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Transient Expression of *lacZ* Gene in Protoplasts of *Orychophragmus violaceus*

Nobukazu TANAKA[1] and Chiaki MATSUI[2]

Plant Pathology Laboratory, Faculty of Agriculture, Nagoya University, Chikusa-ku, Nagoya 464, Japan

Present Address :

[1]*Biochemical Laboratory, Research Center, Daicel Chemical Industries, Ltd.*

1239, Shinzaike, Aboshi-ku, Himeji, Hyogo 671-12, Japan

[2]*Yanagishima Plant Tissue Culture Laboratory, Kinjirushi Wasabi Co., Ltd.*

2-61, Yawata-Hondouri, Nakagawa-ku, Nagoya, 454, Japan

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Transient expression of the reporter gene, β-galactosidase gene (*lacZ* gene), was examined in protoplasts isolated from *Orychophragmus violaceus* suspension culture. The expression vector (pCPLTA) was constructed from 19S mRNA promoter of cauliflower mosaic virus (CaMV), *lacZ* structural gene of *E. coli* fused with ATG -start codon of CaMV ORFVI and mRNA terminator of CaMV. CaMV 35S mRNA promoter sequence was also added in front of the terminator. Constructed pCPLTA was co-precipitated with calcium-phosphate and thereafter introduced into protoplasts with the aid of polyvinylalcohol. Activity of β-galactosidase in protoplast extracts was examined by o-nitrophenyl-β-D-galactoside hydrolysis assay. Extracts from pCPLTA-introduced protoplasts showed β-galactosidase activity at a level 70% higher than that of endogenous ones.

Introduction

Introduction and expression of foreign genes in plant cells has rapidly increased in the last few years[1]. Especially, transient gene expression is widely used as a tool to examine the functional importance of particular DNA sequences for gene expression[2-4]. Regardless of the gene transfer methods, all these examinations have been performed using so-called 'reporter' genes. Genes for chloramphenicol-acetyltransferase (CAT) and neomycin-phosphotransferase are available for chimeric constructions[5,6] and they have been successfully expressed as reporter genes. Recently, luciferase gene of firefly[7] or β-glucuronidase (GUS) gene of *E. coli*[8] have also been introduced into plant cells as a reporter gene.

Although β-galactosidase gene (*lacZ* gene) of *E. coli*[9-11] is most extensively used as a marker gene in organizms other than plants, there have been a few attempts to use this gene as a marker in plants[12-14]. A disadvatage of using *lacZ* gene in plant cells may be the presence of endogenous β-galactosidase[15] which may obscure the expression of the introduced *lacZ* gene. Matsumoto *et al*[13]. and Teeri *et al*[14]., however, ascertained that the expressions of *lacZ* gene in plant cells could be detected not only by biochemical measurement of β-galactosidase activity but also by histochemical identification using 35S promoter of cauliflower mosaic virus (CaMV). Moreover, Matsumoto *et al*[13]. reported that the expression of *lacZ* gene in living cells could be visually detected using an agar medium containing X-Gal, because cell colonies with *lacZ* gene expression

are blue. This result suggests that *lacZ* gene is a usable selection marker gene in direct gene transfer such as PEG-mediated DNA uptake, electroporation or particle gun. After direct gene transfer, for example, plant cells with the expression of introduced *lacZ* gene may be differentiated from those without gene expression by incubation in an X-Gal medium.

In this report, we examined whether the *lacZ* gene was usable as a reporter gene of transient expression in *Cruciferae* plant protoplasts. For this purpose, we constructed an expression vector, pCPLTA with the *lacZ* gene directed by a strong promoter sequence (CaMV 19S). The results show that β-galactosidase activity from introduced *lacZ* gene was considerably higher than that of endogenous ones.

