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Characterization of gamma-irradiated seeds of a wild Namibian marama bean (*Tylosema esculentum*) with microsatellite markers

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Legumes have been used as food by indigenous people of Africa yet their full potential as food sources has not been completely realized. With modern technologies such as marker-assisted selection and mutational breeding, there is scope for rapid improvement of germplasm in these underutilized species. One approach is to induce genetic mutations through irradiation of plant tissues with neutrons or gamma rays generating molecular changes. The irradiation leaves residual radiation in the exposed tissues creating offspring with new features. Genetic mutations in plants can improve their performance, germination, quality, size and disease resistance. The effects of radiation applied to marama bean (*Tylosema esculentum*) were explored. The aim of this study was to screen individuals of *T. esculentum* derived from seeds irradiated with gammarays using microsatellite markers. There were two irradiation treatments -50 and 100 gamma ray irradiations that were compared to non-irradiated seeds as controls. For each treatment, seeds were imbibed and germinated. Seed emergence rates and percentages were ascertained. Each set of seeds was genotyped using a SSR marker - MARA039, located in a sub-unit of a polygalacturonase – an enzyme that mainly regulates seed germination. In regard to emergence, significant differences between the irradiated seeds and non-irradiated counterparts were noted. Irradiated seeds germinated faster and in adosage-dependent manner compared to non-irradiated counterparts (p>0.05). Genotyping revealed that the total number of AGA repeats in the microsatellite region shifted from its original five in the controls to between four and seven in the irradiated seeds. Furthermore, there were several point mutations in the irradiated seeds as compared to the controls. The study findings, suggest that irradiation may induce beneficial mutational changes such as faster germination rate of the marama bean, considering that the mutated microsatellite repeat region is located in gene encoding the enzyme regulating seed germination in plants.

Key words: Marama bean, *Tylosema esculentum*, mutational breeding, irradiation, seed cycle, SSR.

INTRODUCTION

Although legumes have been used by the indigenous peoples of Africa for centuries, their full potential as sources of food has never been realized. However, with the advent of modern technologies such as marker-assisted selection and mutational breeding, there is scope for the rapid improvement of plant germplasm in

these underutilized species (Sprent et al., 2010). Among the grain crops, legumes (also known as pulses or food legumes) rank third in the world production behind cereals and oilseeds, and constitute an important dietary constituent for both humans and animals especially where dietary protein is an expensive commodity.

Grain legumes play a crucial role in the sustainability of agricultural systems and in food protein supplies in the developing world. The production of cereals dominates world food production while pulses play an often under estimated role as a break crop that fixes nitrogen (Popelka et al., 2004). Several constraints that limit crop production or the quality of grain legumes have been addressed by conventional breeding and enhanced management, but there are situations where the existing germ plasm still lacks the required traits. Genetic transformation could help provide solutions to certain constraints, and thus improving food security in developing countries (Popelka et al., 2004). With the ever increasing population growth, the demand for food and feed is also increasing, while natural resources are often becoming more and more constrained. Erratic rainfalls, sudden and severe drought conditions as well as excessive floods, often related to climate changes, even further negatively impact on crop production conditions (Reynolds et al., 2009). Thus the yield potential of crop plants has to be significantly increased in order to combat this worsening food security situation.

Marama bean is an arid zone, long-lived perennial legume that generates from an underground tuber (Lawlor, 2004). The scientific name for its species is *Tylosema esculentum*, a member of the Caesalpinioiodae, in the sub-family of Fabaceae (Castro et al., 2005). Naturally wild and native to the Kalahari sand regions of Namibia and Botswana as well as parts of South Africa (North West and Gauteng Provinces), *T. esculentum* is part of the staple diet of the indigenous people of these regions (Coetzer and Ross, 1977). The bean compares very well in protein and oil content with both soya and peanut and has been suggested as an alternative crop for domestication in the arid and semi-arid regions of Africa, particularly in the face of climatic changes where rainfall patterns are becoming erratic, and with the conventional crops also failing to meet the demands of people for both food and feed (Chimwamurombe, 2008).

