Regeneration of winter oilseed rape and trials of transformation mediated by *Agrobacterium tumefaciens*

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1. Introduction

Rapid development of cell biology in the area of totipotention together with the use of new methods for introduction of genes to the plant cells is now commonly observed in plant breeding. Agrobiotechnology may be used to achieve more abundant harvest and better quality of crops and at the same time to eliminate the use of herbicides. Modification of agricultural plants using genetic engineering methods includes many steps that must be taken to achieve proper regeneration of the plant of interest. After co-cultivation, regeneration of the plant must be performed according to the very precise description of selective media, that should be used, and then the fact of positive transformation must be confirmed with the use of reporter genes, the PCR reaction with suitable primers and numerous other important tests. Unlike transformation of spring rape varieties, have been reports about transformation of the winter rape varieties (1-5). Methods used for spring varieties include co-cultivation of cut petioles. In the case of winter varieties, plant regeneration after such treatment is very difficult (2,3). However, high efficiency of regeneration of winter oilseed rape varieties achieved in our previous experiments with cut fragments of stems (6) shows that this method is very useful for transformation studies. In the Plant Breeding and Acclimatization Institute breeding programs for the most important Polish oil crops have been expanded to include such issues as transformation trials and regeneration of winter oilseed rape.

2. Materials and methods

2.1. Donor plants growing

Seeds of winter oilseed rape, Valesca, Lirajet and Bor varieties, were sown in the infection field conditions of ZDHAR-Borowo (Poland, 1996/97, 1997/98). After 3.5 months of growth in natural conditions (including vernalization), plants were dug up, moved to pot-soils and placed in a greenhouse. Most of them were used for fitopathological studies and only some of them were used for transformation trials and regeneration experiments.

Tissue culture: the plants were in the D2 stage — before flowering (7), 20 cm long fragments of stems were cut off and sterilized in ethyl alcohol followed by washing three times in sterile water. Stalks prepared as described above were cut into 1 cm or 0,5 cm long fragments and put on agar B5 medium containing 10% sucrose, 0.1 mg/l of NAA and 0.1 mg/l of 2,4D. They were incubated in the dark at 28°C for 24 hours. Four days before inoculation, *Agrobacterium tumefaciens* LBA 4404 (pAL 4404, pBI 121) culture was renewed every 24 hours using agar LB medium with 50 mg/l of kanamycin and 50 mg/l of rifampicin. After the bacterial culture reached the log phase, it was transferred to the liquid B5 medium diluted 10 times. 15-20 μl of the medium containing bacteria were put on the cut point of incubated explants, which were then kept at 27°C for 78 hours. After co-cultivation, cut winter oilseed rape stems were placed on MS medium containing 4 mg/l of BAP, 20 mg/l of rifampicin, 15 mg/l of kanamycin A and 500 mg/l of carbenicillin for cv. Valesca, Lirajet and MS medium containing 2 mg/l of BAP, 20 mg/l of rifampicin, 15 mg/l of kanamycin A and 800 mg/l of carbenicillin for cv. Bor. Petri dishes with explants were placed in the growth chamber where the average temperature was 20°C with a 14 hour long photoperiod. The regenerants obtained were cut off from the parent tissue and transferred alternately on the new MS or B5 medium every two weeks, together with the reduction of the BAP concentration to 2 mg/l after one month of cultivation.

