

Full Length Research Paper

Characteristics of molecular changes in lespedeza plants after seeds been flown on satellite

Ren Weibo^{1*}, Deng Bo², Han JianGuo², Zhang YunWei², Xu Zhu¹ and Wang Zhaolan¹

¹Grassland Research Institute, Chinese Academy of Agricultural Sciences, Huhhot, 010010, China.

²Institute of Grassland science, China Agricultural University, Beijing, 10094 China.

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To investigate the effect of space flight factors on lespedeza seeds, dry seeds of *Lespedeza bicolor* were loaded into recoverable satellite for space flight in 2003. After the retrieval, key agronomic traits were tested. Among the 65 plants we tested in the first generation (SP1), ten plants showed changed phenotype characters as prematurity, late-maturity, tall, dwarf, multi-ramify and etiolation. The mutation rate varied from 1.5 to 4.6% among different traits. 60 primers were screened in RAPD analysis to evaluate DNA variation between mutations and their ground controls. Results showed that 272 amplified bands produced by 55 primers. Among them, 12 primers amplified 41 polymorphic bands. Pattern of bands showed that absence of common bands were observed in mutated plants compared with the ground control. Moreover, similar band pattern was observed in the same mutation phenotype. Conclusion was that space flight factors could induce inheritable mutagenic changes on bush clover seeds, and verified these changes in genetic material in the mutants. These mutations may due to the breakage of chromosome fragments which was induced by complex traits of space flight, especially the cosmic radiation and microgravity. More work is being conducted to select the stable mutants with favorable traits and used them in developing new cultivars.

Key words: *Lespedeza bicolor*, spaceflight, RAPD.

INTRODUCTION

Response of plant to spaceflight-associated stress has been a hot topic of investigation since the beginning of human space exploration. Within the past few decades, a number of reports indicated that exposure of dry seeds to space flight factors, especially to the high linear energy transfer (LET) cosmic ray particles, would have serious biological effects, such as morphological aberrations (Kranz et al., 1990), alteration in cell shape (Antipov et al., 1967), chromosome aberration (Gartenbach et al., 1994), and gene mutation (Mei et al., 1998; Yu et al., 2007). Although basic research in cosmic radiation is primarily concerned with the protection of both humans and plants from the harmful effects of space, unprotected space flight of seeds could be advantageous in producing useful genetic mutation (Xu et al., 1999).

Based on this ideas, some stable mutants with promising traits, from the progenies of space flown dry seeds, that is, rice (Ma et al., 2007; Li et al., 2007), tomato (Nechitailo et al., 2005), maize (Mei et al., 1998) etc., had been selected and used in the breeding of new cultivar, which has been named as 'Space Mutation Breeding' (Jiang, 1996; Cyranoski, 2001; Dennis and Ding, 2002). In addition, the genetic characteristic of these mutation were also analyzed by RAPD (Mei et al., 1998), AFLP (amplified fragment length polymorphism) (Li et al., 2007; Yu et al., 2007) and so on.

Bush clover (*Lespedeza bicolor*) is a kind of perennial legume shrubs, which is very nutritious to livestock and can be utilized as ornamental plant for its beautiful flower, or as windbreaks. Space flight plants of bush clover showed changed phenotype characters as prematurity, late-maturity, tall, dwarf, multi-ramify and etiolation. The mutation rate varied from 1.5 to 4.6% among different traits (Ren et al., 2006). However, changes in genetic materials of these mutants have been poorly documented.

*Corresponding author. E-mail: rppcaucau@163.com. Tel: +86 0471 4926884.

Randomly amplified polymorphic DNA (RAPD) (Williams et al., 1990), as one type of molecular genetic markers, has proven quite useful in such area as genetic mapping, plant discrimination, genetic diversity and so on. Genetic analysis of DNA with RAPD has been reported in many crops such as maize, bean (Mei et al., 1998; Wang et al., 1996).

To uncover the molecular nature of mutation induced by space flight factors, some of plants with morphological changes were chosen for RAPD analysis. Our aim was to detect polymorphisms between mutation plants and ground controls or within mutated individuals and to generate specific DNA markers in order to discriminate individuals with mutated locus from those normal plants.

