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**Review**

# Micro-Tom mutants for functional analysis of target genes and discovery of new alleles in tomato

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**Abstract** Tomato is currently the model plant for fleshy fruit development and for *Solanaceae* species. Recent genomic approaches including transcriptome, proteome and metabolome analyses and genetic mapping have produced a wealth of candidate genes whose function needs to be assessed. The recent development in model and crop plants of TILLING (Targeting Induced Local Lesions IN Genomes), which reveals allelic series corresponding to several independent point mutations, and the current availability of deep sequencing tools further increase the interest of generating artificially-induced genetic diversity in tomato. We describe here the generation and use of EMS (ethyl methanesulfonate) tomato mutants in the miniature cultivar Micro-Tom and provide as example the identification of new fruit size and morphology mutants. We further propose new deep sequencing-based strategies for the discovery of mutations underlying phenotypic variations observed in mutant collections that will considerably increase the interest of exploiting Micro-Tom mutant collections for gene discovery in tomato.

**Key words:** Tomato, mutants, TILLING, forward genetics, deep sequencing, phenotype, database.

Tomato (*Solanum lycopersicum*) is a member of the *Solanaceae* family that includes other major crop species such as potato, pepper, eggplant and tobacco and ornamental plants such as petunia. In the last 50 years, tomato production increased more than five-fold, especially in Asia, and tomato now represents the first cultivated fruit worldwide before banana and watermelon. Other tomato features such as short life cycle, easy growth in open fields and greenhouses, availability of well-characterized genetic resources and easy genetic transformation has triggered the use of tomato as a model for both Solanaceaous crop species and fleshy fruits. In the last 10 years, numerous studies have been published on tomato genetics, physiology, development and pathology and a wealth of publicly accessible tomato genomics data have been obtained.

Thanks to these recent advances, linking genotypic variations to associated phenotypic changes is now more accessible. Two strategies, usually named the forward genetic and the reverse genetic approaches, are used to reach this goal. The objective of forward (traditional) genetic approach is to discover the allelic variation(s) responsible for single Mendelian traits or variations of quantitative traits previously identified through phenotypic screening of populations displaying genetic diversity. Reverse genetics approach mainly aims at confirming and/or unraveling the physiological role of a target gene and at establishing its effect on plant phenotype. In the recent years, the identification of candidate genes possibly involved in the control of biological processes or traits of interest, such as fruit quality traits, has been greatly facilitated by the increased availability of large genomic datasets. In tomato fruit, analysis of the correlations between gene expression in given organs, tissues and/or developmental stages and phenotypic changes (e.g., developmental patterns or chemical composition of the fruit) recently enabled the identification of genes of unknown function implicated in the regulation of fruit flavonoids (Ozaki et al. 2010) and of major developmental and metabolic shifts

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occurring during fruit development (Mounet et al. 2009; Rohrmann et al. 2011). Recent developments in deep sequencing combined with laser microdissection of tomato fruit cell layers further allowed the inventory and quantification of all transcripts present in specific fruit cells, thus giving the possibility to infer their function in fruit developmental processes e.g. the cuticle formation in the fruit (Matas et al. 2011). In the same way, the identification of genes underlying traits of interest has been considerably facilitated by the development and analysis of genetic materials such as ILs (Introgression Lines) and RILs (Recombinant Inbred Lines) (Fernie and Klee 2011), of large SNP (Single Nucleotide Polymorphism) genotyping arrays and high-density genetic maps (Sim et al. 2012) and by the recent availability of genomic sequences of the cultivated tomato *S. lycopersicum* and of wild relative *S. pimpinellifolium* (Tomato genome Consortium 2012).

Following their discovery, the relationships between the candidate genes or the genetic variations identified and the processes or traits of interest must be confirmed. Because the generation of large enough transfer DNA (T-DNA) or transposon-tagged lines remains out of reach in tomato (Emmanuel and Levy 2002), functional analysis *in planta* of the target genes is usually done using stable genetic transformation (over-expression, RNA interference and chimeric repressor silencing technologies) with constitutive or tissue-specific promoters (Fernandez et al. 2009) or transient expression by agro-infiltration or VIGS (Virus Induced Gene Silencing) (Orzaez et al. 2009; Quadrana et al. 2011). Recently, association mapping has also been shown to be an effective tool for assessing the molecular basis of fruit quality traits in tomato (Xu et al. 2013).

