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

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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.

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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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

Table 1
Size and origin of the M_2 populations used for DNA isolation.

Mutagen	Dose	No. of plants	
		M_1	M_2
MNU	0.5 mM/3 h	237	310
	1.0 mM/3 h	316	583
	1.5 mM/3 h	295	479
	Total	848	1372
Gamma rays	150 Gy	310	880
	180 Gy	147	309
	210 Gy	293	564
	Total	750	1753

International Atomic Energy Agency (IAEA) Laboratory, Seibersdorf, Austria. Cobalt (^{60}Co) was used as the source of radiation. Three doses of the chemical mutagen N-nitroso-N-methyl urea (MNU): 0.5, 1.0 and 1.5 mM per 3 h and three doses of gamma irradiation: 150, 180 and 210 Gy were applied to the barley seeds. The treatment doses were chosen on the basis of previous experiments in which the somatic and genetic effects of both mutagens were evaluated (data not shown). Batches of the ~1000 seeds per each treatment combination were used. During treatment with MNU, seeds were first pre-soaked in 500 ml of deionised water (ddH_2O) for 8 h at room temperature. Then, the water was replaced by an appropriate MNU solution (0.5 ml/seed) and seeds were gently shaken for 3 h at room temperature. After the 3 h treatment, the seeds were rinsed under running tap water three times. Next, the seeds were transferred to Whatman paper and stored overnight at 4 °C prior to sowing.

2.3. DNA extraction

One young leaf from each individual M_2 plant was collected and dried in a plastic bag filled with silica gel (POCH, Gliwice, Poland) for 48 h. After grinding in a mill (Retch MM 200), total DNA was extracted using the hexadecyl trimethyl-ammonium bromide (C-TAB) method [48]. DNA quality and concentration were estimated using a NanoDrop ND-1000 spectrophotometer and 1.5% agarose gel electrophoresis.

2.4. TILLING

Aliquots of the DNA were diluted to a final concentration of 50 ng/μl for PCR using ddH_2O and arranged in five-fold pools for mutation screening. To facilitate the selection of putative mutants, each genomic DNA was represented in two different pools constructed using the two-dimensional pooling approach. Two genes were tested using the TILLING strategy: *HvEXPB1* (Accession No. AY351785), the root-specific gene for β-expansin 1 responsible for root hair development [49], and the *HvBRI1* (Accession No. AB088206), the gene coding the transmembrane receptor of brassinosteroids [50]. The entire sequence of the *HvEXPB1* gene, starting from −518 to +1952 bp divided into three fragments (EXP1; EXP2 and EXP3) and two fragments of the *HvBRI1* gene (+1639 to +2994 bp) were screened. The sets of primers for PCR were designed using Jellyfish software (http://jellyfish.labvelocity.com/) (Table A1). The first round of PCR amplification was performed in a TGradient (Biometra) cycler as follows: 95 °C for 5 min; 30 cycles of 95 °C for 45 s (denaturation), T_m −5 °C for 45 s (annealing) and 72 °C for 1 min 20 s (extension); 72 °C for 5 min. One hundred ng of genomic DNA was used as a template in a 15 μl volume of PCR reaction (1U DyNAzyme™ polymerase (Finnzymes); 100 μmol each of dNTPs; 0.2 μM of forward and reverse nested primers and 10× buffer for polymerase). Then, PCR products were 5-fold pooled and used as a template in the second round of PCR reaction using nested fluorescent-labeled primers (Table A1).

Heteroduplex formation of the second PCR product was performed using a TGradient cycler as follows: 95 °C for 3 min; 95–80 °C decrementing 3 °C/min; 80–55 °C decrementing 1 °C/min; 55 °C for 20 min; 55–45 °C decrementing 1 °C/min and 45–25 °C decrementing 2 °C/min. After heteroduplex formation, CEL I digestion was performed at 45 °C for 15 min. The CEL I reaction mix included 0.02 μl of enzyme and 1.5 μl 10× CEL I buffer, 5 μl of PCR product and 8.5 μl water. The CEL I used in the experiment was a crude celery juice extract provided courtesy of Prof. Edwin Cuppen, the Hubrecht Laboratory, the Netherlands Institute for Developmental Biology (NIOB). The amount of enzyme per sample was established experimentally. Reaction was stopped by adding 5 μl of 75 mM EDTA and mixing thoroughly. Samples were purified using 96% and 70% ethanol. For PAG electrophoresis, 1 μl samples were run for 4 h (1300 V, 30 mA, 30 W at 45 °C) in 25 cm LI-COR gels.

Sequencing of the putative mutants was performed using a SequiTherm EXCEL™ II DNA Sequencing Kit-LC (EPICENTRE) according to the manufacturer's directions on a LI-COR automated sequencer (LI-COR® 4200). Sequences were analysed using AlignIR 2.0 (http://www.licor.com/bio/Discov/Aligner.jsp) and Jellyfish 1.5.

2.5. AFLP

The genomic DNA of individual M_2 plants (250 ng per sample) was used for AFLP reactions. Restriction, ligation, pre-amplification and selective amplification were performed essentially as described in the original AFLP protocol of Vos et al. [44]. A total of 7 sets of fluorescent-labeled (IRD800) AFLP primers (*Eco*RI + 3 bases/*Mse*I + 3 bases) were used: E + ATA/M + CAG; E + ATC/M + CTA; E + ACT/M + CTC; E + AAC/M + CTG; E + ACC/M + CAT; and E + AAT/M + CTA. The products from the selective amplification were separated on 25 cm × 0.25 mm 6% denaturing polyacrylamide gels in a 1.0 × TBE buffer using a LI-COR automated sequencer 4200. For estimation of the frequency of DNA changes induced by mutagens, AFLP fragments were visually scored as present (1) or absent (0) to create a binary data set.

2.6. Cloning and sequencing of polymorphic AFLP bands

The longest polymorphic AFLP bands were cloned and sequenced. For the samples in which the polymorphic bands were detected, the restriction, adaptor ligation and pre-amplification reactions were repeated. The seven *Eco*RI primers were end labeled with ^{33}P using T4 polynucleotide kinase (Fermentas). The products from the selective amplification, with radioactive-labeled primers were electrophoresed. The gels were transferred to Whatman 3MM paper and dried using a SlabGelDryer SGD 5040 (SAVANT) at 80 °C for 1 h. The dried gels were stapled to Kodak diagnostic X-OMAT film and alignment holes were made in the corners of both the paper filter and film. After 16 h of exposure in the dark at room temperature, the Kodak film was separated from the Whatman paper, developed and fixed in the appropriate Kodak solutions. Dried autoradiograms were analysed for the presence of polymorphic bands. Specific AFLP bands were excised from the Whatman paper filter with a film behind using sterilised needles. Then, the needles were transferred to PCR tubes containing 10 μl of ddH_2O and incubated for 15 min at room temperature. An aliquot of 7.6 μl was re-amplified using the appropriate selective primer combination and the temperature profile. The amplification product was loaded on 1.5% agarose gel in a 0.5 TBE buffer. The DNA band was excised from agarose gel and purified using a QIAEX II Agarose Gel Extraction Kit (Qiagen). Next, it was cloned into a pGEM-T Easy Vector system II (Promega) according to the recommendations of the manufacturer. The component *Escherichia coli* JM209 cells were transformed with 2 μl of the ligation mix and recombinant clones were identified by blue/white selection. For each polymorphic AFLP band, the sequences from three to twelve positive colonies were analysed. DNA sequencing of the cloned AFLP bands was carried out commercially (Genomed, IBB, Poland).

2.7. Genome walking strategy

For two polymorphic AFLP bands, the flanking sequences for both 5′ and 3′ sites were obtained using a GenomeWalker Universal Kit (Clontech Laboratories). A genome walking strategy was performed according to the manufacturer's directions. Sets of primers for PCR were designed using Invitrogen software OligoPerfect™ Designer (http://toolsinvitrogen.com) based on the AFLP product sequences (Table A2).

2.8. Bioinformatic tools

Sequences were analysed using the available bioinformatics software: CLUSTALW 2 (http://www.ebi.ac.uk/Tools/msa/clustalw2), BLAST (http://blast.ncbi.nlm.nih.gov), JALVIEW 2.6 [51], AlignIR 2.0 (http://www.licor.com/bio/Discov/Aligner.jsp) and Jellyfish 1.5. For testing the homoplasy of the AFLP bands, the PCR fidelity calculator (http://www.finnzymes.com/pcr/fidelity_calc.php) was used. The effect of the discovered mutations on protein function and creation of gene models were carried out using PARSESNP (Project Aligned Related Sequences and Evaluate SNPs; http://www.proweb.org/parsesnp). The PSSM difference factor (Position-Specific Scoring Matrices) [52] and the SIFT (Sort Intolerant From Tolerant) program [53] were used for validation of the detected mutations in terms of their potential effect on protein function.

3. Results

3.1. Frequency of point mutations induced by MNU and gamma treatment

3.1.1. Screening of selected genes

The M_2 population after MNU treatment was screened for mutations in the *HvEXPB1* and *HvBRI1* genes. In total 4,537,117 bp were analysed using the TILLING strategy. Altogether, 9 nucleotide changes were identified. Based on this data, the mutation frequency induced by MNU in the analysed genes can be calculated as 2 mutations per million base pairs (Mb). The average mutation density, estimated by dividing the total number of base pairs screened by the number of identified mutations, was 1 mutation per 504 kb (Table 2). In both analysed genes, the dose-dependent effect was observed. The highest mutation frequency was obtained after

Table 2
The mutagenic effect of MNU and gamma rays in selected barley genes.

Mutagen	Dose	Gene	No. of M_2 plants analysed	Size of analysed sequence (bp)	No. of analysed nucleotides	No. of mutations detected	Mutation density (1 mutation/No. of kb)	Mutation frequency (No. of mutations per Mb)
MNU	0.5 mM/3 h	*HvEXPB1*	277	1951	540,427	0	0	0
	1.0 mM/3 h		579		1,129,629	2	564	1.8
	1.5 mM/3 h		471		918,921	2	459	2.2
	0.5 mM/3 h	*HvBRI1*	310	1422	440,820	0	0	0
	1.0 mM/3 h		583		829026	2	415	2.4
	1.5 mM/3 h		477		678,294	3	226	4.4
Total/Average			1348	3373	4,537,117	9	504	2.0
Gamma rays	150 Gy	*HvEXPB1*	819	1951	1,597,869	0	0	0
	180 Gy		309		602,859	0	0	0
	210 Gy		516		1,006,716	1	1007	1.0
Total/Average			1644	1951	3,207,444	1	3207	0.3

treatment with the highest applied dose of MNU; it was 4.4 and 2.2 mutations per one Mb, for *HvBRI1* and *HvEXPB1* respectively. No mutation was detected in the M_2 population treated with the lowest MNU dose (0.5 mM/3 h) in either of the analysed sequences.

The M_2 population after gamma ray irradiation was screened for mutations in only the *HvEXPB1* gene. Contrary to the results observed in the MNU-treated populations, an analysis of 3,207,444 bp revealed only 1 nucleotide change. The mutation was found after irradiation with 210 Gy, the highest dose of gamma rays applied in the study. The average frequency of point mutations calculated on the basis of the sequence screened was 0.3 per 1 Mb. The mutation density after gamma treatment was 1/3207 kb (Table 2).

3.1.2. Analysis at the whole genome level

3.1.2.1. AFLP polymorphism. AFLP analysis was carried out on 920 and 833 M_2 plants obtained after mutagenic treatment with MNU and gamma rays, respectively, along with 50 non-treated plants. Based on the results obtained from the TILLING of selected genes, treatment with the lowest dose of gamma rays (150 Gy) was excluded from the analysis. There were 54–80 amplified fragments generated per the employed primer combination. The AFLP fragment length ranged from approximately 50 to 500 bp with a majority of the polymorphism being distributed between 150 and 400 bp. Plants with changes in the AFLP profile were observed for all of the applied doses of both mutagens.

