Expression and integration of a foreign gene in orange (*Citrus sinensis* Osb.) protoplasts by direct DNA transfer¹⁾

Shozo KOBAYASHI* and Hirofumi UCHIMIYA**

*Akitsu Branch, Fruit Tree Research Station, Akitsu, Hiroshima 729-24

**Institute of Biological Sciences, University of Tsukuba,
Tsukuba City, Ibaraki 305

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ABSTRACT

Protoplasts isolated from suspension cultured cells of orange (*Citrus sinensis* Osb.) were treated with a bacterial plasmid DNA carrying a chimeric gene consisting of the nopaline synthase promoter, the aminoglycoside phosphotransferase II [APH(3')II] structural gene from the bacterial transposon Tn 5 and a terminator region from cauliflower mosaic virus DNA. Colonies capable of proliferating in a medium containing kanamycin (25 µg/ml) possessed APH(3')II activity and an intact foreign gene. The transformation frequency was recorded to be in the order of 10^{-6} .

1. INTRODUCTION

The recent progress in techniques for the manipulation of isolated protoplasts has made possible not only the production of somatic hybrid plants, but also the transfer of foreign DNA directly into plant protoplasts (Davey et al., 1980 ; Krens et al., 1982). The application of these techniques to citrus would be of great value for the improvement of this kind of fruit tree. Vardi et al. (1982) and we (Kobayashi et al., 1983) reported plant regeneration from orange protoplasts, and we demonstrated the uniformity of citrus protoclonal lines (Kobayashi, 1987). This citrus protoplast culture system has been successfully employed in producing somatic hybrid plants (Ohgawara et al., 1985; Kobayashi et al., 1988) and cybrid plants (Vardi et al., 1987). As an alternative means of genetic modification of citrus protoplasts, we have attempted to produce transformants by direct DNA transfer.

Here we report the transfer of foreign DNA into orange protoplasts, and the stable expression and presence of a foreign gene in the cultured cells.

2. MATERIALS AND METHODS

Plant materials and protoplast isolation

Suspension cultured cells of 'Trovita' orange (*Citrus sinensis* Osb.) which

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originated from nucellus tissues were maintained in a Murashige and Tucker [MT] (1969) liquid medium supplemented with 10 mg/l 6-benzylaminopurine [BA] as described earlier (Kobayashi et al., 1985). Two-week-old cells were transferred to a hormone-free MT liquid medium (denoted to MT basal medium). After culturing in the same medium for 2 weeks, the cells were collected and subjected to protoplast isolation using the procedure described earlier (Kobayashi et al., 1985).

DNA transfer

The method employed for DNA transformation was basically a modification of the method of Krens et al. (1982) and Paszkowski et al. (1984). Briefly, 0.9 ml protoplasts (2.2×10^6), suspended in 0.6 M mannitol, were mixed with a 0.1 ml DNA solution containing 10 μ g of the circular form of the bacterial plasmid pCT2T3, which contained the selectable marker gene [aminoglycoside-phosphotransferase II: APH(3')II] (Morota and Uchimiya, 1987; Uchimiya et al., 1986b). The plasmid was prepared without RNase treatment according to the method of Birnboim and Doly (1979).

After 5 min, a 0.5 ml solution of 40% (w/v) polyethylene glycol 6,000, dissolved in a F medium (Krens et al., 1982), was added to the protoplast-DNA mixture. After 30 min, the incubation mixture was diluted with an equal volume of a F medium, with an osmotic value adjusted to 790 mosmol/kg $\cdot$ H₂O by additional glucose. A similar stepwise dilution was performed three times every 10 min.

Protoplast culture and selection of transformants

Protoplasts (10^5 /ml) were cultured in a 3 ml medium, which consisted of a MT basal medium containing 0.35 M mannitol in a Falcon Petri dish (60 $\times$ 15 mm). The plates were sealed with Parafilm, and maintained under 16 h/day illumination with a cool fluorescent light (500 lux) at 26°C. After 2 weeks, the medium was diluted with an equal volume of a MT basal medium supplemented with 0.25 M mannitol. After 2 weeks, the medium was solidified by an equal volume of a MT basal medium supplemented with 0.25 M mannitol, 50 mg/l kanamycin sulfate (Meiji Seika Co., Tokyo, Japan) and 1.2% agarose (Sea Plaque, LMT, Marine Colloids, USA). After 2 months, colonies sustaining cell division under these conditions were transferred onto a MT basal medium containing 0.8% Bacto-agar and 25 μ g/ml kanamycin sulfate for the second selection.

