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Full Length Research Paper

A new time-saving transformation system for *Brassica napus*

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By optimizing different parameters for infection, an efficient Agrobacterium-mediated transformation system for *Brassica napus* was developed. A series of combinations of optical densities, infection durations, concentrations of acetosyringone and silwet L77 were evaluated. Maximum transformation efficiency reached 18.93% when cotyledonary nodes were infected for 10 min with an *Agrobacterium* suspension of 0.8 OD and 200 µM acetosyingone and 0.02% silwet L77 in the culture medium. In addition, the concentrations of 6-benzyladenine and naphthalene acetic acid were also optimized. Meanwhile, maximum transformation efficiency for green plantlet was 94.38% when cotyledonary nodes were cultivated in the medium containing 4 mg/L 6-benzyladenine and 0.04 mg/L naphthalene acetic after co-cultivation. Transformation was confirmed by polymerase chain reaction analysis, southern blotting analysis and green fluorescent protein assay.

Key words: *Agrobacterium tumefactiens*, cotyledonary node, 6-benzyladenine, naphthalene acetic acid, transformation, Silwet L 77.

INTRODUCTION

Brassica napus is one of the most important oil crops in China, Northern Europe, Canada and Australia, ranking third only to soybean and palm in global oil production (De Block, 1989; Cardoza, 2003). It is very common to use *B. napus* oil for cooking, and it is preferred because it has the lowest content of saturated fat. In order to increase its yield and seed quality, it is necessary to improve agronomically its important traits, such as herbicide resistance, disease resistance, tolerance to several biotic and abiotic stress factors and fatty acid compositions (Katiyar, 1986; Malik, 1990).

Genetic improvement of *B. napus* has been achieved mainly by conventional plant-breeding methods. Techniques in plant genetic engineering have advanced rapidly and opened new avenues for crop improvement, such as modification of oil composition (Knutzon, 1992), herbicide tolerance (De Block, 1989), altering protein composition (Altenbach, 1992) and insect resistance (Stewart, 1996). These achievements were accomplished by genetic transformation technique. Among all of the transgenic methods, *Agrobacterium* mediated transformation is the most common, simple and efficient one that has been employed in over 80% of all transformation practices. This method has many advantages such as an efficient introduction and integration of exogenous gene into the host genomes, the transfer of relatively large fragments of DNA and the integration of single/low copy numbers of gene (McCormac, 1998). *Agrobacterium*-mediated transformation of *Brassica* plants has been reported to be successful with *B*rassica *carinata* (Babic et al., 1998), *Brassica juncea* (Mathews, 1990), *B. napus* (Radke, 1988; De Block, 1989; Moloney, 1989), *Brassica oleracea* (De Block, 1989) and *Brassica campestris* (Mukhopadhyay, 1992; Radke, 1992; Takasaki, 1997). Various explants are also used for *Agrobacterium* mediated transformation, such as isolated microspores (Chuong, 1987), stem

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Abbreviations: AS, acetosyringone; **6-BA**, 6-benzyladenine; **CaMV35S**, cauliflower mosaic virus 35S; **CTAB**, cetyl trimethyl ammonium bromide; **GFP**, green fluorescent protein; **NAA**, naphthalene acetic acid; **NOS**, nopaline synthase; **OD**, optical density.

