*Plant Biotechnology* 24, 39–44 (2007)

**Original Paper**

# Generation of gamma irradiation-induced mutant lines of the miniature tomato (*Solanum lycopersicum* L.) cultivar 'Micro-Tom'

Chiaki Matsukura[1], Isao Yamaguchi[1,a], Mami Inamura[1], Yusuke Ban[1], Yoshie Kobayashi[1], Yong-gen Yin[1], Takeshi Saito[1], Chikara Kuwata[2], Shunsuke Imanishi[3], Shigeo Nishimura[1,*]

[1] Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan;
[2] Crop Breeding Institute, Chiba Prefectural Agriculture Research Center, Chosei, Chiba 299-4335 Japan;
[3] National Institute of Vegetable and Tea Science, National Agriculture and Food Research Organization, Tsu, Mie 514-2392, Japan
* E-mail: nshigeo@agbi.tsukuba.ac.jp Tel & Fax: +81-29-853-6620

Received November 24, 2006; accepted January 10, 2007 (Edited by Y. Hotta)

**Abstract** One direct way to identify a gene and its function is a forward genetic approach based on mutation analyses. To accumulate genetic resources for breeding and functional genomics in the tomato, we generated 6,347 lines of an $M_2$ population with 300 Gy of gamma-ray irradiation in the inbred miniature dwarf variety 'Micro-Tom.' In total, 6,301 $M_2$ lines were screened based on morphological alteration and brix-aberration, and 237 lines were selected as mutant candidates at the first screening. Subsequent screening of the self-fertilized $M_3$ and $M_4$ progeny yielded 24 lines of morphological mutants and 11 lines of aberrant brix mutants. Segregation data suggested that most of the mutant lines had single recessive mutations, with the exception of two lines. The chlorophyll mutation ratio in germinated $M_2$ seedlings was 0.37% and the actual mutant frequency was 0.5%. The selected mutant lines exhibited a wide range of mutations, including whole plant properties with a severe phenotype, which allowed for more efficient screening of knockout mutants. Three characterized mutants, *pale leaf*, *pink*, and *short root* are also described.

**Key words:** Fruit brix, gamma-irradiated mutagenesis, Micro-Tom, *Solanum lycopersicum* L.

Globally, the tomato (*Solanum lycopersicum* L.) (Solanaceae) is one of the most important crops to the fresh vegetable market and the food processing industry. It is also an excellent model plant for analyzing fruit development, ripening, and metabolism of novel metabolites in species having a fresh berry fruit. Additionally, the tomato has many advantages to other Solanaceae, including a small diploid genome (930 Mb, n=12) with numerous mapped traits, developed DNA markers, and EST clones (Tanksley 1993). For these reasons, the International Solanaceae Genomics Project (SOL) was established in 2004, and the genome sequence of tomato is the next target, after *Arabidopsis* and rice. Full-length EST sequencing revealed that about 30% of tomato cDNAs are specific to tomato and do not share significant similarity with *Arabidopsis*, but the function of most of the genes is still unknown (Van der Hoeven et al. 2002; Yamamoto et al. 2005). Upon completion of the genome sequence, the next challenge will be to elucidate gene function, which can be directly estimated by analyzing the phenotype of a mutant in which a given gene is mutated. This classical forward genetics approach remains important.

In tomato, nearly 1,000 monogenic mutants have been collected in the Tomato Genetics Resource Center at the University of California, Davis (see the URL http://tgrc.uctavis.edu), and more than 3,000 mutations have been cataloged on the Web site of the Solanaceae Genome Network (http://Zamir.sgn.cornell.edu/mutants/) (Menda et al. 2004). However, since a current rough estimation suggests that the number of genes in the tomato genome ranges from 30,000 to 35,000, the current mutant population is insufficient for saturated mutagenesis. Considerable efforts have been made to generate a large $M_2$ population through chemical (e.g., EMS), physical (e.g., X-ray or fast-neutron irradiation), and insertional (e.g., transposable elements or T-DNA) mutagenesis (Hildering and Verkerk 1965; Knapp et al. 1994; Thomas et al. 1994; Meissner et al. 1997; Li et al. 2001; Emmanuel and Levy 2002; Menda et al. 2004). The mutagenesis methods vary in mutant frequency, spectrum, advantages in screening, and the usability of

