African Journal of Biotechnology Vol. 4 (1), pp. 45-49, January 2005
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2004 Academic Journals

Full Length Research Paper

Study on transformation of cowpea trypsin inhibitor gene into cauliflower (*Brassica oleracea* L. var. botrytis)

Lv lingling[1]*, Lei Jianjun[2], Song-Ming[3], Li Liyun[4] , Cao Bihao[5]

[1]Southern Subtropical Crop Research Institute, Zhan Jiang, Guang Dong , 524091 China.
[2]South China Agricultural University, Guang Zhou, Guang Dong, 510642 China.
[3]Southwest Agricultural University, Chong Qing, 400716 China.
[4]Agriculture & Industry & Commerce Secondary professional School, Zhan Jiang, Guang Dong , 524091China.

Accepted 26 August, 2004

Cowpea Trypsin Inhibitor (CpTI) gene was transferred into cauliflower by *agrobacterium*-mediated transformation method, and 14 transgenic cauliflower plants were obtained. Cotyledons and hypocotyls were used as explants. The putative transformants were assayed by PCR and Southern blotting analysis. The results indicated that CpTI gene was transferred into cauliflower successfully. The result of preliminary insect-resistant assay showed that the transgenic plants were more resistant to *Pieris rapae* than non-transgenic plants.

Key words: cauliflower (*Brassica oleracea* L. *var. botrytis*), *agrobacterium,* mediated genetic transformation CpTI gene, transgenic plant.

INTRODUCTION

Cauliflower belongs to cruciferaceae vegetable and contains several kinds of vitamins. It is cultivated all over the country China and other parts of the world. However, it is easily attacked by Lepidoptera pests (such as *Pieris rapae and Plutella xylostella*) during its growth and breeding. Although chemical insecticide can reduce losses in production, the residual chemicals are harmful to human and environment. Cowpea Trypsin Inhibitor (CpTI) gene is one of insect-resistant genes and can confer resistance to many pests, such as Lepidoptera, Coleoptera and Orthoptera. In this paper, CpTI gene was transferred into cauliflower by *agrobacterium*-mediated method and transgenic plants were obtained. This will lay foundation for insect control and breeding of new varieties without environment contamination.

*Corresponding author. E-Mail: lianzi9381@163.com.

MATERIALS AND METHODS

Plant material and plasmid for transformation

Seeds of cauliflower 0115 and 0116 were bought from a seed company. *Agrobacterium tumefaciens* strain LBA4404, plasmid pGA643 are preserved in our laboratory. NPT-II gene with Kanamycin (kan)-resistance was cloned into plasmid pGA643, so the plant expression vector was kan-resistant.

Regeneration and transformation system of cauliflower

Hypocotyls and cotyledons which are 4-6 days old were used as explants. Many experiments were done to select the suitable hormones and their concentrations, preculture time, co-culture time, concentration of bacterium solution, the time of inoculation with *agrobacterium*, and concentration of Kanamycin and other antibiotics.

PCR detection

Genomic DNA was extracted from leaf tissue of kan-resistant plants and non-transformed plants according to CTAB method (Sambrook et al., 1989).

Table 1. Effects of different hormones on the bud differentiation of explants in *Brassica oleracea* L. *var. botrytis.*

Medium	Bud differentiation ratio (%)			
Concentration of hormone (mg/L)	0115		0116	
	Cotyledons	Hypocotyls	Cotyledons	Hypocotyls
MS+BA2.0+NAA0.2	65.0	75.0	66.7	75.0
MS+BA3.0+NAA0.2	33.3	66.7	35.0	71.4
MS+BA2.0+NAA0.1	45.4	66.7	50.0	71.4
MS+BA3.0+NAA0.1	44.4	71.4	57.1	58.3

Table 2. Comparison of regeneration capacity of cauliflower explants.

