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DNA Binding and Uptake by Nuclei Isolated from Plant Protoplasts

FACTORS AFFECTING DNA BINDING AND UPTAKE[1]

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ABSTRACT

DNA binding and uptake by nuclei isolated from soybean (*Glycine max* L. Merr.) protoplasts were investigated using radioactive homogeneous DNA prepared from soybean cells. DNA binding to nuclei was found to decrease drastically with increased incubation time. Total uptake and acid-precipitable uptake reached a maximum after 20 minutes of incubation. Optimum DNA binding and uptake occurred at pH 6 and the process was enhanced by increasing the incubation temperature to 40 C. *Salmonella typhimurium* DNA and poly([dA-dT]-[dA-dT]) competitively inhibited DNA binding whereas calf thymus DNA was less competitive; however, *Micrococcus lysodeikticus* DNA stimulated DNA binding and tobacco mosaic virus RNA had no effect. DNA binding and uptake was enhanced by addition of Mg ions, Ca ions, poly-L-lysine, and ATP. Increasing amounts of EDTA appeared to decrease DNA binding. Pronase strongly inhibited DNA binding and uptake.

Genetic modification of higher plants by feeding exogenous DNA has been reported (4, 8, 14). Because plant protoplasts lack a cell wall they may have distinct advantages over normal plant cells for exogenous DNA uptake (5, 11, 13, 15). Incorporated DNA may be degraded and reutilized for *de novo* DNA synthesis prior to genetic expression. Exogenous DNA must be stabilized as well as replicated before the genetic information can be expressed in the cell. A possible mechanism for stabilization is the integration of DNA into host genome. Exogenous DNA must therefore penetrate through the cytoplasm, and must be adsorbed to nuclear membrane without being degraded.

Some investigators have observed that DNA taken up by cells was bound to the nuclei using radioautographic techniques (6, 7) and by analyzing nuclei isolated from protoplasts fed labeled DNA (15). This paper describes some of the factors affecting *in vitro* DNA binding and DNA uptake by nuclei isolated from soybean protoplasts.

MATERIALS AND METHODS

Cell Culture and Preparation of Protoplasts. Soybean cells (*Glycine max* L. Merr.) (SB-1) grown in 1-B5 medium (3) were used throughout this work. Protoplasts were prepared by enzymic removal of cell wall as described previously (10).

Isolation of Nuclei from Protoplasts. Protoplasts washed with 0-B5 medium (sucrose-free) containing 0.275 M sorbitol were suspended to 0-B5 (sucrose-free) medium containing 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, and 0.5% Triton X-100 (medium A) plus 0.275 M sorbitol. The protoplasts were disrupted by a Dounce homogenizer with 10 gentle strokes. The homogenate was passed once through a layer of Miracloth and twice through a triple layer of Miracloth. Filtrate was layered on 4 ml of medium A containing 0.4 M sorbitol and centrifuged at 400*g* for 5 min in an International model HN centrifuge. The pellet was suspended in 2 ml of medium A containing 0.275 M sorbitol. This step was repeated twice to remove debris, cytoplasmic organelles, and starch granules. The nuclei suspension was layered on stepwise gradients of 5 ml of 0.5 M sorbitol in medium A and 2 ml of 1 M sorbitol in medium A, and centrifuged at 100*g* for 3 min. The top layer containing the nuclei was withdrawn and spun down at 400*g* for 5 min, and the pellet suspended in 2 ml of medium A containing 0.275 M sorbitol. This process was repeated until larger particles such as small unbroken and dead protoplasts were completely removed. Nuclei were finally suspended into 2 ml of 0.01 M tris-HCl buffer (pH 7) containing 0.275 M sorbitol, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, and 0.1% of Triton X-100. This nuclei suspension was used for the DNA binding and uptake experiments. The nuclei number was counted in a hematocytometer.

DNA Binding and Uptake by Isolated Nuclei. Standard DNA binding and uptake mixture consisted of a total volume of 0.5 ml; 0.1 ml of nuclei suspension (10^6 nuclei), 0.1 ml of 1 M sorbitol solution, 0.1 ml of 0.05 M tris-HCl (pH 7) containing 10 mM $CaCl_2$, 0.1 ml of distilled H_2O, and 0.1 ml of ^{3}H-DNA isolated from SB-1 cells (80,000 cpm, specific radioactivity 3.7 × 10^6 dpm/μg; kindly supplied by A. Kodouri, University of Utah) and incubated at 30 C for 20 min.

