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Are the T-DNA mutants amenable to standard recombination analysis?

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Abstract

Genetic analysis with T-DNA mutants often brings difficulties resulting from instability of the transgenic phenotype. In this work three different *Arabidopsis thaliana* T-DNA embryonic lethals and one T-DNA morphological mutant were analyzed in F2 progeny after 15 different crosses with marker lines for individual chromosomes. F2 analysis of 44 segregation ratios revealed segregation distortion of similar character consisting in abnormal excess of nontransgenic plants to the detriment of transgenic ones. We quantified this phenotypic drift (*d*) on the basis of phenotypic ratios given the respective formulas. The *d* values indicate the rate of F1 gametes which loose the T-DNA mutation or ability of its expression. The obtained *d* value were relatively high, 0.4 to 0.9 for individual crosses. It makes the standard recombination analysis with insertional mutants very problematic or even impossible.

Additional key words: *Arabidopsis*; F2 recombination; phenotypic drift.

Introduction

T-DNA insertional mutagenesis in plants is a common approach for the molecular identification and isolation of tagged genes, as well as for understanding their role in plant growth and development (Koncz *et al.* 1992). A transgene technique is also currently used in various crop plants for improvement of agronomic characters (Rogers and Parkes 1995, Rissler and Mellon 1998).

Nevertheless, work with transgenic plants often brings difficulties resulting from instability of the transgenic phenotype, so that the number of progeny expressing the transgene is sometimes fewer than expected (Mittelsten Scheid *et al.* 1991) and genetic analysis, based on Mendelian rules of inheritance, of transgenes and their phenotype, is therefore not straightforward.

The difficulty in genetic analysis with T-DNA loci can be caused by various factors. In some cases the silencing of the transgene can be epigenetic (Mittelsten Scheid *et al.* 1998). Risseeuw *et al.* (1997) found that the instability of a tagged gene in *Nicotiana* was induced in tissue culture during the regeneration of the transgenic plants. In other cases, different chromosomal aberrations associated with T-DNA insertion in *Arabidopsis* were found (Errampalli *et al.* 1991). Similarly, Castle *et al.* (1993) and Franzmann *et al.* (1995) identified several insertional embryo-defective mutations exhibiting linkage on two different chromosomes, probably due to a chromosomal translocations. Problems with cosegregation analysis of mutant gene and selectable marker occur frequently (Forsthoefel *et al.* 1992, Feldmann *et al.* 1997). Feldmann *et al.* (1997) found that about 10 % of transformed T-DNA *Arabidopsis* lines with exceptional segregation, manifested deficiency of individuals with the selectable marker (Kan^R). The Kan^R marker was transmitted unequally through the gametes since T-DNA tagged genes were associated with gamete functions in these lines. Howden *et al.* (1998) also found that in *Arabidopsis* T-DNA tagged male and female gametophytic mutants segregation distortion occurred for the selectable marker. Similarly, according to Ulian *et al.* (1996), it is difficult to predict the inheritance and the expression of foreign genes in the progeny of transgenic *Petunia* plants. Other examples of unusual segregation for insertional mutations, especially in *Arabidopsis*, can be enumerate but sometimes the exceptional segregation results in their exclusion from the experiment.

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According to the published data the instability of the transgene in plants does not to be an exception but rather a rule. Similarly, our own results reported here show abnormal segregation ratios in the progeny of insertional mutants crossed with other mutants in *Arabidopsis*. Moreover, this deviation from the expected phenotypic ratios was of similar character. In this work we analyze this segregation ratio distortion.

Materials and methods

Three different *Arabidopsis thaliana* T-DNA embryonic lethals (VIII-41-1, VIII-75-1 and VIII-82-1) and one T-DNA morphological mutant (*scaf*) were used in the experiment. The T-DNA lines where we identifed the embryonic lethal mutations were obtained from C. Koncz, MPI Köln, Germany. The T-DNA morphological mutant (*scaf*) was detected in the Department of Plant Molecular Biology in České Budějovice by M. Ondřej and has changes in flower morphology, with higher numbers of stamens and carpels. In all the mutants the hygromycin phosphotransferase (*hpt*) selectable gene was included in the T-DNA.

