The Influence of Fruit Load on the Tomato Pericarp Metabolome in a *Solanum chmielewskii* Introgression Line Population[1][W][OA]

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It has been recently demonstrated, utilizing interspecific introgression lines of tomato, generated from the cross between *Solanum lycopersicum* and the wild species *Solanum pennellii*, that the efficiency of photosynthate partitioning exerts a considerable influence on the metabolic composition of tomato fruit pericarp. In order to further evaluate the influence of source-sink interaction, metabolite composition was determined by gas chromatography-mass spectrometry in a different population. For this purpose, we used 23 introgression lines resulting from an interspecific cross between *S. lycopersicum* and the wild species *Solanum chmielewskii* under high (unpruned trusses) and low (trusses pruned to one fruit) fruit load conditions. Following this strategy, we were able to contrast the metabolite composition of fruits from plants cultivated at both fruit loads as well as to compare the network behavior of primary metabolism in the introgression line population. The study revealed that while a greater number of metabolic quantitative trait loci were observed under high fruit load (240) than under low fruit load (128) cultivations, the levels of metabolites were more highly correlated under low fruit load cultivation. Finally, an analysis of genotype × fruit load interactions indicated a greater influence of development and cultivation than genotype on fruit composition. Comparison with previously documented transcript profiles from a subset of these lines revealed that changes in metabolite levels did not correlate with changes in the levels of genes associated with their metabolism. These findings are discussed in the context of our current understanding of the genetic and environmental influence on metabolic source-sink interactions in tomato, with particular emphasis given to fruit amino acid content.

Recent years have witnessed the widespread adoption of forward genetic screens aimed at the identification of the genetic basis of metabolite accumulation (Causse et al., 2004; Fridman et al., 2004; Keurentjes et al., 2006; Lisec et al., 2006, 2008; Schauer et al., 2006, 2008; Rowe et al., 2008). While association mapping-based approaches have begun to be advocated for this purpose (Fernie and Schauer, 2009; Sulpice et al., 2009) and examples even exist where it has been successful, such as the mapping of genes underlying provitamin A content (Harjes et al., 2008) and kernel composition (Wilson et al., 2004) in maize (*Zea mays*), the majority of studies to date have relied on quantitative trait locus (QTL)-based methods (Keurentjes et al., 2006; Lisec et al., 2006, 2008; Schauer et al., 2006, 2008; Rowe et al., 2008; Zanor et al., 2009a). This is particularly true for metabolomics-scale approaches in which tens to hundreds of metabolites are measured simultaneously. Moreover, the vast majority of metabolomics studies to date have focused on the primary or secondary metabolism of Arabidopsis (*Arabidopsis thaliana*; Keurentjes et al., 2006; Lisec et al., 2006, 2008; Rowe et al., 2008). That said, several recent studies have evaluated natural variance in the metabolomes of rice (*Oryza sativa*; Kusano et al., 2007), maize (Harrigan et al., 2007), *Brassica napus* (Magrath et al., 1993), and even sesame (*Sesamum indicum*; Laurentin et al., 2008), whereas considerable research effort has been expended on determining QTLs for primary metabolites, acyl sugars, carotenoids, and volatile organic components of tomato (*Solanum lycopersicum*) fruits (Causse et al., 2004; Fraser and Bramley, 2004; Schauer et al., 2006, 2008; Tieman et al., 2006b; Stevens et al., 2007; Dal Cin et al., 2009; Mathieu et al., 2009; Schilmiller et al., 2010).

Work in tomato fruits has largely focused on those metabolites thought to be important to either taste (such as sugars, organic acids, and volatile organic compounds [Stevens, 1972; Baldwin et al., 2000; Saliba-Colombani et al., 2001; Schauer et al., 2006, 2008; Tieman et al., 2006a, 2006b]) and/or nutrition (such as essential amino acids and vitamins [Schauer et al., 2006, 2008; Stevens et al., 2007]). To date, a wide range

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of QTLs have been determined that affect either primary metabolites or volatile organic compounds, and despite the fact that there seems to be little correlation between primary metabolites and volatile organic compounds, both compound classes have been demonstrated to be associated with various taste parameters (Causse et al., 2002; Zanor et al., 2009a). When the data documented in these studies are assessed from an applied perspective, several important observations can be made concerning the utility of such data for plant breeding (Giovannoni, 2006; Fernie and Schauer, 2009). The facts that clearly identifiable QTLs for metabolites putatively involved in taste or nutrition were determined and that metabolite QTLs exhibit reasonable heritability demonstrate that the populations analyzed were appropriate for such studies (Causse et al., 2002; Schauer et al., 2006, 2008; Dal Cin et al., 2009; Mathieu et al., 2009). These observations clearly suggest that screens of natural metabolic variation (such as that described by Schauer et al. [2005b]) will likely prove essential means of identifying germplasms that are likely to be of strategic importance in breeding programs.

Lines homozygous for the *Solanum pennellii* introgression displayed an inverse correlation between harvest index and pericarp metabolite content, particularly in the case of amino acids. This raises interesting questions regarding the regulation of carbon partitioning in tomato. Comprehensive analysis of the *S. pennellii* introgression lines alongside a broader allelic series of wild tomato introgressions has provided very strong evidence that phloem unloading of sugars in tomato fruit is apoplastic (Fridman et al., 2004; Baxter et al., 2005). This finding is in keeping with recent transgenic analysis of tomato lines deficient in the expression of either the apoplastic invertase LIN5 (Zanor et al., 2009b) or the Suc transporter SUT2 (Hackel et al., 2006). Here we used a gas chromatography-mass spectrometry-based metabolite profiling approach to evaluate the metabolite composition of introgression lines resulting from a similar interspecific cross between *S. lycopersicum* and *Solanum chmielewskii*. In order to further evaluate the influence of source-sink interaction, metabolite composition was determined for 23 introgression lines under high (HL; unpruned trusses) and low (LL; trusses pruned to one fruit) fruit load conditions. We present 240 and 128 metabolite QTLs under HL and LL cultivation, respectively, for 62 metabolic traits and discuss their interrelation and interaction with respect to the experimental manipulations in source-sink balance. These data are further discussed in the context of transcriptomic data collected on a subset of the population used here (Prudent et al., 2009, 2010) in order to evaluate the relative importance of genetic, developmental, and cultivation-specific changes in the fruit metabolome. The fact that dramatic alterations were observed in the amino acid content, without consistent alterations in transcript abundance of genes associated with either amino acid metabolism or protein degradation, suggests an elevated import of amino acids from the leaves via the phloem. This observation is discussed in the context of our current understanding of source-sink interactions in tomato.

RESULTS

Analysis of Metabolic Changes in a *S. chmielewskii* Introgression Line Population Cultivated under Different Fruit Load Conditions

To investigate metabolic QTLs apparent under two different fruit load treatments (HL, in which tomato fruits were allowed to develop naturally, and LL, where all but one fruit per truss was removed), we carried out extensive metabolic profiling of the primary metabolism of tomato fruit pericarp in a previously characterized *S. chmielewskii* introgression line population (Prudent et al., 2009) using an established gas chromatography-mass spectrometry-based method (Lisec et al., 2006). For this study, we used a total of 23 introgression lines, namely the 20 used by Prudent et al. (2009) and three additional genotypes, C2b, C2d, and C3c (chromosomal positional information and zygosity status are provided in Supplemental Fig. S1). We accurately quantified, by means of dose-response curves that were run alongside each sample set, 62 metabolites of known chemical structure in every experiment. These compounds included amino acids, organic acids, sugars, sugar alcohols, sugar phosphates, fatty acids, and vitamins C (ascorbate) and E (tocopherol). The full data sets from this metabolite profiling study are presented as overlay heat maps for HL and LL conditions (Figs. 1 and 2), while similar heat maps for the individual harvests are provided in Supplemental Figures S2 and S3 for HL condition and in Supplemental Figures S4 and S5 for LL condition. The maps represent, using a false-color scale, increases or decreases in metabolite content relative to the *S. lycopersicum* 'Moneyberg' parental line in a plot of metabolite content versus genotype for the results of 2006 and 2007 field harvests from Avignon, France. Both HL and LL harvests exhibited considerable overlap in metabolite changes between the two harvests. However, there were also a number of opposite changes in metabolite levels (i.e. metabolite levels of a given introgression and under a given cultivation method increased in one year and decreased in the other), with 4.2% of changes under HL cultivation and 0.2% of changes under LL cultivation exhibiting such behavior. It is important to note, however, that these values were considerably lower than the 8% of nonidentical changes reported previously for a *S. pennellii* introgression line population grown under HL cultivation in a field in Israel (Schauer et al., 2006).