TABLE 1

MEDIA USED FOR TRANSFORMATION EXPERIMENTS AND REGENERATION OF WINTER OILSEED RAPE CV. LIRAJET AND VALESCA (1996/97)

Experiment steps	Mineral composition	Sugar per cent	Growth regulators (mg/l)	Antibiotics (mg/l)	Other (mg/l)
Bacterial cultures	LB	Glucose 0.5	—	Kanamycin 50.0 Rifampicin 50.0	—
Co-culture and induction	B5	Sucrose 10.0	2.4D 0.1 NAA 0.1.	—	m-Inositol 100.0 L-Serine 100.0 L-Glutamine 800.0
Shoots formation	MS	Sucrose 3.0	BAP 4.0	Kanamycin 15.0 Rifampicin 20.0 Carbenicillin 500.0	m-Inositol 50.0 L-Serine 50.0 L-Glutamine 400.0
Selection of regenerants	B5	Sucrose 3.0	BAP 2.0	Kanamycin 15.0 Carbenicillin 500.0	m-Inositol 25.0 L-Serine 25.0 L-Glutamine 25.0
Shoots elongation	MS	Sucrose 3.0	Kinetin 1.0 NAA 0.05 2.4D 0.05	Kanamycin 15.0 Carbenicillin 400.0	$AgNO_3$ 2.5
Repeated selection of regenerants	MS	Sucrose 3.0	NAA 0.125 2.4D 0.125	Carbenicillin 200.0 Geneticin 25.0	Vitamin B_5 1.0 $AgNO_3$ 2.5
Roots formation	B5	Sucrose 3.0	—	Geneticin 25.0	—

TABLE 2

MEDIA USED FOR TRANSFORMATION EXPERIMENTS AND REGENERATION OF WINTER OILSEED RAPE CV. BOR (1997/98)

Experiment steps	Mineral composition	Sugar per cent	Growth regulators (mg/l)	Antibiotics (mg/l)	Other (mg/l)
Co-culture and induction	B5	Sucrose 10.0	2.4D 0.1. NAA 0.1	—	m-Inositol 100.0 L-Serine 100.0 L-Glutamine 800.0
Shoots formation	B5	Sucrose 3.0	BAP 4.0	Kanamycin 15.0 Rifampicin 20.0 Carbenicillin 800.0	m-Inositol 100.0 L-Serine 100.0 L-Glutamine 800.0
Selection of regenerants	MS	Sucrose 3.0	BAP 4.0	Kanamycin 15.0 Carbenicillin 700.0	m-Inositol 25.0 L-Serine 25.0 L-Glutamine 400.0
Shoots elongation	B5	Sucrose 3.0	BAP 2.5	Kanamycin 15.0 Carbenicillin 500.0	m-Inositol 25.0 L-Serine 25.0 L-Glutamine 200.0
Roots formation	MS	Sucrose 3.0	—	Geneticin 25.0 Carbenicillin 400.0	m-Inositol 25.0 L-Serine 25.0 L-Glutamine 100.0

2.2. PCR — SCAR analyses

Leaves from winter oilseed rape regenerated plantelets grown on the medium were collected, put on ice and ground to powder in a mortar with liquid nitrogen. DNA was extracted as it was described by (8) with small modifications. The powder was poured with hot (65°C) extraction buffer 2 × CTAB [2%(w/v) CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 1%(w/v) PVP, 1%(v/v) β-mercaptoethanol]. Probes were incubated at 65°C for 30 min and then, after mixing with one volume of chloroform-octanol 24:1 (v/v), they were centrifuged for 10 min at 11500 × g. DNA from the collected aqueous phase was precipitated by addition of two thirds volume of isopropanol. After 10 min. of centrifugation the supernatant was discarded and the pellet was rinsed with 70% ethanol. In the following step the DNA was resuspended in TE buffer containing 40 μg/ml of RNAse A and incubated at 37°C for 1 hour. After digestion of RNA contaminations, DNA was again precipitated and rinsed with 70% ethanol. Then the pellet of DNA was resuspended in TE buffer and the concentration of the probe was tested using electrophoresis in 1.4% agarose gel.

The PCR reaction mixture contained:

100 μM each of dATP, dGTP, dCTP and dTTP (Sigma), 1.5 pmoles of K1 primer (5' AGC TGT AGC TAC GAC GTT 3'), 1.5 pmoles of K2 primer (5' GGT GGG CGA AGA ACT CCA GC 3'), 0.5 units of *Taq* Polymerase (Eurobio), 2.5 μl of 10 × *Taq* Polymerase buffer (Eurobio), 1.5 mM $MgCl_2$, 25 ng of genomic DNA.

