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A comparative analysis of membrane intactness and genome integrity in pea, barley, and wheat in response to UVC irradiation

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Abstract: The maintenance of plant genome integrity plays a critical function in the processes of DNA replication, transcription, and repair. Short-wave UV radiation (UVC) is among the most harmful agents known to affect genome stability and to induce DNA damage, including double-strand breaks (DSBs). Most previous studies in plants addressed the effects of UVC radiation at the physiological level; however, little research effort has been put into genome sensitivity across different plant species. Here, we made use of the trypan blue exclusion test and neutral comet assay to assess nuclear membrane and genome integrity in response to UVC radiation in monocot and dicot plants. We found that UVC radiation substantially affects nuclear membranes and the level of DSBs in a dose-responsive manner. Furthermore, differential sensitivity across plant species was observed, with monocot plants being less vulnerable to DSBs. This allows us to speculate that plant species with larger genomes may better tolerate UVC radiation.

Key words: Ultraviolet radiation, genome integrity, trypan blue exclusion test, neutral comet assay, DNA double-strand breaks

1. Introduction

Plants are continuously exposed to solar radiation. Ultraviolet (UV) radiation, one of the components of sunlight, can be divided into three categories: long-wave UVA (315–400 nm), medium-wave UVB (280–315 nm), and short-wave UVC (100–280 nm). The ozone layer efficiently absorbs UV radiation up to about 310 nm as it shields all UVC and more than 95% of UVB. The most comprehensive data are available about the effect of UVA and UVB radiation on plants. The physiological and genetic response of plant cells to UVA radiation has been observed during stem extension, leaf development, and phototropism (Kunz et al., 2006). Most biological macromolecules are targets of UVB radiation. Alterations in important processes like photosynthesis, photomorphogenesis, seed germination, growth and development, and secondary metabolism have been observed (Mpoloka, 2008). Several studies reported an impact on membranes, phytohormones (Frohnmeyer and Staiger, 2003), and the activation of transposable elements (Qüesta et al., 2010).

UVC light is the most energetic and harmful photolytic agent that has the potential for inducing DNA damage, even at very short exposures. Similarly to UVB, the effects of UVC radiation on the plant genome can be of direct or indirect origin, detected mainly as pyrimidine dimers (adjacent thymine and cytosine), photoproducts (intrastrand cyclobutane-type pyrimidine dimers), which have the capacity to block DNA replication and transcription in plants cells. These lesions are repaired mainly by excision repair; however, incomplete processes can result in the formation of single-stranded DNA gaps sensitive to endonuclease attack (Myllyperkiö et al., 2000). Hence, DNA double-strand breaks (DSBs) also accumulate as a result of these described processes and are followed by chromosomal damage (Ma et al., 2009). In addition, UVC radiation contributes to the formation of DSBs in dividing cells most often through the production of intercellular reactive oxygen species (ROS) (Zemp et al., 2012). Several studies have reported the accumulation of endogenous DSBs caused by "cutting effects" or by the occurrence of a sufficient amount of adjacent single-strand breaks in human cells (Bogdanov et al., 1997; Tashiro, 2000). The effects of UVC irradiation on DNA depend on cell type and proliferation status, DNA repair capability, and the presence of endogenous and exogenous photosensitizers (Stapleton, 1992). Since monocotyledonous (monocot) plants have vertical patterns of leaf growth they tend to

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capture less direct light than dicotyledonous (dicot) plants, whose leaves tend to grow horizontally. In this regard, monocots are suggested to be more tolerant to UV irradiation than dicot plants (reviewed by Kakani et al., 2003).

Despite the accumulated data on plants addressing the effects of UVC radiation, little research effort has been put into genome sensitivity across different plant species. The aim of the present study was to assess the performance of the trypan blue dye exclusion test and the neutral comet assay for detection of UVC-induced alterations affecting nuclear membrane intactness and genome integrity in monocot and dicot plants.

2. Materials and methods

2.1. Growth of plant material and isolation of nuclei

Plants at the 2nd true leaf stage from the dicot *Pisum sativum* L. and the monocots *Triticum aestivum* L. and *Hordeum vulgare* L. were raised under controlled conditions: 26/22 °C day/night temperature, 16/8 h (day/night) photoperiod, 150 μmol $m^{-2} s^{-1}$ photon flux density, and 60% air humidity. From 15 to 20 irradiated and nonirradiated (control) plants were used in all experiments. Individual leaves were placed in a cold Sörensen buffer, pH 6.8, and were gently sliced with a razor blade into a 'fringe' to release the nuclei (Gichner and Plewa, 1998). The nuclear suspension was filtered through 30 μm nylon mesh and centrifuged at 550 × *g* for 5 min at 4 °C.

