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Intercalation of psoralen into DNA of plastid chromosomes decreases late during barley chloroplast development

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ABSTRACT

We have used a DNA crosslinking assay to measure intercalation of the psoralen derivative HMT (4'-hydroxymethyl-4,5',8-trimethylpsoralen) into barley (*Hordeum vulgare*) plastid chromosomal DNA during chloroplast and etioplast development. Intercalation into DNA in intact plastids *in vivo* and in plastid lysates *in vitro* shows that chromosomal DNA in the most mature chloroplasts intercalates HMT less efficiently than DNA in younger chloroplasts. In contrast, there is no change in HMT intercalation during etioplast differentiation in the dark. Our results also show that DNA in higher plant plastid chromosomes is under superhelical tension *in vivo*. The lower susceptibility to HMT intercalation of DNA in the most mature chloroplasts indicates that late during chloroplast development the superhelical tension or the binding of proteins to the DNA or both change.

INTRODUCTION

In prokaryotes, specific proteins that regulate gene expression, replication and recombination recognize DNA that is under superhelical tension (1–3) even when this DNA is not naked but is associated with more general proteins that influence the superhelical tension (4,5). Such proteins, together with topoisomerases which change the linking number of the DNA (6,7), maintain superhelical tension in the DNA within a certain range. Additionally, processes which unwind DNA at least transiently change superhelical tension locally (8,9). Certain environmental (10–12) and developmental (13) signals elicit changes in the linking number of DNA. If unconstrained superhelical tension also changes under these conditions (see Discussion), activities of regulatory proteins could be influenced (14,15).

Circular, deproteinized DNA isolated from chloroplast chromosomes is negatively supercoiled (15a). We have used an HMT crosslinking assay (15b) (see below) to show that *Chlamydomonas* chloroplast chromosomes, like bacterial chromosomes, contain unconstrained superhelical tension *in vivo* (15c). We have also shown that in fully developed chloroplasts of this alga, the susceptibility of chloroplast DNA to HMT intercalation *in vivo* changes rapidly and reversibly in response to light vs. darkness (15c). Here we have used this assay to ask if intercalation of HMT into plastid chromosomal DNA of higher plants changes during chloroplast and etioplast development.

The chloroplasts of higher plants develop from proplastids which lack the prominent thylakoid membranes of mature chloroplasts (for reviews see references 16 and 17). Proplastids contain low levels of DNA, RNA, ribosomes and proteins (18,19). They divide largely in the meristematic cells. As cells derived from the meristem elongate, their immature chloroplasts remain small and continue to divide until the cells finish elongating. Subsequently, the chloroplasts increase in size, and accumulate large amounts of the thylakoid membranes in which pigments and proteins necessary for photosynthesis are embedded. Chloroplast transcription and translation and import of cytoplasmic proteins all occur at high rates at this stage (16,17). As the chloroplasts mature the rate of transcription of many, but not all (20), plastid genes declines significantly (21–23).

In the absence of light, proplastids do not mature into chloroplasts but instead become etioplasts (16,24). Under these conditions, overall plastid transcription remains high (22).

We have used an HMT crosslinking assay to measure intercalation of HMT into plastid chromosomal DNA during barley chloroplast and etioplast development. Chloroplasts at different stages of maturation can be readily obtained from different segments of the same barley leaf (16). Following HMT intercalation into DNA, subsequent brief irradiation with near UV light (UVA) leads to crosslinking of the DNA (25). The number of HMT crosslinks in a given DNA segment can then be determined as described in the Results and in reference 15b. Intercalation of psoralens increases with increasing superhelical tension of underwound DNA (1,26,27). DNA binding proteins can also influence intercalation of psoralen into DNA (28,29). A change in the frequency of HMT crosslinking would thus be indicative of a change in one or more of these factors.

We show here that intercalation of HMT into chloroplast chromosomal DNA changes during chloroplast development. This change does not occur in etioplasts.

MATERIALS AND METHODS

Growth of plants

Barley, *Hordeum vulgare* var. Barsdy, seeds were planted in Metro Mix 200 growth medium (Grace Agricultural Products) at a depth of ca. 1 cm. Light-grown plants were kept in a light-

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tight room with a 14 h light, 10 h dark cycle. White light was furnished by two 75 W Gro N Sho incandescent bulbs (General Electric). These plants were watered every other day and reached a height of 8 cm above the soil level on the sixth day after sowing. Dark-grown plants were kept in light-tight cabinets in the same light-tight room. These etiolated plants were not watered because the soil retained sufficient water. They reached a height of 10 cm above the soil level on the sixth day after sowing.

