Lelystad, The Netherlands) and stored at -80 °C until further analysis.

Chemicals

All chemicals and substrates were purchased from Sigma-Aldrich Ltd. (Gillingham, UK), excepted acetyl coenzyme A, adenosine-5’-triphosphate (ATP), dithiothreitol, leupeptin, nicotinamide adenine dinucleotide (NAD), NADH, nicotinamide adenine dinucleotide phosphate (NADP), NADPH and phosphoenolpyruvate that were purchased from Roche Applied Science (Meylan, FR). All enzymes were purchased from Roche Applied Science (Meylan, FR) excepted aldolase (from rabbit muscle), citrate synthase (from porcine heart), glycerokinase (from E. coli), phosphoglycerokinase (from S. cerevisiae), phosphoglucomutase (from rabbit muscle), triose phosphate isomerase (from rabbit muscle) that were purchased from Sigma-Aldrich Ltd. (Gillingham, UK). Bradford reagent was purchased from Bio-Rad (Marnes-la-Coquette, FR).
Enzyme activity measurements

Aliquots of about 20 mg fresh weight (FW) were extracted by vigorous shaking with 500 µl of extraction buffer composed of 20% (v/v) glycerol, 0.25% (w/v) bovine serum albumine, 1% (v/v) Triton-X100, 50 mM HEPES-KOH pH 7.5, 10 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM ε-aminocaproic acid, 1 mM benzamidine, 10 mM leupeptin, 0.5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride, which was added just prior to extraction. Enzyme activities were assayed using a robotised platform as described in (Gibon et al., 2004; Studart-Guimarães et al., 2005; Gibon et al., 2006; Gibon et al., 2009) and (Steinhauser et al., 2010).

UGPase activity was assayed using a protocol adapted from (Appeldoorn et al., 1997). The assay consisted in 5 µl of extract in 100 mM Tricine-KOH (pH 8.0), 10 mM MgCl2, 2 mM EDTA, 1.2 mM NADP+, 1 mM UDP-glucose, 1 U ml⁻¹ glucose-6-phosphate dehydrogenase, 1 U ml⁻¹ phosphoglucomutase and 0.05% (v/v) Triton X100. The reaction was started by the addition of inorganic pyrophosphate (PPi) to a final concentration of 2.5 mM and a final volume of 100 µL.

Triose phosphate isomerase was assayed in the direction of dihydroxyacetone phosphate formation (Pichersky and Gottlieb, 1984). The assay consisted of 10 µL de-salted extract in 100 mM HEPES-NaOH (pH 8.0), 0.2 mM NADH, 5 mM EDTA and 1 U ml⁻¹ glycerol 3-phosphate dehydrogenase. The reaction was started by the addition of glyceraldehyde 3-phosphate to a final concentration of 1.5 mM.

Enolase was assayed in the direction of phosphoenolpyruvate production as described by (Burrell et al., 1994). The assay consisted of 10 µl of desalted extract in 100 mM HEPES-NaOH (pH 7.5), 10 mM MgCl2, 0.2 mM NADH, 2.7 mM ADP, 5 U ml⁻¹ pyruvate kinase and 6 U ml⁻¹ lactate dehydrogenase. The reaction was started by the addition of 2-phosphoglycerate to a final concentration of 0.5 mM.

NAD and NADP dependent Malic Enzyme activities were assayed using a protocol adapted from (Wheeler et al., 2005). The assay consisted in 2 µl of extract in 100 mM HEPES-KOH (pH 7.5), 10 mM MgCl2, 1 mM NADP⁺ or 6 mM NAD⁺ and 0.05% (v/v) Triton X100. The reaction was started by the addition of malate to a final concentration of 10 mM and a final volume of 20 µL. After 40 min of incubation at 25°C, the reaction was stopped by the addition of 20 µL of HCl 0.5 M / Tricine-KOH 0.1 M (pH 9). After mixing and waiting for 10 min, the acid was neutralized by the addition of 20 µL of 0.5 M NaOH. The
quantification of phosphoenolpyruvate accumulated during the incubation was then performed by the addition of 100 mM HEPES-KOH (pH 7.5), 10 mM MgCl₂, 0.05% (v/v) Triton X100, 1 mM NADH for a final volume of 110µl. The absorbance at 340 nm was read until stabilized then 2 µL of lactate dehydrogenase 100 U ml⁻¹ were added to start the determination.

