Running title: System biology of pepper ripening and development

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Integrative comparative analyses of transcript and metabolite profiles from pepper and tomato ripening and development stages uncovers species-specific patterns of network regulatory behaviour

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Abstract

Integrative comparative analyses of transcript and metabolite levels from climacteric and non-climacteric fruits can be employed to unravel the similarities and differences of the underlying regulatory processes. To this end, we conducted a combined gas chromatography-mass spectrometry and heterologous microarray hybridization assays in tomato (climacteric) and pepper (non-climacteric) fruits across development and ripening. Computational methods from multivariate and network-based analyses successfully revealed the difference between the covariance structures of the integrated data sets. Moreover, our results suggest that both fruits have similar ethylene-mediated signaling components; however, their regulation is different and may reflect altered ethylene sensitivity or regulators other than ethylene in pepper. Genes involved in ethylene biosynthesis were not induced in pepper fruits. Nevertheless, genes downstream of ethylene perception such as cell wall metabolism genes, carotenoids biosynthesis genes and never ripe receptor were clearly induced in pepper as in tomato fruit. While signalling sensitivity or actual signals may differ between climacteric and non-climacteric fruit, evidence described here suggests activation of a common set of ripening genes influence metabolic traits. Also, a coordinate regulation of transcripts and the accumulation of key organic acids, including: malate, citrate, dehydroascorbate, and threonate, in pepper fruit were observed. Therefore, the integrated analysis allows us to uncover additional information for the comprehensive understanding of biological events relevant to metabolic regulation during climacteric and non-climacteric fruits development.
Introduction

Fruits are generally classified into two physiological groups, “climacteric” or “non-climacteric”, according to their respiration pattern and reliance on ethylene biosynthesis during ripening. Climacteric fruits, such as tomato and banana, show an increase in respiration rate and ethylene formation. Non-climacteric fruits, such as strawberry and grape, exhibit neither the respiratory burst nor an elevated ethylene synthesis during ripening (Giovannoni, 2001). Pepper has typically been considered a non-climacteric, although varying patterns of ethylene production and respiratory rates have been reported (Villavicencio et al., 1999). Pepper is also considered a good source of metabolites possessing health-promoting properties, such as: vitamins C, E and B complex, provitamin A, oxygenated carotenoids and other phytochemicals such as flavonoids with high antioxidant activities (Howard et al., 2000; Howard and Wildman, 2007). During maturation, fruit undergo transformations in colour, aroma, nutrient composition, flavour and softening. During this process production of reactive oxygen species (ROS) plays an important role, particularly in the biosynthesis of carotenoids and in the transformations of chloroplasts to chromoplasts (Bouvier et al., 1998; Marti et al., 2009). These transformations are results of dynamic processes that involve a complex series of molecular and biochemical changes under genetic regulation and/or in response to environmental perturbations. To better understand fruit development and ripening mechanisms, numerous studies have been focused on studies on transcript and metabolite levels in climacteric fruits, such as tomato (Roessner-Tunali et al., 2003; Alba et al., 2005; Lemaire-Chamley et al., 2005; Vriezen et al., 2008; Wang et al., 2009) or peach (Lombardo et al., 2011), and in non-climacteric fruits, such as strawberry (Aharoni and O'Connell, 2002; Aharoni et al., 2002; Fait et al., 2008; Osorio et al., 2008; Bombarely et al., 2010), pepper (Lee et al., 2010) and grape (Deluc et al., 2007; Grimplet et al., 2007). Integration of genomics, gene expression and chemical composition data during fruit development and ripening can give important insight into gene-regulatory and metabolic events associated with these processes. In recent years, a number of studies on fruits have begun to integrate these extensive data sets and, while a number of them focused on the non-climacteric grape and strawberry systems, most used the climacteric model of tomato fruit (Carrari et al.,
In this study, we employed similar approaches; however, we have focused on a non-climacteric member of the Solanaceae family (of which tomato is also a member) to facilitate comparative analyses that may provide insights into relationships between climacteric and non-climacteric ripening. Specifically, we have integrated transcript and metabolic data using different development and ripening stages of pepper (*Capsicum chilense* cv. Habanero). While some peppers are climacteric, *Capsicum chilense* is not. Metabolomic profiling was assessed by gas chromatography–mass spectrometry (GC-MS), whilst transcriptomic analysis was performed using the tomato TOM1 array which was demonstrated applicable for assessing transcript levels in pepper (Moore et al., 2002). Lee et al., (2010) have compared climacteric tomato and non-climacteric pepper fruit ripening at the transcriptomic level and identified genes in the ethylene signalling pathway that are induced in pepper, suggesting a non-ethylene regulator influences of these genes in non-climacteric peppers. Here, we conducted integrative comparative analysis of transcriptomics and metabolomics data from tomato and pepper fruits to gain a broader systems perspective and to identify additional common as well as distinct molecular regulatory events during development and ripening.

**RESULTS**

**Metabolic profiling during pepper fruit development and ripening**

In order to assess metabolic profiles reflecting primary metabolism of pepper fruit we selected the following stages of development and ripening: 14, 20, 34, 51 (breaker-1; Br-1), 52 (breaker; Br), 53 (breaker+1; Br+1), 55 (breaker+2; Br+2), 57 (breaker+5; Br+5), 62 (breaker+10; Br+10) and 68 (breaker+16; Br+16) days after pollination (DAP). The normalized data set obtained in this study by GC-MS as well as metabolite data of development and ripening of tomato from Osorio et al., (2011) were examined by two types of multivariate methods: (1) congruence of the covariance matrices, given by the Rv coefficient, and (2) similarity between the principal components (PCs) of all periods spanning four stages (see Materials and Methods). For the comparative analysis of the metabolite profiles we used the
profiles of the 26 metabolites measured over 8 and 10 different developmental and ripening stages in tomato and pepper, respectively. The R_v coefficient takes values between 0 and 1, and small values of the coefficient indicate large difference between the data sets. For the metabolomic data sets of tomato and pepper the R_v coefficient has a value of 0.003, although this value is not statistically significant at significance level 0.05 (P-value = 0.38). Next, we considered all periods spanning four of the investigated stages, and calculating the similarity based on the three PCs explaining maximum of the variance. Figure 1 A confirms that the investigated 5 and 7 periods in tomato and pepper, respectively, are highly dissimilar. Moreover, the considered periods exhibit high but varying similarity, allowing to distinguish the transition between early and late period in the development and ripening at 43DAP (Br+1) for tomato and 52-53 DAP for pepper. Finally, Figure 1 A suggests that for the 26 metabolites, there is similarity between the middle and later periods in the development and ripening of tomato and pepper fruit, but this is not the case for the beginning stages (e.g., see pepper 20 DAP – 51 DAP and tomato 27 DAP – 42 DAP).

