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Evidence for a type II topoisomerase in wheat chloroplasts

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Western blotting of total cell extracts from the young developing wheat leaf with an antibody to yeast topoisomerase II locates two proteins of molecular mass 96–101 kDa which also appear on blots of chloroplast proteins. The maximal levels of both proteins are present in chloroplasts in which chloroplast DNA replication is occurring prior to chloroplast division.

Topoisomerase; Chloroplast DNA; Chloroplast development; Western blotting; (Mesophyll cell)

1. INTRODUCTION

Topoisomerases are enzymes that modify DNA and are classified as type I enzymes, which make a single-stranded nick and are ATP-independent, and type II enzymes which make a double stranded nick and are ATP-dependent. Whilst they have been studied extensively in mammalian and bacterial systems [1] and have been shown to coordinate several topological changes in DNA, topoisomerases in plants have been little studied. A topoisomerase type I activity has been described in wheat embryo mitochondria [2], carrot cells [3] and in spinach [4] and pea chloroplasts [5,6] and a topoisomerase II activity has been reported in cauliflower inflorescence cells [7] and a gyrase activity in pea chloroplasts [5].

The chloroplast contains a negatively supercoiled closed circular genome which codes for many polypeptides [8] and attains high copy number in the developing plastids [9]. The mode of replication of the chloroplast DNA (ct DNA) molecule and its control is unclear but useful insights into the cellular changes in ctDNA levels have come from using the young wheat leaf as a model system. The developing wheat leaf has a basal meristem and consists of an ontogenetic gradient of cells of differing ages along its length with the youngest cells at the base and the oldest cells at the tip (see axis of fig.3). Within this gradient, cells and chloroplasts are developing in a regular and consistent manner both spatially and temporally. Chloroplast replication occurs only in the basal 4 cm of the leaf [9–12]. Prior to chloroplast division ctDNA replication occurs so that the ctDNA copy number per plastid is highest immediately prior to chloroplast division and much reduced in each of the two daughter chloroplasts [9].

Using this developmental system we report evidence of a putative topoisomerase type II enzyme in developing wheat chloroplasts which specifically binds yeast topoisomerase type II antibodies and accumulates in cells in which chloroplast DNA replication is maximal.

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2. EXPERIMENTAL

2.1. *Plant material and cell sample preparation*

Seven day old wheat leaves (*Triticum aestivum* cv. Maris Dove) were grown under controlled conditions [9] and harvested as in [11]. Sections of different developmental stages cut from 50 leaves [11] were frozen in liquid nitrogen and ground in a cold mortar with 1 ml grinding buffer containing 25 mM Tris-HCl (pH 8), 1 mM EDTA, 10 mM β-mercaptoethanol and 2 mM phenylmethylsulphonylfluoride (PMSF). One ml of loading buffer was added containing 60 mM Tris-HCl (pH 6.8), 0.64 M β-mercaptoethanol, 0.292 M sucrose, 0.001% (w/v) bromophenol blue and an SDS content ranging between 0.07 M and 0.389 M and adjusted so that the

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ratio of SDS added to the number of mesophyll cells in each leaf section was constant at 3.6 μmol/10^6 cells. Samples were left to stand for half an hour and then boiled for 2 min. After spinning at 9000 × g for 10 min, the supernatant was decanted and stored at −20°C. Mesophyll cell numbers were estimated by digestion with chromium trioxide and counting in a haemocytometer [12] and gels were loaded with the same number of mesophyll cells per track.

2.2. *Chloroplast preparation*

Two hundred leaf sections were finely chopped on ice in 12 ml of buffer containing 0.4 M sorbitol, 0.75 mM $MgCl_2$ and 50 mM Hepes (pH 7.6). The slurry was filtered through 8 layers of 25 μm nylon bolting cloth and layered onto 1 ml of buffer containing 0.4 M sucrose, 0.75 mM $MgCl_2$ and 50 mM Hepes (pH 7.6). After spinning at 900 × g for 5 min at 4°C, the pellet was resuspended in 150 μl of sorbitol buffer. Plastid numbers were estimated by counting in a haemocytometer prior to addition of 150 μl loading buffer containing 60 mM Tris-HCl (pH 6.8), 10% sucrose, 5% (w/v) β-mercaptoethanol, 0.001% bromophenol blue and SDS concentrations of 10.6 μmol SDS/10^6 plastids. After standing for 30 min, samples were boiled for 2 min and frozen at −20°C. Gels were loaded with the same number of chloroplasts in each track.

