Theor Appl Genet (1987) 74:733–738

Somaclonal variation versus chemically induced mutagenesis in tomato (*Lycopersicon esculentum* L.)

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Received February 28, 1987; Accepted May 25, 1987
Communicated by F. Salamini

Summary. A comparison was made of the type and frequency of mutational events found in the progeny of tomato plants regenerated after one passage in vitro with those induced by chemical mutagenesis with ethyl methane sulphonate. Several mutants were recovered in the progeny of regenerated and mutagenized plants of two cultivars of tomato. They can be grouped into the following categories: seedling lethality, male sterility, resistance to *Verticillium*, short stature, change in number of lateral shoots or in leaf shape. The results indicate that the two sources of variability differ in their effect, changing the spectrum and frequency of the mutants as well as, at least in some cases, their pattern of segregation.

Key words: Tomato – Somaclonal variation – Chemical mutagenesis – Mutants

Introduction

The main sources of variability for the breeder have been mutation and crosses with unrelated or wild species followed by selection for the desired characters. More recently regeneration from in vitro culture has disclosed another source of exploitable variation, generally referred to as somaclonal variation (Larkin and Scowcroft 1981). In fact, a number of different factors, such as pre-existing genetic variations uncovered during in vitro tissue differentiation, stress due to the in vitro culture and selection for specific genotypes during plant regeneration appear to determine genetic dissimilarity in regenerated plant populations. The molecular basis of this new form of variation has not yet been fully established although a number of explanations have been proposed (Evans et al. 1984) and alterations at the DNA level have been demonstrated (Landsmann and Uhrig 1985).

Somaclonal variation is appealing to the breeder because it often occurs at a higher frequency than chemically induced mutagenesis. For instance, 72% variants have been reported in rice regenerants (Sun et al. 1983). On the other hand, it has not yet been adequately established whether the two sources of variability are also qualitatively different. This topic is of particular interest since differences in the genetic target may ultimately produce different classes of mutants and thus contribute differently to plant breeding.

In an attempt to isolate useful variability among commercial varieties of tomato and to learn more about the nature of somaclonal variation, we compared the genetic variability recovered in the progeny of plants regenerated after one passage in vitro to that induced by chemical mutagenesis. The comparison includes type and frequency of mutational events as well as segregation patterns.

The results indicate that somaclonal variation and chemical mutagenesis differ in their effects, changing the spectrum and frequency of mutants as well as, at least in some cases, the pattern of transmission of the mutant character.

Materials and methods

Sources of material

Two commercial varieties of *Lycopersicon esculentum* L. kindly provided by ORIS, Milano, have been used. They were established after selfing for at least seven generations and differ in their marketing, the first (L50) being a variety for processing, the second (L87) for fresh market.

Chemical mutagenesis

Ethyl methane sulphonate (EMS) was applied to seeds and/or mature pollen. Seeds were soaked for 18 h in an aqueous solution containing the mutagen (2‰), then washed carefully and sown in soil. Freshly collected pollen was suspended for 1 h 40 min in paraffin oil containing EMS (0.1‰ or 0.2‰); pollination was accomplished immediately after by applying the pollen suspension to emasculated flowers with a fine brush. Ten flowers were used for each treatment. For the double mutagenic treatment (pollen and seeds) pollen was first treated and the resulting seeds (M1) were further mutagenized at the concentration given above.

In vitro regeneration

Seeds were sterilized with 1% sodium hypochlorite and 0.04% Teepol 610 for 20 min in a vacuum, washed in sterile water and sown in 0.5 strength MS medium (Murashige and Skoog 1962) containing 10 g/l sucrose and 0.8% agar (pH 5.8). Growth was for 8–10 days at 26 °C and 4,000 lux. Cotyledons were excised, sectioned with two diagonal cuts and plated on MS medium containing 30 g/l sucrose, 0.8% agar, 2×10^{-5} M IAA and 2×10^{-6} M zeatin riboside (pH 5.8). Incubation was at 26 °C and 10,000 lux. Calli were produced out of the injured regions and shoots differentiated from the calli within 8–12 days. After reaching about 1 cm in height (2–4 weeks), shoots were cut and transferred for rooting to 0.5 strength MS medium containing 10 g/l sucrose and 0.8% agar (pH 5.8). After about 30 days plantlets were ready for acclimatization in Jiffy pots in the green house. In order to separate mutational events, only one regenerated shoot was transferred to soil from each cotyledonary section.