[a] Present address: Plant Genome Center Co., Ltd., Tsukuba, Ibaraki 305-0856, Japan
Abbreviations: cDNA, complementary DNA; EMS, ethyl methane sulfonate; EST, expressed sequence tag; LD; lethal dose.
This article can be found at http://www.jspcmb.jp/

the treatment. Therefore, it is very important to generate a wide range of mutant populations induced by various mutagenesis methods.

For physical mutagenesis, X-rays, gamma rays, and fast and thermal neutrons are widely used. In this paper, we report the generation of a gamma-irradiated $M_2$ population and the screening of the mutants using the miniature dwarf tomato cultivar 'Micro-Tom'. Gamma-ray mutagenesis can be expected to yield severe phenotypic mutations because it causes large-scale deletions, and occasionally, chromosome reconstitution. Additionally, low and medium doses of gamma rays cause a relatively high proportion of useful mutants with normal yielding properties (Dumanović et al. 1968). Micro-Tom was used in this work because it is suitable for large-scale and high-throughput mutagenesis due to its small size (approximately 15 cm) and rapid life cycle (70–90 days; Scott and Harbaugh 1989; Emmanuel and Levy 2002). The $M_2$ population and the mutant library generated in this work will contribute to the genetic resources for breeding and functional genomics in tomato.

## Materials and methods

### *Mutagenesis with gamma-ray irradiation*

In total, 6,000 'Micro-Tom' seeds were irradiated with 300 Gy of gamma rays from a cobalt source at the Institute of Radiation Breeding, National Institute of Agrobiological Sciences (NIAS; Ohmiya, Ibaraki, Japan) in 2003. The radiation dose was determined based on the lethal dose (LD) causing a 30% reduction in seed germination. The $M_1$ seeds were sown in culture soil in nursery cell trays in a greenhouse. Two weeks after sowing, the germination ratio and number of chlorophyll mutants were determined to examine the mutation frequency affecting those events. $M_1$ seedlings were transplanted 3 weeks after germination and cultivated in the field at the Agricultural Research Center of the University of Tsukuba and in the greenhouse at the Crop Breeding Institute, Chiba Prefectural Agriculture Research Center. $M_2$ seeds were gathered from the first to third flower truss of each $M_1$ plant as independent lines. A total of 6,347 $M_2$ lines was obtained (Figure 1).

### *Mutant screening in the $M_2$ and $M_3$ generations*

Mutant screening on the $M_2$ population was carried out in collaboration with the National Institute of Vegetable and Tea Science, National Agriculture and Food Research Organization and the Chiba Prefectural Agriculture Research Center from 2004 to 2005. Three indivisuals from each $M_2$ line were transplanted to the field at these institutions with nonirradiated control plants and screened as shown in Figure 2A and 2B. The selected mutant candidates were phenotyped morphologically based on following categories: leaf morphology, leaf color, fruit morphology, fruit size, fruit color, ripening, flower morphology, plant size, and other plant phenotype (e.g., branching, internode length, root morphology). We also carried out brix-based screening on all of the $M_2$ lines to obtain sugar-content mutants. Fruit brix (%) at the mature red stage was measured by a refractometer in the field. The lines deviating more than 2% from control fruits cultivated in a same block were selected as mutant candidates. To determine the reproducibility of the phenotypes and for seed multiplication, self-fertilized $M_3$ and $M_4$ (only for the brix mutation lines) progeny from each $M_2$ candidate were cultivated in a hydroponics system in the greenhouse, which evened out the cultivation conditions (Figure 2C). Four indivisuals from each line were cultivated with nonirradiated control plants and the inheritance of the mutant phenotypes was confirmed.