**Metabolite measurements**

Aliquots of about 20 mg FW were fractionated as in (Hendriks et al., 2003). Sucrose, glucose and fructose (Jelitto et al., 1992), malate (Nunes-Nesi et al., 2007), citrate (Tompkins and Toffaletti, 1982), as well as G6P, F6P and G1P (Gibon et al., 2002) were determined in the ethanolic supernatant. Starch (Hendriks et al., 2003) and protein (Bradford, 1976) contents were determined on the pellet resuspended in 100 mM NaOH. Assays were prepared in 96 well microplates using Starlet pipetting robots (Hamilton, Villebon sur Yvette, France) and absorbances were read at 340, 570 or 595 nm in MP96 microplate readers (SAFAS, Monaco).

Individual amino acids analysis was carried out following the AQC-tag method. Derivatization was performed using AccQ-Fluor reagent kit (Waters, Milford, MA, USA) according to manufacturer instructions. Amino acid derivatives were separated on an Acquity BEH C18 column (2.1 x 100mm, 1.7 µm) (Waters). Solvents were AccQ-tag Ultra eluentA (Waters) and acetonitrile/water (25v:75v). Detection was performed at 473 nm after excitation at 266 nm. Quantities of individual amino acids were calculated with external calibration curves prepared from commercial amino acid standards.

**Statistical Analysis**

All ANOVA, Principal Component Analysis and Tukey’s Test (p-value<0.05) analyses were performed using R Software (http://www.r-project.org/) and the package FactomineR (Lê et al., 2008). Hierarchical clustering and Heat-Map were performed on mean-centered data scaled to unit variance, using MEV software v4.8.1. (Saeed et al., 2003) with Pearson’s correlations and complete linkage.
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References


35


Figure Legends

Figure 1. Growth of fruits of *S. lycopersicum* var. Moneymaker obtained under different environmental conditions. (A) Fruit weight is expressed in g ± SD (n=9) at various development stages, under optimal conditions (solid line), 50 % water shortage (dashed line), and under a net removing 60 % of the photosynthetically active radiation (dotted line). (B) Illustration of the nine developmental stages harvested (8, 15, 21, 28, 34 days post anthesis), Mature Green (MG), Turning (T), Orange (O) and Red Ripe (RR). Fruit ripening under shading was delayed by 5 to 8 days.

Figure 2. Maximal activities of 36 enzymes of central metabolism throughout tomato fruit development. Activities were measured in fruit pericarps harvested between 8 and 55 days post anthesis (DPA), at substrate saturation and at 25°C and are expressed as nmol min$^{-1}$ g$_{FW}$ ± SD (n=5). Abbreviations: Acid Inv, acid invertase; AGPase, ADP-glucose pyrophosphorylase; Ald, aldolase; AlaAT, alanine aminotransferase; AspAT, aspartate aminotransferase; cFBPase, cytosolic fructose-1,6-bisphosphatase; CS, citrate synthase; FK, fructokinase; G6PDH, glucose-6P dehydrogenase; GK, glucokinase; NAD-GAPDH, NAD-glyceraldehyde-P dehydrogenase; NAD-GluDH, NAD-glutamate dehydrogenase; NAD-ME; NAD-IDH, NAD-isocitrate dehydrogenase; NAD-malic enzyme; NAD-MDH, NAD-malate dehydrogenase; NADP-GluDH, NADP-glutamate dehydrogenase; NADP-IDH, NADP-isocitrate dehydrogenase; NADP-ME, NADP-malic enzyme; NADP-GAPDH, NADP-glyceraldehyde-P dehydrogenase; Neutral Inv, neutral invertase; PEPC, phosphoenolpyruvate carboxylase; pFBPase, plastidial fructose-1,6-bisphosphatase; PFK, ATP-phosphofructokinase; PFP, PPI-phosphofructokinase; PGK, phosphoglycerokinase; PGM, phosphoglucomutase; PK, pyruvate kinase; SPS, sucrose phosphate synthase; Succ-CoA, succinyl-CoA ligase; SuSy, sucrose synthase; TPI, triose-P isomerase; UGPase, UDP-glucose pyrophosphorylase

