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PEG-mediated plastid transformation: a new system for transient gene expression assays in chloroplasts

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Summary. Evidence is presented for the introduction of functional copies of the GUS-reporter gene with plastid regulatory signals into chloroplasts after treatment of *Nicotiana plumbaginifolia* leaf protoplasts with PEG. GUS-activity is found in cells derived from protoplasts treated with PEG in the presence of plasmids harbouring the GUS-gene under the control of plastid promoter and terminator signals (plastid-specific reporter gene constructions). The activity is maintained after chloroplast isolation and incubation with the protease thermolysin under conditions sufficient to completely remove the much higher transient nuclear/cytoplasmic expression of a GUS-gene carrying the CaMV 35S-promoter. Likewise, GUS-activity derived from a plasmid coding for the nuclear/cytoplasmic expression of the reporter gene with a plastid transit presequence is also maintained after these procedures. These results indicate that PEG-treatment is a suitable protocol by which to introduce DNA into chloroplasts for the study of transient gene expression.

Key words: Chloroplast transformation – β-Glucuronidase – *Nicotiana plumbaginifolia* – PEG – Transient expression

Introduction

In view of the possible impacts that genetically engineering the plastome of higher plants will have on both fundamental and applied research (Löffelhardt 1987), it is not surprising that the development of techniques for transforming chloroplasts represents a major challenge in the cellular and molecular engineering of plants. In many species of higher plants transformation at the nuclear level is now a routine procedure (for a recent evaluation see Potrykus 1990). Until very recently (Svab et al. 1990) attempts at chloroplast transformation, however, have met with only limited success (de Block et al. 1985; Cornelissen et al. 1987; Haring and de Block 1990). The apparent main difficulties to be overcome for successful stable plastid transformation are firstly, the high copy number of plastid chromosomes in a higher plant cell, and secondly the double-layered envelope of the organelle. With respect to the first difficulty, highly efficient intraorganellar and intracellular selection systems are required. It may be significant that successful intra- and intercellular selection have recently been demonstrated. Moll et al. (1990) showed strong and rapid selection for either of two plastomes carrying genes resistant to streptomycin or lincomycin, and Eigel and Koop (1990; see also Eigel et al. 1991) have reported the regeneration of green plants after transfer of a single green plastid into a plastome albino recipient cell via subprotoplast/protoplast-microfusion. The second difficulty, i.e. the need for foreign DNA to pass not only the plasma membrane but also the outer and inner organelle envelopes, could be overcome by microinjection into the organellar compartment (Abel et al. 1989) or by using microprojectile bombardment (Klein et al. 1987). Indeed, chloroplast transformation was first achieved in *Chlamydomonas* (Boynton et al. 1988; Blowers et al. 1989) and is now even possible in higher plants (Svab et al. 1990) using this approach. In this article we present evidence for the transient expression in plastids of genes introduced by PEG-treatment. In contrast to transient assays after particle bombardment (Daniell et al. 1990), in our system DNA is initially introduced into the cytoplasm and not directly into the organelles. Entry of DNA

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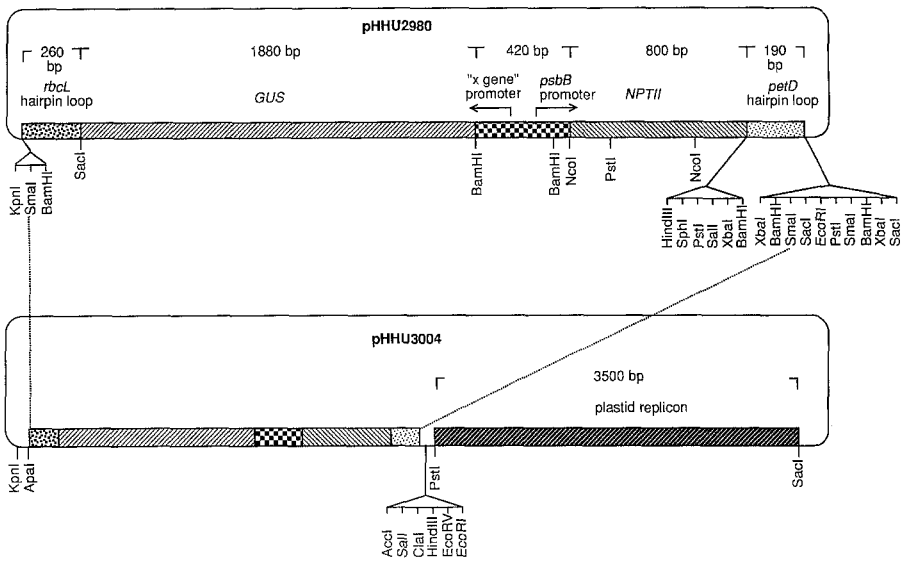

Fig. 1. Structure of chloroplast expression vectors pHHU2980 and pHHU3004. For details see the Materials and methods

through the chloroplast membranes therefore appears not to be a problem. PEG-treatment thus provides a simple procedure for establishing transient expression assays in higher plant protoplasts.