## Results

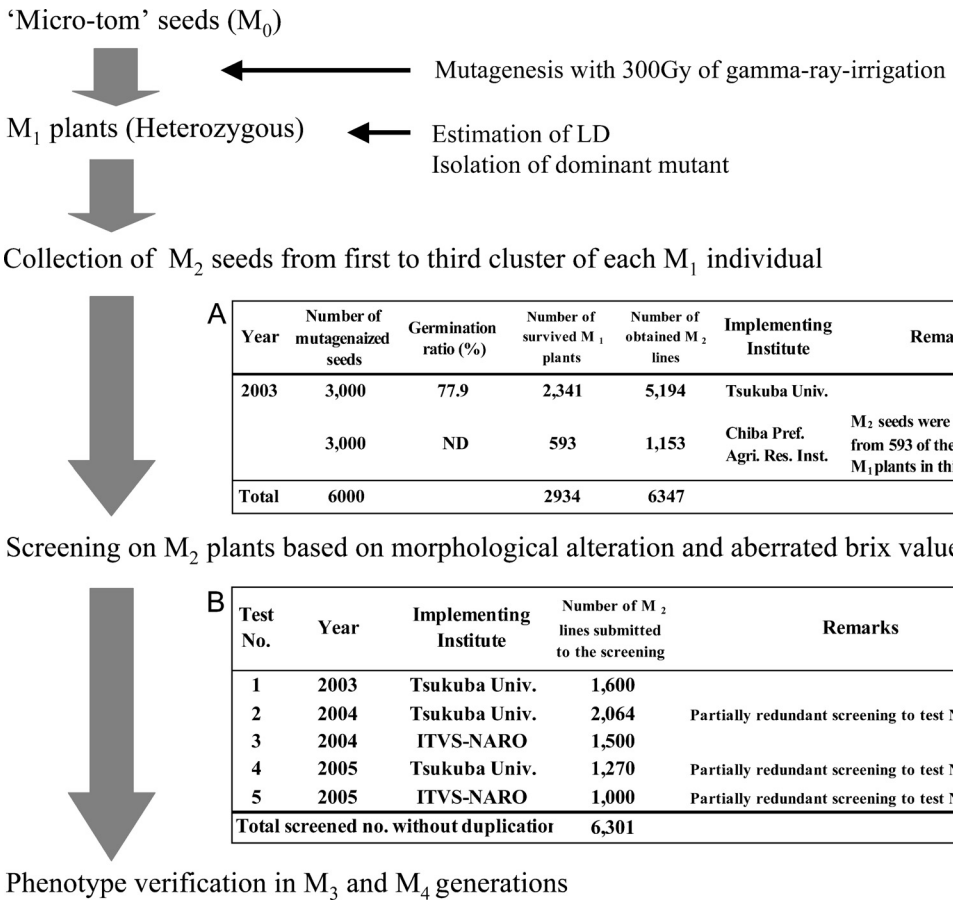
### *Generation of the $M_2$ population*

Before constructing the mutant population, we estimated the LD dose effect of gamma irradiation on Micro-Tom seeds. Three conditions, 200, 300, and 400 Gy, were tested and 300 Gy was determined as the optimal irradiation dosage resulting in a 30% reduction in seed germination (data not shown). The actual germination ratio of $M_1$ seeds in this study was 77.9%. We obtained 6,347 $M_2$ lines in total from 2,934 $M_1$ individuals in 2003. We subjected 6,301 $M_2$ lines to the mutant screening (Figure 1, 2A and 2B). Of the germinated $M_2$ seedlings, 0.37% were chlorophyll mutants. A total of 237 lines, 84 showing morphological alterations and 153 showing aberrant brix values, was selected as mutant candidates at the first screening. These mutant candidates were categorized as follows: leaf morphology, 12 lines; leaf color, 7 lines; fruit morphology, 14 lines; fruit size, 19 lines; fruit color, 4 lines; ripening, 1 line; flower morphology, 2 lines; plant size, 11 lines; other plant phenotype, 14 lines; high-brix value, 119 lines; and low-brix value, 34 lines.

