3

Genetic Transformation in Tomato: Novel Tools to Improve Fruit Quality and Pharmaceutical Production

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1. Introduction

Tomato is one of the most important vegetable crop worldwide with a total production of around 141 million tons on a cultivated area of around 5 million hectares (FAOSTAT, 2009, http://faostato.fao.org). Among the most representative countries, Italy contributes with more than 6 million tons to the world production, on a cultivated area of around 117.000 hectares, both in open fields and greenhouses (FAOSTAT, 2009). This crop represents also one of the major products of the food industry worldwide and Italy ranks first for processing tomato production among Countries of the Mediterranean Region (World Processing Tomato Council, 2009, www.wptc.to). Indeed, the high variability of tomato fruits, ranging from the cherry type to the big round or elongated berry, supplies both fresh market and processing products, such as paste, juice, sauce, powder or whole. In the last years, tomato consumption has further increased since it was demonstrated that tomato fruit could protect against diseases, such as cancer and cardiovascular disorders, due to its antioxidant properties (Rein et al., 2006). Tomato fruits are particularly rich of nutritional compounds such as lycopene and alfa-carotene, vitamin C, flavonoids and hydroxycinnamic acid derivatives whose intake would account for health benefits.

The cultivated tomato (*Solanum lycopersicum*) belongs to the *Solanaceae* family that includes more than 3.000 species, among which 12 represent tomato wild relatives. These species exhibit a wide variety of adaptation to diverse habitats, plant morphology, fruit size and colour, the latter varying from green to white, yellow, pink, red, brown, depending mainly on the metabolites fruit content. The wild related tomato species represent a potential reservoir of useful genes that have been greatly used in breeding programs (Bai & Lindhout, 2007; Gur & Zamir, 2004). Indeed, this vegetable is one of the most investigated crop both at genetic and genomic level not only because of its economic importance but also because it is one of the best characterized plant systems. It has diploid genetics (24 somatic chromosomes), a small genome size (950 Mb per haploid nucleus), is self-pollinated, has a short generation time, is easily reproduced by seed and vegetative propagation and is cross-compatible with many wild species. All these characteristics make it amenable to genetic analysis.

The huge amount of researches focused on tomato allowed the development of new tools and platforms for genetics and genomics analyses (Barone et al., 2008). Since tomato is considered the model species among the *Solanaceae*, these novel techniques have been also exploited for other economically important crops, such as potato, pepper, and eggplant. Moreover, due to the high sinteny existing among *Solanaceae* species, tomato was chosen as reference genome to be completely sequenced by the International Tomato Genome Sequencing Consortium at the end of the year 2003 (Mueller et al., 2005). Molecular comparative mapping studies revealed a high level of conserved gene content and order within this family (Wu & Tanksley, 2010), as well as within other families (i.e grasses, crucifers, legumes). Indeed, a high level of microsinteny amongst the genomes of tomato, potato, pepper and eggplant was observed. Therefore, determination of the tomato genome sequence could allow extending information among species, thus creating a common map-based framework of knowledge. This could allow inferring the sequence organization of other *Solanaceae* crops as basis for understanding how plants diversify and adapt to new and adverse environments.

The recent release of the tomato genome sequence (Mueller et al., 2009), together with the powerful genetic and genomic resources available today for this species, allowed plant biotechnologists to implement novel methods to obtain new genotypes that could answer to new consumer, producer and processor requirements. These resources, in fact, could help the transfer of useful genes among species and/or improved genotypes through assisted breeding programs as well as through genetic transformation technologies.

In the present review, after providing some information on tomato genetic and genomic resources, we will give an overview of genetic transformation techniques and biotechnology applications investigated in this species. Several recent review reported new studies on tomato genetic transformation as a tool for the improvement of resistance to pests and pathogens (Balaji & Smart, 2011; Khan et al., 2011; Panthee & Chen, 2010; Wu et al., 2011; Zhang et al., 2010). Therefore, after a short description of main transformation techniques to which tomato is well adapted, herein we will focus on the use of genetic transformation for fruit quality engineering and pharmaceutical production.

2. Genetic and genomic resources

Among cultivated species, tomato is one of the richest in genetic and genomic resources (Table 1 and Table 2), including information now available from the complete genome sequencing that was released in the last year in a preliminary version. All these tools, used together or separately, are having a great impact on tomato breeding and genetics (Barone et al., 2009; Foolad, 2007).

This cultivated species could count on a number of wild and related species, on a wide collection of naturally or induced mutants and on many well-characterized genetic stocks, such as cultivars and landraces, cytogenetic stocks and pre-bred lines. Today this germplasm is publicly available (Table 1). In the miscellaneous group, the Backcross Recombinant Inbreds and Introgression Lines are particularly useful for the identification of genes and/or QTLs, since they constitute "immortal" population to be used for quantitative analyses (Grandillo et al., 2008). In addition, they also represent exotic libraries that allow to better exploit biodiversity exhibited by wild species. Indeed, the IL population is composed by many lines, each carrying a single homozygous genomic region from the wild species, altogether covering the whole wild genome (Eshed & Zamir, 1995; Fridman et al., 2004).

GENETIC RESOURCE	NOTES	WEBSITE
Wild species	More than 1.100 accessions	TGRC (http://tgrc.ucdavis.edu), NPGS (www.ars-usda.gov)
Monogenic mutants	More than 600 mutants	TGRC
Miscellaneous stock	More than 1.500 accessions	TGRC, NPGS
Introgression lines (IL)	from *S. pennellii*, *S. habrochaites*, *S. lycopersicoides*	TGRC
Backcross Recombinant Inbreds (RIL)	*S. lycopersicum* x *S. pimpinellifolium*	TGRC
Induced-mutant stocks	More than 3.400 induced-mutants from *cv* M82	SGN (http://zamir.sgn.cornell.edu/mutants)
	Around 1000 induced-mutants from Micro-Tom	TOMATOMA (http://tomatoma.nbrp.jp)
	More than 5.000 mutants from *cv* Red Setter	LycoTill (www.agrobios.it/tilling/index.html)

Table 1. Tomato genetic resources publicly accessible via web

Currently, IL populations that derive from various wild species are available, even though others are being generated (Barone et al., 2009). The first population (from *S. pennellii*) has been so far widely used to localize QTLs (Lippman et al., 2007) and to clone them (Frary et al., 2000; Fridman et al., 2000).

