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Original Paper

Ethylmethanesulfonate (EMS) mutagenesis of *Solanum lycopersicum* cv. Micro-Tom for large-scale mutant screens

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Abstract Sequencing a whole genome, cataloguing full-length cDNAs and ESTs, and construction of a comprehensive mutant population are essential steps in genome projects of individual model species. Tomato is one of the most important model crops to be undertaken for study over the next few decades, and the Solanaceae Genomics Project (SOL) has begun genome projects as international collaborations. The *Solanum lycopersicum* cultivar Micro-Tom shares several advantages with *Arabidopsis*, including its small size, short life cycle (approximately 90 days), and growth normally under artificial light. Here we demonstrate an initial attempt to generate a comprehensive mutant population in Micro-Tom. A total of 3,839 M2 families derived from ethylmethanesulfonate (EMS) mutagenesis were visually phenotyped, and putative mutants were classified into 15 primary and 48 secondary categories based on the SOL database, "The Genes That Make Tomatoes".

Key words: Ethylmethanesulfonate (EMS), mutant, *Solanum lycopersicum* cv. Micro-Tom.

Tomato (*Solanum lycopersicum*), one of the world's most important crops, has a diploid genome with 12 chromosomes and a total of 950 MB of sequence, and is easily inbred. The Solanaceae genomes are highly conserved, allowing easy comparison of information from tomato with that of other solanaceous species (SOL 2004). The International Solanaceae Genomics Project (SOL) is currently underway. The SOL regards tomato as the genetic centerpiece of the Solanaceae family, since genetic and metabolic information from this species can aid in the investigation of biological mechanisms in various fruit plants.

The miniature tomato cultivar Micro-Tom, which bears small fruits, was bred by crossing the home-gardening cultivars Florida Basket and Ohio 4013-3 (Scott and Harbaugh 1989) (Figure 1). The pedigree of Micro-Tom suggests that its phenotype is caused by two major recessive mutations: *dwarf* (*d*) and *miniature* (*mnt*) (Meissner et al. 1997). It has also been suggested that the cultivar has a mutation in the *SELF-PRUNING* (*SP*) gene, leading to its determinate phenotype (Pnueli et al. 1998) (Table 1).

Micro-Tom possesses many advantages as a model system for plant genomics. Its small size, about 15 cm in height, allows dense cultivation of up to 1,357 plants per square meter (Meissner et al. 1997), and mature fruits can be harvested 70 to 90 days after sowing (Emmanuel and Levy 2002). Micro-Tom grows normally under artificial light. The small size of Micro-Tom allows experiments in confined and controlled environments that comply with safety regulations for genetically modified organisms (GMO). In addition, a highly efficient transformation procedure for Micro-Tom using *Agrobacterium* has been established (Sun et al. 2006).

Several thousand tomato mutants have been collected by the Tomato Genetics Resource Center at the University of California, Davis, and the Solanaceae Genome Network (Menda et al. 2004). However, most of these mutants are unsuitable for cultivation in experimental rooms or plant incubators because of their large size. The number of tomato genes has been estimated to range from 30,000 to 40,000, and a larger mutant population is required for saturation mutagenesis (Emmanuel and Levy 2002).

The numerous mutagenesis techniques available, including insertional mutagenesis, X-rays, gamma rays, and fast and thermal neutrons, can be categorized by their physical properties and mutagenic effects.

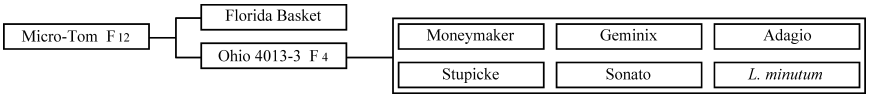


Figure 1. Pedigree of the Micro-Tom cultivar. The crossing order to produce the Ohio 4013-3 cultivar is unclear.

Table 1. Mutations that generated the Micro-Tom phenotype. The *mnt* mutation is putative and still uncharacterized. *mnt* does not affect GA metabolism but is likely to be associated with GA signaling (Marti et al. 2006).

