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Agrobacterium-mediated transformation and regeneration of kiwi fruit

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Summary. Genetically transformed kiwi fruit (Actinidia deliciosa) plants were obtained from hypocotyl and stem segments co-cultured with Agrobacterium tumefaciens strain EHA101 harboring a binary vector, pLAN411 or pLAN421, which contained the neomycin phosphotransferase II (nptII) gene and the β-glucuronidase (GUS) gene. After co-culturing with the A. tumefaciens, the hypocotyl or stem segments were cultured on a selection medium containing 25μg/ml kanamycin and 500μg/ml Claforan. After one month in culture, shoots had regenerated from the cuttings. Green shoots were analyzed for NPTII activity and GUS activity. Eighty-five percent of the green shoots examined expressed the nptII and GUS genes. GUS histochemical assays revealed strong GUS expression in guard cells, mesophyll cells, and trichomes.

Introduction

Kiwi fruit (Actinidia deliciosa) (Ferguson 1990) originated in China, and was introduced into New Zealand in the early 20th century. Agricultural production of kiwi fruit began in 1930s, and its history is therefore very short compared with many other fruit trees. Kiwi fruit production in 1988 was approximately 220,000 tons in New Zealand, with lesser amounts produced in Italy, Japan, and the U.S.A.(International Kiwifruit Organization Conference 1988). Strong increases in production and area under cultivation have taken place during the last few years, and this trend is expected to continue for some time. Generally it is difficult to transport fresh fruits because of softening. Kiwi fruit, however, requires an after ripening before eating, allowing it to be transported long distances. Thus, demand for kiwi fruit is expected to increase around the world.

Efforts to improve kiwi fruit have been on-going mainly in New Zealand since 1937, and most of the modern cultivars were selected from seedlings. Further improvements of such characteristics as sweetness, flavor, and insect or disease tolerance will be required.

In woody plants, genetic heterogeneity and long life cycles have been barriers to genetic improvement by cross breeding. Thus the introduction of agronomically important characteristics by DNA transformation offers alternative methods for such improvement. Because most woody plants are vegetatively propagated, once the desirable characteristics have been introduced into these plants, the transformant can be multiplied as a clone by cuttings or grafting without requirement that the introduced genes be fixed by repeated back crossing.

An Agrobacterium-mediated transformation system has been used routinely for genetic engineering in dicotyledonous annual species (Gasser and Fraley 1989; Horsch et al. 1984; McCormick et al. 1986). In woody plants, considerable effort has been made to establish genetic transformation systems with many species, such as poplar (Parsons et al. 1986; Pythoud et al. 1987; Fillatti et al. 1987), alder and birch (Mackay et al. 1988), willows (Vahala et al. 1989), walnut (McGranahan et al. 1988, 1990), apple (James et al. 1989), grape vine (Baribault et al. 1989), loblolly pine (Sederoff et al. 1986), douglas-fir (Dandekar et al. 1987), and white spruce (Ellis et al.1989). However, the number of successful transformations has been limited to poplar (Fillatti et al. 1987), walnut (McGranahan et al. 1988, 1990), and apple (James et al. 1989).

Fillatti et al.(1987) introduced the aroA and nptII genes into a hybrid poplar. McGranahan et al. (1988, 1990) obtained transformed walnut plants using a repetitive embryogenesis system and co-cultivation with A. tumefaciens. Transformed apple shoots were regenerated from leaf discs co-cultured with A. tumefaciens by James et al. (1989).

In this report we describe successful transformation of kiwi fruit mediated by A. tumefaciens harboring a binary vector containing the nptII and GUS genes.

Materials and methods

Agrobacterium strain. Disarmed A.tumefaciens strain EHA101 harboring a binary vector pLAN411 or pLAN421 was used(Fig.1). Plasmid pLAN411 contains the nptII gene linked to the CaMV 35S promoter and the nopaline synthase (NOS) terminator. Whereas plasmid pLAN421 contains the nptII gene linked to the NOS promoter and the NOS terminator. In both plasmids the GUS gene is linked to the 35S promoter and the NOS terminator.