In addition to a collection of natural mutants available at TGRC (Tomato Genetic Resource Centre), wide collections of induced mutants were generated in different genetic backgrounds, by chemical or physical mutagenesis (Emmanuel & Levy, 2002; Menda et al., 2004; Watanabe et al., 2007). These mutants were widely phenotyped for many traits and contributed to better understand some developmental processes, such as growth habit, flowering and fruit ripening (Giovannoni, 2007; Pineda et al., 2010; Saito et al., 2011). In addition, induced mutagenesis has often been implemented with gene-specific detection of single-nucleotide mutations to generate TILLING platforms. So far, TILLING was developed for the cv. M82 (Piron et al., 2010), Red Setter (Minoia et al., 2010), Tpaadasu (Gady et al., 2009) and Micro-Tom (Saito et al., 2011) and its use has allowed the pinpointing of mutations in genes of interest.

The variability displayed by the different sources of germplasm available for tomato could be explored to search for new genes or favourable alleles to be transferred by conventional breeding and/or genetic transformation in selected genotypes to obtain new varieties.

In recent years, genetic resources combined with tomato specific genomic tools (Barone et al., 2009) allowed to successfully achieve various objectives, including the development of new varieties resistant to biotic and abiotic stresses and with improved fruit quality traits and yield. Most of these resources are also publicly available for the scientific community and are accessible *via* web (Table 2).

GENOMIC RESOURCE	NOTES	WEBSITE
Molecular markers	Thousands markers (i.e RFLP, AFLP, SSR, COS, CAPS, SNP)	SGN (http://solgenomics.net)
Molecular maps	10 genetic maps involving crosses among different species and varieties	SGN
Physical map	from *S. lycopersicum*	SGN
Complete genome sequence	released version SL2.40 January 2011	SGN
EST collections	Around 300.000 from various tissues and developmental stages	SOLESTdb (http://biosrv.cab.unina/solestdb) Tomato Gene Index (http://compbio.dfci.harvard.edu/tgi), plantGDB (http://www.plantgdb.org), MiBASE (http://www.kazusa.or.jp/jsol/microtom)
transcriptomic array	TOM1 (approx. 8000 unigenes)	Tomato Functional Genomics database (http://ted.bti.cornell.edu)
	TOM2 (approx. 11.000 independent genes)	TFGD
	Affimetrix (approx. 10.000 genes)	(http://www.affymetrix.com)
	Combimatrix TomatoArray1.0 (more than 20.000 probes)	Functional Genomic Center (http://ddlab.sci.univr.it)
Metabolomic platforms	Metabolites from *S. pennellii* and *S. habrochaites* ILs , metabolomics of tomato fruit from 96 cultivars	TFGD, MoToDB (http://appliedbioinformatics.wur.nl)
TILLING platforms	From *cv.* Red Setter, M82	LycoTill, UTill (http://urgv.evry.inra.fr/UTILLdb), (http://tilling.ucdavis.edu/index.php/TomatoTilling)
SNP array	SolCAP approx. 8000 SNPs from 6 genotypes	SolCAP (http://solcap.msu.edu)
Bioinformatic platforms	Data mining and integration, genome annotation	SGN, TFGD

Table 2. Tomato genomic resources publicly accessible via web

Since the beginning of 1990s, the contribution of molecular markers and maps to tomato breeding and gene identification has been widely documented (Foolad, 2007; Frary et al., 2005; Gupta et al., 2009), and more than 15.000 different markers are collected in the SGN database, where markers can be searched by name, chromosome position and mapping population. Moreover, cytological and cytogenetic maps are also available, as well as a detailed physical map, which was the foundation for the tomato genome sequencing project (Mueller et al., 2005). Contemporarily, gene expression analyses performed on different tissues and developmental stages, as well as on genotypes that differ in their answer to environmental *stimuli*, have dramatically raised the number of ESTs available at various websites. Consequently, several microarray platforms have being designed and are being used for transcriptional profiling, thus contributing to the identification of novel genes (Baxter et al., 2005b; Di Matteo et al., 2010). In addition bioinformatics resources aiming at integrating the forthcoming tomato genome sequence, wide collections of ESTs and data from transcriptomic, proteomic and metabolomic platforms available for tomato will enhance the design and management of genetic transformation approaches, such as those pointing at fruit quality engineering and production of pharmaceutical proteins.

3. Techniques for tomato genetic transformation

Since the 1980s several *Agrobacterium*-mediated transformation protocols have been developed in tomato, using cotyledons or leaves (Pino et al., 2010; Sharma et al.; 2009; Van Eck et al., 2006). Transformation efficiencies obtained in various cultivars range from 10 to 41%. Many factors were believed to be crucial for tomato transformation using *Agrobacterium tumefaciens*, including the application of nurse cells or acetosyringone to the culture or pre-culture media, the type of explants, the *Agrobacterium* strain used and its concentration, co-cultivation period and the concentration of thiamine, 6-benzylamino purine (BAP), zeatin and indole acetic acid (IAA). Also, new transformation procedures have been developed for tomato varieties with low *in vitro* regeneration capacity (Fuentes et al., 2008) and alternative transformation methods, such as floral dip, have been tested (Yasmeen et al., 2009). In addition, novel resources for temporal and tissue-specific manipulation of gene expression in tomato plants are now available for the scientific community. In this regard, it is noteworthy the work from Fernandez et al. (2009) and Estornell et al. (2009) that created new *Solanaceae* genetic toolkit for targeted gene expression and silencing in tomato fruits.

