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# Segregation of T-DNA inserts in the offspring of *Arabidopsis thaliana* after *Agrobacterium* transformation

M. ONDŘEJ*, T. KOCÁBEK**, S. RAKOUSKÝ* and D. WIESNEROVÁ*

*Institute of Plant Molecular Biology, Academy of Sciences of the Czech Republic*, Faculty of Biological Sciences, University of South Bohemia**, Branišovská 31, České Budějovice, CZ - 370 05, Czech Republic*

## Abstract

Using various transformation methods, T-DNA constructions for insertional mutagenesis were introduced into *Arabidopsis thaliana* and the pattern of segregation of hygromycin resistance selectable marker was followed in succeeding generations in individual transgenic lines up to $T_4$ generation. Despite the low frequency of transformation, T-DNA was often inserted in two or more independent sites. Mendelian segregation ratios 3:1, 15:1, and irregular segregation ratios were observed. We have also shown continuous decrease of the expression of the resident hygromycin resistance transgenic trait in some lines.

*Additional key words:* hygromycin resistance, insertional mutagenesis, root transformation, seed transformation, vacuum infiltration.

## Introduction

The goal of the international *Arabidopsis thaliana* genome project is the sequencing of the whole genome and the determination of function and main interactions of all genes. Important tool in studies of unravelling gene functions is insertional mutagenesis. Insertions of T-DNA constructs of *Agrobacterium tumefaciens* or modified transposons into the *A. thaliana* gene sequences lead to the induction of mutations. The mutated genes can be cloned based on homology with inserted T-DNA or transposon sequences. It seems to be realistic to saturate the *Arabidopsis* genome with insertional mutations because *Arabidopsis* has about 20 000 genes and

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*Abbreviations*: BAP - 6-benzylaminopurine; *hpt* - hygromycin phosphotransferase; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; IPAR - $N^6$-2-isopentenyladenosine riboside; R/S - resistant/sensitive, T-DNA - transferred DNA.

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Fax: (+420) 38 41475, e-mail: ondrej@umbr.cas.cz

only a low fraction of T-DNA insertions are found in repetitive DNA (Aramuganthan and Earle 1991).

Numerous *Arabidopsis* transformation protocols have been reported: for root tissue (Valvekens *et al.* 1988, Koncz *et al.* 1994), leaf tissue (Schmidt and Willmitzer 1988, Van der Graaf and Hooykaas 1996), cotyledons (Lloyd *et al.* 1986), hypocotyl tissue (Akama *et al.* 1992), embryo tissue (Sangwan *et al.* 1991), seeds (Feldmann and Marks 1987) and even whole plants (Bechtold *et al.* 1993, Chang *et al.* 1994, Katavic *et al.* 1994).

For the purpose of insertional mutagenesis, callogenesis and regeneration in tissue culture methods is burdened by possibility of induction of somaclonal variability, which could induce mutations outside T-DNA insertions. *In planta* methods, developed especially for *Arabidopsis* offer two main advantages: tissue culture and the resulting somaclonal variations are avoided and only short time is required in order to obtain individual transformants.

While most transformation methods include the insertion of T-DNA of the Ti plasmid of *A. tumefaciens* into the cell nuclei of the somatic tissues, an important specific feature of *A. thaliana* is the possibility of T-DNA insertions into germ line. It has been first demonstrated by Feldmann and Marks (1987) who found that treating of germinating seeds by *A. tumefaciens* results in T-DNA insertions in the next generation. The bacteria probably survive in the treated plants up to the germ cell stage. Probably the late premeiotic, meiotic or perhaps even postmeiotic cells are transformed, as no homozygous T-DNA inserts have ever been found in the offspring (Feldmann 1991). Bechtold *et al.* (1993) have shown that vacuum infiltration of *A. tumefaciens* into flower shoots leads to more efficient transformation. In our laboratory we have shown that this approach leads also to a high level of transient expression of GUS genes in *A. thaliana* leaf tissues (Rakouský *et al.* 1997). Bechtold *et al.* (1993) came to the conclusion that the stage at which the T-DNA transfer takes place is either the end of gametogenesis or the zygote stage. Chang *et al.* (1994) developed another *in planta* method. Young inflorescences are cut off and the wounded surfaces are inoculated with *Agrobacterium*. The frequency of transformants in the progeny of such inoculated plants is relatively low and very variable. Despite these methods avoiding tissue culture also somaclonal variability can be localized outside T-DNA. It is probably due to an erroneous, uncompleted genetic repair process supporting T-DNA integration which leads to mutations (Koncz *et al.* 1992).

