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DNA replication after mutagenic treatment in *Hordeum vulgare*

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ABSTRACT

The temporal and spatial properties of DNA replication in plants related to DNA damage and mutagenesis is poorly understood. Experiments were carried out to explore the relationships between DNA replication, chromatin structure and DNA damage in nuclei from barley root tips. We quantitavely analysed the topological organisation of replication foci using pulse EdU labelling during the S phase and its relationship with the DNA damage induced by mutagenic treatment with maleic hydrazide (MH), nitroso-N-methyl-urea (MNU) and gamma ray. Treatment with mutagens did not change the characteristic S-phase patterns in the nuclei; however, the frequencies of the S-phase-labelled cells after treatment differed from those observed in the control cells. The analyses of DNA replication in barley nuclei were extended to the micronuclei induced by mutagens. Replication in the chromatin of the micronuclei was rare. The results of simultanous TUNEL reaction to identify cells with DNA strand breaks and the labelling of the S-phase cells with EdU revealed the possibility of DNA replication occurring in damaged nuclei. For the first time, the intensity of EdU fluorescence to study the rate of DNA replication was analysed.

1. Introduction

Physical and chemical mutagens induce a wide variety of DNA damage. The S-phase of the cell cycle has proven to be very sensitive to mutagenic factors. Precise genome replication is crucial in maintaining the stability of genomes so any replication errors are critical for living cells. The proper checkpoints coordinate the cell cycle progression with the DNA damage response. The checkpoints can block the cell cycle in G1, S or G2 in response to genotoxic stress in order to allow cells to repair DNA lesions. The S-phase checkpoints reduce the rate of DNA synthesis by minimising the risk of damage being fixed into the mutations before it can be repaired [1]. This response enhances genomic stability by providing time for cells to repair DNA damage [2]. It is known that DNA damage can be also passed through mitosis and thus propagates the mutations to daughter cells. Translesion DNA synthesis (TLS) is defined as the incorporation of a nucleotide across DNA damage followed by an extension of the potentially mispaired primer-template, which can be error-free or error-prone [3]. TLS is mediated by specialised DNA polymerases that have poor discrimination ability for correct base pairs and thus enables lesion sites to be bypassed and replication fork stalling to be prevented. Therefore, the cell replication and DNA damage can simultaneously be tolerated in the same cells.

DNA replication is a temporary and spatially ordered process, in which specific genome compartments replicate at different times. Knowledge about replication in eucaryotic cells mainly comes from studies on yeast and animal cell cultures [4]. A limited number of plant species has been analysed regarding the spatial distribution of DNA synthesis. The quantitative analysis of spatiotemporal patterns of DNA replication was recently characterised in detail in maize [5]. Resolving the relationships between the timing of DNA replication, chromatin structure and DNA damage is an important task in plant mutagenesis. Detection of DNA synthesis in proliferating cells is possible through the incorporation of labelled DNA precursors into cellular DNA during the S-phase of the cell cycle. Until recently, bromodeoxyuridine (BrdU) has been used to identify replicated chromatin. One major disadvantage of using BrdU is that cells and tissues need to be subjected to strong denaturation that degrades the DNA structure. The next disadvantage is that the specific antibodies used for the BrdU detection increase the size of the signals. In spite of these problems, the BrdU labelling method has been useful for DNA replication analysis for the last few years because modern labelling techniques with higher resolutions to examine spatiotemporal patterns in more detail were lacking. Nowadays, the "click" reaction with 5-ethynyl-2'-deoxyuridine (EdU) can be applied [6,7]. The chromatin structure is well preserved using this technique, which makes this method universally convenient to study both monocts and dicots [8].

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Little is known about the temporal and spatial properties of DNA replication in plants in relation to DNA damage and mutagenesis. However structural and functional relations between spatial and temporal modification of chromatin structure with specific function of chromatin domains were shown previously in control cells. The replication time, nuclear organisation and histone acetylation patterns of distinct chromatin domains of barley during the cell cycle have been studied. The results indicate that replication of the chromatin domains is temporally ordered and reflects the polar nuclear organisation [9].

The aim of the study was to characterise the relationships between DNA replication, chromatin structure and DNA damage in plant nuclei. In the present study, we used barley (*Hordeum vulgare,* 2n = 14) as the model plant species. Barley is a species that is characterised by a relatively large genome, like most cereals. The characteristic spatial architecture of the nucleus, including the polarised organisation of the interphase chromatin, the so-called Rabl configuration, and the specific heterochromatin distribution make barley a convenient species for the analysis of the spatio-temporal properties of DNA replication [10,11], especially in the context of mutagenesis.

In order to meet the aim of the study, we characterised the dynamics of chromatin replication in a control and mutagen-treated barley cells. The localisation of the replication sites in *H. vulgare* root tip nuclei was analysed using pulse EdU labelling in the control and mutagen-treated cells. Maleic hydrazide (MH), nitroso-N-methyl-urea (MNU) and gamma ray were used as the mutagens for treatment. We analysed the topological organisation of the replication foci during the S phase quantitavely. The relationship of DNA replication with DNA damage induced by mutagenic treatment in barley cells was also analysed. To accomplish this, a simultanous TUNEL reaction to identify cells with DNA strand breaks [12] and the labelling of S-phase cells with EdU were applied. For the first time, the intensity of EdU fluorescence in order to study the rate of DNA replication was analysed.

