An efficient *Agrobacterium* – mediated transformation method used in Chinese cabbage (*Brassica rapa* ssp. *pekinensis*)

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Abstract

An efficient method was developed for producing transgenic Chinese cabbage inbred lines Kenshin by co-culturing cotyledonary explants with *Agrobacterium tumefaciens* strain LBA4404 which carried 'pathogenicity quenching factor' genes (Pqf1, Pqf2 and Pqf3). Vector pCAMBIA1301 which contains kanamycin and hygromycin resistance genes was used for transformation. The highest infection frequency was detected when cotyledonary explants were first infected by *Agrobacterium* for 15 min and subsequently co-cultivated with *Agrobacterium* for 2 days in co-cultivation medium supplemented with 50 mg/l acetosyringone. The media for shoot regeneration and selection contained 5.0 mg/l and 10.0 mg/l hygromycin, respectively. More than 60 putative transformants have been obtained from this method in Chinese cabbage inbred line Kenshin. The transformants were confirmed by GUS staining, PCR, Southern blot, Northern blot analysises and progeny test.

Key words: Chinese cabbage, *Brassica rapa* ssp. *pekinensis*, Genetic transformation, *Agrobacterium tumefaciens*, Cotyledonary explant

Introduction

Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) is an important vegetable that is cultivated extensively in Asia, particularly in China, Japan and Korea. Traditionally, genetic improvement of Chinese cabbage has been achieved mainly by conventional plant breeding methods, but recent advances in gene transformation techniques have opened new avenues for crop improvement. Successful exploitation of the technique warrants the availability of an efficient procedure for the introduction of foreign DNA into plant genomes. The *Agrobacterium*-mediated method of genetic transformation has certain advantages over the direct DNA delivery techniques, such as a high frequency of stable genomic integration, the transfer of relatively large segments of DNA and single/low copy numbers of a gene(s) can be achieved (McCormac, Fowler, Chen & Ellioot, 2001)

Agrobacterium-mediated transformation of *Brassica* crops has been reported in *B. napus* (De Block, De Brouwer & Tenning, 1989; Moloney et al., 1989; Radke et al., 1988). *B. juncea* (Mathews et al., 1990), *B. carinata* (Babic, Datla, Scoles & WA, 1998) and *B. campestris* (Mukhopadhyay, Arumugan & Nandakumar, 1992; Radke, Tuener & Facciotti, 1992; Takasaki et al., 1997). Only few studies have been undertaken to develop transgenic Chinese cabbage (Cho et al., 2001; Cho et al., 2003; Christey et al., 1997; Jun et al., 1995; Min et al., 2006; Takasaki et al., 1997; Zhang, Takahata & Watanabe, 2000). This is because of the fact that transformation of Chinese cabbage (*B. rapa* ssp. *pekinensis*) has been proved difficult because of its recalcitrant nature (Zhang et al., 1998), and more so when inbred lines are used. Recently, an effective plant regeneration protocol has been reported in Chinese cabbage using cotyledonary explants of inbred lines (Yang et al., 2004), in which shoot regeneration frequency was over 40%. However, during the process of transformation of Chinese cabbage, regeneration ability is adversely affected by the infection of *Agrobacterium*. Using inbred lines of Chinese cabbage (Kim et al. 2003), *Agrobacrium* mediated transformation frequency ranged from 0.4% to 0.8%. This necessitates improving the Agrobacterium-mediated transformation protocol for developing transgenics of Chinese cabbage. Here, we report an efficient transformation system in Chinese cabbage inbred line.

Material and methods

1. Plant materials and Agrobacterium *strain*

Inbred line Kenshin of Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) were used for the transformation experiment. Seeds of Chinese cabbage were sterilized with 70% ethanol for 1 min, followed by 2% sodium hypochlorite for 40 min, and then washed thrice in sterilized distilled water. The seeds were cultured in petri plates on MS (Murashige T & Skoog F, 1962) medium containing 3% sucrose and solidified with phytogel (2 g/l). The seeds were incubated at 25°C and 16/8 hour day/night photoperiod regime, till cotyledons were fully expanded (four days). The cotyledons were then dissected into pieces avoiding hypocotyls for use as explants.

Agrobacteriurn tumefaciens strain LBA4404 harboring a binary vector pCAMBIA1301 (CAMBIA, Australia) containing

Pqf1, 2 and 3 gene in the T-DNA was used in this research (Figure 1). In the vector, hygromycin resistant gene was designed as putative transgenic plant selection maker and kanamycin resistant gene as the *Agrobacterium* selection reagent. Also there is GUS expression gene with an intron constructed into T-DNA part for transformant selection.

