GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Assessment of genetic diversity and variation of *Robinia pseudoacacia* seeds induced by short-term spaceflight based on two molecular marker systems and morphological traits

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Genet. Mol. Res. 11 (4): 4268-4277 (2012)
Received March 5, 2012
Accepted November 29, 2012
Published December 17, 2012
DOI http://dx.doi.org/10.4238/2012.December.17.2

ABSTRACT. The black locust (*Robinia pseudoacacia*) is a forest legume that is highly valued as a honey plant and for its wood. We explored the effect of short-term spaceflight on development of *R. pseudoacacia* seedlings derived from seeds that endured a 15-day flight; the genetic diversity and variation of plants sampled from space-mutagenized seeds were compared to plants from parallel ground-based control seeds using molecular markers and morphological traits. In the morphology analysis, the space-mutagenized group had

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apparent variation compared with the control group in morphological traits, including plant height, basal diameter, number of branches, branch stipular thorn length, branch stipular thorn middle width, leaflet vertex angle, and tippy leaf vertex angle. Simple sequence repeat (SSR) and sequence-related amplified polymorphism (SRAP) molecular marker analyses showed a slightly higher levels of genetic diversity in the space-mutagenized group compared to the control group. In the SRAP analysis, the space-mutagenized group had 115 polymorphic bands *vs* 98 in the controls; 91.27% polymorphic loci *vs* 77.78% in the controls; 1.9127 ± 0.2834 alleles *vs* 1.7778 ± 0.4174 in the controls; Nei's genetic diversity (*h*) was 0.2930 ± 0.1631 *vs* 0.2688 ± 0.1862 in the controls, and the Shannon's information index (*I*) was 0.4452 ± 0.2177 *vs* 0.4031 ± 0.2596 in the controls. The number of alleles was significantly higher in the space-mutagenized group. In the SSR analysis, the space-mutagenized group also had more polymorphic bands (51 *vs* 46), a greater percentage of polymorphic loci (89.47% *vs* 80.70%); *h* was also higher (0.2534 ± 0.1533 *vs* 0.2240 ± 0.1743), as was *I* (0.3980 ± 0.2069 *vs* 0.3501 ± 0.2412). These results demonstrated that the range of genetic variation in the populations of *R. pseudoacacia* increased after spaceflight. It also suggested that the SSR and SRAP markers are effective markers for studying mutations and genetic diversity in *R. pseudoacacia*. The data provide valuable molecular evidence for the effects of the space environment on *R. pseudoacacia* and may contribute to future space-breeding programs involving forest trees.

Key words: Genetic diversity; Molecular markers; Spaceflight; Morphological traits; *Robinia pseudoacacia*

INTRODUCTION

The unique environment of outer space, including weightlessness, high charge, high radiation, high-energy particles, and features not present on Earth, can directly or indirectly affect metabolic activities and cause heritable DNA mutations in plants (Mashinsky and Nechitailo, 2001). However, the molecular effects of space environments on plants are largely unknown. Molecular-marker techniques have been widely used for detecting genetic mutations after space flight, including inter-simple sequence repeat markers (Gao et al., 2009; Wu et al. 2011), simple sequence repeat (SSR) markers (Zhou et al., 2007; Xiao et al., 2009; He et al., 2010; Lu et al., 2010), amplified fragment length polymorphism markers (Yi et al., 2002; Li et al., 2007; Lu et al., 2010; Sun et al., 2010), random amplification of polymorphic DNA (RAPD) markers (Liu et al., 1999; Gao et al., 2000; Yi et al., 2002; Nechitailo et al., 2005; Xie et al., 2010), and sequence-related amplified polymorphism (SRAP) markers (Chen et al., 2009; Wang et al., 2009). Among these molecular markers, SSRs are often employed because of their reproducibility, codominant inheritance, relative abundance, multiallelic nature, good genome coverage, and high frequency of polymorphism detection (Powell et al., 1996;

McCouch et al., 2002; Lu et al., 2010). However, the generated polymorphisms are limited to defined chromosomal segments. In contrast, SRAP markers combine reliability, simplicity, moderate throughput, and easy sequencing of selected bands. Furthermore, SRAP targets coding sequences in the genome and results in a moderate number of codominant markers (Li and Quiros, 2001). Thus, SRAP and SSR markers are complementary techniques for polymorphism analyses.

