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TRANSIENT GENE EXPRESSION AND INFLUENCE OF PROMOTERS ON FOREIGN GENE EXPRESSION IN *ARABIDOPSIS THALIANA*

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SUMMARY

The influence of a variety of parameters was investigated on polyethylene glycol (PEG)-mediated transient *nptII* and *gus* gene expression in mesophyll protoplasts of *Arabidopsis thaliana* ecotype, Estland, in order to develop a suitable transient gene expression system. The investigation revealed that a combination of 20% PEG, incubation time of 15 min, 20–30 μg plasmid concentration per ml along with 50 μg carrier DNA m/l, and inclusion of calcium and magnesium ions during transfection followed by a culture period of 24 h registered maximum NPTII activity. Of the various promoters used for driving expression of the *gus* gene, the ubiquitin promoter from *A. thaliana* was the most efficient followed by *35S* promoter of the CaMV and the actin promoter of rice. For comparison, similar studies in protoplasts of rice, wheat, and *Brassica* also revealed the differences in strength of these promoters. *Arabidopsis* ubiquitin promoter was the most effective in *Brassica*, and the rice actin1 promoter was the most effective in rice and wheat.

Key words: actin; *Brassica*; *gus*; mesophyll protoplasts; *nptII*; PEG; rice; wheat; ubiquitin.

INTRODUCTION

Arabidopsis thaliana has emerged recently as a powerful model system for investigating plant gene regulation and morphogenesis at the molecular level. Parallel to identifying genes for various phenotypic mutants, there is a growing interest in the study of control of expression of the gene and a demand for methods to analyze various *cis*-acting sequences. Transient gene expression offers a simple and elegant approach for such work as the expression is not biased by position effects, by DNA methylation, or other influences (Nicolaisen and Poulsen, 1993 and references cited therein). Yet few studies of this kind have been made although a number of procedures have been reported to stably transform *Arabidopsis* and regenerate transgenic plants. Transient gene expression has been characterized in detail in cell suspension-derived protoplasts (Axelos et al., 1992; Doelling and Pikaard, 1993), but the establishment and maintenance of cell cultures continues to demand intensive care and is influenced by various exogenous factors. Leaf mesophyll protoplasts are a good alternative in providing a homogeneous population of cells for such investigations (Abel and Theologis, 1994; Hoffman et al., 1994). Towards this end and as a part of a larger program of work on photoregulation of genes in *Arabidopsis*, we have optimized PEG-mediated transient gene expression in mesophyll protoplasts of *A. thaliana* ecotype Estland. The ecotype Estland was chosen as it has been used in the laboratory to isolate mutants through EMS and T-DNA insertion mutagenesis (*see* Khurana et al., 1996; Gandhi et al., 1997). To identify promoters for high level expression of foreign genes, we also investigated the influence of homologous (*UBQ1*) and heterologous (*35S* and *Act1*) promoters in this system and for comparison, in one dicot (*Brassica napus*) and two monocot systems (rice and wheat).

MATERIALS AND METHODS

Plasmids. The *nptII* gene coding for neomycin phosphotransferase was used as the reporter gene for most of the work. The plasmid pHP23 contained *nptII* flanked by CaMV *35S-19S* promoter and *35S* termination signal (Paszkowski et al., 1988). On the other hand, plasmids pBI221 (Jefferson et al., 1987), pAct1-F (McElroy et al., 1990) and pUBQ1gus (Callis et al., 1990) harbored the *gus* gene under the control of CaMV *35S*, rice *Act1* (has an exon 1 and intron 1 also) and *Arabidopsis UBQ1* promoters, respectively. These were used for comparison of transgene activity in different plants. Plasmids were isolated according to the procedure of Birnboim and Doly (1979) and purified by cesium chloride density gradient centrifugation or PEG precipitation.

