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INDIRIZZO MIGLIORAMENTO GENETICO E ORTICOLTURA

GENETIC MECHANISMS UNDERLYING TOMATO QUALITY TRAITS

CANDIDATA
DOTT.SSA ADRIANA SACCO

RELATORE
CHIAR.MO PROF. LUIGI FRUSCIANTE

COORDINATORE
CHIAR.MO PROF. MATTEO LORITO

CORRELATORE
DOTT. ANTONIO DI MATTEO

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1. Introduction

1.1 The tomato

Tomato (*Solanum lycopersicum*) belongs to the nightshade family Solanaceae. The extremely diverse and large Solanaceae family is believed to consist of 96 genera and over 2800 species distributed in three subfamilies, Solanoideae (to which *Solanum* belongs), Cestroideae, and Solanineae (Knapp *et al.*, 2004). Solanaceae is one of the most economically important families of angiosperms and contains many of the commonly cultivated plants, including potato, tomato, pepper, eggplant, petunia, and tobacco. This family is the most variable of all crop species in terms of agricultural utility, the 3rd economically most important crop family, exceeded only by grasses and legumes, and the most valuable in terms of vegetable crops (van der Hoeven *et al.*, 2002). The cultivated tomato, *Solanum lycopersicum* Mill., a fruit that is often treated as a vegetable, is widely grown around the world and constitutes a major agricultural industry. Worldwide, it is the second most consumed vegetable after potato (FAOSTAT 2005; http://faostat.fao.org) and unquestionably the most popular garden crop. In addition to tomatoes that are eaten directly as raw vegetable or added to other food items, a variety of processed products such as paste, whole peeled tomatoes, diced products, and various forms of juice, sauces, and soups have gained significant acceptance. There are more varieties of tomato sold worldwide than any other vegetable. Although a tropical plant, as already mentioned, tomato is grown in almost every corner of the world from the tropics to within a few degrees of the Arctic Circle. It is grown in greenhouses where outdoor production is restricted due to cool temperatures. Major tomato producing countries in descending orders include China, USA, India, Turkey, Egypt, and Italy (Fig. 1.1).

1. China : 31.644.040
2. USA : 11.043.300
3. Turkey : 9.700.000
4. India : 7.600.000
5. Egypt : 7.600.000
6. Italy : 7.187.000

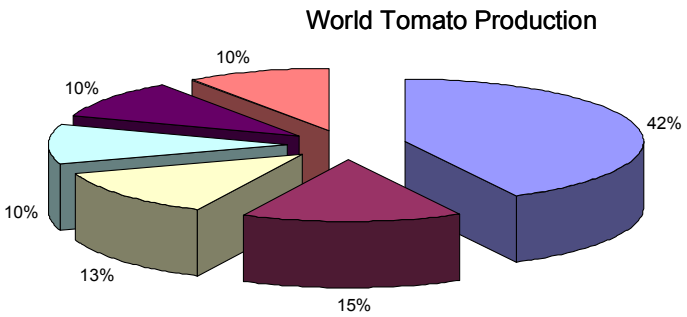


Figure 1.1 World tomato production. Major tomato producing countries in the world (http://faostat.fao.org).

Other leading countries include Spain, Brazil, Iran, Mexico, Greece, and Russia (http://faostat.fao.org). Tomatoes are the most highly consumed vegetables in Italy, with the highest average consumption among European countries (NETTOX, 1998). A study on the Italian food consumption patterns, reported that the consumption of tomatoes (both ripe and for salad) is estimated in 75.5 g/day/capita (Turrini *et al*., 2001). Most the production (62,9%) is concentrated in Southern Italy, the 29,2% in the North and only the 7,9% in the central part of Italy. Actually tomatoes are one of the main component of the traditional Mediterranean diet, which has been associated with health protection and longevity.

In addition to the cultivated species *S. lycopersicum* and its wild form *S. lycopersicum* var. *cerasiforme* (Dun.) Gray (wild cherry), there are eight related wild species, including *S. pimpinellifolium* (Jusl.) Mill. (currant tomato), *S. cheesmanii* Riley, *S. chmielewskii* Rick, Kes., Fob. and Holle, *S. chilense* Dun., *S. neorickii* Rick, Kes., Fob. and Holle, *L. peruvianum*(L.)Mill., *S. habrochaites* Humb. and Bonpl., and *S. pennellii* (Corr.)D'Arcy (Rick, 1976). All tomato species are diploid (2n = 2x = 24) and are similar in chromosome number and structure. Fruit colour varies depending on the species, from red to yellow to green. The cultivated tomato has limited variability, largely because of several population bottlenecks in the forms of founder events and natural and artificial selections that occurred during domestication and evolution of modern cultivars. Compared with the rich reservoir in wild species, the cultivated tomato is

genetically poor. It is estimated that the genomes of tomato cultivars contain 5% of the genetic variation of their wild relatives (Miller and Tanksley, 1990). The lack of diversity in the cultivated tomato can be visualized using DNA technologies. Very few polymorphisms within the cultivated tomato genepool have been identified, even using sensitive molecular markers (Van der Beek *et al.*, 1992; Villand *et al.*, 1998; Park *et al.*, 2004; Garcia-Martinez *et al.*, 2005; Tam *et al.*, 2005).

1.2 The tomato quality

The improvement of crop species has been a fundamental human pursuit since cultivation began. Tomato is a major economic important crop, displaying several characteristics which have established it as a model system for dissection of genetic determinants of quantitative trait loci (QTL). In tomato numerous wild-related species have been demonstrated to be untapped sources of valuable genetic variability, including pathogen-resistance genes, and nutritional and industrial quality traits. Actually, crop improvement strategies are focused not only on the traditional areas of yield enhancement and disease resistance but, driven by recent medical research, also on fruit compositional quality for human health. In particular, during the past century most research were focused on the enhancement of antioxidant compounds, given that antioxidants present in fruit and vegetables may help prevent chronic diseases such as cancer, arthritis and heart disease (Fernie *et al.*, 2006; Harrigan *et al.*, 2007a). Genetic determinants of nutritional quality have long been studied. However, it is only recently that these studies have largely focused on single, or at most, a handful of metabolites, such as carotenoid content in tomato (Liu *et al.*, 2003a), protein content in maize (*Zea mays*) (Moose *et al.*, 2004), starch content in potato (*Solanum tuberosum*) and rice (*Oryza sativa*) (Fernie and Willmitzer, 2004), and tocopherol levels in *Arabidopsis* (Gilliland *et al.*, 2006). Today, fruit quality is a major focus of most tomato breeding programs. In particular, major fruit quality traits of interest to both fresh market and processing tomato industries include fruit size, shape, total solids, colour,

firmness, nutritional quality and flavor. Other important fruit quality characteristics of tomato include pH, titratable acidity, and vitamin contents (Foolad, 2007). Tomato fruit is an important source of lipid-soluble antioxidants in the human diet because of their relatively high content of carotenoids. Lycopene presence in plasma has been related to a reduction in the risk of prostate cancer (Giovannucci *et al.*, 1995). Other carotenoids present in ripe tomato fruits include β-carotene and small amounts of phytoene, phytofluene, δ-carotene, ζ-carotene, neorosporene, and lutein (Khachik *et al.*, 2002). Tomato fruit is also an important source of the water-soluble antioxidants, particularly ascorbic acid (AsA) and phenols. Because tomatoes represent a major contribution to dietary nutrition worldwide, there is a growing interest in the potential of genetic improvement for tomato antioxidant levels either by traditional breeding methods (Ronen *et al.*, 1999, 2000; Zhang and Stommel 2000) or by transgene incorporation (Giuliano *et al.*, 2000; Romer *et al.*, 2000). Improvement in tomato nutritional traits also offers the opportunity to determine basic information about the regulation of antioxidants in fruit crops.

1.2.1 Apocarotenoid volatiles

Apocarotenoids are terpenoid compounds derived from the oxidative cleavage of carotenoids. They are generated when double bonds in a carotenoid are cleaved by molecular oxygen, forming an aldehyde or ketone in each product at the cleavage site. Carotenoid can be cleaved at any of their conjugated double bonds, resulting in a diverse set of apocarotenoids. This structural diversity is the consequence of the large number of carotenoid precursors (more then 600) and subsequent modifications such as oxidation, reduction, and conjugation (Vogel *et al*., 2008). Apocarotenoids perform essential biological functions in plants, animals and photosynthetic bacteria. Examples of biologically active apocarotenoids include the phytohormone abscisic acid (ABA) and retinol (vitamin A), an essential component of the visual cycle in animals. Economically, apocarotenids are valued as colorants and spices. Examples are bixin, an apocarotenoid used as a colorant in foods and cosmetics, and saffron,

a spice extracted from the styles of crocus flowers (Winter-halter and Rouseff, 2002). In particular, in tomato fruit these compounds form a part of the fruit flavour. In fact, tomato fruit flavour is the sum of the interaction between sugars, acids, and multiple volatile compounds. While several hundred volatiles have been identified in tomato, only about 15-20 actually impact our perception of the fruit. Most of these important volatiles are derived from the oxidative cleavage of carotenoids. These include: β-ionone, β-damascenone and 6-methyl-5-heptene-2-one. Additional carotenoid-derived volatiles are geranylacetone and pseudoionone. Despite their importance to flavour of tomato and many other fruit and vegetables, less work has focused on the formation and the role of apocarotenoids in plant, except ABA (Tieman *et al.*, 2006). The first gene encoding a carotenoid cleavege dioxygenase was isolated from the maize (*Zea mays*) ABA-deficient viviparous mutant, *vp 14*. VP14 catalyzes the first step in ABA biosynthesis, the cleavage of either of two 9-cis-epoxycarotenids, violoxanthin or neoxanthin at the 11,12 double bond. Because of its preferred substrate, VP14 is called a 9-cis-epoxycarotenid dioxygenase (NCED) (Schwartz *et al*., 1997). In the *A. thaliana* genome, nine putative carotenoid cleavage dioxygenases have been identified based on sequence homology to VP14. Four of the Arabidopsis dioxygenases (NCED2, NCED3, NCED6 and NCED9) have the same activity as VP14 and are designated NCEDs. NCED5 has high homology to VP14, though its activity has not been determined (Iuchi *et al.*, 2001; Tan *et al.*, 2003). The remaining four proteins diverge from the NCEDs and have been given the generic designation carotenoid cleavage dioxygenases (CCDs). These include CCD1, CCD4, CCD7 and CCD8 (Auldridge *et al.*, 2006). Even if, in the last years more works have focused on the role of these enzymes and their substrates and final products, much more should be done to manipulate the biosynthetic pathways to enhance the production of a desirable volatile.

1.2.2 Total soluble solids

There are two main markets for tomato as a food crop, as a fresh fruit product

and as a processed foodstuff (primarily tomato pastes and sauces) (Gould, 1992). Economic success in the latter market is dictated in part by a combination of total fruit yield and fruit soluble solids content (°Brix). Ripe fruit with high soluble solids require the removal of less water to produce tomato-based food products of the appropriate consistency and taste. In addition, since sugar is a major constituent of total soluble solids, such fruit are also likely to be sweeter and therefore require the addition of less sugar during processing. These processing savings can have a significant bearing on the profitability of processed tomato products and, thus, from a commercial standpoint, there is considerable interest in manipulating the soluble solids content of tomato varieties (Baxter *et al.*, 2005). The total solids of the cultivated tomato comprise 4–7.5% of its fresh weight, though this percentage can be much higher in some wild species (Fridman *et al.*, 2000). The total solids are composed of all fruit components except water and volatiles. In the cultivated tomato, the soluble (SS) and insoluble solids (ISS) account for about 75% and 25%, respectively, of the total solids. Reducing sugars glucose and fructose are the major components of the SS. Sucrose is also present but in very small quantities, although some wild species of tomato, including *S. chmielewskii* and *S. habrochaites*, have higher concentration of sucrose. The remaining soluble solids are composed of organic acids, lipids, minerals, and pigments. The ISS include proteins, cellulose, hemicellulose, pectins, and polysaccharides, which determine fruit viscosity. Quality of tomato juice, catsup, sauce, soup, and paste are influenced by viscosity of the product. Both SS and ISS are related to yield of concentrated tomato products, and yield and quality of certain processed products are determined by sugar contents of the fruit. For tomato products that are sold on the basis of solids content, the higher the solids of the raw products the greater the value of crop yields. For example, an increase in solids of just 1% represents ~20% increase in yield of certain processed products. High sugar content also increases the overall taste and flavor of the fresh fruit. For these reasons, increasing fruit solids content has been the focus of numerous tomato breeding programs (Foolad, 2007).

1.2.3 Fruit firmness

The fruit and vegetable market has become an international market. Consumers demand well developed produce at the peak of ripeness and possess the organoleptic characteristics expected for the species and variety. The development and condition of the produce must be such as to enable them to withstand transport and handling, and to arrive in satisfactory condition at their destination. Texture is one of the critical components for the consumer's perception of tomato fruit quality (Causse *et al.*, 2003; Serrano-Megias and Lopez-Nicolas, 2006). Many traits are involved in fruit texture, mainly sensory attributes such as flesh firmness, mealiness, meltiness, juiciness, and crispness (Harker *et al.*, 1997; Redgwell and Fischer, 2002; Szczesniak, 2002). Major changes in texture occur during fruit ripening, mainly associated with softening which considerably influences post-harvest performance, i.e. transportation, storage, shelf life and pathogen resistance (Brummell and Harpster, 2001). So far, particularly important is fruit firmness, an overall estimation of fruit resistance to compression, to be more precise a combination of skin resistance and flesh firmness (Grotte *et al.*, 2001). A number of works have been published on tomato fruit firmness measurements, and different instruments for measuring firmness has been illustrated (Batu, 1998). Instrumental measurements are generally used to evaluate firmness related to the mechanical properties of fruit tissues. The most widely used measurement of fruit texture is the Magness–Taylor firmness test, which assesses the maximum force needed to compress the sample in a specific way (Abbott, 2004). This system was mainly developed to evaluate commercial quality with respect to the tactile assessment of fruit firmness by consumers at the time of purchase. Many other types of destructive or non-destructive mechanical measurements exist, including compression, puncture, tension, and vibration. Rheological measurements inform about the mechanical properties of the fruit or of a localized part of the fruit (peel, pericarp, etc.).

1.2.4 L-ascorbic acid

Fresh fruit and vegetables are the principal source of ascorbic acid (AsA or vitamin C) for humans, primates and a few other mammals and passerines who are unable to synthesise this vitamin because of mutations in the enzyme catalysing the final step of its biosynthesis, L-gulono-1,4-lactone dehydrogenase. The vitamin C has numerous properties, including as an antioxidant and an enzyme cofactor for example in collagen synthesis (Arrigoni and De Tullio, 2002). Ascorbic acid is also an essential compound for plants, having a primary role as an antioxidant preventing oxidative stress as well as playing a role in plant development and hormone signalling (Pastori *et al.*, 2003), the activation of the cell cycle (Potters *et al.*, 2002) and possibly cell wall loosening during cell expansion or fruit ripening (Fry, 1998). Fruit ascorbic acid content is also valuable from an agronomic point of view, as well as documented evidence exists that the molecule can contribute to both biotic and abiotic stress tolerance response, and also to post-harvest fruit quality (Stevens *et al.*, 2008). However, the mechanism of how AsA levels are regulated in plant cells remains unknown. Plants contain variable amount of L-ascorbic acid that may range from low micromolar to 0.3 molar concentration, depending on the plant species, organ, tissue, cell, and the environmental conditions. The variability of ascorbate content is related to specific functions of vitamin C in plants and, probably, reflects the activity of different biosynthetic but also catabolic pathways in a given plant cell or even cell compartment (Wolucka and Van Montagu, 2007). In plants the major ascorbic acid biosynthesis pathway involves activated forms of the sugars GDP-D-mannose, GDP-L-galactose and L-galactose, before finally L-galactono-1,4-lactone is derived and converted to L-ascorbic acid (Wheeler *et al.*, 1998; Valpuesta and Botella, 2004; Wolucka *et al.*, 2005). The identification of low ascorbic acid (vtc) mutants in Arabidopsis (Conklin *et al.*, 2000) has helped to confirm the intermediates of the pathway and the essential role of enzymes such as GDP-mannose pyrophosphorylase (GMP; vtc1) (Conklin *et al.*, 1999) and L-galactose-1-P phosphatase (vtc4) (Conklin *et al.*, 2006). An alternative pathway has been proposed that uses GDP-gulose and L-gulose instead of the corresponding galactose sugars

(Wolucka *et al.*, 2005) and in strawberry a third pathway has been identified involving the conversion of D-galacturonic acid to L-ascorbic acid via L-galactono-1,4-lactone (Agius *et al.*, 2003). A recycling pathway also exists for ascorbic acid: because of its role as an antioxidant, reduced ascorbate is oxidised into an unstable radical (monodehydroascorbate) which disproportionate into ascorbate and dehydroascorbate, the latter representing the second oxidised form. Dehydroascorbate is also unstable and rapidly degrades so the ascorbate pool can be depleted if the oxidised forms are not recovered by two reductases: monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) (Noctor and Foyer, 1998; Smirnoff and Wheeler, 2000). Modulation of DHAR activity may control the levels of ascorbate in tissues. Overexpression of this enzyme in tobacco increases ascorbic acid levels 2- to 4-fold (Chen *et al.*, 2003) and significantly increased the ascorbate redox state (Chen *et al.*, 2003; Kwon *et al.*, 2003). The regulation of ascorbate levels in cells is therefore tightly controlled by the level of synthesis and recycling as well as degradation (Pallanca and Smirnoff, 2000; Green and Fry, 2005) and the transport of this molecule within the cell (Horemans *et al.*, 2000) although little is known about the details of the latter two processes.

1.2.5 Phenolic compounds

Phenylpropanoid-derived compounds are ubiquitous plant secondary products. These compounds are involved in a broad range of physiological and ecological processes, ranging from recruitment of pollinators and seed dispersers, interactions with pathogens, nitrogen-fixing bacteria and parasitic roots in the rhizosphere, control of male fertility, UV-tolerance, antioxidant based defence, auxin transport, and defence against microbes and grazers (Grotewold, 2006). This wide range of biological functions is matched by an equally vast structural diversity. It has been estimated that around 8000 different phenolic compounds are synthesized by plants, via a variety of polymerization, hydroxylation, methylation, glycosylation, acylation, prenylation, and condensation reactions (Pourcel *et al.*, 2006). The accumulation of phenolic compounds is a carefully

controlled process with both the levels of phenolics and the composition of the phenolic pool varying considerably among organisms, tissues, developmental stages and in relation to environmental conditions (Winkel-Shirley, 2002). Although a large variety of plant phenols exist, most share a common origin: the amino acid phenylalanine. Phenylalanine is deaminated to cinnamic acid, which enters the phenylpropanoid pathway. A key step in this biosynthetic route is the introduction of one or more hydroxyl groups into the phenyl ring, thus producing phenols. As a result, these phenols are derived from a common building block in their carbon skeleton: the phenylpropanoid unit $C_6 – C_3$. Biosynthesis according to this pathway produces the large variety of plant phenols: cinnamic acids ($C_6 – C_3$), benzoic acids ($C_6 – C_3$ or $C_6 – C_1$), flavonoids ($C_6 – C_3 – C_6$), proanthocyanidins ($(C_6 – C_3 – C_6)_n$), stilbenes ($C_6 – C_2 – C_6$), coumarins ($C_6 – C_3$), lignans ($C_6 – C_3 – C_3 – C_6$), and lignins ($(C_6 – C_3)_n$). Polyphenols are the most abundant antioxidant in our diet, since the average daily intake is about 1 g, which is almost 10-fold the intake of vitamin C, 100-fold the intake of vitamin E, and 500-fold the intake of carotenoids (Georgè *et al.*, 2005). As dietary component, the phenolic compounds are thought to have health-promoting properties, probably due to their high antioxidant capacity (Duthie and Crozier, 2000). This function/activity is supported by their ability, *in vitro*, to induce human protective enzyme system and by a number of epidemiological studies which suggest a protective effect against cardiovascular disease in particular, but also against cancer and other age-related diseases such as dementia (Nijveldt *et al.*, 2001). Chlorogenic acid (5′-caffeoylquinic acid), a hydroxycinnamic acid conjugate, is the main phenol in tomato. Rutin (quercetin-3-rhamnosylglucoside) and naringenin are representative flavonoids of tomato, respectively conjugated and nonconjugated, and flavonols such as myricetin are also found in tomato and its products. More recently, resveratrol, which is found primarily in the grape skin and reported to possess cancer prevention properties based on *in vitro* assay results, is also found to exist in both nontransgenic and transgenic tomatoes (Nicoletti *et al.*, 2007).