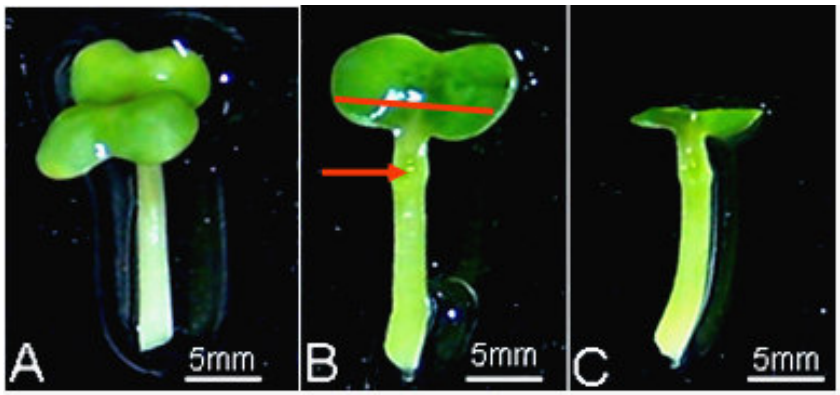

Figure 1. Cotyledonary node. **A:** Whole plantlet; **B:** tearing the epicotyl and vegetative pole form arrow and cutting cotyledon from line; **C:** cotyledonary node.

internodes (Fry, 1987), stem segments (Pua, 1987), cotyledonary petioles (Moloney, 1989), hypocotyls segments (Radke, 1988; Moloney, 1989; Stewart, 1996; Cardoza, 2003) and protoplasts (Chuong, 1987). As explants, cotyledonary nodes have several important advantages over others. Production of transgenic plantlets only takes about 4-5 weeks without a need to develop roots. The transformation efficiency can reach to about 19%. The transformation is not time consuming, laborious and expensive. However, if other types of explants are used, shoot and root regeneration will at least take about 7-8 weeks. Therefore, it is beneficial to further optimize the transformation system with cotyledonary nodes as explants.

In this paper, we reported an optimized transformation system with cotyledonary nodes as explants. This system was achieved by adding two biochemical compounds (acetosyingone and silwet L77) to *Agrobacterium* suspension and co-cultivation medium and subsequently optimizing concentrations of 6-BA, concentrations of NAA, OD of *Agrobacterium* suspension and duration of infection. These measures resulted in a notable improvement in the *Agrobacterium* mediated transformation for *B. napus*.

MATERIALS AND METHODS

Plant material

B. napus cv. "Ningyou 16" seeds were used for transformation. The seeds were surface-sterilized for 2 min with 75% ethanol, and 6 min with 15% sodium hypochlorite and 0.2% Tween-20. Then, the seeds were washed thoroughly with sterile distilled water and germinated for 2 d in the dark on half-strength MS basal medium containing 15 g/L sucrose, solidified with 8 g/L Agar in 10-cm Petri dishes at 22°C. Germinating seedlings were further incubated for additional 2 - 3 days at 22°C under a 16/8 h (light/dark) photoperiod. The seeds were sown at a density of 60 - 70 seeds per Petri dish.

Agrobacterium strain and binary vector

Agrobacterium tumefactiens strain LBA4404 was used. Binary vector pCAMBIA1303 contains the HPTα encoding hygromycin phosphotransferase which allows for the selection of plants resistant to hygromycin and a *gus-mgfp*-his6 fusion encoding the *Aequoria Victoria* green fluorescent protein (Siemering, 1996; Khan, 2008). The HPT□ is placed under the control of cauliflower mosaic virus (CaMV35S) 35S promoter and CaMV-35S polyA termination signal (Tada). The gene encoding green fluorescent protein (GFP) is controlled by CaMV-35S promoter and the nopaline synthase (NOS) terminator. As a selectable marker, GFP can be used to identify transgenic plants. Binary vector pCAMBIA1303 was transferred into *Agrobacterium* strain LBA4404 by liquid nitrogen freeze-thaw method (Holsters, 1978). *Agrobacterium* growing to an OD_{600} of 0.4-0.5 was centrifuged and re-suspended with liquid half-strength MS medium containing 15 g/L sucrose for 30-60 min prior to inoculation. For the optimization of infection conditions, the OD of *Agrobacterium* suspension was measured and adjusted to 0.2, 0.4, 0.8, and 1.0, respectively.

