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Transcriptional regulation of tocopherol biosynthesis in tomato

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Abstract Tocopherols, compounds with vitamin E (VTE) activity, are potent lipid-soluble antioxidants synthesized only by photosynthetic organisms. Their biosynthesis requires the condensation of phytyl-diphosphate and homogentisate, derived from the methylerythritol phosphate (MEP) and shikimate pathways (SK), respectively. These metabolic pathways are central in plant chloroplast metabolism and are involved in the biosynthesis of important molecules such as chlorophyll, carotenoids, aromatic amino-acids and prenylquinones. In the last decade, few studies have provided insights into the regulation of VTE biosynthesis and its accumulation. However, the pathway regulatory mechanism/s at mRNA level remains unclear. We have recently identified a collection of tomato genes involved in tocopherol biosynthesis. In this work, by a dedicated qPCR array platform, the transcript levels of 47 genes, including paralogs, were determined in leaves and across fruit development. Expression data were analyzed for correlation with tocopherol profiles by coregulation network and neural clustering approaches. The results showed that tocopherol biosynthesis is controlled both temporally and spatially however total tocopherol content remains constant. These analyses exposed 18 key genes from MEP, SK, phytol recycling and VTE-core pathways highly associated with VTE content in leaves and fruits. Moreover, genomic analyses of promoter regions suggested that the expression of the tocopherol-core pathway genes is trancriptionally coregulated with specific genes of

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the upstream pathways. Whilst the transcriptional profiles of the precursor pathway genes would suggest an increase in VTE content across fruit development, the data indicate that in the M82 cultivar phytyl diphosphate supply limits tocopherol biosynthesis in later fruit stages. This is in part due to the decreasing transcript levels of geranylgeranyl reductase (*GGDR*) which restricts the isoprenoid precursor availability. As a proof of concept, by analyzing a collection of Andean landrace tomato genotypes, the role of the pinpointed genes in determining fruit tocopherol content was confirmed. The results uncovered a finely tuned regulation able to shift the precursor pathways controlling substrate influx for VTE biosynthesis and overcoming endogenous competition for intermediates. The whole set of data allowed to propose that 1-deoxy-D-xylulose-5-phosphate synthase and *GGDR* encoding genes, which determine phytyl-diphosphate availability, together with enzyme encoding genes involved in chlorophyll-derived phytol metabolism appear as the most plausible targets to be engineered aiming to improve tomato fruit nutritional value.

Keywords Tomato · Metabolism · Tocopherol · Vitamin E · Transcriptional regulation

Introduction

Plants, like all aerobic organisms, have evolved a complex antioxidant system to regulate the intracellular redox status while avoiding the deleterious oxidation of cellular components caused by reactive oxygen species (ROS) (Apel and Hirt 2004). Tocopherols and tocotrienols, collectively referred to as vitamin E (VTE), are non-enzymatic lipid-soluble antioxidants synthesized only by photosynthetic organisms. Beside other roles, VTE compounds inhibit lipid peroxidation and protect photosystem II from oxidative damage by scavenging lipid peroxyl radicals and singlet oxygen. (Munné-Bosch and Alegre 2002; Falk and Munné-Bosch 2010; Takahashi and Badger 2011; Loyola et al. 2012). VTE compounds are tocochromanols; thus, they contain a polar chromanol group originated from homogentisate which derives from shikimate (SK) pathway and an isoprenoid-derived chain which results from the plastidial methylerythritol phosphate (MEP) pathway. The first step of the tocopherol-core pathway, catalyzed by 4-hydroxyphenylpyruvate dioxygenase (HPPD), involves reduction of 4-hydroxyphenylpyruvate to homogentisate, which further is decarboxylated and then condensated with a prenyl donor by a specific prenyltransferase; homogentisate phytyl transferase (VTE2) (reviewed by Munné-Bosch and Alegre 2002). It has been demonstrated that the prenyl donor for tocopherol biosynthesis, phytyl diphosphate, can also be originated from phytol recycling after chlorophyll degradation by a phytol kinase (VTE5) (Valentin et al. 2006). There are four natural forms of tocopherol (α, β, γ and δ) which differ in the position and number of methyl groups on the chromanol ring and are products of the reactions catalyzed by 2-methyl-6-phytyl-1,4-benzoquinol methyltransferase (VTE3), tocopherol cyclase (VTE1) and γ-tocopherol methyl transferase (VTE4) (Munné-Bosch and Alegre 2002). Despite all tocochromanols are potent antioxidants in vitro, α-tocopherol presents the highest VTE activity in humans and animals (Traber and Sies 1996).

Although a considerable amount of information has been accumulated, the knowledge concerning the molecular mechanisms regulating tocopherol biosynthesis remains fragmentary. Enzyme encoding genes involved in the MEP pathway have been found to be transcriptionally activated during plant development in Arabidopsis, suggesting the presence of common transcription factor binding motifs in the promoter regions of these genes (Guevara-García et al. 2005). In this sense, it has been demonstrated that MEP pathway genes are coordinately upregulated by light in Arabidopsis seedlings (Córdoba et al. 2009). On the other hand, regulation of carbon flux through the SK pathway is much less well understood. Recently, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS) has showed to be a rate-limiting factor in this pathway (Tzin et al. 2012). Besides, some transcription factors have been revealed to influence MEP and SK pathways but the mechanism by which this is achieved remains unclear. In tomato, the overexpression of the MYB transcription factor *ODORANT1* led to a significant increase in phenylpropanoid content concomitantly with transcriptional upregulation of several SK pathway genes (Dal Cin et al. 2011). Furthermore, downregulation of *DET1,* which is involved in the suppression of light responses in the absence of light in tomato fruit, resulted in increased contents of carotenoids, flavonoids and tocopherol (Enfissi et al. 2010). Regarding the last steps in tocopherol biosynthesis, several reports have described the effect of gene overexpression on tocopherol profiles, highlighting the role of *HPPD* and *VTE2* in determining total tocopherol content, and *VTE1*, *VTE3* and *VTE4* in shaping tocopherol composition (reviewed by Dellapenna and Mène-Saffrané 2011). However, systematic studies about the spatio-temporal regulatory network of gene expression and VTE contents have not been boarded. Nor a comparative analysis of the *cis*-regulatory motifs in the promoter regions of the MEP, SK and tocopherol-core pathway genes have been studied to date.

Cultivated tomato (*Solanum lycopersicum*) is one of the most globally consumed vegetables and has been intensively used as model system of plants bearing fleshy fruits (Yelle et al. 1991; Rose et al. 2004; Alba et al. 2005; Carrari et al. 2006; Klee 2010). Measurements of tocopherol in ripe tomatoes (Moco et al. 2007; Meléndez-Martínez et al. 2010) suggest that this vegetable is an important source of VTE. Likewise, genes involved in the MEP, SK

and tocopherol-core pathways have been recently identified and mapped onto the tomato genome. In addition, quantitative trait loci (QTL) for tocopherol content have been identified and some of them co-localize with tocochromanol biosynthetic genes, emerging as strong candidates for exploring the allelic natural variation present in this plant species (Almeida et al. 2011). This will not only represent an important resource for biotechnological applications but also likely prove instrumental in improving our understanding of the intricate regulation circuit that operates in these pathways (Fitzpatrick et al. 2012).

In this work, we developed a dedicated qPCR array for a global analysis of the transcript profiles of all tocopherol biosynthetic genes in photosynthetic tissues and across tomato fruit development. Results were analyzed in parallel with tocopherol and pigment profiles. Network analyses provided insights into the relationship between tocopherol content and spatio-temporal mRNA accumulation of the biosynthetic pathway genes, exposing key regulatory points that were confirmed in Andean landrace genotypes. Furthermore, bioinformatics tools allowed the identification of *cis*-regulatory motifs in the promoter regions of coregulated genes which could be acting as binding sites for shared transcription factors. In sum, the results allowed to suggest possible targets for metabolic engineering of this important nutraceutical trait in tomato.

Results

Vitamin E and pigment contents in tomato

To study the spatial and temporal variation of tocopherol levels in tomato (cv M82), the quantitative profile of α, β, γ and δ forms was determined in source and sink leaves, and in pericarp along fruit development (in green, mature green, breaker and ripe stages). Additionally, the levels of total carotene, lycopene and chlorophylls were determined at the same stages of fruit development (Table 1). Since only traces of tocotrienols were detected in these samples, as in agreement with previous reports (Horvath et al. 2006a), these compounds were not considered here.