Preparation of plant material. Seeds were collected from the cultivar 'Hayward' fruit purchased at a

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market and were washed well under tap water to remove the pericarp. Then they were soaked in 2% sodium hypochloride solution and shaken vigorously for 25 min. The seeds were rinsed ten times with sterilized water. Sterilized seeds were plated on the MS (Murashige and Skoog 1962) medium without plant growth regulators solidified with 0.8% agar. About one month later, hypocotyls which had elongated to 3 to 4cm, were cut into 5mm segments and infected with A. tumefaciens.

Stem cuttings were prepared as follows. Newly elongated shoots were collected from a field grown tree, cultivar 'Hayward', planted in the Natori farm (Yamanashi prefecture) in June 1989. Stems were cut to about 5cm length and their surface was washed well with neutral detergent. The cuttings were then soaked in the neutral detergent solution for 5min, washed under running water for 5min, and soaked in 70% ethanol for 5min. They were sterilized twice with 1% sodium hypochloride solution for 10min, and rinsed three or four times with sterilized water. The sterilized cuttings were cut into segments about 3mm in thickness and infected with A. tumefaciens.

Agrobacterium infection and shoot regeneration. A. tumefaciens strains EHA101/pLAN411 and EHA101/pLAN421 were grown in 5ml of LB (Sambrook et al. 1989) medium containing 50µg/ml kanamycin, 25µg/ml chloramphenicol and 50µg/ml spectinomycin at 30 °C overnight and then diluted with 20ml of sterilized water. Hypocotyl segments or stem segments were put into the Agrobacterium suspension and shaken vigorously for 20 min. The segments were separated from the Agrobacterium suspension by filtering the mixture through a sieve.

Segments were blotted on sterilized paper towels, and plated onto culture medium without antibiotics. Two culture media were examined, one medium, MS4PU, was composed of MS medium containing 1.0mg/l N-(2-chloro-4-pyridyl)-N-phenylurea (4PU), and the other medium, B5Z, was composed of B5 (Gamborg et al. 1968) medium containing 3.0mg/l zeatin. All media used for tissue culture in this study were solidified with 0.2% gelrite.

After one day co-cultivation with the A. tumefaciens, segments were transferred to the MS4PU or B5Z medium, each media were supplemented with 500µg/ml of Claforan (Cefotaxime sodium, Hoechst Aktiengesellschaft) to remove bacteria.

One week after co-cultivation, callus formation began at the cut surface of the segments. After two weeks culture on the Claforan containing medium, all segments were transferred to the selection media, MS4PU or B5Z, supplemented with 25µg/ml kanamycin and 500µg/ml Claforan. Hypocotyl segments regenerated shoots after two weeks of culture on the selection medium, and stem segments did after four weeks of culture.

When the regenerated shoots had reached about 5mm in length, they were cut off from the callus tissue or segments and transferred to freshly prepared selection medium. After four or five weeks, shoots had grown to about 1cm, and they were transferred to another selection medium composed of MS medium with 25µg/ml kanamycin and 100µg/ml Claforan without plant growth regulators. Regenerated shoots were subcultured every four or five weeks on a freshly prepared selection medium, which was half-strength MS medium with 25µg/ml kanamycin and 100µg/ml Claforan. When roots had formed on the regenerated shoots, the plantlets were transplanted to sterilized vermiculite for acclimatization. All culture were kept at 25°C and 16hr light under fluorescent light.