The absolute difference in the content of any given metabolite ranged between 0.06 and 4.54 times the value observed in Moneyberg under HL cultivation and between 0.31 and 6.56 times the value observed in Moneyberg under LL cultivation (Supplemental Tables S1 and S2). For both conditions, these values are

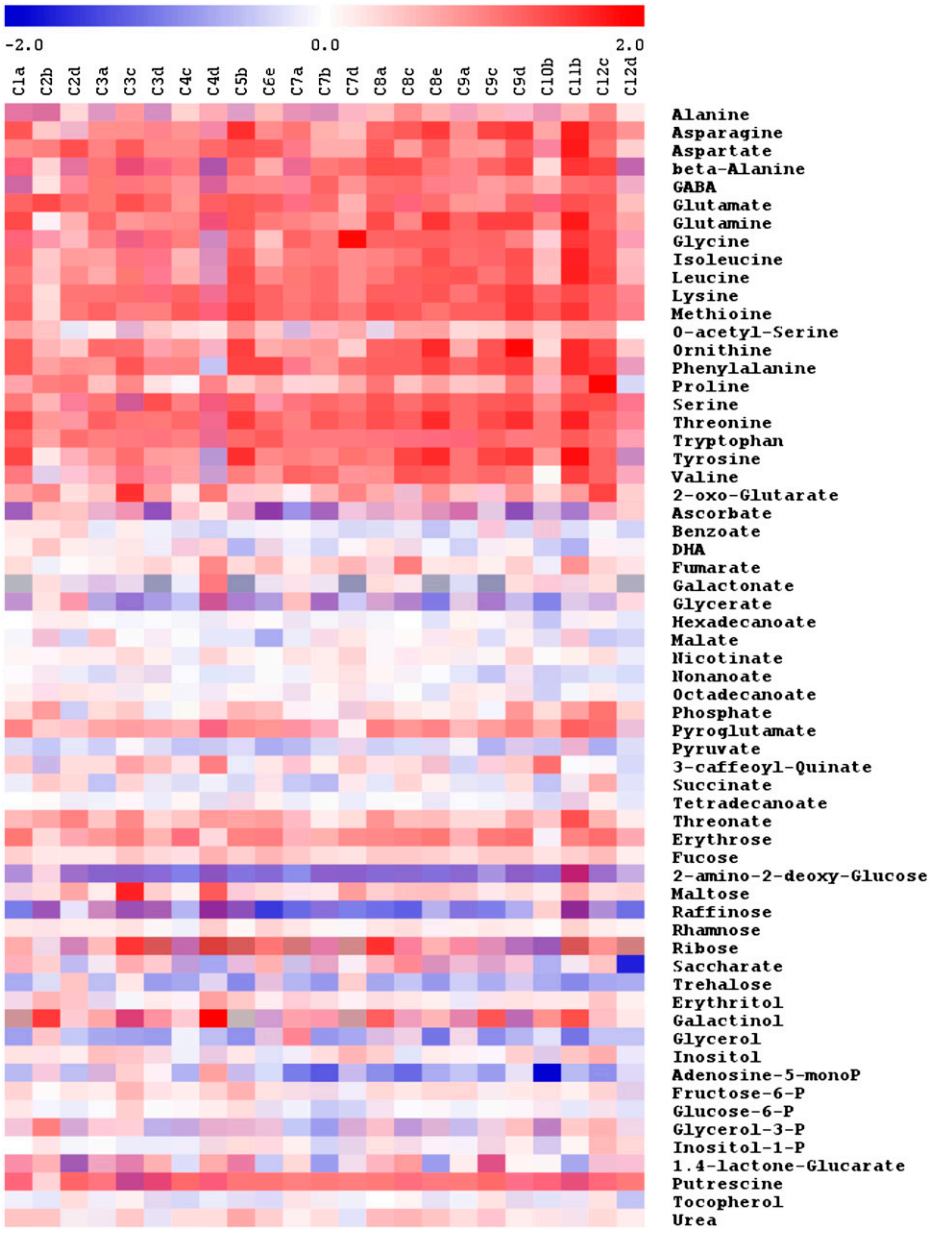

Figure 1. Overlay heat map of the metabolite profiles of the introgression lines in comparison with the parental control under HL condition. Each square represents the effect of chromosomal segment substitution on the amount of every metabolite using a false-color scale. Regions of red or blue indicate that the metabolite content is increased or decreased, respectively, after the introgression of *S. chmielewski* LA1840 segments. For each harvest, gas chromatography-mass spectrometry was used to quantify 62 metabolites, including amino acids, organic acids, fatty acids, sugars, sugar alcohols, sugar phosphates, and vitamins. Fully annotated heat maps for the individual data sets of each harvest are given in Supplemental Figures S1 and S2.

considerably lower than those previously reported by Schauer et al. (2006), for which variation was documented to be between 0.18 and 17.67 times the values of the *S. lycopersicum* control. Nevertheless, the variation is considerable, suggesting that the *S. chmielewski* introgression line population represents useful genetic material for the identification of primary metabolite QTLs. Furthermore, these results imply that the general metabolic variation across the population is not greatly affected by the cultivation conditions, but specific metabolites display much greater effects.

Given that fruit thinning is known to reduce the competition for carbohydrate source, and thus promote fruit size and sugar content in several species including peach (*Prunus persica*; Morandi et al., 2008), apple (*Malus domestica*; Link, 2000), mandarin orange (*Citrus reticulata*; Kubo et al., 2001), papaya (*Carica papaya*; Zhou et al., 2000), and tomato (Heuvelink, 1997; Prudent et al., 2009), we next directly compared the levels of each metabolite between HL and LL cultivation. Many significant changes could be observed in this comparison (see the heat map in Fig. 3 or the individual data sets of different harvests in Supplemental Tables S3 and S4). Most prominent among these were the high increases of amino acids and sugar phosphates and the organic acids glycerate, galactonate, and threonate under LL cultivation, although there were also, more minor, increases in sugar alcohols and sugars. By contrast, citrate and malate levels were slightly decreased under LL cultivation. While the elevated sugar content has been described in this material (Prudent et al., 2009), the other findings are, to

