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Segregation in the progeny of transformed rapeseed (*Brassica napus*)

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Abstract

The primary transformant of spring rapeseed cv. HM-81 contained TL- and TR-DNA of agropine plasmid pRi of *Agrobacterium rhizogenes* 15834. The presence of TL-DNA corresponds to visible transformed phenotype in its progeny; the leaves are wrinkled and the plants are shorter than normal plants. R_1, R_2 and R_3 generations have mostly transformed phenotype. The normal phenotype appears in a low frequency in F_1 generation. Autogamised F_1 plants segregate in F_2 transformed and normal phenotype in 3:1 ratio. It is possible to suppose that TL-DNA is present in two different *loci* of one pair of homologic chromosomes. The recombination frequency is 12 % (microsporogenesis) or 6 % (microsporogenesis and macrosporogenesis). In some crosses the transformed phenotype has a maternal type of inheritance. Maternal inheritance influences also several growth characteristics, *e.g.* length of plants and number of seeds/pods.

Introduction

Rapeseed is an oil plant with growing importance (Bauer and Röbbelen 1989, Scarisbrick *et al.* 1989). It is supposed that genetic engineering will contribute to its future breeding (Knauf 1987). Genome changes with the use of *Agrobacterium* plasmids offer great possibilities. Ooms *et al.* (1985), Guerche *et al.* (1987) and Dusbábková *et al.* (1989) incorporated unchanged T-DNA of pTi or pRi plasmids into rapeseed. Some others (Pua *et al.* 1987, Fry *et al.* 1987, Charest *et al.* 1988, Radke *et al.* 1988) transformed rapeseed with disarmed T-DNA marked with signal and/or other genes.

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Ooms *et al.* (1985) incorporated into rapeseed T-DNA of *A. rhizogenes* LBA 9402 containing plasmid pRi 1855; the transformed plant was entirely sterile. On the contrary, the transformants produced by Guerche *et al.* (1987) with the use of *A. rhizogenes* A4 were fertile; the number of seeds in individual pods was, however, lowered. In this case the recipients of T-DNA were winter cultivars Brutor, Bienvenue and Darmor. The incorporation of T-DNA of plasmids pRi does not interfere with the regeneration of whole plants from transformed roots. The structure and the function of agropine plasmids pRi is sufficiently known (Jouanin 1984, Birot *et al.* 1987). TL-DNA of agropine strains *A. rhizogenes* causes a morfological syndrome with wrinkled leaves, low root geotropy, decreased apical dominance, shortening of internodia, heterostyly and lowered viability of pollen grains. These effects are caused through the function of *rol* genes (Schmülling *et al.* 1988).

In previous papers we described some characters of the transformant HM-81-JZ (Dusbábková *et al.* 1989, Hrouda *et al.* 1988) containing TL and TR of pRi 15834 in its genome. Later we studied the inheritance of its "transformed" phenotype in its progeny obtained after autogamisation and backcrossing. Here we present the results of these studies.

Material and methods

Plant material: Transformation of rapeseed (*Brassica napus* L.) cv. HM-81 with T-DNA of *Agrobacterium rhizogenes* 15834 and the regeneration of the transformant HM-81-JZ (JZ) is described elsewhere (Dusbábková *et al.* 1989). Spring cultivar HM-81 and winter cultivars SL-502, Silesia and Arabela were used for crossing as untransformed parents. Seeds were germinated and plants cultivated in pots in a greenhouse for determination of transformed or normal phenotypes. Autogamised or castrated flowers were isolated with muslin bags.

Classification of phenotypes: The visible phenotype of transformed or normal plants is very different and easy to determine. The leaves of plants with transformed phenotype (T) are wrinkled. Plants with normal phenotype (N) have not wrinkled leaves and are higher than transformants. The phenotypes were checked 6 weeks after germination of seeds. The difference between T and N phenotype is constant till maturity of plants (Fig. 1).

Plants for determination of growth characteristics were cultivated on experimental fields (Breeding Station Slapy near Tábor, South Bohemia). Normal technics of plant breeding praxis were used.

