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Cell cycle stage-specific differential expression of topoisomerase I in tobacco BY-2 cells and its ectopic overexpression and knockdown unravels its crucial role in plant morphogenesis and development

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ABSTRACT

DNA topoisomerases catalyze the inter-conversion of different topological forms of DNA. Cell cycle coupled differential accumulation of topoisomerase I (Topo I) revealed biphasic expression maximum at S-phase and M/G_1-phase of cultured synchronized tobacco BY-2 cells. This suggested its active role in resolving topological constrains during DNA replication (S-phase) and chromosome decondensation (M/G_1 phase). Immuno-localization revealed high concentrations of Topo I in nucleolus. Propidium iodide staining and Br-UTP incorporation patterns revealed direct correlation between immunofluorescence intensity and rRNA transcription activity within nucleolus. Immuno-stained chromosomes during metaphase and anaphase suggested possible role of Topo I in resolving topological constrains during mitotic chromosome condensation. Inhibitor studies showed that in comparison to Topo I, Topo II was essential in resolving topological constrains during chromosome condensation. Probably, *Topo II* substituted *Topo I* functioning to certain extent during chromosome condensation, but not vice-versa. Transgenic *Topo I* tobacco lines revealed morphological abnormalities and highlighted its crucial role in plant morphogenesis and development.

1. Introduction

Topoisomerases are ubiquitous nuclear enzymes that play a crucial role in resolving the complex topological constrains generated while unwinding of duplex DNA during replication, transcription and recombination [1,2]. Different types of topoisomerases have been identified in higher eukaryotes including plants wherein they are broadly categorised into IA, IB, IIA and IIB. These topoisomerases functionally complement each other to some extent for resolving topological constrains in DNA, however, each form of topoisomerase may be specialized to perform specific functions *in vivo* [3,4]. The type I topoisomerases (*Topo I*) are mainly responsible for relaxation of transcription and replication induced supercoiling [5–7], whereas, the type II topoisomerases (*Topo II*) are required mainly for resolving entangled DNA strands during replication and recombination [8,9]. *Topo I* relaxes excess supercoiling of DNA by creating a single-strand break to allow rotation of cleaved strand around the double helix axis to resolve the excess superhelical torsion, and eventually re-ligates the cleaved strand to re-establish the intact duplex DNA [10,11].

The topoisomerases are known to regulate different cell cycle checkpoints as a surveillance mechanism when conditions are inappropriate, that arrests or delays cell division progression to couple the essential events of cell division [9,12–14]. The temporal sequence of many cell cycle events must be maintained to protect the genome integrity and faithful transmission of genetic material to the daughter cells [15,16]. The *in vivo* roles of *Topo I* were initially revealed by analysis of loss of function mutants in lower eukaryotes [17]. However, such topoisomerase mutants are not viable in higher eukaryotes including plants [18,19]. The demand for Topo I activity and content varies as a function of the prolif-

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; GUS, β-glucuronidase; PCNA, proliferating cell nuclear antigen; NOS, nopaline synthase.

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erative and/or physiological status of the cell [20]. Proliferating cells have a much higher Topo I content and activity than the cells that are quiescent. However, the cell divisions are highly regulated, both spatially and temporally during plant development [21–26]. In mature plant, most cell proliferation is restricted to only specialized regions called meristems. Therefore, it is difficult to establish the role of Topo I in different stages of plant cell divisions. Not much is known about the regulation of topoisomerase expression and the functional role of its activity in the cell cycle progression and plant development.

In this study, we investigate the role of *Topo I* during various stages of cell cycle using synchronised suspension cultures of tobacco BY-2 cell line as a model system. We monitored the role of Topo I during interphase and mitotic division by immunofluorescence. Moreover, we established the role of tobacco *NtTopo I* in plant development by ectopic expression of this gene by sense and antisense transgenic approach.

2. Materials and methods

2.1. BY-2 culture and synchronization

Suspension culture of BY-2 cells were maintained by weekly dilution (5:100) into fresh MS medium and cultured at 26 °C and 120 rpm in dark. For synchronization of cell divisions in suspension culture, fully-grown culture was diluted 1:5 in fresh MS medium containing 5 μg ml^{-1} aphidicolin and grown for 24 h. The cells were harvested and washed extensively with MS medium. Further the cells were re-suspended into fresh medium and after 6 h, 1.6 μg ml^{-1} propyzamide was added to arrest the cell growth at G_1/S phase. The propyzamide was removed by extensive washing of cells. The G_1/S phase arrested cells were grown on fresh MS medium for synchronized cell growth [27].

2.2. Incorporation of [^{3}H] thymidine

The synchronized tobacco BY-2 cell suspension culture was incubated with 1 μCi [^{3}H] TTP (NEN Dupont) at 27 °C on rotatory shaker. After incubation, 1 ml of culture was withdrawn at 1 h time intervals and monitored for incorporation of radioactive thymidine into the replicative DNA. The cell suspension culture (1 ml) was mixed with 4 ml of ice-cold ethanol and cells were pelleted down by centrifugation at 2000 × *g* for 10 min. The pellet was washed twice with ice-cold ethanol (80%) and then with 0.2 M ice-cold perchloric acid. Nucleic acids were solubilized by heating in 0.5 M perchloric acid at 80 °C for 15 min. The extract was centrifuged and radioactivity of an aliquot of the supernatant was quantified using liquid scintillation counter [28].

