Gamma-rays induced genome wide stable mutations in cowpea deciphered through whole genome sequencing

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Abstract

Gamma-rays are the most widely exploited physical mutagen in plant mutation breeding. They are known to be involved in development of more than 60% of global cowpea mutant varieties. Nevertheless, the characteristics of genome wide mutations induced by gamma-rays has not been studied in cowpea (*Vigna unguiculata* (L.) Walp.). In the present investigation, mutations elicited by gamma-rays in three cowpea mutant lines in M_6 generation were characterized through whole-genome sequencing. Gamma-rays induced a relatively higher frequency (91.1%) of single base substitutions (SBSs) with an average transition to transversion ratio (Ti/Tv) of 2.82. A > G transitions including its complementary T > C transitions predominated the transition mutations, while all the four types of transversion mutations were detected with almost equal frequencies (5.6−7.4%). Indels constituted about 9% of the total induced variation wherein small insertions (5.3%) were relatively more prominent than small deletions (3.7%). Among the indels, single base indels and in particular those involving A/T bases showed preponderance, albeit indels of up to five bases were detected in low proportions. Distributed across all 11 chromosomes, only a fraction of SBSs (19.3%) and indels (12.7%) potentially altered the encoded amino-acids/peptides. The inherent mutation rate induced by gamma-rays in cowpea was observed to be in the order of 1.76×10^{-6} per base pair. Therefore, gamma-rays with greater tendency to induce SBSs and to a lesser extent indels could be efficiently and effectively exploited in cowpea mutation breeding.

Introduction

The utility of ionizing radiations like x-rays and gamma-rays for induced mutagenesis and genetic improvement of crops gained momentum subsequent to the demonstration by Stadler (1928) of the capability of X-rays to induce heritable changes or mutations in barley and corn. Since then, ionizing radiations in combination with other chemical and physical mutagens have been widely exploited in mutation breeding for crop improvement. The success of mutation breeding is perceivable from the large number of mutant varieties (3,402) released worldwide (https://mvd.iaea.org/). Globally, about 77% (2642) of the released varieties have been developed through physical mutagens, of which 1782 varieties have been primarily through gamma-rays. In India, an almost similar trend is reflected as 72% of the mutant varieties released for commercial cultivation involved use of gamma-rays directly or indirectly (https://mvd.iaea.org/). Gamma-ray is an ionizing radiation that brings about the mutations either due to their direct effect (ionization and excitation) or through their indirect effect involving radiolysis of water and formation of the reactive oxygen species or free radicals resulting in disruption of chemical bonds of biomolecules like nucleic acids (Lagoda 2012; Mba et al. 2012). Gamma-rays induce DNA damages through alterations in the bases leading to base substitutions, or cause breaks in single strands (SSBs) or both the strands of the DNA (DSBs) resulting primarily in insertion or deletion mutations (Lomax et al. 2013). DNA ligases mend most of the SSBs while the repair of DSBs via the homologous recombination (HR) or nonhomologous end joining (NHEJ) is error prone with consequences of base substitutions, insertions, deletions or chromosomal rearrangements (Kitamura et al. 2022). The gamma-rays are characterized with low energy deposition per unit travel path length (0.2 keV/μm) often referred to as the linear energy transfer (LET) in comparison to other physical mutagens like heavy-ion beams (Kazama et al. 2017). Conversely, gamma-rays being chargeless in nature are accorded with higher penetration power. The mutagenic effectiveness (frequency of mutations per unit irradiation dose) and efficiency (frequency of

mutations per unit percent lethality, injury or sterility) of gamma-rays have been widely studied in a range of crops (Ambavane et al. 2015; Dube et al. 2011; Goyal et al. 2020; Julia et al. 2018; Raina et al. 2022). The mutagenic effectiveness largely affected by the LET is low for gamma-rays as against that of heavy-ions (Yamaguchi et al. 2009). However, the mutagenic efficiency of gamma-rays vis-à-vis that of heavy-ions is widely debated with contrasting reports and is largely dependent on the nature of material used for irradiation. In *Arabidopsis*, as revealed by Yoshihara et al. (2010) C-ion irradiation exhibited induction of large indels (> 3 base pairs), while gamma-rays predominantly induced small indels (≤ 2 base pairs). Studies aimed at understanding the molecular effects of the physical mutagens at whole-genome level is increasingly being pursued with the advent of rapid technological developments in the field of genomics such as the next-generation sequencing (NGS). NGS has been used to unravel the molecular effects of spontaneous mutations (Weng et al. 2019) as well as those induced by various physical mutagens like gamma-rays (Hase et al. 2020), X-rays (Nath et al. 2020), ion-beams (Hase et al. 2018; Kazama et al. 2017), proton beams (Lee et al. 2021), and neutrons (Belfield et al. 2012). This coupled with superior bioinformatic algorithms provide a holistic approach in novel understanding of the mutagenic effects of various physical mutagens in different genomic backgrounds. For instance, the preponderance of small and large deletion mutations (2−4 kb) has been traditionally ascribed to be induced by gamma-rays (Morita et al. 2009) and fast-neutrons (Li et al. 2001), respectively. Contrarily, single base substitutions (SBS) and single base deletions, rather than large deletions, predominated the aftermath of exposure to physical mutagens in different crops (Belfield et al. 2012; Li et al. 2016a, 2017; Shirasawa et al. 2016). In addition, ion beams like C-ion beam predominantly induce SBSs and small indels (< 100 bp) in *Arabidopsis*, while Ar-ion beams induce chromosomal rearrangements or large deletions (≥ 100 bp) (Kazama et al. 2017). However, the effects of these radiations cannot be generalized across the species and need to be assessed for each of the mutagens.

The insight into molecular effect of gamma-rays irradiation at the whole genome level is lacking in cowpea and hence, the present study was aimed at characterizing the mutation effects of gamma-rays (250 Gy) irradiation at a dose of 90 Gy min^{-1} by *de novo* whole genome sequencing of the parent ('CPD103'/'CDS') and whole genome resequencing of its three mutants. Since the short-read sequencing technology has a limited ability to evaluate the frequency and detailed structure of the structural variations (SV) (Hase et al. 2018), the study excluded SVs while comparing the characteristics of mutations.