MATERIALS AND METHODS

Plant materials

Dry seeds of bush clover (*Lespedeza bicolor*) were obtained from the Institute of Grassland Science, China Agricultural University. Seeds (8 g) was carried into space aboard on recoverable satellite 'JianBing No.4', which was launched on 3 November, 2003 and recovered on 21 November, 2003. The space flight lasted for 18 days at an orbit of 63°, 200 km/350 km, the microgravity level $10^{-5} \times g$, the radiation level was 10.2 mGy/d. Some of the seeds from the same stock were kept on the ground and used as control.

In 2004, both flight and CK seeds were planted in the field. During 2004-2006, field observation was carried out to evaluate morphological changes of plants developed from flight seeds. Six types of changed characters (pre-maturity, late-maturity, giant, dwarf, multi-ramify, etiolation) were observed in SP1 generation (Ren et al., 2006). The progenies from the cross-bred plants from the SP1 generation were also investigated. Such mutated characters as giant, pre-maturity, late-maturity segregated in the SP2 and SP3 generation. In 2006, leaves of three mutation TF2 (pre-maturity), TF3 (late-maturity), TT1 (giant) in SP1 generation and ten plants from ground controls were collected for DNA extraction.

DNA extraction

DNA was extracted basically, according to Edwards et al. (1991) protocol with the minor modification. The final DNA was resuspended in Tris-EDTA (10 mM Tris, pH 8.0, and 1 mM EDTA) and quantified with a DNA Fluorometer (Model TKO-100, Hoefer Scientific instruments) and by 0.8% agarose gel separation. The DNA stock was diluted to 50 ng/ μ l and was stored at -20°C for use in PCR reaction. In addition, genomic DNA of plants from ground control were extracted respectively and bulked together with the same amount as PCR templates.

Polymerase chain reaction

Sixty arbitrary oligonucleotide primers was designed by DNAMAN and synthesized by Augct company (Beijing, China). The 25 μ l PCR reactions included 2 μ l genomic DNA (50 ng/ μ l), 2.5 μ l 10 \times buffer (Mg²⁺ free), 2 μ l 2.5 mM dNTPs, 0.5 μ l each of 25 μ M primer and 0.125 μ l Ex-Taq polymerase. dNTP_{mix} and PCR buffer were provided by TakaRa company (Dalian, China). PCR amplification reactions were run on a PTC-200 ThermalCycler (MJ Research, Watertown, MA). PCR reaction was programmed as an initial denaturation for 3 min at 94°C and 40 cycle of 45 s at 94°C, 30 s at 36°C and 45 s at

72°C. After the last cycle a final extension was done at 72°C for 10 min.

The PCR template consisted of TF2 (pre-maturity), TF3 (late-maturity), TT1 (giant), CK1 (bulk DNA from five plants of ground controls) and CK2 (bulk DNA from another five plants of ground controls). The amplification for primers screened was conducted two times independently, with the same procedure, to verify that the RAPD markers were reproducible and consistent.

Gel electrophoresis

The PCR products (7.5 μ l) with 1.5 μ l of 6 \times loading buffer (30% glycerol, 0.125% bromophenol blue, 20 mM Tris-HCl, pH 8.0) was fractionated by electrophoresis using a 1.5% (w/v) agarose gel in Tris-acetate (TAE) buffer at a constant voltage of 115 V for 0.5 h. Gels were stained with ethidium bromide. DNA fragments were then visualized under UV light and photographed using ToYOBO FAS II (TOYOBO Biochemicals, Osaka, Japan). The sizes of amplified products were estimated by comparing with a 200 bp DNA ladder marker (TianGen co. Ltd., Beijing, China).

Band recording and data analysis

Each clear, well defined and reproducible band of the replications were recorded as potential RAPD markers using the primer and its band numbers. For example, R15-1 represented the first marker of primer R5 (Chen and Yamaguchi, 2005; Kaundun et al., 2000). Each individual primer-specific amplification product was considered to be represent the dominant allele at a unique RAPD locus. The percentage of polymorphic bands was calculated as the method described by Wachira et al. (1995). Unique, specific RAPD markers, band patterns were also recorded.

