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Cellular genetic study of a somatic instability in a tobacco mutant: in vitro isolation of valine-resistant spontaneous mutants

M. A. Grandbastien, C. Missonier, J. Goujaud, J. P. Bourgin, A. Deshayes and M. Caboche

Laboratoire de Biologie Cellulaire, Institut National de la Recherche Agronomique, F-78026 Versailles Cedex, France

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Summary. A chlorophyll-deficient mutant line of tobacco (*Nicotiana tabacum*), named *tl*, displays spontaneously on leaves green, white, and twinned green/white somatic variations at high frequencies (10^{-3} to 10^{-2}). The frequency of cell events leading to the somatic variations has been shown to be closely dependent on the stage of differentiation of cells during plant development. The activity of transposable elements is suspected in the *tl* genotype, and a study of its mutagenic ability was performed by selecting in vitro new mutant cellular types. The cellular marker chosen was the resistance to toxic doses of valine conferred by a permeation deficiency. A reproducible method allowing efficient selection of valine-resistant mutant clones from haploid tobacco mesophyll protoplast-derived cells was used. In 10 out of 12 experiments, the frequency of spontaneous valine-resistant clones obtained with the wild-type control was null for cell populations tested to the 10^6. On the other hand, spontaneous valine-resistant clones were repeatedly isolated at variable and sometimes high frequencies (greater than 10^{-3}) from cell populations of the *tl* type. Valine resistance of plants regenerated from these clones was transmitted to the progeny as a single monogenic mutation. These results indicate an increased mutagenic ability of the *tl* genotype, as compared to the wild-type line.

Key words: Somatic instability – Valine resistance – *Nicotiana tabacum* – Protoplast

Introduction

Transposable elements induce somatic instabilities at the locus at which they are inserted. They have also been shown to transpose and induce new alleles at other loci (for a review, see Nevers et al. 1986). The presence of active transposable elements in a genotype can, therefore, be detected by this genotype's ability to induce new types of mutants at high frequency in the absence of any external mutagenic agent. Selection procedures developed for the recovery of biochemical mutants from cultured cells of model species such as tobacco (*Nicotiana tabacum*) have proved efficient and reproducible (Grandbastien et al. 1985), and provide a powerful tool for such a study. The aim of the present work was to determine if new cellular types detectable by an in vitro biochemical selection could be obtained at increased frequencies in cell cultures isolated from a chlorophyll deficient tobacco mutant carrying a somatic instability, in which the presence of active transposable elements is suspected. As a marker, we have used the resistance to toxic doses of valine and we have selected spontaneous and UV-induced mutants from populations of haploid mesophyll protoplast-derived cells.

The chlorophyll-deficient unstable mutant

The *tl* tobacco line, isolated after seed treatment with the mutagenic agent, ethyl methane sulfonate (EMS) (Dulieu 1965, unpublished results), has been previously described (Deshayes 1973, 1976, 1978, 1979). The *tl* allele is transmitted as a Mendelian semidominant character and causes chlorophyll deficiency, that is a pale yellow phenotype, associated with the spontaneous appearance of green somatic sectors, usually corresponding to a return to the wild-type phenotype. Other types of somatic variations are also observed (white, as well as twinned green/white), although at much lower frequencies than green somatic sectors. The biochemical function of the wild-type *TL* allele is unknown, but the chlorophyll deficiency

seems due to an imperfect association between chlorophyll pigments and protective lipoproteins in the *tl* chloroplasts (Lemoine 1976), leading to chlorophyll photodestruction, as shown by the strong correlation observed between the leaf yellowing and the light intensity applied during early stages of leaf development (Deshayes 1973). Sectoring is, however, unrelated to the growth conditions. The variations appear as single unconnected areas, and the size of each sector is relatively constant on a given leaf, indicating that the cellular events leading to somatic variegation are not random, but closely dependent on the stage of differentiation of the cell. The sector frequencies also depend on other developmental characteristics, such as the leaf position: sector frequencies increase consistently up to the first prefloral leaf, the appearance of which is correlated with the transition of the shoot apex from the vegetative to the flowering stage (Deshayes 1973).

