Induced Mutagenesis and Natural Genetic Variation in Tomato 'Micro-Tom'

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Abstract

Tomato is a good example of a successful use of mutations affecting major genes for plant breeding, which are also valuable for gene discovery and physiological studies. However, mutant alleles are only currently known for an insignificant fraction of the about 35,000 genes in the tomato genome. Large scale mutagenesis and introgression of natural genetic variation from the wild *Lycopersicon* species into a genetic model system, such as 'Micro-Tom' (MT), can be useful to fill this gap. MT has the advantages of little space requirements and rapid cycle. Here we present the use of MT to explore natural genetic variation in tomato as well as a reassessment of the methodology for large scale tomato mutagenesis based on different agents (chemical and physical) and targets (seeds and pollen). The best parameter for dose adjustment of mutagen was the fertility of M1 plants, whereas the germination of treated-seed and that of pollen were found to be poor indicators of mutagenic efficiency. Using seeds treated with 0.7% EMS and pollen treated with 80 Gy gamma-ray it was possible to isolate a large number of mutants, including some dominant non-chimeric ones in the M1 generation derived from mutagenized pollen. Some mutations were allelic to previously known ones, but many others are novel and could be use for tomato breeding (e.g. high Brix) and basic studies. Furthermore, natural genetic variation affecting diverse developmental process and metabolic pathways was introgressed into MT from wild species. MT was a suitable model for a fast an inexpensive screening and characterization of advantageous mutations and natural genetic variation, which could be further transferred to elite cultivars or hybrids.

INTRODUCTION

Tomato is a good example of the successful use of mutations affecting major genes for plant breeding. Among such genes stand *self-pruning* (*sp*), *ovate* (*o*), *uniform fruit* (*u*) and *jointless2* (*j2*), whose recessive alleles are widely utilized in the tomato processing industry to produce determinate compact plants (*sp*) with elongated fruits (*o*) lacking green shoulders (*u*), which are also more adequate for mechanical harvesting (*j2*) (Rick, 1986; Stevens and Rick, 1986). In some cases, such mutations lead to the discovery of their corresponding gene function, which are important molecules controlling plant development (Pnueli et al., 1998; Mao et al., 2000; Liu et al., 2002).

Despite the interest of mutations in tomato for basic and applied research, only a few dozen mutants have been heretofore characterized (http://tgrc.ucdavis.edu/), whereas the number of genes in the tomato genome is estimated at 35,000 (Van der Hoeven et al., 2002). Large-scale generation of mutants and their subsequent characterization have been performed to start filling this gap (http://zamir.sgn.cornell.edu/mutants/; Menda et al., 2004). The demand for mutants will become increasingly higher in order to assign a function to the large volume of sequences that the tomato genome sequencing project is currently generating (http://www.sgn.cornell.edu/). A small and fast-growing genotype, such as 'Micro-Tom', could be useful as a genetic model to fulfill this task (Meissner et al., 1997). 'Micro-Tom' (MT) is a miniature dwarf determinate tomato cultivar, originally

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bred for home gardening purposes (Scott and Harbaugh, 1989). It differs from standard tomato cultivars primarily by two recessive genes: dwarf (*d*), and probably, miniature (*mnt*). The determinate phenotype of MT is caused by a mutation in the *sp* gene (Martí et al., 2006). On top of the relatively small tomato genome (950 Mb) MT shares features with *Arabidopsis* that make it a suitable model system, such as small size (8 cm when grown in 50-100 ml pots) and short life cycle (70-90 days from sowing to fruit-ripening).

