DNA AND CHROMOSOMAL DAMAGE AS A HALLMARK OF THE INDUCED GENOMIC INSTABILITY IN BARLEY

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Summary: Barley was utilized as a model crop for a complex characterization of the cytogenetic and molecular dimensions of the genomic instability produced in plant genome by mutagenic and clastogenic agents such as ionizing and ultraviolet radiation, Li-ions, bleomycin and restriction endonucleases. The induced genomic alterations on the DNA and chromosomal level were evaluated by a set of molecular and cytogenetic approaches. It was established that effective mechanisms preserving genomic integrity and stability both on chromosomal and DNA level dependable on a variety of factors such as the type of damage, chromosomal constitution, chromatin organization and transcriptional activity are fully operational in barley genome. The data obtained indicate that both the processes of damage induction as well as those governing DNA repair appear to be essential for understanding of the short- and long-term genetic consequences of mutagenic insults that hamper the genomic status of higher plants.


Keywords: Barley; genomic instability; chromosomal and DNA damage.

Abbreviations: BLM – bleomycin; clCPD – clustered CPD; CPD – cyclobutane pyrimidine dimers; DSB – double-strand breaks; ESS – enzyme-sensitive sites; FPG – formamidopyrimidine DNA glycosylase; IR – ionizing radiation; NORs – nucleolus organizing regions; REs – restriction endonucleases; SSB – single-strand breaks; TILLING – Targeting Induced Local Lesions In Genomes; UV – ultraviolet radiation.

Introduction

Living organisms are constantly exposed to a variety of exogenous and endogenous, cytotoxic and genotoxic compounds with proved potential to induce genomic damage. Ultraviolet (UV) radiation primarily generates pyrimidine dimers and cross-links in DNA whereas alkylating agents most frequently attack specific DNA bases. Ionizing radiation (IR), in addition to the highly toxic DNA double-strand breaks (DSB), is thought to generate the majority of its mutational burden by the free radical production. Pattern of the endogenously occurring
DNA damage resulting from the permanent contiguity of DNA with the metabolically active cell environment consists mainly of abasic sites, base damage and single-strand breaks (SSB). Genomes can also suffer DNA damage resulting from the operation of a number of essential cellular processes such as replication, transcription and repair (Aguilera 2002).

Genomic instability is generally attributed to the occurrence or elevated rate of mutation induction in the case when some key processes engaged in genome maintenance such as DNA damage detection and repair become malfunctioned or dysfunctional (Roy 2014). Instability in the eukaryotic genome is manifested by diverse biological endpoints such as chromosomal destabilization, aneuploidy, micronucleus formation, sister chromatid exchanges, gene mutation and amplification, cellular transformation, activation of the mobile genetic elements and DNA repair pathways (Russo et al. 2015). This phenomenon has been also strongly implicated in the processes of aging (Lopez-Otin et al. 2013).

The motionless lifestyle of plants constantly exposes them to extremely high levels of abiotic stress that threatens their genomic integrity, leading to mutations, developmental arrest and/or cell death. As plants lack a reserved germ-line and enter meiosis after significant vegetative growth, mutations occurring in somatic cells can be easily transmitted to the next generation (Stapleton et al. 1997). Therefore, plants during evolution have been forced to develop effective DNA protective mechanisms in order to ensure the stability of their genetic status. It is also important to note that the growth and productivity of plants depends a lot on their capacity to eliminate the consequences of exposure to various environmental factors such as UV and ionizing radiation, pollution-associated ion toxicity, etc., thus making DNA repair an essential defense mechanism protecting both wild and cultivated plants from the harmful influence of the environment (Frohnmeyer and Staiger 2003; Kaiser et al. 2009; Manova and Gruszka 2015).

