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Uptake and expression of bacterial and cyanobacterial genes by isolated cucumber etioplasts

(gene transfer/*in organello* transcription-translation/β-lactamase/ribulose bisphosphate carboxylase/chloramphenicol acetyltransferase)

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ABSTRACT **The uptake and expression by plastids isolated from dark-grown cucumber cotyledons (etioplasts) of two pUC derivatives, pCS75 and pUC9-CM, respectively carrying genes for the large and small subunits of ribulose bisphosphate carboxylase/oxygenase of *Anacystis nidulans* or chloramphenicol acetyltransferase, is reported. Untreated etioplasts take up only 3% as much DNA as that taken up by EDTA-washed etioplasts after 2 hr of incubation with nick-translated [^{32}P]-pCS75. The presence or absence of light does not affect DNA uptake, binding, or breakdown by etioplasts. Calcium or magnesium ions inhibit DNA uptake by 86% but enhance binding (23–200%) and breakdown (163–235%) of donor DNA by EDTA-treated etioplasts. Uncouplers that abolish membrane potential (ΔΨ), transmembrane proton gradient (ΔpH), or both do not affect DNA uptake, binding, or breakdown by etioplasts. However, both DNA uptake and binding are severely inhibited by ATP. Presumably this results from the hydrolysis of ATP, because the poorly hydrolyzable analog adenyl-5′-yl imidodiphosphate does not inhibit the uptake or binding of DNA by etioplasts. β-Lactamase specified by the ampicillin resistance gene of pCS75 can be detected only in EDTA-treated etioplasts that have been incubated with the plasmid pCS75. After the incubation of EDTA-treated etioplasts with pCS75, immunoprecipitation using antiserum to the small subunit of ribulose bisphosphate carboxylase/oxygenase from *A. nidulans* reveals the synthesis of small subunits; these are smaller by 2 kDa than the cucumber small subunit encoded by the nuclear genome. Treatment of etioplasts with 10 mM EDTA shows a 10-min duration to be optimal for the expression of chloramphenicol acetyltransferase encoded by pUC9-CM. A progressive increase in the expression of this enzyme is observed with an increase in the concentration of pUC9-CM in the DNA uptake medium. The plasmid-dependent incorporation of [^{35}S]methionine by EDTA-treated organelles declines markedly during cotyledon greening *in vivo*.**

Most strategies for gene transfer in plants involve the introduction of foreign DNA into protoplasts to enable its integration into the nuclear genome (1–3). However, many of the economically important gene products (e.g., the protein conferring atrazine resistance) either are chloroplast encoded or, if they are nucleus encoded, are functional within the chloroplasts (e.g., *enol*-pyruvylshikimate-phosphate synthase, which confers resistance to glyphosate) (4) or mitochondria (e.g., aryl acylamidase, which confers resistance to propanil) (5, 6). Furthermore, the 1000-fold higher copy number of chloroplast genes relative to nuclear genes (7–9) makes feasible the introduction of multiple copies of foreign genes into plant cells, should the foreign genes become stably integrated into the chloroplast genome.

To obtain gene transfer into chloroplasts, the isolation of intact organelles capable of efficient uptake, transcription, and translation of foreign DNA is essential. As a first step towards achieving this goal, Daniell and Rebeiz isolated plastids from dark-grown cucumber cotyledons (etioplasts) capable of synthesis of protochlorophyllide (10) and chlorophyll (11–13) at extremely high rates. Also, etioplasts that had been loaded with prothylakoid proteins by treatment of etiolated cucumber cotyledons with hormones (14) converted prothylakoids into macrograna when illuminated in a cofactor-enriched medium (15). Daniell and colleagues also demonstrated the development of electron transport coupled to photophosphorylation in concordance with the synthesis of required polypeptides in isolated etioplasts (16, 17). Finally, they also observed linear biosynthesis of pigment and translation of endogenous messages for 8 hr (18). These observations collectively establish that etioplasts of cucumber cotyledons are both metabolically very active and unusually stable in their capacity for protein synthesis, marking them as exceptional targets for gene incorporation and expression. We now report the uptake and expression of pUC derivatives carrying genes for the large and small subunits of ribulose bisphosphate carboxylase/oxygenase (RuBisCO) of *Anacystis nidulans* or chloramphenicol acetyltransferase (CAT) of *Escherichia coli*.

