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Identification of candidate genes for phenolics accumulation in tomato fruit

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ABSTRACT

Phenolics are antioxidants present in tomato fruit that confer healthy benefits and exhibit crucial roles for plant metabolism and response to environmental stimuli. An approach based on two genomics platforms was undertaken to identify candidate genes associated to higher phenolics content in tomato fruit. A comparative transcriptomic analysis between the *S. pennellii* Introgression Line 7-3, which produced an average higher level of fruit phenolics, and the cultivated variety M82, revealed that their differences are attributed to genes involved in phenolics accumulation into the vacuole. The up-regulation of genes coding for one MATE-transporter, one vacuolar sorting protein and three GSTs supported this hypothesis. The observed balancing effect between two ethylene responsive factors (ERF1 and ERF4) was also hypothesized to drive the transcriptional regulation of these transport genes. In order to confirm such model a TILLING platform was explored. A mutant was isolated harbouring a point mutation in the *ERF1* cds that affects the protein sequence and its expected function. Fruits of the mutant exhibited a significant reduced level of phenolics than the control variety. Changes in the expression of genes involved in sequestration of phenolics in vacuole also supported the hypothesized key-role of ERF1 in orchestrating these genes.

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1. Introduction

In the last few years, medical research has highlighted the importance of the compositional quality of vegetable crops for human health [1–3]. Health benefits conferred by vegetables have been mainly attributed to the presence of health-promoting phytochemicals with antioxidant properties. Given its fruit chemical composition and extensive consumption worldwide, tomato (*Solanum lycopersicum*) is an important source of antioxidants for human nutrition, such as phenolics, carotenoids, tocopherols, and ascorbic acid. Regular consumption of tomato fruits has been associated with a decreased incidence of chronic degenerative and proliferative diseases such as some types of cancer and cardiovascular diseases [4,5]. In particular, among phenolics, flavonoids prevent heart diseases, decrease blood viscosity, reduce inflammatory responses and allergic reactions; in addition they have been reported to have anti-tuberculosis, antiviral and antimalarial activities [6].

Phenolics are a large group of molecules that also have important functions in plants [7]. Among these, stilbenes, coumarins, and isoflavonoids are implicated in defence mechanisms and are comprehensively defined phytoalexins. Flavonoids are UV protectants and anthocyanins are pigments responsible for colours of flower, fruit, vegetables, while salicylic acid (SA) is a signalling molecule involved in plant–microbe interactions and monolignols are the building units of lignin, the second most prevalent biopolymer on earth after cellulose. Phenolics content in plant tissues varies with species, varieties, organs, soil properties and climatic conditions. These secondary metabolites accumulate, generally, in all plant organs (roots, stems, leaves, flowers and fruits) although preferentially in the aerial organs. In the fruit, the outer tissues are richer in flavonoids than other tissues, while chlorogenic acid and coumarines are more evenly distributed. In tomato fruit, flavonoids are typically present in the epidermal tissues at

Abbreviations: ERF1, ethylene responsive factor 1; ERF4, ethylene responsive factor 4; FW, fresh weight; GAE, gallic acid equivalents; GSH, reduced glutathione; GSSG, oxidized glutathione; GSH + GSSG, total glutathione; GST, glutathione S-transferase; HPRG, histidine proline rich glycoprotein; IL, introgression line; MATE, multidrug and toxic compound extrusion; PR, pathogen related; QE, quercetin equivalents; TC, tentative consensus; TILLING, targeting induced local lesions in genomes; VSP, vacuolar sorting protein.

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different levels depending on the developmental stage [8]. In addition to the flavonoid naringenin chalcone that accumulates up to 1% dry weight of the tomato fruit cuticle, various other flavonoids accumulate in tomato fruit such as rutin (quercetin-3-rutinoside), kaempferol-3-O-rutinoside and a quercetin-trisaccharide [9,10].

Processes controlling the level of phenolics in plant tissues include biosynthesis and degradation/utilization of intermediates as well as vacuole sequestration. In tomato fruits several authors have extensively reported the wide panorama of phenolics production, with particular attention being paid to the flavonoids biosynthesis and storage [11–14]. Although genes that operate in the biosynthesis of flavonoids are already known, additional insights are required to elucidate the regulative mechanisms of their production and accumulation in tomato fruits.

At sub-cellular level, the two main sites of accumulation of phenolic compounds are the cell wall, where the lignin is deposited, and the vacuole, where different classes of phenolics are stored, particularly flavonoids. Since this compartmentalization is important for local maintenance of high metabolite concentrations, mechanisms for flavonoids and anthocyanins storage have been widely investigated and recently reviewed [15–18]. Briefly, among others, Grotewold and Davies [19] proposed two models to describe the vacuolar compartmentalization of flavonoids *via* intracellular movement from cytosol, the so-called vesicular (VT) and ligandin transport (LT) models. In the first, the transport is mediated by vesicle trafficking involving protein components, such as vacuolar sorting and cargo proteins, whereas the second model relies on membrane transporters, such as MATE and MRP-type ABC transporters. The latter mediates sequestration to the vacuoles of glutathione conjugates, whose conjugation is catalyzed by glutathione-S-transferases (GST), hence through a glutathione-dependent mechanism [20]. In the frame of the LT model, GSTs can also operate as carrier proteins in the transport of GST-flavonoid complexes through a glutathione-independent mechanism [21].

Multiple levels of transcriptional and post-transcriptional regulation have been implicated in controlling the biosynthesis and accumulation of phenolics. In particular, it has been demonstrated that ethylene and other hormones can modulate the intensity and the direction of regulative effects on metabolism and the final level of phenolics in plant tissues. Several authors reported that the production of phenolic compounds can be affected by exogenous treatments with ethylene [22–24] and/or by biotic and abiotic stresses, and that this effect could be mediated by ethylene responsive elements (EREs) and MYB factors [24]. Indeed, by using various Arabidopsis mutants, it has been recently demonstrated that auxin and ethylene regulate flavonol biosynthesis through MYB12-mediated signalling and that ethylene modulates flavonoid accumulation in the roots.

The accumulation of phenolics in tomato fruit is a complex trait controlled by various QTL [25,26] and strongly affected by the environment. In tomato the dissection of complex traits is enabled by genetic resources that have been available for a long time, such as introgression lines (ILs). These are homozygous lines with single chromosome segment substitutions from one wild relative [27]. The combined use of ILs and transcriptional profiling [28] might facilitate the rapid identification of candidate genes involved in the accumulation of fruit phenolics. The aim of this work was to identify regulative mechanisms and genes controlling the accumulation of phenolics in tomato fruit in the *S. pennellii* introgression line IL7-3, which harbours a positive QTL for phenolics content. For this purpose, a comparative transcriptomic approach was carried out on mature fruit from this introgression line and its cultivated parental line (M82). In particular, among candidate genes we identified an ethylene responsive factor 1 (*ERF1*) gene that was up-regulated in the *S. pennellii* IL7-3, which might directly or indirectly regulate a more efficient transport of phenolics to the vacuole. Characterization of a tomato *erf1* mutant line isolated through TILLING (targeting induced local lesions in genomes) technology [29,30] allowed us to suggest a role for the *ERF1* gene in coordinating the accumulation of phenolics in tomato fruits.