Gamma-irradiation was historically the most frequently used tool to mutagenize the plant genomes. Mutagenic potential of any DNA damage is primarily dependent on the efficiency of its elimination by the cell (Britt 1999). Higher plants are thought to be more tolerant to IR than mammalian cells. Studies performed with cultured tobacco cells suggested that in addition to the lower amounts of induced DSB, more efficient repair of DNA damage might also underlie such a high radiation tolerance. Nevertheless, the capacity of plant cells to repair IR-induced DNA damage has been analyzed in a limited number of plants (Yokota et al. 2005). Little is known about the influence of chromatin organization and transcriptional activity on the induction and repair of the different types of radiation-induced DNA lesions in plant cells. The need for intensive studies on the formation and repair of UV-C-induced DNA damage in plants is also supported by the data showing that UV-C radiation could be used as an alternative of IR in order to obtain valuable germplasm (Vlahova et al. 1997). It might be also of substantial practical interest to reveal how the host plant genome maintains the integrity of the expressed transgenic DNA which makes the knowledge on the repair capacity of essential plant genes quite
important in order to predict the outcome of such a directed genome manipulations. An intriguing finding in this respect is that the observed non-random integration of transgenes preferably in the gene-rich regions of barley genome is associated with their enhanced sensitivity to DSBs (Salvo-Garrido et al. 2004).

At present the recovery potential of plants is considered as an effective barrier for introduction of transient or permanent genetic alterations and a primary guardian of their genomic stability (Kohli et al. 2010, Gill et al. 2015). Learning more about the intrinsic nature of this housekeeping cellular function seems essential for better understanding of the mechanisms that ensure proper transmission of the hereditary information and functioning of the eukaryotic genome including that of higher plants.

Barley as a model crop plant

Barley is acknowledged as an important agricultural crop not only worldwide but also in Bulgaria where its share is about 12% of all the cultivated cereals. Barley genome was widely explored as a model system for basic and applied studies on the regional specificity of mutagens due to its well characterized chromosomal complement. In this respect a set of reconstructed barley karyotypes have been created by means of gamma-irradiation and successfully utilized for studies on the chromosome position effects accompanying the action of specific mutagenic agents mainly of chemical nature (Gecheff 1989, Gecheff 1991). The existing collection of barley karyotypes with variable but stably transmitted in the progeny chromosomal constitution was an essential prerequisite to look further on the various cytological and molecular dimensions of the induced genomic instability in barley genome (Gecheff 1996). By means of in situ hybridization with DNA probes, covering defined repeated regions of the barley chromosomal complement, several reconstructed barley lines have been thoroughly characterized in respect to the specific localization of rearrangement breakpoints induced by the initial gamma-rays treatment (Georgieva and Gecheff 2013; Georgieva 2014). Utilizing this methodology, a precise physical mapping of the chromosomal segments engaged in the radiation-mediated reconstruction was achieved which significantly increases the resolution power of this model system for various aspects of barley cytogenetics and molecular genetics. An example of this approach is presented on Fig. 1.

DNA double-strand breaks are the ultimate lesions leading to chromosomal alterations

Elucidation of the molecular mechanisms governing the origin of chromosomal aberrations has been focused by various research groups mainly on three interdependent factors: the nature of the initial DNA lesions, the type and efficiency of the cellular DNA repair systems responsible for their recovery and the way of transformation of these lesions into stable chromosomal alterations. After development of the effective experimental procedures for incorporation of bacterial restriction endonucleases (REs) into the eukaryotic cells based on electroporation and treatment with Streptolysin “O” it was unequivocally established, that the ultimate lesion, leading to the chromosomal aberration formation are DNA double-
Fig. 1. (A) - Multicolor FISH–based visualization of the metaphase chromosomes from the reconstructed barley karyotype PK 19x180 with repetitive DNA sequences: GAA (green signal); Afa family (red signal) and pTa71 (yellow signal). (B) - Putative positions of the rearrangement breakpoints in PK 19x180 (marked with ►) and distribution of the GAA, Afa, pTa71 repeats and Giemsa bands along the individual barley chromosomes.

strand breaks (Natarajan and Obe 1984; Bryant 1984). The key conclusion achieved was that DSBs are the major initial event provoking the formation of chromosomal aberrations, supported strongly from the experiments with mutagenized cells treated with Neurosopora endonuclease, i.e an agent with clear substrate specificity against single-stranded DNA (Natarajan and Obe 1978).