Gene	Gene Name	Description	References
d	*dwarf*	*DWARF* gene was isolated by transposon tagging and encodes a P450 protein catalysing the C-6 oxidation of 6-deoxocastasterone to castasterone in rassinosteroid biosynthesis.	Bishop et al. 1996, 1999
mnt	*miniature*	The biological process and molecular function are unknown.	Meissner et al. 1997
SP	*SELF-PRUNING*	*SELF-PRUNING* belongs to the *CETS* family of regulatory genes (so named for the founding members *CEN*, *TFL1*, and *FT*, which are all regulators of flowering and shoot architecture) that determine the potential for continuous growth of the shoot apical meristem.	Pnueli et al. 2001

Collections of mutants induced by γ rays, which are usually created by gene truncation, are easier to screen for knockout mutants than EMS-induced mutants (Sato et al. 2006). The low mutation frequency observed in γ-ray mutagenesis is caused by high lethality due to the large scale of the deletions induced, up to 6 Mb (Naito et al. 2005). Alkylating agents such as EMS are highly effective, resulting in various types of mutants. These agents form adducts with nucleotides, causing them to mispair with their complementary bases, thus resulting in base changes after replication (Haughn and Somerville 1987; Ashburner 1990). A detailed report of mutagenesis strategies and methods has been published by Emmanuel and Levy (2002).

In the present study, we generated an EMS-induced mutation library and screened for mutants using the miniature dwarf tomato cultivar Micro-Tom, to contribute to advanced genetic studies in plant science and applied molecular breeding.