2.2. UVC irradiation

Freshly isolated nuclear suspensions in a monolayer were irradiated using a BLX 254 UV crosslinker (Life Technologies, GIBCO, BRL UV Crosslinker) with peak emission at 254 nm. The UVC dose was adjusted by varying the duration of the exposure. For pea nuclei exposures ranged from 5 min to 12 min, corresponding to doses between 5 kJ/m^2 and 9 kJ/m^2. For barley nuclei, exposure times were from 5 min to 30 min, corresponding to doses between 5 kJ/m^2 and 25 kJ/m^2. For wheat nuclei, exposure times ranged from 17 min to 24 min, corresponding to doses between 14 kJ/m^2 and 18 kJ/m^2. Irradiation doses for pea nuclei were chosen based on the morphological effect of UVC on intact plants observed by Todorova et al. (2013). Irradiation doses for monocots were chosen according to Kakani et al. (2003).

2.3. Measurement of nuclear membrane intactness

The nuclear membrane integrity after UVC exposure was determined using the trypan blue dye exclusion test described by Nikolova et al. (2013). This parameter was presented as a percentage of the number of nuclei excluding trypan blue to the total number of nuclei for each plant species (more than 100 nuclei were counted for each experimental check point). The nonparametric Kruskal–Wallis test was used for the statistical analysis. The Duncan test was subsequently applied for pair-wise comparisons. The level of statistical significance was set at $P < 0.05$. The calculated values were presented as a mean with standard deviation (±SD) from at least 3 independent experiments.

2.4. Neutral comet assay protocol

Immediately after the UVC exposure, nuclei were processed for the comet assay. The procedure followed the protocol of Georgieva and Stoilov (2008). Embedded nuclei were incubated in lysis buffer at 4 °C in dark for 5 min, 15 min, and 60 min for pea, wheat, and barley nuclei, respectively. Electrophoresis was performed in an electrophoresis buffer precooled to 4 °C with 1X TAE (pH 8) at 0.5 V/cm (pea nuclei), 1 V/cm (wheat nuclei), or 9 V/cm (barley nuclei). The gels were dehydrated consecutively, air-dried, and stained with acridine orange (10 μg/mL). From each slide, at least 50 randomly chosen nucleoids were inspected under a Zeiss Jenamed-2-fluorescence microscope with an excitation filter of 510 nm, then captured by a digital camera (Samsung Digimax V50) and analyzed using the Comet Score software (Tritec Corporation, USA). The percentage of DNA in the comet tail reflects the number of DNA strand breaks and thus represents a quantitative measure of DNA damage. This parameter was presented as a mean value with a standard error (±SE). Each experiment was performed in three replicates and three slides were analyzed for each experimental group. Generalized linear model (GLM) analysis and Tukey HSD multiple comparison implemented in SPSS 15.0 were used to evaluate the differences between all groups (Lovell et al., 1999).

3. Results

3.1. Analysis of nuclear membrane intactness

In this study, a series of dose-response experiments were performed in order to determine the dose effect of UVC exposure on the nuclear membrane integrity of pea, wheat, and barley nuclei. In the trypan dye exclusion test undamaged nuclei displayed a bright blue color, indicating an intact membrane, while damaged nuclei were colored in dark blue, indicative of a loss of membrane intactness. A representative microphotography of damaged and undamaged nuclei isolated from pea leaves is presented in Figure 1.