RESULTS

Genetic polymorphism of mutation induced by space flight factors

Totally 272 products, that is, visible bands on electrophoresis gel, were produced by 55 primers out of 60 with the percentage of 92%. Out of 55 oligonucleotide primers used, only 13 primers generated polymorphic loci, which were listed in Table 1. In total, they generated 41 polymorphic bands out of 52 reproducible products, corresponding to 78.8% polymorphism. The number of products generated by each primer varied from 2 to 5 with the average of 4 (Table 1). The size of the amplified products ranged from 500 to 1600 bp. Data in Table 1 showed that:

- (1) Numbers of polymorphism bands amplified in space mutation was much higher than that in ground control, which meant that changes occurred on genomic DNA between space flight than ground control.
- (2) Number of polymorphism products differed between different type of space flight mutations (TT1, TF2 and TF3), which suggested that the effect of space flight factors on genomic DNA differed with types of mutation.
- (3) Number of polymorphic bands of TF2 was closed to that of TF3; Figure 1 showed part of band patterns amplified by RAPD from genomic DNA of space flight mutation

Table 1. Number of amplification products generated with 13 random sequence primers.

Primer	Sequence (5'-3')	Total no. of RAPD products	Numbers of polymorphic bands	No. of polymorphic bands				Percentage of polymorphic bands (%)
				TT1	TF2	TF3	CK	
R4	AGCCTCCCTT	3	3	1	1	2	2	100
R7	TGAACCGACC	3	3	1	3	2	0	100
R10	AGACGATGTT	3	3	2	1	0	0	100
R13	GATTGCCTCT	2	2	0	1	1	1	100
R15	TGTGATACGG	5	4	0	3	4	1	80
R19	AGACGTTCTC	5	4	0	4	4	3	80
R20	AGGAACAGGC	5	4	3	2	2	0	80
R27	TCCGGTCCCA	5	4	2	4	3	0	80
R35	CAAACCAGTC	3	2	0	1	2	0	66
R38	GCGGCAGCTT	4	2	2	2	2	0	50
R42	CATTCGTGCC	4	3	1	3	3	1	75
R54	ATGACGTCGA	5	3	1	2	3	1	60
R57	ACTAGAGCAA	5	4	2	3	4	0	80
Total		52	41	15	28	32	9	
Polymorphism (%)				36.6	68.3	78.0	21.9	78.8

TT1, Giant mutation; TF2, mutation with pre-maturity; TF3, late-maturity mutation; CK, bulked ground controls.

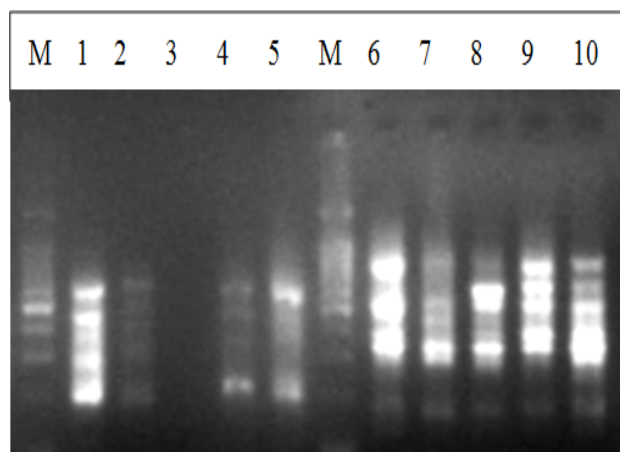


Figure 1. Band patterns of RAPD amplification products. Lanes 1-5: TT1, TF2, TF3, CK1, CK2 band patterns generated by primer R19; lanes 6-7: TT1, TF2, TF3, CK band patterns generated by primer R20. M is 200 bp DNA ladder.

(TT1, TF2 and TF3) and ground controls.

Specific markers related to mutated characters

The genetic variability was detected using two methods: (a) unique RAPD markers and (b) specific band patterns.

By specific band patterns, as showed in Figure 2, three primers R13, R18, R42 produced similar band patterns between TF2 (pre-maturity) and TF3 (late-maturity) whereas these three primers produced different band

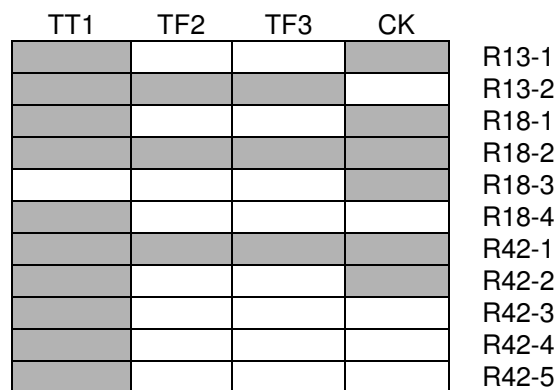


Figure 2. DNA fingerprinting of four mutations based on RAPD markers. Shaded blocks represent the presence of RAPD markers. The right first lane indicates the specific marker for the discrimination.

pattern between TF2 (or TF3), TT1 and CK. This outcome reconfirmed that effect of space flight factors differed with the types of mutation.