2. Material and methods

2.1. Mutagenic treatment

Seeds of the barley (*Hordeum vulgare,* 2n = 14) "Start" variety were used as the plant material. Maleic acid hydrazide (3 mM or 4 mM MH; Sigma, CAS 123–3301), N-nitroso-N-methylurea (2 mM or 3 mM MNU; CAS 684-93-5) and a gamma ray (175 Gy and 225 Gy) were used for mutagenic treatment. The mutagen doses used in the study were applied in previous experiments in which their cytogenetic effects were estimated [13,14]. Before chemical treatment, the seeds of barley were pre-soaked in distilled water for 8 h and then treated with MH or MNU for 3 h. After the treatment, the seeds were washed three times in distilled water and then germinated in Petri dishes at 21 °C in the dark. The irradiation was performed at the International Atomic Energy Agency, Seibersdorf Laboratory, Austria. After irradiation, the seeds were pre-soaked in distilled water for 8 h and germinated in Petri dishes at 21 °C in the dark. The mutagenic treatment procedure was repeated twice.

2.2. EdU detection

The barley seedlings were incubated for 1 h in the dark in a 10 mM EdU solution (5-ethynyl-2'-deoxyuridin; Click-iT EdU Imaging Kits Alexa Fluor 647, Invitrogen). After EdU incorporation, the seedlings were rinsed in distilled water 2 × 5 min and fixed in 3.7% paraformaldehyde in PBS for 30 min. The fixed seedlings were washed 3 × 5 min in PBS. The roots of seedlings were used as the source of meristems for the investigations. For nuclei preparation, the material was washed with 0.01 mM sodium citrate buffer (pH 4.8) for 30 min and digested with 2% cellulase (w/v, Onozuka, Serva) for 1 h at 37 °C. After digestion, the material was washed again with a sodium citrate buffer for 30 min. Squash nuclei preparations were made in a drop of PBS. After freezing and removal of the coverslips, the slides were dried.

Prior to EdU detection, the nuclei slides were permeabilised with 0.5% Triton X-100 for 20 min, and then washed in PBS at RT. The slides were incubated for 30 min at RT in an EdU reaction cocktail (Click-iT EdU Imaging Kits Alexa Fluor 647, Invitrogen), which was prepared according the manufacturer's procedure. For one sample reaction, the following components were added: 43 μl of a 1 x Click-iT reaction buffer, 2 μl of $CuSo_4$ (Component E, 100 mM), 0.12 μl Alexa Fluor 647 azide (Component B) and 5 μl reaction buffer additive (Component F). After 2 × 5 min washes, the slides were stained with 2 μg/ml DAPI (Sigma), washed with PBS and mounted in a Vectashield medium (Vector). The frequencies of EdU-labelled nuclei in the early, middle and late S-phase were analysed.

2.3. TUNEL reaction

The TUNEL reaction for the analysis of DNA damage was applied after EdU detection. Prior to the TUNEL reaction, slides were air dried and permeabilised by incubating the preparations in 0.1% Triton X-100 (Sigma) in 0.1% sodium citrate at 4 °C for 2 min. Then, the preparations were rinsed with PBS. For the positive control, a slide was treated with a DNAse solution (1U) for 30 min at 37 °C in a humid chamber. DNA fragment labelling was carried out with the TUNEL reaction mixture (*in situ* Cell Death Detection Kit, Fluorescein, Roche). 50 μl of the TUNEL reaction mixture (enzyme solution—terminal transferase: label solution, 1:9 v/v) was applied to the preparations and incubated in a humid chamber in the dark for 1 h at 37 °C. As a negative control of the TUNEL reaction, a reaction mixture without any enzyme was used. Preparations were rinsed 3 x with PBS and stained with DAPI (2 μg/ml), air dried and mounted in a Vectashield medium (Vector Laboratories).

2.4. Analysis

Preparations were examined with a Zeiss Axio Imager.Z.2 wide-field fluorescence microscope equipped with an AxioCam Mrm monochromatic camera. For the analyses of the S-phase in barley, images were captured and processed using Adobe Photoshop 4.0. For each analysed endpoint 6 plants were monitored, taking into consideration 2 treatment experiments and number of plants used for one treatment (3 plants).

The frequencies of nuclei with Alexa Fluor 647 signals were calculated. For each experimental group, 1000 cells on each of three slides (each slide made from two meristems from one plant) were evaluated. The detailed distribution of the signals was analysed on the same slides; 300 nuclei were analysed in total for each experimental group. The micronuclei have been analysed on the same slides as nuclei.

The average intensity of the fluorescence of Alexa Fluor 647 after EdU detection, which were labelled the S-phase cells, control and after mutagenic treatment, was analysed. The quantitative acquisition and analysis were performed using a high-content screening system (ScanR̂, Olympus) based on an Olympus IX81 wide-field microscope equipped with an ORCA-ER CCD camera (Hamamatsu Photonics) and an MT20 illumination system based on a 150-W xenon mercury lamp. The automated segmentation of nuclei was based on the threshold values (the border value of the fluorescence intensity of the pixels between the background and the object). The levels of fluorescence were measured as the average value from the total Alexa 647 fluorescence intensities, which were carried out on at least 1000 nuclei, which were measured for the con-

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