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Transformation of Melon (*Cucumis melo* L.) with *Agrobacterium rhizogenes*

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Transformation of melon was conducted using *Agrobacterium rhizogenes* harboring plasmid pBI121 which contains a kanamycin resistant (NPT II) gene and β-glucuronidase (GUS) gene in addition to the resident Ri-plasmid of this strain. Melon leaf segments were inoculated with the bacterium and induced hairy roots were positively selected in the presence of kanamycin. By examining the β-glucuronidase activity of kanamycin-resistant hairy roots, four GUS-positive lines were obtained. An integration of the GUS gene to the host chromosome was further verified by southern blot analysis of the DNAs of these lines. Successful shoot differentiation was achieved by culturing hairy root-derived callus tissues with an MS medium containing 50 μg/*l* of IAA and BAP. Both NPT II and GUS genes were stably expressed at the different stages of shoot differentiation. Thus, the present study suggests that the transfer of foreign genes by *A. rhizogenes* is applicable to the production of transgenic plants in melon.

Introduction

Many dicotyledonous plant species infected with *Agrobacterium rhizogenes* or *A. tumefaciens* produce adventitions hairy roots[7] or crown gall tumor[4], respectively, at infection sites. The production of these extrinsic tissues results from the expression of hormone-producing genes coded in the T-DNA regions which were transferred to the host chromosomal DNA. Trulson *et al.*[12] reported the transformation of cucumber with *A. rhizogenes* and revealed that selected marker genes of the vector derived from *A. tumefaciens* could be transferred to plant cells together with the T-DNA of the resident Ri-plasmid. This methodology made it simple and effective to obtain transgenic plants because transformants were easily selected in the presence of antibiotics from hairy roots produced by Ri-plasmid. In order to establish an efficient system for producing transgenic melon, the present paper describes the possible application of this method. That is by testing the expression of foreign genes, kanamycin resistance (NPT II) and β-glucuronidase (GUS) genes co-transferred with the T-DNA of Ri-plasmid to host plant cells.

Materials and Methods

Bacterial strains, MAFF 07-20001 and 07-20002 are cultures isolated by T. Shiomi, and ArM-123 and ArR-248, of *A. rhizogenes*, were kindly given by Dr. K. Ohta, Shizuoka Agricultural Experiment Station, Shizuoka, Japan, respectively. For examining the infectivity of the strains, bacteria were

shake-cultured at 30°C in L-broth (10g Bactotrypton, 5g yeast extract, 10g NaCl, 1g glucose in 1 *l* of water, *pH* 7.0) for 24 hr. The bacterial suspensions were used for inoculation into melon leaf segments. Young upper leaves of one-month-old seedlings of melon (*Cucumis melo* L. cv. Earl's Favourite) were harvested and surface-sterilized with 70% ethanol and then with 2% sodium hypochlorite. After washing several times with sterilized water, the leaves were cut into small segments (1×1cm) and dipped into bacterial suspension (10^8 cells/m*l*) for inoculation. After orbitally shaking at 26°C for 10 min, the inoculated segments were laid on a 1% agar-solidified plate, then transferred to hormone-free Murashig-Skoog[8] (MS) medium containing 500 μg/m*l* carbenicillin, and cultured at 26°C under continuous illumination of 4000 lux.

Adventitious roots generated from inoculated leaf segments were excised and transplanted to a hormone-free MS medium containing carbenicillin. These vigorouly growing roots were subcultured on the hormone-free medium which was changed for a fresh supply of the same medium several times over a 5-day interval. Isolated hairy roots were cultured with MS medium containing various concentrations of IAA and 6-benzylaminopurine (BAP). Callus tissues induced were subcutured with a fresh supply of the same medium for 2-3 repetitions in order to obtain differentiated shoots.

The virulent strain of *A. rhizogenes* was transformed with plasmid vector, pBI121, which contains NPT II gene (furnished with nos promoter and terminator) and GUS gene (with CaMV 35S promoter and nos terminator) flanked by the border sequences of Ti-plasmid[5]. Transformation of the bacterium was conducted according to the triparental mating method[2]. Transformed bacteria were inoculated into leaf segments by the method mentioned above, and isolated hairy roots were cultured with hormone-free MS medium containing 100-300μg/m*l* kanamycin in order to examine kanamycin resistance. For verifying successful co-transformation of GUS gene with NPT II, the β-glucuronidase activity of kanamycin-resistant lines was fluorogenically assayed according to the methods described by Jefferson[5].