Genetic variability is a very basic asset for crop domestication and improvement, as well as for genetic research (Gepts, 2004). Marama bean has been found to display a very high intra-population diversity and a low inter-population diversity in several studies conducted over the past two decades and using several marker systems including the ribosomal deoxyribonucleic acid (rDNA) (Nepolo et al., 2010), simple sequence repeats/microsatellites (SSR) (Takundwa et al., 2010), random amplified polymorphic DNAs (RAPDs) (Naomab, 2004; Halloran and Monaghan, 1996), and amplified fragment length polymorphism (AFLP) markers (Naomab, 2004). However, relatively very low genetic diversity has been reported in certain populations, such as Omitara of Namibia (Nepolo et al., 2010).

Increasing crop yield to ensure food security is a very huge and important challenge world-wide (Azam-Ali, 1996). Plant breeding requires genetic variation of useful traits for crop improvement but yet and quite often, the desired variation is usually lacking (Ruane and Sonnino, 2006). Utagenic agents, such as radiation and certain chemicals, can be used to induce mutations and generate genetic variations from which desired mutants may be selected and developed (Dattaet al., 2009). Mutation induction has become a proven way of creating variation within crop varieties and mutagenesis is indeed an important tool in crop improvement and development, and is free of the regulatory restrictions commonly imposed on genetically modified (GM) organisms (Seetohul et al., 2009). This forward genetic approach enables the identification of improved or novel phenotypes that can be exploited in conventional breeding programmes (Parry et al., 2009).

Exploiting natural and/or induced genetic diversity is a proven strategy in the improvement of all major food crops, and the use of mutagenesis to create novel variation is particularly valuable in those crops with restricted genetic variability (Seetohul et al., 2009). Even though high intra-population diversity has been shown in marama bean, its natural populations are unfortunately still under very huge and immense pressure from both grazing and the human exploitation of its seed (Naomab, 2004; Chimwamurombe, 2010). Thus under this very delicate circumstance, it is therefore also very important and crucial to generate strategies for conserving and further developing the remaining wild germplasm of this very important and highly promising leguminous crop.

Whilst mutations occasionally occur spontaneously in nature, the frequency of such mutations is too low to be typically relied on, and particularly for the accelerated plant development and breeding systems. However, mutations induced by physical and chemical mutagens applicable to all plant and animal species can then be taken advantage of (van Harten, 2007). Mutations may

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be major, such as large scale deletions of DNA, or relatively very minor and involve only point mutations. Mutation can be induced by irradiation with non-ionizing (for example, UV) or ionizing radiation (for example, X and gamma rays, alpha and beta rays, fast and slow neutrons). These physical mutagens often result in the larger scale deletion of DNA and changes in chromosomal structures (Girija and Dhanavel, 2009). By contrast, chemical mutagens most often only affect single nucleotide pairs (Parry et al., 2009). For plants, some of the most widely and commonly used mutagens include ethylmethane sulphonate (EMS), methylmethanesulphonate (MMS), hydrogen fluoride (HF), sodium azide, N-methyl-N-nitrosourea (MNU) and hydroxylamine (Netto et al., 2011).

Apparently, the degree of mutation is often dependent on the type of tissue and the level of mutagen exposure. Mutations at single nucleotide pairs are generally of the most interest to breeders because large-scale changes to chromosomal structures usually have severely negative results (Parry et al., 2009). Critically, mutations in important traits or genes involved innutritional quality, resource use efficiency, architecture or phenology, can be readily exploited by plant breeders without the legislative restrictions, licensing costs, and societal opposition applied to GM approaches. This is despite the fact that transcriptomic analyses have since shown that large-scale plant mutagenesis may even induce greater changes in gene expressional patterns than the transgene insertions(Batista et al., 2008).

The study presented here describes a molecular exploration on the potential usage of gamma radiation in the induction ofuseful and most preferable germplasm mutational changesof a wild African marama bean, for the possible adoption of this very important legume into modern domestication and its subsequent future participation in mutational and marker-assisted breeding programmes.