Among the 920 M_2 plants derived from MNU treatment, 55 plants (6.0%) carried changes in the AFLP profile. In the majority of plants with changes, only one additional band was present (Fig. 1), but a few plants carrying 2–3 polymorphic AFLP loci were also observed. The polymorphic M_2 plants presented different AFLP fingerprints, which indicates a lack of preferential mutation sites. AFLP analysis identified 488 loci of which 63 (12.9%) were polymorphic. In total, 426,329 AFLP fragments were generated with 65 polymorphic bands (0.015%) (Table 3). As each AFLP band is the product of a specific combination of 16 nucleotides that form restriction and priming sites at the fragment ends (6 bp recognised by *Eco*RI + 3 selective bp + 4 bp recognised by *Mse*I + 3 selective bp) in total, 6821 kb (426,329 AFLP bands × 16 bp) were scanned for AFLP polymorphism in the entire M_2 population. Assuming that each polymorphic band resulted from a single nucleotide change, this indicates that 1 mutation per 105 kb (mutation density) or 9.5 mutations per 1 Mb (mutation frequency) was induced by MNU treatment in the barley genome. Almost all changes (98%) resulted in the creation of a new AFLP band in an M_2 plant. No clear dependence between the applied dose of MNU and mutation frequency was observed.

Gamma irradiation gave approximately a 3-times lower AFLP polymorphism as compared to MNU treatment. Among the 833 M_2 plants derived from gamma ray treatment, only 12 plants (1.4%) carried changes in the AFLP profile. AFLP analysis identified 435 loci of which 18 (4.1%) were polymorphic (Table 3). None of the polymorphic changes (additional or lack of a band) occurred in the same AFLP locus in two different M_2 plants. In total, 350,018 AFLP fragments were generated in the M_2 population with 18 polymorphic bands (0.005%). The AFLP fragments together represented 5600 kb of the genome scanned for AFLP polymorphism. Providing that each polymorphic band resulted from a single point mutation, the observed polymorphism indicated 1 mutation in every 311 kb or 3.2 mutations per 1 Mb screened. Similar to the MNU treatment, the majority of polymorphic AFLP products (72%) were additional bands. The mutation frequency increased with the dose of gamma rays applied. The parameters, such as the percentage of M_2 plants with changes in their AFLP profile, the percentage of polymorphic AFLP loci and bands were about 1.5–2.5 times higher in a

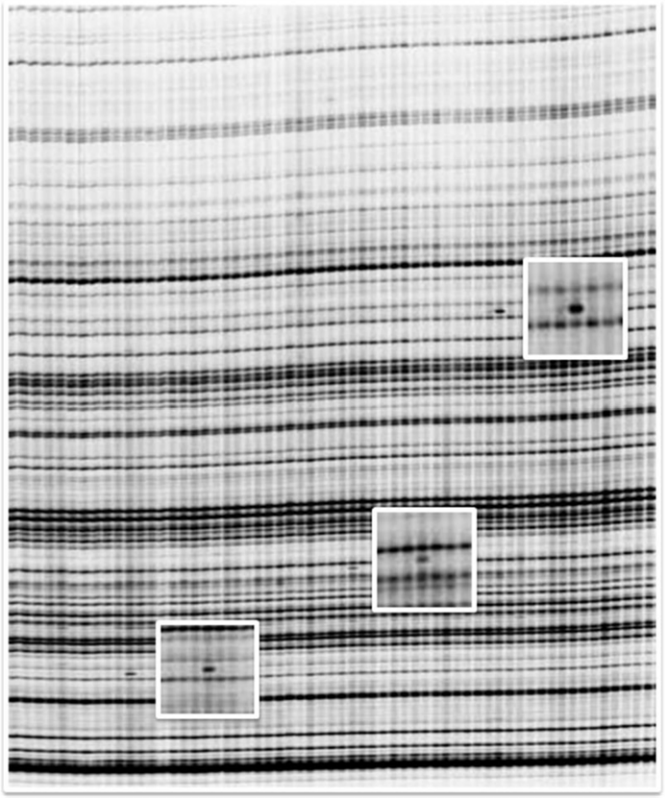

Fig. 1. AFLP polymorphism in the M_2 barley generation after mutagenic treatment with 1.0 mM/3 h MNU. Each line represents an individual M_2 plant. The primer combination M-CTA/E-ATC was used for selective PCR amplification.

Table 3
Evaluation of AFLP polymorphism in barley the M_2 generation after mutagenic treatment with MNU and gamma rays.

Mutagen	Dose	M_2 plants			AFLP loci			AFLP bands			Change type	
		No. analysed	With changes		No. analysed	Polymorphic		No. analysed	Polymorphic		Lack of band	Additional band
			No.	(%)		No.	(%)		No.	(%)		
MNU	0.5 mM/3 h	309	18	5.8	488	21	4.3	144,324	20	0.015	0	21
	1.0 mM/3 h	304	14	4.6	484	17	3.5	141,683	19	0.013	0	19
	1.5 mM/3 h	307	23	7.5	481	25	5.2	140,322	25	0.018	1	24
Total/Average	920	55	6.0	488	63	12.9	426,329	65	0.015	1	64	
Gamma rays	180 Gy	303	3	1.0	414	5	1.2	124,233	5	0.004	1	4
	210 Gy	530	9	1.7	435	13	3.0	225,783	13	0.006	4	9
Total/Average	833	12	1.4	435	18	4.1	350,018	18	0.005	5	13	

No. of analysed bands was estimated as the number of monomorphic loci multiplied by the number of analysed plants plus the number of polymorphic loci for each dose of the mutagen.

Fig. 2. The heterogeneity of AFLP bands.

combination treated with 210 Gy than in that treated with a 180 Gy dose.

In total, 77 polymorphic AFLP bands (additional fragments) were detected in the presented study, 64 (83%) among the M_2 plants obtained after chemical treatment and 13 (17%) after irradiation with gamma rays. The length of the polymorphic AFLP bands, determined using a size marker during PAGE electrophoresis, ranged from 65 to 380 nucleotides with the majority below 150 nucleotides.

3.1.2.2. Heterogeneity of AFLP bands. Thirty-five well-resolved, long polymorphic bands detected in the M_2 plants were excised from the gels and cloned. The length of the sequences ranged from 109 to 342 nucleotides per fragment when measured without the adaptor/primer sequences. The majority (32) of cloned AFLP bands derived from the M_2 plants treated with MNU; only 3 cloned bands (9%) originated from the M_2 plants after gamma rays treatment.

At least three to twelve clones from one polymorphic AFLP band were sequenced. The AFLP adaptor/primer sequences were used to orient and align the fragments. All analysed sequences contained the expected nucleotide sequences, including selective bases and indicating no occurrence of the mismatch of selective bases during PCR amplification. A total of 232 clones were sequenced and aligned, giving 114 independent AFLP products (amplicons).

On average 6.6 clones were analysed for each polymorphic AFLP band, which yielded 3.3 different sequences (AFLP products/amplicons) (Fig. 2). Homogenic AFLP bands represented only 6% of those analysed. The majority of AFLP bands were heterogenic with some bands containing up to 8 different amplicons. The largest group (88%) represented bands with 2 to 5 different AFLP products. For some AFLP amplicons derived from the same band, length differences up to 3 nucleotides were also observed, even though they had the same adaptor/primer sequences at both ends. Examples of sequence identity and heterogeneity among amplicons derived from the same polymorphic AFLP band are presented in Fig. A1 and Fig. A2, respectively. No large insertion/deletion variation among clones derived from the same AFLP band was observed. The sequences of all of the detected AFLP amplicons described in this paper have been submitted to the GenBank library under Accessions No. GS923622 to GS923735.

3.1.2.3. BLASTN analysis of polymorphic AFLP products. The sequenced AFLP amplicons were analysed for annotation in the National Center for Biotechnology Information (NCBI) database using the BLASTN tool and one of three available algorithms: megablast (for 46 AFLP products), discontiguous megablast for 29 (35.4%) and blastn for 7 (8.5%). The best hits presented a maximum identity of 68% to 100%. The results for all of the analysed AFLP

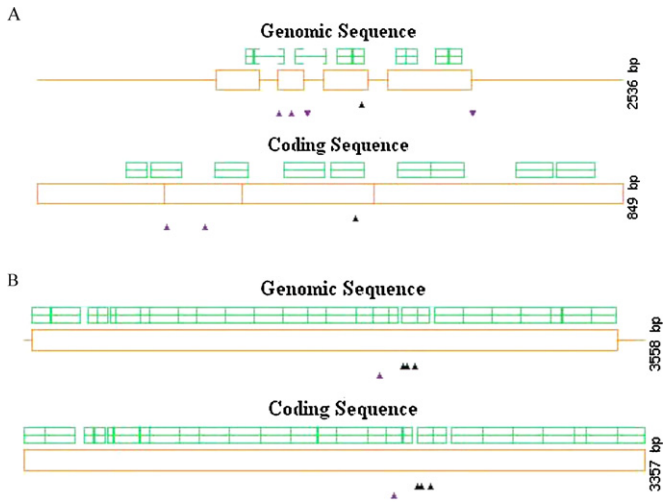

Fig. 3. PARSESNP output for the barley genes *HvEXPB1* (A) and *HvBRI1* (B) showing the mutations induced by MNU treatment. The gen model is shown with red boxes corresponding to exons and lines for introns. Triangles are a sign of the location and type of mutation found. A purple triangle denotes a silent change and a black one represents missense changes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

amplicons along with the highest score during BLAST analysis and an *E* value lower than 0.05 are presented in Table A3.

Among the 114 independent AFLP products sequenced, the majority (98) were obtained after MNU treatment and only 16 after gamma ray irradiation. For this reason, further analysis was only conducted for the MNU-treated population. For the majority (75%) of AFLP products derived from MNU mutagenesis, the sequence similarity search using BLASTN was completed with success and for only 24 sequences (25%) no significant similarity was found in NCBI database using the applied algorithms. The most frequent type of the sequences identified in the barley genome were repetitive sequences (Table 4). The ratio of sequences belonging to Class I (retrotransposons) and Class II (transposable elements) was established as 19:1. Among Class I, LTR elements were present most often (89.5% of all repetitives). Gene and gene-related sequences were detected at a frequency of 23%, and among these, the 5′ and 3′ gene flanking sequences were most frequent (Table 4). The best hits were also found for some coding sequences: the chloroplastic *petA* coding the cytochrome f protein, the mitochondrial gene coding 18S rRNA and three cDNA sequences with unknown functions.

3.2. Mutation types

The analysis of two genes: *HvEXPB1, HvBRI1* and two AFLP fragments extended using the Genome Walking technique (GenBank Accession No. HN149916 and HN149923) revealed 12 nucleotide changes, most of them in the MNU-treated populations (Table 5, Fig. 3). Only one change (C > A transversion resulting in a silent mutation) was detected after gamma ray treatment. Among the mutations induced by MNU in the gene sequences, five missense, three synonymous and two in non-coding sequences were identified. All missense mutations were expected to change the protein function as was indicated by the PSSM difference factor and the SIFT program (Table 5). Two AFLP products (260 and 332 bp long), extended using Genome Walker strategy to 1348 and 1398 bp, respectively, carried the C > T mutation that led to the appearance of a polymorphic AFLP band. In the first case, the mutation led to the appearance of an additional restriction site for the *Eco*RI enzyme; in the second, it created a sequence complementary to the *Mse*I primer used during selective amplification. Both mutations resulted in an additional AFLP band in the respective M_2 plant. One of these plants also carried a 238 bp deletion in the 3′ site of the HN149916 fragment.

The majority (63.6%) of the identified MNU-induced nucleotide changes were transitions. Among them there were G > A (three) and C > T (four) substitutions (Table 6). A relatively high level (36.4%) of different types of transversions: one A > C, one C > A and two T > G were also observed.

4. Discussion

In the presented study, we compared the frequency of point mutations induced in the barley genome by MNU, an alkylating agent and gamma rays irradiation. By using a doubled haploid line as the parent material for mutation induction, we ensured that any change observed in the DNA sequence was the result of mutagenic treatment rather than any possible intra-varietal variability. An M_2 population of ca. 3013 individuals was developed in such a way that each M_2 plant originated from a different M_1 spike and thus represented a separate mutagenic event. The M_2 generation so created served as the plant material to search for changes between M_2 plants and non-treated control (parental DH line 'H930-36') at the DNA level.