Materials and methods

Plasmid constructions

pUCNK1. The plasmid 'pUCNK1 (HindIII)', which contains the neomycine phosphotransferase (NPTII) gene from Tn5 under control of eukaryotic promoter and polyadenylation signals (pNos, nopaline synthase promoter and ocspA, octopine synthase polyadenylation signal), was obtained from L. Willmitzer, Berlin.

pHHU2980, pHHU3004. The chloroplast expression plasmids pHHU2980 and pHHU3004 (Fig. 1) were constructed in the stepwise procedure outlined below. Correct fusion of the fragments was confirmed by restriction or, if necessary, by nucleotide sequence analysis. *E. coli* DH5α served as host in the cloning experiments.

Step 1. Fusing the promoter and the entire 5'-untranslated leader of the spinach plastid *psbB* gene to the coding region of the NPTII gene. To this end a 420 bp SalI/NcoI fragment was excised from plasmid pSo729. This fragment encompasses the *psbB* and, in addition, the 'x gene' promoter that drives transcription in opposite direction to *psbB* (Westhoff 1985). The dual promoter fragment was mixed with an equimolar mixture of the 570-bp NcoI and the 230-bp NcoI/HindIII fragments, which comprise the complete coding region of the NPTII gene and which were obtained from plasmid pRT100neo (Töpfer et al. 1987). The fragments were ligated with SalI/HindIII-restricted pBluescrip M13+, and the recombinants were isolated by selection for both ampicillin and kanamycin resistance. One of the positive transformants, designated pHHU2837, was used for further construction.

Step 2. Linking the β-D-glucuronidase (GUS) gene to the 'x gene' promoter. Plasmid pHHU2837 was cut with SalI, rendered blunt-end by fill-in synthesis with Klenow polymerase and redigested with KpnI. The linearized plasmid was ligated to the 1880-bp SmaI/KpnI fragment that contained the entire GUS gene (Jefferson et al. 1987) and was excised from pRT102gus (Töpfer et al. 1987). The plasmid pHHU2849 obtained contains the NPTII and GUS genes transcribed in opposite direction by the chloroplast *psbB* and 'x-gene' promoters, respectively.

Step 3. Inserting the 3'-located hairpin loop of the spinach *rbcL* gene behind the GUS reading frame. The 3'-untranslated region of the spinach *rbcL* gene containing a 260-bp EcoRI/BamHI fragment (Whitfeld and Bottomley 1980; Zurawski et al. 1981) was obtained from plasmid p3'-4 by EcoRI digestion (Grüne, personal communication). The resulting fragment was blunted by fill-in synthesis and then cut with KpnI. This KpnI site is provided by the multiple cloning site of pBluescript M13+, which was used for the construction of p3'-4 (H. Grüne, personal communication) and is located at the 5'-flanking part of the *rbcL* hairpin loop. Plasmid pHHU2849 was linearized with Asp718 and rendered blunt-end with Klenow polymerase. The GUS/'x gene'-*psbB*-promoter/NPTII construct was excised by HindIII digestion and recovered by agarose gel electrophoresis. Both fragments were ligated with KpnI/HindIII-restricted pBluescript M13+. Plasmid pHHU2860 was found to contain the *rbcL* trailor region inserted in the proper orientation behind the GUS gene.

Step 4. Fusing the 3'-located hairpin loop of the spinach *petD* gene to the trailor region of the NPTII gene. The 3'-untranslated region of the *petD* gene (a 124-bp StuI/XbaI fragment (Heinemeyer et al. 1984) was obtained after several subcloning steps. First, the 1109-bp XbaI fragment of spinach plastid DNA encompassing the entire *petD* gene was inserted into pSP64, resulting in plasmid pSoP931. From this plasmid the fragment containing the *petD* hairpin loop was excised by StuI/EcoRI digestion. It was recloned into SmaI/EcoRI-restricted pBluescribe M13+ yielding plasmid pSoP1735, and finally recovered by digestion with EcoRI and HindIII. The fragment was ligated with HindIII/EcoRI-cut pHHU2860, and positive clones were selected for by colony hybridization. Correct insertion of the *petD* hairpin loop-containing fragment behind the NPTII gene was ascertained by sequence analysis. One of the positive clones was designed pHHU2980 (Fig. 1).