MATERIALS AND METHODS

Cucumber seeds (*Cucumis sativus Linnaeus*) were germinated in moist Vermiculite at 32°C for 3 days in the dark. *E. coli* strain HB101 harboring the plasmid pCS75 was a gift from F. R. Tabita (University of Texas at Austin) and was grown in TYE medium at 37°C (19). The plasmid pUC9-CM (incorrectly printed as pUC9M in ref. 20), a derivative of pUC9 with an insert of a nucleotide 1192–2480 *Hae* II–*Sau*3A fragment from pACYC 184 containing the coding sequence of the CAT gene with its promoter, was a gift from G. An (Washington State University, Pullman). Plasmids pCS75 and pUC9-CM were isolated as described (21). All solutions used for treatment of cotyledons and isolation or treatment of etioplasts were sterile and manipulations were carried out under aseptic conditions. All pH adjustments were done at 25°C and centrifugations were carried out at 4°C.

Four to eight batches (50 each batch) of 3-day germinated cotyledons were excised with hypocotyl hooks and each batch was incubated in the dark at 32°C in 10 ml of 0.5 mM kinetin (prepared as a 15 mM stock, solubilized with NaOH) and 2 mM gibberellic acid in large (10-cm) deep Petri dishes for 20 hr. After the hooks had been removed, each batch

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Abbreviations: RuBisCO, ribulose bisphosphate carboxylase/oxygenase; CAT, chloramphenicol acetyltransferase; Tes, 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid.

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was hand homogenized with 10 pestle strokes in a prechilled mortar and pestle in 20 ml of the isolation medium, which consisted of 0.5 M sucrose, 15 mM Hepes, 30 mM 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid (Tes), 1 mM $MgCl_2$, 1 mM EDTA, 5 mM cysteine, 2 mM dithiothreitol, and 2% bovine serum albumin at a final pH of 7.7. The homogenate was passed through four layers of autoclaved chilled cheesecloth. The homogenate was then centrifuged at 200 × g for 3 min at 4°C and the plastids were sedimented by centrifuging the resultant supernatant for 7 min at 1500 × g. Suspensions of etioplasts from different batches were pooled and purified by layering on 35 ml of 0.6 M sucrose prepared in the homogenization medium and centrifuging at 500 × g for 15 min (22). The plastids, after treatment in the presence or absence of EDTA (as described in the next section), were resuspended in an ice-cold medium A, which consisted of 0.5 M sucrose, 15 mM Hepes, 30 mM Tes, 40 mM NAD, 2.5 mM EDTA, and 1% bovine serum albumin with a final pH of 7.7. This medium was derived from the previously described translation medium for cucumber etioplasts (18), after deletion of ATP and metallic ions, which we found to inhibit DNA uptake.

EDTA-washed etioplasts were prepared according to Daniell and Rebeiz (13) by suspending etioplasts pelleted from the 0.6 M sucrose homogenization buffer in an EDTA wash buffer, pH 7.7, which consisted of 0.5 M sucrose, 15 mM Hepes, and 30 mM Tes containing 10 mM EDTA. After 10 min of incubation at 0–4°C in the dark, the suspended etioplasts were pelleted at 1500 × g for 7 min and resuspended in medium A as described in the preceding section.

Etioplasts, untreated or treated with EDTA, were incubated in 3.0 ml of medium A containing 1 μg of [^{32}P]DNA per ml, which had been labeled by nick-translation of pCS75 using [α-^{32}P]dCTP and DNA polymerase 1 (23). Incubations were carried out at 27°C for different durations with cool white fluorescent light [intensity: 30 $\mu E \cdot m^{-2} \cdot s^{-1}$; 1 E (einstein) = 1 mol of photons] on a reciprocating shaker operated at 60 oscillations per min in a water bath. Binding, uptake, and breakdown of DNA were measured as described previously (23).