Isolation of mesophyll protoplasts. Seeds of *Arabidopsis thaliana* ecotype Estland were surface sterilized for 5 min in 3.5% sodium hypochlorite containing 0.1% Triton X-100 and washed subsequently five or six times with sterile distilled water and germinated on B5 basal medium (Gamborg et al., 1968) under aseptic conditions. Protoplasts were isolated from leaves of 3-wk-old seedlings maintained at 25 ± 2° C under a 16-h photoperiod, with 24 μmol m^{-2} s^{-1} photon flux density provided by fluorescent lamps (Philips TL 40 W/54). Leaves were incubated overnight in an enzyme mixture of pH 5.7 comprising CPW salts (Frearson et al., 1973), 1% cellulase Onozuka R-10, 0.5% macerozyme R-10 (Yakult Pharmaceuticals, Japan), 0.4 *M* sucrose, and 0.1% morpholineethanesulfonic acid (MES) at 25° C. Protoplasts were sieved through a 45-μm stainless steel mesh to remove undigested material and centrifuged at 100 ×g for 3 min. Protoplasts floating on the top were collected and washed twice by flotation in SCa buffer of pH 5.7 (0.4 *M* sucrose, 15 m*M* $CaCl_2$, 0.1% MES) and their density adjusted to 3.0×10^6/ml. Viability was assessed with fluorescein diacetate staining (Widholm, 1972).

Brassica napus ISN706 protoplasts were isolated from cotyledons of 7-d-old seedlings [raised on MS basal medium (Murashige and Skoog, 1962), under growth conditions similar to those of *Arabidopsis*] in an enzyme mixture of pH 5.7 comprised of CPW salts, 1% cellulase, 0.5% macerozyme, 0.4 *M* mannitol, and 5 m*M* MES. The cotyledons were incubated overnight and

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protoplasts were first sieved through a 45-μm steel mesh to remove undigested material. They were then purified by two steps of pelleting at 100 ×g for 3 min with MCa of pH 5.7 (0.4 *M* mannitol, 15 m*M* $CaCl_2$, 0.1% MES) as a wash buffer. The protoplast suspension in MCa buffer was then layered on 21% sucrose (also containing 15 m*M* $CaCl_2$ and 5 m*M* MES) and centrifuged at 100 ×g for 4 min to form a ring of protoplasts at the interface of the MCa buffer and sucrose solution. This ring was carefully removed with the help of a Pasteur pipette. The protoplasts were finally suspended in MCa buffer at a density of 3.0 × 10^6/ml and transfected with different *gus* constructs by a procedure similar to that used for *A. thaliana*.

The rice (*Oryza sativa* cv. Rasi) protoplasts were isolated from the basal 3-cm portion of 7-d-old seedlings after the coleoptile was removed. The seedlings were raised on MS basal medium under conditions essentially similar to those for *Arabidopsis*. The tissue was shredded and incubated for 6 h in an enzyme mixture of pH 5.7 comprised of CPW salts, 1% cellulase (RS grade), 0.1% pectolyase, 0.7 *M* mannitol, and 5 m*M* MES. The protoplasts were purified by two rounds of pelleting at 100 ×g for 4 min with MCa of pH 5.7 comprised of 0.7 *M* mannitol, 15 m*M* $CaCl_2$, 5 m*M* MES as a wash buffer. The protoplasts were then layered on 34% sucrose (with 15 m*M* $CaCl_2$ and 5 m*M* MES) and centrifuged at 100 ×g for 4 min. The protoplasts were finally suspended in MCa buffer at a density of 3.0 × 10^6/ml and transfected with different *gus* constructs by a procedure similar to that used for *A. thaliana*.

Wheat (*Triticum aestivum* cv. HD2329) protoplasts were isolated from the basal 2 cm of the second leaf of 7-d-old seedlings raised on MS basal medium under growth conditions similar to those for *Arabidopsis*. After peeling the leaves were incubated in an enzyme mixture of pH 5.7 comprised of CPW salts, 2% cellulase (R10), 2% macerozyme (R10), 0.6 *M* mannitol, and 5 m*M* MES for 5 h. Protoplasts were purified and transfected by a procedure similar to that adopted for rice.