Step 5. Adding a putative plastid origin of replication. A SacI/PstI fragment of about 3.5 kb was isolated from pHvcP1 (Day and Ellis 1985) and cloned into pBluescript M13+. One of the positive clones (pHHU2999) was cut with XhoI and treated with Klenow polymerase. The blunt and linearized pHHU2999 was ligated with the insert of pHHU2980 excised by digestion

with SmaI. Plasmid pHHU3004 was found to contain a correct fusion of both fragments. Its composition is shown in Fig. 1.

pHHU3005. pHHU3005 is identical to pHHU3004 except for an inverse orientation of the SacI/PstI fragment derived from pHvcP1.

pTP30. pTP30, a plasmid containing the maize "waxy" transit presequence linked to the GUS gene (Klösgen et al. 1989; Klösgen and Weil 1991), was kindly provided by R. B. Klösgen, München.

Protoplast preparation, PEG treatment

For each experiment 10^6 leaf protoplasts isolated from axenic cultures of *Nicotiana plumbaginifolia* line 'LR 400' (Cseplö and Maliga 1982) as described in Tyagi et al. (1989) were incubated in a total volume of 500 µl of incubation mixture (compare Tyagi et al. 1989) for 30 min with 12% PEG 6000 (Merck, Darmstadt) in the presence of 80 µg plasmid DNA per milliliter in the case of plasmids pUCNK1, pTP30, pRT102gus and pHHU2980. For plasmids pHHU3004 and pHHU3005, twice the amount of plasmid DNA was applied in order to compensate for the higher molecular weights of these constructions. Routinely, 50% of the protoplasts survived this treatment. After PEG-plasmid incubation 2.5 ml of culture medium (Eigel and Koop 1989; Tyagi et al. 1989) was added to give a final cell density of approximately 8×10^4 cells per millilitre.

Chloroplast isolation and thermolysin-treatment

After 48 h of protoplast culture at dim light and 25 °C, the newly formed cell walls were removed by incubating the cells from two PEG-treatments for 2 h at room temperature in protoplast incubation medium (Tyagi et al. 1989) containing 2% cellulase R10 and 2% macerozyme R10 (Yakult Honsha, Tokyo). After flotation, reprotoplasted cells were washed with chloroplast isolation medium (CIM: 0.45 *M* sorbitol, 4 m*M* EDTA, 0.5 m*M* KH_2PO_4, 1 m*M* $MgCl_2$, 10 m*M* $MnCl_2$, 10 m*M* NaCl and 44 m*M* MES/KOH, pH 7.0; modified after Marienfeld et al. 1989) and broken by repeated passage through a syringe needle (0.45 mm inner diameter). Intact chloroplasts were prepared by centrifugation (10 min, 13,000 U/min, Sigma centrifuge 201 M) of the broken cells through a two-step percoll gradient (85% and 50%, respectively, in CIM). The band of chloroplasts was retrieved from the interphase, diluted for washing with 2 ml CIM and pelleted (50 *g*, 10 min). The pellet representing 50% of the chloroplasts originally present in the reprotoplasted cells was taken up in 80 µl CIM, and an equal volume of lysis buffer (Jefferson et al. 1987) was added. After a thorough mixing the mixture was cleared by centrifugation (10 min, 13,000 U/min, Sigma centrifuge 201 *M*). The supernatant was used for assaying GUS-activity.

Where appropriate, an additional treatment of isolated chloroplasts with thermolysin (Cline et al. 1984) was used. For this procedure 100 µl CIM, 5 µl 0.1 *M* $CaCl_2$ and 2.5 µl (430 U/ml) thermolysin (Sigma, St. Louis) were added to the pelleted chloroplasts, and incubation was carried out on ice for 1 h. More than 95% morphologically intact chloroplasts were found before and after thermolysin treatment, as checked by phase contrast microscopy.

GUS-assay

The assay mixture contained 40 µl of protein extract, representing about 300 µg of total protein (protein assay according to Bradford 1976), when the GUS activity of whole cells was being determined and approximately 30 µg of protein in the case of isolated chloroplasts. 4-Methylumbelliferyl-glucuronide (10 nmol) was added from a stock solution (1 m*M*) in lysis buffer, and the total assay volume was adjusted to 100 µl with the same buffer. Incubations were carried out for 30 min to 48 h at 37 °C. After the reaction was stopped by the addition of 900 µl 0.2 *M* Na_2CO_3, fluorescence was determined in a Hoefer TK100 fluorimeter (Hoefer Scientific, San Francisco). If necessary, samples were diluted by the further addition of 0.2 *M* Na_2CO_3.