### *Mutant screening of the $M_2$ and $M_3$ generations*

The mutant candidates were subsequently subjected to a second screening of the $M_3$ and $M_4$ progeny in the greenhouse. We have checked the inheritance of the mutant phenotype in about 156 lines to date and selected 24 lines as morphological mutants (Table 1) and 11 lines as brix-value mutants (Table 2 and Figure 2C). Two lines of the morphological mutants also showed aberrant fruit brix values. Segregation data suggested that most of the mutant lines had single recessive mutations, except for two lines isolated from the same $M_1$ plant that exhibited pigtail-shaped fruit. The mutant phenotypes for each category in Table 1 are shown in Figure 3.

Among these lines, we have further characterized several lines. "*Pale leaf*" (Figure 3B) exhibited a lighter and more green–yellow leaf and fruit color than wild-type plants. Interestingly, the carotenoid content and expression of carotenoid biosynthetic genes in the mutant were similar to those of control plants (Imanishi




A

| Year | Number of mutagenaized seeds | Germination ratio (%) | Number of survived $M_1$ plants | Number of obtained $M_2$ lines | Implementing Institute | Remarks |
|---|---|---|---|---|---|---|
| 2003 | 3,000 | 77.9 | 2,341 | 5,194 | Tsukuba Univ. | |
| | 3,000 | ND | 593 | 1,153 | Chiba Pref. Agri. Res. Inst. | $M_2$ seeds were haevested from 593 of the survived $M_1$ plants in this work. |
| Total | 6000 | | 2934 | 6347 | | |

B

| Test No. | Year | Implementing Institute | Number of $M_2$ lines submitted to the screening | Remarks |
|---|---|---|---|---|
| 1 | 2003 | Tsukuba Univ. | 1,600 | |
| 2 | 2004 | Tsukuba Univ. | 2,064 | Partially redundant screening to test No.1 |
| 3 | 2004 | ITVS-NARO | 1,500 | |
| 4 | 2005 | Tsukuba Univ. | 1,270 | Partially redundant screening to test No.1 |
| 5 | 2005 | ITVS-NARO | 1,000 | Partially redundant screening to test No.1 and 2 |
| Total screened no. without duplicatior | | | 6,301 | |

Figure 1. Flow diagram showing the generation of gamma irradiation-induced mutant lines. Panels indicate the number of mutagenized Micro-Tom seeds and the $M_2$ lines obtained (A) and the number of $M_2$ lines and years of the first mutant screening (B). ITVS-NARO: National Institute of Vegetable and Tea Science, National Agriculture and Food Research Organization.

et al. unpublished data). "*Pink*" (Figure 3F) set pink-colored fruits. Measurement of the external fruit color gave a "b" value, which indicates a blue to yellow scale, and did not increase after the breaker stage and reached only one-half that of wild-type fruit even at the mature red stage. Pigment content in the pericarp was also low in mutant fruits compared to control fruits, resulting in the surface color (Imanishi, unpublished data). "*Short root*" exhibited extremely shortened roots and a spindly stem. The average pollen fertility of the homozygous mutant was less than 10%, resulting in male sterility (Figure 3J). We will continue to characterize the remaining mutants isolated in this work.

## Discussion

We generated 6,347 lines in the gamma-irradiated $M_2$ population. Our morphological and brix-based screening of the population yielded 24 lines of morphological mutants and 8 lines of brix mutants. In order to confirm that these mutations arose from new alleles, further phenotyping and genetic analyses of the mutated loci are needed. In this mutagenesis, 0.37% of the germinated $M_2$ seedlings were chlorophyll mutants. The actual mutation frequency was about 0.5% (32/6,347) and is a rough index of the mutagenesis frequency of gamma irradiation. Sato et al. (2006) reported the estimated rate of gamma-ray mutagenesis to be one per 6.19 Mb. This rate is relatively lower than the mutagenesis rates of chemical mutagens such as EMS. Most of the gamma ray-induced mutants are caused by large-scale deletions of up to 6 Mb (Naito et al. 2005). The lower mutation frequency may be due to the lethality of such large deletions. However, since most of the gamma ray-induced mutations involve gene truncation, it allows for more efficient screening of knockout mutants than EMS mutagenesis (Sato et al. 2006). In contrast, EMS treatment mostly causes random G/C to A/T transitions and the frequency of truncated mutations is under 5% (Greene et al. 2003), which should result in leaky mutations in a large percentage. It is highly likely that undetected mutants remained in the population because of deficient screening. Further screening will yield additional mutants.