Southern blot analysis was conducted to confirm the integration of GUS gene in the chromosomal DNA of GUS-positive hairy roots. The DNA was isolated from 2g (fresh weight) of roots according to the procedure of Saghai-Maroof *et al.*[11], digested with some restriction endonucleases, electrophoresed on a 1% agarose gel, and hybridized with probe DNA (GUS gene flanked to CaMV 35S promoter and nos terminator) labeled with horseradish peroxidase (HRP) according to the instruction protocol for ECL gene detection system supplied by Amersham International (Buckinghamshire, England).

Results and Discussion

Transformation of muskmelon with *A. tumefaciens* has been reported by Fang and Grumet[3]. However, it was not obvious whether the melon cultivar used in the present study could be infected with *A. rhizogenes*. The authors, therefore, inoculated some strains of *A. rhizogenes* into melon leaves and examined the capability of the bacteria to produce hairy roots. The results indicated that the strain, MAFF 07-20001, was highly infective to the present cultivar and produced hairy roots vigorously growing in hormone-free MS medium (**Fig. 1-A**).

Plant regeneration from hairy roots is a prerequist for the production of transgenic plants. In some plant species, hairy roots were effectively redifferentiated to intact plants in the absence of plant hormone[1,7,9], while some plants required the addition of hormones in order to promote shoot formation from hairy roots[10,12]. The authors first examined regeneration of hairy roots with hormone-free medium, but did not succeed in obtaining regenerated plants. So they cultured hairy

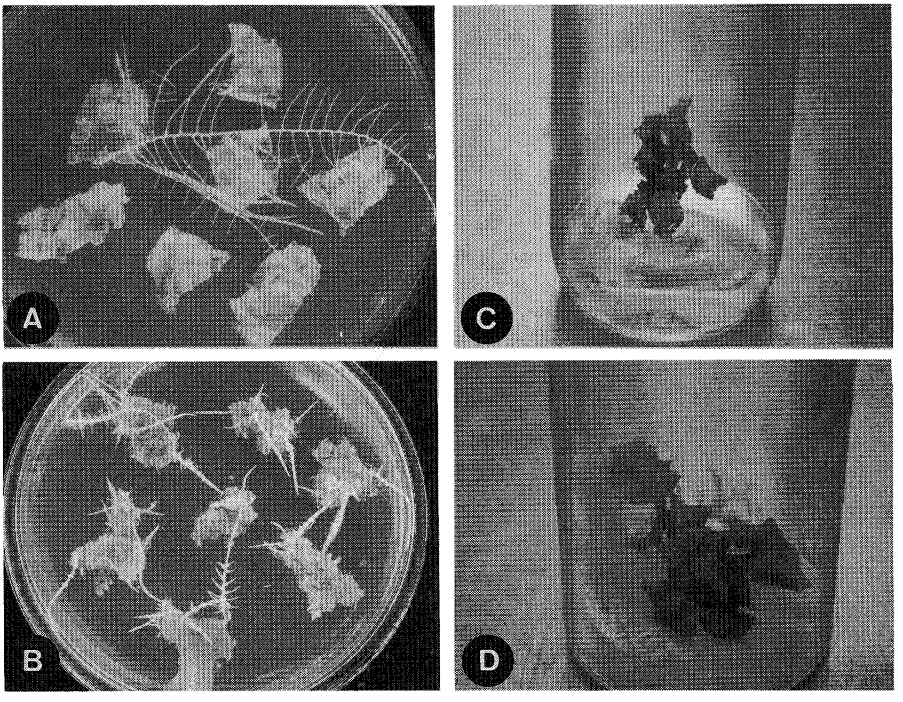

Fig. 1. Callus formation and shoot differentiation from hairy roots originated from melon leaves inoculated with *A. rhizogenes* MAFF 07-20001.
Leaf segment initiated the formation of hairy root after 7 days of inoculation and vigorously elongated the root (A). Hairy roots induced callus tissues (B) 5-7 days after transfer to MS medium containing 50μg/l of IAA and BAP. Shoot (C) was differentiated by subculturing callus tissues with the same fresh medium for 2-3 repetitions over an interval of 5 days. Differentiated shoots secondarily produced hairy roots (D) when transferred to hormone-free MS medium and then cultured for 2-3 days.

