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Transient expression of chimaeric genes in dividing and non-dividing cereal protoplasts after PEG-induced DNA uptake

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ABSTRACT

Transient expression of chimaeric genes (neomycin phosphotransferase fused to four different promoters) was detected in suspension culture derived protoplasts of maize, barley and rice, in mesophyll protoplasts of maize, rice, rye, and root protoplasts of maize. The introduction and expression of foreign genes could be performed with both dividing and non-dividing protoplasts by applying the PEG transformation method. The significance of this method for the functional analysis of genes was demonstrated by the differential expression of a regulated gene in protoplasts of different tissues in agreement with its expression in the donor tissue.

ABREVIATIONS

PEG: polyethylene glycol

NPT: neomycin phosphotransferase

2,4-D: 2,4-dichlorophenoxyacetic acid

caseinh.:caseinhydrolysate

INDRODUCTION

Stable transformation of Gramineae has been achieved by direct gene transfer into protoplasts using PEG (Lörz et al., 1985, Potrykus et al., 1985, Uchimiya et al., 1986) or electroporation (Fromm et al., 1986) and by injection of DNA into young floral tillers (De la Pena et al., 1987). The first method depends on the availability of efficient protoplast culture systems, whilst the last relies on a precise knowledge of plant development and is, so far, of low efficiency only. No general and easily applicable method has been found yet to transform cereal plants, although recent data suggest that Agrobacterium tumefaciens may yet be developed as a vector system for graminaceous species (Graves and Goldmann, 1986, Grimsley et al., 1987).

Gene transfer into protoplasts independent of stable gene integration can be shown by assaying for enzymatic activity of the gene product coded for by the transferred DNA a few days after DNA treatment. This phenomenon of transient gene expression has been reported to occur in cereals either after electric field mediated gene transfer in tissue culture derived protoplasts of maize (Fromm et al., 1985), wheat, sorghum and leaf protoplasts of rice (Ou-Lee et al., 1986) or after PEG-facilitated direct gene transfer into wheat tissue culture protoplasts (Werr and Lörz, 1986). This technique can serve as support for the time-consuming experiments for stable genetic transformation by testing new gene constructions in protoplasts of the plant species of interest. By itself, transient expression can be used as a tool to study promoters quickly and efficiently avoiding the complication of position effects (Howard et al., 1987).

In this report we show the extension of the easy and uniform transformation method based on PEG treatment for transient expression studies to a representative variety of cereal protoplasts, isolated from four different species and three types of tissues.

MATERIAL AND METHODS

Protoplasts: Culture conditions and isolation procedure of suspensions and protoplasts have been recently described for maize (Zea mays L.) by Junker et al. (1986), barley (Hordeum vulgare L.) by Lührs and Lörz (1986) and rice (Oryza sativa L.) by Zimny and Lörz (1986) and Göbel et al. (1986). For the isolation of leaf and root protoplasts surface-sterilized seeds were germinated under sterile conditions; plant material from 10-20 days old seedlings was incubated in enzyme solution after cutting it into small pieces. Protoplasts were isolated in the following way: 2-8 hours incubation in enzyme solution (1.0% Cellulase RS, Onozuka, 0.5% Macerocym R10, Serva, 0.05% Pectolyase Y23, Seishin, 5mM $CaCl_2$, 0.5mM Na_2HPO_4, pH 5.8, mannitol as osmoticum), sieving and extensive washing with seawater diluted to the appropriate osmotic value, floating on 0.6M sucrose, final washing in seawater. Protoplasts were transformed immediately after isolation and plated in the media according to Table 1.

Plasmids: Plasmids were isolated and examined by restriction enzyme patterns according to standart molecular biology techniques.

"35S-NPT": The 35S promoter from cauliflower mosaic virus fused to the coding region of the NPT II gene from Tn5. The Hind III-fragment from pGLVneo1103 (Hain et al., 1985) was fused to the ATG of gene 2 of cauliflower mosaic virus and cloned into the polylinker of pDH51 (Pietrzak et al., 1986, K. Kraus pers. comm.).

"1'-2'-NPT": is described as pCAP212 by Velten and Schell (1985). The NPT coding region from Tn5 is fused to the 2'-promoter of the T-DNA of Agrobacterium tumefaciens.

