Targeted gene disruption coupled with metabolic screen approach to uncover the *LEAFY COTYLEDON1-LIKE4* (*L1L4*) function in tomato fruit metabolism

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ABSTRACT

Tomato fruits from mutant lines bearing targeted disruption of the heterotrimeric Nuclear transcription factor Y (NF-Y) transcription factor (TF) gene *LEAFY-COTYLEDON1-LIKE4* (*L1L4*, *NF-YB6*), a master regulator of biosynthesis for seed storage proteins and fatty acids, were evaluated for metabolites content and morphology. Metabolic screens using LC-MS/MS-

based analysis and physico-chemical methods in different L1L4 mutants of the fourth generation allowed a comparative assessment of the effects of the TF disruption. Mutagenesis resulted in fruits phenotypically similar to wild-type with subtle shape differences in the distal end protrusion and symmetry. Conversely, mutant fruits from independent lines had significant variation in moisture content, titratable acidity and overall metabolite profiles including oxalic and citric acid, fructose, β-carotene, total polyphenols and antioxidants. Lines 6, 7 and 9 were the richest in β-carotene and antioxidant activity, line 4 in ascorbic acid and lines 4 and 8 in succinic acid. The reduced content of the anti-nutrient oxalic acid in several mutant fruits suggests that *L1L4* gene may regulate the accumulation of this compound during fruit development. Detailed LC-MS/MS analysis of mutant seeds showed substantial differences in bioactive compounds compared to wild-type seeds. Taken together, the results suggest that the L1L4 TF is a significant regulator of metabolites both in tomato fruit and seeds providing a molecular target for crop improvement. Elucidation of the candidate genes encoding key enzymes in the affected metabolic pathways aimed at facilitating a better exploration of the L1L4 gene networks and to expedite the use of systems biology approaches in tomato fruit.

KEY MESSAGE

Functional analysis of tomato L1L4 master transcription factor resulted in important metabolic changes affecting tomato fruit quality.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is an important fresh produce crop. The fruit is produced in large scale and consumed all over the world. World's tomato production reached 137 MT in 2012 (http://faostat.fao.org) ranking it as one of the most economically important horticultural

crops. Tomato fruit for consumption, as either fresh or processed, is an important source of nutrients, such as vitamins, carotenoids and phenolics (Antunes, et al. 2013; Breksa, et al. 2015; Odriozola-Serrano, et al. 2008). Sugars and organic acids and their balance are the main contributors to the tomato's fruit taste. In particular, ascorbic acid (vitamin C) along with the carotenoids lycopene and β-carotene and phenolic compounds (flavonoids and hydroxyxinnamic acids) are responsible for the tomato antioxidant properties which are health promoting phytochemicals in human diet (Ilahy, et al. 2011). Therefore, enhancing the tomato fruit quality is of great research interest for both nutraceutical and horticultural industries (Rigano, et al. 2013; Rosati, et al. 2000).

Appropriate cultivars must be selected to reach the demand of growers and consumers. Markets are today interested in differentiating products by attributes related to taste, aroma, acidity, sugar content and vitamins (Causse, et al. 2002; Powell, et al. 2012; Serrano-Megías and López-Nicolás 2006). Traditionally, tomato breeding has been focused on the improvement of yield, disease resistance, tolerance to abiotic stress and sugar content [(López, et al. 2015) and references therein]. Yield enhancement was achieved by crosses with wild relative species (Swamy and Sarla 2008). However, many quantitative trait loci (QTL) for yield and related traits as fruit weight, total soluble solids or lycopene content were found non-randomly distributed in the genome (Fulton, et al. 1997; Fulton, et al. 2000; López, et al. 2015). Domestication and breeding of tomato plants resulted in a variety of phenotypes with increased fruit weight, variability in fruit shape and color (Paran and van der Knaap 2007), as well as differences in fruit quality characteristics which have been largely attributed to genetic factors (Breksa, et al. 2015; Choi, et al. 2014). Phenotypic tomato fruit diversity may range from round to elongated and from

small to large. Thus far, the elongated shape was linked to the loci *sun* (Van der Knaap, et al. 2004; Van der Knaap and Tanksley 2001), *ovate* (Ku, et al. 1999; Liu, et al. 2002; Van der Knaap, et al. 2002) and *fs8.1* (Grandillo, et al. 1996; Ku, et al. 2000).

The downside of the conventional breeding approaches is that they remain a time-consuming (9-15 years for cultivar improvement) and laborious effort considering the relatively long life cycle of tomato (90-120 d) and the fact that, most of the times, these approaches are not targeted to a single genetic locus. As a result of this, classic research in tomato has characterized only a few mutants at molecular level. Recent efforts to increase the available resources for tomato research centered on tomato Heinz 1706 genome sequencing project which after its completion (Tomato Genome 2012), provided clues for fruit evolution and fueled functional genomics studies in this species. The advantages of tomato Heinz 1706 are its compact growth, high production, determinate growth pattern and adaptability to both controlled environments and field conditions. The molecular-based breeding in tomato could be accelerated by using gene targeting technologies, such as the Zinc Finger Nuclease (ZFN) technology, to identify genes required for a particular trait. In our previous studies in tomato, we found that *L1L4*, a member of the *L1L* gene family, was expressed in flowers and green developing fruit (Hilioti, et al. 2014) while a ZFN-based disruption of the coding sequence of the TF affected fruit phenotype (Hilioti, et al. 2016). Notably, the *L1L4* (or *NF-YB6*), encodes the B subunit of CCAAT binding heterotrimeric transcription factor NF-Y, an embryo-specific transcription factor involved in plant development and regulation of seed storage proteins and fatty acid biosynthesis (Kagaya, et al. 2005; Lotan, et al. 1998; Mu, et al. 2008).

We studied the function of *L1L4* TF in tomato fruit development and quality and evaluated both fruit tissues and seeds in an effort to establish a relationship between metabolites in fruits and seeds of each mutant line. The integrative analyses allowed us to identify crucial content variation in major metabolites which was attributed to the regulatory role of L1L4.

RESULTS AND DISCUSSION

Selection of different *L1L4* disruption variants

Tomato, apart from being an economically important food crop, serves as a model system for complex processes, such as fruit development, ripening (Giovannoni 2004) and seed development (Hilhorst, et al. 1998). In a previous study, we used ZFN technology to target the developmental TF *L1L4*. Disruption of L1L4 coding sequence revealed that the TF is a global regulator of tomato development, affecting flower fruit setting, fruit and seed morphology (Hilioti, et al. 2016). In the current study, plants homozygous for *L1L4* of the M_4 generation were produced, as the cultivar Heinz 1706 displays cleistogamy and is self-pollinated. Independent plants from each mutant line were studied for fruit quality. In *Arabidopsis*, LEC1/*L1L* is an essential gene regulating plant development (Kwong, et al. 2003). HRM analysis was used to identify 8 lines carrying different ZFN-induced and NHEJ-mediated mutations (indels) in the target locus (Figure 1A). The presence of indels in the target locus produced different melting curves compared with the one from the non-mutated amplified target DNA flanking the end of exon 1 and beginning of exon 2. Sequencing of the *L1L4* locus amplified from cDNA by PCR using a forward and a reverse primer in exons 1 and 2, respectively, revealed the exact genotypes of the mutant lines (Figure 1B). Interestingly, nucleotide changes were found in lines 2 and 4 while lines 5 and 7 have a 1-bp insertion which results in frameshift. Line 3 had a 2-bp deletion

and line 6 had two 1-bp insertions in addition to nucleotide changes. Line 8 had nucleotide changes and deletions while line 9 had a 1-bp deletion and insertion at the end of exon 1. The unique coding sequence polymorphism in mutant lines lied upstream of the DNA binding domain of the TF and the highly conserved among eukaryotes central region of NF-YB subunit (Li, et al. 1992; Mantovani 1999). Unlike other TFs, three subunits (NF-YA, NF-YB and NF-YC) of NF-Y are required to create the DNA binding domain (Maity and de Crombrugghe 1992; McNabb, et al. 1995; Sinha, et al. 1995). Therefore, one would expect that *L1L4* mutations could potentially affect one or more of the following: the recognition ability of L1L4 to target the promoters of its target genes, the interaction of the L1L4 protein with the NF-YC subunit, and NF-Y complex formation. In the last two cases the effect of the mutation would lead to a functional knockout. Substitutions and other specific characteristics of the *L1L4* mutations in coding region were revealed by comparison with the wild-type sequence via multiple sequence alignment (Supplemental File 1). Indels in lines 3, 5 and 7 led to premature nonsense (stop) codons in *L1L4* and in truncated proteins lacking the central B and C-terminal C domains of the transcription factor (Supplemental File 1). The truncated versions of the TF are expected to be non-functional. However, a well-conserved domain is present in the truncated isoforms and other mutants from residues 1 to 19, where potential phosphorylation sites for serine may be present. If the truncated proteins are stable and substrates for phosphorylation, then they can compete with other substrates causing interference in other phosphorylation-dependent pathways. In line 2, nucleotide substitution mutations led to E34N, L35R, P36H amino acid substitutions, which are also rare as the exchanged residues have very different structures and properties. Mutant line 4 constitutes a unique case as it carries a single amino acid substitution of aspartic (abbreviated as Asp or D) to tyrosine (abbreviated as Tyr or Y) residue at position 23. The D23Y substitution is

also fixed in line 9, in addition to the presence of three more amino acid substitutions (T20P, P21L, A22Q) in this line. The aspartic acid to tyrosine substitution is rare as the two amino acids have very different structures, properties and biological functions. An Aspartic acid residue is negatively charged prefers to be on the surface of proteins, and it can be a phosphomimetic chemically similar to phospho-serine. From the other hand, Tyr residue is aromatic and prefers to be buried in protein hydrophobic cores. This replacement is expected to maximally alter the hydrophofic surface of L1L4 and consequently the accessibility of kinases to possible serine residues located nearby Tyr-23, altering, in such scenario, L1L4 protein regulation. Further, it is possible that tyrosine itself is a target for phosphorylation by tyrosine-specific kinases and this phosphorylation is crucial for a signal transduction process in the cells. Line 6 carries a block of six different amino acid residues (EPGTDN) compared to wild-type (ELP) in the amino acid region between 33 and 38, which is expected to alter the folding and properties of the protein. Notably, the Ser-32, which is quite conserved in other lines, has been substituted with glutamic acid (E) in this line. The insertion and deletion of nucleotides in line 8 changed the reading frame only of the region between P20-L40, resulting in a completely different translation from the wild-type. Overall, the amino acid substitutions in L1L4 appeared between the residues 20 and 38 of the protein, which suggests that the region is under relaxed selection and mutations can be tolerated.