2. Materials and methods

2.1. Plant material

Two tomato accessions (LA4066 and LA3475) were used in this research and seeds were provided by the Tomato Genetics Resource Centre, University of California (Davis, USA). The accession LA4066 identifies the introgression line (IL) 7-3 which harbours a 32 cM single homozygous chromosome segment from the wild species *S. pennellii* (LA0716) in the genomic background of the processing tomato variety *S. lycopersicum* cv. M82 (LA3475). Plants were grown in greenhouse for four consecutive years. After seed germination, six seedlings *per* accession were transplanted into 20 cm pots containing a mixture of medium sandy soil and compost and transferred in a cold greenhouse of the Department of Agricultural Sciences at the University of Naples (Portici, Italy). Plants were randomly distributed, fertilized and irrigated daily until they reached maturity. Fruits were harvested when about 75% of them reached the red-ripe stage. Within a single trial, three fruit samples *per* each genotype were obtained from individual plants by pooling fully red-ripe fruits collected from the same plant. Samples were obtained from whole fruits discarding the seeds, jelly parenchyma, columella and placenta tissues and then were frozen by liquid nitrogen, grinded and stored at −80 °C.

2.2. Fruit measurements

Total phenolics were assayed using a modified procedure of the Folin–Ciocalteu's test [31]. In brief, 250 mg of frozen ground tissue were homogenized in a mortar with pestle and extracted using 1 ml of 60% methanol. Samples were transferred to a 1.5 ml tube and left on ice for 3 min in the dark. Crude extracts were transferred in a 15 ml tube and volume was increased to 5 ml adding 60% methanol. The samples were centrifuged at 3000 × *g* for 5 min; then, 62.5 μl of the supernatant, 62.5 μl of Folin–Ciocalteu's reagent (Sigma) and 250 μl of deionised water were mixed and incubated for 6 min; 625 μl of 7.5% sodium carbonate and 500 μl of deionised water were added to the samples and incubated for 90 min at room temperature in the dark. Absorbance was measured at 760 nm. The concentration of total phenolics was expressed in terms of mg of gallic acid equivalents (GAE) *per* 100 g of fresh weight (FW).

The flavonoid content was measured as reported by Marinova et al. [32]. In particular, 5 ml of 80% met/H_2O were added to 0.5 g of sample. 100 μl of distilled water and 30 μl of 5% $NaNO_2$ were added to 100 μl of extract. 30 μl 10% $AlCl_3$ were added 5 min later. After 6 min, 200 μl of 1 M NaOH were added and the total was made up to 1 ml with distilled water. Absorbance was measured at 510 nm. Total flavonoid content was expressed as mg of quercetin equivalents (QE) per 100 g FW.

Reduced (GSH) and oxidized (GSSG) glutathione were assessed using Glutathione Assay Kit (BioVision – Catalogue number K264-100) following the procedure suggested by the manufacturer. Fluorescence (Ex/Em 340/420 nm) of the samples was read with a VICTOR^{TM} *X* Multilabel Plate Reader (PerkinElmer). Concentrations were expressed as mg *per* g of FW.

Phenotypic data were statistically processed by using the Statistical Package for Social Sciences, version 18 (SPSS Inc Chicago, Illinois). Univariate ANOVA was applied to determine the effect of genotype and trial as fixed factors. The effect of the genotype

Table 1
Primer pairs used for qPCR validation of genes involved in the accumulation of phenolics that are differentially expressed in IL 7-3 and M82.

TIGR ID	Annotation	Primer sequence	
TC170110	Glutathione S-transferase-like protein	fw	5′-AGCAACGGGAGACGAACATC-3′
		rv	5′-ATCTCCAAGTGCTCCCTCCA-3′
TC170138	Ethylene-responsive transcription factor 4	fw	5′-GTCATCCAGCGGAGAAACGG-3′
		rv	5′-GCAGGTGGAAGTCGTCGCCG-3′
TC172097	Vacuolar sorting protein	fw	5′-TTGTGGCGACCTTATGATCC-3′
		rv	5′-GGCTTTATCTCCCAGGATTCAG-3′
TC172606	Glutathione S-transferase	fw	5′-TCCTTATGAAAGAGCCCATGCT-3′
		rv	5′-TTGTTGTCCATACCAATCTCCCT-3′
TC172943	Hydroxyproline-rich glycoprotein	fw	5′-TACCGGAAAAAGTGGTGGTTG-3′
		rv	5′-ACTGGTTTAGGAGGCTGAGGC-3′
TC174512	Ethylene-responsive factor 1	fw	5′-CGCGTAATGGAATTAGGGTTTG-3′
		rv	5′-CATTGATAATGCGGCTTGATCA-3′
TC176201	Glutathione S-transferase	fw	5′-TCTGCAGGTTCAACTGGAAGAA-3′
		rv	5′-GGAGGAGCGAAGGGAAAGAA-3′
TC177287	O-acyltransferase WSD1	fw	5′-TCCCATGCTGAGGCAACTTC-3′
		rv	5′-GGGCAATTCCATCTCCAAGAG-3′
TC177388	Osmotin-like protein	fw	5′-ACGTCTCAATCGAGGCCAAA-3′
		rv	5′-ACGTGCCATCTTAGTTCCCCT-3′
TC178155	Chymotrypsin inhibitor-2	fw	5′-TTTGTCCAGGTGTGACAAAGGA-3′
		rv	5′-GCAAACTTAGCTGGTGTTCCAAG-3′
TC178409	Multidrug resistance protein mdtK	fw	5′-GCTTTGCTTGGATATGTGGC-3′
		rv	5′-CTCTTCATCCCAATCTGTTTTCC-3′
TC179186	Pathogenesis-related family protein	fw	5′-CGTGGGAAGTGATCAACGTG-3′
		rv	5′-AAAGAAACCCCAATGCCTGA-3′
TC185053	Glutathione S-transferase/peroxidase	fw	5′-GCAAAGGCTGAGTGTCCAAAG-3′
		rv	5′-GACTTGGCCACACTGTCCCT-3′
TC186230	Serine/threonine protein kinase	fw	5′-AACCGAGGCATGTGCTTCTG-3′
		rv	5′-TGAAACATGACGATGCTTTTTCC-3′

was confirmed within each trial by Student's *t*-test coupled with bootstrap using 1000 samples and $\alpha = 0.05$.