The final reaction volume of 25 μl was obtained by adding double volume of distilled water.

Amplification was conducted in the Perkin-Elmer GeneAmp PCR System 2400 thermocycler with the following reaction parameters:

• **Initial denaturation step**	**Number of cycles: 1**
Temperature: 94°C	Time: 5 min
• **DNA amplification step**	**Number of cycles: 45**
Denaturation Temperature: 94°C	Time: 1 min
Annealing Temperature: 53°C	Time: 2 min
Polymerization Temperature: 72°C	Time: 3 min
• **Final polymerization step**	**Number of cycles: 1**
Temperature: 72°C	Time: 5 min

The reaction products were analyzed using electrophoresis in 1.4% agarose gel. Separated fragments were visualized under ultraviolet light after ethidium bromide staining. The size marker used was Lambda DNA digested by *Hin*dIII and *Eco*RI (Eurobio).

3. Results

It was observed that the medium with 35.0 mg/l of kanamycin and 45.0 mg/l of geneticin can completely block shoots regeneration after co-cultivation. Kanamycin caused gradual flavescence and then whitening of the explants. In the case of geneticin, first we could observe bronzing of the injured places (cuts) and then, after 7 days, bronzing of all the leaf blade. Such antibiotics as carbenicillin and rifampicin had a good influence on the regeneration process of winter oilseed rape. Concentrations of selective antibiotics were determined experimentally: the right value for kanamycin was 15.0 mg/l and for geneticin it was 25.0 mg/l. It was also experimentally established that BAP cytokinin at the concentration of 4.0 mg/l could be a good stimulator of shoots formation at the first stage of cultivation. During the whole cultivation period the concentration of cytokinin was gradually reduced from 4.0 mg/l to 2.0 mg/l and the concentration of auxins such as NAA and 2,4D, stimulating roots formation, was increased. The concentration of carbenicillin in agar medium was also decreased and reached the value of 200 mg/l, but the concentration of selective antibiotic geneticin was kept unchanged at the level of 25 mg/l. The medium compositions on different stages of cultivation are shown in Table 1 and 2.

TABELA 3
REGENERATION VARIETIES OF WINTER OILSEED RAPE AFTER CO-CULTIVATION WITH *Agrobacterium tumefaciens* LBA 4404 (PAL 4404, PBI 121)

Information about regeneration	Variety of winter oilseed rape		
	Vlesca 1996/97	Lirajet 1996/97	Bor 1997/98
Number of plants used for experiments	3	3	9
Used explants	10	10	18
Regenerated plants, including:	98	353	160
Green plants	29	167	99
White plants	60	154	54
Sectorial chimeras	9	32	7
Plants before vernalization	0	22	35

The nptII gene was detected using the PCR — SCAR analysis carried out on leaves of completely regenerated plants.

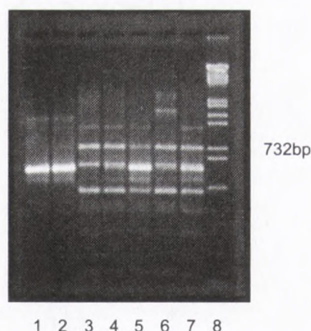

Fig. 1. PCR — SCAR analysis specific for nptII gene made on leaves of plants regenerated after co-cultivation with *Agrobacterium tumefaciens* LBA 4404 (pAL 4404, pBI 121).

1. *A. tumefaciens* — mother culture; 2. *A. tumefaciens* — used for transformation trials; 3. Albinotic leaf alive; 4. 5. 6. 7. Green leaf of winter oilseed rape 732bp; 8. DNA size marker.

