Mutation Research 738–739 (2012) 52–70

Contents lists available at SciVerse ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

Molecular analysis of point mutations in a barley genome exposed to MNU and gamma rays

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ARTICLE INFO

Article history:
Received 21 March 2012
Received in revised form 27 July 2012
Accepted 24 August 2012
Available online 22 October 2012

Keywords:
TILLING
AFLP
Barley
MNU
Gamma rays
Mutagenesis

ABSTRACT

We present studies aimed at determining the types and frequencies of mutations induced in the barley genome after treatment with chemical (N-methyl-N-nitrosourea, MNU) and physical (gamma rays) mutagens. We created M_2 populations of a doubled haploid line and used them for the analysis of mutations in targeted DNA sequences and over an entire barley genome using TILLING (Targeting Induced Local Lesions in Genomes) and AFLP (Amplified Fragment Length Polymorphism) technique, respectively. Based on the TILLING analysis of the total DNA sequence of 4,537,117 bp in the MNU population, the average mutation density was estimated as 1/504 kb. Only one nucleotide change was found after an analysis of 3,207,444 bp derived from the highest dose of gamma rays applied. MNU was clearly a more efficient mutagen than gamma rays in inducing point mutations in barley. The majority (63.6%) of the MNU-induced nucleotide changes were transitions, with a similar number of G > A and C > T substitutions. The similar share of G > A and C > T transitions indicates a lack of bias in the repair of O^6-methylguanine lesions between DNA strands. There was, however, a strong specificity of the nucleotide surrounding the O^6-meG at the −1 position. Purines formed 81% of nucleotides observed at the −1 site. Scanning the barley genome with AFLP markers revealed ca. a three times higher level of AFLP polymorphism in MNU-treated as compared to the gamma-irradiated population. In order to check whether AFLP markers can really scan the whole barley genome for mutagen-induced polymorphism, 114 different AFLP products, were cloned and sequenced. 94% of bands were heterogenic, with some bands containing up to 8 different amplicons. The polymorphic AFLP products were characterised in terms of their similarity to the records deposited in a GenBank database. The types of sequences present in the polymorphic bands reflected the organisation of the barley genome.

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1. Introduction

The high efficiency of classical mutagenesis to generate mutations has been widely documented and reflected in the official release of more than 3000 mutant varieties, including 200 species, as indicated in the FAO/IAEA Mutant Varieties Database (http://mvgs.iaea.org/Search.aspx). Many of these mutant varieties have made an enormous economic impact on agriculture and food production that is currently valued in billions of dollars and millions of cultivated hectares [1]. The rice mutant variety 'Zefu 802' has been grown in China on a cumulative area of 10,6 million hectares over a ten year period. Another rice mutant variety 'Calrose 76', developed in the USA, was widely used in crosses leading to the release of semidwarf varieties that dominate the rice production in California and Australia. The allele *denso* obtained through the X-ray treatment of malting variety 'Valticky' has become the main source of semidwarfness in barley and has led to the release of more than 150 barley varieties that are grown on all continents. In a similar way, induced mutations have contributed to the improvement of sunflower, wheat, rice and many other crop varieties [2].

The vast majority of mutant varieties were obtained following mutagenic treatment with radiation (gamma rays, X-rays, fast neutrons) thus highlighting the importance of physical mutagens. Gamma rays, the electromagnetic waves of very short wavelengths obtained by the disintegration of the radioisotopes ^{60}Co or ^{137}Cs, have been utilised most frequently for breeding purposes. Gamma sources such as gamma cells, gamma rooms or even gamma fields have been installed in many countries [3]. Of the chemical mutagens, alkylating agents such as ethyl methane-sulphonate (EMS) and methyl- or ethyl-nitroso urea (MNU or ENU) have been applied most often. However, while the agronomic potential of induced mutation is well understood, the knowledge of the precise effects of different mutagenic agents on the DNA sequence in plants is

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http://dx.doi.org/10.1016/j.mrfmmm.2012.08.008

rather limited. Furthermore, in recent years novel reverse genetics technologies, such as TILLING, have spurred renewed interest in induced mutation. For these new applications, it is necessary to understand the types of mutations generated by the different classes of mutagens more clearly and to measure their frequency and distribution along the plant genome.