2.3. Microarray hybridization

During two consecutive trials, three fruit samples *per* each genotype have been chosen for further transcriptomic analysis. Total RNA extraction was obtained using the CTAB (hexadecyltrimethylammonium bromide) method [33]. Total RNA was quantified using a NanoDrop ND-1000 Spectrophotometer. RNAs with absorbance ratio A230/260 and A260/A280 higher than 2.0 were tested for integrity using the Agilent 2100 Bioanalyzer (Agilent).

Transcriptomic analysis was performed on a 90 K TomatArray1.0 microarray synthesized using the CombiMatrix platform at the Plant Functional Genomics Center of the University of Verona (http://ddlab.sci.univr.it/FunctionalGenomics/) as previously described [34]. Expression data were normalized using the SPSS software package and probes with significant change (*P*-value <0.01) in their hybridization signals were identified by running a two-factor ANOVA module included in the TIGR Multiple Experiment Viewer Software v4.0 (http://www.tm4.org/). Co-regulations were investigated by Pearson metric. Functional annotation of differential expressed genes was performed by using the ITAG2.3 database [35] available at the Solgenomics network website (http://solgenomics.net). Detailed information about microarray data is available in Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under the series accession GSE37568.

2.4. Quantitative Real-Time PCR (RT-qPCR)

The relative expression of candidate mRNAs for controlling accumulation of phenolics in IL7-3 fruit was validated using a 7900HT Fast Real-Time PCR System (Applied Biosystems). Total RNA was retrotranscribed by transcriptor High-Fidelity cDNA Synthesis kit according to manufacturer's protocol. Real-Time RT-qPCR assay was done in 12.5 μl reaction volumes through SYBR Green PCR Master Mix (Applied Biosystems) with three replications per each reaction. Plate assembling was automated by using a Freedom EVO 1.50 (TECAN) liquid handler station. Quantification was performed according to the $\Delta\Delta$Ct method [36]. Primers were validated using a standard curve over a dilution range $1–10^{-3}$ ($R^2 > 0.98$; slope close to -3.32). A list of primer sequences is provided in Table 1.

2.5. Tentative consensus mapping

Sequences of the 21,550 tomato tentative consensus sequences (TCs) retrieved from TIGR database Gene Index Release 11.0 (June 21, 2006) were mapped on the tomato chromosomes. Advanced mode of the SGN BlastN tools (http://solgenomics.net/tools/blast/index.pl), were used to align TCs with pseudo molecules of the Tomato WGS Chromosomes (SL 2.40) database. Only TCs producing alignment with expectation values $<10^{-10}$ were considered. Also, TCs mapping to the wild *S. pennellii* introgression 7-3 were identified by anchoring the sorted list of TCs mapping to the pseudo chromosome 7 to markers that define the 7-3 segment in the RFLP Tomato-Expen 2000 v.52 markers, that are SGN Marker T1719B and SGN Marker TG20.

2.6. Screening of tomato TILLING mutants

The tomato TILLING platform at Metapontum Agrobios [37] was used for screening a population of M3 mutagenized Red Setter tomato families in order to detect mutations in the coding sequence of the *ERF1* gene. Amplicons were amplified by nested-PCR according to the experimental conditions previously described [38]. The external primer pair (fw: 5′-TACCCACCCAAGAGTCAT-3′ and rv: 5′-CCCTACACCCAAACAAAC-3′) produced a 1428 bp fragment. Internal primers (fw: 5′-CACTATTTCTCTTTCCTCATCC-3′ and rv 5′-CAACTCATCAAAAGCTCTTC-3′) were 5′ labelled with IRDye 700 and IRDye 800 dye (LI-COR®, Lincoln, NE, USA) respectively and produced a fragment of 994 bp. Unlabeled internal primers were also used for priming sequencing reactions. Induced point mutations were identified by means of the mismatch specific endonuclease ENDO 1 (Serial Genetics) in the experimental conditions suggested by the supplier. Digested and labelled DNA fragments were separated on the LI-COR 4300 (LI-COR®, Lincoln,

Table 2
Levels of total phenolics detected in fruit of M82 and IL7-3 grown in greenhouse over 4 consecutive trials. Total phenolics are expressed as mg of gallic acid equivalents *per* 100 g of fresh weight. Mean value, Standard Errors (SE), and relative level in IL7-3 *vs*. M82 (% of control) are shown. Within each trial, asterisk indicates significant differences between IL7-3 and M82 at Student's *t* test coupled with bootstrap (1000 samples and alpha 0.05).

Trial	Genotype	Mean	SE	Relative level (%)
I	M 82	31.99	1.68	129
	IL7-3	41.30*	3.09	
II	M 82	31.98	4.73	171
	IL7-3	54.84*	1.41	
III	M 82	30.60	3.10	154
	IL7-3	47.21*	1.98	
IV	M 82	34.07	2.01	168
	IL7-3	57.31*	6.34	

NE, USA) using denaturing polyacrylamide gels and gel images were analyzed using Adobe Photoshop software (Adobe Systems Inc., San José, CA, USA). Once identified, mutations were validated by sequencing the *erf1* target fragment.

Prediction analysis of the relevance of the mutation for the protein to keep the function was achieved by using SIFT (http://sift.jcvi.org/) [39], and I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) a tool enabling prediction of protein conformational changes [40].

3. Results

3.1. Fruit phenotyping

In our laboratory a population of introgression lines harbouring single homozygous segments of the *S. pennellii* genome in the *S. lycopersicum cv*. M82 background has been analysed over consecutive growing seasons in order to investigate QTL controlling tomato fruit quality. Among these lines, IL7-3 was selected for further analysis because it stably maintained over four trials higher levels of fruit total phenolics compared to M82 (Table 2). Overall, the level of total phenolics was 32.16 ± 1.51 (mean ± SE) and 50.14 ± 2.32 mg of GAE *per* 100 g FW in red-ripe fruits of M82 and IL7-3, respectively. According to the Univariate ANOVA test, the difference was statistically significant ($P < 0.001$) with IL7-3 expressing on average a level of total phenolics 56% higher than M82 and the interaction *genotype* × *trial* was not significant. In addition, the higher accumulation of total phenolics in IL7-3 fruit compared to M82 was confirmed within each trial based on Student's *t*-test ($P < 0.05$) reiterated on 1000 samples generated by bootstrap. When compared to M82, IL7-3 accumulated in the fruit a higher relative level (per cent of control) of total phenolics. In the last trial, total flavonoids were also evaluated. Their level was 8.48 ± 0.54 and 5.06 ± 0.41 mg of QE *per* 100 g FW in red-ripe fruit of IL7-3 and M82, respectively. The higher value in IL7-3 was statistically validated by Student's t test coupled with bootstrap 1000 samples and alpha 0.05.