2.3. *Electrophoresis and Western blotting*

Volumes of extracts equivalent to equal numbers of mesophyll cells or chloroplasts of different developmental stages were separated on SDS-PAGE using 15% acrylamide and 0.1% bis-acrylamide according to [13]. After electrophoresis overnight at 6 mA, gels were electroblotted onto nitrocellulose filters which were then blocked and washed according to [14]. The filters were then probed with a 1:2000 dilution of a polyclonal antibody raised against purified yeast topoisomerase II [15] in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS; 0.157 M NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4) for 1 h at room temperature. After three washes in PBS + 0.1% (v/v) Tween 20 for 12 min followed by one wash in PBS for 12 min, the filter was probed with 37 kBq of ^{35}S Protein A (7.4–26 TBq/mmol) (Amersham, England) in 1% BSA (w/v) in PBS for 1 h. The washing procedure was repeated, the filter dried and autoradiography performed using Beta-max film (Amersham, England) at room temperature (exposure 60 h). For molecular size determinations, molecular weight markers (Sigma, St. Louis) were run on the initial gel and located on the filter by Indian ink staining [16]. Sizing of bands in reference to molecular mass markers was done using a non-linear regression programme [17]. Relative levels were determined by scanning bands with a Joyce Loebel Chromoscan 3 (Vickers Ltd. York) using a 546 nm filter.

3. RESULTS AND DISCUSSION

An antibody raised against purified yeast topoisomerase II was used to probe total cell extracts on Western blots. The antibody was a gift from Dr L.F. Liu, John Hopkins University, Baltimore, USA. This antibody recognises the 150 kDa topoisomerase II protein on Western blots of yeast proteins (not shown). On Western blots of total cell wheat proteins, the antibody showed specific binding to a high molecular mass band (fig.1), which would be discerned on the autoradiograph as a close doublet. Sizing of this band gave an apparent molecular mass from two experiments of 105 kDa. Since equivalent numbers of wheat mesophyll cells were loaded per track, changes in the band intensity are directly related to changes in topoisomerase II content per mesophyll cell. This 105 kDa band was not detected in the basal leaf section where the cells are just post-meristematic but was present in all older leaf cells and increased steadily until the leaf mesophyll cells were about 46 h old (4–4.5 cm from the leaf base) but then declined. In a second experiment the gel was run at 8 mA overnight to achieve greater separation of the tight doublet band observed on the cell blots. On these second blots (fig.2), the doublet is observed as two bands in both chloroplast and cell extracts with apparent molecular masses of 101 and 96 kDa. The developmental profile indicated that the highest levels of the putative topoisomerase II in chloroplasts is in cells 36 h old (2–2.5 cm from the leaf base) and thereafter the amount slowly declines (fig.2). The consistent binding of the yeast topoisomerase type II antibody to these two bands and its appearance amongst the chloroplast polypeptides lead us to believe that this band represents a topoisomerase type II enzyme in the chloroplast.

The size of the putative chloroplast topoisomerase II band with two bands of 96 and 101 kDa would not be inconsistent with the presence of a bacterial-like gyrase protein, since this enzyme in *E. coli* has subunits of 95 and 105 kDa [18]. The bacterial gyrase also shows considerable sequence homology with yeast topoisomerase II [19] used to raise the antibody employed in the present studies. Indeed the use of topoisomerase inhibitors [20] in in vitro chloroplast transcription assays [5] has previously suggested that chloroplasts may possess a prokaryotic gyrase-like enzyme capable of negatively supercoiling ctDNA.

A particularly important aspect of our results is the demonstration of topoisomerase accumulation during the phase of rapid chloroplast DNA replica-

Fig.1. Western blot of whole cell extracts from sections of different developmental stages of the primary wheat leaf probed with yeast topoisomerase II antibody. Samples of an equal number of mesophyll cells (19000) from different developmental stages of the leaf are loaded onto each lane: 1, cells from leaf sections taken 0–0.5 cm from the leaf base; 2, at 0.5–1 cm; 3, 1–1.5 cm; 4, 2–2.5 cm; 5, 3–3.5 cm; 6, 4–4.5 cm; 7, 6–6.5 cm; 8, 8–8.5 cm. The putative topoisomerase II and molecular mass standards are shown.

