Phytochemistry, Vol. 28, No. 2, pp. 323–327, 1989.
Printed in Great Britain.

0031–9422/89 $3.00 + 0.00

USE OF OSMOTICALLY PERMEABILIZED PLANT PROTOPLASTS TO STUDY DNA SYNTHESIS

HOWARD L. VANZEGEREN, ADRIAN J. CUTLER and MOHAMMED SALEEM

Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Saskatchewan S7N 0W9, Canada

(*Received in revised form* 22 *July* 1988)

Key Word Index—*Bromus inermis*; Gramineae; bromegrass; protoplasts; biosynthesis; DNA; deoxyribonucleotides.

Abstract—A method has been developed to measure DNA synthesis in protoplasts freshly isolated from bromegrass cell suspension culture. The method involves exposing the protoplasts to a transient hypotonic shock in the presence of magnesium, substrate deoxyribonucleotides and ATP as an energy source. Incorporation was linear for up to 45 min and was not reduced by addition of thymidine. The optimal pH was 6.5. Incorporation was decreased by over half in the presence of aphidicolin, a potent inhibitor of replication.

INTRODUCTION

The application of many modern genetic engineering techniques to plants requires the use of totipotent (regenerable) protoplasts. Although protoplasts from many dicotyledonous species can be cultured easily, cereal protoplasts frequently show no mitotic activity under similar conditions [1]. In view of these problems, it is of particular interest to study fundamental aspects of mitosis in cereals.

The molecular and cellular processes involved in plant cell division are as yet poorly understood. An important component of these processes is DNA replication in S-phase. More extensive studies on replication have been carried out in animal cells for which a variety of *in vivo* and *in vitro* techniques have been developed. In particular, *in vivo* DNA synthesis has been investigated by means of permeabilized cells [2], in which the synthetic apparatus is maintained intact, but can be probed by reagents that, under normal circumstances, are impermeable. These reagents include specific inhibitors (including antibodies) and potential growth factors. In this way, for example, the role of diadenosine 5′,5‴-P^1,P^4-tetraphosphate in triggering S-phase in animal cells was clearly demonstrated [3]. Early methods for permeabilizing animal cells frequently employed detergents or other chemicals likely to perturb metabolic function [2]. Recently, less stressful techniques such as hypotonic shock [4] and electroporation [5] have been employed for these kinds of experiments.

We have recently investigated methods for permeabilizing plant protoplasts and shown that this can be accomplished conveniently by a transient hypotonic shock [6]. Preliminary evidence that the method was appropriate for studies of DNA synthesis and transcription was also obtained [7]. In this report, data is presented concerning the requirements for maintaining DNA synthesis in permeabilized bromegrass protoplasts.

RESULTS

In order to establish appropriate permeabilization conditions, protoplasts were exposed to various degrees of hypotonic shock in the presence of the fluorescent dye calcein. When significant permeabilization had occurred, protoplasts became brightly fluorescent due to the presence of the dye inside the cell. The results presented in Fig. 1A show that the fraction of the surviving protoplasts that were fluorescent increased as the degree of hypotonic shock increased. However, when results are expressed as a percentage of the initial number of protoplasts (Fig. 1B), the total number of fluorescent protoplasts increased to a maximum when a 300 mOsm treatment was given and decreased with more drastic shocks. It was clear that treatments of 100 and 200 mOsm produced a large increase in the number of dead cells and that this outweighed any increased permeabilization. In subsequent experiments, permeabilization solutions were made up to 250–300 mOsm such that after addition to the protoplasts, and dilution by residual buffer, the final osmotic strength would be as close as possible to 300 mOsm. It should be noted that there was no difference in viability between osmotically stressed (300 mOsm) and control (500 mOsm) protoplasts as measured by phenosafranin staining [8] after they had been returned to control media.

Initial experiments in which incorporation of TTP into DNA was measured employed trichloroacetic acid precipitation, and resolubilization in sodium hydroxide. However, more consistent incorporation was obtained using the ethanolic precipitation procedure described in Experimental.