Recently, as the information provided by the tomato genome sequencing become available, the demand for efficient functional genomics tools are increasing. Functional genomics studies of the tomato plant require the use of high-throughput methods for functional analysis of many genes including simple and easily reproducible plant transformation systems. The miniature tomato cultivar MicroTom is a rapid-cycling cherry tomato variety that differs from standard tomato cultivars primarily by two recessive genes that confer the dwarf genotype (Dan et al., 2006). MicroTom shares some traits with the model plant *Arabidopsis thaliana* such as the small size, short life cycle (70-90 days from sowing to fruit-ripening) and small genome (950 Mb) and it is therefore considered a model cultivar for tomato genetics and functional genomics. Several studies investigated the production of improved protocols for *Agrobacterium*-mediated MicroTom transformation obtaining a transformation efficiencies ranging from 20 to 56% (Dan et al., 2006; Qiu et al., 2007; Sun et al., 2006). Recently, Pino et al. (2010) developed an efficient and inexpensive method for

MicroTom transformation using a new tomato genotype harbouring the allele *Rg1* that greatly improves tomato *in vitro* regeneration.

Another breakthrough in the field of tomato genetic transformation was the development of a system for stable genetic transformation of tomato plastids (Ruf et al., 2001). In comparison with conventional nuclear transformation, the integration of transgenes in the plastid genome presents several advantages: 1) high expression levels of recombinant proteins attainable owing to the high ploidy level of the plastid genome (up to 10,000 plastid genomes per cell); 2) efficient transgene integration since integration into the plastid genome relies on homologous recombination between the targeting regions of the transformation vector and the wild-type plastid DNA; 3) absence of epigenetic effects (gene silencing); 4) increased biosafety due to the biological containment of transgenes and recombinant products owing to maternal inheritance of plastid and plastid transgenes and absence of dispersal in the environment through the pollen; 5) possibility to express multiple transgenes from prokaryotic-like operons, thus simplifying engineering metabolic pathways (Bock & Warzecha; Cardi et al., 2010; Ruf et al., 2001; Wurbs et al., 2007).

The availability of a technology for transgene expression from the tomato plastid genome opened up new possibilities for metabolic engineering and the use of plants as bioreactors for the production of pharmaceuticals (Ruf et al., 2001, Wurbs et al., 2007). The group of Ralph Bock investigated the possibility to elevate the pro-vitamin A content of tomatoes using the chloroplast transformation technology (Apel & Bock, 2009; Wurbs et al., 2007). Apel & Bock (2009) introduced the lycopene β-cyclase genes from the eubacterium *Erwinia herbicola* and the plant daffodil (*Narcissus pseudonarcissus*) into the tomato plastid genome in order to enhance carotenoid biosynthesis inducing lycopene-to-provitamin A conversion. The expression of the enzyme from the higher plant daffodil in fruits of transplastomic tomato plants triggered efficient conversion of lycopene to β-carotene and resulted in a >50% increase in total carotenoid accumulation. Zhou et al. (2008) studied the feasibility of producing human immunodeficiency virus (HIV) antigen in transplastomic plant and demonstrated that the HIV antigens p24 and Nef in the plastid could be expressed in plastid of tomato plants.

Today, the technology of stable plant transformation is successful in tomato; however, the lack of an efficient, simple and reliable protocol and the length of time required to produce transgenic lines complicate the analysis of gene function. In alternative, transient assays could provide a rapid tool for the functional analysis of transgenes and have been often used as an alternative to the analysis of stably transformed lines (Wroblewsky et al., 2005). A powerful tool for fast reverse genetics is the virus-induced gene silencing (VIGS) technology (Orzaez & Granell, 2009). Using this method, recombinant virus vectors carrying host-derived sequences are used to infect the plant; systemic spreading of this recombinant virus causes specific degradation of the endogenous gene transcripts by PTGS (post-transcriptional gene silencing) (Dinesh-Kumar et al., 2003; Liu et al., 2002). In 2002, Liu and colleagues demonstrated that a tobacco rattle virus (TRV)-based VIGS vector could be used in tomato to silence genes efficiently. To shorten the time and simplify the functional analysis in fruits, Orzaez et al. (2006) developed a methodology that allowed transient expression of transgenes directly in fruit tissues. However, the identification and quantification of non-visual phenotypes could be hampered by the irregular distribution of fruit VIGS. In a recent paper Orzaez et al. (2009) developed an anthocyanin-guided VIGS in order to overcome the limitations of this technique such as its irregular distribution and efficiency. To develop a visually traceable system the authors developed a method

comprising: 1) a tomato line expressing *Rosea1* and *Delila* transcription factors under the control of the E8 promoter that showed a purple-fruited phenotype and 2) a modified TRV VIGS vector incorporating partial *Rosea1* and *Delila* sequences agro-injected in the transformed lines and that was able to restore the red-fruited phenotype.

4. Biotechnology applications

4.1 Fruit quality engineering

Tomato fruit quality includes several aspects that may be grouped into two categories: organoleptic properties and nutritious contents. Organoleptic quality involves color and texture of the fruit, but also taste and aroma, whereas nutritional quality refers to the content of metabolites contributing to the intake of nutritious such as sugars, carotenoids, flavonoids, ascorbic acid and folate.

Most of the quality traits show a continuous variation, are attributed to the joint action of many genes and are strongly induced by environmental conditions. Beside their complex inheritance, fruit quality traits have often been engineered in tomato through approaches of reverse genetics, such as genetic transformation and mutagenesis, pointing at controlling the expression of single major genes involved in the regulation of a desirable phenotype. In addition, genetic transformation has often been successful in enhancing fruit quality-related traits in tomato investigating simultaneously the role of candidate genes in specific biological processes in the fruit.