We recently generated highly-mutagenized tomato EMS (ethyl methanesulfonate) mutant resources using the miniature tomato cultivar Micro-Tom for reverse and forward genetics approaches in tomato. In this mini-review, we will first briefly describe the main features of the Micro-Tom EMS mutant collections developed in both Japan and France. We will then provide examples of how Micro-Tom mutant collections can be used for identifying novel fruit size mutants in tomato. Last, we will give some hints on how current deep sequencing technologies may be used in the near-future for identifying unknown mutations responsible for phenotypic changes in Micro-Tom mutants.

### *Generation of Micro-Tom EMS mutant collections*

Micro-Tom is a miniature dwarf determinate tomato cultivar, originally bred for home gardening purposes, which differs from standard tomato cultivars by two recessive genes (Campos et al. 2010; Dan et al. 2007; Martí et al. 2006; Meissner et al. 1997). One of these mutations affects the biosynthesis of the brassinosteroid hormone in the vegetative tissues (Martí et al. 2006; Nomura et al. 2005), thereby giving to the plant its dwarf appearance. Due to its small size, short life cycle and high density culture, Micro-Tom is particularly suitable for large-scale mutant screens. This cultivar was therefore proposed in the nineties as a model for functional genomics in tomato by the group of A. Levy, who first developed collections of Micro-Tom EMS mutants, fast-neutron mutants and transposon-tagged lines (Emmanuel and Levy 2002; Meissner et al. 1997). Since then, a wealth of Micro-Tom genetic resources has been developed including activation-tagged lines (Mathews et al. 2003) and isogenic lines carrying a wide range of introgressed hormonal mutations (Carvalho et al. 2011).

We recently developed highly-mutagenized EMS mutant collections in the miniature cultivar Micro-Tom for three main objectives. First objective was to cope with the increasing need for functional analysis of target genes in tomato, which is hardly compatible with the throughput of the current tomato genetic transformation systems (Dan et al. 2007; Rothan and Causse 2007). Second objective was to generate new sources of genetic diversity that can be used for the isolation of genes underlying traits of interest in tomato. Third objective was to produce allelic series including strong and weak alleles that can be used for mutation breeding of plant and fruits with improved traits, as already done in other *Solanaceaous* species (Julio et al. 2008). EMS is a mutagenic agent producing point mutations, which frequencies are expected to be similar whatever the size of the plant genome (Greene et al. 2003), rendering this approach applicable to most crop species. However, the desired EMS mutation frequencies usually result from a compromise between the requirement of high mutation frequencies for reducing mutant screening effort, and of low mutations frequencies for maintaining plant development and fecundity. Actually, published plant EMS mutation densities detected using various techniques display a wide range of variation from one mutation per Mb in barley (Caldwell et al. 2004) to one mutation per 170 kb in Arabidopsis (Greene et al. 2003) and one mutation per 25 kb in the polyploid (hexaploid) wheat (Slade et al. 2005). High mutation frequency in wheat is likely linked to the tolerance of polyploid species to mutations due to gene redundancy but other factors such as the physiological state of the treated seed and the dose and duration of EMS treatment will also affect mutation density.

The main features of the Micro-Tom EMS mutant collection generated in Japan have been described in recent papers (Okabe et al. 2011; Saito et al. 2011). This collection comprises 3,052 lines generated in the presence of either 0.5% or 1.0% EMS and the mean mutation frequency estimated from the screening of approximately 15.3 kb varies from one mutation

per 1,710 kb (0.5% EMS) to one mutation per 737 kb (1% EMS) (Okabe et al. 2011). In addition, a mutant database called TOMATOMA (http://tomatoma.nbrp.jp/) distributing Micro-Tom mutant collections has been created (Saito et al. 2011).