Consequences of *L1L4* disruption on tomato fruit appearance

Fruit quality, as determined by the appearance of the fruit including its shape, size, color, synchronous ripening and absence of defects, remains an important quality criterion for fresh market industries. To investigate the tomato fruit morphological effects of the *L1L4* coding sequence disruption, we assessed the variation of shape in fertile lines carrying different *L1L4*

mutations in relation to the wild-type species. Based on visual observation, the phenotype of the fruits from mutant lines showed subtle shape differences in the distal end protrusion and symmetry (Figure 2), which did not significantly affect the size of the fruit in terms of diameter, length and weight (Table 1). In contrast to wild-type plants that produced elongated and three-loculed fruit, *L1L4* mutant fruits had usually not more than two locules (data not shown). Previous studies have linked mutations in the *LOCULE NUMBER* (*LC*) (Barrero, et al. 2006) and *FASCIATED* (*f* or *fas*) genes (Lippman and Tanksley 2001) to the increase in locule number of tomato fruit. Further, the characteristic distal end protrusion of Heinz 1706, which is related to localized cell division events, was either more or less pronounced in mutants compared to wild-type (Figure 2). Specifically, lines 2, 3, 4, 8 and 9 showed a pronounced distal end protrusion compared to wild-type. This suggested that the fruit shape trait is under *L1L4* TF control while the genes regulated by this TF remain to be identified. A previous study in tomato determined that mutations in the *SUN*, *OVATE*, *LC* and/or *FAS* genes can explain as much as 71% of the fruit shape diversity (Rodríguez, et al. 2011). In particular, *SUN* gene product has been hypothesized to control elongated fruit shape by acting as positive regulator on growth to alter hormone or secondary metabolite levels (Xiao, et al. 2008).

Tomato color correlates with maturity and the a/b ratio, which measures the relative amounts of red (a) and yellow (b), has been used as an indicator of maturity (Arias, et al. 2000). The results of this study revealed that tomato color was almost not influenced by *L1L4* gene disruption (Figure 2). In addition, color parameters L and a* had similar values among all tomato lines (Table 1) except line 3 (Table 1). Nevertheless, none significantly differed from wild-type fruit at the ripe stage.

Fruit weight ranged from 29.35 g (line 3) to 40.51 g (line 4) showing a 1.4-fold variation from lowest to highest weights, being significantly different only between lines 3 and 4. Nevertheless, fruit diameter and length did not show significant differences among lines and wild-type (Table 1).

Although consumers buy on the basis of appearance and textural quality, their satisfaction and repeat purchases are dependent upon good eating (flavor) quality. Firmness was also not affected by the *L1L4* gene disruption, being all lines and wild-type with values which did not significantly differ (Table 1).

Fruit compositional changes related to *L1L4* gene disruption

Tomato ripening is a highly regulated developmental process involving changes in fruit color and alterations in its texture-a result of cell wall remodeling-as well as changes in metabolism in terms of accumulation of sugars, organic acids, flavonoids, carotenoids and ascorbic acid which can influence the flavor quality of the fruit. An autocatalytic increase in respiration and ethylene biosynthesis precedes the initiation of ripening in tomato while a number of transcription factors encoded by the *NON-RIPENING* (*NOR*), *COLORLESS NON-RIPENING* (*CNR*), and *RIPENING INHIBITOR* (*RIN*) regulate the ripening process in concert with the gas hormone ethylene [reviewed in (Giovannoni 2007)].

To determine whether fruit quality characteristics were different in *L1L4* mutant tomato lines and wild-type, several attributes were evaluated. Tomato firmness is a texture attribute that determines postharvest shelf-life and is required for shipping to distant markets. Maintenance of

firmness was observed in all mutant fruits with values which did not significantly from the wild-type (Table 1). In contrast to fruit firmness, a strong variation in fruit soluble solids content (SSC) was detected for the mutants in Heinz 1706 background with values between 3.6 and 4.68% (Table 1), which were greater than the range (3.57 to 3.75) of SSC values reported by Ferreira (Ferreira 2001) and lower than that (5.18 to 7.88) of the Mexican cultivars (Mahakun, et al. 1979). Importantly, all mutant fruits had increased SSC when compared to wild-type. SSC content is of prime importance in fresh-market tomatoes and a breeding target (Stevens 1986), as it affects flavor (Simandle, et al. 1966) and nutritive value. An increase in SSC is also desirable in processing tomatoes and it has been estimated that an increase of 1% in fruit SSC represents a 20% increase in yield of processed product (e.g. paste, purees) which translates in significant financial profit (Wood 1992). In line 4, the single D23Y substitution was sufficient to increase SSC content over wild-type levels while the additional substitutions present in line 9 did not contribute significantly to SSC content. The SSC contents in lines 3 and 5 carrying the truncated forms of the TF were similar to those in lines 4 and 9 carrying amino acid substitutions between residues 20 and 23. The acidity of the fruit is very important for the flavor and storability of processed tomato. Genetic factors are the major acid content determinants in tomato fruits, with great variation occurring among genotypes (Stevens and Rick 1986). Fruit titratable acidity (TA) varied greatly in the mutant lines with line 9 having the highest value which did not significantly differ from lines 4, 6 and 7, while wild-type had the lowest value which was similar to lines 2, 3, 5 and 8 (Table 1). Specifically, TA values ranged from 0.49 to 0.68 (wild-type) and were similar to values reported for the Mexican tomato cultivars (Méndez I, et al. 2011). The single D23Y substitution in line 4 was sufficient to reduce significantly the TA in fruits. Taste is one factor that affects perception of flavor. The taste index was calculated using the values of SSC and TA

and applying the equation determined by Navez et al. (Navez, et al. 1999). The mutants had values between 0.89 and 1.0 (Table 1), thus being adequate for fresh consumption. According to Navez et al. (Navez, et al. 1999) if the value of the taste index is higher than 0.7, tomato is considered as tasty. Lines 3 and 8 had the lowest values (0.89 and 0.90, respectively), in contrast to line 7, with the highest value (1.0) (Table 1). Fruit moisture (Table 2) ranged from 93.2 (line 9) to 95.34% (wild-type), which was similar in magnitude to the values obtained by some tomato cultivars grown in Tenerife (Suárez, et al. 2008). Fruit fiber content is another important quality characteristic that was affected in lines 4, 5 and 8 with values significantly greater than in wild-type (Table 2), while wild-type values were similar to the ones obtained for other tomato cultivars (Suárez, et al. 2008). These results indicate that L1L4 regulates fiber content in tomato which is a beneficial health parameter.

Tomato mutant lines investigated in this work did not show variation in ash content (Table 2). On the contrary, great variation in the crude protein content (free and protein-bound amino acids) was detected in the mutants with values from 0.48% (line 2) to 0.81% (wild-type) (Table 2). Mutations in lines 2, 3, 4 and 9 resulted in significantly lower protein content than wild-type and lines 6, 7 and 8. The crude protein content of the wild-type background was similar to Korean cherry tomato fruits (Choi, et al. 2014).

Fruit flavor is greatly affected by sugars as they contribute to sweetness. The sugars glucose and fructose are the most prominent fractions of the SSC in the domesticated tomato (*S. lycopersicum*). On the contrary, some wild tomato species (e.g. *S. chmielewskii*) accumulate mostly sucrose (Yelle, et al. 1991). The soluble sugar contents (glucose, fructose and sucrose)

were determined in mature fruit tissues. As anticipated from previous studies, fructose was present in high concentration followed by glucose, in agreement with results in other tomato cultivars (Antunes, et al. 2013; Breksa, et al. 2015). Remarkably, the fructose to glucose ratio was lower (1.3) in wild-type than in the *L1L4* mutants (≥1.5) (Table 2). The remarkably contrasted 'high-fructose-to-glucose' phenotype that contributes to sweetness in mutant lines suggests that the TF regulates fruit fructose content. The mechanism of this effect is not obvious, but needs to be further investigated in tomato fruits from individual lines. Overall, SSC showed a significant and positive correlation with the fructose concentration ($r=486$, $p<0.01$). Fruit flavor is also affected by the degree of sourness and the level of citric acid. Among the identified fruit acids in the current study, citric acid level was significantly reduced in 5 out of 8 mutant lines, with values ranging from 7.66 to 21.88 g/kg DW, which reflects a reduction in wild-type levels by 64.8 and 26.66 %, respectively (Table 2). The persistence of the dramatic reduction in citric acid levels in mutant fruits provided compelling evidence for the involvement of the TF in the regulation of citric acid production.

The presence of oxalic acid in tomato has been previously reported (Islam, et al. 1996; Suárez, et al. 2008). The plant metabolite oxalic acid when present in relative high amounts can be considered as an anti-nutrient because it may inhibit the correct absorption of calcium due to the formation of insoluble salts with calcium, particularly if the ratio of oxalic acid to Ca is higher than 2.25. In such cases, food is considered to be decalcifying (Suárez, et al. 2008). In the present study, oxalic acid was reduced in lines 2 and 5 by 70-80 % of wild-type levels, although it was also reduced in lines 3, 4, 6 and 9 to a smaller extent (Table 2). A reduction in the levels of oxalic acid in tomato fruit is desirable as it may facilitate the absorption of micronutrients.

Ascorbic acid, vitamin C, is a powerful antioxidant and a low intake may result in adverse health effects. Tomato fruits from line 4 showed the highest ascorbic acid content with a 2.7-fold increase above wild-type level. On the contrary, ascorbic acid was not detectable in line 5. Since ascorbic acid is an essential dietary component, line 4 could be used for breeding cultivars enriched in ascorbic acid. Further, line 6 exceeded in quinic acid content by 1.72-fold the wild-type level, while quinic acid was not detected in line 2 (Table 2). Shikimic acid concentrations increased over wild-type levels in 6 out of 8 *L1L4* mutant lines. Although succinic acid was not detected in wild-type fruit, *L1L4* lines produced fruits with increased levels (0.16-1.25 g/Kg DW) of succinic acid, which provides a foundation for developing tomato fruit as a system for producing succinic acid. Succinic acid has a specialty chemical market which includes surfactants, detergents, foods and pharmaceuticals (Zeikus, et al. 1999). Specifically, mutant lines 8 and 4 had the highest succinic acid content. Succinic (Suárez, et al. 2008) and shikimic acids (Osvald, et al. 2001) were also previously reported in tomato samples. The sum of the 6 acids content, herein identified, showed a negative correlation with tomato fructose concentration ($r=-0.535$, $p<0.01$).