Genes introduced into the plant genome by transformation are usually transmitted to the progeny in a Mendelian segregation 3:1 (McCormick *et al.* 1986). Sometimes, however, T-DNA is inserted into two or more sites of the plant genome. According to Feldmann (1991), most of the *A. thaliana* transformants exhibit Mendelian segregation pattern of the dominant selectable antibiotic resistance marker. Of those, about one half contains one functional insert (3:1 ratio), one quarter segregates 15:1, which corresponds to the integration of T-DNA into two sites and the rest segregates in different irregular patterns, which include several genetic and epigenetic phenomena. The last category has usually been neglected in *Arabidopsis* insertion mutagenesis studies.

In the study presented here, we have followed in detail the segregation ratios of antibiotic (hygromycin) resistance in several succeeding generations of individual transformation lines of *A. thaliana* obtained by different transformation experiments. Special attention has been paid to the irregular segregation ratios. Among them also new type of irregularity - gradual decrease of transgene expression has been found.

## Materials and methods

**Plants:** Two *Arabidopsis thaliana* (L.) Heynhold ecotypes have been used: Columbia (Col-0) and C24.

***Agrobacterium* strains:** The *Agrobacterium tumefaciens* strains used have been constructed by Koncz *et al.* (1989) and obtained thanks to C. Koncz and M. Hrouda. They belong to the Plant Cloning Vectors (PCV) series and consist of two functional units: a conditional mini-RK2 and T-DNA. The T-DNA carries hygromycin resistance gene (*hpt*) as a plant selectable marker and a segment of pBR322 plasmid with ColE1 replication origin together with β-lactamase gene (for plasmid rescue and other techniques in *E. coli* and for selection in *Agrobacterium*). Vector plasmids pPCVR-tx and pPCVRN4 have been used (Koncz *et al.* 1994). The pPCVR-tx T-DNA has 35S promoter directed outwards located closed to the right borderline sequence and is able to activate silent genes if inserted behind the plant gene promoter. The pPCVRN4 T-DNA carries tetramer of 35S promoter enhancer to activate silent genes in the vicinity of the sites of integration. Of course, they can induce also recessive mutations, in dependence on the site of integration. The vector plasmids are harboured in *A. tumefaciens* strain GV3101 (pMP90RK). This strain harbours a C58C1 bacterial chromosome carrying rifampicin resistance gene and helper Ti plasmid pMP90RK, which is derivative of pTiC58 with deleted T-DNA, with bacterial gentamycin resistance and kanamycin resistance genes. Bacteria were grown overnight in liquid media according to Langley and Kado (1972) supplemented with 100 mg $dm^{-3}$ of rifampicin and 50 mg $dm^{-3}$ of kanamycin at 28 °C.

**Plant transformation:** Root transformation method of Valvekens *et al.* (1988) modified by Koncz *et al.* (1994) has been used. *Arabidopsis thaliana* C24 root culture was established in the liquid medium. Cut roots were cocultivated with *Agrobacterium* for a short period and transferred to a solid callogenesis medium (2.0 mg $dm^{-3}$ IAA, 0.5 mg $dm^{-3}$ 2,4-D, 0.2 mg $dm^{-3}$ kinetin, 0.2 mg $dm^{-3}$ IPAR) with antibiotics to suppress *Agrobacterium* growth *Claforan* (cefotaxim) 200 mg $dm^{-3}$ and *Ticarpen* (ticarcillin) 500 mg $dm^{-3}$. After two weeks, the roots with growing calli were transferred to shoot inducing medium (0.2 mg $dm^{-3}$ BAP, 0.1 mg $dm^{-3}$ IBA, 0.05 mg $dm^{-3}$ IAA) with 15 mg $dm^{-3}$ hygromycin. Auxin conditioning in the previous medium followed by cytokinin treatment leads to quick regeneration of shoots.

For the seed transformation method of Feldmann and Marks (1987) modified by Pavingerová and Ondřej (1995) was used. Seeds presoaked for 12 h were treated by

*A. tumefaciens* suspension for another 12 h and than sown into the soil. Transformations were detected as a low proportion of hygromycin resistant seedlings in the progeny of plants grown from the treated seeds.