Figure 2 shows the response of plant nuclei from pea, barley, and wheat to various UVC doses. A background level of membrane-disrupted nuclei was observed in all controls (nonirradiated) in the range of 7% to 8% (Figure 2). This is likely to be due to the mechanical isolation of nuclei or to the use of plant material without synchronized cell cycles. Significant decrease of nuclear membrane intactness was observed in all plant species after UVC

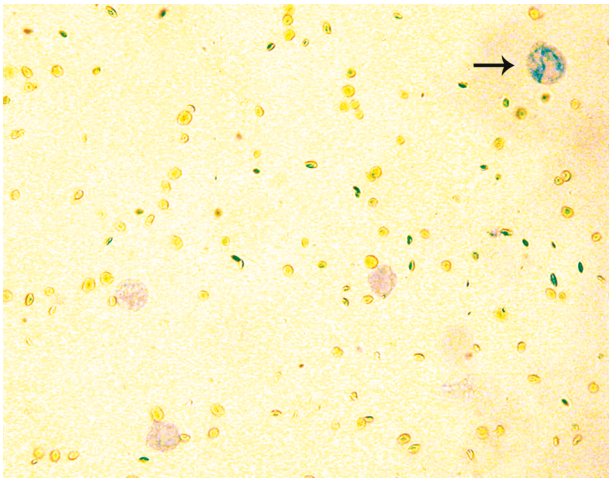

Figure 1. Visual representation of the trypan blue exclusion test performed on pea nuclei. Nuclei with intact membranes are colored in bright blue (negative test). A typical nucleus with lesions (dark blue spots) in the structure of the membrane (positive test) is depicted by an arrow.

irradiation, but after different doses (7 kJ/m^2 for pea, 15 kJ/m^2 for barley, 16 kJ/m^2 for wheat). The threshold lesion doses were 7 kJ/m^2 for pea (Figure 2A), 5 kJ/m^2 for barley (Figure 2B), and 16 kJ/m^2 for wheat (Figure 2C). The similar threshold value for pea and barley is indicative of their similar sensitivity to UVC irradiation.

3.2. Analysis of UVC-induced DNA damage by neutral comet assay

The level of UVC-induced DSBs at nucleoid level increased in a dose-dependent manner in pea (Figure 3A). The percentage of DNA in the tail was 22.14 ± 0.81% after exposure of *P. sativum* nuclei at a dose of 5 kJ/m^2. Exposure at 9 kJ/m^2 resulted in an increase of DSBs to 38.06 ± 0.85%.

Monocot-type nuclei accumulated different levels of UVC-induced DNA strand breaks (Figures 3B and 3C). The lowest dose of UVC (5 kJ/m^2) increased the tail DNA up to 20.17 ± 0.75% in barley (Figure 3B), which is comparable to the values of the parameter in pea. This effect strongly correlated with statistically significant results from the trypan blue dye exclusion test for nuclear membrane intactness at the same dose (Figure 2). In addition, a much higher dose (25 kJ/m^2) was required to reach the maximum level of DSBs (46.27 ± 0.99%) in barley compared to pea (Figure 3B). These results are indicative of the lower sensitivity of barley genome as compared to pea in regard to the level of induced DNA strand breaks.

UVC-treated wheat nuclei populations exhibited significantly less percentage of DNA damage (14.88 ± 0.55%) even at a higher UVC dose of 18 kJ/m^2 (Figure 3C). The level of DSBs is comparable to background level of the DNA damage observed in control barley samples (Figures 3B and 3C).

