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A genetic analysis of mutations recovered from tomato following *Agrobacterium*-mediated transformation with the maize transposable elements *Activator* and *Dissociation*

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Summary. The maize autonomous transposable element, *Activator* (*Ac*), and the nonautonomous element *Dissociation* (*Ds*), were introduced into the tomato cultivars VF36 and VFNT Cherry by *Agrobacterium*-mediated transformation. Progeny families from 145 primary transformants were scored at the seedling stage for phenotypically variant individuals. When VF36 was transformed, 20% of families had progeny with aberrant phenotypes. The mutation frequency in VFNT Cherry transformants was lower; in this cultivar 7% of the transformants had progeny segregating for seedling mutations. The majority of the mutations showed monogenic inheritance in the R_1 population, suggesting that the mutations occurred early in the transformation/regeneration process. One mutation, however, was recovered at low frequency in the R_1, suggesting a late mutagenesis event. When tomato was transformed with either the *Ac* or *Ds* elements, no differences in mutation frequencies were observed. Since *Ac* is transpositionally active in tomato transformants while *Ds* is not, these numbers indicate that the mutation frequency inherent to the transformation process is higher than the mutational activity of *Ac*. These results demonstrate that efficient gene tagging using heterologous transposable elements will require screening for transposon-induced mutations in later generations.

Key words: *Lycopersicon esculentum* – Transformation – Transposable elements – Somaclonal mutation

Introduction

One of the most powerful approaches for cloning genes with uncharacterized gene products is transposon tagging (Doering 1989; Shepherd 1988). Transposable elements are capable of causing mutations in genes into which they insert. If the transposable element causing the mutation has been cloned and characterized, it can be used as a probe to isolate sequences from the mutated gene. These can then be used as probes to isolate the intact gene from a genomic library. To date, transposon tagging has been successful only in those plant species in which endogenous transposable elements have been characterized, maize and *Antirrhinum* (Fedoroff et al. 1984; Martin et al. 1985). The recent finding that the maize transposable element *Ac* is capable of transposition when introduced into unrelated plant species suggests that it may be possible to use *Ac* elements as tags in plant species lacking endogenous elements (Baker et al. 1986; Van Sluys et al. 1987; Yoder et al. 1988).

The utility of heterologous transposable elements as gene tags will depend upon the frequency with which insertional mutations are recovered. This frequency depends upon a number of factors: (1) the frequency and timing of transposition, (2) the frequency with which an insertion event is transmitted to progeny, (3) the stability of the element following insertion, (4) the copy number of the element, and (5) the randomness of insertion sites. Transposition frequencies can be estimated from the frequency of excision because transposition of *Ac* requires excision (Greenblatt and Brink 1962). Baker et al. (1987) estimated that *Ac* excised from the donor T DNA in 25%–70% of transformed tobacco calli. In transgenic tomato plants, evidence of *Ac* excision in somatic tissue was detected in every transformant that contained an intact *Ac* (Yoder et al. 1988). Using an *Ac* excision assay that allowed visualization of revertant sectors, Jones et al. (1989) demonstrated that between 1% and 10% of self progeny from primary tobacco transformants were germinal revertants. Belzile et al. (1989) showed that at

least 60% of primary tomato transformants transmitted at least one, and occasionally more, transposed *Ac's* to R_1 progeny. The stability of transposed *Ac's* in progeny varied between families, in some families the same *Ac* insertion band was found in different progeny, in other families different siblings had different insertion bands. This study also demonstrated that the same *Ac* element remained active for at least three generations following transformation (Belzile et al. 1989). There has been little work to date on the question of transposon insertion site preferences in foreign species.

The most direct estimate of insertional mutation rates comes from measuring the frequency with which monogenic mutations are recovered from a mutagenized population. Mutation rates are readily determined by selfing plants that contain active elements and scoring resultant progeny for visible phenotypic changes. This is more efficient than determining mutations rate at selected loci because a number of loci can be simultaneously scored, and biases resulting from insertion site preferences will be minimized. In maize, 3%–7% of progeny derived from a line containing active *Mu* transposable elements will segregate for new mutations (Robertson 1978).

There is a complicating factor when determining mutation frequencies in transformed plants; regenerated plants generally display more phenotypic variation than non-regenerated controls. While both genetic and epigenetic effects have been observed (Evans 1989), genetic effects are the most troublesome because they are transmitted to progeny. The most common genetic changes occur to chromosome number or structure (Lee and Phillips 1988). Since tomato has a low tolerance for chromosomal changes (Khush and Rick 1967) the majority of these will not be genetically transmitted because of reduced fertility and seed set. Of primary concern in mutation frequency estimates are single gene mutations that arise during the course of regeneration. Evans and Sharp (1983) reported that 5% of tomato plants regenerated from leaf explants yielded progeny with monogenic mutations. To our knowledge, there are no reports about the frequency with which somaclonal mutations are obtained following transformation.