Vacuum infiltration transformation according to Bechtold *et al.* (1993) has been used. In our modification, plants (Col-0) were grown in the soil in pots. When shoots with first flowers appeared, *A. tumefaciens* suspension was vacuum infiltrated into developing inflorescences. The pots were turned upside down and the shoots immersed in vessel with *Agrobacterium* suspension with approximate density $10^9$ mm$^{-3}$ in the infiltration medium, placed in exsiccator. Plants were put under vacuum ($10^4$ Pa) for 15 min in vacuum chamber. After the treatment, plants were cultivated up to the seed stage and seeds were collected. The plants were cultivated in cultivation cabinets with all precautions avoiding the spread of A. *tumefaciens*.

**Growth of plants:** Seeds were surface-sterilized and placed on the MSAR (Koncz *et al.* 1994) medium containing 10 mg dm$^{-3}$ hygromycin. After two weeks hygromycin resistant plants were selected and transferred into the soil. In the infiltration experiments, the growing hygromycin resistant plants have been transferred first to agar medium without hygromycin and, after 10 d, to the soil in pots. The soil after experiments was autoclaved.

**Genetic analyses:** The segregation ratio of hygromycin resistant ($Hyg^R$) and hygromycin sensitive ($Hyg^S$) seedlings was scored. Generations of T-DNA transformed plants are named as follows: $T_0$ = *Agrobacterium* treated plants, $T_1$ = first generation of hygromycin resistant plants, $T_2$ = first segregating generation.

**The PCR detection:** The verification of the the presence of hygromycin phosphotransferase (*hpt*) selectable transgene in purified DNA (Nucleon Phytopure Plant DNA Extraction kit, *Amersham Life Sciences*, Aylesbury England) was performed by PCR. The primers for PCR were *hpt*1: 5'- GTC CTG CGG GTA AAT AGC TGC GCC and *hpt*2: 5'- GTG TAT TGA CCG ATT CCT TGC GG and determined amplification of 295 bp fragment (Scheid *et al.* 1994). PCR reactions were performed in 0.025 cm$^3$ using Taq DNA polymerase (*Promega*, Madison, USA). DNA samples were denatured at 94 °C for 5 min, and amplified using 35 cycles (94 °C for 30 s, 55 °C for 2 min, 72 °C for 8 min) followed by elongation at 72 °C for 10 min.

**Chemicals:** Antibiotics *Hygromycin B* was purchased from *Boehringer*, Mannheim, Germany, *Ticarpen* from *Beecham Pharmaceuticals*, Singapore, *Claphoran* from *Roussel UCLAF*, Paris, France.

## Results

Using three different *Arabidopsis* transformation methods 49 independent transformed lines were induced. The main results of induction of T-DNA insertions and types of *hpt* segregation ratios are given in Table 1. The proportion of

independent lines segregating in a regular Mendelian pattern 3:1 or 15:1 and in irregular ratios correspond roughly to the data of other authors (Feldmann 1991, Chang *et al.* 1994).

Table 1. Types and frequencies of transformed lines segregating in different segregation ratios in the offspring of transformants induced by different methods of transformation.

| Type of segregation (ratio $Hyg^R$:$Hyg^S$) | Root transformation | Seed transformation | Vacuum infiltration | Total | % |
|---|---|---|---|---|---|
| 3:1 | 9 | 7 | 4 | 20 | 41 |
| 15:1 | 9 | 3 | 3 | 15 | 31 |
| >15:1 | 3 | 0 | 0 | 3 | 6 |
| 3:1< $\chi^2$<15:1 | 3 | 1 | 1 | 5 | 10 |
| < 3:1 | 2 | 3 | 1 | 6 | 12 |

Table 2. The pedigree of the insertion line 1, obtained by vacuum infiltration, showing 3:1 segregation ratio in $T_2$ generation (39:13, $\chi^2 = 0.00$).