4.1. Comparison of MNU and gamma rays as sources of induced mutations in plants

Scanning the barley genome with AFLP markers revealed ca. a three times higher level of AFLP polymorphism (additional or lacking AFLP bands) in MNU-treated as compared to the gamma-irradiated population. Searching for mutations in selected genes showed even greater difference between the frequency of nucleotide changes induced by MNU and gamma rays. After gamma irradiation, only one mutation (C > A transversion) was found in the total sequence of 3207 kb of the *HvEXPB1* gene screened, which gives 0.3 mutations per 1 Mb. The frequency of nucleotide substitutions induced by MNU treatment in the same gene was 2.2 mutations per Mb, more than seven times higher.

It can be concluded that N-methyl-N-nitrosourea is a more efficient mutagen than gamma rays in inducing point mutations in barley. In addition to the analysis of AFLP polymorphism and TILLING of selected genes, we estimated the frequency of chlorophyll mutations in the M_2 generation. The highest dose of gamma irradiation, 210 Gy induced 0.6% of chlorophyll seedlings in M_2, while the lowest dose of applied MNU treatment, 0.5 mM per 3 h, resulted in 2.1% of chlorophyll mutants. After treatment with the highest MNU dose, 1.5 mM per 3 h, 5.6% of chlorophyll seedlings were observed. The range of doses of MNU and gamma rays used in this study was based on our previous experience where both the genetic effect of a mutagen, measured by the frequency of chlorophyll mutations in the M_2 generation and the somatic effects in M_1, expressed as a reduction in the emergence, height, fertility and survival of M_1 plants, were evaluated for a range of barley varieties (unpublished data). Doses of MNU below 0.5 mM per 3 h resulted in a low mutation frequency and even growth stimulation in M_1. On the other hand, irradiation with doses of gamma rays higher than 210 Gy caused high sterility of M_1 plants and high survival reduction for majority of varieties tested. It should also be stated that the doses of gamma irradiation used in this study were in a dose range recommended by Manual on Mutation Breeding [54] and well documented by the classical work of Frydenberg and Sandfaer [55]. The so-called 'useful doses' of gamma radiation in barley are between 100-250 Gy. Taking into account the results obtained in the presented study, it is unlikely that even the application of the highest recommended dose of gamma irradiation can induce a frequency of point mutations similar to the level of changes induced by MNU.

Table 4
Types of sequences with an annotation in NCBI databases identified among polymorphic AFLP products after MNU treatment.

	Repetitive sequences				Genes and gene-related sequences			Without a detailed description	Total
	Retrotransposons			DNA transposons	CDS/exon	Intron	Gene flanking		
	LTR		Non-LTR						
	Copia	Gypsy-like							
No.	6	28	2	2	5	1	11	17	72
(%)	(8.3)	(38.9)	(2.8)	(2.8)	(6.9)	(1.4)	(15.3)	(23.6)	(100)
No.	36			2	17				
(%)*	(65.5)			(3.6)	(30.9)				

CDS – coding sequence; LTR – long terminal repeats. Copia: *BARE-1*, *Maximus*; Gypsy-like: *Laura*, *Gyp3*, *Cereba*, *Lolaog*, *Nusif*, *Wham*, *Sokkula*, *Sabrina*, *BAGY-2*, *Fatima*, *Camillia*, *Wilma*, *Vegabond*, *BAGY-1*; Non-LTR: *Karin*, *Miuse*; DNA transposons: *Mandrake*, *Caspar-2*.
* Percentage in relation to the sequences with an NCBI annotation.

Table 5
Mutations identified in the M_2 populations treated with MNU or gamma rays.

Mutagen	Dose	Target	Nucleotide change	Position	Amino acid change	PSSM value
MNU	1.0 mM/3 h	HN149916*	C > T	–	–	–
		HN149923*	C > T	–	–	–
	1.0 mM/3 h	*HvEXPB1*	C > T	1045	G63=	–
			C > T	1404	A154V+	10.8
	1.5 mM/3 h		G > A	1170	intron	–
			C > A	1884	non coding	–
	1.0 mM/3 h	*HvBRI1*	A > C	2040	P666=	–
			T > G	2237	L732W+	10.6
	1.5 mM/3 h		G > A	2171	R710K+	10.4
			G > A	2192	S717N+	10.7
			T > G	2237	L732W+	10.6
Gamma rays	210 Gy	*HvEXPB1*	C > A	1099	A81=	–

= Synonymous change; + Missense change predicted to be damaging to the encoded protein by the PSSM value.
* AFLP fragment extended by Genome Walker strategy.

The high degree of efficiency of MNU as a potent mutagen was demonstrated in other plant species. TILLING populations of rice cv. 'Taichung 65' [42] and soybean cv. 'Williams 82' [41], produced after MNU mutagenesis, exhibited a very high rate of mutations. Analysis of three genes in rice (total sequence 3.3 Mb) and 7 genes in soybean (total 7.7 Mb) revealed the highest density of mutations in a diploid species – 1 mutation per 135 kb and 140 kb, respectively. This is about 3.5 times higher than in the presented study of barley (1 mutation per 504 kb). It should be noted, however, that a direct comparison of mutation rates induced by the same mutagen in different species is difficult. Different species and genotypes within species exhibit a large variability in terms of sensitivity to the action of the same mutagen [56]. The effectiveness of chemical mutagenesis can be modified not only by the concentration of the mutagen and time of treatment but also by the method of the application of the mutagen. The high mutation rate reported in rice might be partially related to the unique method of mutagenic treatment applied by Suzuki et al. [42], where flowers (M_0 generation) were treated by MNU at the single-cell zygote stage. On the other hand, in soybean, the large number of duplicated sequences present in the genome may cause a higher tolerance to mutagen action. Regardless of the mutagen applied, the mutation frequencies for tetraploid and hexaploid wheat reported by Slade et al. [57] and Uauy et al. [58] were five to ten times higher than the mutation rates found in diploids, such as barley [59–61], *Lotus japonicus* [62] and Arabidopsis [63,64].

Among the nucleotide substitutions detected after MNU treatment in this study, G > A transitions accounted for 25% and C > T transitions for 33.3% of the observed changes. In the studies performed on rice, G > A and C > T transitions were predominant and accounted for 50% and 42% of all mutations detected, respectively [42]. Similarly, in soybean, 90% of the observed nucleotide changes formed these two types of transitions [41]. They both arise from alkylation of guanine at the O^6 position, which leads to the formation of O^6-meG – the DNA lesion with the strongest mutagenic property [31]. Alkylation of guanine in the nontranscribed (sense) DNA strand leads to its mispairing with thymine and after replication to replacement by adenine (G > A transition). Alkylation of guanine in the transcribed (antisense) strand results in the C > T transition. The similar share of G > A and C > T transitions reported in barley (this study), rice and soybean (see above) indicates a lack of bias in the repair of O^6-meG lesions between DNA strands. Contrary to these results, G > A transitions were the predominant base pair alterations induced by MNU in the *hprt* (hypoxanthine-guanine

Table 6
The types of nucleotide changes identified in the M_2 population after MNU treatment.

Method of detection	Mutation type				
	Transitions		Transversions		
	G > A	C > T	A > C	C > A	T > G
AFLP	–	2	–	–	–
TILLING	3	2	1	1	2
Total	7		4		
(%)	(63.6)		(36.4)		

phosphoribosyltransferase) gene in rat fibroblasts, which suggests that the DNA repair systems in rat remove O^6-meG much faster from the transcribed than from the nontranscribed DNA strand [35].

G/C to A/T transitions are predominant among the mutations induced by other alkylating agents, such as EMS applied in plants [58,63–65] or ENU used in animal mutagenesis [66]. In many cases, they account for up to 100% of all observed changes [57,67,68]. In the presented study, in addition to G/C to A/T transitions, a relatively high level of transversions: A > C, C > A and T > G was detected. As the parent material used for mutagenic treatment was completely homozygous (DH line), we concluded that the non-GC/AT changes discovered in the TILLING screens did not result from any possible residual variability of the parental line, but were new allelic variants generated by the MNU action. Similarly, a high rate of transversions was obtained by Minoia et al. [69] in the TILLING population of tomato treated with 1% EMS. In this study, five types of transversions accounted for 55% of the observed nucleotide substitutions. Using BLAST analysis of the natural sequence variation of the tilled genes and EcoTILLING of 150 tomato varieties, the authors ruled out the probability that natural polymorphisms, introduced through pollen or seed contamination, could be responsible for the non-GC/AT changes observed in their mutant populations.

The available data set on DNA sequences in which MNU-induced mutations were detected allow for an investigation of whether the G/C to A/T transitions are distributed randomly in plant genes or whether the local bias in the nucleotide composition surrounding the methylated guanine (O^6-meG) exists. A nonrandom distribution of mutations caused by MNU was reported in studies with model systems: *Esherichia coli* [33] and rat [34]. In both cases, the majority of methylated guanine occurred at the middle position of the sequence 5′-GG(A or T)-3′. Methylation at this position accounted for 82% and 68% of G/C to A/T transitions in the *E. coli* and *H-ras* gene (v-Ha-ras Harvey rat sarcoma viral oncogene homolog) in rat, respectively. The nearest two neighbors on either side of the guanine mutated by another alkylating agent – EMS, also showed strong skews in experiments carried out on *Arabidopsis thaliana* [63]. There was an excess of purines with adenines slightly favored over guanines. The authors explained this bias by the differences in repair rates depending on the local DNA environment.

We examined 5′-NGN-3′ sequences for 64 G/C to A/T transitions caused by MNU action in barley (this study), rice [42] and soybean genes [41] (Table A4). The analysis clearly demonstrated the strong specificity of the nucleotide surrounding the methylated guanine at the −1 position. Purines formed 81% of nucleotides observed at the −1 site with predominant guanine (43.8%, Table 7). Contrary to this and to the MNU action in *E.coli* and rat, all four nucleotides flanking the O^6-meG at the +1 site were distributed randomly. Based on these data, it can be concluded that MNU-induced G/C to A/T transitions occur in plant genomes mostly in the sequence 5′-(G/A)GN-3′ but with the same frequency in both DNA strands.

In contrast to the large amount of information regarding the frequency of mutations induced in plant DNA sequences by alkylating agents, mostly EMS, data related to the effect of gamma radiation are very scarce. Based on a TILLING analysis of 25 gene regions in rice (*O. sativa*), Sato et al. [27] estimated the rate of mutation induced by gamma rays as 1 mutation per 6190 kb. This rate is about two times lower than in the presented study and the difference can be attributed to the different species, doses and genes analysed in both studies. Additionally, the authors used an endonuclease extract from the petioles of *Brassica rapa* and agarose gel electrophoresis for mutation detection, which could affect its efficiency as compared to the standard TILLING procedure. Among the detected mutations, there were four single nucleotide substitutions and two 2–4 bp deletions. In another study on rice, based on sequence analysis of 24 gamma ray-induced mutants, deletions of different sizes were the main type of mutations (79.2%) while base substitutions comprised only 12.5% of the observed changes [28]. Similarly, the study of Naito et al. [26] performed on *A. thaliana* indicated that deletions were the major type of mutations induced by gamma rays, although in this case they were mostly large deletions, the majority of which were not transmitted to progeny. In the presented study, no deletions were observed in the *HvEXPB1* gene after gamma ray treatment. It should be pointed out, however, that the CEL I enzyme used for mutation detection in this study recognises the mismatches caused by nucleotide substitutions and small insertions/deletions but not large deletions. Such deletions may have been the cause of the AFLP polymorphism observed as a lack of an AFLP band in some M_2 plants, although point mutations in the restriction/priming sites as a source of polymorphism cannot be excluded.

