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Transformation of Taro (*Colocasia esculenta* Schott) Using Particle Bombardment

Nobuko FUKINO (ITO)[1*], Kaoru HANADA[2], Hidetoshi AJISAKA[3], Junichi SAKAI[2], Hirohiko HIROCHIKA[4], Masashi HIRAI1, Takashi HAGIO[5] and Sueo ENOMOTO[6]

[1,3,6] Department of Vegetable Breeding, National Research Institute of Vegetables, Ornamental Plants and Tea (Ano, Mie, 514-2392 Japan)
[2] Department of Crop Breeding, Kyushu National Agricultural Experiment Station (Nishigoshi, Kumamoto, 861-1192 Japan)
[4] Department of Molecular Genetics, National Institute of Agrobiological Resources (Tsukuba, Ibaraki, 305-8602 Japan)
[5] Department of Biotechnology, National Institute of Agrobiological Resources (Tsukuba, Ibaraki, 305-8602 Japan)

Abstract

Transgenic taro (*Colocasia esculenta* Schott) Plants were obtained by high-velocity particle bombardment. The plasmid pREXHGUS, carrying the hygromycin resistance gene, was used as a selection marker. Highly regenerative taro callus was obtained from the apical meristem, maintained in liquid culture. The callus was chopped into small fragments with a forceps, then transformed and selected on LS medium containing BA, NAA and hygromycin. Since the transformed calli obtained showed vigorous growth on LS-BN medium with 20 mg/L hygromycin, this concentration was considered to be suitable for the selection of transgenic taro. The transformants were confirmed by amplification of the GUS gene, and Southern hybridization. The expression of the foreign gene was demonstrated by the GUS activity.

Discipline: Plant breeding
Additional key words: callus, hygromycin resistance gene, GUS gene

Introduction

Taro originated in India and adjacent areas of Southeast Asia, and is now widely cultivated in Asia and Oceania[18]. In some Oceanian countries, this crop is one of the main sources of starchy food. Since taro propagates mainly with tubers, the occurrence of virus diseases is one of the main problems affecting its cultivation, along with taro leaf blight in tropical countries[12]. Once a clone is infected with virus, its productivity may be reduced. It is difficult to remove the virus under natural conditions of cultivation. Although shoot tip culture *in vitro* has been used to remove virus particles, the problem of reinfection with virus still remains. Recent biotechnological research has revealed that transformation with the coat protein gene or some other viral genes, confers resistance to virus diseases[1,4]. The objective of the present study was to breed a virus-resistant cultivar of taro. Since taro is a monocotyledon, *Agrobacterium*-mediated transformation is considered to be difficult. Although protoplast culture of taro has been reported[13], this technique is very laborious and it is difficult to obtain regenerated plants. In addition, somaclonal variation may occur more frequently. Therefore, protoplast culture is not suitable for direct transformation of taro, and instead

Present address:
[3] Leaf Tobacco Research Laboratory, Japan Tobacco Inc. (Oyama, Tochigi, 323–0808 Japan).
[6] Department of Research Planning & Coordination, National Institute of Agrobiological Resources (Tsukuba, Ibaraki, 305–8602 Japan).
* Corresponding author: fax +81–59–268–1339, e-mail nbk@nivot.affrc.go.jp
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particle bombardment is most appropriate for the transformation of taro. Few studies on the transformation of taro have been carried out. Here we report the transformation of taro using a particle bombardment system.

Materials and methods

1) Plant material

A triploid cultivar of taro (*Colocasia esculenta* Schott), 'Eguimo', was used in the present study. Eguimo is one of the major cultivars used in Japan, and callus induction and shoot regeneration of this cultivar have been developed[6].

2) Plasmid

A plasmid, pREXHGUS, was constructed based on the pUC18 plasmid (Fig. 1). A selection marker, the hygromycin phosphotransferase (hpt) gene, was driven by a 35S promoter harboring one copy of the enhancer, and a beta-glucuronidase (GUS) gene containing the phaseolin intron 1 gene was driven by the 35S promoter containing 7 copies of the cauliflower mosaic virus (CaMV) 35S enhancer region[11].