Plastid isolation

Plastids were isolated from the 0−2 cm, 2−4 cm, 4−6 cm and 6−8 cm leaf sections of the light/dark grown 8 cm plants and from the 6−10 cm leaf section of the 10 cm etiolated plants described above (30). Approximately 10 g of each leaf section were cut and placed in ice water for 10 min. The sections were then transferred to 20 ml of ice-cold grinding buffer (330 mM sorbitol, 50 mM Hepes-KOH pH 8.0, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5% BSA, and 4 mM ascorbic acid) and ground for ca. 4 s in a Brinkman Polytron homogenizer at the highest setting. The homogenate was filtered through two layers of Miracloth and centrifuged for 5 min at 3000 rpm in a Sorvall HB4 rotor (1500×g) at 4°C. The pellet was resuspended in 0.5 ml of ice-cold grinding buffer and layered on a 4.5 ml 40−85% Percoll step gradient containing 2 mM EDTA, 1 mM $MgCl_2$, 50 mM Hepes-KOH pH 8.0, 333 mM sorbitol, 5 mM sodium ascorbate, 500 μM sodium glutathione and 40% or 85% PBF-Percoll. PBF-Percoll contains 3% polyethylene glycol 4000, 1% bovine serum albumin, and 1% Ficoll in Percoll. Following centrifugation for 10 min at 8000 rpm in a Sorvall HB4 rotor (10,000×g) at 4°C, the intact undamaged plastids which banded at the interface were collected and resuspended in 5 ml of resuspension buffer (330 mM sorbitol, 50 mM Hepes-KOH pH 8.0, 2 mM EDTA, 1 mM $MgCl_2$, and 0.96 mM dithiothreitol). This suspension was centrifuged for 5 min at 3000 rpm in a Sorvall HB4 rotor at 4°C. The pellet was resuspended in 100 μl of resuspension buffer. An aliquot, diluted ten-fold, was counted in an inverted Leitz microscope in a Petroff-Hauser chamber. Only morphologically undamaged plastids were used in the crosslinking experiments.

HMT crosslinking of plastid DNA *in vivo* (15b,15c)

The DNA in isolated intact plastids was crosslinked by resuspending the plastids in resuspension buffer at a concentration of 2×10^8 plastids in 20 ml and adding HMT from a 0.025 mg/ml stock solution in ethanol to the appropriate concentration. This suspension was left on ice for 10 min. Irradiation of the plastid suspensions with UVA light was as described previously (15c). The dose rate was ca. 3 W/m^2 at 350 nm.

Following irradiation, the plastids were collected by centrifugation at 5000 rpm for 5 min in 30 ml Corex tubes. The pellet was resuspended in 200 μl of resuspension buffer and total nucleic acids were extracted and precipitated. The RNA was digested with 20 μg of DNase-free RNase A, and the DNA was re-precipitated. The DNA was resuspended in 100 μl of TE buffer and a 10 μl aliquot was cut with *Hind*III or *Eco*RI. (Equal volumes of the different DNA preparations were used rather than equal A_{260} units because the DNA preparations contained varying amounts of oligonucleotides from the RNA digestion). The frequency of HMT crosslinks in the *rbc*L and pBar2 regions of the barley genome (see below) was then determined from the proportion of DNA that was not crosslinked and migrated as single-stranded DNA, assuming crosslinks follow a Poisson distribution (15b,15c). The accuracy of this determination is optimal when there are approximately equal proportions of single- and double-stranded DNA on the Southern blot. This estimate is not affected by the absolute amount of plastid DNA analyzed (i.e., loaded on a given lane). At the UVA dose used in all our experiments with intact plastids (see above), HMT concentrations in the range 0.05−0.10 μg/ml were optimal for these measurements. To compare crosslinking in different restriction fragments, the results were expressed as crosslinks per 10 kb.

As expected under our assay conditions, HMT crosslinking of the plastid DNA increased with increasing HMT concentrations (compare lanes 3 in Fig. 1A and Fig. 2). Crosslinking was also proportional to the UVA dose at these optimal HMT concentrations (data not shown).