Metabolism of sugars and sugar alcohols during pepper fruit development and ripening

The major sugars, Fru, Glc and Suc displayed a continuous increase during development of pepper until 52 DAP (breaker) (Figure 2). The contents of these sugars increased dramatically at the onset of ripening (51 to 52 DAP). However, after 52 DAP, all three major sugars showed similar patterns of decline in content (Figure 2). Indeed the behaviour of the major sugars during pepper ripening is opposite to many other characterized fruits including tomato cv. Moneymaker (Carrari et al., 2006), cv. Ailsa Craig (Osorio et al., 2011), strawberry (Fait et al., 2008), grape (Deluc et al., 2007) and peach (Lombardo et al., 2011). With regard to sugar alcohols, galactinol, myo-inositol-1P and myo-inositol were detected. In the case of myo-inositol, the same behaviour as for the major sugars was observed (Figure 2). Myo-inositol gradually increased during development with a peak at 51 DAP. In the case of myo-inositol-1P, the opposite pattern during early developmental and ripening stages compared to myo-inositol was observed (Figure 2). Myo-inositol-1P decreased during early developmental stages to its minimum value at 34 DAP, while it increased during ripening initiation (51 DAP) and onwards. Galactinol levels were
highest at earlier developmental stages with a peak at 34 DAP (Figure 2) and after this peak galactinol content dramatically decreased.

Xylose was the only cell-wall-related sugar detected and decreased during early developmental stages and then dramatically increased during ripening (Figure 2). This trend is consistent with many reports describing modifications and disassembly of hemicellulosic and pectin cell wall polysaccharides during fruit ripening (Sakurai and Nevins, 1993; Seymour, 1993).

**Metabolism of organic acids during pepper fruit development and ripening**

The variation in the levels of the various organic acids measured during pepper development and ripening processes were complex (Figure 2). Citrate and dehydroascorbate levels dramatically increased during early developmental stages (approximately 40 and 15 fold, respectively) followed by small reductions during ripening stages (Figure 2). Malate, saccharate and threonate levels were increased during pre-ripening development, until 34 DAP for malate and 51 DAP for saccharate and threonate. Moreover, distinct accumulation patterns of these three organic acids during ripening and as compared to each other were observed. Malate levels decreased during later developmental stages (from 34 to 51 DAP), although a substantial increase was observed during ripening (approx. two-fold). Saccharate levels were essentially constant during pepper ripening fruit, while threonate levels declined. Quinate levels decreased through development and ripening - although this decline was not as pronounced during ripening (Figure 2).

**Metabolism of amino acids during pepper fruit development and ripening**

A total of twenty amino acids across all characterized stages were analyzed. During the fruit development phase (from 14 to 51 DAP), three distinct patterns of amino acids accumulation were observed: (1) those that increased through development including Ala, Asn, GABA, Gin, Ile, Arg, Asp, Glu, Lys, Phe, Thr, Ser and Val, (2) those which remained relatively constant including Gly, Met, Tyr and Pro, and (3) Trp which decreased during pre-ripening development. Eighteen dramatically increased at the onset of ripening and all except GABA and Glu declined lasting the final ripe
stage (68 DAP) analysed. Only Trp showed no changes during pepper ripening (Figure 2).

**Network-based analysis of primary metabolite data**

Networks of metabolites were created from Pearson correlations of the corresponding profiles while ensuring a false discovery rate (FDR) of 0.05 (see Materials and Methods). Again, we focused on the analysis of 26 metabolites detected in both tomato and pepper over all observed stages. Threshold values of 0.905 and 0.830 for the absolute value of the correlations ensured the imposed FDR rate in tomato and pepper, respectively. These resulted in 18 and 25 significant correlations, of which 14 and 20 were positive, for the tomato and pepper metabolome, respectively.

The distribution of edges resulted in two major components in the tomato network, containing 8 and 4 metabolites, respectively. The first component comprised the four amino acids (threonine, phenylalanine, aspartate, and pyroglutamate), while the second included three sugars (Fru, Glc, and xylose), three organic acids (malate, quinate, and dehydroascorbate), as well as two amino acids (alanine and glutamate). The remaining 14 metabolites were not involved in significant correlations, yielding isolated nodes (not shown in the Figure 3). The connected components could be further split in four communities (clusters), yielding a modularity value of 0.443 for the tomato network (see Figure 3 A). As expected, major sugars (sucrose, fructose, and glucose) were highly correlated with each other and clustered together; this community also included glutamate and quinate. Moreover, threonine and phenylalanine as well as pyroglutamate and aspartate clustered together. On the other hand, malate was found to negatively correlate to xylose, glutamate, alanine, and dehydroascorbate, and formed a cluster with the latter two.

The pepper network contained three major components, containing 10, 4, and 4 metabolites respectively (Figure 3 B). The first was composed of amino acids, including: alanine, tyrosine, valine, and phenylalanine, which were not significantly correlated to any other of the investigated metabolites. The second component could be further split into two communities, one involving two amino acids (GABA and glutamate) and the other, phosphoric acid and myo-Inositol. Finally, the third and
The largest component could be divided into three clusters, of which the first included, as expected, the three sugars (Glc, Fru, Suc), the second comprised malate, citrate, and ornitine, while the third also included a combination of amino and organic acids. Compared to the tomato network, here quinate (and not malate) formed the only significant negative correlation between organic acids and sugars (i.e., Fru). The community structure of the pepper network is more pronounced compared to that of tomato, as indicated by the modularity value of 0.56.

The correlation of Fru and Glc was the only preserved between the tomato and pepper network at FDR level of 0.05. Moreover, the network-based investigation suggest that there is a major restructuring of the underlying metabolic and regulatory processes in climacteric and non-climacteric fruits since the metabolites without any significant correlations in the former formed relationships in the latter.

**Transcript levels during pepper fruit development and ripening**

To identify differentially expressed genes in pepper pericarp, we used the TOM1 cDNA microarray and a time-series loop design. The microarray-based analysis was performed in the same tissues used for metabolomics (the time-series included eleven stages: 14, 20, 26, 34, 51, 52, 53, 55, 57, 62 DPA and 68 DAP). To profile transcript abundance we used two-colour simultaneous hybridizations to the TOM1 array containing 12,899 EST probes representing ~8,500 tomato unigenes, and using mRNA prepared from each stage listed above. Genes at any fruit developmental stage showing a two-fold or greater expression level change as compared to the first (reference) stage (14 DAP) and after Benjamini-Hochberg correction with P-value cut-off of 0.05 were noted as significant (Supplemental Table 1).