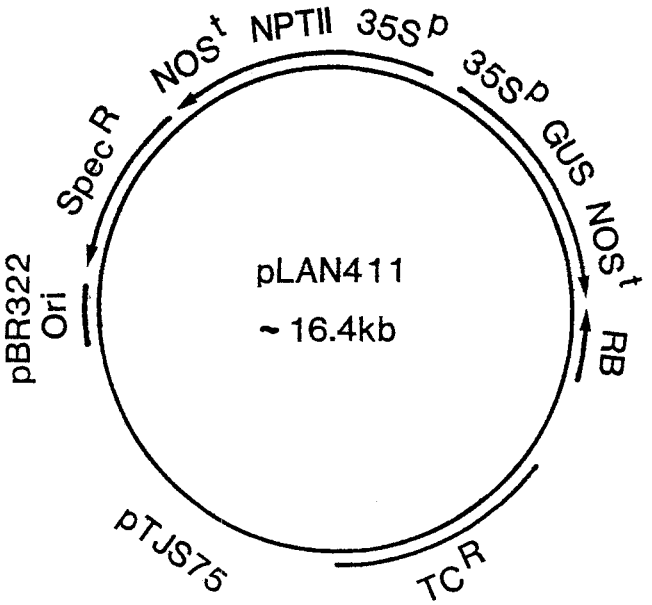

Fig. 1. Binary vector pLAN411 contained 35S^p-nptII-NOS^t and 35S^p-GUS-NOS^t. 35S^p-nptII was replaced by NOS^p-nptII in pLAN421.

NPTII activity assay and GUS assay. NPTII activity was analyzed by the dot blot assay according to McDonnel et al.(1987). GUS activity was examined by the fluorometric assay using 4-methyl umbelliferyl glucuronide (4-MUG) as a substrate according to Jefferson(1987). Localization of GUS expression in the transformed plants was evaluated by a 5-bromo-4-chloro-3-indolylglucuronide (X-Gluc) histochemical assay (Jefferson 1987). Hand-cut sections of leaf, petiole or stem cutting with a razor blade were incubated with the reaction buffer (0.1M sodium phosphate buffer, pH7.0) containing 10mM X-Gluc at 37 °C overnight. GUS expression was detected as a blue precipitate. When chlorophyll masked the observation, sections were soaked in 70% ethanol for 30min after the GUS reaction to decolor the chlorophyll and then observed under a microscope.

RESULTS

Plant tissue culture

Callus formation was observed at the cut end of segments after one week culture on the medium with Claforan. Two weeks after the infection, the segments were transferred to selection medium containing 25µg/ml kanamycin and 500µg/ml Claforan. On the hypocotyl segments, shoot regeneration was observed after two weeks of culture on the selection medium (Fig.2a), and on the stem segments, shoots were observed after three weeks of culture on the selection medium. Green and albino shoots were regenerated. In some cases both types of shoots were obtained from one segment. These regenerated shoots were cut off from the callus tissue and subcultured every four or five weeks onto selection medium. Most of the green shoots continued to grow, but some turned pale green or brown (Fig.2b). Albino shoots did not continue growing and turned brown.