The results presented in Fig. 2A show that with a complete assay system containing ATP, PEP (phosphoenolpyruvate), magnesium chloride and the deoxynucleotides, incorporation was linear for up to 45 min. Furthermore, subsequent treatment of the lysed extract

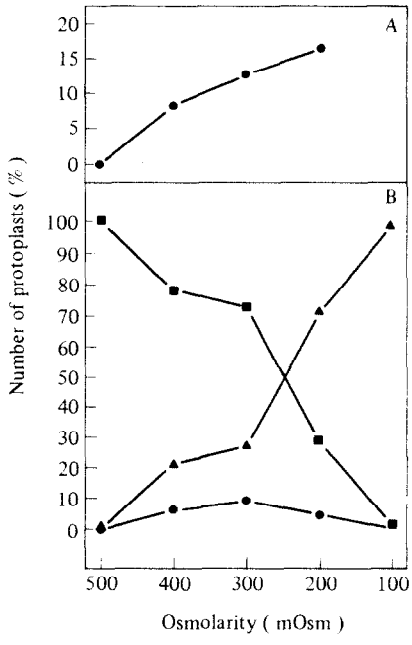

Fig. 1. Optimising hypotonic shock conditions. Permeabilization was performed in standard buffer containing 5 mM calcein and adjusted to the appropriate osmotic strength with sorbitol. After a 30 min incubation protoplasts were washed thoroughly with standard buffer and counted in a haemocytometer. The percentage of the surviving protoplasts that had been permeabilized (fluorescent) was estimated by counting under fluorescent illumination. This technique has been described in detail elsewhere [4]. 1A; percentage of the surviving protoplasts that were fluorescent. 1B; results expressed as a fraction of the protoplasts in the 500 mOsm control: ■—■, number of protoplasts surviving the treatment; ▲—▲, number of protoplasts killed by the treatment; ●—●, number of fluorescent protoplasts.

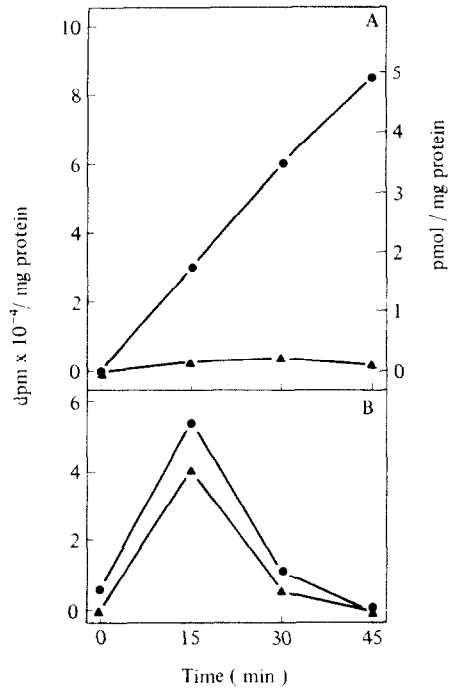

Fig. 2. Sensitivity of alcohol precipitable radioactivity to DNAse. 2A; permeabilization buffer contained the components described in Experimental except that the ATP concentration was 1.25 mM. 2B; $MgCl_2$ was omitted from the components in Fig. 2A. Assays were performed as described in Experimental with (▲) or without (●) DNAse treatment.

with DNAse demonstrated that all of the observed incorporation was into DNA. Incorporation is expressed in molar terms for comparison and shows a rate of 0.67 pmol of TTP (thymidine triphosphate) per hour per mg protein. This is equivalent to 1.1 pmol per 10^7 protoplasts. In the absence of any of the components of the complete system non-linear incorporation was observed. This is illustrated in Fig. 2B with respect to the requirement for magnesium chloride. DNAse treatment revealed that under these conditions, the observed incorporation was not into DNA. The nature of the material formed after 15 min is unknown.