4.2. The usefulness of AFLP markers for the detection of mutations across the barley genome

Arbitrarily primed DNA markers, such as AFLP, ISSR (inter-simple sequence repeats) and ASAP (arbitrary signatures from amplification profiles) have commonly been applied for assessing spontaneous genetic variability in plants (for review see [70]). They are also examples of using marker technology to characterise induced variation in plants, e.g. mutations in somatic and radiation-induced sports of *Chrysanthemum morifolium* [71], genome-wide mutation rates in vegetatively propagated bermudagrass [72] and somaclonal variation induced *in vitro* in *A. thaliana* [73], oil palm [74], nephthytis [75], peach palm [76], grapevine [77] and barley [78]. In the presented study, we used the AFLP technique to uncover genome-wide genetic differences in the DNA between M_2 plants derived from MNU and gamma ray treatment and a non-treated control in barley. In order to check whether AFLP markers can really scan the whole barley genome for mutagen-induced polymorphism, 114 different AFLP products, derived from 35 well-resolved, polymorphic bands detected in M_2, were cloned and sequenced. Most of them came from MNU treatment and only those were the subject of bioinformatic analysis. Among the sequences with NCBI annotation, repetitive sequences with prevalent LTRs were the most frequent type of sequences detected using the AFLP technique. The proportion of repetitive elements in which AFLP polymorphism occurred was slightly lower in our study (69.1%) than their share in the total barley genome. Although the full barley genome has not been sequenced yet, the proportion of different types of DNA sequences in the barley genome has been estimated by many authors. According to Wicker et al. [79], Schulte et al. [80] and Wicker et al. [81], repetitive elements form the main part of the barley genome (80%) and among them retrotransposons are the most numerous [82,83]. These estimates were confirmed by Mayer et al. [84], who sequenced the barley chromosome 1H using next generation sequencing technology, GSFLX Roche. The content of repetitive sequences was determined as 77.1% of the total 1H barley sequence with 71% retrotransposons and 6% DNA transposons. LTR retrotransposons are the most abundant and widespread components of cereal genomes. The bioinformatic analysis of LTRs detected by AFLP analysis in this study showed the highest similarity of 6 AFLP products (15%) to the *copia*-like superfamily and 28 (70%) to the *gypsy*-like superfamily. The same proportion between *copia*- and *gypsy*-like elements in chromosome 1H of barley have been reported by Mayer et al. [84]. In total, the polymorphic AFLP products that belong to repetitive sequences were classified to 16 LTR families, 2 LINE families and the DNA transposon superfamily CACTA. No helitrons have been identified.

It is estimated that genes account for at most 5% of the barley genome [85] and their total number was calculated as ~32,000 [86]. The share of identified coding sequences among the analysed AFLP

Table 7
The most frequent nucleotides flanking meG identified on the basis of the presented study and the previously reported results [41,42].

	Nucleotides flanking O^6-meG in position							
	−1				+1			
	G	A	C	T	G	A	C	T
No.	28	24	7	5	16	20	15	13
(%)	(43.8)	(37.5)	(10.9)	(7.8)	(25.0)	(31.3)	(23.4)	(20.3)

products, 5.1%, is in agreement with these estimates. In conclusion, the performed bioinformatic analysis of the sequenced polymorphic AFLP products indicates that they reflect the organisation of the barley genome and therefore can be used for genome-wide mutation screens in this species.

The vast majority of polymorphic AFLP bands (98%) derived from MNU treatment were the additional bands present in M_2 plants, while only 2% of the lacking bands were detected. These results are in agreement with the restriction enzymes used to generate the AFLP bands and with the molecular mechanism of MNU action. *Eco*RI and *Mse*I endonucleases recognise and cut the 5′GAATTC3′ and 5′TTAA3′DNA sequence, respectively. The lack of an AFLP band can originate from a mutation that causes a nucleotide substitution in either of the restriction sites, while an additional band may result from a mutation that would create a new restriction site. It is well documented that the primary mutagenic action of MNU is O^6 methylation of guanine [33–35]. O^6-methylguanine (O^6-meG) preferentially pairs with T during DNA replication and as a result, the transition G/C to A/T appears. In both restriction sequences, among the 10 nucleotides recognised by the restriction enzymes, there was only one G that could have serve as a target of MNU action. This indicates that a lack of a band in an M_2 plant may result from the methylation of a single G at the *Eco*RI restriction site. The site for *Mse*I (5′TTAA3′) does not have any G, and therefore it cannot be a target of a mutagenic event leading to the loss of an AFLP band. However, a new *Mse*I site can appear through the methylation of a G in one of the following sequences that are possibly present in barley genome: 5′TTGA3′, 5′TTAG3′ or 5′TTGG3′. Furthermore, a new restriction site for *Eco*RI (5′GAATTC3′) might be created through MNU-induced methylation of the second, the third or both Gs in one of the following sequences: 5′GGATTC3′, 5′GAGTTC3′, 5′GGGTTC3′. In any case, the target sequence for MNU action leading to the creation of a new AFLP band has to be located at a distance 100–500 bp from an existing site, as only AFLP products 100–500 bp long were analysed in this study. The probability that a new restriction site for *Eco*RI and/or *Mse*I can be created through methylation of G in the sequences presented above is enhanced by the fact, that in the majority of these sequences, the target G is surrounded by a purine at −1 position. The same reasoning can be applied to the target sequences complementary to the selective nucleotides of AFLP primers which contained a higher amount of A and T nucleotides than G and C.

There are two other probable reasons for the higher frequency of additional AFLP bands that can be relevant to both, MNU and gamma treatment. Firstly, AFLPs are dominant markers, therefore M_2 plants with additional bands represent mutations both in homozygous and heterozygous state, while plants lacking an AFLP band carry homozygous mutations only. Secondly, the high heterogeneity of the analysed AFLP bands revealed in this study could lead to the decreased frequency of polymorphisms that are described as lacking bands. The AFLP amplicons of the same size as the disappearing band, and thus observed as the same AFLP locus, could mask the mutation which led to the loss of the product.

As was mentioned above, the majority of sequenced AFLP bands (94%) were heterogenic. The co-migration of non-homologous AFLP fragments (homoplasy) was observed by several authors, however, only a few studies have examined homoplasy by sequencing AFLP fragments. A genetic diversity study in garlic revealed up to 5 different products in one AFLP band and heterogenic bands constituted 21.8% of all of those analysed [87]. The probability that more amplicons would exist in one AFLP band is higher in large genomes [88] and this is a direct consequence of the number of observed bands per genome and the GC content [89]. There also appears to be a negative relationship between the degree of homoplasy and fragment size [90]. At the individual level, the heterogeneity of AFLP bands may result from the co-migration of similar but not identical repetitive sequences that share the same size but not the same location in the genome [91]. In our study, an average polymorphic AFLP band, 109-342 bp long, carried 3 different sequences differing in length by 0–3 bp. In Meksem et al. research [92] conducted on soybean, the bands ranged in size by 1–2 bp, and one AFLP band could be composed of up to 15 different products. On the basis of interspecific studies of *Echinacea*, Mechanda et al. [93] concluded that the sequence homology decreases and homoplasy increases with increasing taxonomic rank. The authors found much less sequence identity among polymorphic fragments than in monomorphic fragments. The co-migration of non-homologous AFLP fragments can cause errors when this method is used for gene mapping, genotyping or evaluation of genetic variability studies, as homoplasy results in an underestimation of genetic diversity among samples [94]. In model species, the use of so-called *in silico* AFLP allows AFLP profiles to be predicted in order to check the possibility of the appearance of homoplasy before an experiment [91].

5. Conclusions

- Within the range of doses commonly used in barley mutagenesis, gamma rays showed very low effectiveness in inducing nucleotide substitutions in the analysed DNA sequences.
- N-methyl-N-nitrosourea (MNU) is a much more efficient mutagen than gamma rays in inducing point mutations in barley. The average density of MNU-induced substitutions in the selected sequences was 1 mutation per 504 kb or 2.2 mutations per 1 Mb.
- The majority (63.6%) of the MNU-induced nucleotide changes were transitions, with a similar number of G > A and C > T substitutions.
- MNU-induced G/C to A/T transitions occur in plant genomes mostly in the sequence 5′-(G/A)GN-3′ but with the same frequency in both DNA strands.
- The types of sequences present in the polymorphic AFLP bands reflect the organisation of the barley genome and therefore can be used for genome-wide mutation screens in this species.
- The co-migration of non-homologous AFLP fragments (homoplasy) should be taken into account when the AFLP technique is used for diversity studies.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A.

Tables A1–A4.
Figs. A1–A2.

Table A1
Sequences of primers used in TILLING.

Name:	Sequence 5′–3′	T_m
EXP1nesF	GATGAAGGCACCATGAAGG	57 °C
EXP1nesR	GAAAAAGAGTAAGGCTCGGTCT	58 °C
EXP2nesF	GACGGTGCTCGTCATCCTAT	58 °C
EXP2nesR	CATCCCGTTGTACTTGCAG	58 °C
EXP3nesF	CGGACAACTCAGGATCCACT	58 °C
EXP3nesR	CGTGGCCATTTTTCGTTTAC	59 °C
BRI1nesF	CTATGTGTATCTTCGCAACG	51 °C
BRI1nesR	ATGCTGAGAGCATTTGTACC	53 °C
BRI2nesF	ATAGCCATCGAGAGCAAGA	49 °C
BRI2nesR	TTATGGTCCTCACCAAAATC	48 °C
EXP1F	IRD700 - TTACCTTCGGGGGACATCGT	63 °C
EXP1R	IRD800 - GAATGTACGCGAGGCTCACC	61 °C
EXP2F	IRD700 - GGTGAGCCTCGCGTACATTC	61 °C
EXP2R	IRD800 - CGCCTTCAGCAAGCACACTC	63 °C
EXP3F	IRD700 - CGTGCGTGACACTGGTTAGT	58 °C
EXP3R	IRD800 - AAGCCTGAGCGATAATTGGA	59 °C
BRI1F	IRD700 – CGGTCCGAGGACCTTAGTC	58 °C
BRI1R	IRD800 – CAGAATGTGACCGGCTATCA	60 °C
BRI2F	IRD700 – GGAGGCAGAAGAATGACGAG	62 °C
BRI2R	IRD800 – CAGCAACACAACACCGTAGC	62 °C

Table A2
Sequences of primers used in the GenomeWalking strategy.

Name	Sequence 5′–3′	T_m (°C)
GW6631F	TAATCTTTGTTGATCTGCTTCACAACGAGC	69
GW6631R	TGACACCTAGACCAGACCAAGGAGTTCA	69
NGW6632F	TGAGAGTCTCCTGTGATGATCAAATTCTTG	68
NGW6632R	TCTCCCCGGCAGGGAAGCATCTAA	72
GW8421F	AATGCCAAATGCCACTTCTAGGGAGATC	70
GW8421R	CAGAATATCCTTTGACTTGGAGTCGAGAGG	69
NGW8422F	CCTCCCAATAGGATATGTTGACTTGCGAG	70
NGW8422R	GATAACCCTCCCTGTATTCACAGCTCGG	70

Table A3
The types of sequences identified during analysis of polymorphic AFLP products using BLASTN.