Co-cultivation and selection

Cotyledonary nodes (Figure 1), about 1 cm in length, excised from 4-to 5-day-old seedlings, grown under an aseptic condition, were infected for 5, 10, 15 and 20 min by immersing them into *Agrobacterium* suspension containing AS (50, 100, 200 and 400 µM) and silwet L77 (0.01, 0.02 and 0.04%). After infection, they were wiped with sterile filter paper and co-cultivated for 2 days in the dark on MS medium containing 30 g/L sucrose, 4 mg/L 6-BA, 0.04 mg/L NAA, and 5 mg/L $AgNO_3$ solidified with 8 g/L Agar (co-cultivation medium) at 22°C. Explants immersed in *Agrobacterium* suspension and co-cultivated on co-cultivation medium were transferred to MS medium containing 6-BA (1.00, 2.00, 4.00, and 6.00 mg/L), NAA (0.01, 0.02, 0.04, and 0.06 mg/L) (Daneshian 2005; Chai, 2006), 30 g/L sucrose, 500 mg/L carbenicillin and 10 mg/L hygromycin solidified with 8 g/L Agar (pre-selective mesium) and preselected for 7 days at 22°C under a 16/8 h (light/dark) photoperiod. An additional selection was taken for 7 d with 20 mg/L hygromycin. After a 14-day selection explants were transferred to MS medium containing 30 g/L sucrose, 2 mg/L 6-BA, 0.01 mg/L NAA, 5 mg/L $AgNO_3$, and 500 mg/L carbenicillin solidified with 8 g/L Agar (plantlet growth medium) and cultivated for 2 - 3 weeks at 22°C under a 16/8 h (light/dark) photoperiod.

Polymerase chain reaction analysis

The putative transgenic plants were analyzed by a polymerase chain reaction (PCR) to confirm the presence of the transgenes.

Table 1. Effect of different combinations of the infection durations and the OD of *Agrobacterium* on transformation frequency.

Infection duration (min)	OD of *Agrobacterium*	No. of plantlets examined	No. of GFP positive plantlets	Transformation frequency (%)*
20	0.2	77	0	0.00
15	0.4	166	4	2.41
10	0.8	178	10	5.62
5	1.0	72	0	0.00

Cotyledonary nodes infected with Agrobacterium were co-cultivated for 2 d in the dark on co-cultivation medium without AS and silwet L77; and then selected on selective medium with 4 mg/L 6-BA and 0.04 mg/L. Each treatment was replicated 3 times. (*Transformation (%) = no. of GFP positive plantlets/no. of plantlets examined).

DNA extraction was carried out using cetyl trimethyl ammonium bromide (CTAB) method(Doyle). The following primers were employed: *GFP*F [5'-ACTGAACTGGCAGACTATCC-3'] and *GFP*R [5'-CTCTTTAATCGCCTGTAAGTG-3']. PCR was carried out as the following: the DNA template was denatured at 94°C for 5 min, then amplified by 35 cycles of amplification (1 min at 94°C, 1 min at 55°C and 1.5 min at 72°C), and a final extension was performed at 72°C for 10 min. PCR products were separated by eletrophoresis in 1% agarose gel.

Southern blotting analysis

Southern blotting analysis was used to confirm the integration of exogenous gene into the host genomes and to estimate the copy numbers of the *GFP* in transgenic plants. Total genomic DNA was extracted with CTAB method. Genomic DNA (10 µg) was digested by *Hind*III overnight, then ethanol-precipitated and resuspended in 30 µl of water. The electrophoresed was performed on a 0.8% agarose gel at 1 V cm^{-1} for 20 h, and transferred onto a nylon membrane (Hybond N^+) using the downward alkaline (0.4 M NaOH) capillary method (Chomczynski, 1992). As a probe, the MGFP5 gene was labeled with [^{32}P]-dCTP (the Random Primer DNA Labeling Kit Ver. 2.0 was used, TaKaRa) according to the manufacturer's instructions. After a 16 h hybridization at 65°C, membranes were washed and exposed to film (Kodak MS) at -70°C.