For determination of DNA binding, nuclei were washed three times with medium A containing 0.275 M sorbitol (5 ml each time) and lysed by the addition of 2% SDS solution. The lysate was counted with 5 ml of toluene-Triton X-100 containing 2-(4-*tert*-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxidiazole (butyl-PBD, 5 g/l) in Nuclear-Chicago scintillation counter.

For DNA uptake determination, washed nuclei were suspended in 0.4 ml of 0.01 M tris-HCl (pH 7) containing 0.275 M sorbitol, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.1% Triton X-100, and 0.1 ml of DNase solution (1 mg/ml, Worthington) added, and incubated at 30 C for 5 min. Nuclei then were washed three times with medium A containing 0.275 M sorbitol and lysed in 0.5 ml of 2% SDS solution. Radioactivity in 0.1 ml of lysate was counted for total uptake. To the rest of the lysate 0.5 ml of 10% trichloroacetic acid was added. The precipitate was collected on a glass fiber filter and washed with cold 5% trichloroacetic acid, ethanol, and ether, successively. The filter was then air-dried and counted to measure acid-precipitable uptake.

RESULTS

Incubation Time of DNA Binding and Uptake. Figure 1A shows that maximum DNA binding to nuclei took place in less time than was required to wash the nuclei, and was nearly complete in 45 min. Total uptake and acid-precipitable uptake

[1] NRCC No. 15963.

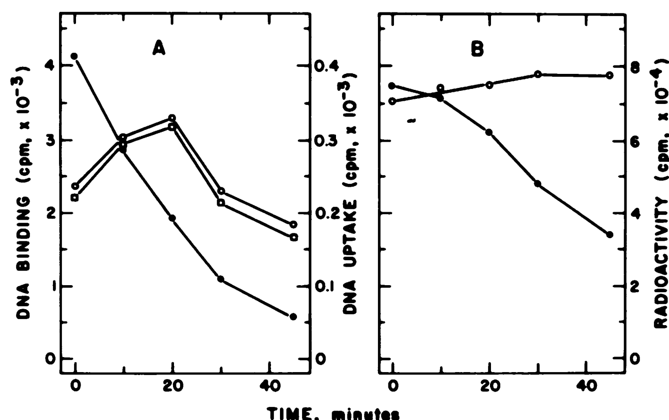

FIG. 1. A: kinetics of soybean ^{3}H-DNA binding and uptake by isolated nuclei from soybean protoplasts. (●——●), ^{3}H-DNA binding; (○——○), total uptake of ^{3}H-DNA; (□——□), acid-precipitable uptake. B: DNase activity in isolated nuclei. (●——●), acid-precipitable ^{3}H-DNA in first washing medium; (○——○), total radioactivity remaining in first washing medium. Average of duplicate experiments.

reached a maximum after 20 min incubation followed by a gradual decrease. These results suggest that isolated nuclei contain nucleases, which are active during DNA binding and uptake. Figure 1B shows the total and acid-precipitable radioactivity remaining in the washing medium. The high mol wt DNA (acid-precipitable radioactivity) appeared to decrease to 45% of initial amount by 45 min although total radioactivity remained unchanged.

Effect of DNA Concentration. Both DNA binding and uptake experiments have been performed by addition of nonradioactive soybean DNA (isolated from protoplasts and purified by CsCl centrifugation) to a constant amount of soybean ^{3}H-DNA. The DNA binding and uptake were calculated on the basis of specific radioactivity of total input DNA. Both DNA binding and uptake by isolated nuclei increased with increasing amounts of DNA up to 20 μg/ml, and apparently saturation occurred above 50 μg/ml (Fig. 2).

Effect of pH. DNA binding was found to be very low at pH 8 and 9. Maximum DNA binding occurred at pH 6. Total uptake and acid-precipitable uptake appeared to be maximum at about pH 6 (Fig. 3).

Effect of Incubation Temperature. DNA binding was profoundly influenced by the temperature. DNA binding to nuclei increased linearly from 0 C up to 40 C (Fig. 4). Total uptake increased sharply in the range of 30 to 40 C. Incorporation into the acid-precipitable fraction also showed a linear relationship but the rate was greatly reduced.