HMT crosslinking of plastid DNA *in vitro*

Chloroplasts from the 6−8 cm and 0−2 cm leaf sections of light/dark grown plants were purified as described above and resuspended in 200 μl and 100 μl, respectively, of resuspension buffer at 0°C. Twenty-five μl of each chloroplast suspension were lysed in 1 ml of 0.1% Triton-X 100, 50 mM Hepes pH 8.0, and the A_{350} was determined. The concentration of A_{350} units in the two samples was then equalized by adding an appropriate amount of resuspension buffer, usually to the chloroplasts from the apical 6−8 cm leaf section.

Either 10 μl or 4 μl of each chloroplast suspension were added to 100 μl of lysis buffer at 0°C. The lysis buffer contained 50 mM Hepes pH 8.0, 100 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1% Triton-X 100, 20 μg/ml herring sperm DNA, 0.5 μg/ml linearized pGEM 1 plasmid DNA, and HMT at 0.3 or 0.6 μg/ml. The samples were then immediately transferred to a single row of flat-bottom wells (0.4 ml/well) in a Falcon Micro Test III Assay Plate at room temperature. The plate was put on aluminum foil and the UVA light source (a cylindrical Sylvania fluorescent bulb) was placed directly over the row of samples. The distance between the light bulb and sample surfaces was ca. 7 mm. Irradiation was for 1.5 min at room temperature. The samples were then processed like the irradiated chloroplasts, except that the RNase digestion step was omitted. The final nucleic acid pellets were resuspended in 20 μl 1×*Hind*III buffer and digested with *Hind*III. Further analysis of the HMT crosslinked chloroplast DNA was as described above.

Linearized pGEM 1 DNA was added to each sample to monitor possible trivial effects of lysate components on intercalation of and crosslinking induced by HMT. In a series of preliminary experiments we found equal crosslinking of the pGEM standard DNA in different lysates when we used the same concentration of A_{350} units. We chose, somewhat arbitrarily, to normalize for A_{350} unit concentrations because 350 nm was the emission maximum of our UVA light source.

The optimal HMT concentrations for the *in vitro* reactions (0.3−0.6 μg/ml) were slightly higher than the concentrations used *in vivo* (0.05−0.1 μg/ml). This is not surprising, because several factors that influence HMT crosslinking were different in the *in vivo* and *in vitro* reactions: the concentrations of cellular components was 10-fold higher in the *in vitro* than *in vivo* reactions, the surface areas during irradiation were different, and the pigments fluoresced in the lysates but not in the intact chloroplasts.

To each lysate sample we also added 2 μg of herring sperm DNA to ensure that the overall DNA concentration was similar, even if different lysates contained different concentrations of

chloroplast DNA. This consideration turned out not to be important. Similar crosslinking differences, such as those shown in table 2, were found when the HMT crosslinking reactions did not contain the herring sperm carrier DNA (data not shown).

Plasmids

Plasmid # 143 from Dr. J.Mullet contains a 1.5 kb *Hind*III/*Pst*I barley plastid DNA fragment containing the *rbc*L gene. Plasmid pBar2 contains a 2.5 kb *Eco*RI restriction fragment of barley plastid DNA cloned into the *Eco*RI site of vector pGEM Blue. This 2.5 kb *Eco*RI fragment was mapped on the plastid chromosome (Fig. 1B) by hybridizations to Southern blots of *Pst*I, *Pvu*II and *Sal*I digests of purified barley plastid DNA (31). pGEM 1 and pGEM Blue were obtained from Promega Biotech.

Enzymes and chemicals

Restriction enzymes and DNA polymerase I Klenow fragment were from Bethesda Research Laboratories. RNAase A was from Boehringer Mannheim. Random primers were from Pharmacia. Novobiocin, Ficoll, PEG 4000 and Percoll were from Sigma. HMT was from HRI Associates, Berkeley, CA. [α-^{32}P]dCTP (800 Ci/mol) was from Amersham.