Pollen grains in plants with normal and transformed phenotype were stained with carmine in propionic acid (Picken 1984).

Extraction of DNA and Southern blotting: Plant DNA was isolated *via* a procedure described by Taylor and Powell (1983), slightly modified according to Amasino *et al.* (1984). Hind III restriction enzyme from ÚSOL (Praha) was used to digest the DNA. The DNA was analyzed using the Southern blot technique as described in Maniatis *et al.* (1982). The clone pNW 44 was used as probe to determine the

presence of the left-hand (TL) portion of the Ri plasmid T-DNA (Huffman *et al.* 1984). The whole plasmid was labelled with ^{32}P(-deoxycytidine 5'-triphosphate) (111 TBq mmol^{-1}) by nick translation kit obtained from *Amersham*.

Fig. 1. Normal phenotype of rapeseed cv. HM-81 (*left*) and transformed phenotype of its transformant HM-81-JZ (*right*).

Flow cytometric analysis of nuclear DNA content: Cell nuclei were isolated from young leaves by chopping them with a scalpel in LB 01 buffer (Doležel *et al.* 1989). Released nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) at the final concentration of 1.4 μM. When necessary, cell nuclei isolated from young leaves of one haploid (n=24) plant of *Nicotiana tabacum* were added to samples prior to staining and were used as an internal reference. Nuclear DNA content was analysed on a *Leitz MPV Compact Flow Cytometer*. Histograms were analysed on a microcomputer using a *Flowstar* software (Doležel 1989). Each sample was analysed five times. A minimum of 10 000 nuclei were measured in each run.

Relative 2C nuclear DNA content was expressed by the DNA index (DI) as the ratio of the mean of the relative DNA content of G_1 nuclei of the sample divided by the mean of the relative DNA content of the G_1 nuclei of the control plant. DNA index was calculated by the following formula :

$$DI = \frac{X}{T} \times \frac{T}{C}$$

where X, T, and C are means of the relative DNA content of unknown sample, internal reference *(N. tabacum)*, and control plant (HM-81), respectively.

Results

The correlation between phenotype T and production of agropine and/or mannopine is not absolute. This situation described already Wullems *et al.* (1981) and Tepfer (1984). The most probable reason of it is the methylation of cytosine (Amasino *et al.* 1984, Brown 1989). Therefore we did not test the production of opines in progenies of primary transformant JZ.

Table 1. Phenotypes in autogamised progeny of primary transformant JZ. T = transformed phenotype, N = normal phenotype

Plant	Generation	Phenotype	
		T	N
JZ	R_1	97	0
JZ-4	R_2	5	25
JZ-14	R_2	12	0
JZ-21	R_2	13	0
JZ-28	R_2	25	0
JZ-36	R_2	14	0
JZ-21/10	R_3	20	0
JZ-28/6	R_3	20	0
JZ-36/8	R_3	20	0

The primary transformant JZ had TL- and TR-DNA of pRi15834 in its genome (Hrouda *et al.* 1988). We tested the presence of only TL-DNA in progenies of the transformant JZ. In more than fifty plants analyzed by Southern blotting the presence of TL-DNA sequences was always in correlation with T phenotype. In plants with N phenotype we did not detect TL-DNA. Example of Southern analysis is on Fig. 2. The bands which did not correspond to internal fragments of TL-DNA and which were present also in non-transformed plants corresponded to nonspecific hybridization with mitochondrial and chloroplast DNA (data not shown). It was impossible to check the presence of absence of TL-DNA in all plants in individual experiments with R_1, R_2, R_3, F_1 and F_2 generations.

The pollen "viability" (staining with carmine) in primary transformant JZ was 62 %, in nontransformed control plants 98.7 %. We did not find lowered pollen

viability in R_1, F_1 and later generations. The heterostyly and other flower abnormalities (Schmülling *et al.* 1988) were not observed.