"NOS-NPT": Chimaeric gene construction from pGLVneo1130

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(nopaline synthase promoter from the T-DNA of Agrobacterium tumefaciens fused to the coding region of NPT of Tn5, Hain et al., 1985) cloned as EcoRI-SalI-fragment into the polylinker of pUC8 (De la Pena, pers. comm.).
"SuSy-NPT": is described as pSKAN1 by Werr and Lörz (1985). The sucrose synthase promoter from maize and part of exon1 ars fused to the coding region of NPT of Tn5. The sucrose synthase gene and its regulation are described by Springer et al. (1986). In short: high expression in endosperm, medium level in roots, shoots, embryos and very reduced expression in green leaves.
Transformation and NPT II assay: For transformation the procedure according to Krens et al. (1982) was used: immediately after isolation the protoplasts were suspended in 1ml F-medium at concentrations ranging from 0.4-2x10^6/ml, circular plasmid DNA (1mg/ml) was added to the protoplasts, followed immediately by the addition of 0.4ml PEG1500 dissolved in F-medium. The mixture was incubated at room temperature for 30 minutes and a total of 10ml of F-medium was added stepwise during the next 30 minutes. The protoplasts were pelleted and plated in the appropriate media (see Table 1). F-medium was adjusted to the necessary osmotic value with mannitol or H_2O respectively.

The NPT assay followed the procedure described by Reiss et al. (1984), and Schreier et al. (1985). Protein extraction could be optimized by freezing the pelleted protoplasts in liquid N_2 and extracting twice by grinding the protoplasts with seasand. The storage of harvested protoplasts at -70°C up to 6 weeks did not affect the enzyme activity of NPT.

RESULTS

Using NPT as a marker gene, the prerequisite for our studies was to isolate at least 0.5x10^6 viable protoplasts from a given tissue in order to obtain a reliable expression signal. Prior to transformation studies we established culture conditions under which approximately 70% of the plated protoplasts were still viable after 2 days of culture. The PEG transformation procedure reduced the viability after 2 days to 30-50% of orginally plated protoplasts. In all cases of randomly chosen protoplasts this range of survival could be established by sreening a few basic tissue culture media, such as N6, CC, MS, C8 (Table 1) covering the osmotic values of 400 to 800mOs/kg H_2O, by comparing mannitol versus glucose as osmotica, and sucrose versus glucose as a source of sugar. At least one of the tested combinations fulfilled the requirements in all cases we investigated (see table 1). Light or dark culture influenced viability in some cases as well. Capacity for division did not affect the ability of the protoplasts to express a foreign marker gene (Figure 1). Parameters such as the number of protoplasts, fresh weight of extracted material or protein content did not correlate with the strength of the expression signal when different types of protoplasts were compared to each other. Within one type of protoplasts these data showed a good correlation within one preparation and only slight deviations within different preparations of protoplasts. Nevertheless aliquots of one preparation of protoplasts were used for the comparison of different parameters.

Activity of the gene product neomycin phosphotransferase could be measured in the crude extract of treated protoplasts from day 1 to day 5 after transformation. Generally the best signal was obtained on day 2.

SOURCE OF PROTOPLASTS GENOTYPE	BASAL MEDIUM REFERENCE	OSMOTICUM OSMOTIC VALUE (mOs/kg H_2O)	ADDITIONS TO MEDIUM	DARK/LIGHT CULTURE	SOURCE OF SUGAR
maize suspension BLACK MEXICAN SWEET	N6 Ozias-Akins and Vasil, 1985	mannitol 500	6mM proline 2mg/1 2,4-D	both possible	6% sucrose
barley suspension GOLDEN PROMISE	CC Potrykus et al., 1979	mannitol 700	1g/1 caseinh. 2mg/1 2,4-D	light	2% sucrose
rice suspension THAIPEI 309	CC Potrykus et al., 1979	mannitol 720	2mg/1 2,4-D	both possible	2% sucrose
maize leaves BLIZZARD	MS Ozias-Akins and Vasil, 1985	glucose 660	10% coconutwater 2mg/i 2,4-D CC vitamins 0.5% caseinh.	light	10% glucose
rice leaves THAIPEI 309	C8 Dudits et al., 1977	glucose 720		light	2% sucrose 12% glucose
rye leaves KARLSHUDER	C8 Dudits et al., 1977	glucose 720		light	2% sucrose 12% glucose
maize roots BLIZZARD	N6 Ozias-Akins and Vasil	mannitol 700	6mM proline 2mg/1 2,4-D 0.6% caseinh.	dark	6% sucrose

Table1:Culture conditions for the protoplasts used in this study.

The amount of NPT activity in the extracts of transformed protoplasts depended on the amount of DNA used for transformation. Aliquots of $1x10^6$ protoplasts isolated from maize suspensions and maize leaves, respectively, were transformed with 5, 10, 20, 50, 100ug of circular plasmid DNA carrying the chimaeric gene NOS-NPT. In both cases the signal strenght increased with the amount of added DNA. Saturation was not reached with these amounts of DNA.