In the second and third trials, red-ripe fruits from IL7-3 and M82 were also assayed for glutathione content (Table 3). According to Student's *t*-test on a bootstrap population of 1000 samples, IL7-3 expressed a statistically significant higher accumulation ($\alpha < 0.05$) of total glutathione (GSH + GSSG) and oxidized glutathione (GSSG) than M82 in both trials, whereas the variations in the level of the reduced glutathione (GSH) was significant only in the second trial.

3.2. Transcriptomic analysis

In order to identify mRNAs regulated at different levels in M82 and IL7-3, biological replicates of total RNA were extracted from three samples taken from each genotype within both second and third trials. Differential transcript accumulation was documented using single-colour hybridization on the TomatArray 1.0 microarray followed by a two-factorial ANOVA test ($P < 0.01$) with the M82 transcriptome as a reference. The comparative transcriptomic approach allowed identifying 260 sequences (1% of those represented on the TomatArray1.0) that were differentially expressed at the red-ripe stage (Supplementary Table S1), of which 129 (49.6%) were up-regulated and 131 (50.4%) were down-regulated in IL7-3 and only 2.7% of them did not match with any annotated predicted gene. They were distributed over all tomato chromosomes with a proportion of TCs mapping to each of the twelve chromosomes out of all differentially expressed TCs ranging from 2.7% (7 TCs) for chromosome 11 to 23.8% (62 TCs) for chromosome 7.

In order to explain the higher content of phenolics in the fruit of IL7-3, we directed our attention to genes belonging to processes controlling the level of phenolics in plant tissues, which include biosynthesis and degradation/utilization of intermediates as well as vacuole sequestration. We initially analysed transcriptomic data searching for changes in the steady state mRNA levels of genes required for biosynthesis and degradation of phenolics. Among genes implicated in controlling biosynthesis of phenolics, 88 TCs (corresponding to 55 predicted genes according to ITAG release 2.3, Supplementary Table S2) putatively encoding for key enzymes were probed on the microarray slide and they did not show differential expression between IL7-3 and M82. Consequently, we focused our attention on a different group of genes that showed high differences in expression level between IL7-3 and M82 and selected them for further analysis. The identified sub-set of transcripts (Table 4) was included in "ethylene signalling pathway", "conjugation/compartmentalization pathway" and "response to stress". Among up-regulated genes there were: one *ERF1* (TC174512, Solyc05g051200), two *GST* (TC170110 and TC185053, Solyc07g056420 and Solyc07g056440, respectively), one gene coding for a vacuolar sorting protein (*VSP*) (TC172097, Solyc06g005080), one gene coding for a multidrug resistance protein belonging to MATE family (TC178409, Solyc10g081260) in addition to a group of genes involved in stress responses. Among the down-regulated genes there were

Table 3
Fruit content of reduced glutathione (GSH), total glutathione (GSH + GSSG) and oxidized glutathione (GSSG) in M82 and IL7-3 over the second and third trials. Content levels are expressed as mg *per* g of fresh weight. Mean value, standard errors (SE), and relative level in IL7-3 *vs*. M82 (% of M82) are shown. Within each trial, asterisk indicates significant differences between IL7-3 and M82 at Student's *t* test coupled with bootstrap (1000 samples and alpha 0.05).

Trial	Genotype	GSH		Relative level (%)	GSH + GSSG		Relative level (%)	GSSG		Relative level (%)
		Mean	SE		Mean	SE		Mean	SE	
II	M82	0.049	0.005	106	2.675	0.089	112	2.629	0.085	112
	IL7-3	0.052	0.005		3.002*	0.041		2.950*	0.040	
III	M82	0.046	0.004	119	2.086	0.105	148	2.052	0.108	140
	IL7-3	0.055*	0.002		3.092*	0.089		2.883*	0.161	

Table 4
Subset of differentially expressed TCs involved in phenolics accumulation and stress response. For each TC the ID of ITAG2.3 annotation, its functional description, the fold change (IL7-3 *vs.* M82) from the microarray (see also Supplementary Table 1) and the RT-qPCR analyses is reported.

Code[a]	TC ID	ITAG2.3 gene ID	Chr	Annotation	IL7-3 *vs.* M82			erf1 *vs.* RS	
					Microarray	RT-qPCR		RT-qPCR	
						Mean	SE	Mean	SE
Ethylene signalling pathway									
1	TC174512	Solyc05g051200.1.1	5	Ethylene-responsive factor 1A	3.04	1.98*	0.72	−0.80	0.51
2	TC170138[b]	Solyc07g053740.1.1	7	Ethylene-responsive transcription factor 4	−7.65	−1.04*	0.11	1.97*	0.32
Conjugation/compartmentalization pathway									
3	TC170110[b]	Solyc07g056420.2.1	7	Glutathione S-transferase-like protein	3.65	0.59*	0.03	−8.30*	1.66
4	TC172606[b]	Solyc07g056510.2.1	7	Glutathione S-transferase	−7.18	−12.83*	0.20	−1.62*	0.68
5	TC176201[b]	Solyc07g056490.2.1	7	Glutathione S-transferase	−5.27	2.53*	0.17	0.80*	0.39
6	TC185053[b]	Solyc07g056440.2.1	7	Glutathione S-transferase-like protein	2.76	4.28*	0.27	−0.36	0.58
7	TC172097	Solyc06g005080.2.1	6	Vacuolar sorting protein	1.20	0.52*	0.03	0.73*	0.05
8	TC178409	Solyc10g081260.1.1	10	Multidrug resistance protein mdtK	1.30	1.63*	0.35	0.49	0.32
Stress response									
9	TC172943	Solyc02g063420.2.1	2	Hydroxyproline-rich glycoprotein	2.68	2.07*	0.30	−0.61*	0.44
10	TC177287	Solyc01g095930.2.1	1	O-acyltransferase WSD1	2.12	1.81*	0.23	0.93*	0.36
11	TC177388	Solyc08g080640.1.1	8	Osmotin-like protein	2.39	2.42*	0.70	−2.50*	0.51
12	TC178155	Solyc08g080630.2.1	8	Chymotrypsin inhibitor-2	3.40	2.79*	0.42	−6.66*	1.26
13	TC179186	Solyc02g031950.2.1	2	Pathogenesis-related protein	3.53	3.49*	1.41	0.82*	0.24
14	TC186230	Solyc05g041420.2.1	5	Serine/threonine protein kinase	0.68	1.20*	0.20	−0.25	0.13

[a]The same codes for the TCs listed in column 2 are used in Figs. 1 and 3.
[b]TCs mapping on the introgression region 7-3.
*Statistically significant differences at $P < 0.05$.