Besides induced mutations, natural genetic variation is a valuable resource for functional genomics in tomato, especially for the loci where cultivated tomatoes already harbor knock-out (i.e. non-functional) versions of the genes (Alonso-Blanco and Koornneef, 2000). Such variation can be found in the wild *Lycopersicon* species, most of which are inter-fertile and amenable to crossing with cultivated tomato (Stevens and Rick, 1986). These species are valuable sources of quantitative trait loci (QTL) and allelic variation for major genes (Bai and Lindhout, 2007). These wild relatives of tomato evolved in a tropical region with a large altitudinal range (the Peruvian Andes), thus, representing an invaluable source of genes related to different adaptation strategies (Warnock, 1991). MT is a candidate model system to study this natural variation in a single genetic background. Although MT has been used intensively for induced mutagenesis and genetic transformation (Meissner et al., 1997; Mathews et al., 2003), there is only one report on the introduction of natural variation into this cultivar (Lima et al., 2004).

Here we present results describing the use of MT to explore natural genetic variation in tomato for basic and applied research as well as a reassessment of the methodology for large scale mutagenesis on this cultivar based on different agents (chemical and physical) and targets (seeds and pollen).

MATERIALS AND METHODS

EMS and Gamma-Ray Treatments

For EMS (ethyl methanesulfonate, Sigma –Aldrich, St. Louis) treatment, seeds of MT (*Lycopersicon esculentum* Mill. [*Solanum lycopersicum* L.] cv. Micro-Tom) were soaked in distilled water for 8 h at room temperature and then incubated in 200 ml (for each batch of 2,000 seeds) of a freshly prepared EMS solution for 12 h under gentle stirring. The EMS solution was removed and the seeds were washed in running water for 10 min. Four different doses of EMS were applied on 500 seeds each: 0.5, 0.7, 0.9 and 1% (w/v) (2,000 seeds were used for the 0.7% treatment). M1 seeds were grown as described previously (Lima et al., 2004). At the end of the growth cycle, fruits were harvested and M2 seeds collected in bulk (6-plant batches). Seeds were treated with bread yeast (*Saccharomyces cerevisae*, Fermix, Brazil) overnight, then washed and dried at room temperature for 12-24 h.

For gamma-ray treatment, doses of 600 to 1200 Gy and 40 to 160 Gy were tested on seeds and pollen, respectively. The irradiance was performed at the Centro de Energia Nuclear na Agricultura, Universidade de São Paulo (CENA/USP, São Paulo, Brazil) using a cobalt source (Gamma Cell 220 Excel). For seed mutagenesis, batches of 200 seeds were treated for each dose. The procedure for M1 plant cultivation and M2 seed harvesting was as described above. In the case of pollen mutagenesis, anthers were removed from 30 MT plants, pollen was collected and treated with doses of 40, 80, 120 and 160 Gy. MT flowers were emasculated and fertilized with irradiated pollen. M1 seed was collected from the resulting fruits.

Pollen Collection and In Vitro Germination

Anthers were collected from newly-opened MT flowers grown in the greenhouse. The anthers were dried overnight at room temperature and then transferred to 1 ml eppendorf tubes and vortexed to release the pollen grains. The anthers were discarded and treated as well as non-treated (control) pollen was germinated in a modified medium described by Song et al. (2001), containing 5% (w/v) sucrose, 15% (w/v) polyethylene

glycol 4000, 1 mM KNO_3, 3 mM $Ca(NO_3)_2.4H_2O$, 0.8 mM $MgSO_4.7H_2O$ and 1.6 mM H_3BO_3 and pH 7.0. After 4 h of dark incubation, 20 µl of medium were mounted on a slide and observed in a light microscope (Zeiss Axioskop 50HBO). Germination rate was determined. Pollen grains were counted as germinated when the tube length exceeded the grain diameter. Four to five random microscopic fields were counted out for each treatment.

Mutant Screening in M1 and M2 Generations

Seeds derived from treated pollen grains were grown in greenhouse to screen for dominant mutations in M1. In order to screen for mutations in M2, plants derived from EMS-treated seeds were cultivated in the greenhouse and in the open-field. Field screening was carried out in collaboration with Nunhems, Brazil. A series of putative alterations in the vegetative and reproductive development (Fig. 1F) was observed in the two environments, including total soluble solids (TSS) content in fruits (Brix). TSS was determined using a refractometer (PR101α, Atago, USA). Mutants selected in M1 and M2 were selfed for seed production and subsequent confirmation of phenotype in M2 and M3 generations, respectively.