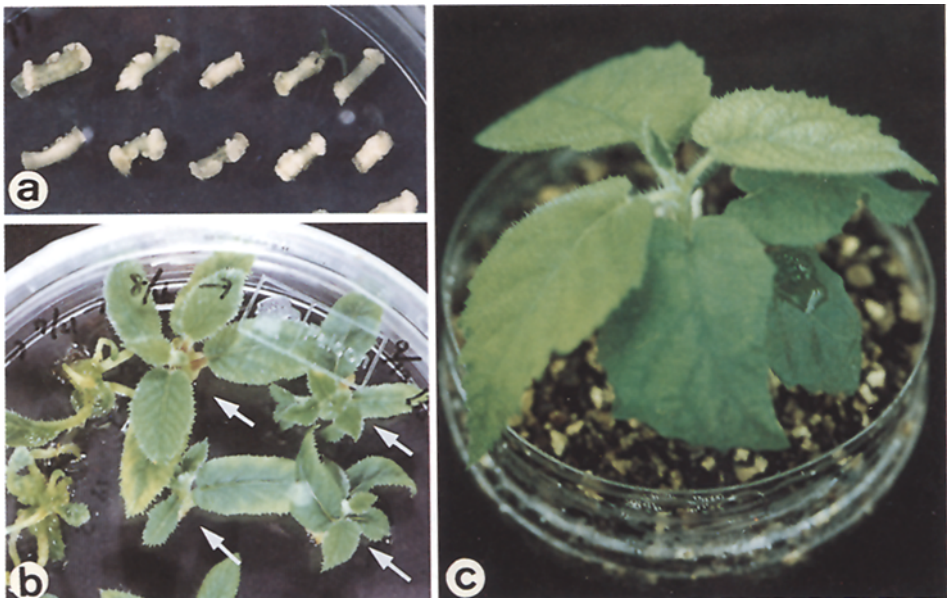

Fig.2. **a**: Callus formation and shoot regeneration from hypocotyl segments on kanamycin containing medium (25 µg/ml), 3 weeks after infection with Agrobacterium strain EHA101/pLAN421.
b: Shoots regenerated from stem segments co-cultured with Agrobacterium strain EHA101/pLAN411 were cultured on the selection medium containing 25µg/ml kanamycin for 8weeks. Arrows indicate transformed shoots, which grew vigorously. Non-transformants didn't expand morphologically nomal leaf and stopped the growth.
c: One of the acclimatized transformants, eight months after Agrobacterium infection.

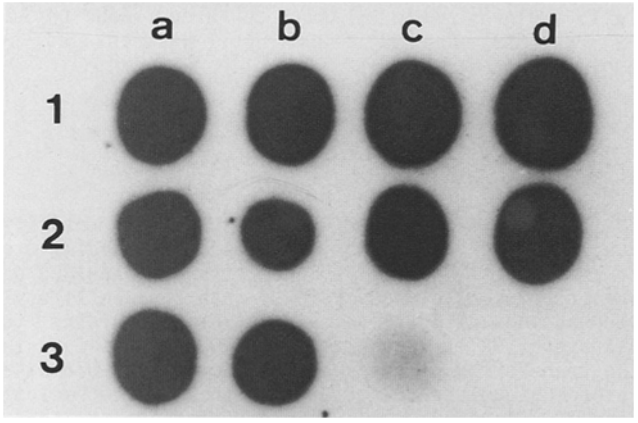

Fig.3. Dot blot analysis of NPTII enzyme activity of regenerated shoots obtained from hypocotyl segments. 1a:1-1, 1b:2-3, 1c:3-1, 1d:3-2, 2a:6-1, 2b:7-1, 2c:9, 2d:15-2, 3a:16-1, 3b:19-1, 3c:untransformed control. All ten shoots examined showed strong NPTII activities.