Protoplast transformation. Protoplasts (1 × 10^6) suspended in a volume of 400 μl SCa buffer were placed in a 15-ml graduated conical centrifuge tube to which 100 μl of SCa buffer containing 50 μg plasmid DNA (unless otherwise mentioned) and 50 μg sonicated calf thymus DNA was added. The plasmid DNA and calf thymus had been precipitated with sodium acetate and ethanol, washed with 70% ethanol twice, dried in laminar flow hood and finally dissolved in 1 × TE (10 m*M* Tris-HCLCl and 1 m*M* EDTA; pH 8.0). Then 500 μl PEG 6000 solution of pH 5.9 (40% PEG, 0.4 *M* sucrose, 15 m*M* $CaCl_2$, 0.1% MES) was added, producing a 20% PEG concentration. The tubes were gently swirled to mix the contents and incubated for 30 min (unless otherwise mentioned), followed by gradual dilution of the suspension with 10 ml of SCa buffer over a period of 15 min. This diluted suspension was layered with 1 ml of culture medium [KM (A) salts of Kao and Michayluk (1975) containing 0.4 *M* glucose, 1 mg 2,4-dichlorophenoxyacetic acid per l, 0.5 mg benzylaminopurine per l, and 0.1 mg α-naphthaleneacetic acid per l; pH 5.7] and centrifuged at 100 ×g for 3 min. The protoplasts at the interface were collected and cultured in 1.5 ml of culture medium in 35-mm petri dishes (Nunc, Germany, or Tarsons, India) and incubated in diffuse light for 24 h unless otherwise indicated. The procedure for culture after transformation in the case of *Brassica*, rice, and wheat slightly differed from the above; in these cases, the diluted protoplast suspension after PEG treatment was pelleted at 100 ×g for 3 min (as their buffers contained mannitol) and the protoplast pellet was suspended in 1.5 ml of culture medium and cultured in 35-mm petri dishes.

All the solutions were autoclaved before use except the enzyme mixture and culture media which were filter-sterilized through a 0.2-μm filter. All chemicals used for this work were of analytical grade purchased from Qualigens, India or Hi-media Laboratories, India. The fine chemicals were purchased from Sigma Chemical Company (St. Louis, MO) or United States Biochemicals (Cleveland, OH).

Enzyme assays. The NPTII assay was performed as described by Roy and Sahasradbudhe (1990) with minor modifications. Protoplasts were pelleted in a microcentrifuge tube and protein was extracted with 100 μl extraction buffer (100 m*M* Tris-HCl, pH 7.0; 10 m*M* EDTA, pH 8.0; 0.1% Triton X-100). Protein was quantified by the Bradford method with bovine serum albumin as a standard. The reaction mixture consisted of 50 μl of extraction buffer containing 10 μg protein and 100 μl of assay buffer [100 m*M* Tris-HCLCl, pH 7.8; 10 m*M* $MgCl_2$; 50 μg kanamycin sulfate; 1 μCi [γ^{32}P]ATP (3000 Ci/mmol); 15 μ*M* ATP]. The radionucleotide [γ^{32}P]ATP was purchased from the Board of Radiation and Isotope Technology, India. Following the enzyme reaction (for 30 min), the samples were blotted onto P81 phosphocellulose paper as described by Platt and Yang (1987). The P81 paper was washed first with double-distilled water and then several times with 0.1 *M* phosphate buffer, pH 7.0. The blot was exposed to X-ray film (Kodak X-Omat™, Eastman Kodak Co., Rochester, NY) for autoradiography and later we quantified the activity with a scintillation counter (Beckman Instruments, Inc., Irvine, CA). Values for NPTII-specific activity given in the figures represent the value from which nonspecific NPTII activity observed in controls, i.e., PEG treatment without DNA or DNA treatment without PEG (the overall mean value being 2 × 10^4 CPM/mg protein), has been deducted.