Materials and Methods

1. Construction of pCPLTA

All DNA manipulations were performed according to Maniatis *et al*[16]. Chimeric plasmids, pABDI[17] and pDOB612[18] which were constructed in Dr. Hohn's laboratory at Friedrich Miescher Institut were obtained through Dr. M. Ikegami of Tokyo University of Agriculture. *Pst*I-*Hind*III fragment (0.4 kb) containing CaMV 19S promoter and a part of ORFVI (including ATG-start codon) was isolated from pABDI, and then subcloned in pUC18 (pCP, see Fig. 1). *Sma*I site of pMC1871[19] (Pharmacia LKB Biotechnology) was exchanged into *Hind*III site using 8 bases *Hind*III linker, and then a *Hind*III fragment of this plasmid (3.9 kb) containing *lacZ* structural gene (no ATG-start codon) was inserted into the *Hind*III site of pCP. The resulting construct contains the *lacZ* structural gene with CaMV 19S promoter and ATG-start codon (pCPL, see Fig. 1). These codon frames of pCPL were confirmed by β-galactosidase expression in *Escherichia coli* JM109 strain using *lacZ* promoter of pUC18.

On the other hand, pCTA containing CaMV 35S promoter and mRNA terminator was constructed by the exchange of *Eco*RV site of pDOB612 to *Sal*I site using 6 bases *Sal*I linker (Fig. 1). Subsequently, *Sal*I fragment (3.7 kb) of pCPL with CaMV 19S promoter, ATG-start codon and *lacZ* structural gene was inserted into the *Sal*I site of pCTA (Fig. 1). After about 0.2 kb of needless sequences were removed with *Pst*I digestion, the resulting sequences were self-religated (pCPLTA, see Fig. 1).

2. Isolation of protoplasts

The suspension culture of *Orychophragmus violaceus* O. E. Schulz, a member of *Cruciferae*, was kindly prepared by Dr. Y. Fukunaga, Faculty of Science, Nagoya University. Three-to-five days after subculture, The cultured suspension was digested in an enzyme mixture containing 1% Cellulase Onozuka RS (Yakult Pharmaceutical Co.), 0.1% Pectolyase Y-23 (Seishin Pharmaceutical Co.) and 0.5 M D-mannitol (pH 5.5) for 60 min at 30°C. The isolated protoplasts were washed twice with 0.5 M D-mannitol.

3. pCPLTA introduction and culture of protoplasts

For pCPLTA introduction into the protoplasts, we used the protocol of Hain *et al*[20]. with modification. Fifty μg DNA mixture containing pCPLTA and salmon testis DNA as a carrier was dissolved in 0.5 m*l* of HEPES-buffered saline solution (NaCl, 8 g/*l*; KCl, 0.37 g/*l*; $K_2HPO_4 7H_2O$, 0.125 g/*l*; glucose, 1 g/*l*; HEPES pH 7.05, 5 g/*l*). The final concentration of 125 mM $CaCl_2$ was added to the DNA solution using 2 M $CaCl_2$ and incubated for 20 min at room temperature. The DNA co-precipitated with calcium-phosphate was mixed with 4×10^6 protoplasts in 0.5 m*l* of 0.5