Our experiments are directed towards learning whether transformants containing *Ac* exhibit a higher mutation frequency than control populations. For the control population, we needed to use plants that were transformed with a construction similar to *Ac* but were incapable of transposition. We had previously shown that non-autonomous *Ds* elements are stable in transgenic tomato plants (Lassner et al. 1989). Since the *Ds* constructions were prepared in the same Ti-based vectors as the *Ac* constructions and both were introduced into tomato using the same selective regimes, we used *Ds* transformants as a control for mutation frequencies in primary transformants. Because *Ds* is stable in transgenic lines and *Ac* is active, a comparison of mutation frequencies between these two transformed lines should determine whether *Ac* increases mutation rates in transformed plants.

We examined progeny of primary transformants containing either the autonomous transposable element, *Ac*, or the stable nonautonomous element, *Ds*, for mutations. When either *Ac* or *Ds* was transformed into different tomato cultivars, the mutation frequencies were similar. In VFNT Cherry, mutations were detected in 7% of the progeny while 27% of progeny obtained from VF36 transformants were segregating for phenotypic variants. A genetic description of these mutations will be given here. We analyzed a number of mutant progeny from *Ac* transformations by Southern hybridization; in no case did a transposed *Ac* segregate with a mutation (Belzile et al. 1989). These two lines of evidence suggest that the mutations were not generated by the *Ac* but probably arose during regeneration.

Materials and methods

The plasmid pMAC was prepared by subcloning the maize transposable element *Ac7* into the Ti-based vector pMON200 (Fraley et al. 1985) as described by Yoder et al. (1988). A similar construction bearing the nonautonomous *DS1* element (Sutton et al. 1984) cloned into pMON200 was called pDS203 (Yoder et al. 1988). Each of these plasmids was introduced into *Agrobacterium tumefaciens* strain GV3111, containing the avirulent Ti-plasmid pTIB6S3-SE, by the triparental mating procedure as described (Fraley et al. 1985).

Cotyledons of either VF36 or VFNT Cherry were transformed with *Agrobacterium* GV3111, containing either pMAC or DS203, by the procedure adapted from Fillatti et al. (1987) as described (Yoder et al. 1988). Explants were maintained on media containing zeatin for 1–4 weeks, during which time regenerating shoots were excised and rooted. Aneuploids were identified by counting chloroplasts in leaf guard cells (Jacobs and Yoder 1989) and were subsequently discarded.

Transformants were grown to maturity in soil in a greenhouse. Flowers were manually self-pollinated, fruit was harvested, seed was collected, and between 25 and 50 self seed of each transformant were sown into wooden flats for scoring. Primary transformants are termed R_0, progeny obtained from selfing these plants are R_1, and progeny obtained from selfing the R_1 are R_2 (Chaleff 1981). R_1 progeny were grown in flats in the greenhouse with supplemental lighting with 1,000-W halide lamps. From emergence on, seedlings were visually scored for chlorophyll deficiencies, anthocyanin deficiencies, and growth habit and leaf morphology aberrations, which segregated in a family. Because of the necessity of growing these plants under recombinant DNA containment conditions, it was not possible to grow large enough populations to maturity to identify mutation past the seedling stage.

Results

Mutation frequencies in VF36 and VFNT Cherry transformed with either pMAC or pDS203

We used *Agrobacterium* transformation to introduce pMAC, a plasmid containing the autonomous transpos-

Table 1. Mutation frequency following transformation with pMAC or pDS203

Plasmid	Host	No. of exps.	No. of families screened	No. of mutations	Mutation frequency
pMAC	VF36	10	36	8	22%
pMAC	VFNT	12	48	3	6%
pDS203	VF36	4	6	1	17%
pDS203	VFNT	13	55	4	7%
Total		35[a]	145	16	11%

[a] In four experiments, both VFNT Cherry and VF36 were transformed

able element *Ac*, into tomato. To eliminate polyploids at an early stage in regeneration, the number of chloroplasts per stomatal guard cell was determined, and plants with aberrant numbers were discarded. Kanamycin-resistant plantlets from 22 separate transformation experiments were grown to maturity in the greenhouse and self-pollinated. Sterile plants were eliminated from the analysis at this time. Viable seed was harvested from 36 VF36 and 48 VFNT Cherry transformants, sown in flats, and between 25 and 50 progeny were scored for the segregation of phenotypic variants. When aberrant phenotypes were observed, a few plants were grown to maturity, self-pollinated, and R_2 progeny were examined to verify the genetic nature of the phenotype. Only mutations that were transmitted into the R_2 generation are included in the mutation rate determination.