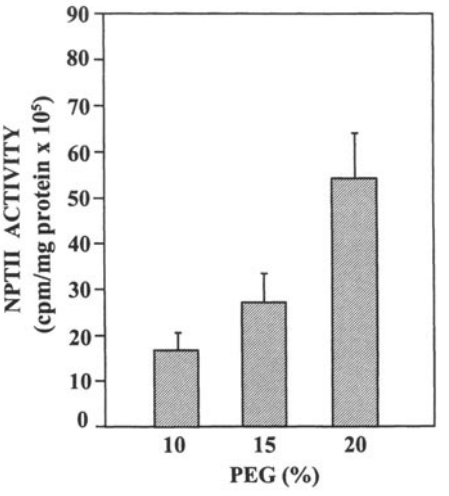

FIG. 1. Effect of different concentrations of PEG during transfection on transient expression of *nptII* as assessed by NPTII activity in mesophyll protoplasts of *Arabidopsis thaliana* after 24 h of culture. *Error bars* reflect the standard error of four independent experiments.

We assayed GUS activity fluorometrically after 24 h of culture as described by Jefferson et al. (1987) using a Shimadzu spectrofluorophotometer Model RF 540 after 14 h of incubation. The 14-h time point was chosen as it was shown earlier in our laboratory that in transient gene expression assays, GUS activity increased linearly for up to 16 h of incubation with the substrate after which it declined (Chaudhury et al., 1993). The relative fluorescence was converted into nmoles methylumbelliferone (MU) produced/mg protein/h with an MU standard. For comparison of GUS activity in different species, the maximum GUS activity obtained with any one promoter in a species was taken as 100 and activity of other promoters was expressed relative to 100.

RESULTS

Optimization of conditions for transient gene expression. Plasmid pHP23 containing the *nptII* gene under the control of the *35S-19S* fusion promoter was used to investigate PEG-mediated transient *nptII* activity. *Arabidopsis* mesophyll protoplasts were obtained in high numbers (up to 2 × 10^7/g fresh wt of tissue) with a viability of more than 90% from 3-wk-old plants. Transient NPTII activity was observed to be a direct function of PEG concentration, 20% PEG displaying the maximum NPTII activity (Fig. 1); higher concentrations were not tested due to practical problems in handling such viscous solutions. When the incubation period with 20% PEG was varied, even a 20-sec incubation was found to be sufficient for detection of high activity (Fig. 2). Longer incubation did not enhance activity; rather it resulted in clumping of protoplasts and proved detrimental for their survival. Thus, in all subsequent experiments, a 15-min incubation time was used, as it registered activity similar to that of 20 sec without affecting viability of protoplasts and also facilitated sample handling.

Transient gene expression was also observed to be influenced by the concentration of plasmid DNA used. When the plasmid DNA

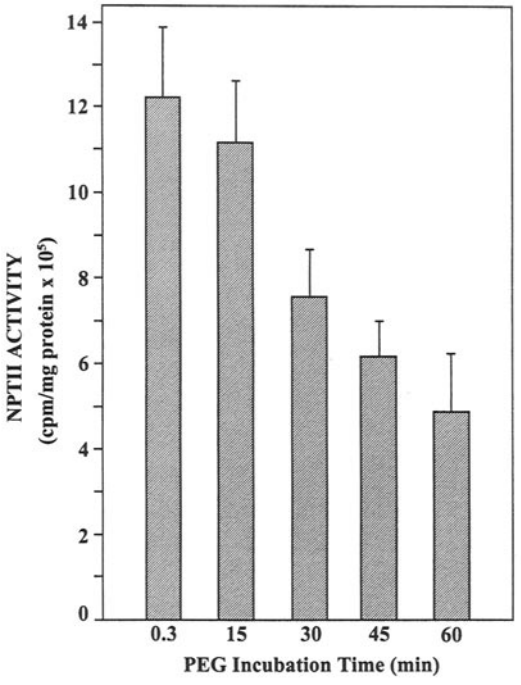

FIG. 2. Effect of duration of PEG (20%) treatment on transient expression of *nptII* as assessed by NPTII activity in mesophyll protoplasts of *Arabidopsis thaliana* after 24 h of culture. *Error bars* reflect the standard error of four independent experiments.