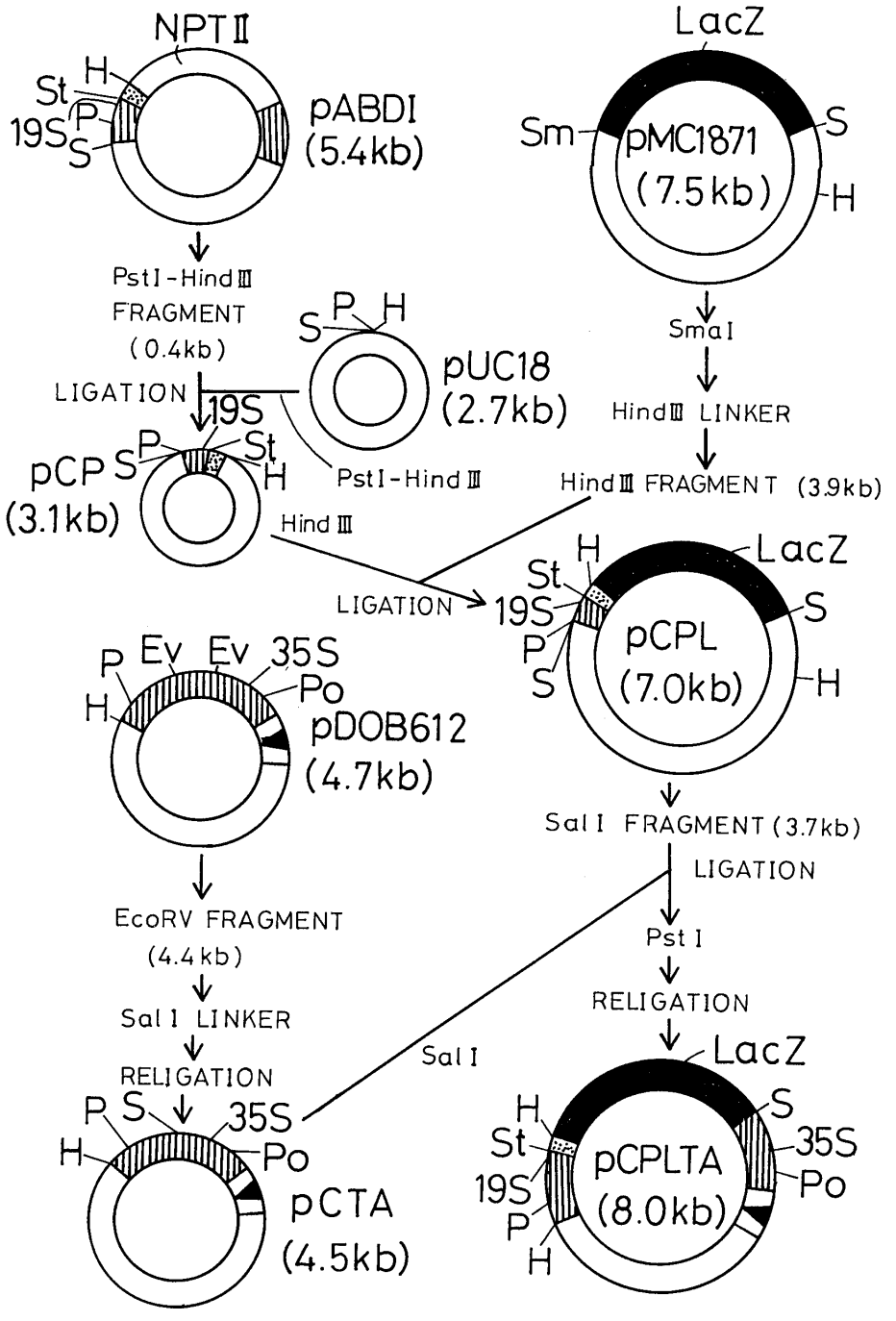

Fig. 1. Strategy for Construction of pCPLTA (7965bp).
The procedures were described in Materials and Methods. Restriction sites are labeled: H. *Hind*III; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; Ev, *Eco*RV. Other abbreviations: ▥, sequence of CaMV DNA; ▩, sequence of CaMV ORFVI; ▼, GAP1 of CaMV; ■, *lacZ* gene; 19S, 19S promoter of CaMV; 35S, 35S promoter of CaMV; St, ATG start codon; Po, polyadenylation signal of CaMV 35S.

M D-mannitol. After the addition of 1 m*l* of 20% (wt/wt) polyvinylalcohol (PVA, degree of polymerization 300) in 0.5 M D-mannitol, the mixture was mixed thoroughly and incubated for 10 min at room temperature. Subsequently, the mixture containing the treated protoplasts was diluted gradually with 0.5 M D-mannitol solution supplemented with 50 mM glycine and 50 mM $CaCl_2$ (pH 10.5). The treated protoplasts were washed twice with 0.5 M D-mannitol and cultured in Murashige-Skoog's medium[21] supplemented with 0.5 M D-mannitol at 25°C in the dark. The protoplasts treated with the carrier DNA (salmon testis DNA) only were used as controls.