In general, there are three main goals of engineering strategies in plants (Verpoorte et al., 2000): the enhancement of a desired trait, the decrease in the expression of a specific unwanted trait, and the development of a novel trait (i.e. a molecule that is produced in nature but not usually in the host plant, or a completely novel compound). Strategies aimed at inducing changes in the expression of a trait changing the synthesis of a specific metabolite are referred to as metabolic engineering. Approaches for achieving the redirection of metabolic fluxes include the engineering of single steps in a pathway to increase or decrease metabolic flux to target compounds, to block competitive pathways or to introduce short cuts that divert metabolic flux in a particular way. However, this strategy has only limited value because the effects of modulating single enzymatic steps are often absorbed by the system in an attempt to restore homeostasis. Recently, strategies aimed at targeting multiple steps in the same pathway are gaining increasing interest because they help to control metabolic flux in a more predictable manner. This might involve up-regulating several consecutive enzymes in a pathway; up-regulating enzymes in one pathway while suppressing those in another competing pathway; or using regulatory genes such as transcription factors (TF) to establish multipoint control over one or more pathways in the cell. Since technical hurdles limits the number of genes that can be transferred to plants and pyramiding of transgenes by crossing transformants for single targets is a highly time-consuming approach, researchers developed new transformation methods to introduce multiple transgenes into plants and express them in a coordinated manner (Navqvi et al., 2009). In addition, controlling the expression of a single TF or a combination of TFs provides attractive tools for overcoming flux bottlenecks involving multiple enzymatic steps, or for deploying pathway genes in specific organs, cell types or even plants where they normally do not express.

A schematic description of successful metabolic engineering for enhancement of fruit quality in tomato is provided in Table 3 and Table 4. Genetic transformation targeting a single TF has been used to successfully engineer tomato for inducing development of parthenocarpic and

seedless fruit. Parthenocarpy enables fruit set and growth to be independent from pollination, fertilization and seed development circumventing the environmental constraints on fruit production and ensuring yield stability. Seedless fruits enhance consumer appeal and could also be a valuable trait for industrial tomatoes because parthenocarpy increases the content of soluble solids, improves yield and flavour of paste and reduces processing costs. Reported applications involved the overexpression of an auxin response factor 8 (*ARF8*) from *Arabidopsis* (Goetz et al., 2007) and downregulation of *Aux/IAA9* transcription factor (Wang et al., 2005) to promote fruit parthenocarpic development.

Modifications of fruit softening and of the overall firmness have been achieved mostly by engineering genes controlling single enzymatic steps in cell wall-associated pathways. In particular, polygalacturonase (Kramer et al., 1992; Langley et al. 1994; Smith et al., 1990), pectin methylesterase (Tieman & Handa, 1994), expansin (Brummell et al., 1999) and β-galactosidase (Smith et al., 2002) genes showed effectiveness in controlling fruit firmness and softening in transgenic tomato plants. A dosage series of the gene *fw2.2,* a negative regulator of cell division (Frary et al., 2000) was generated in tomato by genetic transformation allowing to modulate fruit weight in tomato without affecting cell size in pericarp and placenta tissues (Liu et al., 2003).

Two examples of successful metabolic engineering modifying tomato fruit flavour relayed on heterologous single-gene expression to introduce in tomato untypical traits. In the first example, a biologically active thaumatin, a sweet-tasting, flavour-enhancing protein from the African plant *Thaumatococcus daniellii* Benth was expressed in transgenic tomatoes that produced sweeter fruits with a specific aftertaste (Bartoszewski et al., 2003). In the second example, the lemon basil geraniol synthase (*GES*) gene was overexpressed under the control of the strong fruit-ripening-specific tomato polygalacturonase promoter (*PG*). *GES* encodes the enzyme responsible for the production of geraniol from GDP and its expression caused the plastidial terpenoid biosynthetic flux to divert, leading to a reduced lycopene accumulation and to dramatic changes in the aroma and overall flavour of the transgenic fruits (Davidovich-Rikanati et al., 2007).

In another study, the overexpression of either *LeAADC1A* or *LeAADC2*, encoding for phenylalanine decarboxylases that are involved in the synthesis of 2-phenylethanol from phenylalanine, resulted in fruits with up to 10-fold increased emissions of the products of the pathway, including 2-phenylacetaldehyde, 2-phenylethanol, and 1-nitro-2-phenylethane. On the other hand, antisense reduction of *LeAADC2* significantly reduced emissions of these volatiles (Tieman et al., 2006).

In addition to organoleptic fruit quality, nutritional attributes of tomato fruit have recently received increasing attention by molecular biologists. For instance, the fruit soluble solid content was engineered by using an RNAi approach to generate transgenic plants that were exclusively altered in the expression of a specific isoform of the cell wall invertase *LIN5* (Baxter et al., 2005a; Fridman et al., 2000, 2004; Schauer et al., 2006; Zanor et al., 2009).

Several attempts have been made also to engineer higher carotenoid contents in tomato fruit and a number of tomato lines have been generated with enhanced levels of lycopene, β-carotene and xanthophylls (mainly zeaxanthin and lutein) and low levels of non-endogenous carotenoids such as ketocarotenoids (Fraser et al., 2009). One of the most interesting achievements is the HighCaro (HC) tomato plant (D'Ambrosio et al. 2004), a transgenic line carrying the tomato lycopene β-cyclase (*tLcy-b*) cDNA. Carotenoid biosynthetic pathway is a highly regulated, interconnected, compartmentalized, membrane bound pathway that can be successfully engineered to enhance carotenoids in crop plants

circumventing homeostasis. Carotenoids are biosynthetically related to gibberellins via geranyl-geranyl pyrophosphate and isopentenyl pyrophosphate and this often caused in transgenic plants unpredictable phenotypes. For example, in transgenic lines overexpressing the endogenous gene *Psy-1*, besides an effect on gibberellins formation, the levels of other isoprenoid derived phytohormones were altered in vegetative tissues as well as chlorophyll and tocopherol contents in fruit (Fray et al., 1995). In order to minimize these detrimental effects, engineering approaches to enhance carotenoids in tomato have recently focused on the use of tissue-specific promoters. The use of tomato ripening enhanced promoters allowed a controlled expression at this stage facilitating co-ordination with endogenous carotenoid formation and reducing competition with other branches of the isoprenoid pathway. On the other hand, transcriptional up-regulation of a gene does not always correlate to increased protein or enzyme activity and forward-feed regulation mechanisms could operate within the pathway to maintain homeostasis. For example, in tomato lines expressing a bacterial derived phytoene synthase (*CrtB*) the subsequent desaturation step in the pathway was reduced (Fraser et al., 2002). Moreover, transgenic lines expressing a bacterial desaturase had a reduced phytoene synthase transcription and enzyme activity. In addition, tomato lines overexpressing deoxy-D-xylulose 5-phosphate synthase (*Dxs*) showed elevated phytoene formation in ripe fruit, however desaturation limited progression through the pathway (Enfissi et al., 2005). Finally in tomato lines overexpressing *Psy-1* a lycopene cyclase (*CYC-B*) is induced resulting in increased enzyme activity generating β-carotene as an unintended end-product (Fraser et al., 2007). By contrast, feedback inhibition could also limit accumulation of end-products as was the case of tomato lines expressing the *CrtI* enzyme where the elevated β-carotene levels reduced phytoene synthase (Romer et al., 2000). In contrast with results obtained in rice and potato, multiple step engineering strategies in the carotenoid and isoprenoid precursor pathways in tomato were only partially successful (Diretto et al., 2007). Finally, the simultaneous expression of an Arabidopsis *LCY-B* gene and a pepper *CHY-B* gene resulted in the production of xanthophylls, while the expression of *CrtW* and *CrtZ* from *Paracoccus* spp. leaded to the formation of low fruit levels of ketocarotenoids (Dharmapuri et al., 2002; Ralley et al., 2004). Innovative strategies for carotenoid engineering in tomato fruit consist in alteration of cryptochromes and components of the light signal transduction pathway. These approaches have the advantages of elevating the carotenoid content of the fruit and also other important health related phytochemicals such as phenylpropanoids and flavonoids (Davuluri et al., 2005).