The content of antioxidants in tomato fruits from wild-type and mutant lines growing under controlled conditions in the greenhouse was also investigated. Total phenol content was consistently higher in all of the *L1L4* mutant tomatoes, as compared to the wild-type (Table 3). Line 9 had the highest phenol content which was 1.48-fold higher than the wild-type level. Our results for phenol content in mutant lines are consistent with the results from Toor et al. (Toor and Savage 2005) ranging from 7.41 to 21.7 mg GAE/kg FW, which indicates that the genotype

significantly affects total phenol content in tomato (Dumas, et al. 2003; George, et al. 2004). According to Toor et al. (Toor and Savage 2005), flavonoids represent the major component of the total phenol content in tomato fruit. No differences were detected in flavonoid content among mutant lines. Tomato fruit is an important source of carotenoids in the human diet (Beecher 1998). The β-carotene levels were higher (approximately 3.5-fold increase) in lines 6, 7 and 9 compared to wild-type (Table 3), with concentrations (~0.5 mg/100 g FW) consistent with those reported on tomato cultivars from Italy (Ilahy, et al. 2011) and China (Juroszek, et al. 2009). Lycopene, a carotenoid, is the most prominent (90-99%) (Dumas, et al. 2003) lipophilic pigment in tomato fruits known for its strong antioxidant role associated with its ability to act as free radical scavenger (Müller, et al. 2011). Lycopene levels in mutant lines did not differ significantly from wild-type levels. Nevertheless, lycopene values observed were higher than the ones described for fresh tomatoes from China (Chang, et al. 2006), but slightly lower than in Italian varieties (~10 mg/100 g FW) (Ilahy, et al. 2011). The general correlation between lycopene and a*/b* ratio, a measure of redness, has been noted previously (Arias, et al. 2000) and it was also found in this study ($r=0.675$, $p<0.05$). The metabolites listed above (phenols, flavonoids, lycopene, β-carotene) have the ability to inhibit or retard oxidation processes which are associated with human aging and development of pathologies such as metabolic syndrome, cancer or degenerative disorders (Hamid, et al. 2010). In this context, lines 6 and 9 are of better quality concerning those quality characteristics. The antioxidant activity of the wild-type tomato fruits, determined by both the TEAC and the ORAC methods, was lower, as compared to the mutants. According to Dumas et al. (Dumas, et al. 2003) and Atanassova et al. (Atanassova, et al. 2007) the antioxidant contents in tomato fruit vary in relation to genotype and environment. As all mutant lines were grown under similar conditions, the higher antioxidant content was

attributed to *L1L4* gene disruption. Positive correlations were found between these antioxidant activities (ORAC) and the contents of all bioactive compounds referred above but they were only significant for total phenolics ($r=0.598$, $p<0.001$) and β-carotene contents ($r=0.581$, $p<0.01$).

Principal component analysis (PCA) was used to explore the similarities among all the lines with respect to the analyzed quality parameters. The data showed that three principal components explained 67% of the total variation (Figure 3A). The first, second and third components explained 31.16%, 21.0% and 14.84%, respectively, of total variation. Variable analysis made possible to group the tomato lines in 3 groups in order to express and show the similarities and differences (Figure 3). Group I consisted of wild-type alone characterized mainly by the protein and moisture contents. Group II consisted of lines 2, 3, 4 and 8 which were closer because of their similarity in fiber, color values (a*, b*, C* and hue), pH, ascorbic and succinic acids content. Finally, group III consisted of lines 5, 6, 7 and 9 due to similarities in antioxidant activity, lycopene, β-carotene, glucose, shikimic acid content, a*/b* ratio and fruit dimensions. A dendrogram confirmed the results of PCA (Figure 3B). The results of PCA further confirmed the previous analyses showing that the fruit quality parameters varied considerably with the *L1L4*-specific mutations. Based on the overall results in fruit composition from lines 3, 5, and 7 carrying truncated and non-functional L1L4 proteins, the TF appears to be a negative regulator of fiber, fructose, succinic and shikimic acid biosynthesis and a positive regulator of citric and oxalic acid biosynthesis.

Hence, the single gene mutation breeding accompanied by fruit quality assessment provided a unique link between genotype, phenotype and fruit quality, which is currently missing for tomato

species. The results may provide a forward looking perspective on designing and producing specialized products in plants.

Profiling of metabolites in seeds based on LC-MS/MS analysis

Tomato seeds represent a major (about 40%) byproduct of the tomato processing industry. The seeds contain about 34% oil (Giannelos, et al. 2005) and posses antioxidant properties (Toor and Savage 2005). To further address the role of L1L4 TF in seed metabolism, we screened, in parallel, seeds from the same mutant fruits and wild-type for metabolites. The rational of this approach was that L1L4 may have some effect on known or unknown metabolites predominantly present in seeds. Notably, studies on tomato seed composition remain rather scarce; hence, our analysis contributes new knowledge to what is already known from previous studies (Hilhorst, et al. 1998; Mounet, et al. 2007; Persia, et al. 2003; Voelker and Kinney 2001). Typically, ripe-fruits from mutants and wild-type tomato plants were harvested, and the seeds were then analyzed by LC-MS/MS for the presence of carotenoids, flavonoids and non-volatile acids. The untargeted analysis, revealed a decrease in the levels of apigenin in mutant seeds compared to wild-type (Figure 4). Further, a pronounced reduction in luteolin levels was detected in all mutant seeds while the metabolite was practically undetectable in line 7. These results suggest that L1L4 may regulate luteolin levels in tomato seeds. Luteolin was previously detected in tomato fruits (Chassy, et al. 2006), seeds and skins (Kalogeropoulos, et al. 2012). Apigenin and its derivatives have been previously identified in bell pepper (Miean and Mohamed 2001) and tomato products (Vallverdú-Queralt, et al. 2011), but to the best of our knowledge, this metabolite has not been reported previously for tomato seeds. The flavone apigenin and its structural analogue luteolin are known to have anti-inflammatory (Hougee, et al. 2005; Rezai-

Zadeh, et al. 2008), free radical scavenging properties (Yao, et al. 2004) and cancer-preventing effects in humans (Chen, et al. 2006; Le Marchand 2002).

Citric acid is the predominant organic acid in tomato fruits (Davies 1966) and contributes to the total acidity. A comparison of flavonol profiles in wild-type and mutant seeds showed that the accumulation of citric acid and citric acid derivative were severely affected by the disruption of the TF. Specifically, citric acid derivative rose above wild-type levels in mutant seeds except in line 5. On the contrary, the levels of citric acid were dramatically decreased in all mutant seeds compared to wild-type. These results uncover a potential regulatory role for the TF in citric acid biosynthesis and conversion to organic derivatives in seeds, in addition to its role in regulating citric acid in tomato flesh (Table 2). Previous studies have determined that the processes involved in the metabolism and accumulation of citric acid are under both genetic and environmental control (Etienne, et al. 2013), while during tomato ripening citric acid levels increase (Anthon, et al. 2011).

Hydroxycinnamic acids (HCAs) are secondary metabolites and are widely distributed in plant species. They are health promoting components as a result of their antioxidant activities. In tomatoes, *p*-coumaric acid, caffeic acid and ferulic acid are the most abundant hydroxycinnamic acids (Luthria, et al. 2006) contributing to the beneficial and health promoting effects of tomato products. *L1L4* mutations led to dramatically decreased levels of *p*-coumaric acid in lines 3 and 4 while it was nondetectable in lines 2, 5, 6, 7, 8 and 9, pointing to a regulatory role of L1L4 in *p*-coumaric accumulation. Similarly, in all mutant seeds except those in line 6, the level of caffeic

acid hexoside dimer decreased compared to wild-type. Phenolic compounds such as caffeic acid and derivatives contribute to the bitter taste of immature green tomatoes.

Compared with wild-type, the L1L4 mutants showed a decrease in the level of glucaric acid in seeds, with the exception of line 3 where an increase was detected. Notably, glucaric acid content in seeds from lines 2, 4, 7 and 8 was reduced to 40% of wild-type level. Glucaric acid is formed by the oxidation of glucose. The metabolite has been detected in camelina seeds, another oilseed crop (Li, et al. 2015). D-glucaric acid (or saccharic acid) and its derivatives, normally present in fruits and vegetables (Dwivedi, et al. 1990), have been associated with therapeutic uses including cholesterol reduction (Walaszek, et al. 1996), diabetes treatment (Bhattacharya, et al. 2013) and cancer prevention (Walaszek 1990).

Homoveratric acid, a biophenol compound, has been detected previously in tomato (Gómez-Romero, et al. 2010) and olive millwaste (Obied, et al. 2007). In the present study, the level of homoveratric acid was reduced in mutant seeds of lines 4 and 8 while it was increased above wild-type levels in seeds of lines 3, 5, 6 and 9. Only lines 2 and 7 maintained wild-type levels of homoveratric acid.

Overall, it became evident that the single D23Y substitution present in line 4 was sufficient to lower most of the seed metabolites examined, suggesting a negative interference in their biosynthesis.

Hence, the parallel investigation of ZFN-based targeted *L1L4* mutations revealed the significant role of the TF in regulating tomato fruit quality while the effects on appearance were subtle in the selected mutant lines and mostly related to the distal end protrusion and symmetry. Fruits from lines 5, 6, 7 and 9 of the PCA group III, had good nutritional properties in terms of antioxidant activity, though with a reduced protein content. Importantly, an improved cultivar pertains not only to enrichment in nutrients but also to the low content of metabolites recognized as anti-nutrients such as oxalic acid. In this context, our study links for first time L1L4 function to oxalic acid accumulation in tomato fruits.

In silico reconstruction of putative biosynthetic pathways of L1L4-dependent metabolites

Due to the importance of tomato in human nutrition, we examined the in silico reconstruction of selected portions of specific biosynthetic pathways involved in tomato metabolism as identified by the analysis of metabolic profiles among the L1L4 mutant lines. In particular, the citrate, galactose, ascorbate and carotenoids contents were significantly affected in several mutant lines, suggesting a transcriptional regulation of the corresponding biosynthetic pathways by the TF.

Based on the current data, we initially employed a reductionist computational approach supported by the PMN software and the current biochemical knowledge to reconstruct three putative metabolic pathways for the production of β-carotene, L-ascorbate and succinate by determining the initial and end compound. A model of the metabolic pathways is shown in Figure 5. The L-ascorbate pathway (L-galactose pathway) has only been recently proposed in plants (Wheeler, et al. 1998) and uses D-glucose as metabolic input. In our model, L-ascorbate is formed from D-glucose in ten steps by Hexokinase (HXK), glucose-6-phosphate isomerase (EC 5.3.1.9), mannose-6-phosphate isomerase (EC 5.3.1.8), phosphomannomutase (EC 5.4.2.8), mannose-1-phosphate guanylytransferase (GDP, EC 2.7.7.22), GDP-mannose 3,5 epimerase (EC

5.1.3.18), GDP-L-galactose phosphorylase (EC 2.7.7.69), sugar-phosphatase (EC 3.1.3.93), L-galactose dehydrogenase (EC 1.1.1.316) and L-galactonolactone dehydrogenase (EC 1.3.2.3).