The level of enzyme activity also depended on the promoter fused to the marker gene. Aliquots of different types of protoplasts were transformed with 20ug of circular plasmid DNA with 35S-NPT, 1'-2'-NPT, NOS-NPT and SuSy-NPT. IN all tested cases, which were not all the possible combinations, expression strengh ranked in the order as listed above.

Transforming $1x10^6$ protoplasts isolated from maize suspensions and maize leaves with 20ug of plasmid DNA carrying either NOS-NPT or SuSy-NPT lead to a difference in expression pattern (Figure 2). In the extracts of both types of protoplasts NPT activity could be found when the gene was under the control of the NOS-promoter. However, only in suspension derived protoplasts, and not in leaf protoplasts, could expression be found, when the gene was controlled by the SuSy-promoter. The result was unchanged, whether sucrose in the medium of suspension protoplasts was replaced by glucose or whether the suspension protoplasts were cultured in dark or light. In leaf protoplasts no expression signal was detected after the addition of 20ug SuSy-NPT DNA to $1x10^6$ protoplasts. After adding 100ug SuSy-NPT DNA to $1x10^6$ leaf protoplasts, an expression signal as strong as the one produced by 10ug DNA with NOS-NPT in $1x10^6$ leaf protoplasts could be found. In the plant there is also a reduction of transcription of the sucrose synthase gene of about 80% in leaves as compared to other tissues. Conversely the NOS-promoter expresses genes relatively equally in different parts of transformed plants.

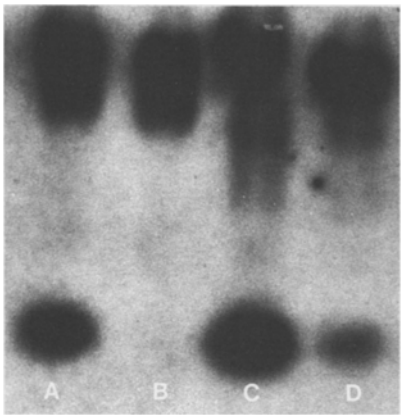
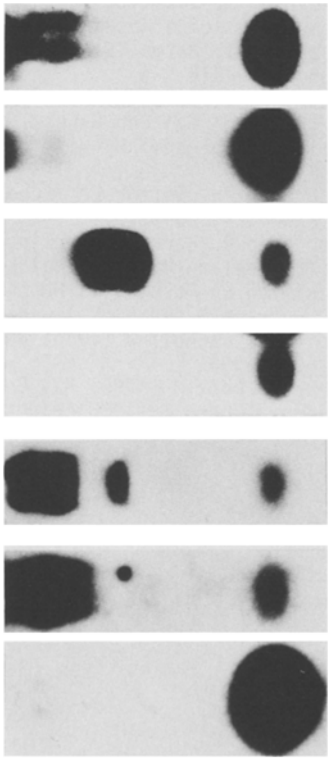


Figure 2:
Activity of neomycin phosphotransferase in the extracts of treated maize protoplasts two days after transformation. Each lane represents $1x10^6$ protoplasts which were transformed with 20ug of circular plasmid DNA. No signals were detected in untreated controls.

Lane:	Source of protoplasts:	DNA:
A	maize leaves	NOS-NPT
B	maize leaves	SuSy-NPT
C	maize suspensions	NOS-NPT
D	maize suspensions	SuSy-NPT

Figure 1:
Activity of neomycin phosphotransferase (➔) in the extracts of different cereal protoplasts. The amount of treated protoplasts is listed in the right lane. Transformation was achieved with 20ug of circular plasmid DNA with NOS-NPT. The transformed material was extracted two days after treatement. The controls showed no signals.



MAIZE SUSPENSIONS	DIVIDING	$0.6x10^6$
BARLEY SUSPENSIONS	DIVIDING	$2.0\ x10^6$
RICE SUSPENSIONS	NON-DIVIDING	$0.7x10^6$
RICE LEAVES	NON-DIVIDING	$0.5x10^6$
RYE LEAVES	NON-DIVIDING	$0.5x10^6$
MAIZE LEAVES	NON-DIVIDING	$0.7x10^6$
MAIZE ROOTS	NON-DIVIDING	$0.5x10^6$

DISCUSSION

The protocol for PEG-mediated gene transfer described originally for tobacco (Krens et al., 1982) could be extended without major modifications to tissue culture derived cereal protoplasts for stable transformation (Lörz et al., 1986; Uchimiya et al., 1986) and for transient expression studies (Werr and Lörz, 1986). The method was shown here to be applicable for all types of cereal protoplasts tested. The application of electroporation for stable and transient expression studies in cereals has also been published recently (Fromm et al., 1985; Fromm et al., 1986; Ou-Lee et al., 1986). A comparision of the efficiencies of the two different methods is difficult at the present stage, since protoplasts, gene constructions and ratios plasmid DNA/protoplasts were different in the various studies. The advantage of PEG is certainly that no expensive technical equipment is needed and that no major adaptation for different protoplasts has to be done.