Effects of Divalent Cations. Both Ca and Mg ions stimulated DNA binding and uptake when added at concentrations of 10 and 20 mM. EDTA was not inhibitory but at low concentrations produced a slight stimulation of DNA binding and uptake (Table I).

Effect of Poly-L-Lysine. High mol wt poly-cations are known to increase DNA uptake by plant protoplasts (11, 13, 15) as well as to stimulate infection of viral RNA (1). As shown in Table II at a concentration of 1 μg/ml, poly-L-lysine (mol wt 150,000) considerably enhanced DNA binding and uptake. Strikingly, most of the DNA bound to nuclei was found to be DNase-resistant and over 80% of radioactivity was accounted for in the acid-precipitable fraction. However, at 5 μg/ml the poly-L-lysine had no effect on binding but bound DNA was again DNase-resistant.

Effects of Various Substances. Carbon sources are known to stimulate DNA uptake and transformation in microorganisms (12). In the present soybean nuclei system adding glucose to the incubation medium had no apparent effect on DNA binding and uptake (Table III). The ATP generating system strongly stimulated DNA binding and uptake (Table III). Figure 5 shows the effect of adding ATP at various concentrations. There was a parallel increase in uptake and incorporation of DNA with increase in ATP. Pronase strongly inhibited DNA binding and uptake (Table III). Increasing amounts of pronase significantly reduced DNA binding and concomitantly, DNA uptake (Fig. 6). Tobacco mosaic virus (TMV) RNA did not compete with DNA for binding and uptake at a concentration of 4 μg/ml (Table III).

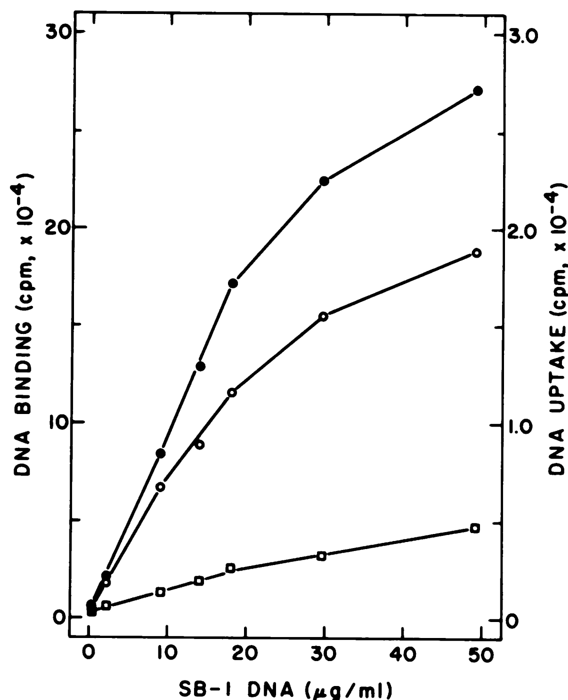

FIG. 2. Effect of DNA concentration on soybean ^{3}H-DNA binding and uptake by isolated nuclei. (●——●), ^{3}H-DNA binding; (○——○), total uptake of ^{3}H-DNA; (□——□), acid-precipitable uptake of ^{3}H-DNA. Average of duplicate experiments.

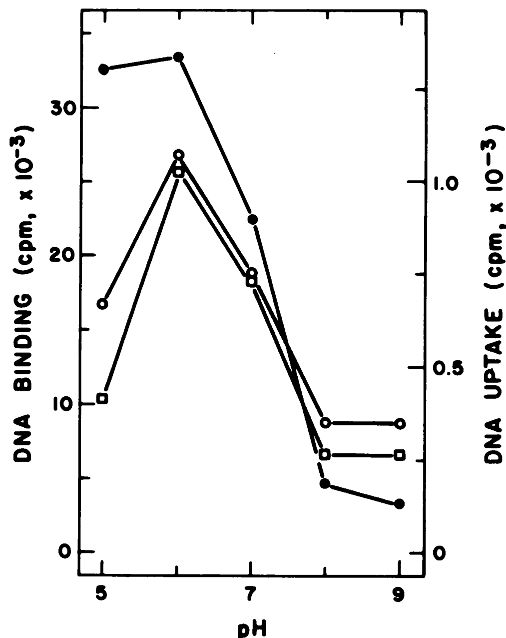

FIG. 3. Effect of pH on soybean ^{3}H-DNA binding and uptake by isolated nuclei. (●——●), ^{3}H-DNA binding; (○——○), total uptake of ^{3}H-DNA; (□——□), acid-precipitable uptake of ^{3}H-DNA. 0.01 M MES buffer was used for pH 5 and 6 and 0.01 M tris-HCl buffer used for pH 7, 8, and 9. Average of duplicate experiments.