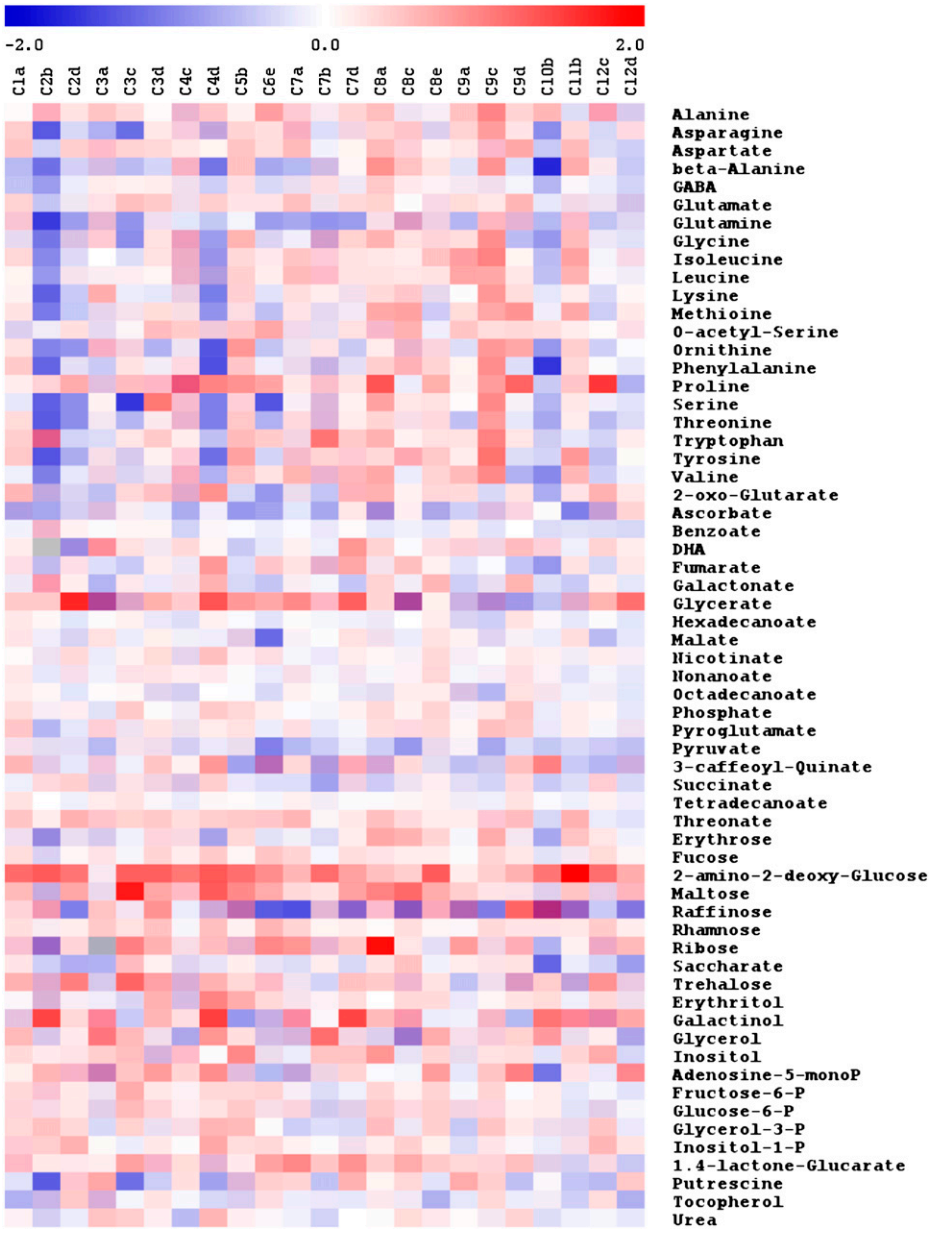

Figure 2. Overlay heat map of the metabolite profiles of the introgression lines in comparison with the parental control under LL condition. Each square represents the effect of chromosomal segment substitution on the amount of every metabolite using a false-color scale. Regions of red or blue indicate that the metabolite content is increased or decreased, respectively, after the introgression of *S. chmielewski* LA1840 segments. For each harvest, gas chromatography-mass spectrometry was used to quantify 62 metabolites, including amino acids, organic acids, fatty acids, sugars, sugar alcohols, sugar phosphates, and vitamins. Fully annotated heat maps for the individual data sets of each harvest are given in Supplemental Figures S3 and S4.

our knowledge, all novel and suggest either that tomato largely imports preformed amino acids from source tissues or that it regulates de novo synthesis in the pericarp tissue in a similar manner to that described previously for potato (*Solanum tuberosum*) tuber parenchyma (Roessner-Tunali et al., 2003b).

QTL Analyses

In order to determine QTLs, we applied a two-way ANOVA test followed by a Dunnett multiple comparison test. Using a permissive significance threshold of 0.05, we identified 240 single-trait QTLs and 128 single-trait QTLs under HL and LL cultivation, respectively (Supplemental Tables S1 and S2). This number is considerably lower than that reported by Schauer et al. (2006), who documented 889 QTLs in the *S. pennellii* introgression line population. When this data set was evaluated in terms of compound class, we found a total of 131 QTLs for amino acids, 44 QTLs for organic acids, 65 QTLs for sugars, seven QTLs for sugar alcohols, five QTLs for sugar phosphates, one QTL for fatty acids, but no QTL for vitamin C and E under HL. By contrast, we found a total of 53 QTLs for amino acids, 19 QTLs for organic acids, 30 QTLs for sugars, 10 QTLs for sugar alcohols, five QTLs for sugar phosphates, but no QTL for fatty acids or vitamin C and E under LL. A total of 45 QTLs were conserved between HL and LL (corresponding to 22% of the HL QTLs and 35% of the LL QTLs). These conserved QTLs included 21 for amino acids, seven for organic acids, 10 for sugars, two for sugar alcohols, and three for sugar

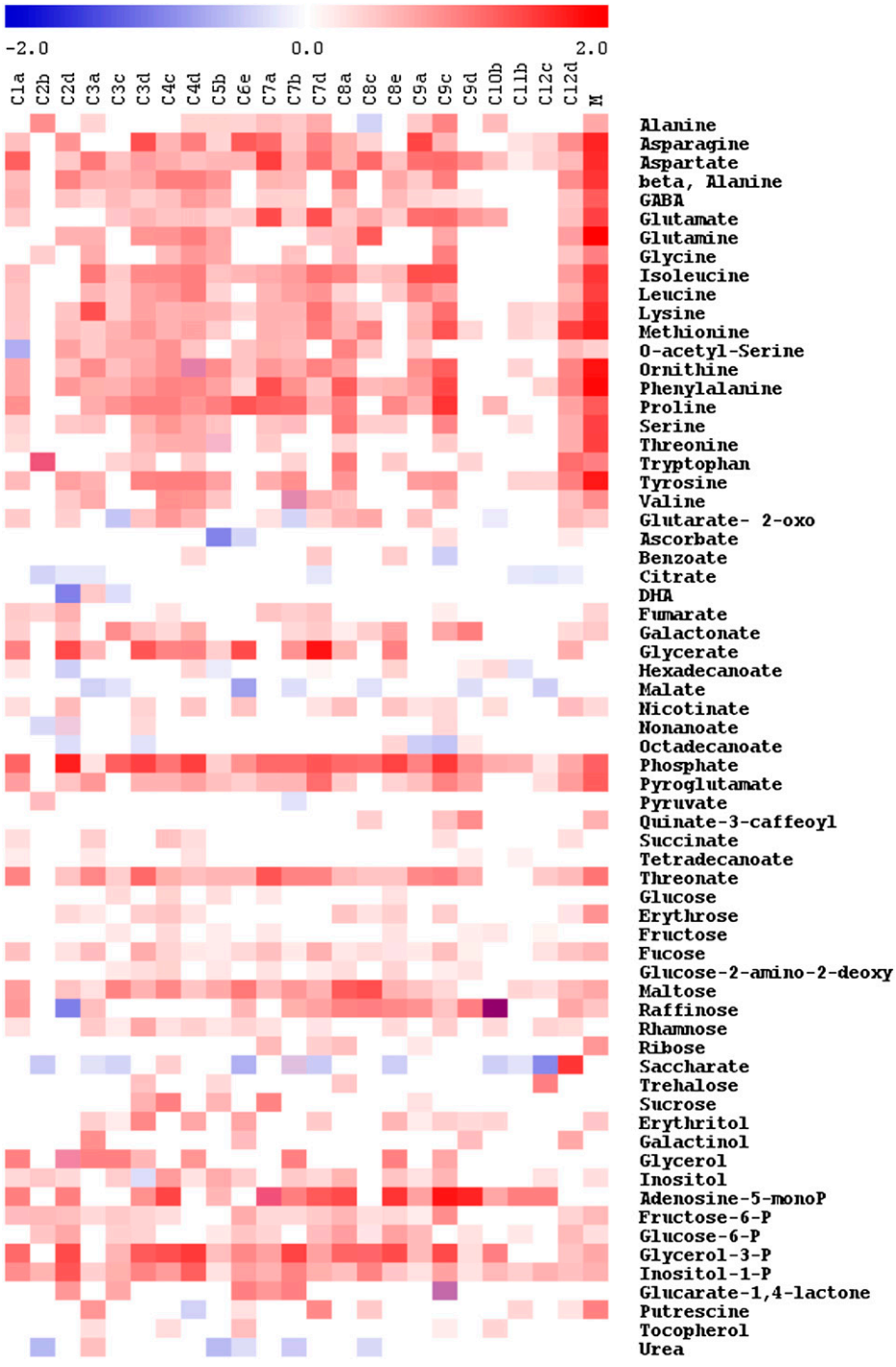

Figure 3. Overlay heat map of the metabolite changes under LL condition compared with HL condition. Each square represents the effect on metabolite level under LL in comparison with HL, at the significance level of $P < 0.05$, using a false-color scale. Regions of red or blue indicate that the metabolite level is increased or decreased, respectively. Very dark coloring indicates that a large change in metabolite content was conserved across harvests, and purple indicates that the metabolite was increased in one harvest but decreased in the other. The full data set for each harvest is given in Supplemental Tables S5 and S6.

phosphates (Supplemental Tables S1 and S2), constituting between 10% and 75% of the QTLs observed under LL cultivation.