The original *tl* mutant line was characterized by a low number of somatic sectors: a few hundred per plant, that is, a frequency of variation averaging 10^{-7} to 10^{-4} per leaf cell. This phenotype was maintained after both vegetative multiplication and sexual reproduction. Several new lines, however, were derived after in vitro bud neoformation from leaf fragments of homozygous *tl/tl* plants (Deshayes 1976, 1979). These new lines were characterized by high frequencies of variation: 10^{-3} to 10^{-2} per leaf cell, leading to a few hundred sectors per leaf. The expression of the somatic instability on these new highly unstable lines is controlled by physiological factors in the same way as the original *tl* mutant. Each of these new lines, however, displays a different pattern of variegation, definable in terms of frequency and size of the somatic sectors. These patterns are heritable with the *tl* allele as codominant markers.

The tobacco *tl* instability displays, therefore, many similarities to other mutable systems attributed to transposable elements (Deshayes 1979; Fedoroff 1983). The activity of plant transposable elements has often been revealed after genomic stresses such as mutagens, and it has recently been demonstrated that tissue culture can also activate cryptic inactive elements in maize (Peschke et al. 1987). It is, therefore, suspected that the *tl* unstable mutation could be due to a transposable element activity revealed after EMS treatment and in vitro culture.

The valine resistance marker

We have used the cellular marker of resistance to toxic doses of valine, due to a permeation deficiency. Such a mutant, named *Val^r-2*, was previously obtained after UV-irradiation of haploid tobacco mesophyll protoplasts (Bourgin et al. 1985). The *Val^r-2* character is determined by two partially dominant unlinked loci, *vr2* and *vr3*, and expresses a high level of resistance from early stages of cell culture (5–6 days after protoplast isolation). Relationships between the two *vr2* and *vr3* loci have not been determined. It is, however, hypothesized that they represent two homoeologous loci present in the tobacco amphidiploid genome (Bourgin et al. 1985). The *Val^r-2* mutant was used in reconstruction experiments, in order to define the optimal selective condition needed for a reproducible selection system for valine-resistant mutants (Grandbastien et al. 1985).

Monogenic lines carrying only one of the two independent mutated loci *vr2* or *vr3* have been constructed. They show low (*vr3*) or intermediate (*vr2*) levels of resistance. *Val^r-2*-type double mutants have been selected from cell cultures of the *vr3* monogenic line (Grandbastien et al. 1985), but not from cell culture of the *vr2* monogenic type, since the level of valine resistance of the *vr2* type is too high to allow efficient selection in the selective conditions chosen to insure reproductibility. We have, moreover, shown that these selective conditions were sensitive enough to allow also the selection of monogenic mutants of the *vr2* type from wild-type cell culture (Grandbastien et al. 1985). This was an important prerequisite for the study reported here, since mutation of two independent loci in the same cell was expected to be a low frequency event.

In this report, the mutagenic ability of the *tl* genotype has been studied at the *vr2* and *vr3* loci. Evidence is presented that the *tl* genotype displays increased mutation frequencies for valine resistance. The genetic analysis of the progeny of some of these spontaneous mutants confirmed that the mutation occurred at one or the other of the two loci involved in the permeation deficiency.

Material and methods

Plant material

tl lines. The original *tl* mutant was obtained after EMS treatment on seeds of *Nicotiana tabacum* cv Samsun. New lines displaying high frequencies of variation were derived from the original mutant after in vitro bud neoformation (see introduction). All *tl* lines were backcrossed to *N. tabacum* cv Xanthi, in order to introduce the character of hypersensitivity to tobacco mosaic virus (TMV). Different homozygous (*tl/tl*) lines displaying high frequencies of variations were obtained. Haploids were produced from these plants by anther culture. Five haploid *tl* plants displaying different variegation patterns were selected for in vitro selection experiments.

