

## SHORT COMMUNICATION



# WHIRLY1-deficient chloroplasts display enhanced formation of cyclobutane pyrimidine dimers during exposure to UV-B radiation

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## Abstract

The single-stranded DNA/RNA binding protein WHIRLY1 is a major chloroplast nucleoid-associated protein required for the compactness of nucleoids. Most nucleoids in chloroplasts of WHIRLY1-knockdown barley plants are less compact compared to nucleoids in wild-type plants. The reduced compaction leads to an enhanced optical cross-section, which may cause the plastid DNA to be a better target for damaging UV-B radiation. To investigate this hypothesis, primary foliage leaves, chloroplasts, and nuclei from wild-type and WHIRLY1-knockdown plants were exposed to experimental UV-B radiation. Thereafter, total, genomic and plastid DNA were isolated, respectively, and analyzed for the occurrence of cyclobutane pyrimidine dimers (CPDs), which is a parameter for genome stability. The results of this study revealed that WHIRLY1-deficient chloroplasts had strongly enhanced DNA damages, whereas isolated nuclei from the same plant line were not more sensitive than nuclei from the wild-type, indicating that WHIRLY1 has different functions in chloroplasts and nucleus. This supports the hypothesis that the compaction of nucleoids may provide protection against UV-B radiation.

## 1 | INTRODUCTION

Plants are dependent on sunlight for their survival and cannot avoid the simultaneous exposure to potentially harmful UV-B radiation (UV-BR). High solar irradiance, as other environmental stress situations, triggers the production of ROS. ROS have many detrimental effects on plants (e.g., oxidation of DNA, proteins, lipids) and thereby reduce growth (Frohnmeier & Staiger, 2003; Takahashi et al., 2002). In addition to these indirect effects, UV-BR has a direct photophysical damaging impact on genome integrity, leading to the inhibition of cell division and even cell death (Jansen et al., 1998).

UV-BR gives rise to different types of DNA damage, which can be mutagenic and eventually lethal by impeding replication and

transcription (Strid et al., 1994; Takahashi et al., 2011). The most common damage of DNA induced by UV-BR is the formation of cyclobutane pyrimidine dimers (CPDs). CPDs account for approximately 75% of DNA lesions, while a group of diverse other pyrimidine adducts account for the remainder of the DNA lesions. Upon absorption of UV-BR by the aromatic ring of the bases, CPDs are induced between adjacent pyrimidines on the same DNA strand through dimerization (Mitchell & Nairn, 1989; Takahashi et al., 2011). In plant cells, DNA is distributed among nuclei, mitochondria, and plastids (ptDNA). UV-BR-induced CPD formation can occur in organellar DNA as well as in nuclear DNA (Takahashi et al., 2011).

Plastids are descendants of cyanobacteria and contain multiple copies of a remnant prokaryotic genome. The multiple copies of the

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genome, together with RNA and proteins, are organized in structures that resemble bacterial nucleoids. These structures are remarkably variable in number, shape, and distribution within different types of plastids (Powikrowska et al., 2014). For example, in proplastids, nucleoids are attached to the envelope membrane. During chloroplast development, nucleoids change their position and become attached to thylakoid membranes where unpacked ptDNA could be damaged by ROS formed as a byproduct of photosynthetic electron transport (Powikrowska et al., 2014). Chloroplast nucleoids, however, contain numerous DNA-binding proteins mediating the shaping and organization of the nucleoid (Krupinska et al., 2022; Melonek et al., 2016). These so-called nucleoid-associated proteins (NAPs) have replaced the histone-like proteins of bacteria such as the heat unstable (HU) and histone-like nucleoid-structuring proteins (H-NS) (Kobayashi et al., 2016), which in bacteria determine the shape and topology of nucleoids in response to internal and external factors (Hołowka & Zakrzewska-Czerwińska, 2020). These histone-like proteins are abundant proteins binding unspecifically to DNA. They help the cell to quickly react to changing conditions by coating and condensing the nucleoid, thereby protecting the DNA from damage and creating physical protection for the DNA (Hołowka & Zakrzewska-Czerwińska, 2020). In a *Deinococcus radiodurans* mutant lacking one of these histone-like proteins, the survival rate during UV exposure was eightfold reduced (Wang et al., 2012).