Impacts of Genotype and Fruit Load Treatment on Combined Networks of Metabolic and Phenotypic Traits

In an attempt to better understand the global changes observed in the data sets acquired here, we next implemented an algorithm written by Guimerà and Nunes-Amaral (2005) that identifies functional modules within complex networks (Fig. 4). The resulting cartographic network comprises three large, highly connected modules under both HL and LL cultivation conditions. The networks for the different cultivation types were markedly different. In the HL network, one module comprises most of the amino acids, another mainly sugars and sugar alcohols as

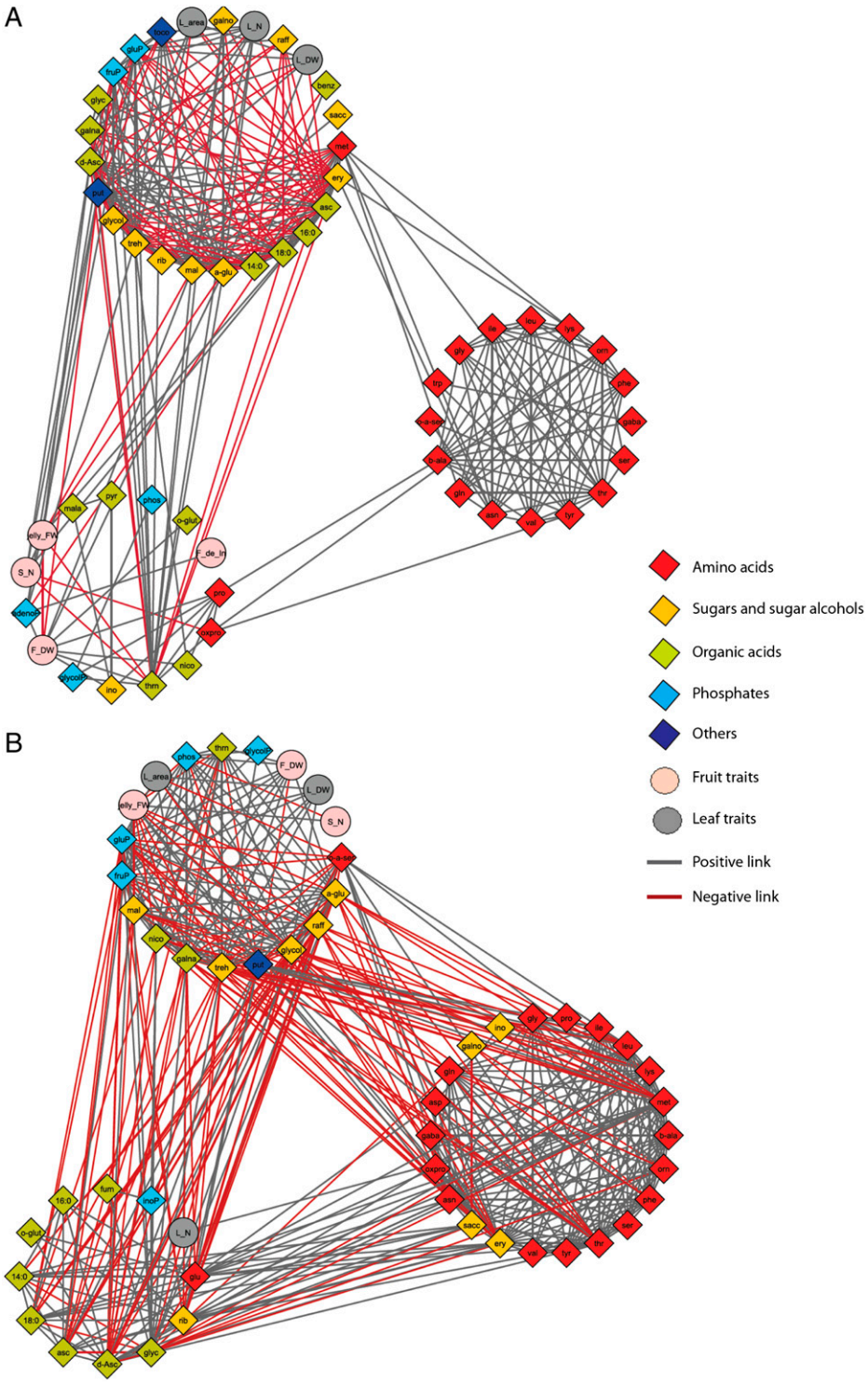

Figure 4. Cartographic representation of the combined metabolic and phenotypic network of the tomato under HL (A) and LL (B) conditions. Each trait (node) is represented by a shape (diamonds for metabolite, circles for phenotype) and color as follows: red, amino acid; yellow, sugar and sugar alcohols; green, organic acids; blue, phosphates; dark blue, miscellaneous metabolites; pink, fruit traits; gray, leaf traits. Interactions are indicated with lines: gray represents positive correlations, and red represents negative correlations. Abbreviations are as follows: F_DW, fruit dry weight; F_de_In, fruit deformation index; jelly_FW, jelly fresh weight; L_area, specific leaf area; L_DW, leaf dry weight; L_N, leaf number; S_N, seed number; 14:0, tetradecanoate; 16:0, hexadecanoate; 18:0, octadecanoate; adenoP, adenosine-5-monophosphate; a-glu, Glc-2-amino-2-deoxy; asc, ascorbate; asn, Asn; asp, Asp; b-ala, β-Ala; benz, benzoate; d-Asc, dehydroascorbate; ery, erythrose; fruP, Fru-6-P; fum, fumarate; gaba, GABA; galna, galactonate; galno, galactinol; gln, Gln; glu, Glu; gluP, Glc-6-P; gly, Gly; glyc, glycerate; glycol, glycerol; glycolP, glycerol-3-phosphate; ile, Ile; ino, inositol; inoP, inositol-1-phosphate; leu, Leu; lys, Lys; mal, maltose; mala, malate; met, Met; nico, nicotinate; o-a-ser, *O*-acetylserine; o-glut, 2-oxoglutarate; orn, Orn; oxpro, pyroglutamate; phe, Phe; phos, phosphate; pro, Pro; put, putrescine; pyr, pyruvate; raff, raffinose; rib, Rib; sacc, saccharate; ser, Ser; thr, Thr; thrn, threonate; toco, tocopherol; treh, trehalose; trp, Trp; tyr, Tyr; val, Val.

well as some phosphates, organic acids, and leaf traits, and the third module comprises all fruit traits as well as some organic acids and phosphates and the amino acids Pro and oxyproline (Fig. 4A). This pattern is highly reminiscent of that observed in the *S. pennellii* introgression line network (Schauer et al., 2006, 2008), which is perhaps not surprising given that this population was also grown under HL cultivation. To provide an even more simple comparison, we replotted the data for *S. chmielewski* and *S. pennellii* populations using only traits common to both studies (Supplemental Fig. S6). By contrast, although in the LL network the first module still comprises mainly amino acids, it additionally includes four sugars or sugar alcohols,

while the second and third modules are dramatically different, comprising leaf and fruit traits, sugars, sugar alcohols and phosphates, and organic acids (Fig. 4B).

A detailed assessment of the connectivity of the various metabolites under the different fruit load conditions revealed that under LL more metabolites are highly connected, with four metabolites being correlated to at least 30 other metabolites and 21 metabolites being correlated to at least 20 other metabolites. By contrast, under HL, only eight metabolites are correlated to at least 20 other metabolites, with none being correlated with more than 23. However, it is interesting that under HL condition, the *S. chmielewskii* population has a denser network topology than the *S. pennellii* population (Supplemental Fig. S6).