At INRA Bordeaux (France), two Micro-Tom EMS mutant populations with different mutation frequencies have been generated by our group. In both cases, mutagenesis with 1% EMS was carried out as described (Dan et al. 2007; Okabe et al. 2011). In addition, to further increase the mutation frequency in one of these populations, M2 seeds collected from mutant M1 plants were again submitted to a second round of mutagenesis with 1% EMS. The M2 seeds collected from mutant M1 plants resulting from one or two rounds of EMS mutagenesis were sown (4 to 12 plants per family according to the population), and leaf material from the two populations (~8,000 M2 families) was collected for DNA isolation. In addition, plant and fruit phenotypes from the most highly mutagenized population (ca. 3,500 M2 families, 12 plants per family) were analyzed along plant development, from seed germination to ripe fruit. The resulting phenotypic annotations were stored in a web-searchable phenotypic mutant database called MicroTom Mutant Database (MMDB, not yet publicly searchable), which currently includes >30,000 annotations for more than 150 phenotypic categories. The mean mutation density of the INRA Bordeaux combined mutant collections was estimated to be close to one mutation per 663 kb by screening 49.9 kb of DNA sequence on 7,296 M2 families, using *Endo1* endonuclease (Okabe et al. 2011; Triques et al. 2007). However, this result is probably a large under-estimate because our TILLING experiments identified up to one mutation per 125 kb for some genes (Dan et al. 2007) and that the recent whole genome sequencing of an EMS mutant line from the INRA Bordeaux collection uncovered higher mutation frequencies (data not shown).

### *Reverse genetics approach using Micro-Tom EMS mutant collections*

One primary objective of the generation of Micro-Tom EMS mutant collections was to develop genetic resources for the functional analysis of target genes in tomato. Genetic transformation is easy but remains tedious and is time-consuming in this species, which limits the development of other useful technologies commonly used in *Arabidopsis* such as insertional T-DNA mutagenesis. Besides, EMS mutagenesis is well adapted to tomato. Mutations are distributed at random over the whole genome. High mutation frequencies can be observed in EMS mutant collections, as described above, meaning that saturation mutagenesis can be reached with a limited number of mutant lines and that large allelic series can be obtained for a given gene-of-interest. Indeed, since knockout mutations of certain genes can be lethal, or produce strong phenotypes that cannot be used to decipher the physiological role of a gene or, at the opposite, give weaker phenotypes than those observed with missense mutations producing dominant-negative mutants, the isolation of series of allelic variants appears as the most appropriate strategy for evaluating the function of a gene in the context of the plant. The identification of unknown point mutations in regions of interest of target genes by TILLING produces ~45% of silent mutations, ~5% of nonsense and ~50% of missense mutations which may affect protein function (Greene et al. 2003). Increasing the size of the mutant population therefore increases the chance to isolate allelic variants of interest such as splicing site, non-sense or non-conservative missense mutations.

To date, identification of mutations in tomato by TILLING using various technologies has been reported for several EMS mutant collections generated in either the determinate tomato cultivars M82, Red Setter, and TPAADASU used for processing tomatoes (Gady et al. 2009; Menda et al. 2004; Minoia et al. 2010; Rigola et al. 2009) or in the semi-determinate cultivar Arka Vikas used for fresh market (Sreelakshmi et al. 2010). Recently published results highlight how powerful this approach can be in providing new alleles for deciphering the function of tomato genes involved in virus resistance (Piron et al. 2010; Rigola et al. 2009), plant growth and architecture (Busch et al. 2011; MacAlister et al. 2012; Martín-Trillo et al. 2011) and fruit nutritional quality (Di Matteo et al. 2013; Gady et al. 2012; Jones et al. 2012). In Micro-Tom, screening 3,052 EMS mutants from the Japan Micro-Tom collection for allelic series of 10 genes involved in ethylene signaling (the *SlETR1*, *SlETR2*, *SlETR3*, *SlETR4*, *SlETR5* and *SlETR6* genes), $\gamma$-aminobutyric acid (GABA) metabolism (the *SlSSADH* and *SlGABAT1* and *SlGABAT3* genes) and fruit softening (the *SlPL* gene) allowed the identification of up to 35 allelic mutants (Okabe et al. 2011). Among these, two novel mutant alleles of the ethylene receptor gene *ETHYLENE-RESPONSE1* (*SlETR1*) displaying amino acid substitutions in the predicted transmembrane domain were isolated. Plants carrying the mutant alleles displayed the typical alterations in ethylene responses in the ethylene-insensitive plants, including delayed fruit ripening. In the INRA Bordeaux Micro-Tom populations, screening up to 7,296 M2 families for 33 different genes led to the identification of 291 mutated alleles, which were further confirmed by sequencing (data not shown). Strong missense or nonsense mutations were identified for most genes. Among these were missense, truncation and splice junction mutations in three different genes involved in ascorbate biosynthesis, which resulted in reduced tolerance to high light intensity in the ascorbate-deficient mutants. These

results are described in a companion paper (Baldet et al. 2013).