In tobacco, the sulfite reductase has acquired a specific DNA binding motif conferring nucleoid compaction (Cannon et al., 1999). In contrast, in barley, and likely in other monocot species, WHIRLY1 is the major protein determining nucleoid architecture. WHIRLY1 belongs to the small family of WHIRLY ssDNA-binding proteins and contains the DNA-binding motif KGKAAL, whose presence in the WHIRLY protein is specific for angiosperms (Krupinska et al., 2022). Using an antibody against maize WHIRLY1, nucleic acid co-immunoprecipitation revealed that the protein binds to ptDNA in a sequence-independent manner and, hence, was compared to the bacterial HU (reviewed by Krupinska et al., 2022). In barley, WHIRLY1 was shown to be responsible for the compaction of chloroplast nucleoids. DNA staining in leaf cells of barley plants with an RNAi-mediated knockdown of the WHIRLY1 gene revealed that the nucleoid population is much more heterogeneous and that most nucleoids are significantly less compact than those of wild-type plants (Krupinska et al., 2014). Accordingly, it was shown that WHIRLY1 is required for the compact structure of nucleoids mediated by a proline-rich motif (i.e., Pro-Arg-Ala-Pro-Pro, PRAPP), which specifically exists in members of the Poaceae family (Krupinska et al., 2014; Oetke et al., 2022). Besides its role in the architecture of chloroplast nucleoids, WHIRLY1 has also been shown to act as a transcription factor in the nucleus (Krupinska et al., 2022).

In line with the function of WHIRLY1 in compacting chloroplast nucleoids, WHIRLY1 deficiency in barley WHIRLY1 knockdown plants (W1) resulted in nucleoids with heterogeneous sizes which were mostly loosely packed (Krupinska et al., 2014). The target theory says that the probability that a given target is hit by radiation is proportional to the volume of the target (Ballarini, 2010). Hence, it was hypothesized that higher compaction of ptDNA in nucleoids mediated by WHIRLY1 lowers the susceptibility of ptDNA toward UVBR-induced

photodamage. In this study, the potential impact of the chloroplast/nucleus-located WHIRLY1 protein on the resistance of DNA to UV-BR in chloroplasts and nuclei was assessed.

## 2 | MATERIALS AND METHODS

### 2.1 | Plant material and growth conditions

*Hordeum vulgare* L. cv. “Golden Promise” as wild-type (WT) and the RNAi-mediated WHIRLY1 knockdown line of the same barley cultivar having a minute amount of WHIRLY1 (RNAi-W1-7 = W1-7) (Krupinska et al., 2014) were used. Growth conditions were as described before by Saeid Nia et al. (2022). Plants were grown side by side and under irradiances of about 250–350  $\mu\text{mol photon m}^{-2} \text{s}^{-1}$  measured by a quantum sensor (Li-185 A, Li-Cor Biosciences) in the leaf plane.

### 2.2 | UV screening pigments

In vivo UV screening of the adaxial side of primary foliage leaves was determined with a Xenon-PAM fluorometer (Walz) before UV-B exposure. The technical information about the setup of optical filters, light source, sample holder and fluorescence detector has been described by Burchard et al. (2000) and Pescheck and Bilger (2019). Detached primary leaves were kept in Petri dishes and on wet filter paper for this measurement.