DNA analysis

DNAs were extracted from the callus according to the method of Rogers et al. (1985). Ten micrograms DNA digested with *Eco*RI and *Bgl*II were subjected to agarose gel electrophoresis. After electrophoresis, the gel was treated with alkali, then blotted to a nitrocellulose filter. The dried filter was hybridized with biotin-labelled probe DNA. DNA fragments [2 kb *Eco*RI-*Bgl*II, containing the

APH(3')II gene] prepared from plasmid pCT2T3 were used as the probe. Biotin-labelling of the probe DNA was carried out using biotin-11-dUTP and a nick-translation reagent kit (Bethesda Res. Labs. Inc., USA). Visualization of the probe-target DNA hybrid was carried out using streptavidin-alkaline phosphatase conjugate, nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate, p-toluidine salt (BCIP).

APH(3')II assay

The analysis of APH(3')II was carried out according to the methods of Reiss et al. (1984) and Schreier et al. (1985).

3. RESULTS AND DISCUSSION

The plating efficiency of orange protoplasts was approximately 20% in the medium used. No colony was produced in the control cultures where protoplasts, not treated with DNA, were maintained under the selective conditions. Ten colonies, of the size greater than 1 mm, were obtained from 9×10^5 DNA-treated protoplasts by the first selection (Fig. 1). Upon transferring those colonies to the second kanamycin-containing medium, 8 colonies survived and increased their size. Then, these clones were transferred onto a kanamycin-free MT basal medium supplemented with 5 mg/l BA and 0.8% agar. After subculture for 6 months, DNAs isolated from 8 such clones were tested by Southern hybridization with a probe containing a chimeric APH(3')II gene [2 kb *Eco*RI-*Bal*II fragment of

Fig. 1. A colony derived from DNA-treated citrus protoplasts after first selection. Kanamycin sulfate (25 μ g/ml) was added to the agar solidified medium.

Fig. 2. Southern blot analysis of DNA from kanamycin-resistant clones. A sample of 10 micrograms DNA digested with *EcoRI-BglII* was loaded on each track. The probe was the *EcoRI-BglII* 2 kb fragments of pCT2T3 (Morota and Uchimiya, 1987; Uchimiya et al. 1986b) containing the APH(3')II gene. Lane 1, control; lanes 2-9, kanamycin-resistant clones, R1, R2, R4, R5, R6, R7, R8, and R10, respectively; lanes 10 and 11, 20 picograms and 100 picograms, respectively, of pCT2T3. The numerals indicate kbp.

Fig. 3. Growth response of control callus and that of transformed clones to kanamycin. About 30 mg 8-month-old calli, which subcultured on a kanamycin-free medium, were cultured on the medium containing various concentrations of kanamycin sulfate. The fresh weight was recorded after 2 months. The % growth is the percentage increase in the fresh weight of a callus grown on a medium containing kanamycin sulfate at a given concentration as compared to the weight of a callus grown on a medium lacking kanamycin sulfate.

pCT2T3]. An intact 2 kb fragment containing the chimeric APH(3')II gene was detected in 2 clones (R5 and R7) (Fig. 2). The other DNA fragment (15 kb) homologous to the probe DNA was also detected in the clone R5, and slightly in clones R1, R2 and R8. DNAs from clones R4, R6, R10 and those from control callus did not show such a hybridization signal (Fig. 2). This finding shows that the clones R5 and R7 are transformant. However, it is not clear whether or not the other clones R1, R2 and R8 are also transformant. The transformed clones R5 and R7 were tolerant to exogenous kanamycin until the concentration of 25 µg/ml (Fig. 3). The APH(3')II enzyme activity was detected in the clone R7 (Fig. 4), but not in the clone R5. The clone R5 probably does not produce a detectable amount of the enzyme.

Fig. 4. APT(3')II activity analysis. Lane 1, kanamycin-resistant *Oryza sativa* transformed cells T14 (Uchimiya et al., 1986b); lane 2, control; lane 3, kanamycin-resistant R7 clone. The arrow indicates the position of APT(3')II enzyme.

DNA-mediated transformation using protoplasts has been reported for dicotyledonous plants such as moth bean (Köhler et al., 1987) and tobacco (Paszkowski et al., 1984; Uchimiya et al., 1986a), and for graminaceous monocots including Italian Ryegrass (Potrykus et al., 1985), maize (Fromm et al., 1986), rice (Uchimiya et al., 1986b), sugarcane (Chen et al., 1987) and one-grained wheat (Lörz et al., 1985). In woody plants, however, DNA-mediated transformation has not yet been reported. In this report, we demonstrated that protoplasts of fruit tree *Citrus sinensis* could be transformed through direct DNA transfer. Regeneration of whole plant from such cell line will be needed in order to facilitate genetic manipulation of citrus.

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