Total tocopherol was approximately twofold more abundant in leaves than in fruits and; all the samples analyzed showed detectable amounts of each tocopherol form, although at variable levels. The main VTE-active molecule, α-tocopherol, was the most abundant species both in leaves and fruits, contributing up to 97 % of the total VTE in the mature green fruits but essentially being invariant across tissues. While the profile of the least abundant tocopherol forms kept invariant between source and sink leaves, it varied along fruit development (Table 1).

As expected, whereas total carotene and lycopene displayed a massive increase at the fruit ripe stage, 6- and 29-fold higher than that observed at the mature green stage, respectively; the chlorophyll contents decreased during fruit development down to undetectable levels, reflecting the transition from chloroplasts to chromoplasts and the consequent metabolic changes during the ripening of tomato fruit (Table 1).

Expression profile of the genes encoding vitamin E biosynthesis enzymes

Almeida et al. (2011) identified, characterized and mapped the enzyme encoding genes involved in VTE biosynthetic pathways of tomato. On the basis of this information, a qPCR array was developed to address the variation of

Table 1 Tocopherol, lycopene, total carotene and chlorophylls content in tomato

	Leaves		Fruits			
	Source	Sink	Green	Mature green	Breaker	Ripe
α-tocopherol	17.92 ± 2.99[a]	15.45 ± 2.83[(a,b)]	9.11 ± 1.55[(b)]	7.43 ± 1.01[(b)]	10.54 ± 0.44[(b)]	9.74 ± 0.65[(b)]
β-tocopherol	0.60 ± 0.12[(a)]	0.63 ± 0.15[(a)]	1.13 ± 0.44[(a)]	0.13 ± 0.05[(b)]	0.14 ± 0.00[(b)]	0.15 ± 0.01[(b)]
δ-tocopherol	2.62 ± 0.77[(a)]	4.43 ± 1.04[(a)]	0.26 ± 0.04[(a)]	0.02 ± 0.00[(b)]	0.02 ± 0.01[(b)]	0.11 ± 0.03[(a)]
γ-tocopherol	0.79 ± 0.17[(a)]	1.26 ± 0.21[(a)]	0.17 ± 0.05[(b)]	0.09 ± 0.03[(b)]	0.72 ± 0.10[(a)]	1.64 ± 0.49[(a)]
Total tocopherol	21.93 ± 2.49[(a)]	21.77 ± 10.10[(a)]	10.67 ± 1.46[(b)]	7.67 ± 1.07[(b)]	11.42 ± 0.55[(b)]	11.64 ± 0.28[(b)]
Lycopene	–	–	3.02 ± 1.12[(a)]	1.86 ± 0.56[(a)]	4.60 ± 1.28[(a)]	54.02 ± 11.32[(b)]
Total carotene	–	–	6.76 ± 0.79[(a)]	2.43 ± 0.35[(a,b)]	3.41 ± 0.16[(b)]	15.26 ± 2.29[(c)]
Chlorophyll a	532.98 ± 29.99[(a)]	426.18 ± 22.80[(b)]	12.16 ± 0.95[(c)]	9.08 ± 1.22[(c)]	3.57 ± 1.39[(d)]	nd
Chlorophyll b	142.89 ± 21.99[(a)]	110.77 ± 11.73[(a)]	3.76 ± 0.38[(b)]	4.05 ± 0.88[(b)]	3.76 ± 2.29[(b)]	nd

Tomato plant samples (cv. M82) were grown in greenhouse under controlled conditions as described in "Materials and methods". Values are expressed in μg gFW^{-1} and are means ± SE from 4 to 6 replicates. Different letters indicate statistically significant differences between tissues detected by the Tukey tests ($p < 0.05$)

nd non detected, – non measured

mRNA levels of these genes along with the fluctuation in tocopherol and pigment contents. For this purpose, specific primer pairs were designed for 16 MEP, 16 SK, 9 tocopherol-core, and 6 tocopherol-related pathway genes. Figure 1 displays a heat map presenting a global view of the normalized expression patterns for all VTE biosynthetic pathway genes in source and sink leaves as well as in green, mature green, breaker and ripe fruits from the *S. lycopersicum* cv M82. It can be observed that, with the exception of *DXS(2)* and *TYRA(2)* at the breaker and mature green stages, respectively, the complete set of genes were expressed in all the samples analyzed (Table S1). Interestingly, paralogous genes showed differences in expression profiles, suggesting a spatio-temporal specificity. In this sense, for five pairs of paralogs (*IPI*, *GGPS*, *CS*, *CM* and *TAT*) the correlation analysis displayed opposite patterns of expression (Fig. S1).

It is worth mentioning that both precursor pathways of VTE synthesis, MEP and SK, also provide intermediates for a plethora of plastid-synthesized compounds whose levels are tightly controlled during plant development (Vranová et al. 2012). Thus, it is expected that the contents of the end-products depend, at least in part, on the control of mRNA levels of the enzyme encoding genes that supply the needed precursor metabolites. This is suggested by the principal component analysis (PCA) of the gene expression data, where the first two dimensions explained 67.8 % of the data variance for all the samples analyzed and separated both organs and developmental stages (Fig. 2).

In summary, the results presented here clearly reveal that there are several steps along the carbon flux through tocopherol biosynthesis that are both temporally and spatially regulated at the mRNA levels of the enzyme encoding gene.

Coordinated changes in gene transcript levels and tocopherol contents in tomato leaves and fruits

To better understand the impact of the variation in gene mRNA levels on VTE content, two different approaches were taken. First, a weighted coregulation network analysis including mRNA and metabolites quantification data from leaves and fruits was performed (Zhang and Horvath 2005). This approach assigns a connection weight to each gene/metabolite pair showing a coordinated expression and allowed the identification of one well defined cluster consisting of genes highly associated to pigments and VTE content (Fig. 3). This cluster showed a median number of neighbors of 6 (Table S2) and, as expected, chlorophyll a and b were centrally positioned in the network. Eleven genes (out of those 18 weighted by the analysis) showed at least 6 links. Interestingly, *VTE3(2)* gene resulted in the most interconnected node with 20 directed edges. Furthermore, it was highly linked to tocopherol, but neither with lycopene nor with total carotene thus, exposing this gene as the major hub in the regulatory circuit of VTE contents in tomato tissues. *VTE4* and *HPPD(1)* of the tocopherol core-pathway; *DXR, GGDR* and *GGPS(1)* of the MEP; *CM(2), TAT(2)* and *PAT* of the SK pathway as well as *CLH* and *FPGS* also showed a number of links above the median. Hence, these results would suggest that these genes are key players of the VTE content modulation when considering both photosynthetic and fruit tissues.

The second approach to link gene expression patterns with tocopherol variations entailed the application of a neural clustering model through a self-organizing map (*omeSOM; Milone et al. 2010) that grouped genes and metabolites according to their variations exclusively across fruit development and ripening. Three main patterns were identified (Fig. S2). The first group included 13 genes, whose expression varied from early developmental stages until breaker stage and kept constant afterwards. In contrast, the second group clustered 19 genes, γ-tocopherol and chlorophylls, showing fairly constant expression patterns until mature green or breaker stages, increasing or decreasing drastically later on. Finally, the third cluster included 13 genes peaking at breaker and decreased towards ripe stage. Although no pathway-specific pattern was detected, some interesting observations could be made when the expression profiles of the individual pathways were analyzed across fruit development (Fig. S2 and Table S1). Most of the MEP genes were upregulated from green fruits to their breaker stage. The exceptions were *CMS* and *GGDR*. For the latter, the reduction in transcript levels from green fruit onwards coincided temporally with the decrease in chlorophyll biosynthesis at later stages of fruit development. By contrast, the transcript profiles of SK genes were more variable. Remarkably, the genes encoding enzymes downstream of shikimate-3P were upregulated at least until breaker stage, with the only exception of the *PAT* gene, which remained constant. Despite no significant alterations were detected in total tocopherol content throughout fruit development, the core pathway genes showed altered mRNA levels. While *VTE*1, *VTE*3*(2)*, *VTE*4 and *VTE*5 were downregulated, *VTE*2 was upregulated. Additionally, *HPPD* and *VTE*3*(1)* reached the highest mRNA accumulation at breaker stage. Finally, it is worth pinpointing the decrease of *GGDR* and *VTE5* expression across fruit ripening, both of which directly limits phytol diphosphate input supply towards VTE biosynthesis.

