

Uptake and Transient Expression of Chimeric Genes in Seed-Derived Embryos

Reinhard Töpfer, Bruno Gronenborn, Jeff Schell,¹ and Hans-Henning Steinbiss

Max-Planck-Institut für Züchtungsforschung, Abteilung Genetische Grundlagen der Pflanzenzüchtung, D-5000 Köln 30, Federal Republic of Germany

Uptake of DNA in dry and viable embryos of wheat by imbibition in DNA solution was detected by monitoring the transient expression of chimeric genes. Gene expression vectors used in this study contained a neomycin phosphotransferase (NPT) II reporter gene fused to various promoters. Some of the chimeric “neo” genes were shown to yield reproducibly NPT II activity in germinating embryos. This NPT II activity was increased markedly when the neo genes were carried by a vector capable of autonomous replication. Dimers of wheat dwarf virus, a monopartite gemini virus, were thus shown to be effective in amplifying the transient expressed NPT II activity in embryos of several cereals. These and other observations indicate that the observed transient expression really results from DNA uptake and expression in plant embryo cells and is not due to contaminating microorganisms.

INTRODUCTION

The stable genetic transformation of a number of plant species can now be achieved by routine procedures and has been used extensively to investigate gene regulation and gene function at the molecular level (for review see Schell, 1987). A less laborious and more rapid strategy to study the molecular basis of gene expression in plants takes advantage of the phenomenon of transient expression in plant protoplasts (e.g. Fromm et al., 1985; Hauptmann et al., 1987; Pröls et al., 1988a, 1988b). Different protoplast systems (Hauptmann et al., 1987; Junker et al., 1987), reporter gene systems (Töpfer et al., 1988), and various promoter sequences have been characterized (Ebert et al., 1987) and compared (Junker et al., 1987; Boston et al., 1987) by measuring transient gene expression. This method has also been used to study the replication and gene expression of wild-type and mutant plant viral genomes (V. Matzeit, M. Kammann, S. Schaefer, H.-J. Schalk, J. Schell, and B. Gronenborn, manuscript submitted for publication).

To achieve DNA transfer across the plasmalemma of protoplasts, one needs to treat them either physically (e.g. by electroporation; see Fromm et al., 1985; Hauptmann et al., 1987), or chemically (e.g. by polyethylene glycol; see Ballas et al., 1987; Pröls et al., 1988a, 1988b). In addition, microprojectiles have been used recently to introduce tobacco mosaic virus (TMV) RNA or DNA carrying a chimeric chloramphenicol acetyltransferase gene into epidermal cells of *Allium cepa* (Klein et al., 1987). By means of this technique, DNA can pass through both the cell wall and the plasma membrane.

The uptake of DNA of dry plant tissue through membranes, whose physiochemical characteristics change while natural desiccation occurs (Simon, 1974), was thought to be an alternative and very simple way of gene transfer. This general approach has been used previously (Hess, 1969, 1970; Ledoux and Huart, 1969; Ledoux et al., 1971, 1974; Soyfer, 1980), but the results of these early studies were discussed controversially in the literature (Kleinhofs et al., 1975; Kleinhofs and Behki, 1977; Soyfer and Titov, 1981). In our study, defined molecular markers were used and their expression was followed over a short time interval. The presence, in an active form, of a defined reporter enzyme coded for by the DNA offered for uptake was used to monitor transfer of DNA. Experiments were designed to test explanations other than uptake and expression in plant cells. In particular, the possibility that the observed transient expression might result from uptake of the reporter gene by contaminating bacteria was considered. Besides this, the question of DNA penetration through the cell wall and/or plasma membrane is addressed.

RESULTS

DNA Uptake and Expression by Dry Embryos

When this work was initiated, small batches of 20 to 30 seed-derived dry wheat embryos were imbibed in solution containing the plasmid pRT100neo carrying the 35S RNA promoter of cauliflower mosaic virus (CaMV) fused to neomycin phosphotransferase (NPT) II gene. After 3 days

¹ To whom correspondence should be addressed.

of cultivation, such embryos gave rise occasionally to an NPT II-dependent phosphorylation product (data not shown). Because of this promising observation, we decided to attempt to devise an experimental protocol that would allow the use of this system as a reproducible transient assay system. The technique that was developed is outlined in "Methods."

Figure 1 illustrates the results of NPT II assays performed with extracts of embryos and endosperm imbibed with different gene constructs (plasmids pRT101cat, pRT100neo, pCAP212, and pLGV1103neo). The amount of kanamycin phosphate, the product of the NPT II-specific phosphorylation of aminoglycoside antibiotics, reflects the strength of different promoters: the 35S RNA promoter (pRT100neo) being stronger than the P₁' P₂' promoter (pCAP212), which in turn is stronger than the NOS promoter (pLGV1103neo) (Junker et al., 1987). NPT II expression was observed only if embryos were treated with DNA coding for this phosphotransferase, whereas no NPT II activity was found in untreated embryos or in embryos treated with DNA not coding for NPT II (Figure 1, minus DNA, pRT101cat). Moreover, pieces of endosperm were unable to yield any NPT II activity (Figure 1, endosperm).