Materials And Methods

Material

From our previous study, three cowpea mutants were identified following irradiation of cowpea cultivar 'CPD103' (henceforth referred as 'CDS') with 250 Gy of gamma-rays at a dose rate of 90 Gy/m in gamma chamber GC5000 having ^{60}Co as its source. These include cowpea aphid borne mosaic disease resistant ('CDR'), large seed size ('LSS') and small seed size ('SSS') mutants that were identified in M_2/M_3 generation in 2014−2015. The three mutants were subjected to progeny testing up to M_6 generation and the stability and true breeding nature of these mutants were confirmed. The selection of dose was based on our previous studies and successful outcomes in cowpea mutation breeding at our institute (Souframanien et al. 2020).

The parent along with the three mutants (Fig. 1) were chosen in this study for whole genome resequencing (WGR).

Dna Extraction And Quality Control

Genomic DNA was extracted in duplicate from young leaves using Qiagen DNeasy Plant Mini kit. Briefly, young leaves were homogenized in TOMY Microsmash homogenizer and 400µl of preheated AP1 Buffer was added; vortexed at high speed for 5 minutes. 20 µl of 20 mg/ml RNase (MP Biomedicals) was added and incubated at 65°C for 25 minutes. Further 130 µl of P3 buffer was added and incubated on ice for 5minutes.The lysate was centrifuged to remove debris. The supernatant mixed with 1.5 volume of AW1 buffer was loaded onto Qiagen DNeasy column and further steps were followed as per manufacturer's guidelines. DNA was eluted with 50 µl of 10mM Tris-Cl, pH 8.0. The quantification and quality of the genomic DNA was assessed using Nanodrop2000 (Thermo Scientific, USA), Qubit (Thermo Scientific, USA) and agarose gel electrophoresis.

Illumina Library Preparation And Sequencing

Library construction was carried out at M/s Genotypic Technology, Bengaluru, using the Illumina compatible NEXTflex Rapid DNA sequencing Bundle (BIOO Scientific, Inc. U.S.A.) according to manufacturer's instructions. 500 ng of Qubit quantified DNA was sheared using Covaris S220 sonicator (Covaris, Inc. USA) to generate specific fragments with size range of 200–250 bp. The fragments were purified using HiPrep cleanup beads. Purified fragments were end-repaired, adenylated and ligated to Illumina multiplex barcode adaptors as per NEXTflex Rapid DNA sequencing bundle kit protocol. Adapter-ligated DNA was purified using HiPrep clean up beads. Resultant fragments were amplified for 4 cycles of PCR using Illumina-compatible primers provided in the NEXTflex Rapid DNA sequencing Bundle. Final PCR product (sequencing library) was purified with HiPrep cleanup beads followed by library quality control. Sequencing library was quantified by Qubit fluorometer (Thermo Fisher Scientific, MA, USA) and the fragment size distribution was analyzed on Agilent 2200 Tape Station. The libraries were equimolar-normalized and pooled for multiplexed, paired-end sequencing which was carried out on HiSeq XTen, for 150 cycles, following manufacturer's instructions.

Nanopore Library Preparation And Sequencing

The DNA (1.5µg) were end-repaired (NEBnext ultra II end repair kit, New England Biolabs, MA, USA) and purified using 1x AmPure beads (Beckmann Coulter, USA). Adapter ligation (AMX) was performed at RT (20 C) for 20 minutes using NEB Quick T4 DNA Ligase (New England Biolabs, MA, USA). The reaction mixture was purified using 0.6X AmPure beads (Beckmann Coulter, USA) and sequencing library was eluted in 15 µl of elution buffer provided in the ligation sequencing kit (SQK-LSK109) from Oxford Nanopore Technology. Sequencing was performed on GridION X5 (Oxford Nanopore Technologies, Oxford, UK) using SpotON flow cell R9.4 (FLO-MIN106) in a 48 hrs sequencing protocol to obtain long reads of 'CDS' with 20-fold depth. Nanopore raw reads (*'fast5'* format) were base called (*'fastq5'* format) using Guppy basecaller3-v2.3.4 (https://bio.tools/guppy).

Bioinformatic Analysis

The hybrid genome assembly for cowpea sample 'CDS' was performed using MaSuRCA4-v3.4.2 assembler (Zimin et al. 2013). The assembled genome was processed for repeat region masking using RepeatModeller5-v2.0 (Flynn et al. 2020) and RepeatMasker6-v4.0.6 (Smit et al. 2015). The draft genome assembly validation was carried out using BUSCO7-v3.0.2 (Felipe et al. 2015) tool to retrieve percentage of complete genes annotated. For the purpose of variant analysis, the raw Illumina reads from cowpea samples ('CDR', 'LSS', 'SSS') were trimmed for removal of adapter sequences and low-quality reads (reads with quality score(Q) < 30 and length < 20 bases) using Trim galore12-v to generate good quality processed reads (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). The good quality reads were then mapped against draft genome (used as reference) in Bowtie213-v2.2.5 (Langmead and Salzberg 2012) tool and variants (SNPs and Indels) were predicted using Picard14-v1.102 (https://broadinstitute.github.io/picard/) and GATK15-v4.1.4.1 (McKenna et al. 2010) pipeline. The predicted variants were annotated using snpEFF16-v3.3h tool (Cingolani et al. 2012).

Data Availability

The Illumina NGS data files have been submitted to NCBI Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/sra) and are accessible under accession number PRJNA858559.