Marker lines for individual linkage groups originate from M. Koornneef and are available in NASC Nottingham, England. The following marker lines were used: NW4 (*ch-1, ap1-1, gl2-1*), NW151 (*sti, cp2-1, er-1*), NW6 (*cp2-1, as-1, cer8-1*), NW7 (*hy2-1, gl1-1, tt5-1*), NW8 (*bp-1,cer2-2, ap2-1*), NW9 (*ttg-1, yi-1*).

The scheme of crosses for F2 recombination analysis of embryonic lethals was as follows:
e^+e^- *mm* (marker line) x e^-e^- m^+m^+ (heterozygote for embryonic lethal mutation): selfpollination of F1 genotype e^-e^+ m^+m: F2 analysis of four phenotypes: A = e^+e^+ m^+-, B = e^-e^+ m^+-, C = e^-e^- *mm*, D = e^-e^- *mm*. The segregation ratio 3A: 6B : 1C : 2D is expected in case of independent assortment. F1 and F2 embryonic lethal heterozygotes (e^-e^+) were identified by the presence of siliques containing one quarter of defective seeds, *i.e.* segregating homozygous lethal embryos of the next generation (Müller 1963).

The scheme for F2 recombination analysis of the morphological recessive mutation was as follows: *scaf scaf mm* (marker line) x *scaf scaf* m^+m^+ (morphological T-DNA mutant): selfpollination of F1 genotype *scaf scaf* m^+m: F2 analysis of four phenotypes: E = *scaf*- m^+-, F = *scaf scaf* m^+-, G = *scaf*- *mm*, H = *scaf scaf mm*. The segregation ratio 9E : 3F : 3G : 1H is expected in case of independent assortment.

In all crosses emasculated marker lines were used as the maternal parent. Plants were grown in cultivation chambers in pots with soil, at temperature 20 - 23 °C, 12-h photoperiod, and irradiance 70 μmol m^{-2} s^{-1}.

Results

Ten different crosses were performed with the three different T-DNA insertional embryonic lethal mutations and 5 different crosses with the one morphological T-DNA mutation. Standard homozygotes and heterozygotes in 1:2 ratio are expected among F1 progeny plants from crosses with the embryonic lethals. However, more homozygous plants (or fewer of heterozygotes) were obtained together in F1 (*e.g.*, 79 homozygous e^+e^+ and 122 heterozygous e^+e plants from all 10 crosses performed with embryonic lethal mutations). In the F2 generation 44 segregation ratios of insertional mutant and individual marker genes were analyzed. In all cases, the F2 analysis revealed unusual segregation ratios (Tables 1, 2). Interestingly, the frequency of plants exhibiting the transgene phenotype (B + D, F + H, respectively) was dramatically reduced while the frequency of the non-transformed phenotype (A + C, E + G, respectively) was increased.

Since distortion in the frequency of the F2 phenotypic classes was similar in all the T-DNA crosses studied (embryonic lethal as well as morphological, *scaf*), it was possible to construct a mathematical model to describe this situation.

Mathematical model for segregation ratio distortion in T-DNA mutants: The model is based on the presumption of a certain phenotypic drift (*d*) in F2 progeny after the crossing. The phenotypic drift can be due to different causes of segregation distortion which is always manifested in F2 generations as an excess of non-transgenic plant phenotypes over transgenic plant phenotypes.

According to the model the rate of phenotypic drift can be estimated on the basis of the F2 segregation ratio.

For embryonic lethals the four phenotypic classes in F2 are: A (e^+e^+ m^+-), B (e^+e m^+-), C (e^-e^- *mm*) and D (e^+e *mm*), where *e* is the insertional recessive lethal embryonic mutation and *m* is the recessive visible marker gene. Factor *d* is estimated according to the formula:
$d = (2q-1) / (2q+1)$, where q = A/B = C/D.
In individual progeny it is better to use the phenotypic ratio A/B than C/D, since it is based on higher plant numbers and is independent on variable viability of homozygous genotypes carrying different marker gene alleles. Other possibilities for estimating the *d* value are to use the combined data, where q = (A+C) / (B+D), or, or even better, the combined weighted estimate, where

Table 1. F2 segregation ratios produced by the cross of individual *Arabidopsis* embryonic lethal T-DNA mutants with marker lines. The expected ratio with no linkage is 3A : 6B : 1C : 2D (where A = $e^+e^+m^+$-, B = $e^+e\,m^+$-, C = e^+e^+ *mm*, D = e^+e *mm*).