EDTA-treated etioplasts were incubated in a water bath in resuspension medium A without or with plasmid DNA (50 μg/ml) at 27°C for 2 hr with cool white fluorescent light (30 $\mu E \cdot m^{-2} \cdot s^{-1}$) on a reciprocating shaker operated at 60 oscillations per min. At the end of the incubation, the plastids were pelleted at 1500 × g for 7 min and the incubation medium was discarded. Etioplasts were washed thoroughly with ice-cold medium B (0.5 M sucrose/15 mM Hepes/30 mM Tes, pH 7.7) and were resuspended in transcription-translation medium, which contains the following components at a final pH of 8.2: 0.4 M sucrose [RNase-, DNase-, and protease-free (Schwarz/Mann)], 50 mM Tris acetate, 60 mM potassium acetate, 11 mM ammonium acetate, 14 mM magnesium acetate, 20 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 0.5 mM UTP, 0.5 mM cAMP, 10 mM phospho*enol*pyruvate, type III pyruvate kinase (Sigma, 6.4 units/ml of final incubation mixture), *E. coli* tRNA (0.125 μg/ml), 19 amino acids (0.2 mM each), 0.14 mM pyridoxine·HCl, 0.1 mM FAD, 0.1 mM NADP, 0.06 mM *p*-aminobenzoic acid, 1.6 mM dithiothreitol, 1% polyethylene glycol 6000, and 20 μCi 1(1 Ci = 37 GBq) of [^{35}S]methionine per 100-μl reaction mixture. Each component was stored independently as a 10× stock solution at −20°C. Storage of combined reaction mixture at −20°C resulted in rapid loss of transcription-translation. Incubations were carried out for 2 hr at 27°C. At the end of each incubation, the plastids were centrifuged, washed once with ice-cold medium B, and finally frozen in liquid nitrogen in suitable aliquots.

β-Lactamase was assayed by a spectrophotometric method (21) in which the β-lactam ring of nitrocefin (λ_{max} = 390 nm) is hydrolyzed to the ring-opened product (λ_{max} = 490 nm). Immunoprecipitation of small subunits of RuBisCO with antiserum to the *A. nidulans* protein (raised in rabbits by Jose Torres-Ruiz of our laboratory) was done essentially as described previously (21). CAT was assayed in etioplast sonic extracts as reported in ref. 24 except that the amount of [^{14}C]chloramphenicol was decreased to 50 nCi per sample.

RESULTS

Preliminary experiments on incubation of etioplasts with nick-translated ^{32}P-labeled pCS75 (3.8 × 10^8 cpm/μg of plasmid) showed a linear increase in uptake or binding of DNA at 27°C for at least 120 min. Therefore, all incubations of DNA with etioplasts were carried out for 2 hr. DNase treatment of etioplasts ensured that the high (Table 1) and continuing uptake of DNA observed was due to intact organelles, confirming earlier results (15) with an organelle fraction isolated identically. The rate of uptake of [^{32}P]pCS75 by etioplasts in the light, 0.86 × 10^4 cpm/μg of protein, was comparable to a value of 1.35 × 10^4 cpm/μg of protein calculated for "permeaplasts" of *A. nidulans* when [^{32}P]-pBR322 of approximately identical specific radioactivity were used (22). Permeaplasts are cells with partially digested cell wall obtained by treatment of cells with lysozyme and EDTA (22). Etioplasts that had been subjected to 10-min incubation at 0–4°C in the dark in the sucrose/Hepes/Tes buffer lacking EDTA showed only 3% of as much uptake of DNA as was observed with EDTA-washed etioplasts after 2 hr of incubation with nick-translated [^{32}P]pCS75 (Table 1). On the other hand, 42% as much binding of DNA as had been observed with EDTA-washed etioplasts was observed with etioplasts washed in the absence of EDTA under identical conditions of incubation. The presence or absence of light did not affect DNA uptake, binding, or breakdown in etioplasts washed in the presence or absence of EDTA (Table 1). Cations such as calcium or magnesium ions significantly inhibited DNA uptake (86%) in EDTA-washed etioplasts but enhanced binding (23–200%) and breakdown (163–235%) of DNA (Table 1).