Further investigation on metabolic genes putatively involved in the above pathways will enable the engineering of tomato metabolite accumulation rationally in order to produce fruits with specific nutritional compositions. The great diversity of metabolites produced in fruits as well as the presence of gene families involved in metabolite biosynthesis pose limitations in the holistic representation of the fruit system. Current techniques for reconstructing metabolic networks have depended heavily on sequence homology searches (Bono, et al. 1998). PMN searches of genes coding for key enzymes in the selected metabolic pathways against the entire tomato genome enabled us to identify 31 gene models of *S. lycopersicum* fruit metabolism (Figure 5). Notably, most of the predicted genes in the gene models are not functionally characterized in tomato while multi-gene copies exist as in the case of mannose-1-phosphate guanylytransferase (*Solyc03g096730.2.1*, *Solyc03g113790.2.1*, *Solyc09g011220.2.1*, *Solyc06g051270.2.1*). Another gene family encoding HXK has been previously characterized in tomato (Dai, et al. 2002; Kandel-Kfir, et al. 2006; Menu, et al. 2001). Interestingly, in the HXK gene family only *LeHXK4* was expressed in young fruits (Kandel-Kfir, et al. 2006). Genes that are directly regulated by L1L4 TF should be enriched for the CCAAT consensus LEC1 (CBF) binding element in their promoters. The biological validity of the computational prediction of gene candidates shown in Figure 5 will be verified in future experiments using L1L4 mutant backgrounds.

CONCLUSIONS

Targeted genome technologies are a young field in metabolic engineering of plants. The results of the current study in tomato fruits attempt to fill this gap in knowledge by demonstrating that ZFN-mediated targeted disruption of the master developmental regulator *L1L4* affects major fruit and seed metabolites which were hard to predict. The mutations were found between the residues 20 and 38 of L1L4 protein, which suggests that the region is under relaxed selection and mutations can be tolerated. This in turn, generates novel insight on *L1L4* function in fruit quality. The identification of identical phenotypes (e.g. metabolic and morphologic) in several mutant fruits shows the robustness of the ZFN technology. The current data establish a relationship between high-order regulators of gene expression and physiological-metabolic phenotypes of tomato fruit. L1L4 TF appears to be a negative regulator of fiber, fructose, succinic and shikimic adic biosynthesis and a positive regulator of citric and oxalic acid. The single gene-based variations in metabolite composition may direct breeding for specific nutritional and/or anti-nutritional needs, such as fruits with high fructose content or low oxalate for diabetic diets. While *L1L4* mutagenesis effect on fruit quality may be specific to tomato, it may be common or provide guidance for other fleshy-fruited plants.

MATERIALS AND METHODS

Plant material

Tomato seeds of *S. lycopersicum* (cv. Heinz 1706), control (wild-type/WT) and eight different *L1L4* gene disruption lines from M_4 generation, obtained at Institute of Applied Biosciences (Thessaloniki, Thermi, 57001, Greece) were sown in a plastic greenhouse at the University of Algarve (located in Faro, Portugal), and the resulting seedlings were transplanted to pots. Plants were grown in the greenhouse under normal culture procedures until the end of fruit harvest

period in June-July 2013. The greenhouse was ventilated when the temperature exceeded 24 ºC. For each tomato line and replication, 6 to 10 completely red and firm ripe fruits were harvested randomly from different plants of the same genetic line.

ZFN-based *L1L4* mutant lines

ZFN technology was used to develop tomato lines in which the *L1L4* has been disrupted in the coding region (Hilioti, et al. 2016). Different *L1L4* disruption lines of M_4 generation were selected by genotyping.

RNA extraction and cDNA synthesis

RNA from leaf tissue (100 mg) was extracted using the NucleoSpin RNA plant kit (Macherey-Nagel). cDNA synthesis was performed using the PrimeScript first strand cDNA synthesis kit (TaKaRa). To detect DNA changes at target site, cDNA isolated from mutant lines of M4 generation and wild type (control) was used as template in PCR-based analysis.

Detection of ZFN-induced modifications at *L1L4* locus

High Resolution Melting (HRM) analysis on founder plants and selected progenies (M_4 generation) was performed using F1: GCTATCGCAGATCTCCACAACCAACC and R2: CGTTTGGTATGACTTCGTCTTGCTCCTG primers flanking the *L1L4* (*Solyc05g005350*) target site to amplify a 131 bp product in cDNA. HRM analysis was performed in a total volume of 15 µL on a Rotor-Gene 6000 real-time 5P HRM PCR Thermocycler (Corbett Research, Sydney, Australia). The reaction mixture contained 20 ng genomic DNA, 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 300 nM forward and reverse primers, 1.5 mM Syto® 9 green fluorescent nucleic acid stain (Life Technologies Corp., Paisley, UK) and 1 U Kapa Taq DNA

polymerase (Kapa Biosystems, Cape Town, South Africa). The PCR protocol was conducted in a 36-well carousel, using an initial denaturing step of 95 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 20 sec, annealing at 60 °C for 20 sec, extension at 72 °C for 20 sec and final extension at 72 °C for 10 min. All samples including the wild-type were plotted according to their melting profiles. Shape differences in melting curves relative to wild-type control were indicative of sequence changes within the amplicon analyzed. For sequencing data, primers F4: ATGGATGGTGGTTCAGGAAATG and R2 (as above) were used in PCR reactions with cDNA as template.

Fruit size, color evaluation and fruit quality parameters

Following fruit maturity, a minimum of 18 ripe tomatoes (3 replication with 6 fruits each one) from each line were evaluated for a series of phenotypic traits related to fruit size: fruit weight, in grams; fruit length was obtained by measuring, in centimeters, from stem to blossom end; fruit width was measured, in the equatorial zone of the fruit, using a caliper.

Tomato surface color was determined at 3 points around the fruit using a Chroma meter CR-300 series (CE Minolta, Japan) in CIE L* a* b* color space. The L* value indicates lightness (black=0 and white=100), a* changes from green (negative values) to red (positive values) and b* from blue (negative values) to yellow (positive values). The a* and b* readings were converted to the vectorial coordinates hue angle (h) and chroma (C*) using the equations h = arc tan b*/a* and C* = (a*2 + b*2)1/2, respectively (McGuire 1992).

Firmness was determined on two opposite sides of each fruit using a Chatillon Force TCD 200 and Digital Force Gauge DFIS 50 (Jonh Chatillon & Sons, Inc., Largo, FL, USA), by measuring

the force required for a 6-5 diameter probe (conical for the last 3 mm) to penetrate a tomato fruit to a depth of 7 mm through the skin.

After quantification of the parameters referred to above, the fruits from each replication were longitudinally cut into quarters and all the seeds were extracted for separate analysis. Three quarters of each fruit were stored at -80 ºC for later use. The remaining quarter of each fruit in each replication was ground using an UltraTurrax mixer T18 (IKA, Starfen, Germany) and the juice was extracted by squeezing the fruit mixture through cheesecloth. The juice aliquot was collected and frozen at -80 ºC until use. The remaining juice obtained was used to quantify soluble solids content (SSC) and titratable acidity (TA). SSC was measured in juice using a digital refractometer (Model PR-100, Atago Co., Tokyo, Japan).

Titratable acidity was determined by titration of the juice with 0.1M of NaOH to the end-point of pH 8.2 and the results expressed as percentage of citric acid.

A taste index (TI) was calculated from the SSC data and the titratable acidity, as previously described (Navez, et al. 1999), using the following expression: TI = [SSC pulp/(20*titratable acid)] + titratable acidity.

Fruit composition

Moisture, fiber, protein and ash content

Moisture was determined by desiccation at 105 ºC for about 24 h till constant weight (Horwitz, et al. 2000). Total dietary fiber (TDF) was evaluated using a commercial kit (Sigma-Aldrich, St. Louis MO, USA). This assay determines the TDF content using a combination of enzymatic and

gravimetric methods (Horwitz, et al. 2000). Lyophilized tomato samples (1 g) were gelatinized with a heat-stable α-amylase (pH 6, 100 °C, 15 min) and then enzymatically digested sequentially with protease (pH 7.5, 60 °C, 30 min) and amyloglucosidase (pH 6, 60 °C, 30 min). TDF was precipitated with ethanol, and after washing and drying, the residue was weighed. Part of the residue was analyzed for nitrogen content by the Kjeldahl method (Bradstreet 1954). Nitrogen content was multiplied by a conversion factor of 6.25 to calculate protein content. Another part of the residue was used for ash analysis by combustion in a furnace at 550 ºC for 5 hours. TDF values were recorded after subtracting protein and ash contents.

Extraction, quantification of sugars and sweetness index

Extraction and quantification of sugars (fructose, glucose and sucrose) were based on a method described by (Terry, et al. 2007) and modified as described previously (Magwaza, et al. 2012). Briefly, a 150 ± 0.5 mg of fruit powder was extracted in 3 mL 62.5% (v/v) aqueous methanol. Following extraction, the concentrations of fructose, glucose and sucrose were determined in an HPLC binary pump system (L-2130, Elite LaChrom series, Hitachi, Japan). Ten µL of a diluted sample solution (1:10) was injected into a Purospher Star NH_2 (amino) column (4.6 mm diameter × 250 mm, 5 µm particle size; Merck Millipore, Germany) with an amino guard column (LiChroCART 4-4 Merck Millipore, Germany). The thermostatic column compartment temperature was set at 35 ºC. The mobile phase used was HPLC-grade water at a flow rate of 1.0 mL/min and the presence of carbohydrates was detected on a refractive index detector (RID, L-2490, Elite LaChrom series, Hitachi, Japan). Sugars were quantified from a linear standard curve (0.05–1.25 mg/mL; average R2= 0.99).

Extraction and quantification of non-volatile organic acids

Non-volatile organic acids (ascorbic, citric and oxalic acid) were extracted and determined using a method described previously (Crespo, et al. 2010) with slight modifications (Magwaza, et al. 2013). Briefly, 50 ± 0.5 mg of freeze dried samples were cold extracted for 5 min in 3 mL of HPLC water. The flocculate was filtered through a 0.2 m syringe filter before HPLC analysis. Ascorbic, citric and oxalic acid concentrations were determined on a HPLC binary pump system equipped with a diode array detector (DAD, L-2455, Elite LaChrom series, Hitachi, Japan) with multiple wavelength detector, degasser and cooled autosampler. The filtered sample extract (20 µL) was injected into a Purospher Star RP-18 column (4.6 mm diameter × 250 mm, 5 µm particle size, Merck Millipore, Germany) with an organic acid guard column (LiChroCART 4-4 Merck Millipore, Germany). Temperature of the column was set to 35 ºC using a thermostated column compartment (L-2300, Elite LaChrom series, Hitachi, Japan). The mobile phase used was 0.2% HPLC-grade aqueous metaphosphoric acid at a flow rate of 1.0 mL/min. Non-volatile organic acids were detected at 210 nm except for ascorbic acid which was detected at 245 nm and quantified using linear standard curves (0.01–1.25 mg/mL; average R2= 0.99).