For the comparative analysis of the transcript profiles, we used the profiles of the 318 genes found to have pronounced differential expression. For the transcriptomic data sets of tomato and pepper the R_v coefficient has a significant value of 0.154 (P-value = 1.09e^{-314} < 0.05), indicating that the transcript levels the two types of fruit differ but less so compared to the metabolite levels. Next, analogously to what was performed for the metabolite profiles, we considered all periods spanning four of the investigated stages, and calculating the similarity based on the three PCs explaining maximum of the variance confirms that the investigated 5 and 7 periods in tomato
and pepper, respectively, are highly dissimilar (Figure 1 B). Moreover, the considered periods exhibit high but varying similarity, allowing to distinguish the transition between early and late period in the development and ripening at 42 DAP for tomato and 26-34 DAP for pepper. Finally, Figure 1 B suggests that for the analyzed transcripts, there is similarity between the later periods in ripening of tomato and pepper fruit (e.g., see pepper 55 DAP – 68 DAP and tomato 42 DAP – 47 DAP), but this is not the case for the beginning stages (e.g., see pepper 20 DAP - 51 DAP, pepper 26 DAP – 52 DAP and tomato 27 DAP – 42 DAP, tomato 39 DAP – 43 DAP).

In addition, all genes showing significant (P<0.05) differential expression values were classified in different functional categories using the MapMan classification system (Usadel et al., 2005) (Figure 4) and reflected the physiological and biochemical changes that occur during pepper development and ripening fruit. The functional distribution of differentially expressed genes revealed several interesting facts. For instance, 25 – 50% of the differentially expressed genes are of yet unknown function. The categories “photosystem” and “major CHO metabolism” included the most down-regulated genes during ripening (from 53 DAP onwards). Interestingly, the category “RNA” was well represented in both up and down-regulated genes and for all studied stages, although the percentage of down-regulated genes from this category decreased during development and ripening and increased for up-regulated genes in the same stages. Genes involved in cell wall synthesis, degradation and modification considered as a global category were predominantly down-regulated during ripening (51 DAP onwards) and some genes of this category were the most differentially expressed during ripening stages. These genes represented an abundant class of either up- or down-regulated at 26 DAP (Figure 4). It is well documented that cell wall restructuring and disassembly genes (endo- β-1,4-glucanases from glycosyl hydrolase (GH) family, expansins, xyloglucan transglucosylase hydrolases (GH16) and a range of pectinases) are canonically associated with fruit development and ripening (Rose and Bennett, 1999; Rose et al., 2004; Brummell, 2006) in addition to earlier stages of fruit development (e.g. as characterized by the 26 DAP stage) (Srivastava et al., 2010; Rose et al., 1998; Ranwala et al., 1992). Genes belonging to the “development” classification were more highly represented among genes up-regulated through the ripening process (from 51 DAP onwards). It is also noteworthy that genes involved in protein synthesis and degradation showed the same
distribution between categories of up-dowregulated genes among all studied stages (Figure 4).

PageMan (Usadel et al., 2006) and MapMan (Usadel et al., 2005) mapping files, described in Materials and Methods, were used to study the development and ripening of pepper pericarp by identifying significantly over-represented functional groups, using the earliest developmental stage (14 DAP) as baseline and on the basis of Fisher’s exact and Wilcoxon tests for each category. This analysis facilitated the analysis of the global activation and/or repression of metabolic pathways and gene regulatory networks of the pericarp. Individual gene responses can be viewed in MapMan (Supplemental online file). Using PageMan we identified metabolic pathways that were enriched during fruit development (Figure 5). As anticipated, photosynthetic genes expression declined dramatically during later ripening stages (55 DAP onwards). However, a broader biphasic response was also noted with a significant decline occurring during earlier developmental stages (26 and 34 DAP) as well, which may reflect the transition of the fruit from a photosynthetic to a sink tissue (Figure 5). As also was expected in an organ displaying transient starch synthesis (Beckles et al., 2001; Carrari et al., 2006), a large reduction during ripening of genes involve in starch synthesis was observed coincident with the ripening transition (from 51 DAP onwards). Degradation of sucrose, was also up-regulated at the onset of ripening (53 and 55 DAP). Interestingly, genes associated with the TCA cycle were up-regulated during fruit development (26 and 51 DAP; Figure 5) which contrasts the trend described for tomato fruit (Carrari et al., 2006). Similar to ripening tomato fruit (Carrari et al., 2006), in ripening pepper fruit, the transcript levels involved in cell wall restructuring and disassembly showed a clear tendency to be increased which was corroborated by qRT-PCR in some genes such as expansin1 (EXP1), expansin precursor (EXP3), xyloglucan endotransglycosylase/hydrolase (XTH5) and polygalacturonase (PG). The same behaviour was observed in some genes involved in carotenoid metabolism such as phytoene synthase (PSY1), and carotene desaturase (ZDS) to be increased in pepper (Figure 6). Others metabolic pathways that were enriched prior to the onset of fruit ripening were those associated with hormone metabolism in which we specifically observed a strong reduction of auxin-responsive genes (from 34 to 52 DAP) as well as strong up-regulation of genes involved in biosynthesis of ribosomal proteins (from 26 to 51 DAP), although these
genes showed a decrease in expression during ripening (at 55 DAP) (Figure 5). Finally, we in all studied stages a general up-regulation of ubiquitin protein degradation process (Figure 5) as was also observed in tomato fruit (Osorio et al., 2011).

Hormones biosynthesis and responses in pepper fruit

The effects of plant hormones on fruit maturation differ for climacteric and non-climacteric fruit. Tomato is a model system to study climacteric fruit ripening and numerous efforts have been undertaken with the goal of understanding ethylene biosynthesis and response including the steps involved in ethylene perception and signal transduction (reviewed in Klee and Giovannoni, 2011). Ethylene biosynthesis includes conversion of Asp to Met, conversion of Met to ethylene, and the Met recycling pathway (Yang and Hoffman, 1984). Ethylene is synthesized from \textit{S}-adenosyl methione (SAM) by the sequential action of two ethylene-biosynthetic enzymes, namely, 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase. SAM can participate in polyamine as well as ethylene biosynthesis (Mattoo et al., 2007). In climacteric tomato fruits, ACC synthase and ACC oxidase are elevated during ripening and genes (Carrari et al., 2006; Osorio et al., 2011). In contrast, no changes in transcript levels of ACC synthase and ACC oxidase during non-climacteric habanero pepper development and ripening processes were observed. However, SAM-synthetase, the catalyst of SAM biosynthesis, showed up-regulation in later ripening stages (57-68 DAP; Supplemental online file). This result would suggests that polyamine biosynthesis may pre-dominate the flux through SAM metabolism rather than ethylene biosynthesis in pepper.