MATERIALS AND METHODS

Seed material and germination regimes

Seeds for this study were collected from a wild population of a Namibian marama bean plant, and the irradiation process was done by the International Atomic Energy Agency (IAEA) Laboratories in Vienna (Austria) prior to germination with normalization of seed moisture content. There were three experimental groups in this study: 50 seeds irradiated with 50 gamma rays (50Gy), 50 seeds irradiated with 100 gamma rays (100Gy), and 50 non-irradiated seeds (controls). For each experimental group, five batches of seeds in collections of 10 seeds per batch were surface-sterilized by transferring the 10 seeds into a sterile 50 mL Falcon tube followed by their flood-soaking with10 mL 70% ethanol for 30 s.

The seeds were then vortexed for 90 s and allowed to stand for another one and half minute. Ethanol was discarded and the seeds repeatedly washed (5 times) with sterile distilled water. The seeds were then submerged into 10 ml of sterilization buffer (50% bleach and 0.1% Triton X-100) and immediately vortexed for 3 to 5 min. The sterilization buffer was removed and seeds then thoroughly washed (5 times) with sterile distilled water. About 10 ml of 0.1% Type ‘M’ agar were then added to the washed seeds in the Falcon tube ensuring that all the seeds were fully submerged.

The tube was then incubated for 3 days at 4°C and in order to stratify the seeds for rapid and improved uniform germination rates. After stratification, the seeds were transferred to vials lined with sterile moist filter papers and then incubated (as is shown in Figure 1) for 14 days (the natural germination period of marama bean seeds) in a Plant Growth Chamber (Labex-LabconGC-300, Maraisburg, RSA) set at a constant temperature of 23°C, under an 18 h light and 6 h dark cycle. The time of emergence as well as the percentage germination of each of the three experimental groups were then evaluated and determined by respectively monitoring the exact time at which each of the various embryonic axes was produced as well as noting the exact number of embryonic axes that had successfully been produced by each group.

Extraction of total genomic DNA and its purification for polymerase chain reaction

The DNA material that was used for the molecular screening of any possible gamma ray-induced genetic mutations in the treated seeds was extracted from their emerging embryonic axes (Figure 2). Each embryonic axis coming out of a germinating seed was physically dissected with a sterile scalpel blade to expose its active mitotic tissue, and from which the genomic DNA was then extracted. This tissue (embryonic axis mitotic tissue), which includes the initial shoot and initial root of a new plant, has a very high DNA content to fresh weight ratio and thus the overall yield of DNA from this tissue is usually very excellent. In the study case, the targeted total genomic DNA was extracted using a Zymo ResearchPlant/Seed DNA Mini-prep kit and in accordance with the manufacturer’s instructions (Zymo Research Corporation, California, USA). The extracted DNA was then quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Maryland, USA), and also further resolved onto a 0.8% agarose gel stained with 1 µg/mL ethidium bromide in Tris-borate-EDTA (TBE) buffer and viewed under a UV illumination system (UV Transilluminator 2000, Bio-Rad Laboratories, Munich, Germany), before being used for the subsequent PCR amplifications.

Polymerase chain reaction

Out of screening 80 known primers for marama, MARA 0001-MARA080 for differences between irradiated and non-irradiated seeds, MARA 039 was found to detect differences. Polymerase chain reaction (PCR) amplifications were performed using a Thermo Scientific DreamTaq Green PCR Master mix (2X) (Thermo Scientific, Maryland, USA) in 25 µL reaction volumes using the following reconstitutions: 12.5 µL 2X DreamTaq Green PCR Master mix, 1.0 µL forward primer (MARA039; L-TCATTAAAGGGCTCCATTGC), 1.0µL reverse primer (MARA039; R-ATGCCCAAAATCACCAACAT), 8.5 µL sterile water and 2.0µL DNA template. The primer set (MARA039) specifically targeted and amplified the AGA repeat and microsatellite region of the extracted seed embryonic axis DNA. The PCR conditions on the thermocycler (C1000 Touch™, Bio-Rad Laboratories, Munich, Germany) were as follows: 1 cycle of Pre-denaturation: 95°C for 4 min; 35 cycles of Denaturation: 95°C for 30 s, Annealing: 55°C for 1 min, and Extension: 72°C for 2 min; 1 cycle of Final Extension: 72° for 5 min and a sample holding period at 4°C. The amplified PCR products were viewed on a 2% agarose gel in TBE buffer stained with 1 µg/mL

Figure 1. Germination of the marama seeds. The surface-sterilized seeds were germinated in transparent plastic vials lined with sterile moist filter papers under controllable and measurable growth chamber conditions.