Introgression of Natural Genetic Variation from Wild *Lycopersicon* Species into 'Micro-Tom'

The wild *Lycopersicon* species *L. esculentum* var. *cerasiforme* (Dun.) Gray, *L. pimpinellifolium* (Jusl.) Mill., *L. cheesmanii* Riley, *L. parviflorum* Rick, Kes., Fob., & Holle., *L. chmielewskii* Rick, Kes., Fob., & Holle., *L. hirsutum* Humb. and Bonpl., *L. peruvianum* (L.) Mill., *L. chilense* Dun., and *L. pennellii* D'Arcy were used as pollen donors for crosses with MT. For self-compatible species, the F1 plants were selfed to obtain a recombinant F2 population which was selected for small size, as described in Lima et al. (2004), and various novel recombinant phenotypes due to the presence of alleles from wild species. The selected plants were backcrossed (BC) with MT and those phenotypes showing monogenic dominant (1:1 segregation in BC1) or recessive (3:1 segregation in BC1F2) inheritance were backcrossed up to the sixth generation (BC6), with selfing every second generation to screen for homozygous recessive alleles. After BC6F2 the resulting genotypes were be considered near isogenic lines. For natural genetic variation present in *L. peruvianum*, *L. chilense* and *S. lycopersioides*, all of which show breeding barriers with cultivated tomato, intermediary hybrids and cultivars derived from these species were used for crosses with MT. These seeds were kindly provided by the Tomato Genetics Resource Center (Davis, USA).

RESULTS AND DISCUSSION

EMS and Gamma-Ray Mutagenesis

For both chemical (EMS) and physical (Gamma-ray) mutagenesis, a critical factor is dosage of the mutagenic agent. High doses, in spite of producing a higher mutation rate, have the downfall of producing mutations in multiple loci, some of which can lead to sterility or lethality (Koornneef, 2002). In the present study, we tested doses of 0.5, 0.7, 0.9 and 1% EMS and 600, 800, 1000 and 1200 Gy of gamma-radiation on seeds. No dose of either treatment had an expressive effect on M1 seed germination (data not shown), which suggests that, although commonly used for tomato (Menda et al., 2004) and also *Arabidopsis* (Koornneef, 2002), this parameter is not the best indicator of the optimum dose for 'Micro-Tom'.

M1 seedling height varied proportionally to the gamma-ray dose applied, whereas this effect was not as pronounced for EMS (Figs. 1A and B). Despite 50% reduction in plant height to be a useful parameter for gamma-ray dose, it was inadequate for EMS treatment in tomato. We assessed fertility instead, measured as the rate of M1 plants producing viable seeds, as a parameter for EMS mutagenic effectiveness. This lead us to choose the 0.7% EMS dose, while not deleterious for germination of treated seeds,

produced a 15% reduction in the fertility of M1 plants. Chemical mutagens are extremely toxic and, therefore, require more care in the manipulation of treated material, compared to physical mutagens, which is not residual. The latter can be easily applied to pollen (Liharska et al., 1997) and then be used to screen for dominant and non-chimeric mutants in M1. It can also be used to screen for additional alleles of existing recessive mutants when they are pollinated with mutagenized wild-type pollen. The rapid and high throughput in vitro pollen germination screen is the obvious parameter to determine the adequate dose for mutagenesis. However, we discovered that even high gamma-ray doses (1000 Gy) did not impair pollen germination (Figs. 1C and D). Thus, we decided to test the capacity for M1 seed formation in fruits derived from plants pollinated with gamma-ray treated pollen. By doing this we determined that 80 Gy was enough to produce a 50% reduction in seed formation (Fig. 1E), which was therefore the best criterion to define gamma-ray dosage for pollen mutagenesis.