Antioxidant composition

Extraction and assay of total phenols (Folin-Ciocalteau)

The total phenolic content was determined using the Folin-Ciocalteau reagent and gallic acid as standard as described by Slinkard and Singleton (Slinkard and Singleton 1977). Tomato juice (0.2 mL) or gallic acid concentration was mixed with 0.8 mL of an aqueous sodium carbonate solution (75 g L^{-1}) and were added to 1 mL of 10% (v/v) Folin-Ciocalteau reagent. After 30 min of reaction at room temperature, the absorbance was measured at 765 nm in a Shimadzu, UV-visible recording spectrophotometer model UV-160A (Shimadzu Corporation, Kyoto, Japan).

β-Carotene and lycopene

β-Carotene and lycopene were determined following a procedure previously described (Nagata and Yamashita, I 1992; Pinela, et al. 2012), measuring the absorbance at 453, 505, 645 and 663 nm. Contents were calculated according to the following equations: β-carotene (mg/100 ml) = $0.216 \times A_{663} - 1.220 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}$; lycopene (mg/100 ml) = $-0.0458 \times A_{663} + 0.204 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}$, and further expressed in mg per 100 g fruit. A_{663}, A_{645}, A_{505} and A_{453} are absorbance at 663nm, 645nm, 505nm and 453nm, respectively.

Flavonoid content

Flavonoid content was quantified as described by (Ahn, et al. 2007). Briefly, 0.5 mL of 2% $AlCl_3$-ethanol solution was added to 0.5 mL of sample or standard. After 1 h at room temperature, the absorbance was measured at 420 nm. Quercetin was used as standard for the construction of calibration curve.

Evaluation of antioxidant activity

Oxygen radical absorbance capacity (ORAC)

The antioxidant activity by the method ORAC measures the ability of samples for scavenging peroxyl radicals. The ORAC method used, with fluorescein (FL) as the fluorescent probe, was that described previously (Ou, et al. 2001). As the ORAC assay is extremely sensitive, the samples must be diluted appropriately before analysis to avoid interference. In each well, 150 µL of fluorescein working solution and 25 µL sample previously diluted, blank (75 mM phosphate buffer) or standard (Trolox) were placed. The plate was covered with a lid and incubated in the pre-heated (37 °C) Tecan Infinite M200 Microplate Reader for 10 min with a previous shaking of 3 min. The 2, 2-Azobis-2-methyl-propanimidamide dihydrochloride (AAPH) was added to

each well of the plate, except for the control and blank. The final volume of the assay was 200 µL. The fluorescence was read every minute for 90 min at excitation of 485 nm and emission of 527 nm. The ORAC values are calculated according to a previous work (Huang, et al. 2002). Briefly, the net area under the curve (AUC) of the standards and samples was calculated. The standard curve was obtained by plotting Trolox concentrations against the average net AUC of the two measurements for each concentration. Final ORAC values were calculated using the regression equation between Trolox concentration and the net AUC and were expressed as mmol Trolox/100 g fresh weight.

Trolox equivalent antioxidant activity (TEAC)

The preformed radical monocation of 2, 2-azinobis-(3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS) was generated according to the modified method of Re et al. (Re, et al. 1999), as described elsewhere (Antunes, et al. 2010). For the assay, 10 µL of the extract was added to 990 µL of ABTS radical cation solution. The absorbance was monitored spectrophotometrically at 735 nm for 6 min (Shimadzu spectrophotometer 160-UV). The antioxidant activity of each sample was calculated using the following equation: scavenging effect % (IA%) = (1 − Af/A0) × 100, where A0 stands for the absorbance of the control and Af for the absorbance in the presence of the sample. The values were compared with the standard curve constructed with a series of Trolox concentrations and the values were expressed as mM Trolox equivalent antioxidant capacity.

LC-MC/MS-based metabolic profiling in tomato seeds

Wild-type and *L1L4* mutant seeds of the M_4 generation were ground in liquid nitrogen and stored at -80 °C. Mature seed tissue was extracted in 80% methanol (HPLC grade) and then vortexed

for about 20 s. Subsequently, the samples were centrifuged at 11,000 g for 15 min at 4 °C to remove protein pellets. Next, the cleared supernatants containing extracted metabolites were transferred into aliquots for LC-MS/MS analyses. Metabolites were identified by triplicate analysis of single seed extracts.

Statistical analysis

Analysis of variance (ANOVA) was performed on the data and, when needed, means were compared using Duncan's Multiple Range Test at $P < 0.05$. Pearson correlation was used to test whether two traits varied together for all possible pairwise comparisons. Software utilized for the statistical treatments was SPSS (SPSS Inc., Chicago, USA). Principal component analysis (PCA) and hierarchical cluster analysis (HCA) were performed using the statistical software Chemoface version 1.5 (Nunes, et al. 2012).

Metabolic and gene pathway inference

The metabolic pathways as well as the gene identities in each pathway have been computationally predicted by PMN (http://pmn.plantcyc.org/) using species-specific pathway databases, selection of the initial and end metabolite and specification of the number of maximum path length.

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AUTHOR CONTRIBUTION STATEMENT

Z.H. and. D. A designed, performed and supervised experiments, analyzed data and wrote the article; C.G. and V.D. performed experiments; analyzed data and wrote parts of the article; K. P., A. G., and G. M. performed experiments of the project and edited the article.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Table legends

Table 1. Quality parameters of tomato fruit from wild-type and *L1L4* disruption lines.

Table 2. Composition of fruit from wild-type tomato and *L1L4* disruption lines.

Table 3. Antioxidant composition and antioxidant activity from wild-type tomato fruit and *L1L4* disruption lines.

Figure legends

Figure 1. **Selection of lines carrying different single gene mutations**

(A) Mutation scanning at *L1L4* locus using HRM analysis. A 131-bp fragment (exon 1 end – exon 2 beginning) was amplified from each cDNA sample of 8 ZFN-*L1L4* tomato lines of M_4 generation and a wild-type background. Shown are the normalized temperature HRM curves (WT: red, Line 2: magenta, Line 3: orange, Line 4: beige, Line 5: olive, Line 6: pink, Line 7: blue, Line 8: cyan, Line 9: green). The eight different genotypes of *L1L4* gene at the specified region can be seen as curves with shape differences. (B) Genotypes of the *L1L4* disruption lines. Target site sequences of wild type (WT) and mutant lines (2-9) are aligned. ZFN binding sites are shown in bold letters. A deletion is depicted with dash, an insertion with parenthesis and nucleotide change with red color.

Figure 2. Tomato fruit phenotyping

Tomato fruit phenotypes from wild-type and M_4 ZFN-*L1L4* lines.

Figure 3. Principal component analysis of fruit quality parameters

(A) Scores and loading plot for PC1, PC2 and PC3 for wild-type tomato fruit and 8 *L1L4* gene disruption lines. The percentage of total variance explained from each principal component is shown in parentheses. WT=wild-type; 2=line 2; 3=line 3; 4=line 4; 5=line; 6=line 6; 7=line 7; 8=line 8; 9=line 9. a=a* value; b= b* value; C*= chroma; L*=lightness; h=hue angle; fd=fruit diameter; fl=fruit length; fw=fruit weight; fm=firmness, TA=titratable acidity; TI=taste index; mo=moisture; pr=protein; fib=fiber; fru=fructose; glu=glucose; fla=flavonoids; ly=lycopene; bc=β-carotene; tp=total phenols content; asc=ascorbic acid; cit=citric acid; oxa=oxalic acid; qui=quinic acid; shi=shikimic acid; suc=succinic acid; TE=trolox equivalent antioxidant activity; OR=oxygen radical absorbance capacity. (B) Dendogram of wild-type tomato fruit and 8 *L1L4* gene disruption lines based on the measured quality and physicochemical variables.

Figure 4. Seed metabolic profiling

Metabolic profiles in tomato mature seeds from *L1L4* disruption lines and wild-type based on LC-MS/MS data. Relative amounts of metabolites in mature seeds from different mutant lines and wild-type background are expressed as % of the highest amount, which was set as ´100´. Results are the means ±SD.

Figure 5. In silico reconstruction of putative biosynthetic pathways

Inference of putative metabolic pathways affected by *L1L4* coding sequence disruption in lycopene, ascorbate and citrate biosynthesis. The predicted identities of genes encoding key enzymes in the metabolic pathways are shown. Key metabolites in each pathway are shown with squares and end products with circles. Significantly affected metabolites are shown with thick border.

Table 1. Quality parameters of tomato fruit from wild-type and *L1L4* gene disruption lines.

	Fruit quality parameters										
lines	Diameter (mm)	Length (mm)	Weight (g)	Lightness	a*	b*	a*/b*	Firmness (N)	Soluble solid content (%)	Titratable acidity	Taste index
WT	34.32±0.44**a**	51.35±0.4**a**	32.66±1.52**ab**	46.08±2.48**a**	22.03±2.98**a**	19.65±2.57**abc**	1.12±0.02**ab**	5.37±0.56**a**	3.6±0.17**d**	0.68±0.04**a**	0.95±0.03**abc**
2	34.06±1.38**a**	53.17±1.08**a**	35.77±5.25**ab**	44.56±1.92**a**	22.57±1.68**a**	22.33±3.09**ab**	1.04±0.10**ab**	4.46±0.36**a**	3.85±0.06**cd**	0.65±0.03**ab**	0.95±0.01**abc**
3	32.7±0.38**a**	51.55±1.00**a**	29.35±0.55**b**	46.13±2.66**a**	21.67±1.67**a**	24.76±1.88**a**	0.94±0.02**b**	4.92±0.9**a**	3.9±0.4**cd**	0.49±0.01**d**	0.89±0.02**c**
4	35.79±0.29**a**	56.31±1.23**a**	40.51±1.86**a**	42.15±1.69**a**	22.17±2.07**a**	20.35±1.83**abc**	1.09±0.00**ab**	4.84±0.34**a**	4.55±0.1**ab**	0.56±0.01**bcd**	0.97±0.01**ab**
5	34.34±1.6**a**	55.18±3.29**a**	37.01±5.51**ab**	47.73±0.19**a**	17.37±0.11**a**	14.77±0.99**c**	1.19±0.09**a**	5.21±0.36**a**	3.93±0.03**cd**	0.56±0.03**cd**	0.91±0.01**bc**
6	36.02±1.11**a**	53.57±1.53**a**	38.18±2.9**ab**	45.82±1.77**a**	19.48±1.97**a**	16.24±2.21**bc**	1.21±0.04**a**	5.28±0.84**a**	4.2±0.1**bc**	0.62±0.04**abc**	0.96±0.02**ab**
7	36.69±0.35**a**	52.11±1.48**a**	38.61±1.02**ab**	43.25±1.87**a**	19.57±1.20**a**	17.35±2.34**bc**	1.17±0.15**a**	4.65±0.35**a**	4.58±0.2**ab**	0.65±0.01**ab**	1.00±0.00**a**
8	35.6±2.57**a**	53.73±1.62**a**	31.77±2.4**ab**	44.42±2.02**a**	22.28±2.55**a**	21.01±2.13**abc**	1.06±0.07**ab**	5.4±0.74**a**	3.81±0.51**cd**	0.55±0.04**cd**	0.90±0.04**c**
9	35.66±1.08**a**	55.12±2.01**a**	38.98±3.44**ab**	44.09±1.47**a**	21.72±1.51**a**	17.65±2.13**abc**	1.25±0.08**a**	4.55±0.4**a**	4.68±0.46**a**	0.55±0.02**cd**	0.98±0.02**ab**

Values are mean±SE. The values followed by the same letter, in the same column/ parameter are not significantly different (Duncan's new multiple range test at $P<0.05$)

Table 2. Composition of fruit from wild-type (WT) tomato and *L1L4* gene disruption lines.

