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MicroTom – A New Model System for Plant Genomics

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ABSTRACT

The application of genetic modifications to economically important plant species primarily includes tolerance to broad-spectrum herbicides such as glufosinate or glyophosate, resistance to pest and diseases, high yield, biofuel production and better quality food products for consumption. Plant model systems have played an important role in understanding plant biology that leads to economic trait development in crops. The most highly developed plant model system is *Arabidopsis* because of its small size, rapid life cycle, small genome, and transformability. MicroTom is a miniature dwarf determinate tomato cultivar, originally bred for home gardening purposes, which shares the major features with *Arabidopsis* that make it successful as a model system, including small size (up to 1357 plants/m^2), short life cycle (70-90 days from sowing to fruit-ripening), relatively small genome (950 Mb) and transformability. In this article we review the current status of the genetic transformation, genome sequencing, functional genomics, reverse genetic tools and improvement of fruit nutrition and flavor quality in MicroTom/tomato, which provides a vision of a new plant model system for functional genomics and its application to economic trait development for crops.

Keywords: functional genomics, high-throughput, metabolite database, reverse genetics, transformation

CONTENTS

INTRODUCTION

Tomato (*Solanum lycopersicum*) is a member of the family Solanaceae that includes more than 3000 species and is the third most economically important plant taxa, exceeded only by the grasses and legumes. The family contains several important crop species such as tomato, potato, pepper, and eggplant as well as ornamental plants such as petunia and Nicotiana. Tomato is a major vegetable crop throughout the world, with an annual production of approximately 108 million metric tons in 2002 (FAS/USDA 2003). Due to its importance as a human food source and its biological features like relatively small genome and transformability, tomato has long served as a model system for plant genetics,

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Invited Review

Fig. 1 A MicroTom plant in a pot.

development, pathology, and physiology, resulting in the accumulation of substantial information regarding its biology.

Plant model systems have played an important role in understanding plant biology. *Arabidopsis* is the most successful plant model system because of its small size, rapid life cycle, small genome, and transformability (Meyerowitz and Sommerville 1994; Meinke *et al*. 1998). MicroTom is a miniature dwarf determinate tomato cultivar, originally bred for home gardening purposes (Scott and Harbaugh 1989) (**Fig. 1**). It differs from standard tomato cultivars primarily by two recessive genes, which confer the dwarf phenotype (Meissner *et al*. 1997). MicroTom shares some features with *Arabidopsis* that make it successful as a model system, such as small size (up to 1357 plants/m^{-2}), short life cycle (70-90 days from sowing to fruit-ripening), and small genome (950 Mb). In addition MicroTom possesses some unique and useful features. Tomato is susceptible to a wide range of pathogens (Webb *et al*. 1973). For example, MicroTom is susceptible to *Phytophthora infestans* (Rommens 2000; Dan *et al*. 2001), but the susceptibility to *P. infestans* has not been reported in *Arabidopsis*. Therefore MicroTom has unique potential as a susceptible model system to study certain types of plant disease. MicroTom responds to glyphosate, an herbicide, in a similar manner as cotton, particularly with respect to reproductive tolerance, and therefore has been used as a model plant to study glyphosate tolerance (Sidorova *et al*. 2001). Because of reasonable size of MicroTom's fruits compared with the very tiny size of *Arabidopsis* seeds, MicroTom has an advantage to be a model system to study the yield trait. MicroTom has been used as a model system to dissect tomato molecular genetics through techniques such as mutagenesis, and gene tagging, trapping and knockouts (Meissner *et al*. 1997; Mathews *et al*. 2003). Recently, tremendous progresses in genetic transformation, genome sequencing, functional genomics, reverse genetic tools and improvement of fruit nutrition and flavor quality in MicroTom/tomato further provide a great value for using MicroTom as a new plant model system for functional genomics and economic trait development in crops.

TOMATO GENETIC TRANSFORMATION

Transformation of regular tomato genotypes

Most of tomato transformation methods use *Agrobacterium tumeficiens*. Since the first report of tomato transformation (McCormick *et al*. 1986), there have been numerous publications on the progress of tomato *Agrobacterium*-mediated transformation (Koornneef *et al*. 1986; Fillatti *et al*. 1987; Hinchee *et al*. 1994; Vidya *et al*. 2000; Hu and Phillips 2001; Krasnyanski *et al*. 2001; Park *et al*. 2003). To date, the majority of transformation frequencies ranging from 0.72% to 37% for regular tomato genotypes have been reported (Hamza and Chupeau 1993; van Roekel *et al*. 1993; Frary and Earle 1996; Ling *et al*. 1998; Jiang *et al*. 1999; Vidya *et al*. 2000; Hu and Phillips 2001; Park *et al*. 2003; Shahriari *et al*. 2006).

Different factors were found to play important roles in tomato transformation. They were *Agrobacterium* cell density (Murray *et al*. 1998; Velcheva *et al*. 2005), regeneration parameters including explant types and plant growth regulators, and co-cultivation conditions (Chyi and Phillips 1987; Frary and Earle 1996; Murray *et al*. 1998; Costa *et al*. 2000; Drori and Altman 2001; Hu and Phillips 2001), acetosyringone and cell competence after wounding (Davis *et al.* 1991; Murray *et al*. 1998), selective agents and gene constructs (Krasnyanski *et al*. 2001). Cotyledons have been the common explant source in tomato transformation (Shahin *et al*. 1986; Fillati *et al*. 1987; Hamza and Chupeau 1993; van Roekel *et al*. 1993; Ultzen *et al*. 1995; Frary and Earle 1996; Ling *et al*. 1998; Hu and Phillips 2001; Ellul *et al*. 2003; Park *et al*. 2003), but transgenic plants were also generated from stem segments (Chyi and Phillips 1987), hypocotyls (Frary and Earle 1996; Park *et al*. 2003) and leaves (Koornneef *et al*. 1986; McCormick *et al*. 1986; Jacobs and Yoder 1989; Park *et al*. 2003). Different concentrations of BAP, IAA, NAA and zeatin were used for tomato direct shoot regeneration in the studies described above. The neomycin phosphotransferase II (*nptII*) gene, which confers resistance to kanamycin, was mostly used for selection of stable transformants for tomato transformation. However one alternative approach utilized mannose as a selective agent (Sigareva *et al*. 2004) because many plants cannot utilize mannose as a carbohydrate source, and such cells will not grow when cultured on mannose containing media. However, when cells are transformed with the phosphomannose isomerase (PMI) gene, they are able to survive by utilizing mannose as a carbohydrate. Average transformation frequencies ranging from 2.0 to 15.5% for three tomato varieties were obtained when using 1% mannose in combination with 0.1-0.5% glucose depending on the construct, genotype and type of tissue used for transformation. Carbenicillin and cefotaxime have been commonly used as effective antibiotics for elimination or suppression of *Agrobacterium* cells for tomato transformation described above. They have a broad spectrum of activities against bacteria, but a low toxicity to eukaryotes. However both carbenicillin and cefotaxime reduced callus growth, shoot regeneration and transformation efficiency when using cefotaxime at 500 mg/ l and carbenicillin at 250-1000 mg/l (Lin *et al*. 1995; Vergauwe *et al*. 1996; Nauerby *et al*. 1997). Therefore, Timentin was used as an alternative for growth suppression of *Agrobacterium* cells for tomato (Vergauwe *et al*. 1996; Cheng *et al*. 1998; Ling *et al*. 1998). Timentin is a semisynthetic antibiotic widely used in human and animal clinics to inhibit gram-positive, gram-negative, aerobic and anaerobic bacteria. Timentin is a mixture of ticarcillin and clavulanic acid. Another alternative antibiotic for suppressing *Agrobacterium* cells during tomato transformation is Augmentin® (Ieamkhang and Chatchawankanphanich 2005). The efficiency of Augmentin compared with Timentin to suppress the growth of *Agrobacterium* was at concentrations of 300 and 100 mg/l, respectively. In addition, the high concentration up to 500 mg /1 of both antibiotics showed no significant toxicity to shoot regeneration. The overgrowth of *Agrobacterium* was observed on tomato shoots regenerated on medium containing cefotaxime even at high concentration up to 500 mg/1. Moreover, shoot regeneration from medium containing cefotaxime was lower than the one from medium containing Timentin and Augmentin. Augmentin at the concentration of 300 mg/l with fresh preparation prior to use was recommended for tomato transformation (Ieamkhang and Chatchawankanphanich 2005).