| $T_3$ | | | | | $T_4$ | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Line | $Hyg^R$:$Hyg^S$ | R/S | Theoretical segregation ratio tested | $\chi^2$ | Line | $Hyg^R$:$Hyg^S$ | R/S | Theoretical segregation ratio tested | $\chi^2$ |
| 1 | 55: 0 | - | 1:0 | 0.00 | 1-2 | 49: 0 | - | 1:0 | 0.00 |
| 2 | 24: 7 | 3.42 | 3:1 | 0.10 | | | | | |
| 3 | 49:12 | 4.08 | 3:1 | 0.92 | 3-1 | 139: 0 | - | 1:0 | 0.00 |
| | | | | | 3-3 | 83:19 | 4.37 | 3:1 | 2.21 |
| | | | | | 3-4 | 179: 0 | - | 1:0 | 0.00 |
| 4 | 81: 0 | - | 1:0 | 0.00 | | | | | |
| 5 | 67:16 | 4.19 | 3:1 | 1.45 | 5-1 | 71:27 | 2.63 | 3:1 | 0.34 |
| 6 | 41:14 | 2.93 | 3:1 | 0.01 | 6-1 | 91:35 | 2.60 | 3:1 | 0.52 |
| 7 | 44:10 | 4.40 | 3:1 | 1.21 | 7-1 | 133:49 | 2.71 | 3:1 | 0.36 |
| | | | | | 7-2 | 148:39 | 3.79 | 3:1 | 1.71 |
| | | | | | 7-3 | 161: 0 | - | 1:0 | 0.00 |
| 8 | 5: 0 | - | 1:0 | | 8-1 | 78:23 | 3.39 | 3:1 | 0.37 |
| | | | | | 8-2 | 191: 0 | - | 1:0 | 0.00 |
| | | | | | 8-3 | 150: 0 | - | 1:0 | 0.00 |
| | | | | | 8-4 | 126: 0 | - | 1:0 | 0.00 |
| 9 | 18: 7 | 2.57 | 3:1 | 0.12 | 9-1 | 24: 7 | 3.43 | 3:1 | 0.10 |
| | | | | | 9-2 | 135: 0 | - | 1:0 | 0.00 |
| | | | | | 9-3 | 127:39 | 3.26 | 1:0 | 0.20 |
| 10 | 39:16 | 2.44 | 3:1 | 0.49 | 10-1 | 189: 0 | - | 1:0 | 0.00 |
| | | | | | 10-2 | 159: 0 | - | 1:0 | 0.00 |
| | | | | | 10-3 | 96:33 | 2.91 | 3:1 | 0.02 |

During the experiments, altogether 130 248 seedlings in 1088 segregation ratios were followed. As only hygromycin resistant plants have been grown to the flower and seed stage, the offspring of hygromycin sensitive plants was never obtained. The values of segregation ratios were sometimes intermediate between 3:1 and 15:1 and

also other irregularities of segregation occurred. Thus the segregation ratios can be divided into the following groups:

*1*) The ratio of hygromycin resistant and hygromycin sensitive plants ($Hyg^R$:$Hyg^S$) is 3:1. The *hpt* selectable transgene is present in a single site in the original transformed genome (Table 2).

Table 3. The pedigree of insertion line 2, obtained by root transformation, segregating 15:1 in $T_2$ generation (30:2, $\chi^2 = 0.00$).