Although significant achievements have been made in spaceflight-induced mutation breeding, most studies have focused on crops; research on spaceflight-induced mutations in forest trees is in its infancy. Furthermore, the effects of space environments on plants, especially forest trees, at the molecular level are largely unknown. This limits the application of spaceflight-induced mutation on forest-tree breeding.

Robinia pseudoacacia displayed superior growth rates, energy yields, and other attractive traits (Stringer and Carpenter, 1986). In addition, *R. pseudoacacia* has advantages such as alkali resistance, drought and cold tolerance, and extensive adaptability; therefore, it has become an important ecological plant in China. However, for the traditional breeding of *R. pseudoacacia*, its breeding cycle is too long and produces limited variations. *R. pseudoacacia* can be propagated by asexual reproduction; therefore, once a beneficial mutation is obtained from spaceflight, it can be quickly produced and utilized.

Here, we present the first comparative analysis of genetic diversity among space-induced mutagenesis and ground-based control populations of *R. pseudoacacia* using SSR and SRAP molecular markers and morphological traits. Our objectives were to determine differences in genetic diversity between ground-based control and space-mutagenized seedlings of seeds that endured a 15-day flight, both at the molecular and morphological levels.

MATERIAL AND METHODS

Plant materials

Healthy, mature seeds of *R. pseudoacacia* from open-pollinated plants were collected from Henan Province of China in 2005. The seeds were randomly divided into 2 groups. One group was flown on the 'Shijian No. 8' breeding satellite for a 15-day spaceflight (September 9-24, 2006). The satellite had a 355-h flying time within 187 km for perigee and 463 km for apogee. The other group was kept on the ground and was later used as the ground-based control. Both the spaceflight and the ground-based control seeds were planted at the Experimental Station of Yanqing Sandstorm Source Centre in Beijing in 2007 under the same conditions. The field trial was conduced using a complete randomized block design with 3 replicates.

A total of 120 individual plants were randomly selected, including 60 space-mutagenized plants (SM) and 60 ground-based controls (OS). Young leaves were collected from each of the selected trees in May 2010 and were transported in a foam box containing ice bags and stored at -80°C for genomic DNA extraction.

Isolation of genomic DNA

Total genomic DNA from each leaf sample was extracted from 100 mg young leaves using the DNAsecure Plant Kit (TIANGEN, Beijing) according to manufacturer instructions.

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The genomic DNA was quantified on 1.0% agarose gels, and the DNA concentration was determined using a NanoVue ultramicrospectrophotometer. The samples were then diluted to a final concentration of about 20 ng/μL and stored at -20°C before polymerase chain reaction (PCR) amplification.

SSR amplification and selection of optimum primers

A total of 21 SSR primers [Generay Biotech (Shanghai) Co., Ltd., China] described by Lian and Hogetsu (2002), Lian et al. (2004), and Mishima et al. (2009) were initially screened in 10 samples from the SM and the OS individuals. Primers were excluded from the study if they did not show different band sizes or consistently failed to amplify products in 10 samples. A final set of 12 SSR primers that produced clear and reproducible fragments were selected for further analysis (Table 1).

Table 1. Sequences and statistical data of 12 SSR primers.