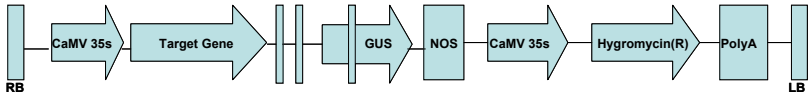

Figure 1. T-DNA regions of the vector pCAMBIA 1301, CaMV 35s: cauliflower mosaic virus 35s promoter, Target Gene: Pathogenicity quenching factor genes (Pqf1, Pqf2 and Pqf3), GUS: ß-glucuronidase gene, NOS: Nopaline synthase terminator (T-NOS), PolyA: Terminator. RB: Right border, LB: Left border, Hygromycin(R): Hygromycin resistant gene

.2. Transformation procedure

In transformation, tissue culture media of Chinese cabbage were made according to Yang et al and modified to use in transformation by testing the factors influencing transformation frequency. Cotyledonary explants, excised from the seedlings of Chinese cabbage were cultured on pre-culture medium in petri plates. At the same time, 5 µl of *Agrobacterium* stock was cultured in 5 ml YEP (Sherman F, 1991) medium containing 100 mg/l kanamycin. After 24 h culture in a rotary shaker at 250 rpm and 28°C, 10 µl aliquot was sub-cultured in 10 ml YEP medium till OD was 0.6. The bacterial cultures were then centrifuged at 3,500 rpm for 10 min and the pellet suspended in 10 ml resuspension medium. After pre-culture, the cotyledonary explants were immersed in resuspension medium containing *Agrobacterium* inoculum in culture tube for 15 min. These were then transferred on a sterile filter paper for 1 min to get rid of the *Agrobacterium* remainder and co-cultivated in petri plates in co-cultivation medium, at 25°C under dark. After co-cultivation, the explants were rinsed thrice in sterilized water and once in *Agrobacterium* elimination medium for 20 minutes, and placed on sterile filter paper for 1 min to remove the remaining *Agrobacterium* and then transferred to shoot regeneration medium containing 5 mg/l hygromycin. After incubation for 4 weeks, well-developed shoots were rinsed in sterile water, followed by carbenicillin (250 mg/l) to eliminate remaining *Agrobacterium* again and transferred onto selection medium for selection of transformants. After another 4 weeks of culture on selection medium, the survival plants were transferred to rooting medium. In one month, the number of rooted plantlets was recorded. The rooted plantlets were transferred to pots and covered with polyethylene bags with small holes for one week in growth chamber and then polyethylene bags were removed. After two weeks of acclimation, as needed some of the pots were transferred to 4°C culture chamber one and half months for vernalization. Finally, the plants were transferred to greenhouse, and grown to maturity.

Results

Putative transformants of Chinese cabbage were obtained in Chinese cabbage inbred line Kenshin. In our research, Pqf1, Pqf2 and Pqf3 were utilized in transformation. After selection, the regenerated putative transformed plants were counted (Table 2). The regeneration frequencies of putative transformants were 3.34, 3.58, and 3.01 for Pqf1, Pqf2 and Pqf3 genes individually.

Table 2. The putative transformants regeneration frequencies of Pqf1, 2 and 3 genes in Chinese cabbage inbred line Kenshin

Genes	Pqf1	Pqf2	Pqf3	Average
Explants number	1348	1395	1293	4036
Regenerated shoots number	47	58	60	165
Shoot regeneration frequency (%)	3.48	4.16	4.64	4.09
Regenerated plantlets number	45	50	39	134
Plants regeneration frequency (%)	3.34	3.58	3.01	3.32
Survival plants after acclimation	25	22	10	57

The putative transformants were tested by GUS staining, PCR, Southern and Northern blotting analysises and progeny test. The results shows that Pqf1, 2 and 3 genes were transferred into Chinese cabbage inbred line.

Discussion

In comparison to other *Brassica* species, *B. rapa* has been reported to be recalcitrant to tissue culture (Murata and Ortan 1987). This species has the lowest regeneration frequency among the basic diploids and their amphidiploids (Narsimhulu and Chopra 1988). This has been the major impediment in the development of *Agrobacteroium* -mediated transformation in *B. rapa*, especially inbred lines. The problem of regeneration in *B. rapa* was overcome by an improved protocol in inbred lines of Chinese cabbage with regeneration frequency of 40% using cotyledonary explants of inbred lines. We used this protocol in genetic transformation of Chinese cabbage.

In our research, the transformants were got in Chinese cabbage inbred line Kenshin. In Chinese cabbage transformation, there are many factors influencing transformation. By analysis these factors, Optimal *Agrobacterium* mediated transformation conditions were obtained for Chinese cabbage inbred line Kenshin. Also, the universality of this established Chinese cabbage inbred line transformation method was investigating in other Chinese cabbage lines.

Conclusion

This Chinese cabbage inbred line transformation system was established by the confirmation of DNA and RNA lever existence of transformed foreign gene. The procedure described herein provides a simple, efficient and reproducible *Agrobacterium*-mediated gene transfer method in Chinese cabbage inbred line Kenshi. To obtaining an integer data, research on progeny separation ratio and stability of the gene on progeny are proceeding.

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