Impact of Fruit Development

In order to assess the impact of ripening stage on the metabolite content on pericarp metabolite content under HL and LL conditions, samples were harvested from the Moneyberg genotype at five stages of fruit development (21, 28, 35, and 42 days post anthesis [DPA] and mature fruit). Data were normalized to the early stage of development (21 DPA) in order to visualize changes in metabolism during fruit development under either HL or LL cultivation. Under HL cultivation, cv Moneyberg displayed a large increase at the final stage of maturation in glucuronate, galacturonate, ascorbate, Glu, and Asp. By contrast, raffinose, Rha, dehydroascorbate, Tyr, Met, Lys, Thr, Asn, Leu, Ser, Gly, erythritol, and maltose increased across the developmental period, while tricarboxylic acid intermediates also displayed mild increments. The hexose phosphates Glc-6-P and Fru-6-P and the sugar alcohol mannitol, however, decreased over this period (Supplemental Fig. S7A). Under LL cultivation of Moneyberg, metabolic changes were largely conserved, with the same large increases in glucuronate, galacturonate, ascorbate, Asp, and Glu on final maturation (Supplemental Fig. S7B). Moreover, similar decreases in Glc-6-P and Fru-6-P and mannitol were observed. In addition, the levels of maltitol decreased under LL, and several metabolites, namely maltose, Gly, Leu, Val, Lys, Pro, Gln, raffinose, glycerol-3-phosphate, Trp, and Phe, behaved oppositely under the two cultivation regimes, while many remained largely unaltered across the maturation period under LL as opposed to HL. These changes were similar to those previously described for the maturation of the Moneymaker (Carrari et al., 2006) and Ailsa Craig (Fraser et al., 2007) *S. lycopersicum* cultivars; however, some cultivar-specific differences are apparent.

Impact of Fruit Load at Different Developmental Stages

In order to simplify the comparison of metabolite levels under HL and LL for each given developmental stage at each time point (21, 28, 35, and 42 DPA and maturity), the metabolite level under LL was divided by that under HL to highlight changes in metabolism due to fruit load treatment across development of the parental line. These data are presented in Figure 5 and revealed that many of the metabolite levels remained relatively similar between LL and HL cultivation. For example, the major sugars and many ascorbate-associated metabolites were unaltered, as were glycerol, the fatty acids, and shikimate and its derivatives quinate and benzoate. There were also a number of changes especially in amino acids and to a lesser extent in tricarboxylic acid cycle intermediates, the ascorbate metabolite dehydroascorbate, and in the free pools of cell wall-associated minor sugars such as Xyl and Ara. With the exception of Gln and Orn, for which the differences were maximal at maturation, most of the metabolites showed the greatest difference at 35 DPA (e.g. Met, Arg, Phe, and oxoproline). The general trend is of milder increases under HL than under LL, with only malate levels being slightly decreased under this cultivation condition.

Interaction of Fruit Load, Fruit Development, and Genotype on Primary Metabolism of Tomato Fruit Pericarp

To investigate the interaction among fruit load, development stage, and genotype on the primary metabolism of tomato fruit pericarp, we compared the three genotypes, Moneyberg, IL C9d, and IL C12d, under HL and LL across the exact same developmental experiment described above. These specific introgression lines were chosen because, in a previous study (Prudent et al., 2009), it was documented that they exhibited contrasting behavior in response to the change in fruit load. For C9d, QTLs for fresh weight and sugar content were opposite yet conserved across both fruit loads, whereas for C12d, QTLs for fresh weight and sugar content were dependent on fruit load, only being apparent under LL cultivation. Principal component analysis (Supplemental Fig. S8) revealed a clear separation between HL and LL conditions, as well as between early and mature fruit development stages, but not between the genotypes, suggesting that fruit load and fruit developmental stage have greater influence in defining the global primary metabolite profile than genetic factors. ANOVA *P* values, which express the statistical significance of changes in primary metabolites as related to genotype, fruit load, and fruit development as well as the interaction between these three features, are provided in Supplemental Table S5. These data were consistent with our interpretation of the principal component analysis data in stating that fruit load and the ripening process play more important roles in defining the general primary metabolite profile of the fruit than genetic variations. They also allowed us to extend this conclusion to state that fruit load and the ripening process are also more important in terms of defining the metabolite profile than the interaction between any of the features. Particularly striking from this analysis

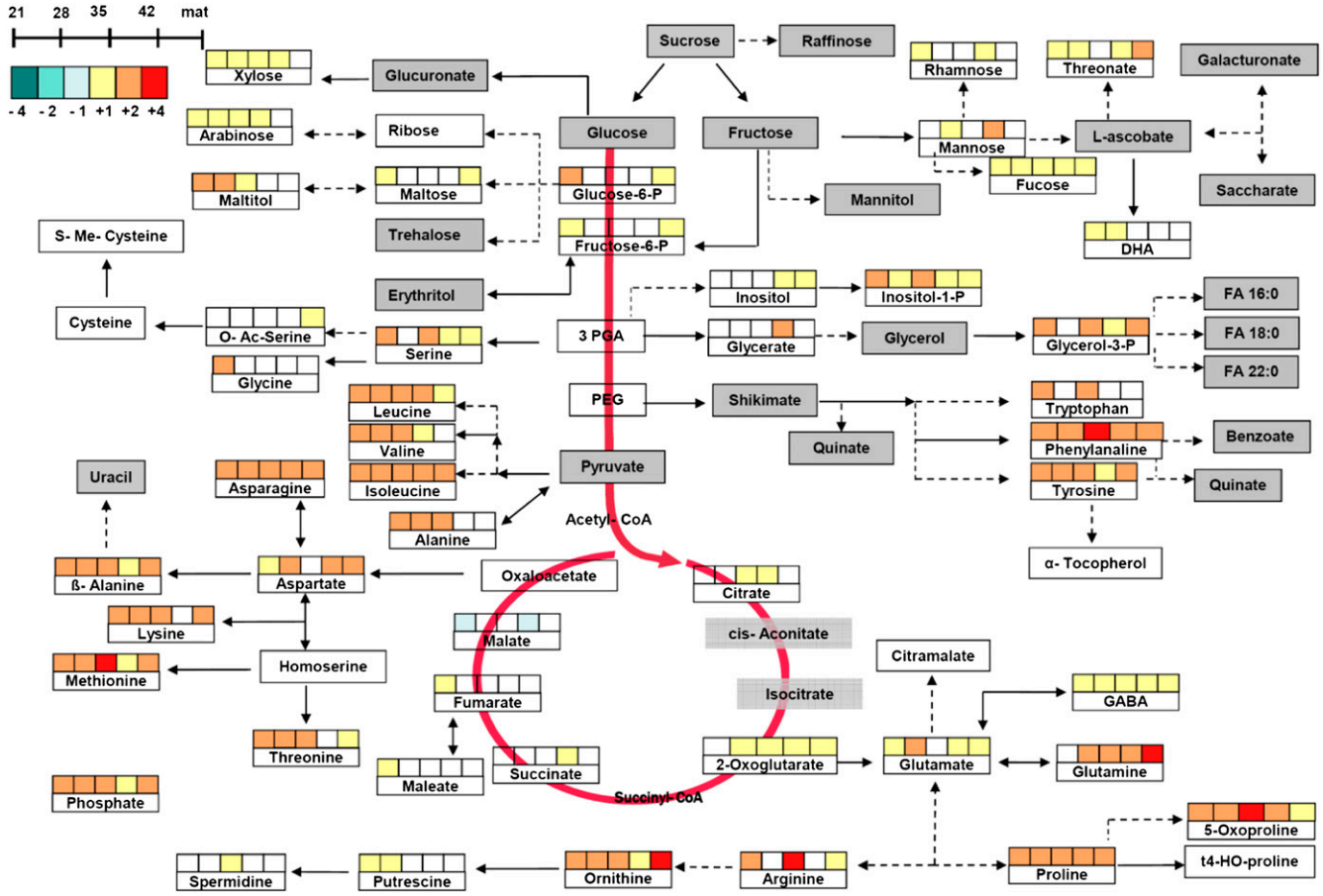

Figure 5. Schematic representation of the metabolic changes occurring in the transition from development to ripening processes in tomato fruits of Moneyberg under LL condition compared with HL condition. Each box represents a single time point of fruit development: 21, 28, 35, and 42 DPA and mature stage (from left to right). Boxes marked in yellow, light red, and dark red indicate an increase in metabolite level with increased magnification. Those marked in light blue, blue, and dark blue indicate a decrease in metabolite level with decreased magnification. Boxes in gray and white indicate no change and not determined, respectively.

was the clear effect of fruit load and fruit age on amino acids, sugars, and sugar phosphates.

