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Movement of DNA across the chloroplast envelope: Implications for the transfer of promiscuous DNA

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Dedication

To Dan Amon, a superb scientist whose findings opened up enormous fields of research in photosynthesis, and a good friend.

Abstract

Little is known about the mechanistic basis for the movement of promiscuous nucleic acids across cell membranes. To address this problem we sought conditions that would permit the entry of plasmid DNA into isolated, intact pea chloroplasts. DNA uptake did not occur normally, but was induced by hypotonic treatments, by incubation with millimolar levels of Mg^{2+}, or by heat shock at 42 °C. These results are consistent with DNA movement being permitted by conditions that transiently alter the permeability of the chloroplast envelope. Plant cells are subject to osmotic tensions and/or conditions inducing polymorphic changes in the membranes, such as those used in the present study, under several environmental stresses. In an evolutionary time frame, these phenomena may provide a mechanism for the transfer of promiscuous nucleic acids between organelles.

Keywords: chloroplast envelope, DNA entry, promiscuous DNA

Abbreviations: PEG, polyethylene glycol; T-DNA, transferred DNA

Introduction

There is extensive evidence for the movement of nucleic acids across cellular membranes inside eukaryotic cells. The endosymbiotic origin of mitochondria and chloroplasts (Cedergren et al. 1988; Gray 1989), contrasted with the small size of their present genomes (Palmer 1985; Levings and Brown 1989; Palmer 1990), implies that a massive transfer of genetic material must have taken place from these organelles into the nucleus. There is now good evidence for this process (Scott and Timmis 1984; Ayliffe et al. 1988; Schuster and Brennicke 1988; Gray 1989; Baldauf et al. 1990; Gantt et al. 1991; Nugent and Palmer 1991). Moreover, transfer of DNA from the mitochondrion to the nucleus has been experimentally demonstrated in yeast (Thorsness and Fox 1990 and 1993).

The existence of promiscuous DNA (Ellis 1982) in mitochondria is also well documented. Plastid and nuclear sequences have been found in the mitochondrial genomes of several species (Stern and Lonsdale 1982; Schuster and Brennicke 1987; Nugent and Palmer 1988; Schuster and Brennicke 1988; Joyce and Gray 1989). However, evidence for transfer of foreign DNA into the chloroplast genome is limited to a putative nuclear fragment found in the plastome of *Acetabularia mediterranea* (Li-Weber et al. 1987), and several species-specific sequences of unknown origin present in subclover chloroplast DNA (Milligan et al. 1989). In addition, most of the introns shared by many land plant chloroplast genomes were acquired by the plastome after its endosymbiotic origin (Manhart and Palmer 1990), implying horizontal transfer of genetic information. Because of the compact structure of the chloroplast genome in land plants, with little non-coding space (Palmer 1985; Ohyama et al. 1986; Shinozaki et al. 1986; Hiratsuka et al. 1989; Palmer 1990), the relative absence of foreign DNA sequences in the plastome probably reflects

the lack of sites into which these sequences can insert without causing a lethal mutation (Nugent and Palmer 1988; Schuster and Brennicke 1988). Alternatively, chloroplasts may be less prone to take up foreign DNA sequences due to the structural integrity of the envelope membrane. In any case, the mechanism(s) that allows the inter-organelle movement of promiscuous DNA remains unknown, although in yeast there appear to be nuclear-coded factors that control escape of DNA from mitochondria (Thorsness and Fox 1993).

There is convincing evidence that several plant virus nucleic acids, and T-DNA from *Agrobacterium tumefaciens* can become localized in chloroplasts (DeBlock et al. 1985; Gröning et al. 1987; Schoelz and Zaitlin 1989; Gröning et al. 1990; Venkateswarlu and Nazar 1991). However, it is not known whether a vector mediated mechanism might be involved in the transport of these nucleic acids into chloroplasts.

