Studies on transferring Δ6 – fatty acid desaturase gene(D6D) into *Brassica napus* L. plants

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Abstract

The desaturase of Δ6-fatty acid (D6D) is a rate-limiting enzyme in the production of γ-linolenic acid(GLA) and it can converts linoleic acid(LA) into α-linolenic acid(ALA) and GLA. In this study, a Δ6-fatty desaturase gene was cloned from *Rhizopus stolonifer* and the plasmid pCNR was constructed by inserting D6D gene into plant high-efficient expression vector pCAMBIA2301G. This construct was used for *Brassica napus* L. transformation mediated by *Agroubacterium tumefaciens* LBA4404. Results from GUS assay and PCR analysis of transformed plants indicated that Δ6-fatty acid desaturase gene from the *Rhizopus nigricans* was integrated into *Brassica napus* genomes.

Key Words: *Brassica napus L.*, regeneration, transformation, transgenic plants.

Introduction

Rhizopus stolonifer is a kind of filamentous fungi which can product γ-linolenic acid, because it carried a rate-limiting enzyme (the desaturase of Δ6-fatty acid) that can converts linoleic acid(LA) into α-linolenic acid(ALA) and GLA. The γ-linolenic acid (GLA) is a precursor substance for the synthesis of prosteglidine (PG) in the human body. We isolated a novel enzyme with specific activity of γ-linolenic acid from *Rhizopus stolonifer* and cloned its ORF(Sequence number from NCBI: AY795076) in the Lab, and constructed the plasmid pCNR. We hope that *Brassica napus L.* will be transformed into this gene, then rape can product that special polyunsaturated fatty acid benefit for the healthy. Transformation protocols reported so far, are relatively specific for var Westar and have not been used for yellow-seeded winter varieties. The efficiency of *A. tumefaciens* mediated transformation technique in oilseed rape is influenced by cultivar donor plant age , explant type (Poulsen, 1996) , cultured condition and so on. Although genetic transformation protocols are now available for most of the major crop species, the protocols are applicable within each species to only a few genotypes/ varieties that regenerate in vitro at high frequency.The main objectives of the present study were: to establish an efficient system for high frequency shoot regeneration in two geno-types of *Brassica napus* L. by investigating some influence factors ; to optimize genetic transformation protocol for *B. napus* line GH01 with A. tumefaciens strain LBA4404 harboring a binary vector pCNR; to gain massive transgenic rapes inducted into Δ6 – fatty acid desaturase gene.

Materials and Methods

Bacterial strain and plasmid

The strain of *A. tumefaciens* used in this study was LBA4404 (Hood et al., 1986) harboring pCNR (Hiei et al., 1994). pCNR is a binary vector (Figure 1) that contains a kanamycin-resistant gene and a GUS-gene (Ohta et al., 1990).

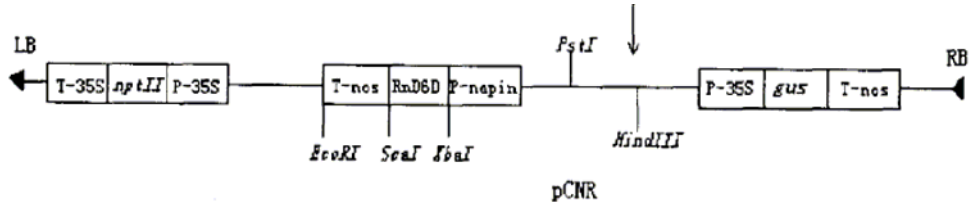

Fig1. Schematic diagram of a part of the T-DNA region of transformation vector pNR. RB, right border; LB, left border; P-napin, promoter for encoding seed storage protein; NPTII, gene for neomycin phosphotransferase; T-NOS, terminator of nopaline synthase;, P-35S promoter of cauliflower mosaic virus; intron, the first intron of catalase gene of caster bean; GUS, gene for β-glucuronidase; HPT, gene for hygromycin phosphotransferase

Plant material

Callus culture was carried out using four self-hybrid lines *Brassica napus L.* , which derived from two yellow-seeded rape GH01,SH01and two black-seeded rape D2,96V44. Four lines were used to investigate the regeneration frequency from hypocotyls. Hypocotyls were excised from 7-day old aseptic seedlings and cut into 5-mm-long segments for the explants.