To determine the reproducibility, the DNA introduction experiments were repeated at least three times.

4. Assay for β-galactosidase activity

The rapid assay for β-galactosidase activity was performed according to the modified method of Matsumoto *et al*[13]. Cultured protoplasts into which pCPLTA or carrier DNA had been introduced were washed twice with 0.5 M D-mannitol by centrifugation at 1,500 rpm for 5 min. The washed protoplasts resuspended in 0.25 M Tris-HCl (pH 7.8)-5 mM DTT solution were disrupted by sonication for 10 sec, and then frozen in a dry-ice-ethanol bath and rethawed to facilitate protoplast breakage. This procedure was repeated three times. After centrifugation at 10,000 rpm for 10 min, 100 μl of cell extract was diluted with 1 ml of buffer containing 0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M 2-mercaptoethanol, pH 7.0 (Z buffer). The reaction was started by adding 200 μl of 2 mg/m*l* *o*-nitrophenyl-β-D-galactoside (ONPG) in 0.1 M potassium phosphate buffer (pH 7.0) to the extract. After incubation at 37°C, 500 μl of 1 M Na_2CO_3 was added to stop the reaction. The mixture was centrifuged to remove insoluble materials, and absorbance at 420 nm of supernatant was measured with a spectrophotometer.

The per cent β-galactosidase activity was calculated as follows. A/B×100, wherein A was obtained by the absorbance of the reaction mixture from pCPLTA-introduced protoplasts and B was the absorbance from control protoplasts.

5. DNA extraction and dot blot hybridization

DNA was extracted from protoplasts using the method as follows. Cultured protoplasts were washed twice with 0.5 M D-mannitol and harvested by centrifugation at 1,500 rpm for 5 min. The protoplasts resuspended in Tris-HCl buffer (pH 8.0) containing 10 mM EDTA, 5 mM 2-mercaptoethanol and 0.5% SDS were disrupted by freeze-thaw repeats. After addition of 25 μl of 10 mg/m*l* proteinase K, they were incubated for 5 min at 60°C and over night at 37°C. After centrifugation for 10 min at 10,000 rpm, the supernatant was extracted three times with phenol, and was precipitated with ethanol. The isolated DNA was washed with 70% ethanol, dried, and dissolved in 10 μl of TE buffer, pH 8.0.

For dot blot hybridization, 1 μl of extracted DNA solution was spotted on a nitrocellulose membrane, and denatured with 0.5 M NaOH followed by neutralization with 1 M NaCl and 1 M Tris-HCl, pH 7.0. After baking for 4 hr and prehybridization with hybridization buffer for 4 hr, the membrane was incubated in hybridization buffer containing pCPLTA probe labeled with biotin-dUTP using the Nick Translation System kit (BRL Inc.) for 16 hr at 42°C. Colorimetric detection with streptavidin-alkaline phosphatase was performed using BluGene kit (BRL Inc.). The Densities of the blue-colored spots which appeared were measured at 400 nm by TLC chromatoscanner (Shimazu Inc.).

Results and Discussion

1. Endogenous β-galactosidase activity in *O. violaceus* protoplasts

Plant cells have a wide range of endogenous β-galactosidase activities and some of them are active at neutral pH, near the optimum of exogenous one[15]. Matsumoto *et al*[13]. also found that tobacco cells had their own endogenous β-galactosidase activity. *O. violaceus* protoplasts may have endogenous β-galactosidase and it must interfere assay of β-galactosidase activity directed by *lacZ* gene of the introduced pCPLTA. For examination of endogenous β-galactosidase activity in protoplasts, an absorbance at 420 nm of extract from the control protoplasts was measured after ONPG hydrolysis reaction. Absorbance of the control protoplast extract increased linearly during 30 min (Fig. 2). This result showed β-galactosidase apparently existed in *O. violaceus* cells. Therefore, for estimation of true β-galactosidase activity directed by introduced *lacZ* gene, endogenous β-galactosidase activity must be subtracted from the total activities.