Transformation of Micro-Tomato genotypes

Agrobacterium-mediated transformation method for Micro Tom was first reported by Meissner *et al*. (1997), but no details on the transformation experiments were described although up to 80% of transformation frequencies were reported. Mathews *et al*. (2003) reported an *Agrobacterium*-mediated MicroTom transformation method with transformation rates of 40 to 60% using leaves, stems, hypocotyls and shoot tips as starting explants followed by kanamycin selection. However there were no details on the transformation protocol development.

A new tomato genotype (Micro-MsK), which possess high regeneration capacity, and small size and short life cycle, has been developed by crossing MicroTom with MsK genotype (Lima *et al*. 2004). MsK genotype derived from *Lycopersicon peruvianum* (Koornneef *et al*. 1986) has high regeneration capacity, which was attributed to two loci, named *Rg-1* and *Rg-2* (Koornneef *et al*. 1987). Apparently, regeneration from root explants and callus is controlled by the same loci, although the behavior of some MsK lines suggested that one dominant allele at one locus can be sufficient for shoot regeneration from root explants (Koornneef *et al*. 1993). Stable and non-segregating F6 plants of this genotype have higher regeneration ability than MicroTom. Regeneration capacities of cotyledon and hypocotyl explants were tested for Micro-MsK and MicroTom. No significant differences were found regarding the percentage of regenerating explants when comparing cotyledon explants from the two genotypes 15 days after culturing. However, the percentage of regenerating Micro-MsK hypocotyls explants was significantly ($P < 0.001$) higher than those from MicroTom. About 73% of the hypocotyls from Micro-MsK regenerated into shoots in comparison to 12% of the Micro Tom under the same conditions. When the number of shoots formed per explant was compared, Micro-MsK had better response for both types of explants. The regeneration capacity of Micro-MsK was four times (300%) higher than that of MicroTom. The observation that Micro-MsK genotype is superior to Micro-Tom in terms of regeneration capacity when the explants are hypocotyls but not cotyledons, may provide a hypothesis in order to understand the physiological function of the *Rg-1* locus. The presence of *Rg-1* allele seems to be sufficient for high regeneration from hypocotyl and root explants but not from cotyledons or callus (Koornneef *et al*. 1993). Since hypocotyl and root explants have pre-existing meristematic cells, while cotyledons and callus don't, the *Rg-1* allele appears to act in meristems, but it may not be sufficient to enhance the induction of new meristems. The observation that the shoot formation in MsK roots is in the pericycle (Peres *et al*. 2001), a meristematic tissue, supports this hypothesis. However, since Micro-MsK is not near-isogenic to MicroTom, these assumptions need to be further tested. Previous study by Peres's group showed that the presence of *Rg-2* locus was necessary for high regeneration from cotyledon explants (Lima *et al*. 2004). Therefore, the Micro-MsK probably lacks the *Rg-2* locus. Micro-MsK will be useful in understanding molecular mechanisms for plant regeneration pathway via tissue culture and as well as exploring functional genomics in tomato by insertional mutagenesis.

A high-throughput *Agrobacterium*-mediated transformation model system, which used both *nptII* (*neomycin phosphotransferase* II) and *cp4* (the 5-enolpyruvylshikimate-3-phosphate synthase) genes, has been developed in MicroTom (Dan *et al*. 2006). With the NPTII selection system, transformation frequency calculated as independent transgenic events per inoculated explant ranged from 24 to 80% with an average of 56%, in industrial production scale transformation experiments. For CP4, with glyphosate selection, the average transformation frequency was 57%. Stable transformation frequency was positively correlated with transient expression (R=0.85), and variable with the genes of interest. DNA integration and germline transformation were confirmed by biological assay, Southern blot analysis and R_1 phenotype segregation. Transgene expression was observed in leaf, root, stem, flower and fruit tissues of the transgenic plants. Ninety-five percent of transgenic events co-expressed two introduced genes based on *β-glucuronidase* (GUS) and NPTII expression. Seventy-five percent of transgenic events contained 1 to 2 copies of the introduced *uidA* (*GUS*) gene based on Southern analysis. Transgenic plants from the cotyledon explants to the transgenic plants transferred to soil were produced within about two to three months depending on the genes of interest. This MicroTom transformation system possesses five elements of a high throughput transformation system: 1) large scale production capability with an average transformation frequency of 56% for both kanamycin and glyphosate selections, 2) rapid production cycle, which took 2 to 3 months from cotyledon explants to transgenic plants which were ready to soil depending on the gene constructs, 3) minimal production complexity, which had a simple protocol with three single steps (shoot induction usually without callus phase, shoot elongation and rooting) and no sub-culture transfer to fresh medium in each step, 4) inexpensive DNA delivery-method, which was *Agrobacterium*-mediated transformation and 5) high quality of transgenic plants with high co-expression and low copy number (Dan *et al*. 2006).

Sun *et al*. (2006) reported a highly efficient *Agrobacterium*-mediated transformation protocol for MicroTom. Cotyledon explants from 7- to 10-day-old seedlings were inoculated with *A. tumefaciens* C58C1RifR harboring pIG121-Hm. The explants were placed on co-cultivation medium with the abaxial side down, and incubated in the dark for 3-4 d. The explants were then sub-cultured in callus induction medium containing 100 mg/l kanamycin, 1.5 mg/l zeatin and 375 mg/l Augmentin. Transgenic callus initiation was observed 2-3 weeks after inoculation. After 3-4 weeks, calli with small shoot buds were induced from the cut end of the explants. The explants generated a mass of chimeric non-transgenic and transgenic adventitious buds. Repeated shoot elongation from the mass of adventitious buds on selection media resulted in the production of multiple transgenic plants that originated from independent transformation events. The transformation efficiency was 48.8%.

A transient transformation method using MicroTom, which was named fruit agroinjection, has been developed by Orzaez *et al*. (2006). MicroTom fruits at mature green stage (22-25 days after anthesis) were agroinjected with *Agrobacterium* strains LBA4404 harboring a plasmid pBIN-YFP/GUS containing yellow fluorescence protein (YFP) and GUS genes directed by the 35S promoter using a 1-ml syringe. Needle was introduced 3 to 4 mm in depth into the fruit tissue through the stylar apex, and the infiltration solution was gently injected into the fruit. High levels of glucuronidase activity were detected in agroinjected fruits 4 days after agroinfiltration. GUS activity decreased thereafter until ripening. At ripen stages (9 days post injection), measurement of GUS activity using standard techniques was unreliable probably due to endogenous activity. Under UV light, high levels of yellow fluorescence were clearly visible around the placenta tissue of 4 days post injection fruits. Confocal microscopy confirmed nucleocytoplasmic localization of plant expressed YFP. The fruit agroinjection method was applied to three case studies, including 1) the heat shock regulation of an *Arabidopsis thaliana* promoter, 2) the production of recombinant IgA antibodies as an example of molecular farming, and 3) the virus-induced gene silencing of the carotene biosynthesis pathway. The studies showed that this technology was efficient as a tool for fast transgene expression in tomato fruits.

Tomato plastid transformation

Chloroplast transformation provides unique advantages in plant biotechnology, including high level protein expression of target genes, absence of epigenetic effects, and no transgene transmission through pollens. However, a wide application of plastid genome engineering in biotechnology

has been largely restricted by both the lack of chloroplast transformation systems for major crops and the low plastid gene expression in non green tissues such as fruits, tubers, and other storage organs. Ruf *et al*. (2001) have for the first time developed a plastid transformation system for tomato. Young sterile leaves from 3 to 4 week old tomato plants were bombarded with plasmid DNA-coated gold particles of 0.6 μm diameter using the DuPont PDS1000He biolistic gun and 1,100 p.s.i. rupture disks. The plasmid DNA contained a chimeric *aadA* gene driven by the rRNA operon promoter. Bombarded leaf samples were transferred to RMOP medium containing spectinomycin (300-500 mg/l), and incubated under dim light (25 μE; 16 h light, 8 h dark) for three to four months. Primary spectinomycin-resistant lines were identified as yellow or pale green growing calli. For plant regeneration, homoplasmic callus tissue was transferred onto the surface of agar-solidified MS medium containing 0.2 mg/l IAA and 3 mg/l BAP. Alternatively, shoot induction was obtained with the same medium, but 2 mg/l zeatin to replace BAP. It took approximately two years to produce the first ripe transplastomic tomatoes from the biolistic bombardment of the leaves. There were four important parameters that were required for the successful transformation of tomato chloroplasts, including 1) the use of extreme low-light conditions (25 μE) during the entire selection phase, 2) the extension of the primary selection phase to three or four months compared with three to five weeks in tobacco, 3) the small size (optimal size 3 × 3 mm) of the leaf pieces exposed to the tissue culture medium during primary selection as compared to tobacco plastid transformation and 4) the optimization of the selection and plant regeneration scheme. This study shows that chromoplasts in the tomato fruit express the transgene to ~50% of the expression levels in leaf chloroplasts. Since the generally very high foreign protein accumulation in transgenic chloroplasts (>40% of the total soluble protein), this system provide a great potential to efficiently produce edible vaccines, pharmaceuticals, and antibodies in tomato.