From our studies we feel confident that any type of protoplasts which can be isolated in sufficient quantities, independent of species, line or tissue, can be used for transient expression studies. With the NPT gene the minimal number for reliable results is around 0.5×10^6 protoplasts. The only other prerequisite is that protoplasts can be kept viable in culture for several days. In our case, for randomly chosen protoplasts the establishment of culture conditions could be achieved in a short time.

The amount of DNA and the promoter fused to the NPT gene clearly influence the expression signal. The protoplasts seem to be the most critical and undeterminable parameter. The physiological state of the protoplasts affects the level of expression and the regulation mechanism of a promoter. Variations in culture conditions can be thought to be reflected in the expression strength of an introduced foreign gene as well and need to be studied in more detail.

Tissue-specificity seems to be retained within a certain range in the transformed protoplasts. This proves at least that studies with protoplasts, even when they do not have a capacity for division and further development, show characteristics of the original tissue they were isolated from. Further experiments on control and induction of endogenous genes in protoplasts and comparisons of transformed protoplasts and transgenic plants of the same species with the same regulated gene construction will help to clarify these observations.

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REFERENCES

De la Pena A, Lörz H, Schell J (1987) Nature 325: 274-276
Dudits D, Hadlaczky G, Levi E, Fejer O, Haydu Z, Lazar G (1977) Theor Appl Genet 51: 127-132
Fromm M, Taylor LP, Walbot V (1985) Poc Natl Acad Sci USA 82: 5824-5828
Fromm ME, Taylor LP, Walbot V (1986) Nature 319: 791-793
Göbel E, Ozias-Akins P, Lörz H (1986) IN: Alderson and Withers (eds) Plant tissue culture and its agricultural applications. Butterworth, Kent, pp 395-365
Graves ACF, Goldmann SL (1986) Plant Mol Biol 7: 43-50
Grimsley N, Hohn T, Davis JW, Hohn B (1987) Nature 325: 177-179
Hain R, Stable P, Czernilofsky AP, Steinbiß HH, Herrera-Estrella L, Schell J (1985) Mol Gen Genet 199: 161-168
Howard EA, Walker JC, Dennis ES, Peacock WJ (1987) Planta 170: 535-540
Junker B, Baker B, Lörz H (1986) In: Horn et al (eds) Genetic manipulation in plant breeding. De Gruyter, Berlin, pp 827-830
Krens FA, Molendijk L, Wullems GJ, Schilperpoort RA (1982) Nature 296: 72-74
Lörz H, Baker B, Schell J (1985) Mol Gen Genet 199: 178-182
Lührs R, Lörz H (1986) In: Horn et al (eds) Genetic manipulation in plant breeding. De Gruyter, Berlin, pp 483-486
Ou-Lee TM, Turgeon R, Wu R (1986) Proc Natl Acad Sci USA 83: 6815-6819
Ozias-Akins P, Vasil IK (1985) In: Vasil IK (ed) Cell culture and somatic cell genetics. Academic Press, London, pp 129-147
Pietrzak M, Shillito RD, Hohn T, Potrykus I (1986) Nucleic Acids Research 14: 5857-5868
Potrykus I, Harms CT, Lörz H (1979) Theor Appl Genet 54: 209-214
Potrykus I, Saul MW, Petruska I, Paszkowski J, Shillito RD (1985) Mol Gen Genet 199: 183-188
Reiss B, Sprengel P, Will H, Schaller H (1984) Gene 30: 217-223
Schreier PH, Seftor EA, Schell J, Bohnert HJ (1985) EMBO J 4: 25-32
Springer B, Werr W, Starlinger P, Bennett Dc, Zokolica M, Freeling M (1986) Mol Gen Genet 205: 461-468
Uchimiya H, Fushimi T, Hashimoto H, Harada H, Syono K, Sugawara Y (1986) Mol Gen Genet 204: 204-207
Velten J, Schell J (1985) Nucleic Acids Research 19: 6981-6998
Werr W, Lörz H (1986) Mol Gen Genet 202: 471-475
Zimny J, Lörz H (1986) Plant Cell Reports 5: 89-92.