Control lines. The wild-type is the SH6 haploid line obtained through anther culture of a *N. tabacum* cv Xanthi plant, or its corresponding diploidized fertile line, named XHFD8 (Bourgin and Missonier 1973).

Valine-resistant lines. The SH6 haploid line was originally used for the isolation of the *Val^r-2* mutant (genotype *vr2/vr2; vr3/vr3*). The two monogenic lines *vr2* (genotype *vr2/vr2; +/+*) and *vr3* (genotype *+/+; vr3/vr3*) were produced as described previously (Grandbastien et al. 1985), and their monogenic nature was confirmed later by test-crossing experiments. The three mutant types have been used both as haploids and diploids.

Protoplast culture and subculture of protoplast-derived cells

Plants were cultured in a shaded greenhouse. Under these conditions, the *tl* chlorophyll deficiency is not expressed and plants are green, except for the few white somatic sectors. Protoplasts were isolated from the mesophyll of entire fully expanded young leaves, and cultured as described previously (Grandbastien et al. 1985). For the *tl* genotypes, the protoplast culture medium was slightly modified by lowering the mannitol concentration to 7.5%. This lowered mannitol concentration was used in subsequent culture steps for all genotypes. The plating efficiency of subcultured cells was expressed as the percentage of divided cells that gave rise to visible colonies, and will be referred to as the relative plating efficiency (RPE). RPE was usually between 35% and 45%.

Mutagenesis

UV-mutagenesis was performed on protoplasts after 24 h of culture and each dose-response curve was obtained by irradiating, at increasing doses, different samples of the same original protoplast population.

Selection and regeneration of valine-resistant clones

These steps have also been detailed previously (Grandbastien et al. 1985). Selection was performed by adding 2 m*M* of L-valine 6 or 7 days after protoplast isolation (1 or 2 days after subculture of divided cells). Presumptive resistant colonies were scored after 6–8 weeks and transferred to nonselective medium to allow further growth. Resistance tests were performed on calli, and colonies whose resistance was confirmed were regenerated into plants. These plants were transferred to the greenhouse for subsequent analysis. Diploidization often occurred during these in vitro steps. When this was not the case, however, haploid plants were diploidized by culture of small leaf fragments onto solidified R4MO medium; regenerated shoots were rooted onto medium B (Bourgin et al. 1979). Approximately 30% of regenerated plants were fertile diploid.

Seedling resistance tests

Transmission and segregation of valine resistance in the progeny were scored by germination tests. Seeds were surface-sterilized and laid on top of solidified medium B containing 2 m*M* of L-valine. Valine effect on sensitive seedlings could be visualized as early as 10 days after sowing. Sensitive seedlings displayed a yellow and typically distorted phenotype. Only fully resistant seedlings (*vr2/vr2; vr3/vr3*) can develop normal leaves (Bourgin et al. 1985). Results were scored after 6–8 weeks.

Results

Spontaneous and UV-induced resistant frequencies

We have carried out several independent series of selection of valine-resistant colonies, with or without mutagenesis, on haploid cell populations of wild-type, monogenic *vr3*, and *tl* lines. Results on wild-type and monogenic lines have already been presented (Grandbastien et al. 1985) and are mentioned here in connection with results obtained with the *tl* lines. For each point in each independent experiment, a minimum of 10^6 divided cells was submitted to selection. Results are presented in Fig. 1.