Locus	Motif	Primer sequence	Total number of alleles	Size ranges (bp)	PIC
Rops08	$(CA)_8TA(CA)_3$	TTCTGAGGAAGGGTTCCGTGG GTTAAAGCAACAGGCACATGG	7	191-205	0.5631
Rp102	$(GA)_{12}$	CCAAATCTCAAAATGTGCTAAGTAGC ACTTGGGCTATGGTATTGCA	3	205-211	0.5296
Rp206	$(GT)_9$	GCCAAATCCCATTAGATCACAGTTGA AGAAGTTAGACTTACGTGCTGC	7	222-246	0.5637
Rops16	$(CT)_{13}$	AACCCTAAAAGCCTCGTTATC TGGCATTTTTTGGAAGACACC	5	195-223	0.6663
Rops05	$(AC)_2GC(AC)_7$	TGGTGATTAAGTCGCAAG GTGGTTGTGACTTGTACGTAAGTC	3	120-138	0.5780
Rops15	$(CT)_{20}$	GCCCATTTTCAAGAATCCATATATTGG TCATCCTTGTTTTGGACAATC	8	112-254	0.8202
Rops150	$(TC)_3TT(TC)_{12}$	TCGTTGGATCAACATGCATGG ACAGAACCCTAACCCTAGCA	7	199-217	0.7180
Rops06	$(GT)_3ACA(GT)_{11}$	CTAAGGAGGTGCTGACCCTC TTAATCTGTGATGGGACACTG	3	117-144	0.5542
Rops02	$(AC)_{13}(AT)_4$	CAGAACTGTGGAGAATAATTCTGAACCG CGCCATCTGTTAGTTTGTTGC	2	107-138	0.4998
Rops18	$(AC)_8$	AGATAAGATCAAGTGCAAGAGTGTAAG TAATCCTCGAGGGAACAATAC	3	135-219	0.4763
Rp109	$(AG)_{17}$	GAGGAATCACAAAACCGTTTGG TGGGATTTGAGAGAGTGGTGGTG	5	136-160	0.7066
Rp200	$(AG)_{23}$	GGTTTCTTTGTTCACCTGCTCTGG ACCTACGTGTCCACGGCTCT	4	160-198	0.8493
Total			57		
Mean			4.5		0.6271

PIC = polymorphic information content.

SSR amplification was carried out by an ABI 9700 thermal cycler in a 12.5-μL reaction mixture containing 20 ng genomic DNA, 1X Taq buffer, 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.15 μM of each primer pair, and 0.25 U Taq DNA polymerase. The amplification reaction was performed with an initial denaturation step at 94°C for 5 min, 10 cycles of 94°C for 30 s, 63° to 53°C for 30 s (decreasing at 1°C per cycle), and 72°C for 90 s, then 20 cycles at the annealing temperature (53°C), and finally an extension at 72°C for 10 min. The amplified products were electrophoresed on 8.0% non-denaturing polyacrylamide gels run at 200 V in 1X TBE buffer, visualized by silver staining (1 g/L), and photographed with GS-800 Calibrated Densitometer (Bio-Rad). The molecular sizes of the amplified fragments were estimated by comparison to a DL2000 DNA marker.

SRAP amplification and selection of optimum primers

Using the optimized SRAP-PCR system previously reported (Yuan et al., 2011), 12 primer combinations that generated strong and clear amplified bands were selected for further population investigations (Table 2). The SRAP-PCR conditions were reported previously (Yuan et al., 2011). The reaction volume of 12.5 µL consisted of 0.30 µM of each primer pair, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.75 U Taq DNA polymerase, and 30 ng genomic DNA. DNA amplifications were performed with an initial step at 94°C for 5 min, 5 cycles of 1 min at 94°C, 1 min at 35°C, and 1.5 min at 72 °C, then 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min, and a final extension at 72°C for 10 min (Li and Quiros, 2001). The PCRs were carried out in an ABI 9700 thermal cycler. The SRAP-PCR products were analyzed on 8.0% non-denaturing polyacrylamide gels in 1X TBE buffer run at a constant voltage of 200 V for 70 min, silver stained, and then photographed with a GS-800 Calibrated Densitometer (Bio-Rad).

Table 2. Number of polymorphic bands obtained from 12 sequence-related amplified polymorphism primer combinations.