2.3. Total RNA isolation and RT-PCR analysis

Total RNA from *Nicotiana tabaccum* cultured cells and/or leaf tissues were isolated according to Chomczynski and Sacchi [29]. Poly (A)$^+$ RNA was purified after annealing it to 5′-biotinylated oligo-dT$_{(18)}$ primer and subsequently immobilized on streptavidin-linked paramagnetic beads followed by magnetic separation. First strand cDNA synthesis was carried out using 5 μg total RNA and 200 U of superscript II, a reverse transcriptase enzyme. The relative abundance of *Topo I* transcript was monitored along with the expression of two cell cycle marker genes PCNA and cyclin-B by RT-PCR analysis. PCR was carried out in a reaction volume of 50 μl containing dNTPs (200 μM), Taq DNA polymerase (2.5 U) and 150 ng of specific primers (Table 1) using 20 ng of cDNA as template with reaction conditions as 94 °C 1 min; 53 °C 1 min and 72 °C 1 min for 30 cycles. The relative expression values of *Topo I*, PCNA and cyclin-B were normalized using the expression values of tobacco cdc2a as a reference gene, which constitutively expressed throughout the cell cycle [30]. Relative abundance of *Topo I* transcript was monitored in the sense and antisense transgenic plants by RT-PCR. The expression levels were normalized using actin gene as internal standard. Three replicates were performed for each analysis.

2.4. Developing antibodies

We cloned the complete coding sequence of *NtTopo I* in expression vector pET28a using the forward and reverse primers 5′-GAGACATATGATGGCTGTTGAGGCTTTTTCCA-3′ and 5′-GAGACTCGAGTGGGAACCTATGGCGCTCACA-3′ flanked by *Nde* I and *Xho* I restriction sites and eventually expressed as an N-terminally His-tagged fusion protein in *Escherichia coli* strain BL21 (DE3). Polyclonal antibody against purified recombinant tobacco topoisomerase I was raised in rabbit. Pre-immune serum was obtained by collecting blood from the ear lobe of the rabbit in a clean sterile dry glass tube. The blood was allowed to stand for 1 h at room temperature and then incubated overnight at 4 °C. The clot was carefully separated from the walls of the tube with the help of a glass rod and straw-coloured serum was separated from the clot by centrifugation at 500 × *g* for 15 min. The supernatant was collected, aliquoted in eppendorf tubes and stored at −20 °C. Purified recombinant polypeptide (250 μg) was mixed with an equal volume of Freund's complete adjuvant (Sigma Chemical Co, USA) and this emulsified sample was injected subcutaneously into New Zealand white rabbit. The primed rabbit was given first booster after one month of primary immunization with 150–200 μg of protein emulsified with Freund's incomplete adjuvant. Subsequent booster doses were administered monthly as described for the first. Test bleeding was done after two week of each booster and titre of the antibodies was checked using western blotting. Blood (50 ml) was collected from the immunized rabbit and antiserum was separated as described earlier.

2.5. Immunofluoresence staining and confocal microscopy

Exponentially growing BY-2 suspension cells of tobacco were fixed and permeabilized according to Proust et al. [31] and layered onto poly-L-lysine coated cover slips. The cells were immunostained with tobacco topoisomerase specific primary antibody in 1:2000 and Alexafluor-488 labeled secondary antibody (Molecular Probes, USA) in 1:1000 dilutions. The cells were counterstained with DAPI (0.2 μg ml^{-1}) for 20 min just before mounting the slide in anti-fade solution (Fluroguard, Bio Rad, USA). Confocal laser scanning (Radiance 2100, Bio-Rad, USA) was performed using a Nikon microscope (objective Plane Apo 60×/1.4 oil, Japan). The excitation wavelength for Alexafluor fluorescence was 488 nm (argon laser) and fluorescence was detected through emission filter HQ515/30 (High quality band pass, centered at 515 nm) with bandwidth of 30 nm. DAPI fluorescence was excited by blue diode (405 nm) and detected through emission filter HQ442/45. Image processing was carried out using LazerSharp (Bio-Rad, USA) and PhotoShop (5.5) (Adobe systems, San Jose, CA) was used for the final image assembly. The inhibition of Topo I and II by their inhibitors campotothecin (10 μM) and nogalamycin (20 μM), respectively was monitored to reveal their significant role in chromosome packaging and condensation at different stages of mitosis in cultured tobacco BY-2 cells.

2.6. Tobacco plant transformation

The complete ORF of *Topo I* was amplified using the forward primer 5'-GAGAGCTAGCATGGCTGTTGAGGCTTTTTCCA-3′ and the reverse primer 5'-GAGAGCTAGCTGGGAACCTATGGCGCTCACA-3′