ERF4 (TC170138, Solyc07g053740) and three *GST* genes (TC171442, TC172606, and TC176201, Solyc07g056420, Solyc07g056510, and Solyc07g056490, respectively). TC170110 and TC171442 revealed to represent the same *GST* predicted gene (corresponding to Solyc07g056420), and following detailed sequence analysis (see below) only one (TC170110) was chosen for subsequent investigations.

Since the down-regulation of genes mapping into the wild introgressed region 7-3 (TC170138, TC170110, TC172606, TC176201, TC185053) might also be due to mismatches occurring in the region where the 35mer microarray probe and/or the qPCR primers anneal, we searched for polymorphisms in these genes, using the sequences obtained from the ongoing *S. pennellii* genome sequencing project. For *ERF4* (TC170138), we could verify that four nucleotide mismatches occurred between IL7-3 and M82 in the region where the microarray probe annealed. Such mismatches could conceivably affect the expression level revealed by the microarray hybridization (Supplementary Fig. S1), thus explaining the negative fold change (−7.65 in IL7-3 *vs.* M82) observed, since the probe was designed on a TC coming from the cultivated tomato. Only one mismatch was detected in the probes for two *GSTs* (TC172606, TC176201) but these would not influence the hybridization signal, whereas mismatches occurred between IL7-3 and M82 with the probe TC171442, thus leading us to select TC170110 as the specific EST related to Solyc07g056420. In any case, for a better expression level estimation of all these genes, the qPCR primer pairs were designed on conserved regions between the wild genotype *S. pennellii* and the cultivated *S. lycopersicum*. Consequently, the negative fold change estimated by qPCR for *ERF4* (−1.04) was more reliable, as well as those estimated for the *GSTs*. qPCR confirmed the higher expression level of the genes coding for ERF1, GST, MATE and VSP proteins (Table 4). The up-regulation of a group of stress-related genes was also confirmed, as well as the down-regulation of *ERF4* and one *GST* (TC172606). A discrepancy between microarray and Real-Time expression data was found only for TC176201.

Finally, promoters within these genes were screened for the presence of GCC-boxes. Such elements were found at position −360 (complementary strand) from the start codon of the *MATE* gene, and at position −79 of the *VSP* gene. The chymotrypsin inhibitor, the osmotin-like protein and the pathogenesis-related protein promoters also include GCC boxes, however, no GCC-box was found in the promoter of any of the analyzed *GSTs*.

Focusing on the selected transcripts, we propose that the higher level of phenolics in IL7-3 is the result of a more efficient conjugation/compartmentalization of these metabolites, driven by the higher expression level of a *MATE*, a *VSP*, and of the over-expressed *GST* genes (Fig. 1). In our model we also propose a key-role of ERF1 as a potential regulator of the mechanisms that control phenolics compartmentalization in the vacuole. In order to validate the role envisaged for this ethylene responsive factor, we explored a functional TILLING platform available for the tomato cultivar Red Setter to find mutants for the *ERF1* gene.

3.3. Isolation and characterization of an erf1 mutant

Screening of 5221 EMS-treated M3 families resulted in the identification of a mutant line harbouring a mutation in the sequence of the *erf1* gene (TC174512). The mutation was a cytosine to thymine transition at position 521 of the cds (Fig. 2) and caused in the ERF1 protein sequence the amino acid change at position 174 from Serine to Phenylalanine (S174F). SIFT algorithm allowed the prediction of whether this amino acid substitution affects protein function. The prediction was based on the degree of conservation of amino acid residues in a sequence alignment derived from 27 ERF1 homologues proteins (Supplementary Fig. S2). According to the predicted output, the S in position 174 showed 100% conservation and its substitution with F was expected to be deleterious (Fig. 2). The impact of the mutation on the secondary structure of the protein has been also evaluated by I-TASSER showing that this substitution in the mutant protein causes the coiled-coil to change in alpha-helix structure.

Ten M5 plants of the *erf1* mutant line were used in our analyses. The homozygosity for the mutation in the *erf1* gene was validated by sequencing. These plants were grown in a greenhouse and characterized in comparison with Red Setter control plants (Table 5). On average, fruits from the *erf1* mutant showed a significant reduced level of total phenolics compared to Red Setter fruits. In particular, Red Setter fruits showed an average content in total phenolics of 44.39 ± 3.65 whereas fruits from the *erf1* mutant

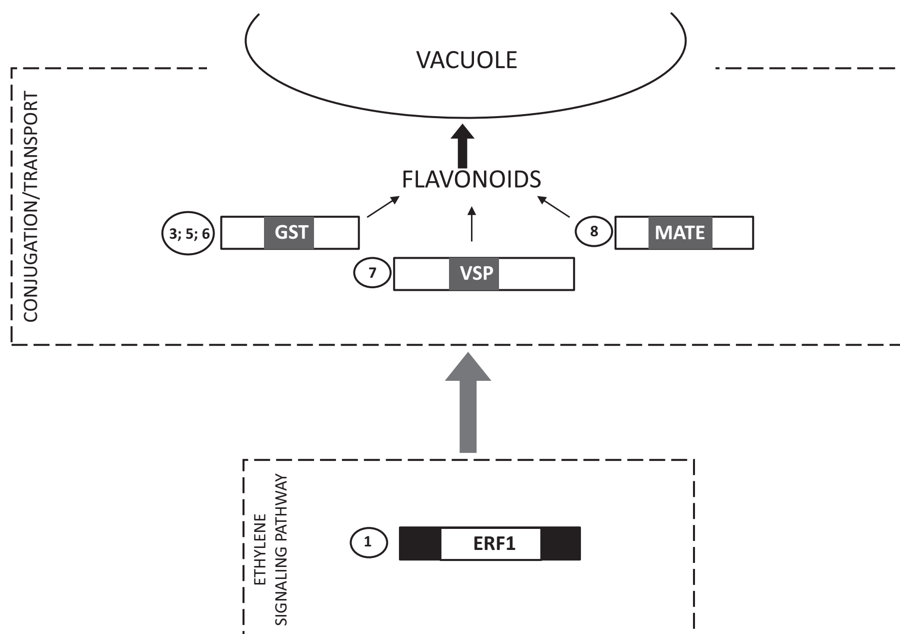

Fig. 1. A schematic representation of phenolics vacuolar accumulation in tomato fruit of the IL7-3.
The main candidate genes involved in the sequestration of phenolics to the vacuole are reported and numbered according to codes indicated in Table 4. ERF1: ethylene responsive factor 1; GST: glutathione-S-transferase; VSP: vacuolar sorting protein; MATE: multidrug and toxic compound extrusion.

showed the significant lower content of 27.63 ± 1.31 (α < 0.05). Also flavonoids were significantly reduced in the *erf1* mutant compared to Red Setter (3.83 mg ± 0.23 *vs.* 7.74 ± 0.48 of QE *per* 100 g FW). The fruit levels of GSH, GSH + GSSG and GSSG did not show any significant variation in the *erf1* mutant line compared to Red Setter.