Data demonstrating the requirement for ATP is shown in Table 1. In the absence of an exogenous energy source, incorporation was at the background (zero time) level and a concentration of 5 mM was necessary to maximize incorporation.

Table 1. ATP requirement for DNA synthesis in permeabilized protoplasts

ATP concn (mM)	Incorporation (%)
0	0
1.25	65
5	100*

* 7.8×10^4 dpm/mg protein.

Experiment performed as described in Experimental except that the permeabilization solution contained 10 mM $MgCl_2$ and 0.25 mM unlabelled dNTP's. Incorporation is reported after a 30 min incubation, with the dpm at zero time subtracted.

Initial experiments were performed with the permeabilization solution at a pH of 5.8, a standard pH for protoplast culture. The experiment reported in Fig. 3 shows that a more appropriate pH for measuring *in vivo* DNA synthesis in protoplasts in 6.5. In addition, when permeabilization solutions contained Tris–HCl (2-amino-2(hydroxymethyl)-1,3-propandiol), non-linear incorporation often resulted (data not shown). MES [2-(*N*-morpholino)ethanesulphonic acid] buffer and a pH of 6.5 were used in all subsequent experiments.

Other experiments to optimize the system (data not shown) showed that addition of PEP was not absolutely required but gave optimal results. No requirement for manganese chloride could be demonstrated and potassium chloride (50 mM) was inhibitory. No benefit was obtained by increasing the concentration of the unlabelled deoxyribonucleotides to a higher level than that

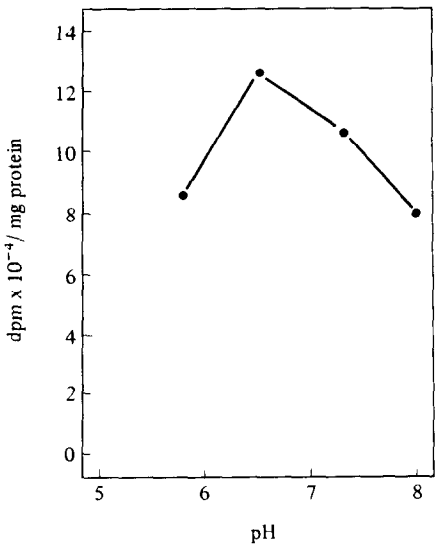

Fig. 3. Sensitivity of incorporation to pH. Experiments were performed as described in Experimental except that the pH of the permeabilization buffer was adjusted as indicated using either MES buffer (up to pH 6.5) or Tris–HCl (above pH 6.5). Incubation time was 30 min.

of the ^{3}H-TTP (0.1 mM). Addition of ribonucleotides (GTP, CTP, UTP, all at 0.1 mM) to the permeabilization solution did not result in increased incorporation.

In order to demonstrate that incorporation was directly from TTP, and not after degradation of TTP to thymidine, the experiment reported in Table 2 was performed. Addition of an excess of thymidine to the permeabilization solution did not significantly inhibit incorporation of labelled TTP into DNA after a 30 min incubation.

DNA synthesis in freshly isolated protoplasts was characterized using inhibitors (Table 3). Neither ddTTP (dideoxythymidinetriphosphate) nor NEM (*N*-ethylmaleimide) can cross the plasma membrane of unpermeabilized cells. Preliminary experiments were performed to establish the concentration at which each inhibitor produced maximum effect (results not shown). Using the previously established concentrations, aphidicolin produced a 55% inhibition, NEM a 75% inhibition, and ddTTP a 17% inhibition. Combinations of inhibitors produced no more than a 69% inhibition.

Table 2. Effect of thymidine on incorporation of TTP into DNA

Addition	Incorporation (%)
None	100*
Thymidine	91

* 74.6×10^4 dpm.

Procedure was as described in Experimental except that 1 mM thymidine was added to the permeabilization solutions where indicated. Incorporation is reported after a 30 min incubation.