1.3 Tomato breeding

Breeding new cultivars of tomato with improved characteristics started more than 200 years ago in Europe (mainly in Italy). Although the process of plant breeding is theoretically simple, it does create novelty. The art of tomato breeding is identifying and combining the specific traits for each market. The goals of public and private tomato breeding programmes vary widely depending on location, need and resources. In general, breeding goals in tomato have gone through four phases: breeding for yield in the 1970s, for shelf-life in the 1980s, for taste in the 1990s and for nutritional quality currently (Bai and Lindhout 2007). Recently, recovery of these quality phenotypes in food crops has benefited from renewed research activity (Goff and Klee 2006), a trend driven both by the efforts of public health agencies and health professionals to add more nutritious, 'functional' foods to our diets and by the willingness of consumers to pay for them. The key challenge is to subsequently introduce this specific genetic material (DNA) into elite production varieties through breeding while retaining their performance attributes (Giovannoni, 2006). The advent of genomics has brought a real boost to the generation of data, knowledge and tools that can be applied to breeding, and that transformed breeding from a rate individually based activity to a multidisciplinary teamwork which is most suited to exploit genes from tomato germplasm in an efficient way. As a result, it is expected that the improvement of tomato cultivars will continue in the future. With advances in genome mapping and quantitative genetic analyses, the genetic basis is being dissected for traits that are related to domestication in many crops (Poncet *et al.*, 2004). The advent of molecular markers and linkage maps has made it possible to find associations between markers and phenotypes. Breeders can use a known association of molecular markers with a trait or a chromosome segment to select the presence of molecular markers rather than the phenotype. Nowadays, dozens of genes, important for tomato breeding, have been mapped and molecular markers have been made available online (http://sgn.cornell.edu). Breeders use these markers to a great extent with the main aim of increasing the efficiency of breeding programmes. Via marker-assisted selection, the paradigm of plant breeding has changed from

selection of phenotypes towards selection of genes, either directly or indirectly. With the advance of tomato genome sequences and genomics, the genetic basis of plant growth and development are expected to be better understood. Knowing the candidate genes for important traits and having the knowledge of exact functional nucleotide polymorphism within the gene, breeders can easily identify useful alleles in the wild germplasm and create novel genotypes by introgressing and pyramiding favourite unused natural alleles and/or even by shuffling and re-organization of genomic sequences. Learning from domestication and with more and more available knowledge on genomics, plant breeders might consider manipulating transcription and regulation factors in the genome to generate a pool of new trait variation. Very recently, Davuluri *et al*. (2005) demonstrated that manipulation of a plant regulatory gene can influence the production of several phytonutrients generated from independent biosynthetic pathways and lead to a novel genotype that cannot be achieved by a conventional breeding approach

1.4 Biotechnological approaches to tomato breeding for quality traits

Tomato has been an excellent model system for both basic and applied plant research. This has been due to many reasons, including ease of culture under a wide range of environments, short life cycle, photoperiod insensitivity, high self fertility and homozygosity, great reproductive potential, ease of controlled pollination and hybridization. Additionally, tomato is a diploid species with a rather small genome (0.95 pg/1C, 950 Mbp), lacks of gene duplication, has an amenable to asexual propagation and whole plant regeneration, allows to develop haploids, and account for availability of a wide array of mutants and genetic stocks (including wild species; http://tgrc.ucdavis.edu; http://www.sgn.cornell.edu). Tomato's regenerative plasticity also allows easy grafting, an attribute that facilitates certain developmental and practical studies. Recent availability of high molecular weight insert genomic libraries, including both YAC and BAC libraries, has facilitated map-based or positional cloning. Furthermore, members of *Solanum* are easily transformed, and transgenic

tomatoes are routinely produced using co-cultivation with *Agrobacterium tumefaciens*. Tomato was the first food crop in the U.S. for which a genetically engineered variety was marketed and also for which a disease resistance gene was positionally coloned (Fooland, 2007). Tomato is not only a major crop but also a model for fruit development with a wealth of data available at physiological and genetic levels. Research on this species is set to continue with the current genome-sequencing project (Mueller *et al.*, 2005). The plant lends itself to studies on fruit architecture, ripening and all aspects of fruit quality; for this reason different ILs populations have been created and evaluated (Eshed and Zamir, 1995; Causse *et al.*, 2002). Particularly, many efforts have been addressed to improve both organoleptic and nutritional quality in tomato fruit (Verhoeyen *et al.*, 2002; Fraser and Bramely, 2004; Levin *et al.*, 2004). Strategies to achieve the goal focused mainly on transgenic approaches and on characterization of mutant or quantitative trait loci with pronounced effects on such traits. Consistent with public concerns and policy limitation on widespread cropping of genetically modified plants, alternative approaches to breeding for quality traits are desirable. In particular, the –omics technologies could help to resolve the complex traits in major genes and link higher performing genotypes to polymorphic QTLs. This may allow tracking quality-associated superior allelic combinations in assisted breeding programs, despite QTL show low heredity and high environmental interaction. Many –omics approaches used in tomato for dissecting genetic determinants of fruit quality have focused on ripening processes because of the high magnitude of metabolic modifications they imply. In fact, ripening confers desirable flavour, colour, and texture, increases fruit pathogen susceptibility, imparts numerous quality and nutritional characteristics including fiber content and composition, lipid metabolism and antioxidant composition. The ability to understand key control point in global ripening regulation or within specific ripening processes, such as carotenoid, flavonoid, vitamin, and flavour volatiles, will allow for manipulation of nutrition and quality characteristics associated with ripening. Among –omics strategies, comparative transcriptomic performed on phenotypes of interest is a powerful strategy for identification of key genetic regulatory mechanism.

1.5 Tomato introgression lines and quantitative traits loci

Traditionally, quantitative trait mapping studies in plants and animals involve whole genome segregating populations, but epistatic interactions in F2 or recombinant inbred lines (RILs) make it difficult to fully define and characterize individual loci. Introgression lines (ILs) are a set of nearly isogenic lines developed through a succession of backcrosses, where each line carries a single marker-defined chromosome segment from a divergent genome (Zamir, 2001). The term ILs, often used in plant biology, is synonymous with chromosome substitution strains (CSS) or 'congenics', which have been constructed for mice (Lhote *et al.*, 2007; Singer *et al.*, 2004). A complete IL population reconstitutes the donor parent genome in overlapping chromosomal segments and is permanent since it can be maintained by self-pollination. Consequently, these populations are very effective in identifying and stabilizing quantitative traits loci (QTL), because any phenotypic difference between an IL and the recurrent parent is attributed solely to one or more donor parent genes within the introgressed chromosomal segment (Lippman *et al.*, 2007). The principles of the IL approach were first demonstrated in tomato. *Solanum lycopersicum* (domesticated tomato) is one of the 17 core species in the tomato clade. The small green-fruited desert species *Solanum pennellii* is a distant relative of *S. lycopersicum*, having evolved unique adaptations in terms of morphology, mating system, chemistry (especially secondary compounds) and responses to biotic/abiotic stress. Despite these drastic ecological differences, *S. pennellii* is sexually compatible and produces fertile hybrids with *S. lycopersicum*, making it the founding donor parent of the first IL population used for interspecific QTL identification, cloning, and plant breeding. The ILs, representing whole-genome coverage of *S. pennellii* in overlapping segments in the genetic background of *S. lycopersicum* (cv. M82) presently consists of 76 genotypes. The *S. pennellii* ILs have been publicly available and have been phenotyped for hundreds of traits including repeated measurements of the same traits, thus allowing for the identification of 2795 QTL (Lippman *et al.*, 2007). The stability of the ILs has allowed multiple years of phenotyping over different environments, which has led to the reproducible identification of QTL

for more integrated traits. The *S. pennellii* ILs have recently gone beyond standard QTL identification studies, and ventured into a multifaceted systems-level analysis to address a classical biological question relating to plant architecture and physiology. A large-scale association study linking plant structure and biochemistry was carried out by phenotyping the ILs for a wide range of plant morphology and fruit metabolic profiles (Schauer *et al.*, 2006). Plant geneticists, realizing the value of wild species diversity to the genetic improvement of plants, have generated over the past decade numerous populations that segregate for genetically mapped complex phenotypic variation. IL populations are now available for many wild tomato species (Brouwer and St Clair, 2004; Finkers *et al.*, 2007; Frary *et al.*, 2004; Monforte and Tanksley, 2000; and Peleman and van der Voort, 2003). Similarly, the power of the IL approach is now being realized in other model systems, including major food and biofuel crops such as pepper (Ben Chaim *et al.*, 2003), rice (Ashikari and Matsuoka 2006), barley (von Korff *et al.*, 2004), wheat (Liu *et al.*, 2006), maize (Szalma *et al.*, 2007), soybean (Concibido *et al.*, 2003) and Arabidopsis (Keurentjes *et al.*, 2007). These populations are being phenotyped widely, but only a small fraction of the raw data finds its way to existing databases. Achieving multilayered phenotypic integration in all model systems will be necessary in the future to realize the full discovery potential of genomics-assisted comparative QTL studies.

1.6 Aim of the study

Tomato fruit quality for fresh consumption is determined by tomato organoleptic quality, which can be defined by physical-chemical and sensorial parameters that make the product satisfactory for consumers, and by nutritional quality, characterized by a number of parameters responsible for properties related to human health.

Organoleptic quality involves taste and flavour, but also the colour and texture of the fruit. Most of these quality traits show a continuous variation, strongly induced by environmental condition. The genetic variation of such traits was

attributed to the joint action of many genes (QTLs), which can be mapped on the genome with genetic markers.

Tomato nutritional quality is characterized by the biomolecules (carotenoids, vitamins, and phenols) with antioxidant proprieties present in fruits, leading to the beneficial role of tomato intake in human diseases. In the last few years, one of the main object in tomato breeding programmes was to select genotypes with better total quality characteristics.

The aims of the present thesis were:

1. to study the AtCCD7 and AtCCD8 genes involvement in the volatile apocarotenoid production to clarify the key point of the biosynthetic carotenoid pathway using *E. coli* as model biological system in which genes could be over-express
2. to identify genomic regions controlling the antioxidant capacity in tomato fruit by detecting new QTLs for ascorbic acid, phenolic compounds, soluble solids content, and fruit firmness in the *S. pennellii* IL population that can be introgressed into desirable genetic backgrounds;
3. to clarify by means of comparative transcriptomics analysis the plant biosynthetic AsA pathway in order to identify key sequences directly involved in fruit ascorbate content or genes underlying the accumulation regulative mechanisms.

2. Materials and Methods

2.1 Plant material and fruit sampling

Seeds from 76 independent ILs of *S. pennellii* population, and 8 wild *Solanum* accessions were kindly provided by C.M. Rick, Tomato Genetics Resource Center (TGRC) (http://tgrc.ucdavis.edu/) (Tab. 2.1). Each IL includes single marker defined introgressed genomic regions from the wild green-fruited species *S. pennellii* (LA0716) in the genomic background of the cultivated *S. lycopersicum* (inbred variety M82, Acc LA3475). Overall, the IL population provide a complete coverage of the wild-species genome (Fig. 2.1).

Table 2.1 Plant material

ILs	All 76 ILs of *Solanum pennellii* population
Wild accessions	*S. pimpinellifolium* LA0722
	S. pimpinellifolium LA2904
	S. chmielewskii LA1306
	S. habrochaites LA1777
	S. neorickii LA2133

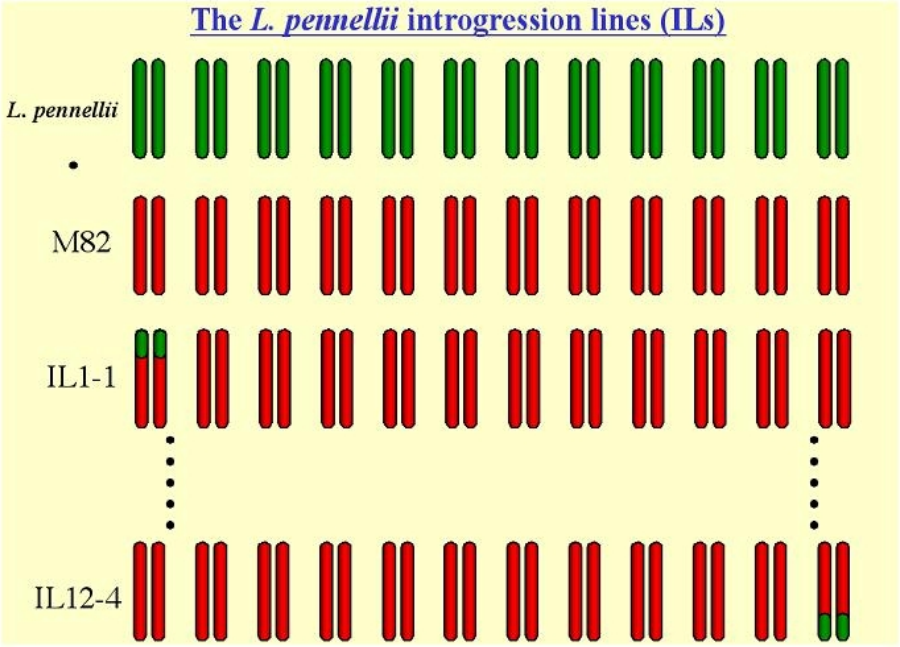


Figure 2.1 Development of *S. pennellii* introgression lines. The ILs have been produced through successive introgression backcrossing and marker-assisted selection to generate a set of recurrent parent lines with a single homozygous introgressed segment (Eshed and Zamir, 1995). Sublines were obtained as the product of backcrossing selected introgressed lines with M82, so generating smaller introgressed sections. The *S. pennelli* ILs thus subdivide the tomato genome into 107 "bins".

Plants were grown in a controlled cold greenhouse at 20°C temperature. Ten seeds for each line were placed on water-soaked paper in Petri disc and incubated in growth chamber at 25°C in the dark until germination. Then the *plantule* were transplanted in a tray until 10-15 cm of height was reached. Three to five plants from each genotype were transplanted in cold greenhouse, located in Portici (NA) Italy, for three consecutive summer trials (2006, 2007 and 2008). Individual tomato plants were grown in pots (20cm diameter) containing sterilized soil supplemented with Nitrophoska slow release fertilizer.

Plants were laid down according to a completely randomized design and were watered daily. In order to provide an estimate of *S. pennellii* homozygote and heterozygote values the F_1 hybrid (*S. pennellii* X *S. lycopersicum* cv. M82) was

also grown. Samples were made by pooling red ripe fruit from the same plant. At least three samples per IL were collected and each sample consisted of a number of fruit as higher as possible.
Intact fruit were used for measuring firmness and soluble solids content. 50-200 g of whole fruit were also longitudinally cut, seeds and locular jelly removed, and flesh frozen by immersion in liquid nitrogen. Frozen tissues were ground by a laboratory WARUING® Commercial blander. Resulted powder was frozen again in liquid nitrogen and stored at -80 °C until required for AsA and phenols measurement and RNA extraction and purification.

2.2 Phenotypic measurements

°Brix

Ripe fruit mesocarp was squeezed and the soluble solids content of the resulting juice measured on a portable refractometer ATAGO Model ATC-1. Soluble solids content were expressed on a °Brix scale. For each genotype the average of 10-20 measures was considered.

Fruit firmness

The firmness value of tomatoes was determined by using a T.R. TURIONI penetrometer. A probe was applied at as many as possible points on the fruit equator, the displacement of the probe when compressing the fruit was recorded, and the average of 10-20 measures was used for each genotype.

Ascorbic acid and total ascorbic acid

Ascorbic acid (AsA) and total ascorbic acid (AsA + dehydroascorbate) were measured using a modified version of the enzymatic method described by Kampfenkel et al. (1995). The assay is based on the reduction of Fe^{3+} to Fe^{2+} by AsA and the spectrophotometric detection of Fe^{2+} complexed with 2,2'-dipyridyl. Deydroascorbate is reduced to AsA by preincubation of the sample with dithiothreitol (DTT). Subsequently, the excess DTT is removed with N-ethylmaleimide (NEM), and total AsA is determined by 2,2'-dipyridyl method.

250 mg of frozen powder were weighted in a 1.5 ml tube with a bead and 200 µl of ice-cold 6% trichloroacetic acid (TCA) added. Samples were homogenized twice in a Tissue Lyzer (Qiagen) at 50 Hz for 1 min. Tubes were centrifuged for 25 min at 25000 g at 4°C. Recovered supernatant was brought to 500 µl with 6% TCA and 50 µl were used in the assay. The assay procedure is shown in Table 2.2. The absorbance was read at 525 nm using water as reference with a UV spectrophotometer (Beckman DU-640). The ascorbic acid concentration was expressed as µmol g^{-1} of fresh weight based on the following standard curve: Abs = 3,6593 x µmol AsA designed over a dynamic range 0 to 0.7 µmol AsA (R^2 = 0.9982). The total AsA In the same way the total AsA concentration was expressed as µmol g^{-1} of fresh weight based on the standard curve: Abs = 0.495 x µmol DHA designed over a dynamic range 0 to 0.7 µmol DHA (R^2 = 0.97).

Table 2.2 Pipette scheme in protocol used for AsA and total AsA determination

	AsA		Total AsA	
Component in assay (ml)	Test	Blank	Test	Blank
Standard/Sample	0.05	-	0.05	-
10 mM DTT	-		0.05	0.05
6% TCA	-	0.05	-	0.05
0.2 M phosphate buffer (pH 7.4)	0.15	0.15	0.1[a]	0.1[a]
0.5% NEM	-	-	0.05[b]	0.05[b]
H_2O double distilled	0.05	0.05	-	-
10% TCA	0.25	0.25	0.25	0.25
42% H_3PO_4	0.2	0.2	0.02	0.02
4% 2,2'-dipyridyl	0.2	0.2	0.02	0.02
3% $FeCl_3$[c]	0.1[d]	0.1[d]	0.1[d]	0.1[d]

[a] Mix and incubate 15 min at 42 °C in water bath

[b] Mix and incubate 1 min at room temperature

[c] Mix immediately and vigorously after addition of $FeCl_3$, otherwise turbidity results

[d] Incubate at 42 °C in water bath for 40 min and read absorbance at 525 nm

Total phenols

The amount of total phenolics compound was determined according to Folin-Ciocalteu's procedure (Singleton and Rossi, 1965). 1 ml of 60% methanol was added to 250 mg of ground tissue in a 1.5 ml tube with a bead. Samples were homogenized twice in a Tissue Lyzer (Qiagen) at 50 Hz for 1 min. Extracts were placed on ice 3 min in the dark and vortexed. The extraction was transferred in a 15 ml tube and volume brought to 5 ml with 60% methanol. Samples were centrifuged at 3000 g for 5 min. 62.5 microliters of the supernatant, 62.5 µl of Folin-Ciocalteu's reagent (Sigma), and 250 µl of deionizated water were incubated for 6 min. After addition of 625 µl of 7.5% sodium carbonate and 500 µl of deionizated water, samples were incubated for 90 min at room temperature in the dark. Absorbance was measured at 760 nm. The total phenolics concentration was expressed in terms of µg of gallic acid equivalents mg^{-1} of fresh weight, based on a gallic acid standard curve designed over a dynamic range 0 to 125. Abs 760nm = (0.0234 x µg gallic ac.) – 0.0776 (R^2 = 0.995).

2.3 Statistical analysis of phenotypic data

Physical and chemical data collected on the IL population and wild accessions were analyzed by mean of parametric test by using SPSS (Statistical Package for Social Sciences) Package 6 version 15.0. Significance of QTL was determined by comparing mean values of individual ILs to the control M82 through a factorial analysis of variance (One-way-ANOVA) with LSD post-hoc test at significance level of 0.05. ANOVA was performed to assess year and genotype effects. Analysis of variance was used to study the differences between ILs and M82 for firmness, soluble solids content, ascorbate and total phenolics concentration. The presence of a QTL was hypothesized when an introgression had a significant effect at least over two trial seasons.

2.4 Gene expression analysis

2.4.1 RNA isolation

Total RNA was isolated from homogenized powdered tomato fruit stored at -80°C according to Griffiths and co-workers (1999) procedure. Approximately, 4 g of ground flesh were added to 12 ml of Extraction Buffer (Solution A) and an equal volume of phenol/chloroform (Solution B) in an Oakridge tube. Tubes were vigorously shaken and then centrifuged at 10000 rpm (15 min, Room Temperature (RT)). 11 ml of the aqueous phase was then transferred to a clean Oakridge tube, the nucleic acid was precipitated in the aqueous phase for 1 h at -20°C with the addition of 27.5 ml of ice-cold 100% ethanol and 1.1 ml of 3 M sodium acetate (pH 6.0). Following centrifugation at 10000 rpm (15 min, 4 °C) the pellet was washed in 70% ethanol. The samples were then redissolved in 2 ml of DEPC-treated water, followed by the addition of 2 ml of 2X cetyltrimethylammonium bromide (CTAB) Extraction Buffer (Solution C). Nucleic acids were precipitated by the addition of 4 ml of CTAB Precipitation Buffer (Solution D) and centrifuged at 12000 rpm (30 min. RT). The pellet was re-suspend in 400 µl of 1.4 M NaCl, followed by the addition of 1 ml of ice-cold 100% ethanol and precipitated at -20°C for 1 h to over-night. The samples were then centrifuged at max speed (10 min, 4 °C), the pellets washed in 500 µl of 70% ethanol, redissolved in 400 µl of DEPC-treated water and incubated at 50 °C for 5 min. 400 µl of phenol/chloroform (Solution B) were added twice. The nucleic acid was precipitated in 0.1 volume of 3 M sodium acetate (pH 6.0) and 3 volumes of ice-cold 100% ethanol for 1 h to over-night at -20 °C. The samples were washed with 500 µl of 70% ethanol. The pellet was redissolved in 180 µl of DEPC-treated water, followed by addition of 20 µl of RQ1 DNase Reaction Buffer (Solution E) and 1 µl of RQ1 DNase (Promega)

and incubated at 37°C for 30 min. The RNA samples were finally collected for spectrophotometric quantification and stored at -80 °C. The purity of the RNA was determined using a Bio Rad Smart Spec 3000 spectrophotometer and integrity was confirmed using the AGILENT 2100 Nano Bioanalyzer with RNA 6000 nano chip

RNA Extraction Buffer (Solution A)

6% (w/v) 4-aminosalicylic acid
1% (w/v) 1,5-naphthalenedisulphonic acid
50mM Tris-HCL pH 8.3
5% (w/v) Phenol Solution

Phenol Solution

100 g Phenol crystals
14 ml m-cresol
0.1 g 8-hydroxy-quinoline
30 ml DEPC-treated water

Phenol/Chloroform Solution (Solution B)

500 g Phenol crystals
0.5 g 8-hydroxy-quinoline
500 ml Chloroform
20 ml Iso-amyl alcohol
200 ml 100mM Tris-HCLpH 8.0

2X CTAB Extraction Buffer (Solution C)

1.4 M NaCl
2% (w/v) CTAB
0.1 M Tris-HCL pH 8.0
20 mM EDTA pH 8.0

CTAB Precipitation Buffer (Solution D)

1% CTAB (w/v)
50 mM Tris-HCL pH 8.0
10 mM EDTA pH 8.0

10X RQ1 DNase Reaction Buffer (Solution E)

400 mM Tris-HCL pH 8.0
100 mM $MgSO_4$

10 mM $CaCl_2$

2.4.2 Synthesis antisense RNA

The aRNA was synthesised using SuperScript™ Indirect RNA Amplification System Kit (Invitrogen Catalog no. L1016-02) and labeled with fluorescent dye Alexa Fluor 647 Reactive Dye. In particular, the first step was to synthesise the cDNA. In a 1.5-ml RNase-free tube, was added:

Component	*Sample*
1 µg of mRNA	
T7-Oligo(dT) Primer	1 µl
DEPC-treated water	to 10 µl

The mix was incubated at 70°C for 10 min, and then placed on ice for 1 min, and centrifuged briefly to collect the contents. After that was added:

Component	*Volume*
5X First-Strand buffer	4 µl
0.1 M DTT	2 µl
10 mM dNTP Mix	1 µl
RNaseOUT (40 U/µl)	1 µl
SuperSript III RT (200 U/µl)	2 µl
Total Reaction Volume	20 µl

The tube was centrifuged briefly and then incubated at 46°C for 2 min, following an incubation at 70°C for 10 min. After incubation the synthesis of the Second-Strand cDNA was performed To the tube placed on ice was added:

Component	*Sample*
DEPC-treated water	91 µl
5X Second-Strand buffer	30 µl

10 mM dNTP Mix	3 µl
E. coli DNA Polymerase I (10 units/µl)	4 µl
E. coli DNA Ligase (10 units/µl)	1 µl
E. coli RNase H (2 units/µl)	1 µl
Total Volume	150 µl

The reaction mixture was incubated at 16°C for 2 hours, and then placed on ice. To purify the cDNA 500 µl of cDNA Loading Buffer were added to the reaction tube, and then the mixture was loaded directly onto the Spin Column. The samples were then centrifuged at 6000 g at RT for 1 min, and the flow-through discarded. cDNA was washed twice with 700 µl of Wash Buffer, following two centrifugation steps at 6000 g at RT for 1 min and 2 min respectively. To eluate the cDNA 24 µl of DEPC-treated water were added and incubation was done at RT for 2 min. Finally, samples were centrifuged at 10000 g at RT for 1 min to collect the purified cDNA. To obtain the aRNA the *in vitro* transcription was performed adding the following components at room temperature:

Component	*Sample*
100 mM ATP	1.5 µl
100 mM CTP	1.5 µl
100 mM GTP	1.5 µl
100 mM UTP	0.75 µl
50 mM aa-UTP	2 µl
10X T7 Reaction Buffer	4 µl
T7 Enzyme Mix	7 µl

The samples were incubated at 37°C over-night. aRNA Binding Buffer was added to bring the total volume to 200 µl, and then 100 µl of 100% ethanol. The entire aRNA/Buffer Solution was loaded directly onto the Spin Column and centrifuged at 12000 g for 15 sec at RT. The flow-through was discarded and 500 µl of a RNA Wash Buffer added, following centrifuge at 12000 g for 15 sec. This step was performed twice. To eluate the aRNA 100 µl of DEPC-treated water were added and then incubated at RT for 1

min and centrifuged at 12000 g for 2 min. The aRNA was labelled with Alexa Fluor 647 Reactive Dye. 3 µg of aRNA were placed in a speed-vacum to evaporate the samples at low heat until the volume was reduced to ≤ 3 µl. The dye was resuspended in 8 µl of 2X Coupling Buffer and dye solution was added to the aRNA. The solution was mixed thoroughly. The samples were incubated at RT in the dark for 30 min. To purify the dye-coupled aRNA the same procedure mentioned above for the RNA purification was used.