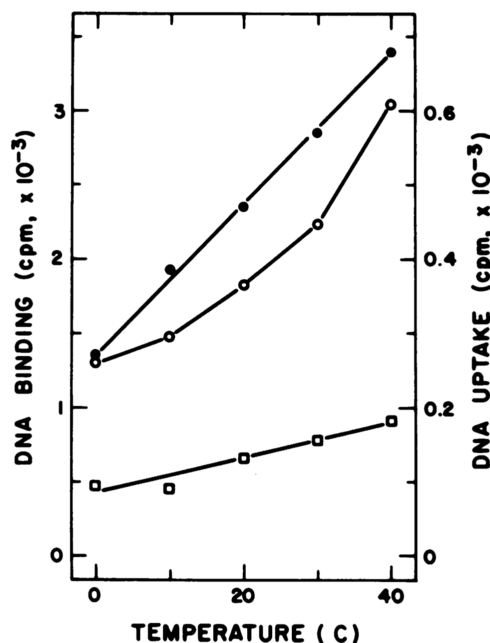

FIG. 4. Effect of incubation temperature on soybean ^{3}H-DNA binding and uptake by isolated nuclei. (●——●), ^{3}H-DNA binding; (○——○), total uptake of ^{3}H-DNA; (□——□), acid-precipitable uptake of ^{3}H-DNA. Average of duplicate experiments.

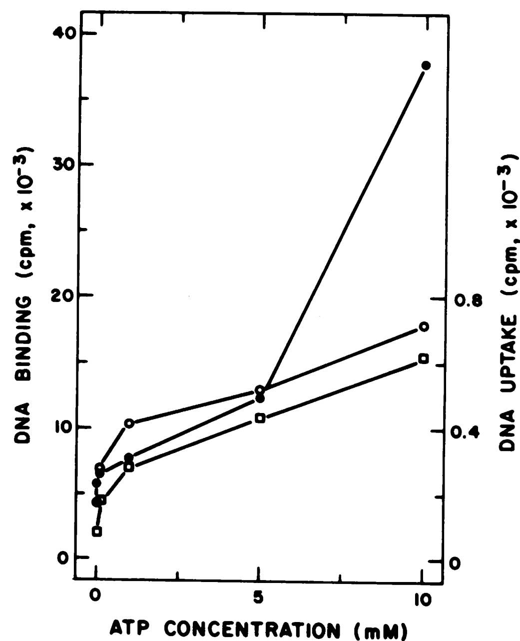

FIG. 5. Effect of ATP concentration on soybean ^{3}H-DNA binding and uptake by isolated nuclei. (●——●), ^{3}H-DNA binding; (○——○), total uptake of ^{3}H-DNA; (□——□), acid-precipitable uptake of ^{3}H-DNA. Average of duplicate experiments.

Table I. Effects of cations on DNA binding and uptake by isolated nuclei

The data are the average of duplicate experiments.

	mM	DNA binding	DNA uptake Total	DNA uptake Acid-precipitable
		cpm	cpm	
None		4059	154	75
Mg^{2+}	1	4624	276	159
	10	11689	586	325
	20	15174	806	437
Ca^{2+}	1	6179	220	127
	10	24173	495	297
	20	19490	598	421
EDTA	1	7691	319	139
	10	7277	174	83
	20	5471	167	83

Table II. Effects of poly-L-lysine on DNA binding and uptake by isolated nuclei

The data are the average of duplicate experiments

µg/ml	DNA binding	DNA uptake Total	DNA uptake Acid-precipitable
		cpm	
None	5356	142	149
0.1	8315	4364	3479
1.0	15425	15900	13506
5.0	4943	4773	1690

Table III. Effects of various substances on DNA binding and uptake by isolated nuclei from soybean protoplasts

The data are the average of duplicate experiments

Compounds	DNA binding	DNA uptake Total	DNA uptake acid-precipitable
		cpm	
None	3436	166	81
Glucose (2%)	3820	162	78
ATP generating system (ATP, PEP and PEP kinase)[1]	7362	838	253
Pronase (2 µg/ml)	1743	71	15
RNA (TMV-RNA, 4 µg/ml)	3144	131	70

[1] ATP generating system consists of 1mM ATP, 0.3mM PEP, and 10 µg/ml PEP kinase.