Forced uptake of DNA into plastids by biolistic means, resulting in chloroplast transformation, has been observed repeatedly (Svab et al. 1990; Ye et al. 1990; Newman et al. 1991). However, the exact physical pathway for entry is not known. The entering particle might penetrate the chloroplast envelope, depositing DNA inside. Alternatively, DNA deposited in the cytosol might be able to enter the plastid by an unknown mechanism. This could have been the case when entry of DNA into chloroplasts occurred following PEG-promoted DNA uptake by protoplasts (Spörlein et al. 1991; O'Neill et al. 1993). Unlike the situation in many eubacteria (Porter 1986; Stewart and Carlson 1986; Maynard Smith et al. 1991), there is no evidence for a specific DNA-uptake mechanism in the chloroplast envelope. An alternative is DNA-uptake by chloroplasts permitted by a transient alteration of the envelope membrane permeability that does not cause irreversible damage to the organelle. Significantly, forced uptake of DNA involving formation of a temporary hole in the envelope was achieved with the use of a controlled laser beam (Weber et al. 1989).

The classic procedure for transformation of *Escherichia coli* by plasmids (i.e. heat shock at 42 °C of bacterial cells preincubated at 0 °C in the presence of high concentrations of Ca^{2+} or Ba^{2+}) induces polymorphic changes in the membrane including the formation of non-bilayer structures (hexagonal H_{II} phase), as well as intermembrane and intercellular contacts followed by membrane fusion (Borovjagin et al. 1987). There appears to be a correlation between these morphological changes in the membrane and the induced DNA uptake. It is very suggestive that the main component of thylakoid membranes, monogalactosyl diacylglycerol (MGDG), is a non-bilayer forming lipid (Douce et al. 1984; Gruner et al. 1985; Gounaris et al. 1986; Williams and Quinn 1987). Indeed, non-bilayer structures (hexagonal H_{II} phase) can be induced in thylakoid membranes by a variety of treatments: mild heat shock, charge shielding by divalent cations or low pH, or reduction in water activity (Carter and Staehelin 1980; Gounaris et al. 1984; Thomas et al. 1985; Williams and Quinn 1987; Williams 1988). Such structures may occur in plants under conditions of environmental stress, when the normal interactions between the membrane components are disrupted (Gounaris et al. 1984; Steponkus 1985; Williams and Quinn 1987; Williams 1988; Steponkus et al. 1990). The lipid composition of envelope inner membranes from higher plant chloroplasts is qualitatively similar to that of the thylakoid membranes (Douce et al. 1984; Joyard et al. 1991). Thus, it is tempting to speculate that the inner envelope membranes which are the main barrier to entry of solutes, will behave like the thylakoid membranes with respect to the polymorphic phase preference of the lipids.

Therefore, movement of nucleic acids across the chloroplast envelope could take place under conditions causing transient breaches in the membrane, equivalent to the reversible pore formation caused by electroporation (Borovjagin et al. 1987; Chang and Reese 1990) or a laser beam (Weber et al. 1989). In support of this interpretation, we report here that heat shock, the divalent cation Mg^{2+}, and hypotonic treatments allow entry of DNA into isolated pea chloroplasts used as a model system.

Materials and methods

Protoplast preparation and chloroplast isolation

Protoplasts were prepared from pea seedlings (cv. Progress No. 9) as described in Nivison et al. (1986).

The purified protoplasts were washed once (5 min, 75 g) with chloroplast isolation buffer (Nivison et al. 1986), and broken by five passages through 10 μm nylon mesh. Intact chloroplasts were isolated by centrifugation of the homogenate on discontinuous Percoll gradients (Nivison et al. 1986). Chloroplasts were also directly isolated from leaves as previously described

(Nivison et al. 1986). Purified chloroplasts were finally resuspended in incubation buffer (50 mM Hepes-KOH, pH 7.5; 350 mM sorbitol; 1 mM KCl; 0.3 mM $MgCl_2$; 0.3 mM $MnCl_2$; 1 mM DTT; and 0.05% BSA) to a final concentration of approximately 2 mg chlorophyll/ml.