It has been previously suggested that the calcium-dependent breakdown of donor DNA, reflected in the perchloric

Table 1. Characterization of DNA uptake, binding, and breakdown by etioplasts

	cpm × 10^{-4}/mg plastid protein (%)		
Experimental conditions	Uptake	Binding	Breakdown
Untreated etioplasts			
Control, light	28 (100)	215 (100)	297 (100)
Control, dark	28 (100)	194 (90)	282 (95)
+ gramicidin	30 (107)	187 (87)	325 (100)
+ gramicidin + NH_4Cl	30 (108)	203 (95)	319 (110)
+ valinomycin	29 (103)	204 (95)	337 (108)
+ ATP	7 (25)	22 (10)	297 (114)
EDTA-washed etioplasts			
Control, light	856 (100)	465 (100)	154 (100)
Control, dark	788 (92)	456 (98)	139 (90)
+ magnesium acetate	124 (14)	570 (123)	516 (335)
+ $MgCl_2$	124 (14)	681 (146)	470 (305)
+ $CaCl_2$	119 (14)	1392 (300)	404 (263)
+ gramicidin	822 (96)	471 (101)	156 (263)
+ gramicidin + NH_4Cl	813 (95)	470 (101)	160 (104)
+ valinomycin	805 (94)	446 (96)	167 (108)
+ ATP	197 (23)	51 (11)	152 (99)

Etioplasts were incubated in medium A with 1 μg of nick-translated ^{32}P-labeled pCS75 under various conditions. The additional components were added at the following concentrations: gramicidin, 10 μM; NH_4Cl, 5 mM; valinomycin, 10 μM; ATP, 10 mM; magnesium acetate, 20 mM; $MgCl_2$, 20 mM; and $CaCl_2$, 20 mM.

acid-soluble fraction, may be an essential requirement for transformation of *Bacillus subtilis* (25–27). However, in studies of yeast protoplasts, binding of plasmid DNA was not accompanied by its degradation (28). We have demonstrated recently that DNA uptake in permeaplasts of *A. nidulans* is unrelated to the breakdown of donor DNA (22). In the present work on etioplasts, cations inhibited DNA uptake but enhanced breakdown (Table 1), suggesting that the two processes may be unrelated in this case also.

Protonmotive force, which consists of a membrane potential ($\Delta\Psi$) and proton gradient (ΔpH), has been shown to be the driving force for DNA uptake in the transformation of some bacteria (29). Therefore, the effect of various reagents that inhibit ATP synthesis or uncouple photophosphorylation from photoelectron transport was investigated. Gramicidin forms an aqueous transmembrane channel through which protons pass slowly but does not inhibit electron transfer even at very high concentrations; gramicidin inhibits phosphorylation by abolishing $\Delta\Psi$ without having a major effect upon the transmembrane ΔpH (30). To achieve total uncoupling by abolishing both $\Delta\Psi$ and ΔpH, we used the "uncoupler couple" NH_4Cl plus gramicidin. Neither gramicidin alone nor gramicidin together with NH_4Cl had any effect on DNA uptake, binding, or breakdown by etioplasts (Table 1). In *B. subtilis*, valinomycin, a dissipator of the $\Delta\Psi$ component of the protonmotive force, inhibited DNA binding (29), but this compound had no effect on DNA uptake or binding by etioplasts (Table 1). In *B. subtilis*, DNA uptake or binding is an active process driven by protonmotive force (29), whereas in yeast protoplasts (28), cyanobacterial permeaplasts (22), or etioplasts (Table 1) each is a passive process.

Both DNA uptake and DNA binding were severely inhibited in etioplasts by ATP, but the breakdown of DNA was essentially unaffected (Table 1). The effect was almost certainly due to the hydrolysis of ATP, because the nonhydrolyzable (or very poorly hydrolyzable) ATP analog adenyl-5′-yl imidodiphosphate did not inhibit uptake or binding of DNA (Fig. 1). The striking inhibition of DNA binding (and binding-dependent uptake) by ATP is reminiscent of the effect of ATP on the same phenomena observed with permeaplasts of *A. nidulans* (22). Because this ATP analog is noninhibitory, we conclude that externally added ATP may phosphorylate one or more etioplast membrane components that are involved in DNA binding.