TOMATO GENOME SEQUENCING

Currently genome sequencing of three plant species, Arabidopsis (Arabidopsis Genome Initiative 2000), rice (International Rice Genome Sequencing Project, 2005) and poplar (Tuskan 2006), has been completed or nearly completed, while genome sequencing of several other plant species, including maize, *Lotus japonicus*, and *Medicago truncatula*, is under way. Comparing to the above sequenced or to be sequenced species, which are from rosid and monocot clade on the family tree of flowering plants, Solanaceae represent a distinct and phylogenetically distant set of clades, euasterid I and euasterid II (Soltis *et al*. 1999). Sequencing of Solanaceae genomes will provide an excellent opportunity for comparative genomics to unveil distinct and common aspects of plant evolution.

The Solanaceae family is unique in that there have been no large-scale duplication events (e.g. polyploidy) early in the radiation of this family as most Solanaceae species have the same basic chromosome number (x = 12) and are diploid. EST (Expressed Sequenced Tag) and cDNA microarray analysis indicated that the coding region among different Solanaceae species is well conserved (Rensink *et al*. 2005; Shanna *et al*. 2005). In addition, comparative sequence analysis of a set of selected BACs (Bacterial Artificial Chromosomes) and comparative genetic linkage map analysis (Doganlar *et al*. 2002) indicated a high conservation both at microsynteny (gene content and order) and macrosynteny (genome structure) levels among different Solanaceae species. Despite highly similar in gene content, the Solanaceae species show markedly different phenotypes, making the family an ideal system to explore the basis of diversity and adaptation. The highly conserved microsynteny and macrosynteny amongst Solanaceae family allow us to select a reference for genome sequencing. The resulted high quality reference genome sequences will enable us to predict regions between genomes that are identical by descent and to study the evolution of sequence and function of orthologous genes. Tomato has been selected as the reference genome for sequencing due to its moderate genome size, the availability of large collections of mutants and inbred lines, being a model system for fleshy fruit development and ripening, more general plant genetics, disease response and numerous aspects of physiology, and having a wealth of genomics and functional genomics resources.

Tomato genome comprises of 12 chromosomes and has a size of 950 Mb (Arumuganathan and Earle 1991), which is approximately six times of Arabidopsis genome (157 Mb; Bennett *et al*. 2003). Approximately 75% of tomato genomic DNA is in the pericentric heterochromatin region which is rich in repetitive sequences and largely devoid of genes. The largely continuously stretches of gene-rich euchromatin, which correspond to less than 25% (~220 Mb; 1.4 times of Arabidopsis genome) of the genome and sit on the distal portion of each chromosome, contains mostly single or low copy sequences and more than 90% of the estimated 35,000 tomato genes (van der Hoeven *et al*. 2002). Instead of sequencing the 950 Mb of whole tomato genome, a strategy to sequence a minimal tiling path (MTP) of BAC clones through only the 220 Mb euchromatin has been employed by the tomato genome sequencing consortium. This strategy has been proved to be successful in sequencing the *Medicago trancatula* genome (http://www.medicago.org/genome). Compared to the whole genome shot-gun or the methyl-filtration sequencing approach, in which gene order would be largely lost, the strategy of sequencing a tiling path through the euchromatin will allow recover both gene content and gene order information, while at the same time it remains cost-effective. As a reference genome of the Solanaceae family, it is essential to retain gene order information for tomato. Having gene order information will help us to predict both gene content and gene order in other solanaceous species and related taxa connected through comparative genetic maps, to facilitate positional cloning in solanaceous species, and to allow the organization of tomato genome to be compared with other sequenced organisms (SOL initiative white paper; http://www.sgn.cornell.edu/solanaceae-project/).

Currently the tomato genome is being sequenced by an international consortium which includes 10 different countries, including USA (chromosome 1, 10, 11), South Korea (chromosome 2), China (chromosome 3), UK (chromosome 4), India (chromosome 5), The Netherlands (chromosome 6), France (chromosome 7), Japan (chromosome 8), Spain (chromosome 9) and Italy (chromosome 12). As described above, a BAC-by-BAC approach was chosen to sequence the euchromatin region of the tomato genome. Three BAC libraries, *Hind*III (15X coverage), *Mbo*I (7X), and *Eco*RI (9X), are now available for BAC selection and extension. Approximately 95,000 BACs from *Hind*III library, 50,000 from *Mbo*I and 50,000 from *Eco*RI have been deep sequenced from both ends, which generated more than 330,000 sequences. In addition, an ordered tomato fosmid genomic library with an average insert size of 40 kb and a minimum of 200,000 clones (8X coverage) is being created which will be used to fill less-than-BAC size gaps during tomato genome sequencing. These clones will also be sequenced from both ends. An ultra-high density tomato genetic map, based on a *S. pennellii* and *S. lycopersicum* F2 population, has been extended to more than 4000 markers. Over 800 genes from this map were screened as overgo probes on the tomato *Hind*III BAC library, of which 700 markers have been unambiguously anchored to the high-density map. An additional 250 anchored BACs have been identified through BAC end sequence analysis. From these anchored points, a total of 950 seed BACs were identified, which serve as starting points for tomato sequencing. A minimum tiling path will be generated by extending the seed BAC in both directions using the deep BAC end sequence information and the fingerprinted contig (FPC) map information, which is currently available for *Hind*III and

*Mbo*I libraries. Recently, Syngenta mapped 17,000 BACs, which represent a total of 763 anchored BAC contigs, to the bin positions of *S. pennellii* introgression lines. This is a valuable resource which will help to facilitate new seed BAC identification and BAC extension to generate MTP. FISH analysis is used to localize BACs near the euchromatin/heterochromatin boundaries and near telomeres, to define the size of gaps in sequenced DNA, and to confirm the proper tiling path extension. As of July 2007, approximately 28% of the tomato euchromatin has been sequenced and the sequencing of the whole tomato euchromatin is expected to be completed in 2009. Besides the euchromatin, the plastid genome of tomato has been sequenced (Kahlau *et al*. 2006) and sequencing the mitochondrial genome is underway. The genome sequences of these organelles will provide much information on their gene structure and evolution.

TOMATO FUNCTIONAL GENOMICS

Although complete genome sequences can provide a wealth of information on gene structure and its physical position on the genome and give us new insights into areas such as genome content, architecture and organization, they do not tell us the expression of genes under different conditions and developmental stages and how genes work together to comprise functioning cells and organisms. The development of a complex organism and its interaction with the environment are mostly related to dynamic changes of gene activities, which lead to downstream changes of protein activity and metabolite accumulation, and ultimately phenotype changes. Investigating the dynamics of transcriptome, proteome, metabolome, and/or phenome and their interactions and relationships is the basis of functional genomics.

In recent years, rapid progresses have been made in the area of functional genomics, both theoretically and technologically. In tomato, most functional genomics studies focused on transcriptome and its dynamics. As a basis, a large collection of ESTs has been generated to capture the sequence information of tomato transcriptome. Currently, the collection contains ~250,000 ESTs which represent ~35,000 unigenes (http://www.sgn.cornell.edu). ESTs are created by partially sequencing randomly isolated gene transcripts that have been converted into cDNA and cloned (Adams *et al*. 1991). ESTs have been successfully applied to accelerating gene discovery including gene family expansion, elucidating phylogenetic relationships, building physical and genetic maps, inferring intron-exon boundaries and identifying alternative spliced and polyadenylated transcripts, and facilitating large-scale gene expression analysis (reviewed in Rudd 2003). It has been proved that the occurrence of ESTs from non-normalized, non-subtracted cDNA libraries represents the relative expression of genes from which the ESTs are derived. Through the comprehensive analysis of a large public tomato EST dataset which contains ~150,000 ESTs from 27 different tissues representing a range of developmental programs and biological responses, a global coordinated gene expression atlas was created for 6,758 genes, and a number of tissue specific genes and genes differentially expressed during various developmental programs such as fruit and flower development and upon different biological responses such as pathogen challenges were able identified (Fei *et al*. 2004). In addition, through comparative analysis of tomato and grape EST collections, Fei *et al*. (2004) were able to identify common regulators induced by ripening process of both tomato (a climacteric fruit) and grape (a non-climacteric fruit), which provided new insights into molecular mechanisms of fruit ripening processes. Using EST as an approach of expression profiling in tomato has advantages over microarray in that microarray suffers from the cross-hybridization of closely related sequences. However, the overall effort and cost of EST sequencing are major hurdles of this approach. This situation might be changed soon due to recent advances of new sequencing technologies. One such technology is 454 sequencing by synthesis technology (Margulies *et al*. 2005), which can generate 400,000 ESTs with an average length of 250-300 base pairs in a single 7.5-hour instrument run. In addition, 454 sequencing does not require labor intensive cloning effort and its cost can be reduced by more than 10 fold when compared to conventional Sanger technology. The continuing advances of new sequencing technologies combining with the rapid progress of bioinformatics tools make it possible to use deep EST sequencing approach as a general tool in tomato for expression profiling analysis in the future while currently microarray technology is still dominant in gene expression analysis.