In a complementary approach, we performed hierarchical cluster analysis of the entire data set collected in this three-component study. The result is presented in the combined heat map in Figure 6. This analysis facilitated the division of the metabolite data into seven clear clusters as follows: cluster 1, metabolites that are unchanged across the experimental conditions studied and accumulate only to a low level; cluster 2, metabolites that decrease during fruit development; cluster 3, metabolites that are unchanged but present at a considerably higher level than those in cluster 1; cluster 4, metabolites that increase during fruit development and accumulate considerably under LL cultivation; cluster 5, metabolites whose levels sharply increase at the mature stage and accumulate somewhat more under LL than under HL cultivation; cluster 6, metabolites whose levels decrease at the mature stage and accumulate somewhat more under LL than under HL cultivation; and cluster 7, metabolites whose levels decrease slightly during early fruit development but increase slightly at the mature stage. When the composition of these clusters was analyzed, some clear trends in compound class became apparent. Cluster 1 comprised the highly abundant metabolites Fru, Glc, isocitrate, and malate, cluster 2 largely sugar derivatives, cluster 3 fatty acids and to a lesser extent organic acids, and cluster 4 almost exclusively amino acids. Similarly, cluster 5 is dominated by ascorbate-related metabolites and cluster 6 by metabolites that are closely related to mitochondrial energy metabolism. By contrast, cluster 7 includes a very diverse range of compounds that appear to be functionally unrelated.

DISCUSSION

Here, we evaluated primary metabolite composition under two different fruit load conditions in a *S. chmielewskii* introgression line population in which the ma-

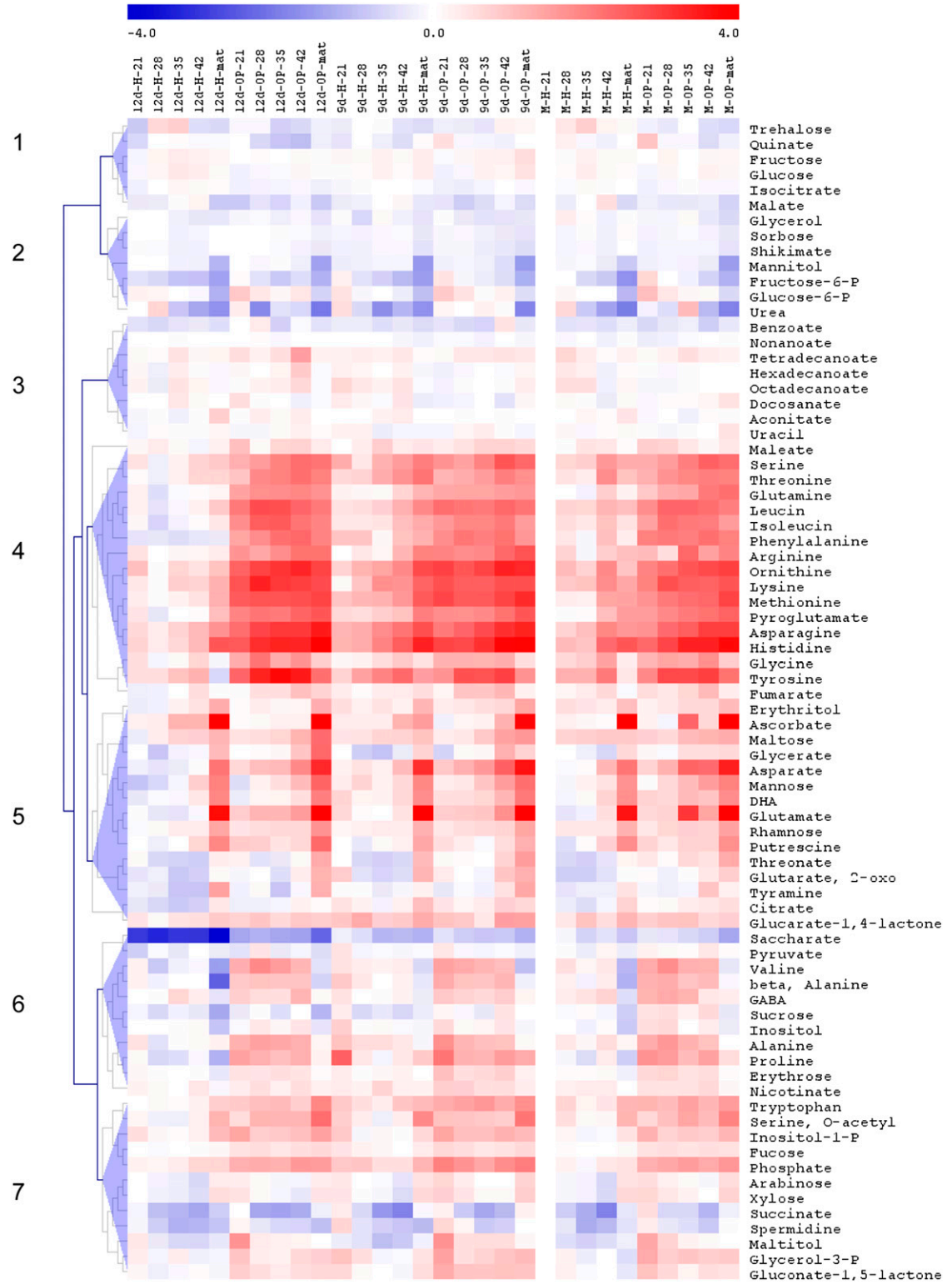

Figure 6. Changes in metabolite levels during fruit development of three genotypes under HL and LL conditions. Color indicates increase (red) or decrease (blue) in metabolite contents. Data were normalized to Moneyberg under HL condition at 21 DPA. Clustering was performed using complete linkage.

jority of the *S. chmielewskii* genome was substituted in the background of *S. lycopersicum* 'Moneyberg' (Prudent et al., 2009). Additionally, we compared the metabolite profiles of two of the introgression lines, IL C9d and IL C12d, with the parental Moneyberg line under both HL and LL cultivation conditions at five different developmental stages. A previous report details the transcriptional response of this population and subpopulation under the same experimental conditions (Prudent et al., 2010), thus allowing us to assess whether metabolic changes in the fruit pericarp are highly influenced by the transcriptional programs or rather under the control of photosynthate supply from source tissues. Given that relatively few data sets exists that provide a comprehensive overview of the primary metabolite content of fruit pericarp in introgression lines of tomato, it is interesting to analyze how these vary. The data presented in this study displayed considerably less variation than previously observed in a *S. pennellii* introgression line population (Schauer et al., 2006), both at the level of quantitative variation within a trait and in the total number of QTLs observed. However, it is important to note that this comparison is somewhat biased by the facts that the *S. chmielewskii* introgression line population studied here only accounts for 59% of the *S. chmielewskii* genome and that we studied only 62, as opposed to 74, metabolites here. A secondary consideration is that the population structure somewhat differs, with the *S. chmielewskii* introgression line population constituting a mixture or single and multiple introgressions and, in the minority of instances, the introgressions being present in the heterozygous state (Prudent et al., 2009). Thus, we must take into consideration that on the one hand the data presented here may be more subject to epistatic effects, while on the other hand we underestimate the total number of QTLs, since we would not observe some of the recessive QTLs in this population. However, given that the total size of wild species introgressions are of a similar range and that our previous study indicated only a minority of metabolite QTLs to be recessively inherited, we think these issues are not likely to greatly influence our comparison. Indeed, we found between zero and 28 and between zero and six QTLs for each metabolite measured under HL and LL cultivation, respectively. Nevertheless, *S. pennellii* is phylogenetically more distant from *S. lycopersicum* than *S. chmielewskii* (Spooner et al., 2005; Peralta et al., 2008), suggesting that these data may also be influenced by the greater allelic diversity of *S. pennellii*. In accordance with this theory, the number and magnitude of volatile QTLs observed in a *Solanum habrochaites* introgression line population were also considerably lower than those determined for the *S. pennellii* introgression line population (Dal Cin et al., 2009; Mathieu et al., 2009). However, such a relationship between trait and genomic variation cannot be simply assumed, since many of the changes we previously observed in the *S. pennellii* population were the result of transgressive behavior and could not have been predicted from our screen of fruit pericarp primary metabolite content across the tomato clade (Schauer et al., 2005b).

It is additionally possible that the reduced number of QTLs was due to the controlled nature of the growth conditions in this study as opposed to the field growth of the *S. pennellii* lines. It is our experience that greenhouse-grown populations harbor more reproducible QTLs due to the lower influence of genotype (e.g. contrast Maloney et al. [2010] with Schauer et al. [2006, 2008]); however, we are as yet unable to formally exclude this possibility. Moreover, the number of QTLs observed in the *S. chmielewskii* population is not massively above that previously recorded in intraspecific crosses of *S. lycopersicum* (Zanor et al., 2009b), further indicating that the largest changes in a trait are not necessarily conferred by having the most diverse parental genotypes.