The introduction and expression of foreign DNA was measured by assaying β-lactamase encoded by the plasmid pCS75, using a spectrophotometric method in which a spectral shift from 390 nm to 490 nm accompanies hydrolysis of the β-lactam ring of nitrocefin (23). Whereas sonic extracts of etioplasts incubated in the absence of DNA did not hydrolyze nitrocefin, those incubated with DNA hydrolyzed nitrocefin (Fig. 2). The initial rate of nitrocefin hydrolysis by transformed etioplasts with 0.12 A_{490} unit/min per mg of protein, may be compared with a value of 0.4 obtained for pBR322 transformants of permeaplasts of *A. nidulans* (23). In transformed higher plant protoplasts, nitrocefin hydrolysis was extremely slow and could be detected only after an 18-hr incubation (31).

In these experiments, special precautions were taken to minimize the possibility that bacterial contaminants of etioplasts could account for these results. To address this question, bacteria were enumerated at 30°C and 37°C in etioplast fractions (with or without EDTA treatment) incubated in the presence or absence of pCS75. Bacterial contamination was estimated in terms of the ratio of bacterial protein to etioplast protein. The high ratios of etioplast to bacterial protein (data not shown) established that bacterial contamination was minimal. Moreover, ampicillin-resistant bacteria could not be detected after plating etioplast fractions on LB medium containing ampicillin. In this connection, it should also be pointed out that in *B. subtilis* and *E. coli* DNA uptake and binding are quenched by EDTA and uncouplers (29), in contrast to our results with etioplasts (Table 1). Moreover, with those bacteria, Ca^{2+}-dependent breakdown of donor DNA may be required for transformation (25–27).

The plasmid pCS75 carries the genes for the large and small subunits of RuBisCO from *A. nidulans*. Immunoprecipitation of the translation products with antiserum to the small subunit of RuBisCO from *A. nidulans* suggested the presence of labeled 12-kDa small subunits in etioplasts incubated with

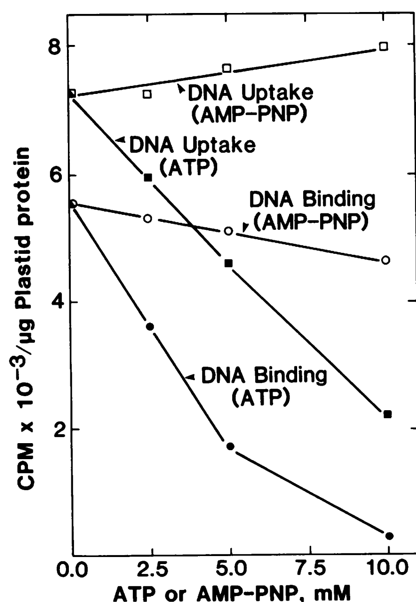

FIG. 1. Effect of ATP or its analog adenyl-5′-yl imidodiphosphate (AMP-PNP) on the uptake or binding of labeled donor DNA by etioplasts. Etioplasts were incubated with the indicated concentrations of ATP or its analog (pH 7.0) in the presence of nick-translated ^{32}P-labeled pCS75 for 2 hr in the light at 27°C.