2.5 Chip design

The 90k TomatoArray1.0 microarray chip has been synthesized on Combimatrix platform to the Facoltà di Scienze Matematiche Fisiche e Naturali at the Università di Verona (Italy), in the frame of a collaboration with prof. M. Pezzotti (Dipartimento di Scienze Tecnologie e Mercati della Vite e del Vino) and prof. M. Delledonne (Dipartimento Scientifico e Tecnologico). The TomatoArray1.0 chip contains 90k siliceous electrodes supporting 20200 *in situ* synthesized DNA probes with 4 replications. Each probe consists of 35-mer oligonucleotides designed to be specific for a different Tentative Consensus (TC) from TIGR *S. lycopersicum* Gene Index Release 11.0 (June 21, 2006). As negative control nine bacterial oligonucleotide sequences provided by Combimatrix were utilized. The Custom Array™ 90K was designed with four replicates of each probe randomly distributed across the array to allow measurement of the variability within the array.

2.6 Hybridization and imaging

Labeled aRNA was hybridized on the TomatoArray 1.0. The microarray slides were pre-hybridized, onto the rotisserie in the hybridization oven, for 30 min at 45°C in the Pre-hybridization Solution (Solution A). The microarray was then

hybridized filling the hybridization chamber with the Hybridization Solution (Solution B), , and incubated for 14 h at 45°C in the hybridization oven. After hybridization the slides were iteratively washed with different Wash Solution (Solution C, D, E) until the final wash with PBST Wash Solution (Solution F), and incubated at room temperature for 1 min. Three hybridization *replica per* each genotype (M82, IL 12-4) *per* two years (2007-2008) for a total of six *replica per* genotype were performed. In particular within each genotype, an hybridization *replica* was done using RNA extracted from fruit coming from a single plant. Slides were then scanned using a Perkin Elmer ScanArray 4000 XL scanner and acquisition software (ScanArray Express Microarray Analysis System Version 4.0) according to the manufacturer's instructions For more detailes see CustomArray® 90K Microarray - Hybridization and Imaging Protocol (PTL020) available at website http://www.combimatrix.com/support_docs.htm.

Pre-hybridization Solution (Solution A)	Volume for 120 µl
2X Hyb Solution stock[a]	60 µl
Nuclease-free water	41 µl
50X Denhart's solution	12 µl
Salmon sperm DNA (10 mg ml^{-1})	1 µl
1% SDS	6 µl

[a]2X Hyb Solution stock	Volume for 10 ml
20X SSPE	6 ml
10% Tween-20	100 µl
0.5 M EDTA	560 µl
Nuclease-free water	3.34 ml

Hybridization Solution (Solution B)	Volume for 120 µl
2X Hyb Solution stock[a]	60 µl
DI Formamide	30 µl
Labeled RNA	10 µl
Salmon sperm DNA (10 mg ml^{-1})	1 µl
1% SDS	5 µl
Nuclease-free water	to 120 µl

6X SSPET Wash Solution (Solution C)	Volume for 10 ml
20X SSPE	3 ml
10% Tween-20	50 µl
Nuclease-free water	6.95 ml

3X SSPET Wash Solution (Solution D)	Volume for 10 ml
20X SSPE	1.5 ml
10% Tween-20	50 µl
Nuclease-free water	8.45 ml

0.5X SSPET Wash Solution (Solution E)	Volume for 10 ml
20X SSPE	250 µl
10% Tween-20	50 µl
Nuclease-free water	9.7 ml

PBST Wash Solution (Solution F)	Volume for 10 ml
10X PBS	2 ml
10% Tween-20	100 µl
Nuclease-free water	7.9 ml

2.7 Data extraction, normalization and filtring

After scanning microarray TIF images were processed to generate numerical data using the CombiMatrix Microarray Imager Software version 5.8.0 copy@right 2001. The Quick Start Guide or the Microarray Imager User's Manual is available at the website https://webapps.combimatrix.com. Signal probe medians and standard deviations were further imported in SPSS software.

Normalization between arrays was achieved by correcting each probe median based on the ratio between the median of the array and the average median of arrays. Following data normalization and quality control all values were log transformed (log base 2).

Finally, probe signals having a variability coefficient higher than 0.5, as well as spikes and factory probes, were filtered out. Also, probe showing the 10%

uppermost and the 10% lowest signal intensity were deleted. To visually check normalization Box plot were provided. The median of the data is represented by the line in the center of the rectangular box; the two ends of the rectangles represent the upper quartile and the lower quartile. The other two values always shown are the maximum and minimum value of the data set (Fig. 2.2).

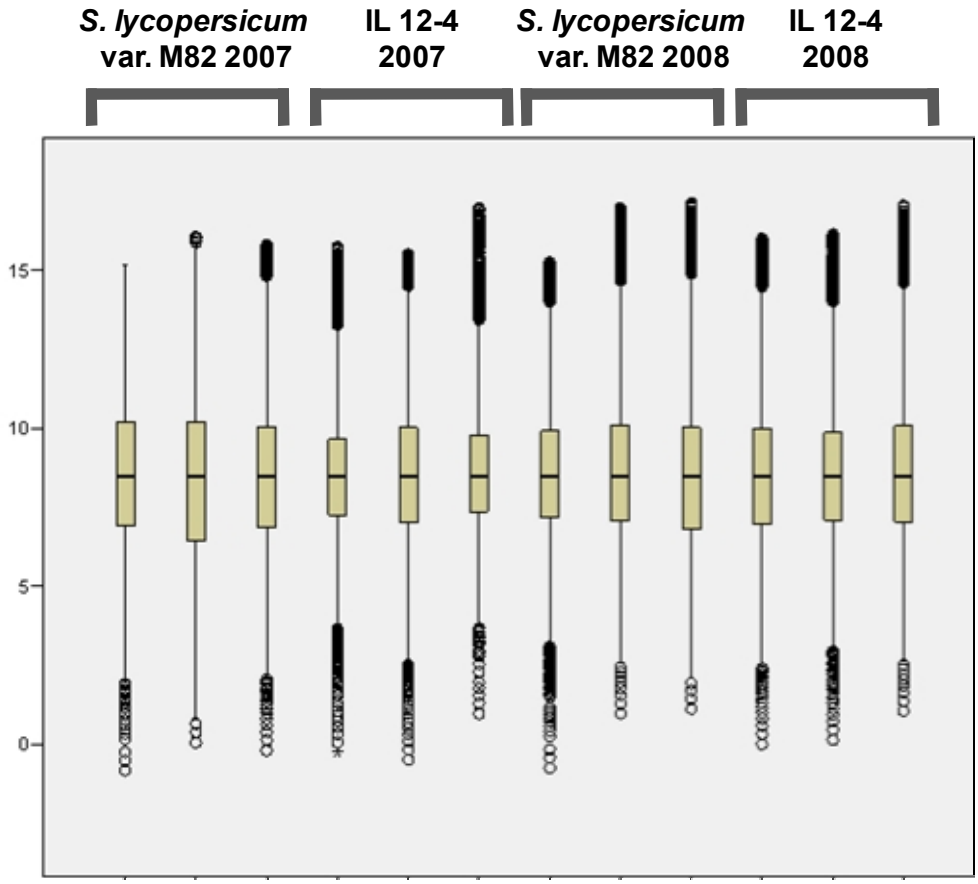


Figure 2.2. Box plot of the microarray experiment. Each rectangle represent one hybridization experiment. Line inside the box represent the median of a signal registered from a single hybridization.

2.8 Statistical analysis and bioinformatics procedures

Differentially expressed signals were identified using the t-test module contained within the TIGR Multiple Experiment Viewer version 4.0 (http://www.tigr.org/software/tm4/ - Saeed *et al.*, 2003). In particular, the between-subject algorithm with a statistical significance of $P < 0.01$ was used for comparing transcriptomic profiles of IL12-4 with the M82 counterpart (Tusher *et al.*, 2001).

Blast2GO (http://blast2go.bioinfo.cipf.es/ - Conesa *et al.*, 2005) was used to provide automatic and high-throughput annotation, gene ontology mapping, and categorization of TCs showing differential transcription signal. An expectation value < E 10^{-10} was used.

2.9 *E. coli* transformation

pDEST-15 empty vector (Invitrogen) and pDEST-15 derived expression clone containing a coding sequence (CDS) cassette of the genes AtCCD7 and AtCCD8 were transformed into chemically competent *E. coli* BL21-AI (Invitrogen) cells harbouring plasmids encoding carotenoid biosynthetic genes (Cunningham and Gatt, 2001, 2007) (Tab. 2.3).

In BL21-AI cells the *ara*BAD promoter regulated by L-arabinose is used to control expression of T7 RNA polymerase promoter present into pDEST-15 vector. Cells have been transformed by a Heat Shock procedure. 50 μl of *E. coli* cells were thawed on ice, then 50 ng of vector were added and tubes were incubated on ice for 10 min. The samples were incubated into water bath at 42°C for 45 seconds and back on ice for 2 min. 900 μl of SOC broth (Solution A) were added and the samples were incubated at 37°C with shaking at ~250 rpm for 1 h. 100 μl of resulting culture were spreaded in plate on LB containing 100 μg ml^{-1} carbencillin (Sigma) and 34 μg ml^{-1} chloramphenicol (Sigma) and incubated over-night at 37 °C.

SOC broth 1 (Solution A)	1 Litre
Bacto-tryptone	20 g
Bacto yeast extract	5 g
NaCl	0.6 g
KCl	0.5 g
$MgCl_2$	10mM
$MgSO_4$	10mM
Glucose	20mM

Table 2.3 *E. coli* strains producing different carotenoids

E. coli strain code	Type of carotenoid
BL21-AI a	GGPP
BL21-AI b	Phytoene
BL21-AI c	Zeta-carotene
BL21-AI d	Lycopene
BL21-AI e	Delta-carotene
BL21-AI f	Beta-carotene
BL21-AI g	Zeaxanthin

2.10 Volatile analysis from *E. coli* cultures

Three individual colonies for each transformed bacterial strain were used to inoculate three different 3-ml cultures of liquid LB containing 100 μg ml^{-1} carbenicillin and 34 μg ml^{-1} chloramphenicol. The 3 ml cultures were grown overnight at 37°C. On the next day, 1 ml of overnight culture was used to inoculate 100 ml of LB containing 100 μg ml^{-1} carbenicillin and 34 μg ml^{-1} chloramphenicol in a 250-ml baffle flask. The cultures were grown at 200 rpm for 2.5–3 h at 33°C in darkness until an absorbance at 600 nm of 0.8–1.0 was reached. The flask was then chilled on ice for 5 min and induced with a final concentration of 0.1% L-arabinose (Fischer Scientific).

The cultures were placed back on the shaker and grown for an additional 3 h (200 rpm at 25°C). The cultures were then placed on a bench and capped with a rubber stopper, and partially purified air (the filter consisted of 0.5 mg of activated charcoal in a 0.6 x 10 cm glass tube) was bubbled through the cultures (~175 ml min^{-1}) for 2 h using a dual diaphragm air pump (General Hydroponics, Sebastopol, CA). The volatile head space was collected on a 30 mg Super Q column (80/100 mesh; Alltech Associates, Inc., Deerfield, IL).
After volatile collection, the A_{600} was recorded. The Super Q columns were eluted with 150 µl of methylene chloride (Fischer Scientific) after addition of 400 ng of nonyl acetate (in 5 µl of methylene chloride) as an internal standard. Nitrogen was blown over the samples to evaporate solvent until they reached a volume of ~300 µl. Separation of volatiles was performed on an Agilent DB-5 column (Palo Alto, CA) and an Agilent 6890N gas chromatograph according to Schmelz *et al*., (2001). The volatiles were compared with known standards (Sigma-Aldrich) and calculated as nmol A_{600}^{-1} h^{-1}. Any background present in control reactions was subtracted, and values were normalized on the percent recovery of each volatile. Percent recovery was calculated as the average amount of volatiles recovered from flasks with 100 ml of LB treated exactly as described above except than at the point of "induction" a known amount of each volatile compound was added. The identity of each peak was confirmed by gas chromatography/mass spectrometry using an Agilent 5975 mass detector in electron ionization mode.

2.11 Protein blot analysis

One ml of bacteria samples from each culture was recovered after volatiles collection. A_{600} was determine and samples were re-suspended in an amount of 2X SDS-PAGE Buffer (Solution A) equal to culture optical density. 20 µl of sample was then loaded onto 10% Ready Gel Tris-HCL (Bio-Rad), Electrophoresis chamber was filled with 1X Running Buffer (Solution B) and the run set at 200V until tracking dye reached the bottom of gel. Proteins were transferred to a nitrocellulose membrane using the Bio-Rad Mini Trans-Blot cell.

Membranes were blocked overnight in 10% Carnation milk/TBST at 4°C (Nestle; http://www.nestle.com). Membranes were washed twice for 5 min in TBST, and then incubated with primary anti-GST(1:2000) or anti-HIS (1:5000) antibody diluted in 5% Carnation milk/TBST for 1 h. Membranes were subsequently washed three times for 10 min in TBST, and then incubated with peroxidase-conjugated goat anti-rabbit (1:5000) secondary antibody (Kirkegaard & Perry Laboratories; http://www.kpl.com) diluted in 5% Carnation milk/TBST for 45 min. Membranes were finally washed three times for 10 min in TBST. Visualization of signal was performed using Amersham ECL detection reagents (http://www.amershambiosciences.com/) before exposure to film for 30 secons.

2X SDS-PAGE Buffer (Solution A)	Volume for 8 ml
Deionized water	2.9 ml
0.5 M Tris-HCL pH 6.0	1.0 ml
Glycerol	2.0 ml
10% SDS	1.6 ml
β-mercaptoethanol	0.4 ml
1.0% bromophenol blue	0.1 ml

10X Running Buffer (Solution B)	Volume for 500 ml
Tris base	15.0 g
Glycine	72.0 g
SDS	5.0 g
Deionized water	500 ml

3. RESULTS

3.1 QTL analysis in tomato *S. pennellii* IL population

In order to screen genetic resources for QTLs involved in controlling AsA and phenols concentration, total soluble solids content, and fruit firmness, chemical and physical analysis were performed on red ripe fruit of *S. pennelli* IL population and parental line. Missing genotypes are caused by lack of plant or fruit replications *per* trial. This was due to different constraint such as biotic and abiotic stress. Moreover, for fruit firmness the reduced size of fruit produced by different ILs often caused the measurement to fail. Because the flash collapse before been inflicted by the penetrometric probe. AsA accumulation and total phenols content were also analyzed in different tomato wild accessions.

3.1.1 Total soluble solids

On average, total soluble solids into *S. lycopersicum* cv. M82 fruit was 4.24 °Brix. In the IL population the total soluble solids content of red ripe fruit showed to vary over the range from 3.07 °Brix (IL 2-1) to 6.5 °Brix (IL 2-3). One-way ANOVA test displayed no significant interaction between genotype and year (Tab 3.1). Analysis of the total soluble solids content in the IL population over two growing seasons (2007 and 2008) allowed to identify eight lines expressing QTLs for different fruit °brix content (Fig 3.1).

Table 3.1 One-way ANOVA results of total soluble solids in fruit from IL population and S. lycopersicum cv. M82 grown over two years trials.

Source	Sum of Square	df	Maen Square	F	P
Corrected Model	69,751	39	1,788	4,73	0,00
Year	0,186	1	0,186	0,49	0,49
Genotype	59,124	37	1,598	4,23	0,00
Year * Genotype	$0{,}571\ 10^{-4}$	1	$0{,}571\ 10^{-4}$	0,00	0,99
Error	44,968	119	0,378		
Total	3739,78	159			

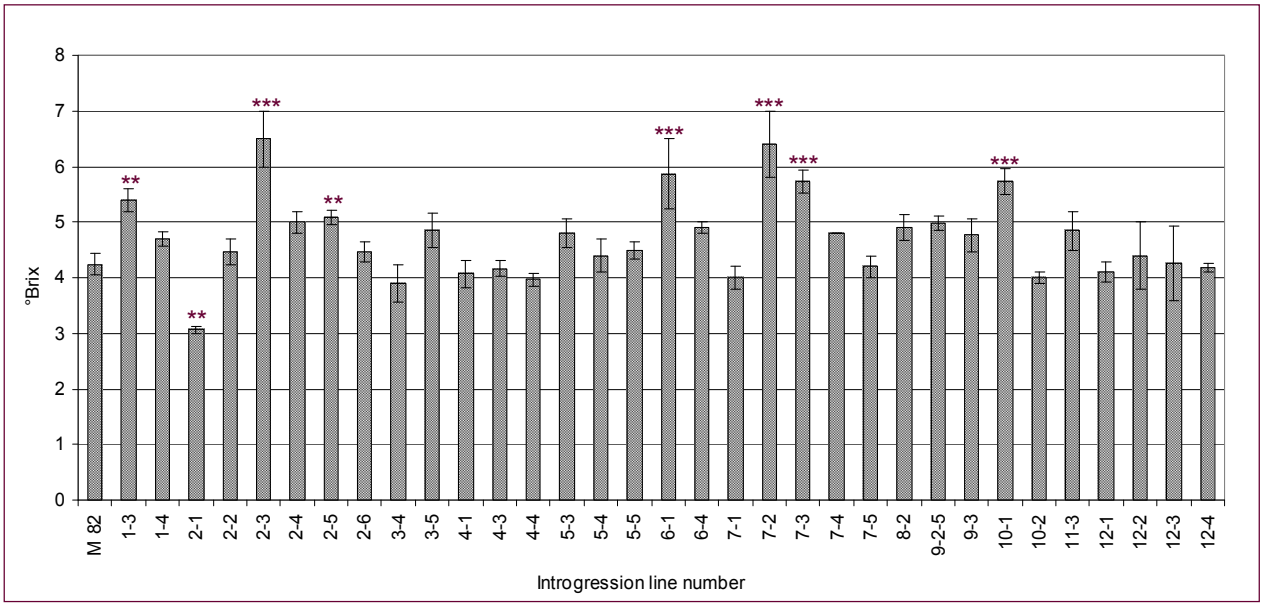


Figure 3.1 Total soluble solids content (°Brix) of red ripe tomatoes from *S. pennellii* IL population and *S. lycopersicum* cv. M82 control. Data shown are comprehensively referred to two growing seasons (2007-2008) with three replica per line each year. Mean value and standard error are represented. Asterisks indicate ILs accumulating fruit soluble solids levels statistically different from M82 at LSD post-hoc test: *: P<0.05; **: 0.01< P < 0.05; ***: P<0.001.

Seven detected QTLs showed a positive effect, whereas one a negative effect. A summary of the identified QTLs for °Brix are provided in Table 3.2. As observed the °Brix content in the fruit of IL 2-1 expressing the only negative QTL was 27% lower than the *S. lycopersicum* cv. M82 control. The positive QTLs ranged from 51% to 20% higher than the control.

Table 3.2 Summary of ILs expressing QTLs for total soluble solids content in the fruit

Introgression line	°Brix	% of M82
Increase		
1-3	5,40	27
2-3	6,50	53
2-5	5,08	20
6-1	5,87	38
7-2	6,40	51
7-3	5,73	35
10-1	5,73	35
Decrease		
2-1	3,07	-27

3.1.2 Fruit firmness

S. lycopersicum cv. M82 showed on average penetrometric index equal to 22.46 N mm^{-2}. One-way ANOVA test for fruit firmness displayed no significant interaction between genotype and year (Tab 3.3). Over the two trial seasons (2007-2008) five ILs with decreased fruit firmness were identified (Fig. 3.2). In particular, the lowest penetrometric index was recorded for IL 5-4 (on average 15.48 N mm^{-2}; $P<0.05$), that is 31 % less than *S. lycopersicum* cv. M82, whereas the IL 10-1 performed on average a penetrometric index of 18.98 N mm^{-2} ($P<0.05$), that is only 15% lower than the M82 (Tab. 3.4).