When the GUS activities in total cells, isolated chloroplasts and chloroplasts additionally treated with thermolysin were being compared, the respective protein extracts were derived from two transformation treatments. The activity derived from one-fifth of the reprotoplasted cells was compared to those determined from two-fifths each used for chloroplast isolation and thermolysin treatment in order to compensate for the loss of 50% of the chloroplasts during isolation. From the total extract of 160 µl (80 µl suspension plus 80 µl lysis buffer) 40 µl was used for each GUS-assay. Thus, in the determination of the GUS activity in plastids, extracts represented 5×10^4 cells or their plastids for each measurement.

Results

Construction of chloroplast-specific vectors

Aiming at achieving a stable transformation of higher plant plastids using various transformation techniques we developed a series of plastid-specific reporter gene constructions (Fig. 1). The three vectors used, i.e. pHHU2980, pHHU3004 and pHHU3005, all contain the β-glucuronidase and NPTII reporter genes under the control of the plastid *psbB* and 'x gene' promoters. The carboxi termini of the chimeric genes are flanked by plastid sequences derived from the 3'-untranslated parts of the spinach *rbcL* and *petD* genes, which are capable of forming stem-and-loop structures upon transcription into RNA. It has been shown that such hairpin loops can confer enhanced stability to RNA upstream segments (Stern and Gruissem 1987). Plasmids pHHU3004 and pHHU3005 additionally carry a fragment of barley plastid DNA that may include plastid replicon sequences (Day and Ellis 1985).

Expression of chloroplast-specific vectors in total cell extracts

Applying the very sensitive (Jefferson et al. 1987) transient GUS-assay after PEG-treatment of *Nicotiana plumbaginifolia* protoplasts in the presence of plastid-specific GUS-constructions we measured low activity after prolonged incubation of total cell protein extracts with the substrate. In a number of experiments this activity was always higher than the background activity measured after cells were transformed under the same conditions with a control plasmid (Table 1), indicating that the increased activity was indeed caused by transformation with sequences coding for the GUS gene. Since it has been shown previously that plastid-specific vectors like *psbA*-promoter-driven reporter genes (Cornelissen and

Table 1. GUS-activity in protoplasts after incubation with chloroplast vector pHHU3004

Experiment number	Duration of incubation (h)		
	6	18	48
1	–	150	590
2	–	130	160
3	–	195	300
4	–	170	–
5	–	255	–
6	430	–	–
7	187	–	–

Relative amounts of 4-methylumbelliferone produced by the enzyme activity of total cell extracts from 5×10^4 protoplast-derived cells of *Nicotiana plumbaginifolia* 48 h after transformation with plasmid pHHU3004 in various independent experiments and after different times of incubation with the substrate. Background activities determined after using plasmid pUCNK1 in the same experiments was taken as 100%, and was in the range of 2 pmol MU per hour

Vandewiele 1989) may lead to unexpected expression in the nuclear/cytoplasmic genetic compartment, it was necessary to test our constructions for nuclear activity.

Localization of GUS-activity in chloroplasts

Assuming that a nucleus-derived enzyme activity should be lost or at least greatly reduced after isolation of the chloroplasts, we determined the GUS-activity in isolated chloroplasts. Activity was still found in isolated organelles (Figs. 2, 3), indicating that the expression should have been due to organellar transcription and translation. This assumption was further supported by the finding that additional incubation of isolated plastids with a proteinase intended to remove contaminating cytoplasmic enzyme activity did not lead to a detectable loss of activity. Both a positive and a negative control were included to prove the plastid origin of transient GUS-activity. In the positive control experiment the activity of β-glucuronidase imported into plastids via transit presequence-mediated import into the organelle (Klösgen et al. 1989; Klösgen and Weil 1991) was also completely maintained after organelle isolation and thermolysin incubation procedures. In the negative control experiment, i.e. transformation with a 35S, promoter driven GUS-gene, however, almost all of the expression found in total cell extracts was lost after organelle isolation and thermolysin incubation (Figs. 2, 3). This proves the suitability of our methods for isolating chloroplasts devoid of cytoplasmic contamination. Note that total cell expression of PRT102gus (321 pmol 4-methylumbelliferone) was about 50 times higher than the expression of pTP30 (6.85 pmol) and pHHU3004 (7.2 pmol).