RESULTS

The HMT crosslinking assay

HMT intercalates into DNA and forms covalent adducts with pyrimidines when exposed to UVA light (25). A monoadduct can subsequently crosslink with an appropriately positioned pyrimidine on the opposite DNA strand. After cutting with a restriction enzyme, alkali-denaturation and neutralization, crosslinked restriction fragments will rapidly reanneal and migrate as double-stranded species, whereas the fragments containing no crosslinks will migrate as single-stranded species (15b,15c). As pointed out in the Introduction, the extent of HMT intercalation into a given DNA segment depends on the torsional stress of the DNA and proteins bound to the DNA. A similar HMT crosslinking assay has been independently developed by other workers (32).

A representative experiment demonstrating HMT crosslinking of etioplast chromosomal DNA is shown in Figure 1A. *Hind*III-restricted DNA from HMT-treated or untreated etioplasts was alkali-denatured and rapidly renatured. After electrophoretic separation, blotting, and probing of the Southern blot with the *rbc*L probe (Fig. 1B), the two expected bands corresponding to the double- and single-stranded form of the 6.0 kb *Hind*III fragment, and only these bands, were detected (Fig. 1A, lane 3). As expected, non-denatured restriction fragments migrated as double-stranded DNA (Fig. 1A, lane 1) and alkali-denatured DNA which was not crosslinked migrated exclusively as single-stranded DNA (Fig. 1A, lane 2).

For theoretical and technical reasons discussed in Materials and Methods, all subsequent comparisons were done at optimal HMT concentrations and UVA doses (i.e., under conditions where the proportions of the double- and single-stranded DNA species could be accurately determined).

HMT crosslinking of plastid DNA changes during chloroplast development

We have arbitrarily divided 8 cm barley leaves from light/dark grown plants into four 2 cm sections. Chloroplasts in consecutive sections are at more advanced stages of development. Rapid cell elongation and plastid division occur within the 0–2 cm leaf section (22). The most immature chloroplasts obtained from this section contained a few single thylakoid membranes in the stroma, while the most mature chloroplasts contained a few small grana consisting of no more than three appressed thylakoid disks (our electron micrographs, not shown). The mature chloroplasts from the apical 6–8 cm of the same plants all contained many large grana stacks and were much larger than those from the 0–2 cm leaf section.

A representative Southern blot comparing HMT crosslinking in the *rbc*L region of chromosomal DNA of the young, immature and the most mature chloroplasts is shown in Figure 2, lanes 1 and 2. DNA in the immature chloroplasts experienced an average of 1.97 crosslinks/10 kb. DNA in the most mature chloroplasts experienced an average of 1.25 crosslinks/10 kb. Thus the crosslinking ratio for chloroplast DNA from these leaf sections (6–8 cm/0–2 cm) was 0.63. In six independent experiments,

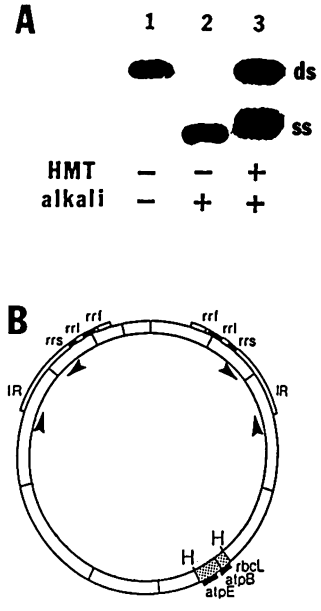

Figure 1. HMT crosslinking of barley etioplast chromosomal DNA. Panel A. Purified etioplasts were irradiated with UVA light in the presence of HMT to induce interstrand crosslinks in the chromosomal DNA (see Materials and Methods for details). After treatment, DNA was isolated and restricted with *Hind*III. The DNA was then alkali-denatured and separated in neutral-agarose gels. The DNA was blotted to nitrocellulose and probed with a labeled 1.5 kb plastid DNA restriction fragment containing the *rbc*L gene. This probe hybridizes to a unique 6.0 kb *Hind*III fragment which contains the *rbc*L gene (see Panel B). Lane 1, non-denatured *Hind*III-digested plastid DNA from control etioplasts; Lane 2, denatured *Hind*III-digested plastid DNA from control etioplasts; Lane 3, denatured *Hind*III-digested plastid DNA from etioplasts irradiated with UVA light (1.5 min) in the presence of HMT (0.05 μg/ml). Panel B. Map of barley plastid DNA (31) showing the location of the regions probed with the HMT crosslinking assay. *Pst*I restriction fragments are shown. The 6.0 kb *Hind*III fragment which hybridizes to the *rbc*L probe used above is stipled. For simplicity, this 6.0 kb stretch of DNA will be referred to as the *rbc*L region even though it also contains the *atp*B and *atp*E genes. In experiments analogous to the one shown in panel A, the plastid DNA was *Eco*RI-restricted and the Southern blots probed with the insert of plasmid pBar2. This plasmid contains a 2.5 kb *Eco*RI plastid DNA restriction fragment which is contained entirely within the inverted repeat (IR) between the arrowheads. We could not map this *Eco*RI fragment more accurately because an *Eco*RI restriction map of barley plastid DNA is not in the literature. For simplicity, this 2.5 kb stretch of DNA will be referred to as the pBar2 region. Although no known genes have been mapped to this region, it has been reported to give rise to a large number of low abundance transcripts (31). Ribosomal RNA genes are indicated by rrs, rrl and rrf.