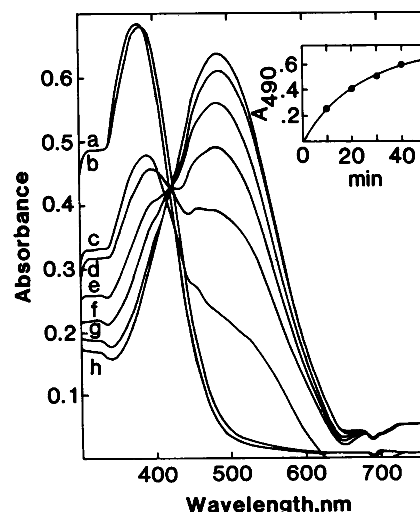

FIG. 2. Assay of β-lactamase based on hydrolysis of the β-lactam ring of nitrocefin in sonic extracts of etioplasts. The spectral shift (λ_{max} = 390 nm to λ_{max} = 490 nm) after hydrolysis catalyzed by β-lactamase establishes that the reaction was almost complete in a 60-min incubation at 20°C. The traces represent nitrocefin alone (a) or incubated with etioplast sonic extract (b) or with sonic extract from etioplasts that had been incubated with supercoiled pCS75 and assayed for 10, 20, 30, 40, 50, or 60 min (c–h, respectively). Spectral scan times were 30 sec. (*Inset*) Change of A_{490} with time, reflecting data from traces c–g.

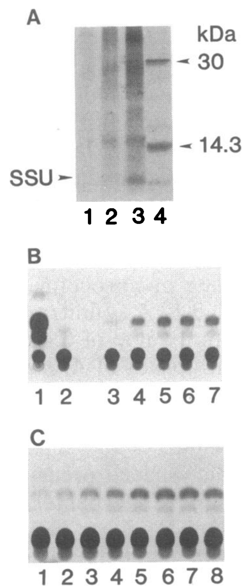

FIG. 3. (*A*) Autoradiograph reflecting immunoprecipitation of the small subunit (SSU) of RuBisCO from [^{35}S]methionine-labeled etioplasts. Sonic extracts (100 μl, each containing 850 μg of plastid protein) of EDTA-washed etioplasts were treated with preimmune serum and subsequently immunoprecipitated with specific antiserum to the small subunit of *A. nidulans* RuBisCO, after incubation of etioplasts minus pCS75 (lane 2) or plus supercoiled pCS75 (lane 3). Lane 1 reflects the treatment with preimmune serum of sonic extracts of EDTA-washed etioplasts that had been incubated with pCS75. Lane 4 represents [^{14}C]methylated protein standards, with molecular masses shown in kDa. (*B*) Expression of CAT with pUC9-CM in etioplasts that had been treated for different durations with 10 mM EDTA. CAT was assayed by thin-layer chromatography of [^{14}C]chloramphenicol and its faster-migrating acetylated products. Lanes were as follows: 1, purified *E. coli* CAT (Pharmacia); 2, negative control, minus etioplast extract; 3–7, EDTA treatment for 0, 5, 10, 20, and 30 min, respectively. Except as noted, all incubation mixtures contained 20 μg of supercoiled pUC9-CM and 50 μg of calf thymus carrier DNA and etioplasts containing 850 μg of protein. (*C*) Expression of CAT with pUC9-CM: Effects of gene dosage, ribonuclease, and protease. Lanes reflect the following: 1–5, etioplasts that had been incubated with 0, 2, 4, 6, and 10 μg of supercoiled pUC9-CM, respectively. Etioplasts in lanes 6–8 had been incubated with 10 μg of supercoiled pUC9-CM. Each incubation mixture contained 50 μg of calf thymus carrier DNA and etioplasts (900 μg of protein). Etioplasts were treated with (lane 6) or without (lane 7) thermolysin (100 μg/ml) for 30 min at 4°C after coupled transcription-translation; the reaction was stopped by the addition of 10 mM EDTA and the etioplasts were washed once in the isolation medium prior to breakage. Alternatively, etioplasts were incubated in the presence of ribonuclease (500 μg/ml) during transcription-translation (lane 8).

pCS75 (Fig. 3*A*), a molecular mass that is in accord with that for the small subunit of RuBisCO from *A. nidulans* (19). To check for contamination of etioplasts by cytoplasmic messages, experiments were carried out in cotyledons labeled with [^{35}S]methionine *in vivo*. Etioplasts preincubated with pCS75 showed a translation product 2 kDa smaller than that of cucumber encoded by its nuclear genome (data not shown). Our immunoblots further revealed that cucumber small subunit strongly crossreacts with the small subunit of RuBisCO from *Chromatium vinosum* but not with that from *A. nidulans*. It is known that newly synthesized stromal proteins of low molecular mass are almost completely degraded in chloroplasts in the presence of ATP (32, 33). The imported RuBisCO small subunit is also destroyed rapidly by proteolysis when conditions for assembly with the large subunit are limiting (34). Therefore, studies of the expression of *A. nidulans* small subunit (Fig. 3*A*) were not pursued.