Identification of common *cis*-regulatory elements in VTE biosynthetic pathway gene promoters

Having demonstrated that VTE biosynthesis genes are modulated at transcript level in tomato in a spatial and

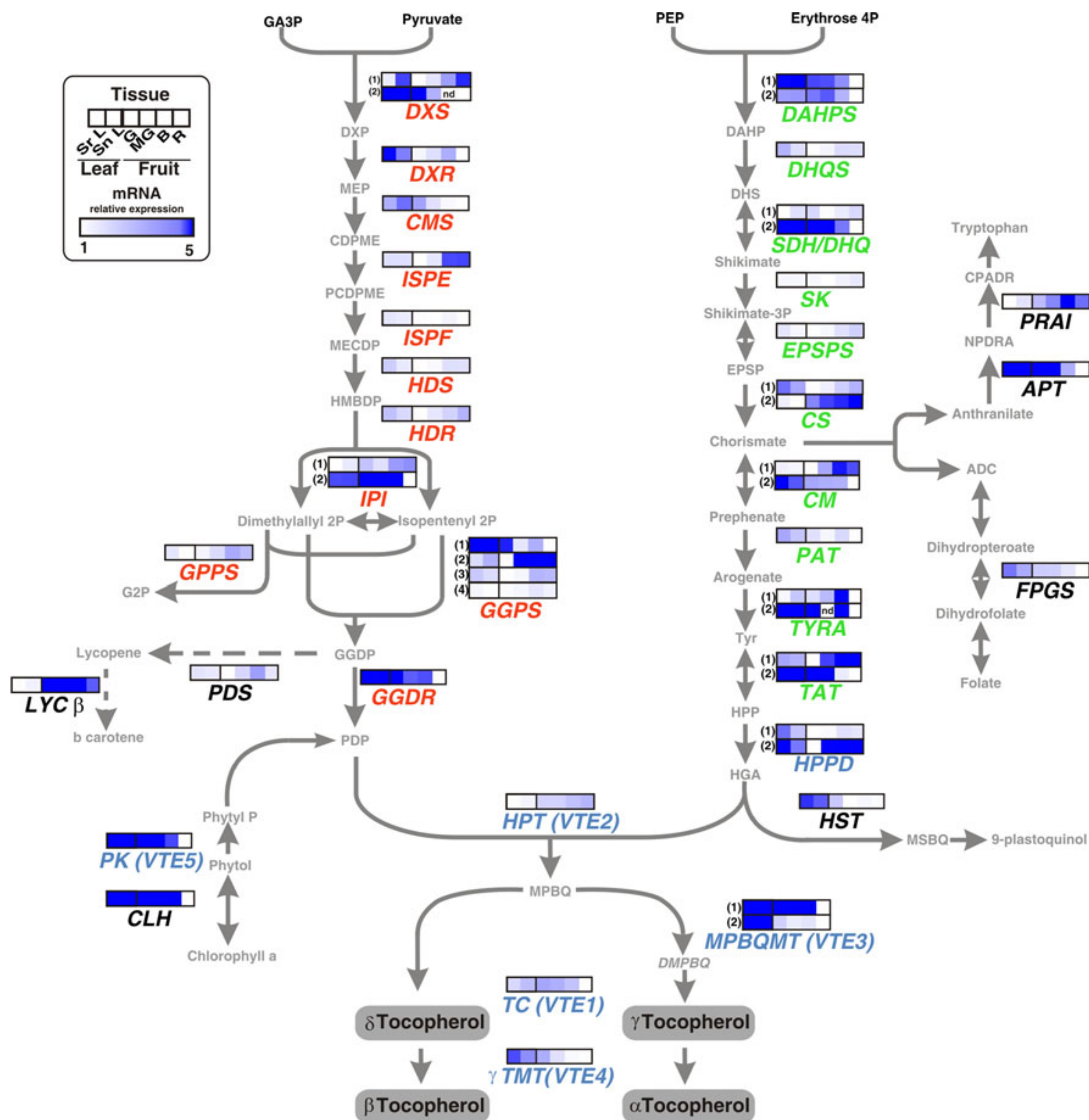

Fig. 1 Expression profile of VTE biosynthetic pathway genes in tomato. Schematic view of the methyl erythritol (MEP), shikimate (SK), vitamin E-core and -related pathways with the corresponding enzymes indicated in *red*, *green*, *blue* and *black*, respectively. Intermediate compounds are indicated in *gray*. Gene expression profiles of each enzyme-encoding gene were measured by qPCR in samples from source (SrL) and sink (SnL) leaves, and green (G), mature green (MG), breaker (B) and ripe (R) fruits (n = 3). Relative gene expressions are indicated above the name of each enzyme by a *color scale* representing the median expression normalized to the sample showing the lowest relative expression. Enzyme encoding genes are named according to the following abbreviations: 1-deoxy-D-xylulose-5-P synthase (*DXS*); 2-C-methyl-D-erythritol 4-phosphate synthase (*DXR*); 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (*CMS*); 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase (*ISPE*); 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (*ISPF*); 4-hydroxy-3-methylbut-2-enyl- diphosphatesynthase (*HDS*); 4-hydroxy-3-methylbut-2-enyl- diphosphate reductase (*HDR*); isopentenyl diphosphate d–isomerase (*IPI*); geranyl pyrophosphate synthase (*GPPS*); geranylgeranyl pyrophosphate synthase (*GGPS*); geranylgeranyl reductase (*GGDR*); 3-deoxy-D-arabino-heptulosonate-7-P synthase (*DAHPS*); 3-dehydroquinate synthase (*DHQS*); shikimate dehydrogenase/3-dehydroquinate dehydratase (*SDH/DHQ*); shikimate kinase (*SK*); 5-enolpyruvylshikimate-3-P synthase (*EPSPS*); chorismate synthase (*CS*); chorismate mutase (*CM*); prephenate aminotransferase (*PAT*); arogenate dehydrogenase (*TYRA*); tyrosine aminotransferase (*TAT*) 4-hydroxyphenylpyruvate dioxygenase (*HPPD*); homogentisate solanesyl transferase (*HST*); homogentisate phytyl transferase (*VTE2*); 2,3-dimethyl-5-phytylquinol methyltransferase (*VTE3*); tocopherol cyclase (*VTE1*); c -tocopherol C-methyl transferase (*VTE4*); phytol kinase (*VTE5*); anthranilate phosphoribosyltransferase (*APT*); phosphoribosylanthranilate isomerase (*PRAI*); folylpolyglutamate synthase (*FPGS*); chlorophyllase (*CLH*); lycopeno β-cyclase (*LYCβ*); phytoene desaturase (*PDS*). Paralogous genes are indicated by *different numbers*

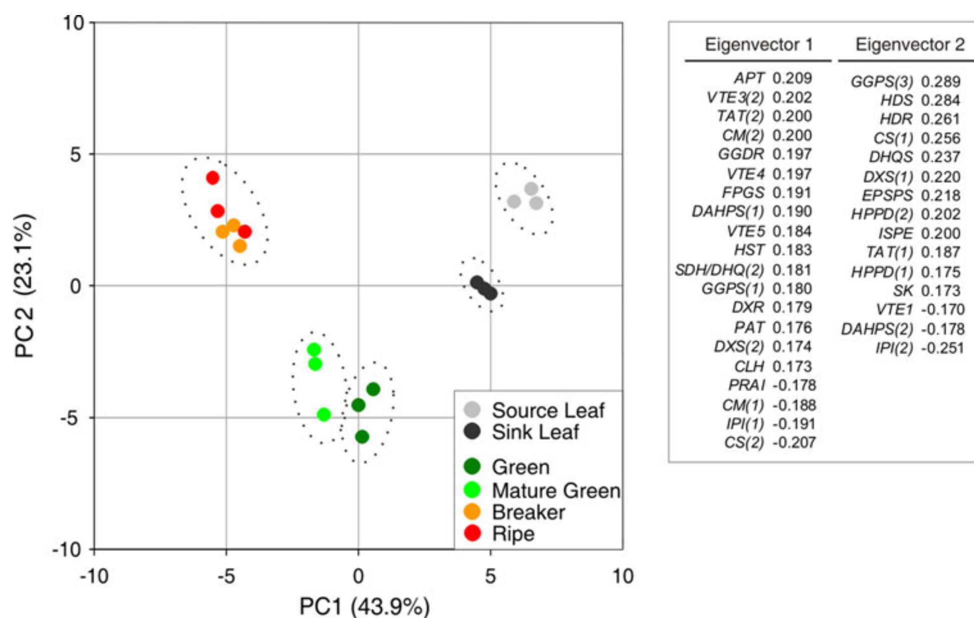

Fig. 2 PCA of the expression profile of VTE biosynthetic pathway genes in tomato leaves and fruits. A principal component analysis was performed with the expression data of the 47 VTE biosynthetic genes assayed in different developmental stages of tomato leaves and fruits. The highest coefficients (cut off |0.170|) of the first two eigenvectors are shown on the *right*