Mutant Isolation in the M1 and M2 Generations

Using 0.7% EMS for seed mutagenesis and 80 Gy for pollen mutagenesis we screened for mutants in different categories in M1 and M2 (Fig. 1F). We found 78 mutants with morphological and Brix alterations in 2,800 M2 plants derived from EMS seed mutagenesis. Screening 280 M1 plants derived from gamma-ray pollen mutagenesis we obtained 31 mutants. These results represent a mutagenesis efficiency of 2.8 and 11% for EMS and gamma-ray pollen mutagenesis, respectively. If one considers that the mutants found in M1 are only the dominant ones and that many other recessive mutants could be found in M2, the high efficiency and advantages of pollen mutagenesis becomes clear.

Among the new mutants found in greenhouse (Fig. 2A) or field (Fig. 2B) screenings, some appear to be allelic to mutations already known in tomato, such as *ovate* (Fig. 2D, Liu et al., 2002), *jointless* (Fig. 2F, Mao et al., 2000), *high pigment1* or *dark green* (Fig. 2H, Kendrick et al., 1997) and *never ripe* (Fig. 2I, Wilkinson et al., 1995). We are currently performing allelism tests with those mutants. Since MT harbours the *sp* allele, which is usually epistatic to *jointless1* and *2*, it may as well be that the phenotype observed in Figure 2F is representative of a novel locus or a strong allele causing inhibition of abscission zones on pedicels and the characteristic reversion of reproductive organs (formation of leaves in inflorescences) commonly observed in *jointless* mutants (Szymkowiak and Irish, 2006). We also found, among other, putative novel mutants with a much reduced plant size (Fig. 2E), shortened pedicels (Fig. 2G) and small fruits (Fig. 2C). Variations in both extremes were found for the agronomically relevant trait Brix (total soluble solids in the fruit). The occurrence of mutants with high Brix in both M1 and M2 (Fig. 1F) indicates that this complex agronomic trait could be also controlled by major genes, some of them dominant.

Isolation of Monogenic Traits from Wild Tomato Species

The wild *Lycopersicon* species evolved in a restricted region from southern Ecuador to northern Chile (Warnock, 1991), which comprises different habitats and environmental conditions, such as drylands, high altitudes with very low temperatures at night and salinity at the sea shore (Taylor, 1986). These environments determined the existence of species with resistance to drought (*L. pennellii* and *L. chilense*), cold (*L. hirsutum*) and high salinity (*L. cheesmanii*) in the genus (Taylor, 1986). Although the genetic basis of abiotic stress resistance is most likely polygenic, major genes from wild *Lycopersicon* species has been found that control complex traits, such as fruit size (Frary et al., 2000), fruit sugar metabolism (Schaffer et al., 1999) and in vitro regeneration capacity (Lima et al., 2004). These observations prompted us to cross MT with wild *Lycopersicon* species and introgress those traits that could potentially be monogenic in further crosses or backcrosses (BCs). In this way, we produced novel near isogenic lines harbouring dark green fruit (Fig. 3B), small plant size (Fig. 3C), and increased number of flowers and fruits (Figs. 3D and G).

Our results are in accordance to the fact that many important monogenic mutations known in the cultivated tomato actually are allelic variation from wild *Lycopersicon* species. Examples are beta-carotene (*B*) and delta (*Del*), which are present in some *Lycopersicon* species (e.g. *L. hisurtum*) and are normally involved in the conversion of lycopene to other types of carotenoids (Ronen et al., 2000). In the same way, *atroviolacea* (*atv*) and intense pigment (*Ip*) show altered response to light (Kendrick et al., 1997) and were introduced into the cultivated tomato from *L. cheesmanii* and *L. chmielewskii*, respectively. Monogenic resistance to diverse pathogens are also examples of natural genetic variation derived from wild species such *L. pimpinellifolium* and *L. peruvianum* (Taylor, 1986; Bai and Lindhout, 2007). Among such pathogen resistance genes, *Ve*, *I*, *Mi* and *Tm-2* are found in the so-called VFNT cultivars, which, therefore, have resistance to fungi *Verticillium* and *Fusarium*, nematode *Meloidogyne* and *Tobacco mosaic virus* (TMV). In the present work, some of these already known monogenic natural genetic variations were also introgressed into MT, such as the leaf architecture variant *Petroselinum* (Fig. 3A), anthocyanin variant *Abg* (Fig. 3F) and virus resistant variant *Tm-2* (Fig. 3F).