Figure 2. Extraction of total genomic DNA from embryonic axes. The panel presents an embryonic axis emerging from a germinating seed. The total genomic DNA was subsequently extracted from the active mitotic region of this tissue.

ethidium bromide under UV illumination to check their sizes and qualities. The yielded amplicons were then cleaned up and concentrated for subsequent sequencing using a Zymo Research, DNA Clean and Concentrator-5kit (Zymo Research Corporation,

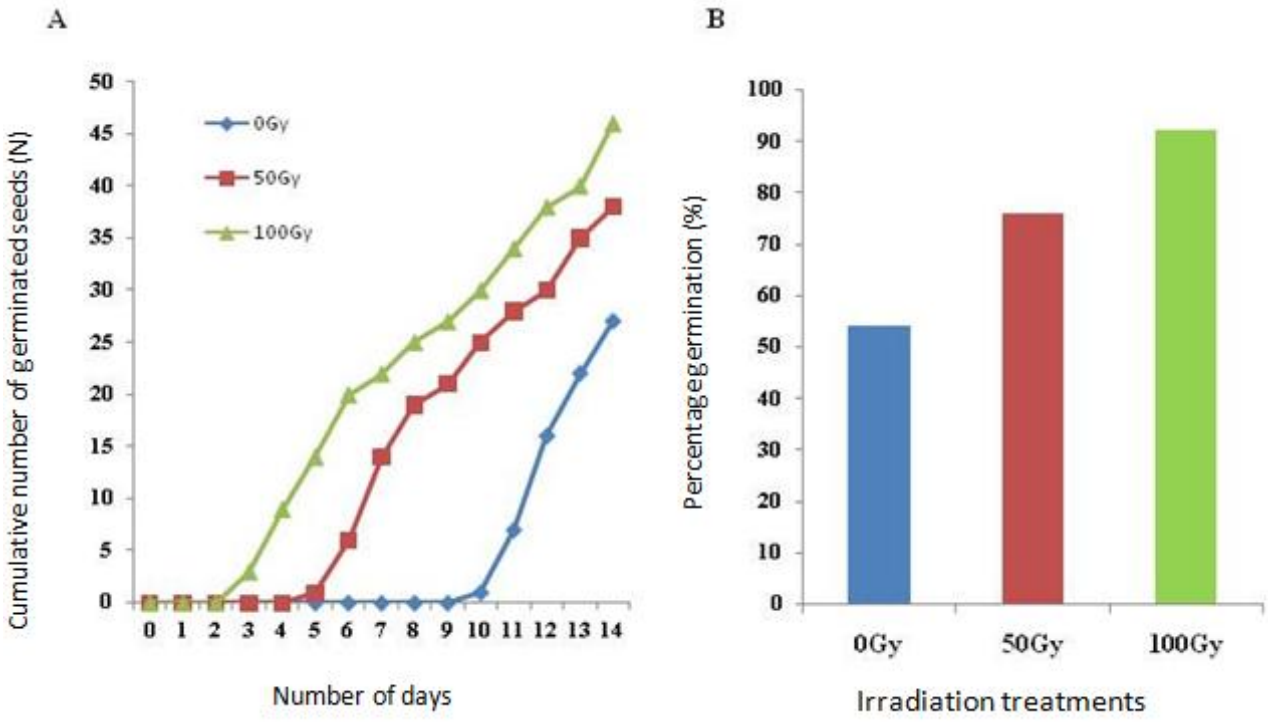

Figure 3. Emergence rates and percentage germinations. (A) A comparative analysis of the average time taken for seeds to germinate among three different groups of marama bean seeds. (B) A comparative analysis of the total number of seeds germinated per group (n=50, p>0.05).

California, USA). Sequencing was then carried out at Inqaba Biotechnology in Pretoria, South Africa.