the average ratio was 0.51 ($P<0.01$) (Table 1, line 4). HMT crosslinking of chromosomal DNA in the two chloroplast populations was also compared in the pBar2 region of the genome (Fig. 1B). In this case the average crosslinking ratio in five independent experiments was 0.49 ($P<0.01$). These results indicate that the frequency of HMT crosslinks in two widely separated regions of the chloroplast chromosome is, on average, significantly lower in the most mature chloroplasts as compared with the young, immature chloroplasts.

The above and all subsequent HMT crosslinking reactions employing intact plastids were carried out with very dilute plastid suspensions (2×10^8 plastids in 20 ml). Absorption of the UVA light by these suspensions was below detectable limits (data not shown).

To get a general idea of where on the leaf the frequency of HMT crosslinks decreases, intact chloroplasts from intermediate leaf sections were compared with those from the bottom (0–2 cm) and top (6–8 cm) leaf sections. Chloroplasts from the 2–4 cm, 4–6 cm and 6–8 cm leaf sections were morphologically indistinguishable in the electron microscope, but, as mentioned above, they appeared different than those from the 0–2 cm leaf section (data not shown). In contrast, the frequency of HMT crosslinks in chromosomal DNA of chloroplasts from the intermediate leaf sections clearly was not reduced as compared with the 0–2 cm leaf section (Table 1, lines 2 and 3). Only DNA in chromosomes of the most mature chloroplasts from the top leaf section showed a significant decrease in the frequency of HMT crosslinks (Table 1, line 4).

The data in Table 1, line 2, suggest that the frequency of HMT crosslinks in the *rbc*L region of the chloroplast chromosome might actually increase slightly in the 2–4 cm vs the 0–2 cm leaf sections. The statistical significance of this difference is borderline. Additional experiments are needed to verify a possible difference for this or other regions of the genome.

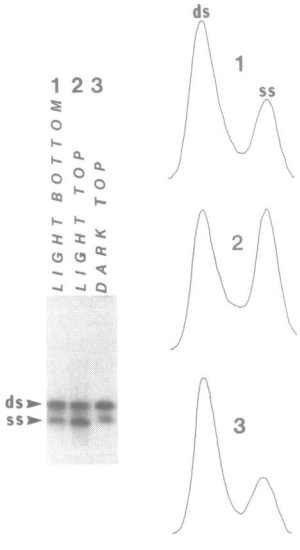

Figure 2. HMT crosslinking of chromosomal DNA in different plastid types. A representative Southern blot probed to detect crosslinking in the *rbc*L region of the plastid chromosome is shown (ds, double-stranded DNA; ss, single-stranded DNA). In analogous experiments, HMT crosslinking in the pBar2 region of the chromosome was analyzed. Light bottom (0–2 cm), young immature chloroplasts; light top (6–8 cm), fully mature chloroplasts; dark top (6–10 cm), etioplasts. Densitometer scans of lanes 1–3 are shown at the right. The HMT concentration was 0.10 μg/ml. In control experiments (data not shown), there was no significant difference in the frequency of HMT crosslinks in etioplast chromosomal DNA in the top 2 cm and the bottom 2 cm leaf sections of etiolated plants. Therefore, we used the top 6–10 cm of etiolated plants in this and all subsequent experiments.