To confirm the involvement of the *ERF1* gene in the regulative mechanisms controlling the level of total phenolics in the IL7-3 tomato fruits, we assayed by RT-qPCR the relative abundance of candidate transcripts in the *erf1* mutant compared to Red Setter (Table 4). As expected, no significant reduction in the transcript

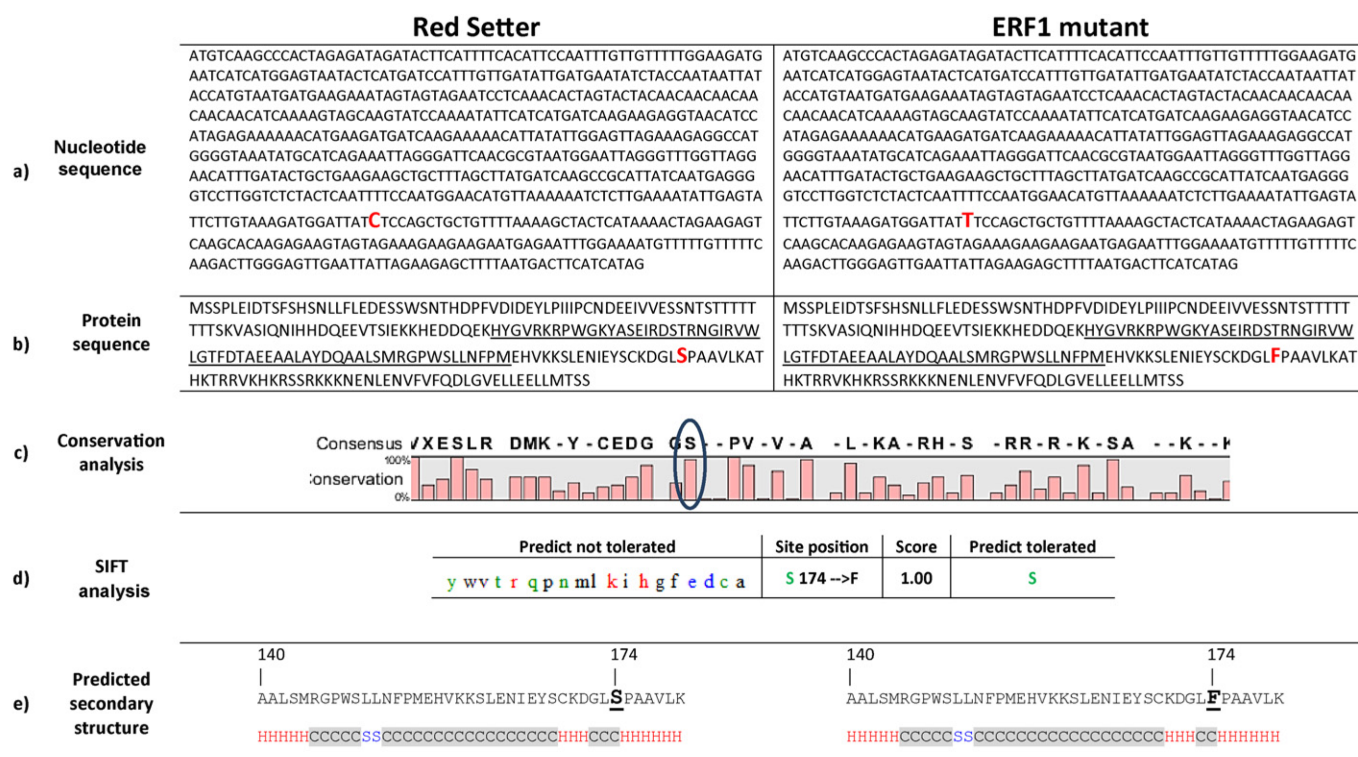

Fig. 2. Analysis of mutated *erf1* gene isolated by TILLING.
The figure shows: (a) nucleotide sequences of *erf1* gene in Red Setter and erf1 mutant showing a cytosine to thymidine transition at 521 bp position; (b) change of the aminoacid 174 from Serine (S) to Phenylalanine (F) in the protein sequence (the underlined sequence identify the AP2/ERE domain); (c) conservation in the ERF1 protein assessed by a sequence comparison of 26 different homologues proteins (see also supplementary Fig. 2); (d) SIFT algorithm output; (e) predicted secondary structure by I-TASSER analysis where H: helix; C: coil and S: sheet.

Table 5
Fruit phenotyping of *erf1* mutant and Red Setter. Fruit content of total phenolics, total flavonoids, reduced glutathione (GSH), total glutathione (GSH + GSSG) and oxidized glutathione (GSSG) in the *erf1* mutant and Red Setter. Mean value and Standard Error (SE) are shown. Asterisk indicates significant differences between *erf1* mutant and Red Setter at Student's *t* test coupled with bootstrap (1000 samples and alpha 0.05).

	Red Setter		*erf1* mutant	
	Mean	SE	Mean	SE
Total phenolics (mg GAE × 100 g^{-1} FW)	44.39	3.65	27.63*	1.31
Total flavonoids (mg QE × 100 g^{-1} FW)	7.74	0.47	3.83*	0.23
GSH (mg g^{-1} FW)	0.03	0.00	0.03	0.00
GSH + GSSG (mg g^{-1} FW)	2.82	0.11	2.88	0.10
GSSG (mg g^{-1} FW)	2.73	0.11	2.79	0.10

level of the target *erf1* (TC174512) gene was observed between the Red Setter and the *erf1* mutant genotype. By contrast, *ERF4* (TC170138) showed a 1.97 fold increased expression in the mutant compared to the wild type. As for genes involved in the transmembrane transport of phenolics, red fruits from the *erf1* mutant line showed a significant lower abundance of mRNAs related to two *GST* genes (TC170110, TC172606), whereas a similar level of expression in the relative transcript abundance of the *MATE* gene (TC178409) and a higher expression of the *VSP* gene (TC172097). Finally, the lower expression of stress-related genes exhibited by the mutant also supported the reduced efficiency of the mutated *erf1* gene in promoting their expression.

4. Discussion

In the present work we investigated the molecular mechanisms that lead to a higher tomato phenolics content in a *S. pennellii* introgression line by combining currently available -omics resources for this species such as the transcriptomic Combimatrix array and the TILLING gene functional platform. The first approach allowed the identification of a group of genes contributing to the increase of vacuolar sequestration of phenolics and led us to hypothesize a key-role of the gene *ERF1* in orchestrating the mechanisms involved therein. The second allowed the identification and characterization of one *erf1* mutant that supports the role of *ERF1* gene in controlling the level of phenolics/flavonoids in the fruit of the selected tomato introgression line.