Table 3.3 One-way ANOVA results of firmness in fruit from IL population and *S. lycopersicum* cv. M82 grown over two years trials.

Source	Sum of Square	df	Maen Square	F	P
Corrected Model	795,064	16	49,691	3,291	0,00
Year	0,280	1	0,280	0,37	0,37
Genotype	795,064	16	49'691	3,291	0,00
Year * Genotype	0,008	1	0,029	0,26	0,83
Error	1721,408	114	15,1		
Total	57833,579	131			

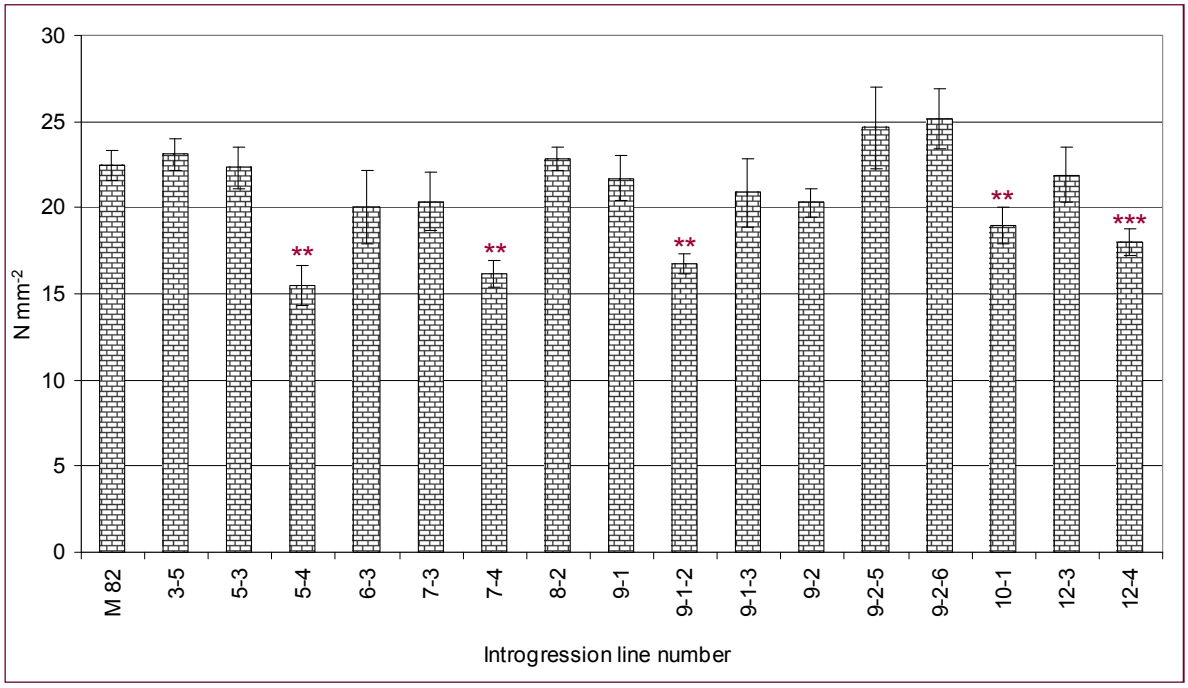


Figure 3.2 Fruit firmness of red ripe tomatoes from *S. pennellii* IL population and *S. lycopersicum* cv. M82. Data shown are comprehensively referred to two growing seasons (2007-2008) with three *replica per* line each year. Mean value and standard error are represented. Asterisks indicate ILs with fruit firmness statistically different from M82 at LSD post-hoc test: **: 0.01< P < 0.05; ***: P<0.001.

Table 3.4 Summary of ILs expressing QTLs for fruit firmness

Introgression line	Fruit firmness	% of M82
Decrease		
5-4	15,48	-31
7-4	16,13	-28
9-1-2	16,73	-25
10-1	18,98	-15
12-4	17,99	-20

3.1.3 Ascorbic acid and total ascorbic acid content

The average ascorbic acid (AsA) concentration observed was 0.71 µmol g^{-1} fresh weight (FW) in ripe fruit from *S. lycopersicum* cv. M82, while was 2.13 µmol g^{-1} FW in *S. pennellii* LA0716 fruit. Fruit from interspecific hybrid F_1 showed a higher ascorbic acid accumulation (3.34 µmol g^{-1} FW) than it was in fruit of *S. lycopersicum* cv. M82, as well as higher than the wild parent fruit. One-way ANOVA test for AsA content displayed significant interaction between genotype and year (Tab 3.5).

Table 3.5 One-way ANOVA results of AsA content in fruit from IL population and S. lycopersicum cv. M82 grown over three years trials.

Source	Sum of Square	df	Maen Square	F	P
Corrected Model	85,382	81	1,054	18,01	0,00
Year	1,335	2	0,667	11,40	0,00
Genotype	35,180	59	0,596	10,19	0,00
Year * Genotype	8,597	20	0,430	7,34	0,00
Error	23,530	402	0,059		
Total	452,942	484			

Thirteen lines with significantly different ascorbic acid concentration were identified through the IL population over a three years (2006, 2007 and 2008) of greenhouse trials (Fig. 3.3). Among these thirteen lines four QTLs were detected that affect ascorbic acid concentration compared to the control. Three were positive QTLs (IL 7-3; IL 8-2; IL 12-4), corresponding to an increase of AsA concentration, while the other one was negative (IL 10-1), corresponding to a decrease in AsA concentration. As shown in Figure 3.3 two of these QTLs were observed during two years experimental seasons, whereas the other two were detected in all growing trials. The highest AsA concentration was observed in the IL 7-3 fruit ($0.01<P<0.05$) displaying an average of 1.40 µmol g^{-1} FW, that is 97% higher than the *S. lycopersicum* cv. M82 control. Conversely, the lowest

AsA concentration was detected in the IL 10-1 (0.01<P<0.05) with 0.51 µmol g^{-1} FW, that is 28% lower than the control. As shown in Table 3.6 the percentage increase of AsA concentration in ILs containing positive QTLs ranged from 33% to 97% compared to *S. lycopersicum* cv. M82, mirroring a high phenotypic variability.

Table 3.6 Summary of ILs expressing QTLs for fruit ascorbic acid content

	AsA	
Introgression line	**µmol AsA g^{-1} FW**	**% of M82**
Increase		
7-3	1.40	97
8-2	0.95	33
12-4	1.09	53
Decrease		
10-1	0.51	28

The variation of ascorbic acid content in whole red ripe fruit was also evaluated in wild accessions of tomato (Fig. 3.4). Not surprising, when the AsA accumulation was measured into the wild genotypes a great amount of ascorbic acid concentration was observed, indeed four wild accessions showed an increase statistically significant with a P<0.001. In particular, *S. pimpinellifolium* LA0722 showed the highest average concentration (2.19 µmol g^{-1} FW); followed by *S. pennellii* LA0716 (2.13 µmol g^{-1} FW); *S. pimpinellifolium* LA2904 (2.11 µmol g^{-1} FW); *S. neorickii* LA2133 (1.66 µmol g^{-1} FW).

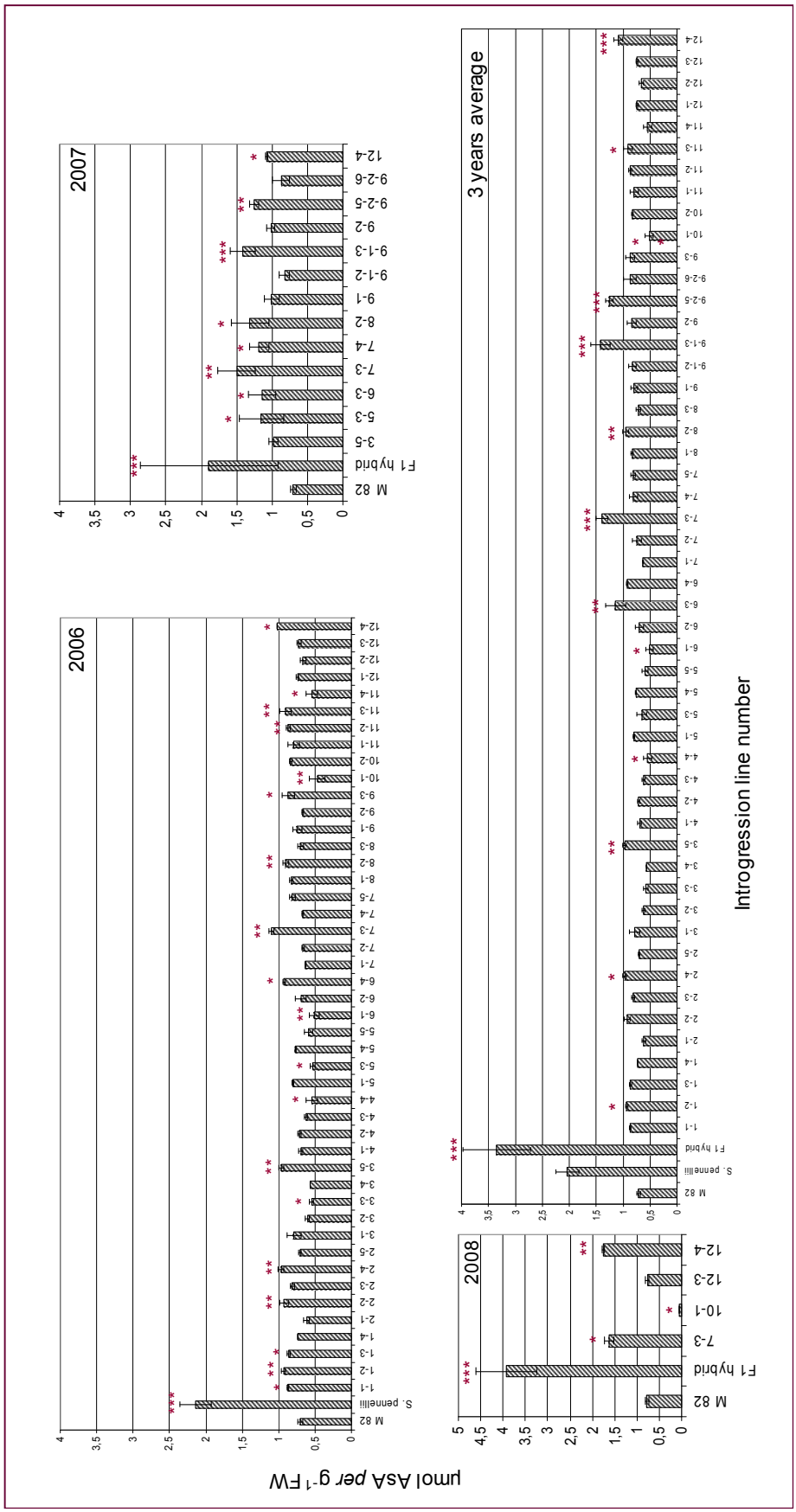


Figure 3.3 Ascorbate content in ripe tomatoes from *S. pennellii* IL population, parental line *S. lycopersicum* cv. M82, and interspecific F_1 hybrid. Data shown are referred to three growing seasons (2006-2007-2008) with three replica per line each year. AsA concentration over all trials is also shown. Mean value and standard error are represented. Asterisks indicate ILs accumulating fruit ascobate levels statistically different from M82 at LSD post-hoc test: *: P<0.05; **: 0.01< P < 0.05; ***: P<0.001.

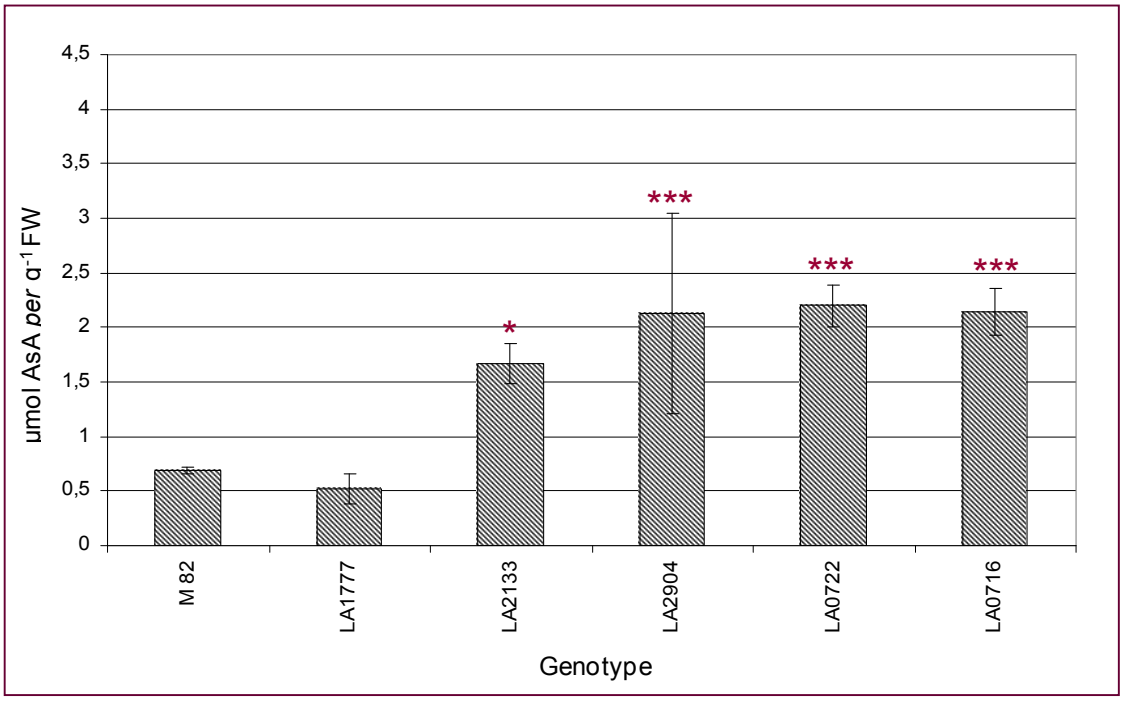


Figure 3.4 Ascorbic acid (AsA) content of ripe tomatoes from wild accessions and *S. lycopersicum* cv. M82. Bars represent mean values and standard errors. Asterisks indicate difference statistically significant at LSD post-hoc test for P<0.05 (*) and P<0.001 (***) compared to *S. lycopersicum* var. M82. Three replicas *per* genotype were used.

IL population fruit were also profiled for total AsA concentration. The average total AsA concentration was 3.53 µmol g^{-1} FW in ripe fruit from S. lycopersicum cv. M82; 9.94 µmol g^{-1} FW in fruit from *S. pennellii*; and 16.01 µmol g^{-1} FW in fruit from F_1 hybrid. As for the AsA content one-way ANOVA test displayed significant interaction between genotype and year ab. 3.7). Two QTLs were detected (IL 7-3; IL 12-4) affecting total AsA concentration over two trial years (2006-2007) (Fig.3.5).

Table 3.7 One-way ANOVA results of total AsA content in fruit from IL population and *S. lycopersicum* cv. M82 grown over two years trials.

Source	Sum of Square	df	Maen Square	F	P
Corrected Model	1357,756	64	21,215	20,42	0,00
Genotype	1047,536	59	19,765	19,02	0,00
Year	56,931	2	28,466	27,40	0,00
Year * Genotype	121,250	9	13,472	12,97	0,00
Error	380,279	366	1,039		
Total	9141,834	431			

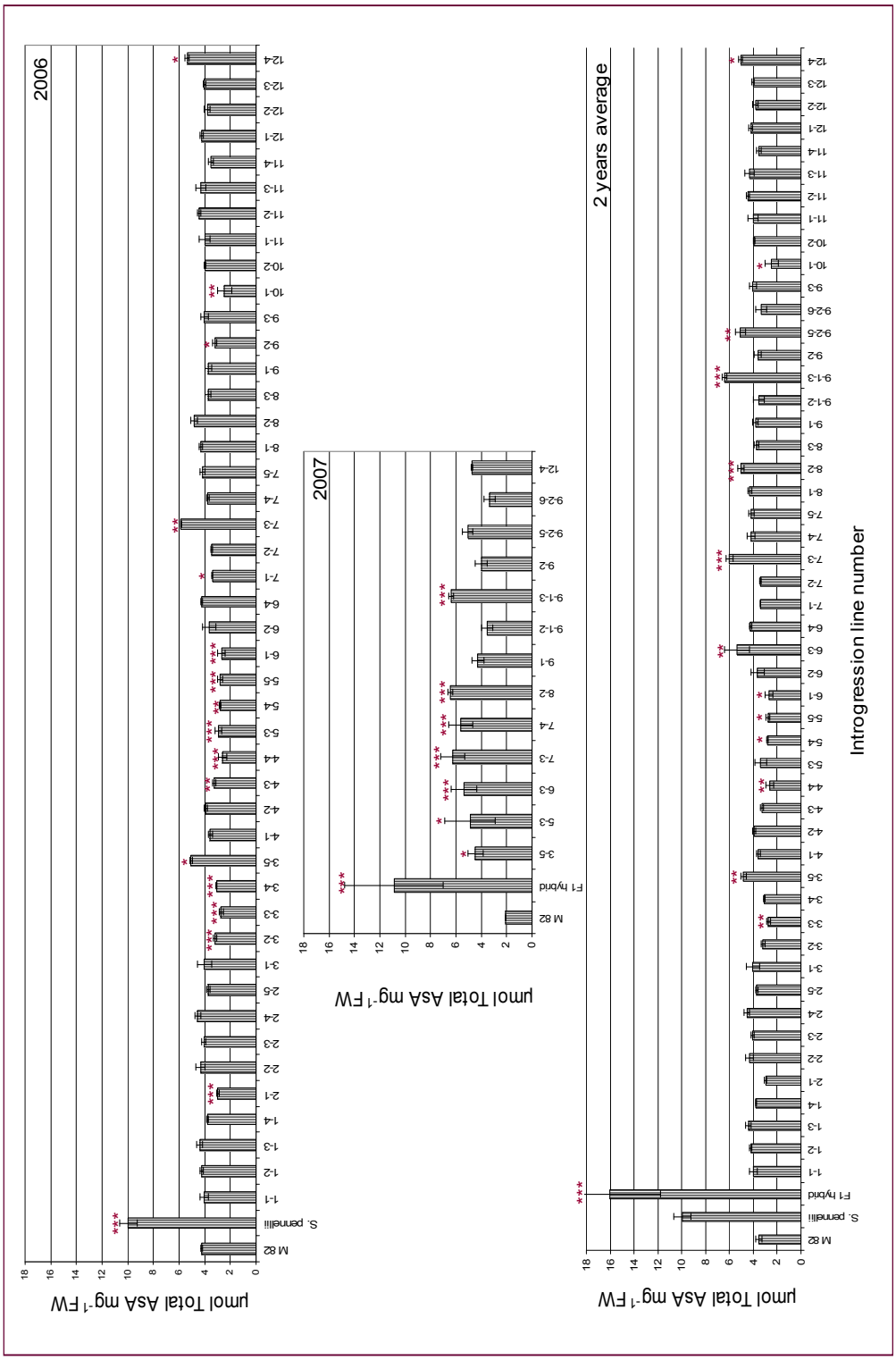


Figure 3.5 Total ascorbate content in ripe tomatoes from *S. pennellii* IL population, parental line *S. lycopersicum* cv. M82, and interspecific F_1 hybrid. Data showed are referred to two growing seasons (2006-2007) with three replica per line each year. Mean value and standard error are represented. Asterisks indicate ILs accumulating fruit ascobate levels statistically different from M82 at LSD post-hoc test: *: P<0.05; **: 0.01< P < 0.05; ***: P<0.001.

The total AsA concentration in the fruit of the two ILs 7-3 (6.00 µmol g^{-1} FW) and 12-4 (5.05 µmol g^{-1} FW) expressing a QTL was 69% and 43% higher than the control, respectively. As observed for the AsA concentration, the highest total AsA concentration was found in fruit from wild genotypes (Fig. 3.6) Particularly, *S. pimpinellifolium* LA 2904 (6.11 µmol g^{-1} FW) showed an increase statistically significant with a P<0.001.

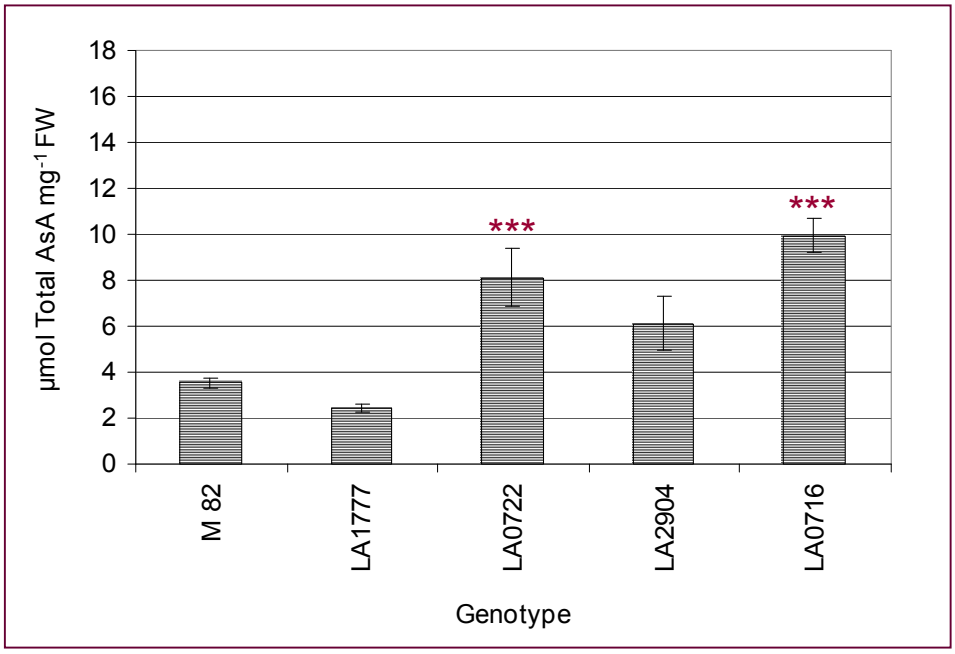


Figure 3.6 Total Ascorbic acid (AsA) content of ripe tomatoes from wild accessions and *S. lycopersicum* cv. M82. Bars represent mean values and standard errors. Asterisks indicate difference statistically significante at LSD post-hoc test for P<0.001 (***) compared to *S. lycopersicum* cv. M82. Three replicas *per* genotype were used.

