Transformation of Brussels Sprouts (*Brassica oleracea* var. *gemmifera* Zenk.) by *Agrobacterium rhizogenes* Harboring a Reporter, β -Glucuronidase Gene

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Summary

Transfer of β -glucuronidase (GUS) gene into Brussels sprouts was made using *Agrobacterium rhizogenes* vector-system. Plasmid pBI 121 was transferred to *A. rhizogenes* IFO 13257 by the freeze-thaw method. When leaves of Brussels sprouts were inoculated with *Agrobacterium*, 34 hairy roots were produced with GUS activities. From seven root clones with GUS activities, 14 shoots were regenerated from calli on Murashige-Skoog (MS) medium supplemented with $1 \text{ mg} \cdot \text{liter}^{-1}$ α -naphthaleneacetic acid (NAA) plus $5 \text{ mg} \cdot \text{liter}^{-1}$ zeatin. Seven shoots obtained from four root clones again showed GUS activities, suggesting that they were transformants. In two root clones, shoots with and without GUS activity were regenerated from the same callus. Four shoots from three root clones showed no activity. Thus, half of GUS genes were stably transmitted from hairy roots to regenerated plants.

Introduction

Brussels sprouts (*Brassica oleracea* var. *gemmifera* Zenk.) are one of the important vegetables which belong to the Cruciferae family. However, this vegetable is susceptible to cabbage moth, common cabbage worm, bacterial soft rot, and black rot (Akutsu and Itoh 1987; Iijima 1987). If resistant genes against these pests/diseases were introduced to Brussels sprouts, breeding and their cultivation would be greatly facilitated. Transformation, using *Agrobacterium*-vector system, has been shown to be promising for introducing desirable genes into target plants. For example, TMV coat protein gene and insect-resistant gene of *Bacillus thuringiensis* were introduced into tobacco (Gasser and Fraley, 1989). Transformation studies of *Brassica oleracea* were successful in cabbage (Berthomieu and Jouanin, 1992), cauliflower (David and Tempe, 1988), ornamental kale (Hosoki et al., 1989), broccoli (Hosoki et al., 1991) and Brussels sprouts (Hamada et al., 1989). In these studies, however, only Ri plasmid, intrinsic gene in *Agrobacterium rhizogenes* was transferred to plant cells. Transfer of foreign genes has not been re-

ported except for one recent report on broccoli (Sato et al., 1993).

The objective of the present experiment is to transfer plasmid pBI 121 (a reporter, β -glucuronidase (GUS) gene and a selective kanamycin-resistant gene, nptII) into *A. rhizogenes* followed by transferring the plasmid to Brussels sprouts. To check the success, expression of GUS gene in the plant tissue was observed. Such an *Agrobacterium*-vector system would be useful when desirable genes instead of just markers are transferred.

Materials and Methods

1. Transfer of pBI 121 into *Agrobacterium rhizogenes*.

Because a conventional method of tri-parental mating (Ditta et al., 1980) was not successful in transferring a plasmid pBI 121 (Clontech Lab. Inc.) to *A. rhizogenes* IFO 13257 (Institute for Fermentation Osaka, Osaka, Japan), a freeze-thaw method (An et al., 1988) was adopted. Namely, *A. rhizogenes* was incubated at 28°C in 50 ml IFO 802 medium consisting of 1% polypeptone, 0.2% yeast extract, 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.2% glucose at pH 7.0 (Hosoki, 1991) and shaken at 40 rpm for 24 hr.

Suspension culture of *Agrobacterium* was chilled on ice for 30 min and centrifuged at 3000 rpm for 5 min. The supernatant was discarded and pellet with *Agrobacterium* was re-suspended in ice-cold 1 ml solution of 20 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. A 100 μl suspension was transferred to an Eppendorf tube to which 10 μl (4 μg pBI 121 dissolved in 40 μl Tris buffer, pH 7.5) were added and chilled on ice for 30 min. Each Eppendorf tube containing *Agrobacterium* was then frozen at -70°C with dry ice/methanol for five min and thawed at 37°C . One ml of IFO 802 medium was added and *Agrobacterium* was incubated at 28°C for five min. The *Agrobacterium* suspension was then centrifuged at 9,000 rpm for five min. The supernatant was discarded and *Agrobacterium* pellet was re-suspended in 50 μl IFO 802 medium. The suspension was plated onto a Petri dish containing solid IFO 802 medium (1.5% agar) supplemented with 200 $\text{mg} \cdot \text{liter}^{-1}$ kanamycin sulfate and incubated at 28°C for four days.

2. Transfer of pBI 121 to Brussels sprouts mediated by *Agrobacterium rhizogenes*.

After the above colony of *A. rhizogenes* with GUS activity was incubated at 28°C for two days, the bases of Brussel sprout leaves were inoculated with *Agrobacterium* as described before (Hosoki et al. 1989). These leaves were then individually inserted upside down to test-tube (2×15 cm) containing 15 ml of solid MS (Murashige and Skoog, 1962)(0.8% agar) medium and incubated at 28°C for two days. Each leaf was then transferred to MS medium supplemented with 500 $\text{mg} \cdot \text{liter}^{-1}$ carbenicillin sodium to stop excessive infection with *Agrobacterium*.