The genotype we analysed (IL7-3) exhibited an average level of fruit total phenolics higher than its cultivated parental line M82 over four greenhouse trials. The higher content of phenolics in fruits of IL7-3 *vs.* M82 was confirmed over two field trials (data not shown). In a recent work carried out on the same plant materials used in the present paper, it has been established by HPLC analysis that the level of the flavonol rutin was significantly higher in the fruit of IL7-3 compared to M82 [41]. Accordingly, our preliminary data revealed a higher level of total flavonoids in the IL7-3 fruit. Comprehensively, these data allowed the confirmation of a major QTL for fruit phenolics accumulation mapping to the introgressed region 3 of chromosome 7 that was expressed independently of the trial and environmental conditions. Indeed, in the Tomato Expression Database (http://ted.bti.cornell.edu) [42] this line was already reported as exhibiting a fruit relative level higher than M82 (139.7% of control) and a very low level of carotenoids. Therefore, in order to use this introgression line as source of favourable alleles for higher phenolics in the fruit it would be necessary to disrupt the linkage between the gene(s) that promote phenolics content and those that contribute to lower carotenoids. Nowadays, genomics tools such as transcriptomic profiling and genome sequencing/annotation might enable the accurate dissection of this quantitative trait in candidate genes to be used in breeding for fruit phenolics accumulation. Altogether, the introgression line IL7-3 was used in a comparative transcriptomic analysis with respect to its cultivated parental line M82.

The differential gene expression evidenced in our experiments strengthens the hypothesis that increased transport and vacuolar sequestration of phenolics in IL7-3 led to the higher phenolics level in its fruits rather than to an elevated biosynthetic flux. We thus assumed that in IL7-3 the sequestration of phenolics into vacuoles is regulated according to mechanisms previously described in other species, such as maize, petunia, Arabidopsis and grape [43–48]. Indeed, the higher expression in the IL7-3 of genes coding for three GSTs, one VSP and one MATE fits with the co-operative contribution of these proteins for the accumulation of anthocyanins in the vacuole, recently evidenced in grapevine [49].

In particular, the model we proposed is based on the central role of one *ERF1* gene mapping on chromosome 5, which is distinct from the *TERF1* mapping on chromosome 3 that enhances osmotic stress tolerance in tomato [50]. The over-expression of *ERF1* in IL7-3 makes it a good candidate for the regulation of processes involved in fruit phenolics accumulation. We reasoned that the over-expression of *ERF1* might balance the evidenced down-regulation of *ERF4* since no polymorphism is expected between IL7-3 and M82 in the *ERF1* gene mapping on chromosome 5. Indeed, down regulation of *ERF4*, that maps in the introgression region, might be due to polymorphisms evidenced in the "wild" *pennellii* promoter of *ERF4* and/or to different regulators acting in the wild and the cultivated tomato genotypes in concert with the *ERF* genes. This balance between ethylene responsive factors would ensure proper ethylene functions during the development of IL7-3 fruit through a homeostatic cellular mechanism, even though these two factors might also have different specific roles in the fruit cells. Indeed, no specific functions have been up till now assigned to the numerous members of tomato ERF family and a high functional redundancy has been proposed for them [51]. Following our hypothesis, in the genotype IL7-3 the higher expression of *ERF1* might drive an increased transcriptional regulation of genes coding for some GSTs, one MATE and one VSP that results in an increase of vacuolar compartmentalization of phenolics, specifically flavonoids. Hence, among members of the AP2/ERF family, ERF1 integrates signals from ethylene and jasmonic acid signalling pathways and ultimately triggers a defence response against pathogen attack by inducing the expression of defence genes, such as PR proteins, HPRGs and GSTs, usually by binding the specific DNA target GCC box [52–54]. Here, the regulatory action of ERF1 through the GCC-box binding could be assumed for the *MATE* and the *VSP*, as well as for some stress-related genes that also displayed up-regulation. Unexpectedly, this *cis*-element was not detected in promoters of the *GST*s up-regulated in IL7-3. This might be similar to what was previously observed for the transcription factor *Pti4*, which appeared to regulate gene expression in tomato by binding GCC box but also non-GCC box *cis* elements of many gene promoters [55]. In addition, recently it was demonstrated in Arabidopsis that the AtERF2 protein specifically binds to the promoter of the *AtGST11* where no GCC-box was detected [56]. These observations could thus support our hypothesis that ERF1 might directly regulate expression of genes involved in the transport of flavonoids to the vacuole. It is important to note, however,