4. Discussion

Shoots regeneration in *Brassica napus* L. and the significance of such factors as the type of explants, genotype or hormone concentration have already been studied (9). It has been demonstrated that both the type of explants and their genotype affected the amount of regenerated shoots. However, handly any attention was paid to the role of hormone concentration, therefore its effect was not found. In our studies flowering shoots fragments were used as a source of explants (10). The morphogenetic potential of the varieties was expressed as the efficiency of shoots formation per explant. As it is shown in Table 3, the efficiency of shoots formation was high for both varieties but higher for Lirajet than Bor. Shoots regeneration could be mainly observed at injured places (cuts). New shoots usually developed from epidermal cells which are strongly susceptible to *Agrobacterium* infection (1). The transformation process is closely associated with the selection of transformed tissues on the media containing selective factors such as kanamycin A and geneticin (which are aminoglycoside antibiotics of the neomycin group). Npt II gene, located on plasmid pBI 121 and coding neomycin phosphotransferase II (NPT II), is a very common reporter and selection system. The product of this gene catalyses an ATP mediated phosphorylation reaction that causes inactivation of antibiotics mentioned above (1,11-15). T-DNA fragment of the plasmid also contains sequences complementary to primers K1 and K2 giving PCR product of 732 bp present both in bacteria and transformed plants. In many reports (13,14,16,17) it has been noticed that among transformed shoots there are some albinotic ones or some sectorial chimeras, that were also observed in our studies. Using the hybridization method, the presence of multiple copies of npt II gene can be detected in shoots (14). This can be due to inaccurate integration of T-DNA left flank with the plant genomic DNA (18,19). To ensure that the amplification of 732 bp DNA fragment is not caused by the bacteria living in the plant tissue, cultivation of regenerated plants should be conducted on agar medium as long as carbenicillin, which at the first stage inhibited the growth of *A. tumefaciens*, is completely eliminated. It has been experimentally established that under the *in vitro* conditions *A. tumefaciens* could occupy intercellular spaces of callus tissue from stems and leaves for as long as

three or four months. Only after the confirmation of the lack of bacteria in these tissues, can the DNA analysis be performed. It must be pointed out that at this stage there is no clear evidence that such plants are genetically modified and that such modification will be observed in the progeny.

5. Conclusions

- After co-cultivation with *Agrobacterium tumefaciens* LBA 4404 (pAL 4404, pBI 121) a large number of regenerated plants of winter oilseed rape *Brassica napus* L. cv. Lirajet, Valesca and Bor were obtained.
- Many of these plants cv. Lirajet, Valesca (1996/97; 214 white, 41 sectorial chimeras and 196 green) and cv. Bor (1997/98) were developing quite well on the selective medium containing kanamycin A and geneticin, and that may indicate positive transformation.
- In order to ensure that the regenerated plants are free from bacteria residing in the intracellular spaces, certain steps have to be taken: first, it is necessary to transfer the plants on to a medium without carbenicillin; if there is no bacteria growth observed the following step is the analysis of the DNA using the PCR method.
- It seems that the 732 bp fragment of the reporter gene npt II detected in the plant material using PCR reaction with primers K1 and K2 can also be used as an indicator of positive transformation.

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Summary

The effects of one strain *Agrobacterium tumefaciens* LBA 4404/pBI 121, conditions of co-cultivation and growth regulators on regeneration were studied and trials of transformation were performed on winter oilseed rape *Brassica napus* L. cv. Lirajet, Valesca and Bor. Strong shoots regeneration was observed after co-cultivation without acetosyringone. Among antibiotics that were used in the experiments, kanamycin A and geneticin hampered whereas carbenicillin and rifampicin stimulated shoots regeneration. The presence of npt II gene was confirmed by PCR and K1, K2 primers which generate a fragment of 732 bp in length.

Key words:
winter oilseed rape, regeneration, transformation, *Agrobacterium tumefaciens*.

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