Etioplasts treated with EDTA (10 mM) for various times

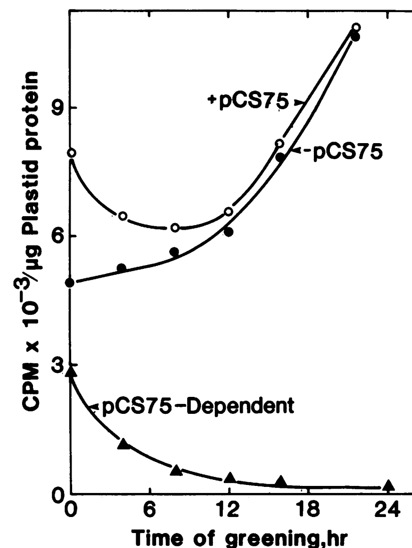

FIG. 4. Translation competence of etioplasts during greening. Incorporation of [^{35}S]methionine into the hot trichloroacetic-acid-insoluble fraction after incubation of cucumber etioplasts isolated at different stages of greening: with (○) or without (●) the plasmid pCS75. Also shown is DNA-dependent translation (▲), which is the difference between translation with and without plasmid.

showed that 10 min was optimal for the expression of CAT (Fig. 3*B*). The background activity observed in etioplasts in the absence of DNA (Fig. 3*C*, lane 1) could have been due to a low level of endogenous acetylase activity found to be present in all plant species tested (35). A progressive increase in the expression of CAT was observed with an increase in the concentration of pUC9-CM in the DNA uptake medium (Fig. 3*C*). Furthermore, treatment of etioplasts with ribonuclease during incubation in the transcription-translation reaction mixture or with thermolysin after coupled transcription-translation did not affect the expression of CAT (Fig. 3*C*) or incorporation of [^{35}S]methionine (data not shown). These results with thermolysin and ribonuclease confirm our conclusion that etioplasts are indeed intact during translation.

All of the present studies (Figs. 1–3) were conducted on etioplasts isolated at zero-time greening. *In organello* translation of endogenous mRNA in etiochloroplasts isolated from cucumber at different stages of greening showed a continuous increase as greening progressed (Fig. 4). In contrast, DNA-dependent incorporation of [^{35}S]methionine decreased dramatically as the tissue matured, and only 5% of the transcription/translation activity towards foreign DNA remained after 24 hr of greening. It seems likely that the overall enhancement in translation of 60% observed in pCS75-treated etioplasts (Fig. 4) was due to protection of endogenous mRNA by incorporated plasmid. In this connection, carrier DNA has been used in addition to plasmid DNA to increase translation by plant protoplasts that were subjected to electroporation (35, 36).

DISCUSSION

The present studies establish that DNA binding and uptake by etioplasts are greatly enhanced by EDTA treatment. The striking 30-fold stimulation of DNA uptake by treatment of etioplasts with EDTA or expression of foreign genes in EDTA-treated etioplasts presumably reflects chelation of inhibitory endogenous divalent metal ions, although the exact molecular basis is unclear. Indeed, we have observed extensive degradation of endogenous mRNA and of foreign DNA in etioplasts in the absence of EDTA treatment (unpublished observation). In this connection, EDTA treatment has been

found to irreversibly inactivate a nuclease from mung bean seedlings (37).

Introduction and stable expression of multiple copies of foreign genes in plant cells may become a reality now that the uptake and expression of foreign DNA has been achieved with isolated etioplasts. The uptake of large numbers of chloroplasts by protoplasts and regeneration of green tobacco plants from albino protoplasts that had taken up normal chloroplasts have also been reported (38). The use of polyethylene glycol significantly improves the efficiency of chloroplast uptake by protoplasts (39, 40). All of these findings augur well for the stable expression of foreign genes after their introduction into isolated etioplasts as presently described. Thus an approach may be opened to major advances in genetic engineering of higher plants.

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