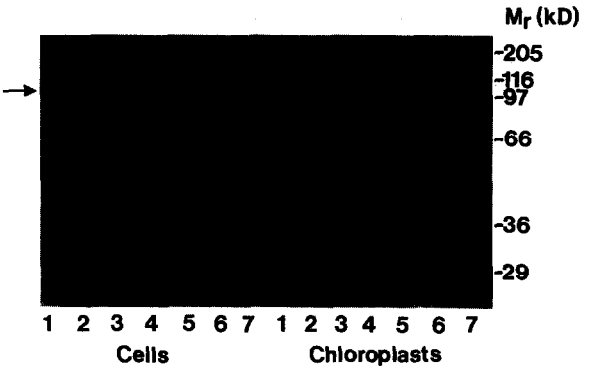

Fig.2. Western blots of whole cell extracts and chloroplast extracts from leaf sections of different developmental stages of the primary wheat leaf probed with yeast topoisomerase II antibody. Samples of an equal number of mesophyll cells (28 500) or chloroplasts (21×10^6) from different developmental stages of the leaf were loaded onto each lane: 1, cells or chloroplasts from sections taken 0–0.5 cm from the leaf base; 2, 1–1.5 cm; 3, 2–2.5 cm; 4, 3–3.5 cm; 5, 4–4.5 cm; 6, 6–6.5 cm; 7, 8–8.5 cm. The putative topoisomerase II and the molecular mass standards are shown.

tion in wheat leaf cells. Chloroplast DNA replication within the developing and dividing chloroplast occurs between 24 and 36 h after cell division ceases in cells between 1 and 2.5 cm from the leaf base (9,12) at the time when the topoisomerase II content per chloroplast is maximal (fig.3). During the subsequent chloroplast division phase (indicated by the decline in plastid genome copy number (fig.3)) the topoisomerase II content also rapidly declines. There are barely detectable levels of topoisomerase II in chloroplasts which have ceased to divide (above 4 cm).

The period of maximal topoisomerase II accumulation also coincides with the period of major increase in transcription of chloroplast genes as measured by in vitro translation of chloroplast proteins [21] and exemplified by a five-fold increase in the production of ribulose bisphosphate carboxylase large subunit transcripts in 24–36 h cells (Leech et al., unpublished).

The profiles of the change in levels of the topoisomerase bands expressed as per mesophyll cell and as per chloroplast differ since the mesophyll cell profile is a product of changes in level per plastid and also the increased chloroplast number per cell after chloroplast division. In mature leaf cells the topoisomerase II bands are not seen in the chloroplasts.

We conclude from these experiments that topoisomerase type II enzyme is associated with the chloroplasts in young wheat leaves, and that the amount of enzyme per chloroplast is highest at the time when chloroplast DNA replication is maximal. The apparent molecular weights and dual

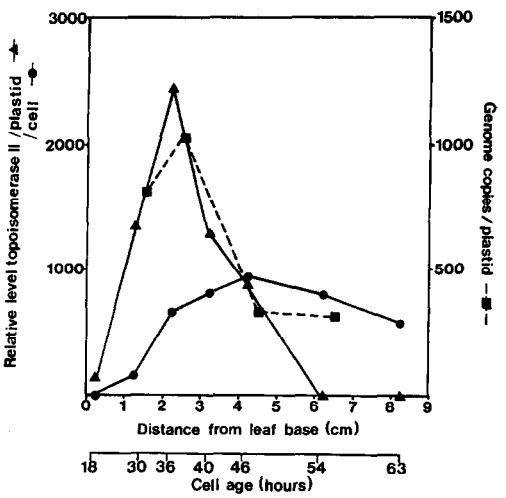

Fig.3. Relative levels of topoisomerase II per chloroplast (▲) and topoisomerase II per mesophyll cell (●) from different developmental stages in the young developing wheat leaf as determined from scanning of autoradiographs of Western blots plotted with the changes in chloroplast DNA levels per plastid (■) from [9]. Cell ages are taken from [11].

bands of this putative chloroplast topoisomerase II suggest that the protein may be similar to a bacterial gyrase type II enzyme with two subunits. The precise roles of topoisomerases in the chloroplast are unclear but the balance between topoisomerase type I and gyrase activities on the ctDNA has been shown to be important in controlling transcription of some chloroplast genes [5,22].

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