Type of explants	Total number of explants	the number of differentiated explants	bud differentiation ratio
0115 cotyledons	50	31	62.0
hypocotyls	50	37	74.0
0116 cotyledons	90	60	66.7
hypocotyls	100	75	75.0

Primer 1 is 5' GATGATGGTGCTAAAGGTGT-3'; Primer 2 is 5'-CTTACTCATCATCTTCATCC-3'. The 25 µl PCR reaction contains 25 ng DNA template, of 5 µmol/L of each primer, 10 mmol/L dNTPs and 1 U Taq. Initial denaturation was 94°C for 4 min, Cycling parameters were 94°C (denature) for 1 min, 56 °C (anneal) for 1 min and 72°C (extension) for 1 min. After 35 cycles, it was kept under 72°C for 10 min.

Southern blotting analysis

2.5 µg genomic DNA of eight PCR positive samples were completely digested with EcoR I at 37 °C overnight in a total volume of 200 µl, then transferred onto nylon membrane by capillary transfer technique. Probe for CpTI gene was prepared by separation of the fragments after PCR on 0.8% of agarose and purification of 326 bp band from the gel. Southern blot was prepared and hybridized using standard procedure (Gao et al., 1998). The method of southern blotting analysis was carried out according to the introduction of DIG label and detection kit (company that makes kit).

RESULTS

Regeneration of cauliflower explants

Effects of hormones on the bud differentiation of explants

MS (mineral salts) was used to as base medium. Different hormones were added to MS, bud differentiation ratio of explants was calculated 32 days later. Results showed that when 2.0mg/L of 6-BA and 0.2mg/L of NAA were added to MS, the bud differentiation ratio was the highest (Table 1).

Comparison of regeneration capacity of explants

Cotyledons and hypocotyls of cauliflower were cultured in regeneration medium (MS + 2.0 mg/L of 6-BA + 0.2 mg/L of NAA), and 32 days later, bud differentiation ratio was calculated. Results indicated that the differentiation capacity of hypocotyls was higher than that of cotyledons. As to hypocotyls, about 74.5% of calli had differentiated into green buds; Cotyledons' was about 64.0% (Table 2). At the same time, the difference of regeneration capacity between two varieties was small as evident in Tables 1 and 2. Thus, only the 0116 variety was used in subsequent experiments.

Transformation of explants

The cotyledons and hypocotyls of cauliflower were sensitive to kan. When different concentration of kan (0, 5, 10, 15, 30 and 50 mg/L) were added to regeneration medium, the differentiation of explants was different. When the concentration of kan was 5 mg/L, some explants could form calli and some buds survived; when it was 10 mg/L, most of the explants died before forming calli; when it was higher than 15 mg/L, all of the explants died before differentiation (Table 3). So 15 mg/L was used to select transgenic plants. During the transformation of cauliflower, when explants were inoculated with *agrobacterium* without preculture, the wound of explants would turn brown easily and cells would die. It affected the differentiation of explants. In this study, explants were

Table 3. Effect of kan concentration on callus inducement and bud differentiation of explants in cauliflower.

Concentration of mg/L	Number of explants	number of explants forming calli	Number of explants kan differentiating into buds
0	40	40	30
5	40	15	6
10	40	10	0
15	40	0	0
30	40	0	0
50	40	0	0

Figure 1. Kan-resistant transgenic plant in root regeneration medium.

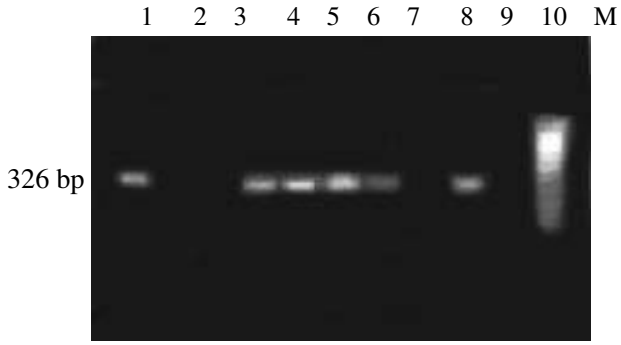

Figure 2. PCR detection of transgenic plants. M: DNA marker, 1-7: genomic DNA of transformed plant, 8: non- template (negative control), 9: plasmid pGA643 (positive control), 10: genomic DNA of non-transgenic plant (negative control).

precultured 2 and 3 days respectively before they were inoculated with *agrobacterium*. This decreased the number of explants which turned brown. The effect of preculture for 3 days was better.