RESULTS

Optimization of the infection conditions

Our experiments revealed that the OD of *Agrobacterium* suspension and the duration of infection had a direct influence upon transformation efficiency. It showed clearly from Table 1 that an OD of 0.8 and infection duration of 10 min was the best combination and resulted in a transformation efficiency of 5.62%. Necrosis was observed when explants were immersed in a more concentrated *Agrobacterium* suspension and incubated for a longer time, resulting in failure of transformation. Our previous experiments showed that AS and silwet L77 could increase transformation efficiency. When added to *Agrobacterium* suspension and co-cultivation medium, AS increased transformation efficiency from 5.62 to 11.90% and 11.90 to 18.93% for silwet L77 (Table 2). It was clear from Table 2 that 200 µM AS and 0.02% silwet L77 gave the best transformation efficiency of 18.93%. Interestingly, AS had more positive impact on transformation efficiency in the presence of silwet L77 than in the absence of it.

Optimization of concentrations of 6-BA and NAA

Our previous experiments showed that 6-BA and NAA had a direct effect on the development and growth of new plantlets and transformation efficiency. In order to determine the optimal concentrations of 6-BA and NAA, eight combinations of 6-BA and NAA concentrations were designed (Table 3). It was found from Table 3 that the maximum green plantlet efficiency (94.38%) was obtained in the medium supplemented with 4 mg/L 6-BA and 0.04 mg/L NAA. In this medium, the new plantlets developed from apical meristem, and 5.62% hypocotyls juxtaposing apical meristem became intumescent. In the absence of 6-BA and NAA, there were no new plantlet development and growth from apical meristem, and most cotyledonary nodes were necrotic. Therefore, 6-BA and NAA are critical factors to obtain transgenic plants.

Selection and identification of transgenic plantlets

Genomic DNA from independently obtained hygromycin-resistant plantlets and a control (non-transgenic) plant was subjected to PCR with MGFP5-specific primers. The result of PCR using hygromycin resistant plantlets showed that samples (lanes 1-8) gave the expected DNA band of 771 bp for the MGFP5 gene (Figure 2). No DNA amplification was detected in the control samples. Southern blotting analysis was used to confirm the transgenic nature of the PCR-positive plantlets and to estimate the copy numbers of MGFP5 (Ce´sar, 2008; Skalicky, 2008). It shows clearly from Figure 2 that there were four single hybridizing bands and two double hybridizing bands. The single hybridizing bands may indicate one copy of *GFP*. The variation in size of the hybridizing bands suggests MGFP5 be inserted into different location of the host

Table 2. Influence of AS and silwet L77 on transformation.

Co-cultivation AS (µM)	Medium silwet (%)	No. of explants examined	No. of GFP positive plantlets	Transformation frequency (%)*
0		178	10	5.62
50		76	4	5.26
100		101	9	8.91
200		126	15	11.90
400		92	6	6.52
200	0.01	98	12	12.24
200	0.02	206	39	18.93
200	0.04	104	7	16.35

Cotyledonary nodes were infected for 10 min 0.8 OD of Agrobacterium. After co-cultivation, explants were selected on selective medium with 4 mg/L 6-BA and 0.04 mg/L. Each treatment was replicated 3 times. (* Transformation (%) = no. of GFP positive plantlets/no. of plantlets examined).

Table 3. Effect of 6-BA and NAA on green plantlets frequency.

6-BA (mg/L)	NAA (mg/L)	No. of explants examined	NO. of regeneration shoots	Regeneration frequency (%)*
1.00		138	27	19.52
2.00		156	51	32.69
4.00		147	119	80.95
6.00		135	88	65.19
4.00	0.01	207	173	83.57
4.00	0.02	234	191	81.62
4.00	0.04	249	235	94.38
4.00	0.06	213	193	90.61

Cotyledonary nodes were infected for 10 min with 0.8 OD of *Agrobacterium*; and then co-cultivated for 2 d in the dark on co-cultivation medium with AS and silwet L77. Each treatment was replicated 3 times. (* Green plantlet frequency (%) = no. of green plantlets/no. of plantlets examined).