### 2.3 | Isolation of intact chloroplasts

Ten days after sowing, about 250 primary leaves from each genotype were harvested in the morning before the onset of illumination to minimize the formation of starch granules. Isolation of intact chloroplasts was done in dim light and in a cold room at 4°C with the method described by Grussem et al. (1986) using Percoll step gradients consisting of 40% (wt/vol) and 80% (wt/vol) Percoll, respectively, to purify the chloroplasts. Intact chloroplasts from each genotype were counted three times using a hemocytometer (Thoma) under a brightfield microscope system (Zeiss Axiophot with a Plan Apochromat, 20×/0.45 objective). Imaging was done by Olympus DP7 camera CELL'F software version 5.1. Before UV-B exposure, the isolated chloroplasts were diluted in 1× GM-mix (including 330 mM sorbitol and 50 mM HEPES, adjusted to pH 6.8; for more details, see Grussem et al., 1986) to a final concentration of about  $12 \times 10^5$  chloroplasts per  $\mu\text{l}$ .

### 2.4 | Isolation of nuclei

About 250 primary foliage leaves of each genotype were harvested at the same time of the day as described for chloroplasts. Leaves were kept on ice and the isolation of nuclei was done in dim light in a cold room (4°C) according to Pilartz and Jeske (2003).

Quantification of isolated intact nuclei was done with a Thoma counter chamber after 5 min DNA staining with 0.5 µg/ml DAPI (4',6-diamidino-2-phenylindole; D1306, Thermo Fisher). Intact nuclei per square were analyzed by a fluorescence microscope system (Zeiss Axiophot with filter tube 02 consisting of G365, FT395, LP420 and with a Plan Apochromat 20×/0.45 objective; imaging done by Olympus DP7 camera and CELL'F software version 5.1). This procedure was repeated three times. For exposing intact nuclei to UV-B, the isolated nuclei were kept in 1× GP buffer (as described by Pilartz & Jeske, 2003) at a final concentration of about  $13 \times 10^3$  nuclei per µl.

## 2.5 | UV-B exposure

For the induction of DNA damage, chloroplasts or nuclei were pipetted in multiwell plates (Cell culture plate, 24-well, Sarstedt), while in experiments with detached primary leaves, the latter were placed on wet filter paper in Petri dishes. Exposure to UV-BR was done for 1 h. To minimize the repair of DNA damage by the photolyase enzyme via UV-A and blue light-dependent photoreactivation, UV-B lamps with a low contribution of UV-A and blue light were used. UV-BR was provided by fluorescent tubes (TL40/12RS, Philips) installed in a growth cabinet (GroBanks, CLF plant climatics). Desired irradiances were obtained by a variable distance toward the fluorescent tubes, whose output was tuneable. A portable UV-B sensor (Veit et al., 1996) was used to measure UV-B irradiance. This sensor was calibrated against a DM150 double monochromator spectroradiometer (Bentham Instruments, Ltd.). Incident UV-B irradiances were 13, 6 and 13 W m<sup>-2</sup> for leaves, chloroplasts and nuclei, respectively. Each exposure experiment was conducted three times.

In the case of chloroplasts or nuclei, each marked well in the multiwell plates was filled with 1 ml of either isolated chloroplasts or nuclei suspension in a random arrangement. Multiwell plates were kept on ice during the UV-B exposure. Plates were turned clockwise and the solution in the wells was also very gently mixed every quarter of an hour.

During UV-B exposure, plates and Petri dishes were covered with WG 295 glass filters (Schott), whereas controls were covered with UV-excluding plexiglass filters (GS 321, Röhm, T<sub>50%</sub> at 390 nm). After UV-B exposure of primary leaves, segments of ca.1 cm in length were excised 1.5 cm below the tip of primary leaves and leaf segments were immediately frozen in liquid nitrogen. In case of chloroplasts or nuclei, the suspensions from at least 6 wells for each genotype were pooled in one tube and were centrifuged at 2000g for 2–3 min at 4°C to sediment the chloroplasts or nuclei, respectively. Afterwards, the supernatants were discarded and pellets were immediately frozen in liquid nitrogen. Frozen samples were kept at –80°C until DNA isolation.