According to conference 2 and 10, explants were inoculated with *agrobacterium* for 30-60 s and 5-10 min, respectively. Results indicated that the contamination of explants was very high when inoculation time was 5-10 min, and the rate of bud differentiation was lower than those inoculated for 30-60 s. So inoculation time of 30-60 s was used in subsequent experiments. In addition, the concentration of *agrobacterium* solution affects transformation of foreign gene. When kan concentration is too low, the foreign gene is difficult to get into explants; If it is too high, explants will die easily because of toxins. Making the O.D. of *agrobacterium* solution 0.3-0.5 is optimum. Explants were co-cultivated for 1, 2 and 3 days. Results indicated that the rate of bud differentiation was lowest when co-cultivated for 3 days.

However, when contamination of explants is taken into consideration, it is best to co-culture for only 2 days.

In order to select the transgenic plants, buds were transferred to two new selected medium, and the concentration of Carbencillin was decreased to 200 mg/L. When the height of buds was 3 cm, buds were transferred to root regeneration medium I (MS + cef250 mg/L + kan15 mg/L), roots could be seen after about 20 days. When buds were transferred to root regeneration medium II (MS + NAA0.2 mg/L + kan15 mg/L), roots could be seen after 7-10 days, making root regeneration medium II better. In this study, 305 buds were transferred to selection medium, 14 kan-resistant plants were obtained (Figure 1).

Health transgenic plants were later transplanted into sand. They were cultured at 25-27^{o}C and 90% relative humidity in an incubator. Hoagland' solution was used to provide nutrition. 7-10 days later, they were transplanted to field. Of the 120 transgenic plants transplanted to field, 24 (20%) survived.

PCR detection

PCR was used to verify the presence of introduced CpTI in the transgenic plants. The results indicated that 8 in 14 plants were positive, the non-transgenic plant and the

transgenic plants with no template were negative. The PCR results of 7 randomly selected transgenic plants are shown in Figure 2.

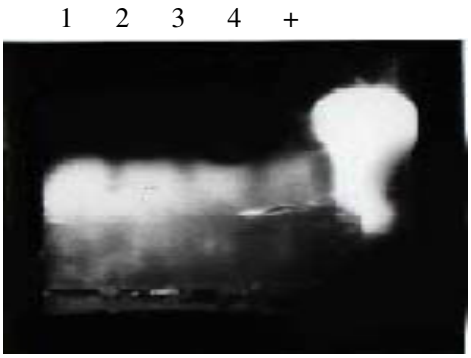

Figure 3. Southern blotting analysis of transgenic plants. +: PCR product of plasmid pGA643 digested with EcoRI (positive control); 1-4: genomic DNA of transgenic plants digested with EcoRI.

Figure 4. Preliminary insect-resistant assay of transgenic plants. The left leaves were detached from non-transformed plants, and the right leaves were detached from transgenic plants .

Southern blotting analysis

Using PCR band of plasmid pGA643 as positive control. The results showed that CpTI gene had been integrated into cauliflower genome. Some results were showed in Figure 3. The transformation efficiency was 2.62% (8/305).

Preliminary insect-resistant assay

Young leaves were detached from the top of transgenic cauliflower plants, and young leaves at the same stage from non-transformed plants were used to as negative control. Leaves were placed into a box with 2 wet filter paper on bottom and the control was in left. Each side was placed ten *P. rapae* larvae of the second instar, and the extent of leaf consumed was recorded 3 days later. Results showed that transgenic plants had some resistance to *P. rapae* (Figure 4).