Sequence analysis

The nucleotide sequences obtained by Inqaba Biotechnology (Pretoria, South Africa) for the primer set MARA039, were manually edited from their established chromatograms in the Chromas Light and BioEdit program. In order to obtain the regions of overlaps, the forward and reverse sequences of the amplicons were aligned using complements of their sequences. All overlapping sequences were then trimmed and the resulting consensus sequences created in the BioEdit Sequence Alignment Editor for each of the obtained samples. The consensus sequences were then used to create BLAST searches of the NCBI database using CLUSTAL Xto align with organisms in the database and seeking the most likely gene identity of the obtained amplicons.

Rates of emergence and percentage germinations

For all the three experimental groups (controls, 50Gy, and 100Gy), the exact time of embryonic axis emergence per seed as well as the actual number of germinated seeds per group were physically monitored and manually recorded over the 14-day experimental period. For each group, the exact time of emergence was noted and then regarded as the rate of emergence while the total number of germinated seeds per group was expressed as the percentage germination.

RESULTS

Emergence rates and percentage germinations

When comparing the non-irradiated (control) seeds and the irradiated (50 Gy and 100 Gy) seeds, both relatively higher emergence rates and percentage germinations were observed in irradiated seeds than in the non-irradiated seeds (Figure 3A and B). In terms of the emergence rates, the earliest time of embryonic axis emergence was observed in seeds treated with 100 Gy (3 days), followed by the seeds treated with 50 Gy (5 days) and then the control seeds (10 days). Once again, the highest number of seeds to germinate was observed in seeds treated with 100 Gy (92%), followed by the seeds treated with 50 Gy (76%) and then the control seeds (54%). This observation signifies a dosage-dependent inductive effect of the applied radiation that preferably favored both enhanced emergence rates and high percentage germinations.

Polymerase chain reaction

From the PCR work undertaken, the targeted and most desired AGA microsatellite repeat region of the marama

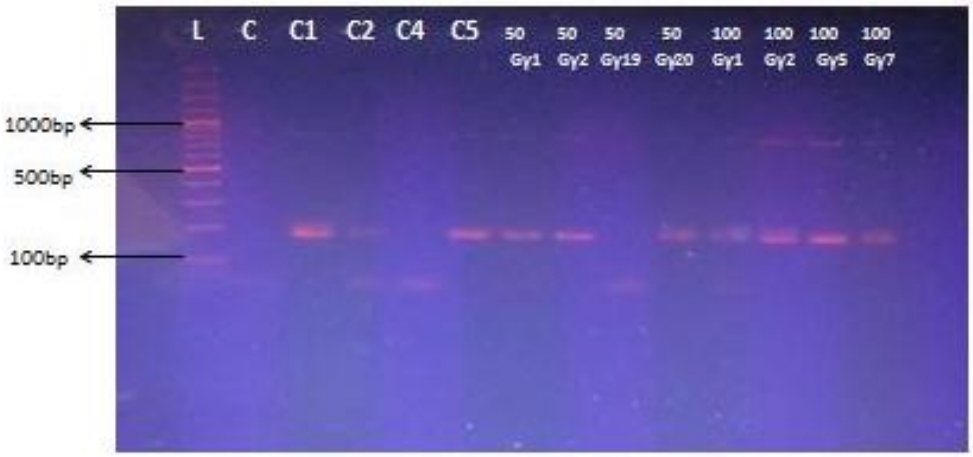

Figure 4. Electrophoresis gel of PCR products amplified in the AGA microsatellite repeat region of marama bean by the MARA039 primer set. The amplified products were resolved on a 2% agarose gel stained with 1µg/mL ethidium bromide and run in 1X TBE buffer at 90 V for 1 h. L is the 100 bp ladder, C represents the non-irradiated control seeds, 50Gy represents seeds irradiated with 50 gamma rays and 100Gy represents seeds irradiated with 100 gamma rays.