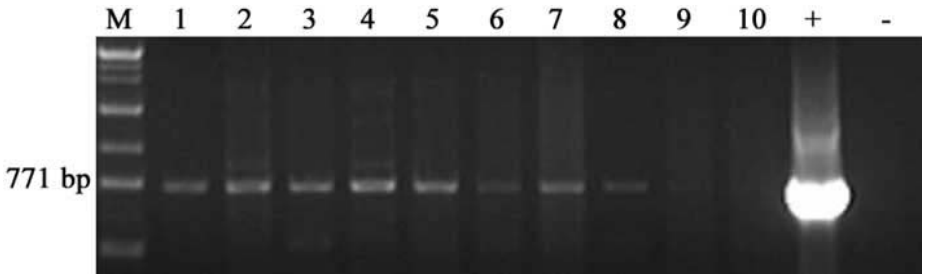

Figure 2. PCR analysis to detect the presence of MGFP5 gene in transgenic plants. Electrophoresis was carried out with 1% agarose gel. The lane "M" indicates DNA size marker. Lanes 1-8 showed the PCR fragment of MGFP.5 gene from different transgenic plants, which is consistent with the expected size of 771 bp. Lanes 9-10 showed the PCR results from control plants that they were untransgenic. The lane "+" shows PCR product with plasmid as template. The lane "-" shows PCR product with genomic DNA of untransgenic plantlet as template.

genome. The double hybridizing bands may indicate two copies of *GFP*. Positive plantlets identified with southern hybridization were further tested for specific GFP signal with a fluorescence microscope. 66.67% plantlets emitted

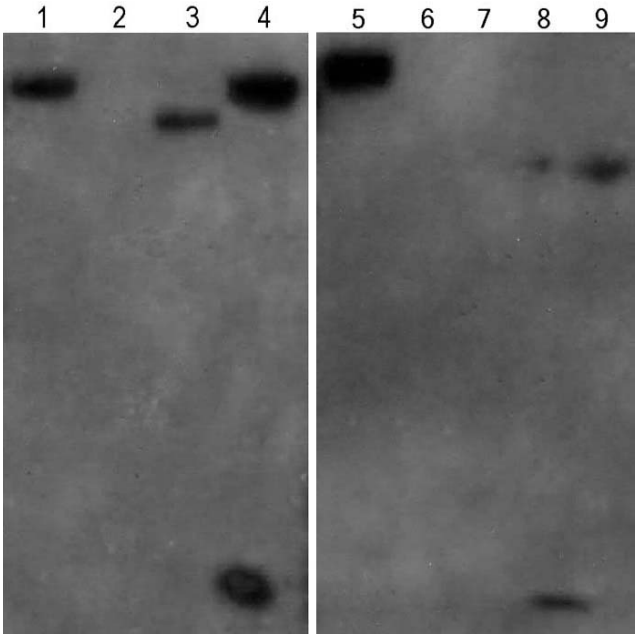

Figure 3. Southern blotting analysis to detect the insertion of MGFP5 gene into transgenic plants. 10 µg genomic DNA was digested overnight with *Hind*III, electrophoresed in 0.8% TAE-agarose gel and hybridized with [^{32}P]-dCTP- labeled PCR probes of MGFP5 gene. Lanes 1, 3, 5 and 9 showed single hybridizing bands. Lanes 4 and 8 showed double hybridizing bands. Lanes 2, 6 and 7 did not show any hybridizing band.

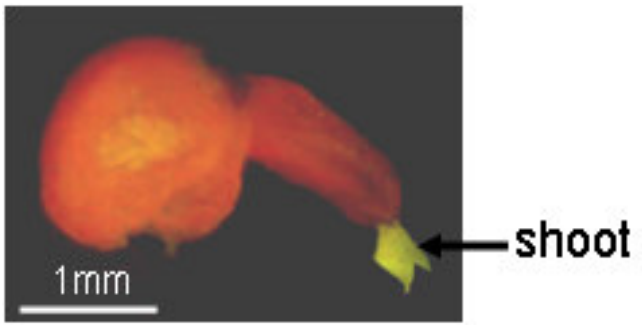

Figure 4. The expression of MGFP5 gene in plantlet. The plantlet was observed with blue light under the fluorescence microscope. The green shoot in figure was fluorescent, indicating the positive expression of GFP and successfully transformation.

green fluorescence (Figure 4).