A tomato cDNA array (named TOM1 array) which contains ~13,000 unique spots representing 8,500 tomato unigenes, and later a long oligo-nucleotide array (named TOM2 array) containing ~12,000 tomato unigenes were generated based on the large public tomato EST collection (Alba *et al*. 2004; http://bti.cornell.edu/CGEP/CGEP.html). These two publicly available arrays along with other homemade tomato cDNA arrays have been used extensively to investigate gene expression profiling among different genotypes, during various developmental processes, and during responses to different environmental stimuli such as biotic or abiotic stresses. A milestone of such analyses is the creation of gene expression maps during the fruit development and ripening of a wild type tomato and a tomato mutant (*Never-ripe* [*Nr*]), which contains a mutation in an ethylene receptor, consequently has a reduced ethylene sensitivity and fails to ripen completely (Alba *et al*. 2005). A total of 869 genes that are differentially expressed in wild type tomato developing pericarp were identified and the expression of 37% of them was altered in *Nr* mutant (Alba *et al*. 2005). Further comparison of expression patterns for genes encoding enzymes in fruit development pathways to the measured products of said pathways (e.g., ethylene, carotenoids, and ascorbate) identified novel fruit development regulatory points influenced by ethylene (Alba *et al*. 2005).

In an assay of comparing transcriptomic changes in six fruit introgression lines producing fruit with elevated Brix were selected from a population of 76 lines carrying defined introgressions from *S. pennellii* in a *S. lycopersicum* background (Zamir IL lines; Eshed and Zamir 1994). Baxter *et al*. (2005) were able to identify candidate genes which helped to reveal underlying regulatory mechanisms of fruit metabolism that lead to high Brix.

Microarray technology has also been successfully applied to investigate tomato responses to endogenous or environmental stimuli. For example, to investigate the molecular roles of the type III secretion system (TTSS) effectors in disease formation, Cohn and Martin (2005) used TOM1 array to analyze the transcriptome changes of a susceptible tomato host in response to wild-type *Pseudomonas syringae* pv. *tomato* strain DC3000 and a mutant lacking a functional TTSS. They were able to identify 306 differentially expressed genes and many of them encode proteins associating with hormone response or hormone biosynthesis pathways (Cohn and Martin 2005). Similarly, by monitoring host transcriptional changes in response to the TTSS effector proteins AvrPto and AvrPtoB, they found both effectors induced a set of genes involved in ethylene biosynthesis and signaling (Cohn and Martin 2005).

Currently a tomato Affymetrix array which contains ~10,000 probe sets representing approximately 8,000 unique tomato genes is available (http://www.affymetrix.com/). However, this array has not gained much popularity in tomato community probably due to its high cost and low number of genes on the array.

A tomato expression database (TED; http://ted.bti.cornell.edu) has been developed (Fei *et al*. 2006), which serves as a central repository for raw gene expression data derived from public tomato arrays in addition to archive various normalized and processed gene expression data derived either from microarray analysis or digital (EST occurrence) analysis. A set of tools which facilitate data mining and visualization were implemented in TED (Fei *et al*.

2006).

High-throughput analysis of differential gene expression through tag or microarray approach has been proved to be a powerful tool for gaining information about a certain biological process on a genomic scale. However, some discrepancies between mRNA and its corresponding protein levels can exist. Thus coupling transcriptome with proteome studies will help us to identify post-transcriptional events and lead to better knowledge of gene networks. Unfortunately, currently available reports on tomato proteome analysis are very limited. Rocco *et al*. (2006) performed a comparative proteomic analysis on tomato fruits from a regional and commercial elite ecotype during maturation. A number of constitutive and differentially expressed proteins were identified in both varieties. Recently Faurobert *et al*. (2007) generated the proteome maps at six developmental stages during cherry tomato pericarp development and ripening using 2-DE and MS approaches. They identified a total of 1,791 distinct spots from 2-DE gels, of which 148 spots were significantly variable during pericarp development and ripening. When comparing the proteome results to the previously published transcriptome results, some discrepancies were noted, indicating certain genes undergo posttranscriptional modifications (Faurobert *et al*. 2007).

Currently more and more studies on tomato focus on metabolome analysis. Schauer *et al*. (2006) investigated the content of 74 known metabolites including amino and organic acids, sugars, sugar alcohols, fatty acids and vitamins C and E, in the Zamir IL lines in two different seasons. Using a combined metabolite profiling and phenotypic analysis, they were able to identify correlations among numerous metabolites and important fruit quality and yield traits of tomato. In a recent study, Carrari *et al*. (2006) investigated the changes of abundance of a broad range of metabolites, as well as transcriptome changes, during tomato fruit development and ripening. Several distinct patterns of metabolite profiles during fruit development were identified. They also identified some correlations between specific transcripts and metabolites, of which several new associations could provide potential targets for manipulation of fruit composition traits.

To study gene function, a comprehensive and saturated mutant population is indispensable. Mutants of tomato have been collected over several decades and currently 1,017 monogenic stocks (at 622 loci) are catalogued in the C.M. Rick Tomato Genetics Resource Center (http://tgrc.ucdavis.edu/). Recently, a large tomato mutant library containing a total of 13,000 M_2 families was developed through EMS and fast-neutron mutagenesis (Menda *et al*. 2004; http://zamir. sgn.cornell.edu/mutants). All these mutants add a great value to tomato functional genomics. Once an interesting mutant is identified, the integration of phenomics data with data sets from transcriptome, proteome and metabolome analyses will help to obtain a clear picture of the mutant gene function.

REVERSE GENETICS APPROACHES USING MICROTOM

Reverse genetics approaches aim at determining the function of a target gene in the context of a plant by analyzing the phenotype of the plant showing allelic gene variations for that gene. Various strategies can be used to evaluate the physiological function of a target gene in a plant and/or its effect on an agronomical trait. Target gene can be inactivated by insertional mutagenesis with mobile elements (transposons) or with T-DNA transferred from *Agrobacterium*, by deletion mutagenesis with physical agents (fast neutrons, X-rays; reviewed by Tadege and Mysore 2006; Wang *et al*. 2006), or by creating truncation mutations (nonsense and splicing site mutations) with chemical agents such as EMS. The EMS-induced point mutations may also alter the function of the protein encoded by the target gene (loss-of-function or gain-of-function mutations) as a result of a change in amino acid identity and function due to a missense mutation. The T-DNA or transposons inserted into a plant genome may also carry transcriptional enhancers that induce over-expression of genes in their vicinity (activation-tagging) or reporter genes that are expressed when inserted under the control of regulatory elements in the genome (promoter- or enhancer-traps). Several other approaches rely on transient gene inactivation or over-expression in various organs of a plant. Towards this end, different technologies using *Agrobacterium* injection or virus infection (Virus Induced Gene Silencing or VIGS) have been developed for transient expression assays.

Insertional mutagenesis by T-DNA and transposons

T-DNA tagging

In *Arabidopsis* and rice, insertional mutagenesis by T-DNA tagging has proved to be a powerful tool for high throughput gene discovery and functional analysis of target genes. Several populations of tens of thousand of mutagenized plants can be screened by PCR or simply by BLAST search for identifying knockout mutants in given target genes, as reviewed in Ostergraard and Yanofsky (2004). The phenotype of several independent mutants, often in different ecotypes, can thus be analyzed in order to decipher the functional role of the target gene in the plant. However, very large numbers of mutagenized plants are needed to reach near saturation of the collection (Krysan *et al*. 1999) in order to increase the probability of finding loss-of-function mutants in all the genes. In *Arabidopsis*, for example, the probability of achieving an insertion mutant in a gene less than 1 kb is less than approximately 60% for a collection of 100,000 lines (Ostergraard and Yanofsky 2004). Another disadvantage is that insertional mutagenesis of T-DNA or of any other element (e.g. transposon) leads to complete loss of gene function, in contrast to chemical mutagenesis that creates allelic series.