Results

Whole genome resequencing of parent and mutants

Hybrid (Illumina and nanopore) whole genome sequencing approach was carried out to assemble cowpea parental genome ('CDS') and was used as the reference genome for variant calling. A total of ~241 million Illumina short reads along with ~7.7 million nanopore long reads were generated. The sequencing coverage for Illumina data was ~120x and for nanopore data was ~20x. The hybrid genome assembly for cowpea sample 'CDS' resulted in haploid genome of size ~325 MB which covers ~87% of the haploid genome estimated by KmerGenie program. The assembled genome was processed for repeat region masking to generate final draft assembly genome. The assembled draft genome was validated by read utilization and identification of single copy genes to ensure the completeness of the draft assembly (haploid). About 94% percent of read utilization and 93.4% of BUSCO completeness (C: 93.4% (S: 92.0%, D: 1.4%), F: 1.5%, M: 5.1%, n: 5366) confirmed good draft assembly (Fig.2). A total of 188 million high-quality (Q>30 and length >20 bases) processed Illumina reads was generated for the three mutants 'CDR', 'LSS' and 'SSS' and about 96% - 98% of the reads mapped to the assembled reference genome with a sequencing coverage of 20x-30x. The mapping statistics are given in Table 1.

Variant identification

Variant analysis was performed using good quality Illumina reads from cowpea samples ('CDR', 'LSS', 'SSS') which were mapped against the assembled draft genome (used as reference) to identify the

variants. Alignment data were used for predicting variants in GATK HaplotypeCaller pipeline. The variants identified were of 3 categories (SNPs, insertions and deletions). A total of 2491 variants were identified from the three mutants and are summarized in Table 2. Of these, 2466 (98.9%) variants were observed to be in homozygous state. Single base substitutions (SBS) constituting 91.1% (2270) predominated the type of mutant variants induced genome wide by gamma-rays, followed by small insertions (133, 5.3%) and deletions (88, 3.5%).

Single base substitutions (SBSs):

Gamma-rays induced a relatively large frequency of SBSs, in the range of 579 (90.3% in 'LSS') to 925 (91.4% in 'SSS') of all the mutations in each of the mutants with an average of 756.67 per mutant (Table 2). Both types of substitutions (transition of purine to purine or pyrimidine to pyrimidine and transversion of purines to pyrimidines or vice versa) were induced in all the three mutants. Gamma-rays were found to induce a relatively larger proportion of transitions in comparison to transversions (Table 3). The percent induced transitions in each of the mutants ranged from 71.8% ('SSS') to 76.2% ('CDR') with an average of 73.8%. The mutant-wise yield of transition to transversion ratio (Ti/Tv) ranged from 2.54 ('LSS') to 3.21 ('CDR') with an average of 2.82 (Table 3). Depending on the type of transition or transversion, the mutant variants were classified into the following 12 categories: A>G, A>T, A>C, G>A, G>T, G>C, T>A, T>G, T>C, C>A, C>T and C>G. In all the three mutants, the predominant kind of transitions were A>G and T>C transitions (Fig 3) accounting for 22.02% and 20.09% of SBSs, respectively, while the G>A and C>T transitions constituted 16.12% and 15.59% of SBSs, respectively. Among the transversions, C>A, A>C, G>T and A>T showed preponderance (average of 28.17%) over G>C, C>G, T>G and T>A transversions (average of 21.33%).

Small deletions

Gamma-rays were also found to induce small deletions at low frequency in all the three mutants. Small deletions accounted for 3.6% of the total induced variation (Table 2). On an average, 29.33 deletions (Table 2) were observed in each of the mutant and 77.27% of the deletions induced were of single base deletions (Table 4). On analysis of the single base deletions, it was found that the majority of deletions involved A or T bases in comparison to G or C bases. Fifty-two of the 68 single base deletions induced across the mutants involved A/T bases, while only 16 were of C/G bases. Multiple bases (2 - 5) were deleted in 22.73% of induced deletion mutations. About 5 – 10 deletions were observed in each of the mutants involving two or more bases. In 'CDR' and 'LSS' mutants, small deletions involving maximum of 3 bases were detected, while in 'SSS' mutant deletions up to 5 bases were also observed.

Small insertions

In addition to deletions, gamma-rays also elicited small insertions at comparatively higher frequency than that of small deletions. These small insertions constituted 5.3% of the total induced variations (Table 2). In each of the mutants, 44.33 small insertions were induced on an average and predominantly involved single bases (88.72% of total insertions) (Table 4). As that of deletions, single base insertions primarily involved A or T bases (87) as against C or G bases (31). Insertions involving multiple bases (2 – 5) varied from four to seven in each of the mutants. Small insertions up to 3 bases were induced in 'LSS' and 'SSS' mutants, while a maximum of 5 bases were inserted in 'CDR' mutant.

Annotation of induced mutations

The various mutations induced by gamma-rays in all the three mutants vis-à-vis the parent were annotated. A total of 1049 (46.21%) of the 2270 SBSs induced by gamma-rays occurred in the intergenic regions, 656 (28.90%) in exons (inclusive of 1.32% in start/stop codons and 0.22% in splice regions), and 565 (24.89%) in introns (Table 5). Of the 656 SBS mutations induced in the exons, 437 (19.3% of SBSs) resulted in non-synonymous mutations (altered proteins or peptides) while the remaining 219 (9.65% of SBSs) exonic mutations represented synonymous mutations unaffecting the amino acid sequences of the proteins they coded for. Most of the deletions and insertions were also observed to occur in the intergenic (67.1% of deletions and 57.9% of insertions) and intronic regions (22.7% of deletions and 27.8% of insertions). Only a small proportion of deletions and insertions were induced in the exonic (9.1% and 13.5%, respectively) and splice sites (1.1% and 0.8%, respectively). Six of the eight deletions and 17 of the 18 insertions induced in the exons were resultant of frameshift mutations due to deletion or insertion of single or two bases. The remaining mutations in exons were the resultant of codon deletion or codon insertion involving three bases.