One disadvantage of gamma-ray mutagenesis is that the reverse genetics approach is not available for

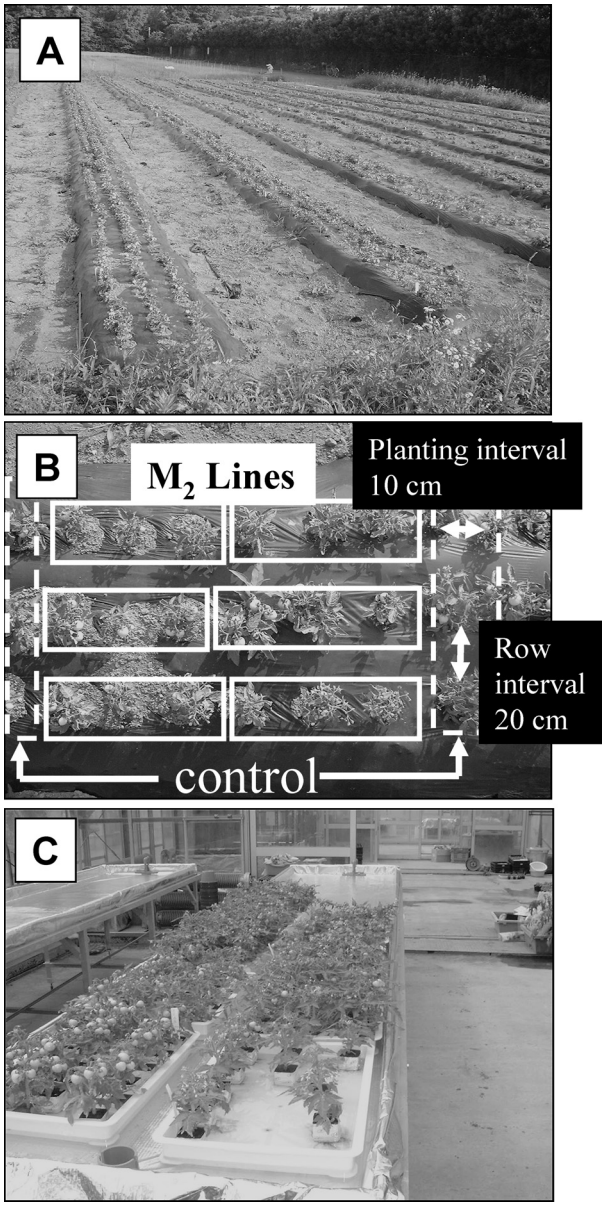



Figure 2. Mutant screening in the field and the greenhouse. The first screening is of the $M_2$ population in the field (A). Three indivisuals from each $M_2$ line were transplanted in the fields with nonirradiated control plants and screened based on morphological and fruit-brix alterations (B). The self-pollinated $M_3$ and $M_4$ progeny of each $M_2$ mutant candidate were cultivated in a hydroponics system with nonirradiated control plants in the greenhouse to verify the inheritance of the mutant phenotypes (C).

identifying and isolating a deletion-mutated gene. However, more recently, the TILLING (targeting induced local lesions in genome) method was developed to identify EMS-induced point mutations (McCallum et al. 2000a, b) and applied to several crops, including tomato (Emmanuel and Levy 2002; Till et al. 2004). The TILLING method is effective at detecting gamma ray-induced 2–4-bp deletions (Sato et al. 2006). Furthermore, Li et al. (2001) demonstrated the effectiveness of a PCR-based reverse genetic approach