Average gene length is longer in tomato than in *Arabidopsis* (van der Hoeven *et al*. 2002), increasing thus the probability to hit a target gene by insertional mutagenesis in tomato. However, given the size of the tomato genome (950 Mb compared to 157 Mb in *Arabidopsis*) and the absence of very high throughput transformation technologies in this species, the task of producing a near saturation tomato mutant collection by insertional mutagenesis with T-DNA remains challenging. To our knowledge, an unique collection of more than 10,000 activation-tagging transgenic lines has been created in tomato, using MicroTom as a model because this cultivar is suited for high-throughput mutagenesis. Since the activation-tagging strategy used is based on the insertion of T-DNA in tomato genome, it can also produce loss-of-function mutants resulting from the integration of a T-DNA in coding or regulatory regions of a gene. In this collection, the lines over-expressing tomato genes under the control of a transcriptional enhancer allowed the discovery of genes controlling important agronomic traits such as fruit shape and colour. Among them was a MYB transcription factor controlling anthocyanin biosynthesis, modification and transport, which was identified by analyzing an activation-tagging line over-expressing the *ANT1* gene and displaying purple vegetative organs and fruits (Mathews *et al*. 2003).

Transposon tagging

Gene inactivation by insertional mutagenesis with mobile elements can be a solution for the overwhelming task of producing the large numbers of transgenic tomato plants by somatic embryogenesis required by T-DNA tagging. Because the mobile elements have the advantage to excise and to reinsert in a nearby gene (Emmanuel and Levy 2002), a lower initial number of transformants are required than for a T-DNA mutagenesis approach. In tomato, the interest of transposon-tagging for gene discovery and functional analy-

sis of target genes was highlighted by the discovery of the *Dwarf* gene (Bishop *et al*. 1996), which is actually one of the mutations presented in MicroTom. Since then, several transposon-based insertional mutagenesis strategies have been devised. They mostly rely on the use of transposons from heterologous species such as the non autonomous mobile elements Activator (Ac)/Dissociation (Ds) of maize, which are very effective for creating knockout mutants (Meissner *et al*. 1997, 2000; Gidoni *et al*. 2003). A transposon-tagged pilot collection based on this system was developed in MicroTom. The collection included about 3,000 lines carrying 2-3 inserts per line, which represented approximately 7500 Ds insertions (Meissner *el al*. 1997, 2000). In this strategy, only one step of initial transformation by somatic embryogenesis is required to obtain on the one hand lines carrying Ds elements and on the other hand lines carrying transposase (Ac) under the control of a constitutive promoter (CaMV35S). The Ds and Ac lines are then crossed to finally obtain germinally stable transposition: excision, re-insertion and stabilization mutant lines by selecting against transposase. Besides the inactivation of target genes, the vectors used for generating these lines can include other features. For example, the use of promoter-trap and enhancer-trap may allow the identification of organ and tissue-specific promoters (Meissner *et al*. 2000), which are necessary for functional analysis of candidate genes in tomato. However, despite the increased number of the insertional mutants using mobile elements, the objective of near-saturation insertional mutagenesis tomato collection remains probably out of reach for most tomato consortiums or companies. Indeed, considering the size of tomato genome, Emmanuel and Levy (2002) estimated that near to 200,000 to 300,000 lines would be necessary to obtain 95% saturation of the tomato genome.

RNAi and miRNA silencing in tomato through stable transformation

Large scale sequencing of tomato cDNAs allowed the generation of tomato EST databases comprising more than 250,000 ESTs. A large collection of full-length cDNA sequences of MicroTom is publicly available at Kazusa Institute in Japan (http://www.pgb.kazusa.or.jp/kaftom/). In addition, the current tomato sequencing project should allow the identification of the tomato genes in the coming years.

Therefore, new strategies based on the systematic over-expression of target genes or on their silencing by RNA interference (RNAi) or micro RNA (miRNA) (Alavarez *et al*. 2006) can now be envisaged in tomato. New site-specific recombination-based cloning vectors, which allow efficient and high throughput insertion of inverted repeats of a gene sequence in plant transformation vectors (Karimi *et al*. 2002; Waterhouse *et al*. 2003; Karimi *et al*. 2005), can be used to specifically target a candidate gene or the different members of multigene families. Since silencing often results in a range of more or less severe phenotypes, this strategy can be useful when analyzing large gene families or classes of genes. In addition, new promoters, which drive gene expression in specific organ, tissue or developmental stages in order to silence (RNAi, miRNA) or to over-express target genes, are currently developed in tomato (P Hilson, pers. comm.). Systematic over-expression or silencing of approximately 35,000 genes of tomato (van der Hoeven *et al*. 2002) still relies on the availability of easy and high throughput transformation protocols in tomato.

Transient methods

Agrobacterium-infiltration

Agrobacterium-infiltration or *Agrobacterium*-injection of various organs of the plant have been used in MicroTom by several groups to silence endogenous genes (Liu *et al*. 2002; Orzaez *et al*. 2006) or to over-express homologous or heterologous genes in tomato (Orzaez *et al*. 2006). *Agrobacterium* can be used to transfer foreign DNA to plant cells that can be transiently transcribed and translated into active protein (Orzaez *et al*. 2006).

Virus Induced Gene Silencing (VIGS)

Virus Induced Gene Silencing is an attractive reverse-genetics tool for the study of gene function in tomato. The high throughput functional analysis of genes by VIGS is now possible in tomato using a viral vector based on *Tobacco Rattle Virus* (TRV). The recombinant TRV infects plants and efficiently induces gene silencing, as has been shown in MicroTom for genes of the ethylene signalling pathway or carotenoid biosynthesis genes (Liu *et al*. 2002), and in tomato roots and in various Solanaceous species (Brigneti *et al*. 2004; Tyu *et al*. 2004). The addition of Gateway recombination system sites to the vector facilitates the cloning of candidate genes to be tested, allowing high throughput screening of candidate genes identified through EST and full-length cDNA sequencing programs. Using these vectors, fruit-specific silencing can be achieved by vacuum infiltration of fruit detached from the plant or by syringe injection to the fruit attached to the plant, allowing the study of fruit development and ripening (Fu *et al*. 2005). However, one of the current limitations of this approach is that gene silencing is unevenly distributed in the various parts of the fruit, as shown for the ethylene signalling-related genes in tomato (Fu *et al*. 2005; Orzaez *et al*. 2006). As a consequence, unless new technologies and vectors are designed to overcome this limitation, the use of VIGS in tomato fruit will probably remain mostly restricted to genes affecting fruit colour (e.g. ripening-related genes) and the identification of the silenced sectors in the fruit.

Physical and chemical mutagenesis in MicroTom

Physical or chemical mutagenesis have been used for decades in tomato to create artificially-induced genetic variability because the generation of large mutant collections is very simple and does not require excessive time and efforts. Artificially-induced mutations have been further exploited for breeding tomato cultivars with improved agronomic traits (FAO Mutant Varieties Database at http://www-mvd.iaea.org/MVD/default.htm). Recent advances in PCR-based methods led to the development of high throughput mutation detection technologies (TILLING, mutants) and the use of tomato mutant collection for reverse genetics approaches.

Chemical agents or ionizing radiations create high density of irreversible mutations ranging from point mutations to very large deletions, depending on the mutagen. Mutation density is independent of the size of the genome, allowing the application of mutagenesis to many crop plants with large genome size, including tomato. As a consequence, only few thousands mutant lines are sufficient to reach genome saturation in tomato, as compared with the hundreds of thousand lines required for T-DNA or transposon-based collections of insertional mutants (Ostergaard and Yanofsky 2004). Moreover, chemical or physical mutagenesis does not require genetic transformation of the plant. The GMO-free plants with improving alleles can thus be included in classical and marker assisted breeding programs for tomato crop improvement.

Several collections of fast-neutron and ethylmethane sulfonate (EMS) mutants have been generated during the recent years in MicroTom (Meissner *et al*. 1997, 2000; Watanabe *et al*. 2007). The mutation detection technologies, which were recently developed such as TILLING (Targeting Induced Local Lesions IN Genomes) using S1-type endonuclease, have been successfully applied to tomato. Therefore, MicroTom mutant collections can now be used for functional genomics and crop improvement.

