High-frequency non-selective transformation method in *Brassica napus* for obtaining marker-free plants

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Abstract

Non-selective transformation was investigated as a method for obtaining marker-free transgenic plants. The hypocotyl explants of Rapeseed (Brassica napus cv Westar) were co-cultivated with a general Agrobacterium tumefaciens strain LBA4404 containing a pBI121-NPTII plasmid in which there is only GUS gene as an interesting gene in the T-DNA and the NPTII gene is deleted. Co-cultivation was carried out under the condition of no culture medium, which mimics natural process of Agrobacterium tumefaciens infecting plants and greatly improves the transformation frequency. According to the X-Gluc histochemical staining, GUS was expressed in 63-89% of the harvested callus and 27-51% of the harvested plants. Furthermore, this transformed nature was confirmed by PCR and Southern blot analysis. These results suggest that not only the isolation of transformants without a selective marker gene is feasible, but high-efficiency could be reached. This method may provide a simple and useful experimental tool to obtain marker-free transgenic plants in rapeseed.

Key words: Non-selective transformation, Marker-free transgenic plants, Brassica napus, Agrobacterium tumefaciens

Abbreviations: GUS ß-Glucuronidase, 2,4-D 2,4-dichlorophenoxy acetic acid, Kt Kinetin, Zt Zeatin, MS Medium Murashig and Skoog medium, NPTII Neomycin phosphotransferase

Introduction

In recent years, concerns about the presence of selectable genes have been raised. The selected genes are mostly based on genes conferring antibiotic or herbicide resistance, which might be an unpredictable hazard to the ecosystem as well as to human health when these transgenic plants are grown in fields or processed into food products, although there is as yet no scientific evidence to support this (Flavell et al. 1992; Fuchs et al. 1993). Furthermore, due to only a limited number of selectable marker genes available for practical use and a large number of desirable traits and genes not worth incorporating into plants, the stacking of multi-interest genes in same plant would be very difficult in technology. Therefore, there have been strong efforts to develop strategies for efficiently eliminating marker genes (Yoder and Goldsbrough 1994; Daniell and Dhingra 2002; Brasileiro and Arogao 2001). Although during the early period of producing transgenic plants, non-selective transformation was proved to be feasible in tobacco (Shillito et al. 1985), it is generally thought that only a limited number of regenerant plant cells are accessible and/or competent for transformation, so transformation of plant cells mediated by Agrobacterium tumefaciens occurs only at a very low frequency (Puchtsa 2000). Therefore, strategies of eliminating selectable marker genes is always limited how to removal them from the plant genome or hide them, such as co-transformation, site specific recombination, homologous recombination, transposition and tissue specific expression, chemically inducible expression of selectable marker genes, after they have fulfilled their purpose - distinguishing between plant cells with an integrated transgene and the bulk of non-transformed cells (Komari et al. 1996; Gleave et al. 1999; Goldbrough et al. 1993; Ebinuma et al. 1997; Dale and Ow 1991; Zuo et al. 2001; Zubko et al. 2000; Daley et al. 1998; Libiakova et al. 2001).

In really recent, Vetten et al. (2003) presented a transformation method for obtaining marker-free plants of a cross-pollinating and vegetatively propagated crop. Without using any selective marker genes, the frequency of PCR-positive transformants by incubation of potato explants with a supervirulent strain AGL0, which contains a DNA region originating from the virulence region of Ti plasmid pTiBo542, ranged between 1.3% and 5.6%. However, when a strain LBA4404 is employed, the frequency is below 0.8%. Here we presented a high-efficiency non-selective transformation method for obtaining marker-free plants in Brassica napus, in which the bacterium was still a general A. tumefaciens strain LBA4404. The most significant change that we had made in the transformation procedures was the co-cultivated condition. The co-cultivation of explants with A. tumefaciens was carried out under the condition of no culture medium, which mimiced natural process of Agrobacterium tumefaciens infecting plants. This new procedure greatly improved the transformation frequency. The ratio of transformed plants among the harvested plants from non-selective medium by using this procedure was between 27.3% and 51.7%.