2. β-galactosidase activity in pCPLTA-introduced protoplasts

After introduction of 30 μg of pCPLTA into protoplasts, the time-course of β-galactosidase activity in protoplasts was measured for 5 days to detect its maximal activity. After 2 days of culture, β-galactosidase activity in pCPLTA-introduced protoplasts was about 40% higher than the control (Fig. 3), and this higher activity of β-galactosidase was continued subsequently for at least 3 days. Thus, the transient expression of *lacZ* gene in the following experiments was examined 2 days after introduction of pCPLTA.

To investigate the correlation between the amount of pCPLTA introduced and β-galactosidase activity, 1, 10, 30, and 50 μg of pCPLTA were introduced into protoplasts and cultured for 2 days

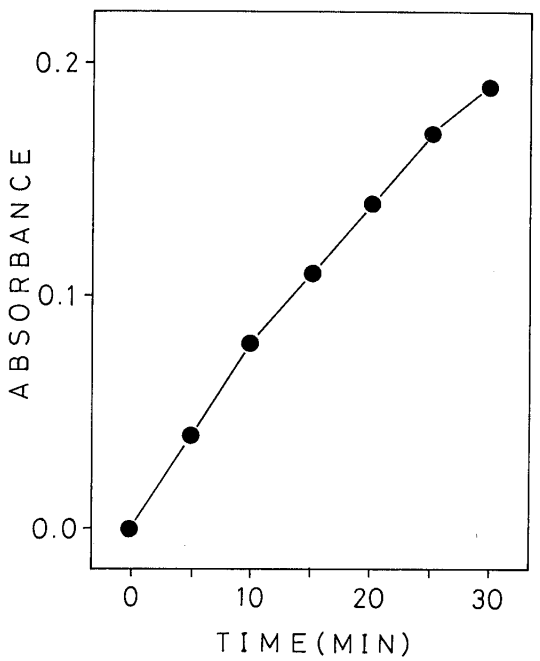

Fig. 2. Endogenous β-galactosidase Activity in *O. violaceus* Protoplasts.
Protoplast extract (50 μl) was incubated with ONPG at 37°C. All absorbances were measured by ONPG hydrolysis at 420 nm in a spectrophotometer.

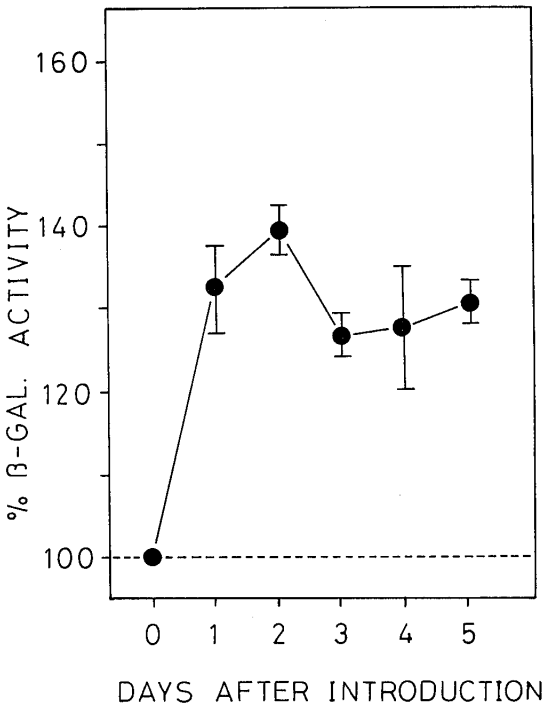

Fig. 3. Time Course of β-galactosidase Activity in pCPLTA-introduced Protoplasts.
Protoplasts were treated with 30 μg of pCPLTA. Bars represent the average of 3 replicates ± standard error.