No.	AFLP product No. clone/clones	Algorithms BLASTN	Accession	Types of sequences	Description	Max identity (%)	*E*-value
1	77/3; 77/4	1	EU534409.1	–	Mitochondrial DNA	96	2e-88
2	77/2	NF	–	–	–	–	–
3	14/1	1	AY268139.1	LTR BARE-1	BAC 184G9	89	4e-56
4	14/3	1	AP009567.1	Gene flanking	Genes for putative iron-deficiency specific 4 protein and putative ethylene-responsive transcription factor	92	4e-81
5	46/1, 46/3, 46/4	2	EF067844.1	LTR Vegabond	Locus *vrs1*; gene *Hox1*	84	2e-27
6	46/2	1	DQ537335.1	LTR Fatima	BAC 1031P08; BAC 754K10; BAC 1344C1	78	2e-30
7	14/4	1	EU812563.1	*RGA1* flanking	Genes *Rpg4*; *RGA1*; *Rpg5*; *PP2C*; *ADF3*	99	3e-67
8	14/2	1	EF067844.1	LTR Laura	Locus *vrs1*; gene *Hox1*	93	1e-76
9	14/5	1	EF115541.1	Gene *petA*	Chloroplastic DNA	99	9e-58
10	46/5	2	AC133398.4	–	BAC OSJNBa0083F15, chromosome 3	81	4e-11
11	68/1	1	AP009567.1	Gene flanking	Genes for putative iron-deficiency specific 4 protein and putative ethylene-responsive transcription factor	95	3e-117
12	68/2, 92/2, 92/4, 100/1, 100/3, 100/4, 100/5	3	AY661558.1	–	Gene *eIF4E*	75	1e-04
13	92/5	3	AF459639.1	LTR Nusif	BAC 116F2 and gene 115G1	80	1e-18
14	92/1	1	EF067844.1	LTR Wham	Locus *vrs1*; gene *Hox1*	95	7e-119
15	36/1, 36/2, 36/4	NF	–	–	–	–	–
16	36/5	2	FJ175362.1	–	Region with repetitive sequences	91	3e-14
17	16/5	3	AM932689.1	LTR Camillia	BAC TA3B63N2, chromosome 3B	76	4e-28
18	37/1	3	AM932689.1	LTR Camillia	BAC TA3B63N2, chromosome 3B	76	4e-28
19	37/4	2	AM932689.1	LTR Camillia	BAC TA3B63N2, chromosome 3B	77	3e-29
20	16/3	2	AM932681.1	LTR Wham	BAC TA3B63B13, chromosome 3B	83	1e-46
21	19/3	2	DQ249273.1	LTR Viva	BAC 631P8	81	3e-65
22	19/5	1	AK248318.1	Gene	FLbaf52b15, cDNA	100	9e-64
23	93/5	1	AY485643.1	LTR Sukkula	BAC 615K1	83	3e-59
24	15/3, 15/5	1	EF067844.1	LTR Maximus	Locus *vrs1*; gene *Hox1*	96	2e-124
25	15/1	1	EF067844.1	LTR Maximus	Locus *vrs1*; gene *Hox1*	96	4e-126
26	15/2	1	FJ477091.1	–	Gene *Hox1*	96	1e-127
27	40/1, 40/2, 40/3, 40/5	1	DQ249273.1	LTR Viva	BAC 631P8	89	8e-89
28	40/4	1	AY853252.1	DNA transpson Caspar	Telomeric region of chromosome 7 H	97	2e-80
29	12/2, 103/1, 103/2, 103/3, 103/4, 103/5	2	AY853252.1	LTR Wham	Telomeric region of chromosome 7 H	86	5e-87
30	12/3	1	AY853252.1	LTR Wham	Telomeric region of chromosome 7 H	93	8e-104
31	25/1, 25/2, 25/3, 25/4, 25/5	3	DQ386610.1	–	Protein kinase ABC1063 gene	73	2e-13
32	24/1	2	EF067844.1	DNA transposon Mandrake	Locus *vrs1*; gene *Hox1*	91	5e-26
33	56/1, 56/2, 56/4, 56/5, 57/2, 57/5	2	AY485643.1	–	BAC 615K1, undescribed region of chromosome 4	73	2e-19
34	57/3	2	AK330913.1	Gene	SET1 A17, cDNA	85	1e-17
35	34/2, 35/1	2	AB058924.1	Intron	Gene *HvPKABA1*	81	4e-34

Table A3 (*Continued*)

No.	AFLP product No. clone/clones	Algorithms BLASTN	Accession	Types of sequences	Description	Max identity (%)	*E*-value
36	34/1, 34/3, 35/2, 35/3, 35/4, 35/5	NF	–	–	–	–	–
37	34/4	1	AF078801.1	LTR Cereba	Degenerated retrotransposon Cereba	96	3e-65
38	34/5	1	AF078801.1	LTR Cereba	Degenerated retrotransposon Cereba	97	7e-62
39	84/2, 84/3, 84/4, 85/2, 85/3, 85/4, 85/5	2	AF028263.1	–	RAPD marker IAS-pHc7-1	73	1e-18
40	84/1	1	AF474071.1	LTR BAGY-2	BAC 745c13	95	6e-115
41	85/1	1	AK252490.1	Gene	FLbaf155o08, cDNA	87	8e-54
42	64/1	NF	–	–	–	–	–
43	65/1	2	EU660903.1	–	BAC 1548L13: cytosolic acetyl-CoA carboxylase (Acc-2) and putative amino acid permease genes	82	4e-55
44	64/5	1	Z14059.1	Gene *18S rRNA*	Mitochondrial DNA	100	6e-79
45	64/4	NF	–	–	–	–	–
46	65/5	2	AY054376.1	LTR Sukkula	Retrotransposon Sokkula	84	1e-30
47	66/1, 66/3, 66/4, 66/5, 67/1	2	EU660899.1	–	BAC 1100E3: plastid acetyl-CoA carboxylase (Acc-1) gene	71	1e-30
48	67/3	2	EU660899.1	–	BAC 1100E3: plastid acetyl-CoA carboxylase (Acc-1) gene	71	1e-39
49	26/1, 27/1, 98/3, 98/4, 98/5	1	EF67844.1	*Hox1* flanking	Locus *vrs1*; gene *Hox1*	94	3e-66
50	99/1	NF	–	–	–	–	–
51	99/2	NF	–	–	–	–	–
52	91/2, 91/4, 91/5,	2	FJ477093.1	–	Genes *Rym4* and *MCT-1*	79	5e-27
53	90/3, 91/3	2	AF446141.1	–	LZ-NBS-LRR class RGA, NBS-LRR class RGA, HCBT-like putative defense response protein, and putative alliin lyase genes	81	5e-21
54	90/2	1	DQ445253.1	LTR Sokkula	Gene *CBF9;* retrotransposon Sabrina T8A-1; retrotransposon Sukkula T8A-1; retrotransposon BARE T8A-1 SoloLTR	94	1e-70
55	30/2, 30/4	1	FJ477091.1	–	Gene *Hox-1*	96	6e-33
56	30/1, 31/2, 31/3	1	EF067844.1	*Hox1* flanking	Locus *vrs1*; gene *Hox1*	94	1e-64
57	1/3, 2/1, 2/2, 2/3, 42/3, 42/4, 42/5	1	AJ270050.1	LTR Gyp3	Retrotransposon Gyp3, partial sequence	79	8e-55
58	33/4	NF	–	–	–	–	–
59	33/5	NF	–	–	–	–	–
60	32/5	NF	–	–	–	–	–
61	97/4, 97/5	1	EF067844.1	LTR Wham	Locus *vrs1*; gene *Hox1*	84	4e-25
62	97/3	NF	–	–	–	–	–
63	97/2	1	DQ900687.1	LTR BAGY-2	BAC 455J22	94	4e-40
64	97/1	3	CT009735.1	LTR Wilma		68	9e-50
65	78/1	NF	–	–	–	–	–
66	79/1, 79/3	NF	–	–	–	–	–
67	78/2	NF	–	–	–	–	–
68	78//3	2	AF459639.1	Non-LTR Miuse	BAC 116F2 and gene 115G1	82	2e-25

Table A3 (*Continued*)

No.	AFLP product No. clone/clones	Algorithms BLASTN	Accession	Types of sequences	Description	Max identity (%)	*E*-value
69	20/1, 20/2, 20/4, 21/1	1	AF509779.1	LTR Sabrina	BAC RSB416 with gene *Rpg1*	95	3e-41
70	20/3	NF	–	–	–	–	–
71	28/2, 29/5, 81/1, 81/5	NF	–	–	–	–	–
72	28/1, 81/4	1	DQ249273.1	LTR Wham	BAC 631P8	93	7e-21
73	28/3, 29/2, 29/3, 81/2	NF	–	–	–	–	–
74	29/4	2	DQ900685.1	LINE Karin	BAC 761F4	74	5e-12
75	81/3	NF	–	–	–	–	–
76	71/1, 101/2, 101/3, 102/3, 102/4, 102/5	2	AY268139.1	LTR Lolaog	BAC 184G9	79	1e-19
77	101/4	2	Y14573.1	LTR BAGY-1	Chromosome 4 H	95	2e-23
78	71/2	2	AM932681.1	LTR Wham	BAC TA3B63B13, chromosome 3B	83	8e-29
79	11/2, 39/1, 39/2, 39/3	NF	–	–	–	–	–
80	39/4	NF	–	–	–	–	–
81	94/1, 94/2, 94/4, 94/5	1	FJ477093.1	–	Genes *Rym4* and *MCT-1*	91	8e-27
82	94/3	2	AF509775.1	LTR Sokkula	BAC RSB409A with gene *Rpg1*	89	3e-55
83	83/2, 83/3	NF	–	–	–	–	–
84	83/1, 83/5	NF	–	–	–	–	–
85	8/1, 8/2, 8/3, 8/4	2	AP009567.1	Gene flanking	Genes for putative iron-deficiency specific 4 protein and putative ethylene-responsive transcription factor	89	3e-40
86	8/5	2	AP009567.1	Gene flanking	Genes for putative iron-deficiency specific 4 protein and putative ethylene-responsive transcription factor	88	1e-38
87	21/2, 21/3, 21/5, 49/3	NF	–	–	–	–	–
88	21/4	2	AP009567.1	Gene flanking	Genes for putative iron-deficiency specific 4 protein and putative ethylene-responsive transcription factor	88	6e-58
89	49/1	3	DQ175896.1	-	Clone 28-34 A-LB	78	5e-21
90	49/2	NF	–	–	–	–	–
91	21/1	2	FJ266026.1	–	BAC 194G19	80	3e-11
92	22/3	1	EU812563.1	*RGA1* flanking	Genes *Rpg4*; *RGA1*; *Rpg5*; *PP2C*; *ADF3*	95	9e-30
93	22/2, 48/1, 48/2, 48/3	1	EU812563.1	*RGA1* flanking	Genes *Rpg4*; *RGA1*; *Rpg5*; *PP2C*; *ADF3*	96	2e-31
94	48/5	1	EF067844.1	LTR Laura	Locus *vrs1*; gene *Hox1*	94	1e-12
95	23/2, 23/4	1	DQ900687.1	LTR Maximus	BAC 455J22	90	5e-59
96	23/1, 23/5, 43/1, 43/2, 43/3, 43/4	1	AY643844.1	LTR Maximus	BAC 799C8; BAC 122A5	97	3e-51
97	43/5	1	EF067844.1	*Hox1* flanking	Locus *vrs1*; gene *Hox1*	90	3e-56
98	17/1, 17/2, 17/3, 17/4, 17/5, 18/1	1	EF067844.1	LTR Maximus	Locus *vrs1*; gene *Hox1*	90	2e-84
99	38/4	1	AY643844.1	LTR Maximus	BAC 799C8; BAC122A5	98	3e-50
100	38/5		AY643844.1	LTR Maximus	BAC 799C8; BAC 122A5	99	7e-52
101	10/3, 10/4, 38/2, 38/3	1	AY643844.1	LTR Maximus	BAC 799C8; BAC 122A5	99	7e-52
102	10/1	1	AY643844.1	LTR Maximus	BAC 799C8; BAC 122A5	98	3e-50

Table A3 (*Continued*)

No.	AFLP product No. clone/clones	Algorithms BLASTN	Accession	Types of sequences	Description	Max identity (%)	*E*-value
103	10/2	1	AY643844.1	LTR Maximus	BAC 799C8; BAC 122A5	98	3e-50
104	88/2, 88/3, 88/4, 89/3	NF	–	–	–	–	–
105	89/1, 89/4	NF	–	–	–	–	–
106	89/2	NF	–	–	–	–	–
107	88/1	NF	–	–	–	–	–
108	89/5	NF	–	–	–	–	–
109	47/3, 95/1, 95/2, 95/3	NF	–	–	–	–	–
110	47/5	1	AY853252.1	LTR Wham	Telomeric region of chromosome 7 H	91	4e-54
111	47/2, 47/4	2	AB058924.1	Intron	Gene *HvPKABA1*	81	1e-23
112	47/1	NF	–	–	–	–	–
113	95/5	NF	–	–	–	–	–
114	95/4	1	EF067844.1	*Hox1* flanking	Locus *vrs1*; gene *Hox1*	93	9e-61

(1) Megablast, (2) Discontiguous megablast, (3) blastn; NF – not found. The products from 1 to 98 were obtained after MNU treatment, from 99 to 114 after gamma rays irradiation.