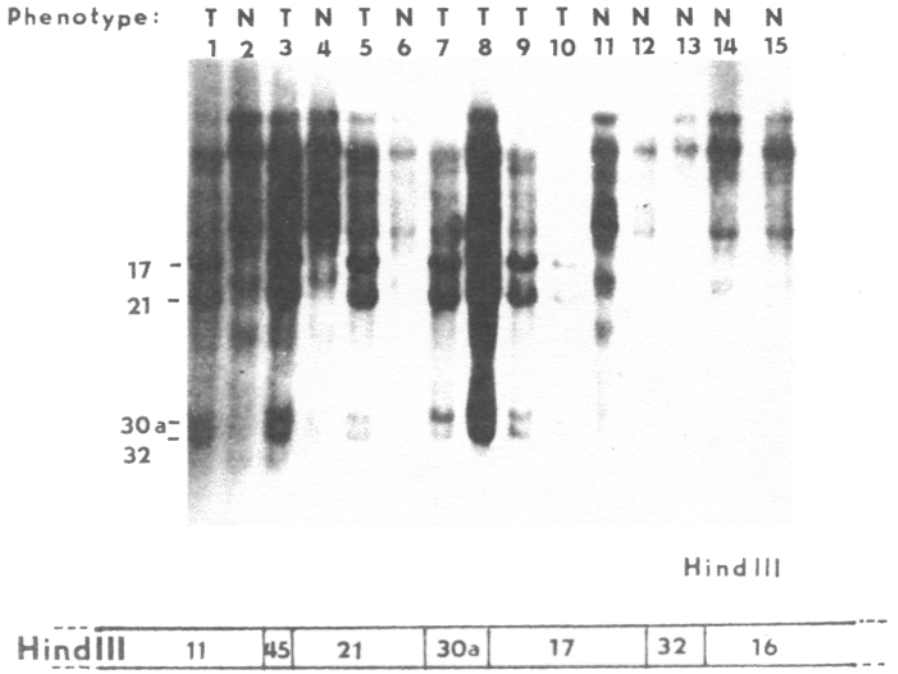

Fig. 2. Southern blot analysis of the DNA from F_1 progenies of transformed plant HM-81-JZ. Correlation between transformed (T) and non-transformed (N) phenotype of the plants and the presence of TL-DNA sequences. 10 µg of plant DNA were digested with Hind III and probed with ^{32}P-labelled pNW 44 (Huffman *et al.* 1984) (specific activity 3×10^{13} Bq mol^{-1} DNA). Lanes 1-5: HM-81 x JZ, plants No. 1, 2, 4, 7, 15. Lanes 6-9: JZ x HM-81, plants No. 1, 2, 7, 9. Lanes 10-14: SL 502 x JZ, plants No. 3, 4, 5, 10, 17. Lane 15: nontransformed plant HM-81. Figures on the left indicate the number of Hind III restriction fragments of TL-DNA and are from the restriction map established by Huffman *et al.* 1984.

Table 1 shows the phenotype segregation of plants after self-pollination of JZ. With the exception of JZ-4 all plants in R_2 and R_3 generation have phenotype T only. The difference in the plant JZ-4 is hardly to explain; maybe there is some instability of T-DNA.

The results of reciprocal crosses of transformant JZ with untransformed plants (cultivars HM-81, Sl-502, Silesia and Arabela) are summarised in Table 2. In the crosses of JZ with HM-81 and JZ with SL-502 (cross 1a, 1b, 2a and 2b) it is possible to see an effect of maternal inheritance. Due to the maternal inheritance length of plants, number of branches, number of pods/plant, mass of 1000 seeds and number

of seeds/pod are lower in the cross JZ × HM-81 than in the cross HM-81 × JZ (results not shown). The difference in the length of plants and number of seeds/pod is statistically significant ($P = 0.05$ and $P = 0.01$, respectively).