## 2.6 | DNA isolation and detection of cyclobutane pyrimidine dimers

DNA extraction from frozen leaf segments, chloroplasts and nuclei was done as described by Pescheck et al. (2014). In the case of DNA

isolation from chloroplasts and nuclei, 500 µl of extraction buffer was added directly to the frozen pellet. The detection and quantification of cyclobutane pyrimidine dimers (CPDs) was done as described by Pescheck et al. (2014). UV-B-induced DNA damage was shown in the graphs as the numbers of detected CPD by immunoblotting per megabase pair of DNA (CPDs Mb<sup>-1</sup>).

## 2.7 | Staining of nucleoids

Leaf cross-sections were produced from the primary foliage leaf of 10-day-old plants by a hand microtome at a 2–2.5 cm distance from the tip. Sections were immediately fixed by 4% (wt/vol) paraformaldehyde in phosphate-buffered saline pH 7.4 (PBS) overnight at 4°C. After washing with buffer, the sections were stained with SYBR®Green (1:5000, S7563 Invitrogen™) for 45 min at room temperature. After washing with 1× PBS, the sections were transferred onto a slide and capped with PBS/glycerol (vol/vol: 1:1).

Imaging was done with a Leica SP5 confocal microscope system (HCX PL APO CS 63.0 × 1.2 W objective). Fluorescence was excited by an argon laser line at 488 nm (6% power) and emission was detected between 510 and 570 nm (HV 768 V) and 690–760 nm (HV 510 V). Z stacks with six layers were taken in a window of 61.57 × 61.57 µm and a depth of 5 µm (1024 × 1024, 8-Bit). A minimum of four stacks out of different regions of the specimen were taken from each sample. Examples of images are shown in Figure S1, Supporting Information.

Pixel counts per nucleoid signal were quantified automatically with a macro written in ImageJ 1.52p. After setting a threshold, a binary mask was created and pixels were counted in areas with a size of 30–2000 pixels and a circularity between 0.02 and 1.0. Four z-stacks with five optical levels were quantified per line.

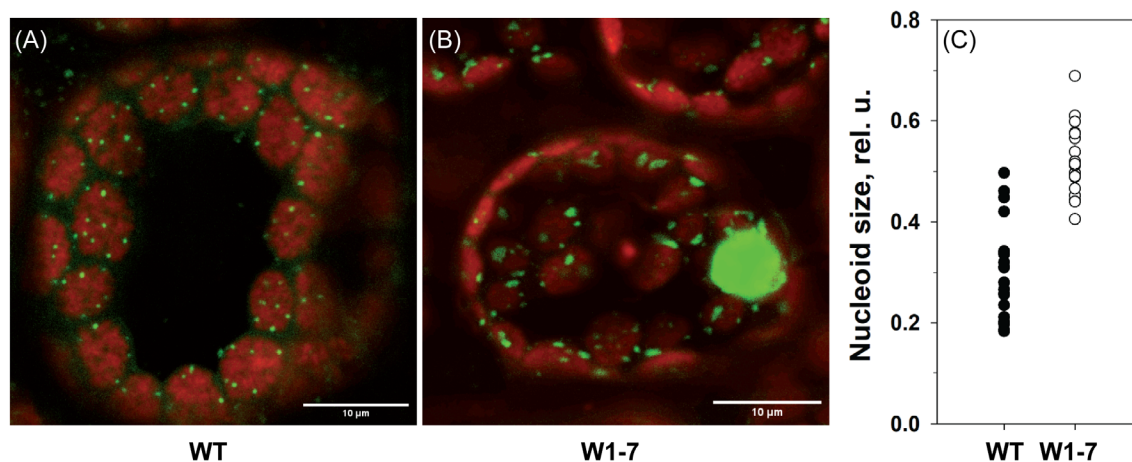
## 2.8 | Statistical analysis

UV-B transmittance and UV-B-induced DNA lesions in total, nuclear and plastid DNA isolated from leaf segments, nuclei and chloroplasts, respectively, were compared in wild-type and transgenic plants by a t-test. In case of failure in the normality test or equal variance test, the Mann–Whitney rank sum test was used by Sigmaplot 13 (Systat Software GmbH).