Figure 3. Pericarp weight (A), protein content (B), fructokinase (C) and phosphoenolpyruvate carboxylase (D) activity during tomato fruit development. Fruit age is expressed as Days Post Anthesis (DPA); pericarp weight is expressed in g ± SD (n=9);
enzyme activities are expressed in \( \text{nmol.min}^{-1}.\text{gFW}^{-1} \) (●) and \( \text{nmol.min}^{-1}.\text{mgprot}^{-1} \pm SD \) (□) (n=5).

**Figure 4. Hierarchical clustering analysis of enzyme activity profiles throughout development and ripening of fruit obtained under optimal growth conditions.** (A) The clustering analysis was performed on activities expressed on a protein basis via Pearson’s correlation on mean-centered and scaled to unit data. Columns correspond to nine developmental stages, rows correspond to enzyme activities. The four main enzyme clusters are highlighted with a colored bar on the left of figure. (B) Simplified drawing of central metabolism in plant. The color code corresponds to the clusters selected in Fig. 4A. Blue, activities highest during cell division and beginning of cell expansion; green, activities highest during cell expansion; orange, activity peaking at late expansion; red, activities highest at ripening. Abbreviations for enzymes are the same as in legend of Figure 2.

**Figure 5. Comparison of enzyme activity profiles obtained in fruits grown on different trusses, under optimal or sub-optimal growth conditions and at different locations by principal component analysis.** Numbers indicating the coordinates correspond to fruit age. (A) Scores Plot obtained for the reference culture conducted in Sainte-Livrade-France (numbers in black) and subsequently used for comparison. (B) Corresponding loadings plot. (C) Scores plots obtained by adding data obtained for trusses 5 (numbers in green), 6 (numbers in blue) and 7 (numbers in pink). (D) Scores plots obtained by adding data obtained under Water Shortage (numbers in red) and Shading (numbers in grey). (D) Scores plots obtained by adding data obtained from fruits grown in Avignon-France (numbers in green) and Oxford-United Kingdom (numbers in orange). Abbreviations for enzymes are the same as in legend of Figure 2.

**Figure 6. Changes in major metabolites throughout tomato fruit development.** Fruit age is given in days post anthesis (DPA). Total amino acids, glucose, fructose, malate and citrate are expressed in \( \mu\text{mol.gFW}^{-1} \pm SD \), and sucrose and starch in glucose-equivalents (\( \mu\text{mol.gFW}^{-1} \pm SD \)). (A) Data obtained from fruits grown in Sainte-Livrade under optimal growth conditions and on trusses 5, 6 and 7 (n=5). (B) Data from fruits grown under control (means for trusses 5, 6 and 7), water limited (n=5) and shaded (n=5) growth conditions. (C) Data
obtained from fruits grown in Sainte Livrade (same as in B), Avignon (n=5) and Oxford (n=5).

**Figure 7. Comparison of changes throughout fruit development in starch, hexoses phosphate and hexoses when expressed on a fresh basis or on a whole fruit pericarp basis.** Fruit age is given in day post anthesis (DPA). (A, C, E, G, I), data are expressed on a fresh weight basis (mol.g⁻¹FW); (B, D, F, H, J), data are expressed on a fruit basis (µmol.fruit pericarp⁻¹).

**Figure 8. Integration of enzyme activities and metabolites accumulation rates in tomato fruits during development and ripening using principal component analysis.** Enzyme activities were expressed on a protein basis and data for metabolite rates per fruit pericarp and per day. Data have been centered and scaled prior to PCA. (A) Loadings plot obtained with control data (fruits grown under optimal conditions). (B) Loadings plot for additional data obtained under water shortage. (C) Loadings plot for additional data obtained under shading. Coordinates for each enzyme activity or metabolite are indicated by text. Text colors indicate the cluster to which enzymes have been assigned using hierarchical clustering (blue for cell division, green for cell early expansion, orange for late expansion and red for ripening; see also Figure 4). Abbreviations for enzymes are the same as in legend of Figure 2.
Supplemental data

Supplemental tables (xlsx file)

Supplemental Table I. Enzyme activities of fruits collected from three different trusses (5, 6 and 7) and at nine stages of development. Activities have been determined on the pericarp and are expressed in nmol.g⁻¹FW.min⁻¹ ± SD (n=3).