	Fruit composition											
	Moisture (%)	Fiber (%)	Protein (%)	Ash (%)	Fructose (g/kg DW)	Glucose (g/kg DW)	Ascorbic acid (g/kg DW)	Citric acid (g/kg DW)	Oxalic acid (g/kg DW)	Quinic acid (g/kg DW)	Shikimic acid (g/kg DW)	Succinic acid (g/kg DW)
WT	95.34±0.21**a**	1.77±0.08**b**	0.81±0.11**a**	0.43±0.02**a**	43.58±1.28**b**	34.17±5.12**a**	1.03±0.01**b**	21.72±3.46**a**	20.67±3.9**a**	6.67±0.12b**c**	0.22±0.004**c**	n.d.
2	94.88±0.11**ab**	1.99±0.18**ab**	0.48±0.03**e**	0.43±0.04**a**	53.2±0.82**a**	32.92±0.32**a**	1.00±0.07**b**	7.66±1.77**d**	5.84±1.11**de**	n.d.	0.22±0.007**c**	0.26±0.08**d**
3	94.25±0.22**bc**	2.08±0.18**ab**	0.57±0.01**de**	0.48±0.05**a**	54.16±1.15**a**	34.17±0.56**a**	1.21±0.01**b**	15.93±1.08**bc**	9.26±0.37**cd**	5.03±1.16**bc**	0.26±0.011**a**	0.36±0.08**d**
4	93.97±0.1**cd**	2.56±0.18**a**	0.63±0.04**bcde**	0.56±0.02**a**	52.32±1.67**a**	33.71±0.52**a**	2.80±0.95**a**	10.96±0.41**cd**	9.03±0.53**cd**	2.66±0.84**d**	0.25±0.009**ab**	1.16±0.03**a**
5	94.18±0.44**bc**	2.39±0.33**a**	0.58±0.04**de**	0.59±0.06**a**	53.65±1.5**a**	33.95±0.35**a**	n.d.	15.73±1.67**bc**	3.74±0.29**e**	3.64±0.25**cd**	0.25±0.006**ab**	0.26±0.01**d**
6	93.56±0.32**cd**	2.10±0.16**ab**	0.71±0.04**abcd**	0.58±0.02**a**	54.1±0.87**a**	34.58±0.31**a**	0.92±0.05**b**	16.63±0.93**abc**	12.71±1.04**bc**	11.51±1.26**a**	0.24±0.001**abc**	0.70±0.03**c**
7	93.4±0.09**cd**	2.12±0.07**ab**	0.75±0.02**abc**	0.51±0.04**a**	52.69±0.51**a**	33.57±0.32**a**	0.98±0.04**b**	20.29±2.63**ab**	17.17±1.49**ab**	3.73±0.22**cd**	0.23±0.005**bc**	0.95±0.06**b**
8	94.14±0.41**bc**	2.36±0.14**a**	0.78±0.06**ab**	0.52±0.07**a**	46.89±1.25**b**	31.51±0.6**a**	0.98±0.09**b**	21.88±0.43**a**	19.19±0.68**a**	4.23±0.11**cd**	0.25±0.004**ab**	1.25±0.09**a**
9	93.2±0.31**d**	2.31±0.14**ab**	0.60±0.02**cde**	0.55±0.07**a**	54.21±0.49**a**	34.26±0.06**a**	1.04±0.11**b**	13.87±0.86**c**	12.78±0.96**bc**	3.23±0.19**cd**	0.25±0.007**ab**	0.16±0.08**d**

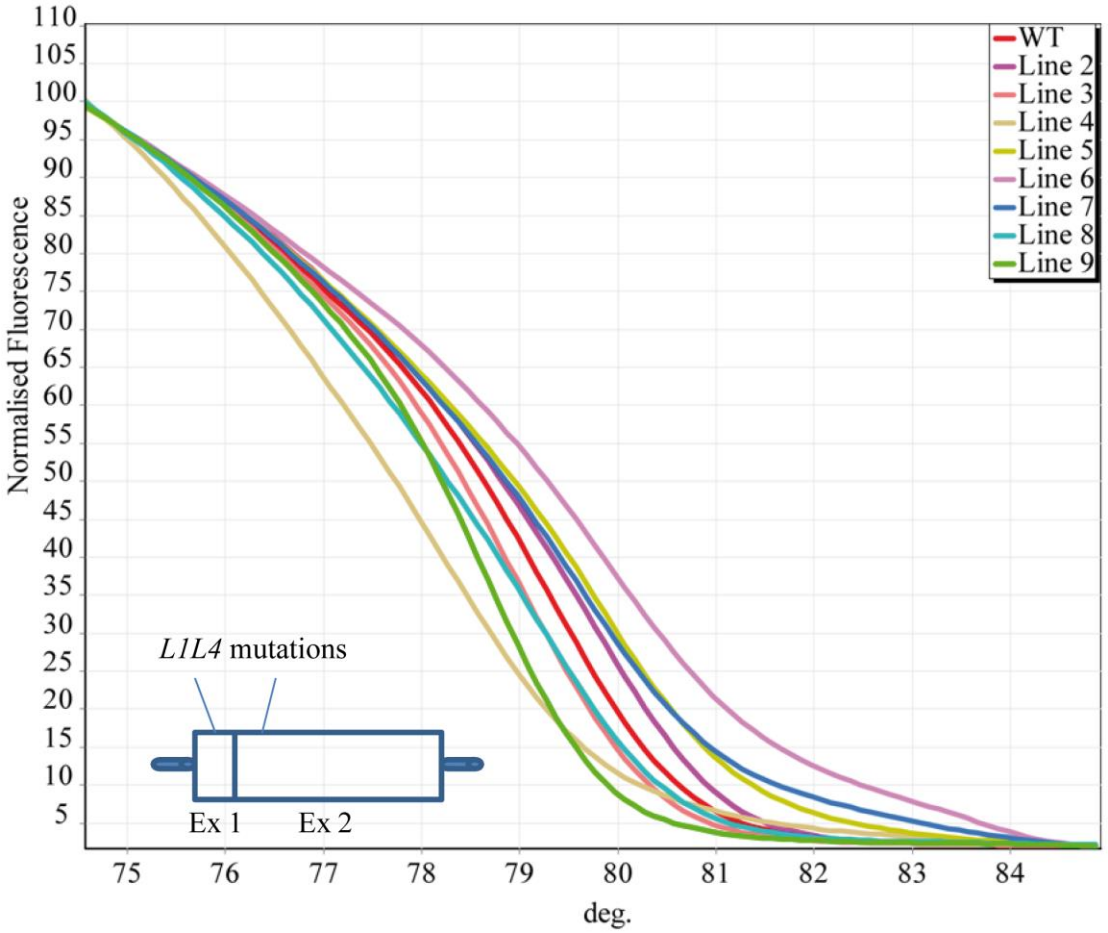
Values are mean±SE. The values followed by the same letter, in the same column/ parameter are not significantly different (Duncan's new multiple range test at P<0.05). n.d = not detected.

Table 3. Antioxidant composition and antioxidant activity from wild-type (WT) tomato fruit, and from 8 more *L1L4* gene disruption lines.

	Antioxidant composition and activity					
	Total phenols (mg gallic acid/100g FW)	Flavonoids (g quercetin/ 100g FW)	Lycopene (mg/ 100g FW)	β-carotene (mg/ 100g FW)	TEAC (µmol TE/100g FW)	ORAC (µmol TE/100g FW)
WT	19.51±0.47**c**	0.54±0.12**a**	6.28±0.72**abc**	0.74±0.1**b**	37.75±3.61**d**	2.2±0.003**c**
2	23.18±0.89**b**	0.5±0.1**a**	5.98±1.02**abc**	0.59±0.07**b**	53.56±3.15**abc**	2.22±0.024**bc**
3	25.16±0.66**b**	0.59±0.13**a**	5.83±0.47**bc**	0.71±0.07**b**	56.66±1.55**ab**	2.27±0.012**ab**
4	23.62±0.93**b**	0.57±0.02**a**	5.66±0.31**bc**	0.44±0.09**b**	45.01±1.68**cd**	2.24±0.025**bc**
5	24.31±1.22**b**	0.58±0.03**a**	7.23±0.09**ab**	0.65±0.05**b**	50.97±3.22**abc**	2.28±0.01**ab**
6	29.31±0.56**a**	0.64±0.03**a**	7.51±0.08**a**	2.66±0.19**a**	42.24±5.11**cd**	2.33±0.04**a**
7	23.34±1.14**b**	0.68±0.05**a**	7.5±0.08**a**	2.32±0.43**a**	60.37±1.21**a**	2.26±0.002**bc**
8	22.64±1.11**b**	0.74±0.1**a**	5.1±0.37**c**	0.57±0.04**b**	45.86±4.26**bcd**	2.23±0.012**bc**
9	29.03±0.4**a**	0.54±0.04**a**	7.12±0.28**ab**	2.66±0.25**a**	48.91±4.79**bcd**	2.27±0.016**ab**

Values are mean±SE. The values followed by the same letter, in the same column/ parameter are not significantly different (Duncan's new multiple range test at $P<0.05$)

