Transformation and Plant Regeneration of Broccoli (*Brassica oleracea* var. *italica*) Mediated by *Agrobacterium rhizogenes*

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Summary

Transformation and plant regeneration of broccoli cv. Early De Cico were studied using *Agrobacterium rhizogenes*-vector system. Hairy roots were induced from inoculated leaf tissue within 3 weeks whereas only callus was induced from uninoculated tissue. Four root clones out of 18 grew well on the hormone-free Murashige-Skoog (MS) medium. Each root clone was sectioned and cultured on the medium supplemented with 0.1 mg·liter⁻¹ or 1 mg·liter⁻¹ α -naphthaleneacetic acid (NAA) in combination with 1 mg·liter⁻¹ or 5 mg·liter⁻¹ zeatin. Two root clones out of 4 produced adventitious buds on the 5 mg·liter⁻¹ zeatin-containing medium.

The other two clones produced only callus. Mannopine, which is indicative that genetic transformation had occurred, was detected in the regenerated shoots and in one callus from a root clone. The compound was not detected from another callus and in other shoots of the control mother plants.

Introduction

Broccoli is one of the most important vegetable crops and its production in Japan has increased sixfold in the past 10 years. Broccoli belongs to the Brassica family and is susceptible to attack by the cabbage moth and common cabbage worm or to infection by bacterial soft and black rots. If resistant genes against these pests/diseases were introduced from another family, breeding of broccoli would be greatly advanced. Recently, somatic cell fusion technique has enabled breeders to obtain hybrids between sexually incompatible species (5). However, this method still poses problems such as selection of only fused cells and that of plant regeneration. Moreover, undesirable genes would be introduced from donor plants, which necessitates repeated back-crossing to eliminate them. Recently, a gene transfer system using *Agrobacterium tumefaciens* and *A. rhizogenes* has been developed (2, 18). This method has an advantage especially when a single or a few desirable genes are required to be introduced to recipient plants (14).

Potato and tomato (*Solanaceae*) and cucumber

(*Cucurbitaceae*) plants have been successfully transformed using *A. tumefaciens* (9, 15) or *A. rhizogenes* (21); likewise, in *Cruciferae*, cabbage and cauliflower have been transformed using these bacterium (3, 4, 17). To date, no transformation of broccoli, which is becoming more and more important vegetable crop, has been reported.

A. rhizogenes harboring Ri plasmid has an advantage over *A. tumefaciens*, because plant cells transformed by the former bacterium form the tissue which is easily distinguishable by the emergence of hairy roots; the explants grow well on hormone-free medium (20). Furthermore, target genes are easily inserted between T_L and T_R regions (6). On the contrary, when *A. tumefaciens* is used as a vector, non-transformed cells are often impossible to distinguish from transformed tumor cells. Hence, this method necessitates the addition of a selectable marker-gene such as kanamycin-resistance near or adjacent to the target gene. For these reasons, we chose to transform broccoli using *A. rhizogenes* rather than *A. tumefaciens*.

Materials and Methods

A strain of *Agrobacterium rhizogenes* (Ar. M-123), which was originally isolated from melon hairy root disease (8, 11), was multiplied at 28°C in the dark

on 1.5% agar-solidified meat/yeast extract culture medium as described earlier (7). Before inoculation of plant material, freeze-stored *Agrobacterium* was transferred to a fresh medium for 3~4 days to reestablish a vigorous colony. Plant material was prepared by harvesting axillary buds of field-grown broccoli (*Brassica oleracea* var. *italica* Plenck) cv. Early De Cico. Immature leaves, 1.5~2.0 cm long, were picked and sterilized with a dilute solution of sodium hypochlorite (active chlorine, 0.7%) for 10 min. After rinsing with sterile water, petiole tips were cut off and *Agrobacterium* was inoculated directly on the fresh cut surface of the leaf. The leaf was then inversely inserted into the medium. The culture medium consisted of Murashige-Skoog (MS) major elements and Fe-EDTA (10), Ringe and Nitsch minor elements and vitamins (12), 2% sucrose and 0.8% agar (pH 5.6). The cultures were kept at 28°C and under 3000 lux supplied by a bank of fluorescent lamps for 16 hr per day. Within one day after inoculation, *Agrobacterium* spread out from the leaf blade into the medium. Infected leaves were then transferred to the same medium supplemented with 500 mg·liter⁻¹ carbenicillin sodium to stop bacterial growth. Additional methodology is described with the results.

For proof of transformation, tissue sections (0.2 g fw) of regenerated buds were homogenized in an Eppendorf tube containing 0.1 ml of 0.1 N HCl and centrifuged at 5000 rpm. A sample of the supernatant layer was subjected to electrophoresis at 350 V for 1 hr (18). After drying the electrophoretogram, mannopine, one of the opines produced by Ri plasmid was made visible by reacting it with an alkaline silver nitrate reagent (18).