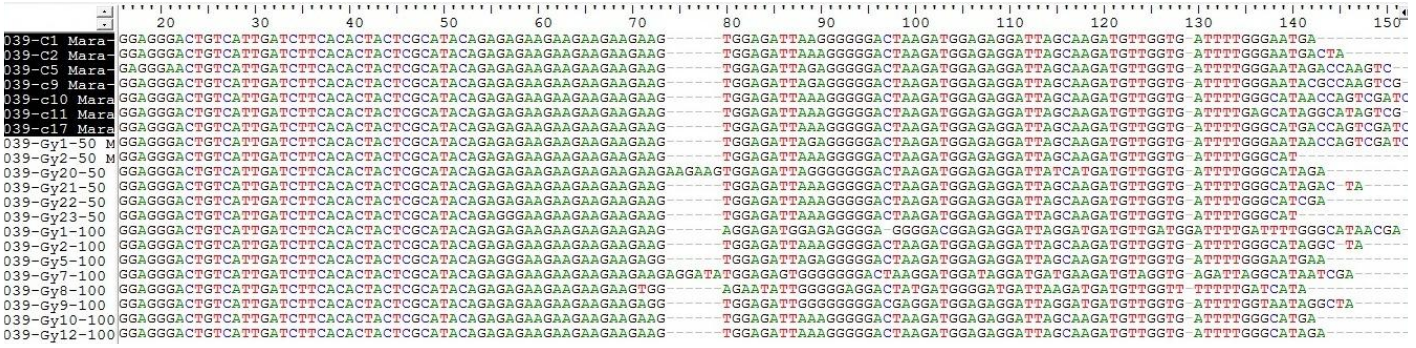

Figure 5. Sequence alignment of the amplified amplicons in the AGA microsatellite repeat region of marama bean.A MARA039 primer set was used to amplify various amplicon fragments of the AGA microsatellite repeat region of the marama bean seeds treated with either0, 50 or 100 gamma rays. The amplified fragments were sequenced and then aligned in the Clustal X program in order to determine their specific point or AGA repeat mutations.

bean plant was successfully amplified for both the irradiated and non-irradiated seeds (Figure 4). Amplifications were carried out using the MARA039 primer set (L-TCATTAAAGGGCTCCATTGC; R-ATGCCCAAAATCACCAACAT), which in all cases, succeeded to amplify the various amplicon fragments close in size to the expected for the marker 176 bp (Figure 4).

Sequence alignment

All obtained amplicons in Figure 4 were carefully excised and cleaned up for subsequent sequencing at the Inqaba Biotech, Pretoria, South Africa. Sequencing was undertaken in order to specifically determine the exact base pair differences between the various amplicons in the targeted microsatellite repeat region. The exact base pair differences were then determined by aligning the sequenced amplicons against each other in the Cluster X program and as is shown in Figure 5. Apparently, the targeted region of interest displayed several point mutations as well as some differences in the number of the AGA repeat units. All control samples retained their original 5 AGA repeat units and had no point mutations while the irradiated samples either gained or lost one to two more AGA repeat units and had several point mutations. This thus indicated that the irradiation system had some profound mutational effects of either changing the number or type of the AGA repeat units in the