3) Induction and maintenance of callus

Linsmaier and Skoog[9] medium (LS medium) supplemented with 2 mg/L benzyladenine, 1 mg/L naphthaleneacetic acid and 3% sucrose was used as a basic medium (LS-BN medium). The shoot tips (0.5 to 1 mm) were removed from surface-sterile tubers and planted on LS-BN medium solidified with 2% gellan gum, then cultured at 25°C (16 h photoperiod, 36 μE m^2 sec^{-1}) to induce callus. The induced calli were maintained on the solidified medium by subculturing every 2 or 3 weeks. As a target for particle bombardment, the calli were chopped into small pieces (2 to 3 mm in diameter) with a forceps, and cultured in liquid LS-BN medium with rotary shaking at 100 rpm at 25°C. These calli were maintained by weekly chopping and subculture.

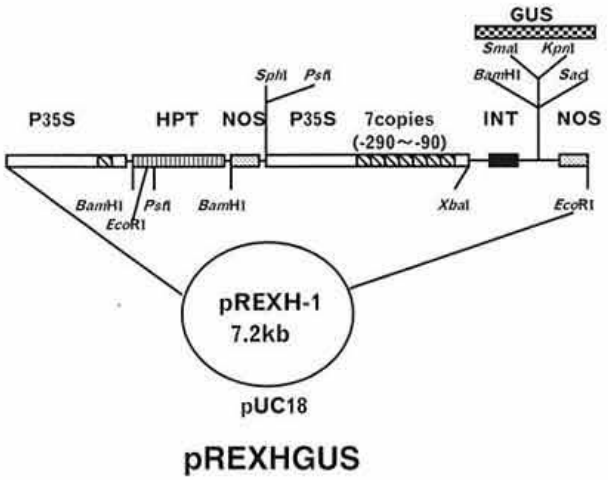

Fig. 1. Diagrammatic representation of the transformation plasmid pREXHGUS

A modified 35S promoter and an intron (intron 1 of the phaseolin gene) synergistically activate the gene expression.

4) Particle bombardment

The calli (ca. 2.0 g) were placed on a sterile filter paper (70 mm in diameter). Then, the filter paper was placed on a 9 cm petri dish filled with solid LS-BN medium and cultured at 25°C for 2~24 h.

Biolistic PDS-1000/He (Bio-Rad) was used. Before the bombardment, the pressure in the chamber was reduced to 660 mm Hg. Gold (1.6 μm in diameter) or tungsten (1.1 μm in diameter) particles were coated onto the plasmids as previously described[7], then delivered from a distance of 9 cm to the taro calli on a filter paper at a pressure of 1,100 psi.

A total of 96 runs were conducted.

5) Optimization of culture duration and hygromycin concentration

The calli were bombarded at 0, 4, 6 and 8 days after chopping. The bombardment procedure was the same as that described above. After 48 h of bombardment, GUS activity was examined as described by Kosugi et al.[8] by counting the number of blue spots. This experiment was repeated twice. The data points were subjected to logarithmic transformation, then analyzed statistically.

For optimization of the hygromycin concentration, the calli (120 to 195) were placed on hormone-free LS medium and LS-BN medium containing 0, 5, 10, 20 and 40 mg/L hygromycin. After 40 days of culture, callus survival was examined. Then, surviving calli were subcultured onto the same medium containing 10 mg/L hygromycin, and after 40 days of culture, the number of surviving calli were counted.