DISCUSSION

There were several reports about regeneration and *Bacillus thuringiensis* (*Bt*) gene transformation of cauliflower (Dong et al., 1997; Cai et al., 2000; Hua et al., 1992; Chen et al., 1994). In addition, David et al. (1988) and Srivastava et al. (1988) obtained transgenic cauliflower plants, but the plants could not grow normally. Reporter gene has also been transferred into cauliflower by He et al. (1991) and others (Chen et al., 1995; De Bloch and De Browwer, 1998) and transgenic plants were obtained, but the transformation efficiency was very low.

In this study, the transformation efficiency was enhanced through following measures. First, suitable concentration of 6-BA and NAA were added to MS, and the bud differentiation ratio of explants increased to 70%. Secondly, explants turned brown quickly after inoculation with *agrobacterium*. Through preculture for 3 days (O.D. of *agrobacterium* 0.3-0.5) inoculation time of 30-60s, and co-culture for 2 days could decrease the number of explants turning brown. Thirdly, cauliflower explants were sensitive to kan. Kan was added to culture medium for selection after explants were cultured for 7-10 days. This decreased the inhibition by kan and enhanced transformation efficiency.

REFERENCES

Cai R-Q (蔡荣旗). Sun D-L (孙德岭), Zhao Q-C (赵前程) (2000). Preliminary Report of Bt Infected in Cauliflower by *Agrobacterium Tumefaciens* [J]. Tianjin Agric. Sci. 6(4): 9-12.

Chen J-H (程继鸿) (1994).. Genetic transformation of insecticidal genes in Brassica Crops[D]. Department of horticulture, Northwest Agricultural University. Yangling, Shanxi.

Chen X-B (陈晓邦), Hua X-J (华学军), Huang Q-M (黄其满) (1995). Intron-GUS gene was transferred into cauliflower by *agrobacterium*-mediated method and transgenic plants were obtained J. Chinese Bulletin of Bot. 12 (Suppl.): 50-52.

David C, Tempe J (1988). Genetic transformation of cauliflower (Brassica oleracea L.var. botrytis) by Agrobacterium rhjizogenes. J. Plant Cell Rep. 7(2): 88-91.

De Bloch M, De Browwer D (1989). Transformation of Brassica napus and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expressing of the bar and neo genes in the transgenic plants. J. Medee Fac Landbouwwer Ri jksuniv Gent 54 (4): 1267-1276.

Dong W-B (董五辈), Tang G-F (唐桂芬), Lan Z-H (兰尊海) (1997). Studies on High Regeneration Frequency system of Cauliflower. J. Henan Sci. 15(1): 70-73.

Gao Y-F (高越蜂), Zhu Z.（朱祯）, Xiao G-F.（肖桂芳）,Zhu Y.朱玉）, Wu Q.（吴茜）, Li X-H （李向辉）(1998). Isolation of soybean kunitz trypsin inhibitor gene and its application in plant insect -resistant genetic engineering. J. Acta Botanica Sinica(植物学报). 40: 405-411.

He Y-K (何玉科), Gong Z-H (攻振辉), Wang F (王飞) (1991). Transformation Efficiency of Brassica Crops with *Agrobacterium Rhizogenes* Harboring the Binary Vector Bin19[J]. Chinese J. Biotechnol. 7(4): 382-385.

Hua X-J (华学军). Chen X-B (陈晓邦), Fan Y-L (范云六) et al. *A. Tumefaciens*-mediated transformation of B.T-endotoxin gene into cauliflower[J]. Scientia Agricultural Sinica. (1992). 25 (4): 82-87.

Srivastava V, Reddy AS, Mukherjee GS (1988). Transformation and regeneration of *Brassica oleracea* mediated by an oncogenic *Agrobacterium tumefaciens*. J. Plant Cell Rep. 7(7): 504-507.

Sambrook J, Fritsch EF, Mainiati T (1989). Molecular Cloning: A Laboratory Manual. M. 2nd editon. New York. Cold Spring Harbor Laboratory Press, pp. 480-481.