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Meiotic Transmission of T-DNA Genes in *Arabidopsis thaliana* Plants and Their Expression After 5-Azacytidine Treatment

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Abstract. *Arabidopsis thaliana* tumors were induced by octopine strain of *Agrobacterium tumefaciens* B6S3 and its derivatives with modified T-DNA. Flowering shoots appeared spontaneously on *in vitro* cultivated tumors and set seeds. R_1 and R_2 progeny of octopine synthesizing plants segregated in opine synthesis activities 3 : 1 and 15 : 1. Octopine synthase activity showed absolute linkage with agropine synthesis in most lines. In R_3 and R_4 progenies, the fraction of octopine synthase and agropine synthesis positive plants was lower than expected, but Mendelian segregation was restored if plants were cultivated on medium with 5-azacytidine. The most probable mechanism of disapearance of opine synthesis is cytosine methylation. The effect of 5-azacytidine lasted for at least next two generations.

Gradual disapearance of the expression of introduced markers in succeeding generations of the progeny of transgenic plants is scarcely mentioned in publications (Norton and Towers 1984, Matzke *et al.* 1989). Here we give description of this phenomenon in *Arabidopsis thaliana*. *A. thaliana* has been shown to regenerate shoots efficiently from tumors after transformation by *Agrobacterium* strains with wild T-DNA of both *A. tumefaciens* (Aerts *et al.* 1979, Pavingerová *et al.* 1983, Ondřej *et al.* 1984) and *A. rhizogenes* (Pavingerová *et al.* 1984). Expression of T-DNA genes in the plant genome is often inactivated by T-DNA hypermethylation and its effects can be eliminated by 5-azacytide (Hepburn *et al.* 1983, Amasino *et al.* 1984). Here we give new data on the repression of opine synthesis in *A. thaliana* plants three generations after transformation and its restoration by 5-azacytidine.

MATERIAL AND METHODS

Plant material

Seeds of *A. thaliana* (L.) Heynh. cv. Dijon were obtained from T. Gichner (Institute of Experimental Botany, Prague) and cv. Landsberg were obtained from J. Braaksma (University of Groningen, Haren).

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Bacterial strains

Transformation of plant tissues was carried out with *A. tumefaciens* B6S3 strain either carrying the original Ti plasmid or its derivatives (Leemans *et al.* 1982) with some T-DNA genes inactivated, pGV2100, pGV2210, pGV2216 and pGV2219 kindly provided by J. Schell (Max-Planck-Institut, Köln).

Transformation and cultivation of *Arabidopsis thaliana*

Plants and transformed tissues were cultivated on PG media (Negrutiu *et al.* 1975) as described in Pavingerová *et al.* (1983) and Ondřej *et al.* (1984). 5-azacytidine (AC) produced by Serva, Heidelberg was added to the media after autoclaving. Sterilization was carried out by ultrafiltration and make final concentration made to 2 mg l^{-1}.

Opine detection

Agropine detection by paper electrophoresis and silver nitrate staining was carried out as described by Petit *et al.* (1983). The octopine synthase (ocs) activities were determined by the method after Otten and Schilperoort (1978).

Southern blot analysis

Plant DNA was prepared by the method of Roger and Bendich (1985). The DNA was analyzed using the Southern blotting technique as described in Maniatis *et al.* (1982). The clones pBam 8/29 and pNW 31,C,2,19 (Thomashow *et al.* 1980), obtained by courtesy of P. Thomashow, Department of Microbiology and Immunology, University of Washington, were used as probes to determinate the presence of T-DNA.

RESULTS

The *A. tumefaciens* strains used for crown gall induction can be divided into three types.The first one is the B6S3 strain with unmodified Ti plasmid and the other two types are derivatives thereof. The second type is the strain carrying Ti plasmid pGV2210, which posses deletion on T-DNA, covering gene 4 (itp) for cytokinin synthesis. The *A. tumefaciens* strains carrying Ti plasmids pGV2100, pGV2216 and pGV2219 form the third type. Their T-DNA region has inactivated auxin synthesis genes 2 (iaaH) and 1 (iaaM), respectively (Leemans *et al.* 1982).

Crown galls appeared 2–3 weeks after inoculation of centres of leaf rosettes. There were no significant differences between crown gall formation on *A. thaliana* races Dijon and Landsberg and results in Table 1 are pooled. The efficiency of crown gall formation by B6S3 was slightly higher than that caused by the other strains. On the other hand, crown galls induced by *A. tumefaciens*

TABLE 1
Induction and differentiation of crown galls

Type	Ti plasmid used	Sites inoculated	Crown galls No.	[%]	Difference from B6S3 χ^2 value	Differentiated* crown galls No.	[%]	Difference fromB6S3 χ^2 value
1	B6S3	610	208	34.1		24	11.5	
2	pGV2210	490	126	25.7	9.1	103	81.7	167.5
3	pGV2100	610	166	27.2	6.8	59	35.5	39.6
	pGV2216	560	136	24.3	13.5	80	58.8	88.0
	pGV2219	560	111	19.8	30.0	82	73.9	124.8