Table A4
The nucleotide positions flanking metG in the nontranscribed and transcribed DNA strand (based on [41,42] and the presented study).

Species	Analysed genes	DNA strand Nontranscribed/(sense)	Transcribed/(antisense)	Author
O. sativa	*PLA1* (Acc.:AB096259.1) *OsSAD1* (Acc.:AB110207.1) *OsAHP1* (Acc.:AB110206.2)	AG*A	TG*C	[42]
		AG*A	AG*C	
		GG*T	CG*A	
		AG*G	CG*G	
		TG*C	AG*T	
		GG*C	AG*G	
		GG*C	CG*C	
		TG*G	CG*T	
		GG*C	GG*T	
			GG*G	
G. max	*CLV1A* (Acc.:AF197946) *NARK* (Acc.:AY166655) *PPCK4* (Acc.:AY568714) *RHG1* (Acc.:AF506516) *RHG4* (Acc.:AF506518) *SACPD A* (Acc.:AY885234)	AG*G	AG*A	[41]
		AG*A	GG*C	
		AG*C	GG*T	
		GG*T	AG*G	
		GG*T	GG*C	
		CG*A	GG*A	
		GG*A	TG*G	
		AG*G	AG*G	
		AG*A	GG*A	
		GG*A	GG*G	
		GG*C	GG*T	
		GG*C	AG*T	
		GG*G	AG*A	
		GG*G	GG*A	
		AG*T	AG*T	
		GG*G	AG*A	
		CG*A	AG*A	
		TG*T	GG*A	
		GG*A	AG*A	
			GG*G	
			AG*A	
H. vulgare	*HvBRI1* (Acc.:AB088206)	AG*G		Presented study
		AG*C		
	HvEXPB1 (Acc.:AY351785)	GG*T	CG*C	Presented study
			GG*C	
Total		31	33	
%		48.4	51.6	

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42/3    AATTCAACTTCAGACTTGCTGAATTTGGCATAAAGCTGGTGCTCTCGAAGCTTCCCTAGG 60
1/3     AATTCAACTTCAGACTTGCTGAATTTGGCATAAAGCTGGTGCTCTCGAAGCTTCCCTAGG 60
2/1     AATTCAACTTCAGACTTGCTGAATTTGGCATAAAGCTGGTGCTCTCGAAGCTTCCCTAGG 60
42/5    AATTCAACTTCAGACTTGCTGAATTTGGCATAAAGCTGGTGCTCTCGAAGCTTCCCTAGG 60
42/4    AATTCAACTTCAGACTTGCTGAATTTGGCATAAAGCTGGTGCTCTCGAAGCTTCCCTAGG 60
2/3     AATTCAACTTCAGACTTGCTGAATTTGGCATAAAGCTGGTGCTCTCGAAGCTTCCCTAGG 60
2/2     AATTCAACTTCAGACTTGCTGAATTTGGCATAAAGCTGGTGCTCTCGAAGCTTCCCTAGG 60
        ************************************************************

42/3    ACCACCCTCAAGTGTTTCTCATGTTCTTCTTCAGACTTGTAGAAGACGAGTTTATCCTCG 120
1/3     ACCACCCTCAAGTGTTTCTCATGTTCTTCTTCAGACTTGTAGAAGACGAGTTTATCCTCG 120
2/1     ACCACCCTCAAGTGTTTCTCATGTTCTTCTTCAGACTTGTAGAAGACGAGTTTATCCTCG 120
42/5    ACCACCCTCAAGTGTTTCTCATGTTCTTCTTCAGACTTGTAGAAGACGAGTTTATCCTCG 120
42/4    ACCACCCTCAAGTGTTTCTCATGTTCTTCTTCAGACTTGTAGAAGACGAGTTTATCCTCG 120
2/3     ACCACCCTCAAGTGTTTCTCATGTTCTTCTTCAGACTTGTAGAAGACGAGTTTATCCTCG 120
2/2     ACCACCCTCAAGTGTTTCTCATGTTCTTCTTCAGACTTGTAGAAGACGAGTTTATCCTCG 120
        ************************************************************

42/3    ATGCAGAAAATGACAAACTTGTCCTGATACTCCATGAAGACTTTGTTCATGAGGTACATG 180
1/3     ATGCAGAAAATGACAAACTTGTCCAGATACTCCATGAAGACTTTGTTCATGAGGTACATG 180
2/1     ATGCAGAAAATGACAAACTTGTCCAGATACTCCATGAAGACTTTGTTCATGAGGTACATG 180
42/5    ATGCAGAAAATGACAAACTTGTCCAGATACTCCATGAAGACTTTGTTCATGAGGTACATG 180
42/4    ATGCAGAAAATGACAAACTTGTCCAGATACTCCATGAAGACTTTGTTCATGAGGTACATG 180
2/3     ATGCAGAAAATGACAAACTTGTCCAGATACTCCATGAAGACTTTGTTCATGAGGTACATG 180
2/2     ATGCAGAAAATGACAAACTTGTCCAGATACTCCATGAAGACTTTGTTCATGAGGTACATG 180
        ************************ ************************************

42/3    AAGTAGGCCATGTCATTAGTCAACCCAAATAACATGACCGTGTACTCATACAGGCCATAC 240
1/3     AAGTAGGCCATGTCATTAGTCAACCCAAATAACATGACCGTGTACTCATACAGGCCATAC 240
2/1     AAGTAGGCCATGTCATTAGTCAACCCAAATAACATGACCGTGTACTCATACAGGCCATAC 240
42/5    AAGTAGGCCATGTCATTAGTCAACCCAAATAACATGACCGTGTACTCATACAGGCCATAC 240
42/4    AAGTAGGCCATGTCATTAGTCAACCCAAATAACATGACCGTGTACTCATACAGGCCATAC 240
2/3     AAGTAGGCCATGTCATTAGTCAACCCAAATAACATGACCGTGTACTCATACAGGCCATAC 240
2/2     AAGCAGGCCATGTCATTAGTCAACCCAAGTAACATGACCGTGTACTCATACAGGCCATAC 240
        *** ************************ ******************************

42/3    CGGGTCATGAATGTCGTCTTTGGAATATCCTGGGGTCTGATCTTCAACTGATAGTACCCA 300
1/3     CGGGTCATGAATGTCGTCTTCGGAATATCCTGGGGTCTGATCTTCAACTGATAGTACCCA 300
2/1     CGGGTCATGAATGTCGTCTTTGGAATATCCTGGGGTCTGATCTTCAACTGATAGTACCCA 300
42/5    CGGGTCATGAATGTCGTCTTTGGAATATCCTGGGGTCTGATCTTCAACTGATAGTACCCA 300
42/4    CGGGTCATGAATGTCGTCTTTGGAATATCCTGGGGTCTGATCTTCAACTGATAGTACCCA 300
2/3     CGGGTCATGAATGTCGTCTTTGGAATATCCTGGGGTCTGATCTTCAACTGATAGTACCCA 300
2/2     CGGGTCATGGATGTCGTCTTTGGAATATCCTGGGGTCTGATCTTCAACTGATAGTACCCA 300
        ********* ********** ************************************

42/3    GACGGGAGCTCGATGTTGGAAAACACCTTGGCACCATTCAGT 342
1/3     GACGGGAGCTCGATGTTGGAAAACACCTTGGCACCATTCAGT 342
2/1     GACGGGAGCTCGATGTTGGAAAACACCTTGGCACCATTCAGT 342
42/5    GACGGGAGCTCGATGTTGGAAAACACCTTGGCACCATTCAGT 342
42/4    GACGGGAGCTCGATGTTGGAAAACACCTTGGCACCATTCAGT 342
2/3     GACGGGAGCTCGATGTTGGAAAACACCTTGGCACCATTCAGT 342
2/2     GACGGGAGCTCGATGTTGGAAAACACCTTGGCACCATTCAGT 342
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LEGEND:

nucleotides recognized by restriction enzymes
nucleotides used during selective amplification

Fig. A1. The alignment between the sequences of seven clones derived from the same AFLP band showing sequence homogeneity. The stars indicate the same nucleotides in all sequences.

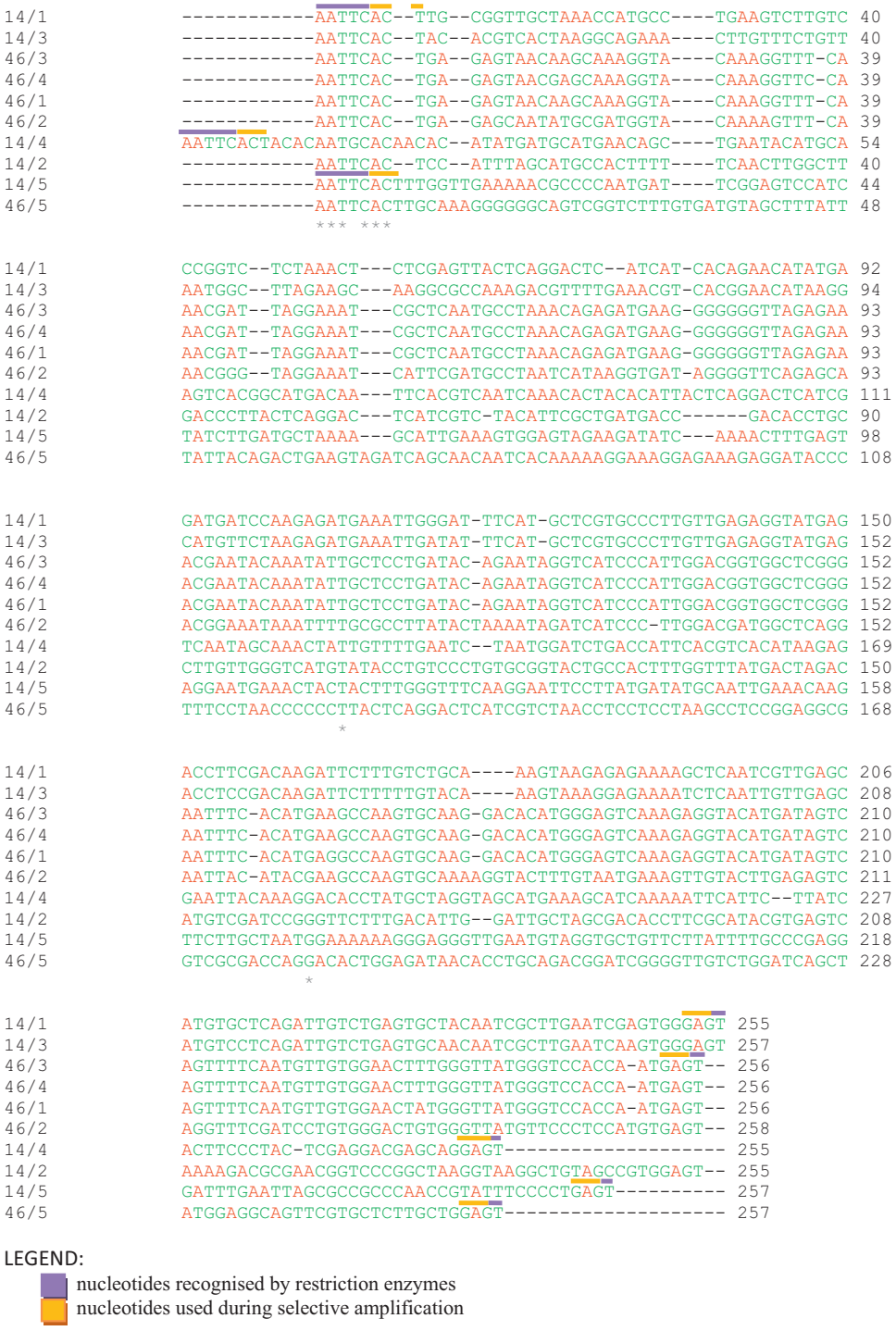

Fig. A2. The alignment between the sequences of ten clones derived from the same AFLP band showing sequence heterogeneity. The stars indicate the same nucleotides in all sequences.