Embryos imbibed for 30 min in a DNA solution and for a subsequent 30 min in buffer expressed the NPT II gene as shown in Figure 2. However, when the sequence of these treatments was reversed (first buffer and then DNA solution), no activity was obtained. This indicates that embryos have to be in a desiccated condition to be able to take up DNA and thus achieve transient gene expression. In a more detailed analysis, NPT II expression was

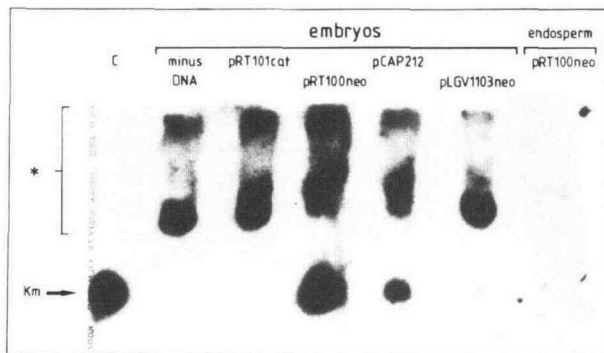


Figure 1. NPT II Blot of Wheat Embryos and Endosperm Treated with Various Plasmid Constructs as Indicated above the Individual Lanes.

Minus DNA and pRT101cat represent negative controls. pRT100neo (35S RNA promoter), pCAP212 (P₁' P₂' promoter), and pLGV1103neo (NOS promoter) describe constructs with various promoters of documented strength. C = control, pRT100neo transformed tobacco callus; * = unspecific background activity; Km = kanamycin phosphate.

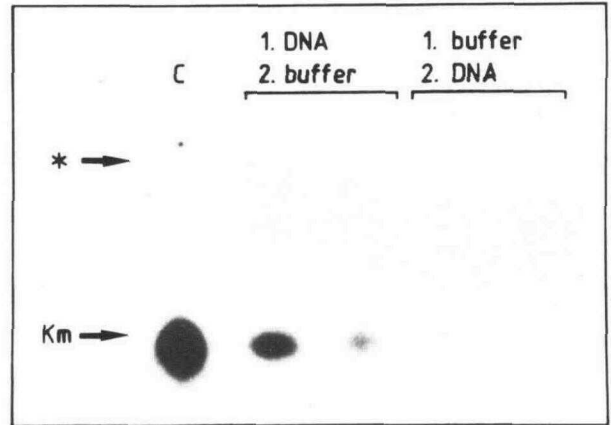


Figure 2. NPT II Blot of Wheat Embryos Treated for 30 min with Incubation Buffer Containing 200 μ g of DNA per Milliliter (1. DNA), and, Subsequently, for 30 min with Incubation Buffer omitting DNA (2. buffer).

Compared with these, embryos were treated for 30 min with incubation buffer free of DNA (1. buffer), followed by a 30-min DNA treatment (2. DNA). C = control, pRT100neo transformed tobacco callus; * = unspecific background activity; Km = kanamycin phosphate.

shown to decline rapidly to zero when DNA was added to embryos preimbibed in buffer for 1, 3, 5, 10, or 15 min.

Expression of Recombinant Wheat Dwarf Virus (WDV) Genomes after Uptake in Wheat Embryos

Assuming that the genome of a DNA plant virus would not be able to replicate unless it is actually taken up by plant cells, the genome of a wheat gemini virus (WDV) was marked with an NPT II reporter gene and used in uptake experiments. A WDV monomer, pWDVneo1, and two WDV dimers, pWDVneo2 and pWDVS2 (V. Matzeit, M. Kammann, S. Schaefer, H.-J. Schalk, J. Schell, and B. Gronenborn, manuscript submitted for publication), were used for these experiments. In both "neo" plasmids, the WDV coat protein coding region was replaced by NPT II. pWDVS2, however, represents a cloned wild-type virus.

The level of expression of NPT II obtained after uptake of vectors containing a WDV dimer was significantly higher than with controls. Thus, pWDVneo2 gave 15 to 20 times higher NPT II activity than a pRT100neo control (data not shown). That this increase in NPT II activity is correlated with the capacity of the vector to replicate was shown by the fact that uptake of a WDV neo monomer (pWDVneo1) did not result in a high level of NPT II activity unless a vector containing a WDV dimer, but no neo gene (pWDVS2), was offered for simultaneous uptake (Figure 3).