The tomato genome harbors six characterized ethylene receptors and five of the six (\textit{ETR1}, \textit{ETR2}, \textit{ETR4}, \textit{ETR5}, \textit{ETR6} and \textit{ETR3/Never-ripe}) have shown to bind ethylene (Klee and Tieman, 2002; Klee, 2002) while expression studies revealed several different profiles. For example, transcript levels of \textit{ETR1}, \textit{ETR2}, and \textit{ETR5} change little during ripening or upon ethylene treatment of mature fruit, while \textit{NR}, \textit{ETR4}, and \textit{ETR6} are strongly induced during ripening and mutant and transgenic studies have shown that all can impact ripening phenomena (Kevany et al., 2007). Additional studies indicate that, as in Arabidopsis, tomato ethylene receptors are
functionally negative regulators of ethylene signalling (Klee and Tieman, 2002; Klee, 2002). Consistent with this model, exposure of immature fruits to ethylene caused a reduction in the amount of ethylene receptor protein and earlier ripening (Kevany et al., 2007). Interestingly, \( Nr \) transcript (SGN-U590044) was up-regulated during pepper ripening (Figure 6 and Supplemental online file) as it is during climacteric tomato ripening (Figure 6). Moreover, in tomato it has been shown that following ethylene interaction with the receptor, the signal is transferred via a MAP kinase cascade to a family of transcription factors (ethylene response factor; ERFs) that influence expression of ethylene-regulated genes including those mediating cell wall disassembly (endo-polygalacturonase, pectin methyl esterase and pectate lyase). During pepper ripening, \( ERF3 \) transcript (SGN-U564955) was up-regulated as well as transcripts related to cell wall restructuring and disassembly (Figure 6 and Supplemental online file). These results are consistent with the hypothesis that ethylene signalling components downstream of the receptors are regulated by a mechanism independent of ethylene (Lee et al., 2010).

Jasmonic acid has been demonstrated to be involved in cross-talk with ethylene signalling components in arabidopsis (Devoto and Turner, 2005) and has been associated with fruit maturation. In climacteric fruits such as tomato and apple, jasmonate levels increase during early fruit maturation, suggesting that they could be involved in the regulation of this process (Fan et al., 1998). Consistent with this hypothesis, exposure of immature apple fruits to jasmonate caused elevated ACC oxidase activity and heightened ethylene production (Saniewski et al., 1986; Miszczak et al., 2000). The increase of jasmonate in tomato and apple in addition to non-climacteric fruits such as sweet cherry occur during the earlier developmental stages could indicate a role for jasmonate in cell division or other early fruit expansion processes (Kondo and Tomiyama, 2000). During early development (26 DAP) of pepper fruit we observed elevated transcript levels for the jasmonate synthesis gene 12-oxophytodienoate reductase3 (SGN-U595636) (Supplemental online file). Moreover, at the onset of ripening (52 to 57 DAP), a lipoxygenase gene (SGN-U594638) associated with jasmonate biosynthesis was also induced suggesting that jasmonate may also play a role in non-climacteric fruit ripening (Supplemental online file).
Network-based analysis of transcript levels

Similarly to the analysis of metabolite profiles, coexpression networks of transcripts were created from Pearson correlations of the corresponding profiles while ensuring a false discovery rate (FDR) of 0.05 (see Materials and Methods). Again, we focused on the analysis of 318 transcripts showing most pronounced differential behaviour in both tomato and pepper over all observed stages. Threshold values of 0.9524 and 0.8425 for the absolute value of the correlations ensured the imposed FDR rate in tomato and pepper, respectively. These resulted in 768 and 4530 significant correlations, of which 660 and 3544 were positive, for the tomato and pepper transcriptome, respectively.

In the tomato coexpression network, the distribution of edges resulted in 57 components, containing at least one edge. The largest of these components contained 75 transcripts, followed by two components composed of 9 transcripts each. Looking at the level of community structure, the tomato coexpression network showed modularity of 0.402 for the 62 communities. The three largest communities, composed of 28, 26, and 21 transcripts, respectively, were subnetworks of the largest component (Figure 7 A). At a significance level of 0.05, the first was enriched for the following functional categories (see Materials and Methods): cell wall components, modification, and degradation, hormone metabolism, major CHO metabolism, redox metabolism, protein activation, targeting, folding and assembly. The second and the third were enriched for all of the abovementioned functional categories, except cell wall degradation and hormone metabolism. The average correlations for transcripts involved in cell wall modification, CHO synthesis and degradation, and protein synthesis and degradation were found to be: 0.980, 0.976, 1, 0.980, and 0.711, respectively. This implies that all but the transcripts involved in protein degradation exhibit statistically significant and high correlations.

The pepper coexpression network contained 5 components, containing at least one edge (Figure 7 B). The largest of these components contained 355 transcripts, followed by a component with only 5 transcripts. These components could be further decomposed into 9 communities (clusters) yielding a modularity value of 0.555 for the pepper coexpression network. The largest of these communities contained 109 transcripts and was significantly enriched for the same functional categories as the
largest community in the tomato coexpression network, except for protein activation and hormone metabolism. The average correlations for transcripts involved in cell wall modification, CHO synthesis and degradation, and protein synthesis and degradation were found to be: 0.978, 0.737, 0.497, 0.848, and 0.850, respectively. This implies that all but the transcripts involved in CHO synthesis and degradation exhibit statistically significant and high correlations, which is in contrast to what was found for the tomato coexpression network. In facts, 10% and 25% of the correlations between genes involved the CHO synthesis and degradation were found to be negative.

While there is preservation of the functional categories enriched in the clusters of the tomato and pepper coexpression networks, the intersection of the two coexpression networks was composed of only 180 edges (i.e., 3% of the total number of significant correlations in both data sets). This indicates that on the transcriptome level, the investigated climacteric and non-climacteric fruits indeed exhibit large differences.