Some of the major cellular effects of radiation, such as induction of mutations, chromosomal aberrations and cell killing, can be successfully mimicked by generation of ‘pure’ double-strand breaks in the cellular DNA via REs, although the chemical structure of the RE-induced DSB ends differs from those induced by X-rays (Bryant 1988). Because of their sequence specificity REs have been very helpful as a tool to study the mechanisms of DSB formation and repair events in the mammalian and plants cells (Pfeiffer et al. 2005; Puchta and Hohn 2012).

The potential of REs to induce chromosomal damage in barley genome in vivo was also unequivocally demonstrated. The data obtained displayed that the efficiency of chromosomal aberration induction depends on several key factors such as the type of the generated DSB and the methylation status of the recognized target DNA sequence (Stoilov et al. 1996, 2000). It was also suggested that the observed biased localization of the RE-induced chromosomal rearrangements in barley nucleolus organizing regions (NORs, Fig. 2) might be associated either with a preferential DSB induction in rDNA or with some specificity of the repair mechanisms operating within the ribosomal gene clusters (Gecheff et al. 1997).

This assumption was later supported by the data for the induction and early recovery kinetics of DSB, produced by the restriction endonucleases MspI and AluI in barley ribosomal DNA assessed by Southern hybridization (Manova and Stoilov 2003; Manova 2007, Fig. 3).
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Figure 2. Distribution of chromatid aberrations, produced by restriction endonucleases HpaII (dark blue columns), Msp I (bright-blue columns) and HaeIII (green columns) along the NOR-bearing chromosomes 6i and 7s from the fully reconstructed barley karyotype PK 88.

The potential of REs to induce DSBs in barley genomic DNA was also clearly demonstrated by treatment of isolated barley nuclei with the restriction endonuclease EcoRI and subsequent application of the single cell gel electrophoresis, or comet assay (Fig. 4). The results obtained so far are strongly favoring the application of REs as an effective tool for directed induction of chromosomal and genomic damage in higher plants (Stoilov and Gecheff 2009).

Figure 3. Hybridization profiles of rDNA bands and histogram representation of the densitometric data obtained after treatment of barley root tips from lines T-1586 and T-35 with Alu I in vivo.
Figure 4. Representative comet images, obtained after application of the neutral comet assay performed with isolated barley nuclei, digested with 0.5 u/ml EcoRI in vitro for 1 hr.

Induction and repair of DNA lesions

It is assumed that DNA damage can be divided into two basic categories: single-stranded damage disturbing only one DNA strand and double-stranded damage which affects both strands of the DNA double-helix. In the case of a single-stranded damage, the second DNA strand still contains native information about the respective sequences and can be utilized as a template for correct repair of the lesion. On the other hand, if the disturbance affects both DNA strands, there is an increased probability to lose the original sequence or change the native genetic information due to the erroneous repair. Pre-mutation sources on the DNA level can be also distinguished based on their intrinsic nature, being either endogenously generated like oxidative damage and depurination or from the external origin like formation of chemical adducts or a consequence from the action of various radiation sources. DNA damage can be even provoked by errors in the action of the natural polymerases or through the operation of the “error-prone” DNA repair activities.

Ionizing radiation as a tool for damage induction

Gamma-rays, X-rays as well as the ion-beams are amongst the most effective DNA damaging sources applied to mutagenize plant genome. IR generates a variety of single-stranded DNA lesions indirectly via production of free radicals. The hydroxyl radical (OH\(^{\cdot}\)) is the most reactive as it is able to extract a hydrogen atom from the deoxyribose, thus generating a DNA radical (Alpen 1998). Free radicals (which may be produced not only by IR, but also by hydrogen peroxide or a variety of radiomimetic agents), interact with the DNA bases and provoke their oxidation, reduction or fragmentation (Cadet et al. 1997), resulting in a range of oxidation products such as 8-oxoguanine (8-Oxo-G), 5-hydroxycytosin, etc., as well as of abasic sites. A disruption introduced in the phosphodiester bond connecting the deoxyribose residues generates a discontinuity in the DNA strand in the form of SSB, which is also mainly formed via indirect ionization. An important characteristic of IR is its ability to directly ionize the DNA molecule thus generating the most genome-threatening DNA lesion, i.e. the DSB. The frequency of IR-induced DSBs as well as their distribution within the eukaryotic genome might be modulated by the chromatin organization and nuclear matrix proteins (Lavelle and Foray 2014).