### *Forward genetics approach using Micro-Tom EMS mutant collections: towards the identification of mutations underlying variations in fruit size*

Mechanisms involved in fruit size/growth are difficult to unravel because of complex interactions between cell specification, polarity, asymmetric division, rearrangement and growth. After fruit set, early growing tomato fruit undergoes a phase of cell division followed by a phase of cell expansion associated with a large increase in cell size and nucleus ploidy level (up to 512 C) due to endoreduplication (Cheniclet et al. 2005). Data mining and functional analyses *in planta* of candidate cell cycle-related genes led to the identification of several key players involved in the control of cell division and in the shift from mitotic cycle to endocycle in tomato fruit (Chevalier et al. 2011) and therefore in fruit size control. However, as demonstrated for the cell division-related *FW2.2* gene controlling the main fruit size QTL in domesticated tomato (Frary et al. 2000) and for the *fasciated* locus controlling tomato carpel number (Cong et al. 2008), forward genetics approach appears as the most powerful for the identification of new genes susceptible to regulate fruit growth in tomato fruit. Indeed, both FW2.2 and the YABBY-like transcription factor (*fasciated*) are responsible for the evolution of extreme fruit size during tomato domestication. However, they would have been hardly identified through reverse genetic approach. To date, the function of FW2.2, a negative regulator of cell division, remains unknown and no evidence has been found in *Arabidopsis* that indicates a control of carpel number by *YABBY2* gene in this species, in contrast to tomato (Huang et al. 2013).

Mutant collections offer invaluable resources for discovering new phenotypes and new allelic variants, as recently demonstrated for the *SFT* gene involved in fruit yield control in tomato (Krieger et al. 2010). We screened ca. 3500 lines of the Micro-Tom EMS mutant collection at INRA Bordeaux for fruit size (small or large fruit) or pericarp thickness (thick or thin) mutants using the MMDB database as shown on Figure 1A. Among these, 36 mutant lines were selected (21 fruit size mutants, 15 pericarp thickness mutants). Following confirmation of the phenotype, 24 mutant lines (13 fruit size mutants, 11 pericarp thickness mutants) were submitted to detailed fruit cytological (number of cell layers, cell size) and nucleus ploidy level analyses. Figure 1B shows one small fruit mutant with thin pericarp and one large fruit mutant with increased pericarp thickness. In the small fruit size mutant the number of pericarp cell layers and maximum cell sizes are clearly reduced (Figure 1C) but flow cytometry profile of breaker stage fruit nuclei is similar to that of wild type fruit (Figure 1D). In contrast,