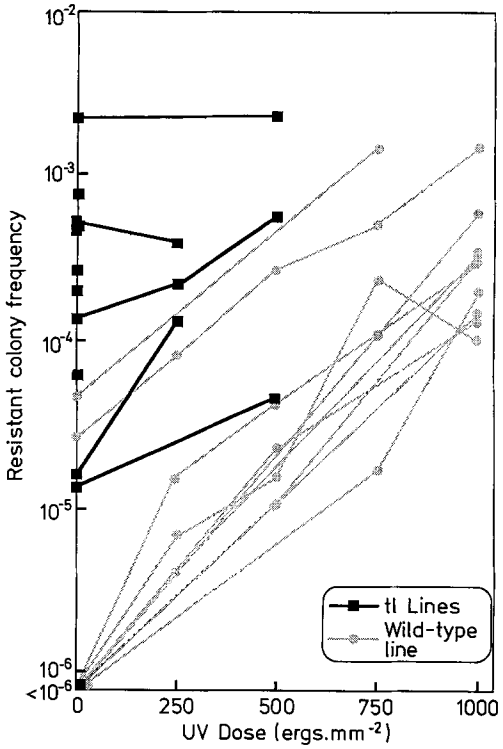

Fig. 1. Semi-logarithmic plot of resistant colony frequencies observed with or without UV-mutagenesis for haploid *tl* and wild-type control lines. Each dose-response curve was obtained by irradiating at increasing doses different samples of the same protoplast population. Protoplast-derived cells were plated at a density of 10^4 cells/ml and 2 m*M* valine was added to the culture medium 0, 1, or 2 days after plating. Resistant colonies were scored 6–8 weeks after plating, and frequencies were estimated in reference to the number of divided cells submitted to selection

Spontaneous valine-resistant frequencies. No spontaneous resistant colony has been isolated in 11 out of 13 independent experiments performed with the wild-type control. Spontaneous resistant mutants were isolated in only two control experiments at frequencies of 3 and 6×10^{-5}, respectively. Each independent experiment was performed on a different cell population isolated from a different SH6 rooting, thus eliminating chances of random sampling. Results described previously (Grandbastien et al. 1985) have shown, however, that spontaneous mutants at the *vr2* locus are repeatedly detected from *vr3* monogenic-type cell cultures at frequencies of around 0.5 to 4×10^{-5}, which are, therefore, frequencies expected in lines devoid of the *tl* instability. Spontaneous mutations obtained from wild-type cells should be at least as frequent as those from the monogenic lines. It was, however, the case in only 2 experiments out of 11, which are discussed later.

On the other hand, spontaneous resistant colonies have been isolated in 11 out of 15 experiments performed with independent cell populations isolated from five dif-

ferent *tl* lines. The frequencies of resistant colonies were variable and sometimes strikingly high (Fig. 1). In several experiments, spontaneous mutants were obtained with *tl* cells at low frequencies (less than 5×10^{-5}), corresponding to the theoretical background frequencies expected from mutational events unrelated to a transposable element activity. However in more than half of the experiments, valine-resistant clones were obtained from *tl* lines at frequencies higher than 10^{-4} and up to 2.3×10^{-3} in one case.

Comparison of spontaneous resistance frequencies obtained with the five different *tl* lines indicated no influence of the variegation pattern of the *tl* line (data not shown).

UV-induced resistant frequencies. UV dose-response curves of ten independent experiments performed with the control line, and six independent experiments performed with *tl* lines are shown in Fig. 1. Dose-response curves carried out with wild-type cells in eight independent experiments (Fig. 1) and *vr3* monogenic-type cells (Grandbastien et al. 1985) demonstrate the good reproducibility of the selection system (for each UV dose, the variability observed for the induced resistant frequencies was less than a factor of 10). In the two control experiments in which spontaneous resistant have been obtained, it has been deduced (Grandbastien et al. 1985), from the comparison of UV dose-response curves with those performed with the wild-type and the monogenic *vr3* lines, that these spontaneous resistant clones were not induced during cell culture, but that leaves collected in these two experiments contained large sectors of cells already mutated at one of the two loci *vr2* or *vr3*, following a spontaneous mutation which had occurred at an early stage of leaf development.