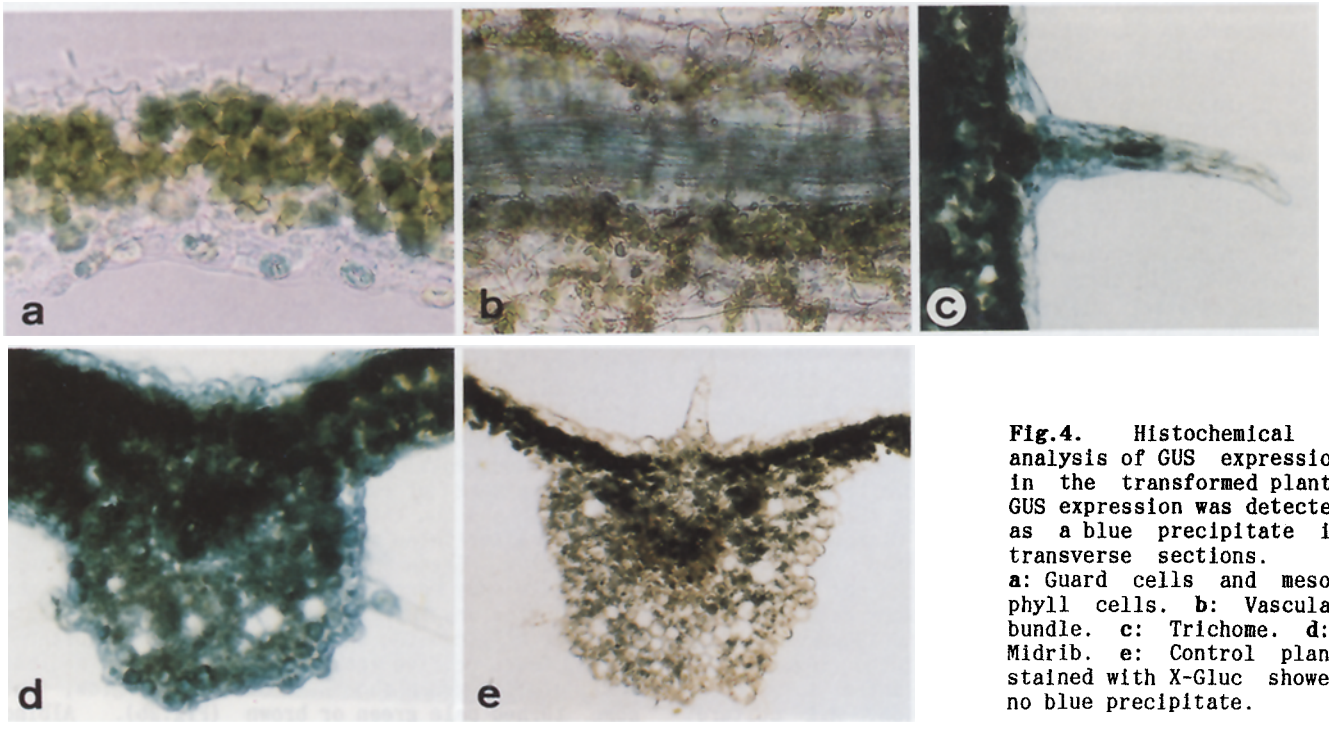

Fig.4. Histochemical analysis of GUS expression in the transformed plant. GUS expression was detected as a blue precipitate in transverse sections.
a: Guard cells and mesophyll cells. **b**: Vascular bundle. **c**: Trichome. **d**: Midrib. **e**: Control plant stained with X-Gluc showed no blue precipitate.

Effect of culture medium and explant tissue

Differences in the regeneration frequency of green shoots were found in both culture media and explant tissue used (Tables 1,2). Shoot induction occurred faster on B5Z medium than on MS4PU medium. The growth of regenerated shoots was also faster on the B5Z medium. A regeneration frequency of 26-62% was obtained from the hypocotyl or the stem segments cultured on the B5Z medium, except for hypocotyl segments inoculated with pLAN411. The regeneration frequency on MS4PU medium ranged from 5.3 to 17.5%.

The stem segments of newly elongated shoots showed a higher regeneration frequency than those from seedling hypocotyl. The maximum regeneration frequency of 62% was obtained from stem segments inoculated with pLAN411 and cultured on B5Z medium containing kanamycin. Leaf segments of sterile seedlings showed the lowest regeneration frequency (<10%; data not shown).

In total we obtained more than 100 regenerated plants from 53 stem segments and about 70 plants from 21 hypocotyl segments. They were grown in Magenta box and some of them were transplanted to sterilized soil.

Foreign gene expression

To identify transformants in the early stages of shoot formation, NPTII enzyme activity and GUS activity were determined. The NPTII activity was assayed for the 13 green shoots obtained from the hypocotyl segments by dot blot assay. Twelve of the 13 shoots examined showed strong activity (Fig.3, Table 3).

GUS activity assay was performed with the same 13 shoots obtained from the hypocotyl segments. The values of the GUS fluorometric assay showed wide variation from 4.8 to 8487.7 pmoles/min/mg protein (Table 3). Control plants which were not infected with Agrobacterium had a weak GUS activity of 4.9 pmoles/min/mg protein. Two shoots, 7-1 and 21-1, showed GUS activity as low as control plants. We consider the GUS gene introduced and expressed in regenerated shoots if their GUS activity was higher than 10 pmoles/min/mg protein. According to this standard, 11 out of the 13 shoots examined contained the GUS gene and expressed it (Table 3).

From the combined results of the NPTII and the GUS activity assays, 11 of 13 shoots (~85%) tested were confirmed to be transformants (Table 3).

Histochemical assay of GUS expression

Localization of GUS expression was analyzed for the GUS positive shoots confirmed by the fluorometric assay. Hand-cut sections of leaves and petioles were incubated at 37°C overnight with X-Gluc as a substrate. A blue precipitate was found in guard cells, mesophyll cells, and trichomes of the leaves (Fig.4).