Treatments allowing entry ofDNA into chloroplasts

Standard procedures were used for plasmid isolation by isopycnic centrifugation in CsCl/ethidium bromide gradients (Sambrook et al. 1989). Each reaction, except where otherwise noted, contained 10 μg of pUC 8 or pTB 27 plasmid DNA (Sugiura et al. 1986) and intact chloroplasts corresponding to 100 μg of chlorophyll (0.5–1.0 $\times$ 10^8 plastids). The final volume and incubation conditions varied as described below.

a) *Hypotonic treatments.* One hundred microliter aliquots of chloroplast suspension, to which pTB 27 had been added, were diluted by stepwise addition of incubation buffer (with the proper sorbitol concentration) to a final volume of 500 μl and the desired final sorbitol concentration (70–350 mM). After incubation at room temperature for 45 min, they were slowly returned to the isotonic condition.

b) Heat shock treatments. Two hundred microliter aliquots of chloroplast suspension were incubated with pUC 8 at room temperature for 30 min. Then, they were heated to 42 °C for 1 to 6 min, followed by rapid cooling on ice for 1 min.

c) *Magnesium chloride treatments.* Two hundred microliter aliquots of chloroplast suspension (200 μg chlorophyll) were adjusted to different concentrations of $MgCl_2$ (0–10 mM), and incubated with pUC 8 at room temperature for 45 min.

d) *Chloroplast concentration treatments.* Aliquots containing the same amount of chloroplasts (125 μg of chlorophyll) were diluted with incubation buffer to different final volumes. Then, plasmid DNA (pUC 8) was added to the same final concentration (30 ng/μl). After 30 min incubation at room temperature, samples were heat shocked at 42 °C for 5 min followed by cooling on ice for 1 min.

e) *Control treatments.* Entry of DNA into the organelles was induced by heat shock treatments, and then samples were adjusted to 900 μl with incubation buffer. In the first control the buffer lacked sorbitol, causing osmotic lysis of the chloroplasts. Triton X-100 to a final concentration of 1% was added to a second sample, and the third and fourth controls received 10 and 100 μg, respectively, of salmon testis DNA to compete out non-specifically bound DNA. Samples were incubated at room temperature for another 30 min after the additions.

Digestion of non-internalized DNA

All treatments were diluted to 900 μl with incubation buffer and $MgCL_2$, where present, was chelated by the addition of an equivalent amount of EDTA. Deoxyribonuclease I and $MgCl_2$ were added, in that order, to final concentrations of 50 μg/ml and 3 mM, respectively. After incubation at 4 °C for 30 min, the reaction was stopped by the addition of half a volume of wash buffer (50 mM Tris-HCl, pH 8.3; 375 mM sorbitol; and 60 mM EDTA). Intact chloroplasts were reisolated by centrifugation through a 40% Percoll cushion.

Purification and detection of DNA

The chloroplast pellet was resuspended in swelling buffer (10 mM Tris-HCl, pH 8.3; 200 mM NaCl; and 25 mM EDTA), and linearized pBR 322 added as an internal standard to account for losses during the subsequent DNA manipulations. DNA was purified as previously described (Kut and Flick 1986), separated by agarose gel electrophoresis and transferred to nylon membranes (GeneScreen Plus, NEN) by standard procedures (Sambrook et al. 1989). The filters were hybridized with a labeled 1.87 Kb Hae II segment, common to pUC 8, pTB27, and pBR 322. The probe had been labeled with [α-^{32}P]-dCTP and DNA polymerase I large fragment using the random oligonucleotide primers procedure (Sambrook et al. 1989). Standard hybridization conditions were used (Sambrook et al. 1989), and the blots were finally exposed to Kodak XAR-5 film. Preliminary experiments revealed that the probe does not cross-hybridize with chloroplast DNA from pea (data not shown).