The late Charles Rick once said that "if *Arabidopsis* is the *Drosophila* of plant genetics, then tomato has become the mouse" (Rick, 1991). Given its small size and its precocity, the true equivalent of mouse for plant scientists, in our opinion, is 'Micro-Tom'. The mutagenesis and introgression of natural genetic variants, presented in this work, not only increase significantly the possibilities for studies at the basic research level, but also paves the way for using MT as a model for tomato breeding. Thus, if one takes into account that MT differs from commercial cultivars in a handful of recessive mutations, the pyramidation of several dominant alleles generated in this cultivar (by means of natural genetic variation introgression and pollen mutagenesis) would yield parental lines which, when crossed to elite varieties, could be screened for commercially valuable normal size F_1 hybrids. Since pyramidation requires the screening of an increasingly higher number of plants during several generations, the small size and fast life cycle of MT become an obvious advantage.

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Figures

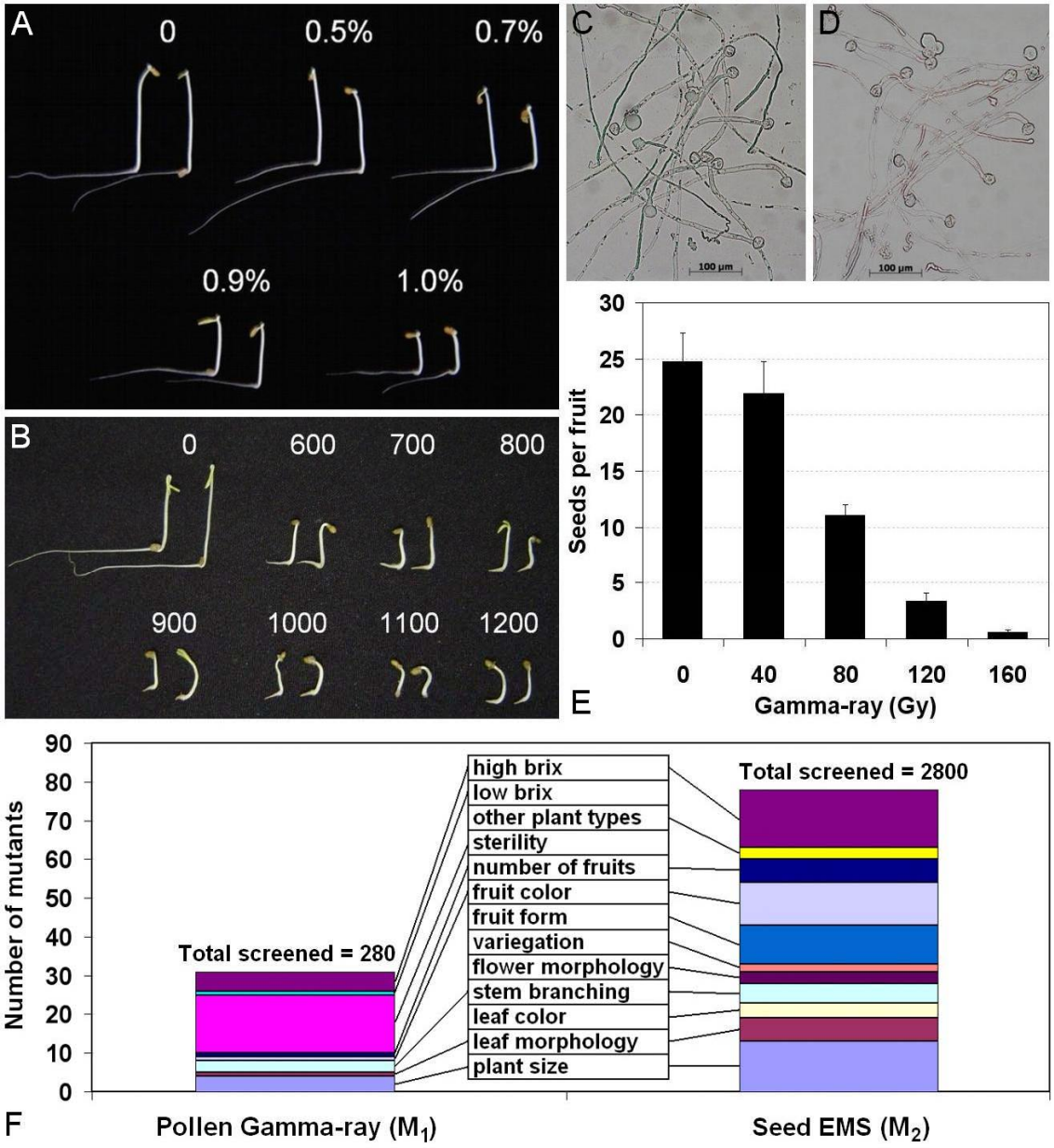