Table 3. Effect of inhibitors on incorporation of TTP into DNA

Inhibitor	Incorporation (%)
None	100*
Aphidicolin	45
NEM	25
ddTTP	83
Aphid + ddTTP	31
Aphid + ddTTP + NEM	33

* 58×10^4 dpm.

Experiments were performed as described in Experimental except that, where indicated, inhibitors were added at the following concentrations. Aphidicolin; 40 μg/ml, NEM; 2 mM, ddTTP; 1 mM.

DISCUSSION

The requirements for assaying DNA synthesis in hypotonically shocked Bromegrass protoplasts were quite different than those in lysolecithin-permeabilized soybean cells [9]. In both cases there was an absolute requirement for magnesium chloride, but in the lysolecithin system neither substrate dNTPs nor ATP (or an ATP generating system) was an absolute requirement. Neither system responded to the presence of NTPs (ribonucleoside triphosphates) as extension primers (DNA primase substrates, [10]). The most striking difference between the assay conditions in the two systems was the pH at which the assays were carried out. In the hypotonic shock system reported here the best pH was 6.5 (Fig. 3); whereas in the lysolecithin system a narrow pH optimum in the range 8.0–8.5 was reported. This latter range is close to the pH optima reported by Dunham and Bryant [11] for partially purified α, β, and γ polymerase activities from *Brassica rapa* (pH 7.8–8.0). This apparently large difference may be explained by the type of permeabilization procedures employed. Lysolipids are incorporated permanently into membranes and invariably cause cell death at high concentrations [2]. Plant protoplasts are not only particularly susceptible to lysolipids [12] but the concentration employed (500 μg/ml) was higher than concentrations typically used with animal cells (eg. 150 μg/ml in ref. [13]). Therefore it is likely that the intra- and extra-cellular pHs equilibrated following lysolecithin treatment so that the assay had to be carried out at the *in vitro* pH optimum. On the other hand we have shown, both in this system and previously in soybean protoplasts [7], that hypotonically shocked protoplasts reseal their membranes after treatment and maintain long-term viability. Consequently, the assay conditions reflect the pH conditions under which plant cells are usually cultured.

The requirements for measuring DNA synthesis in bromegrass protoplasts were similar to those required for analogous experiments with hypotonically shocked animal cells (e.g. [3 and 14]). Although the animal cell assays were carried out at slightly alkaline pH no significance

can be attributed to this since animal cells are usually cultured at a similar alkalinity. Assay mixtures for permeabilized animal cells usually contained either potassium [13, 3] or sodium chloride [14]. Although Dunham and Bryant reported that β and γ type polymerases were stimulated by potassium chloride [11], purified α polymerase from spinach was strongly inhibited [15]. In the bromegrass system KCl had an overall inhibitory effect. Although measurable DNA synthesis was low, two factors have to be taken into account. Firstly, only *ca* 10% of protoplasts are permeabilized by hypotonic shock (Fig. 1) and secondly, many protoplasts may not have entered S-phase.

The enzymes of DNA synthesis have been classified into three categories based largely on work in animals [16]. α polymerases are involved in nuclear DNA replication; β polymerases are involved in nuclear repair and γ polymerases are involved in organellar replication. Aphidicolin has been repeatedly shown to be a specific inhibitor of both plant and animal DNA polymerase α and therefore of replication [10]. Over half (55%, Table 3) of the total observable incorporation can therefore be assigned to replication. By contrast, Cannon *et al.* [9] observed no aphidicolin sensitive incorporation in lysolecithin permeabilized soybean cells. Since the soybean cells were presumably actively growing, this implies that the lysolecithin method leads to inhibition of replication. α type polymerases have also been shown to be inhibited by NEM. In addition, plant β type polymerases are very sensitive to NEM [11, 16] in contrast to β type enzymes from animal sources [16]. Consistent with this, NEM inhibited 75% of the bromegrass activity, suggesting that if 55% of the incorporation was due to replication then 20% was from repair (β) activity. The inhibitor ddTTP reduced activity by 17%. Since a combination of all three inhibitors was unable to completely abolish incorporation it seems most likely that firstly, the γ type (organellar) synthesis is resistant to any of the inhibitors used and secondly, that ddTTP specifically inhibits β type activity. Consistent with this, a combination of aphidicolin and ddTTP produced about the same inhibition as NEM. Wheat β polymerase has been shown to be inhibited by this compound whereas α polymerase from this and other sources (including animals) was resistant. It should be noted, however that γ enzymes from animals are known to be sensitive to ddTTP; however it is by no means clear to what degree plant organellar DNA polymerases fall into the γ category [16].