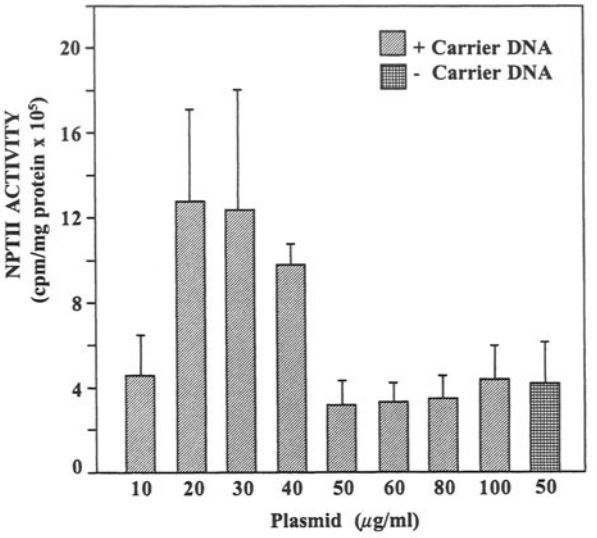

FIG. 3. Effect of plasmid (pHP23) concentration along with a fixed concentration of carrier DNA (50 μg/ml) on PEG-mediated transient expression of *nptII* as assessed by NPTII activity in mesophyll protoplasts of *Arabidopsis thaliana* after 24 h of culture. *Error bars* reflect the standard error of three independent experiments.

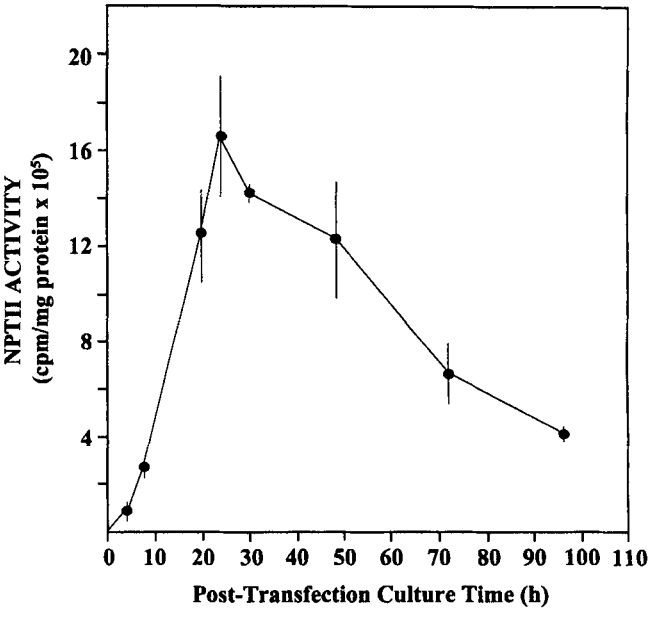

FIG. 4. Effect of duration of culture on transient expression of *nptII* after PEG-mediated transfection as assessed by NPTII activity in mesophyll protoplasts of *Arabidopsis thaliana*. *Error bars* reflect the extent of absolute variation between two independent experiments.