Table 2. Phenotypes in reciprocal crosses of transformed and untransformed plants. T = transformed phenotype, N = normal phenotype

Cross	Parents	Phenotype T	N	Theoretical ratio	chi square
1a	JZ x HM-81	18	1		
1b	HM-81 x JZ	5	20	1 : 1	5.00
2a	JZ x SL-502	9	0		
2b	SL-502 x JZ	23	15	1 : 1	1.68
3a	JZ x Silesia	26	0		
3b	Silesia x JZ	9	0		
4a	JZ 21/10 x Arabela	20	0		
4b	Arabela x JZ 21/10	19	1	3 : 1	4.26
5a	JZ 28/13 x Arabela	20	0		
5b	Arabela x JZ 28/13	19	1	3 : 1	4.26
6a	JZ 36/6 x Silesia	20	0		
6b	Silesia x JZ 36/6	18	1	3 : 1	3.94
7a	JZ 21/11 x HM-81	20	0		
7b	HM-81 x JZ 21/11	20	0		
8a	JZ 28/1 x HM-81	20	0		
8b	HM=81 x JZ 28/1	20	0		
9a	JZ 36/11 x HM-81	20	0		
9b	HM-81 x JZ 36/11	18	2	3 : 1	3.60

For $P = 0.05$ the chi square is 3.84

Table 3. Phenotypes in progeny of hybrids between transformed and normal plants (F_2 generation). T = transformed phenotype, N = normal phenotype

Hybrid plant	Phenotype T	N	Theoretical ratio	chi square
(JZ x HM-81) - 7	34	10	3 : 1	0.12
(HM-81 x JZ) - 1	14	10	3 : 1	3.54
(HM-81 x JZ) -15	34	14	3 : 1	0.44

The results of F_1 generation, shown in Table 2, would be possible (with some limitation) to explain as caused through bifactorial inheritance. The F_2 generation (autogamic progeny of T × N and N × T) segregates, however, in the simple ratio 3:1 (Table 3). The bifactorial model of inheritance is therefore excluded.

In several crosses of transformed plants with untransformed control plants we obtained plants with phenotype N in low frequency (Table 2, cross 1a, 4b, 5b, 6b and 9b). We did obtain these N phenotypes repeatedly so that was no experimental

mistake. Because the R_1 generation is uniform and the crosses of JZ with untransformed plants have nearly uniform progeny it is possible to accept the model of "homozygosity" of primary transformant JZ.

Table 4. Relative 2C nuclear DNA content of transformed plants. The results are expressed as mean DNA indices (ratio of nuclear DNA content of a sample to the DNA content of the control).

Plant	DNA index mean	standard deviation
HM-81 (control)	1.000	
JZ (R_1) No. 1	0.989	0.009

Peerbolte *et al.* (1987) explained maternal inheritance of male sterility and heterostyly in transformed tobacco with aneuploidity of transformed tobacco plants. Our results on relatively 2C nuclear DNA content suggest that no gross changes in ploidy and/or DNA content took place in transformed plants. However, the presence of small karyotype rearrangements cannot be excluded.

We did not observe male sterily and heterostyly in rapeseed transformed with T-DNA of plasmid pRi. Maternal inheritance in our studies is an exceptional event and concerns leaf morphology and length of plants. The supposed effect of cytoplasm on different characters of plants is hardly to explain. We excluded also the possibility of parthenogenetic or matromorphic seed development (Röbbelen 1965, Banga 1968); flowers with amputated stigmas and flowers without pollination do not produce seeds and/or pods.

If the transformant JZ is homozygous for T-DNA, the R_1 plants would be uniform in T phenotype as well as the F_1 (hybrid) plants. With the exception of the plant JZ-4 this expectation nearly corresponds to experimental results (Table 1 and 2). In several crosses of JZ with untransformed parents (Table 2, cross 4b, 5b, 6b and 9b) plants with N phenotype appeared in a small frequency (1 in about 20 plants). It is possible to suppose that TL-DNA is localised in two different loci of one pair of homologic chromosomes in trans arrangement and that the N phenotype is a result of a recombination process. Similar situation found Spielmann and Simpson (1986) in tobacco transformed with NPT-II gene. Using the values of crosses 4b, 5b, 6b and 9b the distance betwen these two *loci* is 12 recombination units. Irrespective of the possible difference in crossing-over frequency at microsporogenesis and macrosporogenesis, the distance is 6 recombination units. Both values are approximate, because the parents have not the same background genotype, the number of plants in individual progenies is relative low *etc.* In experiments of Spielmann and Simpson (1986) the distance between two NPT II genes was 3 recombination units.

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