Table 1. Number of mutant lines in each phenotype category

| Phenotype category | Number of mutant lines |
|---|---|
| Leaf morphology | 3 |
| Leaf color | 3 |
| Fruit morphology | 4 |
| Fruit size | 2 |
| Fruit color | 4 |
| Ripening | 1 |
| Flower morphology | 2 |
| Plant size | 4 |
| Other plant type | 1 |
| High fruit brix | 10 |
| Low fruit brix | 1 |
| Total | 35 |

Table 2. Mutant lines exhibiting aberrant fruit brix

| Characteristic | Line | Brix (%) | Tested generations |
|---|---|---|---|
| Wild type | — | 5.8±1.48** | — |
| High brix | *hbx-1* | 10.1±1.62** | $M_2$-$M_4$ |
| | *hbx-2* | 9.4±1.47** | $M_2$-$M_4$ |
| | *hbx-3* | 9.4±1.05** | $M_2$-$M_4$ |
| | *hbx-4* | 10.0±1.3** | M2-$M_4$ |
| | *hbx-5* | 9.0±1.69** | $M_2$-$M_4$ |
| | *hbx-6* | 8.9±0.49** | $M_2$-$M_4$ |
| | *hbx-7* | 10.9±2.59** | $M_2$-$M_3$ |
| | *hbx-8* | 8.6±1.16** | $M_2$-$M_3$ |
| | *hbx-9* | 8.6±0.51** | $M_2$-$M_4$ |
| | *hbx-10* | 8.6±0.55** | $M_2$-$M_3$ |
| Low brix | *lbx-1* | 3.9±0.37* | $M_2$-$M_4$ |

Values are fruits brix (%)±SE ($n$=5). * P<0.05, ** P<0.01 versus wild type.

for detecting mid-sized deletions (~1 kbp) caused by fast-neutron irradiation. Although it still necessary to expand our detection ability for large-scale deletions, these strategies will be applicable to the identification of gamma ray-induced mutations.

Mutant resources have contributed to plant breeding as well as to functional genetics. Commercial crop varieties bred from mutant resources are limited because most are not of any agronomic interest. Here, we selected fruit-brix mutants (Table 2). QTL analyses using introgression lines of *Lycopersicon pennellii* in the cultivated variety revealed at least two loci involved in the modification of brix in tomato, and subsequent mapping identified one of those loci as a fruit apoplastic invertase (Eshed and Zamir 1995; Fridman et al. 2000, 2002). However, another locus has not been defined and mutants of these loci have not been isolated. Our aberrant-brix mutants will extend the genetic resources with an agronomic value as well as aid in the identification and isolation of brix-regulating genes in tomato. Finally, we will release the $M_2$ population generated in this work through the Japan Solanaceae Consortium in the near future.