While the HL-grown *S. chmielewskii* introgression lines clearly displayed a lesser trait variation than the previously characterized *S. pennellii* population, they still displayed a number of highly interesting features. First, there was an overlap of many of the metabolic changes in similar genomic regions, with, for example, 85 amino acid, five organic acid, four sugar, four sugar alcohol, and one sugar phosphate QTLs being apparently conserved between the *S. chmielewskii* and *S. pennellii* introgression line populations (Supplemental Table S3). Moreover, a total of 36 amino acid, 18 organic acid, one sugar, two sugar alcohol, and two sugar phosphate QTLs revealed opposite behavior in the two populations. The simplest way to explain the opposite behavior between the introgression line populations is to assume that they contain allelic variation within the same gene. As a first step toward defining the genetic basis of these traits, we looked for candidate genes in overlapping chromosome regions between two introgression line populations with a full list of candidate metabolism-associated genes, derived from genetic information published by Bermúdez et al. (2008) and Kamenetzky et al. (2010), presented in Supplemental Table S7. The evaluation of the genomic region corresponding to Bin 4I (following the Bin mapping approach described by Fridman et al. [2002]) of the *S. pennellii* population revealed colocation of a putative hexokinase and an invertase (β-fructofuranosidase) and a QTL for Suc in the *S. chmielewskii* population. While the 9C region harbors a Lys decarboxylase and the Bin 9B-D region a Tyr decarboxylase, QTLs for Lys and Tyr were observable in the respective introgression lines of *S. chmielewskii*. Intriguingly, none of these QTLs was previously identified in the *S. pennellii* population (Schauer et al., 2006). Finally, the QTL for dehydroascorbate found in both populations in a genomic region overlapping Bin 11C colocated to the map position of dehydroascorbate reductase. Interestingly, in the last instance, the QTLs in the different populations displayed opposite allele effects; however, further genetic dissection of the respective populations will be required to evaluate

whether this is due to allelic variation at the same locus. In future studies, it will be intriguing to see if the starch level acts as an integrating parameter in the same manner as previously described for Arabidopsis (Sulpice et al., 2009).

As yet, we have only described single-metabolite QTLs and not considered the interactions between metabolites and indeed between metabolic and morphological traits. Two kinds of pathway QTLs have previously been reported in the *S. pennellii* population: one involved in sugar metabolism and linking citrate to γ-aminobutyrate (GABA) and Pro (Schauer and Fernie, 2006) and the other displaying coordinate control of branched-chain amino acid content (Schauer et al., 2006; Maloney et al., 2010). In the population studied here, at least under HL cultivation, pathway QTLs additionally included those involved in the oxaloacetate-derived amino acids Asn, Asp, Lys, Met, and β-Ala (lines C5b, C8a, C8c, C9d, and C12c) and in aromatic acids (lines C5b, C8c, C9c, C9d, and C12c). That said, this population also harbored pathway QTLs linking branched-chain amino acids (lines C8e, C9d, C11d, and C12c) as well as the metabolites linking Glu metabolism and the tricarboxylic acid cycle (line C12c), similar to those previously described for the *S. pennellii* population, albeit not always at the same chromosomal location. Moreover, environmental conditions, including the exact practice of cultivation, are often described as determinants of fruit growth and quality (Heuvelink, 1997; Gautier et al., 2001; Bertin et al., 2003; Semel et al., 2007). Therefore, in this study, we attempted to evaluate the influence of all the above-mentioned parameters by assessing these features in the differently behaving *S. chmielewskii* introgression lines, IL C9d and IL C12d, and the parental line. These development-dependent analyses have already been carried out at the morphological (Prudent et al., 2009) and gene transcriptional (Prudent et al., 2010) levels, with the latter study revealing the difficulty of linking the two sets of observations. This conclusion is supported by at least two facets of the data presented in this paper. First, while the developmental changes in the metabolome described here are broadly in accordance with those described previously (Roessner-Tunali et al., 2003b; Carrari et al., 2006; Fraser et al., 2007; Wang et al., 2009; Nashilevitz et al., 2010), several cultivar/genotype differences are also apparent. Most notably, changes in sugar and organic acids levels, with the exception of succinate, are less pronounced in the Moneyberg genotype; however, changes in amino acid levels across ripening are highly similar in all genotypes studied. The fact that both the number of metabolite QTLs detected and the density of the metabolic networks observed across the population are greatly different under the different fruit load conditions is also in keeping with a massive role for source-sink relations in the ultimate determination of fruit metabolite composition. This statement is broadly true across the spectrum of metabolites measured here, providing evidence that it is not biased by the behavior of any given compound class.

The genetic control of fruit growth and composition at both plant (Schauer et al., 2006, 2008) and fruit (Fridman et al., 2004; Zanor et al., 2009a, 2009b) levels has previously been demonstrated. However, a large number of studies have also accrued evidence that the development and ultimate composition of sink organs are additionally strongly dependent upon carbon availability and sink strength (Baldet et al., 2006; Hackel et al., 2006; Burstin et al., 2007). In keeping with this statement, this study revealed that only 128 QTLs were found in plants grown under LL cultivation while 240 were found under HL. Conversely, a considerably denser network topology was seen in the LL-grown plants than in the HL-grown plants. This clearly suggests that a cultivation practice managing carbon availability has a massive effect on metabolite content and as such is most likely to influence crop product quality (Fernie et al., 2006). However, the relative paucity of similar analyses (including only Arabidopsis ecotypes [Sulpice et al., 2009], tomato [Schauer et al., 2006, 2008], and smaller scale studies in maize [Harrigan et al., 2007] and rice [Kusano et al., 2007]) makes it, as yet, difficult to generalize. Therefore, we decided to concentrate on the most striking features of the data set that we present here: the dramatic increase in amino acid levels following fruit thinning. While this is somewhat reminiscent of the situation that occurs in potato tubers (Roessner-Tunali et al., 2003a), the mechanism underlying it is clearly quite different. In potato, amino acid levels are up-regulated when the total tuber yield is depressed by a transcriptional enhancement of the levels of key genes of tuber amino acid biosynthesis. By contrast, the up-regulation of fruit amino acids is not associated either with major transcriptional changes in genes associated with fruit amino acid metabolism or protein degradation (Prudent et al., 2010). Changes in the expression of some genes associated with sugar and organic and amino acid metabolisms, such as an up-regulation of GABA transaminase and succinate dehydrogenase and a down-regulation of Fru-2,6-bisP, were apparent but did not always correlate with the observed changes in metabolite levels. However, this likely reflects the fact that protein abundance, let alone metabolite levels, is often uncoupled from transcript abundance (Gibon et al., 2009; Piques et al., 2009; Steinhauser et al., 2010; Sulpice et al., 2010). The comparison we are making here is one between early fruit transcript levels and later fruit metabolite levels, which is likely to further complicate matters. However, two interesting findings of the transcriptomic study were the strong genotype-versus-fruit load interactions for Suc synthase and GAD1 (which play important roles in tomato sink determination and GABA metabolism, respectively [Wang et al., 1993; Studart-Guimarães et al., 2007]). Evaluating the levels of the metabolites Suc, Fru, Glc, and GABA (presented in Supplemental Tables S1–S5) revealed interesting trends. GABA was clearly affected by fruit load and

development, accumulating more under LL than under HL and declining at the mature stage under HL cultivation. By contrast, Suc exhibited an interaction effect with fruit age whereby its level declined at the mature stage (except in line C9d), whereas Fru and Glc were unchanged across the development stage and between cultivation conditions, suggesting that they were under much stronger genetic influence. It thus appears that, when taken together, these data sets suggest that the majority of the differences in primary metabolism described here were not mediated at the level of transcription. While in contrast to the situation for secondary metabolism in Arabidopsis (Kliebenstein, 2009), this observation is perhaps not fully surprising (Gibon et al., 2009; Steinhauser et al., 2010; Sulpice et al., 2010). We strongly believe that these two data sets imply that the dramatic increase in amino acid content in the fruit is the result of an up-regulated transport of amino acids via the phloem. It has long been demonstrated that tomato phloem contains high levels of amino acids and that these levels vary with respect to the age of the fruit (Valle et al., 1998). Given this fact and the lack of clear transcriptional regulation in the fruit of the two introgression lines studied, it seems likely that the sink status is transmitted to the leaf via an as yet unknown mechanism. It remains conceivable that the cloning of the underlying QTLs will shed important light on the nature of this signal.