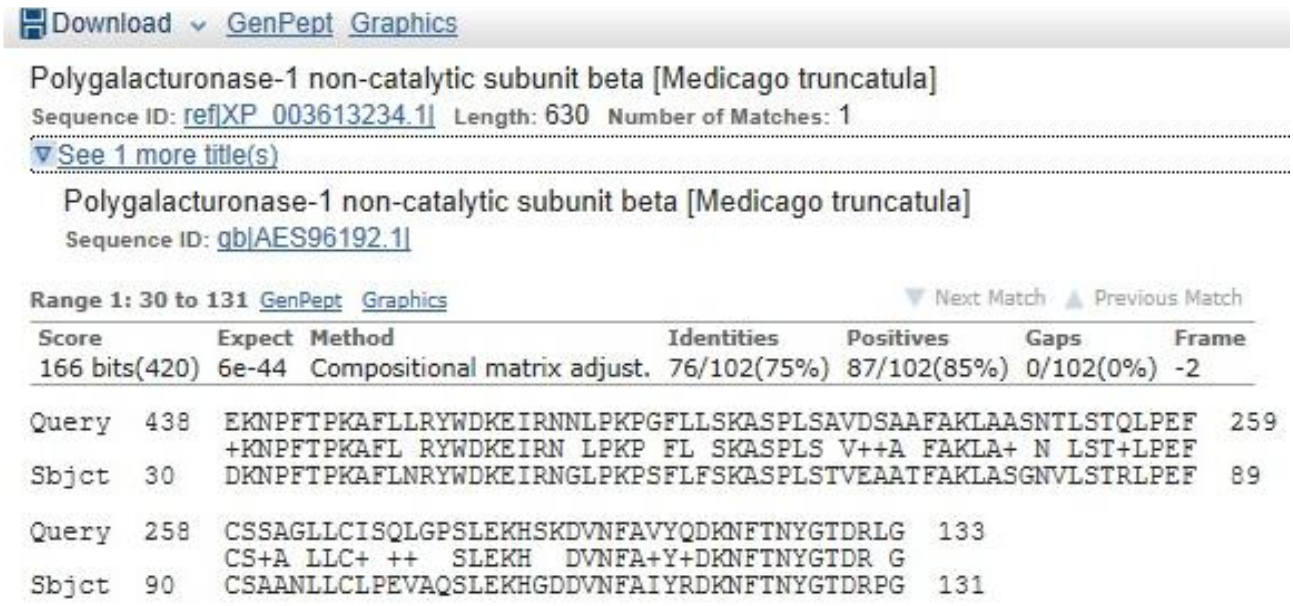

Figure 6. Sequence identity of the AGA microsatellite repeat region in the marama bean. A control amplicon from the non-irradiated marama seeds was used as a search motif to BLAST the NCBI database and it returned an identity that was the non-catalytic beta sub-unit of a *Medicago truncatula* polygalacturonase (PG).

targeted microsatellite region.

BLAST searches

After amplifying the AGA microsatellite repeat region in each of the irradiated or non-irradiated marama bean seeds and determining its irradiation-associated mutational changes, it was then necessary to attempt and establish the exact identity of this region in the marama bean plant. In order to achieve this, a BLAST search in the NCBI database using one of the non-irradiated amplicons was carried out. The search returned a 75% homologous hit which apparently was the non-catalytic beta sub-unit of a polygalacturonase (PG) enzyme in the *Medicago truncatula* genome (Figure 6). This then proposed that the targeted and amplified AGA microsatellite repeat region in the marama bean could also be the non-catalytic beta sub-unit of a polygalacturonase enzyme of this plant.

DISCUSSION

The study presented here describes how the process of gamma irradiation can be practically used as a method to induce detectable but agronomically important mutations in seeds of a wild trans-Kalahari legume, the marama bean (*T. esculentum*). After exposing the seeds to the various dosages of gamma rays (0, 50 and 100 Gy), the mutational effects of the applied radiation onto the exposed seeds were then studied by analyzing the molecular features of an AGA microsatellite repeat region that naturally exists in one specific area of the total genomic DNA of these seeds. Apparently, this particular area was specifically targeted for this study because its sequence-specific microsatellite primers (MARA039 set) have already been developed and the area can be easily amplified by polymerase chain reaction (PCR) (Takundwa et al., 2010).

When the MARA039 primer set was used to target and amplify the desired AGA microsatellite repeat region in the total genomic DNA of both the irradiated (50 Gy and 100 Gy) and non-irradiated (control) seeds, a number of amplicons were successfully amplified and all in the expected size range of between 100 and 200 bp (~176 bp) (Figure 4). A subsequent sequence alignment of those amplified fragments using Cluster X revealed that while DNA from all control seeds had maintained its original 5-AGA repeat, DNA from the irradiated seeds had either gained or lost one to two more AGA repeats (Figure 5), an aspect which typically suggested to us that the used irradiation system was practically capable of inducing some marginal genetic mutations into the exposed seeds. Furthermore, besides just altering the number of AGA repeat units, the irradiation system also could induce several point mutations in the exposed seeds (Figure 5). Apparently, when a single amplicon

from the control seeds was used as a motif to BLASTsearch the NCBI database, it managed to return a single hit to which it had a 75% homologous similarity level, and this hit happened to be the non-catalytic beta sub-unit of a polygalacturonase (PG) enzyme from Medicago truncatula (Figure 6). This then thus proposed to us that the targeted and amplified AGA microsatellite repeat region in the marama bean could also be the non-catalytic beta sub-unit of a polygalacturonase enzyme of this plant.