Due to their presumed health benefits, there is growing interest in the development of food crops with tailor-made levels and composition of flavonoids. The repertoire of case studies aimed at increasing the levels of flavonoids in tomato fruit also offers the wider range of examples of successful engineering strategies ever realized. Herein we will list some of the results recently obtained.

The first strategy is related to engineering single structural genes controlling key steps in the pathway, such as a chalcone isomerase (CHI) (Muir et al., 2001) and a chalcone synthase (CHS) (Colliver et al., 2002). More encouraging results were obtained targeting multiple constitutive genes within the flavonoid pathway. For instance, the concomitant ectopic expression of *Petunia CHS, CHI, F3H* (flavanone hydroxylase) and *FLS* (flavonol synthase) in tomato fruit led to increased levels of flavonols in both peel (primarily quercetin glycosides) and flesh (primarily kaempferol glycosides). In another case, the concomitant expression of both *CHS* and *FLS* had a synergistic effect resulting in a significant accumulation of both

naringenin- and kaempferol-glycosides in tomato flesh (Colliver et al., 2002). Secondly, in order to increase the range of flavonoids produced in tomato fruit, a different strategy was taken that consisted in introducing branches to the pathway leading to the synthesis of atypical flavonoids. The overexpression of a grape stylbene synthase (STS) resulted in the accumulation of resveratrol aglycon and its glucoside in tomato fruit peel, while the level of naringenine chalcone was negatively affected because of a competition effect with the main pathway (Schijlen et al., 2006). Similarly, the concomitant overexpression of a petunia CHS and an alfalfa chalcone reductase (CHR) allowed deoxychalcones to accumulate in the tomato peel. When a gerbera *FNS-II* gene and a *Petunia CHI* gene were simultaneous overexpressed in tomato, flavones (mainly as luteolin aglycon) accumulated in their peel (Schijlen et al., 2006). The third strategy involved engineering of transcription factors to enhance a wider range of flavonoid compounds. Besides the increased level of flavonoids induced in tomato fruit by silencing DET1, the expression in tomato of the transcription regulator AtMYB12 activated flavonol biosynthesis as well as the caffeoylquinic acid biosynthetic pathway (Adato et al., 2009). Also, a 60-fold increase in kaempferol glycosides has been achieved in tomato flesh tissue by simultaneous ectopic expression of the two maize transcription factors Lc and C1 (Bovy et al., 2002). Most surprisingly, the expression of the Delila (*Del*) and Rosea1 (*Ros1*) genes, two transcription factors from the snapdragon *Antirrhinum majus*, in the fruit of transgenic tomatoes induced the accumulation of high levels of anthocyanins in tomato (Butelli et al., 2008) through the activation of a broad range of flavonoid biosynthetic pathway related genes.

In contrast with flavonoid metabolism, so far a reduced number of efforts have been placed into genetic transformation-mediated metabolic engineering of tomato fruit for enhanced ascorbic acid levels. Only few of them succeeded in effectively affect ascorbic acid content and only for a limited number of structural genes within the ascorbic acid pathway. In a fruit systems biological approach, transgenic tomato lines silenced for a mitochondrial ascorbic acid synthesizing enzyme L-galactono-1,4-lactone dehydrogenase performed an increased fruit ascorbic acid level (Garcia et al., 2009) whereas the silencing of an GDP-D-mannose-3′,5′-epimerase resulted in a reduced fruit ascorbic acid accumulation (Gilbert et al., 2009). On the other hand, overexpression of GDP-D-mannose-3′,5′-epimerase genes resulted in enhances ascorbic acid accumulation in tomato fruit (Zhang et al., 2011).

Similarly to ascorbic acid, the opportunity of engineering folate accumulation in tomato fruit has been mostly overlooked and only a few attempts gave rise to successful outcomes. In order to increase pteridines, which act as folate precursors and are synthesized from *p*-aminobenzoate, a GTP cyclohydrolase I was overexpressed and a 2-fold increase in folate level in tomato fruit was gained (de la Garza et al., 2004). A higher folate accumulation (up to 25-fold increase) was achieved in tomato fruit by combining in the same plant the overexpression of an aminodeoxychorismate synthase, the *p*-aminobenzoate-forming enzyme, and the GTP cyclohydrolase I (de la Garza et al., 2007).

Comprehensively, within genetic engineering strategies for crop improvements, the most striking advances so far have involved plants engineered to produce missing nutrients or increase the level of nutrients that are already synthesized. An important trend is to move away from plants engineered to produce single nutritional compounds towards those simultaneously engineered to produce multiple nutrients, a development made possible by the increasing use of multigene engineering or regulative genetic element with pleiotropic effects.