6) Maintenance and selection of transformants

After the bombardment, the calli were cultured for 2 days on the same filter paper at 25°C. The treated calli were then transferred to liquid LS-BN medium supplemented with 20 mg/L hygromycin (Hyg), then cultured for 5 days with rotary shaking at 100 rpm. After 5 days of shaking culture, the calli were transferred to solid LS-BN medium with 20 mg/L hygromycin, then cultured at 25°C under a 16 h photoperiod. Resulting green calli were transplanted onto solid LS-BN medium with 10 mg/L hygromycin for 30 days, then on hormone-free LS medium supplemented with 5, 10 and 20 mg/L hygromy-

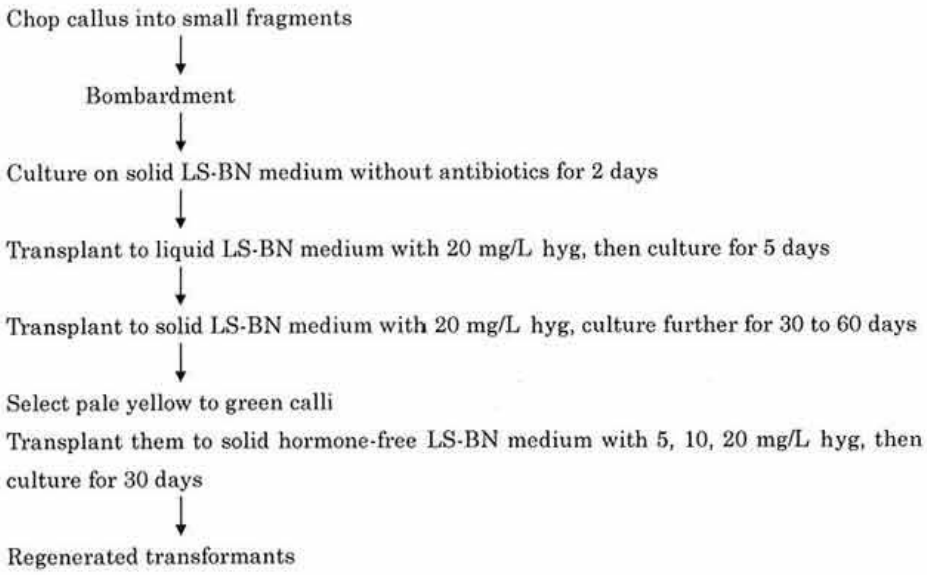

Fig. 2. Flow chart of taro transformation

cin for induction of plantlets.

The flow chart of taro transformation is shown in Fig. 2. Selected transformed plantlets and non-transformed control plants were used for GUS analysis.

7) PCR and Southern blot analysis

For PCR amplification, DNA was isolated from fresh leaf tissue according to the method of Liu et al.[10]. The presence of the transferred gene was assayed by PCR using oligonucleotides specific for the GUS genes. The upstream and downstream primers employed were (5'-ATGTTACGTCCTGTAGAAAC-3') and (5'-GTCCAGTTGCAACCACCTGT-3'), respectively. For Southern blot analysis, genomic DNA was isolated from fresh leaf tissue as previously described[14], digested with *Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I, respectively, then blotted onto a nylon membrane. Plasmid DNA was labeled with digoxygenin using a DIG-DNA labeling kit (Boehringer Mannheim) then used as a probe. Hybridhization and detection were performed according to the manufacturer's protocol.

Results

1) Effect of culture duration after callus chopping

Since taro calli grow as a mass, they need to be chopped into small pieces for efficient delivery of the particles. The effect of the culture duration after callus chopping was examined (Table 1). Bombardment just after chopping resulted in rather poor transient expression of the GUS gene. However, the expression ability recovered almost fully after 4 days of culture (Fig. 3a). Therefore, calli after 4–8 days of culture were used subsequently.

2) Effect of antibiotic concentration on callus growth

Effects of various concentrations of hygromycin in LS medium on callus growth were examined (Table 2). The growth of untransformed callus was effectively suppressed by 20 mg/L hygromycin. After 40 days, surviving calli on the media containing 5 or 10 mg/L hygromycin were further cultured on a new medium containing 10 mg/L hygromycin, for an additional period of 40 days. None of the calli survived.