Competitive Effects of Various DNA Sources. As described above, both DNA binding and uptake with isolated nuclei were stimulated significantly by ATP, and pronase was inhibitory. This suggested that isolated nuclei may have specific DNA-binding sites. Therefore, DNA competition experiments were performed with various sources of heterogeneous DNA. We observed that DNA from *Micrococcus lysodeikticus,* calf thymus, *Salmonella typhimurium* as well as poly([dA-dT]-[dA-dT]) showed various degrees of competition with homogeneous soybean ^{3}H-DNA for binding to the soybean nuclei (Fig. 7). Figure 7 B and C shows competition curves for total uptake and acid-precipitable uptake, respectively. Poly([dA-dT]-[dA-dT]) and *S. typhimurium* DNA showed marked competitive effects for DNA binding. The latter has a higher affinity for the binding sites than the soybean DNA. On the other hand, *M. lysodeikticus* DNA apparently enhanced both DNA binding and uptake, but failed to stimulate acid-precipitable uptake. Calf thymus DNA ex-

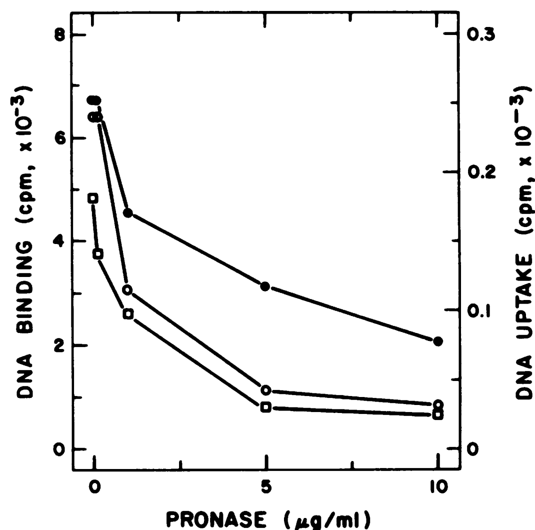

FIG. 6. Effects of preincubation of isolated nuclei with pronase on soybean ^{3}H-DNA binding and uptake. Isolated nuclei were preincubated for 5 min with the concentration of pronase indicated and subsequently washed twice with medium A containing 0.275 M sorbitol before incubation with DNA. (●——●), ^{3}H-DNA binding; (○——○), total uptake of ^{3}H-DNA; (□——□), acid-precipitable uptake of ^{3}H-DNA. Average of duplicate experiments.

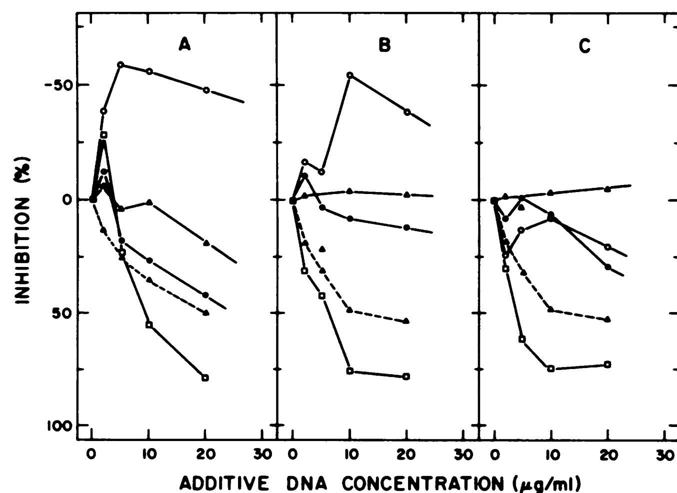

FIG. 7. Competition experiments with soybean ^{3}H-DNA for binding (A), total uptake (B), and acid-precipitable uptake (C) in the presence of different sources of DNA; *M. lysodeikticus* DNA (○——○); calf thymus DNA (▲——▲); poly([dA-dT]-[dA-dT]) (●——●); soybean nonradioactive DNA (△- -△); *S. typhimurium* DNA (□——□). Average of duplicate experiments.

hibited slight competition for DNA bindings, but not for total uptake and acid-precipitable uptake.