Fig. 1. Comparison of chemical and physical treatment of 'Micro-Tom' (MT) seeds and determination of gamma-ray (Gy) dose for pollen irradiation. A-B: 8-days-old M1 seedlings from seeds treated with EMS (A) or gamma-ray (B) were germinated in paper filter. C-E: Comparison of in vitro germination of control (C) and treated (D) with 1000 Gy of gamma-ray pollen as well as M1 seed formation in fruits (n=10) derived from plants pollinated with gamma-ray treated pollen (E). F: Mutants observed in 280 M1 plants from gamma-ray pollen mutagenesis and 2,800 M2 plants from EMS seed mutagenesis of MT. Other plant type includes alterations in the flower peduncle, corolla retention in the fruits and lack of abscission zones on fruit pedicel. Low Brix ≤4.0 and high Brix ≥7.4 (MT's Brix was 5.7±0.2, n=10 fruits).

Fig. 2. Cultivation and phenotypes of EMS-induced mutant lines in 'Micro-Tom'. The M1 population (1,000 plants) was cultivated in a greenhouse (A). The first screening was performed in a field-grown M2 population (B) based on morphological alterations, such as small and abundant fruits (C), elongated fruits (D), similar to *ovate* mutation, small plant size (E), absence of abscission layer in pedicels and presence of leaves in inflorescences (F), similar to the *jointless* mutation, short pedicels (G), high pigmented (dark green) fruits (H) and incomplete ripening fruits (I).