It was recently shown that the
reconstructed barley line D-2946 behaves as a typical radiation-sensitive mutant with hampered ability to maintain its genomic integrity both in respect to DNA and chromosomal damage, including that produced by 7Li-ions on a DNA level (Stoilov et al. 2013). Further studies aimed at thorough characterization of the cytogenetic effects of high-energy 7Li-ion beams in this barley deletion line as well as the parental lines T-29 and T-46 have been performed. An intriguing fact obtained by in situ hybridization was the detection of a translocation between the two satellite chromosomes 5H and 6H resulting in combination of both NORs containing ribosomal repeats in one and the same chromosome of line T-46. Initially this chromosomal reconstruction was found within the cell population of a single primary root germinated from the irradiated seeds which rather speaks in favor of its spontaneous nature. In order to reveal whether this translocation is inherited the respective seedling was grown further up to the mature plant, samples from the obtained seeds were germinated and subjected to metaphase analysis by Feulgen staining. The data obtained are strong evidence that the observed co-localization of both NORs on one and the same chromosome is stably transmitted into the progeny, i.e. this is a new translocation line (termed T46M) with promising potential for further studies on the molecular mechanisms of the intraspecific nucleolar dominance in barley (Fig. 5).

The data obtained so far from these studies clearly indicate that Li-ion beams are potent and prospective S-independent inducers of chromosomal damage in barley chromosomal complement (Nikolova et al. 2015).

Experiments directed towards an estimation of the relative frequency of DNA lesions produced after irradiation of barley genomic DNA with γ-rays were also executed in our Lab. DNA damage detection was performed by an assay based on the Number Average Length Analysis (NALA) (Sutherland et al. 1999), optimized and successfully applied for a quantitative and qualitative assessment of DNA damage and repair at the level of total genomic DNA in barley after treatment with a variety of mutagenic agents. The SSB induced directly by the gamma-irradiation or generated at the sites of IR-induced base DNA damage

![Figure 5. Chromosomal complements of the parental line T-46 (left) and the new stable translocation line T-46-M (right). Arrows indicate the respective satellite chromosomes bearing NORs.](image-url)
after application of the respective lesion-specific enzymes were assessed by agarose gel electrophoresis under alkaline conditions and densitometric analysis of the respective gel images. The enzyme-sensitive sites (ESS) generated by the formamidopyrimidine DNA glycosylase (FPG) reflect radiation-mediated oxidative DNA lesions (mainly oxidized purines) whereas the ESS generated by T4EndoV reflect mainly AP sites and FaPyAde (Fig. 6).

The irradiation of barley DNA in different solutions showed similar relative rates of SSB and oxidized purines obtained in AE- and TE-irradiated genomic DNA, higher rate of FPG ESS compared to the SSB in the genomic DNA irradiated in dH2O, and higher levels of SSB and FPG sites in TE-irradiated lambda DNA compared to the TE-irradiated genomic DNA. The linear dose-response curves obtained demonstrated that the applied approach allowed accurate measurement of DNA damage frequency. Hence, it was further applied for in vivo experiments where the kinetics of damage formation (SSB, DSB, oxidative DNA lesions) has been analyzed in both barley cell suspension cultures and barley root and leaf seedlings exposed to gamma-rays or maleic hydrazid (Gecheff et al. 2008).