In summary, a method for studying *in vivo* DNA synthesis has been developed that should be generally applicable to plant protoplasts. The requirements of the assay were broadly similar to analogous systems used with animal cells. The results clearly showed that freshly isolated bromegrass protoplasts incorporated nucleotides into DNA. Under the conditions of the assay, over half of the incorporation was replicative indicating that at least some of the protoplasts were in S-phase. The remainder of the incorporation was due to a combination of nuclear repair and organellar replication. Further studies will allow a more detailed comparison of DNA synthesis in animal and plant cells.

EXPERIMENTAL

Preparation of protoplasts. Protoplasts were prepared from bromegrass (*Bromus inermis* Leyss) suspension culture line Bg 970 [17] two or three days after transfer to fresh medium. Cell suspensions were maintained in modified Kao's medium [18] containing 1 mg/l 2,4-D, 0.1 mg/l zeatin riboside and 5 g/l glucose. Suspensions were grown in 50 ml of medium in 250 ml flasks on a rotary shaker at 25°. After allowing the cells to settle, the medium was decanted and replaced by 20 ml of enzyme soln containing 1.5% Onozuka cellulase, 0.75% hemicellulase and 1.5% pectinase dissolved in standard buffer MM-1 (3 mM MES 6.8 mM $CaCl_2$, 0.7 mM NaH_2PO_4 and 0.5 M sorbitol pH 5.8). Cells were transferred to two Petri dishes on a shaker (30 rpm) and incubated with enzyme soln overnight. In a typical experiment, up to 8 flasks of cells were used to make protoplasts. Next morning protoplasts were filtered through a sterile nylon mesh (41 μm) with a glass wool pre-filter then divided into aliquots. The protoplasts were pelleted in a bench top centrifuge (100 *g*) and washed with MM-1.

Permeabilization. Protoplasts were resuspended in an equal vol. of MM-1, gently mixed, then 150 μl aliquots (containing *ca* 0.75×10^6 protoplasts) were transferred to microfuge vials and pelleted at 100 *g*. The supernatant was removed and 172 μl of assay soln was added. Assay solns were prepared fresh and contained (unless otherwise indicated) MM-1 components plus 5 mM ATP, 5 mM PEP, 15 mM $MgCl_2$, 0.1 mM of each of the unlabelled deoxynucleotides, 0.1 mM ^{3}H-TTP (15 μCi) and sorbitol to give an osmolarity of 250–300 mOsm, the pH was adjusted to 6.5. Purity of the radioactive substrate was monitored and maintained by TLC.

Measurement of DNA synthesis. After the appropriate incubation time, 25 μl aliquots of the suspension were added to 100 μl TES buffer (100 mM Tris–HCl, 20 mM EDTA, 1% sodium dodecyl sulphate, pH 7.4) and mixed thoroughly. Following this 200 μg of calf thymus DNA was added (40 μl) and, after mixing, 0.5 vol of 7.5 M NH_4OAc (*ca* 83 μl) followed by 2.5 vol of EtOH (*ca* 413 μl). The DNA was then pptd by incubation at $-20°$ for 45 min. After centrifugation (5000 *g*, 5 min), DNA was redissolved in TES and reprecipitated by ethanolic NH_4OAc. Finally DNA was dissolved in 1 ml of TES and transferred to a scintillation vial. After addition of 10 ml of scintillation fluid, radioactivity was measured in a scintillation counter. Protoplasts remaining at the end of the incubations were homogenised in TES buffer and frozen at $-20°$ until protein determinations were performed using the Bradford method [19]. Background incorporation was subtracted from every value reported in this paper. Typically, background accounted for a third of the total counts after a 30 min incubation. Although this is high relative to many other biochemical assays, consistent reproducible results were obtained in all of the experiments reported. All data is expressed in terms of protein, since less variability is associated with the Bradford method than with counting protoplasts in a haemocytometer. However conversion of data to a per cell basis can be made based on the fact that 6×10^6 protoplasts yields 1 mg of protein.