Our results suggest that not only the isolation of transformants without a selective marker gene is feasible, but high-efficiency could be reached for different species if the conditions of co-cultivation are improved. Furthermore, as shown by our results, the dependency of high-efficiency transformation on co-cultivation without culture medium might suggest that the proficiency of A. tumefaciens infecting plants would be fully evoked or cross-talks between plant and microorganism was

enhanced under this condition. However, it remains to be brought to light for their internal causes. Because non-selective transformation method does not require genetic segregation or site-specific DNA-deletion systems to remove marker genes, this procedure may provide a simple and useful experimental method to obtain marker-free transgenic rapeseed.

Materials and methods

Plasmid constructions

Binary vector pBI121 (Clontech) was digested with enzymes PmeI and ClaI to remove the NPTII gene. The sticky end was made blunt-ended by Klenow polymerase treatment, after which the vector DNA was circularized by blunt-end ligation using T4 DNA ligase. This resulted in vector pBI121-NPTII (Fig. 1). Plasmid pBI121-NPTII was then transformed into Agrobacterium tumefaciens strain LBA4404 (Hoekema et al. 1983).

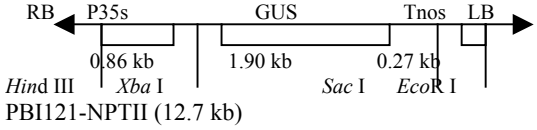

Fig.1 Map of T-DNA regions of pBI121-NPTII (*RB* right border sequence of a T-DNA, *LB* left border sequence of a T-DNA).

The GUS is driven by the CaMV 35S promoter and terminated by the nopaline synthase terminater. Only restriction sites utilized for analysis are shown.

Plant material and culture media

Sterilized seeds of *Brassica napus* cv Westar were spread on M0 germination medium and grown in a dark chamber for seven days at 25±1°C (Fig.2A, 2B). The seedlings were then used to the preparation of explants.

M0 medium: only containing 10g / l agar and 50 mM $CaCl_2$. M1 medium: MS medium with 0.5 mg / l 2,4-D, 0.5 mg / l NAA, 0.5 mg / l 6-BA.. M2 medium: M1 medium with 300 mg / l Carboxyl and 5.0 mg / l $Ag_2S_2O_3$. M3 medium: MS medium with 1.0 mg / l Zt, 0.1 mg / l Kt and 6.0 g / l argrose. M4 medium: 1 / 2 MS medium with 0.1 mg / l IBA and 8.0 g / l agar.

Co-cultivation

Agrobacterium tumefaciens (LBA4404) with plasmid pBI121-NPTII was prepared overnight. Bacteria were harvested at time periods of 16-20 hours. Dilutions of bacteria to concentration of 10^4-10^5 bacteria per ml were prepared in M1 medium. The hypocotyls of seedlings were harvested and cut into pieces 0.3-0.5 cm in length and were soaked into the *Agrobacterium* suspension for about one hour. Then, the explants were dredged up from the infection medium (Fig. 2C, 2E, 2F). The co-cultivation of bacteria and explants was carried out for 60-72 hours without culture medium.

Callus induction and plant regeneration

After 3 days of co-incubation, hypocotyls were cultured in M2 medium for 12-15 days. The cultures then were transferred to M3 medium until shoots were regenerated. The regenerated shoots were transferred to M4 medium for root growth. The regenerated plants were transferred to soil and grown to maturity.

GUS assay

Harvested callus and leaves of regeneration plants were tested for histochemical GUS expression in an X-Gluc solution (Jefferson 1987, Fig. 2I, 2J, 2K, 2L).

PCR and Southern blot analysis

Genomic DNA was isolated from leaves of regeneration plants by a modified CTAB method (Doyle and Doyle 1989) and used for PCR and Southern blot analysis.