In summary, this analysis primarily demonstrated that the majority of the recorded traits were reproducible following similar cultivation but that very few traits could be directly assigned to candidate genes in the populations studied. That said, many genes associated with primary metabolism were associated with each trait, suggesting that creation of recombinant lines harboring smaller and smaller introgressions from the wild species will ultimately be necessary in order to define the genetic basis underlying these QTLs. While it is possible that the genome regions we examined in this study are not representative of the recently released tomato genome sequence (made available as a work in progress by the International Tomato Genome Sequencing Consortium at ftp://ftp.solgenomics.net/tomato_genome/wgs/assembly/), this is unlikely, since these regions were chosen because of their high density of metabolite QTLs.

It was further revealed that the *S. chmielewskii* population contained a substantial variation in primary metabolite composition and that while much of this is also dependent on the cultivation conditions, a considerable proportion is independent of this factor. In particular, this study demonstrated that amino acid content was strongly influenced by cultivation conditions. This finding thus suggests that although populations such as these will ultimately be useful resources to aid the genetic tailoring of plant metabolism (Fernie and Schauer, 2009), a fuller understanding of their exact genetic basis alongside its interaction with cultivation practice is required before this is rendered a routine process.

MATERIALS AND METHODS

Plant Material

Tomato (*Solanum lycopersicum* 'Moneyberg') and 23 introgression lines carrying single or multiple marker-defined genomic regions of the wild species *Solanum chmielewskii* LA1840 in the genetic background of cv Moneyberg was obtained from Keygene. The mapping of the introgressed regions of the introgression lines has been described previously in the literature (Prudent et al., 2009).

Growth Conditions and Experimental Treatments

Plants were grown in a controlled ground bed greenhouse in Avignon in southern France at 24°C/16°C day/night during spring 2006 and 25°C/15°C day/night during spring 2007 at a density of 3.6 plants m^{-2}. Two fruit load conditions were investigated in this study: LL, where trusses were pruned to one fruit, and HL, where trusses were not pruned. The conditions were very similar between the 2 harvest years. Under HL condition, the average number of fruits per truss was very similar (5.5 in 2006 and 5.1 in 2007). Similarly, the mean fruit mass under HL was approximately 92 g in 2006 and approximately 89 g in 2007. For three genotypes, C9d, C12d, and M, individual flowers were tagged at anthesis to accurately follow fruit age through development. Fruits of those genotypes were harvested at five different developmental stages from the cell expansion period through to maturity (21, 28, 35, and 42 DPA and red ripe). For other genotypes, fruits were harvested at red ripe stage. Harvested fruits were weighed, and pericarp was separated from the placental tissue, weighed, and then immediately frozen in liquid nitrogen before being stored at −80°C until further analysis. Phenotypic and gene expressional traits have been described previously for these harvests (Prudent et al., 2009, 2010).

Metabolite Profiling

Sample Preparation, Measurement, and Data Processing

Tomato pericarp tissue was homogenized, and the exact amount used for metabolite extraction was defined. Six replicates for each introgression line under each fruit load condition were measured. Metabolite extraction, derivatization, gas chromatography-mass spectrometry analysis, and data processing were performed as described previously (Roessner et al., 2001; Lisec et al., 2006). Metabolites were identified in comparison with database entries of authentic standards (Kopka et al., 2005; Schauer et al., 2005a).

Data Normalization

All samples were extracted and measured in groups of 50. The huge number of samples led to extraction and measurement periods of weeks per experiment. It is necessary, therefore, to correct for technical errors over the different times. In addition to normalization using an internal standard, an external standard was used. This external standard was a metabolite mix divided into aliquots from the same metabolite extraction pool that was extracted from cultivated tomato samples using a strategy adopted from Sulpice et al. (2009). Five replicates of external standard were measured together with samples that were randomized within days. Two normalization steps were applied. In the first step, metabolite data were normalized to internal standard data. In the second step, the intensity of metabolite *i* was divided by the average intensity of *i* from five external standards measured on the same day.

Statistical Analysis

Statistical analyses were performed using R statistical software (www.r-project.org), SAS version 9.2 (SAS Institute), Multiexperiment Viewer version 4.1 (Saeed et al., 2003), or Microsoft Excel. If two observations are described as different, this means that they were determined to be statistically different ($P < 0.05$) by the performance of Student's *t* test using the algorithm embedded in Microsoft Excel.

QTL Mapping

To map metabolic traits, a two-way ANOVA was used to partition metabolic variation into genotype, year, and genotype × year interaction

effects under each fruit load condition. A significant metabolic effect in a specific introgression line was identified by a Dunnett multiple comparison test (SAS version 9.2). Metabolite QTLs were defined as those that were different in the introgression line from the Moneyberg parent at the significance threshold of 0.05.

Heat Maps

Heat maps were created using Multiexperiment Viewer. False-color imaging was performed on the $\log_2$-transformed metabolite data. The overlay heat maps were generated by using the multiplication functions of Adobe Illustrator (Adobe System) on the single heat maps.

Network Analysis

Correlation between all trait (metabolite and phenotype) pairs was tested by using introgression line mean values under each fruit load condition. Correlation coefficients and significances were calculated by applying a Spearman algorithm using statistical software environment R, version 2.7. Correlation networks were then visualized as a cartographic representation using the algorithm developed by Guimerà and Nunes-Amaral (2005) using a strict significance level of $P < 0.0001$.

Cluster Analysis

Hierarchical cluster analysis and principal component analysis were performed with Multiexperiment Viewer. Hierarchical cluster analysis allows the presentation of cluster results in a dendrogram, where the similarity of samples can be determined from the value on the distance axis at which they join in a single cluster (the smaller the distance, the more similar the sample). Hierarchical cluster analysis was performed on $\log_2$-transformed metabolite data, using complete linkage clustering and the Pearson correlation. Principal component analysis was performed on $\log_2$-transformed metabolite data using a default weighted covariance estimation function, and results were then presented as a two-dimensional graphical display.

Supplemental Data

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

Supplemental Figure S1. Marker positions and genetic variability of the three previously uncharacterized introgression lines C2b, C2d, and C3c.

Supplemental Figure S2. Heat map of the metabolite profiles of the introgression lines in comparison with that of the parental control from the individual data set year 2006 under HL condition.

Supplemental Figure S3. Heat map of the metabolite profiles of the introgression lines in comparison with that of the parental control from the individual data set year 2007 under HL condition.

Supplemental Figure S4. Heat map of the metabolite profiles of the introgression lines in comparison with that of the parental control from the individual data set year 2006 under LL condition.

Supplemental Figure S5. Heat map of the metabolite profiles of the introgression lines in comparison with that of the parental control from the individual data set year 2007 under LL condition.

Supplemental Figure S6. Comparison of the metabolic network obtained from the *S. chmielewskii* introgression line population (A) with the previously reported network obtained from the *S. pennellii* introgression line population (B).

Supplemental Figure S7. Schematic representation of the metabolic changes occurring in the transition from development to ripening processes in tomato fruits of Moneyberg under HL (A) and LL (B) conditions.

Supplemental Figure S8. Principal component analysis of metabolite data obtained from three genotypes during fruit development (21, 28, 35, and 42 DPA and mature stage) under two fruit load conditions (HL and LL).

Supplemental Table S1. Metabolite QTL table under HL condition.

Supplemental Table S2. Metabolite QTL table under LL condition.

Supplemental Table S3. Metabolite changes under LL condition compared with HL condition in individual year 2006.

Supplemental Table S4. Metabolite changes under LL condition compared with HL condition in individual year 2007.

Supplemental Table S5. ANOVA *P* values expressing the statistical significance of the changes in primary metabolite contents under different fruit load conditions and different fruit development stages.

Supplemental Table S6. Comparison of metabolite QTLs detected with the previously reported *S. pennellii* introgression line population (Schauer et al., 2006) and QTL near isogenic lines population (Zanor et al., 2009b).

Supplemental Table S7. A candidate gene survey of shared QTL regions between two introgression line populations, *S. chmielewskii* and *S. pennellii*, affecting chemical composition in tomato fruit.

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