Supplemental Table II. Statistical analysis of enzyme activities measured in fruits of three different trusses. Samples have been grouped into three developmental stages (cell division, cell expansion and ripening). Significant differences are highlighted according to the legend.

Supplemental Table III. Enzyme activities of fruits collected from plants grown under control conditions, water stress or shading and at nine stages of development. Activities have been determined on the pericarp and are expressed in nmol.g⁻¹FW.min⁻¹ ± SD (n=9).

Supplemental Table V. Statistical analysis of enzyme activities measured in fruits collected from plants grown under control, water stress or shading conditions. Samples have been grouped into three developmental stages (cell division, cell expansion and ripening). Significant differences are highlighted according to the legend.

Supplemental Table V. Enzyme activities of fruits collected from plants grown in Sainte-Livrade (FR), Avignon (FR) and Oxford (UK) and at nine stages of development. Activities have been determined on the pericarp and are expressed in nmol.g⁻¹FW.min⁻¹ ± SD (n=9).

Supplemental Table VI. Statistical analysis of enzyme activities measured in fruits collected from plants grown in Sainte-Livrade (FR), Avignon (FR) and Oxford (UK). Samples have been grouped into three developmental stages (cell division, cell expansion and ripening). Significant differences are highlighted according to the legend.

Supplemental Table VII. Metabolite levels and protein content of fruits collected from three different trusses (5, 6 and 7) and at nine stages of development. Metabolites and proteins have been determined on the pericarp and are expressed in µmol.g⁻¹FW ± SD and mg.g⁻¹FW ± SD, respectively (n=3).

Supplemental Table VIII. Statistical analysis of metabolite levels and protein content measured in fruits of three different trusses. Samples have been grouped into three
developmental stages (cell division, cell expansion and ripening). Significant differences are highlighted according to the legend.

**Supplemental Table IX.** Levels of individual amino acids in fruits collected from truss 6 and at nine stages of development. Metabolites have been determined on the pericarp and are expressed in nmol.g⁻¹FW ± SD (n=3)

**Supplemental Table X.** Metabolite levels and protein content of fruits collected from plants grown under control conditions, water stress or shading and at nine stages of development. Metabolites and proteins have been determined on the pericarp and are expressed in µmol.g⁻¹FW ± SD and mg.g⁻¹FW ± SD, respectively (n=9).

**Supplemental Table XI.** Statistical analysis of metabolite levels and protein content measured in fruits collected from plants grown under control, water stress or shading conditions. Samples have been grouped into three developmental stages (cell division, cell expansion and ripening). Significant differences are highlighted according to the legend.

**Supplemental Table XII.** Metabolite levels and protein content of fruits collected from plants grown in Sainte-Livrade (FR), Avignon (FR) and Oxford (UK) and at nine stages of development. Metabolites and proteins have been determined on the pericarp and are expressed in µmol.g⁻¹FW ± SD and mg.g⁻¹FW ± SD, respectively (n=9).

**Supplemental Table XIII.** Statistical analysis of metabolite levels and protein content measured in fruits collected from plants grown in Sainte-Livrade (FR), Avignon (FR) and Oxford (UK). Samples have been grouped into three developmental stages (cell division, cell expansion and ripening).

**Supplemental figures (pdf file)**

**Supplemental Figure 1.** Principal component analysis of metabolite profiles obtained for fruits collected throughout development. Control fruits from Sainte Livrade (in black, n=9) were used as the reference, all other data have been added as supplementary data. **Figure 1a.** Comparison between trusses (Sainte Livrade) Trusses 5, 6 and 7 (n=3) are indicated in blue and averages (n=9) calculated for these trusses in black. **Figure 1b.** Comparison between optimal and sub-optimal growth conditions (Sainte Livrade). Control conditions (Ctrl, black), shading (Shad, blue) or water stress (WS, blue). Data were expressed as means of 9 samples collected on trusses 5, 6 and 7. **Figure 1c.** Comparison of growth locations. Fruits were
collected in Sainte-Livrade (Ctrl, black), Avignon (Avi, blue) or Oxford (Oxf, blue). Data were expressed as means of 9 samples collected on trusses 5, 6 and 7.