Transient Expression in Embryos of Other Crop Plants

In addition to wheat, dry embryos of other cereals and of some grain legumes were tested for their capacity to take up and express chimeric genes transiently. Figure 4 shows the results and provides a list of the plant species tested and of the DNAs applied. Both vectors pWDVneo2 and pRT100neo were used to investigate NPT II expression in cereals (Figure 4a). In all species tested, the NPT II gene was expressed, although to a low level in *Zea mays*.

It is difficult to compare levels of NPT II expression among various plant species. The standard in our experiments was based on dry weight, which in the case of *Z. mays* meant that 60 embryos were treated in comparison with about 300 for the other cereals. The protocol used may not have achieved optimal conditions for DNA uptake. Moreover, different cereals may vary in their response to WDV constructs, and this might explain the differences in NPT II activity.

The three legumes treated with the plasmid pRT100neo showed intensive NPT II signals (Figure 4b). As already shown for wheat, no endogenous phosphorylating activi-

ties were detected when either no DNA or pRT101cat DNA (as a negative control) was offered for uptake.

DISCUSSION

We report on a system for the transient expression of foreign DNA in embryos of a wide variety of plant species. The approach described here is reminiscent of work carried out in the late 1960s and early 1970s, when seeds or seedlings were treated with bacterial or genomic plant DNA. This earlier work proved to be contentious because of the possibility of bacterial contamination, the lack of an effective molecular marker, as well as the lack of reproducibility (Hess, 1969, 1970; Ledoux and Huart, 1969; Ledoux et al., 1971, 1974; Soyfer, 1980). A critical evaluation of the results described in this paper prompts several obvious questions that can be summarized as follows: Are the observed signals in embryos true NPT II-dependent activities resulting from the uptake and expression of DNA in the cells of the plant embryos? Several control experiments were performed to test alternative explanations such as (1) endogenous activities, (2) activities resulting from contaminating microorganisms, or (3) the possibility that the observed signals originated in some sort of cell-free system. The presented data provide evidence against such interpretations.

Endogenous phosphotransferases comigrating with NPT II can be excluded because NPT II activity was not observed in extracts from embryo batches imbibed without DNA (Figure 1, minus DNA) or imbibed with DNA that was not coding for NPT II (Figure 1, pRT101cat). It is also evident that embryos are responsible for the NPT II activity since control experiments performed with equal amounts of wheat endosperm did not result in any NPT II activity. The NPT II reporter enzyme did not give any unspecific background, whereas chloramphenicol acetyltransferase and β -glucuronidase background activities were detected in untreated embryos. Therefore, NPT II was used in all further experiments.

Because it can never be excluded convincingly that the observed activities actually resulted from DNA uptake by either contaminating or symbiotic endophytic microorganisms, it was decided to test whether plant-specific, eucaryotic gene expression signals could drive the transient expression in a manner consistent with the hypothesis that expression took place in plant cells. NPT II gene constructs with different promoters (Figure 1, pRT100neo [35S], pCAP212 [P₁' P₂'], pLGV1103neo [NOS]) were used. They yielded NPT II activities of decreasing intensities as predicted on the basis of the known strength of the different promoters in protoplast systems (Junker et al., 1987). In addition, an NPT II gene in pKM1 controlled by its original Tn 5 promoter (Beck et al., 1982), which is active in *Escherichia coli* but inactive in tobacco SR1 protoplasts, proved to be inactive in embryos (data not shown). If the

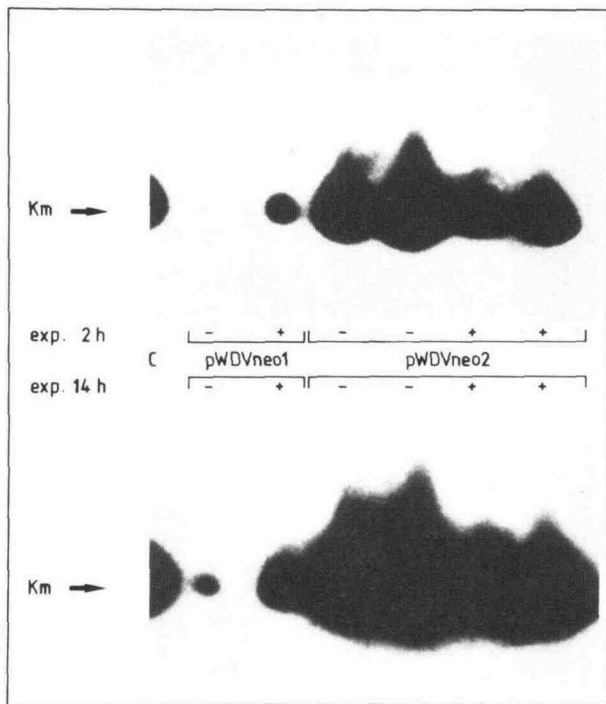


Figure 3. NPT II Blot: Comparison of Embryo Batches, Treated with neo Genes Carried on WDV Genomes.