Chromosomal distribution of induced mutations

To investigate the chromosomal influence on mutational induction of gamma-rays, the average of all the mutations induced in each of the chromosomes of the three mutants were considered to estimate the average length of chromosomes at which the mutations were induced. Apparently, the SBSs and indels were observed to be scattered across all the 11 chromosomes without any ostensible restriction (Fig 4). However, the estimated lengths at which the mutations were induced were skewed towards the higher side on chromosome 10 with mutations occurring every 284 kb and towards the lower side on chromosome 7 with mutations frequenting every 834 kb (The lengths of chromosomes are as per the *Vigna unguiculata* reference genome assembly ASM411807v2 in NCBI). In other words, the number of mutations induced per unit Mb of chromosomes varied from 1.20 in chromosome 7 to 3.62 in chromosome 10 at an average of 1.80±0.21 per Mb of each chromosome (Table 6).

Discussion

The genetic variability in any species could be ascribed to the mutations that have accumulated over several years and the natural selections acting upon these mutations leading to discriminatory perpetuation of those mutations imparting adaptive advantage. The extremely slow mutation frequency under natural conditions could be accelerated by induced mutagenesis, which then could be exploited by the breeders for genetic improvement of crops. Mutagenic effects of physical and chemical mutagens are varying and thus have profound influence on the type of resultant mutation. For instance, the chemical mutagens are known to induce point mutations emanating from transitions and transversions of nucleotide bases consequent to altered base pairing. Contrastingly, the physical mutagens predominantly produce deletions through single strand and double strand breaks and often produce complex DNA damages. Many of the DNA damages are corrected by the inbuilt DNA repair and proofreading mechanisms that strive to maintain the integrity of the genome for ensuring faithful inheritance of the traits over the generations. However, these processes are often incomplete or error prone and often result in the perpetuation of the mutations in the offsprings (Eccles et al. 2011; Esnault et al. 2010; Mahaney et al. 2009; Mladenov and Iliakis 2011). Additionally, the physical

mutagens are also very effective in producing single base substitutions and small indels (Yang et al. 2019). Therefore, it becomes imperative to comprehend the mutagenic machinery of these physical mutagens for enhancing their efficiency.

In the present study, the effect of gamma-rays in inducing stable mutations were investigated through whole genome sequencing (WGS) of the parent and three of its mutants in M_6 generation. The variants generated through physical mutagens in mutant *per se* could be characterized even at single base resolution by employing WGS (Li et al. 2017; McCallum et al. 2000), and it bestows researchers with a potent tool for examining the whole genome spectrum of induced mutations and efficient mapping of such causal mutations. The assembly completeness for gene contents rather than the assembly contiguity plays an influential role in genomic data interpretation and data quality assessment (Felipe et al. 2015). The completeness of genome assembly and gene annotation in the present study was gauged using Benchmarking Universal Single-Copy Orthologs (BUSCO) tool. BUSCO is prominently being referred to as a standard for assessing the completeness of genome assembly and is used by UniProt (The UniProt Consortium 2019) and the United States National Centre for Biotechnology Information (NCBI) (Sayers et al. 2021). BUSCO was able to retrieve 93.4% of complete genes which bolstered the completeness of the assembled genome.

The WGS analysis of three cowpea mutants in the present study that were derived from gamma ray irradiation indicated that SBSs (91.1%) were more predominantly induced in comparison to small insertions (5.3%) and deletions (3.6%). The utility of WGS in identifying induced variants in nuclear (Du et al. 2022; Hase et al, 2020; Kazama et al. 2017; Li et al. 2016a) and cytoplasmic genomes (Zheng et al. 2020a) has been demonstrated in number of crops for different physical mutagens and could fairly augment our knowhow of the nature of induced casual mutations in plants. The preponderance of SBSs as against indels induced by gamma-rays in the present study is in conformity with that of similar reports in other crops (Du et al. 2022; Li et al. 2016a; Yang et al. 2019). Gamma-rays have been observed to predominantly induce SBSs as opposed to deletions ($\geq$ 2 bp) by carbon ions (Hase et al. 2020). Within the SBSs, gamma-rays were observed to induce a relatively higher proportion of transitions ($\bar{x}$=73.82%) as against transversions ($\bar{x}$=26.17%) with an average Ti/Tv of 2.82 in cowpea. This is close to that reported in *Arabidopsis* (Ti/Tv = 2.73) for spontaneous mutations (Ossowski et al. 2010) and in rice (Ti/Tv = 2.78) for Ar ion-beam irradiation (Zheng et al. 2020b). However, the Ti/Tv was reportedly lower for gamma-rays (up to 1.68), fast neutrons (up to 1.4), EMS (up to 1.83), and carbon ion beams (up to 2.22) in other crops like rice and *Arabidopsis* (Li et al. 2016a; Li et al. 2016b; Mohd-Yusoff et al. 2015; Yang et al. 2019). It could be inferred that carbon ion beams and fast neutrons induced relatively higher transversions resulting in lower Ti/Tv ratio. The lower Ti/Tv (1.68) of gamma-rays in rice could be attributed to the difference in the mutant generation (M_2 vs M_6 in this study) taken up for WGS. Advanced mutant generations probably accumulate or inherit much of the transition mutations as could be comprehended from the higher Ti/Tv ratios in the present study as well as in the spontaneous mutation accumulation lines (Ossowski et al. 2010).

In all the three mutants, the predominant kind of transition was A > G (including its complementary T > C transitions) (Table 3, Fig. 3) accounting for 42.11% of SBSs, while the G > A (including its complementary C > T transitions) constituted 31.71% of SBSs. Among the DNA bases, guanine and adenine, have high oxidation potentials (vs. normal hydrogen electrode) of 1.29 and 1.42, respectively, and guanine among the bases is the most susceptible to oxidation (Kino et al. 2020). If oxidation were to be considered for A > G or G > A

transitions, it would be obvious to expect higher frequency of G > A transition in comparison to A > G transition as has been reported in various studies involving ion-beams and fast neutrons (Belfield et al. 2012; Du et al. 2017; Li et al. 2016b). The A > G transitions though not spontaneous, are quite thermodynamically probable under irradiation and may occur through the processes of hydrolytic deamination of adenine, followed by oxidation of hypoxanthine intermediate and finally by the animation of xanthine intermediate to guanine (Tolosa et al. 2019). Interestingly, all the four types of transversions were frequently detected in the present study, albeit, the G > T (along with its complementary C > A) transversions were observed to be most frequent (7.4%) and G > C (along with its complementary C > G) transversions the least frequent (5.6%) among the SBSs (Table 3). Li et al. (2016a) also reported dominance of all four types of transversions following gamma-rays induced mutagenesis in rice. There was no congruency in the predominance of any particular type of transversion reported by various researchers. Prevalence of higher A > T (Du et al. 2017; Hase et al. 2018), G > T (Belfield et al. 2012)d > G (Li et al. 2016a) transversions were reported in *Arabidopsis* and rice following carbon-ion beam, fast neutron, and gamma ray irradiations, while in each case G > C transversions were observed to be less prevalent.