**Radiomimetic agents as an alternative**

Radiomimetic agents induce a spectrum of DNA lesions similar to that of the ionizing radiation. The anticancer drug bleomycin (BLM) is very effective DSB inducer in plants, which explains its frequent utilization as a DNA damaging agent in plant DNA repair studies, and particularly in barley (Manova et al. 2006; Manova et al. 2009; Georgieva and Stoilov 2008; Stoilov et al. 2013; Stolarek et al. 2015). The spectrum of BLM-induced DNA lesions includes SSB and abasic

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**Figure 6.** Alkaline gel visualizing the distribution of γ-irradiated barley genomic DNA diluted in AE* buffer (TE buffer with pH 9). Histogram representations of the absolute frequency of gamma-rays induced DNA lesions in barley gDNA irradiated in different solutions and a comparison of the DNA damage rate measured in barley and lambda DNA irradiated in standard TE.
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sites, being in some cases closely localized to each other on the opposing DNA strands thus forming a clustered DNA lesion, as well as DSB with blunt or non-complementary ends. The formation of BLM-induced DNA lesions is influenced to a high degree by the chromatin status of the DNA. As a result the strand breaks are generated mainly in the linker regions, whereas DNA wrapped in nucleosomes is more resistant to BLM influence. Such preferential action of bleomycin in vivo has been demonstrated in both mammalian and plant cells (Smith et al. 1994; Kuo 1981; Manova et al. 2006).

Ultraviolet radiation

The short-wave UV radiation, such as UV-B and UV-C light, induces predominantly the formation of cyclobutane pyrimidine dimers (CPD) and less frequently pyrimidine-6-4pyrimidone photoproducts (6-4 PP). The relative ratio of these lesions found in UV-B exposed plants is about 9:1, respectively (Dany et al. 2001). The long-wave UV-A spectrum is also effective in generating CPD in mammalian cells and human skin both in vitro and in vivo (Tewari et al. 2012). Natural solar light also contains high UV-A fluence rate which is beneficial for plants because they utilize it for the photorepair reactions; however whether this part of the spectrum is harmful to the plant DNA still remains to be elucidated. The distribution of UV-photoproducts along the DNA is affected by its chromatin conformation, the sequence identities of the surrounding bases and the presence of methylated cytosine (Gale and Smerdon 1990; Mitchell 2000; Law et al. 2013). Moreover, non-histone proteins, for example the transcription factors, when bound to DNA could considerably modify the distribution of UV photoproducts by favoring their formation in the active promoters or decreasing their rate within the inactive ones or other specific DNA regions (Aboussekhra and Thoma 1999). It is logical to expect that similar factors may influence the distribution of CPD and other UV-induced lesions in the plant genome as well; however, no studies have been focused on this issue in plants so far.

CPD formation and repair

An extensive evaluation of the potential of barley genome to repair UV-C induced cyclobutane pyrimidine dimers has been performed in our Lab (Manova et al. in preparation). An example of CPD formation and the light-dependent repair occurring in 4 day-old etiolated barley seedlings is shown on Figure 7. The results revealed that the percentage of CPD remaining in barley genome after 6h sunlight recovery was reduced to ~33% of the initial ones. It is evident that the interval of 6 hours was not sufficient for the photorepair system to completely remove the induced UV lesions.

We were also interested to find out whether UV-C irradiation was able to produce CPD in close proximity to each other, namely clustered CPD (cICPD). Such CPD clusters could be dangerous for the cells if they are many and have to be repaired by excision mechanisms as their simultaneous excision will result in the formation of the most severe lesion in the DNA, namely the DSB. The presence of cICPD can be easily revealed by electrophoresis under neutral conditions of (−) and (+) T4EndoV treated samples without any heating of the DNA.

We were able to establish for the
first time that clCPD might be formed in the plant genome upon their exposure to ultraviolet radiation (data not shown). Indeed, a substantial fraction of CPD induced in barley genomic DNA after UV-C irradiation of the etiolated seedlings (50kJ/m²) is in the form of clusters. It is still not known whether the full removal of such clustered CPD might be more difficult for the photorepair system or for the plant’s excision machinery as it was suggested to be the case for the clustered oxidative DNA damage generated by ionizing radiation in mammalian cells.