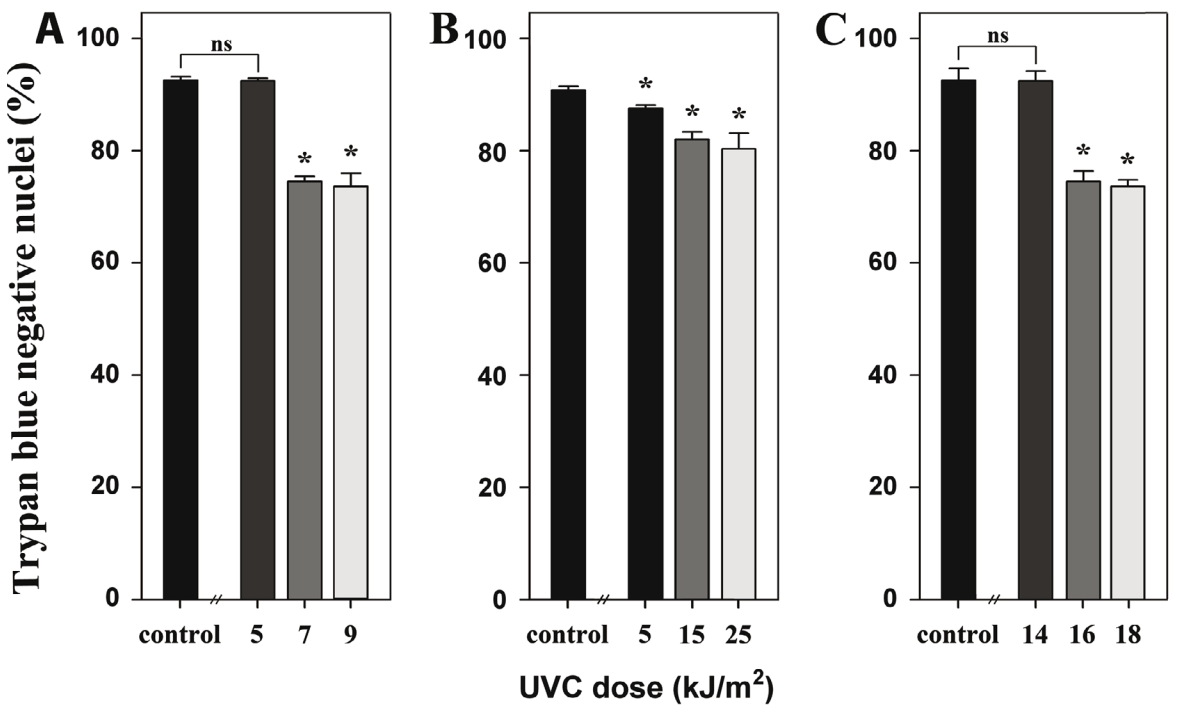

Figure 2. Mean values of membrane integrity (±SD) of pea (A), barley (B), and wheat (C) nuclei exposed to different doses of UVC irradiation and assessed using the trypan blue exclusion test. *P < 0.05 vs. control (Kruskal–Wallis test followed by Duncan's test). ns: not significant.

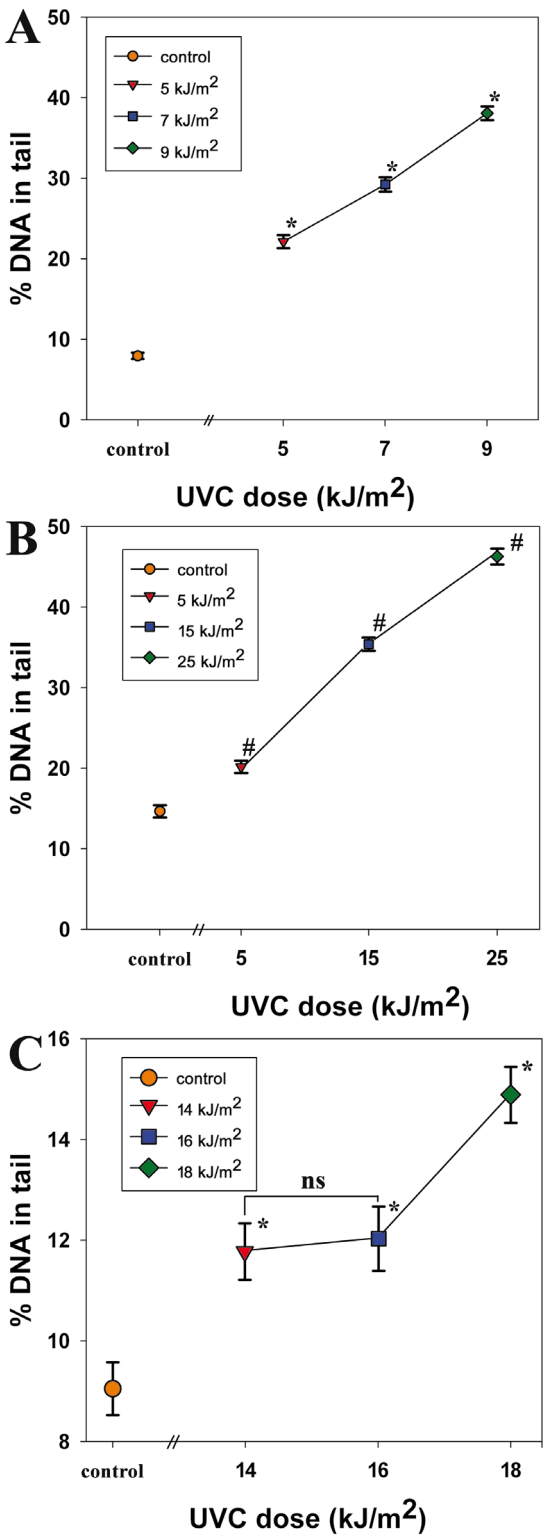

Figure 3. UVC-induced DNA damage in pea (A), barley (B), and wheat (C) nuclei as determined by the neutral comet assay. The graphs represents the mean values. *GLM followed by Tukey HSD multiple comparison ($P < 0.001$; # $P < 0.05$; ns: not significant). Error bars represent standard deviation of mean.