Germination

Lots of 50 or 100 seeds from each M2 or R2 family were surface-sterilized with 2% chlorox for 15 min, rinsed with sterile water, left overnight on a rotary shaker (120 rpm), then transferred on a sterile Petri dish with a wet filter paper disc (Whatman No. 3) below and above the seeds, watered with a fixed volume of Hoagland solution. Seeds were germinated in darkness for 4 days at 25 °C, then exposed to light (6,000 lux).

Mutants screening

With early and late presumed mutants we refer to nonparental phenotypes first observed in the selfed progeny of mutagenized (M2) or regenerated (R2) plants and scorable at the seed or young seedling (early) or grown up (late) stage, respectively. The general scheme adopted for detecting and verifying the induced mutants is outlined in Fig. 1, while Table 1 reports the total number of mutagenic treatments and of M2 and R2 families scored. A description of the experimental approach and of the nonparental phenotypes searched for among early and late presumed mutants is now given.

Early mutants. Lots of 50 or 100 seeds from each R2 and M2 family were germinated in Petri dishes. Seedlings were then scored at fixed time intervals starting from the 4th day after planting up to day 13. During this time seedlings go through different stages, four of which are outlined in Fig. 2. This approach allowed us to calculate the average time required to reach each of the four stages, information necessary to identify seedling lethal mutants (see below). The phenotype of some of the presumed mutants is also found in control families, even though at a low frequency. Thus an arbitrary value of the frequency of nonparental phenotypes was chosen as the lower limit to consider their presence as suggestive of mutant segregation. The following mutants were considered. (a) Brown seeds (bs). Lower limit: 10%. (b) Lack of germination (germ$^-$). Lower limit: 20%. (c) Seedlings with three rather than two cotyledons (tric). Lower limit: 10%. (d) Small seedlings (small), visibly smaller than their siblings. Lower limit: 10%. (e) Dwarfs (df), i.e. short seedlings with dark green curly cotyledons and first leaves. (f) Seedling lethals (leth), a group of nonparentals including all the families where the difference between the frequency of seedlings reaching growth stage 1 and 3 at fixed times is at least 15%.

Late mutants. A sample of 20–30 seeds from each M2 and R2 family was planted in the field and scored at maturity for presence of nonparental phenotypes. These include the following traits: (a) male sterility (ms); (b) suppression or reduction of lateral shoots (shoot$^-$); (c) height reduction (short); (d) early ripening (rip); (e) potato leaf (leaf), an abnormal leaf morphology; (f) resistance to fungal attack (res), judged by visual inspection.

Genetic origin of nonparental phenotypes

A sample of 20–30 seeds of each of the early and late presumed mutants isolated in the M2 of R2 generation was progeny tested to verify transmission of the mutant phenotype. In some cases, if the presumed mutant plants were not available, normal siblings were raised and their selfed progeny scored for mutant segregation: this involved at least three sibling plants' progenies.

Testing for disease resistance

Seedlings obtained by selfing presumed resistant mutants were scored for resistance to *Verticillium dahliae, Fusarium oxysporum* f. sp. *lycopersici* (race 1), nematodes (*Meloidogyne incognita*) and Tobacco Mosaic Virus (TMV) by inoculating the pathogen and scoring for disease symptoms at fixed times.

For *Verticillium* wilt, the inoculum was prepared using 12 days old culture grown at 25 °C on potato-dextrose agar (PDA) Petri dishes, blended (low speed for 60 s) with 60 ml of sterile distilled water to make a thick slurry (Bender and Shoemaker 1984). Tomato seedlings, grown in sterile soil, were inoculated at the second true leaf stage by immersing roots in the inoculum slurry for 4–5 s and then transplanted in sterile soil. After 3 weeks, seedlings were scored for the development of typical foliar symptoms of *Verticillium* wilt (chlorosis, necrosis and wilting).

For *Fusarium* wilt the inoculum was prepared by blending 6-day-old fungal colonies grown in Petri dishes on Malt agar at 26 °C (Cirulli and Ciccarese 1982). Inoculation and scoring was done as described for *Verticillium* wilt. For *Meloidogyne incognita* the preparation of inocula and the inoculation were as described by Hussey and Barker (1973) using eggs and larvae obtained from roots of infected tomato cvs VC 90 and Rutgers. For TMV resistance, strains isolated from tomato (ToMV) were used as inoculum while for symptoms scoring a double diffusion test in presence of sodium dodecylsulphate was adopted (Purcifull and Batchelor 1977).