3.1.4 Phenolic content

The average concentration of total phenolics in fruit from *S.lycopersicum* cv. M82 was 0.99 µg gallic acid equivalents mg^{-1} FW, while fruit from the F_1 hybrid displayed an average total phenolics of 2.13 µg gallic acid mg^{-1} FW. One-way ANOVA test displayed no significant interaction between genotype and year (Tab 3.8).

Table 3.8 One-way ANOVA results of total phenolics in fruit from IL population and S. lycopersicum cv. M82 grown over two years trials

Source	Sum of Square	df	Maen Square	F	P
Corrected Model	10,571	26	0,407	6,71	0,00
Year	0,092	1	0,092	1,51	0,22
Genotype	8,469	22	0,385	6,35	0,00
Year * Genotype	0,087	3	0,029	0,48	0,70
Error	4,060	67	0,061		
Total	110,442	94			

When we compare the ILs with M82 we detect a QTL controlling the accumulation of total phenolics in the IL 7-3 (P <0.05) over two growing seasons (2007 and 2008), with 71% increase content compared to *S. lycopersicum* cv. M82, (Fig. 3.7).

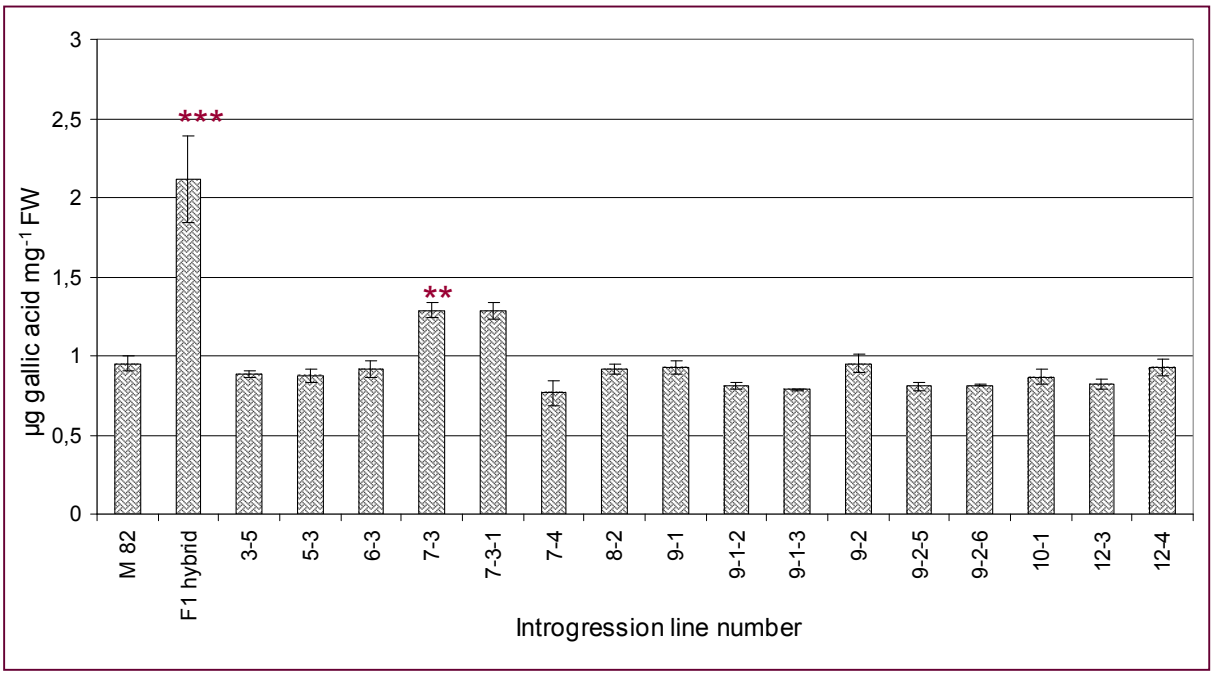


Figure 3.7. Total phenolics content of red ripe tomatoes from *S. pennellii* IL population and *S. lycopersicum* var. M82 control. Data shown are comprehensively referred to two growing seasons (2007-2008) with three replica per line each year. Mean value and standard error are represented. Asterisks indicate ILs accumulating fruit total phenol levels statistically different from M82 at LSD post-hoc test: *: P<0.05; **: 0.01< P < 0.05; ***: P<0.001.

Higher amount of total phenolic compounds was also detected in the wild genotypes, particularly in *S. habrochaites* LA1777 (average concentration of 2.10 µg gallic acid equivalents mg^{-1} FW), *S. neoricki* LA2133 (average concentration of 1.97 µg gallic acid equivalents mg^{-1} FW), and *S. pimpinellifolium* LA0722 (average concentration of 2.66 µg gallic acid equivalents mg^{-1} FW) (Fig.3.8).

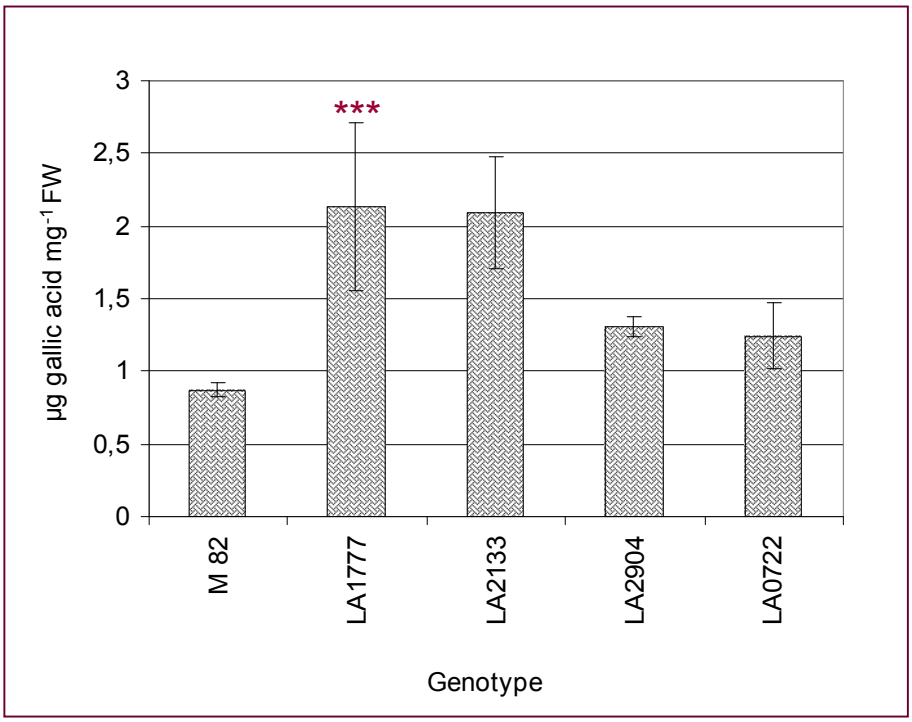


Figure 3.8 Total phenolics content of red ripe tomatoes from wild accessions and *S. lycopersicum* cv. M82 control. Bars represent mean value and standard error over three replica per genotype. Asterisks indicate difference statistically significante at LSD post-hoc test for P<0.001 (***) compared to *S. lycopersicum* cv. M82.

3.2 Microarray analysis of gene expression in fruit of Introgression Line (IL) 12-4

Comparative transcriptomic analysis of IL 12-4 fruit allowed the identification of 155 TCs showing a significant differential expression. In particular, the between-

subject T-test identified 27 probe with higher hybridization signal and 128 with lower signal compared to M82 fruit transcriptome (P<1%). The Volcano plot (Fig. 3.9) shows the difference between the means of log_2 M82 signal (groups A) and log_2 IL 12-4 signal (group B) for each gene plotted against the negative log_{10} of the P-value.

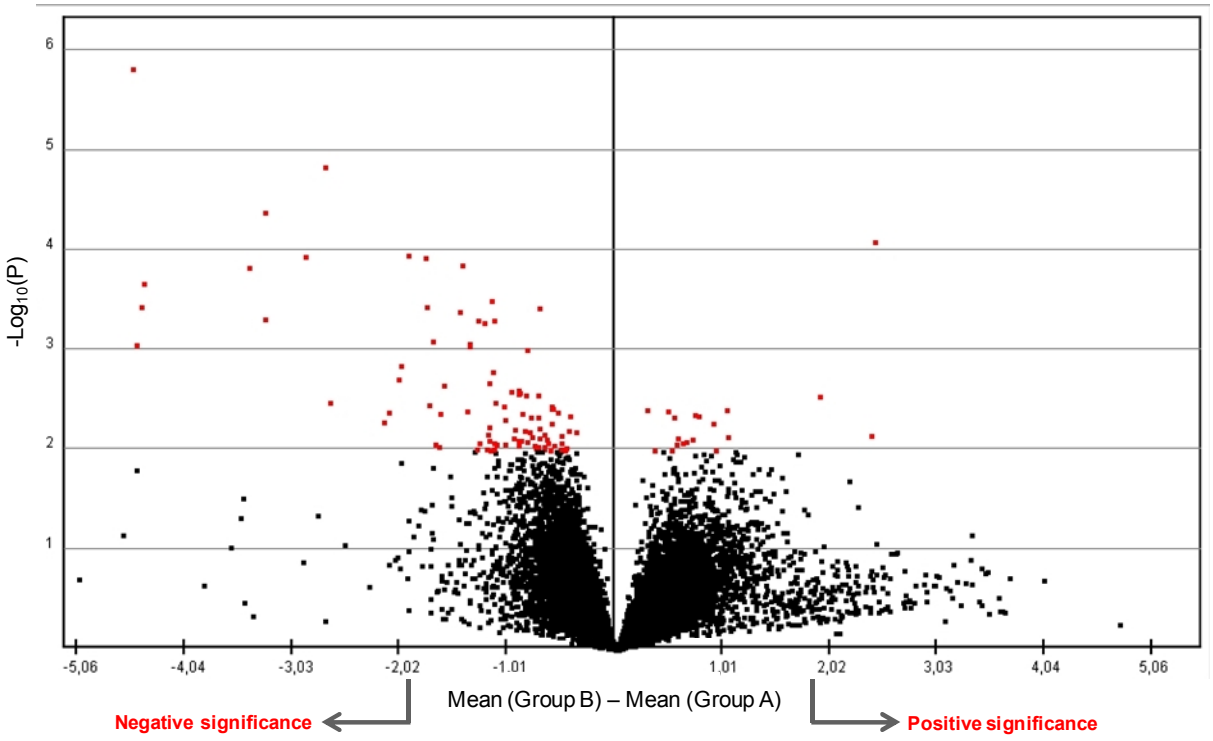


Figure 3.9 Volcano plot. The horizontal axis is the fold change between the two groups (on a log scale, so that up and down regulation appear symmetric), and the vertical axis represents the negative log scale of the p-value for a t-test of differences between samples. The first axis indicates biological impact of the change; the second indicates the statistical evidence, or reliability of the change.

Hierarchical clustering of all the differential transcripts were also grouped according to their signal pattern.(Fig. 3.10). The tree allowed to indentify groups of sequences showing similar expression profiles. Sequences annotated as glutathione reductase (#Probe 11949) and glutathione s-transferase (#Probe 17266) were co-regulated with two transcription factors, (#Probe 138 and

#Probe 3573, respectively). Figure 3.11 shows a graphical categorization of differentially expressed probes. According to cellular component GO vocabulary terms the plastid category accounted for the largest group (29.13%) followed by unknown genes (22.33%) and mitochondrion genes (14.56%). Similarly, in categorization for molecular function GOs the largest group was represented by unknown category (25.27%) and the second largest group was hydrolase activity category (13.19%). Finally, categorization for biological process terms allowed to assign most sequences to the unknown category (15.33%) followed by cellular component organization and biogenesis (10.67%) and transport category (10%). Table 3.9 lists differentially expressed probes, their BLAST annotation, GO mapping, and the rate of transcriptional signal compared to the M82 counterpart as resulted from microarray data processing.

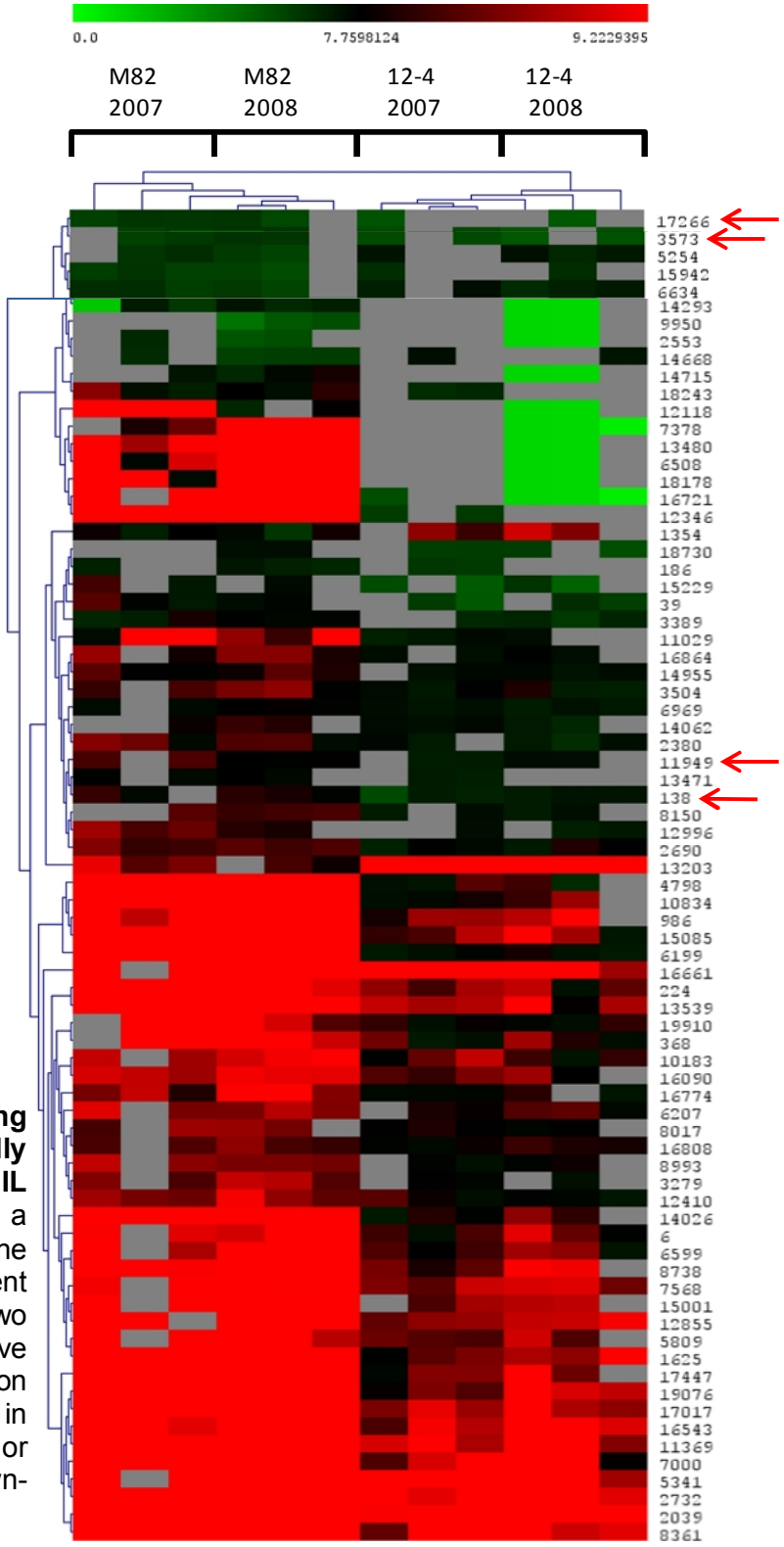


Figure 3.10 Hierarchical clustering of 155 tomato fruit differentially expressed genes in *S. pennelli* IL 12-4. Each gene is represented by a single row of colored boxes. The twelve columns represent the different chip hybridization replica of two different years. The color line above the samples indicates the expression key, the abundance of each gene in the samples correlates with color intensity, goin from green (down-regulates) to red (up-regulates).

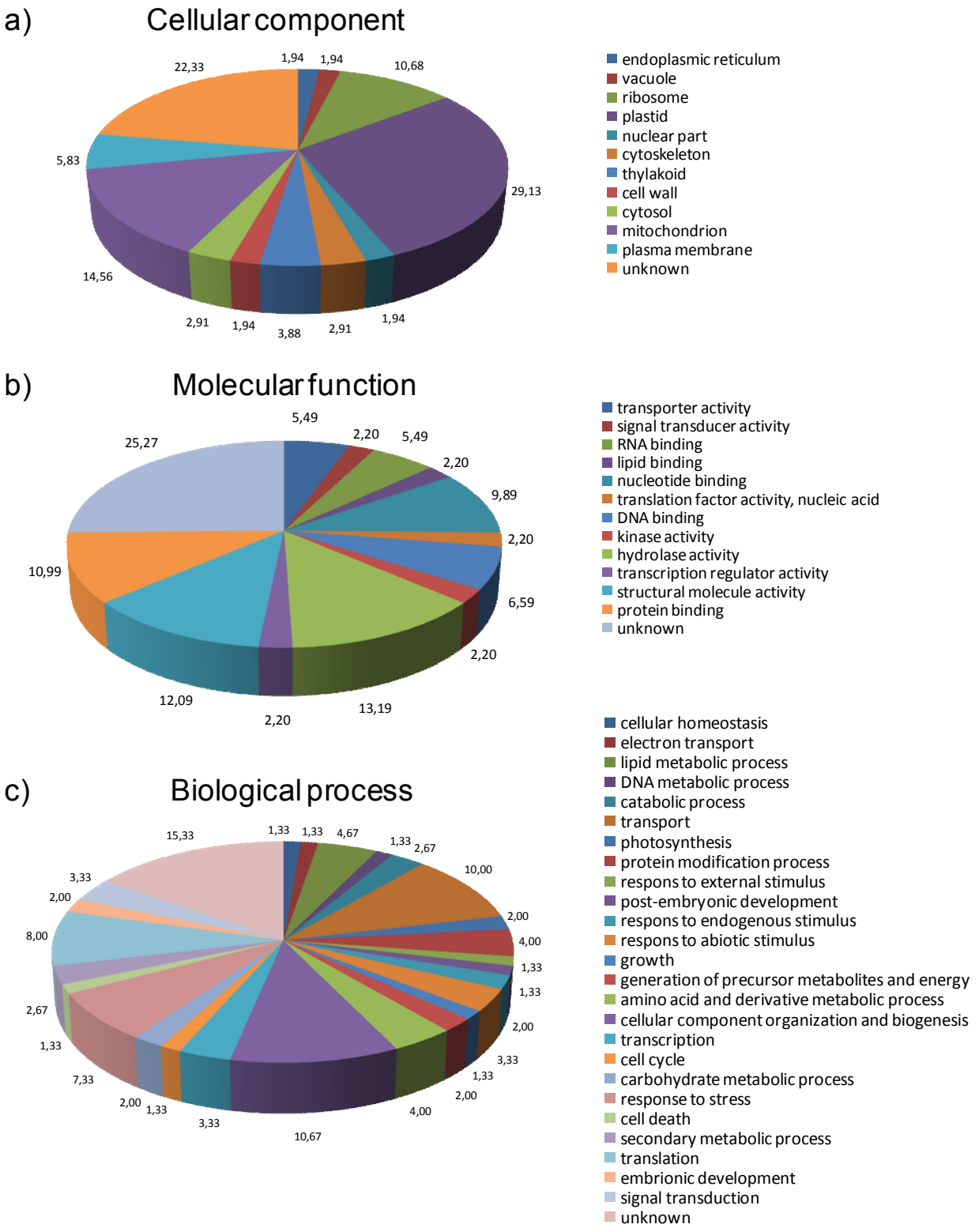


Figure 3.11 Categorization of 155 differentially expressed sequences provided through transcriptomic comparision of fruit from S. pennellii IL 12-4 and the parent line S. lycopersicum cv. M82. Corresponding GOs were classified for terms from: a) cellular component; b) molecular function; and c) biological process vocabularies.

Table 3.9 Sequences differentially expressed in the fruit from IL 12-4 compare to M82 .

Probe #	Fold change 12-4 vs. M82	seq description	sim mean	#GO IDs	Enzyme
13480	-8,64	zinc finger	73,44%	F:GO:0005488	
6199	-4,54	family protein	80,65%	C:GO:0005739 C:GO:0009536 C:GO:0016020	
7378	-7,88	protein	78,55%		-
10834	-2,74	rub1 conjugating enzyme	91,00%	F:GO:0003824 F:GO:0005515 P:GO:0019538 P:GO:0006464	EC:6.3.2.19,
5794	-3,31	ac022472_2pigpen protein from mus musculus gb	69,15%	C:GO:0005737	
7355	6,60	---NA---	-		
13203	2,43	tryptophan synthase subunit beta	84,55%	P:GO:0009058 P:GO:0006519 F:GO:0003824 C:GO:0009536	
3373	-1,96	calmodulin	99,30%	F:GO:0005515 F:GO:0005488 P:GO:0008150	
14223	-2,93	ubc32 (ubiquitin-conjugating enzyme 32) ubiquitin-protein ligase	73,80%	F:GO:0003824 P:GO:0006464	EC:6.3.2.19,
14026	-1,80	sec14 cytosolic factor family protein phosphoglyceride transfer family protein	77,40%	C:GO:0005622 P:GO:0006810	
19498	-1,45	---NA---	-		-
12346	-3,45	nucleotide binding protein	80,80%	F:GO:0000166	
18178	-4,45	phloem-specific lectin	60,60%	P:GO:0000003 P:GO:0009790	
14715	-6,09	slowmo homolog 2	64,95%	F:GO:0003700 F:GO:0005488 C:GO:0005654 P:GO:0006350	
17017	-1,18	quinone oxidoreductase-like protein	84,10%	P:GO:0008152 C:GO:0005634 F:GO:0005488 F:GO:0003824 P:GO:0006950	EC:1.3.1.74,
15085	-1,79	60s ribosomal proteinbbc1 protein	91,35%	C:GO:0005840 C:GO:0005829 P:GO:0016043 F:GO:0005198 P:GO:0006412 C:GO:0005739	EC:3.6.5.3,
1427	-4,47	gcn5-related n-acetyltransferasefamily protein	63,67%	P:GO:0008152	
12996	-0,73	aldo keto reductase	77,40%	C:GO:0016020 C:GO:0009536 C:GO:0009579 F:GO:0003824	
368	-1,47	protein	77,62%	C:GO:0009536	
4798	-3,30	zinc finger protein	80,15%	C:GO:0005622 F:GO:0003677 F:GO:0005488 P:GO:0006350	
5809	-1,15	atp synthase subunit h family protein	89,06%	C:GO:0016020 P:GO:0006810 P:GO:0006091 P:GO:0006139 C:GO:0005737 F:GO:0000166 F:GO:0016787 F:GO:0005215 C:GO:0005739 C:GO:0005622	EC:3.6.3.14,
39	-1,30	homoaconitate hydratase family protein	82,10%	P:GO:0008152 C:GO:0009536	
2732	-1,25	---NA---	75,55%	F:GO:0003674 P:GO:0008150	-
7000	-1,72	ac012190_11 it is a member of an uncharacterised protein family pf	86,45%		-
19076	-1,39	ubiquitin-associated ts-n domain-containing protein	72,90%	F:GO:0003674 P:GO:0008150	-
6508	-4,51	diacylglycerol acyltransferase	57,50%	C:GO:0012505 F:GO:0003674	-
2824	-1,38	nucleotide-binding protein	90,90%	C:GO:0005737 F:GO:0000166 C:GO:0005634	
7541	-0,84	small nuclear ribonucleoprotein e	93,05%	P:GO:0006139 C:GO:0005622 F:GO:0003723	
16964	-5,17	probable photosystem i chain xi precursor	87,80%	C:GO:0016020 P:GO:0006091 P:GO:0015979 C:GO:0009536 C:GO:0009579	

Table 3.9 (Continued from previous page.)