Fast neutron mutagenesis

Fast neutron bombardment creates DNA deletions ranging from a few bases to more than 30 kb, resulting in the creation of knockout mutants. Several genes may be removed by large deletions. This can be particularly useful for inactivating duplicated genes with functional redundancy arranged in tandem repeats. On the other hand, when several independent genes are deleted, it can be problematic for the subsequent genetic analyses of the mutants.

Generation of deletion mutant collection by fast neutron is straightforward (Meissner *et al*. 1997, 2000; Menda *et al*. 2004). In MicroTom, the optimal radiation doses and corresponding mutation rates have been defined in a pilot study by Meissner *et al*. (1997), who demonstrated the feasibility and interest of this approach. The collection of MicroTom deletion mutants obtained in this study yielded a large number of mutant showing phenotypic variations affecting vegetative organ, flower and fruit. Some mutants were further characterized, e.g. the *tangerine* mutant obtained by Meissner *et al*. (1997) which shows bright orange fruits because of a deletion in carotenoid isomerase gene (Isaacson *et al*. 2002). Another collection of MicroTom fast-neutron mutants has been recently been created by the Japanese Solanaceae consortium (JSOL) (Watanabe *et al*. 2007). These results emphasize the potential interest of this strategy for functional studies of target genes in tomato and for improvement of agronomic traits.

In most crops that lack sequence information on target gene and surrounding genomic regions, forward genetics approaches have long been most adapted to isolate mutated alleles in fast neutron mutagenized plant collections. With the current advance of genome sequencing in tomato, high sensitivity PCR-based technologies using pools of thousand deletion lines such as the one devised by Li *et al*. (2001) should allow the efficient screening of MicroTom deletion mutants. In *Arabidopsis*, it was estimated that more than 50,000 mutagenized lines would be necessary to reach near saturation of the collection (i.e. 85% of mutated loci). Thus, in the fleshy fruit tomato where harvesting the seeds is a long and time-consuming process, the task of producing a near saturation fast neutron mutant collection that can be used for high throughput reverse genetics remains probably difficult for most research groups.

TILLING

Targeting Induced Local Lesions in Genomes (TILLING) is a general reverse-genetics strategy first described by McCallum *et al*. (2000) combining random chemical mutagenesis by ethylmethanesulfonate (EMS) and PCR-based methods for detecting unknown point mutations in target genes. It allows the identification of allelic series including truncation mutations (nonsense and splicing site mutations) that lead to gene inactivation, neutral mutations with no effect on the encoded protein, and missense mutations (Henikoff and Comai 2003). Depending on the function of the amino acid affected by the mutation, the missense mutation in a target gene may either result in the inactivation or in the modification of the function of the encoded protein, leading to loss-of-function or to gain-of-function mutants. This is an advantage when knockout mutations can be lethal for the plant (Diévart and Clark 2003). EMS mutagenesis and TILLING have been applied to a large number of plants with diverse genome size and ploidy level (Gilchrist and Haughn 2005; Haughn and Gilchrist 2006), including tomato and more specifically MicroTom because it generates large artificially-induced genetic variability in model crop species and that it allows the identification of unknown mutants.

Creating MicroTom EMS mutant library

The chemical mutagenic agent EMS creates point mutations, which are mostly (>99%) G/C-to-A/T transitions (Greene *et al*. 2003). Similar mutation frequencies are expected regardless of the plant genome size (Korneef *et al*. 1982), as with fast-neutron deletion mutants. Reported mutation densities are however highly variable depending on the species and range from 1 mutation/Mb in barley (Caldwell *et al*. 2004) to 1 mutation/25 kb in tetraploid wheat (Slade *et al*. 2005). According to our own unpublished results, mutation density can reach 1 mutation/125 kb in MicroTom tomato, i.e. in the same range as in *Arabidopsis* (ATP project; Greene *et al*. 2003).

The creation of a MicroTom EMS mutant library for TILLING is detailed in **Fig. 2**. EMS mutagenesis is straightforward and is usually done by soaking overnight the M0 seeds in EMS solution to give the M1 (mutagenized) seeds (Meissner *et al*. 1997). Since the EMS concen-

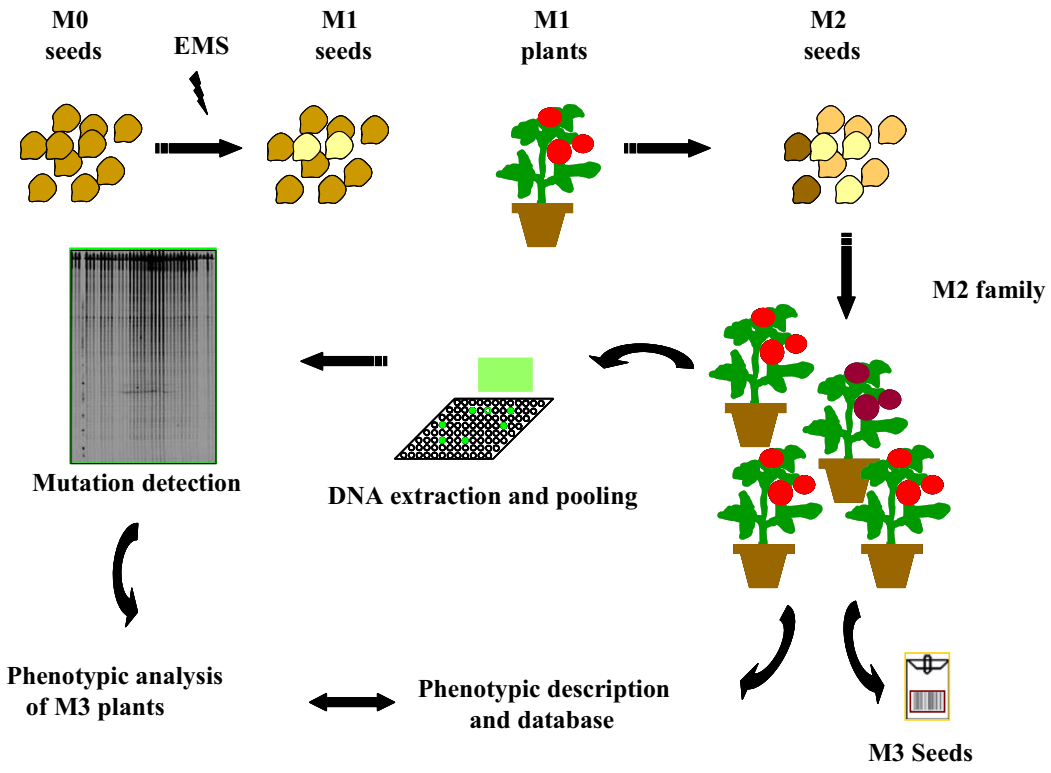

Fig. 2 Creation of Micro Tom mutant collection and detection of mutated alleles. Seeds (M0) are mutagenized with ethylmethanesulfonate (EMS). Mutagenized M1 seeds are sown to give M1 plants. M2 seeds collected from one given M1 plant are sown to give M2 plants (12 seeds for each M2 family). Phenotypic description of the M2 family is stored in a database. M3 seeds are archived for phenotyping and analysis of M3 plants. DNA is extracted from leaf disks collected from M2 family plants, pooled (8 family DNA pools) and used for mutation detection. Phenotypic analysis of the mutants identified is conducted by using phenotypic description database and analysing M3 plants.

trations may vary considerably depending on plant species and even to the seed batch, initial studies with different EMS concentrations (from 0.2 to 1.5%) are usually carried out before large scale mutagenesis to determine the optimum EMS dose. The objective is to reach the highest mutation rate that allows efficient mutation discovery by TILLING without compromising plant survival and fertility. Towards the objective, an EMS dose of 0.8% to 1% is usually used for MicroTom (Meissner *et al*. 1997; Watanabe *et al*. 2007; our own unpublished results). Following EMS mutagenesis, up to 50 to 70% of the seeds may not germinate or give viable plantlets and 40 to 50% of the resulting M1 plants can be sterile. Because M1 plants are chimerical as a consequence of somatic mutations in meristematic cells (only two to three cells of the apical meristem will give rise to reproductive tissues), the phenotypic characterization and mutation analysis are conducted using the M2 plants that were obtained by self-crossing the M1 plants (usually 8 to 12 plants per family). The results provide the first hints on the presence of known mutations and the frequency of mutated alleles in the collection. Phenotypic data on Microtom mutant families can further be stored in a phenotypic description database, as developed by Menda *et al*. (2004) for M82 tomato cultivar (http://www.sgn.cornell.edu/mutants/mutants_web/). However, this step is not a prerequisite for the detection of mutated alleles in the collection, though the phenotypic description of M2 mutant families can be useful for identifying mutants for forward genetic approaches or may be helpful for characterizing the function of a target gene once a mutant has been identified by TILLING. Actually, only one or a few plantlets per M2 families are required for DNA extraction to subsequently detect mutations.