HMT crosslinking in lysed chloroplast suspensions

To show that the decrease in frequency of HMT crosslinks in chromosomal DNA of the mature chloroplasts was not due to some trivial reason such as reduced uptake of HMT, crosslinking assays were performed *in vitro* with chloroplast lysates. Lysis was gentle such that disruption of DNA-protein interactions would be minimal. In a series of experiments, described in Materials and Methods, we first determined the optimal reaction conditions for the *in vitro* HMT assay. Under conditions where crosslinking of the pGEM 1 standard DNA was similar, the frequency of HMT crosslinks in the *rbc*L region of the chloroplast genome was significantly reduced in lysates of chloroplasts from the 6–8 cm leaf section as compared with the 0–2 cm leaf section (Table 2). Quantitatively, this decrease was similar to that observed with intact chloroplasts (Table 1 and Table 2).

The *in vivo* and *in vitro* results together indicate that HMT intercalates less efficiently into chromosomal DNA of the most mature chloroplasts.

Table 1. HMT crosslinking of chromosomal DNA in chloroplasts isolated from different barley leaf sections of light/dark grown plants.

	Ratio of Crosslinks[a]	P value[b]
1) $\frac{0-2\text{ cm}}{0-2\text{ cm}}$	1.00	–
2) $\frac{2-4\text{ cm}}{0-2\text{ cm}}$	1.30[c]	$P<0.10$
3) $\frac{4-6\text{ cm}}{0-2\text{ cm}}$	0.92[d]	$P>0.10$
4) $\frac{6-8\text{ cm}}{0-2\text{ cm}}$	0.51[e]	$P<0.01$

[a]Autoradiograms like those shown in Figure 2 were scanned with a Zenith soft laser densitometer and the tracings were used to calculate the number of crosslinks/10 kb as described in Materials and Methods. These numbers were then used to calculate the indicated ratios. The *rbc*L region of the plastid chromosome was probed (Fig. 1B). Depending upon the experiment, the HMT concentration was 0.05 μg/ml or 0.1 μg/ml.
[b]The Student's t-test was used to determine if the indicated ratios were significantly different than the ratio 0–2 cm/0–2 cm = 1.00.
[c]This value is the mean of three experiments.
[d]This value is the mean of three experiments.
[e]This value is the mean of six experiments.

Table 2. HMT crosslinking of chloroplast chromosomal DNA *in vitro*.

	[HMT] (μg/ml)	μl plastids	A_{350} units[b]	Ratio of Crosslinks[a] $\frac{6-8\text{ cm}}{0-2\text{ cm}}$ cpDNA	pGEM
exp. 1	0.3	10	0.09	0.54	1.19
exp. 2	0.6	10	0.16	0.62	0.95
	0.6	4	0.06	0.56	1.16

[a]Ratios were determined as described in the legend to Table 1. The *rbc*L region of genome was probed.
[b]The values are the A_{350} readings obtained when the indicated volume of plastid suspension is added to 1 ml of lysis buffer. See Materials and Methods for further details.

Intercalation of HMT into etioplast DNA does not decrease during development

In the absence of light, a proplastid develops into an etioplast which is morphologically and functionally different than a chloroplast. Etioplasts contain a prolamellar body instead of the chloroplast thylakoid membranes (24). They lack the pigments, proteins and structures required for photosynthesis. Overall transcription in etioplasts is higher than in fully mature chloroplasts (22). Etioplasts rapidly develop into chloroplasts when dark-grown plants are exposed to light (24). Though there are similarities in the development of etioplasts and proplastids into chloroplasts, their developmental pathways are not identical (16). However, in many respects the greening of etioplasts is considered as a model system for chloroplast development.

We isolated etioplasts from the apical 6−10 cm of dark-grown 10 cm plants. The etioplasts contained typical prolamellar bodies (our electron micrographs, not shown). The chromosomal DNA in etioplasts from the apical section was significantly more crosslinked with HMT than DNA in chromosomes of the most mature chloroplasts (Fig. 2, lanes 2 and 3 and Table 3, line 2). In contrast, crosslinking was not significantly different from that of DNA in young, immature chloroplasts (Fig. 2, lanes 1 and 3 and Table 3, line 3).