Probe #	Fold change 12-4 vs. M82	seq description	sim mean	#GO IDs	Enzyme
7634	-2,03	60s ribosomal protein l29	92,95%	C:GO:0005840 P:GO:0016043 F:GO:0005198 P:GO:0006412 C:GO:0005739	EC:3.6.5.3,
18730	-1,59	non-specific lipid transfer protein	77,50%	P:GO:0006950 F:GO:0008289 P:GO:0006810	
7763	5,80	---NA---	-		-
12118	-8,43	glutathione s-transferase	79,60%	F:GO:0016740 P:GO:0008152 P:GO:0009987	EC:2.5.1.18,
690	-1,16	elongation factor p	82,30%	C:GO:0009536 P:GO:0006412	
10630	4,93	---NA---	-		-
16602	-2,05	potassium channel beta subunit	87,55%	F:GO:0003824 F:GO:0005215 P:GO:0006810 C:GO:0005739 C:GO:0005886	
19914	7,49	histone h2a	96,40%	C:GO:0005622 F:GO:0003677 P:GO:0016043 F:GO:0005515 C:GO:0005634 C:GO:0009536	
18516	-1,19	---NA---	71,75%	F:GO:0003674 P:GO:0008150	-
3055	-1,63	lipoic acid synthetase	87,25%	P:GO:0009791 F:GO:0016740 F:GO:0005488 F:GO:0003824 P:GO:0009790 C:GO:0009536 C:GO:0005739 P:GO:0040007 P:GO:0009058 P:GO:0009987	EC:2.8.1.8,
12855	-0,80	---NA---	79,00%	F:GO:0003674 C:GO:0009507	-
18589	-0,92	glycosyl hydrolase family 17 protein	64,55%	F:GO:0016787 C:GO:0005737 C:GO:0005739 C:GO:0005886 P:GO:0005975	EC:3.2.1,
17793	-0,99	ubx domain-containing protein	63,95%	F:GO:0003674 C:GO:0005575	-
15001	-0,91	at5g14910 f2g14_30	82,58%	P:GO:0006810 C:GO:0005739 C:GO:0009536	
14293	-4,68	protein	74,45%	P:GO:0006464	
2039	-0,92	60s ribosomal protein l36	90,65%	C:GO:0005840 F:GO:0005198 P:GO:0006412 C:GO:0009536 P:GO:0016043	EC:3.6.5.3,
9149	-0,85	pde225 ptac7 (pigment defective 225)	77,47%	C:GO:0009536	
14955	-0,74	proline iminopeptidase	82,10%	F:GO:0016787	EC:3.4.11,
14392	1,91	tryptophan synthase subunit beta	81,15%	F:GO:0003824 F:GO:0005488 P:GO:0009058 P:GO:0006519 C:GO:0009536	EC:4.2.1.20,
8738	-1,36	50s ribosomal protein l13	84,30%	C:GO:0005840 F:GO:0005198 C:GO:0009536 P:GO:0006412 P:GO:0016043	EC:3.6.5.3,
16310	-2,70	translational initiation factor 1	94,75%	F:GO:0003723 F:GO:0008135 C:GO:0009536 C:GO:0005840 P:GO:0016043 P:GO:0006412	
18919	-1,13	ac022472_2pigpen protein from mus musculus gb	69,20%	C:GO:0005737	
6969	-0,45	acyl-thioesterase ii	73,35%	P:GO:0009987 P:GO:0006629 F:GO:0016787	
3279	-0,99	---NA---	75,13%	F:GO:0003674	-
8361	-1,76	rpt2 (root phototropism 2) protein binding	65,60%	C:GO:0005634 F:GO:0005515	
12089	-0,61	leucine-rich repeat family protein	67,75%	F:GO:0005515 C:GO:0005886	
5341	-1,06	pyrophosphate-dependent phosphofructo-1-kinase	87,05%	F:GO:0005515 C:GO:0005829 P:GO:0005975 P:GO:0006091 P:GO:0009056 C:GO:0009536	
19288	-0,60	---NA---	-		-

Table 3.9 (Continued from previous page.)

Probe #	Fold change 12-4 vs. M82	seq description	sim mean	#GO IDs	Enzyme
8150	-1,04	xs domain containingexpressed	60,40%		-
7673	-0,60	amplified in osteosarcoma	63,50%	C:GO:0005737	
15758	0,28	squalene synthase	93,10%	C:GO:0016020 C:GO:0005783 F:GO:0016740 F:GO:0003824 F:GO:0005488 P:GO:0009058 P:GO:0009987 P:GO:0006629 P:GO:0019748	EC:2.5.1.21,
18935	1,04	---NA---	-		-
9696	-1,41	at3g61200 t20k12_100	67,23%	F:GO:0016787 P:GO:0009987 P:GO:0006629	
986	-1,90	fiber protein fb2	69,60%	C:GO:0005634 F:GO:0005515 P:GO:0007165	
18652	0,49	---NA---	76,62%	C:GO:0009536	
13471	-0,70	ornithine aminotransferase	86,35%	P:GO:0006950 P:GO:0009628 P:GO:0006519 F:GO:0005488 F:GO:0016740 C:GO:0005739	EC:2.6.1.13,
16693	-0,55	alpha beta fold family protein	74,00%	C:GO:0005783 F:GO:0016787	
11029	-2,14	pto kinase interactor 1	89,35%	P:GO:0006464 F:GO:0000166 F:GO:0016301 P:GO:0006519	EC:2.7.11, EC:2.7.10,
11949	-0,79	glutathione reductase	87,45%	P:GO:0009987 F:GO:0003824 F:GO:0005488 F:GO:0000166 P:GO:0009628 P:GO:0006950 P:GO:0009056 P:GO:0006259 P:GO:0009058 P:GO:0005975 P:GO0019725 P:GO:0007049 C:GO:0009536 P:GO:0006118 P:GO:0008152	EC:1.8.1.7,
1405	-1,65	alcohol dehydrogenase	81,30%	F:GO:0005488 P:GO:0008152 F:GO:0003824	
17266	-0,88	glutathione s-transferase	79,90%	P:GO:0009056 P:GO:0009987 P:GO:0019748 F:GO:0016740 P:GO:0006950 P:GO:0009719 P:GO:0007165 C:GO:0009536 P:GO:0008152 F:GO:0005515	EC:2.5.1.18,
12870	0,73	---NA---	73,00%		-
5839	0,77	membrane related protein cp5	65,30%	F:GO:0003674 P:GO:0008150	-
12998	-0,43	polyubiquitin	99,50%	C:GO:0005634 P:GO:0006464	
3573	-0,81	dna binding transcription factor	87,69%	C:GO:0005634 F:GO:0003677 P:GO:0006350	
2911	0,54	alpha tubulin	99,40%	P:GO:0006810 P:GO:0016043 C:GO:0005575 F:GO:0000166 F:GO:0016787 C:GO:0005856 F:GO:0005198	EC:3.6.5.1, EC:3.6.5.2, EC:3.6.5.3, EC:3.6.5.4,
1894	-0,73	atp binding domain 1member b	84,95%	F:GO:0000166 P:GO:0009790 C:GO:0005856 P:GO:0009987	
7568	-0,67	ribosomal protein l13	81,25%	F:GO:0005198 C:GO:0005840 P:GO:0006412 C:GO:0005739 P:GO:0016043	EC:3.6.5.3,
5810	-1,05	at5g40670 mnf13_190	76,70%	C:GO:0016020	
8017	-0,74	mrna capping enzyme family protein	74,10%	F:GO:0016740 P:GO:0006139	EC:2.7.7.50,
13362	-2,19	leucine-rich repeat receptor-like kinase	55,05%	C:GO:0005623	
2690	-0,62	protein	68,00%	F:GO:0003674 P:GO:0008150	-
6634	0,91	ac006341_1ests gb	79,05%	C:GO:0005739	
186	-0,78	brassinosteroid signalling positive regulator-related	72,00%	C:GO:0009536	
2731	5,45	peroxidase 1	69,35%	F:GO:0005488 C:GO:0005737 F:GO:0003824 P:GO:0006950	EC:1.11.1.7,
16661	-1,19	vacuolar processing enzyme	87,50%	P:GO:0019538 P:GO:0009987 C:GO:0005773 P:GO:0008219 C:GO:0005829	EC:3.4.22.34,

Table 3.9 (Continued from previous page.)

Probe #	Fold change 12-4 vs. M82	seq description	sim mean	#GO IDs	Enzyme
16864	-0,87	3-hydroxyisobutyrate dehydrogenase	69,50%	F:GO:0005488 P:GO:0008152 P:GO:0009987 F:GO:0003824	EC:1.1.1,
16721	-7,97	at3g16910 k14a17_3	81,90%	C:GO:0012505	-
11739	-0,72	integral membrane protein	84,05%	C:GO:0016020 F:GO:0005215 P:GO:0006810	
18243	-1,16	vap27-2	77,40%	P:GO:0006810 P:GO:0009987	
13539	-0,95	uncharacterised protein family containingexpressed	71,50%		-
1658	-0,45	serine threonine protein kinase	92,35%	F:GO:0016301 P:GO:0006464	
8993	-0,86	dimethyl-8-ribityllumazine synthase precursor	77,70%	F:GO:0016740 P:GO:0009058 P:GO:0009987 C:GO:0005623 C:GO:0009536	EC:2.5.1.9,
17907	-0,81	c9h6orf115 protein	85,05%	F:GO:0003674 P:GO:0008150	-
3504	-0,89	2-phosphoglycerate kinase-related	76,00%	F:GO:0003674 C:GO:0009507	-
6119	-0,38	proteinase inhibitor i	80,45%	C:GO:0005576 F:GO:0030234 P:GO:0009605 P:GO:0006950 F:GO:0016787	
6	-1,27	cytochrome f	97,45%	F:GO:0003674 F:GO:0005488 C:GO:0016020 C:GO:0009579 P:GO:0006810 C:GO:0009536 P:GO:0015979 P:GO:0006118	
138	-1,10	transcription initiation factor	71,93%		-
14062	-0,79	retroelement pol polyprotein	61,55%	C:GO:0005739	
19910	-1,54	hev b 3	67,60%	F:GO:0003674 C:GO:0005737 P:GO:0006950 P:GO:0008150	-
1415	-0,68	atpase-like protein	78,65%	C:GO:0009536	
16774	-1,21	ribosomal protein l18	79,70%	C:GO:0005840 P:GO:0016043 F:GO:0005198 F:GO:0003723 P:GO:0006412 C:GO:0009536	EC:3.6.5.3,
10417	2,39	pectinesterase family protein	60,00%	C:GO:0005618	
13276	-0,52	calcium-binding ef hand family protein	83,90%	C:GO:0005623 C:GO:0005634 F:GO:0005488	
10306	-0,79	tetratricopeptide repeat domain 5	54,95%	F:GO:0005488 P:GO:0006974 P:GO:0006281 C:GO:0005634	-
5254	1,05	tpa: a-type response regulator	78,00%	P:GO:0007165 F:GO:0003677 F:GO:0030528 F:GO:0004871 P:GO:0009719 P:GO:0006350	
6207	-0,70	---NA---	-		-
3849	0,58	---NA---	-		-
19272	-0,96	---NA---	-		-
10183	-0,97	---NA---	46,00%		-
12410	-0,73	chromodomain helicase dna binding protein 1	53,00%	F:GO:0005488	
14668	1,22	auxin influx carrier protein	79,50%	C:GO:0016020 P:GO:0009719 P:GO:0007165 P:GO:0006810 C:GO:0005886 F:GO:0005215	
3389	-0,66	gamma tubulin	84,35%	C:GO:0005635 F:GO:0005198 C:GO:0005737 F:GO:0000166 P:GO:0009653 P:GO:0030154 P:GO:0007275 P:GO:0016043 P:GO:0007049 C:GO:0005575 P:GO:0006810 F:GO:0016787 C:GO:0005886 P:GO:0016049 C:GO:0005618 P:GO:0009791 C:GO:0005856	EC:3.6.5.1, EC:3.6.5.2, EC:3.6.5.3, EC:3.6.5.4,
2592	0,71	proline-rich apg-like protein	82,50%	C:GO:0005739 F:GO:0016787 P:GO:0006629	EC:3.1.1, EC:3.1.1.1,

Table 3.9 (Continued from previous page.)

Probe #	Fold change 12-4 vs. M82	seq description	sim mean	#GO IDs	Enzyme
2380	-0,99	4-nitrophenylphosphatase-like protein	79,10%	F:GO:0016787 C:GO:0005634 C:GO:0009536 P:GO:0008152 P:GO:0009987	EC:3.1.3.18,
1625	-1,20	got1-like family protein	72,84%	C:GO:0016020 P:GO:0006810 P:GO:0009987	
16090	-0,74	vacuolar sorting receptor	83,64%	P:GO:0019538 C:GO:0005737 C:GO:0005794 C:GO:0016020 F:GO:0016787 F:GO:0005488 F:GO:0003676 P:GO:0006810 C:GO:0005886 P:GO:0007165 P:GO:0009987 F:GO:0004872	
11807	-0,90	alanine racemase family protein	82,90%	F:GO:0005488	
6599	-0,92	at3g22430 mcb17_17	69,63%		-
15210	-0,84	protein	92,40%	C:GO:0016020	
15942	0,66	---NA---	-		-
16808	-0,43	mitochondrial substrate carrier family protein	77,25%	C:GO:0016020 C:GO:0005739 P:GO:0006810 P:GO:0009987	
3617	-0,51	heparanase-like protein 2expressed	67,30%	C:GO:0012505	-
90	-1,15	chloroplast photosystem ii reaction center protein	81,40%	C:GO:0016020 C:GO:0009536 C:GO:0009579 F:GO:0005488 P:GO:0015979	
12564	0,63	chaperone protein dnaj-related-like	81,30%	P:GO:0016043 P:GO:0009058 P:GO:0009987 P:GO:0006629 P:GO:0019748 C:GO:0009536	
10583	-1,29	af190634_1udp-glucose: salicylic acid glucosyltransferase	63,30%	F:GO:0016740 P:GO:0008152 F:GO:0016758 C:GO:0005575 F:GO:0016757	-
16623	-0,65	elongation factor beta-1	85,70%	F:GO:0008135 C:GO:0005737 C:GO:0005840 P:GO:0006412	
19108	-1,13	ribosomal protein s14	96,95%	C:GO:0005840 F:GO:0005198 C:GO:0009536 P:GO:0006412 F:GO:0003723 P:GO:0016043	EC:3.6.5.3,
11209	0,56	sulfate transporter	70,85%	F:GO:0005215 C:GO:0016020 P:GO:0006810	
1354	1,23	3-hydroxy-3-methylglutaryl coenzyme a synthase	90,15%	P:GO:0019725 C:GO:0016020 P:GO:0008152 P:GO:0009056 P:GO:0006950 P:GO:0009605 P:GO:0009628 F:GO:0008289 F:GO:0005515 P:GO:0009058 P:GO:0009987 P:GO:0006629 P:GO:0006519 F:GO:0016740 P:GO:0008150	EC:2.3.3.10,
224	-1,70	ornithine carbamoyltransferase	59,80%	F:GO:0016740 F:GO:0005488 C:GO:0005737 P:GO:0006519	EC:2.1.3.3,
11369	-1,05	at5g45690 mra19_8	72,50%	C:GO:0009536	
17387	-0,59	homogentisate phytyltransferase vte2-2	75,45%	C:GO:0016020	
3774	-0,77	ring-h2 finger proteinexpressed	72,60%		-
15229	-2,19	constans-like protein	57,35%	F:GO:0005488	
4601	-0,92	oleosin low molecular weight isoform	87,00%	C:GO:0005737 C:GO:0016020 P:GO:0006629	
17200	-0,74	anthocyanidin synthase	56,15%	P:GO:0019748 F:GO:0003824 P:GO:0008219	EC:1.14.11,
5361	-0,68	mgc82051 protein	66,00%	P:GO:0016043 P:GO:0030154 P:GO:0007275	
17447	-1,67	---NA---	80,05%	P:GO:0006950	
9950	-3,70	dna ligase iv	58,88%	P:GO:0009628 P:GO:0006950 F:GO:0005515 P:GO:0006259	
2553	-4,50	bzip transcription factor	57,13%	F:GO:0003677 P:GO:0006350	
3568	-0,47	rna-binding protein	69,85%	F:GO:0003723 C:GO:0009536 P:GO:0006139	
10142	0,84	---NA---	-		-

Table 3.9 (Continued from previous page.)

Probe #	Fold change 12-4 vs. M82	seq description	sim mean	#GO IDs	Enzyme
7440	-0,76	lg127 30 like gene	72,25%	C:GO:0012505 P:GO:0008150 C:GO:0005575	-
16543	-1,15	synaptobrevin-like protein	85,20%	C:GO:0016020 C:GO:0005773 P:GO:0006950 P:GO:0009628 P:GO:0016043	
17912	-1,22	monoterpene glucosyltransferase	71,30%	F:GO:0016740 P:GO:0008152	EC:2.4.1,
3353	-0,52	---NA---	-		-
14932	-0,68	kelch repeat-containing f-box family protein	74,00%	F:GO:0003674 P:GO:0008150	-
3327	-1,31	60s ribosomal protein l17	99,25%	C:GO:0005840 F:GO:0005198 P:GO:0006412 C:GO:0005739 P:GO:0016043	EC:3.6.5.3,
11174	0,36	---NA---	87,00%		-
8300	-0,62	rna polymerase ii associated protein 3	68,00%	P:GO:0008152 F:GO:0016740	
14312	-0,48	replication factor c subunit 3	83,10%	F:GO:0000166	
14065	-0,53	60s ribosomal protein l27a	91,40%	C:GO:0005840 F:GO:0005198 P:GO:0006412 P:GO:0016043	EC:3.6.5.3,
17394	0,52	---NA---	-		-
9503	0,93	tetracycline transporter	65,87%	C:GO:0016020	
16501	-1,19	ubiquitin associated tsn domain containing protein	76,00%	F:GO:0003674 P:GO:0008150	-

3.3 AtCCD7 and AtCCD8 apocarotenoid volatile products

A variable amount of apocarotenoid volatiles were identified from the culture head space collected from *E. coli* strains accumulating different carotenoids also carring the construct for the expression of AtCCD7 and AtCCD8. As shown in Figure 3.12, GGPP-accumulating strain produced 0.09 nmol OD^{-1} h^{-1} of 6-methyl-5-hepten-2-one (MHO) and 0.10 nmol OD^{-1} h^{-1} of β-ionone as result of the transformation with the double construct for AtCCD7 and AtCCD8. By contrast trace levels of the others volatiles analyzed were found. On the other hand very low level of all apocarotenoid volatiles analyzed were detected into phytoene- and zeta-carotene-accumulating strains. Similarly, zeaxanthin-accumulating strain showed low level of MHO and geranylacetone, whereas the production of β-ionone was detected in 0.15 nmol OD^{-1} h^{-1} and pseudoionone in 0.11 nmol OD^{-1} h^{-1}. Lycopene-accumulating *E. coli* produced the highest production of MHO and pseudoionone reaching 0.65 nmol OD^{-1} h^{-1} and 0.55 nmol OD^{-1} h^{-1} respectively, but there was not production of geranylacetone, β-ionone, and α-ionone. Only delta-carotene-accumulating strain provided a considerable higher production of α-ionone (0.98 nmol OD^{-1} h^{-1}). In addition it showed a little less production of MHO and pseudoionone compared to the lycopene-accumulating strain. Lastly, the beta-carotene-accumulating strain provided the highest amount of β-ionone (2.58 nmol OD^{-1} h^{-1}) and very low production levels of others analyzed apocarotenoid volatiles.

To ensure that the proteins were expressed in the transformed *E. coli* strains a protein blot analysis was performed after volatiles collection using His-tag antibody and GST-tag antibody for AtCCD7 and AtCCD8, respectively. As showed in Figure 3.13, AtCCD7 protein was not detected although the cleavage products were present. By contrast the expression of AtCCD8 was confirmed.