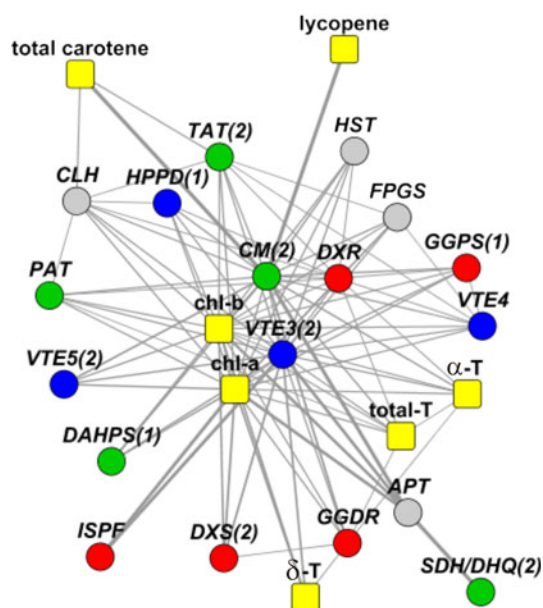

Fig. 3 Gene expression and tocopherol content co-variation network. *Circles and boxes* represent gene and metabolite nodes, respectively. *Red*, *green*, *blue* and *gray circles* indicate MEP, SK, VTE-core and VTE-related genes, respectively. Chl-a and chl–b indicate chlorophyll a and b, respectively. Total-T, δ-T, and α-T indicate total, α and δ-tocopherol, respectively. *Thickness of the lines* is proportional to nodes relative interconnectedness. The network was constructed by using the WGCNA R package and parameters selected as described in "Materials and methods". Cytoscape software (Shannon et al. 2003) was used for network drawing

temporal manner, we next proceeded *(i)* to identify *cis*-regulatory motifs overrepresented in promoter regions of the 47 genes analyzed and *(ii)* to evaluate the conservation of *bona fide* transcription factor-binding sites in orthologous sequences. Out of 38 consensus motifs found in these gene promoter regions, 20 were found to be significantly overrepresented ($p < 0.001$) in the VTE genes promoters when compared to 10,000 randomly selected groups of 47 *S. lycopersicum* promoters (Table S3). Among these overrepresented motifs, 18 showed high similarity (e value $<1e-4$) to previously reported *cis*-regulatory elements annotated in public databases (AGRIS, Yilmaz et al. 2011; AthaMap, Bülow et al. 2009 and; PLACE, Higo et al. 1999), whereas two, VTE12MEME6 and VTE15Av_12, had not been previously described. The two top MNCP scoring motifs (VTE14Av_28 and VTE14MEME5) showed high similarity to described MYB transcription factor binding sites found in phenylpropanoid biosynthetic gene promoters, while the third highest scoring motifs (VTE12MEME9) was similar to a bZIP binding site (Table S3).

Some of the motifs were found in a high number of gene promoters from the three biosynthetic pathways analyzed (VTE10MS10, VTE10Av_27, VTE10Av_29), while others were restricted to a relative few number of genes (VTE14MEME5, VTE15Av_12 and VTE12Av_46). On the other hand, some other motifs were overrepresented in the MEP (VTE12MS7, VTE14MEME8), SK (VTE14 Av_42) or the tocopherol core (VTE12MS2) pathway genes, whereas VTE12MEME4, VTE14MEME6 and VTE14Av_47 were found exclusively in MEP gene promoters (*IPI(1)*, *HDS*, *IPI(2)*, *HDR*, *GPPS*) (Fig. 4a).

In agreement with the fact that most of the paralogous genes showed different expression profiles, they displayed at least four motifs distinct between each other, suggesting potential spatio-temporal specificity (Fig. 1 and Fig. S1).

Since it is well known that the regulation of gene expression may occur through the coordinated action of

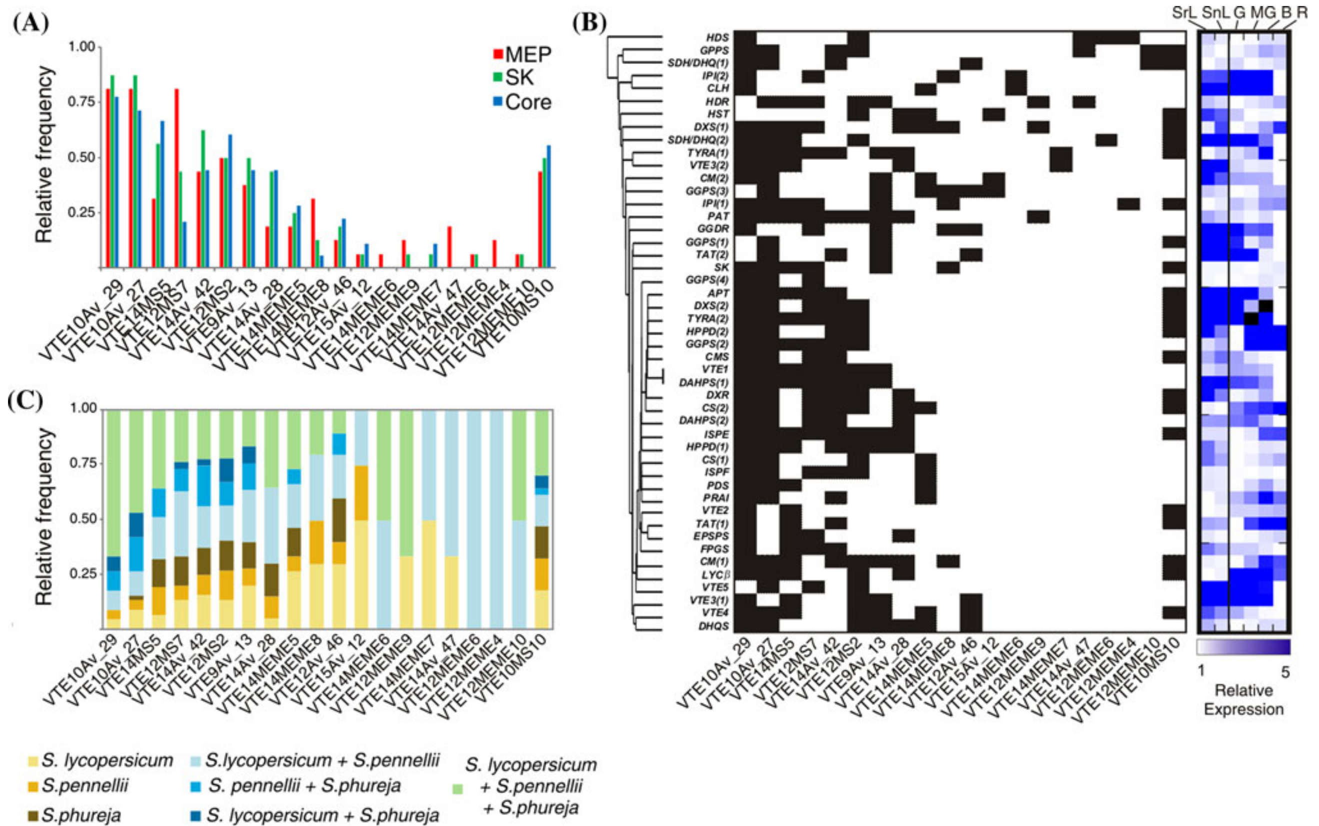

Fig. 4 *Cis*-consensus motifs identified in MEP, SK, VTE-core and VTE-related pathway genes. **a** Relative frequency distribution of the *cis*-consensus motifs identified in promoter regions of MEP- (*red*), SK- (*green*) and VTE core-pathway (*blue*) *S. lycopersicum* genes. **b** Concurrency of consensus motifs in VTE co-expressed genes. Black boxes indicate motif presence, and the expression profile of each gene is indicated by the heat map presented on the right. **c** Relative frequency distribution of the conservation of the identified *cis*-motifs in *S. lycopersicum*, *S. pennellii* and *S. phureja*

multiple transcription factors, a single linkage clustering analysis was performed to group promoters sharing the same motifs (Fig. 4b). Overall it is possible to identify that closely related genes in the cladogram displayed similar transcript patterns. An interesting example is that of *VTE1* and *DAHPS(1)* carrying the same seven motifs in their promoters and both genes displaying similar mRNA accumulation profiles, decreasing along ripening. The *CLH* and *IPI(2)* genes pair shares the ubiquitous VTE10Av_29 and the exclusive VTE14MEME6 motifs and their mRNA levels showed a gradual decrease across fruit development (Fig. 4b and Table S1).