Indels were referred to as insertions/deletions of size 1 bp – 10 bp, while that of size > 50 bp were defined as structural variants (Alkan et al. 2011). The short reads (2 x 150 bp) generated through NGS are effective in discerning SBSs and small indels, but are known to detect structural variations only sporadically (Du et al. 2022). Therefore, only SBSs and small indels were primarily concentrated in this study. Gamma-rays were found to induce indels at low frequencies compared to SBSs. Small insertions and small deletions accounted for 5.3% and 3.7% of the total induced variation, respectively, and cumulatively were responsible for about 9% of the total induced variation. The excess of insertions over the deletions though not a common phenomenon perceived in various studies, is reiterated by the fact that insertions in highly conserved proteins undergo less purifying selection than do deletions and consequently excess of insertions were discerned in all the examined taxa involving plants, animals and fungi (Ajawatanawong and Baldauf 2013). The prevalence of higher insertion mutations was also observed when dry seeds rather than seedlings were used as irradiation material (Hase et al. 2018) as is the case in the present context. The ratio of indels to SBSs were observed to be around 1:10, which is slightly higher than that of earlier reports wherein up to 1:8 have been obtained (Zheng et al. 2020b), presumably due to differences in depth of sequencing and the genomic content. Li et al. (2016a) in their study observed that in rice the gamma-rays induced more SBSs and comparatively less indels that were predominated with single base insertions and deletions. Likewise, single base indels were also more frequent than large indels (≥ 2 bp) in *Arabidopsis* mutants induced by other physical mutagens like carbon-ion beams (Du et al. 2017). Majority of the indels in the present study involved single bases (88.72% of insertions and 77.27% of deletions) primarily A/T bases as against G/C bases. Insertions and deletions of more than two bases and up to five bases were induced at very low frequencies (Table 4). Yoshihara et al. (2010, 2013) also observed the high prevalence of transitions and small deletions of less than 2 bp among the mutations induced by gamma-rays. Du et al. (2022) also reported an almost similar trend induced by gamma-rays in *Arabidopsis* wherein small indels ranging from one bp to ten bp primarily involved single bases with adenine followed by thymine as the most prevalent types.

Both in the case of SBSs and indels, most of the mutations were in homozygous state as would be expected in M_6 generation with average homozygosity of 99% for SBSs and 98.6% for indels. Both SBSs and indels were

found distributed across all 11 chromosomes without any apparent preference although numerically a greater number of mutations were found on chromosome 10 as compared to others (Fig. 4, Table 6). On an average, 68.8 ± 7.6 SBSs and 6.7 ± 0.6 indels were induced per chromosome with a mean length of 43.0 ± 2 Mbp resulting in an average of $1.6 \pm 0.2 \times 10^{-6}$ SBSs and $0.16 \pm 0.01 \times 10^{-6}$ indels per base pair (1 SBS every 678.1 ± 59.3 kbp and 1 indel every 6900 ± 666.0 kbp). The mutation rate (1.78×10^{-6}) induced in the present study was about 7.8x the mutation rate induced by gamma-rays in rice ($2.31 \pm 1.5 \times 10^{-7}$, Li et al. 2019) and about 5x that of carbon-ion beam (3.37×10^{-7}, Du et al. 2017) and fast neutrons (3.6×10^{-7}, Belfield et al. 2012) in *Arabidopsis* and could be ascribed to the differences in the genomes, mutant generation, and nature of the mutagen involved. Additionally, mutants in M_6 generation were used in the present study, and, hence, may not represent the true picture of mutations induced in the M_1 generation. Many of the M_1 mutations (leading to gamete unviability, large deletions/insertions, corrective actions of DNA-repair machineries) would perish over the generations (Kazama et al. 2017; Naito et al. 2005) and only inherent DNA mutations accumulated until M_6 generations were captured. The mutations were found located in the intronic, intergenic, coding, or regulatory regions. Most of the SBSs and the indels were concentrated in the intergenic regions rather than the exonic regions. About 46.2% of the SBSs, 67.0% of deletions and 57.9% of the insertions were spotted in the intergenic regions. Likewise, the abundance of induced mutations in the intergenic region was also reiterated in rice following ion-beam irradiation (Zheng et al. 2020b). Only 28.9% of the SBSs and 12.7% of indels were found to affect the protein coding exons (including start/stop codons and splice site regions). This is in concurrence to the reports of Yang et al. (2019) who studied the effect of gamma-rays irradiation in rice and reported that 25% of the SBSs and 13.24% of the indels prevailed in the exonic regions. Du et al. (2017) also observed that 25% of the mutations induced by carbon-ion beam irradiation in *Arabidopsis* occurred in exons. Only a fraction of 19.3% of SBSs and 12.7% of the indels could potentially alter the encoded amino-acids (non-synonymous mutations) or the encoded peptides (frame-shift mutations or splice site variants) or the functionality of the proteins (start/stop codon mutations).

Hence, the WGS of mutants give a holistic mutation profile of the mutations induced by the physical mutagen and helps in better understanding of the nature and type of mutations induced by gamma-rays. It could be concluded that gamma-rays primarily induce SBSs and small indels (≤ 5 bp) among the stably inherited mutations; bring out more of A > G transitions and all types of transversions along with others in cowpea. These insights aid in the proper selection of mutagen for inducing a desirable type of mutation and for improving mutagenic efficiency.