roots with MS medium containing various concentrations of IAA and BAP. As a result, the growth of hairy roots was promoted with lower levels (2.5-40μg/l) of hormones, though hormone levels inducing the highest growth were different among the hairy root lines tested. On the other hand, hairy roots produced callus tissues when cultured with higher concentrations (50-100μg/l) of IAA and BAP. **Fig. 1-B** shows callus tissues derived from hairy roots with MS medium supplemented with 50μg/l of 2, 4-D and BAP. Shoots were frequently redifferentiated after callus tissues were subcultured for 2-3 repefitions using a fresh supply of the same medium (**Fig. 1-C**). Hairy roots were secondarily produced when shoots were transferred to hormone-free medium (**Fig. 1-D**). The excised tip (1cm long) of these hairy roots showed vigorous growth in the absence of hormone and could repeat the processes for shoot differentiation when the hormone condition was changed. Although the existence of T-DNA was not directly examined in the present study, the repeated production of hairy roots strongly suggested a successful integration of T-DNA to host cell chromosome and stable expression of the genes required to produce hairy roots.

The present study demonstrated an efficient formation of hairy roots and plant regeneration from hairy roots. In the following experiment, therefore, melon leaf segments were actually inoculated with *A. rhizogenes* harboring pBI121, and an expression of both NPT II and GUS genes were examined in the hairy root lines isolated. Eventually, 54 hairy root lines were obtained in the present inoculation and 5 lines showed resistance to kanamycin. Among these lines, KG-11 was highly resistant to the antibiotic and produced elongated hairy roots with an extensive lateral branching

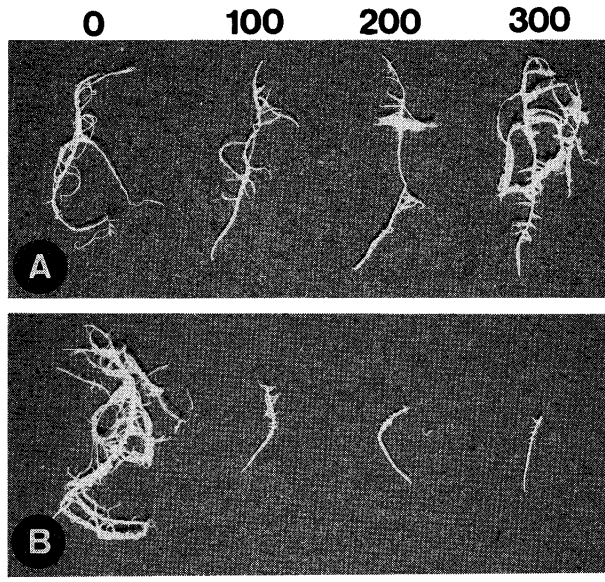

Fig. 2. Kanamycin resistance of hairy root line (KG-11) induced by *A. rhizogenes* MAFF 07-20001 with vector pBI121 carrying NPT II and GUS genes.
Hairy roots of KG-11 (A) and control hairy roots (B) induced by this bacterium without the vector were cultured for 3 days in the selective medium containing various concentrations of kanamycin. The numbers in the figure represent concentrations (μg/m*l*) of kanamycin added to the medium.

in the presence of 300 μg/m*l* kanamycin (**Fig. 2-A**). Kanamycin resistance of the isolated lines was stably expressed even after several repetitions of subculture with kanamycin-free medium. On the other hand, hairy roots (control) produced by *A. rhizogenes* (without pBI121) were highly sensitive to kanamycin and completely ceased the elongation within 3 days after transfer to the selective

Table 1. Kanamycin resistance and β-glucuronidase (GUS) activity in hairy roots of melon

Hairy roots	Kanamycin resistance[b]	GUS activity[a] (4-MU nmol/μg protein/min)
Control[c]		
25 lines	−	− (0.001—0.002)
Isolated lines[d]		
49 lines	−	− (0.001—0.003)
KG-10	+ (100)	+ (0.129)
KG-11	++ (300)	++ (0.243)
KG-12	+ (100)	− (0.003)
KG-23	+ (100)	+ (0.173)
KG-26	+ (100)	+ (0.110)

a) Clarified homogenates of hairy roots were mixed with a buffer containing 4-methyl-umbelliferyl glucuronide and incubated at 37°C for 3hr. Fluorescence intensities of enzymatic product, 4-methyl-umbelliferone (4-MU), were spectrofluorimetrically determined relative to the intensity of authentic 4-MU.
b) Numbers in parentheses represent the highest levels of kanamycin permitting the growth of hairy roots.
c) Hairy roots obtained by inoculation with *A. rhizogenes* (MAFF 07-20001) without the vector.
d) Hairy roots by *A. rhizogenes* (MAFF 07-20001) transformed with the vector pBI121 containing kanamycin resistance and GUS genes.