# 3 | RESULTS AND DISCUSSION

## 3.1 | Morphology of nucleoids in chloroplasts

Using laser scanning microscopy, the compaction of nucleoids in the leaves was investigated (Figure 1). The observed fluorescence signals from chloroplast nucleoids and nuclei were similar to those reported previously (Krupinska et al., 2014). Quantitative microscopic analysis of the nucleoid signal size (Figure 1C) indicated that the major portion



**FIGURE 1** (A) Staining of chloroplast nucleoids in leaf sections from WT and W1-7 plants by SYBR Green. The bar represents 10 µm. (B) Nucleoid sizes in relative pixel counts for WT and W1-7 plants ( $n = 20$ ). Each data point in the diagram represents an arithmetic mean value calculated out of 58–210 nucleoid signal areas measured in a single image. The nucleoid sizes are significantly different with  $p < 0.001$  using Student's  $t$ -test

of the nucleoid population in the chloroplasts of transgenic barley plants with an RNAi-mediated knockdown of WHIRLY1 (W1-7) was significantly less compact than that of wild-type plants (WT). The residual amount of WHIRLY1 in these plants, amounting to about 1% of the wild-type level, likely was responsible for the packaging of a few nucleoids, resulting in a rather heterogeneous nucleoid morphology (Krupinska et al., 2014). Repeated staining of nucleoids confirmed that this trait is preserved in plants of the barley W1-7 line used in this study.

### 3.2 | UV-B screening in leaves

To investigate whether UV-screening of the leaves used for the isolation of chloroplasts and nuclei differed between W1-7 and WT plants, the epidermal UV-B transmittance was investigated. Previously, we had shown that WHIRLY1-deficient leaves are unable to acclimate to high light (Saeid Nia et al., 2022). However, this inability did not extend to epidermal UV-B screening, as epidermal UV-B transmittance was comparatively low and not significantly different between W1-7 and WT leaves (means  $\pm$  standard deviation from three independent experiments with six to seven leaves each:  $6.2 \pm 0.96$  and  $6.3 \pm 0.83$  for WT and W1-7 leaves, respectively). Similarly, and in accordance with this result, it was shown by Saeid Nia et al. (2023) that WT and W1-7 plants did not differ in their content of flavonoids, one of the main groups of screening pigments (Agati et al., 2009). Altogether, these results suggest that the same amount of UV-B could penetrate through the epidermis and reach the mesophyll cells in the WT and W1-7 plants.

