Brassica napus plant regeneration and transformation via *agrobacterium*-mediated-transformation

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Abstract

Gene transformation Technology is a powerful tools for transformation of desirable genes to organisms that use for biology scientists. Transformation of genes which can create a new triat, development of quality or quantity triats had been important for scientist. Rapeseed (*Brassica napus*) is one of the most important oil seed crop in with usage industrial and edible. Genetic manipulation of rapeseed relies on the suitable tissue culture and efficient method for the plant regeneration and need a good transformation system. The results showed that cotyledons were able to multiple shoot formation with high regeneration frequency than hypocotyl in *B. napus*. In this study two *Agrobacterium tumefaciens* strains, LBA_{4404} and C58 (pGV_{3101}), were used for gene transformaion. Cotyledon explants excised from 5 days old seedling in sterile condition and placed onto MS medium containing 4.5 mg/l BAP, 3% sucrose and 0.8% agar pH 5.8 for 1 days. The cotyledons were inoculated with suspension of Agrobacterium for 10 seconds and immediately transferred to the same madium for 2 days in dark and 25 ^{o}C (co-cultivation). Explants were transferred to shoot induction medium with 15 mg/l kanamycine and subcultured each 10 days on the same medium. Green shoots were transferred to elongation medium and after 2-3 weeks green shoots transferred to root induction medium, containing 2 mg/l IBA. Molecular analysis, PCR, and histochemical GUS assay were carried out on the plants. Results showed that 1 day cotyledon preculture was useful for shoot induction and LBA_{4404} strain was better than C58 (pGV_{3101}) strain for gene transformation.

Key words: *Brassica napus*, Tissue culture, gene transformation, reporter gene, *Agrobacterium*

Introduction

Oilseed rape (*Brassica napus*) is now the second largest oilseed crop in the word providing 13% of the word's supply (Raymer, P. L, 2002). *Brassica napus* contain both spring and winter forms that are distinguishing by vernalization requirement. Seeds of *B. napus* contain 40% or more oil and produce meals with 35 to 40% protein. The properties of edible vegetable oils are determined to a large extent by the relative content of triacylglycerol fatty acids (Kinney et al., 2002). *B. napus* is aparticularly interesting plant species as it is both a major crop and a highly manipulable laboratory organism. The species is amenable to a large number of tissue culture techniques. *B. napus* has also proven to be susceptible to a variety of methods leading to production of transgenic plants, of these, *Agrobacterium tumefaciens*-mediated transformation has proven to be the most officient method (de Block *et al*., 1989; Moloney *et al*., 1989; Schroder *et al*., 1994; Stewart *et al*., 1996; Halphill *et al*., 2001; Cardoza and Stewart, 2003).

In this study we focused on some conditions which they affected on plant regeneration and transformation in *B. napus*. One of them is effect of pre-culture time on transfomation rate and the others are: type of variety, kind of explant and *Agrobacterium* strains.

Materials and Methods

Vector

The pBI121 vector contaied the *npt*II gene coding for neomycin phosph transferase, which allows for the selection of plants resistant to kanamycin. This vector as well as contained the GUS gene was under the control of CaMV 35S promoter and a NOS terminator. The plasmid was transformed into *Agrobacterium* strains C58 (pCV_{3101}) and LBA_{4404} according to Sambrook and Russel, 2001 method.

Plant Materials

Seeds of three *B. napus* L. cv, namely, PF, K16 amd Maplus were surface sterilised for 10 min with 10% sodium hypochlorite with 0.1% tween20 added as a surfactant. The sterilization was followed by washed thoroughly with sterile distilled water and germinated on MS (Murashige and Skoog, 1962) basal medium with 20 g/l sucrose, solidified with 8 g/l agar pH 5.8. Seeds were germinated at 25 $^{\circ}$ C in 19/8 h light and dark respectively.

Hypocotyls were excised from 5- to 10 day- old seedling, cut into about 1 cm pieces and pre-conditioned for 24 h on MS medium supplemented with 1 mg/l 2,4-D and 30 g/l sucrose, solidified with 7 g/l agar pH 5.7. Then after contamination with *Agrobacterium* and co-cultivation for two days, explants transffed into selection medium: MS + 1 mg/l 2,4-D + 30 g/l sucrose

+ 200 mg/l Cefotaxime + 15 mg/l kanamycin, solidified with 7 g/l agar pH 5.8. Then explants after a week subcultured into another medium with 4.5 mg/l BAP.

Cotyledons were excised accordng to Moloney *et al*., 1989 method and were placed on MS medium contain 30 g/l sucrose, 4.5 mg/l BAP, solidified with 8 g/l agar pH 5.8 for 24, 48 h as a pre-culture medium or without pre-culture. After contamination with *Agrobacterium* and co-cultivation for two days, cotyledones subcultured into shoot induction medium with above antibiotics.

After 2 weeks, explants were subcultured. The shoots that developed were translated to SEM (shoot elongatiom medium), without any phytohormone, at last them transferred to rooting medium consisting of half-strength MS salts, 20 g/l sucrose, 7 g/l agar, 2 mg/l IBA and antibiotics as above. All the cultures were maintained at 25 ° C under 16/8 (light/dark) photoperiod. The rooted shoots were transferred to soil and grown in a plant growth chamber.

PCR analysis were done with following primers for GUS gene: (forward): 5′ -GGT GGT CAG TCC CTT ATG TTA CG (23 mer) and (reverse): 5′ –CCG GCA TAG TTA AAG AAA TCA TG (23 mer). These primers were amplified a 521 bp fragment and histochemical GUS assay were applied to confirm the presence of the T-DNA in transformation plants.