Phylogenetic conservation of the above-mentioned elements was investigated by mapping all motifs on the orthologous promoter regions from *Solanum pennellii* and *Solanum phureja*. All motifs were found in the wild tomato species, while 15 were also found in the potato orthologs, in agreement with the phylogenetic distance between species. The most widely distributed motifs were VTE10Av_29, VTE10Av_27 and VTE12MEME9, which were found in the three species analyzed in over 40 % of the genes in which they occur. In contrast, a subset of motifs including VTE15Av_12, VTE14MEME7, VTE14Av_47, VTE12_MEME6 and VTE12_MEME4, occurs exclusively in genes from the two species of the Lycopersicon section (Fig. 4c).

Positional preference is a variable determining biological functionality in evaluations of transcription factor-binding motifs (Tharakaraman et al. 2005). Out of the 20 overrepresented motifs, VTE14Av_28, VTE9Av_13 and VTE14MEME6 showed strong positional preference ($p < 0.0001$) when mapped along the first 750 bp on the VTE gene promoters (Table S3). These motifs displayed the highest frequency in the first 200 bp upstream of the putative translation start site. The fact that VTE14Av_28 and VTE9Av_13 also displayed significant p values when mapped on all annotated gene promoters of the *S. lycopersicum* genome reinforces the positional preference of these motifs.

Tocopherol content and qPCR profiles in Andean tomato land-races

To evaluate the variation in the transcript levels of the key genes that regulate fruit VTE contents, 16 genotypes of the

section Lycopersicon from different geographic origins and evolutionary history were analyzed: eleven *S. lycopersicum* Andean landrace genotypes, two wild tomato species (*Solanum habrochaites,* LA407 and *Solanum pimpinellifolium,* LA1589), and four commercial reference cultivars (Fig. 5a). Tocopherol forms, lycopene and total carotene were also quantified in ripe fruits of these genotypes. Six genotypes (LA1589, CHMI, CMP, STUF, C526 and GPEA) displayed increased total tocopherol levels, whereas none showed a statistically reduction with respect to the reference cultivar (M82). By contrast, six other accessions presented decreased lycopene levels and, as expected, the high-lycopene-containing LA1589 accession showed the highest pigment contents (Fig. 5b).

Taking an advantage of the diversity revealed concerning tocopherols and carotenoids content in the analyzed genotypes, we next applied the above-described qPCR array for the 19 enzyme encoding genes whose expression levels might limit tocopherol synthesis in ripe fruit. Although this qPCR platform was established for the M82 cultivar, mRNA levels were reliable detected for most of the genes and genotypes tested. The only exceptions were *DXS(1), VTE2* and *VTE1* for LA407, *VTE1* for CZBU and *DXS(2)* for C525, most likely due to the existence of polymorphisms in the amplicon sequences. Interestingly, all the six high-tocopherol-containing genotypes showed increased amounts of mRNA for tocopherol core-encoding genes (Fig. 5c and Table S4). In particular, these genotypes exhibited higher level of the *VTE3(2)* mRNA, while five of them also displayed increased amount of *HPPD(2)* transcript. Moreover, *VTE*4, *VTE*2 and *VTE*3*(1)* mRNA levels were also high in at least two of the high-tocopherol-containing genotypes. Focusing on the MEP pathway, *DXS(2)* and *GGDR* were upregulated in five and two out of the six genotypes of the VTE elite, respectively. LA1589, the only genotype with high content of both quantified metabolite

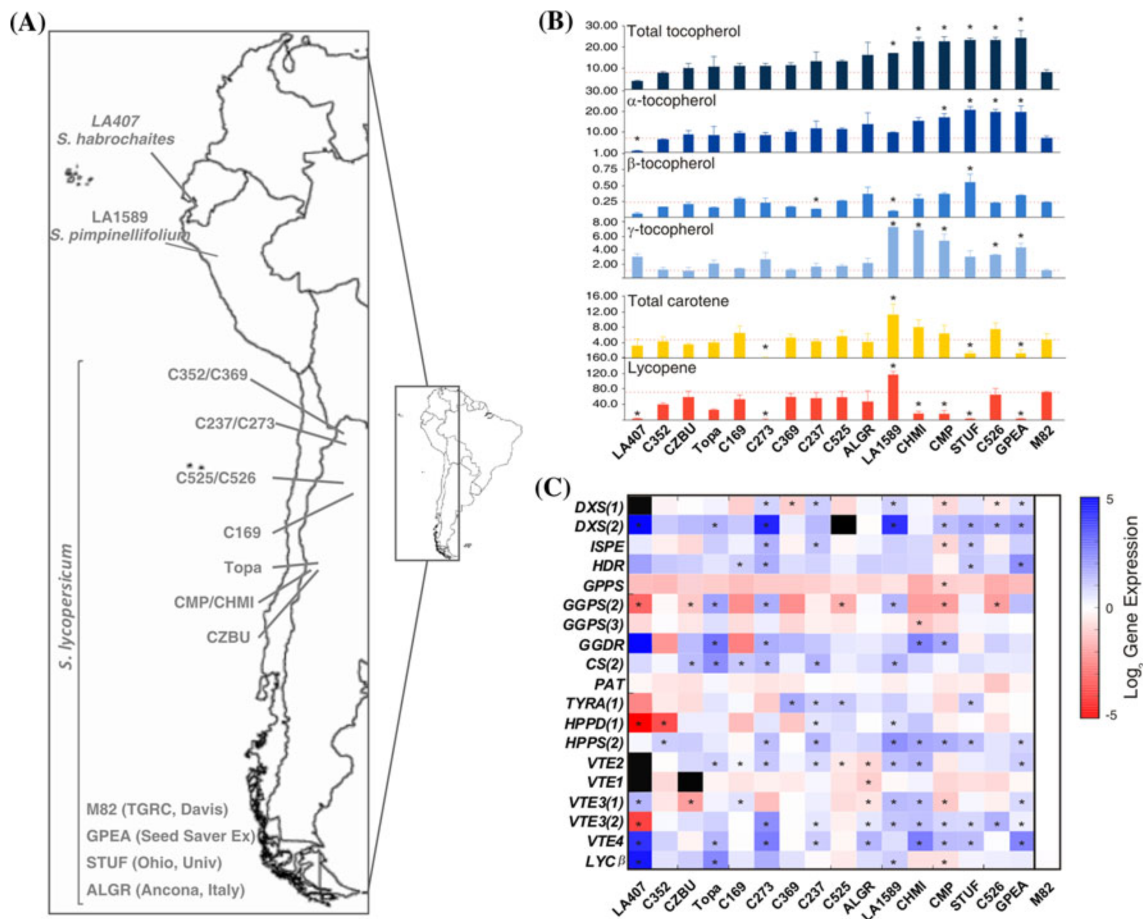

Fig. 5 Natural variation in VTE content regulation in tomato fruits. **a** Geographic distribution of the tomato Andean landrace of *S. lycopersicum* and the wild species *S. habrochaites* and *S. pimpinellifolium* used in this study. **b** Tocopherol and pigment contents in pericarps of ripe fruits. **c** Relative gene expression levels (log_2 median) are shown in a *color scale* where *red* and *blue* indicate decreased and increased transcript levels, respectively with respect to the M82 reference cultivar (*white boxes*). *Black boxes* indicate not detected. *Asterisk* indicates statistically significant differences ($p < 0.05$) with respect to contents measured in fruits from the M82 reference cultivar

families, had increased level of *LYCβ* mRNA, thus confirming the regulatory role of this enzyme in carotenoid biosynthesis (Ronen et al. 2000).

The regulatory role proposed for some of the above pinpointed genes was reinforced by a correlation analysis. *VTE3(2)* and *HPPD* revealed to be positively correlated with tocopherol content. Accordingly, the tight link between the first MEP committed step, *DXS(1)*, and the tocopherol-core pathway was clearly evidenced by its correlation with *HPPD(1), VTE1*, *VTE2* and *VTE3(2)* (Fig. S3).