3) Callus growth after bombardment

Most of the calli on the solid LS-BN medium with 20 mg/L hygromycin turned dark brown 30 to 60 days after bombardment, then died. However, a small number of yellowish to greenish calli were observed among the dark brown calli (Fig. 3b). These calli were transplanted onto hormone-free LS medium with 10 mg/L hygromycin to induce plantlets (Fig. 3c). Antibiotic resistance of

Table 1. Effect of additional culture after chopping the callus

Culture duration after chopping (days)	Number of blue spots per dish[a)]
0	2.3 (2.3)
4	5.3 (0.9)
6	4.4 (0.2)
8	5.1 (0.0)

a): Mean value of 2 replications.
( ): SD.

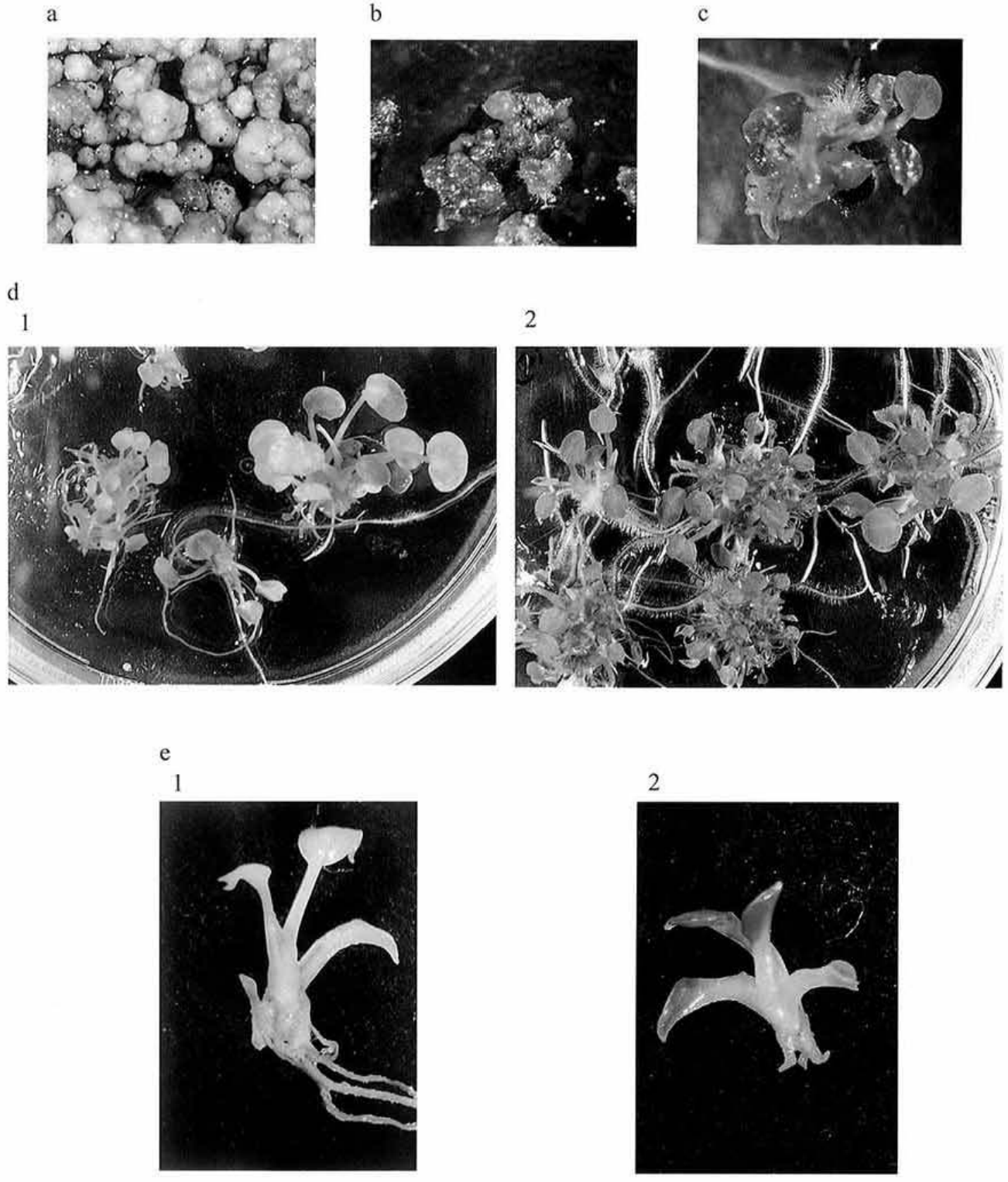

Fig. 3. Transient expression of GUS gene, and selection of transformants

a. Staining of calli for GUS activity 48 h after bombardment.
b. Calli after 40 days of bombardment.
c. Plant regeneration on LS-BN medium containing hygromycin.
d. Plant growth on LS-BN medium with 20 mg/L hygromycin.
1: untransformed, 2: transformed.
e. Expression of GUS gene in regenerated plant.
1: untransformed, 2: transformed.

Table 2. Effect of antibiotic concentration on the growth of untransformed taro callus

Plant hormone	Concentration of hygromycin (mg/L)	Surviving calli (%) [a)]	Surviving calli after subculture (%) [b)]
Present	0	100	–
	5	26.7	0
	10	19.5	0
	20	2.5	–
	40	0	–
Absent	0	100	–
	5	12.8	0
	10	3.6	0
	20	0	–
	40	0	–

A total of 120 to 195 calli were used for each experiment.
a): Callus survival was examined after 40 days of culture.