Declarations

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Tables

Sample Name	Draft Assembly Genomes	Total Processed Reads	Total Mapped Reads	% Mapped Reads
CDR	CDS	71722549	69434600	96.81
LSS	CDS	53351651	52284618	98.00
SSS	CDS	63750894	62648004	98.27

Table 1 Mapping statistics of high-quality processed reads of three mutants (CDR, LSS, SSS) against the CDS (parent) genome

Table 2 Single base substitution (SBS), deletion and insertion mutations identified in three mutants of cowpea

Mutant	Total variants		SBSs		Deletions		Insertions	
	Homo	Hetero	Homo	Hetero	Homo	Hetero	Homo	Hetero
CDR	828	10	756	10	30	0	42	0
LSS	638	3	578	1	28	1	32	1
SSS	1000	12	914	11	28	1	58	0
Total (%)	2491 (100%)		2270 (91.1%)		88 (3.6%)		133 (5.3%)	
Average	830.33		756.67		29.33		44.33	
SE	107.2		100.0		0.33		7.31	

Homo: homozygous; Hetero: heterozygous

Table 3 Transition and transversion single base substitutions (SBSs) identified in three cowpea mutants (M_6)

Type of SBS			Mutant	Total	Mean	% (total)
	CDR	LSS	SSS			
Transitions						
A>G	174	136	190	500	166.67	22.02
T>C	157	111	188	456	152.00	20.09
G>A	133	90	143	366	122.00	16.12
C>T	120	91	143	354	118.00	15.59
Ti (total)	584	428	664	1676	558.67	73.82
	(76.2%)	(73.9%)	(71.8%)	(73.8%)		
Transversions						
C>A	31	18	33	82	27.33	3.61
G>T	28	21	37	86	28.67	3.79
A>C	30	24	31	85	28.33	3.75
T>G	18	14	35	67	22.33	2.95
A>T	20	23	42	85	28.33	3.74
T>A	17	16	30	63	21.00	2.78
G>C	19	16	26	61	20.33	2.69
C>G	19	19	27	65	21.67	2.86
Tv (total)	182	151	261	594	198	26.17
Ti/Tv	3.21	2.83	2.54	2.82	2.82	
TOTAL SBS	766	579	925	2270	756.67	

Table 4 Indel mutations identified in three cowpea mutants

Indels	CDR		LSS		SSS		Mean	% insertion/% deletion
	Base	Frequency	Base	Frequency	Base	Frequency		
Deletion:1 base (SBD)	A	10	A	12	A	8	10.00	
	C	6	C	2	C	1	3.00	
	G	2	G	1	G	4	2.33	
	T	7	T	9	T	6	7.33	
Total SBD		25		24		19	22.67	77.27%
Deletion: ≥ 2 bases	AA	1	AC	1	AC	1		
	AC	1	CC	1	AT	2		
	GC	1	TA	2	CC	1		
	TT	1	TCG	1	CT	1		
	TAA	1			TC	1		
					GAC	1		
					CAA	1		
					TTT	1		
					TATCC	1		
Total (≥ 2 bases)		5		5		10	6.67	22.73%
Total deletions		30		29		29	29.34	
Insertion: 1 base (SBI)	A	14	A	15	A	14		
	C	4	C	2	C	8		
	G	5	G	4	G	8		
	T	15	T	8	T	21		
Total SBI		38		29		51	39.33	88.72%
Insertion: ≥ 2 bases	AT	2	TT	2	AA	1		
	AAG	1	AAG	1	AT	1		
	GTGAC	1	AAA	1	CT	1		
					AG	1		
					TA	1		
					TTT	1		
					TGG	1		
Total (≥ 2 bases)		4		4		7	5.00	11.28%
Total insertions		42		33		58	44.33	
Total indels		72		62		87	73.67	

Table 5 Annotation of induced mutations in three cowpea mutants in M_6 generation

Annotation	CDR	LSS	SSS	Mean	Percent
SBS	766	579	925	756.67	
Intergenic	367	264	418	349.67	46.21
Intron	176	148	241	188.33	24.89
Non-synonymous coding	138	104	160	134.00	17.71
Splice site acceptor	0	0	2	0.67	0.09
Splice site donor	1	1	1	1.00	0.13
Start lost	1	0	1	0.67	0.09
Stop gained	9	6	8	7.67	1.01
Stop lost	3	1	1	1.67	0.22
Synonymous coding	71	55	93	73.00	9.65
Indels	72	62	87	73.67	
Deletions	30	29	29	29.33	
Intergenic	19	18	22	19.67	67.06
Intronic	7	8	5	6.67	22.74
Frameshift	4	1	1	2.00	6.82
Splice site acceptor	0	1	0	0.33	1.13
Codon deletion	0	1	1	0.67	2.28
Insertion	42	33	58	44.33	
Intergenic	26	22	29	25.67	57.91
Intronic	11	6	20	12.33	27.81
Frameshift	5	4	8	5.67	12.79
Splice site acceptor	0	0	1	0.33	0.74
Codon change + codon insertion	0	1	0	0.33	0.74

Table 6 Chromosome-wise mutations (SBSs and indels) induced in three gamma rays induced cowpea mutants (M_6)