Discussion

Tocopherol biosynthetic pathway is ubiquitous and transcriptionally regulated by an intricate network resulting in constant VTE levels across leaf and fruit development

Tocopherol species were detected in young and fully expanded leaves and across fruit development in tomato. The higher tocopherol levels in leaves emphasize the hypothesis of its involvement in the protection of the photosynthetic apparatus in chloroplasts (Krieger-Liszkay and Trebst 2006), which whilst still occurring in green tomato fruits likely does so at much lower rates (Lytovchenko et al. 2011). The profiles presented here showed that α-tocopherol was the most abundant form as previously reported by Abushita et al. (1997) and Moco et al. (2007) and, additionally demonstrated that neither this form nor total tocopherol significantly varied across development. Unlike that observed in leaves, the composition of the minor tocopherol forms is timely regulated in fruits. β-tocopherol dramatically decreased from green to mature green stages (tenfold). Conversely, γ- and δ- species significantly increased upon mature green stage tenfold and fivefold, respectively (Table 1). These sequential changes in tocopherol composition are explained by the reduction observed in *VTE4* mRNA levels and the upregulation of *VTE3(1)* (Table S1). Furthermore, the increase in γ-tocopherol at the ripe stage might reflect its importance during seed development. In Arabidopsis, this form is predominant in mature seeds and its loss results in an increased oxidation of polyunsaturated fatty acids and reduced seed longevity (Sattler et al. 2004; Abbasi et al. 2007).

Variations in tocopherol composition and gene expression levels demonstrated that the three pathways involved in VTE biosynthesis are functionally and transcriptionally regulated in both leaves and fruits, in agreement with RNA-sequencing data recently published by The Tomato Genome Consortium (2012). The observation that most of the paralogous genes showed different, or even contrasting, mRNA accumulation patterns indicates that: *(i)* there is a lack of functional redundancy for the encoded enzymes, *(ii)* the advent of paralogous genes brought novel regulatory mechanisms to the pathway and *(iii)* paralogous genes might participate in different multienzymatic complexes, promoting metabolic channeling improving pathway efficiency as has already been shown in phenylpropanoid (Rasmussen and Dixon 1999; Vranová et al. 2012) and flavonoid (Winkel-Shirley 2001) biosyntheses. This hypothesis is also supported by our PCA and promoter sequences analysis results. In the former, organs and developmental stages are clearly separated. Interestingly, for green and mature green fruits, the variables displayed intermediate values between leaves and ripening fruits, reflecting the partial heterotrophy condition of the reproductive organ at these stages. The second approach revealed that the same set of transcriptional regulatory motifs was not shared by paralogous promoter sequences. A nice example of paralogous functional divergence is *GGPS*. Our data suggest that *GGPS(1)* is involved in supplying isoprenoid precursors for photosynthetic plastidial functions, as evidenced by their abundance and predominant mRNA accumulation in leaves. In turn, *GGPS(3)* and *GGPS(2)* seem to be responsible for the production of terpenoids in chromoplasts. Finally, the constant mRNA levels of *GGPS(4)* together with subcellular localization experiments (Almeida et al. unpublished data), leads us to propose its involvement in the synthesis of ubiquinol into the mitochondria (Table S1).

When the entire data-set (leaves and fruit samples) of gene mRNA profile was analyzed together with the tocopherol contents the resulting network displayed a major cluster with 18 highly interconnected genes (Fig. 3). Thirteen of them were found in the eigenvector 1 of the PCA explaining 43.9 % of data variance (Fig. 2) re-enforcing their key roles in tocopherol metabolism. *VTE3(2)* emerged as a major player in tocopherol biosynthesis being the most interconnected gene in a central position of the network. Interestingly, even when VTE2 is the only tocopherol exclusive enzyme, our data suggested that its mRNA level is not limiting for VTE biosynthesis. While leaves contain twice tocopherol than fruits, *VTE2* did not show higher mRNA levels in photosynthetic organs. However, the constitutive expression of the apple fruit *VTE2* increased tocopherol levels in transgenic tomato leaves and fruits (Seo et al. 2011). This scenario suggests the existence of posttranscriptional regulatory mechanisms or the activity of other prenyltransferase as HST (Sadre et al. 2006; Tian et al. 2007). HST activity might contribute to tocopherol biosynthesis in an environment enriched for phytyl-diphosphate as leaves. This hypothesis is in line with the *HST* spatio-temporal transcriptional profile found

here. The *HST* mRNA levels were significantly higher in photosynthetic tissues than in ripe fruit. The maintenance of constant expression levels from mature green towards ripe might be indicative of the plastoquinone-dependent PDS activity for carotenoid biosynthesis (Norris et al. 1995; Phatthiyaa et al. 2007).

Across fruit development, the transcriptional regulation of VTE biosynthesis and the resultant content of tocopherol forms emerge as a complex intricate network that deeply depends on the changes in the intermediate fluxes throughout branching pathways. Tomato fruit is programmed to accumulate large amounts of many metabolites such as pigments, flavonoids and volatiles (Carrari and Fernie 2006). Indeed, our results evidenced that the transcription level of the MEP and post-chorismate pathway genes increases across ripening. Regarding the latter, this is reflected by the higher amounts of *CS* and *CM(1)* mRNA detected between breaker and ripe stages. Concomitantly, increased mRNA levels of *TYRA*, *TAT(1)* and *HPPD* strengthens the metabolic flux to homogentisate synthesis, suggesting the important role of these genes in tocopherol production. These findings are in agreement with previous reports for *TAT* and *HPPD*. In senescent Arabidopsis leaves, an increase in tocopherol content correlated with both the transcriptional level and enzymatic activity of TAT (Holländer-Czytko et al. 2005). Furthermore, tocopherols were substantially reduced in *tat* Arabidopsis mutants (Riewe et al. 2012). However, *HPPD* overexpression was not enough to strengthen the SK pathway flux towards tocopherol biosynthesis. Instead, bypassing the phenylalanine branching point by overexpressing a yeast prephenate dehydrogenase (*PDH*) together with *HPPD*, a tenfold increase in tocochromanol content was obtained, demonstrating that homogentisate flux is limiting for tocochromanol synthesis in leaves (Rippert et al. 2004). In the case of the MEP pathway, our qPCR array data suggest that the intense isoprenoid production at late ripening is determined by the high mRNA accumulation of *DXS(1), DXR*, *ISPE*, *HDR*, *IPI(1), GGPS(2)* and *GGPS(3).* It is worth mentioning that, with the exception of ISPE, the product of all these genes were found in tomato chromoplast proteome (Barsan et al. 2010). Previous experimental evidences have shown the importance of *DXS* and, to a lower extent, of *DXR* and *HDR* transcripts in MEP-derived plastid isoprenoid accumulation (Lois et al. 2000; Paetzold et al. 2010; Carretero-Paulet et al. 2006; Botella-Pavía et al. 2004). In tomato, analyses of *DXS(1)*-overexpressing plants have demonstrated that this gene is undoubtedly a major controlling step in MEP pathway fluxes (Enfissi et al. 2005). Hence, in this context and in accordance with the coordinately mRNA accumulation of *VTE2* and *VTE3* observed, an increase in VTE content across fruit development would be expected. Nevertheless, total tocopherol remained constant. While induction of MEP pathway genes somehow guarantees GGP supply for carotenoid accumulation, the metabolite flux towards tocopherol synthesis is gradually reduced by the decrease in *GGDR* expression across ripening. The role of GGDR in determining VTE content has been evidenced by the fruit specific downregulation of *DET1* gene in transgenic tomato (Enfissi et al. 2010). These lines exhibited metabolic changes including increased levels of carotenoids and tocopherols though, the antioxidant contents did not correlate consistently with the transcriptional level of the tested genes. However, GGDR was remarkably upregulated in mature green stage correlating with the increment in VTE content observed. Ultimately, the limiting precursor for VTE production in tomato fruit is phytyl-diphosphate, which at late ripening could also be supplied from chlorophyll degradation-derived phytol. VTE5 catalyzes the conversion of the chlorophyll degradation-derived phytol in phytyl-phosphate, which is later phosphorylated, to be finally incorporated in tocopherol (Ischebeck et al. 2006; Valentin et al. 2006). In this sense, the contribution of chlorophyll degradation to tocopherol synthesis is strengthened by the constant levels of VTE and the high number of interconnection between chlorophylls, tocopherols, *GGDR, VTE5* and *CLH* observed in the network analysis. In addition, Schelbert et al. (2009) have described the chlorophyll dephytylation route catalyzed by pheophytinase that could also be involved in ripening process. Again, the absence of correlation between transcript levels and their putative enzymatic activities might suggest posttranscriptional and/or posttranslational controls. For instance, *DXS* is target of a posttranscriptional feedback regulatory mechanism, as suggested by the lack of correlation between protein and mRNA levels in response to different perturbations of MEP pathway flow (Guevara-García et al. 2005; Rodríguez-Villalón et al. 2009; Fraser et al. 2007).