b): Surviving calli were subcultured onto LS-BN medium with 10 mg/L hygromycin. After an additional period of 40 days, the numbers of surviving calli were counted.

the regenerated plants was examined on LS medium supplemented with 5, 10 and 20 mg/L hygromycin. Some plants did not grow, or wilted, on the medium containing 10 mg/L hygromycin (Fig. 3d). Others showed normal and rapid growth on the same medium. Two of these plants were propagated vegetatively.

4) GUS and DNA analysis

The plantlets were stained with X-gluc solution, and the young leaves and tracheal tissue of roots showed a blue color (Fig. 3e). The genomic DNA was extracted from the plants showing normal growth on the medium containing hygromycin. The presence of the introduced gene was examined by PCR. A 650-bp band was observed in these plants after gel electrophoresis. A band of the same size was also observed when the original pREXHGUS plasmid was used as a template (Fig. 4). The presence of the foreign gene was further examined by Southern blot analysis. The DNA was digested with restriction enzymes, separated by gel electrophoresis, then blotted onto a nylon membrane, probed with the digoxygenin-labeled pREXHGUS. The hygromycin-resistant plants showed positive signals while the non-

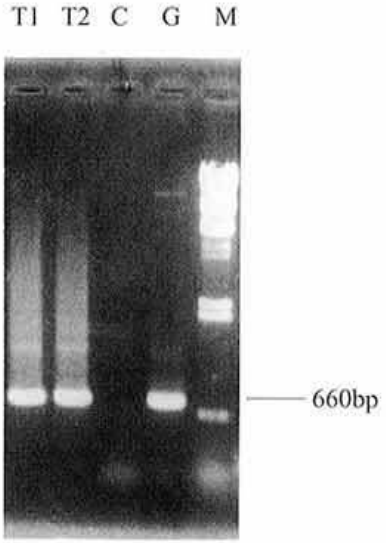

Fig. 4. Amplification of the introduced gene by PCR
T1: transformed plant 1. T2: transformed plant 2. C: untransformed plant. G: plasmid pREXHGUS. M: molecular marker (λ*Hin*dIII).