C. No.	C. length (Mb)*	SBSs			SBSs/C	kb/SBS	Indels			Indels/C	Mb/ Indels	kb/ mutation	Mutations/ Mb
		CDR	LSS	SSS			CDR	LSS	SSS				
1	42.13	93	33	50	58.67	718.08	4	1	7	4.00	10.54	672.25	1.49
2	33.91	60	32	65	52.33	648.00	6	8	5	6.33	5.36	578.80	1.73
3	65.29	53	58	114	75.00	870.53	9	5	5	6.33	10.31	802.78	1.25
4	42.73	50	38	56	48.00	890.21	5	0	10	5.00	8.55	806.22	1.24
5	48.75	76	45	70	63.67	765.67	8	10	9	9.00	5.42	670.84	1.49
6	34.46	124	49	56	76.33	451.46	9	5	2	5.33	6.47	421.99	2.37
7	40.88	33	34	63	43.33	943.46	3	7	7	5.67	7.21	834.29	1.20
8	38.36	58	36	59	51.00	752.16	6	6	11	7.67	5.00	653.83	1.53
9	43.93	37	89	118	81.33	540.15	3	7	7	5.67	7.75	504.94	1.98
10	41.33	118	97	187	134.00	308.43	11	8	15	11.33	3.65	284.39	3.62
11	41.68	64	68	87	73.00	570.96	8	5	9	7.33	5.69	518.86	1.93
Mean	43.04	69.63	52.63	84.09	68.78	678.10	6.55	5.63	7.91	6.70	6.90	613.56	1.80
SE	2.55	9.15	6.92	12.44	7.58	59.29	0.80	0.90	1.05	0.62	0.66	51.96	0.21

C.No.: chromosome number, C.length: chromosome length, SBSs: single base substitutions, SBSs/C: single base substitutions per chromosome, kb/SBSs: kilo base pairs per single base substitution, indels/C: indels per chromosome, Mb/indel: million base pairs per indel; kb/mutation: kilo base pairs per mutation, mutations/Mb: mutations per million base pairs, *:as per *Vigna unguiculata* reference genome assembly ASM411807v2 in NCBI

Figures

Figure 1

Cowpea mosaic disease resistant (CDR), small seed size (SSS) and large seed size (LSS) mutants with its parent (CDS); figures in parenthesis indicate 100 seed weight
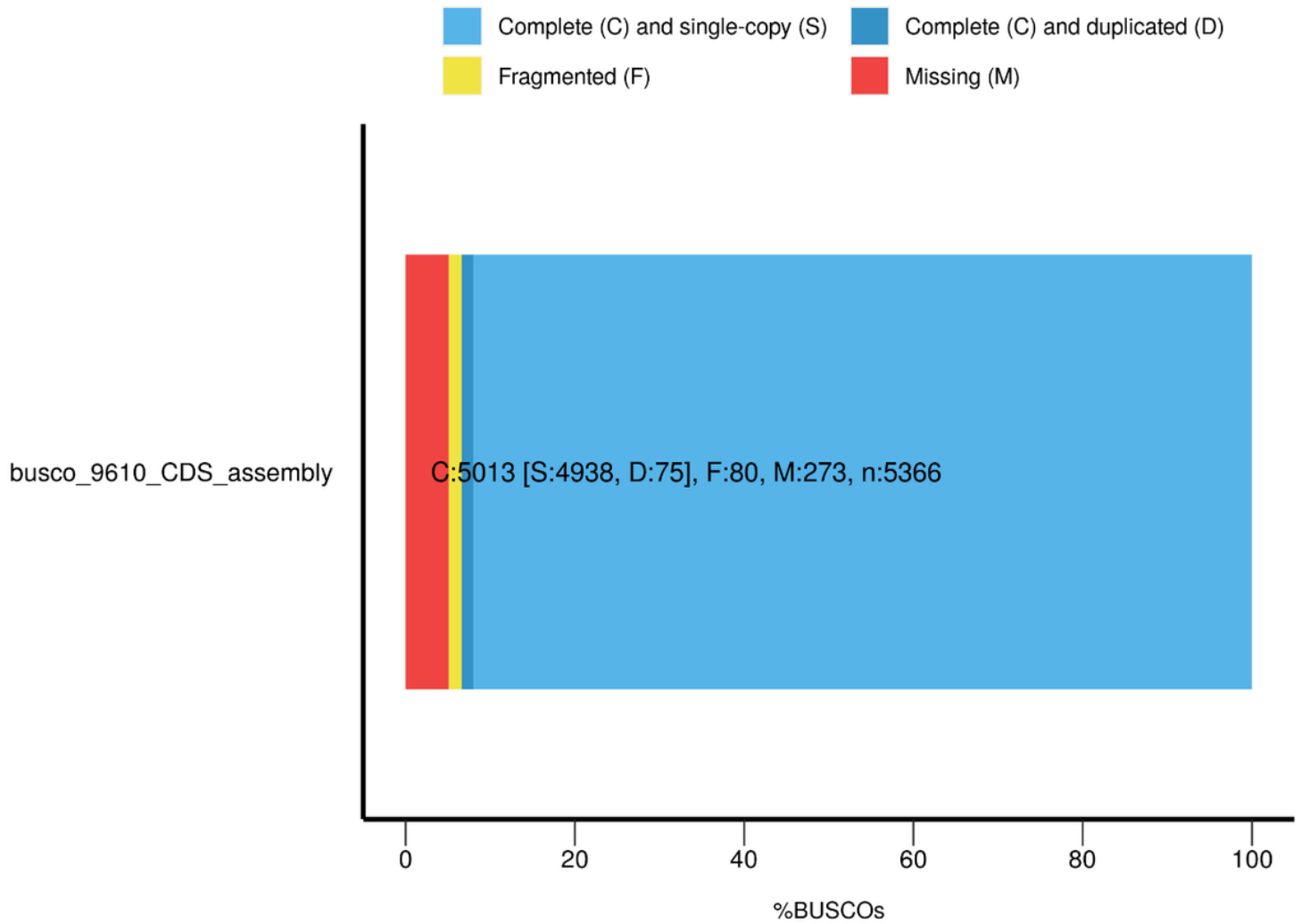
Figure 2