Table 1. Regeneration frequency of green shoots from kiwi fruit hypocotyl segments on selection medium containing 25μg/ml kanamycin after co-culturing with A. tumefaciens.

Agro. strain	medium	no. of segment treated	no.of segment with green shoots*	regeneration frequency (%)	total no. of green shoots**
EHA101/ pLAN411	MS+4PU	49	3	6.1	8
	B5+zeatin	49	1	2.0	1
EHA101/ pLAN421	MS+4PU	43	4	9.3	11
	B5+zeatin	50	13	26.0	49
EHA101	MS+4PU	18	0	0	0

*) Data scored after 3 months in culture.
**) Multiple shoots were obtained in some cases.

Table 2. Regeneration frequency of green shoots from kiwi fruit stem segments on selection medium containing 25μg/ml kanamycin after co-culturing with A. tumefaciens.

Agro. strain	medium	no. of segments treated	no.of segments with green shoots*	regeneration frequency (%)	total no. of green shoots**
EHA101/ pLAN411	MS+4PU	38	2	5.3	9
	B5+zeatin	42	26	61.9	48
EHA101/ pLAN421	MS+4PU	40	7	17.5	22
	B5+zeatin	38	18	47.4	24

*) Data scored after 3 months in culture.
**) Multiple shoots were obtained in some cases.

DISCUSSION

This is the first report on the transformation and regeneration of transgenic kiwi fruit plants. The highest transformation frequency was achieved when stem segments from field grown trees were co-cultured with the Agrobacterium. The regeneration frequency of green shoots on the selection medium was 62%. About 85% of green shoots were confirmed transformants. This frequency was much higher than previously reported for transgenic woody plants such as poplar (Fillatti et al. 1987) and apple (James et al. 1989).

Table 3. Results of NPTII assay and GUS activity of 13 green shoots regenerated on selection medium.

Plant no.	NPTII	GUS (pmoles/min/mg protein)
untransformed control	-	4.9
1-1	+	914.2
2-3	+	43.4
3-1	+	1424.1
3-2	+	244.5
6-1	+	323.0
7-1	+	4.9
9	+	2224.5
15-2	+	96.1
16-1	+	106.1
16-2	+	4031.7
19-1	+	8487.7
19-2	+	82.6
21-1	-	4.8

Though poor plant regeneration from callus tissue have been reported for many species of woody plants, tissue culture of kiwi fruit was found to be easy for both callus formation and shoot regeneration (Shimura et al. 1990). In our preliminary experiments, nearly 100% of the stem segments produced shoots (data not shown). The regeneration frequency varied with the medium used, and B5 medium containing zeatin was most suitable for obtaining transformed shoots (Table 2). The high response of kiwi fruit in tissue culture made it possible to obtain transformed shoots at a high frequency.

GUS activity in the transgenic plants varied from 4.8 to 8487.7 pmoles/min/mg protein as shown in Table 3. The difference in GUS activity might reflect the differences in copy number of GUS genes integrated into the plant genome or genome positions in which the gene integrated. Although the GUS gene was regulated by a constitutive promoter, 35S of CaMV, fluctuation in GUS activity of leaves from a single plant has been observed (data not shown). It might have been caused by differences in growing stage, leaf age or days after subculture. We first checked the GUS activity of regenerating shoots to determine whether or not the regenerant was a transformant. However, due to observed fluctuation of the GUS activity, an NPTII activity assay was then required.

Although the transformed plant No.7-1 had positive NPTII activity, its GUS activity value of 4.9 pmoles/min/mg protein was as low as that of a non-transformant. There could be two explanations for this ; either both the nptII gene and GUS gene were integrated into the kiwi fruit genome, but only the nptII gene was expressed, or only the nptII gene was integrated and expressed. The situation will be resolved by further investigation by southern blotting analysis.

This is the first report about an Agrobacterium mediated kiwi fruit transformation system. Genetic engineering of kiwi fruit using this system will offer a novel alternative to the traditional breeding system.

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