4. Discussion

UVC radiation is a powerful genotoxic and cytotoxic agent capable of inducing severe alterations in the membrane and cytoplasmic structures as well as different DNA lesions. Here, we compared the genetic response of three plant species to UVC irradiation in regard to alterations in the integrity of plant nuclei. We showed that DNA and nuclear membranes, crucial for the maintenance of cell viability, are affected by UVC irradiation in all studied plant species. The number of nuclei with damaged membranes and the percentage of DNA strand breaks were proportional to the applied UVC dose. The nuclear periphery (nuclear envelope and the underlying nuclear lamina) not only provides mechanical integrity but also triggers signaling cascades shaping nuclear architecture and gene regulation (Wong et al., 2014). Cell and nuclear membranes in human cell lines are targets vulnerable to UV radiation and the activation of membrane-bound cell death receptors is related to the process of apoptosis (Kulms and Schwarz, 2002). Our study fills a critical void in the comprehensive understanding of the impact of UVC radiation on membrane integrity in plants and provides an innovative approach for its assessment in plants. The trypan blue dye exclusion test has been efficiently used to assess cell viability (Mou et al., 2000). Here we demonstrate for the first time that this approach can also be successfully applied for screening of nuclear membrane integrity in plants in response to UVC radiation.

The comet assay was primarily developed and mainly applied to animal cells that have a membrane instead of a typical cell wall. This method requires the isolation of intact plant nuclei, which makes the assay more laborious. Recently, the comet assay has emerged as a robust tool for screening mutagenic effects in genetic ecotoxicology, radiation biology, and DNA repair in plants (Cotelle and Férard, 1999; Stoilov et al., 2013). Similarly to nuclear membrane intactness, we also observed dose-dependent induction of DNA breaks after UVC treatment, as reported in another study in barley (Armalyte and Žukas, 2002).

Monocots and dicots, however, differed in their sensitivity to UVC irradiation. Much higher UVC doses were required for the detection of noticeable alterations in the structure of nuclear membranes in wheat and barley nuclei compared to pea. Similarly, the increase of DNA damage induction, assessed by neutral comet assay, was more pronounced in barley and pea compared to wheat. What may account for such differential sensitivity to UVC radiation? The plant species studied here differ in chromosome number, genome size, and ploidy level. The barley and pea genomes are of the same ploidy level and chromosome number (2n = 14); however, they differ in genome size: 5100 Mb for barley and ~4300 Mb for pea (Macas et al., 2007). The hexaploid wheat (2n = 42,

AABBDD) also belongs to monocots, as does barley, but it possesses a much larger genome (17 Gb) resulting from two polyploidization events of diploid progenitor species *Triticum* and *Aegilops* spp. (Salse et al., 2008). It might be speculated that plants with higher genome size (e.g., hexaploid wheat) are less sensitive to DNA damage and better tolerate UVC irradiation compared to plants with smaller genomes (e.g., pea and barley). This finding allows us to argue that genome size variation and ploidy level influence UVC-induced damage accumulation and account for the observed differences between dicots and monocots and within monocots themselves. As previously reported, these host genome characters are involved in promoting genetic differentiation between monocots and dicots (Leitch et al., 2010) and can stand as an indicator of sensitivity to UVC radiation as previously reported (Heddle and Athanasiou, 1975). The current experimental platform, however, does not allow us to make definite conclusions about this hypothesis. More plant species have to be included in the analysis in order to obtain a comprehensive understanding of the causative relationship between genome size and UV sensitivity.

We do not exclude, however, that the observed sensitivity to UVC irradiation may also be related to species-specific accumulation levels of endogenous $O_2^{\cdot-}$, thiolic compounds, the activities of antioxidant enzymes, and repair capacity (Armalyte and Žukas, 2002; Mahdavian et al., 2008). Further investigations at biochemical and genetic levels are required to comprehensively evaluate this link.

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