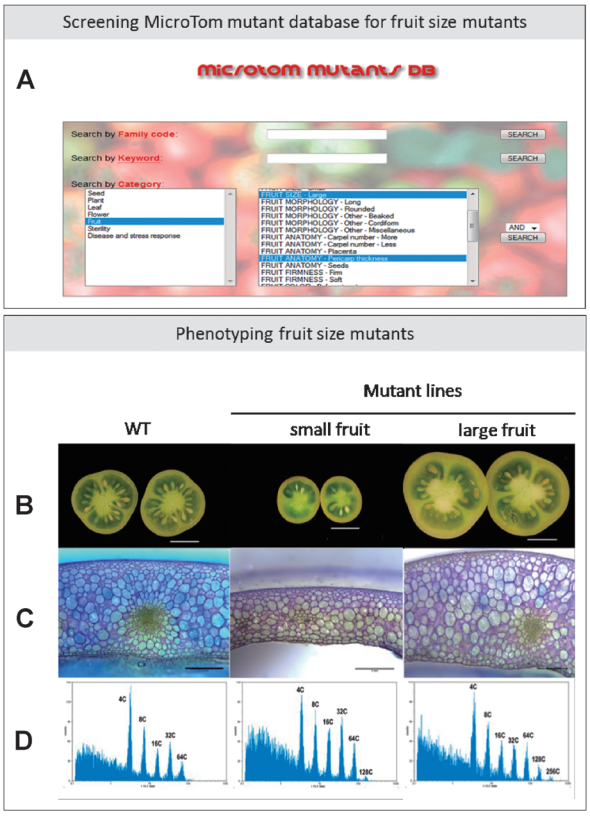


Figure 1. A: Searching Micro-Tom Mutant Database (MMDB) for both "Fruit size" and "Fruit anatomy" categories using the Boolean term "AND" to identify large fruits affected in pericarp thickness. B: Comparison of wild type (WT) and small and large fruit mutants. C: Comparison of toluidine blue stained pericarp sections from wild type (WT) fruit and small and large fruit mutants, which display the reduced number of cell layers and smaller cell size in the small fruit mutant and the increased number of cell layers and larger cell size in the large fruit mutant. D: Comparison of flow cytometry profiles of breaker stage fruit nuclei from wild type (WT) fruit and from small and large fruit mutants. Large fruit size mutant (right panel) shows a clear increase in DNA ploidy levels. Ploidy profiles of nuclei from equatorial region of pericarp from fruits at the breaker stage were obtained as described in Cheniclet et al. (2005).

in the large fruit size mutant, the number of pericarp cell layers, cell sizes and DNA ploidy levels are clearly increased (Figure 1C, D). These results illustrate how screening mutant collections can successfully contribute to the isolation of a large number of new fruit size and yield mutants. Once the initial characterization of the mutant collection and the construction of the database have been done, this strategy is relatively straightforward and can provide new genetic material for deciphering the mechanisms involved in the control of fruit growth and size.

Once mutants have been identified, the next step is the identification of the gene(s) underlying the variations in the phenotypic traits studied. Map-based cloning of Micro-Tom mutations has been greatly facilitated by the discovery and mapping of Micro-Tom SNPs (Shirasawa et al. 2010). This approach is well illustrated

by the recent identification in the INRA Bordeaux collection of a Micro-Tom mutation in the *SlCYP86A69* gene responsible for a cuticle-deficiency phenotype giving a pink appearance to the fruit (Shi et al. 2013). Interestingly, another allelic variant of the same tomato gene was discovered in parallel by the Rose group at the Cornell university (USA) through more classical forward genetics approach, which includes the cross of the cuticle-deficient mutant identified from the M82 mutant collection (Menda et al. 2004) with a more distantly-related species (Shi et al. 2013).

Though the process of map-based cloning has been considerably facilitated by the recent availability of tomato genome sequence (Tomato Genome Consortium 2012) and of large SNP genotyping arrays and high-density genetic maps (Sim et al. 2012), it often requires lengthy and tedious steps involving crosses with more or less distant polymorphic genotypes. However, crossing cultivated tomato, including Micro-Tom, with distantly-related polymorphic plant material may produce F2 offspring with highly variable phenotypic traits, including large fruit size and shape variations (data not shown), which prevents phenotyping the original fruit size mutation. Now that deep sequencing platforms provide an unprecedented possibility to sequence whole genomes with high coverage at reasonable costs, we propose to develop a new strategy to exploit the invaluable genetic diversity present in the Micro-Tom EMS mutant resources. This strategy illustrated in Figure 2 involves successive steps of: (i) crossing the mutant line of interest displaying a recessive mutation with wild type Micro-Tom (Figure 2A); (ii) phenotyping the mutant trait studied in the BC1F2 population issued from this cross. Because the genetic background of the mutant line is identical to that of the wild type Micro-Tom, except for the point mutations introduced by EMS mutagenesis, no undesirable phenotypic segregation should be observed in the BC1F2 population. In addition, using Micro-Tom, BC1F2 populations of 300 to 800 plants can easily been grown on few square meters in greenhouses (Figure 2B), making this strategy affordable to research groups with limited access to large room space in greenhouse or to open fields; (iii) deep sequencing the mutant bulks showing the mutant trait and mapping the sequence reads to the Micro-Tom reference sequence, which has been recently obtained (Aoki, personal communication, and own unpublished results). The mutation of interest is likely linked to the chromosomal region carrying SNPs displaying 100% frequency in the mutant bulk (Figure 2C), one of these SNPs being responsible for the phenotypic variation studied; (iv) once identified, the function of the genes identified can be further characterized, e.g. by analyzing additional mutant lines carrying allelic series identified by TILLING. Such strategies to identify unknown mutations have recently been shown to be highly effective in the model plants *Arabidopsis* (Hartwig et al. 2012; Schneeberger et al. 2009) and rice (Abe et al. 2011).