| $T_3$ Line | $Hyg^R$:$Hyg^S$ | R/S | Ratio tested | $\chi^2$ | $T_4$ Line | $Hyg^R$:$Hyg^S$ | R/S | Ratio tested | $\chi^2$ |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 144: 9 | 16.00 | 15:1 | 0.04 | 1- 1 | 44: 1 | 44.00 | 15:1 | 1.25 |
| 2 | 79:21 | 3.76 | 3:1 | 0.85 | 1- 2 | 48: 0 | - | 1:0 | 0.00 |
| 3 | 64:23 | 2.78 | 3:1 | 0.10 | 1- 3 | 42: 3 | 14.00 | 15:1 | 0.01 |
| 4 | 69:29 | 2.38 | 3:1 | 0.10 | 1- 4 | 121: 3 | 40.30 | 15:1 | 3.11 |
| 5 | 38: 0 | - | 1:0 | - | 1- 5 | 44: 0 | - | 1:0 | 0.00 |
| 6 | 91: 0 | - | 1:0 | - | 1- 6 | 41: 9 | 4.56 | 3:1 | 1.31 |
| 7 | 67:16 | 4.19 | 3:1 | 1.45 | 1- 7 | 33:10 | 3.30 | 3:1 | 0.07 |
| 8 | 87:18 | 4.83 | 3:1 | 3.46 | 1- 8 | 46: 3 | 15.30 | 15:1 | 0.001 |
| 9 | 60:26 | 2.31 | 3:1 | 0.19 | 1- 9 | 50: 5 | 10.00 | 15:1 | 0.76 |
| 10 | 39: 4 | 9.75 | 15:1 | 0.68 | 1-10 | 55: 0 | - | 1:0 | 0.00 |
| 11 | 30: 7 | 4.29 | 3:1 | 0.55 | 1-11 | 27:14 | 1.93 | 3:1 | 1.83 |
| 12 | 34: 0 | - | 1:0 | - | 1-12 | 36: 8 | 4.50 | 3:1 | 1.09 |
| 13 | 50: 4 | 12.50 | 15:1 | 0.12 | 2- 1 | 30: 9 | 3.33 | 3:1 | 0.08 |
| 14 | 124: 6 | 20.70 | 15:1 | 0.59 | 2- 2 | 36: 0 | - | 1:0 | 0.00 |
| 15 | 114:16 | 7.13 | 15:1 | - | 2- 3 | 43: 8 | 5.38 | 3:1 | 2.36 |
| 16 | 51:15 | 3.40 | 3:1 | 0.18 | 2- 4 | 49:15 | 3.27 | 3:1 | 0.08 |
| 17 | 44: 6 | 7.33 | 15:1 | 2.65 | 2- 5 | 51: 0 | - | 1:0 | 0.00 |
| 18 | 61:16 | 3.81 | 3:1 | 0.73 | 2- 6 | 30:12 | 2.50 | 3:1 | 0.29 |
| 19 | 67:21 | 3.19 | 3:1 | 0.06 | 2- 7 | 46:16 | 2.88 | 3:1 | 0.02 |
| 20 | 100:11 | 9.09 | 15:1 | 2.54 | 2- 8 | 35: 0 | - | 1:0 | 0.00 |
| 21 | 51: 4 | 12.75 | 15:1 | 0.10 | 5- 1 | 51: 0 | - | 1:0 | 0.00 |
| 22 | 50: 3 | 16.67 | 15:1 | 0.03 | 5- 2 | 55: 0 | - | 1:0 | 0.00 |
| 23 | 22: 7 | 3.14 | 3:1 | 0.01 | | | | | |
| 24 | 27:11 | 2.45 | 3:1 | 0.32 | | | | | |
| 25 | 89:10 | 8.90 | 15:1 | 2.51 | | | | | |
| 26 | 38:11 | 3.45 | 3:1 | 0.17 | | | | | |
| 27 | 48: 2 | 24.00 | 15:1 | 0.43 | | | | | |
| 28 | 24: 3 | 1.85 | 3:1 | 2.03 | | | | | |

*2*) The ratio $Hyg^R$:$Hyg^S$ corresponds to the theoretical ratio 15:1, which, in terms of classical genetics, is due to duplicite noncumulative factors. It is expected to be the consequence of integration of T-DNA into two unlinked sites. Theoreticaly, in the next generation 4/15 of the lines derived from single hygromycin resistant plants should segregate in the ratio 15:1, 4/15 segregate in the ratio 3:1 and 7/15 show hygromycin resistance but they do not (Table 3).

*3*) The segregation ratio was sometimes lower than 15:1 but higher than 3:1. This segregation ratio was observed in 10 % of the lines. The *hpt* gene was inserted most

probably into two sites of the genome. One possibility of explanation of the distortion of the segregation ratio is that the T-DNA sites were linked on the same chromosome. The other explanation is that the inserts were integrated into two unlinked chromosomes, but the expression of one of the inserts was partly inhibited (Table 4).