A



B

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GCTATCGCAGATCTCCACAACCAACCCCTGCAGATCCCCCTTCCTCTGATGTGGAGATGAGCCTGGAACTGCCTGCACATCTCAACCAGGAAGT   WT
GCTATCGCAGATCTCCACAACCAACCCCTGCAGATCCCCCTTCCTCTGATGTGGAGATGAGCCTGAACCGCCATGCACATCTCAACCAGGAAGT   2
GCTATCGCAGATCTCCACAACCAACCCCTGCAGATCCCCCTTCCT--GATGTGGAGATGAGCCTGGAACTGCCTGCACATCTCAACCAGGAAGT   3
GCTATCGCAGATCTCCACAACCAACCCCTGCATATCCCCCTTCCTCTGATGTGGAGATGAGCCTGGAACTGCCTGCACATCTCAACCAGGAAGT   4
ATCGCAGATCTCCACAACCAACCCCTGCA(G)TATCCCCCTTCCTCTGATGTGGAGATGAGCCTGGAACTGCCTGCACATCTCAACCAGGAAGT   5
GCTATCGCAGATCTCCACAACCAACCCCTGCAGATCCCCCTTCCTCTGATGTGGAGATGGAGCCTGGAACTGAC(A)(A)TGCACATCTCAACC   6
GCTATCGCAGATCTCCACAACCAACCCCTGCAGATCCCCCTTCCTCTGATGTGGAGATGAGCCTGGAACTGCCTGCACATCTCAACCAGG(G)AAG   7
          CAACCACCGCCAGCAGATCCCCCTTCCCCTGATG-GGAGGGGGCC-GGAACTGCCAACACT-ATCAACCA                   8
     CAACCA-CCCCTGCAG(T)ATCCCCCTTCCTCTGATGTGGAGATGAGCCTGGAACTGCCTGCACATCTCAACCAGGAAGT   9
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Figure 1. **Selection of lines carrying different single gene mutations**

(A) Mutation scanning at *L1L4* locus using HRM analysis. A 131-bp fragment (exon 1 end – exon 2 beginning) was amplified from each cDNA sample of 8 ZFN-*L1L4* tomato lines of M_4 generation and a wild-type background. Shown are the normalized temperature HRM curves (WT: red, line2: magenta, Line 3: orange, Line 4: beige, Line 5: olive, Line 6: pink, Line 7: blue, Line 8: cyan, Line 9: green). The eight different genotypes of *L1L4* gene at the specified region can be seen as curves with shape differences. (B) Genotypes of the *L1L4* disruption lines. Target site sequences of wild type (WT) and mutant lines (2-9) are aligned. ZFN binding sites are shown in bold letters. A deletion is depicted with dash, an insertion with parenthesis and nucleotide change with red color.

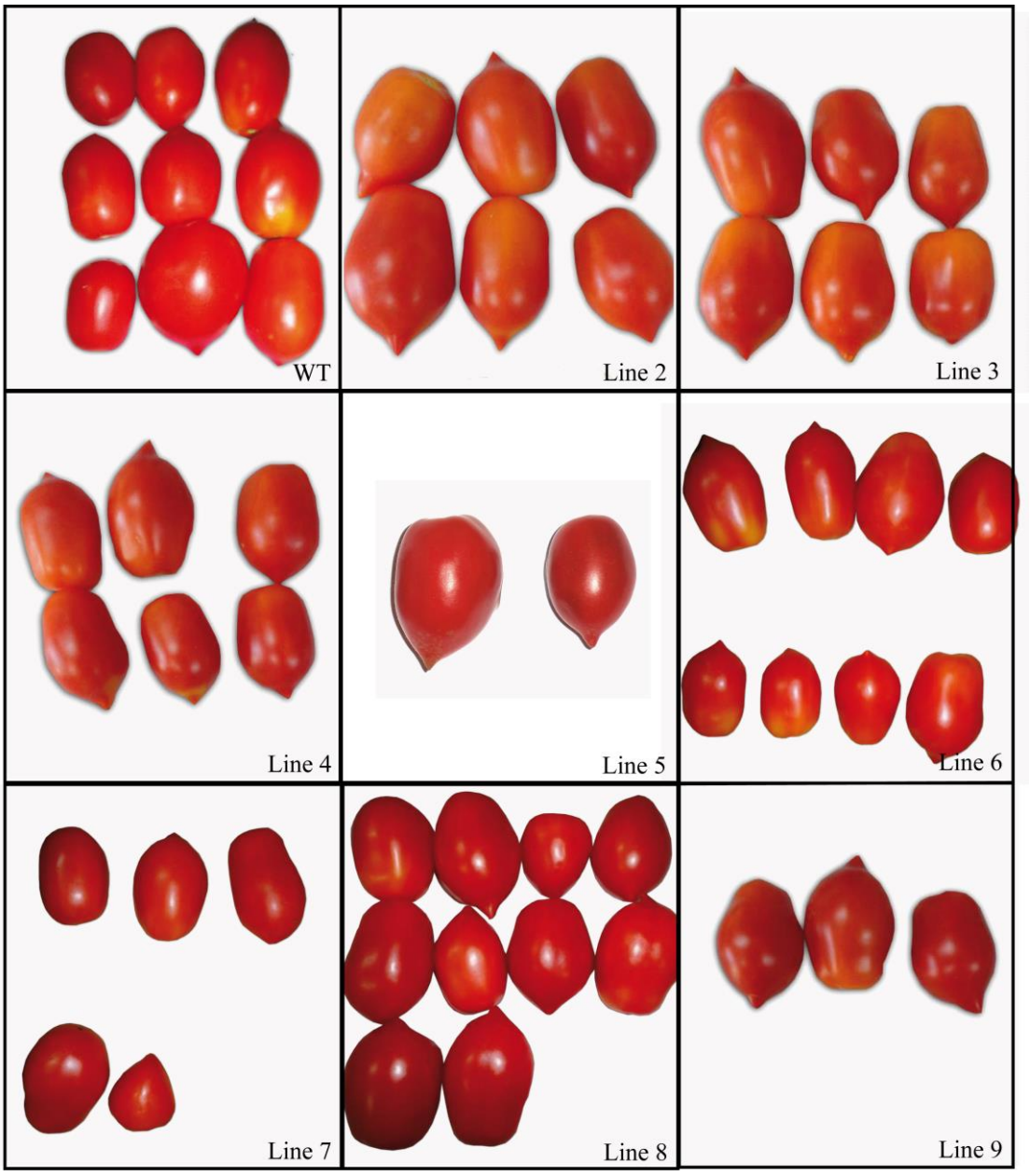

Figure 2. **Tomato fruit phenotyping**

Tomato fruit phenotypes from wild-type and M_4 ZFN-*L1L4* lines.

Figure 3

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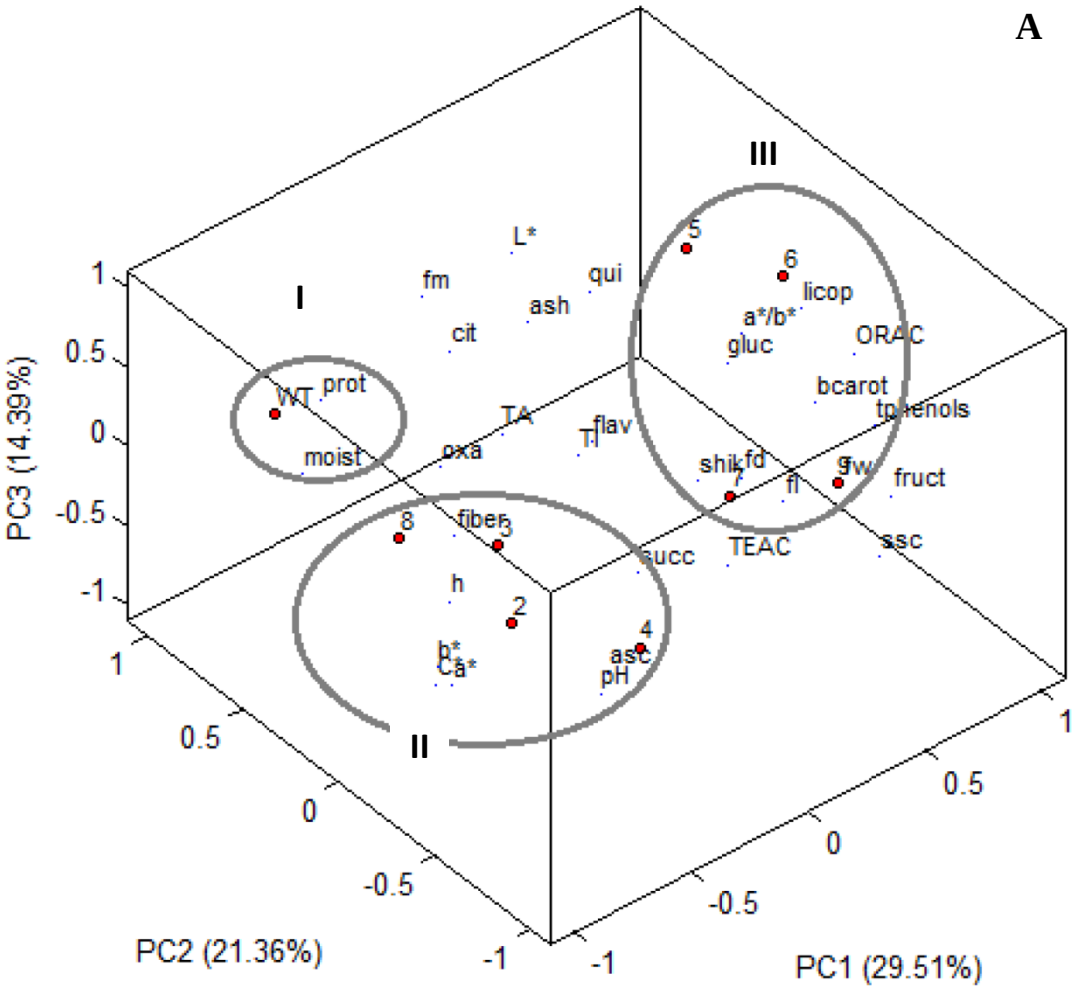