Unique RAPD markers (Table 2) showed that (1) all the space flight mutations (TT1, TF2 and TF3) showed the presence of the specific RAPD marker R13-2 while it is absent in ground controls, which may be caused by small inserted DNA fragments. (2) All the space flight mutation (TT1, TF2 and TF3) lost ten bands R18-3, R27-2, R27-3, R38-3, R38-4, R54-1, R57-1, R57-2 while it is present in ground controls, corresponding 24.3% of total polymorphism bands amplified by RAPD primers, which may be attributed to large DNA fragment deletion occurring during the space flight.

Table 2. Unique markers generated between spaceflight mutation and ground controls.

Unique marker	Length (bp)	Mutation	CK
R13-2	800	Presence	Absence
R18-3	1400	Absence	Presence
R27-2	1100	Absence	Presence
R27-3	1000	Absence	Presence
R38-3	800	Absence	Presence
R38-4	600	Absence	Presence
R54-1	1400	Absence	Presence
R57-1	1600	Absence	Presence
R57-2	1400	Absence	Presence

Mutation bands are the common bands of TT1, TF2 and TF3. CK = bulked ground controls.

DISCUSSION

RAPD marker used as a suitable method for detection of genetic variability between space flight mutation and ground control have been reported on many crops such as maize (Mei et al., 1998), tomato (Han et al., 1996), kidney bean (Zhang et al., 2000) and so on. The degree of polymorphism revealed in space mutations, especially TF2 (pre-maturity) and TF3 (late-maturity), is more extensive than that in ground control, which demonstrated that space flight environment could induce inheritable mutagenic effects on plant seeds. There were similarly reports on maize and erect milkvetch (Mei et al., 1998; Li and Wang, 2002). After erect milkvetch seeds were flown in orbit, pre-maturity mutation was observed heritable in the following generation (Li and Wang, 2002).

In this study, two types of mutation, including fluorescence abnormality (per-maturity and late-maturity) and vegetative organ variance (giant) were observed to generate distinct band patterns when a certain primer was used. Similarly, reports also showed that variation in banding patterns was correlated with specific mutation when RAPD assay was performed (Mei et al., 1998). Therefore, RAPD assay might be used as an effective and economic tool in assessing genetic damage of plant induced by space flight factors (Mei et al., 1998). As for RAPD assay, it is relatively inexpensive, easy to detect and need no sequence information. Hence, it was especially suitable for research of grass plant mutation induced by space flight.

In addition, RAPD fingerprints (specific bands by certain primers) allowed us to discriminate specific mutation types and their progenies line. Moreover, by excision, cloning and sequencing, specific RAPD marker might be transformed into SCARs (sequence characterized amplified regions) marker, which are not only be useful in marker assisted selection of space mutation breeding but also provide sequence information for better understanding of the mechanism of mutation induced by space flight.

There were successful examples for detecting space flight mutation by developing SCARs markers. RAPD assay had been used to detect genetic variance between long-pod mutation and ground controls. Three out of 100 oligonucleotide primers could produce specific bands related to long-pod mutation. Three fragments (OPZ-13₁₄₀₀, OPY-04₂₀₀₀, OPY-07₁₀₀₀) generated by these three primers were cloned and sequenced. Among them, the marker OPY-07₁₀₀₀ was successfully transformed into SCAR markers and used for marker assisted selection in the new cultivar developing (Qiu et al., 1998).

The number of common bands among three space mutations, compared with ground controls, decreased rather than increased, with the ratio of 10:1. The possible reason may attribute to the breakage or deletion of chromosome DNA. Reports indicated that cosmic radiation could bring about breakage or deletion of chromosome DNA in the same time microgravity disturbed the DNA repair pathway (Gartenbach et al., 1994). Vibration and linear acceleration, which occurred at the launch and return of satellite, also caused chromosome aberrations, and brought the breakage of large fragment of genomic DNA compared with the control (Anikeeva et al., 1979; Vaulina and Kostina, 1975). Mutation could be induced by space condition on the whole plant genome, mainly showing chromosome deficiency and duplication (Mei et al., 1998; Yang et al., 2003).

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