**Induction rate and repair kinetics of oxidative DNA damage produced by UV-C light**

UV radiation generates also oxidative DNA lesions mediated mainly by free radicals produced by biological molecules acting as endogenous photosensitizers. We tested the hypothesis whether oxidative DNA damage might be induced in barley genome by UV-C irradiation of intact seedlings. An example of the FPG-sensitive sites detected in barley leaves exposed to acute UV-C treatment in our lab is presented in Fig. 8.

The results showed that the existence of FPG-sensitive sites was easily detectable in the DNA samples immediately after the application of both high and low UV-C doses. Hence, although in the literature there are some data on the presence and formation of oxidative DNA damage in plant genome, there are no studies dealing with their repair in plants. In this respect we initiated experiments aimed to measure not only the rate of formation but also the repair kinetics of these DNA lesions in UV-C irradiated barley seedlings. The frequency of Fpg-sensitive sites induced in barley leaves immediately after irradiation with 5kJ/m² was measured to be about 19/Mb of gDNA. As these lesions are known to be removed by excision mechanisms, we followed their repair kinetics after dark incubation of the irradiated seedlings and
it is evident that they persist in barley leaves even after 24 hours post-irradiation incubation (Fig. 8). These results suggest that oxidative DNA damage might also have potential to increase the UV-associated mutagenicity and genomic instability in plants. However, despite the recent progress in plant DNA repair field, the data about the extent of formation and the efficiency of their repair in UV exposed plant cells are still insufficient (Roldan-Arjona and Ariza 2009).

**Reverse-genetics methodology**

For a long time the ultimate approach in plant genetic studies has been to utilize phenotype characteristics in order to reveal the genetic control of the traits based on the characterization of the respective DNA sequences involved. With the appearance and practical application of the TILLING methodology (Targeting Induced Local Lesions In Genomes) - a reverse genetics strategy that allows screening for mutations in genes with known sequences in a created mutant population, it became possible to accelerate significantly these analyses by going from the gene sequences to their phenotype expression. The method combines high density of the point mutations generated by the traditional chemical mutagenesis with a rapid mutational screening based on PCR amplification and some endonucleases (initially Cel I) that specifically cut DNA heteroduplexes to discover the induced single nucleotide polymorphisms (SNPs). TILLING platforms have been successfully developed for several model crops including barley (Kurovska et al. 2011, 2012). In a joint study with the Genetics Department of the University of Silezia, Poland, screening by TILLING and EcoTILLING for mutations and natural variability within the 3' end of the barley CPD photolyase gene in EMS-induced TILLING population based on cultivar Sebastian and collection of barley reconstructed lines obtained by γ-irradiation has been recently initiated.
The primers were designed based on the genomic DNA sequences of barley CPD photolyase gene (line Freya) already identified by us (GenBank Accession No KC345035).

The data obtained from the experiments conducted so far does not reveal any mutations or polymorphisms in this specific compartment of the barley CPD photolyase gene. Further studies and probably development of new TILLING populations are needed in order to fully utilize the potential of this approach for mutation screening in the barley genome.

**Conclusions**

A number of investigations focused to highlight the molecular and cytogenetic responses of barley genome to mutagenic and clastogenic agents with different characteristics and mode of action have been performed in our Lab during the last two decades. The data obtained revealed that effective mechanisms for alleviation of the induced genetic burden are operating in barley both on chromosomal and DNA level aimed to preserve the native genetic status of the genome and to ensure proper maintenance and transition of the hereditary information into the progeny. They also demonstrate the applicability of the directed chromosomal reconstruction for evaluation of the intrinsic nature of damage response in higher plants and cereal genomes in particular. In addition, the results are pointing to the option that besides the direct mutagenesis, substantial efforts should be made to elucidate the indirect mechanisms of DNA damage response, an emerging and quite challenging field in the area of the eukaryotic genome integrity and barley genetics as well.

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