Using *Xba*I and *Sac*I-digested DNAsamples, PCRs were carried out under standard conditions with 1 min denaturation, 1 min annealing, 2 min extension at 94°C, 60°C, and 72°C, respectively, for 30 cycles. Reaction products were resolved by electrophoresis in a 1.2% (wt / vol) agarose gel. The sequences of the PCR primers for GUS were as follows: 5′-GTGGAATTGATCAGCGTTGG-3′ and 5′-GCACCGAAGTTCATGCCAGT-3′.

For Southern analysis, 10ug of HindIII and EcoRI-digested DNAsamples was separated on a 0.8% (wt / vol) agarose gel, blotted to nylon membranes (Hybond-N, Amersham) and probed with a DIG-labeled coding region of the GUS gene. The probe DNA fragment, a part of the GUS gene, was labeled by PCR using DIG-dUTP, following the supplier's instructions (Boehringer Mannheim). Hybridization, washing and detection were performed using DIG Easy Hyb (hybridization solution) and DIG Wash and Block Buffer Set (Boehringer Mannheim) following the supplier's instructions.

Results

The hypocotyl explants of Rapeseed (Brassica napus cv Westar) were co-cultivated with a general Agrobacterium tumefaciens strain LBA4404 containing a pBI121-NPTII plasmid in which there is only GUS gene as an interesting gene in the T-DNA and the NPTII gene is deleted. Co-cultivation was carried out under the condition of no culture medium. Dependent on this co-cultivation procedure, the callus of hypocotyls of Brassica napus cv Westar behaved very high transformation frequency (Fig. 2I).