**Transcripts and metabolites correlations**

We next assessed the integrated metabolite and transcript profiles based on the global analysis of the covariance structure of the data sets as well as the extracted networks of tomato and pepper. For this purpose, we used the profiles for the already analyzed 26 metabolites and 318 transcripts. The \( R_v \) coefficient exhibited a (non-significant) value of 0.002, which hints that the already observed differences are present at this level of data integration, too. Analysis of the similarities between the principal components of the periods spanning four stages of fruit development and ripening further stressed this finding (Figure 1 C) which confirms that the investigated 5 and 7 sample points in tomato and pepper, respectively, are highly dissimilar revealed by Figure 1 A. The covariance structure of metabolites did, however, reveal internal similarity between the middle and later periods in the development and ripening of tomato and pepper fruits when each species is considered independently, but this is not the case for the beginning stages for tomato (e.g., see tomato 39 DAP – 43 DAP and tomato 27 DAP – 42 DAP).
To construct a network integrating the metabolite and transcript profiles, the threshold values of 0.952 and 0.843 were used for the data sets from tomato and pepper, respectively. These values ensure FDR of 0.05. The representation of the networks in Figure 8, due to their size, was prohibitive for any visual investigations. Therefore, we focused on the strength of the relationships between the transcripts of the following functional categories: starch synthesis and degradation, cell wall precursor synthesis and cellulose synthesis, as well as protein degradation, and the compound classes, involving: amino acids, organic acids, and sugars. The findings are given in Tables 1.

**DISCUSSION**

Extensive efforts have been undertaken for understanding fruit develop and ripening mechanism and in recent years especially at the levels of transcriptional control (Aharoni and O'Connell, 2002; Alba et al., 2005; Lemaire-Chamley et al., 2005; Moore et al., 2005; Grimplet et al., 2007; Vriezen et al., 2008; Wang et al., 2009; Bombarely et al., 2010; Lee et al., 2010) and downstream metabolomic consequences (Fraser et al., 2007; Fait et al., 2008; Fraser et al., 1994; Lombardo et al., 2011). Recently, a small number of studies have been undertaken using systems strategies for combining plant transcriptome and metabolomic data to develop associations in tomato (Carrari et al., 2006; Enfissi et al., 2010; Garcia et al., 2009; Mounet et al., 2009; Osorio et al., 2011) or grape (Carrari et al., 2006; Deluc et al., 2007; Enfissi et al., 2010; Zamboni et al., 2010; Osorio et al., 2011). Here comprehensive transcriptomic and GC-MS metabolomic analysis have been combined to provide novel insights into development and ripening of non-climacteric pepper fruit. These analyses were compared with similar transcript and metabolite analyses through development and ripening of tomato fruit (Carrari et al., 2006; Deluc et al., 2007; Enfissi et al., 2010; Zamboni et al., 2010; Osorio et al., 2011) which allowed identification of similar and distinct regulation at the gene and metabolite levels between non-climacteric (pepper) and climacteric (tomato) fruits.

**Sugars metabolism during pepper fruit ripening and development**
During earlier development (until 52 DAP) and ripening stages we observed considerable changes in sugar metabolism of pepper fruit. Photosynthetic light reactions exhibited up-regulation during early stages followed by down-regulation during ripening. Also during ripening, a dramatic down-regulation in genes involved in synthesis of starch was observed and coupled to up-regulation of sucrose degradation at the onset of ripening. This suggests an activated photosynthesis and carbon assimilation process during early fruit stages followed for reduction of these pathways coincident with ripening. Consistently, we noted gradual accumulation of the major soluble sugars (Glc, Fru, Suc) during early stages followed by their decrease following the onset of ripening. Comparing with climacteric tomato fruits we detected similar expression patterns of sugar-related genes during ripening. However in contrast, these genes were down-regulated during early fruit stages and consistent with stable Fru, Glc, and Suc levels during pre-ripening tomato fruit development (Osorio et al., 2011). Additionally, in tomato fruit, hexoses derived from degradation of Suc were described as key to starch synthesis during early development and at the onset of ripening (Carrari and Fernie, 2006; Centeno et al., 2011). In pepper fruit, consistent with up-regulation of sucrose degradation genes a decrease in Suc levels was observed. However in pepper ripening down-regulation of starch synthesis genes occurred only during ripening which may suggest that starch synthesis regulation is not conserved between climacteric and non-climacteric fruits.

**Ethylene regulation during pepper fruit ripening and development**

Fruits are generally classified into two physiological groups, “climacteric” or “non-climacteric” according their respiratory and associated ethylene biosyntheses profiles during ripening. It is known that climacteric ethylene biosynthesis involves conversion of Asp to Met, conversion of Met to ethylene, and the Met recycling pathway (Yang and Hoffman, 1984) through SAM and the two ethylene-biosynthetic enzymes, ACC synthase and ACC oxidase. SAM also serves as a precursor of polyamine synthesis (Mattoo et al., 2007; Mattoo and Handa, 2008). Immature pepper fruit at 26 DAP, were characterized by SAM synthase up-regulation, however changes in expression of other ethylene-biosynthesis genes associated with climacteric fruits (such as methionine sulfoxide reductase involved in the recycling of Met, ACC synthase and ACC oxidase) did not occur. In addition, during normal pepper ripening, Asp, the
precursor of Met, as well as Met increased between 3 and 8 fold, respectively. In contrast to tomato fruit and as previously describe by Lee et al., (2010), these results suggest that ethylene biosynthesis does not play a significant role in the accumulation of Asp and Met during pepper fruit ripening. On the other hand, SAM was up-regulated during early developmental stages in pepper fruit which may indicate flux through SAM to polyamines even though significant changes in levels of Asp and Met were not observed in these stages.

Ethylene perception represents a point of regulation of response downstream of hormone synthesis. Prior studies have shown that ethylene receptors act as negative regulated of ethylene signalling in climacteric fruits (Klee and Tieman, 2002; Klee, 2002; Osorio et al., 2011) and that following ethylene interaction with the receptors, ripening-related genes including those encoding cell wall-disassembling enzymes and phytoene synthase (PSY1) which catalyzes the formation of phytoene (the first C40 carotene intermediate in carotenoid biosynthesis) are activated. Interestingly, in parallel to the situation in tomato fruit, Nr transcript (SGN-U590044) was up-regulated during pepper fruit ripening. In addition, a number of ethylene/ripening-related genes shown to be up-regulated in climacteric fruits following ethylene perception including cell wall-disassembly genes (polygalacturonases, pectate lyases, and pectin methyl esterases) were up-regulated during normal pepper fruit ripening and indeed represent one of the most evident and well-studied ripening associated changes in tomato fruit (Brummell, 2006). The major carotenoids responsible of red fruit color are lycopene for tomato and capsanthin and capsorubin for pepper (Hornero-Mendez et al., 2000; Hornero-Mendez and Minguez-Mosquera, 2000; Hirschberg, 2001). As previously described, genes involved in carotenoids biosynthses as phytoene synthase 1 (PSY1) and phytoene dehydrogenase were up-regulated during pepper ripening fruit as well as tomato ripening fruit (Lee et al., 2010; Osorio et al., 2011). These results, the up-regulation of both cell wall-disassembly genes and carotenoids-biosynthesis related genes during normal pepper ripening process, non-climacteric fruit, which is also characteristic events during ripening in climacteric fruit, suggest that both fruits conserve ethylene signalling components but both differ in hormonal regulation.
Organic acids metabolism during pepper fruit ripening and development