Results and Discussion

1. Transfer of pBI 121 into *Agrobacterium rhizogenes*

Five colonies of *Agrobacterium* appeared on the medium but only two colonies showed reaction to a reagent, 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) which was stained blue by GUS activity (Jefferson, 1987). Thus, two transformed *Agrobacterium rhizogenes* colonies were obtained while the other colonies were probably kanamycin-resistant mutants which escaped.

2. Transfer of pBI 121 to Brussels sprouts mediated by *Agrobacterium rhizogenes*

Within 2 weeks, 106 hairy roots were produced from the treated leaves but only 34 roots showed positive GUS reaction when root tips were assayed with X-gluc (Jefferson, 1987; Hosoki et al., 1994). The roots with positive reaction were cut out from the bases and cultured on MS medium supplemented with 1 $\text{mg} \cdot \text{liter}^{-1}$ α -naphthaleneacetic acid (NAA) plus 5 $\text{mg} \cdot \text{liter}^{-1}$ zeatin to regenerate shoots. For complete elimination of *Agrobacterium*, 500 $\text{mg} \cdot \text{liter}^{-1}$ carbenicillin sodium was continuously supplemented. Kanamycin sulfate was not supplemented as a selective antibiotic because roots of Brussels sprouts turned out to be resistant to it in a preliminary test. To check for GUS activity in the shoots regenerated from the hairy roots, the shoots were assayed with X-gluc and 4-methyl umbelliferyl β -D-glucuronide (MUG). In the MUG assay, sap extracted from the leaves was incubated at 37°C for two hr in 22 mg MUG dissolved with 50 ml GUS assay buffer (pH 7.0) containing 50 mM NaPO_4 , 10 mM β -mercaptoethanol, 10 mM Na_2EDTA , 0.1% sodium lauroyl sarcosinate and 0.1% Triton X-100 (Jefferson, 1987).

One month after culture on MS medium supplemented with NAA plus zeatin, all roots formed callus. Among 34 calli, seven calli derived from seven root clones produced adventitious shoots, each callus having one to four shoots (Fig. 1). Shoots from seven root clones were assayed with X-gluc and also with MUG which emitted fluorescent light under UV lamp (365 nm) if GUS gene was activated (Jefferson, 1987). Of shoots derived from four root clones (A to D), seven shoots showed positive reactions to the two reagents, although two shoots from C root clone and one shoot from D root clone did not show positive reaction (Table 1)(Figs. 2 and 3). The shoots from the other clones (E to G) showed no positive reaction. Based on these facts, it is concluded that half of the pBI 121 plasmids were stably transmitted from hairy roots to adventitious shoots while in the other half, the plasmid disappeared during the callus-growing stage or while the GUS gene was being inactivated in the plant genome. A similar case was reported by Birot et al. (1987). Although more direct evidence showing integration of pBI 121 into the plant genome by Southern blotting

Fig. 1. A shoot regenerated from hairy root harboring pBI 121.

Table 1. GUS activity of shoots regenerated from 7 root clones.

Hairy root clones	Number of shoots regenerated from each root clone	Number of shoots showing positive reaction to X-gluc MUG
A	2	2
B	2	2
C	4	2
D	2	1
E	2	0
F	1	0
G	1	0

(Southern, 1975) is necessary, we believe that a transfer of a foreign gene into Brussels sprouts was accomplished. Such a technique will be a useful tool when more desirable genes are isolated.

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Fig. 2. A leaf of control shoot shows no reaction to X-gluc (left); a leaf of shoot shows positive reaction (blue stain) to X-gluc (right). Control shoot was obtained from a hairy root to which pBI 121 was not transferred.

Fig. 3. Sap extracted from control shoot showing no reaction to MUG (left); Sap extracted from shoot showing positive reaction (fluorescent light emission under UV) to MUG (right). Control shoot : the same as in Fig. 2.

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β -Glucuronidase 指標遺伝子を有する *Agrobacterium rhizogenes* によるメキャベツの形質転換

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摘 要

メキャベツへの指標遺伝子 β -glucuronidase (GUS) の導入が *Agrobacterium rhizogenes* ベクター系を用いて試みられた。プラスミド pBI 121 は凍結-融解法で *A. rhizogenes* IFO 13257 に導入された。*Agrobacterium* がメキャベツの葉に接種された時、GUS 活性を示す 34 本の毛状根が得られた。これらの根を $1 \text{ mg} \cdot \text{liter}^{-1}$ NAA と $5 \text{ mg} \cdot \text{liter}^{-1}$ zeatin を含む MS 培地で培養すると、7 本の根系からカルスを經由して 14 本の莖葉

が分化した。4 本の根系から得られた 7 本の莖葉は再び GUS 活性を示し、形質転換体と判断された。同一のカルスでも GUS 活性を示す莖葉と活性を示さない莖葉を分化した根系もあった。3 本の根系由来の 4 本の莖葉はまったく活性を示さなかった。以上より、再生植物体の半数は毛状根で認められた GUS 活性を安定して伝えていた。