Influence of the wild-type dimer pWDVS2 on pWDVneo1 and pWDVneo2 expression was investigated. (-) no addition and (+) addition of pWDVS2 DNA to the DNA solution. C = positive control; Km = kanamycin phosphate.

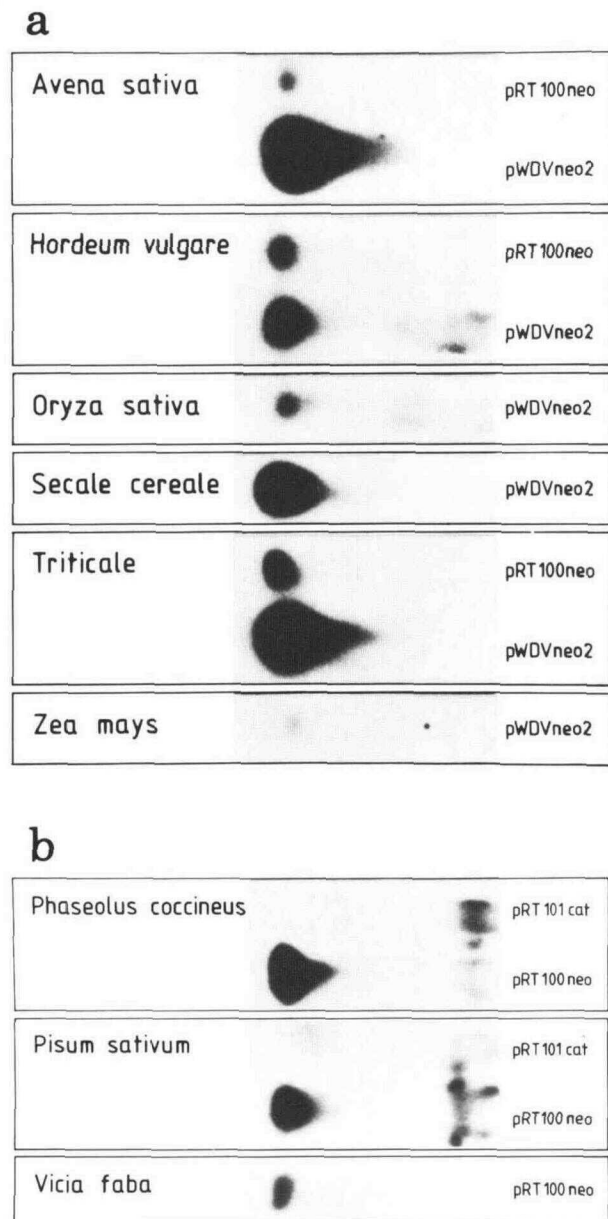


Figure 4. NPT II Blot of Embryos of Various Cereals (a) and Legumes (b) Tested for Transient Expression with the Constructs Indicated.

pWDVneo2 and pRT100neo represent plasmids carrying the NPT II gene; pRT101cat serves as negative control.

signals obtained were due to the presence of procaryotes, one would expect to observe NPT II activity when using the Tn 5-driven NPT II gene.

The experiments illustrated in Figure 2 also are not consistent with the assumption that the observed NPT II activities originate from contaminating microorganisms.

Indeed, NPT II signals were observed only when DNA was offered to dry embryos. Preimbibed embryos or endosperm tissues never yield NPT II activities.

Possibly the single most convincing argument in favor of the idea that the observed NPT II expression is taking place in plant embryo cells is based on the observation that a plant-specific replicon can amplify the NPT II signal (Figure 3). Matzeit et al. (V. Matzeit, M. Kammann, S. Schaefer, H.-J. Schalk, J. Schell, and B. Gronenborn, manuscript submitted for publication) showed that pWDVneo2, a dimer of a recombinant WDV genome carrying a neo gene, when applied as supercoiled DNA to protoplasts of a *Triticum monococcum* suspension culture, was able to replicate in these protoplasts, whereas an equivalent monomer, pWDVneo1, did not replicate. The results illustrated in Figure 3 show that a neo gene carried by pWDVneo2, which is capable of replication, is highly expressed in wheat embryos, whereas a neo gene carried by the nonreplicating pWDVneo1 vector was only expressed weakly. This low level of expression was enhanced, however, by the presence in *trans* of a cloned dimer of the wild-type virus genome (pWDVS2). These results are clearly consistent with the DNA being taken up by plant embryo cells and inconsistent with the DNA being taken up by contaminating microorganisms.