Organic acids are crucial to many aspects of tomato fruit biology and maturation and they correlate strongly with genes associated with ethylene and cell wall metabolism related pathways (Carrari et al., 2006; Centeno et al., 2011; Osorio et al., 2011). In some non-climacteric fruits such as strawberry receptacle, only some TCA cycle intermediates such as succinate, fumarate and 2-oxoglutarate displayed substantial changes during ripening associated with a heavy demand for carbon skeleton components (Fait et al., 2008). Here we report that organic acids, included the two TCA cycle intermediates malate and citrate, were strongly affected across ripening, suggesting that organic acids are regulated at the transcriptional level as in climacteric fruits. Recently, malate metabolism was described as important for transitory starch metabolism in normal tomato fruit development (Centeno et al., 2011). Interestingly, the correlation networks in this study revealed positive correlation between malate levels and genes involved in synthesis of starch. This result raises the possibility that malate metabolism is generally important for transitory starch metabolism and its regulation is conserved between climacteric and non-climacteric fruits.

The levels of ascorbate (vitamin C) have been shown to be dramatically elevated during pepper ripening (Martinez et al., 2005). In this study, ascorbate was not detected; however we observed a high increase in dehydroascorbate (its oxidized form) and relatively minor changes in threonate (a catabolic product of ascorbate) throughout ripening suggest high redox activity and carbon recycling.

Comparison of amino acid profiles during development and ripening of pepper (Supplemental Figure 1) and tomato indicate that both fruits follow distinct metabolic regulation programs. For example, significant differences were detected in tomato where some amino acids increased while others decreased through development and ripening stages. Aromatic amino acids (Phe and Tyr) were observed to accumulate during pepper ripening but not in tomato. These amino acids serve as precursors for various branches of the phenylpropanoid pathway and the biosynthesis plant phenolics 2-phenylethanol and 2-phenylacetaldehyde (Tieman et al., 2006).
In general, correlation network analysis suggests that metabolism during pepper fruit development is highly coordinated though a number of notable associations between transcripts and metabolites occur. For example, organic acids such as citrate, dehydroascorbate and malate are highly correlated to genes associated with starch and cell wall pathways as well as protein degradation, suggesting the importance of these organic acids in pepper fruit development and ripening.

Conclusions

A comparative transcriptome and metabolome analysis during maturation processes of climacteric and non-climacteric fruits (tomato and pepper, respectively) reveal fewer changes in gene expression in pepper fruit as compared to tomato. When comparing our results with those of previous studies on gene expression (Lee et al., 2010) during pepper and tomato ripening, we suggest that both fruits have similar ethylene-mediated signalling components; however the regulation of these genes is clearly different and may reflect altered ethylene sensitivity or regulators other than ethylene in pepper. Genes involved in ethylene biosynthesis in climacteric fruits including ACC synthase and ACC oxidase are not induced in pepper fruits however, as in tomato fruits, genes downstream of ethylene perception such as cell wall metabolism genes, *ERF3* and carotenoids biosynthesis genes are clearly up-regulated during pepper fruit ripening. While signalling sensitivity or actual signals may different between climacteric and non-climacteric fruit, evidence described here suggests activation of a common set of ripening genes influence metabolic traits. We additionally, provide information how phytohormone biosynthesis and response genes, particularly those related to ethylene, auxins and jasmonate display expression patterns consistent with roles in fruit developmental process of non-climacteric pepper fruits, as well as defining the metabolic programs occurring during development. Taken together this data should greatly improve our understanding of a non-climacteric fruit ripening and development as well as providing a basis for hypothesis drive research which will be able to elucidate regulatory events underlying this important biological process.
MATERIALS AND METHODS

Plant material and sampling

Pepper (*Capsicum chilense* cv. Habanero) was grown in the greenhouse at 26°C under 12 h supplemental lighting, followed by 12 h at 20°C. To collect pepper stages prior to ripening, fruits were tagged at 14 days post anthesis (DAP) and harvested at one of the following ten time points: 14, 20, 26, 34, 51 (Breaker-1; Br-1), 52 (Breaker; Br), 53 (Breaker+1; Br+1), 55 (Breaker+2; Br+2), 57 (Breaker+5; Br+5), 62 DPA (Breaker+10; Br+10) and 68 DAP (Breaker+10; Br+16) (Figure 9). The first signs of carotenoid accumulation on the external surface of the fruit were defined the breaker stage. Transcriptome and metabolomic analyses were performed on the same material; 20-40 fruit at each developmental stage were collected and pooled. A minimum of three biological replicates in each time point were considered. All fruits were collected from four individual plants which were grown in the greenhouse and randomly distributed. After tissue selection, pericarp tissue was collected, then frozen in liquid nitrogen, and stored at -80°C until further analysis. For tomato, the analysed time points were: 7, 17, 27, 39 (Mature green; MG), 41 (Breaker-1; Br-1), 42 (Breaker; Br), 43 (Breaker+1; Br+1), 47 (Breaker+5; Br+5), 52 (Breaker+10; Br+10), and 57 DPA (Breaker+15; Br+15) DAP (Figure 9). For tomato transcriptome analysis 8 to 10 individual fruits for each time point as biological replicates were used. These individual fruit replicates came from 10-15 plants. Tomato data is used from Osorio et al., (2011).

Transcriptome analysis

The tomato array (TOM1) was used for tomato and pepper transcriptome analysis. TOM1 contains approximately 8,000 unique elements randomly selected from cDNA libraries isolated from a range of tissues, including leaf, root, fruit, and flowers, and representing a broad range of metabolic and developmental processes. Nucleic acid sequence and annotation data pertaining to the TOM1 microarray are available via the Tomato Functional Genomics Database (http://ted.bti.cornell.edu). Microarray analysis were performed as described in (Alba et al., 2004; Alba et al., 2005). The raw intensity values were normalized using Robin’s default settings for two-colour microarray analysis (Lohse et al., 2010). The obtained P-values were corrected for multiple testing using the strategy described by (Benjamini and Hochberg, 1995)
separately for each of the comparisons made. Genes that showed an absolute log2 fold-change value of at least 1 and a P-value lower than 0.05 were considered significantly differentially expressed. The log2 fold-change cutoff value was imposed to account for noise in the experiment and make sure that only genes that show a marked reaction are recorded. MapMan and PageMan software packages (Usadel et al., 2006; Usadel et al., 2009) were used for visualization. The transcript data for tomato has previously been published (Osorio et al., 2011).