**Supplemental Figure 2.** Principal component analysis of a combined set of enzyme and metabolite data obtained for fruits grown under control conditions and harvested throughout their development. Enzymes were expressed on a mg protein basis and metabolites on a fruit basis. Control fruits from Sainte Livrade (in black, n=9) were used as the reference, all other data have been added as supplementary data. A, scores plot and B, loadings plot. B. **Figure 2a.** Trusses. **Figure 2b.** Growth conditions. **Figure 2c.** Growth locations.

**Supplemental Figure 3.** Principal component analysis of a combined set of enzyme and metabolite data obtained for fruits grown under control conditions and harvested throughout their development. Enzymes were expressed on a mg protein basis and metabolites on a g fresh weight basis. Control fruits from Sainte Livrade (in black, n=9) were used as the reference, all other data have been added as supplementary data. A, scores plot and B, loadings plot. B. **Figure 3a.** Trusses. **Figure 2b.** Growth conditions. **Figure 2c.** Growth locations.

**Supplemental Figure 4.** Principal component analysis of a combined set of enzyme and metabolite data obtained for fruits grown under control conditions and harvested throughout their development. Enzymes and metabolites were expressed on a mg protein basis. Control fruits from Sainte Livrade (in black, n=9) were used as the reference, all other data have been added as supplementary data. A, scores plot and B, loadings plot. B. **Figure 4a.** Trusses. **Figure 4b.** Growth conditions. **Figure 4c.** Growth locations.

**Supplemental Figure 5.** Principal component analysis of a combined set of enzyme and metabolite data obtained for fruits grown under control conditions and harvested throughout their development. Changes in enzymes and metabolites were expressed on a fruit and day basis. Control fruits from Sainte Livrade (in black, n=9) were used as the reference, all other data have been added as supplementary data. A, scores plot and B, loadings plot. B. **Figure 5a.** Trusses. **Figure 5b.** Growth conditions. **Figure 5c.** Growth locations.
Figure 1. Growth of fruits of *S. lycopersicum* var. Moneymaker obtained under different environmental conditions. (A) Fruit weight is expressed in g ± SD (n=9) at various development stages, under optimal conditions (solid line), 50 % water shortage (dashed line), and under a net removing 60 % of the photosynthetically active radiation (dotted line). (B) Illustration of the nine developmental stages harvested (8, 15, 21, 28, 34 days post anthesis), Mature Green (MG), Turning (T), Orange (O) and Red Ripe (RR). Fruit ripening under shading was delayed by 5 to 8 days.
Figure 2. Maximal activities of 36 enzymes of central metabolism throughout tomato fruit development. Activities were measured in fruit pericarps harvested between 8 and 55 days post anthesis (DPA), at substrate saturation and at 25°C and are expressed as nmol min⁻¹ gFW⁻¹ ± SD (n=5).