was 20–30 μg/ml along with 50 μg/ml of carrier DNA, maximum NPTII activity was observed (Fig. 3). Higher concentrations of plasmid DNA resulted in clumping of protoplasts, thereby adversely affecting DNA uptake as was apparent from the gene activity assayed subsequently. Further, it was observed that the NPTII activity brought about by 50 μg of plasmid per ml alone is the same as that obtained by 10 μg of plasmid DNA per ml along with 50 μg of carrier DNA per ml, thus amply substantiating the beneficial role of carrier DNA (Fig. 3). However, note that expression using 50 μg plasmid per ml alone is also equivalent to 50 or 60 μg plasmid per ml along with 50 μg carrier DNA per ml. The lower expression in the first case (i.e., 50 μg plasmid alone) could be due to less template DNA, whereas similar expression in the latter case could be due to higher concentrations of DNA, use of which results in clumping of protoplasts.

The study of kinetics of NPTII expression (Fig. 4) showed that maximum activity is attained between 20–30 h of culture after transfection although activity could be detected as early as 4 h after culture. The decline in NPTII activity after 48 h of culture is rather rapid. This could be due to reasons such as degradation of active templates and instability of transcripts and/or enzyme. Viability of protoplasts during the study period remained largely unaffected, thus ruling this out as the cause of decline in NPTII activity (data not shown).

When the effect of calcium (15 m*M* $CaCl_2$) and magnesium (15 m*M* $MgCl_2$) ions was studied individually and collectively (7.5 m*M* $CaCl_2$ + 7.5 m*M* $MgCl_2$) in the transfection buffer, a combination of both ions gave maximum activity, followed by Ca^{2+} and finally Mg^{2+} individually (Fig. 5). As the final concentration of divalent cations was kept constant at 15 m*M*, the possibility that ionic concentration affected NPTII expression is ruled out.

Activity of different promoters. To identify promoters for high level expression of foreign genes, mesophyll protoplasts of *Arabidopsis* were transformed with equimolar amounts of plasmid constructs containing the *gus* reporter gene under the control of different promoters

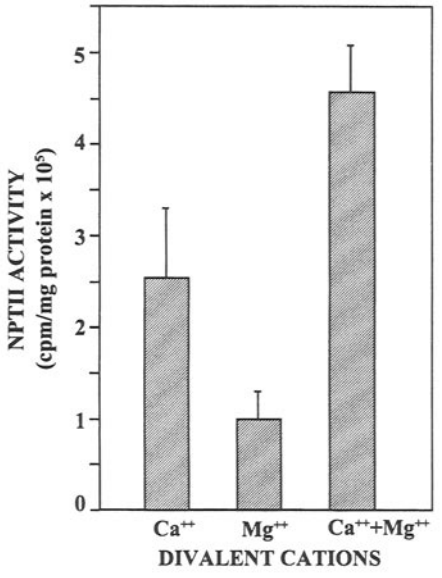

FIG. 5. Influence of divalent cations on PEG-mediated transient expression of *nptII* in mesophyll protoplasts of *Arabidopsis thaliana* after 24 h of culture. *Error bars* reflect the extent of absolute variation between two independent experiments.