Agrobacterium tumefaciens culture and co- cultivation with explants

A single colony of *A. tumefaciens* was inoculated into 50 ml of liquid YEP (Yeast Extract Peptone) medium (An et al., 1988) containing 50 mg l^{-1}kanamycin in an Erlenmayer flask and shaken at 200–210 rpm overnight in the dark at 28 °C. The protocol for transformation is given in Table 1.Hypocotyl segments were pre-cultured on Y1 medium (Table1) for 5-7 day. Overnight culture of *A. tumefaciens* having an OD of 0.4–1.5 was diluted OD_{600} 1:1 with liquid MS medium and poured into small petri plates. The plant material was immersed in bacterial suspension for 5-10 min with constant shaking.Explants were removed blotted dried with sterile filter paper to remove excess bacteria and placed on filter paper on the Y1 medium in petri plates. The plates were sealed with parafilm and co-cultivation was carried out at 22-28 °C for 3–4 days.

Selection

The infected cultures were washed with MS medium containing 300 mg l^{-1} Carbenicillin Na2 to stop the growth of *A. tumefaciens* attached to the explants and transferred to F1-Cab-Km medium . After 15 days of culture, then the selected callus tissues from the hypocotyl explants were removed to F2-Km-cab medium containing 10–200 mg l^{-1}kanamycin and 300 mg l-1 Carbenicillin Na2 for regenerating shoots. After 6-7weeks, regeneration shoots were removed to rooting medium to recover complete plants. green callus formation (as a percentage) was estimated as (no. of explants with green calli) / (no. of explants) × 100. Shoot formation frequency was estimated as (no. of shoots) / (no. of explants) 12 weeks after culture initiation .

Assay of β-glucuronidase (GUS) activity

Histochemical GUS assay was carried out essentially as described by Jefferson (1987).

DNA isolation and polymerase chain reaction(PCR) analysis

Genomic DNA was extracted from leaf tissues transgenic plants and untransformed control plant according to CTAB method (Doyle and Doyle, 1990). Polymerase chain reaction(PCR) analysis was carried out by Lenghong(2006).

Results

Regeneration frequency in two yellow-seeded and two black-seeded white rape

We investigated the frequency of regeneration from hypocotyls in three lines of *Brassica napus L.*. Among two black-seeded lines, the hybrid line D2 showed the highest frequency of green callus formation (100%) and shoot formation (0.9 shoots per explant). But 96V44showed a lower frequency of shoot formation(0.8 shoots per explant).Another side ,The inbred lines GH01 in two yellow-seeded lines showed a higher frequency of green callus formation (98%) and shoot formation (0.5 shoots per explant). SH01 showed a lower frequency of shoot formation(0.18 shoots per explant). Consequently, D2 and GH01 were used in subsequent experiments.

Histochemical GUS transient assay

To examine the efficiency of transformation by *A. tumefaciens*,we used the histochemical GUS assay to investigate preculture period, coculture period, concentration of acetosyringone, and concentration of *Agrobacterium*.

preculture period

We investigated the effect of preculture period on transformation efficiency in hypocotyl explants of D2 and GH01. Those explants precultured for 7 days produced the highest number of GUS-positive (GUS+) explants (Table 2, Fig2). All of the preculture periods differed significantly according to the chi-square test ($P<0.01$). The fewest GUS+ explants were observed in the 0-day preculture. Our results indicate that the length of the preculture period plays an important role in the frequency of infection of Brassica napus L. by *A. tumefaciens*.

Fig2. Expression of GUS gene in hypocotyl explants

coculture period

We investigated the effect of the coculture period on infection ability. The 63-hour ~ 72-hour coculture resulted in the highest infection ability (Table 3). All coculture periods differed significantly according to the chi-square test ($P<0.01$).

Effect of acetosyringone in the cocultivation medium

Acetosyringone is known to enhance the ability of *Agrobacterium* to transform recalcitrant host plants and has been incorporated in various media used for the cocultivation of bacteria and plant tissues (Godwin et al. 1991, 1992; Holford et al. 1992). We investigated four concentrations of *acetosyringone* (0–300 μM) in the cocultivation medium. Medium containing 200μM *acetosyringone* resulted in the highest infection ability (Table 4), and the differences were significant between this

concentration and the other concentrations of *acetosyringone* according to the chi-square test ($P<0.01$). Although there was no significant difference in the shoot regeneration ability of GH01 among the more higher concentrations of *acetosyringone* (data not shown), the hypocotyl segments tended to become browner at higher concentrations, which might affect *Agrobacterium* inoculation.

concentration of Agrobacterium

Concentration of *Agrobacterium* is important of enhancing infection ability. Usually, *Agrobacterium* in the log period has the highest infection ability. We investigated four concentration of *Agrobacterium* (OD_{600}:0.2-1.2) diluted with MS medium. The higher concentration of *Agrobacterium* (OD_{600}:1.0-1.2) benefits for infecting the explant.(Table5)the data also indicated that concentration of *Agrobacterium* directly affect the efficiency of infection.