Eleven mutations were recovered from 84 transgenic plants when pMAC was used as the transformation vector (Table 1). The phenotypes of these mutations will be described in detail below. Of the 11 mutants, 8 were recovered from VF36 transformants (22%) and 3 from VFNT Cherry (6%).

Similar transformation experiments were conducted using a T DNA construction, pDS203, which contains the nonautonomous *Ds* element instead of *Ac*. Fifty-five VFNT Cherry plants and six VF36 plants were regenerated over the course of 13 different transformation experiments using pDS203 as the donor plasmid. Seed were collected and R_1 seedlings were scored as described for the pMAC transformants. One mutation was recovered from VF36 and four were recovered from VFNT Cherry transformants. When pDS203 was used as donor plasmid, the mutation frequency was 17% for VF36 and 7% for VFNT Cherry (Table 1).

Overall, VF36 lines had higher mutation frequencies than VFNT Cherry (20% and 7%, respectively). Within the same cultivar, mutation frequencies were similar when either pMAC or pDS203 was used as the transformation vector. Therefore, there was no difference in mutation frequencies in primary transformants when either *Ac* or *Ds* was included on the transformation vector.

Description of mutations recovered from VF36

A total of nine mutants was recovered from transgenic VF36; six of these had altered chlorophyll pigmentation. The mutation in family 88-14 resulted in completely chlorotic cotyledons and lethality before development of the first leaves. The mutation recovered from 88-07 resulted in pale green cotyledons and leaves and reduced plant vigor. Two mutations resulted in variegated chlorophyll pigmentation. Progeny 88-08 had green-white variegation with well-defined somatic sectors of variegation. This mutant is similar to the ghost mutation described by Brauer and Rick (1956) although the white sectors in 88-08 are smaller. The progeny of transformant 88-13 segregated for a yellow-green variegation. Two mutations had altered chlorophyll pigmentation as an early manifestation but were pleiotropic at later stages. The leaves in plants bearing the mutation recovered from 88-94 had a white speckled color as well as a morphology similar to *entire* (Butler 1952). The mutation in 89-175 resulted in yellow-green leaves as well as reduced anthocyanin expression in hypocotyls and early leaves. A second anthocyanin-deficient mutant, 89-106, was completely lacking in anthocyanin and had leaves of a normal color. Each of the mutations was recessive as determined from the normal appearance of F_1 hybrids prepared between the mutants and wild types, or from the recovery of mutant phenotypes from normal appearing, heterozygous R_1s.

Two mutations that affected plant morphology were recovered from transformed VF36. In plants bearing the mutation recovered from 88-94, the first leaves emerge with an upward curl such that the underside of the leaves are clearly visible. This curling remains in later leaves where it is quite pronounced. Another manifestation of this mutation is an extended peduncle length, extending to over 25 cm in some plants. Progeny from 88-01 had rounded cotyledons and leaves with an obtuse apex (Fig. 1); the degree of rounding varies between plants. In order to quantify this phenotype, the ratio of leaf length to leaf width at the first lobe was determined. We measured the second leaves of young R_2s, wild-type VF36s, and F_1s prepared between the two. Using Duncan's Multiple Range Test, there was significant grouping of each population, the F_1s having values intermediate between either parental. From this analysis, it was determined that the mutation was incompletely dominant.

Eight of the nine mutants segregated in the R_1 as monogenic loci (Table 2). The exception was the mutation recovered from family 88-94. Forty R_1 progeny were examined and only one had the mutant phenotype of chlorosis and entire leaf shape. Twelve R_1s that appeared normal were grown, selfed seed was collected, and at least 20 R_2 seed were sown from each. None of these displayed either chlorosis or the aberrant leaf shape. The

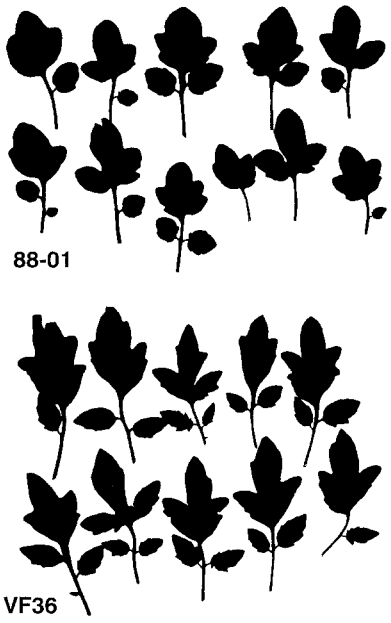

Fig. 1. Rounded leaf phenotype of 88-01. The first leaves of the 88-01 and wild-type VF36 seedlings are shown