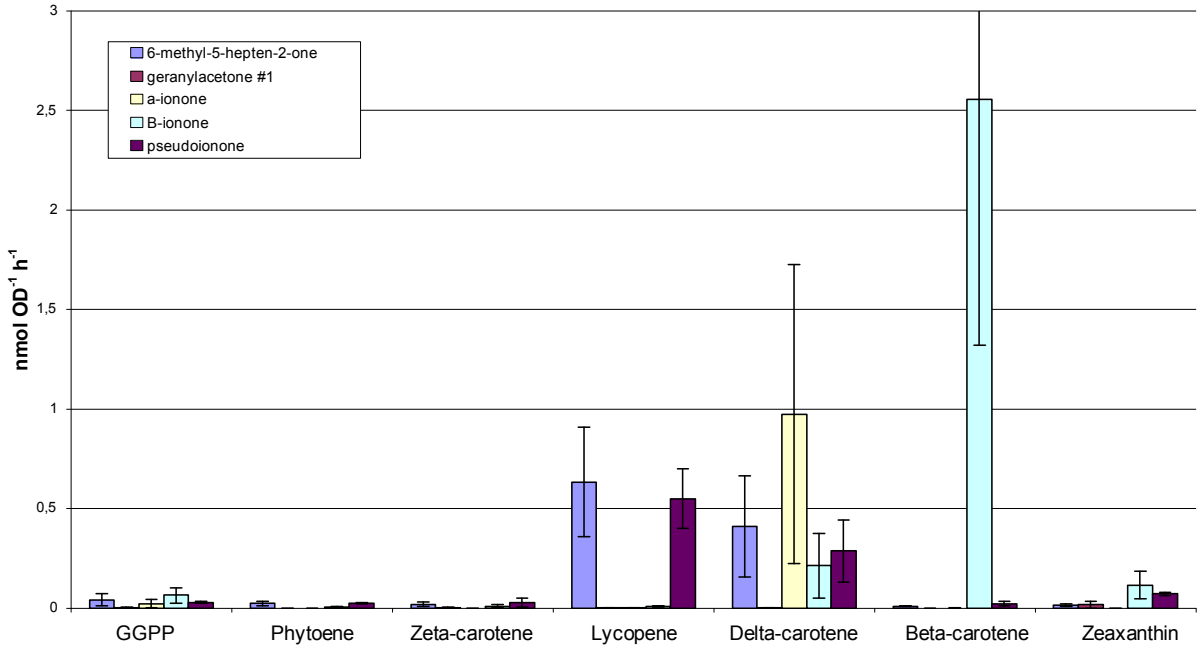


Figure 3.12 Volatiles produced by carotenoid-accumulating *E. coli* strains transformed for co-expression of AtCCD7 and AtCCD8 CDs. Volatiles were collected from *E. coli* strains accumulating different carotenoids and harboring either an empty vector or the expression construct for AtCCD7 and AtCCD8. Separetion of the peaks was performed by GC and GC-MS. Bars showed the mean value of three observations and standard error.

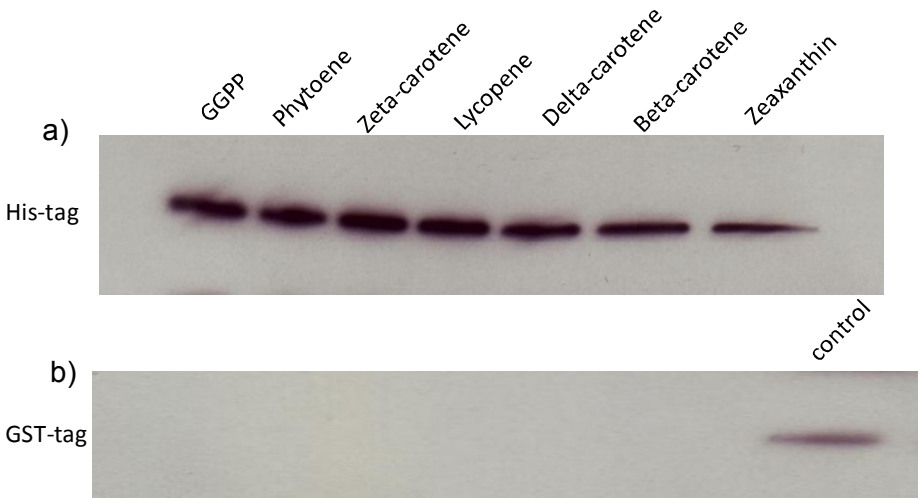


Figure 3.13 Protein blot analysis of AtCCd7 and AtCCD8 expression. a) analysis of AtCCD8 expression postvolatile collection using GST antibody, b)) analysis of AtCCD7 expression postvolatile collection using His antibody

4. Discussion

4.1 QTLs for tomato fruit quality

In recent years, there has been much renewed interest in the possibility of breeding not only higher yielding but also better quality crops. One potential approach to this end is the combined use of transcriptional profiling and introgression breeding. While there has been much interest in influencing fruit size and shape as well as improving the organoleptic properties of tomato (Frary *et al.*, 2003; van der knaap *et al.*, 2004; Chaib *et al.*, 2006), nutritional quality has largely been overlooked in tomato breeding programs. However, the compositional fruit quality is receiving increasing interest, particularly given the results of recent studies highlighting the nutritional importance of lycopene, flavonoids, and chlorogenic acid in the human diet (Davuluri *et al.*, 2005; Dixon, 2005; Niggeweg *et al.*, 2006; Rein *et al.*, 2006). Such improvements are particularly important in tomato, since, in this species, the flavour components associated with nutrition have been depleted through breeding (Goff and Klee, 2006; Morris and Sands, 2006). One such approach to identify genetic material suitable for reintroducing these traits is the introgression approach, whereby wild allelic variance is introduced back into cultivated species by marker-assisted selection of a single chromosome segment substitutions (Zamir, 2001; Giovannoni, 2006). In this study the AsA and phenols concentration, soluble solids content and fruit firmness were evaluated in an IL population in which marker-defined regions of the cultivated variety M82 (*S. lycopersicum*) are replaced with homologous regions of wild species *S. pennellii*. The power for identifying genomic regions that are significantly associated with quantitative traits is higher in the ILs than in populations that segregate simultaneously for multiple QTLs scattered throughout the genome, where independent loci can mask the effect of one another through epistatic interaction (Schauer *et al.*, 2006). In the IL population, the lines are identical across their whole genome except for a single introgressed region. As result, all of the phenotypic variation is associated with the introduced segment and the efficiency for identifying

individual QTLs is increased. In addition, owing to the permanent nature of the population experiments are generally reproducible, thereby facilitating the integration of data from independent groups.

QTL mapping became very popular in tomato genetics and breeding research, where QTL have been identified for numerous agriculturally and biologically important complex traits. Practically, it is difficult to provide a complete account of all genes and QTLs that have been identified and/or mapped in tomato chromosomes and the mapping population that have been used. Most mapping populations have been based on interspecific crosses between the cultivated tomato and relative wild species. In fact, almost all wild species of tomato have been used for gene and/or QTL mapping, although with different frequencies. In particular, the *S. pennellii* interspecific introgression lines population were shown to be powerful material to dissect plant yield and fruit quality (Eshed and Zamir, 1995; Eshed *et al.*, 1996). This population has already been used for mapping candidate genes and QTLs for carotenoids (Liu *et al.*, 2003), fruit weight and composition in sugars and acids (Causse *et al.*, 2004), antioxidant compounds (Rousseaux *et al.*, 2005), volatiles aromas (Tadmor *et al.*, 2002) or various metabolites (Schauer *et al.*, 2006). In the current study, the *S. pennellii* ILs population has been used to identify QTL underlying tomato fruit quality composition that could be used in a breeding program aimed to pyramiding desirable characteristic in a single *elite* variety. The levels annual variability of the different biochemical compounds in the ILs compared in this study point to the importance of making multiple-year comparisons to establish the QTL stability and the role of exogenous factors such as environment. Many studies reported that most of the traits of interest have a continuous variation, strongly influenced by environmental conditions. Temperature and light intensity exert a direct influence on the QTL expression, indeed find the location of such QTL is only the first step, and for “target breeding“ the underlying physiological consequences of genetic variation needs to be defined in terms of specific and general adaptation, so that varieties can be “tuned” to their target environment (Snape et al., 2007). Our results are in agreement with findings reported in literature. Indeed, as concern the QTL detected for each characteristic, some of them have already been related, whereas other have not been mentioned. On

the other hand, some QTL already well-known for fruit AsA content and °brix has not been found in our experimental conditions. Moreover, given the strong effect of the seasons, the expression of a certain phenotypic characteristic was not detected every year.

Total soluble solids

Within soluble solids determinants, sucrose, glucose, and fructose are the major sugars found in tomato fruit with high hexose accumulation being characteristic of domesticated tomato (*S. lycopersicum*). Together with quinic and citric acid, these compounds are the principal quality components for processing tomatoes, determining the soluble solids content or °Brix index (Carrari and Fernie 2006). The genetic bases of the sucrose-accumulation trait of the wild species tomato has highly been studied by means of introgressing wild germoplasms into domesticared cultivars (Yelle *et al.*, 1991; Fridman *et al.*, 2000, 2004). With a large range of variation for fruit composition, several QTLs for sugar and acid content have been mapped (Causse *et al.*, 2002, 2004; Fulton *et al.*, 2002; Lecomte *et al.*, 2004). Eshed and Zamir (1995) have evaluated 50 of the *S. pennelli* ILs in Israel, and reported a minimum number of 23 QTLs for °brix content. These results were compared with those obtained in France with the same subset of ILs. Fewer QTLs were detected in France but, eight among the nine soluble solids content QTLs found were also detected in Israel (Eshed and Zamir 1995). In our work 8 QTLs that increase the °brix content were found, most of them shared with results from Eshed and Zamir (1995). In addition, °brix QTLs detected in our experiment also overlapping those identified by Causse and colleagues (2004) in a *S. pennelli* population grown in the southeast France. Moreover, summarizing all data available in literature *S. pennelli* alleles increased the line mean for all the sugar-related QTLs, whereas positive and negative alleles were found for acids. In particular, Fulton and co-workers (2002) found in several interspecific populations that wild alleles increased the sugar content for the majority of QTLs, whilst both positive and negative effects were found for organic acids. In this study for the first time a negative QTL for total soluble solids was detected. The comparison with other

QTL approaches for acid and sugar-related traits (Sabila-Colombani *et al.*, 2001; Fulton *et al.*, 2002) revealed a few chromosomal regions where QTLs for the same trait could be found in different populations. Some QTLs controlling acid or sugar-related traits were observed in the same bin regions in seven cases for acids and in four cases for sugars (Fulton *et al.*, 2002).

By contrast to results from many studies aimed to look for °brix QTLs in *S. pennellii* IL population the IL 9-2-5 did not show a positive QTL for total soluble solids in our experimental conditions. A detailed biochemical characterization of vegetative and fruit tissues of this introgression line carrying the *Lin5* wild allele and harbouring the moderate °brix QTL was recently reported (Baxter et al., 2005a). Finding of an increased capacity of IL 9-2-5 to take up sucrose from the phloem adds physiological support to the conclusions drawn by Fridman and colleagues (2004) concerning the key role played by the apoplastic invertase LIN5.

Fruit firmness

The fruit morphology and the combination of diverse tissues play a crucial role in fruit texture of which firmness is one of the mainly traits involved (Dupart *et al.*, 1991). In the face of such complexity, very few studies have described fruit texture as a whole (Seymuor *et al.*, 2002). Most studies have focused on a single aspect of the fruit texture (Liebhard *et al.*, 2003), and more specifically on cell wall properties (Waldron *et al.*, 2003; Devaux *et al.*, 2005; Brummell, 2006). Indeed, a decline in fruit firmness typically coincides with dissolution of the middle lamella, depolymerization, and solubilization of hemicellulosic and pectic cell wall polysaccharides and, in some cases, wall swelling (Brummell and Harpster, 2001). Finally, genetic control of such traits in tomato is poorly understood (Fulton *et al.*, 2000; Doganlar *et al.*, 2002; Frary *et al.*, 2003). In order to improve our understanding of the physical component involved in changes of fruit texture the fruit firmness of the *S. pennelli* IL population was analyzed using instrumental measurements, which inform about the mechanical properties of the fruit. Five negative QTLs corresponding to a decrease of fruit firmness were detected. One of them showed by IL 9-1-5. QTLs for fruit

firmness (evaluated by a penetrometer) on chromosome 9 and 4 have been previously detected (Sabila-Colombani *et al.*, 2001) in a population of RILs derived from the cross between a cherry tomato line *S. lycopersicum* var. cerasiforme (Cervil) and a round larger-fruited tomato line *S. lycopersicum* (Levovil). Moreover, QTLs for fruit firmness on chromosome 4 and chromosome 9 were detected in the same regions as fruit firmness (evaluated by hand squeezing) QTLs detected in crosses with *S. pimpinellifolium* (Tanksley *et al.*, 1996) and *S. peruvianum* (Fulton *et al.*, 1997). Recently, results from two QTL-NILs population showed that the idividual effect of QTLs 4 and 9 on texture parameters were generally not significant in QTL-NILs compared with the parental lines (Chaib *et al.*, 2007). Conversely, significant individual effect on firmness was observed for the QTL located on chromosome 9 in the VilB genetic background. The same effect was not detected in a previous study by Chaib and co-workers (2006). These results demonstrated the impact of genetic background and/or environment on the expression of texture-related QTLs and possible interactions between them. This lack of consistency of QTLs for firmness was in part explained by the different instrumental methods that were used in the determination of the firmness. Moreover, such variation, confirm that firmness interacts strongly with environmental conditions such as year of cultivation or location (Bernacchi et al., 1998; Chaib et al., 2006).

Ascorbic acid

AsA is essential for cardiovascular function, immune cell development, connective tissue, and iron utilization. Although plants and most animals can synthesize ascorbic acid, humans lack L-gulono-1,4-lactone oxidoreductase activity, which is required for the final step in AsA synthesis. Because AsA cannot be synthesized and stored in the human body, the vitamin must be acquired regularly from dietary sources (Zou *et al.*, 2005). Tomatoes are an important source of water-soluble antioxidant, particularly AsA. Due to the high consumption levels of tomatoes and the substantial ascorbic acid content (approximately 4 mg/100 g FW), these fruit represent a major contribution to dietary nutrition worldwide (Willcox *et al.*, 2003). In the present work fruit AsA

concentration in the ILs population exhibited the typical distribution of quantitative traits that are controlled by several QTLs. To our knowledge, no null mutants have been identified for AsA content in fruit, and only four mutant loci have been identified in *Arabidopsis* after an ozone screen (Conklin *et al.*, 2000). Of these mutants, vtc1 corresponds to a mutation in the GMP gene (Conklin *et al.*, 1999), and the positional cloning of the vtc2 mutation led to an unknown protein. The vtc4 mutant has been recently shown to encode L-Gal-1-P phosphate, a plant AsA biosynthetic enzyme (Conklin *et al.*, 2006). These mutants contain reduced levels of AsA, and no null mutants are found, presumably because plants without ascorbate would not be viable. In tomato, by screening 118 M82 tomato mutant families, a few mutants with reduced or increased fruit ascorbic acid content compared to M82 have been identified (Stevens *et al.*, 2006). Fruit from the 118 families contained between 6.1 and 31.4 mg ascorbic acid *per* 100 g fresh weight, which is similar to the range of natural variation observed in the population studied here. Indeed, in the *S. pennellii* IL population the AsA content ranging from 8.85 to 24.3 mg ascorbic acid *per* 100 g of fresh weight. Improvement of AsA content may be a target for tomato breeders. Improvement of vitamin content in species of agronomic interest is cited as an important criterion (Agius *et al.*, 2003; Dalvuluri *et al.*, 2005; Paine *et al.*, 2005). Moreover, it is interesting to note that the total AsA (i.e. reduced + oxidized forms) showed the same trend of the ascorbate in the ILs analyzed. Indeed, fruit from IL with a higher AsA content also displayed a higher total AsA concentration, leading to a completely overlapping of the two analysis. For this reason, in the year 2008, the total AsA content of the IL population was not analyzed. The total AsA pool measured in tomatoes in this study reflects what a consumer would expect when eating a fresh tomato. The presence of several transgressive lines has revealed the potential use of the wild relatives to improve this trait. When we compare our QTLs controlling the AsA content with those already related in literature contrasting results were obtained. Probably because as shown by Toor and collegues (2006) fruit AsA content is highly influenced by the environment. Moreover, the experimental conditions for measuring the trait may be subject to variation. Indeed, analysis of the same IL population grown over two years in Israel for a range of fruit

metabolites showed 4 positive QTLs for AsA, of which only 1 (IL 12-4) was common with our study (Schauer *et al.*, 2006). In a previously work focused on IL population grown over two years in California (Rousseax et al., 2005), AsA content was analyzed, and positive QTL was detected in IL12-4, as in our research. Negative QTL were also found in 5 ILs, of which 1 (IL 10-1) was common with the present results. Moreover, Rousseaux and co-workers (2005) did not detect any AsA in *S. pennelli* fruit. On discussing this result they also associated it to the extraction procedure carried out by grinding fresh fruit on ice. They mention that by grinding fresh fruit in liquid nitrogen *S. pennelli* fruit AsA levels were restored to 1.9 mg *per* 100 g, which is still slightly lower that our value. This result is also in disagreement with reported average AsA concentration in *S. pennellii* ripe fruit of 71 mg *per* 100 g of FW (Stevens *et al.*, 2007). Consistent with higher fruit AsA concentration in *S. pennellii*, other papers showed this specie as a good source of vitamins (Schauer *et al.*, 2005) confirming the potential of wild species as a source of genetic variation to bring up crop improvement (Fernie *et al.*, 2006). In agreement with these latter , our results showed an average AsA concentration of 37.2 mg per 100 g of FW in *S. pennellii* red ripe fruit. High AsA levels were also found in the other wild species analyzed. Noteworthy as *S. pennellii* fruit remain green over ripening, it is difficult to evaluate its ripe stage. So the incorrect identification of the ripe stage may be cause of wide differences observed.

Analysis of the same IL population grown in France for three years led to the detection of 12 QTLs for fruit total ascorbic acid content (reduced + oxidized forms) (Stevens *et al.*, 2007). Of these QTLs none was common to QTLs detected in our study. Given that a complete overlapping of AsA and total AsA QTLs was found in the IL population, contrasting results from different studies may be due to the sample selection. Indeed, our quantification was performed on fruit including pericarp and depleted of locular jelly, seeds, and *columnella*. Conversely, Stevens and colleagues (2007) used whole fruit in their study, detecting higher concentrations of ascorbic acid in the locular jelly than in the pericarp of M82 at the red ripe stage.

Recently, fourteen genes associated with tomato ascorbic acid biosynthesis and metabolism were mapped (Zou *et al.*, 2006). The gene map included 15 loci,

mapping on nine chromosomes. Most of the genes were mapped on introgressed regions already known to contain QTL for fruit AsA content.

Total phenols

Despite tomato is the principal source in human diet of phenolic compounds, such as flavonoids and hydroxycinnamic acids, and evidences for their beneficial properties as health-promoting compounds (Duthie and Crozier, 2000; Pietta, 2000; Nijveldt et al., 2001), only limited data are available on their occurrence and distribution in tomatoes. In tomato, most of the studies for phenolic compounds have been focused on over-expression of the genes encoding biosynthetic enzymes (Verhoeyen *et al.*, 2002), or transcription factors (Bovy *et al.*, 2002), or by suppressing regulatory gene (Davuluri *et al.*, 2005). Phenolic compounds present in different tomatoes were evaluated in fruit samples from nine commercial varieties finding a range of distribution from 259.15 to 498.60 mg Kg^{-1} FW (Martinez-Varlverde *et al.*, 2002). These values are tipycal of the extractable phenolic concentration seen in other fruit and vegetables, that can vary from 2 to 500 mg Kg^{-1} (Scalbert and Williamson, 2000). The IL population showed a total phenols concentration ranging from 740 to 1700 mg Kg^{-1} FW. Observed inconsistency may be explained because there is no single analytical method that, collectively and accurately, is able to measure the total polyphenol content of a food. Reason for this, include the structural diversity found amongst phenolic compounds and the large variation in content depending on the nature of the food and the plant part from which it derives (Bravo, 1988). The total phenolics assay used in our experiments, as well as in the quoted study, usually overestimates the content of phenolic compounds, since other reducing agents present in the food, can interfere. Moreover, the distribution of phenolic compounds in tomatoes varies greatly between the various parts of the plant, at the organ, tissue and cellular level (Scalbert and Williamson, 2000). Martinez-Varlverde and co-workers (2002) assayed whole fruit without distinguish between the different parts of the fruit, whereas we assayed the fruit depleted of the locular jelly, seeds and *columella*. Also cultural and environmental factors may affect phenolic

concentration, as showed by Raffo and colleagues (2006) reporting on variations in phenol levels associated with seasonal influences.

Moreover, in contrast to the relative abundant information on QTL for carotenoids, fruit weight and composition in sugars and acids, and various metabolites in tomato fruit, very little has been done about QTL for phenolic compounds in tomato and particularly in IL populations. As far as we know, in only one previous report focused on *S. pennellii* IL population (Rousseax *et al.*, 2005), phenol content was analyzed leading to the identification of nine QTLs, one positive QTL and eight negative QTLs. In the present work one positive QTL (IL 7-3) controlling phenol content was detected but, it was not the same found by Rousseaux and colleagues (2005). Reasons of this inconsistency may be probably due to different environmental conditions used in the trails.

The phenols content of wild accessions of tomato was also analyzed. They all displayed higher phenolics content than cultivated tomato confirming the value of wild species as source of natural variability to improve fruit quality. The metabolic profiles of leaves and fruit of *S. lycopersicum* and five wild species tomatoes that can be crossed with this *elite* variety (*S. pimpinellifolium*, *S. neorickii*, *S. habrochaites*, *S. chmielewskii*, *and S. pennellii*) has been reported by Schauer and colleagues (2005). A tremendous variance in metabolite content in both leaves and fruit of the wild species was showed. In particular, wild species showed higher levels of metabolic variation in fruit than in leaves, probably due to the higher degree of morphological variation in these organs. So far it is evident that these wild species represent a valuable resource to increase tomato nutritional properties.

4.2 Comparative transcriptomic analysis

Physical and chemical assay performed on *S. pennellii* IL population allowed the identification of QTLs involved in fruit organoleptic and nutritional traits. In particular, the IL 12-4 displayed at the same time a QTL for increased fruit AsA concentration and a QTL for decrease fruit firmness. To identify transcripts (or cluster of transcripts) involved in the higher AsA content and lower fruit firmness

showed by IL 12-4 compared to *S. lycopersicum* cv. M82, and gain insight into the role of transcriptional regulation in tomato fruit a comparative microarray analysis on COMBIMATRIX TomatoArray1.0 was performed.