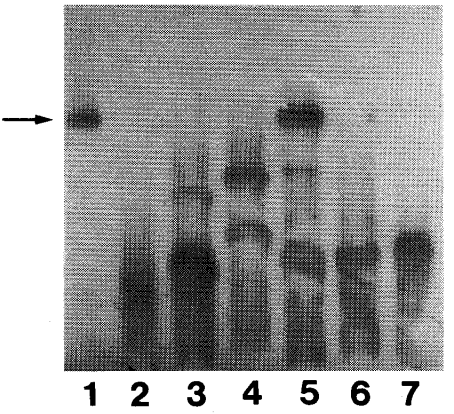

Fig. 3. Southern blot analysis for an integration of GUS gene to host chromosomal DNA of hairy root line, KG-11.
Lane 1, GUS gene (approximate 3 kirobases) (arrow) cut out of the original vector pBI121 by *Hin* dIII-*Eco* RI digestion; Lane 2, non-digested chromosomal DNA of KG-11; Lane 3-7, chromosomal DNA of KG-11 digested with different restriction endonucleases; Lane 3, *Pst* I; Lane 4, *Sph* I; Lane 5, *Hin* dIII and *Eco* RI; Lane 6, *Hin* dIII; Lane 7, *Eco* RI. The hybridization results suggest that more than two copies of GUS genes were integrated into chromosomal DNA of KG-11.

medium containing 100 μg/m*l* kanamycin (**Fig. 2-B**). These results suggest that NPT II gene of pBI121 could be transferred to host cells together with the T-DNA of *A. rhizogenes* Ri-plasmid. Genetic transformation of kanamycin-resistant lines was further verified by examining both the activity of β-glucuronidase and the integration of GUS gene. An enzymatic assay clearly showed that four of kanamycin-resistant lines exhibited β-glucronidase activities approximately 50-120 times higher than control hairy root (**Table 1**). In addition, southern blot analysis of the DNA from GUS-positive hairy root lines confirmed the integration of vector DNA to the host chromosome (**Fig. 3**).

The culture conditions defined in control hairy roots were also applicable to regeneration of the selected lines, and actually allowed us to obtain regenerated plants from kanamycin-resistant, GUS -positive hairy roots. In this experiment, therefore, the authors examined whether both genes could be expressed at the different stages of hairy root differentiation. For this purpose, callus tissues, leaves of shoots, and secondary hairy roots of KG-11 were treated with 100 μg/m*l* kanamycin, and then reacted with 5-bromo-4-chloro-3-indolyl-glucuronide (X-glucuronide) for histochemical detection of β-glucuronidase activity[5]. The tissues were resistant to kanamycin and showed positive reaction for the enzyme activity (**Fig. 4**). These results clearly indicate that both NPT II and GUS genes transferred to primary hairy roots could be stably propagated and expressed at the different stages of redifferentiation.

In the present study, it was demonstrated that foreign genes can be successfully mediated to melon plants by inoculation with *A. rhizogenes*. In addition, the present system revealed that it is not necessarily essential to analyze the resident Ri-plasmid of the bacterial strain, because the infectivity of bacteria was easily examined by testing the production of hairy roots, and because true transformants could be effectively isolated from hairy roots by the positive screening for antibiotic resistance and/or the enzymatic analysis of the translation product of the reporter gene. This may make it possible to use the virulent strains of a newly isolated bacteria promptly, and therefore to generalize this methodology in a broad range of melon cultivars into which the existing strains cannot be infected.