Transformation

Many Kanamycin-tolerant callus were regenerated from explant on selected medium supplemented with 100-200 mgl^{-1} kanamycin after 2 months. Kanamycin-tolerant plantlets were obtained 4 months following culture initiation on shoot differentiation medium supplemented with 60 mgl^{-1} kanamycin. The transformants were confirmed by both PCR and histochemical GUS assay(Fig3,Fig5). In total, we obtained 13 transformants (Fig.4)from about 1,000 explants.(Table 6)The best conditions for *Agrobacterium* inoculation and culture were given by hypocotyl explants precultured on medium for 5-7days, followed by inoculation and coculture with *A. tumefaciens* strain LBA4404 containing 200 μ*M* acetosyringone for 2-4 days. Under these conditions, average transformation efficiency was 1.3 % (4 transformants per 300 explants). PCR analysis of two independently transformed plants revealed that each had been inserted into the goal gene (Fig.5). and GUS expression of root and leaf between the transformant (GH01) and control (non-transformant, GH01) was significantly different (Fig.3).

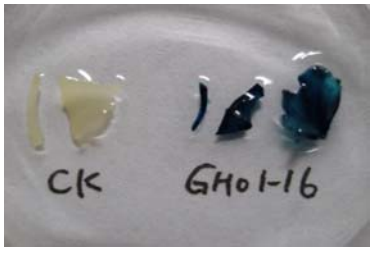

Fig3. Expression of GUS gene in root and leaf
The left is root and leaf of control, the right is transgenic rape

Fig4. Transgene yellow-seeded *Brassica napus L.*

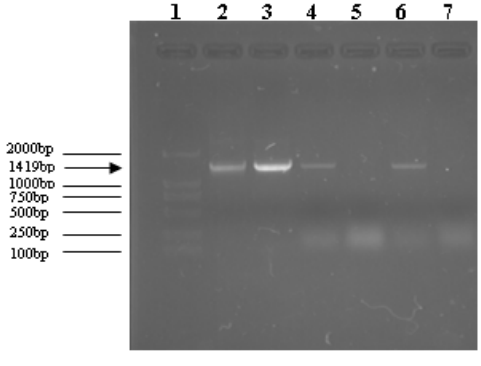

Fig5. PCR analysis to detect the presence of RnD6D gene in transgenic yellow seeded Brassica napus L. Lane1: DNA marker; Lane 2-3: DNA products of plasmid (positive control); Lane 5,7: PCR products of untransformed tall fescue control plant (negative control); Lane 4,6: PCR products of two Km-resistant plants..

Discussion

The data demonstrated that not only plant different varieties but also any culture condition will affect efficiency of plant genetic transformation. For example, the pre-cultured period, the co-cultured period, the concentration of *acetosyringone,* the concentration of *Agrobacterium,* and so on. It is indicated that the longer pre-cultured period make the explant tolerant the higher selected pressure, and the other factors directly result in the efficiency of infection. In different plant and different varieties , there were different condition benefits for their. Moreover, it is confirmed that the vector constructed by our lab can be succeed for transforming the goal gene into *Brassica napus L.*. The GUS gene can examine the transgenic plant effectively because it is ligated with 35S promoter. Then it will be expressed in the any spot of plant. PCR analysis still confirmed that the plants with GUS expression are the transgenic plant with RnD6D.

Conclusion

Here we reported of the first time the higher transformed system (average1.3%)for yellow-seeded *Brassica napus L.*. Although the efficiency of transformation is still lower, histochemical GUS assay and PCR analysis indicated that the goal gene had been inserted into the yellow-seeded rape. In the course of gain the transgenic plant, we investigated some factors affecting the transformation. It confirmed that pre-cultured 5-7 days explants should be infected into *Agrobacterium* (OD_{600}:1.0-1.2) and co-cultured 3-4 days in the medium containing 200μM *acetosyringone*.

In the future, we will confirm the function of the Δ6-fatty acid desaturase gene.

References

Omitted.