DISCUSSION

There are some critical factors affecting the generation of transgenic plants, such as pre-cultivation time of explants, co-cultivation time, and delay in selection and addition of hygromycin, especially use of young seedling explants, the duration of infection and the OD of *Agrobacterium* suspension. In the present study, it was not easy to excise cotyledonary nodes from seedlings, therefore, we did pre-experiments and decided to choose cotyledonary nodes from 4- to 5-d-old seedlings, because those from 3-d-old seedlings became easily necrotic after being infected with *Agrobacterium*, and the transformation efficiency was quite low if using 6-d-old seedlings.

It is known that AS is a potent vir gene inducer and can promote the integration of exogenous gene into the host. Therefore, it would be expected that AS may have positive effect on transformation. It has already been reported that using 50 µM AS in *Agrobacterium* suspension and co-cultivation medium had stronger shoot regeneration (Cardoza, 2003). Yet, this result is contradictory to that of Rashid et al. (Rashid, 1996), where no GUS expression was observed after the addition of 50 µM AS in co-cultivation medium in *Moricandia arvensis.* So the effect of AS on transformation remains elusive. Silwet L77 is like a spreader and can concentrate *Agrobacterium* on apical meristem in order that *Agrobacterium* can efficiently infect apical meristem. Our experiment revealed that the addition of AS and silwet L77 to *Agrobacterium* suspension and co-cultivation medium can greatly increase transformation efficiency from 2.41 to 18.93%.

The optimal concentrations of 6-BA and NAA for transformation of *Brassica* plants varied with genotypes, cultivars and explanted types used in separate studies. For example, Hachey et al. (1991) obtained the highest shoot regeneration efficiency (70%) using 2 mg/L 6-BA and 1 mg/L NAA, whereas Xiang et al. (Xiang, 2000) obtained 40% shoot regeneration efficiency of *Brassica campestris* with 4 mg/L 6-BA and 2 mg/L NAA. Moloney et al. (1989) considered that only 6-BA was crucial for shoot induction and the effect of NAA was not evident. Heide (2002) considered that 6-BA used at the concentration of 3 mg/L not only reduced time needed for the formation of the calli, but also slightly enhanced the regeneration of shoots by 38.2 and 47.3% in 5- and 3-d-old explants, respectively. Wahlroos et al. (2003) thought that cultivation of explants for more than 3 weeks using the medium with 6-BA was detrimental to regeneration process. We did a series of experiments to test the effect of different combinations of BA and NAA. Our data clearly showed that 4 mg/L 6-BA and 0.04 mg/L NAA were the best combination for new plantlet development from apical meristem.

Multiple tools are employed to identify transgenic plantlets, including PCR, Southern hybridization and GFP assay. PCR is a fast and sensitive method, useful for perliminary screening, and should not be used if incredible. Southern blotting analysis of MGFP5 gene as a probe revealed that most plantlets identified had a single gene insertion. It is considered that single gene insertion in the transgenic plants has advantages over multiple genes insertions, because the insertion of multiple copies of T-DNA may reduce the expression of exogenous gene in the host genomes (Takasaki , 1997).

In conclusion, an optimized and efficient *Agrobacterium* mediated transformation system for *B. napus* with cotyle-

donary nodes as explants was established, cotyledonary nodes were infected for 10 min with an *Agrobacterium* suspension of 0.8 OD and 200 µM acetosyingone and 0.02% silwet L77 in the culture medium, and then cultivated in the medium containing 4 mg/L 6-benzyladenine and 0.04 mg/L naphthalene acetic.

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