Detecting unknown mutations

Several mutation detection technologies are available for detecting unknown point mutations and have been described in detail by Yeung *et al*. (2005) and Comai and Henikoff (2006). Generally, three major technologies are commonly used.

(1) Sequencing: it allows a precise and easy identification of point mutations in target genes and becomes more attractive as the price of sequencing decreases. However, it still has limitations, such as the sequencing cost and requirement of screening mutants individually.
(2) Conformation-based methods: technologies such as denaturing HPLC (dHPLC) and Single Strand Conformational Polymorphism (SSCP) allow the discovery of mutants by detecting the conformational changes induced by point mutations. Their main limitations are the size (<500 bp) of DNA fragment and the size (<4 individual or family pools) of the mutant pools that can be analyzed.
(3) Enzymatic detection: the most popular technology uses the mismatch detection enzyme CEL1, an endonuclease of the S1 family that detects mismatches and cleaves at the 3′ side of the mutation. CEL1 can be readily purified from plants (Colbert *et al*. 2001) and is commercially available (Qiu *et al*. 2004). Cleaved and uncleaved labelled DNA fragments are then separated on a sequencing gel. This technology led to a wide usage of TILLING, from *Arabidopsis* to a large number of plants species and even to insects and animals (Henikoff and Comai 2003; Gilchrist and Haughn 2005). It can detect 1 genome in 16 i.e. in eight-fold pool (Comai and Henikoff 2006).

The technology used for high throughput TILLING with CEL1 is very simple. Differentially labelled primers are designed to amplify a DNA fragment of 0.5 to 2 kb of the target gene. Tools such as CODDLE (*Codons Optimized to detect Deleterious Lesions*) software (Henikoff and Comai 2003) have been developed to define the genomic region showing the highest probability of finding severe mutants (knockout or missense mutations). In tomato, however, knowledge of genomic sequences is usually scarce since sequences available are mostly ESTs. Therefore, amplification of genomic DNA with unlabelled primers is usually conducted before TILLING in order to check the primers. After PCR amplification of the target gene with labelled primers, the formation of DNA homoduplexes and heteroduplexes is triggered by submitting the amplicon to a high temperature-denaturation/low temperature-reannealing cycle. The heteroduplexes are then cleaved by CEL1 enzyme while homoduplexes are left intact. The cleaved and uncleaved end-labelled DNA fragments are separated by electrophoresis on denaturing gel, usually on LicOR sequencer (Comai and Henikoff 2006) although capillary sequencers can also be used (Caldwell *et al*. 2004). The use of differentially labelled primers allows the precise location on the gel of the two cleaved fragments and thus the identification of the mutated region in the target gene. To identify the mutant family, the same TILLING approach with CEL1 is applied to perform the deconvolution of the 8 family pools. The identification of the mutation is usually conducted by amplifying and sequencing the mutated allele.

Linking mutation to phenotype

Point mutations induced by EMS are mostly G/C-to-A/T transitions (Greene *et al*. 2003), which may result in silent mutation (no amino acid change), missense mutations (changes in the encoded amino acid that may be conservative or non-conservative) or truncation (induced by the generation of stop codon or mis-splicing) of the target protein. Experimental TILLING results from *Arabidopsis* (Greene *et al*. 2003) indicated 55% of non silent mutations (5% of truncations and 50% of missense mutations) could affect the biological function of the target protein and hence the phenotype of the plant. The allelic series obtained in EMS mutants range thus from more or less severe mutations (truncation mutations, non-conservative missense mutations), which potentially affect the phenotype of the plant.

Once a mutant family has been detected by TILLING, the phenotype of the plants (usually M3 plants) must be analyzed in order to link the mutation to the phenotype and hence to study the function of the target gene in the plant context. The mutation can also be isolated by successive backcrosses (BC4 to BC6; Henikoff and Comai 2003; Slade *et al*. 2005). One common objection to the use of TILLING and EMS mutants for functional analysis of target genes is the very high number of mutations that were found in a given mutant collection. Indeed, in most mutant collections, a number of related or unrelated (due to different mutations) phenotypic traits can be observed in a single family. However, as calculated from *Arabidopsis* TILLING collections (Henikoff and Comai 2003), when the parent M2 plant is heterozygous, the possibility of misattributing a phenotype observed in M3 plants to a mutation in the target gene is only of about 0.05%. Increasing the number of mutated alleles giving rise to the same phenotype is the best way to insure that the phenotypic trait observed is linked to a mutation in the target gene and not to another combination of mutations (Ostergaard and Yanofsky 2004). Given the known frequency of mutations (Greene *et al*. 2003), an allelic series including at least one knockout mutation and ~10 missense mutations affecting the biological function of the protein should roughly comprise 20 mutated alleles. In the existing Microtom mutant collections, this objective represents between 2,500 to 3,000 mutant families to screen for 1 kb of target gene (unpublished results), a screening that can be performed in a short period of time. In conclusion, because of the wide range of mutated alleles and phenotypic variations detected, TILLING allows the discovery of allelic series and is highly informative for understanding the functional role of a target. In addition, TILLING allows the functional analysis of target genes for which severe mutations are lethal.

IMPROVEMENT OF FRUIT NUTRITION AND FLAVOR QUALITY

Fruit flavor and nutrition composition have clear and profound potential for positive human benefit. Nutritive compounds include various antioxidants, vitamins, minerals, fiber, lipids and amino acids, which have direct impact to human health while the modification of fruit flavor, may lead to improved health via increased fruit or vegetable consumption. The nutritional importance of fruit and vegetables is reflected in current USDA recommendations of five or more servings of fruit or vegetables a day for a healthy diet. The World Health Organization and the United Nations Food and Agriculture Organization (FAO) recently launched an effort to enhance fruit and vegetable consumption worldwide as low consumption is considered one of the top ten contributing factors to human mortality (http://www.fao.org/english/newsroom/news/2003/24439-en.html).

Fruit flavor and nutrition have proved to be difficult traits to modify through traditional breeding. Consumers recognize that commercially available tomatoes often leave much to be desired when it comes to flavor. Plant breeders have traditionally ignored flavor due to its genetic complexity and the tomato has suffered from this neglect. Tomato flavor is due to a complex and poorly understood interaction between sugars, acids and ~16 volatile compounds (**Table 1**). These volatiles can have concentration-dependent positive or negative effects on flavor. Currently little is known about the genes involved in either synthesis or regulation of most of the volatiles. In the case of nutritional compounds such as carotenoids, much is known of the synthetic steps and their corresponding genes, yet little is know regarding the molecular nature of pathway regulation. Identification of genes controlling volatile levels and nutrition pathways would open the door to breeders and biotechnologists for significant quality improvements.

Recent progresses on the fields of genomics, functional genomics and bioinformatics provide us a unique opportunity to dissect the molecular mechanism of fruit nutrition and flavor using an integrative systems approach. As stated previously, a collection of 76 tomato (*S. lycopersicon*) lines harbor single, defined and overlapping introgressions from the wild species *S. pennellii* is available. These lines together cover the entire genome of *S. pennellii* and they demonstrate substantial, reproducible line to line phenotypic variations in many aspects. Profiles of ~20 nutrition-related metabolites that include carotenoids, ascorbate, phenolics and tocopherols, and ~40 flavor-related metabolites that include sugars, acids, soluble solids and a set of fruit volatiles have been investigated in the ripe fruit tissue of the 76 *S. pennellii* introgression lines and their parent line, M82, from multiple different seasons. Profiles of selected fruit nutrition and flavor-related metabolites have also been generated for another set of introgression lines derived from wild tomato species, *S. hirsutum* (Monforte and Tanksley 2000). Introgression lines with significant changes in each of the selected metabolites compared to their parent control line were identified. Through this analysis, multiple loci altered in flavor volatile emissions have been discovered (Tieman *et al*. 2006a). In order to identify novel genes involved in or regulating a specific metabolite pathway using systems approaches, gene expression profiling data is also being generated for each *S. pennellii* line using tomato cDNA arrays. Genes significantly changed in each line were identified.