Results

Hairy roots emerged directly from most of the explants on inoculated leaf tissue within 3 weeks (Fig. 1), whereas uninoculated leaf tissue (control) produced only callus on the cut surface. When the roots grew to 4~6 mm long, they were transferred to MS medium supplemented with 500 mg·liter⁻¹ carbenicillin sodium and cultured for 3 weeks to inactivate the *Agrobacterium* completely. The roots were then transferred to antibiotic-free MS medium. Four roots out of 18 resumed growth during the 1-month culture period. The other roots stopped growth and eventually turned yellow. The four actively growing root clones were divided into

9 to 19 sections (4~5 mm long) depending on the length of each root clone. These sections were subcultured on regeneration MS media which contained 0.1 mg·liter⁻¹ or 1 mg·liter⁻¹ NAA combined with 1 mg·liter⁻¹ or 5 mg·liter⁻¹ zeatin. Most of roots produced callus and secondary hairy roots (Fig. 2). Green spots appeared in the callus 6 weeks after transfer into the regeneration medium. Two weeks later most of them had developed into adventitious buds. Five mg·liter⁻¹ zeatin was essential for bud formation (Table 1). Concentration of NAA did not increase the number of buds that formed. Adventitious buds were obtained from two root clones but not from the other two clones. Adventitious buds were initially transferred to MS medium supplemented with 1 mg·liter⁻¹ zeatin and subsequently to a medium containing 0.2 mg·liter⁻¹ zeatin and cultured at 20°C for 3~4

Fig. 1. Hairy root formation (right) from *Agrobacterium* inoculated-leaf cut surface and callus formation (left) from uninoculated cut surface (control) (one month after culture).

Fig. 2. Active growth of secondary hairy root and callus from a hairy root clone.

Table 1. Effect of NAA and zeatin on the formation of callus, secondary hairy roots and adventitious buds from hairy root explant (2 months after culture, 7–19 explants per treatment).

NAA (mg·liter ⁻¹)	Zeatin (mg·liter ⁻¹)	Callus formation (%)	Secondary hairy root formation (%)	Adventitious bud formation (%)	Mean of adventitious buds per explant ^z
0.1	1	100	71	0	0 a ^y
0.1	5	100	100	29	3 b
1	1	100	100	0	0 a
1	5	89	89	26	2 b

^z Adventitious bud number calculated only by explants with adventitious buds.

^y Means followed by the same letter within column did not differ significantly as 5% level by LSD test.

Fig. 3. Plant from transformed tissue (right) and from the control plant (left).

weeks to induce shoot elongation. When the shoots were 2–4 cm in length, they were transferred to a medium with 0.1 mg·liter⁻¹ 3-indolebutyric acid to induce rooting. Eighty percent of the shoots produced roots within 3 weeks (Fig. 3). Most of the 13 plants appeared normal. There were no wrinkled leaves as often found in Ri plasmid-transformed plants (19). However, 3 plants showed stem fasciation.

Root growth was active in most plants, suggesting that they were probably transformed by the introduction of the Ri plasmid.

Regenerated plants from two hairy root clones and calli from the other two hairy root clones were subjected to electrophoresis to verify transformation. All regenerated shoots and one callus showed a presence of mannopine (Fig. 4), which indicates that they were transformed (23). Mannopine was not detected in another callus and control shoots from the mother plants.

Fig. 4. Example of electrophoretogram for detection of mannopine. M: Standard mannopine, T₁ and T₂: Regenerated shoots from hairy roots, C: A shoot from the mother plant (control). Lower spots in T₁, T₂ and C are unknown-silver positive reactants.

Discussion

Introduction of foreign genes from sexually incompatible species may be possible by electroporation or with polyethyleneglycol using protoplast culture system (16). However, there is a problem that plasmids are not firmly integrated into the chromosome (24). *Agrobacterium* vector system is the most reliable method of gene transfer in dicotyledonous species at the present time (13).

In our previous work (7), we succeeded in transforming ornamental kale using *A. rhizogenes* harboring Ri plasmid. In this experiment, we obtained

transformed broccoli plants in which most of the regenerants appear normal because the number of undersirable genes introduced was probably minimal. A few plants, however, showed fasciated stem. It is not clear whether this abnormal morphology was caused by: a) influence of Ri plasmid, b) genetic variation through *in vitro* culture, or c) hormonal effect in *in vitro* callus culture (22). A callus derived from one hairy root clone in which mannopine was detected failed to induce adventitious buds. This observation suggests that the adventitious bud formation is dysfunctional, probably because of its location on the chromosome to which Ri plasmid was integrated. Another callus lacked mannopine because a dominant gene from mannopine production was probably not introduced to the chromosome, whereas other genes on Ri plasmid were transferred (3).

More than 15 rooted plantlets including explants from axillary shoots which were transferred to large glass bottles containing MS medium are growing well. Our next step will be to construct a vector plasmid connected with desirable genes by cointegration (13) or binary vector system (1), and to introduce them to broccoli using the method established here.

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Agrobacterium rhizogenes を用いたブロッコリーの 形質転換と植物体再生

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摘 要

Agrobacterium rhizogenes をベクター系としてブロッコリー 'Early De Cico' の形質転換と植物体再生が検討された。*Agrobacterium rhizogenes* を葉組織に接種すると、3週間以内に毛状根が発生したが、非接種葉からはカルスのみ生じた。発生した18本の根のうち4本はホルモンフリーのMurashige-Skoog(MS)培地でよく生長した。この4本の根(ルートクローン)を切片に分け、NAA(0.1 mg・liter⁻¹と1 mg・liter⁻¹)に Zeatin(1

mg・liter⁻¹と5 mg・liter⁻¹)を組み合わせたMS培地で培養すると、Zeatin 5 mg・liter⁻¹を含む区で2本のルートクローンから不定芽が生じた。再生した不定芽およびカルスからは、形質転換の指標となるマンノピンの反応が認められた。なお母植物体の芽および一部のルートクローンからのカルスからは反応が認められなかった。