Novobiocin and gamma-ray irradiation reduce intercalation of HMT into etioplast chromosomal DNA

In bacteria, the inactivation of DNA gyrase results in an increase in the average linking number (33,34) and a decrease in the average superhelical tension of the chromosomal DNA as measured by psoralen intercalation (1). We have shown previously (15c) that *Chlamydomonas* chloroplast chromosomal DNA is under superhelical tension *in vivo* and that this tension can be reduced *in vivo* by the gyrase inhibitor novobiocin. Higher plant chloroplasts contain a novobiocin-sensitive gyrase (35) and DNA from these chloroplasts is known to be negatively supercoiled following removal of proteins (15a). To determine whether this supercoiling is constrained by binding of proteins *in vivo*, we tested the effect of novobiocin on HMT crosslinking of DNA in chromosomes of isolated etioplasts. We found that novobiocin significantly reduced the frequency of HMT crosslinks within 15 min after addition (Table 4, lines 1 and 2).

Gamma-ray irradiation nicks DNA. It should thereby partially relax plastid chromosomal DNA and reduce HMT intercalation if and only if the DNA contains residual superhelical tension not constrained by proteins (1,36). Gamma-ray irradiation clearly reduced the frequency of HMT crosslinks in etioplast chromosomal DNA (Table 4, line 3) as expected if superhelical tension was reduced.

Although neither of the above approaches alone would give definitive answers, together they provide compelling evidence that higher plant plastid chromosomal DNA is under superhelical tension *in vivo*.

Table 3. HMT crosslinking of chromosomal DNA in etioplasts and fully mature chloroplasts

	Ratio of Crosslinks[a] rbcL	pBar2
1) dark, 6−10 cm / dark, 6−10 cm	1.00	1.00
2) light, 6−8 cm / dark, 6−10 cm	0.56	0.43
3) light, 0−2 cm / dark, 6−10 cm	1.12	–

[a]Ratios were determined as described in the legend to Table 1. Each ratio was from an independent experiment.

Table 4. The frequency of HMT crosslinks in etioplast chromosomal DNA is reduced by novobiocin treatment and gamma-ray irradiation.

	Ratio of Crosslinks[a] (treated/control)
novobiocin[b]	0.50
	0.46
gamma-irradiation[c]	0.39

[a]These ratios were determined as described in the legend to Table 1. Each value is from an independent experiment. The *rbc*L region of the plastid chromosome was probed. The HMT concentration was 0.05 μg/ml.
[b]Purified etioplasts in resuspension medium (see Materials and Methods) were treated with 300 μg/ml novobiocin for 15 min at 25°C.
[c]Etioplasts prepared as above were irradiated with 12.5 krad of gamma-rays over a 15 min period from a cobalt source.

DISCUSSION

We have used a DNA crosslinking assay to measure intercalation of HMT into chromosomal DNA of barley chloroplasts at different developmental stages. As mentioned in the Introduction, in monocots such as barley, chloroplasts from different sections of a leaf are in different stages of development.

Our *in vivo* results with intact plastids and our *in vitro* results with plastid lysates can be summarized as follows: 1) Significantly less HMT intercalates into chromosomal DNA of the most mature chloroplasts (6−8 cm leaf sections) as compared with younger chloroplasts (4−6, 2−4, or 0−2 cm leaf sections). This decrease represents the average among the population of all chloroplast chromosomes. Since each cell contains many chloroplasts and each chloroplast contains multiple chromosome copies, it is likely that there is heterogeneity among chloroplast chromosomes of a given leaf section and that our assay underestimates the decrease in intercalation of HMT into the most affected chromosomes. 2) In contrast, HMT intercalation into DNA did not change during etioplast development of dark-grown plants; it remained similar to that found in chloroplasts from the lower leaf sections. 3) HMT intercalation into etioplast DNA was reduced by two treatments that reduce negative supercoiling of DNA: addition of the DNA gyrase inhibitor novobiocin or gamma-ray irradiation of the isolated plastids.

The latter results indicate that DNA in chromosomes of higher plant plastids, like that of algal chloroplast (15c) or bacterial chromosomes (1), contains unconstrained superhelical tension *in vivo*. The lower susceptibility to HMT intercalation of DNA in the most mature chloroplasts indicates that late during chloroplast development the superhelical tension or the binding of proteins to the DNA or both change.

We have considered several trivial explanations for our results but find them unlikely for the following reasons.