as shown in Fig. 4. β-galactosidase activities in pCPLTA-introduced protoplasts at 1 and 10 μg of pCPLTA were as low as that in the control. The activities increased at 30 and 50 μg of pCPLTA, and were maximal at 50 μg DNA. At 50 μg DNA, β-galactosidase activity was up to 70% higher than that of control, and the reaction mixture changed deep-yellow color which was easily distinguished from the control (data not shown). The standard errors associated with each data point were routinely small.

The present paper is the first to report the transient expression of *lacZ* gene in *Cruciferae* protoplasts. Because of the interference of endogenous β-galactosidase activity, however, ONPG hydrolysis assay can not always detect *lacZ* gene expression. Teeri *et al*[14]. found that pre-treatment of tissues with glutaraldehyde was effective in histochemical detection of β-galactosidase activity because this reagent could inactivate the endogenous β-galactosidase activity without loss of enzymatic activity of *lacZ* gene product.

Many studies on transient gene expression in plant protoplasts have been done using CAT, GUS, or luciferase genes as a reporter[2-8]. However, Balazs and Bonneville[22] reported that some species of *Brassica* plants exhibited endogenous CAT activity. Furthermore, Hu *et al*[23]. found that intrinsic GUS-like activity was detectable in reproductive organs, especially, the embryos of many seed plants. On the other hand, Matsumoto *et al*[13]. found no detectable β-galactosidase activity in *Gynostremma pentaphyllum*, a member of *Cucurbitaceae*. These results suggest that *lacZ* gene is an excellent reporter gene for some plant species as well as CAT or GUS genes.

3. Fate of pCPLTA

The fate of pCPLTA was examined by dot blot hybridization and the result was shown in Fig.

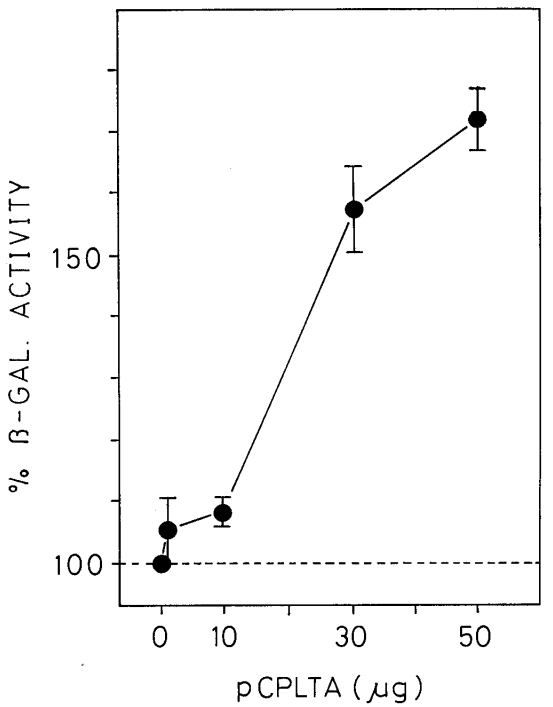

Fig. 4. Correlation between β-galactosidase Activity and Amount of pCPLTA.
Protoplasts were incubated for 2 days after pCPLTA introduction. Per cent β-galactosidase activity was calculated as described in Materials and Methods. Bars represent the average of 3 replicates ± standard error.