Materials and methods

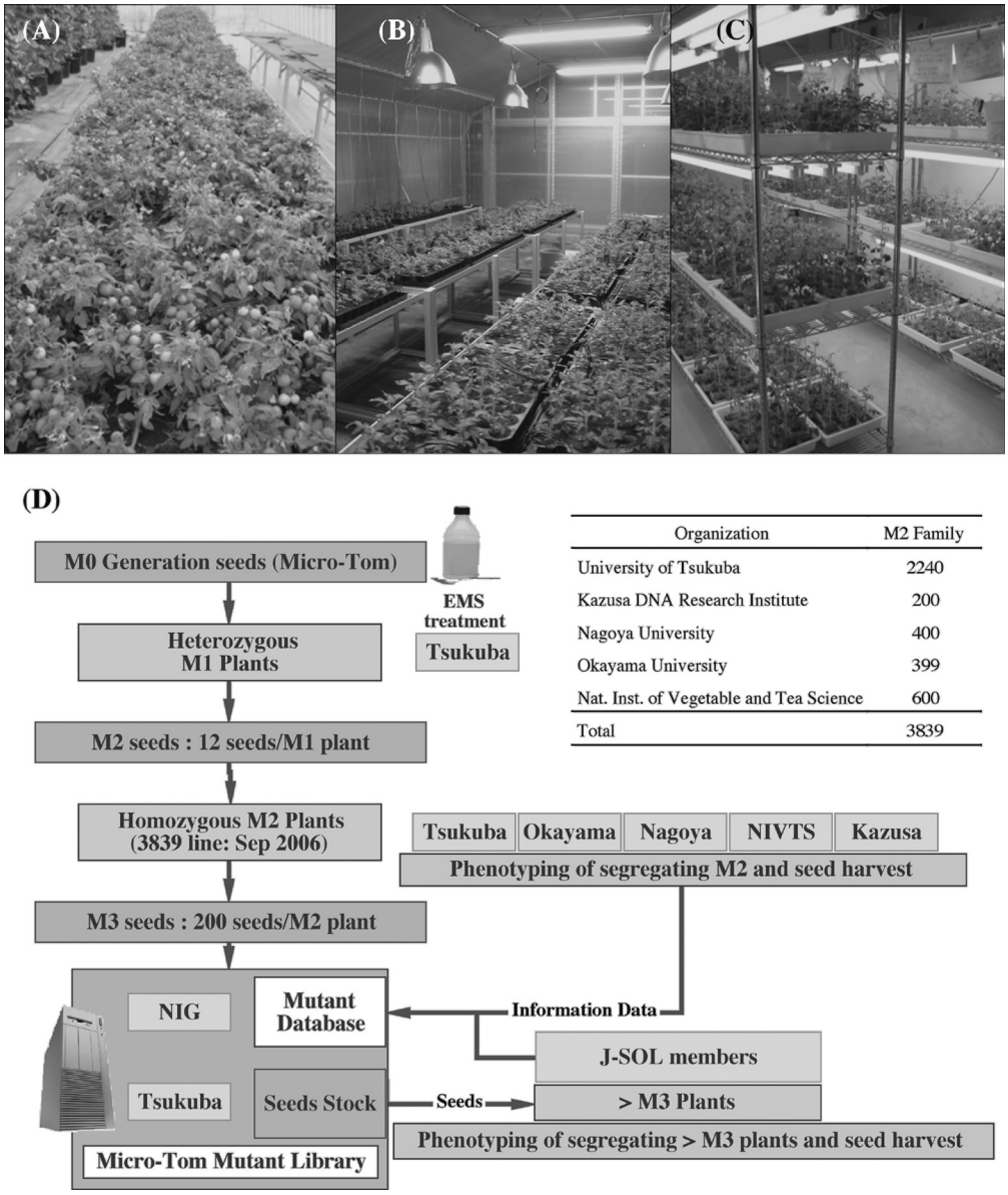
Plant material and EMS treatment

Seeds of *Solanaceae lycopersicum* cv. Micro-Tom, provided by the Kazusa DNA Research Institute, were subjected to ethylmethanesulfonate (EMS) mutagenesis. Batches of 1000–2500 imbibed seeds were sealed in nylon-mesh packs (5×8 cm), and the packs were gently shaken in 200 ml distilled water for 48 h at room temperature. The seeds were then incubated in 100 ml of a solution containing EMS (Sigma-Aldrich, St. Louis) at 0.3, 0.5, or 1% (w/v), with gentle shaking for either 24 or 48 h. In the first year of the study, seeds were treated with 0.3 or 0.5% EMS for 24 h. In the subsequent years, incubation was performed in 0.5 or 1% EMS for 24 or 48 h. After the EMS treatment, the seeds were washed twice in 200 ml of 3% sodium thiosulfate buffer for 20 min at room temperature, with gentle shaking, followed by three washes in 200 ml distilled water as above. The used EMS and wash solutions and all equipment were treated in sodium thiosulfate buffer for three days and then disposed of as regular waste. The mutagenized (M1) seeds were sown in soil in multi-pot plates (5×5 cm per a pot) and grown in a greenhouse or incubation rooms. For M2 seed production, plants were grown in small pots in a greenhouse or field. At the end of the fruit-ripening phase, the M2-generation seeds were collected either from single M1 plants to construct a family design, or in bulk. After being harvested from each fruit using a spatula, the M2 seeds were sealed into a small mesh pack and washed first with 1% hydrochloric acid (v/v) and then water for 10 min in a wash machine. The seeds were then dried at 55°C for 24 h.

Mutant screening in the M2 generation

The M2 seeds were distributed to the main Japanese SOL supporting organizations for screening of the M2 mutants and production of the next mutant generations (Figure 2), and data on the M2 mutants were collected. At the University of Tsukuba, M2-generation families (12 plants each) were planted in soil in multi-pot plates and visually phenotyped in incubation rooms. Each mutant candidate was characterized according to 15 major categories and 48 subcategories: seeds (germination, seedling lethality, slow germination), plant size (extremely small, small, and large plants), plant habit (internode length, branching, aborted growth, or other plant habit), leaf morphology (leaf width, leaf size, leaf complexity, leaf texture, or other leaf development issues), leaf color (purple, white, yellow, yellow-green, dull green/gray, or dark-green leaves or variegation), flowering (timing of flowering), inflorescence (inflorescence structure), flower morphology (homeotic mutation, organ size, organ width, or other flower morphology), flower color (white, pale-yellow, deep-yellow), fruit size (small or large fruits), fruit morphology (long or rounded fruits or other fruit morphology), fruit color (yellow, orange, or dark-red fruits or epidermis or green fruit), fruit ripening (early or late ripening), sterility (partial or full sterility), and disease and stress responses (necrosis, wilting, or other disease responses). (Menda et al. 2004).