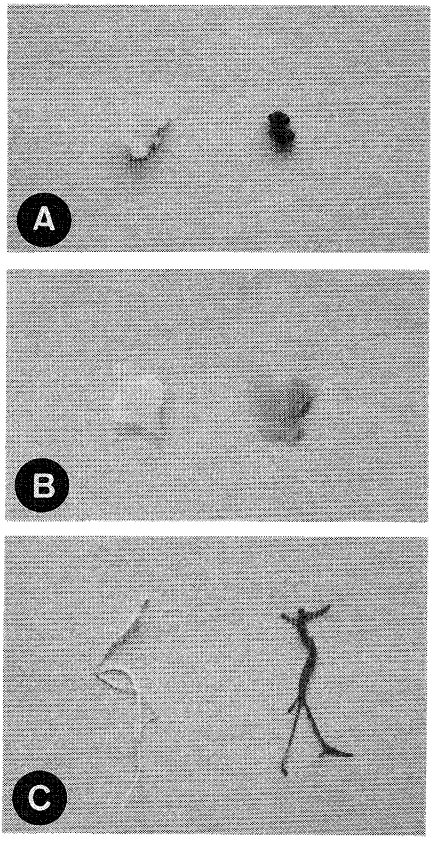

Fig. 4. Positive β-glucuronidase activity in callus tissues(A), leaf developed from shoot(B), and secondary hairy roots(C) of KG-11.
The tissues were fixed on ice for 3 min with 0.3% formaldehyde, washed three times with a buffer(10mM sodium phosphate, pH 7.0, containing 0.05mM potassium ferricyanide and 0.05mM potassium ferrocyanide), and reacted with a buffer containing 1mM X-glucuronide at 37°C for 12 hr in the dark. Leaves were cleared by ethanol before observation. Note that the materials(left) obtained from control hairy root were not stained by this treatment.

Hairy root production was an important factor for efficient selection in our system, but is not always essential characteristic in transgenic progeny. Thus, the forthcoming problem of this work is to genetically analyze the distribution of introduced genes in the progeny, especially the assortment of genes and the T-DNA controlling the production of hairy roots. An additional problem may be the occurrence of somaclonal variation in the progeny. Since it has been generally recognized that various genetic variations are frequently induced in plant tissue cultures[6], the present regenerants obtained through callus cultures may be a source of somaclonal variation in addition to transformed characters. The answers to these questions will be described in a following paper.

References

1) David, C., J. Tempe, 1988. Plant Cell Rept., **7**: 88-91.
2) Ditta, G., S. Stanfield, D. Corbin, D. R. Helinski, 1980. Proc. Natl. Acad. Sci. USA., **77**: 7347-7351.
3) Fang, G., R. Grument, 1990. Plant Cell Rept., **9**: 160-164.
4) Holbrook, L. A., B. L. Miki, 1985. Plant Cell Rept., **4**: 329-332.
5) Jefferson, R. A., 1987. Plant Mol. Biol. Rept., **5**: 387-405.
6) Larkin, P. J., W. R. Scowcroft, 1981. Theor. Appl. Genet., **60**: 197-214.

7) Mugnier, J., 1988. Plant Cell Rept., **7**: 9-12.
8) Murashige, T., F. Skoog, 1962. Physiol. Plant. **15**: 473-497.
9) Noda, T., N. Tanaka, Y. Mano, S. Nabeshima, H. Ohkawa, C. Matsui, 1987. Plant Cell Rept., **6**: 283-286.
10) Rech, E. L., T. J. Golds, T. Husnain, M. H. Vainstein, B. Jones, N. Hammatt, B. J. Mulligan, M. R. Davey, 1989. Plant Cell Rept., **8**: 33-36.
11) Saghai-Maroof, M. A., K. M. Soliman, R. A. Jorgensen, R. W. Allard, 1984. Proc. Natl. Acad. Sci. USA., **81**: 8014-8018.
12) Trulson, A. J., R. B. Simpson, E. A. Shahin, 1986. Theor. Appl. Genet., **73**: 11-15.

《和文要約》

Agrobacterium rhizogenes によるメロン形質転換体の作出

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メロンの形質転換体を作出する目的で，*A. rhizogenes* による遺伝子導入系を検討した．まず，メロン葉片に本菌を接種し，誘導された毛状根からの個体再生条件を検討した．それによると，ホルモン添加培地で毛状根からカルスを誘導すると，そのカルスからshootが高頻度に形成され，再度ホルモンフリーに戻すと，shootから二次毛状根が形成された．この方法で毛状根から再生体を得ることが可能となったので，本菌にNPT IIおよびGUS遺伝子を有するプラスミドを導入し，同様の接種試験を行って，メロンにおける両遺伝子の発現を検討した．すなわち，カナマイシン抵抗性ならびにGUS(+)の毛状根系統を選抜し，選抜系統の染色体DNAをGUS遺伝子をプローブとしたサザンブロット法で解析した．その結果，本遺伝子の染色体への組み込みが証明され，さらに本系統からカルス，shootおよび二次毛状根を誘導し，カナマイシンならびにGUS活性を調べたところ，いずれにおいても両遺伝子の発現が確認され，本法がメロンの形質転換体作出に有効であることが示された．