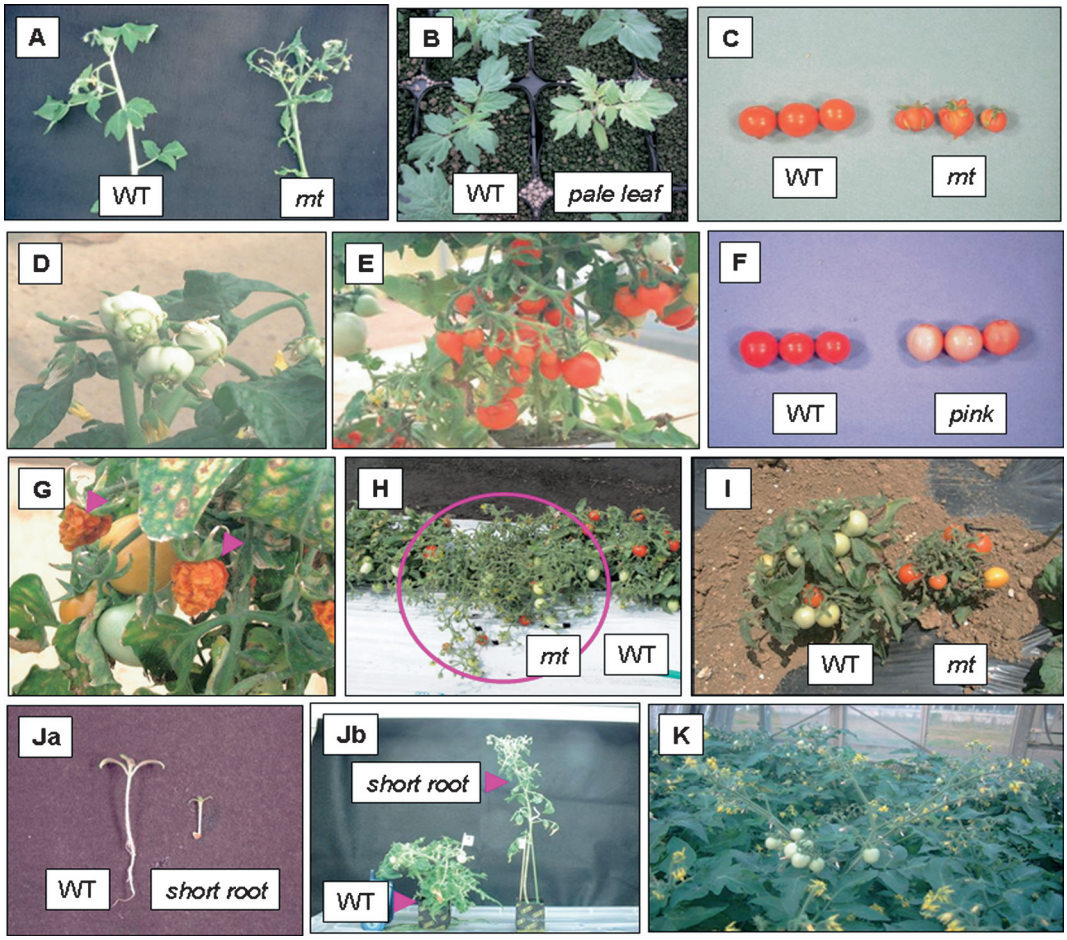



Figure 3. Morphological mutants. Samples represent each category listed in Table 1. (A) leaf morphology: narrow leaf, (B) leaf color: *pale leaf*, (C) fruit morphology: fused sepal with fruit, (D) fruit morphology: imbricate fruit, (E) fruit size: small fruit, (F) fruit color: *pink*, (G) ripening: early ripening and shrunken fruit (indicated by arrows), (H) flower morphology: abnormal inflorescence (inside the circle), (I) plant size: dwarf, (J) other plant phenotype: *short root* (a) and spindly stem (b), (K) other plant phenotype: long peduncle. WT: wild-type 'Micro-Tom'; *mt*: mutant.

## Acknowledgments

We thank the Institute of Radiation Breeding, NIAS, for allowing us to use their equipment. This work was supported in part by the grants "Research projects utilizing advanced technologies in agriculture, forestry and fisheries" from the Japanese Ministry of Agriculture, Forestry and Fisheries, "Grant-in-Aid for Scientific Research" from the Japanese Society for the Promotion of Science and "21st Century COE program" from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

## References

Dumanović J, Denić M, Jovanović C, Ehrenberg L (1968) Radiation-induced heritable variation of quantitative characters in wheat. *Hereditas* 62: 221–238

Emmanuel E, Levy AA (2002) Tomato mutants as tools for functional genomics. *Curr Biol* 5: 112–117

Eshed Y, Zamir D (1995) An introgression line population of *Lycopersicon pennellii* in the cultivated tomato enables the identification and fine mapping of yield-associated QTL. *Genetics* 141: 1147–1162

Fridman E, Pleban T, Zamir D (2000) A recombination hotspot delimits a wild-species quantitative trait locus for tomato sugar content to 484 bp within an invertase gene. *Proc Natl Acad Sci USA* 97: 4718–4723

Fridman E, Liu YS, Carmel-Goren L, Gur A, Shoresh M, Pleban T, Eshed Y, Zamir D (2002) Two tightly linked QTLs modify tomato sugar content via different pathways. *Mol Gen Genet* 266: 821–826