Trait	Engineering strategy	Inserted target	Fruit phenotype	Reference
Partheno-carpy	single TF	*Arf8* *IAA9*	induced parthenocarpy	Goetz et al., 2007; Wang et al., 2005
Firmness	single biosynthetic key gene	*PG*	reduced softening	Langley et al. 1994
		PME	reduced shelf-life	Tieman and Handa, 1994
		EXP1A	reduced firmness	Brummell et al., 1999
		β-galactosidase	increased firmness	Smith et al., 2002
Size	dosage series of a single gene	*fw2.2*	increased size	Frary et al., 2000
Flavour	heterologous single gene	thaumatin	enhanced flavour	Bartoszewski et al., 2003
Flavour and aroma	heterologous single gene for diverting biosynthetic flux	*GES*	changes in flavor & aroma	Davidovich-Rikanati et al., 2007
	single biosynthetic key gene	*LeAADC1A*, *LeAADC2*	Increased/ decreased 2-phenylacetaldehyd, 2-phenylethanol, and 1-nitro-2-phenylethane	Tieman et al., 2006

Table 3. Examples of successful fruit engineering for organoleptic quality trait in tomato. Abbreviations: TF, transcription factor; SG, silencing; SI, serial increase of gene dosage

Trait	Engineering strategy	Inserted target	Fruit phenotype	Reference
Soluble solids content	single biosynthetic key gene	*Lin5*	reduced sugars accumulation	Zanor et al., 2009
Carotenoid content	single biosynthetic key gene	*Dxs*	increased phytoene & carotenoids	Enfissi et al., 2005
		CrtB, *CrtI*, *CrtY*	increased carotenoids	Fraser et al., 2002, 2007; Wurbs et al., 2007
		PSY-1	increased carotenoids	Römer et al., 2000
		CYC-B, *LCY-B*	increased lycopene & β-carotene	Rosati et al., 2000 D'Ambrosio et al., 2004; Ronen et al., 2000
	genes targeting biosynthetic steps	*LCY-B*, *CHY-B*	β-cryptoxanthin & zeaxanthin	Dharmapuri et al., 2002
	single regulative gene	*CRY-2*	increased carotenoid	Giliberto et al., 2005
		DET-1, *COP1LIKE*, *CUL4*	increased carotenoid and flavonoid	Liu et al., 2004; Wang et al., 2008; Davuluri et al., 2005
		FIBRILLIN	increased carotenoids and volatiles	Smikin et al., 2007
	single biosynthetic key gene	*spermidine synthase*	increased lycopene	**Neily et al., 2011**
Flavonoid content	single biosynthetic key gene	*CHI*	increased fruit peel flavonol	Muir et al., 2001
	genes targeting biosynthetic steps	*CHS*, *CHI*, *F3H*, *FLS*	increased flavonols	Colliver et al., 2002
	heterologous gene/genes for diverting flux	*STS*, *CHS*, *CHR*, *FNS-II*, *CHI*	accumulation of resveratrol, deoxychalcones, & flavones	Schijlen et al., 2006
	single TF	*MYB12*	accumulation of flavonols	Adato et al., 2009
	Multiple TFs	*Del*, *Ros1*	high levels of anthocyanins	Butelli et al., 2008

Trait	Engineering strategy	Inserted target	Fruit phenotype	Reference
Ascorbic acid content	single biosynthetic key gene	*GalLDH*, *GME*	increased/ decreased fruit ascorbic acid	Garcia et al., 2009; Gilbert et al., 2011; Zhang et al., 2011
	genes targeting consecutive biosynthetic steps	*GCHI* and/or *ADCS*	increased fruit folate	Diaz de la Garza et al., 2004; 2007

Table 4. Examples of successful fruit engineering for nutritional quality traits in tomato. Abbreviations: TF, transcription factor; SG, silencing; IPP, isopenthenilpyrophosphate

4.2 Production of pharmaceutical proteins

Genetically modified plants are currently being evaluated as promising alternative for the production of recombinant proteins and antigens. Major advantages of plant-made pharmaceuticals include low cost of production, higher scale-up capacity and lack of risk of contamination with mammalian pathogens. Several antigenic proteins have been produced in plant, examples are plant-made vaccines against smallpox, HIV and HPV (Human Papilloma Virus) (Lenzi et al., 2008; Rigano et al., 2009; Scotti et al., 2009). In addition, transgenic plants can represent a suitable vehicle for oral delivery of pharmaceuticals since the plant cell wall protects the recombinant antigen in the harsh condition of stomach and intestine (Sharma et al., 2008a). The delivery of vaccines to mucosal surface makes immunization practise safe and acceptable and is capable of inducing both humoral and cell-mediated immune responses (Salyaev et al., 2010). The production of plant-made mucosal vaccines eliminates needle-associated risks and downstream processing of traditional vaccines such as purification, sterilization and refrigeration. Recently, in addition to other systems, tomato plants have been used as vehicles for the expression and oral delivery of vaccines since tomato is edible, generates abundant biomass at low cost, has flexible growth conditions and contains the natural adjuvant α-tomatine (Salyaev et al., 2010; Soria-Guerra et al., 2011). In this regards, it is noteworthy the work from Zhang and colleagues (2006) that expressed the recombinant Norwalk virus capsid protein in tomato and potato and demonstrated that, although in mice oral immunization with both dried tomato fruit and potato tuber elicited systemic and mucosal antibody responses, the recombinant vaccine in transgenic tomato fruit, especially in air-dried material, was a more potent oral immunogen than potato. The authors speculated that the robust immunogenicity of tomato-derived vaccines was due to natural bioencapsulation by the plant cell matrix and membrane systems, larger amount of smaller 23 nm Virus-like particles and the presence of the natural adjuvant α-tomatine. In this paragraph, we will describe several examples of pharmaceuticals produced in tomato plants focusing on the most recently reported studies.