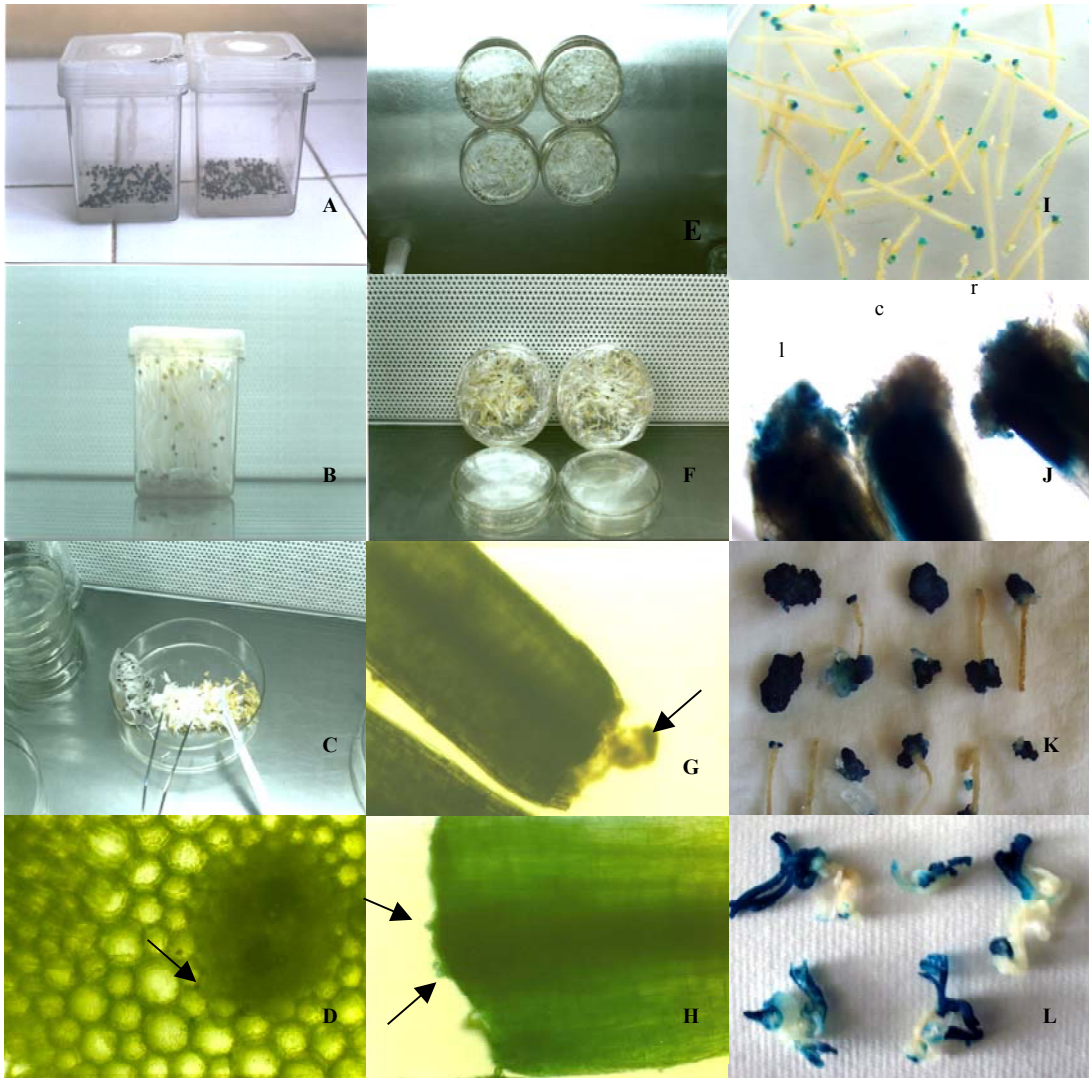

Fig. 2 Non-selective transformation of *Brassica napus*. A. Sowing seeds; B. seedlings; C. Hypocotyl explants. D. Microscopic structure of explant cuts, the arrow indicates the pericycle cells of hypocotyls; E. Infection with *A. tumefaciens*; F. Co-cultivation without culture medium; G. The arrows indicates the colonization of bacterium after three day's co-cultivation; H. The arrows indicate micro-callus originated from the pericycle cells; I. The stained hypocotyls fifteen days after co-cultivation; J. The stained hypocotyls under microscope: l (left). the total calli is stained, c (center). half calli is stained, r (right). only part of calli is stained; K. The stained callus during the regeneration culture; L. The stained shoots.

Callus and plants grown on non-selective medium were analyzed for GUS activity. Sixty-three to eighty-nine percent of the harvested callus and twenty-seven to fifty-one percent of the harvested plants showed GUS activity (Table 1, 2).

Table 1 Frequency of non-selective transformation during the callus stage

No. of tests	No. of hypocotyls	No. of staining positive cuts	Ratio(%)
1	2000	1656	82.8
2	2000	1279	63.9
3	2000	1788	89.4
4	2000	1764	88.2
Amount	8000	6487	81.1

We selected randomly 23 staining-positive plants for PCR and six positive plants for Southern blot analysis, the predicted 1.7 kb fragment was amplified (Fig. 3 A) and 3.0 kb fragment was hybridized with T-DNA-specific probes (Fig. 3 B).

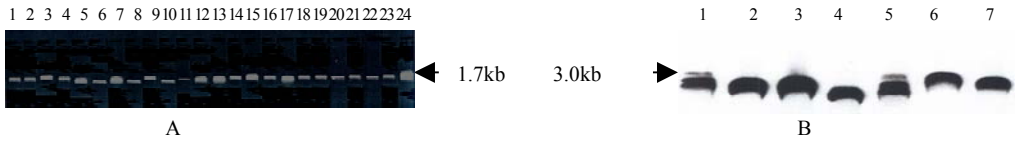

Fig. 3 PCR and Southern blot analysis of transgenic plants: A. Line 1-23, PCR of 23 staining-positive plants; Line 24, positive control of plasmid. B. Line 1-6, Southern blot analysis of 6 positive plants; Line 7, positive control of plasmid.