It may be argued that the NPT II expression occurs on the surface of embryos in destroyed cells. This possibility is unlikely since extensive washing of the embryos prior to plating did not result in a disappearance of the signals. Moreover, where WDV replication has been implicated, one would have to assume that DNA synthesis occurs independently from the normal cell compartmentation.

How, then, can we explain the capacity of cells in desiccated plant embryos to really take up and express foreign DNA? Two barriers exist that might prevent the entry of macromolecules into viable cells: cell wall and plasmalemma. The passage of DNA through cell walls is somewhat unexpected. Carpita et al. (1979) determined that the pore size in cell walls of a cell suspension culture of *Acer pseudoplatanus* is 3.8 to 4.0 nm. This would be too small for the passage of a 4.15-kb (pRT100neo) or an 8.86-kb (pWDVneo2) plasmid. However, during the isolation procedure, the embryos are somewhat damaged, which might lead to imbibition of DNA molecules across plasmodesmata between intact and damaged cells. Consequently, only cells adjacent to damaged cells might obtain foreign DNA and would be responsible for transient expression.

A careful look to the plasmalemma in dry embryos, the second barrier the DNA has to overcome, reveals some possible explanations for the passage of DNA. Transient expression in protoplasts requires the use of chemical agents (Paszowski et al., 1984; Hain et al., 1985; Ballas et al., 1987), or of electric pulses (Fromm et al., 1985; Hauptmann et al., 1987) or of both (Shillito et al., 1985) to overcome the uptake control exercised by the plasma-

lemma. Although we recommend the use of DMSO, which is assumed to interfere with the membrane (Kawai and Nishizawa, 1984) for optimal uptake, no chemical agents were employed in our initial experiments. Since NPT II expression was observed also in these cases, dry embryos seem to have unique properties. The observation that the early imbibition phase is critical can be explained by ultrastructural studies. Webster and Leopold (1977), using aqueous fixatives, reported discontinuity and disorganization in the plasmalemma of unimbibed soybean cotyledons. However, after 20 min of imbibition, the plasmalemma became more intact and uniform. Similarly, Thomson (1979) discussed "ill-defined images of membranes" in cotyledons of dry bean seeds after nonaqueous primary fixation. On the other hand, Öpik (1980), using anhydrous fixation, described the ultrastructure of membranes of coleoptile cells in dry rice as continuous and unbroken. Despite the controversy in the literature about the image of membranes in dry seeds, both Thomson (1979) and Öpik (1980) described that membranes require the presence of about 20% water on a total weight basis for stability, whereas in dry seeds the water content is in the range of 4% to 8% of total weight (Öpik, 1980).

This, as well as our observation that the DNA uptake occurs most efficiently during the first 30 min of imbibition (Figure 2), points to the special properties of membranes in dry embryos. Possibly both explanations, uptake by damaged or through fragile cell walls and the unusual constitution of membranes in dry embryos, are valid for the observed transient expression.

When considering our data along with those of others, one can imagine the following pathway of DNA uptake. Hallam et al. (1972) distinguished three stages of water uptake when imbibing rye embryos: "Phase I a short period (10 min) of physical wetting; Phase II a longer period (1 h) when little further imbibition occurs, followed by Phase III a continuous phase of active water uptake." During phase I and II, embryos leak substances, as can be observed readily with both embryos and seeds during the early imbibition period (Simon, 1974). The most rapid leakage from embryos occurs during the first few minutes of immersion. Our results indicate that phase I and perhaps part of phase II are essential for DNA uptake (see Figure 2). As substances leaking out of embryos and seeds diffuse along concentration gradients, DNA uptake might occur in a similar fashion along a concentration gradient.

The extension of the described procedure to other plant species (Figure 4, a and b) indicates a broad field of applications. Dry embryos are easy tools for obtaining gene expression in a homologous or heterologous plant system. They might be useful for studies of gene function and gene regulation or for studies of virus uptake and virus movement in plants. In addition, the method carries some technical advantages: it does not require any expensive equipment, it is easily learned, and there is no need to maintain plant material in tissue culture.

At present this system cannot be compared with well-characterized protoplast systems for transient expression (Fromm et al., 1985; Werr and Lörz, 1986; Junker et al., 1987; Pröls et al., 1988b) because more detailed analysis of the mechanism of DNA uptake is needed. The mature embryo will germinate easily and give rise to a plant. Therefore, this method of DNA uptake may not only be useful for the study of gene expression in cereals or grain legumes but might also lead to stable transformation of plants. Plantlets have been regenerated from treated embryos of wheat, and experiments are in progress to test whether some of these plantlets and their offspring are transformed.