UV-mutagenesis at 250 and 500 ergs mm^{-2} on populations of *tl* cells increased resistance frequencies in the three experiments where the spontaneous resistance frequencies were the lowest. In the two other mutagenesis experiments performed, spontaneous mutation frequencies were too high to permit the detection of any additional effect of the UV-mutagenesis.

Characterization of spontaneous valine-resistant tl clones

Spontaneous *tl* valine-resistant colonies were picked up from the four independent experiments in which the highest spontaneous mutation rates were observed. Presumptive mutant colonies were subcultured on solid medium without valine, and callus fragments were used for tests on valine-containing solid medium. Proportions of confirmed resistant colonies varied between experiments from 60%–75% on a total of 66 tested clones. After a second similar testing, some confirmed resistant clones were regenerated into plantlets, diploidized if necessary, and transferred to the greenhouse for phenotype study, protoplast isolation, and progeny analysis.

Chlorophyll phenotype of regenerated plants. The phenotype of 14 plants regenerated from 14 different spontaneous *tl* valine-resistant colonies was studied. Two plants out of 14 had reverted to a wild-type green phenotype. The 12 other plants still displayed the characteristic *tl* chlorophyll deficiency. Plants from three clones presented the same variegation patterns as their respective parental line used for the selection, and three others were highly unstable, but display new different patterns. The remaining six valine-resistant clones had lost the parental highly unstable phenotype, and displayed a phenotype similar to the original *tl* line obtained after EMS treatment.

Valine resistance level of the spontaneous tl mutants. Valine resistance level was determined by measuring the effect of valine on the plating efficiency of mesophyll protoplast-derived cells isolated from regenerated plants, plated at low cell density. Results are presented in Fig. 2.

Table1. Testing of the F_2 progenies derived from crosses between different *tl* spontaneous valine-resistant mutants and the two monogenic test lines. Progenies were analyzed by germinating seeds on solidified medium containing 2 m*M* valine: only homozygous *vr2:vr3* seedlings grew unaffected. If the spontaneous mutation has appeared at the locus complementary from the one mutated in the test-line, a ratio of 1/16 of resistant plants is expected in the F_2 progeny

Original F1 cross	No. of F2 seedlings		
	resistant	sensitive	
sp5 × vr2	7	190	NS*
sp5 × vr3	0	201	–
sp6 × vr2	0	350	–
sp6 × vr3	18	201	NS*
sp12 × vr2	0	148	–
sp12 × vr3	9	139	NS*
sp14 × vr2	0	150	–
sp14 × vr3	10	140	NS*
sp21 × vr2	0	124	–
sp21 × vr3	5	109	NS*
sp23 × vr2	0	14	–
sp23 × vr3	7	78	NS*
sp37 × vr2	0	379	–
sp37 × vr3	0	343	–
sp52 × vr2	0	442	–
sp52 × vr3	0	474	–

NS* the probability that observed = expected has been determined to be superior to 0.95 by Chi-square calculation

