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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.

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