**Metabolome Analysis**

Metabolite extraction derivatization, standard addition and sample injection for GC-MS were performed according Osorio et al., (2012). The mass spectra were cross-referenced with those in the Golm Metabolome Database (Kopka et al., 2005).

**RNA extraction and quantification for qRT-PCR**

The RNA and qRT-PCR were determined and analyzed as described by Zanor et al. (2009). Expression of expansin 1 (EXP1), expansin precursor (EXP3), 1-aminocyclopropane-1-carboxylate oxidase (ACO1), 1-aminocyclopropane-1-carboxylate synthase (ACS4), xyloglucan endotransglucosylase/hydrolase (XTH5), polygalacturonase (PG), phytoene synthase (PSY1), phytoene desaturase (PDS), carotene desaturase (ZDS), ethylene response factor 3 (ERF3) and never ripe receptor (Nr) was analyzed by real-time qRT-PCR using the fluorescent intercalating dye SYBR Green in an iCycler detection system (Bio-Rad). Relative quantification of the target expression level was performed using the comparative Ct method. The following primers were used: for analysis of EXP1 (Centeno et al., 2011), transcript levels forward 5´-TACCAATTTCTGCCCACCAAAT-3´ and reverse 5´-GGTTACACCAGCCACCATTGT-3´; for EXP3 (Centeno et al., 2011), transcript levels forward 5´-TTTGCGGAGCTTGCTTTGAA-3´ and reverse 5´-GGAGCACAAAAATTCGTTGCAG-3´; for XTH5 (Centeno et al., 2011), forward 5´-GGATTCAGCCATCTCTTTGGTG-3´ and reverse 5´-GAACCCTGAACCTGTGTTTTGG-3´; for ACO1 (Centeno et al., 2011) forward 5´-CAGAAGGTTAAGGCGCTTGTG-3´; for AC1 (Centeno et al., 2011) forward 5´-
AAATCATGAAGGAGTTTGCTGATAAA-3’ and reverse, 5’-TTTTCACACACA-GCAAATCCAACAG-3’; for ACS4 (Centeno et al., 2011) forward 5’-
CCATCTTTTGTGCACGAAATA-3’ and reverse 5’-
CGATGCTAACGAATTGAGAA-3’; for PSY1 (Lee et al., 2012) forward 5’-
TGGCCCAACGCATCATATA.3’ and reverse 5’- CACCATCGAGCATGTCAATG-
3’; for PDS (Lee et al., 2012) forward 5’- GTGCATTTTGATCATCGCATTGAAC-
3’ and reverse 5’- GCAAAAGTCTCTCCAGATTACC-3’; for ZDS (Lee et al., 2012)
forward 5’- TTGGAGCGTCTGGAGGCAAT-3’ and reverse 5’-
AGAAATCTGCATCTGGTGATAGA-3’; for ERF3 forward 5’-
AGAAGGCTGGAAAAACCATAAG-3’ and reverse 5’- GGTGGAAGGAAAACCATTGGA-
3’. To normalize genes, expression was used the constitutively expressed ubiquitin3
(GenBank accession number X58253) using the following primers: forward, 5’-
ACCACGAAGCTCTCCAGGAG-3’, and reverse, 5’-
CATTGAACCCACCATTGTCACC-3’, (Zanor et al., 2009).

**Rv coefficient and similarity of covariance matrices**

The Rv coefficient was introduced by Escoufier (1973) and is a theoretical tool to
analyze multivariate data. It is a similarity coefficient between positive semi-definite
matrices (e.g., covariance matrices) obtained from the same set of variables. Given
two covariance matrices A and B, the Rv coefficient is defined as:

$$ R_v = \frac{\text{tr}(A^TB)}{(\text{tr}(A^TA)\text{tr}(B^TB))^{1/2}}, $$

Where $^T$ denotes the matrix transpose and tr is the trace of the matrix.

The similarity between two matrices A and B can be analyzed by investigating the
first k principal components explaining maximum of the variance. Suppose that the
first k principal components of A and B are given by $(a_1, ..., a_k)$ and $(b_1, ..., b_k)$,
respectively. Then, a similarity measure between the two matrices A and B can be
formulated by:

$$ \text{sim}(A, B) = 1 - 1/k\Sigma\Sigma <a_i, b_j>^2, $$

where $<a_i, b_j>$ denotes the dot-product of the principal components $a_i$ and $b_j$ Similar
measure has been already considered in Krzanowski (1979).

**Network-based analysis**
Networks were created based on the Pearson correlation between the considered molecular profiles. To control for the number of false positive, a false discovery rate (FDR) of 0.05 was required. To this end, the smallest threshold rendering the required FDR was determined according to a commonly used procedure (Noble, 2009). Only correlations which in absolute value were not smaller than the threshold value were retained in the network.

**Data analysis**
Data normalization and heatmap representation were obtained by using built-in R functions (Gentleman, 1996). The network-based analysis was implemented with the help of functions in the igraph package as well as authors’ implementation in R. The rest of the analyses, including the Rv coefficient and similarity of covariance matrices were implemented by the authors also in R.

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

**Supplemental Table 1.** Transcriptomic data set.

**Supplemental Data online.** MapMan [http://mapman.gabipd.org/web/osorio](http://mapman.gabipd.org/web/osorio)

**Supplemental Figure 1.** Primary metabolite levels during pepper and tomato development and ripening.

**Supplemental Figure 2.** Color coding for the nodes representing transcripts for Figure 7.

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Figure legends

Figure 1. Comparative analysis of the covariance structure of the metabolites and transcript profiles in tomato and pepper fruits.

Similarity analysis of the covariance structure of (A) metabolite, (B) transcript, and (C) integrated (metabolite and transcript) profiles over 8 and 10 development and
ripening stages from tomato and pepper, respectively. A similarity score was calculated for every pair of periods, each spanning four stages in development and ripening, based on the corresponding principal components (see Materials and Methods). The similarity score ranges from 0 to 1, 0 indicating equality of the compared covariance structures. The results are displayed in a form of a heatmap (colour key is included on the bottom).

Figure 2. Primary metabolite levels during pepper development and ripening.
Time-points presented are 14, 20, 34, 51, 52, 53, 55, 57, 62, 68 DAP. Data are normalized to the mean response calculated to the 14 DAP stage (value = 1). Values presented are mean ± standard error of three replicates. (*) denote differences that were determined to be significant by Students t-test analysis (P<0.05) compared to the 14 DAP stage.