Radiation was the first known mutagenic agent. As early as 1928, Stadler reported that X-rays could induce mutations in barley and maize [4,5]. When radiation passes through a tissue, physical events such as ionization – the ejection of electrons from molecules and excitation – the process of raising electrons to a higher energy state occur and this leads to DNA damage [6]. This damage can be caused by the direct effect of the radiation on the DNA sequence – the ionisation of DNA itself and by the indirect radiation effect – the reaction of radicals, formed in the neighbourhood of DNA, with DNA and other cellular macromolecules [7,8]. Chemical events are induced that start with the formation of active molecules, the so-called free radicals ($OH^{\bullet}$ and $H^{\bullet}$), that arise from OH^{-} and H^{+}. If oxygen is present, it reacts readily with radiation-induced free radicals to form peroxy radicals [9]. Numerous experimental data show that the hydroxyl radical ($OH^{\bullet}$) is the main active form of oxygen responsible for the majority of DNA damage [10,11]. Ionising radiation induces the formation of various types of DNA lesions such as single- or double-strand breaks (DSB), a variety of base modifications, AP sites (either apyrimidinic or apurinic) and DNA-DNA and DNA-protein cross-links [12,13]. Ionising radiation can also cause complex damage known as non-DSB clustered DNA damage sites [14]. All of these lesions, when not repaired, can lead to both chromosomal aberrations (deletions, translocations, inversions) and point mutations [15–19]. Among the modified bases, there are products of imidazole ring fragmentation (formamidopyrimidines, FAPY) and 8-oxopurines: 7,8-dihydro-8-oxoguanine (8-oxoG) and 7,8-dihydro-8-oxoadenine (8-oxoA) [20]. The oxidised purines and pyrimidines have either miscoding properties or are blocks for DNA and RNA polymerases during replication and transcription, respectively. The most predominating oxidative DNA damage is the formation of 8-oxoG [21]. 8-oxoG has strong miscoding properties. It has been shown that in addition to correct pairing with cytosine, 8-oxoG can mispair with adenine, resulting in G/C to T/A transversions [22]. Formation of these transversions was demonstrated in experiments carried out on bacteria [23] and mammalian cells [24,25].

In *Arabidopsis thaliana*, gamma ray-induced mutations have been reported to be mostly large deletions of up to 6 Mb, the majority of which were not transmitted to the next generation [26]. Similarly, among offspring of mice treated with different doses of gamma rays, numerous deletions were detected by the direct sequencing of 160 UniSTS markers [13]. The induction of nucleotide substitutions and small deletions (2–16 bp) by gamma ray treatment has been demonstrated in different plant species but the frequency of these changes at the DNA level has been evaluated in only a few studies performed on rice [27,28] and Arabidopsis [29].

N-methyl-N-nitrosourea (MNU) is an alkylating agent that causes DNA damage by transferring a methyl ($-CH_3$) group to the oxygen and nitrogen atoms of nucleotide bases. A wide spectrum of lesions can be obtained with the biological effect of these lesions ranging from less harmful to those leading to cell death. The majority of reactions occur at the N^7 position of guanine [30], but methylation of this position does not change the coding specificity of guanine [31]. However, it was also shown that the methylation of products in DNA: N^7-methylguanine (N^7-meG) and N^3-methyladenine (N^3-meA) can block DNA synthesis *in vitro*, resulting in cell death [32]. The methylation at the O^6 position of guanine has been the most extensively studied type of lesion induced in DNA by alkylating agents. It was demonstrated in *in vitro* experiments carried out on bacteria [33] and mammalian genes: *H-ras* (hypoxanthine-guanine phosphoribosyltransferase) [34] and *hprt* (v-Ha-ras Harvey rat sarcoma viral oncogene homolog) [35] that O^6-methylguanine prefers to pair with thymine over cytosine during DNA replication. In the first replication cycle, O^6-meG mispairs with T and as a result, the transition G/C to A/T appears. The replication efficiency for O^6-meG paired with thymine is 10-fold higher than for O^6-meG paired with cytosine [36]. It is also possible that O^6-meG pairs correctly with cytosine [37] and mutation does not emerge or that this mispairing is recognised by the post-replication mismatch repair (MMR) system, but is not repairable, thus resulting in cell death [38]. Recently, a new *in vivo* study of alkylation-induced damage showed MMR-dependent chromosomal instability in zebrafish embryos [39].