All differences are significant on 1% level

B6S3 showed lower degree of regeneration of shoots than those induced by modified strains independently whether genes iaaH, iaaM or ipt were inactivated. Shoots differentiated from crown galls directly on plants inoculated with *A. tumefaciens* strains of the second and third types, but never on those inoculated with B6S3. Further differentiation took place after subcultivation. Table 1 give the final results. No shoots rooted, but some shoots flowered and set seeds. 360 shoots were obtained and 90 of them showed morfological deviations: either dwarf growth, curled leaves and thick stems, or giant growth and dark green leaves. Almost half (46 %) of regenerated shoots showed octopine synthase (ocs) activity and agropine synthesis. Ocs activity and agropine synthesis of 54 % regenerated shoots were bellow the limit of detection.

Seeds were collected from 10 individual R_0 regenerants showing ocs activity and sown on PG_0 medium. 328 plants, which all formed roots, were obtained and 46 of them showed abnormal phenotype. Only for three lines the number of analysed plants was sufficient for statistically sound interpretation (Table 2). One line of analysed plants (L-2100-5) showed in 89,2 % ocs activity while only in 72,2 % agropine synthesis. No plants showing only agropine synthesis were found.

TABLE 2
Segregation of opine production in R_1 progeny of plants regenerated from crown gall tumors

Progeny line	No. of plants tested	ocs* No.	[%]	ocs* and agr* No.	[%]	Segregation ratio
L-B6S3-1B	23	18	78.3	18	78.3	3:1
L-2100-5	223	199	89.2	161	72.2	3:1 <–>[a] 15:1
L-2100-7	47	42	89.3	40	85.1	15:1

<–>[a] This designation indicates that the segregation ratio was between 3:1 and 15:1 for ocs

TABLE 3
Summary of chi-square analysis of R_2 families

Segregation	ocs activity	agropine synthesis
3 : 1 (one insert)	14	12
15 : 1 (two unlike inserts)	5	5
T-DNA homozygotes	7	5

Offspring of 26 plants from 10 lines of R_1 which showed ocs activity was used in segregation studies (Table 3). Totally 22 out of 26 offspring lines showed both ocs activity and agropine synthesis segregating together as single marker. There were rare exceptional plants showing ocs activity but not agropine synthesis in all generations, which make less than 2 % ocs and agropine synthesis

TABLE 4
Segregastion of ocs activity of offspring of the line D-2216-6/7 in R_4, R_5, R_6 and R_7 generations after cultivation on media with and without 5-azacytidine

R_4			R_5			R_6			R_7		
a	b	c	a	b	c	a	b	c	a	b	c
5/4	0	38 : 26									
5/4	AC	46 : 15	/2	0	87 : 35						
			/2	AC	66 : 18	/2	0	32 : 8	/24	0	5 : 1
									/24	AC	10 : 2
						/2	AC	22 : 6	/13	0	12 : 3
									/13	AC	4 : 1
13/4	0	24 : 4									
13/4	AC	26 : 0	/1	0	36 : 1						
			/1	AC	35 : 0						
			/2	0	42 : 0						
			/2	AC	33 : 0						
13/6	0	22 : 6	/2	0	42 : 1						
			/2	AC	46 : 1						
13/6	AC	34 : 1	/4	0	27 : 2						
			/4	AC	32 : 2						
			/5	0	18 : 4	/1	0	24 : 1	/25	0	16 : 0
									/25	AC	12 : 1
						/1	AC	27 : 1	/16	0	16 : 2
									/16	AC	12 : 2
			/5	AC	11 : 1						

a – offspring subline, b – treatment: AC = azacytidine, 0 = no azacytidine, c – segregation

positive plants. We suppose that they produced agropine in levels bellow the detection limit. On the contrary to this, only ocs but not agropine synthesis was found in remaining four lines, which should be explained by integration of T_L-DNA but not T_R-DNA.

In individual R_3 and R_4 sublines, either homozygosity for T-DNA markers or 3 : 1 segregation was expected. In all lines, markedly lower than theoreticaly expected frequencies of opine synthesis were observed in all sublines scored. The obvious cause is the decrease of the expression of genes for opine syntesis.

Seeds of transgenic opine synthesizing plants were sown on PG_0 medium with AC. The effect of AC on restoration of Mendelian segregation was evident. Expression of opine synthesis genes after AC treatment was investigated also in further three generations (Table 4). In most of cases loss of the regained activity of opine synthesis genes was not observed, even during next three generations without AC.