References

[1] B.S. Ahloowalia, M. Maluszynski, K. Nichterlein, Global impact of mutation-derived varieties, Euphytica 135 (2004) 187–204.
[2] M. Maluszynski, I. Szarejko, Induced mutations in the green and gene revolutions, in: R. Tuberosa, R.L. Phillips, M. Gale (Eds.), In the Wake of the Double Helix: From the Green Revolution to the Gene Revolution, Avenue Media, Bologna, 2005, pp. 403–425.
[3] M. Maluszynski, I. Szarejko, J. Maluszynska, Mutation techniques, in: Encyclopedia of Applied Plant Sciences, Elsevier Science, Oxford, 2003, pp. 186–201.
[4] L.J. Stadler, Mutations in barley induced by X-rays and radium, Science 68 (1928) 186–187.
[5] L.J. Stadler, Genetic effects of X-rays in maize, Proc. Natl. Acad. Sci. USA 14 (1928) 69–75.
[6] A.P. Breen, J.A. Murphy, Reactions of oxyl radicals with DNA, Free Radic. Biol. Med. 18 (1995) 1033–1077.
[7] T. Parman, M.J. Wiley, P.G. Wells, Free radical-mediated oxidative DNA damage in the mechanism of thalidomide teratogenicity, Nat. Med. 5 (1999) 582–585.
[8] C. von Sonntag, The chemistry of free-radical-mediated DNA damage, Basic Life Sci. 58 (1991) 287–317.
[9] A. Kodym, R. Afza, Physical and chemical mutagenesis, in: E. Grotewold (Ed.), Plant Functional Genomics, Humana Press, Totowa, New Yersey, 2003, pp. 189–203.
[10] S.S. Wallace, Enzymatic processing of radiation-induced free radical damage in DNA, Radiat. Res. 150 (1998) 60–79.
[11] A. Kumar, V. Pottiboyina, M.D. Sevilla, Hydroxyl radical ($OH^{\bullet}$) reaction with guanine in an aqueous environment: a DFT study, J. Phys. Chem. B 115 (2011) 15129–15137.
[12] S.S. Wallace, Biological consequences of free radical-damaged DNA bases, Free Radic. Biol. Med. 33 (2002) 1–14.
[13] J. Wu, M. Morimyo, E. Hongo, T. Higashi, M. Okamoto, A. Kawano, Y. Ohmachi, Radiation-induced germline mutations detected by a direct comparison of parents and first-generation offspring DNA sequences containing SNPs, Mutat. Res. 596 (2006) 1–11.
[14] M. Gulston, J. Fulford, T. Jenner, C. de Lara, P. O'Neill, Clustered DNA damage induced by gamma radiation in human fibroblasts (HF19), hamster (V79–4) cells and plasmid DNA is revealed as Fpg and Nth sensitive sites, Nucleic Acids Res. 30 (2002) 3464–3472.
[15] I. Szarejko, M. Maluszynski, Translocations and inversions in barley induced by fast neutrons and N-nitroso-N-methylurea (MNUA), Barley Genet. Newsletter 10 (1980) 67–69.
[16] B.W. Shirley, S. Hanley, H.M. Goodman, Effects of ionizing radiation on a plant genome: analysis of two Arabidopsis transparent testa mutations, Plant Cell 4 (1992) 333–347.
[17] R.K. Sachs, L.R. Hlatky, B.J. Trask, Radiation-produced chromosome aberrations, Trends Genet. 16 (2000) 143–146.
[18] N. Shikazono, C. Suzuki, S. Kitamura, H. Watanabe, S. Tano, A. Tanaka, Analysis of mutations induced by carbon ions in *Arabidopsis thaliana*, J. Exp. Bot. 56 (2005) 587–596.
[19] A. Tanaka, N. Shikazono, Y. Hase, Studies on biological effects of ion beams on lethality, molecular nature of mutation, mutation rate, and spectrum of mutation phenotype for mutation breeding in higher plants, J. Radiat. Res. (Tokyo) 51 (2010) 223–233.
[20] L.V. Konevega, V.L. Kalinin, Mutagenic effects of gamma rays and incorporated 8-3H-purines on extracellular lambda phage: influence of *mutY* and *mutM* host mutations, Mutat. Res. 459 (2000) 229–235.
[21] A.F. Fuciarelly, B.J. Wegher, W.F. Blakely, M. Dizdaroglu, Yields of radiation-induced base products in DNA: effects of DNA conformation and gassing conditions, Int. J. Radiat. Biol. 58 (1990) 397–415.
[22] S. Shibutani, M. Takeshita, A.P. Grollman, Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8oxodG, Nature 349 (1991) 431–434.
[23] G.K. Kuipers, B.J. Slotman, H.A. Poldervaart, C.A. Reitsma-Wijker, M.V.M. Lafleur, The influence of combined Fpg- and MutY-deficiency on the spontaneous and g-radiation-induced mutation spectrum in the *lacZα* gene of M13mp10, Mutat. Res. 461 (2000) 189–195.
[24] J.C. Klein, M.J. Bleeker, C.P. Saris, H.C.P.F. Roelen, H.F. Brugghe, H. van den Elst, G.A. van der Marel, J.H. van Boom, J.G. Westra, E. Kriek, A.J.M. Berns, Repair and replication of plasmids with site-specific 8-oxodG and 8-AAFdG residues in normal and repair-deficient human cells, Nucleic Acids Res. 20 (1992) 4437–4443.
[25] M. Moriya, Single-stranded shuttle plasmid for mutagenesis studies in mammalian cells: 8-xoguanine in DNA induces targeted G:C to T:A transversions in simian kidney cells, Proc. Natl. Acad. Sci. USA 90 (1993) 1122–1126.
[26] K. Naito, M. Kusaba, N. Shikazono, T. Tanaka, T. Tanisaka, M. Nishimura, Transmissible and nontransmissible mutations induced by irradiating *Arabidopsis thalina* pollen with gamma-rays and carbon ions, Genetics 169 (2005) 881–889.
[27] Y. Sato, K. Shirasawa, Y. Takahashi, M. Nishimura, T. Nishio, Mutant selection from progeny of gamma-ray-irradiated rice by DNA heteroduplex cleavage using *Brassica* petiole extract, Breeding Sci. 56 (2006) 179–183.
[28] R. Morita, M. Kusaba, S. Iida, H. Yamaguchi, T. Nishio, M. Nishimura, Molecular characterization of mutations induced by gamma irradiation in rice, Genes Genet. Syst. 84 (2009) 361–370.
[29] R. Yoshihara, Y. Hase, R. Sato, K. Takimoto, I. Narumi, Mutational effects of different LET radiations in rpsL transgenic Arabidopsis, Int. J. Radiat. Biol. 86 (2010) 125–131.
[30] D.B. Ludlum, The properties of 7-methylguanine-containing templates for ribonucleic acid polymerase, J. Biol. Chem. 245 (1970) 477–482.
[31] K. Kleibl, Molecular mechanisms of adaptive response to alkylating agents in *Escherichia coli* and some remarks on O^{6}-methylguanine DNA-methyltransferase in other organisms, Mutat. Res. 512 (2002) 67–84.
[32] A. Klungland, L. Fairbairn, A.J. Watson, G.P. Margison, E. Seeberg, Expression of the *E. coli* 3-methyladenine DNA glycosylase I gene in mammalian cells reduces the toxic and mutagenic effects of methylating agents, EMBO J. 11 (1992) 4439–4444.
[33] K.K. Richardson, F.C. Richardson, R.M. Croby, J.A. Swenberg, T.R. Skopek, DNA base changes and alkylation following *in vivo* exposure of *Escherichia coli* to N-methyl-N-nitrosourea or N-ethyl-N-nitrosourea, Proc. Natl. Acad. Sci. USA 84 (1986) 344–348.
[34] N.M. Mironov, F. Bleicher, G. Martel-Planche, R. Montesano, Nonrandom distribution of O^{6}-methylguanine in *H-ras* gene sequence from DNA modified with N-methyl-N-nitrosourea, Mutat. Res. 288 (1993) 197–205.
[35] J.G. Jansen, G.R. Mohn, H. Vrieling, C.M.M. van Teijlingen, P.H.M. Lohman, A.A. van Zeeland, Molecular analysis of *hprt* gene mutations in skin fibroblast of rats exposed *in vivo* to N-methyl-N- nitrosourea or N-ethyl-N-nitrosourea, Cancer Res. 54 (1994) 2478–2485.
[36] J.J. Warren, L.J. Forsberg, L.S. Beese, The structural basis for the mutagenicity of O(6)-methyl-guanine lesions, Proc. Natl. Acad. Sci. USA 103 (2006) 19701–19706.
[37] P.J. Abbott, R. Saffhill, DNA synthesis with methylated poly(dC-dG) templates. Evidence for a competitive nature to miscoding by O^{6}-methylguanine, Biochim. Biophys. Acta 562 (1979) 51–61.
[38] P. Karran, M. Bignami, Self-destruction and tolerance in resistance of mammalian cells to alkylation damage, Nucleic Acids Res. 20 (1992) 2933–2940.
[39] H. Feitsma, A. Akay, E. Cuppen, Alkylation damage causes MMR-dependent chromosomal instability in vertebrate embryos, Nucleic Acids Res. 36 (2008) 4047–4056.
[40] L.A. Rapoport, N.N. Zoz, S.I. Makarova, T.V. Salnikova, Supermutagenes, Nauka, Moscow, 1966, pp. 272.
[41] J.L. Cooper, B.J. Till, R.G. Laport, M.C. Darlow, J.M. Kleffner, A. Jamai, T. El-Mellouki, S. Liu, R. Ritchie, N. Nielsen, D.K. Bilyeu, K. Meksem, L. Comai, S. Henikoff, TILLING to detect induced mutations in soybean, BMC Plant Biol. 8 (9) (2008), http://dx.doi.org/10.1186/1471-2229-8-9.
[42] T. Suzuki, M. Eiguchi, T. Kumamaru, H. Satoh, H. Matsusaka, K. Morigchi, Y. Nagato, N. Kurata, MNU-induced mutant pools and high performance TILLING enable finding of any gene mutation in rice, Mol. Genet. Genomics 279 (2008) 213–223.
[43] C.M. McCallum, L. Comai, E.A. Greene, S. Henikoff, Targeting induced local lesions in genomes (TILLING) for plant functional genomics, Plant Physiol. 123 (2000) 439–442.
[44] P. Vos, R. Hogers, M. Bieeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, M. Zabeau, AFLP: a new technique for DNA fingerprinting, Nucleic Acids Res. 21 (1995) 4407–4414.
[45] D. Rigola, J. van Oeveren, A. Janssen, A. Bonné, H. Schneiders, H.J. van der Poel, N.J. van Orsouw, R.C. Hogers, M.T. de Both, M.J. van Eijk, High-throughput detection of induced mutations and natural variation using KeyPoint technology, PLoS ONE 4 (2009) e4761, http://dx.doi.org/10.1371/journal.pone 0004761.
[46] H. Tsai, T. Howell, R. Nitcher, V. Missirian, B. Watson, K.J. Ngo, M. Lieberman, J. Fass, C. Uauy, R.K. Tran, A.A. Khan, V. Filkov, T.H. Tai, J. Dubcovsky, L. Comai, Discovery of rare mutations in populations: TILLING by sequencing, Plant Physiol. 156 (2011) 1257–1268.
[47] J. Monson-Miller, D.C. Sanchez-Mendez, J. Fass, I.M. Henry, T.H. Tai, L. Comai, Reference genome-independent assessment of mutation density using restriction enzyme-phased sequencing, BMC Genomics 14 (13) (2012) 72.
[48] J.J. Doyle, J.L. Doyle, A rapid DNA isolation procedure for small quantities of fresh leaf tissue, Phytochem. Bull. 19 (1987) 11–15.
[49] M. Kwasniewski, I. Szarejko, Molecular cloning and characterization of β-expansin gene related to root hair formation in barley, Plant Physiol. 141 (2006) 1149–1158.
[50] D. Gruszka, I. Szarejko, M. Maluszynski, New allele of *HvBRI1* gene encoding brassinosteroid receptor in barley, J. Appl. Genet. 52 (2011) 257–268.
[51] A.M. Waterhouse, J.B. Procter, D.M. Martin, M. Clamp, G.J. Barton, Jalview Version 2 – a multiple sequence alignment editor and analysis workbench, Bioinformatics 25 (2009) 1189–1191.
[52] N.E. Taylor, E.A. Greene, PARSESNP: a tool for the analysis of nucleotide polymorphisms, Nucleic Acids Res. 31 (2003) 3808–3811.
[53] P.C. Ng, S. Henikoff, SIFT: predicting amino acid changes that affect protein function, Nucleic Acids Res. 31 (2003) 3812–3814.
[54] FAO/IAEA, Manual on Mutation Breeding, International Atomic Energy Agency, Vienna, 1995.
[55] O. Frydenberg, J. Sandfaer, The vitality, productivity, and radiosensitivities of recurrently irradiated barley populations, in: The Use of Induced Mutations in Plant Breeding, Report of the FAO/IAEA Technical Meeting held in Rome, FAO/IAEA, Pergamon Press, Oxford, 1965, pp. 175–183.
[56] M. Maluszynski, I. Szarejko, C.R. Bhatia, K. Nichterlein, P.J.L. Lagoda, Mutation techniques, in: E. Guimares, S. Ceccarelli, E. Weltzein, P.G. Rajendran (Eds.), Plant Breeding Book, FAO, Rome, 2009, pp. 159–194.
[57] A.J. Slade, S.I. Fuerstenberg, D. Loeffler, M.N. Steine, D. Facciotti, A reverse genetic, nontransgenic approach to wheat crop improvement by TILLING, Nat. Biotechnol. 23 (2005) 75–81.