Results

We incubated isolated pea chloroplasts with plasmid DNA under different experimental situations, to evaluate whether DNA uptake could be induced by conditions expected to cause a transient alteration in the permeability of the membranes. The non-internalized DNA was digested with DNase I and intact plastids reisolated. After purification, the presence of heterologous plasmid

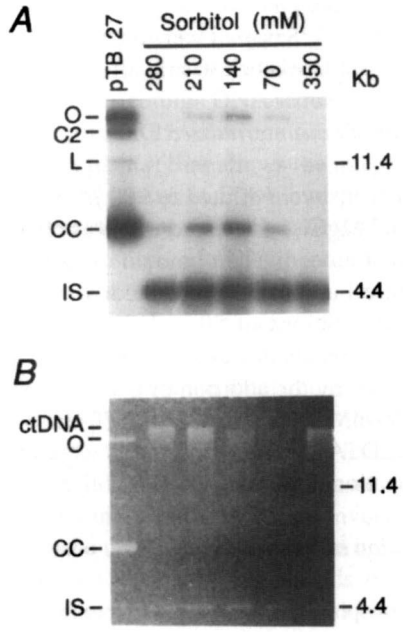

Fig. 1. Hypotonic conditions allow DNA movement across the chloroplast envelope, (a) Autoradiography of Southern blot probed with a [^{32} Pi-labeled Hae II pUC 8 fragment. The slight background in the control lane (350 mM sorbitol) is presumably due to incomplete digestion by the added DNase I. (b) Ethidium bromide stained agarose gel. Abbreviations: CC - covalently closed circular form; C2 - putative dimeric form (closed circular); IS - internal standard (linearized pBR 322); L - linear form; 0 - open circular form; ctDNA - chloroplast DNA.

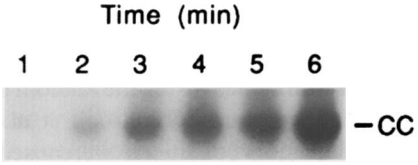

Fig. 2. Entry of DNA into isolated pea chloroplasts is permitted by heat shock treatments. Lanes indicate time at 42 °C. Abbreviations: CC - covalently closed circular form.

DNA was detected by Southern hybridization with a labeled Hae II restriction fragment common to pUC 8 and pBR 322. Linearized pBR 322 was added at the beginning of the isolation procedure, as an internal standard to account for losses during extraction and transfer of the DNA. The detection of plasmid DNA protected from DNase I digestion was taken as evidence of movement into the organelles.

Hypotonic and hypertonic treatments

Under hypotonic conditions, the net entry of DNA increased as the concentration of sorbitol in the incubation medium decreased (Fig. 1a). This also caused partial chloroplast lysis, as revealed by a lower recovery of chloroplast DNA with decreasing concentrations of sorbitol (Fig. 1b). Maximal DNA uptake was observed at 140 mM of sorbitol, also found to be optimal for permeabilization of soybean protoplasts (Saleem and Cutler 1986; Cutler and Saleem 1987). At lower concentrations the net entry of DNA was probably reduced due to significant osmotic lysis of the chloroplasts (Fig. 1). Hypertonic treatments (up to 2000 mM sorbitol) were ineffective in inducing DNA uptake by chloroplasts (data not shown).

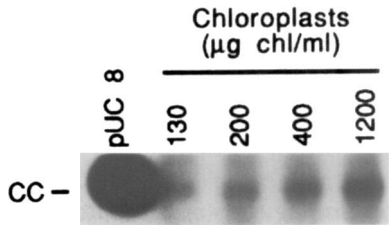

Fig. 3. Effect of chloroplast concentration on the passive DNA uptake induced by heat shock. The heating was at 42 °C for 5 min (see "Materials and Methods" for details). The concentration of plasmid DNA and the number of chloroplasts were kept constant in all treatments, while the chloroplast concentration was varied by using different volumes of incubation. Abbreviations as in Fig. 1.

Heat shock and magnesium chloride treatments

Heat shock at 42 °C allowed DNA uptake by isolated intact chloroplasts as a function of the duration of the treatment (Fig. 2). The recovery of chloroplast DNA was not affected by the treatments, suggesting that up to 6 min of heat shock do not cause significant lysis of the chloroplasts (data not shown). The exogenous DNA must be present during the heat shock treatment, since addition afterwards resulted in no detectable recovery of plasmid DNA (data not shown).