Cross	Marker gene	Number of plants in each F2 phenotypic class			
		A	B	C	D
W4 × VIII-41-1	*ch-1*	141	63	50	10
	ap1-1	161	69	30	4
	gl2-1	142	66	49	7
W151 × VIII-41-1	*sti*	141	67	40	13
	cp2-1	119	63	62	17
	er-1	138	66	43	14
W7 × VIII-41-1	*hy2-1*	108	35	38	6
	gl1-1	107	35	45	5
	tt5-1	130	36	17	4
W8 × VIII-41-1	*bp-1*	107	73	45	12
	cer2-2	127	80	25	5
	ap2-1	118	76	34	9
W9 × VIII-41-1	*tt*	202	14	34	2
	g-1	175	14	61	2
	yi-1	192	16	44	0
W4 × VIII-75-1	*ch-1*	61	35	10	2
	ap1-1	50	35	21	2
	gl2-1	53	31	18	6
W151 × VIII-75-1	*sti*	57	36	25	11
	cp2-1	71	39	11	8
	er-1	73	39	9	8
W9 × VIII-75-1	*tt*	98	35	29	7
	g-1	89	27	38	15
	yi-1	105	40	22	2
W8 × VIII-82-1	*bp-1*	139	50	49	3
	cer2-2	158	47	30	6
	ap2-1	114	42	74	11
W9 × VIII-82-1	*tt*	160	86	16	4
	g-1	137	78	39	12
	yi-1	161	85	15	5

Table 2. F2 segregation ratios after the cross of *Arabidopsis* T-DNA mutant *scaf* with marker lines. The expected ratio with no linkage is 9E : 3F : 3G : 1H (where E = *scaf* - m^+-, F = *scaf scaf* m^+-, G = *scaf* - *mm*, H = *scaf scaf mm*).

Cross	Marker gene	F2 phenotypic classes with number of plants			
		E	F	G	H
W4 × *scaf*	*ch-1*	141	63	50	10
	ap1-1	161	69	30	4
	gl2-1	142	66	49	7
W6 × *scaf*	*cer8-1*	141	67	40	13
	cp2-1	119	63	62	17
W7 × *scaf*	*hy2-1*	108	35	38	6
	gl1-1	107	35	45	5
	tt5-1	130	36	17	4
W8 × *scaf*	*bp-1*	107	73	45	12
	cer2-2	127	80	25	5
	ap2-1	118	76	34	9
W9 × *scaf*	*tt*	202	14	34	2
	g-1	175	14	61	2
	yi-1	192	16	44	0

q = (3A+C) / (3B+D). If d = 0, q = 0.5, but with increasing d the value q increases up to infinity at d = 1.

For morphological visible recessive mutant the factor d is estimated on the basis of four F2 phenotypic classes E, F, G, H mentioned above according to the quadratic formula:

$$d_{1,2} = 1 \pm (2\sqrt{q+1})/(q+1)$$

where q = E/F = G/H and the same rules are valid for it's estimation as in case of embryonic lethals. If d = 0, q = 3. With increasing d the value q increases up to infinity at d = 1.

Application of the mathematical model: On the basis of the crosses (Tables 1,2) mathematical model was used to estimate the phenotypic drift values, d, which are given in Table 3. The value, of d = 0.5, for example, means that 50 % of F1 gametes lost the T-DNA mutation or lost the ability to express the T-DNA phenotype. In all crosses, the calculation was made using both the phenotypic ratio A/B, E/F, respectively and the weighted combined estimate. These values are generally very close and moreover they are independent of the possible differential viability of marker gene phenotypes.