For all EMS treatments, the lethal dose (LD) from each EMS concentration was determined by calculating the survival rate of the plants: the frequency of M1 seedlings in each EMS treatment was divided by the frequency of control seedlings



Organization	M2 Family
University of Tsukuba	2240
Kazusa DNA Research Institute	200
Nagoya University	400
Okayama University	399
Nat. Inst. of Vegetable and Tea Science	600
Total	3839

Figure 2. Establishment and cultivation of mutant lines. The M1 population was cultivated in a greenhouse at the University of Tsukuba (A). Mutant screening of the M2 population at the University of Tsukuba. The M2 population was cultivated in a greenhouse (B) or a cultivation room (C). Flow diagram showing the establishment of the Micro-Tom EMS-mutant lines (D).

treated in distilled water. The frequency of fertile M1 plants among all of the surviving plants was recorded and the germination rate of the M2 seeds assessed.

Results and discussion

Effects of EMS mutagenesis on M1 and M2 plants

In this study, 12,900 Micro-Tom seeds were treated with EMS and 3,839 lines of the EMS-mutagenized M2 population were generated. Four EMS mutagenesis protocols were performed, using 0.3, 0.5, or 1.0% EMS for 12 h or 0.5% EMS for 48 h, shown as 0.3E/12H, 0.5E/12H, 1.0E/12H, and 0.5E/48H, respectively. The frequency of M1 seedlings decreased with increasing EMS concentration. The LDs in each EMS treatment were approximately 10, 20, and 57 when 0.3, 0.5, or 1.0% EMS were used for mutagenesis, respectively (Table 3). The LDs in the *S. lycopersicum* cultivar M82 were 15 and 90 when 0.5 and 1% EMS were used for mutagenesis, respectively (Menda et al. 2004). Although the LD of M1 Micro-Tom seedlings at 1.0% EMS was lower than that of M82 (Menda et al. 2004), the frequency of fertile M1 plants at 1.0% EMS was 19%, resulting in a harvest of below 10% of the M1 seeds (Table 3). Moreover, the germination rate of M2 seeds harvested from M1 plants generated using 1.0% EMS (1.0E/12H) was much lower than with the other conditions (Table 3). Therefore, we chose the 0.5% EMS condition for further EMS mutagenesis.

Table 2. Resources related to tomato mutants and the Micro-Tom cultivar on the Web. The Tomato Mutant Archive database will be released publicly within a few years.

Name	Description	URL
Sol Genomics Network	Center of genomic information about Solanaceae resources including tomato; Cornell University (U.S.)	http://sgn.cornell.edu/index.html
Solaceae Source	The official Web site of the Solanum PBI project, containing species descriptions, specimen data (U.S.)	http://www.nhm.ac.uk/research-curation/projects/solanaceaesource//
TIGR Tomato Gene Index	Tomato gene index in TIGR; ESTs and consensus sequences for plants (U.S.)	http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=tomato
The C.M. Rick Tomato Genetics Resource Center	Large collection of Solanaceae germplasms, with a special focus University of California, Davis (U.S.)	http://tgrc.ucdavis.edu/
Genes that Make Tomatoes	Web site of a large-scale tomato mutagenesis and phenotyping project. 3,417 mutations have been catalogued (Israel)	http://zamir.sgn.cornell.edu/mutants/
MiBASE	EST and unigene dataset from the Microtom tomato variety, including GO annotations and Pathway Viewer. Full-lengch cDNA clones are available on your request from Feb 2006; Kazusa DNA Research Institute (Japan)	http://www.kazusa.or.jp/jsol/microtom/indexj.html
Tomato Mutant Archive	Micro-Tom mutant database presented by the University of Tsukuba and the National Institute of Genetics (Japan)	under construction

Table 3. Effects of EMS treatment on the Micro-Tom cultivar. The lethal dose (LD) in each EMS concentration was determined by calculating the survival rate of plants: the frequency of M1 seedlings in each EMS treatment was divided by the frequency of control seedlings (treated in distilled water). The frequency of fertile M1 plants among all of the surviving plants was recorded and the germination rate of the M2 seeds assessed.