Results and Discussion

For selection of transformed from untransformed shoots, kanamycin were used as a selectable marker. The putative transgenic plants were confirmed to be PCR-positive with GUS-specific primers (Fig. 1). Transgene expression was confirmed by histochemical GUS assay analysis for T_0 plants leaves (Fig. 2).

Effect of *B. napus* varieties (K16, PF and Maplus), kind of explant (Hypocotyl and Cotyledon) and *Agrobacterium* strains (LBA4404 and GV3101) on plant regeneration and transformation:

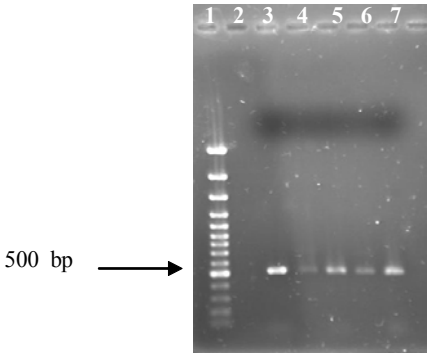

Fig. 1: PCR anaysis of transgenic canola with GUS primers.
Lanes: 1 DNA marker. 2 negative control, 3 positive control, 4-7 DNA from transgenic plants.

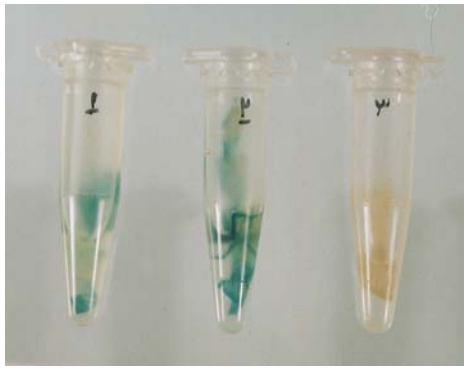

Fig. 2: Histochemical GUS assay. Left to right: 1 and 2 transgenic plants leaves , 3 non transgenic plant.

Table 1: Analysis of variation for transformation frequency.

S.O.V	d.f	MS
Replication	2	5.43**
Variety	2	16.25**
Explant	1	2731.98**
Variety*Explant	2	2.28**
Agro-strain	1	203.77**
Variety*Agro-strain	2	3.75**
Explant* Agro-strain	1	387.79**
Variety*Agro-strain*Explant	2	2.38**
Error	22	0.403
Total	35	
C.V%= 3.7		** significant difference in 1% level

S.O.V= source of variation; d.f= degree of freedom; MS= mean of squares

For determination of the effect of above factors we udes a factorial experiment with three replications in template of RCBD (randomized complete block design). Results of analysis of variation for transformation frequency showed in table 1. According to these results there were significant differences between al factors fot transformation frequency. In order to reach to the best composition we done means comparison by means of Duncan's new multiple range test. Comparison of three varieties showed that *B. napus* cv. Maplus had the most transformation frequency (11.87%) versus PF and K16 (10.78 and 9.34% respectively). Mean transformation frequency ranged from 14.26% for LBA_{4404} strain of *Agrobacterium* and 7.06% for

C58 (pGV_{3101})strain. Study of interaction between three factors showed that the combination of Maplus×cotyledon×LBA_{4404} strain was the best combination with about 29.56% transformation rate. There are some differents reports by nother scientists. No significant difference for the transformation rate was found between the rapeseed genotypes (Menze and Moller, 1999). In contrast, *A. tumefaciens* mediated transformation showed a high genotye dependence (De Block *et al*., 1989 ; Stefanov *et al*., 1994).

Effect of cotyledon pre-culture time on transformed regeneration plants:

We used a factorial experiment with three replications in template of RCBD (randomized complete block design). Results showed a significant difference between all factors exception two way intraction between variety and pre-culture time. Preculturing of cotyledon played an important role in increasing the transformation efficiency. Explants precultured for 48 h and 24 h befor contamination with *Agrobacterium* produced a higher transformaion frequency (18.74 and 18.77 mean squares respectively) than explants without precultured with 8.36. According to these results we selected combination of Maplus ×24 h preculture×LBA_{4404} *Agrobacterium* strain with a mean square of 27.7 for transformaion rate (table 2).

Table 2: Comparion of means of three way interation between *B. napus* varieties, pre-culture time and *Agrobacterium* strains for transformation rate

B. napus varieties, pre-culture time and *Agrobacterium* strains (S.E=0.40)	
[c]14·5	Maplus × 0 h × LBA_{4404}
[f]6·7	Maplus × 0 h × C58 (pGV_{3101})
[a]27·7	Maplus × 24 h × LBA_{4404}
[c]14·3	Maplus × h24 × C58 (pGV_{3101})
[a]27·4	Maplus × h48 × LBA_{4404}
[c]14·1	Maplus × 48 h × C58 (pGV_{3101})
[cd]12·7	PF × 0 h × LBA_{4404}
[Fg]45·	PF × 0 h × C58 (pGV_{3101})
[a]27·1	PF × 24 h × LBA_{4404}
[cd]412·	PF × 24 h × C58 (pGV_{310}1)
[a]26·4	PF × 48 h × LBA_{4404}
[c]12·4	PF × 48 h × C58 (pGV_{3101})
[g]5·0	K16 × 0 h × LBA_{4404}
[fg]5·7	K16 × 0 h × C58 (pGV_{3101})
[b]20·0	K16 × 24 h × LBA_{4404}
[de]910·	K16 × × 24 h C58 (pGV_{3101})
[b]21·6	K16 × 48 h × LBA_{4404}
[e]010·	K16 × 48 h × C58 (pGV_{3101})

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