Several studies reported the production of transgenic tomato plants for the expression of viral antigens. In 2008, Perea Arango and colleagues reported high-level expression of the entire coding region of the nucleoprotein (N) gene of rabies virus in transgenic tomato plants. When mice were immunized both intraperitoneally (i.p.) and orally with the tomato-made N protein, the antibody titer of mice immunized i.p. was at least four times higher

than that of mice immunized orally. In addition, only mice immunized i.p. were partially protected against a peripheral virus challenge. In the same year, Pan et al. (2008) described the production of genetically modified tomato plants that expressed the structural polyprotein, P1-2A, and protease, 3C, from foot-and-mouth disease virus (FMDV). Guinea pigs vaccinated intramuscularly with foliar extracts from the transgenic material developed a virus-specific antibody response and were protected against a challenge infection. Recently, in order to develop a vaccine against HPV Paz De la Rosa et al. (2009) expressed in tomato plants chimeric particles containing the HPV 16 L1 sequence fused to a string of T-cell epitopes from HPV 16 E6 and E7 proteins. L1 fused to the string of epitopes was able to assemble into chimeric VLPs (Virus-like particles); in addition, intraperitoneal administration in mice of the transgenic material was able to induce both neutralizing antibodies against the viral particle and a cytotoxic T-lymphocytes activity against the epitopes. Up to date, several groups investigated the production of a mucosal vaccine against HIV and HBV (Hepatitis B virus) in genetically modified tomato plants (Lou et al., 2007; Salyaev et al., 2010; Zhou et al., 2008). For instance, Shchelkunov et al. (2006) investigated the production of transgenic plants expressing a synthetic chimeric gene, *TBI-HBS*, encoding the immunogenic ENV and GAG epitopes of HIV-1 and the surface protein antigen (HBsAg) of HBV and investigated the immunogenicity of the transgenic material fed to experimental mice. Peña Ramirez and colleagues (2007) investigated the possibility of expressing the HIV-1 Tat protein in fruits of tomato plants. In mice, oral feeding with the tomato-based vaccine was able to raise mucosal IgAs and induce serum IgGs with neutralizing activity. More recently, Cueno at al. (2010) expressed the HIV-1 protein Tat in tomato plants reaching up to 4 µg of recombinant protein per milligram plant protein. In addition, tomato extracts intradermally inoculated into mice were found to induce both humoral and cellular immune responses.

Bacterial antigens have also been expressed in transgenic tomato plants. Alvarez and colleagues (2006) expressed in transgenic tomato plants the FI-V antigen fusion protein for the production of a vaccine against pneumonic and bubonic plague. The authors tested the immunogenicity of the tomato-made vaccine in mice which were primed subcutaneously with bacterially produced F1-V and boosted orally with freeze-dried, powdered transgenic tomato fruit and demonstrated that the vaccine elicited IgG1 in serum and mucosal IgA in fecal pellets. In 2007, Soria-Guerra and collegues expressed in tomato a plant-optimized synthetic gene encoding the recombinant polypeptide sDTP (diphtheria-pertussis-tetanus), containing six DTP immunoprotective exotoxin epitopes and two adjuvants in order to develop an edible multicomponent DPT vaccine. Recently, the same group examined whether immunization of mice fed with freeze-dried tomato material elicited specific antibody responses. Sera of immunized mice tested for IgG antibody response to pertussis, tetanus and diphtheria toxin showed responses to the foreign antigens; in addition, high response of IgA against tetanus toxin was evident in gut (Soria-Guerra et al., 2011). In addition, several studies investigated the feasibility of production of a safe, inexpensive plant-based mucosal vaccine against cholera. For instance, Jang et al. (2007) expressed the Cholera toxin B subunit (CTB) in transgenic tomato fruits and demonstrated the immunogenicity of the tomato-made vaccine in mice. In alternative, Sharma and colleagues (2008b) produced the toxin co-regulated pilus subunit A (TCPA) of *Vibrio cholerae* and its immunogenic epitopes P4 or P6 fused to cholera toxin B subunit (CTB) in tomato plants. In the same year, the same research group reported the production of genetically modified

tomato plants for the expression of accessory colonization factor subunit A (ACFA) of *Vibrio cholerae* and ACFA fused to CTB (Sharma et al., 2008a).

Another advantage of using transgenic plants for the production of recombinant protein of biopharmaceutical and industrial importance is that plant cells are able to perform complex post-translational modification, including glycosylation (Agarwal et al., 2008). In this regard, the feasibility of expression of glycosylated and biologically active recombinant human α-1-antitrypsin (AAT) protein in transgenic tomato plants was demonstrated. In this study, in order to achieve high-level expression of recombinant protein in transgenic plant cells, the gene encoding human AAT protein was optimized by codon adjustment and elimination of mRNA destabilizing sequences. In addition, the synthetic gene was expressed with different signal sequences, translation initiation context sequence, Alfalfa mosaic virus UTR (untranslated region) at 5′ end and ER (endoplasmic reticulum) retention signal sequence (KDEL) at 3′ end. The modified gene driven by CaMV35S duplicated enhancer promoter resulted in high-level expression (up to 1.55% of TSP) of recombinant protein in transgenic tomato plants. Elias-Lopez et al. (2008) described the production of transgenic tomato plants expressing interleukin-12. BALB/c mice were infected with either *Mycobacterium tuberculosis* H37Rv strain or multi-drug-resistant clinical isolate (MDR) and treated with a daily oral dose of transgenic fruit extracts. Oral administration of the transgenic plant material improved protective immunity and induced higher resistance to mycobacterial infection, when administered the day before infection or during late progressive disease induced by virulent mycobacteria. Other therapeutic proteins produced in transgenic tomato plants include the analgesic-antitumor peptide (AGAP) from the venom of *Buthus martensii* Karsch (Lai et al., 2009) and human beta-amyloid for the production of a vaccine against Alzheimer`s disease (Youm et al., 2008).

Another alternative for the production of recombinant antigens in plant cells is transgene expression from the plastid genome. Chloroplast transformation offers a number of advantages, including the potential to accumulate enormous amounts of recombinant protein, uniform transgene expression rates, no gene silencing and transgene containment. Recently, Zhou et al. (2008) expressed HIV antigens p24 and Nef from tomato`s plastid genome. In tomato, antigen accumulation reached values of approximately 40% of total leaf protein. When the authors determined p24-Nef accumulation in fruits they found that although green tomatoes accumulated the HIV antigens to approximately 2.5% of the TSP, there was no expression in ripe fruits. The authors speculated that this was due to the presence in red-fruited tomatoes of chromoplasts that, compared to chloroplasts, are usually less active in plastid gene expression.