Table 2. Mutations recovered from VF36

Family	Plasmid	Phenotype	Segregation	χ^2
88-01	pMAC	Rounded cotyledons and leaves	24 : 10	0.15[a]
88-07	pMAC	Pale green, reduced vigor	32 : 17	2.76[a]
88-08	pMAC	Green-white variegation	84 : 27	0.48[a]
88-13	pMAC	Yellow-green variegation	21 : 4	0.88[a]
88-14	pMAC	Early lethal albino	74 : 22	0.22[a]
88-94	pMAC	Leaves speckled white, entire	40 : 1	10.71
88-98	pMAC	Leaves curl, extended peduncle	118 : 37	0.14[a]
89-175	pMAC	Yellow-green, reduced anthocyanin	68 : 22	0.06[a]
89-106	pMAC	Anthocyanin deficient	52 : 10	3.03[a]

[a] No significant deviation from 3 : 1 ratio ($P > 0.05$)

possibility of seed lot contamination was eliminated because of the unique phenotype of this plant (C.M. Rick, personal communication). The mutation is stably transmitted to R_2 progeny and has been shown to be recessive because the F_1s prepared between the mutant and VF36 are normal.

Description of mutations recovered from VFNT Cherry

Four families had progeny with altered chlorophyll pigmentation (Table 3). The mutations in two of these families, 89-186 and 89-188, were recessives and resulted in complete chlorosis and early death of seedlings. The mutation in 89-186 is lethal very early upon germination; in only a few cases did the seedlings emerge from the soil. We have not yet germinated seeds in vitro and so have not determined the segregation ratio of this mutation. The mutation in 89-188 is delayed in its action; completely chlorotic first leaves emerge but the plants die soon after. Two other mutations with altered chlorophyll pigmentation were less drastic. Leaves from family 89-177 were yellow-green upon emergence and remained so through maturity. The leaves in family 89-144 emerged and remained pale green. Both of these plants were fully fertile at maturity.

Three families were segregating for morphological aberrations. Mutants from family 89-140 have an erect growth habit and leaves with fewer leaflet segments. Family 89-169 segregates for a phenotype in which the hypocotyl terminates in callus tissue; there is no further development of stems or leaves (Fig. 2). Occasionally, viable shoots emerge from the callus tissue and develop into fertile plants. These then transmit the mutant phenotype to 100% of their progeny. Because the plants occasionally grow out of the callus phase, we have not yet properly characterized the segregation ratio of this mutation. The third morphological variant recovered from transformation of VFNT Cherry is a leaf shape variant (89-179) in which the leaves are reduced to the mid-vein filament (Fig. 3). This mutation is similar in appearance to the known wiry mutations (Butler 1952). The appropriate allele tests are currently being done to see if the same loci have been affected.

Table 3. Mutations recovered from VFNT Cherry

Family	Plasmid	Phenotype	Segregation	χ^2
89-140	pDS203	Fewer leaflet segments erect habit	30 : 15	1.93[a]
89-144	pMAC	Light green	47 : 16[b]	0[a]
89-169	pDS203	Hypocotyl terminates in callus	N.D.[c]	
89-177	pDS203	Yellow green	66 : 14	2.40[a]
89-179	pDS203	Leaves reduced to mid-vein filament	80 : 2	22.87
89-186	pMAC	Early lethal albino	N.D.[c]	
89-188	pMAC	Delayed lethal albino	43 : 17[b]	0.36[a]

[a] No significant deviation from 3 : 1 ($P > 0.05$)
[b] Data from segregating R2 were pooled because of insufficient numbers in R1
[c] See 'Results'

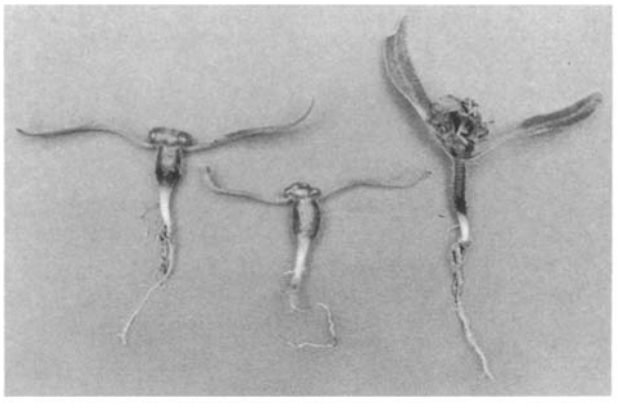

Fig. 2. Callus mutant in family 89-169. Three seedlings from the mutant 89-169 are shown. Shoots are regenerating from the callus in the third seedling; these grew into fertile plants