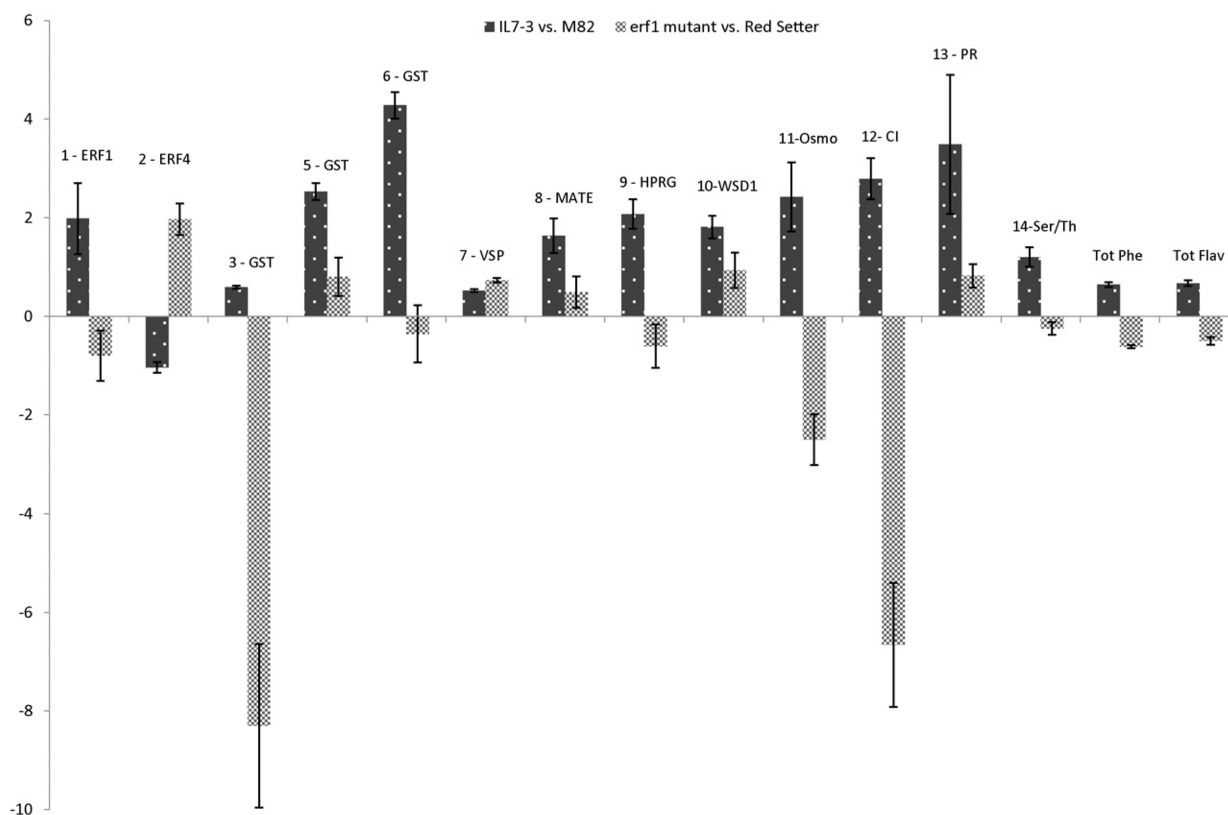

Fig. 3. Transcriptomic and phenotypic trend analysis of the two different genetic systems IL7-3 *vs.* M82 and erf1 mutant *vs.* Red Setter.
Gene expression data are those referred to qPCR analysis (fold change) and genes are numbered according to codes reported in Table 4. Phenotypic data are also reported as fold change. ERF1: ethylene responsive factor 1; ERF4: ethylene responsive factor 4; GST: glutathione-S-transferase; VSP: vacuolar sorting protein; MATE: multidrug and toxic compound extrusion; HPRG: histidine–proline rich glycoprotein; WSD1: O-acyltransferase WSD1; Osmo: Osmotin-like protein; CI: Chymotrypsin inhibitor-2; PR: Pathogenesis-related protein; Ser/Th: Serine/threonine protein kinase; Tot Phe: Phenolics fruit content; Tot Fla: Flavonoid fruit content.

that ERF1 might also indirectly interact with other transcription factors in activating some promoters as suggested by Solano et al. [57] who studied the transcriptional cascade controlling ethylene signalling in Arabidopsis. Implications of an *ERF1* gene controlling phenolics accumulation have been previously reported in photosynthetic tissues of Arabidopsis [54], where the involvement of auxin and ethylene in inducing flavonoids accumulation has been demonstrated. In particular, it was proposed that the increase of expression of a subset of genes of the flavonoid pathway was regulated through the activation of ethylene responsive elements, perhaps with the participation of a MYB transcriptional factor.

To our knowledge, this is the first time that the role of *ERF1* in modulating phenolics accumulation is reported in fleshy fruit, and the selection of a TILLING mutant harbouring a single point mutation in the *erf1* cds permitted us to support such hypothesis. Unfortunately, no functional mutant was found in the *ERF4* gene (data not shown) and only one mutation was detected in the *ERF1* gene. The availability of more than one genotype harbouring mutations in either *ERF1* or *ERF4*, or in both genes, would have allowed confirming their involvement in the regulation of phenolics accumulation in tomato fruit. However, the serine to phenylalanine amino acid substitution observed in the *erf1* mutant line is predicted to induce a modification in the three-dimensional structure of the protein (Fig. 2). Moreover, this replacement involves the C-terminal of the protein that is highly conserved and therefore could be crucial for its proper functioning. Based on these considerations, we presumed that the modified structure might affect the activity of the erf1 protein. In addition, even though other mutations affecting the protein activity could have occurred in the same mutant genotype in other genes in addition to *erf1*, according to the frequency of mutation reported for the Red Setter TILLING platform [37,58] it is very unlikely that such mutations would involve other genes influencing the phenolics biosynthesis and storage.

The subsequent characterization of our *erf1* mutant line at the transcriptional and phenotypic level supported the involvement of this ethylene responsive element in regulating a group of genes that comprehensively might control the level of phenolics in tomato fruit. First of all, the higher expression of *ERF4* might result from a reduced functionality of *erf1* and this would be in line with the balancing between these two *ERF* genes already observed in IL7-3. Moreover, the reduced functionality of erf1 might also be related to the lower expression, in comparison with that evidenced in the line IL7-3, of the *MATE* and of some stress-related genes, whose expression is modulated by the presence of GCC-box in their promoter. At the phenotypic level, a reduced level of total phenolics and flavonoids in the mutant respect to the wild type Red Setter reinforces the role of ERF1 in orchestrating the compartmentalization of these compounds to the vacuole. Comprehensively, when comparing the two systems analysed in the present work (IL7-3 *vs.* M82 and *erf1* mutant *vs.* Red Setter), changes in the expression of genes involved in the conjugation/compartmentalization of phenolics and in stress response appear to follow variations of the phenolics/flavonoids content in the tomato fruit (Fig. 3). This is most probably explained by the reduced activity of the mutated erf1 protein in the TILLING mutant and by the higher expression of *ERF1* gene in the IL7-3 introgression line (both compensated in part by the contrasting expression of *ERF4*). Nevertheless, a further functional validation by silencing these *ERF* genes in IL7-3 or over-expressing them in M82 will need to be carried out before definitively considering them as key-candidates for improving the phenolics content of tomato fruit.

Overall, these data suggest that a more efficient compartmentalization to the vacuole underlies the higher fruit phenolics content of IL7-3. This is likely achieved by transport mechanisms mediated by a MATE, a VSP and some GSTs, and transcriptional results from the *erf1* mutant strongly suggest that the role of one particular GST (TC170110) might be key in affecting phenolics/flavonoids accumulation. Finally, a concerted action of ethylene responsive factors *ERF1* and *ERF4* in directing this transport has been hypothesized, designating them as novel candidate genes to increase phenolics content in tomato fruit *via* genetic manipulation strategies that could enhance/reduce their fruit specific expression levels. We might also assume the putative role of other genes mapping in the introgression region 7-3 as master regulators of *ERF1*. Indeed, the genetic diversity that exists between the cultivated and wild species in this genomic region might reflect differences also in key regulatory elements, which could boost *ERF1* levels in tomato fruit cells. Nowadays, the tomato genome has been completely sequenced [59] and a preliminary annotation of genes mapped on the region 7-3 revealed the presence of putative regulatory elements (data not shown). These genes could directly promote the higher expression of *ERF1* in IL7-3 in a *trans*-acting regulatory mechanism or might be active in modifying *ERF4* expression, thus indirectly inducing a higher expression of *ERF1* through a homeostasis balancing mechanism. Their potential role as additional candidate genes to drive the increase of *ERF1* transcription in the fruit and the consequent accumulation of phenolics should be further investigated. These "wild" regulatory elements could then be transferred into cultivated varieties by sexual hybridization without adopting genetic transformation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2013.02.001.

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