Time (M.Y)	Cultivate condition	EMS treatment (h)	Number of M1 seeds treated	M1 seedlings (%)	LD	Fertile M1 plants (%)	Germination rate of M2 seeds (%)	Number of M2 line
Apr. 2005	Greenhouse	Distilled water (12 h)	200	80.0	Control	75.0	79.0	—
Nov. 2004	Incubation room	0.3% (12 h)	1000	72.2	10	68.4	Collecting data	494
Nov. 2004	Incubation room	0.5% (12 h)	3000	64.8	19	82.3	63.7	1600
Apr. 2005	Greenhouse	1.0% (12 h)	2400	34.7	57	19.0	32.6	158
Apr. 2005	Greenhouse	0.5% (12 h)	2500	64.3	20	55.9	Collecting data	899
May. 2006	Greenhouse	0.5% (12 h)	1500	65.1	19	70.4	Collecting data	688
Sep. 2006	Greenhouse	0.5% (48 h)	2500	52.8	34	Collecting data	Collecting data	Collecting data
		Total/average	12900	57.4	28	51.9	46.9	3839

Storage of data in a web database

We obtained 3,839 M2 lines from 7,404 M1 individual plants and assigned a unique number to each M2 line. Mutant screening of the M2 generation was performed by the JSOL primary organization in 2005 (Figure 2). A total of 382 plants showing morphological alterations were selected as mutant candidates based on the first screening. The screening data, including phenotypic information and photographs of these candidates, were deposited in a pilot database named TOMATOMA (Tomato Mutant Archives) at the National Institute of Genetics (Figures 2, 3). Subsequently, the M3 and M4 progeny of the mutant candidates were subjected to a second screening in incubation rooms. Certain lines were selected as morphological mutants for further analysis, and segregation data suggest that these mutants resulted from monogenic mutations (Figure 3).

Figure 3 shows a representative mutant in each of the primary categories. The screening identified putative Micro-Tom mutants with dark-yellow (line 3139, Figure 3B) and pale-yellow flowers (line 3322; Figure 3C), as compared to the white flowers of wild-type plants (Figure 3A). The trichomes of line 0794 are much shorter than those of wild-type plants (Figure 3D). In the fruit size and morphology categories, lines 0221 and 2233 produce small fruits (Figure 3E, F) and line 0206 produces long fruits (Figure 3G). In the leaf-color category, lines 0066 and 0043 exhibit variegated leaves (Figure 3H, I), and lines 0132 (Figure 3J), 0289 (Figure 3K), 2207 (Figure 3L), and 3139 (Figure 3M) show deformed and developed, white (albino), pale-green, and dark-green leaves, respectively.

In brief, 3,839 M2 populations derived from ethylmethanesulfonate (EMS) mutagenesis were obtained, and some of the populations were visually phenotyped. A comprehensive mutant population is an effective resource for studying the genetic bases of traits. EMS mutagenesis gives rise to a high mutation frequency without preference for specific genomic regions, and generates many alleles that allow the isolation of null phenotypes. The point mutations can be identified by the TILLING (targeting induced local