Up to date, several studies demonstrated the feasibility of using tomato plants as vehicles for the production of pharmaceuticals. One drawback of a tomato-made vaccine could be the short shelf-life of fresh fruits. To provide antigen stability during storage, food-processing techniques, such as freeze-drying, could be applied to transgenic tomato fruits expressing recombinant proteins. Freeze-dried plant material could be stored for long time and consumed without cooking; in addition, this technique could allow to standardize and concentrate the plant-made vaccine. Several studies applied this technique to vaccine produced in transgenic tomato and demonstrated that freeze-dried produced stable formulations for oral delivery (Alvarez et al., 2006; Salyaev et al., 2010; Shchelkunov et al., 2006; Soria-Guerra et al., 2011; Zhang et al., 2006).

5. Conclusion

In the present review, we underlined the role of genetic transformation as method to improve fruit quality and pharmaceutical production. In addition, we highlighted the double role of genetic transformation as tool for biotechnology applications and functional analyses of genes of interest (Figure 1). For tomato, these approaches are feasible following strategies of gene/QTL identification based on the use of genetic and genomic resources today available for this species.

Nowadays, European politicians often debate about perceived risks of genetically modified crops, while ignoring potential benefits; therefore, it is highly unlikely that engineered crops will be adopted in the short-to-medium term.

Considering these constraints, mutants could be envisaged as valid alternative to engineer tomato plants for enhanced fruit quality (Figure 1). Mutants could be selected from natural variation or generated using different approaches. In addition, if the mutant exhibits superior alleles, it could be used as improved genotypes or as donor parent in backcrossing breeding schemes to deliver the desirable trait. The isogenic mutant resources available today for tomato are useful for dissecting the mechanisms underlying mutant phenotypes, and such mutagenized populations are also being used to develop targeting induced local lesions in genomes (TILLING) platforms, which represent a high-throughput genetic strategy to screen for point mutations in specific regions of targeted genes, and to validate gene function (McCallum et al., 2000).

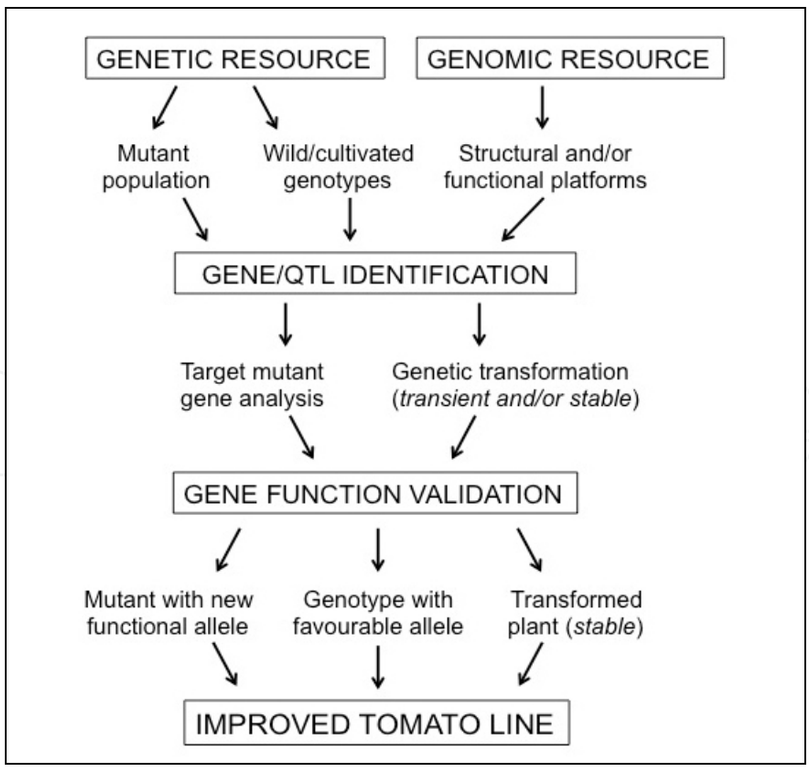

Fig. 1. Flow-Chart of steps from the screening of genetic and genomic resources to improved tomato lines

Another alternative approach to obtain tomato with desirable traits is to discover gene markers that discriminate contrasting alleles in genes or QTLs that control the trait(s) of interest (Figure 1). Following their identification, useful genes or QTLs can be introgressed into desirable genetic backgrounds via Marker Assisted Selection (MAS), where the selection for a trait is based on the genotype rather that the trait itself (Foolad, 2007). The knowledge of the tomato genome sequence dramatically enhances identification of novel molecular markers. Indeed we can envisage that, notwithstanding the implementation of recently developed Next Generation Sequencing technologies, the routine application of markers in tomato breeding will increase (Varshney et al., 2009).

In conclusion, the use of different approaches, such as tomato genetic transformation, exploiting of mutants and identification of allele-specific markers, could not only speed up the process of gene transfer, but it could also allow pyramiding of desirable genes and QTLs from different genetic backgrounds. The rapid integration of new alleles in elite tomato lines will allow new cultivars with desirable traits to enter the market in a shorter time compared to cultivar obtained through traditional breeding.

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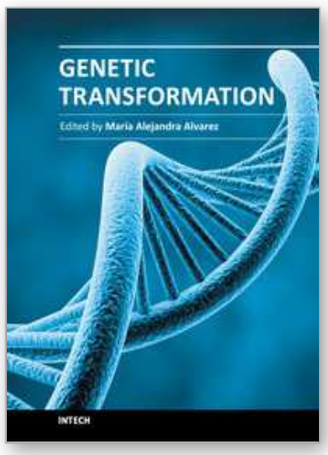

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Genetic transformation of plants has revolutionized both basic and applied plant research. Plant molecular biology and physiology benefit from this power fool, as well as biotechnology. This book is a review of some of the most significant achievements that plant transformation has brought to the fields of Agrobacterium biology, crop improvement and, flower, fruit and tree amelioration. Also, it examines their impact on molecular farming, phytoremediation and RNAi tools.

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