As a proof-of-concept, 16 genotypes including wild species, commercial cultivars and Andean landrace genotypes were evaluated to test whether mRNA levels of the above pinpointed genes were indeed effecting tocopherol production. *HPPD* and *VTE3(2)* showed higher mRNA accumulation in ripe fruits from high-tocopherol-containing genotypes, re-enforcing the major role of these genes in VTE accumulation. Although endosperm plastids are structural and functional different from tomato chromoplast, constitutive expression of the Arabidopsis *HPPD* and *VTE3* in maize plants determined up to threefold increments of γ-tocopherol in kernels (Naqvi et al. 2010). However, due to the expressive carotenoids accumulation in tomato fruit, the phytyl-diphosphate precursor may be limiting and an enhanced input by DXS and/or GGDR is also needed. Most of the high-tocopherol-containing genotypes showed reduced carotenoid amounts, supporting

the idea that precursor competition is the main limiting feature for VTE biosynthesis.

The genotypes evaluated here comprise the wild species *S. pimpinellifolium* and *S. habrochaites*, four domesticated commercial cultivars and Andean landraces of the *S. lycopersicum* species. They have been collected based on variable morphological characters and across a wide geographic distribution with diverse climates and altitudes, which are criteria usually associated with genetic diversity (Marshall and Brown 1975, Witcombe and Gilani 1979). Moreover, Williams and St. Clair (1993) has reported that Andean landrace cultivars preserve larger genetic diversity than modern commercial varieties of tomato. The mentioned variability was evidenced by the differential expression profile observed. Thus, the results presented here demonstrate that coexpression analyses are suitable in the investigation of key regulatory steps in metabolic pathways and allow proposing candidate genes for further functional genomic studies (Fukushima et al. 2012).

Cis-regulatory motifs identification suggests that the transcriptional regulation of VTE core pathway genes is coregulated with specific steps of the precursor pathways

The fact that most of the 20 predicted conserved motifs in the promoter regions of the studied genes displayed high similarity to previously described plant *cis*-regulatory elements, their strong positional preference, and that they are overrepresented in the promoters of VTE pathway genes, suggest that these motifs are indeed functional *cis*-regulatory elements.

Neither the presence of shared motifs nor that of exclusive motifs ensures a coexpression profile. An example of this are the cases of *IPI(2)-CLH* and *VTE3(2)-TYRA(1)*. Both pairs of genes share the common exclusive motifs VTE14MEME6 and VTE14MEME7, respectively, and while the first pair shows a coordinated transcription profile, the second one does not. Moreover, the number of shared motifs seems not to be a key variable to determine coregulation. While *IPI(2)-CLH* shares only two motifs and the genes display a similar expression pattern, *VTE3(2)-TYRA(1)* shares five motifs but the genes do not show coordinated expression. Although most identified motifs were found in different repositories of known *cis*-regulatory elements with proven functions, these results should be taken with care as in most cases the functionality of these motifs depend on the genomic context in which they are inserted (Swanson et al. 2010).

Of particular note are the most highly ranked motifs, namely VTE14Av_28 (MNCP value = 14.05) and VTE14MEME5 (MNPC value = 13.0), showing similarity to described MYB transcription factor binding sites found in phenylpropanoid biosynthetic gene promoters from tobacco and *Antirrhinum majus* (Sablowski et al. 1994). These two motifs have also been found in regulatory regions of phenylalanine ammonia-lyase genes of parsley (Lois et al. 1989) and carrot (Takeda et al. 2002), which is a key step in the phenylpropanoid biosynthetic pathway (Fig. 4a, Table S3). Other evidence that these motifs may have a biological function is the conserved positional preference of VTE14Av_28 and VTE14MEME5 in the first 200 bp of the promoters when mapped on the VTE pathway genes (Table S3). These results allow us to suggest that MYB transcription factors might be main regulatory proteins of the analyzed pathway genes also in tomato.

Interestingly, the fact that among the five tomato specific motifs VTE12MEME6 and VTE15Av_12 had not been previously described in any species suggests these two as regulatory innovations in tomato. In addition, these elements are present in members of paralog groups, *GGPS(3)*, *CM(2)* and *SDH/DHQ(2),* in a specific manner. Moreover, VTE14Av_47 and 12MEME4 are exclusively found in the promoters of MEP gene [*HDR*, *HDS*, *GPPS* and *IPI(1)*] encoding enzymes, whose expression increases across fruit development. These motifs are recognized by ABA-responsive transcriptional factors (Shen and Ho 1995; Busk and Pages 1998; Ezcurra et al. 1999) in agreement with the well known increment of this hormone during this process.

The promoter analyses exposed the lack of exclusive *cis*-regulatory motifs for the VTE-core pathway genes. This finding is in agreement with the role of some of the *vte* genes beyond tocochromanol pathway: *VTE3, VTE1* and *VTE4* also participate in plastoquinol-9 and derived compounds synthesis (Zbierzak et al. 2009). Moreover, this result also reflects the tightly tocopherol biosynthesis dependence on precursors pathways. In this sense, we identified *cis*-regulatory elements shared by *VTE3* and *HPPD* genes, (pointed as major regulatory steps in VTE-core pathway) and with genes from the SK and/or MEP pathways (VTE12Av_46, VTE9Av_13, VTE14Av_42 and VTE12MS2). Given that changes in *cis*-regulatory activity are major sources of phenotypic divergence within and between species their identification and further functional characterization can help to resolve questions about genetic mechanisms of phenotypic evolution (Wittkopp and Kalay 2012).

Final considerations

Although MEP and SK are involved in the production of several different key compounds that dramatically change in abundance across fruit development and ripening, tocopherol level remains constant in tomato fruit. This intriguing issue seems to be related to fruit physiology. In

grape (*Vitis vinifera*), a non-climacteric fruit, tocopherol content declines gradually along its development (Horvath et al. 2006b). Mango (*Mangifera indica* L.), a climacteric fruit like tomato, exhibits an inverse pattern (Singh et al. 2011). The intense respiration and ethylene production along maturation that trigger changes in color, aroma, texture, and flavor (Carrari and Fernie 2006) in climacteric fruits are associated with an oxidative phenomenon which is considered as a functional modified form of senescence (Jiménez et al. 2002). As a result, during fruit ripening, a turnover of ROS is required to balance their levels between production and removal by the antioxidant systems. Within this oxidative environment, the maintenance or increase in tocopherol levels is clearly advantageous. In mango, it has been described that *HPPD* expression increases in concert with the increment in tocopherol and carotenoid levels across fruit ripening (Singh et al. 2011). This study provides evidences for the activation of the tocopherol biosynthetic pathway in fruits, which leads to a ripening-specific increase in VTE content. On the other hand, it is worth mentioning that high contents of tocopherol in later stages of ripening could result from an increase in ascorbate levels, which contribute to the efficient regeneration of tocopheroxyl radicals by the ascorbate–glutathione cycle (Kobayashi and DellaPenna 2008).

The evidences collected in this work point that metabolic engineering aiming the enhancement of tocopherol content in tomato fruit requires not only the manipulation of structural genes in VTE-core pathway, but also the understanding of precursor pathways bottlenecks ensuring intermediates influx. The results showed that the transcription level of the MEP and post-chorismate pathway genes increases across fruit development. However, carotenoids biosynthesis competes for intermediates and GGDR appears as a limiting step for the isoprenoid precursor availability. In this sense, the improvement of VTE content can be reached either by enhancing MEP entry point (i.e. *DXS*), increasing tocopherol-core pathway influx via *GGDR*, or by boosting the alternative source of phytyl-diphosphate via chlorophyll degradation. The link between ripening/chlorophyll degradation and tocopherol content rises as an unexplored metabolic hub for future VTE improvement. Later on, *HPPD* and *VTE3* mRNA levels strongly correlate with tocopherol content and composition in tomato fruits.

Materials and methods

Plant material

Tomato seeds from *Solanum lycopersicum* L. (cv M82) were obtained from the Tomato Genetic Resource Center (http://tgrc.ucdavis.edu). Tomato plants were grown in 20-l pots under greenhouse conditions: 16/8 h photoperiod, 24 ± 3 °C, 60 % humidity, and 300 ± 100 μmol m^{-2} s^{-1} incident photo-irradiance. Source (SrL) and sink (SnL) leaves were collected from eight-week-old plants. Fruits at green (G), mature green (MG), breaker (B) and ripe (R) stage were harvested 30, 45, 50 and 60 days after anthesis, respectively. All samples were obtained from six independent plants, immediately frozen into liquid N_2, and stored at −80 °C until use. Samples were pooled in three replicates for further analyses.