Table2 Frequency of non-selective transformation for regenerating plants

No. of tests	No. of hypocotyls	No. of regeneration plants	Frequency of regenerating	No. of staining positive plants	Ratio of transformed plants among the harvested plants
1	100	22	22.0%	6	27.3%
2	100	17	17.0%	8	47.1%
3	100	28	28.0%	9	32.2%
4	100	25	25.0%	12	48.0%
5	100	29	29.0%	15	51.7%
6	100	31	31.0%	15	48.4%
Average	100	25.3	25.3%	11	42.7%

Discussion

These results indicate that it is feasible to high-efficiently obtain transformants without marker genes by using a general *A. tumefaciens* strain and an optimized co-cultivation procedure. Furthermore, we did not find any chimerism among the 162 transgenic plants by pollen staining analysis (data not shown); these observations indicate that the ratio of chimerism emergence in non-seslective transformation of *Brassica napus* is lower than 0.6%.

Successful genetic plant transformation using *Agrobacterium* relies on optimization of a combination of several factors including plant tissue harvesting and manipulation, bacterial colonization and infection, T-DNA transfer, and post-transformation culture conditions for efficiency plant regeneration. However, the co-cultivation may be the most critical step among all above lists. It concludes the adjusting of temperature, pH, oxygen, concentration of *Agrobacterium* and using of induced agents such acetosyringone (Holford et al. 1992; Fullner and Nester 1996; Dillen et al. 1997; Vernade et al. 1998; Finer and Finer 2000). Compared with previous procedures (De Block et al. 1989), we mainly changed the co-cultivation with culture medium into without culture medium. But this change has greatly improved transformation efficiency of positive transgenic shoots from 30% under selective conditions (De Block et al. 1989) to 51.7% under non-selective conditions (Table 2).

Why could this simple change of transformation procedure have greatly effect on the efficiency of gene transformation? We consider it might be from following three aspects: (1) controlling the over-growth of *A. tumeficiens*, and decreasing cell death caused by *A. tumeficiens*. With the same concentration of *A. tumeficiens* and same co-cultivation period, the cell death ratio of explant cutting surface with culture medium is higher than without culture medium (data not shown), and *A. tumeficiens* can produce a better colonization on the wounded surface of explants by no culture medium co-cultivation (Fig. 2G); (2) under the conditions of plentiful nutrition, for example, in liquid culture medium, the over- growth of *A. tumeficiens* can result in violent change of pH even using 2-(N-Morpholino)ethanesulfonic acid (MES) as buffer agent (data not shown), so the co-cultivation without culture medium may be beneficial to stability of pH during the co-cultivation period, which can affect the expression of *vir* gene (Stachel et al. 1985); (3) the dependency of high-efficiency transformation on co-cultivation without culture medium might suggest that the proficiency of *A. tumefaciens* infecting plants would be fully evoked under the stress of starvation. However, it remains to be brought to light why the starvation stress plays an important role in enhancing cross-talk between plant and microorganism.

For a long time, it is putatively accepted that the transformed cells for a certain explant are limited and the non-transformed cells are major, so the selective agents should be used to help the growth of transformed cells and / or inhibit that of non-transformed cells. In fact, most of non-transformed cells do not directly compete with totipotent transformed cells for propagating because they are extremely differentialled or non-competent for tissue culture. Therefore, only those totipotent non-transformed cells can compete with transformed cells into regeneration plants. In our experiments, only the pericycle cells of hypocotyls are totipotent either for regeneration or transformation (Fig. 2D, H). If most of totipotent cells become transformed, it is naturally unnecessary to use selective agents and selectable marker genes.

Conclusions

We have developed a high-efficiency non-selective transformation method in *Brassica napus* for obtaining marker-free transgenic plants by simply modified co-cultivation between explants and *Agrobacterium tumeficiens* into without culture medium. A better understanding of this process could provide the basis for new strategies to improve the efficiency of plant transformation. Because nonselective transformation method does not require genetic segregation or site-specific DNA-deletion systems to remove marker genes, this procedure may provide a simple and useful experimental method to

obtain marker-free transgenic plants in rapeseed and other species.

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