METHODS

Plasmids Used

Cloning work was performed as described by Maniatis et al. (1982). An NPT-II gene (originally from Tn 5) with GCGGG-ACCCaagcttCGACggatcctctgag following the TGA stop codon at position 2343 according to Beck et al. (1982) was used for cloning. At its 5' end, the gene was essentially identical to pKm22 (Beck et al., 1982). It contains an HindIII linker instead of the original ATG codon. Insertion of an NcoI linker (cccatggg) into the HindIII site (filled in) at the 5' end creates a new ATG codon in-frame to the NPT II gene. Finally, as an NcoI-XbaI fragment, this NPT II gene was inserted into pRT100 (Töpfer et al., 1987) to create pRT100neo shown in Figure 5.

Other plasmids used were pCAP212 (Velten and Schell, 1985), pLGV1103neo (Hain et al., 1985), and pRT101cat (Pröls et al., 1988b) as well as the WDV-based constructs pWDVneo1, pWDVneo2, and pWDVS2 previously described (V. Matzeit, M. Kammann, S. Schaefer, H.-J. Schalk, J. Schell, and B. Gronenborn, manuscript submitted for publication).

Isolation and DNA Treatment of Wheat Embryos

Isolation of dry wheat (*Triticum aestivum*) embryos follows basically the protocol of Johnston and Stern (1957) using cyclohexane/carbon tetrachloride except for the use of dry ice. For optimal expression, 150 mg (about 300) of dry embryos were imbibed for 30 min in 500 μ l of 15 mM NaCl, 1.5 mM sodium citrate, 20%

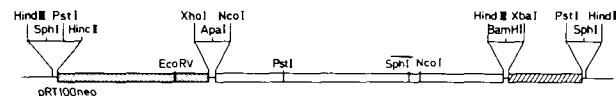


Figure 5. Scheme of pRT100neo.

CaMV 35S RNA promoter (gray area, 420 base pairs [bp]); NPT II gene (white area, 800 bp); CaMV polyadenylation signal corresponding to the 35S RNA promoter (hatched area, 200 bp); plasmid pUC18/19 (Yanisch-Perron et al., 1985).

DMSO, and 100 μg of pRT100neo DNA. Embryos were then washed three times with 1 ml of 15 mM NaCl and 1.5 mM sodium citrate.

Culture of Wheat Embryos

Embryos were placed on filter paper (10 cm diameter) on top of solid (1% agar, Merck) MS medium (Murashige and Skoog, 1962) without hormones. The pH was adjusted to 5.8 using 0.2% Mes and NaOH. To prevent growth of bacteria, the medium was supplemented with 250 μg of Claforan (Hoechst) per milliliter. The embryos were then cultivated for 3 days in a culture room (24°C, 12-hr photoperiod, 800 lux).

Isolation, DNA Treatment, and Culture of Other Cereal Embryos

Embryos of seven different cereals were isolated in the same way as described for wheat. Bran was blown off, and endosperm and embryos were separated by cyclohexane and carbon tetrachloride (Table 1). More or less intact embryos were selected manually. One hundred and fifty mg of embryos from all cereals (i.e. about 50 maize embryos) were imbibed for 2 hr (because optimum of 30 min was elaborated for wheat only). The incubation buffer as well as the culture were carried out as described for wheat.

Isolation, DNA Treatment, and Culture of Legume Embryos

In comparison to most of the cereals used, the selected grain legumes possess much larger seeds, and, consequently, larger embryos. Therefore, we treated 20 to 30 g of dry seeds twice for 1 sec in a Waring Blendor at low speed. The embryos could be collected easily with a pair of forceps under the stereo microscope. To prevent contamination, seeds were surface-sterilized with absolute ethanol (5 min), and all steps were carried out aseptically. For DNA treatment, 300 mg of embryos were imbibed in 1 ml of 15 mM NaCl, 1.5 mM sodium citrate, 20% DMSO, and 200 μg of plasmid DNA for 2 hr. Legume embryos were then plated onto an MS medium (Murashige and Skoog, 1962) supplemented with 0.1% casein hydrolysate, 0.2% Mes (pH 5.8 adjusted with NaOH), 0.4% Gellan Gum (Kelco K 9A40), 1 mg of benzylaminopurine per liter, and both 250 μg Claforan (Hoechst) and 30 μg Fenbendazol (Hoechst) per milliliter. Culture conditions were as recommended for wheat.

Table 1. Plant Material and Ratios for Cyclohexane and Carbon Tetrachloride Used to Isolate Embryos.