Seeds of landrace cultivars were obtained from the germplasm bank of the EEA-INTA-La Consulta (Argentina). Seedlings were grown until four true leaves in 150-ml pots and transplanted to soil under field-production conditions in the Campo Experimental del Instituto de Horticultura, Universidad Nacional de Cuyo, Mendoza, Argentina, 32°50′S, 68°52′W and 900 masl. The field experiment was conducted from October 2008 to March 2009 in a randomized design of three replicates with three plants each. The experiment was protected with an anti-hail mesh and crop irrigation was applied to keep soil available water content (AWC) constant. At ripe stage, two fruits per plant were harvested approximately 60–65 days after anthesis and immediately frozen in liquid N_2 and kept at −80 °C until use.

Tocopherol and pigment quantification by HPLC

Tocopherol was extracted and quantified exactly as described in Almeida et al. (2011). Lycopene, total carotene (α-, β- and γ-carotene) and chlorophylls were extracted and quantified exactly as described in Quadrana et al. (2011).

RNA extraction and cDNA synthesis

Total RNA was extracted from 50 mg of frozen leaves and 100 mg of frozen tomato fruit pericarps with TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. After quantification by Nanodrop spectrophotometer and integrity check by agarose-gel electrophoresis, DNA traces were removed by treatment with amplification-grade DNAse I (Invitrogen) following the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using random primers and the SuperScript III enzyme (Invitrogen) in a final volume of 20 μl. The suitability of each cDNA for PCR reaction and DNA contamination were checked using an intron-flanking pair of primers for the *vte1* gene (forward: GTACCACGTT GCGGG, reverse: GTTAGGTGCAACCTGAGACAA GTC). cDNA samples were 1/10 diluted to a final concentration of 5 ng reverse-transcribed RNA/μl and subsequently used for qPCR reactions.

Primer design, validation, qPCR condition and data analyses

Forty seven gene-specific pairs of primers (Table S5 according to the MIQE guidelines, Bustin et al. 2009) were designed based on tomato unigene sequences (http://solgenomic.net) using the PRIMER3 software (Rozen and Skaletsky 2000). To prevent amplification from traces of genomic DNA, depending on each gene structure, primer pairs spanning introns were selected based on exon prediction. Reactions were carried out in duplicate using 2X SYBR Green Master Mix reagent (Applied Biosystems) in a 20 µl final volume and a 7500 real-time PCR system (Applied Biosystems). The thermal conditions were 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 55/60 °C (Table S5), and 30 s at 72 °C. The amplification of single products was confirmed by melting curve analysis and by agarose-gels electrophoresis. The optimal concentration of PCR primer pairs was determined in a 100–800 nM range based on the lowest Ct values and absence of primer dimer formation. PCR efficiency and Ct for each sample were calculated using algorithms incorporated into the LingReg software package (Ruijter et al. 2009). Primer efficiencies of the 51 genes ranged from 1.924 to 1.694 with values of 1.87 and 1.89 for mean and median, respectively (Table S5 and Fig. S4). Technical reproducibility assayed by linear regression revealed high confident qPCR array setting, as demonstrated by the R2 value of 0.963 (Fig. S5). Samples showing Ct values ≥34 were eliminated for subsequent analyses.

Selection of reference genes

Four reference genes were chosen based on Expósito-Rodríguez et al. (2008): *CAC*, *TIP41, EXPRESSED* and *EF-1α*. Gene stability was tested with NormFinder (Andersen et al. 2004), GeNorm (Vandesompele et al. 2002), and BestKeeper (Pfaffl et al. 2004) softwares. Since all three analyses indicated that the most stable genes were *expressed* and tip41 (Table S6), their mean was subsequently used for gene expression ratio calculations.

Gene expression analysis and statistical tests

Normalized gene expression was calculated by the $^{\Delta\Delta}$Ct method with the improvements proposed by Hellemans et al. (2007). This algorithm allows using several reference genes at the same time leading to a more accurate relative expression data according to the following equation:

$$R = \frac{E_{goi}^{\Delta Ct,goi}}{\sqrt[f]{\prod_0^f E_{ref_0}^{\Delta Ct,ref_0}}}$$

where R indicates the relative gene expression, E is the amplicon efficiency, ΔCt is the Ct difference between the control and the target samples, *goi* indicates the gene of interest, and *ref* the housekeeping genes. The median value of biological replicates was calculated from the mean of two technical replicates and normalized against the sample with the lowest relative expression in Table S1 or the M82 control value in Table S3. A permutation test, which lacks sample distribution assumptions (Pfaffl et al. 2002), was used to detect statistical ($p < 0.05$) differences in expression levels between organs, developmental stages and landrace cultivars using the algorithms incorporated to the fgStatistics software (http://sites.google.com/site/fgstatistics, Di Rienzo 2009, Córdoba, Argentina). Pearson correlation and PCA analyses were performed as described by Carrari et al. (2006) and Quadrana et al. (2011), respectively. Network construction and analyses were performed with expression and metabolite data using the WGCNA R package (Zhang and Horvath 2005). A signed network with threshold power $(\beta) = 6$ was performed according to Zhang and Horvath (2005) and Horvath (personal communication). The network was constructed with genes and metabolites presenting node connectivities ≥0.50 by using an edge-weighted force-directed layout incorporated to the Cytoscape software (Shannon et al. 2003). Coordinated variations between co-expressed genes and metabolites accumulation during fruit development were calculated as Euclidean distances by the omeSOM* software (version v2.27.15, available in http://sourcesinc.sourceforge.net/omesom/). For this analysis, data were normalized as previously described (Milone et al. 2010), and only genes and tocopherol isoforms presenting statistically significant variation along fruit ripening were included. A 5 × 5 map was selected to group coexpresssed genes showing either directed or inverted expression patterns. Results presented in Figure S2 group neighbor neurons with a Vn = 1. ANOVA followed by Tukey's tests ($p < 0.05$) were applied to compare the tocopherol and pigment contents between different tissues and landrace cultivars by using the InfoStat software.

In silico cis-regulatory element identification and evaluation

The IDs and genomic coordinates of 47 unique *S. lycopersicum* genes (Almeida et al. 2011) were retrieved from the ITAG2.3 genome annotation version (Bombarely et al. 2011) and the 750 bp upstream of the putative translation start codon were further analyzed. Highly similar sequences were searched using Purge (threshold 600) from the MEME Suite (Bailey et al. 2009), but no redundancy was

identified. Low-complexity regions and repeats were masked using TANTAN software with default parameters (Frith 2011). Three softwares using different identification and scoring algorithms were applied: MEME (Bailey and Elkan 1994), MotifSampler (Thijs et al. 2002), and Weeder (Pavesi and Pesole 2006). Motifs ranging from 8 to 14 bp for MEME and MotifSampler, and from 6 to 12 bp for Weeder were searched in both strands of the DNA sequences. All identified motifs were clustered, and redundant motifs were averaged using the script "motif_cluster" included in the program Gimmemotifs with default settings (van Heeringen and Veenstra 2011). To reduce the false positive discovery rate, we evaluated if each motif was significantly enriched in the promoter regions of the VTE genes, by using both the ROC-AUC and the MNCP parameters as calculated by the "motif_roc_metrics" script of the Gimmemotifs program. The comparison demonstrated that MNCP was more stringent for our set of data. Then, to determine the statistical significance of a given MNCP value, we generated 10,000 groups of 47 random *S. lycopersicum* promoters of the same length and calculated this parameter for all motifs. The fraction of random rank orders that gives MNCP values higher than those found with the real data were used as an estimate for the one-tailed p value for each motif (Clarke and Granek 2003). The significant overrepresented motifs detected were named as VTE (for vitamin E), a number indicating the consensus nucleotide length and letters standing abbreviations for MotifSampler (MS), MEME and Av for average in cases where redundant motifs were combined into a single motif. To assess the phylogenetic conservation of each motif, the motifs discovered were mapped on the promoters of the orthologous VTE genes (identified by reciprocal Blastn) from *Solanum pennellii* (Usadel and Fernie, unpublished data, sequences available on request) and *Solanum phureja* (The Potato Genome Sequencing Consortium 2011) using the script "pwmscan" as implemented by the program Gimmemotifs. Since binding sites of transcription factors often occur at some preferred distance from the transcription start site in promoter regions, we evaluated the statistical significance of the positional preference for each motif with the script "motif_localization_plots" from the program Gimmemotifs, which calculates a p value as a result of a Kolmogorov–Smirnov test, comparing each motif distribution to a uniform one. The similarity of the over-represented motifs with those present in AGRIS (Yilmaz et al. 2011), AthaMap (Bülow et al. 2009) and PLACE (Higo et al. 1999) databases was assessed with STAMP software using default parameters (Mahony and Benos 2007).

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