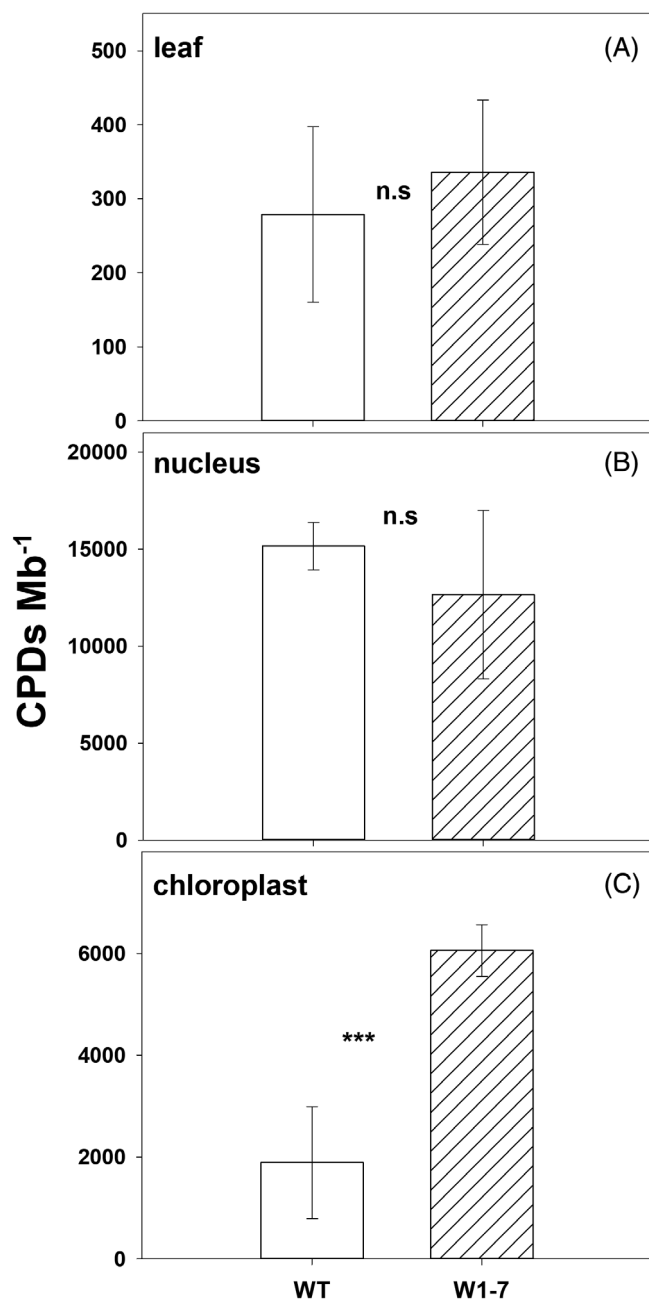
### 3.3 | DNA lesions in plastid, nuclear and total DNA

After UV-B exposure, no significant differences between genotypes in CPDs were detected in total DNA isolated from UV-B-exposed primary leaves (Figure 2A). Exposed to a similar amount of UV-B, nuclei were shown to be 60 times more sensitive than the intact leaves but,

also here, no significant differences between WT and W1-7 transgenic plants were detected (Figure 2B). However, in chloroplasts, W1-7 mutants displayed a three times higher CPD formation than the WT (Figure 2C). WHIRLY1 deficiency caused loosely packed nucleoids with a more outspread structure in the chloroplasts (Figure 1) and an increased optical cross-section exposed to UV-BR. These nucleoids have a higher chance of getting hit by UV-BR since the probability of being hit by radiation is proportional to the volume of the target (Ballarini, 2010). Similar reasoning has been applied to the so-called “package effect”, when the light absorption by a certain amount of chlorophyll in smaller or larger algae is considered (Falkowski & Raven, 2007). Here, the tighter the chlorophyll molecules are packed, the lower the absorption of light per chlorophyll molecule is.

The difference in plastid DNA damage between the two genotypes may also have been caused by a difference in repair rate. The photolyase-dependent repair, which is the major route for CPD removal, requires UV-A or blue light. UV-B treatment was done in the absence of any visible light source and the UV-A/UV-B ratio of the lamps used was much lower than in sunlight. Furthermore, repair activity was minimized by applying low temperature (Pakker et al., 2001). However, it cannot be completely ruled out that the packaging of the nucleoids had a direct influence on the potentially remaining repair activity. The increased CPD formation in W1-7 chloroplasts resembles the situation in the radiation-resistant bacterium *Deinococcus radiodurans*, where a mutant lacking a histone-like protein binding to DNA was much more sensitive to UV exposure than the wild-type (Wang et al., 2012). Coating of DNA by this or other architectural proteins associated with nucleoids in bacteria protects the cells and prolongs their survival (reviewed by Hołowka & Zakrzewska-Czerwińska, 2020).