The DNA uptake induced by heat shock was proportional to the concentration of chloroplasts (Fig. 3), suggesting the involvement of a bimolecular-like reaction. This is consistent with the DNA being internalized by individual plastids rather than by the fusion of two chloroplasts. In the latter case the amount of internalized DNA would be expected to be proportional the square of the plastid concentration. However, even under the best conditions, the amount of internalized DNA is very low. Based on densitometric analysis, we estimate that one plasmid molecule out of 10^4–10^5 is internalized. This argues against the existence of a specific mechanism for DNA uptake.

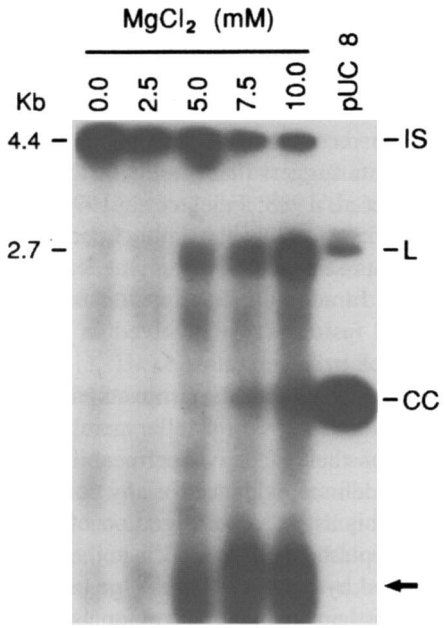

Fig. 4. Movement of DNA across the chloroplast envelope occurs in the presence of magnesium chloride. Note that most of the internalized DNA is present in the linear form or as degraded fragments (arrow). Abbreviations as in Fig. 1.

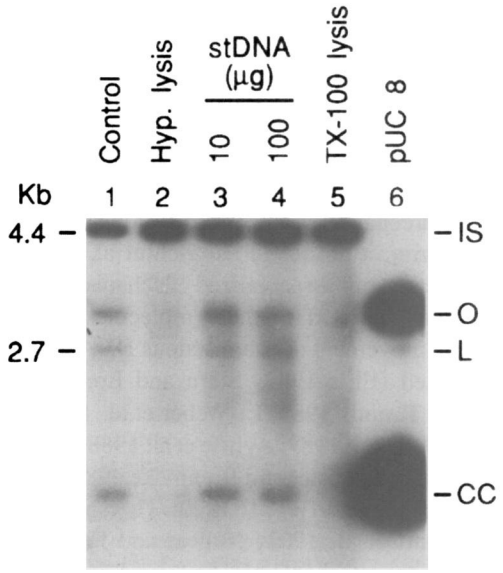

Fig. 5. DNA protected from Dnase I digestion has been internalized. The addition of salmon testis DNA (stDNA), to compete out non-specifically bound DNA, does not affect the amount of detected DNA. However, breakage of the chloroplasts by osmotic lysis or Triton X-100 eliminated detection of any exogenous DNA. Abbreviations as in Fig. 1.

Incubation of the chloroplasts in the presence of $MgCl_2$ also permitted DNA movement across the envelope, as the concentration of the divalent cation increased to 10 mM (Fig. 4). There was an apparent threshold of approximately 5 mM below which there was no detectable uptake. In these experiments, the internalized DNA showed an increased proportion of the open circular and linear forms of the vector, as well as lower molecular weight fragments (Fig. 4). Although we cannot exclude the possibility of preferential uptake of certain DNA forms, it seems unlikely in the absence of a specific mechanism of DNA uptake. More probably this is due to partial digestion of the supercoiled DNA by Mg^{2+} dependent deoxyribonuclease(s) localized in chloroplasts. The existence of nuclease activity in chloroplasts has been reported previously (Shoemaker et al. 1983; McKown and Tewari 1984; Monko et al. 1994). We also detected significant nuclease activity, stimulated by Mg^{2+}, in crude stromal extracts (data not shown).