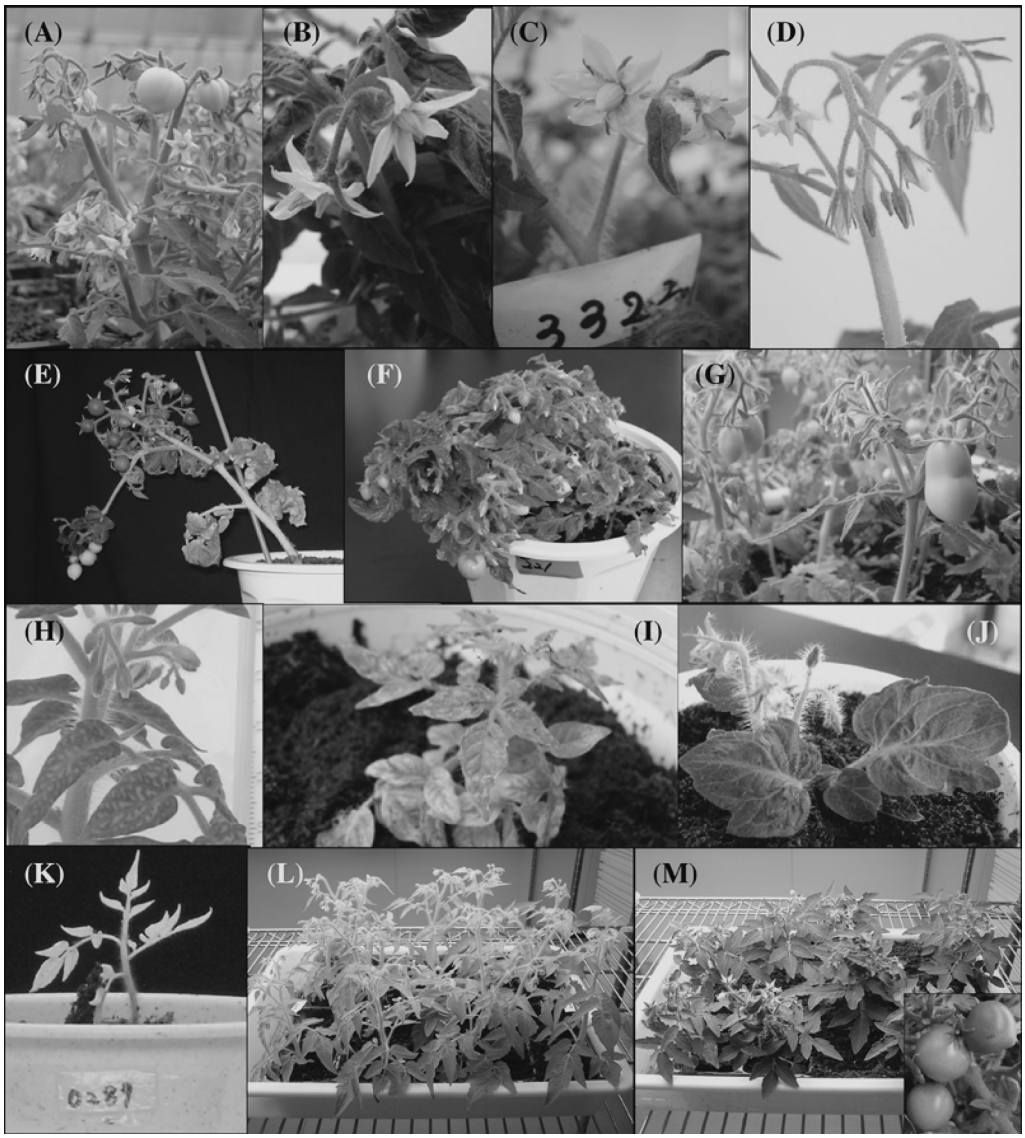


Figure 3. Mutant phenotypes obtained by EMS mutagenesis. (A) Wild type; (B) dark-yellow flowers; (C) pale-yellow flowers; (D) very short trichomes; (E) small fruits; (F) bushy, blanching, and many small fruits; (G) long fruits; (H) variegated leaves; (I) deformed and developed leaves; (J) variegated leaves; (K) albino; (L) pale-green leaves; (M) dark-green leaves.

lesions in genome) method (McCallum et al. 2000a, b), and the extraction of genomic DNA from the M2 and M3 populations for TILLING analysis is currently underway. We will be increasing the EMS-mutagenized M2 population to about 5000 and will continue the phenotyping of the EMS-mutagenized Micro-Tom. The JSOL is planning to integrate the Micro-Tom mutant population, including both the EMS-mutagenized and the γ ray-irradiated mutants (Matsukura et al. this issue), and release them through the JSOL in the near future.

At present, we are establishing a Micro-Tom mutation library by EMS treatment and creating a database with the aim of supplying experimental resources and information to research communities. This article reports the latest progress in our work, undertaken as part of JSOL.

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