Although MNU is considered as a very strong chemical mutagen, sometimes even called a 'supermutagen' [40], a detailed study of the mutation types and frequency induced by this agent in plants has only been performed on soybean [41] and rice [42]. In these studies, the TILLING populations of both species were screened for mutations in selected genes. To the best of our knowledge, the mutagenic effect of MNU on DNA sequences has not been evaluated in other plant species. There is also a lack of reports that compare the mutagenic effect of physical and chemical mutagens in the same plant material at the molecular level.

The main objective of the presented study was to estimate the frequency and types of DNA changes induced by N-methyl-N-nitrosourea (MNU) and gamma rays in barley (*Hordeum vulgare* L.). This has been achieved through a survey of the selected DNA sequences for single nucleotide polymorphism (SNP) and through scanning the entire genome for amplified fragment length polymorphism (AFLP) after mutagenic treatments of the defined plant material. The TILLING strategy (Targeted Induced Local Lesions in Genome) [43] and AFLP analysis [44] were applied to uncover genetic differences in DNA between the non-treated parent and plants of M_2 generation obtained after mutagenic treatment of a doubled haploid (DH) line with different doses of gamma rays and MNU. This information could be useful in designing more efficient forward and reverse mutation screening protocols, especially with the application of next generation sequencing technologies (NGS) which have been already employed for mutation discovery in tomato, rice and wheat, Arabidopsis and rice [45–47].

2. Materials and methods

2.1. Plant material

Seeds of barley DH line 'H930-36' were used for chemical and physical mutagenesis. The DH line was chosen for mutagenic treatments in order to ensure the homogeneity of the starting material. The DH line 'H930-36' was generated using the 'Bulbosum' method from the F_1 generation of the cross between two-rowed, spring barley varieties: 'Klages' and 'Mata' and was provided courtesy of Dr. D. Falk.

The production of M_1 population after mutagenic treatment with MNU was carried in a controlled environment. After treatment, M_1 seeds were sown in pots filled with soil and vermiculite in a 3:1 ratio and the plants were grown in a greenhouse at 20–23 °C, light intensity 350 $\mu mol\ m^{-2}\ s^{-1}$ and a photoperiod of 16/8 h, day/night. M_1 plants were harvested individually and three main spikes from each M_1 plant were kept in individual bags without threshing. The M_2 generation was grown in a greenhouse under the same conditions. Taking into consideration the chimerism of the M_1 generation, up to three spikes from each M_1 plant were used for the creation of the M_2 populations. To avoid mutation redundancy, only one M_2 plant per M_1 spike was grown. Leaf material from each M_2 plant was collected for DNA isolation and then the plants were grown to maturity. In total, DNA was extracted from 1372 M_2 plants obtained from the MNU treatment and 1753 M_2 plants derived from gamma irradiation (Table 1). Additionally, the frequency of chlorophyll mutants was evaluated among the M_2 seedlings derived from each treatment combination. M_3 seeds were harvested from M_2 individuals and kept in a seed depository.

2.2. Chemical and physical mutagenesis

Chemical mutagenesis was conducted at the Department of Genetics, the University of Silesia, Poland, while physical treatment was performed at the

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