Species	Cultivar	Cyclohexane:CCl ₄
<i>Avena sativa</i>	Rollo	1:2.5
<i>Hordeum vulgare</i>	Gerbel	1:2.5
<i>Oryza sativa</i>	IR 36	1:2.5
<i>Secale cereale</i>	Karlshuder	1:3
<i>Triticale</i>	Clercal	1:3.3
<i>Triticum aestivum</i>	Sokrates	1:2.5
<i>Zea mays</i>	Brutus	1:2

NPT II Assay

The NPT II assay was performed as described previously (Reiss et al., 1984; Schreier et al., 1985) using 100 μCi of ³²P-ATP (Amersham Corp.) per assay. The samples were prepared according to the following procedure: Germinated embryos were homogenized in 2 ml of extraction buffer (62.5 mM Tris-HCl, pH 6.8, 5% 2-mercaptoethanol) in a mortar on ice. The homogenate was centrifuged at 100,000g for 1 hr. The supernatant was first adjusted to 30% (NH₄)₂SO₄ and centrifuged at 9,000g at 4°C for 15 min. It was then made to 45% of (NH₄)₂SO₄ and centrifuged again. To set the (NH₄)₂SO₄ concentration, a cold saturated solution was used. The protein pellet was washed carefully with 0.25 ml of extraction buffer and dissolved on ice in 50 μl of gel loading buffer (62.5 mM Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, 10% glycerol, 0.025% bromophenol blue).

Received October 26, 1988.

REFERENCES

- Ballas, N., Zakai, N., and Loyter, A.** (1987). Transient expression of the plasmid pCaMVCAT in plant protoplasts following transformation with polyethylenglycol. *Exp. Cell Res.* **170**, 228–234.
- Beck, E., Ludwig, G., Auerswald, E.A., Reiss, B., and Schaller, H.** (1982). Nucleotide sequence and extract localisation of the neomycin phosphotransferase gene from transposon Tn 5. *Gene (Amst.)* **19**, 327–336.
- Boston, R.S., Becwar, M.R., Ryan, R.D., Goldsbrough, P.B., Larkins, B.A., and Hodges, T.K.** (1987). Expression from heterologous promoters in electroporated carrot protoplasts. *Plant Physiol.* **83**, 742–746.
- Carpita, N., Sabulase, D., Montezinos, D., and Delmer, D.P.** (1979). Determination of the pore size of cell walls of living plant cells. *Science* **205**, 1144–1147.
- Ebert, P.R., Ha, S.B., and An, G.** (1987). Identification of an essential upstream element in the nopaline synthase promoter by stable and transient assays. *Proc. Natl. Acad. Sci. USA* **84**, 5745–5749.
- Fromm, M., Taylor, L.P., and Walbot, V.** (1985). Expression of genes transferred into monocot and dicot plant cells by electroporation. *Proc. Natl. Acad. Sci. USA* **82**, 5824–5828.
- Hain, R., Stabel, P., Czernilofsky, A.P., Steinbiss, H.-H., Herrera-Estrella, L., and Schell, J.** (1985). Uptake, integration, expression and genetic transmission of a selectable chimeric gene by plant protoplasts. *Mol. Gen. Genet.* **199**, 161–168.
- Hallam, N.D., Roberts, B.E., and Osborne, D.J.** (1972). Embryogenesis and germination in rye (*Secale cereale* L.) II. Biochemical and fine structural changes during germination. *Planta (Berl.)* **105**, 293–309.
- Hauptmann, R.M., Ozias-Akins, P., Vasil, V., Tabaeizadeh, Z., Rogers, S.G., Horsch, R.B., Vasil, I.K., and Fraley, R.T.** (1987). Transient expression of electroporated DNA in monocotyledonous and dicotyledonous species. *Plant Cell Rep.* **6**, 265–270.