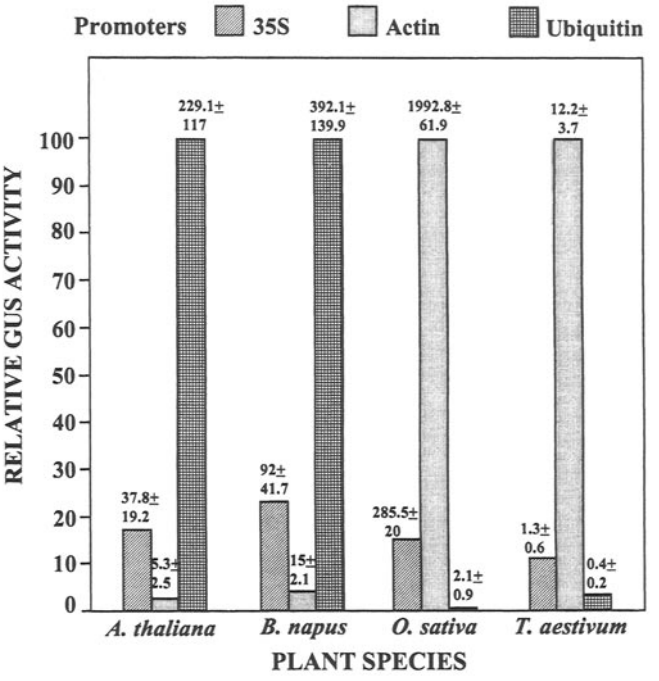

FIG. 6. Effect of various promoters on transient expression of *gus* in leaf protoplasts of various plant species after PEG-mediated gene delivery. GUS activity was determined fluorometrically after 24 h of culture and converted into relative activity by taking maximum activity in a particular species as 100 and equating other values accordingly. *Numerals* above the *bar* represent mean values ± standard error of GUS-specific activity (nmoles MU/mg protein/h) in three or four experiments.

like *35S* of CaMV, actin1 (*Act1*) of rice, and ubiquitin (*UBQ1*) of *Arabidopsis*. Use of the construct containing *gus* under the control of *UBQ1* promoter brought about highest expression of the gene followed by *35S* and *Act1* promoters (Fig. 6). *UBQ1*-driven GUS expression was 23–61 times higher than that with *Act1* promoter and 4–16 times higher than that with the *35S* promoter.

Transient *gus* expression under the control of dicot promoters—*35S* of CaMV and *UBQ1* of *Arabidopsis*—and a monocot promoter, *Act1*, from rice, was also analyzed in protoplasts from leaf bases of *Oryza sativa* and *Triticum aestivum* as well as from cotyledons of *Brassica napus*. The conditions of transformation were similar for all species, the only difference being the type and concentration of osmoticum used during transfection and culture, which was 0.4 *M* sucrose during isolation and transfection of *Arabidopsis* protoplasts, 0.4 *M* mannitol for *B. napus*, 0.7 *M* mannitol for *O. sativa*, and 0.6 *M* mannitol for *T. aestivum*. The osmoticum in the culture medium consisted of 0.4 *M* glucose for *Arabidopsis* and *Brassica*, 0.6 *M* for *T. aestivum* and 0.7 *M* for *O. sativa*. In *O. sativa*, the use of equimolar amounts of different plasmids containing *gus* under the control of different promoters reflected maximum *gus* expression under the control of the *Act1* promoter as assessed by GUS activity fluorometrically. The GUS activity obtained in rice using *Act1* promoter was 6–8 times higher than with *35S* promoter and 550–2000 times higher than with the *UBQ1* promoter. Similarly, in *T. aestivum*, highest *gus* expression was observed with *Act1* which was 10–47 and 8–13 times higher than with *UBQ1* and *35S* promoters, respectively (Fig. 6). This trend of differential expression due to promoters was, however, reversed in the case of cotyledon protoplasts of *B. napus*, where *UBQ1* brought about the highest activity which was 1.5–11 and 5–40 times higher than that of *35S* and *Act1* promoters, respectively (Fig. 6). As the transfection procedure was not optimized for individual species, it was not possible to compare the absolute values of GUS activity brought about by different promoters in different species. Thus, for the comparison shown in Fig. 6, the maximum value of GUS activity obtained in a particular species was considered as 100 and the activity of other promoters calculated accordingly.