Tomato genomic resources have been widely exploited for developing new microarray-based technologies. Many arrays are currently available worldwide for tomato transcriptome analysis, each combining different technologies and properties. TOM1 array was developed by the Center for Gene Expression Profiling (CGEP) of the Boyce Thompson Institute for Plant Research and made publicly available. Each spot on its glass slide corresponds to a single unigene. Each gene was random selected from different cDNA libraries including a range of tissues as leaf, root, fruit, and flower. Recently, the CGEP has released the new tomato array TOM2. Tomato transcriptome analysis is also feasible using the Affymetrix GeneChip Tomato Genome Array wherein sequence information was selected from public data sources including *S. lycopersicum* UniGeneBuild #20 (October 3, 2004) and GenBankR mRNAs up to November 5, 2004. TOM1, TOM2 and Affimetrix arrays have already been used for functional genomics studies, whose results are available at the Tomato Functional Genomics Database website (http://ted.bti.cornell.edu). On the other hand, Agilent tomato gene expression microarrays are synthesized on demand as custom microarrays. Finally, TomatArray1.0 provides through COMBIMATRIX technology combines phosphoramidite chemistry and semiconductors to digitally control probe synthesis at the chip surface. The 90k TomatArray1.0 contains 90,000 siliceous electrodes (features) overall supporting 20200 in situ synthesized DNA probes with 4 replications. Probes have been designed to match specifically with 21550 Tentative Consensus (TCs) from TIGR database *S. lycopersicum* Gene Index Release 11.0 (June 21, 2006). Combimatrix slides can be easily stripped and re-hybridized 4 to 6 times, so economizing the costs of experiments.

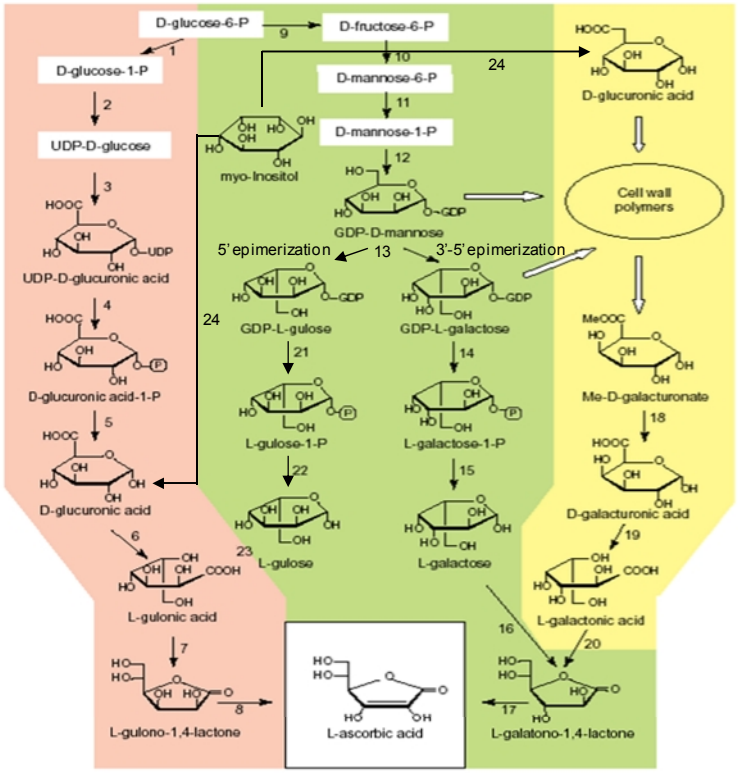
The tomato transcriptomic analysis through Combimatrix chip led to the identification of 155 differentially expressed sequences between IL 12-4 and M82. Actually, the expansion in the use of functional genomics technologies, such as microarray experiments, in biological research had specific consequences in computational biology. Functional interpretation is a key step

in the analysis of this data which cannot be done without the availability of extensive functional annotation of the datasets. This makes standardized functional annotation essential. The most widespread and probably most extensive functional annotation schema for gene and protein sequences is the Gene Ontology (GO) (Ashburner *et al.*, 2000) which has become the standard in nearly all public data-bases. The accurate assignment of functional information to gene products is a complex, laborious and time-consuming task often performed manually. Moreover, the functional annotation of uncharacterized sequences is the balance between accuracy and intensity. All these elements have been driving guidelines in the development of Blast2GO application (Gotz *et al.*, 2008). Indeed, through Blast2GO bioinformatic suite, blast annotation, GO mapping, and graphical categorization of all different expressed sequences can be performed automatically. Using Blast2GO program, categoritation of the 155 differentially expressed transcripts was performed. In particular, cellular component categorization showed that the largest groups of transcripts belonged to the mitochondrion and plastid category. From the literature we know that ascorbate occurs in the cytosol, chloroplasts, vacuoles, mitochondria and cell wall (Rauten-Kranz *et al.*, 1994). Indeed, AsA is present at high-millimolar levels in all subcellular compartements; in particular, it is found in chloroplasts, at concentrations of 20 mM or more (Ishikawa and Shigeoka, 2008). It has also become apparent that AsA has multiple roles in metabolism particularly as an *in vitro* electron donor for photosynthetic and mitochondrial electron transport (Smirnoff, 1996). Moreover, it is interesting to note, amongst the differentially expressed sequences, the presence of two down-regulated glutathione-s-transferase (# Probe 17266 and 12118), and one up-regulated peroxidase (# Probe 2731) (Tab. 3.9). Indeed, AsA pool size is the result of the balance between the rates of synthesis/catabolism and turnover. Turnover results from further metabolism or from oxidation followed by non-enzymatic degradation. AsA is readily oxidized to monodehydroascorbate (MDA) as part of its antioxidant function. Oxidation is catalysed by two enzymes: ascorbate peroxidase (APX) and ascorbate oxidase (AO). MDA disproportionates to dehydroascorbate (DHA) and AsA if it is not immediately reduced. DHA is unstable above pH 7 and

irreversibly delactonizes to 2,3-diketogulonate (Loewus, 1988; Smirnoff, 1995). Under normal circumstances the AsA pool is at least 90% reduced. This is achieved by the action of two enzymes: NAD(P)-dependent monodehydroascorbate reductase (MDAR) and glutathione (GSH)-dependent DHAR. The observation that reduced glutathione (GSH) was capable of reducing DHA to AsA led Foyer and Halliwell (1976) to propose a role of GSH in the regeneration of AsA. Recycling of GSH is achieved by glutathione reductase reducing glutathione disulphide (GSSH) by consumption of NADPH: In contrast, glutathione transferase reduce the GSH level leading to an increase of GSSH. Probably, the results obtained from comparative transcriptomic analysis may indicate an involvement of a reduced activity of the glutathione transferases in enabling more GSH availability for the regeneration of AsA.

Also, microarray data can potentially be used to identify regulatory genes associated with coordinating expression of the pathways of interest (Janseen *et al.*, 2008) such as, in this study, AsA metabolism. Indeed, using hierarchical clustering, two transcription factors (# Probe 3573 and 138) (Tab. 3.9) were identified with a similar expression pattern to glutathione reductase (# Probe 11949) and glutathione transferase (# Probe 17266) both down-regulates into IL 12-4.

Finally, sequences annotation allowed to identify a pectin-methyl esterase (# Probe 10417) among the up-regulates differentially expressed genes probably involved in the different AsA content and fruit firmness showed by IL12-4. Besides previous evidences (Carrari *et al.*, 2006) focused on the central role of the Smirnoff-Wheeler pathway (Wheeler *et al.*, 1998) in the tomato fruit ascorbate control, alternative AsA pathway (Valpuesta and Botella, 2004) may lead to metabolic modifications of AsA level observed into IL 12-4. These include processing involving D-glucurono-1-4-lactone (GlcUL), D-galacturonate (GalUA), methyl D-galacturonate (MeGalUA), and L-gulono-1,4-lactone (GulL), putative intermediates first considered by Isherwood *et al.*, (1954) and more recently, in the case of GulL, in transformed plants (Jain and Nessler, 2000) (Fig. 4.1)



TRENDS in Plant Science (Valpuesta and Botella, 2004).

Figure 4.1 Biosynthetic pathways of L-ascorbic acid in animals (reactions 1–8) and plants (reactions 9-24). Enzymes catalyzing the numbered reactions are: 1, phosphoglucomutase; 2, UDP-glucose pyrophosphorylase; 3, UDP-glucose dehydrogenase; 4, glucuronate-1-phosphate uridylyltransferase; 5, glucurono kinase; 6, glucuronate reductase; 7, aldono-lactonase; 8, gulono-1,4-lactone dehydrogenase; 9, glucose-6-phosphate isomerase; 10, mannose-6-phosphate isomerase; 11, phosphomannomutase; 12, GDP-mannose pyrophosphorylase (mannose-1-phosphate guanylyltransferase); 13, GDP-mannose-30,50-epimerase; 14, phosphodiesterase; 15, sugar phosphatase; 16, L-galactose dehydrogenase; 17, L-galactono-1,4-lactone dehydrogenase; 18, methylesterase; 19, D-galacturonate reductase; 20, aldono-lactonase; 21, phosphodiesterase; 22, sugar phosphatase; 23, L-gulose dehydrogenase; 24, myo-Inositol oxygenase.

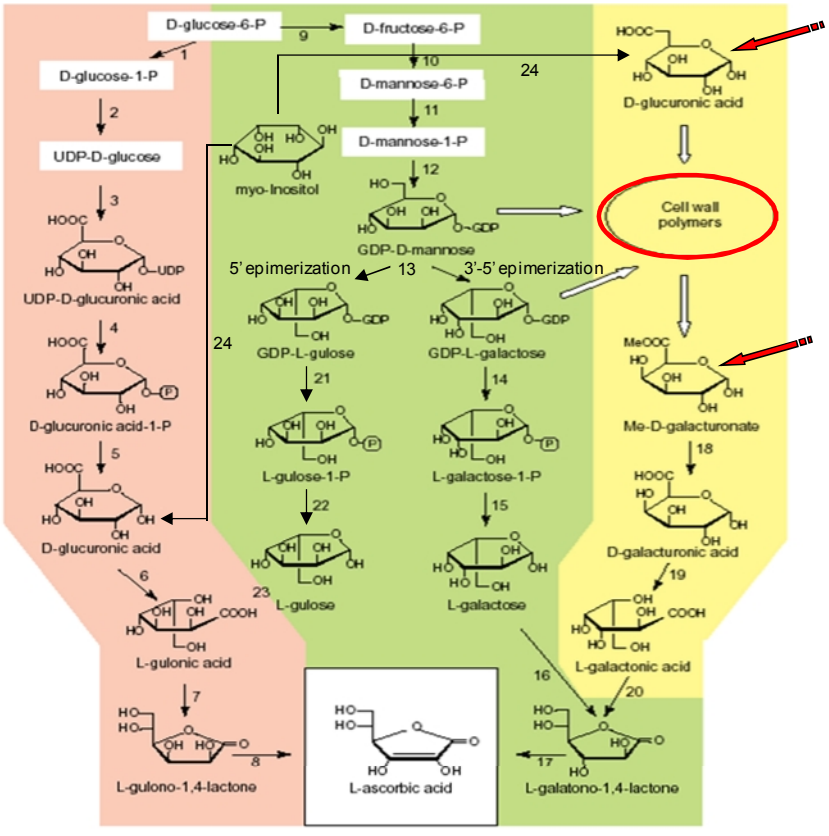
In particular, feeding experiments using precursors have shown that the methyl ester of D-galacturonic acid causes a significant increase in the L-ascorbic acid content of cress seedlings and Arabidopsis cultured cells (Davey *et al.*, 1999). It is also known that D-galacturonic acid-1-^{14}C is metabolized to L-ascorbic acid-6-^{14}C by an inversion pathway in detached ripening strawberry fruit (Loewus and Kelly, 1961). A uronic acid pathway that accommodates both findings, the

role played by methyl ester of D-galacturonic acid as precursor and the occurrence of an inversion pathway in some plant tissues, was previously proposed (Smirnoff *et al.*, 2001). Molecular evidences for this pathway came with the cloning and characterization of a D-galacturonic acid reductase from strawberry fruit (Agius *et al.*, 2003). In this pathway, pectin-derived D-galacturonic acid is reduced to L-galactonic acid, which in turn is spontaneously converted to L-galactono-1,4 lactone. This compound is the substrate of the L-galactono-1,4-lactone dehydrogenase enzyme (Fig. 4.1, yellow background). It is likely that the flux through this branch is dependent upon the availability of the substrate D-galacturonate. This compound appears as a product of the turnover of cell wall pectins in senescing cells. D-glucuronic acid, can follow two pathways in plant cells, either the biosynthesis of L-ascorbic acid or the supply of precursors for the synthesis of cell wall polymers such as pectin and hemicellulose (Loewus and Murthy, 2000).

That said, we supposed that the higher AsA content of the IL 12-4 compared to M82 may also be due to the increased pectin-methyl esterase activity leading to a major availability of AsA precursors (Fig. 4.2). This would also explain the less fruit firmness showed from IL 12-4. Indeed, the primary plant cell wall consists of cellulose, hemicellulose, pectin and proteins (Carpita and Gibeaut, 1993). Cellulose and hemicellulose are the main load-bearing polysaccharides in the cell wall, maintaining the cell shape and turgor pressure. Pectin is not load bearing, but may control the mobility and access of enzymes to load-bearing hemicellulose molecules (.Blamey, 2003; Filisetti-Cozzi and Carpita, 1991; Konno *et al.*, 1999; and Ma *et al.*, 1999), thereby modulating cell elongation. Pectin contains negatively charged galacturonic acid residues, which contribute to the cell wall cation exchange capacity. Cation binding to pectin therefore affects the charge, pH, swelling behaviour (pore size) and buffer capacity of the cell wall. This can have direct effects on the mobility of cell wall-degrading enzymes and cell elongation (Cosgrove, 2000). Modifications in cell wall polymers are intricate and considered to involve the co-ordinated and interdependent action of a range of cell wall-modifying enzymes and proteins such as polygalacturonase (PG), pectin-methyl esterase (PME), β-galactosidase, α-L-arabinofuranosidase, endo-(1,4) β-D-glucanase, expansin,

and xyloglucan endotransglycosylase (Brummell and Harpster, 2001). Given that, the breaking up of cell wall by pectin-methyl esterase activity may lead to the release of galacturonic acid with consequences for both fruit firmness and AsA accumulation.

Currently, efforts are being undertaken to validate the differential transcriptional profile of single mRNA by Real-Time qPCR. Further investigations, by means of functional analysis, will focus on testing the hypothesis that fruit ascorbate biosynthetic flux may increase through the poligalacturonate pathway.



modified from *TRENDS in Plant Science*

Figure 4.2 Proposed involvement of pectin-methyl esterase activity into AsA biosynthetic pathwas.

4.3 AtCCD7 and AtCCD8 cleave multiple carotenoids to generate apocarotenoid volatiles

In recent years, a family of enzymes that cleave carotenoid substrates at different double positions have been described in plants. This family, the carotenoid cleavage dioxigenases (CCDs), are specific for the location of double bond in the molecule they cleave, but many are promiscuous in their carotenoid substrate choice and for other the specific substrate is still unclear (Auldridge *et al.*, 2006). Particularly, the hypothetical proteins AtCCD7 and AtCCD8 are the most disparate members of this protein family in Arabidopsis. It has been shown that two carotenoid cleveage dioxygenases, LeCCD1A and LeCCD1B, can cleave multiple linear and cyclic carotenoids at the 9,10 position generating geranylacetone, pseudoionone, and β-ionone. Antisense reduction of the tomato genes led to ~50% reduced emissions of geranylacetone and β-ionone, confirming roles of these enzymes. By contrast, no QTLs for any of these volatiles was associated with the map positions of LeCCD1A or LeCCD1B in the *S. pennellii* IL population. Despite great importance has been given to these enzymes because of their involvement in the production of important fruit flavour volatiles as geranylacetone, pseudoionone, and β-ionone (Tieman *et al.*, 2006), their biochemical characterization is an important step in identifying the biologically active products. Previous finding demonstrated that AtCCD7 cleave multiple carotenoid substrates leading to the mainly production of the apocarotenoid volatile β-ionone (Booker *et al.*, 2004; Schwartz *et al.*, 2004). Different studies indicated that CCD1 and CCD7 have similar activities, the former oxidatively cleaving multiple carotenoids symmetrically at the 9,10 and 9',10' positions and the latter only at the 9,10 position (Booker *et al.*, 2004; Schwartz *et al.*, 2001, 2004). Conversely, studies carried out in the Klee's laboratory (University of Florida), tested the activity of AtCCD8 on carotenoid substrates by expressing the Arabidopsis CCD8 genomic sequence in *E. coli* strains engineered to accumulate β-carotene, lycopene or zeaxanthin. When expression of AtCCD8 was induced, accumulation of each of these carotenoids was significantly reduced. Despite this reduction in carotenoid accumulation the cleavage product was not detected, either by HPLC analysis of cell or growth

media extracts or by gas chromatography analysis of the collected volatiles (unpublished data). Moreover, because the *max3* and *max4* mutants in *A. thaliana* resulted from lesions in AtCCD7 and AtCCD8 genes, and both mutants display a dramatic increase in lateral branching (Booker *et al.*, 2004), it is likely that the two gene products function in the same pathway. For these reasons, a construct for the co-expression of AtCCD7 and AtCCD8 was transformed into the carotenoid-accumulating strains.

Particularly, to determine the substrate specificities and bond cleavage preferences of AtCCD7 and AtCCD8, we undertook a systematic analysis of the volatile products generated by the enzymes in various carotenoid-accumulating strains of *E. coli*, because the volatile products are indicative of these cleavage activities (Tab. 4.1).

Table 4.1 Volatiles predicted to be generated by carotenoid cleavage at various bond positions

	Position of the double bond		
	5,6 or 5',6'	**7,8 or 7',8'**	**9,10 or 9',10'**
GGPP	6-Methyl-5-hepten-2-one		Geranylacetone
Phytoene	6-Methyl-5-hepten-2-one		Geranylacetone
ζ-Carotene	6-Methyl-5-hepten-2-one		Geranylacetone
Lycopene	6-Methyl-5-hepten-2-one	Citral	Pseudoionone
δ-Carotene		Citral	α-Ionone, pseudoionone
β-Carotene		β-Cyclocitral	β-Ionone
Zeaxanthin		3-Hydroxy-β-cyclocitral	3-Hydroxy-β-ionone

Besides the protein expression analysis did not detect the AtCCD7 protein in the double engineered strains, the presence of the final products derived from AtCCD7 activity was enough to suppose that the gene was expressed.

Work by Schwartz and colleagues (2004) showed the presence of a new C_9 product in experiment of co-expression of the two genes AtCCD7 and AtCCD8, but this product has not yet been identified. Referring to the Table 4.1, the results indicate a preferential substrate for AtCCD7 enzyme. In particular, given

the so high production of β-ionone in beta-carotene-accumulating strain, we could state the preferential 9,10 or 9',10' bond cleavage of AtCCD7 protein using beta-carotene as substrate, such as already reported in literature. Unfortunately, we did not detected any new volatile compound as expected from the cleavage action of AtCCD8. It is likely that the product was further catabolised in E. coli, as it has been previously observed by others von Lintig and Vogt (2000).

5. Conclusion

The objective of this study was to identify genomic regions involved in the control of tomato fruit quality traits. In particular, this study focused on fruit antioxidant and soluble solids content and fruit firmness. Also, aimed to elucidate molecular mechanisms of the phenotypic variation. Evaluated traits showed a continuous distribution over wide ranges in genetic resources tested. Total soluble solids varied from 3.07 °Brix to 6.5 °Brix on average. Fruit firmness ranged from 25.14 N mm^{-2} to 15.48 N mm^{-2}. AsA and phenols content ranged, from 0.51 µmol g^{-1} FW to 1.40 µmol g^{-1} FW, and from 0.76 µg gallic acid equivalents mg^{-1} FW to 1.28 µg gallic acid equivalents mg^{-1} FW, respectively.
Moreover, IL fruit with physical and chemical properties significantly different from the M82 parental line, were detected. In particular, eight QTLs for total soluble solids, five QTLs for fruit firmness, four QTLs for AsA content and one QTL for total phenols content were identified.
Wild introgressions showed to be effective either in increasing and decreasing AsA, whereas only increasing and decreasing effects, respectively for total phenols and fruit firmness, were detected. As resulted from the phenotypic profiling, the IL 12-4 showed at the same time a QTL for increased fruit AsA content and a QTL for reduced fruit firmness. This IL was selected for comparative transcriptomic analysis aimed at the identification of transcripts underlying the phenotypic difference observed. In particular, microarray hybridization was performed on the Combimatrix 90K TomatArray1.0 slides. This chip was designed and synthesized on Combimatrix platform to the Center for Plant Functional Genomics of the University of Verona in the frame of a collaboration with Prof. Massimo Delledonne and Prof. Mario Pezzotti.
Comparative transcriptomic of IL12-4 fruit allowed the identification of transcripts potentially relevant to AsA biosynthesis and cell wall metabolism in tomato fruit. Among all, a differential hybridization signal was observed for:

- two glutathione s-transferase (# Probe 17266 and 12118), and one glutathione reductase (#Probe 11949) that were down-regulated
- one peroxidase (# Probe 2731) up-regulated
- one pectin-methyl esterase (# Probe 10417) up regulated

Currently, validation of expression patterns through Real-Time qPCR are in progress and further functional characterization of differential expressed sequences will help in elucidating their involvement in fruit quality-related traits. However, the identification of ILs carrying QTLs for fruit quality traits, suggest strategies for improving tomato through introgression breeding by pyramiding desirable characteristic in a single *elite* variety.

In addition, two available sequences, AtCCD7 and AtCCD8, previously involved in the apocarotenoid volatiles metabolism, were functionally investigated through co-expression in *E. coli*. Results provided evidences for the preferential substrate for AtCCD7 enzyme. In particular, given the so high production of β-ionone in β-carotene-accumulating *E. coli* strain, it was possible to state the preferential 9,10 or 9',10' bond cleavage of AtCCD7 protein using β-carotene as substrate, such as already reported in literature. Unfortunately, we did not detected any new volatile compound as expected from the cleavage action of AtCCD8 had been detected. It is likely that the product was further catabolised in *E. coli*, as this was previously observed.

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