In the plant family, polygalacturonases (PGs) are a group of enzymes which chiefly degrade cell wall pectin compounds (Swain et al., 2011) by primarily hydrolyzing the α-1,4 glycoside bonds that naturally exist between two residues of galacturonic acid in the pectin chain (Swain et al., 2011; Ghianiet al., 2011). PGs are always strongly associated with the various and very important plant developmental processes such as seed germination (Kanai et al., 2010), pollen tube growth (Clarke and Gleeson, 1981; Mu et al., 1994), fruit maturation (Ghianiet al., 2011), lateral root outgrowth (Peretto et al., 1992), organ abscission (Taylor et al., 1990; Bonghi et al., 1992), seed pod dehiscence (Jenkins et al., 1996), and the intrusive growth of non-articulated laticifers (Wilson et al., 1976). In the process of seed germination, the hydration of a seed by water usually results in increased metabolic activities, which either stimulate the synthesis of new enzymes or literally enhance the activation of pre-existing ones, with the mobilization of reserves and digestion of the cell wall, weakening it and causing a consequent rupture of the integument by the radicle (Baskin and Baskin, 1998). Typically, the developing radicle always requires a weakening of the endosperm tissue opposite its tip (Clarke and Gleeson, 1981; Mu et al., 1994) as well as a softening of the integument for its successful protrusion (Baskin and Baskin, 1998; Kanai et al., 2010) and consequently, both processes are primarily controlled by PGs (Karssen et al., 1989).

Now considering the proposed possible link between the AGA microsatellite repeat region and the non-catalytic beta sub-unit of the polygalacturonase gene in the marama bean, it was therefore not unusual for us to speculate for some possible phenotypic changes in the germination profiles of those seeds whose microsatellite regions had been altered by exposure to the applied gamma irradiation. Not surprisingly, all irradiated seeds somewhat showed some relatively high emergence rates (Figure 3A) as well as some relatively enhanced germination percentages (Figures 3B) when compared to their non-irradiated counterparts, and thus suggesting to us for a possible induction of some relatively positive and somewhat favorable mutational effects onto the exposed seeds by the applied gamma irradiation, which eventually improved their emergence and germination efficiencies.

In this regard, it appears as though that the generated and acquired mutational changes (both the repeat unit changes and point mutations) in the microsatellite repeat region of the marama bean seeds could have had favored some conformational and structural changes in the non-catalytic domain of the polygalacturonase gene that then eventually enhanced the catalytic activity of this enzyme and finally resulting in improved emergence and germination profiles of the irradiated seeds.

By summing up all these findings, it is conceivable to state that the process of gamma irradiation can be potentially used as a practical method in both the marker-assisted and mutational breeding programs of the marama bean for its ultimate improvement as an indigenous legume of the Kalahari regions of Africa. Notably, the fact that the observed induced mutations tended to target the microsatellite region, MARA 039, which seems to be part of an enzyme with central roles in various important plant developmental processes, typically makes this suggestion an imminent possibility. Apparently and in some previous domestication efforts of the marama bean, researchers have frantically been trying to search for ways to reduce the seed cycle of this legume, which apparently appears to be not less than 18months (unpublished data) but to no success. However, and based onto this study findings here, the approach of mutational breeding may be a possible option to attempt and reduce this lag period by producing new mutants with early and improved germination efficiencies.

Furthermore, the fact that the PG enzyme is also primarily involved in earlier fruit maturation means that its potential modification to favor both the early seed germination and early fruit maturation would strongly augment the practical shortening of its seed cycle in the developed marama bean mutants and probably making this wild legume an ideal candidate for possible domestication and becoming one of the mainstream crops for the -Kalahari regions of Africa.

Conclusion

Plant breeding has evolved and is no longer limited to simple selection, but is also concerned with the creation, selection, evaluation and multiplication of desirable genotypes. Apparently, the process of mutation breeding can now be shortened by new techniques such as in vitro culture, molecular markers as well as the use of mutagenesis to increase the genetic variability of plants. The mutation breeding technique has led to the recent significant increase in cultivars released and applying it to emerging crops like the marama bean will eventually benefit its production. Thus based on findings of this study, the use of gamma irradiation in the marama bean

for the induction of various favorable mutations seems a very feasible approach that will result in the generation and establishment of mutants with very important and most desirable agronomic traits such as early seed germination and early fruit maturation.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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