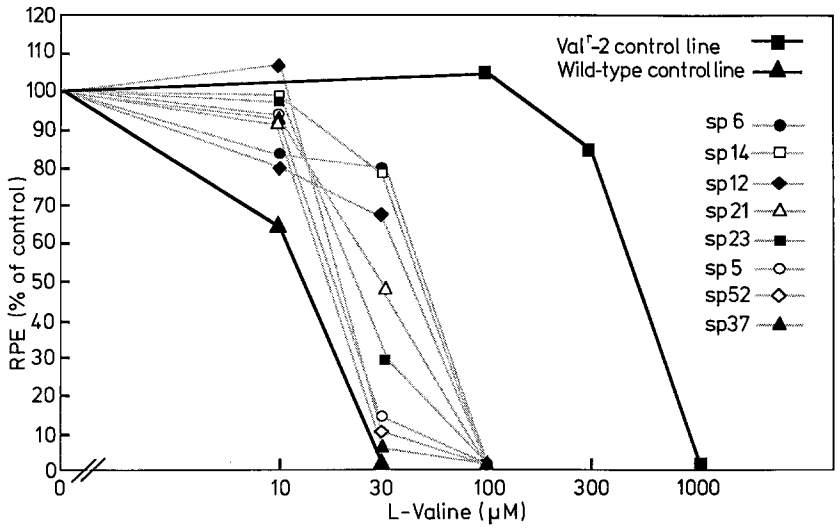

Fig. 2. Effect of valine on plating efficiency of protoplast-derived cells isolated from leaves of regenerated spontaneous *tl* valine-resistant clones, plated at low density (100 divided cells/ml)

Most mutants displayed intermediate levels of resistance, and none of them was similar to the *Val^r-2* double mutant. This event, requiring the simultaneous inactivation of two genes in the same cell, was indeed expected to occur at low frequency. Some of the mutant plants (clones *sp6, sp12, sp14, sp21,* and *sp23*) displayed a resistance level similar to that of the *vr2* monogenic lines. Other plants displayed a lower level of resistance: clones such as *sp5, sp37,* and *sp52* seemed to be more similar to the *vr3* monogenic lines. Finally, a number of mutant clones did not clearly show any resistance level as compared with the wild-type control (data not shown).

F2 progeny testing of spontaneous tl mutants. To determine the transmission pattern of valine resistance to the progeny, several confirmed *tl* spontaneous resistant clones have been test-crossed with both *vr2* and *vr3* monogenic lines. F2 seeds of these crosses have been scored for the appearance of *Val^r-2*-type double-mutants by germination on medium containing valine (Table 1). Eight clones were tested. For six of them, there was complementation for valine resistance between one of the two test lines and the spontaneous mutants, demonstrating the transmission of valine resistance to the progeny and, therefore, the mutational origin of the *tl* spontaneous resistant colonies. Complementation of the *vr3* locus by *sp6, sp12, sp14, sp21,* and *sp23* indicates that the spontaneous mutation is located on the *vr2* locus, as expected from the resistance level of these mutants (Fig. 2). Similarly, complementation of the *vr2* locus by *sp5* demonstrates that this spontaneous mutation is located on the *vr3* locus. The two clones, *sp37* and *sp52,* however, did not complement any of the two mutated loci. We assume that either the mutation is not transmitted to the progeny or that they are mutants affected at a different locus, not involved in the *Val^r-2* permeation deficiency.

Discussion and conclusion

(1) The results reported here show that spontaneous valine resistant clones are repeatedly obtained in cell cultures of tobacco line carrying the *tl* somatic instability: spontaneous valine resistant clones have been obtained in 11 out of 15 independent experiments performed with *tl* lines, while such resistant clones were obtained in only 2 out of 13 independent experiments performed with a control tobacco line devoid of the somatic instability. Moreover, spontaneous valine resistant clones were obtained in *tl* cell cultures at variable and often very high frequencies (more than 10^{-4} in 8 experiments out of 15, and up to 2.3×10^{-3} in one experiment). The variability often encountered after tissue culture cannot explain these frequencies, since previous results have shown that spontaneous valine resistant clones were expected to be at the most 5×10^{-5} at both *vr2* and *vr3* loci, in lines devoid of the *tl* somatic instability.

The high variability observed for *tl* spontaneous resistant frequencies (up to a factor of 1,000) was not due to the selection system, since dose-response curves carried out with wild-type cells and monogenic *vr3* cells demonstrated its good reproducibility. There is no way of determining whether the spontaneous appearance of resistant colonies is attributable to events induced by the in vitro culture step, or reflects events occurring during the leaf development. The observed variability suggests, however, that most of these events occurred prior to protoplast isolation, since the selection was performed 6 days, that is, only two cell generations after protoplast isolation.