The values of phenotypic drift are given for individual crosses (Table 3). The variation is due to the slight differences in distribution of the standard homozygotes and heterozygotes for T-DNA mutation among phenotypically standard plants for different marker genes. This intercrossing variation does not exceed 0.1 (with one exception). The values of factor d for individual crosses is characteristic and varies from approximately 0.5 to 0.9.

Table 3. Estimated phenotypic drift values (d) using segregation ratios in F2 progeny of different crosses of four *Arabidopsis* insertional mutants with marker.

T-DNA mutant type	Marker line	d values
Embryo lethal VIII-41-1	W4	0.62 to 0.65
	W151	0.58 to 0.62
	W7	0.72 to 0.76
	W8	0.49 to 0.52
	W9	0.92 to 0.93
Embryo lethal VIII-75-1	W4	0.48 to 0.55
	W151	0.52 to 0.59
	W9	0.68 to 0.74
Embryo lethal VIII-82-1	W8	0.69 to 0.74
	W9	0.56 to 0.58
Morphological *scaf*	W4	0.46 to 0.58
	W6	0.48 to 0.51
	W7	0.42 to 0.52
	W8	0.59 to 0.64
	W9	0.62 to 0.63

Discussion

A standard recombination analysis which has been used for decades by geneticists, as the main tool for gene mapping, is sometimes impaired by different effects (other than linkage) that cause the unusual segregation ratios. Some of these effects are due to differential viability of genotypes and methods of calculating these are known.

In this report similar unusual F2 segregation ratios were found in all experiments involving 15 different crosses of four different T-DNA mutants. The distortion of segregation ratios had the character of phenotypic drift and lead to an excess of nontransgenic plants to the detriment of transgenic plants in all cases. The distortion was more marked with the embryonic lethal T-DNA mutants. These changes in the F2 phenotypic ratios may make the calculation of possible linkage problematic even if correction for vitality or certation (Koornneef and Stam 1987) was applied.

The ratios obtained (Tables 1 and 2) can be theoretically explained by unreliable transmission or expression of the transgene in the next generations. The resulting phenotypic drift, d was quantified and the d figures obtained indicate the rate at which F1 gametes either loose the T-DNA mutation or lose the ability of its expression. For example, the d factor of 71.5 % can change the F2 segregation ratio 3:6:1:2 which is valid for lethal mutations to curriously exactly 9:3:3:1, the typical F2 ratio for recessive morphological mutations. In several crosses with embryonic lethals this drift was statistically proved in our experiments (Table 1, f.i. crosses W7 with marker genes *hy2-1* or *gl1-1*). Another figures of d factor fit to other of our F2 segregation ratios.

Different causes have been ascribed to the difficulties when T-DNA transformed lines are used in genetic analysis (Maessen 1997). Instability at the transgene locus can be due to the loss of the insert, the transgene silencing or unequal transmission through the gametes. In this work we only characterize and quantify the course of deviations from the expected phenotypic segregation ratios without analysis of the causative factor(s).

Experiments reported here with T-DNA *Arabidopsis* mutants show that the drift in the frequency of transgenic and non-transgenic phenotype in F2 is characteristic of individual crosses. According to the theoretical model, the deviations towards the lower values of d can be due to the linkage between the transgene and the marker gene. Since decreasing frequency of recombinants also decreases the estimated q value (the phenotypic ratio A/B, E/F, respectively) and consequently decreases the value of phenotypic drift, d. Theoretically, extremly low d value could indicate the linkage of the transgene with the corresponding marker. According to the given

formulas the influence of linkage in lowering the *d* values is higher in the cases of embryonic lethal than in morphological T-DNA mutations. Nevertheless, the lowest value of *d* was 0.42 (Table 3) and it was the case based on the segregation ratio insertional mutant *scaf* with marker gene *gl1-1* on the chromosome 3 where the linkage (*P* = about 0.3) was detected in another experiment using molecular markers (data not shown).

Our results showed dramatic disruption of F2 phenotypic segregation ratios after the crossing of transgenic *Arabidopsis* mutants. The phenotypic drift *d* was quantified. The high estimates of the *d* found in our experiments makes the standard recombination analysis with T-DNA mutants very problematic or even impossible.

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