In order to measure sensitivity of EtOH precipitable material to DNAse, protoplasts were osmotically lysed in the absence of SDS and treated with 2 mg/ml of pancreatic DNAse (freshly prepared) in the presence of 4.2 mM $MgCl_2$ for 60 min before EtOH pptn. No significant degradation of DNA occurred in the absence of DNAse under otherwise identical conditions.

Equipment and chemicals. Osmolarities were measured with a micro Osmette freezing point osmometer (Precision Systems Inc. Natick, MA). [Me-^{3}H]TTP (75 Ci/mmol) was obtained from New England Nuclear. Onozuka R-10 cellulase was obtained from Kanematsu-Gosha, Vancouver, B. C., fungal pectinase was obtained from Fluka AG, Terochem Labs, Edmonton, Alta., hemicellulase was from Rohm and Haas Co., Canada Ltd., West Hill, Ontario. All other chemicals were obtained from Sigma Chemical Co.

Acknowledgement—This publication is National Research Council of Canada No. 28384.

REFERENCES

1. Vasil, I. K. (1987) *J. Plant Physiol.* **128**, 193.
2. Felix, H. (1982) *Anal. Biochem.* **120**, 211.
3. Grummt, F. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 371.
4. Okada, C. Y. and Rechsteiner, M. (1982) *Cell* **29**, 33.
5. Sokoloski, J. A., Jastreboff, M. M., Bertino, J. R., Sartorelli, A. C. and Narayanan, R. (1986) *Anal. Biochem.* **158**, 272.
6. Cutler, A. J. and Saleem, M. (1987) *Plant Physiol.* **83**, 24.
7. Saleem, M. and Cutler, A. J. (1986) *J. Plant Physiol.* **124**, 11.
8. Widholm, J. M. (1972) *Stain Technol.* **47**, 189.
9. Cannon, G. C., Heinhorst, S. and Weissbach, A. (1986) *Plant Mol. Biol.* **7**, 331.
10. Sala. F. (1985) in *Molecular Form and Function of the Plant Genome.* (Vloten-Doting, L. van., Galli, M. G. and Hall, T. C., eds), p. 141. Plenum Press, New York.
11. Dunham, V. L. and Bryant, J. A. (1986) *An. Botany* **57**, 81.
12. Nagata, T., Eibl, H. and Melchers, G. (1979) *Z. Naturforsch.* **34c**, 460.
13. Castellot, J. J., Miller, M. R., Lehtomaki, D. M. and Pardee, A. B. (1979) *J. Biol. Chem.* **254**, 6904.
14. Griffiths, T. D. and Carpenter, J. G. (1980) *Exp. Cell Res.* **130**, 470.
15. Misumi, M. and Weissbach, A. (1982) *J. Biol. Chem.* **257**, 2323.
16. Litvak, S., Castroviejo, M. (1985) *Plant Mol. Biol.* **4**, 311.
17. Kao, K. N., Gamborg, O. L., Michayluk, M. R., Keller, W. A. and Miller, R. A. (1973) *Colloq. Intern. CNRS* **212**, 207.
18. Kao, K. N. (1982) in *Plant Tissue Culture Methods.* (Wetter, L. R. and Constabel, F., eds), p. 49. National Research Council, Saskatoon.
19. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248.