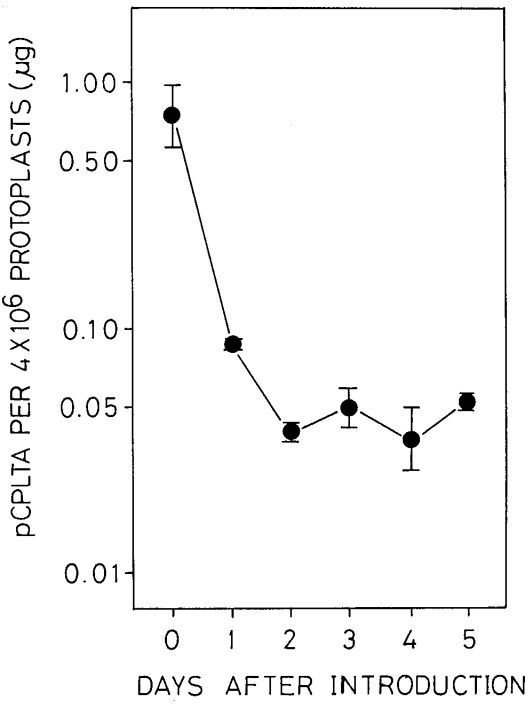

Fig. 5. Fate of pCPLTA Introduced into Protoplasts.
Protoplasts were treated with 50 μg of pCPLTA. Total DNA was extracted from protoplasts and then dot blot hybridization analysis was employed for detection of pCPLTA. Bars represent the average of 3 replicates ± standard error.

5. Just after the treatment with 50 μg of pCPLTA, about 0.8 μg of pCPLTA existed in the cytoplasm or on the plasma membrane of 4×10^6 protoplasts. After one day of incubation, however, the amount of pCPLTA decreased to about 0.1 μg. This value should be the true amout of pCPLTA introduced into protoplasts. Presumably, DNA which adhered on the plasma membrane was digested completely by DNase within a day's incubation. Thereafter, 0.04-0.05 μg of pCPLTA were maintained stably in the protoplasts. Thus, it is clear that β-galactosidase activity in Fig. 3 was directed by the *lacZ* gene of intracellular pCPLTA.

In the present study, 0.08-0.10% of pCPLTA could be introduced into 4×10^6 protoplasts by calcium-phosphate co-precipitated DNA transfer system with the aid of PVA. Since the weight of 1 copy of pCPLTA was about 8.7×10^{-18} g, and $1.0\text{-}1.2\times10^{-14}$ g of pCPLTA existed in a protoplast, it was assumed that about 1300 copies of pCPLTA were introduced into each protoplast.

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《和文要約》

ムラサキハナナ培養細胞プロトプラストにおけるβ-ガラクトシダーゼ遺伝子の発現

田中伸和[1)]，松井千秋[2)]

名古屋大学農学部植物病理学教室
〒464　名古屋市千種区不老町
[1)]現住所：ダイセル化学工業株式会社総合研究所生物化学研究所
〒671-12　兵庫県姫路市網干区新在家1239
[2)]現住所：金印わさび株式会社柳島植物組織培養研究所
〒454　名古屋市中川区八幡本通り2-61

アブラナ科ムラサキハナナ（*Orychophragmus violaceus* O. E. Schulz）の懸濁培養細胞プロトプラストを使用して，β—ガラクトシダーゼをレポーター遺伝子としたキメラ・プラスミドの形質発現実験を行った．キメラ・プラスミドとして，CaMV19Sプロモーター，CaMV ORFVIと*lacZ*の融合遺伝子，CaMV35Sプロモーター，およびターミネーター（ポリ-Aシグナル）より構築した発現ベクターpCPLTAを作成した．これを，リン酸カルシウムと共沈澱後，ポリビニルアルコールを用いてムラサキハナナプロトプラストに導入し，ONPGの加水分解による420 nmの吸光度変化により細胞中のβ-ガラクトシダーゼ活性を測定した．50 μgのpCPLTAを導入して2日間培養した4×10^6個のプロトプラストでは，内在のβ-ガラクトシダーゼ様活性より70%以上高い活性が認められ，導入したベクターの*lacZ*遺伝子が発現したことが示された．