Control treatments

Several control experiments suggest that the DNA protected from deoxyribonuclease digestion is actually internalized by the chloroplasts. Breakage of the organelles by osmotic lysis or Triton X-100, after the inducing treatments but prior to the addition of DNAse I, completely prevented detection of any exogenous DNA (Fig. 5). Potentially the DNA could be protected from digestion by non-specific binding to proteins and/or to membranes rather than being taken up by the chloroplasts. To compete out any non-specifically bound DNA, we added up to 10-fold excess of salmon testis DNA after the inducing treatments. Samples were incubated for another 30 min to allow for exchange between the putative bound and competing DNAs to take place. Finally, DNAse I was added to digest free DNA and intact chloroplasts isolated following the standard protocol. There was no difference in the amount of detected DNA compared with a similar sample lacking the competing DNA (Fig. 5).

Discussion

The need to import certain small RNAs, required for RNA processing and translation in mitochondria and chloroplasts, has been recognized or implied in several species (Boer and Gray 1988; Chang and Clayton 1989; Maréchal-Drouard et al. 1990; Morden et al. 1991; Taylor et al. 1991; Dietrich et al. 1992). Presumably, there is a specialized mechanism(s) for the continuous transport of these molecules. The inter-organelle movement of promiscuous DNA is also well documented (Ellis 1982; Stern and Lonsdale 1982; Scott and Timmis 1984; Li-Weber et al. 1987; Schuster and Brennicke 1987; Ayliffe et al. 1988; Nugent and Palmer 1988; Schuster and Brennicke 1988; Joyce and Gray 1989; Baldauf et al. 1990; Thorsness and Fox 1990; Gantt et al. 1991; Nugent and Palmer 1991). However, the mechanism(s) involved remains as yet only speculative.

Several hypotheses have been postulated to explain the movement of promiscuous nucleic acid across membranes: (a) vector-mediated transfer, involving plasmids, transposons, or viruses (Ellis 1982; Nugent and Palmer 1988; Schuster and Brennicke 1988), (b) a specific DNA uptake mechanism (Gray 1989a), similar to the one operating in many eubacteria (Porter 1986; Stewart and Carlson 1986; Maynard Smith et al. 1991), (c) fusion between different organelles allowing their genomes to come into contact and recombine (Ellis 1982; Stern and Lonsdale 1982; Nugent and Palmer 1988; Schuster and Brennicke 1988), or (d) transient alterations in the permeability of the organellar membranes (Schuster and Brennicke 1988; Thorsness and Fox 1990).

To date, there is no evidence for the operation of the first two mechanisms in chloroplasts. Indeed, our results argue against the existence of a specific DNA uptake system in the chloroplast envelope. Isolated pea chloroplasts incubated with a variety of plasmids, including one (pTB27) containing 7 Kb of chloroplast sequence, do not take up DNA at a detectable level. The addition of ATP to 1 mM final concentration or incubation in the light had no effect on this lack of uptake (data not shown). Similarly, untreated cucumber etioplasts are very inefficient at DNA uptake (Daniell and McFadden 1987).

Fusion between mitochondria and chloroplasts has been suggested based on some early studies with both phase contrast cinematography and transmission electron microscopy (Wildman et al. 1962; Crotty and Ledbetter 1973; Montes and Bradbeer 1976; Wellburn and Wellburn 1979). However, the membranes of these organelles are now known to be very different in composition (Douce et al. 1984; Joyard et al. 1991), which would make fusion difficult. Moreover, experiments designed to recover chloroplast DNA recombinants in land plants suggest that plastid fusion is very rare (Bowman et al. 1988; Fejes et al. 1990). Therefore, the possibility of chloroplast/mitochondrion fusion in higher plants seems unlikely or, at least, extremely infrequent. In addition, our results strongly suggest that plastid fusion is not involved in the uptake of DNA by isolated pea plastids.