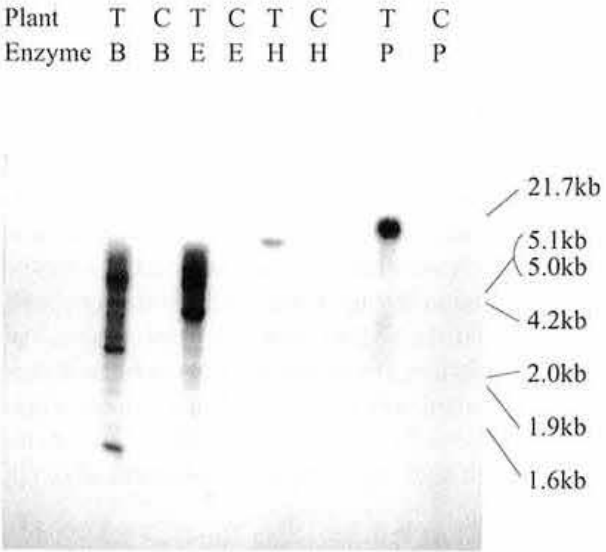

Fig. 5. Southern blot analysis of the GUS gene
T: transformed plant, C: untransformed plant, B: *Bam*HI, E: *Eco*RI, H: *Hin*dIII, P: *Pst*I.

transformed controls did not show any bands (Fig. 5).

Discussion

1) Regeneration system for taro

The system used in the present study was originally developed by Karube et al.[6] using a triploid cultivar, Eguimo. The calli of Eguimo showed a very high regeneration ability in this system. Moreover, the calli were easily maintained on solid or liquid LS-BN medium. They readily differentiated plantlets on the hormone-free medium, and were considered to be a suitable target for transformation. A similar regeneration system can be established in other triploid cultivars. However, taro shows a wide range of genetic diversity[5,17], and cultivars are classified into 2 varieties: var. *antiquorum* and var. *esculenta*. Cultivars grown in tropical areas are diploid and classified into var. *esculenta*[18]. Using a cultivar from the Solomon Islands, a regeneration system for taro has also been reported[15,16,21,22]. Although a detailed description of the cultivar was not given, cultivars from the Solomon Islands should be diploid and classified into var. *esculenta*[3]. With minor modifications, the transformation system described here is considered to be suitable for other cultivars including diploid ones. To maintain a fine suspension of callus, the callus should be chopped when subcultured. However, the results of bombardment just after chopping were not satisfactory, and therefore the calli may have been under some stress, inducing a low transformation competence.

2) Selection of taro transformants

Choice of a selection marker gene and the concentration of selective agents are key factors for successful transformation. Kanamycin is widely used for the transformation of various plant species. However, Wilmink and Dons[20] have reported that hygromycin is more suitable for the selection of monocot transformants. In wheat, rice and barley, hygromycin is commonly used as a selection marker for transformants. Moreover, in our preliminary experiment, taro calli survived in a medium containing up to 200 mg/L kanamycin (data not shown). Therefore, in the present study, we tried to select taro transformants using hygromycin. This antibiotic induced a clear suppression of callus growth at a concentration of 20 mg/L (Table 2). Therefore, selection of transformed calli with 20 mg/L hygromycin is considered to be optimal.

Some of the untransformed calli survived during 40 days of culture, but finally died when transplanted onto new medium containing 10 mg/L hygromycin. To obtain transformants rapidly, regeneration medium containing 10 mg/L hygromycin was used. Since some of the untransformed plants may possibly survive under these conditions, regenerated taro plants showing rapid growth and budding from the base on medium containing 10 mg/L hygromycin should be selected as transformants.

Efficiency of transformation is still rather low: a total of 2 independent transformants were obtained from 96 runs. Each run involved one petri dish containing 2.0 g of calli. The number of blue spots also varied considerably in each experiment, and this may have been due to the physiological status of the calli used. More detailed studies are required to obtain more efficient and stable conditions for transformation.

3) Breeding of disease-resistant taro cultivars

Since taro is widely cultivated in Asia and Oceania, the present study has shown that this technique is applicable to the breeding of taro. The occurrence of virus diseases is one of the main problems for taro growers. For example, dasheen mosaic virus and cucumber mosaic virus have been detected in taro cultivated in Japan[12,19]. Both viruses have been well studied at the molecular biological level. Transformation of these viruses with coat protein (CP) genes may enable to confer virus resistance to taro. Another possibility is to breed taro varieties resistant to leaf blight, which is caused by a fungus, *Phytophthora colocasiae,* and is a serious problem in tropical areas[12,18]. Transformation with chitinase genes has been reported to produce plants resistant to fungal diseases[2]. This technique may be useful for breeding disease-resistant taro varieties.

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