Table 4. The pedigree of insertion line 3, obtained by root transformation, segregating between 3:1 and 15:1 (174:24, in $T_2$ generation).

| $T_2$ Line | $Hyg^R$:$Hyg^S$ | R/S | Ratio tested | $\chi^2$ | $T_3$ Line | $Hyg^R$:$Hyg^S$ | R/S | Ratio tested | $\chi^2$ |
|---|---|---|---|---|---|---|---|---|---|
| 3 | 174:24 | 7.25 | 15:1 | 11.7* | 1 | 101: 35 | 2.89 | 3:1 | 0.040 |
| | | | | | 2 | 67: 17 | 3.94 | 3:1 | 10.200* |
| | | | | | 3 | 46:142 | 0.32 | | |
| | | | | | 4 | 207: 23 | 9.00 | 15:1 | 5.520* |
| | | | | | 5 | 177: 29 | 6.10 | 3:1 | 13.100* |
| | | | | | 6 | 324: 87 | 3.72 | 3:1 | 3.220 |
| | | | | | 7 | 358: 24 | 14.90 | 15:1 | 0.001 |
| | | | | | 8 | 85: 26 | 3.27 | 3:1 | 0.150 |
| | | | | | 9 | 101: 39 | 2.59 | 3:1 | 0.610 |
| | | | | | 10 | 47: 6 | 7.83 | 15:1 | 2.330 |
| | | | | | 11 | 105: 14 | 7.50 | 15:1 | 6.180* |
| | | | | | 12 | 96: 38 | 2.53 | 3:1 | 0.810 |
| | | | | | 13 | 77: 29 | 2.66 | 3:1 | 0.310 |
| | | | | | 14 | 204: 42 | 4.86 | 3:1 | 8.240* |
| | | | | | 15 | 108: 44 | 2.45 | 3:1 | 1.260 |
| | | | | | 16 | 162: 20 | 8.10 | 15:1 | 6.980* |
| | | | | | 17 | 67: 12 | 5.58 | 3:1 | 4.060* |
| | | | | | 18 | 45:124 | 0.36 | | |
| | | | | | 19 | 115: 49 | 2.35 | 3:1 | 2.080 |
| | | | | | 20 | 69: 29 | 2.38 | 3:1 | 1.100 |
| | | | | | 21 | 107: 21 | 5.10 | 3:1 | 5.040* |
| | | | | | 22 | 118: 8 | 14.75 | 15:1 | 0.002 |
| | | | | | 23 | 92: 15 | 6.13 | 3:1 | 6.880* |
| | | | | | 24 | 57: 19 | 3.00 | 3:1 | 0 |
| | | | | | 25 | 55: 13 | 4.23 | 3:1 | 1.260 |
| | | | | | 26 | 60: 19 | 3.16 | 3:1 | 0.040 |
| | | | | | 27 | 146: 23 | 6.35 | 3:1 | 11.700* |
| | | | | | 28 | 133: 19 | 7.00 | 15:1 | 10.100* |
| | | | | | 29 | 111: 10 | 11.10 | 15:1 | 0.840 |
| | | | | | 30 | 134: 56 | 2.39 | 3:1 | 2.030 |

* $\chi^2$ tests against hypotheses were not significant.

*4*) The ratio of $Hyg^R$ and $Hyg^S$ plants is higher than 15:1. This segregation ratio appeared especially after root transformation and most probable explanation is presence of three or more independent sites of insertion, *i.e.* 63:1 for three inserts, 255:1 for four inserts etc. There are also other possibilities of explanation, like somatic crossing over in the chromosome pair which carries the transgene.

*5*) Only a small proportion (or no proportion) of the progeny of hygromycin

Table 5. The pedigree of insertion line 4, obtained by seed transformation, with decreased expression of *hpt* gene.

| $T_2$ Line | $Hyg^R$:$Hyg^S$ | R/S | $T_3$ Line | $Hyg^R$:$Hyg^S$ | R/S | $T_4$ $Hyg^R$:$Hyg^S$ |
|---|---|---|---|---|---|---|
| 1 | 13:71 | 0.18 | 1 | 6: 93 | 0.06 | All |
| | | | 2 | 15:106 | 0.14 | plants |
| | | | 3 | 21: 73 | 0.29 | $Hyg^S$ |
| | | | 4 | 0: 58 | 0.00 | |
| | | | 5 | 2:111 | 0.02 | |
| | | | 6 | 2: 95 | 0.02 | |
| | | | 7 | 0:102 | 0.00 | |
| | | | 8 | 0:130 | 0.00 | |
| | | | 9 | 2:106 | 0.02 | |
| | | | 10 | 4: 84 | 0.05 | |
| | | | 11 | 16: 39 | 0.11 | |
| | | | 12 | 5:143 | 0.03 | |

* plants were analysed by PCR, and results are shown in Fig. 1.