Greene EA, Codomo CA, Taylor NE, Henikoff JG, Till BJ, Reynolds SH, Enns LC, Burtner C, Johnson JE, Odden AR, Comai L, Henikoff S (2003) Spectrum of chemically induced mutations from a large-scale reverse-genetic screen in *Arabidopsis*. *Genetics* 164: 731–740

Hildering GJ, Verkerk K (1965) Chimeric structure of the tomato plant after seed treatment with Ems and X-rays. In: *The Use of Induced Mutations in Plant Breeding*. Pergamon Press, Oxford, pp 317–320 (Rep FAO and IAEA)

Knapp S, Larondelle Y, Robberg M. Furtek D, Theres K (1994) Transgenic tomato lines containing Ds elements at defined genomic positions as tools for targeted transposon tagging. *Mol Gen Genet* 243: 666–673

Li X, Song Y, Century K, Straight S, Ronald P, Dong X, Lassner M, Zhang Y (2001) A fast neutron deletion mutagenesis-based reverse genetics system for plants. *Plant J* 27: 235–242

McCallum CM, Comai L, Greene EA, Henikoff S (2000a) Targeted screening for induced mutations. *Nature Biotechnol* 18: 455–457

McCallum CM, Comai L, Greene EA, Henikoff S (2000b) Targeting induced local lesions in genomes (TILLING) for plant functional genomics. *Plant Physiol* 123: 439–442.

Meissner R, Jacobson Y, Melamed S, Levyatuv S, Shalev G, Ashri A, Elkind Y, Levy A (1997) A new model system for tomato genetics. *Plant J* 12: 1465–1472

Menda N, Semel Y, Peled D, Eshed Y, Zamir D (2004) In silico screening of a saturated mutation library of tomato. *Plant J* 38: 861–872

Naito K, Kusaba M, Shikazono N, Takano T, Tanaka A, Tanisaka T, Nishimura M (2005) Transmissible and nontransmissible mutations induced by irradiating *Arabidopsis thaliana* pollen with γ-rays and carbon ions. *Genetics* 169: 881–889

Sato Y, Shirasawa K, Takahashi Y, Nishimura M, Nishio T (2006) Mutant selection from progeny of gamma-ray-irradiated rice by DNA heteroduplex cleavage using *Brassica* petiole extract. *Breeding Sci* 56: 179–183

Scott JW, Harbaugh BK (1989) A miniature dwarf tomato. MICRO-TOM – a miniature dwarf tomato. *Agricultural Experiment Station, Institute of Food and Agricultural Sciences, University of Florida, Circular* S-370: 1–6

Tanksley S (1993) Linkage map of tomato (*Lycopersicon esculentum*) (2N=24). In: O'Brien J (ed) *Gengetic Maps: Locus Maps of Complex Genomes*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp 6.39–6.60

Thomas CM, Jones D, English JJ, Carroll BJ, Bennetzen JL, Harrison K, Burbidge A, Bishop GJ, Jones JDG (1994) Analysis of the chromosomal distribution of transposon-carrying T-DNAs in tomato using the inverse polymerase chain reaction. *Mol Gen Genet* 242: 573–585

Till JB, Reynolds HS, Weil C, Springer N, Burtner C, Young K, Bowers E, Codomo AC, Enns CL, Odden AR, Greene AE, Comai L, Henikoff S (2004) Discovery of induced point mutations in maize genes by TILLING. *BMC Plant Biol* 4: 12

Yamamoto N, Tsugane T, Watanabe M, Yano K, Maeda F, Kuwata C, Torki M, Ban Y, Nishimura S, Shibata D (2005) Expressed sequence tags from the laboratory-grown miniature tomato (*Lycopersicon esculentum*) cultivar Micro-Tom and mining for single nucleotide polymorphisms and insertions/deletions in tomato cultivars. *Gene* 356: 127–134

Van der Hoeven R, Ronning C, Giovannoni J, Martin G, Tanksley S (2002) Deductions about the number, organization, and evolution of genes in the tomato genome based on analysis of a large expressed sequence tag collection and selective genomic sequencing. *Plant Cell* 14: 1441–1456