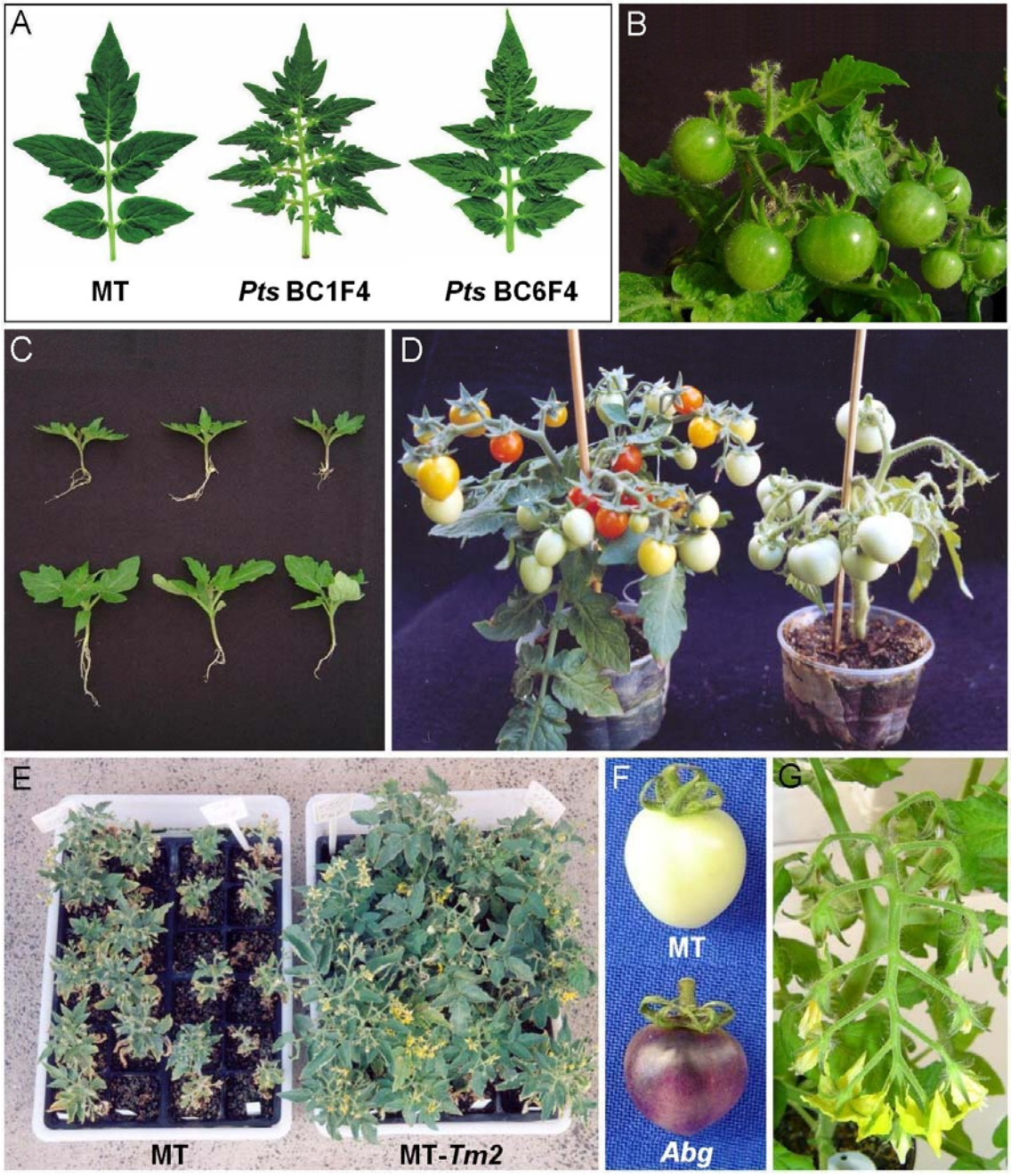

Fig. 3. Natural genetic variation introgressed into 'Micro-Tom' (MT). The *Petroselinum* (*Pts*) allele from *Lycopersicon cheesmanii* var. *minor* produces excessively divided leaves (A). The loci for hairy (high trichome number) and high pigmented fruits (B) as well as seedling dwarfism (C) are also from *L. cheesmanii* var. *minor*. Large number of fruits per plant is characteristic of MT plants derived from *L. pimpinellifolium* (D). The *Tm-2* gene (resistance to the TMV) was introgressed into MT from a VFNT cultivar (E). Both trays containing 35-days old plants were sprayed with a TMV solution. The *Aubergine* (*Abg*) gene was introgressed into MT from a cultivar derived from *Solanum lycopersicoides*. It confers purple fruit epidermis (F), particularly when exposed to direct light. MT derived from *L. pimpinellifolium* presenting 13 flowers per inflorescence (G), which is twice of that observed in MT.