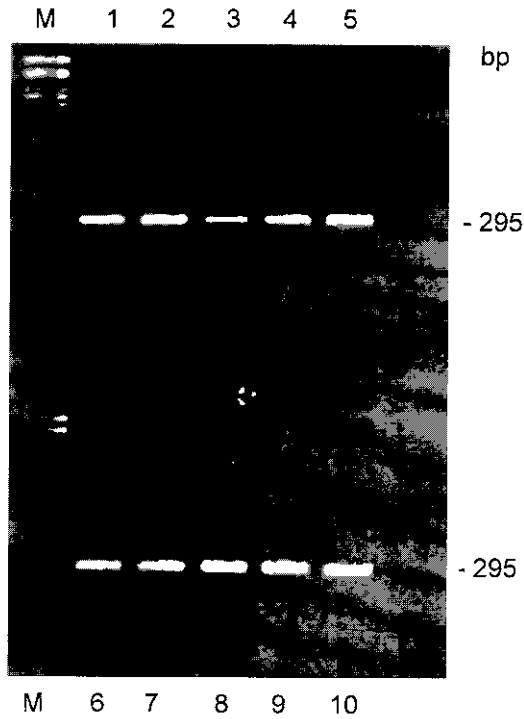


Fig. 1. PCR screening for the presence of hygromycinephosphotransferase gene (*hpt*) region in transformed *A. thaliana* sublines of insertion line 4 in $T_4$ generation. M - DNA size marker (λ/Pst I); 1 - 10 - amplified 295 bp region of *hpt* gene from individual transformed lines.

resistant transformant shows resistance to hygromycin. It could result from variety of mechanisms. One of them is the gradual decrease of transgene expression in the progeny, which was found in one of our offsprings followed (Table 5). In the $T_4$ generation, the offsprings of individual $T_3$ lines was divided into two pairs. One of them has been sown on MSAR media in Petri dishes with hygromycin to test for the hygromycin resistance. Here, no clear-cut hygromycin resistant plants have been observed, all plants showed almost full degree of sensitivity to hygromycin. The other half was sown on MSAR medium without hygromycin. Five weeks old plants (with fully developed leaf rosette and begining of flowering stage) have been subjected for detection of the presence or absence of the *hpt* gene by PCR. The results showed that hygromycin sensitive plants still carried the whole transgene. The amplified *hpt* gene fragment was detected in 48 out of 50 plants analysed (Fig. 1).

## Discussion

Among 49 lines tested, 72 % segregated in monohybrid or dihybrid Mendelian segregation, 6 % of the lines segregated for more than two T-DNA inserts and 22 % showed non-Mendelian patterns of T-DNA inheritance. There are several possible causes of erroneous Mendelian segregation, such us chromosomal rearrangements and other phenomena which often cause lethal embryonic mutations (Castle *et al.* 1993, Nacry *et al.* 1998). Feldmann *et al.* (1997) have shown that there is also a fraction of *A. thaliana* transformants in which the selectable antibiotic resistance trait fails to segregate in expected Mendelian ratio - the proportion of antibiotic resistant plants in the offspring is variably reduced in the first segregating generation and this reduction is stabilized also in succeeding generations. Such reduction is due to an absence of T-DNA in the sensitive plants and it is accompanied by the reduction of the number of seeds per silique. Feldmann *et al.* (1997) have shown that it is due to the failure of transmission of T-DNA insertion through the gametophyte stages either microgametophyte, megagametophyte or both. This phenomenon was explained by inactivational insertional mutations of genes important in gametogenesis and failure of development of a proportion of gametes carrying the mutated chromosomes.

Our transformed line No. 4, obtained by infiltration method of transformation, is of different type. We have proven that T-DNA is still present, but antibiotic resistance marker expression is decreased and still gradually decreases in succeeding generations so that in $T_4$ it is almost not expressed at all. The gradual decrease of expression of the transgene is frequent phenomenon (Mlynárová *et al.* 1996, Stam *et al.* 1997), especially in *A. thaliana* (Pavingerová and Hrouda 1991, Scheid *et al.* 1991). It is usually caused by gene silencing due to gene methylation (Furner *et al.* 1998). Here we brought an example of this phenomenon, which contributes to irregular segregation of the selectable trait in *A. thaliana* insertional mutagenesis.

Among the insertion lines scored, one insertional mutation with several malformations in flower development has been found and its detailed study will be the the subject of another publication.

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