Plant material for Southern blotting was cultivated as leaf-derived callus tissues on PG_2 medium. The T-DNA structure was analysed in the span of three years to determine the stability of exogenous integrated DNA sequences (Fig. 1). Although the structure of integrated T-DNA sequences differ from Ti plasmid T-DNA (Fig. 1C), no change in the position and number of bands was detected. The fate of T-DNA in the progeny of regenerated transformed plants was followed through several generations and the stability of exogenous sequences during meiosis was proved in most cases. For example, in the fourth generation of the progeny of a regenerant 2219-15A showing ocs activity the structure of T-DNA was similar as in the original R_0 plant (Fig. 2). In one of the samples of the fourth generation, however, two new fragments appeared (Fig. 2, line 6). The appearance of the new fragment can be explained by duplication or amplification of certain region of T-DNA.

DISCUSSION

In most independently transformed lines. gene for ocs activity and agropine synthesis genes were found, by segregation data, localized in a single linkage groups of the plant genome. The T-DNA of most octopine Ti plasmids is composed of two regions, T_L and T_R. The ocs gene is localized on T_L (Leemans *et al.* 1982) and agropine synthesis is coded by three genes on T_R (Ellis *et al.* 1984). In tobacco, T_L and T_R are integrated mostly independantly (Dahl *et al.* 1983). Situation of octopine T_L- and T_R-DNA integration in our experiments with *A. thaliana* is quite different. Plant species dependent mechanism not only from the point of view of T-DNA integration, but also T-DNA excision in *A. tumefaciens* cells (Peralta and Ream 1985) should be supposed.

The inactivation of T- DNA genes, as the result of T-DNA hypermethylation, which can be removed by AC has already been found by Hepburn *et al.* (1983)

and Amasino *et al.* (1984). Such inactivation might be general cause of regeneration of transformed plants from crown galls, as described first in *Nicotiana tabacum* (Amasino *et al.* 1984). Regeneration of whole plants from crown galls induced by unmodified Ti plasmids was described also in *Bidens alba* (Norton and Towers 1984) and tomato (Nečásek *et al.* 1988). In both studies, the offspring of transformed lines was followed and reduced proportion of opine synthesizing plants was found. Here we showed dissapearance of opine synthesizing activities in part of the plants in the third generation of offspring. The most striking feature of this process is the duration of opine synthesis for three generations and then its partial loss simultaneously in independently transformed lines. The possibility of repression of the activity of transgene after several seed generations points to the importance of following the expression of transgenes, especially those which are not selected for, during several succeeding generations.

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Figs. 1 and 2 at the end of the issue.

Lamb, C. J., Beachy, R. N. (ed.): Plant Gene Transfer. UCLA, Symp. Molec. and Cellul. Biology. New Ser., Vol. 129. Wiley-Liss, New York–Chichester–Brisbane–Toronto–Singapore 1990. 345 pp. ISBN 0-471-56739-6

The new volume of traditional UCLA Symposia on Molecular and Cellular Biology summarizes proceedings of the Symposium on Plant Gene Transfer held at Park City, Utah April 1–7, 1989. The numerous authors deal with a wide range of different fields of plant molecular biology, represented by individual chapters in the book; Transformation, Gene Isolation, Gene Regulation, Receptors and Signal Transduction, Protein Engineering and Targeting, Engineering Stress Resistance and Metabolic Engineering. Each chapter comprises four to five contributions bringing the newest information in the respective fields. Several contributions describe new techniques and strategies as *e.g.* transformation technologies (Cao *et al.*), strategies for physical mapping of complex genomes (Evans and Evans) or for transposon tagging (Baker and Hehl), strategies for modifying plant lipid composition (Browse *et al.*), for engineering a new flower colour in Petunia (Meyer *et al.*), for targeting to subcellular organelles as vacuoles (Tague *et al.*) or mitochondria (Schmitz *et al.*) and use of antisense RNAs in physiological studies (Rothstein *et. al.*, Cannon *et al.*). The results of studies of several important physiological processes are reported. Using transgenic *rin* mutants of tomato Giovannoni *et al.* showed, that expression of polygalacturonase gene is not sufficient for the completion of ripening process. In studies of plant defense genes homologies in promoters of genes for chalcone synthase and phenylalanine-ammonia lyase were found (Dixon *et al.*). Partial purification and identification of fusicoccin receptor is reported (Weiler *et al.*) as well as structure of site I auxin-binding site from corn (Palme *et al.*). In this material presence of ras- and YPT-related genes coding for GTP-binding proteins was demonstrated. An interesting approach to obtain heavy metal resistant plants by introducing gene for human metallothionein II is reported by Misra and Gedamu.

The first chapter on transformation is closed by a discussion summary. I find it a very good idea and it is a pity, that it was not done also in the other chapters.

The contribution by J. E. Mullet on Reversible Inhibition of Hypocotyl Growth in Soybean Seedlings Exposed to Water Deficit, although very interesting, does not seem to match the book content.

The new volume of UCLA Symphosia brings broad, highly professional information on new achievements and possibilities in the field of plant gene transfer. As such, it will be very useful for all research workers in this field.

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