It is likely that the *in vivo* movement of promiscuous nucleic acids across organellar membranes requires alteration of their selective permeability. However, there is no definite evidence for any consistent mechanism for this as yet. The migration of nucleic acids from chloroplasts or mitochondria to the nucleus could be explained by occasional breakage of an organelle, releasing nucleic acids into the cytoplasm (Ellis 1982; Nugent and Palmer 1988; Thorsness and Fox 1990). Subsequently, these macromolecules could move into the nucleus through the nuclear pores or become incorporated after the dissolution and reforming of the nuclear envelope in the normal cell cycle. But integration of foreign DNA into mitochondria or chloroplasts must require a transient alteration of the enclosing membranes, to avoid irreversible damage to the organelles.

In this work several treatments permitted DNA entry into the chloroplasts. Hypotonic conditions clearly allowed internalization of DNA, with optimal net uptake at 140 mM sorbitol. Such a treatment has also been shown to induce uptake of DNA by soybean protoplasts, without significantly affecting their viability (Saleem and Cutler 1986; Cutler and Saleem 1987). Hypotonic conditions probably cause osmotic expansion of the chloroplasts. Because elastic expansion of any membrane is limited, especially without incorporation of new material (Steponkus 1985; Steponkus et al. 1990), large tensions likely occur in the plane of the membrane causing transient pore formation and passive DNA uptake.

Heat shock at 42 °C (for 1 to 6 min) or the presence of Mg^{2+} (from 5 to 10 mM) also permitted movement of DNA across the chloroplast envelope. However, the amount of internalized DNA was low, suggesting the involvement of a non-specific mechanism in allowing this DNA movement. Similar treatments have been shown to induce the formation of nonbilayer structures in thylakoid membranes (Carter and Staehelin 1980; Gounaris et

al. 1984; Thomas et al. 1985; Williams and Quinn 1987; Williams 1988). Considering that the chloroplast inner envelope is very similar in lipid composition (Douce et al. 1984; Joyard et al. 1991), a transient induction of the lamellar to hexagonal H_{II} phase transition could explain our results.

It has been suggested that the transitory formation of inverted lipid micelles (intermediary structures between the lamellar and H_{II} phases) could be involved in the transport of polar molecules across membranes (Cullis et al. 1980; Verkleij 1984; van't Hof et al. 1991). These structures have been directly implicated in DNA uptake during *E. coli* transformation (Borovjagin et al. 1987). Mitochondrial membranes also contain lipids which can adopt the H_{II} phase (Gruner et al. 1985; Joyard et al. 1991), and such a phenomenon has been observed upon treatment with divalent cations (Verkleij 1984). Moreover, DNA migration from mitochondria to the nucleus in yeast has been found to increase under conditions stressful for the membranes, such as high temperature or freezing (Thorsness and Fox 1990). PEG treatment of protoplasts has been shown to allow the introduction of DNA into chloroplasts (Spörlein et al. 1991; O'Neill et al. 1993). Again, it has been suggested that PEG destabilizes membranes, potentially inducing nonbilayer structures (Boni and Hui 1987).

Our results suggest that the entry of DNA into chloroplasts takes place under stressful conditions that transiently alter the permeability of the envelope. Cell membranes are subject to large osmotic stresses at freezing temperatures (Steponkus 1985; Steponkus et al. 1990). Freezing has also been shown to induce the formation of nonbilayer structures in the plasma membrane (Steponkus 1985; Steponkus et al. 1990). Polymorphic changes in the membranes of mitochondria and thylakoids are induced by a variety of treatments such as heat shock, divalent cations, or reduction in water activity (Carter and Staehelin 1980; Gounaris et al. 1984; Verkleij 1984; Thomas et al. 1985; Williams and Quinn 1987; Williams 1988). These phenomena very likely occur in whole plants from time to time under stressful environmental conditions, and would provide a mechanism for the movement of promiscuous nucleic acids across membranes.

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