DISCUSSION

This study comprised two components: (1) optimization of conditions for transient expression, and (2) a test of efficacy of different promoters. A few points invite comparison with earlier studies. We found the following conditions to be optimal for maximal expression of the transgene in *Arabidopsis*: (a) 20% PEG, with as little as 20 sec being adequate for incubation with the plasmid and protoplasts, (b) 20–30 μg of plasmid along with 50 μg of carrier DNA, (c) presence of both Ca^{2+} and Mg^{2+}, and (d) 24 h posttransfection incubation. Our work is most comparable with that of Hoffman et al. (1994) who also used mesophyll protoplasts of *Arabidopsis* and reported the following conditions for maximal expression: (a) 9% PEG (6000), (b) 100 μg plasmid DNA with 50 μg carrier DNA, (c) 48 h posttransfection incubation, (d) use of linearized plasmid DNA, and (e) PEG pH above 6. Whereas we used the *nptII* gene for most of our work and Hoffman et al. (1994) experimented with the *gus* gene, the optimal time of incubation after transfection can understandably be at variance (for example, different enzymes may have different stabilities). However, the other differences are not so easily explained. However, one should note that the earlier work is on the Columbia

type strain rather than on Estland. Also, Hoffman et al. did not specifically study optimal incubation time of protoplasts with DNA and PEG, nor their requirement for Mg^{2+}. However, researchers using other transgene reporters and recipient plant materials have reported incubation times as short as 2–5 min (e.g., Negrutiu et al., 1990 with tobacco) and higher transient foreign gene expression with Mg^{2+} than with Ca^{2+} (Maas and Werr, 1989 in maize). Clearly, conditions for transient gene expression need careful standardization, and our study should be of value in this regard for future work on *Arabidopsis*.

With respect to the efficacy of different promoters to drive transgene expression in *Arabidopsis*, the *Arabidopsis UBQ1* promoter was far superior to CaMV *35S* which has been commonly used in genetic engineering, whereas the *Act1* promoter (belonging to distantly related rice) gave the lowest expression. Conversely, in rice protoplasts, rice *Act1* promoter gave the maximal expression whereas expression from the *UBQ1* promoter was barely detectable. Although this study is not extensive, our results agree with those of previous reports concerning lower efficiency of heterologous promoters. The requirement of some degree of homology between promoters and host cells is supported also by transfection studies on *Brassica*, rice and wheat protoplasts. *Brassica* is a crucifer related to *Arabidopsis* and the *UBQ1* promoter worked well in protoplasts of this plant too, whereas the *Act1* promoter was almost as effective in wheat as in rice (both belong to Gramineae). The *Act1* promoter contains an intron (which has been reported to increase transgene expression) and the high efficiency in monocots could be due to more efficient splicing in monocots than in dicots. The lower efficiency of monocot-specific promoters has been reported in dicots, in cases in which the promoters used also carried a monocot-specific intron in the untranslated leader sequences of the transcribed RNA molecule (Callis et al., 1987). This lower efficiency could result from inefficient splicing of monocot introns in a dicot system (Callis et al., 1987; Maas et al., 1991) due to differences in the gap junctions between monocot and dicot introns (Hanley and Schuler, 1988). The low expression of *gus* using rice *Act1* promotor in *Arabidopsis* & *Brassica* is also in conformity with studies of McElroy et al. (1991) in which no GUS expression was obtained with the rice *Act1* promoter (with or without intron) in tobacco suspension culture cells and reduced expression of GUS using *35S* promoter when it was hooked to an intron of the rice *Act1* gene. In the same report, very high GUS activity was observed in rice and maize cells with the rice *Act1* promoter.

Holtorf et al. (1995) reported lower *gus* expression with *Arabidopsis UBQ1* promoter than with CaMV *35S* promoter in *Arabidopsis* transformants. However, their study reports results of stable rather than transient expression, and there could be other explanations for this apparent contradiction. For example, in stable transformation, expression is known to depend also on methylation, and site of integration of foreign gene and position effect comes into play. These points were exemplified as large differences in GUS expression levels in the entire seedlings as compared to the leaves of *35Sgus* and *UBQ1gus* transgenic lines. Further, in the present investigation, the *35Sgus* construct used has a *nos* terminator whereas the one used by Holtorf et al. (1995) had *35S* terminator sequences. Thus, our study and theirs are not strictly comparable.

Acknowledgments

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