To facilitate correlation of metabolite and gene expression data, we are developing a public online database, Tomato Metabolite Database (TOMET; http://tomet.bti.cornell.edu). Besides metabolite and gene expression data, TOMET includes representative photographs of ripe fruit from each introgression line and lists of analyzed metabolites and related phenotypes. A number of data retrieval and analysis functions have been implemented in TOMET which allow to: 1) sort the introgression lines and control parent according to levels of a selected metabolite in a specific season; 2) identify introgression lines with specific metabolite properties; 3) mine fruit gene expression data for a selected line as a function of expression in the M82 control to identify significantly changes genes; 4) identify gene expression ratios that correlate with accumulation of a selected metabolite in all or a subset of introgression lines for a given trial. Additional tools such as tools to cluster and classify gene expression and/or metabolite profiles are under development. Using the metabolite profiling data and data mining tools currently available in TOMET, Tieman *et al*. (2006b) successfully identified the first step of biosynthesis pathway of 2-phenylethanol and 2-phenylacetaldehyde, both of which are important to tomato fruit flavor and aroma, through an integrated biochemistry, molecular biology, functional genomics and bioinformatics approach.

One major goal of TOMET is to identify key regulators of fruit flavor and nutrient composition through the integration of genotype, metabolite and gene expression data. Currently, several meaningful or expected correlations can be identified in TOMET (**Fig. 3**). In the meantime, we have observed a number of novel, significant correlations between metabolite and gene expression. Some genes involved in the correlations are transcription factors, which may be candidate regulators of fruit flavor and nutrition pathways. The relationship between the candidate genes and fruit flavor and nutrition will be characterized by transfer respective genes into tomato plants using the state-of-the-art RNA interference (RNAi) technology. By identifying the key regulators that control the flavor and nutritional value of the tomato, it will not only help to improve the way tomatoes are grown but will also contribute to how this crop is being used and developed in other biotechnology research programs. Researchers and breeders will also be able to directly apply the findings to other important crops.

Table 1 The 16 most significant flavor volatiles of tomato. Adopted from Goff and Klee (2006) with modifications.

Volatile	Precursor	Odor
cis-3-Hexenal	Lipid	tomato/green
β-ionone	carotenoid	fruity/floral
Hexanal	Lipid	green/grassy
β-Damascenone	carotenoid	fruity
1-Penten-3-one	carotenoid	floral/green
2+3-Methylbutanal	Ile/Leu	musty
trans-2-Hexenal	Lipid	green
2-Isobutylthiazole	Leu	tomato vine
1-nitro-2-Phenylethane	Phe	musty, earthy
trans-2-Heptenal	Lipid	green
Phenylacetaldehyde	Phe	floral/alcohol
6-Methyl-5-hepten-2-one	carotenoid	fruity, floral
cis-3-Hexenol	Lipid	green
2-Phenylethanol	Phe	nutty
3-Methylbutanol	Leu	earthy, musty
Methyl salicylate	Phe	wintergreen

CONCLUSION

Our review has examined the biological and technological advantages of MicroTom/tomato, including transformation, genome sequencing, functional genomics, reverse genetic tools and improvement of fruit nutrition and flavor quality in MicroTom/tomato. The major features of MicroTom comprehend:

(1) Sharing the intrinsic advantages of tomato such as a wealth of publicly available data in various fields such as plant physiology, biochemistry, pathology and genetics.
(2) Possessing small size (up to 1357 plants/m^{-2}), short life cycle (70-90 days from sowing to fruit-ripening), relatively small genome (950 Mb) and high throughput transformation technologies.
(3) 28% of tomato euchromatin sequenced and the sequences of entire tomato euchromatin expected to be completed in 2009.

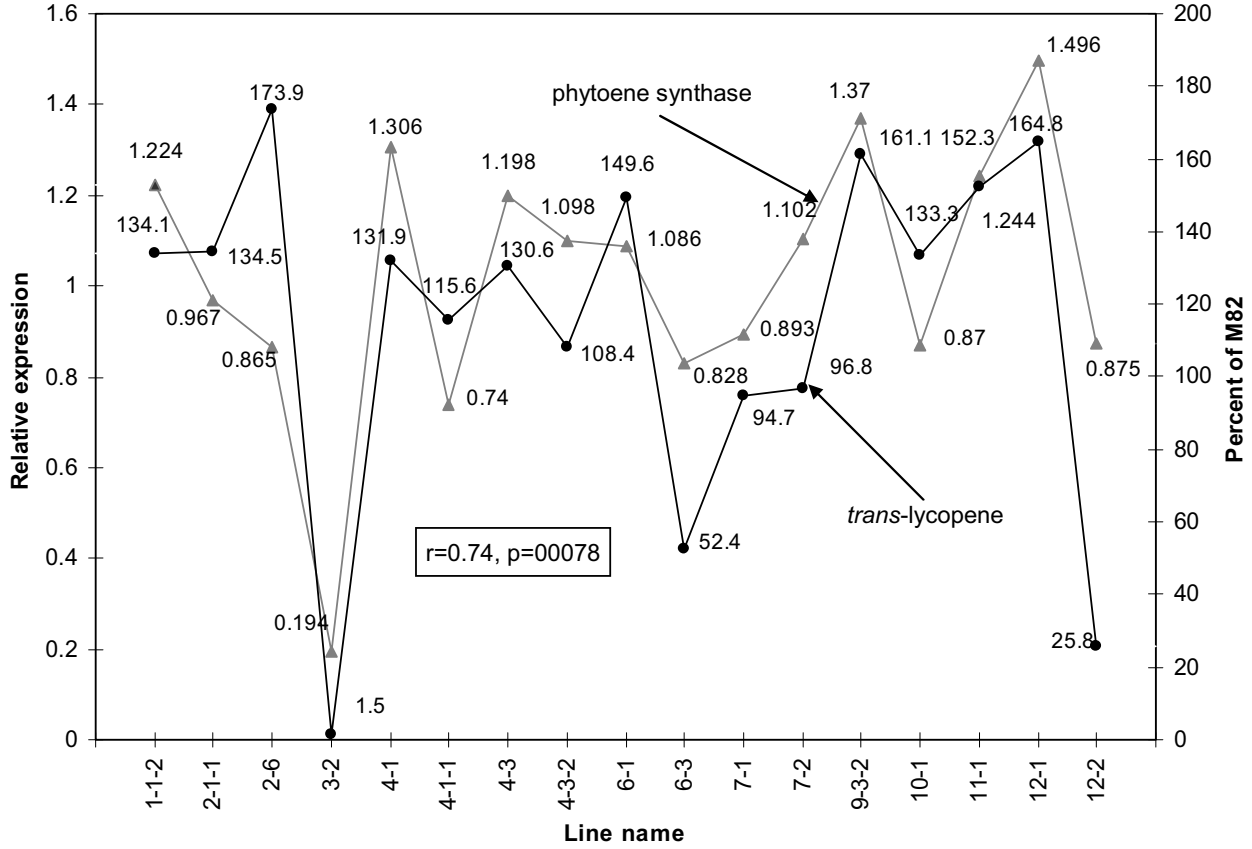

Fig. 3 Correlation between profile of *trans*-lycopene (right axis) and expression profile of a phytoene synthase (left axis), an upstream enzyme in *trans*-lycopene biosynthesis pathway, across 17 tomato IL lines.

(4) Public availability of large functional genomic databases, including more than 40,000 ESTs, 8000 full-length cDNAs (MiBASE at http://www.kazusa.or.jp/jsol/microtom/) and hundreds of Single Nucleotide Polymorphisms (SNPs) (Yamamoto *et al*. 2005) in MicroTom and ~250,000 ESTs representing ~35,000 unigenes (http://www.sgn.cornell.edu), a cDNA array representing 8,500 unigenes, a long oligo-nucleotide array containing ~12,000 unigenes (Alba *et al*. 2004, http://bti.cornell.edu/CGEP/CGEP.html), an Affymetrix array containing ~10,000 probe sets representing approximately 8,000 unique genes (http://www.affymetrix.com/) and an expression database (TED; http://ted.bti.cornell.edu) in tomato.

(5) Availability of diverse reverse genetic tools using Micro Tom.

(6) A public online Tomato Metabolite Database (TOMET; http://tomet.bti.cornell.edu), which provides the information of metabolite and gene expression for studying fruit flavor and nutrition.

(7) Public availability of large tomato/MicroTom mutant library containing 1,017 monogenic stocks (http://tgrc.ucdavis.edu/) and 13,000 M2 families (http://zamir.sgn.cornell.edu/).

The biological features and technological advances in MicroTom/tomato described above provide unique advantages to MicroTom as a new plant model system for plant genomics and development of economically important traits to improve crops.

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