Figure 3. Networks from the primary metabolite data set of tomato and pepper fruits.
Networks were obtained by determining the significant correlations of the metabolite profiles from (A) tomato and (B) pepper, guaranteeing a false discovery rate of 0.05. Positive correlations are indicated with red edges, while negative are displayed with blue edges. The gray edges denote the relation between the communities (clusters) of metabolites in the network. The colour coding of the nodes, representing the metabolites, denote the compound classes: amino acids (red), organic acids (light blue), sugars and sugar alcohols (dark blue), and others (green).

Figure 4. Functional distribution of expressed genes.
Functional distribution of all genes showing significant (P<0.05) expression values in base of MapMan classification (Usadel et al., 2005) in pepper developmental and ripening stages (stages: 14, 20, 34, 51, 52 (breaker stage), 53, 55, 57, 62 and 68 DAP).

Figure 5. Expression analysis in pepper development and ripening.
A condensed PageMap display of altered pathways. Gene expression data is presented as log2 fold changes in comparison to the first harvested time-point (14 DAP). The analysed time-points were 20, 34, 51, 52, 53, 55, 57, 62, 68 DAP. The data was subjected to a Wilcoxon test in PageMan and the results are displayed in false-colour code. BINs coloured in red are significantly up-regulated, whereas BINs coloured in blue are significantly down-regulated.

**Figure 6.** Quantitative PCR of cell wall, carotenoids and ethylene related genes of tomato and pepper fruits across developmental and ripening stages. Cell wall related genes; expansin1 (*EXP1*), expansin precursor (*EXP3*), xyloglucan endotransglucosylase/hydrolase (*XTH5*) and polygalacturonase (*PG*). Carotenoids related genes; phytoene synthase (*PSY1*), Phytoene desaturase (*PDS*) and carotene desaturase (*ZDS*). Ethylene related genes; 1-aminocyclopropane-1-carboxylate oxidase (*ACO1*), 1-aminocyclopropane-1-carboxylate synthase (*ACS4*), Never ripe receptor (*Nr*). The values represent the means ± SE of four individual plants. An asterisk indicates values determined by *t* test to be significantly from the first analyzed stage (P<0.05).

**Figure 7.** Networks from selected transcripts of tomato and pepper fruits. Transcripts involved in cell wall metabolism, hormone metabolism, redox regulation, major carbohydrate metabolism, protein synthesis, protein targeting, protein posttranslational modification, protein degradation, protein folding and protein assembly and cofactor ligation were used in the analysis. Color coding for the nodes can be found in Supplemental Figure 2. Transcripts were grouped by functionality on the basis of MapMan gene ontology. Networks were obtained by determining the significant correlations of the transcript profiles from (A) tomato and (B) pepper, guaranteeing a false discovery rate of 0.05. Positive correlations are indicated with red edges, while negative are displayed with blue edges. The gray edges denote the relation between the communities (clusters) of metabolites in the network. The color code for the nodes, representing the selected transcripts, corresponds to the MapMan bins and indicates the different function categories.

**Figure 8.** Network-based representation of the integrated metabolomics and transcriptomic data set of tomato and pepper.
Analogous procedure as that used to obtain Figures 3 and 7 was employed on the combined data sets. Nodes denoting metabolites are drawn in squares, while those representing transcripts are given in circles for (A) tomato and (B) pepper. The color coding of nodes represented by circles corresponds to the MapMan bins. The color coding of nodes represented by squares follows that used in Figure 3 for the network of metabolites and nodes represented by circles follows that used in Figure 7 for the network of transcripts. Red edges denote positive, while blue edges represent negative correlations. The gray edges denote the relation between the communities (clusters) of metabolites in the network.

Figure 9. Experimental design.
To collect pepper (*Capsicum chilense* cv. Habanero) prior to ripening, fruits were tagged at 14 days post anthesis (DAP) and harvested at one of the following ten time points: 14, 20, 26, 34, 51 (Breaker-1; Br-1), 52 (Breaker; Br), 53 (Breaker+1; Br+1), 55 (Breaker+2; Br+2), 57 (Breaker+5; Br+5), 62 DPA (Breaker+10; Br+10) and 68 DAP (Breaker+10; Br+16). For tomato (*Solanum lycopersicum* cv. Ailsa Craig), the analysed time points were: 7, 17, 27, 39 (Mature green; MG), 41 (Breaker-1; Br-1), 42 (Breaker; Br), 43 (Breaker+1; Br+1), 47 (Breaker+5; Br+5), 52 (Breaker+10; Br+10), and 57 DPA (Breaker+15; Br+15) DAP

Table 1: Average correlations between gene function categories and compound classes in the integrated networks for pepper and tomato. NA indicates that no significant correlations were observed for the particular pair of gene function category and compound class. The included values indicate that sugars and sugar alcohols are in general more negative correlated to transcripts involved in starch synthesis in pepper compared to tomato, thus resulting in a smaller value for the average correlation compared to the used threshold of 0.843. Analogous reasoning implies that organic acids, on the other hand, are more positively correlated to transcripts involved in protein degradation in pepper compared to tomato.

| Pepper | Starch synthesis | Starch degradation | Cell wall precursor synthesis | Cell wall cellulose synthesis | Protein synthesis | Protein degradation |
|--------|------------------|--------------------|-------------------------------|-------------------------------|-------------------|---------------------|
| Amino acids | -0.735 | -0.739 | NA | NA | -0.570 | 0.864 |
| Organic acids | -0.765 | -0.852 | NA | NA | -0.640 | 0.355 |
| Sugars & sugar alcohols | 0.317 | NA | NA | NA | 0.293 | 0.784 |
|-------------------------|-------|----|----|----|-------|-------|
| **Tomato**              |       |    |    |    |       |       |
| Amino acids             | NA    | NA | -0.969 | NA | NA | 0.969 |
| Organic acids           | NA    | NA | NA | NA | NA | 0.001 |
| Sugars & sugar alcohols | 0.968 | NA | NA | NA | NA | NA |
**Figure 1. Comparative analysis of the covariance structure of the metabolites and transcript and profiles in tomato and pepper fruits.**

Similarity analysis of the covariance structure of (A) metabolite, (B) transcript, and (C) integrated (metabolite and transcript) profiles over 8 and 10 development and ripening stages from tomato and pepper, respectively. A similarity score was calculated for every pair of periods, each spanning four stages in development and ripening, based on the corresponding principal components (see Materials and Methods). The similarity score ranges from 0 to 1, 0 indicating equality of the compared covariance structures. The results are displayed in a form of a heatmap (colour key is included on the bottom).
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