[58] C. Uauy, F. Paraiso, P. Colasuonno, R.K. Tran, H. Tsai, S. Berardi, L. Comai, J. Dubcovsky, A modified TILLING approach to detect induced mutations in tetraploid and hexaploid wheat, BMC Plant Biol. 9 (115) (2009), http://dx.doi.org/10.1186/1471-2229-9-115.
[59] D.G. Caldwell, N. McCallum, P. Shaw, G.J. Muehlbauer, D.F. Marshall, R. Waugh, A structured mutant population for forward and reverse genetics in barley (*Hordeum vulgare* L.), Plant J. 40 (2004) 143–150.
[60] V. Talame, R. Bovina, M.C. Sanguineti, R. Tuberosa, U. Lundqvist, S. Salvi, TILLMore, a resource for the discovery of chemically induced mutants in barley, Plant Biotechnol. J. 6 (2008) 477–485.
[61] S. Lababidi, N. Mejlhede, S.K. Rasmussen, G. Backes, W. Al-Said, M. Baum, A. Jahoor, Identification of barley mutants in the cultivar 'Lux' at the *Dhn* loci through TILLING, Plant Breeding 128 (2009) 332–336.
[62] J. Perry, A. Brachmann, T. Welham, A. Binder, M. Charpentier, M. Groth, K. Haage, K. Markmann, T.L. Wang, M. Parniske, TILLING in *Lotus japonicus* identified large allelic series for symbiosis genes and revealed a bias in functionally defective ethyl methanesulfonate alleles toward glycine replacements, Plant Physiol. 151 (2009) 1281–1291.
[63] E.A. Greene, C.A. Codomo, N.E. Taylor, J.G. Henikoff, B.J. Till, S.H. Reynolds, L.C. Enns, C. Burtner, J.E. Johnson, A.R. Odden, L. Comai, S. Henikoff, Spectrum of chemically induced mutations from a large-scale reverse-genetic screen in *Arabidopsis*, Genetics 164 (2003) 731–740.
[64] B.J. Till, S.H. Reynolds, E.A. Greene, C.A. Codomo, L.C. Enns, J.E. Johnson, C. Burtner, A.R. Odden, K. Young, N.E. Taylor, J.G. Henikoff, L. Comai, S. Henikoff, Large-scale discovery of induced point mutations with high-throughput TILLING, Genome Res. 13 (2003) 524–530.
[65] Y. Okabe, E. Asamizu, T. Saito, C. Matsukura, T. Ariizumi, T. Mizoguchi, H. Ezura, Tomato TILLING technology: development of a reverse genetics tool for the efficient isolation of mutants from Micro-Tom mutant libraries, Plant Cell Physiol. 52 (2011) 1994–2005.
[66] E. Wienholds, F. van Eeden, M. Kosters, J. Mudde, R.H. Plasterk, E. Cuppen, Efficient target-selected mutagenesis in zebrafish, Genome Res. 13 (2003) 2700–2707.
[67] B.J. Till, S.H. Reynolds, C. Weil, N. Springer, C. Burtner, K. Young, E. Bowers, C.A. Codomo, L.C. Enns, A.R. Odden, E.A. Greene, L. Comai, S. Henikoff, Discovery of induced point mutations in maize genes by TILLING, BMC Plant Biol. 4 (12) (2004), http://dx.doi.org/10.1186/1471-2229-4-12.
[68] E. Himelblau, E.J. Gilchrist, K. Buono, C. Bizzell, L. Mentzer, R. Vogelzang, T. Osborn, R.M. Amasino, I.A. Parkin, G.W. Haughn, Forward and reverse genetics of rapid-cycling *Brassica oleracea*, Theor. Appl. Genet. 118 (2009) 953–961.
[69] S. Minoia, A. Petrozza, O. D'Onofrio, F. Piron, G. Mosca, G. Sozio, F. Cellini, A. Bendahmane, F. Carriero, A new mutant genetic resource for tomato crop improvement by TILLING technology, BMC Res. Notes 3 (69) (2010), http://dx.doi.org/10.1186/1756-0500-3-69.
[70] M. Agarwal, N. Shrivastava, H. Padh, Advances in molecular marker techniques and their applications in plant sciences, Plant Cell Rep. 27 (2008) 617–631.
[71] R.W. Trigiano, M.C. Scott, G. Caetano-Anolles, Genetic signatures from amplification profiles characterize DNA mutation in somatic and radiation-induced sports of chrysanthemum, J. Am. Soc. Hort. Sci. 123 (1998) 642–646.
[72] G. Caetano-Anolles, High genome-wide mutation rates in vegetatively propagated bermudagrass, Mol. Ecol. 8 (1999) 1211–1221.
[73] C. Polanco, M.L. Ruiz, AFLP analysis of somaclonal variation in *Arabidopsis thaliana* regenerated plants, Plant Sci. 162 (2002) 817–824.
[74] M. Matthes, R. Singh, S.C. Cheah, A. Karp, Variation in oil palm (*Elaeis guineensis* Jacq.) tissue culture-derived regenerants revealed by AFLPs with methylation-sensitive enzymes, Theory Appl. Genet. 102 (2001) 971–979.
[75] J. Chen, R.J. Henny, P.S. Devanand, C.T. Chao, AFLP analysis of nephthytis (*Syngonium podophyllum* Schott) selected from somaclonal variants, Plant Cell Rep. 24 (2006) 743–752.
[76] D.A. Steinmacher, N.G. Krohn, A.C. Dantas, V.M. Stefenon, C.R. Clement, M.P. Guerra, Somatic embryogenesis in peach palm using the thin cell layer technique: induction, morpho- histological aspects and AFLP analysis of somaclonal variation, Ann. Bot. 100 (2007) 699–709.
[77] P. Schellenbaum, V. Mohler, G. Wenzel, B. Walter, Variation in DNA methylation patterns of grapevine somaclones (*Vitis vinifera* L.), BMC Plant Biol. 8 (78) (2008), http://dx.doi.org/10.1186/1471-2229-8-78.
[78] P.T. Bednarek, R. Orłowska, R.M.D. Koebner, J. Zimny, Quantification of the tissue-culture induced variation in barley (*Hordeum vulgare* L.), BMC Plant Biol. 7 (10) (2007), http://dx.doi.org/10.1186/1471-2229-7-10.
[79] T. Wicker, R. Guyot, N. Yahiaoui, B. Keller, CACTA transposons in Triticeae. A diverse family of high-copy repetitive elements, Plant Physiol. 132 (2003) 52–63.
[80] D. Schulte, T.J. Close, A. Graner, P. Langridge, T. Matsumoto, G. Muehlbauer, K. Sato, A.H. Schulman, R. Waugh, R.P. Wise, N. Stein, The International Barley Sequencing Consortium – at the threshold of efficient access to the barley genome, Plant Physiol. 149 (2009) 142–147.
[81] T. Wicker, S. Taudien, A. Houben, B. Keller, A. Graner, M. Platzer, N. Stein, A whole-genome snapshot of 454 sequences exposes the composition of the barley genome and provides evidence for parallel evolution of genome size in wheat and barley, Plant J. 59 (2009) 712–722.
[82] N. Rostoks, Y.J. Park, W. Ramakrishna, J. Ma, A. Druka, B.A. Shiloff, P.J. SanMiguel, Z. Jiang, R. Brueggeman, D. Sandhu, K. Gil, J.L. Bennetzen, A. Kleinhofs, Genomic sequencing reveals gene content, genomic organization, and recombination relationships in barley, Funct. Integr. Genomics 2 (2002) 51–59.
[83] Y.J. Park, A. Dixit, J.W. Yoo, J. Bennetzen, Further evidence of microcolinearity between barley and rice genomes at orthologous regions, Mol. Cells 17 (2004) 492–502.
[84] K.F.X. Mayer, S. Taudien, M. Martis, H. Simkova, P. Suchankova, H. Gundlach, T. Wicker, A. Petzold, M. Felder, B. Steuernagel, U. Scholz, A. Graner, M. Platzer, J. Dolezel, N. Stein, Gene content and virtual gene order of barley chromosome 1H, Plant Physiol. 151 (2009) 496–505.
[85] A.H. Schulman, R. Kalendar, A movable feast: diverse retrotransposons and their contribution to barley genome dynamics, Cytogenet. Genome Res. 110 (2005) 598–605.
[86] K.F. Mayer, M. Martis, P.E. Hedley, H. Simková, H. Liu, J.A. Morris, B. Steuernagel, S. Taudien, S. Roessner, H. Gundlach, M. Kubaláková, P. Suchánková, F. Murat, M. Felder, T. Nussbaumer, A. Graner, J. Salse, T. Endo, H. Sakai, T. Tanaka, T. Itoh, K. Sato, M. Platzer, T. Matsumoto, U. Scholz, J. Dolezel, R. Waugh, N. Stein, Unlocking the barley genome by chromosomal and comparative genomics, Plant Cell 23 (2011) 1249–1263.
[87] M. Ipek, A. Ipek, P.W. Simon, Sequence homology of polymorphic AFLP markers in garlic (*Allium sativum* L.), Genome 49 (2006) 1246–1255.
[88] D.M. Althoff, M.A. Gitzendanner, K.A. Segraves, The utility of amplified fragment length polymorphisms in phylogenetics: a comparison of homology within and between genomes, Syst. Biol. 56 (2007) 477–484.
[89] A. Caballero, H. Quesada, Homoplasy and distribution of AFLP fragments: an analysis *in silico* of the genome of different species, Mol. Biol. Evol. 27 (2009) 1139–1151.
[90] X. Vekemans, T. Beauwens, M. Lemaire, I. Roldán-Ruiz, Data from amplified fragment length polymorphism (AFLP) markers show indication of size homoplasy and of a relationship between degree of homoplasy and fragment size, Mol. Ecol. 11 (2002) 139–151.
[91] M. Paris, B. Bonnes, G.F. Ficetola, B.N. Poncet, L. Despres, Amplified fragment length homoplasy: *in silico* analysis for model and non-model species, BMC Genomics 11 (287) (2010), http://dx.doi.org/10.1186/1471-2164-11-287.
[92] K. Meksem, E. Ruben, D. Hyten, K. Triwitayakorn, D.A. Lightfoot, Conversion of AFLP bands into high-throughput DNA markers, Mol. Genet. Genomics 265 (2001) 207–214.
[93] S.M. Mechanda, B.R. Baum, D.A. Johnson, J.T. Arnason, Sequence assessment of comigrating AFLP bands in *Echinacea* – implications for comparative biological studies, Genome 47 (2004) 15–25.
[94] H.M. Meudt, A.C. Clarke, Almost forgotten or latest practice? AFLP applications, analyses and advance, Trends Plant Sci. 12 (2006) 106–117.