Results

Three classes of mutagenized plants are studied in the present work: the first include plants regenerated from in vitro culture, while the second and the third include plants obtained after chemical mutagenesis (EMS) of

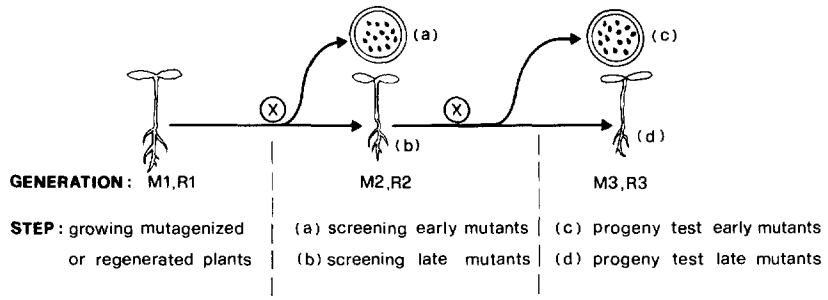

Fig. 1. Scheme for selection of presumed early and late mutants in the second (M2 and R2) generation and identification of their genetic origin in the third (M3 and R3) generation

seeds and pollen, respectively. In an independent experiment pollen was first treated with EMS and the resulting seeds (M1) were further mutagenized with the same chemical (Table 1).

Scoring of nonparental phenotypes was performed in the R2 and M2 generation, as outlined in Fig. 1, in order to recover recessive mutations and to overcome problems related to chimerism in first generation plants. In fact, only in the case of mature pollen grains the target of the mutagenic treatment is represented by a single cell, while treatment to seeds is expected to induce chimeric M1 mutant plants because of the multicellular composition of the dormant embryo shoot. The same is true for regenerated (R1) plants since these arise from a few initial cells which differentiate shoots out of the undifferentiated callus cells (unpublished data). Furthermore, scoring in the R2 and M2 generation allowed us to restrict the analysis to fertile variants and to eliminate first generation nonparental phenotypes due to physiological or environmental stresses. The experimental approach and the classes of early (scorable at the seed or young seedling level) and late (scorable in grown up plants) variants selected at this stage are given in "Materials and methods".

In order to better understand their genetic bases, progeny tests were then performed on second generation variant phenotypes. Spectrum and frequency of verified mutants affecting early functions such as germination (germ$^-$), seedling growth (dwarfs, df) and seedling lethals (leth) are reported in Table 2. Results show that both the in vitro culture and the EMS treatments are responsible for a significant level of mutational events, even though the distribution of mutants within each group and their frequency differ significantly for the two treatments. Out of 14 cases of early mutants analysed, 9 showed the segregation expected for a single recessive gene, while in the remaining five cases there was an excess of mutant over normal sibling segregation, suggesting the presence of a semi-dominant gene mutant.

Variant phenotypes affected in early function, such as brown seeds (bs), tricotiledons (tric) and small seedlings (small) were also found in both the R2 and M2 plants, but progeny tests proved that these variants were not transmitted. These variant phenotypes with no genetic bases were thus not considered further.

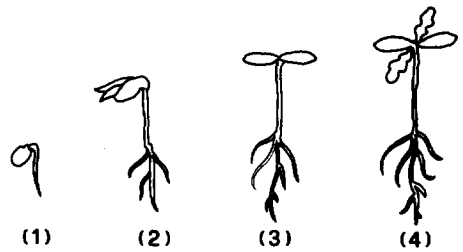

Fig. 2. Stages of growth of tomato seedlings during the first 13 days of germination, from root emission at day 4 (stage 1), to the onset (stage 2) and full expansion (stage 3) of cotyledons and to growth of first leaves (stage 4)

Table 1. Summary of mutagenic treatments and of number of M2 or R2 families tested

Line	Treatment	n^a	No. families analysed (M2 or R2)
50	Control	60	60
87	Control	60	60
50	Regeneration	323	102
87	Regeneration	261	21
50	EMS to seeds	1,000	355
87	EMS to seeds	500	226
87	EMS to pollen	530	464
87	EMS to pollen + seeds	530	77

[a] Total number of in vitro regenerated plants, mutagenized seeds or seeds derived from pollen treatment

Spectrum and frequency of verified mutants affecting late functions are reported in Table 3. Here again it is evident that both treatments yield mutants, in vitro plant regeneration being quantitatively more efficient than the tested EMS treatments. However, qualitative differences are also evident. In particular, regeneration was quite effective in inducing the leaf mutation, an abnormal leaf morphology called "potato leaf", which was