DISCUSSION

In genetic transformation with exogenous DNA, the following three steps are essential: (*a*) adsorption (binding) to the plasma membrane, and if cells were used, the cell wall would pose another barrier for DNA entry; (*b*) passage into the cells and through the cytoplasm where the DNA may be degraded; (*c*) binding and passage through the nuclear membrane into the nucleus. Recently, several workers have reported that exogenous DNA fed to plant protoplasts was detected in the nucleus (6, 7, 15). This indicates that exogenous DNA may penetrate the cytoplasm and the nucleus without complete degradation.

This paper is the first attempt to designate the factors responsible for *in vitro* DNA binding and uptake by isolated nuclei from plant protoplasts. Incubation of DNA with isolated nuclei beyond 45 min caused a rapid reduction in DNA binding, while both total uptake and incorporation into the acid-precipitable fraction appeared to reach a peak after 20 min incubation. This observation suggests the gradual release and activity of endogenous DNase in the nuclei preparation. Up to 55% of the added DNA in the binding mixture was degraded with 45 min of incubation. Also endogenous DNase activity may account for the observation that the ratio of DNA binding to DNA uptake varies with the nuclei preparation (Figs. 2, 3, and 4).

The DNA binding and uptake linearly increased with concentration up to 20 μg/ml of homologous DNA. The distinct pH optimum for DNA binding and uptake, and complete reduction at pH higher than 8 suggest that ionic charges of the nuclear membrane may be a significant factor in the binding processes.

Genetic transformation in *Escherichia coli* requires high concentrations of Ca ions (2). In the soybean nuclei system, Ca and Mg ion similarly stimulated DNA binding and uptake. EDTA appeared to enhance rather than inhibit at low concentration. This may be due to inhibitory action of EDTA against DNase which degrade nuclear membrane-bound DNA. The results resemble those reported in *Pneumococci* genetic transformation (12).

DNA uptake by plant protoplasts is known to be promoted by addition of poly-L-lysine (11, 13, 15). The beneficial effects of poly-L-lysine are due to its protective action on the nuclear membrane-bound DNA against DNase. At the higher concentration of poly-L-lysine disruption of the nuclear membrane possibly reduced DNA binding.

Glucose in the incubation mixture did not stimulate DNA binding and uptake as had been observed with *Pneumococci* (12). ATP substantially enhanced the DNA binding and uptake. A similar ATP stimulation has been observed in the *E. coli* DNA uptake by animal cells (9). However, further investigation would be required to establish if indeed DNA uptake by plant nuclei is an energy-requiring process.

Pronase inhibition may reflect disruption or digestion of nuclear membrane. In genetic transformation experiments with *Pneumococci* cells, trypsin treatment inhibited the DNA binding to specific proteins (12).

There have been no previous reports on DNA specificity in DNA binding and uptake experiments with a particular receptor material. It was unexpected that *S. typhimurium* DNA and poly([dA-dT]-[dA-dT]) should show a high degree of competition with soybean ^{3}H-DNA in binding and uptake, while calf thymus DNA exhibited a negligible competition. The basis for the enhancement of soybean DNA binding and uptake by *M. lysodeikticus* DNA is not clear. It is possible that the *M. lysodeikticus* DNA may have provided a preferred substrate or in some other way protected the ^{3}H-DNA against DNase. Additional evidence for DNA-binding specificity is seen in the lack of competition by TMV-RNA, indicating that nuclei have a limited range of binding and uptake sites. Further studies are in progress to determine the effects of DNA size, strandedness, and guanine-cytosine (G-C) content on DNA binding and uptake by isolated nuclei.

This paper described some of the parameters which affect DNA binding and uptake in isolated plant nuclei and is the first report on factors responsible for *in vitro* DNA binding and uptake by isolated nuclei of any eukaryote. The results are of particular interest since they provide evidence for DNA competition, ATP stimulation, and pronase inhibition on DNA binding and uptake. Further studies on the fate of nuclear membrane-bound DNA and analyses of the incorporated DNA in the nuclei are required to elucidate details on the DNA uptake and mechanisms of integration into the genome of the plant cells.

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