BUSCO assessment for completeness of draft assembly in cowpea
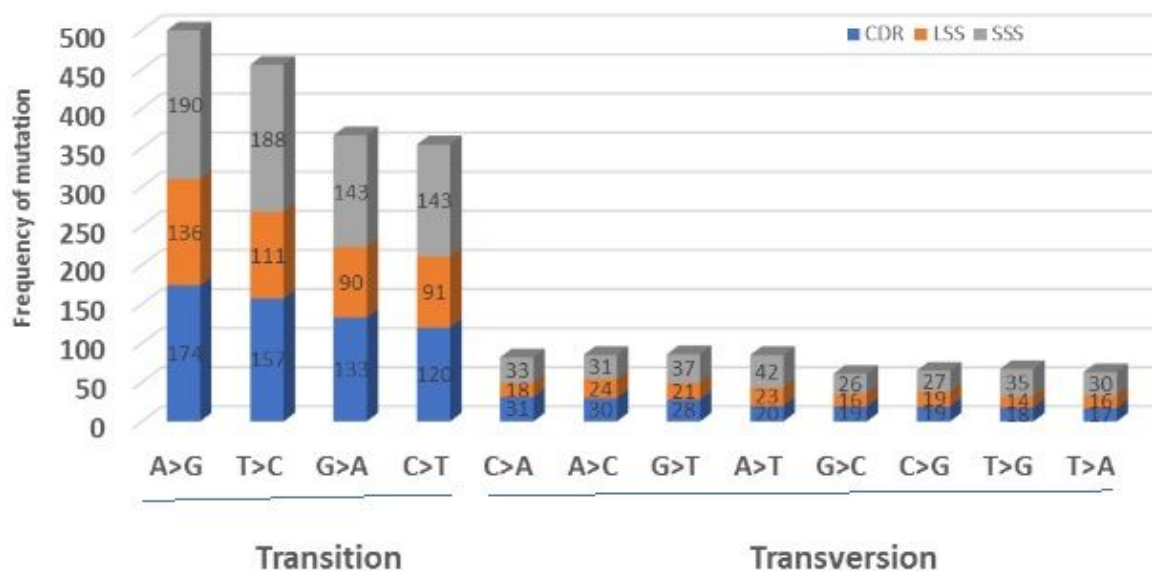
Figure 3

Frequency of different transition/transversion genome-wide mutations in three cowpea mutants
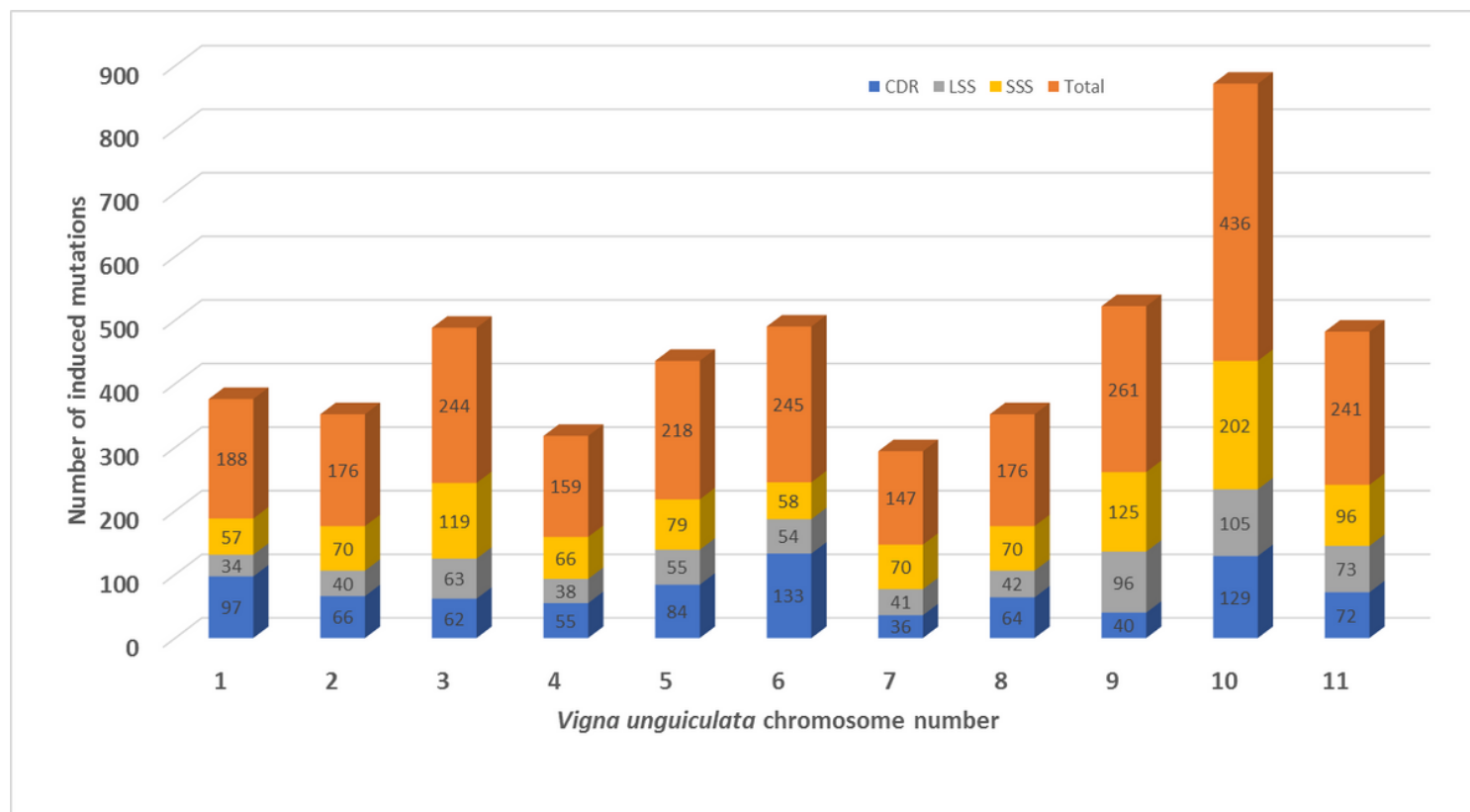

Figure 4

Chromosomal distribution of induced mutations (SBSs and indels) in three cowpea mutants