Abbreviations: Acid Inv, acid invertase; AGPase, ADP-glucose pyrophosphorylase; Ald, aldolase; AlaAT, alanine aminotransferase; AspAT, aspartate aminotransferase; cFBPase, cytosolic fructose-1,6-bisphosphatase; CS, citrate synthase; FK, fructokinase; G6PDH, glucose-6P dehydrogenase; GK, glucokinase; NAD-GAPDH, NAD-glyceraldehyde-P dehydrogenase; NAD-GluDH, NAD-glutamate dehydrogenase; NAD-IDH, NAD-isocitrate dehydrogenase; NAD-ME; NAD-IDH, NAD-malic enzyme; NAD-MDH, NAD-malate dehydrogenase; NADP-GluDH, NADP-glutamate dehydrogenase; NADP-IDH, NADP-isocitrate dehydrogenase; NADP-ME, NADP-malic enzyme; NADP-GAPDH, NADP-glyceraldehyde-P dehydrogenase; Neutral Inv, neutral invertase; PEPC, phosphoenolpyruvate carboxylase; pFBPase, plastidial fructose-1,6-bisphosphatase; PFK, ATP-phosphofructokinase; PFP, PPI-phosphofructokinase; PGI, phosphoglucone isomerase; PGK, phosphoglycerokinase; PGM, phosphoglucomutase; PK, pyruvate kinase; SPS, sucrose phosphate synthase; SuSy, sucrose synthase; TPI, triose-P isomerase; UGPase, UDP-glucose pyrophosphorylase; cFBPase, cytosolic fructose-1,6-bisphosphatase; pFBPase, plastidial fructose-1,6-bisphosphatase;
Figure 3. Pericarp weight (A), protein content (B), fructokinase (C) and phosphoenolpyruvate carboxylase (D) activity during tomato fruit development. Fruit age is expressed as Days Post Anthesis (DPA); pericarp weight is expressed in g ± SD (n=9); enzyme activities are expressed in nmol min⁻¹ g⁻¹ FW (□) and nmol min⁻¹ mg⁻¹ Protein ± SD (□) (n=5).
Figure 4. Hierarchical clustering analysis of enzyme activity profiles throughout development and ripening of fruit obtained under optimal growth conditions. (A) The clustering analysis was performed on activities expressed on a protein basis via Pearson correlations on mean-centered and scaled to unit data. Columns correspond to nine developmental stages, rows correspond to enzyme activities. The four main enzyme clusters are highlighted with a colored bar on the left of figure. (B) Simplified drawing of central metabolism in plant. The color code corresponds to the clusters selected in Fig. 4A. Blue, activities highest during cell division and beginning of cell expansion; green, activities highest during cell expansion; orange, activity peaking at late expansion; red, activities highest at ripening. Abbreviations for enzymes are the same as in legend of Figure 2.
Figure 5. Comparison of enzyme activity profiles obtained in fruits grown on different trusses, under optimal sub-optimal growth conditions and at different locations by Principal component analysis. Numbers indicating the coordinates correspond to fruit age. (A) Scores Plot obtained for the reference culture conducted in Sainte-Livrade-France (numbers in black) and subsequently used for comparison. (B) Corresponding loadings plot. (C) Scores plots obtained by adding data obtained for trusses 5 (numbers in green), 6 (numbers in blue) and 7 (numbers in pink). (D) Scores plots obtained by adding data obtained under Water Shortage (numbers in red) and Shading (numbers in grey). (D) Scores plots obtained by adding data obtained from fruits grown in Avignon-France (numbers in green) and Oxford-United Kingdom (numbers in orange). Abbreviations for enzymes are the same as in legend of Figure 2.
Figure 6. Changes in major metabolites throughout tomato fruit development. Fruit age is given in days post anthesis (DPA). Total amino acids, glucose, fructose, malate and citrate are expressed in μmol·g FW⁻¹ ± SD, and sucrose and starch in glucose-equivalents (μmol·gluc·g FW⁻¹ ± SD). (A) Data obtained from fruits grown in Sainte-Livrade under optimal growth conditions and on trusses 5, 6 and 7 (n=5). (B) Data from fruits grown under control (means for trusses 5, 6 and 7), water limited (n=5) and shaded (n=5) growth conditions. (C) Data obtained from fruits grown in Sainte Livrade (same as in B), Avignon (n=5) and Oxford (n=5).
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Figure 8. Integration of enzyme activities and metabolites accumulation rates in tomato fruits during development and ripening using principle component analysis. Enzyme activities were expressed on a protein basis and data for metabolite rates per fruit pericarp and per day. Data have been centered and scaled prior to PCA. (A) Loadings plot obtained with control data (fruits grown under optimal conditions). (B) Loadings plot for additional data obtained under water shortage. (C) Loadings plot for additional data obtained under shading. Coordinates for each enzyme activity or metabolite are indicated by text. Text colors indicate the cluster to which enzymes have been assigned using hierarchical clustering (blue for cell division, green for cell early expansion, orange for late expansion and red for ripening; see also Figure 4). Abbreviations for enzymes are the same as in legend of Figure 2.