- Hess, D.** (1969). Versuche zur Transformation an höheren Pflanzen: Induktion und konstante Weitergabe der Anthocyan-synthese bei *Petunia hybrida*. Z. Pflanzenphysiol. **60**, 348–358.
- Hess, D.** (1970). Versuche zur Transformation an höheren Pflanzen: Mögliche Transplantation eines Gens für Blattform bei *Petunia hybrida*. Z. Pflanzenphysiol. **63**, 461–467.
- Johnston, F.B., and Stern, H.** (1957). Mass isolation of viable wheat embryos. Nature (Lond.) **179**, 160–161.
- Junker, B., Zimny, J., Lührs, R., and Lörz, H.** (1987). Transient expression of chimaeric genes in dividing and non-dividing cereal protoplasts after PEG-induced DNA uptake. Plant Cell Rep. **6**, 329–332.
- Kawai, S., and Nishizawa, M.** (1984). New procedure for DNA transfection with polycation and dimethyl sulfoxide. Mol. Cell. Biol. **4**, 1172–1174.
- Klein, T.M., Wolf, E.D., Wu, R., and Sanford, J.C.** (1987). High-velocity microprojectiles for delivering nucleic acids into living cells. Nature **327**, 70–73.
- Kleinhofs, A., and Behki, R.** (1977). Prospects for plant genome modification by nonconventional methods. Annu. Rev. Genet. **11**, 79–101.
- Kleinhofs, A., Eden, F.C., Chilton, M.-D., and Bendich, A.J.** (1975). On the question of the integration of exogenous bacterial DNA into plant DNA. Proc. Natl. Acad. Sci. USA **72**, 2748–2752.
- Ledoux, L., and Huart, R.** (1969). Fate of exogenous bacterial deoxyribonucleic acids in barley seedlings. J. Mol. Biol. **43**, 243–262.
- Ledoux, L., Huart, R., and Jacobs, M.** (1971). Fate of exogenous DNA in *Arabidopsis thaliana*. Eur. J. Biochem. **23**, 96–108.
- Ledoux, L., Huart, R., and Jacobs, M.** (1974). DNA-mediated genetic correction of thiaminless *Arabidopsis thaliana*. Nature (Lond.) **249**, 17–21.
- Maniatis, T., Fritsch, E. F., and Sambrook, J.** (1982). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).
- Murashige, T., and Skoog, F.** (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Plant Physiol. **15**, 473–497.
- Öpik, H.** (1980). The ultrastructure of coleoptile cells in dry rice (*Oryza sativa* L.) grains after anhydrous fixation with osmium tetroxide vapour. New Phytol. **85**, 521–529.
- Paszowski, J., Shillito, R.D., Saul, M., Mandák, V., Hohn, T., Hohn, B., and Potrykus, I.** (1984). Direct gene transfer to plants. EMBO J. **3**, 2717–2722.
- Pröls, M., Schell, J., and Steinbiss, H.-H.** (1988a) Critical evaluation of electro-mediated gene transfer and transient expression in plant cells. In Electroporation and Electrofusion in Cell Biology, E. Neumann, A.E. Sowers, and C.A. Jordan, eds (New York: Plenum Publishing Corp.), in press.
- Pröls, M., Töpfer, R., Schell, J., and Steinbiss, H.-H.** (1988b). Transient gene expression in tobacco protoplasts: I. Time course of CAT appearance. Plant Cell Rep. **7**, 221–224.
- Reiss, B., Sprengel, R., Will, H., and Schaller, H.** (1984). A new sensitive method for qualitative and quantitative assay of neomycin phosphotransferase in crude cell extracts. Gene (Amst.) **30**, 211–218.
- Schell, J.** (1987). Transgenic plants as tools to study the molecular organisation of plant genes. Science **237**, 1176–1183.
- Schreier, P.H., Seftor, E.A., Schell, J., and Bohnert, H.J.** (1985). The use of nuclear-encoded sequences to direct the light-regulated synthesis and transport of a foreign protein into plant chloroplasts. EMBO J. **4**, 25–32.
- Shillito, R.D., Saul, M.W., Paszowski, J., Müller, M., and Potrykus, I.** (1985). High efficiency direct gene transfer to plants. Biotechnology **3**, 1099–1103.
- Simon, E.W.** (1974). Phospholipids and plant membrane permeability. New Phytol. **73**, 377–420.
- Soyfer, V.N.** (1980). Hereditary variability of plants under the action of exogenous DNA. Theor. Appl. Genet. **58**, 225–235.
- Soyfer, V.N., and Titov, Y.B.** (1981). The absence of a definite gene-specific effect in wheat after seed treatment with exogenous DNA. Mol. Gen. Genet. **182**, 361–363.
- Thomson, W.W.** (1979). Ultrastructure of dry seed tissue after a non-aqueous primary fixation. New Phytol. **82**, 207–212.
- Töpfer, R., Matzeit, V., Gronenborn, B., Schell, J., and Steinbiss, H.-H.** (1987). A set of plant expression vectors for transcriptional and translational fusions. Nucleic Acids Res. **15**, 5890.
- Töpfer, R., Pröls, M., Schell, J., and Steinbiss, H.-H.** (1988). Transient gene expression in tobacco protoplasts: II. Comparison of the reporter gene systems for CAT, NPT II, and GUS. Plant Cell Rep. **7**, 225–228.
- Velten, J., and Schell, J.** (1985). Selection-expression plasmid vectors for use in genetic transformation of higher plants. Nucleic Acids Res. **13**, 6981–6998.
- Webster, B.D., and Leopold, A.C.** (1977). The ultrastructure of dry and imbibed cotyledons of soybean. Am. J. Bot. **64**, 1286–1293.
- Werr, W., and Lörz, H.** (1986). Transient gene expression in a Gramineae cell line—A rapid procedure for studying plant promoters. Mol. Gen. Genet. **202**, 471–475.
- Yanisch-Perron, C., Vieira, J., and Messing, J.** (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene (Amst.) **33**, 103–119.