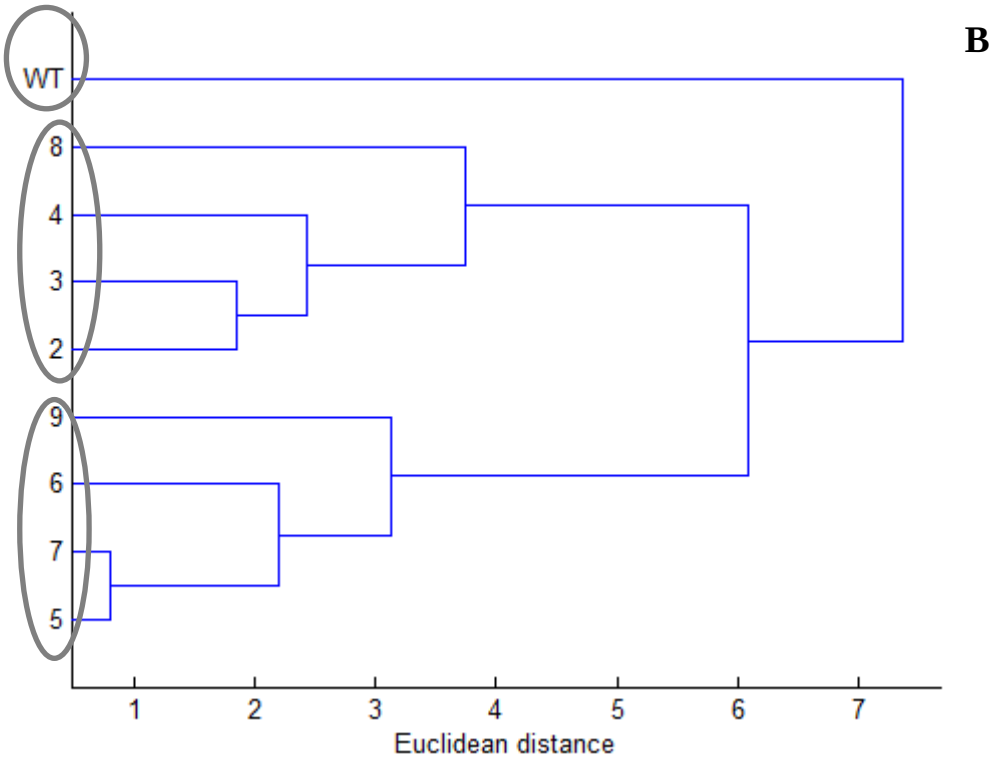

Figure 3. **Principal component analysis of fruit quality parameters**

(A) Scores and loading plot for PC1, PC2 and PC3 for wild-type tomato fruit and 8 *L1L4* gene disruption lines. The percentage of total variance explained from each principal component is shown in parentheses. WT=wild-type; 2=line 2; 3=line 3; 4=line 4; 5=line; 6=line 6; 7=line 7; 8=line 8; 9=line 9. a=a* value; b= b* value; C*= chroma; L*=lightness; h=hue angle; fd=fruit diameter; fl=fruit length; fw=fruit weight; fm=firmness, TA=titratable acidity; TI=taste index; moist=moisture; prot=protein; fruct=fructose; gluc=glucose; flav=flavonoids; licop=lycopene; bcarot=β-carotene; tphenols=total phenols content; asc=ascorbic acid; cit=citric acid; oxa=oxalic acid; qui=quinic acid; shik=shikimic acid; succ=succinic acid; TEAC=trolox equivalent antioxidant activity; ORAC=oxygen radical absorbance capacity. (B) Dendogram of wild-type tomato fruit and 8 *L1L4* gene disruption lines based on the measured quality and physicochemical variables.

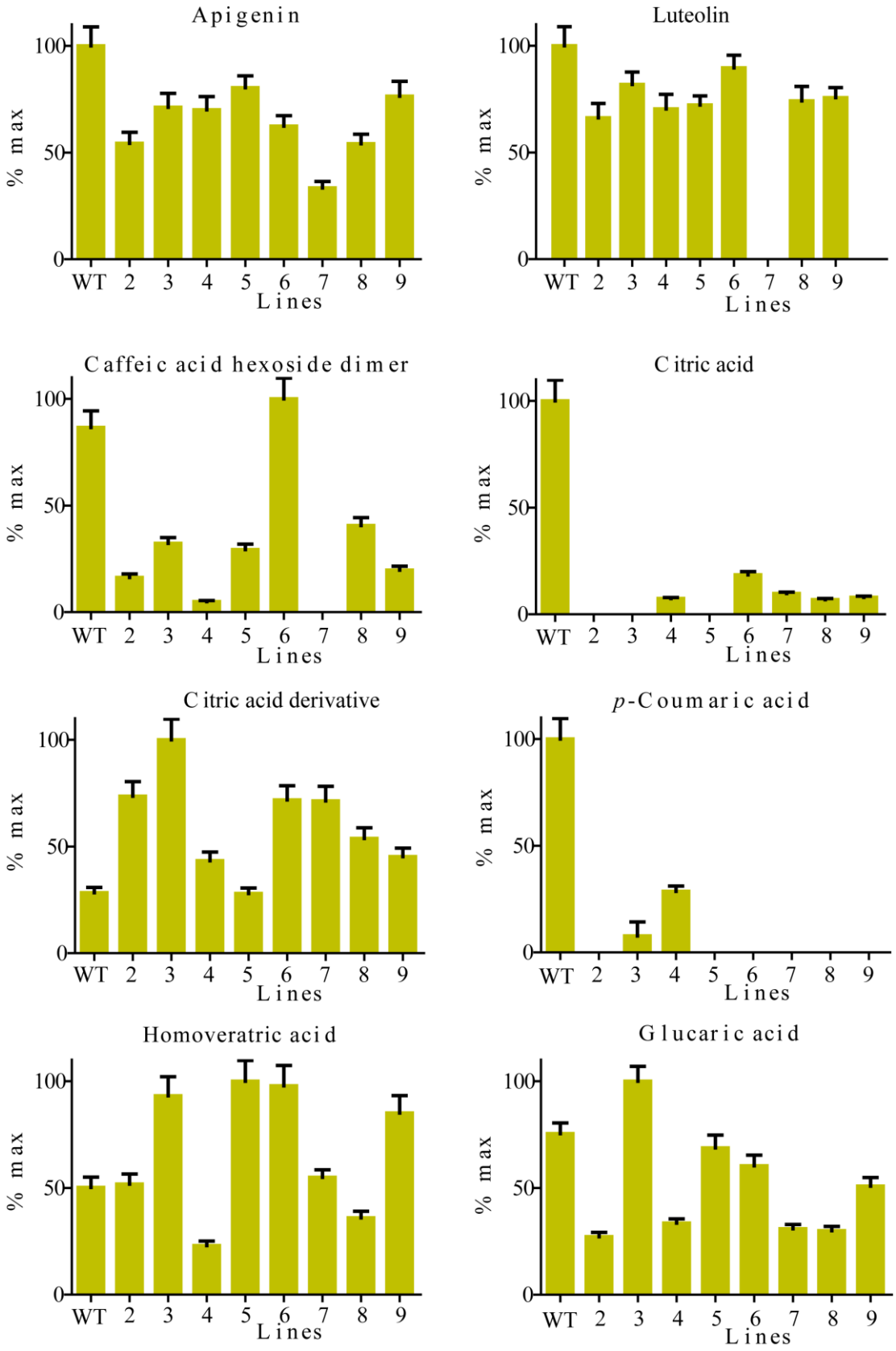

Figure 4. Seed metabolic profiling

Metabolic profiles in tomato mature seeds from *L1L4* disruption lines and wild-type. Metabolites identified in mature seeds based on LC-MS/MS data. Relative amounts of metabolites in mature seeds from different mutant lines and wild-type background expressed as % of the highest amount, which was set as ´100´. Results are the means ±SD.

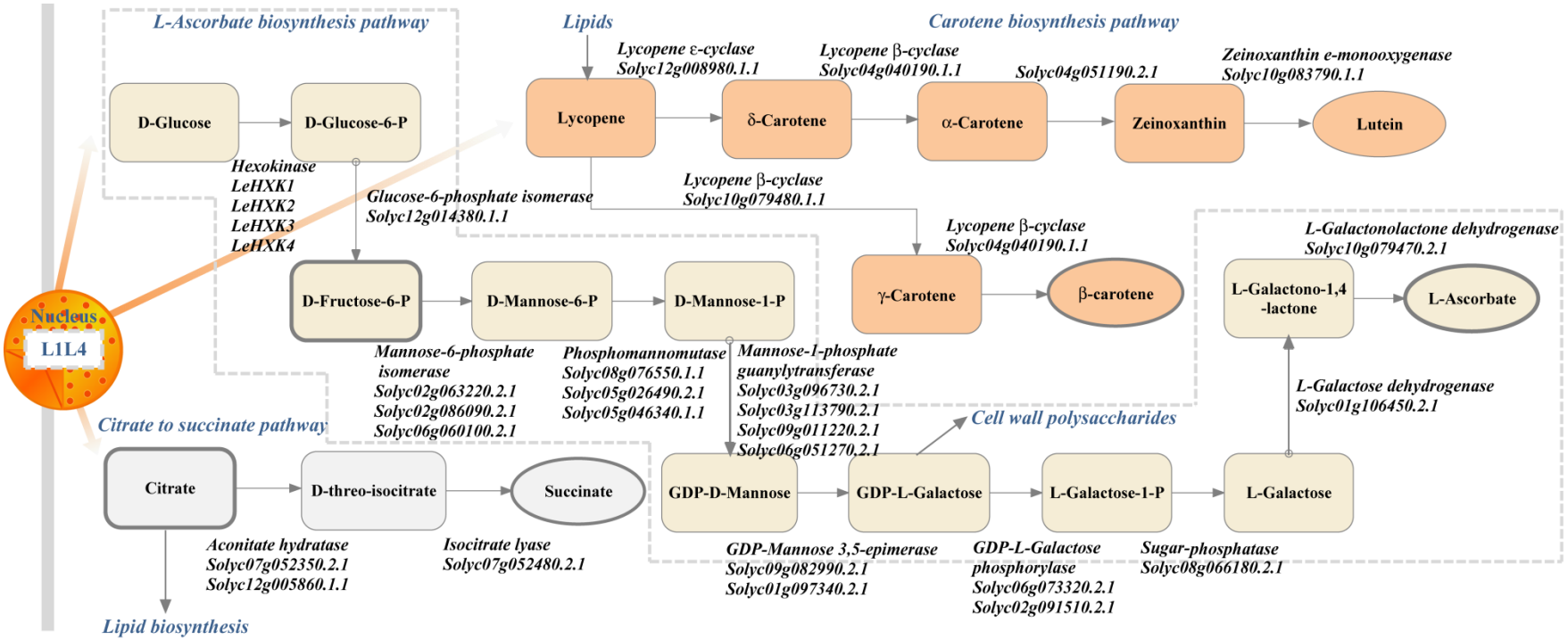

Figure 5. In silico reconstruction of putative biosynthetic pathways

Inference of putative metabolic pathways affected by *L1L4* coding sequence disruption in lycopene, ascorbate and citrate biosynthesis. The predicted identities of genes encoding key enzymes in the metabolic pathways are shown. Key metabolites in each pathway are shown with squares and end products with circles. Significantly affected metabolites are shown with thick border.