1) Most importantly, we found quantitatively similar differences in HMT crosslinking between immature and the most mature chloroplasts when we probed DNA in intact chloroplasts or in lysed chloroplasts. This agreement excludes the possibility that differential uptake of HMT or shielding of the DNA by thylakoid membranes were responsible for our results. We

consider it unlikely that other trivial reasons would give the quantitative agreement of the results shown in Tables 1 and 2, using intact or lysed plastids, respectively.

2) The differences in HMT crosslinking are not due to differences in UVA absorbing pigments in the intact plastids because there was no detectable attenuation of the UVA light by the plastid suspensions and no correlation of pigment content and the frequency of HMT crosslinking of the chromosomal DNA in etioplasts or chloroplasts of different developmental stages (data not shown).

3) The method of plastid purification (see Materials and Methods) and the tests for intactness ensured that intact plastids from the different leaf segments were subjected to the HMT assay.

4) Differences in DNA content of plastids from different leaf sections do not affect our conclusions, since under our assay conditions, we measure the *proportion* of DNA that can be crosslinked after HMT intercalation, regardless of the DNA concentration. Addition of carrier DNA to the lysates did not change those proportions.

The differences in HMT intercalation that we found can be explained by changes in superhelical tension and by binding of proteins to DNA. These are related to each other in a complex way. As a first approximation, superhelical density of DNA is determined by relative activities of different topoisomerases which change the linking number of DNA in opposite directions (7). Superhelical tension is maintained within a physiological range by homeostasis (6), i.e. in *E.coli*, synthesis and activities of DNA gyrase are reduced, and those of topoisomerase I are enhanced with increasing negative superhelicity in the DNA (7). Transcription or other processes that unwind the DNA can locally distort superhelical density without changing the average linking number, by generating positive supercoils ahead of and negative supercoils behind the unwinding complexes (8,9). Such local distortions in superhelicity due to DNA unwinding mechanisms can lead to changes in linking numbers only if topoisomerases compensate for the localized excessive positive and negative superhelical tension with different rates.

Similarly, wrapping of the DNA around proteins (4,5) can reduce or increase the superhelical tension (depending on the direction of wrapping), again without directly altering linking numbers. If topoisomerases alter the linking number in response to the altered superhelical tension, binding of proteins can ultimately lead to changes in the linking number of extracted DNA.

Certain environmental signals (e.g., osmotic stress), by altering the relative activities of relaxing and supercoiling topoisomerases, cause superhelical tension in DNA to come to a new steady-state level (12,37). Certain developmental pathways (e.g., sporulation in *Bacillus*) also lead to stable changes in linking number of DNA although in this case it is not clear to what degree superhelical tension changes (13).

Kochel and Sinden (27) have shown that a reduction in average HMT crosslinking *in vitro* of small deproteinized plasmid DNA is approximately proportional to the reduction in superhelical density of the DNA. Therefore, there is at most a two-fold difference in unconstrained supercoils between DNA of the most mature and younger chloroplasts if all difference in HMT intercalation were due to differences in unconstrained superhelical tension. As discussed above, such a decrease would require a change in relative topoisomerase activities (i.e. gyrase vs. relaxing topoisomerases). This might be sufficient, however, to change relative rates of transcription initiation from different promoters.

Several plastid DNA binding proteins have been described (38–40). If chloroplast DNA-protein interactions change during development, this could lead to the differences in intercalation of HMT into the DNA. By analogy, during differentiation of *B.subtilis* cells into spores, DNA binding proteins change the structure of DNA (from the B to the A form) (41).

Binding of sequence-specific repressor or activator proteins is unlikely to be responsible for the differences in HMT crosslinking that we observed. Such proteins would not be expected to affect intercalation of HMT to similar extents in different regions of the chromosome, at the low levels of crosslinking used in our experiments (1–2 crosslinks/10 kb).

A change in linking number of deproteinized circular chloroplast DNA would be indicative of at least a transient change in superhelical tension *in vivo*. Unfortunately, this is difficult to ascertain because of the large size of these molecules and the difficulties in isolating them intact. We have looked for changes in linking number of DNA from the different types of plastids, using both equilibrium CsCl/ethidium bromide density gradients (42) and pulse-field gel electrophoresis, but obtained no conclusive results.

We find it intriguing that the decrease in intercalation of HMT into chromosomal DNA of the most mature chloroplasts could be related to the change in transcription patterns which occur during development of chloroplasts in growing leaves (22).

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