In comparison to chloroplasts, CPD formation in nuclei was not significantly different between WHIRLY1-deficient and WT plants. This indicates that the function of WHIRLY1 in the nucleus is different than in chloroplasts. WHIRLY1 is known as the repressor or activator of nuclear genes and has been found to bind to promoters



**FIGURE 2** Total DNA lesions, detected as CPDs Mb<sup>-1</sup>, induced by UV-B exposure of primary leaves (A), isolated nuclei (B) and isolated chloroplasts (C). Columns are means  $\pm$  standard deviation of 12–13 leaves, 14 nuclei samples (each analyzed sample is a pool of at least six exposed suspensions of nuclei) and six chloroplast samples (each analyzed sample is a pool of at least six exposed chloroplast suspensions) from three independent UV-B exposure experiments, each comprising 4–5 leaves, 4–5 nuclei samples and 2 chloroplast samples, respectively. Statistical significance was tested for CPDs in chloroplasts using a *t*-test. For CPDs detected in leaves and nuclei, a Mann–Whitney rank sum test was used. n.s., not significant; \**p* < 0.001

of stress responses and senescence-associated genes (Krupinska et al., 2022). WHIRLIES might contribute to the regulated accessibility of promoters for transcription factors, but they do not play a role as chromatin architects in the nucleus (Krupinska et al., 2022); the

packaging of DNA in the nucleus is mediated by histones and the chromatin compaction is regulated by posttranslational modifications of histones. Most probably, the presence or absence of the WHIRLY1 protein in the nuclei (in WT and W1 plants) might not have any effect on UV-BR-induced CPD formation in nuclear DNA.

The absolute CPD formation was in intact leaves much lower than in the isolated organelles. This difference reflects the presence of UV screening compounds and the complex optical properties within leaves. The similar CPD formation in intact W1-7 and WT leaves may, in part, be explained by a larger proportion of nuclear (and mitochondrial) DNA in comparison to DNA located in plastids. In addition, in intact leaves, only the uppermost cell layers are responsible for the majority of detected UV-BR-induced DNA lesions. Thus, the dilution by undamaged DNA from the rest of the leaf blade probably diminished the difference between the genotypes even more.

Comparison between UV-BR-induced CPD formation in nuclear and plastid DNA showed that, in the WT, nuclear DNA was more sensitive, even when the damage was corrected for the difference in UV-BR exposure of the two organelles. An explanation of the reduced damage in chloroplasts could be the absorption of UV-BR by chlorophyll. The absorption spectra of chlorophyll *a* and *b* extend well into the UV-B spectral region (Bilger et al., 2001) and it has even been speculated that chlorophyll might initially (evolution-wise) have served as UV-protectant before it was recruited by photosynthesis as a light-harvesting pigment (Mulkidjanian & Junge, 1997). Therefore, the chlorophyll in the chloroplast suspension may have competed effectively with the plastid DNA for absorption of UV-BR.

It is well-known that chloroplast nucleoids are dynamic structures changing in shape and organization during plastid development and likely also in response to environmental change. In this study, it has been shown for the first time that high compactness of nucleoids may reduce the formation of CPDs, the most important DNA lesions induced by UV-BR. The importance of nucleoid restructuring during plant responses to environmental change deserves more attention in research.

## AUTHOR CONTRIBUTIONS

Monireh Saeid Nia, Wolfgang Bilger and Karin Krupinska conceived and designed the research. Material preparation and conduction of experiments (except detection of nucleoids of fluorescence signal areas, which was done by Christine Desel) were performed by Monireh Saeid Nia with assistance of Frauke Pescheck. Graphs and statistical analysis were performed by Monireh Saeid Nia. The first draft of the manuscript was written by Monireh Saeid Nia. All authors commented and revised the first version of the manuscript. All authors read and approved the final manuscript.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

The raw datasets in this study are available from the corresponding author on reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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