It is not clear why no spontaneous resistant clones were obtained in 11 experiments performed with at least 10^6 cells of the wild-type line, since spontaneous resistant clone frequencies are expected to be around 5×10^{-5} at

vr2 or *vr3* loci. In 8 of these experiments performed with wild-type cells, UV-mutagenesis experiments were also performed, and the good correlation found between UV dose and induced resistant frequencies demonstrated that selective conditions were appropriate. It seems, therefore, that in contrast with valine resistant clones selected from *vr3* monogenic lines, spontaneous resistant clones are poorly selected from cells containing both wild-type permease loci. This observation gives additional meaning to the fact that in most experiments performed with the *tl* cells, spontaneous resistant colonies were obtained at high frequencies.

(2) Although only valine resistant colonies whose resistance was confirmed by two successive callus testings have been regenerated into plantlets, the resistance level of some of the regenerated plants, determined by the effect of valine on protoplast-derived cells, was not higher than the wild-type control. Since we assume that two successive rounds of callus testing should have eliminated most of the wild-type cells in possible chimera colonies, we can only hypothesize that these clones could be either leaky or highly unstable mutants, in which valine resistant cells have been lost during early stages of callus growth on nonselective medium.

Most clones displayed, however, intermediate levels of resistance, and this resistance was transmitted in most cases to the progeny. There was a good agreement between the observed level of resistance and the locus (*vr2* or *vr3*) which was shown to be mutated.

(3) Most of the regenerated plants did retain the *tl* character of chlorophyll deficiency, but often displayed variegation patterns different from the parental pattern. Such events were also observed after the in vitro anther culture which produced the haploid *tl* plants used for the selection experiments presented in this report ('Material and methods'). Most haploid *tl* plants did not display the same variegation patterns as the diploid parents, and part of them had returned to a low instability phenotype. In a few cases, we have also observed a loss of the chlorophyll deficiency and a return to the wild-type. These results indicate that *tl* variegation patterns are not reproduced with high fidelity after in vitro culture, whereas they are usually transmitted through sexual or vegetative reproduction.

Our results constitute an additional argument in favor of the presence of active transposable elements in the genome of the tobacco *tl* lines. We suspect that these elements are the cause of the increased mutagenic ability of the *tl* genotypes. Their activation by tissue culture could also explain the peculiar behavior of the *tl* instability after various in vitro steps, in ways similar to the maize transposable elements (Gorman and Peterson 1978; Culley 1986; Peschke et al. 1987).

A proof of the insertional nature of the *tl* spontaneous mutations at *vr2* and *vr3* loci should be given by the demonstration of instability for these mutations. We have, however, not been able to develop a reproducible procedure for selecting cells which have reverted to the wild-type, and the scoring of revertant seedlings in F3 progenies has not proven successful. The high level of *tl* somatic instability is, in fact, not correlated to a high level of germinal instability (around 1/10,000 for heterozygous *tl*/*TL* plants), and this should prevent an easy detection of any reversion event in progeny.

Experiments are under way for demonstrating the transposition of these putative transposable elements into the *nia* locus coding for the nitrate reductase apoenzyme. Mutations at this locus produce a phenotype selectable in vitro and a molecular probe is available (Calza et al. 1987), thus making possible a subsequent molecular isolation of the transposable element.

This work also confirms the usefulness of protoplast-derived cells as an appropriate material for cell genetic studies. New types of biochemical mutants can easily be selected at the cell level, instead of screening for individual plants or seeds. So far, plant transposable elements have been characterized from plant mutants expressing a limited range of phenotypic instabilities, mostly seed coloration or starch content. Few species, however, possess the advantage of maize, which allows the detection of instability on individual seeds. In most cases, transposable element activity can be visualized only by instabilities for floral coloration or chlorophyll expression. The type of approach described in this paper could become a useful tool for taking advantage of all of the possibilities afforded by transposon-tagging in higher plants.

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