Table 2. Spectrum and estimated frequency of early mutants affecting seed and seedling development as recovered in M2 and R2 generation and verified in the third generation. germ$^-$: lack of germination; df: dwarfs; leth: seedling lethals

Line	Treatment	Generation	No. families	Estimated frequency ($\times 10^{-2}$) of mutants		
				germ$^-$	df	leth
50	Control	–	27	0.0	0.0	0.0
87	Control	–	24	0.0	0.0	0.0
50	Regeneration	R2	118	8.5	2.5	18.7
87	Regeneration	R2	21	23.8	0.0	12.9
50	EMS to seeds	M2	41	0.0	2.4	46.1
87	EMS to seeds	M2	62	0.0	0.0	4.3
87	EMS to pollen	M2	158	0.6	0.0	14.8
87	EMS to pollen + seeds	M2	25	12.0	0.0	10.8

Table 3. Spectrum and estimated frequency of late mutants as recovered in the M2 and R2 generation

Line	Treatment	Generation	No. families	Estimated freq. ($\times 10^{-2}$) of mutants[a]					
				ms	rip	short	shoot$^-$	leaf	res
50	Control	–	60	0.0	0.0	0.0	0.0	0.0	0.0
87	Control	–	60	0.0	0.0	0.0	0.0	0.0	0.0
50	Regeneration	R2	102	2.9	11.8	4.3	0.0	18.6	0.0
50	EMS to seeds	M2	355	0.6	1.7	0.0	0.0	0.0	0.0
87	EMS to seeds	M2	226	2.6	0.9	0.9	1.3	0.0	0.0
87	EMS to pollen	M2	564	1.2	0.5	0.3	0.3	0.0	0.3
87	EMS to pollen + seeds	M2	77	0.0	1.3	0.0	00	0.0	0.0

[a] For mutant categories designation see "Materials and methods"

Table 4. Overall segregation of different plant mutants as determined in the second (M2, R2) and third (M3, R3) generation. Segregation expressed as percent of mutants (m%) over total number (n) of scored plants

Mutant phenotype	No. of independent mutational events	Origin		Generation			
		M2	R2	Second		Third	
				n	m%	n	m%
Short with curly leaves	1	+	–	20	20.0	240	18.7
Short	7	+	+	ND[a]	–	77	41.5
Male sterility (ms)	7	+	+	160	20.6	105	23.8
Early ripening (rip)	16	+	+	128	27.3	103	44.7
Lateral shoot reduction (shoot$^-$)	3	+	–	58	51.7	60	55.5
Higher vigour	1	–	+	21	100.0	ND[a]	–
Potato leaf (leaf)	18	–	+	426	100.0	202	100.0
Potato leaf (leaf)	1	–	+	32	65.6	26	80.8

[a] *ND:* Not determined

never induced by any of the tested chemical mutagenic treatments. Other traits of great agronomic relevance, such as early ripening (rip) and height reduction (short) were also significantly more abundant among regenerated plants. The same was true, although to a lower degree, for male sterility (ms).

As for the genetic bases of late mutants, some indication may be obtained from the segregation data (Table 4). In order to verify the stability of mutant expression, data collected in two successive summers for the second and third generations, are shown. It appears that only ms and short behave as single gene recessive mutants, while the segregation ratio for short, rip, shoot$^-$ show an excess over the expected one-quarter. However, caution should be taken when interpreting the data since expression of these mutants is less well defined than that of the first two groups and probably more sensitive to environmental changes.

Table 5. Homozygous mutants recovered in the second generation (R2) of regenerated plants

Mutant phenotype	Mutational events	Mutant: normal in R2
Higher vigor	1	21:0
Potato leaf	18	426:0
No germination	1	100:0

Table 6. Response to inoculated pathogens of progeny seedlings obtained by selfing M2 plants selected as presumed mutants for disease resistance. +: resistant; –: susceptible; +/–: segregating resistance

Line	Cod. No.	Origin	Resistence to:			
			Fusarium	*Verticillium*	TMV	Nematodes
50	50	Control	+	–	–	–
87	87	Control	+	–	–	–
50	A125	EMS to seeds	+	–	–	–
87	A 89	EMS to pollen	+	–	–	–
87	A 90	EMS to pollen	+	–	–	–
87	A 91	EMS to pollen	+	+/–	–	–
87	A 92	EMS to pollen	+	–	–	–
87	A 93	EMS to pollen	+	+/–	–	–

Table 4 also shows that mutants affected in leaf morphology exhibit a very unusual pattern of inheritance: in fact, out of 19 tested cases, 18 breed true in R2 as if the mutant was already present in a homozygous state in the parental R1 plant. This unusual pattern of transmission is also observed for greater vigour and two early mutants and is confined to plants obtained through regeneration. A summary of cases so far detected of breeding true mutants in the second generation of regenerated plants is presented in Table 5.