Fig. 3. Leaf shape mutant in 89-179. The leaves of this mutant are reduced to only the mid-vein, similar to the known wiry type mutants

Discussion

We examined progeny from 145 transformed tomato lines for segregating mutations. Sixteen families had siblings with chlorophyll deficiencies, reduced anthocyanin pigmentation, or altered growth morphology. Transmission ratios of the mutant phenotypes in R_1 progeny were consistent with the vast majority of mutations being monogenic. This was also observed by Evans and Sharp (1983) for mutations induced in nontransformed regenerating shoots. While the majority of somaclonal mutations are generally chromosomally based (Lee and Philips 1988), these were selected against in our transformation experiments at two stages. Transformants with aberrant chloroplast numbers were rogued at an early stage to eliminate polyploids (Jacobs and Yoder 1989). Also, because regenerated plants needed to have sufficient seed set in order for their progeny to be screened, plants with abnormal chromosome complements were selected against, since aneuploidy reduces fertility in tomato (Khush and Rick 1967). The monogenic ratios also suggest that the mutation events occurred early in the transformation/regeneration process. An exception was detected in family 88-94, in which only 1 of 40 R_1 progeny had the mutant phenotype. The event leading to the mutation in this family presumably occurred late in development so that only so small fraction of gametes were affected.

The overall mutation frequency in these populations was 11%. This is about twice that reported for somaclonal mutations in unselected, regenerated tomato (Evans and Sharp 1983). We raise three hypotheses for this difference: (1) the transposable elements cloned into the transformation vector are responsible for the increased mutation rate, (2) the transformation regime itself results in more variation than regeneration regimes, or (3) the lines we used for transformation are inherently more susceptible to tissue-culture-induced mutations than those used by Evans and Sharp (1983).

The overall frequency of mutations in primary tomato transformants is similar to that reported for active *Mu* elements in maize (Robertson 1978). There was, however, no difference in mutation frequency in plants transformed with either *Ac* or *Ds*; both plasmids resulted in equal numbers of aberrant progeny. Since *Ac* is an active transposable element in tomato while *Ds* is not (Yoder et al. 1988), this suggests that the mutations are not caused by the mobilization of *Ac*. In order to study this more closely, we examined aberrant progeny from families 88-01, 88-08, 88-14, and 88-94 by Southern hybridization (Belzile et al. 1989). In this analysis, *Ac*- and T DNA-specific fragments were used as probes on filters containing DNA from progeny that either did or did not segregate for a mutation. If insertion of a transposed *Ac* or inserted T DNA was responsible for the mutation, we expected to see correlation between those plants having an aberrant phenotype and a particular *Ac* or T DNA insertion band on the blot. Of those mutant progeny examined, none correlated to either an *Ac* insertion or a T DNA insertion (Belzile et al. 1989). We conclude that mutations recovered in these R_2 are not caused by the insertion of either *Ac* or T DNA, but rather result from the transformation process. In order to select insertional mutations induced by *Ac*, it will be necessary to score subsequent generations of families that did not originally transmit aberrant phenotypes.

From a number of studies it is clear that the hormonal regime, time in culture, and source of explant material can affect the types and rates of chromosomal variation (Bayliss 1980). Therefore, the difference in regeneration versus transformation regimes may affect somaclonal mutation rates. For example, transformation requires selection of transformed cells in antibiotics. It is our experience that transformed plants grow slower under selection than nonselected regenerating plants, with the result that transformed plants are in culture longer than unse-

lected plants. Other components of transformation may also affect mutation rates.

There was a significant difference in the number of mutations recovered from VFNT Cherry and VF36 transformants. When either the *Ac*- or the *Ds*-containing plasmids were used, the mutation frequency in VFNT Cherry transgenics was 6%–7%. This is in contrast to VF36 transformants, where mutants were recovered in about 20% of the families. Differential mutation rates following regeneration of different cultivars has been previously described (Bayliss 1980; Tempelaar et al. 1985). Clearly, selection of a cultivar for transformation studies should include a consideration of the somaclonal rates inherent in the line and not just the ease with which the plant can be transformed.

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