A number of experiments involving total cell extracts as well as the analysis of isolated chloroplasts was also

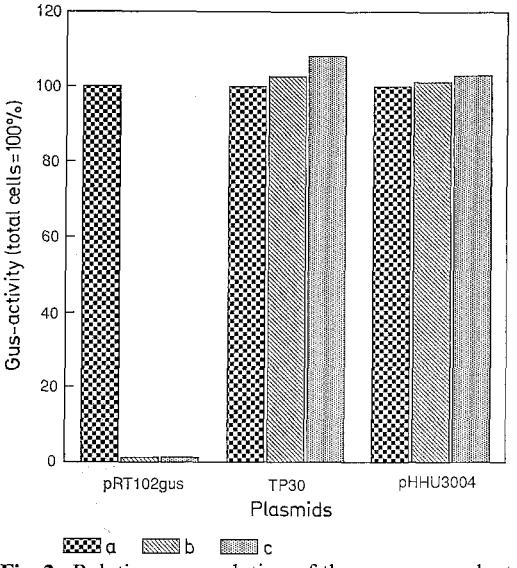

Fig. 2. Relative accumulation of the enzyme product 4-methylumbelliferone after 48-h incubation of protein extracts prepared from the total cells (*a*), isolated chloroplast (*b*) and thermolysin-treated, isolated chloroplasts (*c*) of 10^5 protoplast-derived cells of *Nicotiana plumbaginifolia*, each 48 h after transformation with plasmids pRT102gus (promoter: CaMV 35S, structural gene: GUS, polyadenylation signal: CaMV 35S), pTP30 (promoter: CaMV 35S, structural gene: "waxy" transit sequence, GUS, polyadenylation signal: CaMV 35S) and pHHU3004 (promoter: plastid "x gene"/psbB, structural gene: GUS, terminator: rbcL hairpin loop) respectively. Total cell extract activities were taken as 100%. Note that activity was about 50 times higher in the case of pRT102gus than in pTP30 and pHHU3004, respectively

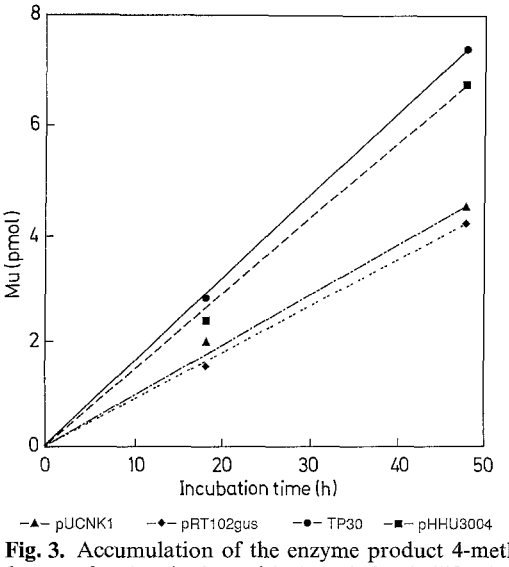

Fig. 3. Accumulation of the enzyme product 4-methylumbelliferone after incubation with 4-methylumbelliferyl-glucuronide of protein extracts from isolated and thermolysin-treated chloroplasts prepared from 5×10^4 protoplast-derived cells of *Nicotiana plumbaginifolia* 48 h after transformation with plasmids pUCNK1 (control, with pNos/NPTII/ocspA gene), pRT102gus (nuclear GUS construct, no plastid-targeting presequence), pTP30 (nuclear GUS construct, with plastid-targeting presequence) and pHHU3004 (plastid GUS construct), respectively

performed using plasmids pHHU2980 and pHHU3005. About the same activity as with pHHU3004 was produced from pHHU3005, indicating that the orientation of the SacI/PstI fragment containing a putative plastid replicon does not measurably influence the level of expression. Transformation with pHHU2980, however, yields only about one-third of the expression found with pHHU3004. A similar increase in transient organellar expression in a higher plant by the presence of putative replicons in the vector has also been described by Daniell et al. (1990) after particle bombardment of *Nicotiana* suspension cells.

Discussion

Since the only significant progress in organelle transformation has been the development of the particle bombardement technique (Klein et al. 1987; Boynton et al. 1988; Johnston et al. 1988; Blowers et al. 1989; Daniell et al. 1990; Svab et al. 1990), it is generally assumed that delivery of plasmids into the plastid compartment is a prerequisite for chloroplast transformation. Our data demonstrate the transient expression of plastid-specific vectors after introduction into protoplasts by PEG-treatment. Expression in chloroplasts is confirmed by organelle isolation followed by proteinase incubation, which is shown to abolish cytoplasmic reporter gene activity but to maintain enzymatic activity derived through import into the organelle. This indicates the autonomous introduction of at least some functional DNA into the chloroplasts once it has been taken up into the cell. Thus, to achieve transformation of higher plant plastids it may not be necessary to use particle bombardment or microinjection.

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