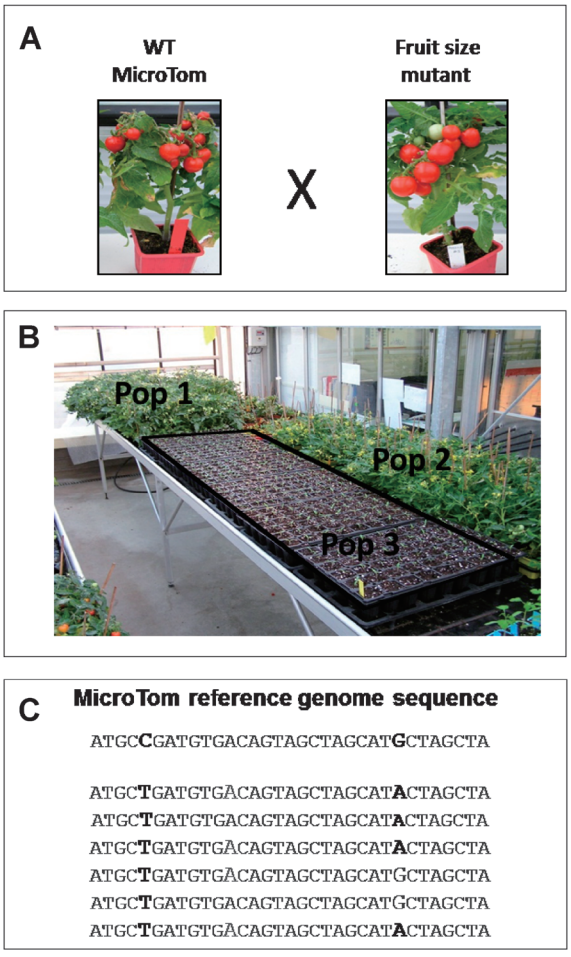


Figure 2. A: Crossing wild type plant with homozygous mutant plant displaying large fruit size/increased yield. B: View of the greenhouse showing three BC1F2 populations of ca. 300 plants each, issued from a cross between wild type Micro-Tom and different Micro-Tom fruit size mutants. Pop 1, Pop2 and Pop3 each refer to a given population at different development Pop 2 stages of Pop 3 plant culture i.e. cotyledon-first leaves (Pop 1), flowering (Pop 2) and fruiting (Pop 3) stages. C: Identification of a mutation linked to a mutant phenotype through deep sequencing. In that theoretical example, the sequences obtained by deep sequencing of a BC1F2 bulk displaying a recessive mutant phenotype (below) are compared to the Micro-Tom reference sequence (above). For the sake of clarity, only two mutations are shown. SNPs linked to the mutation responsible for the mutant phenotype (C to T) display a 100% frequency in the sequence reads while unlinked SNPs (G to A) display a lower frequency.

### *Conclusion*

The Micro-Tom EMS mutants available in both France and Japan display a large genetic diversity that makes them invaluable resources for reverse genetics approach using TILLING in tomato. Thanks to new insights into tomato genome (Tomato Genome Consortium 2012), including Micro-Tom genome (Asamizu et al. 2012; Shirasawa et al. 2010), the map-based cloning of

mutations of interest has been considerably facilitated. In addition, new strategies based on deep sequencing of EMS mutants are currently being developed. This will greatly facilitate and scale up the exploitation by forward genetic approaches of Micro-Tom EMS mutant collections for plant and fruit biology studies and for mutation breeding.

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