For one character, potato leaf, inheritance was followed for three succeeding generations without noticeable changes in inheritance pattern. Six families were selected out of a total of 1,444 second generation (M2 and R2) field tested families for showing fewer signs of fungal or nematode attack. Five were from plants obtained from EMS-treatment of pollen and one from treated seeds but none from regenerated plants.

Progeny seedlings from selfed plants of each of these six families were tested for resistance to *Fusarium, Verticillium,* TMV and nematodes (Table 6). All seedling retained the *Fusarium* resistance already present in parental lines, while two segregated for resistance to *Verticillium.* Both originated from EMS treatment of pollen. Single plants of these two families were selfed and their progeny seedlings retested for resistance. For each of the two isolates, six progeny families were analysed. The results (Table 7) fit the hypothesis of a mutant single dominant gene conferring resistance to *Verticillium,* if one assumes that the few susceptibles recovered in the two families which show a majority (over 90%) of resistant seedlings are due to misclassification.

Table 7. Segregation values for resistance to *Verticillium dhaliae* as determined in selfed M4 progeny of two independently isolated mutants. R: resistant; S: susceptible

Line	Cod. no. of M3 family	No. of M4 families scored	Overall segregation		Inferred family genotype for resistance
			R	S	
87	A 91	5	70	45	Heterozygous
87	A 91	1	20	1	Homozygous
87	A 93	5	75	47	Heterozygous
87	A 93	1	58	6	Homozygous

Discussion

The results presented in this paper add further strength to the proposed use of somaclonal variation to recover, at high frequency, new genetic variability from existing crop varieties (Buiatti et al. 1985; Evans and Sharp 1986; Fox 1986).

In fact, regeneration from in vitro culture leads to a higher number of mutations, in the case of both early and late mutants, than application of the chemical mutagen to either seeds or pollen or both. The pattern of mutants is also different, with some mutants classes (e.g. potato leaf) arising exclusively in the case of somaclonal variation.

Completely unexpected is the frequent recovery of homozygous "solid" mutants in the R2 generation. Evidence is lacking on their origin, and the phenomenon is confined to regenerated plants and affects developmental functions such as leaf shape and vigor. Thus the plant regeneration process may act as a sieve against mutants of genes expressed in vitro while allowing the recovery of mutant genes expressed during plant development. In addition, the potato leaf character breeds true for at least 3 generations in 18 out of the 19 isolates tested, excluding the contribution of a maternal effect as described in barley in the case of seg mutants (Jarvi and Eslick 1975). Whatever the basis of this phenomenon, which is not new in the literature (Evans and Sharp 1983; Larkin et al. 1984; Oono 1985), the possibility of isolating homozygous mutants in the selfed progeny of regenerated plants represents a rather attractive tool for breeders to accelerate the selection scheme.

On the other hand, our data suggest that plant regeneration does not offer evident advantages in inducing disease resistance. In fact, the only two cases of resistance found refer to EMS-induced mutagenesis to pollen. Data are too scanty to conclude that this treatment is preferable when induction of disease resistance is desired, however this observation is worth deeper investigation, also in view of the definition of appropriate methodologies for the induction of desirable mutants. Additionally, of relevance to the breeder are the dominant resistance gene mutants obtained after EMS treatment of pollen.

One class of mutants found at high frequency in all mutagenic treatments is lethal seedling. It might well be that the adopted selection scheme leads to the recovery of a heterogeneous spectrum of mutants including partial or complete auxotrophs, those with hormone balance and growth rate affected or with chromosome numerical changes or rearrangements. The latter class would account for segregations of mutants in a ratio significantly different from the expected one-quarter. However, further studies on the inheritance pattern as well as complementation tests to known gene markers with similar phenotype are needed to elucidate the genetic basis of the mutants so far isolated.

Acknowledgements. This investigation was supported by funds of the "Biotechnology Action Programm" of the Commission of the European Communities (Contract BAP No. 0084-I) and of C.N.R., Italy, Special Grant IPRA, sub project 1.

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