Primer combinations	No. of bands	No. of polymorphic bands	Polymorphism (%)
Em6-Me6	8	7	87.50
Em9-Me11	9	7	77.78
Em6-Me4	12	11	91.67
Em5-Me12	9	9	100.00
Em5-Me9	11	10	90.91
Em4-Me12	10	9	90.00
Em12-Me11	8	7	87.50
Em11-Me11	13	12	92.31
Em5-Me6	11	10	81.82
Em5-Me5	9	9	100.00
Em4-Me13	9	9	100.00
Em4-Me11	17	16	94.12
Total	126	116	
Mean	10.5	9.7	91.13

Morphological data

Twenty-two morphological traits were measured from each single tree of the 3 replicates of the field trial during 2 growing seasons of 2009-2011. These traits included plant height (PH), basal diameter (BD), number of branches (NB), knot spacing, trunk prickle length (TPL), trunk prickle base width, trunk prickle middle width (TPMW), branch stipular thorn length (BSTL), branch stipular thorn base width (BSTBW), branch stipular thorn middle width (BSTMW), number of leaflets of pinnate fronds, pinnate frond petiole length, leaflet length, leaflet width, leaflet petiole length (LPL), leaflet vertex angle (LVA), leaflet base angle, tippy leaf length, tippy leaf width, tippy leaf petiole length (TLPL), tippy leaf vertex angle (TLVA), and tippy leaf base angle. The means, maximum and minimum values, standard deviation, and coefficients of variation (CV) were calculated for each of the 22 morphological characteristics. These traits were chosen and measured according to the literature (The People's Republic of China Ministry of Agriculture, 2002).

Data analysis

The amplified fragments that were clear and well defined for SRAP were scored as either absent (0) or present (1) for all specimens. The genetic parameters of observed number of

alleles (N_O), effective number of alleles (N_E), number of polymorphic bands (*P*), percentage of polymorphic loci (*PPL*), Shannon's information index (*I*), and Nei's gene diversity index (*h*) were calculated using the POPGENE32 software. For SSR analysis, the bands were scored for each of the microsatellite primer pairs in each specimen based on presence or absence of the allele at a given locus, generating a matrix of 1 and 0. The PIC value of SSR loci was measured using the formula developed by Nei, (1973): $PIC = 1 - \sum X_k^2$, where X_k is the frequency of the k^{th} allele in the set of species investigated. SPSS (version 16, Chicago, IL, USA) was used for statistical analysis.

After the homogeneity of sample variances was verified using the Levene test, an independent sample *t*-test was used to compare means between the SM and OS groups for all morphological characteristics using the SPSS 16.0 software.

RESULTS

SSR analysis

Of 21 SSR primers, only 12 (57%) showed polymorphisms among SM and OS groups. The remaining 9 markers were either unstable or monomorphic in the present study. The polymorphic SSR markers showed 2 (in Rops02) to 8 (in Rops15) alleles with an average of 4.5 alleles per locus (Table 1). The PIC value varied from 0.4763 to 0.8493 with an average of 0.6271 per marker. Low PIC values were observed for the primers of Rops18 (0.4763) and Rops02 (0.4998). The PIC values of the remaining microsatellite loci were above 0.50 (Table 1).

At the group level, the SM group showed more polymorphic bands (51) and a slightly higher percentage of polymorphic loci (89.47%) than the OS group (46, 80.70%) (Table 3). In addition to *P* and *PPL*, *h* (Nei, 1973) and *I* were also calculated to estimate the level of genetic diversity. At the group level, the SM group showed higher *h* and *I* values (0.2534 ± 0.1533 and 0.3980 ± 0.2069) compared to the OS group (0.2240 ± 0.1743 and 0.3501 ± 0.2412) (Table 3).

Table 3. Genetic variations of *Robinia pseudoacacia* based on SSR marker.

Group	Sample size	*h*	*I*	*PL*	*PPL* (%)
SM	60	0.2534 ± 0.1533	0.3980 ± 0.2069	51	89.47
OS	60	0.2240 ± 0.1743	0.3501 ± 0.2412	46	80.70

Data are reported as means ± SD. *h* = Nei's gene diversity; *I* = Shannon's information index; *PL* = number of polymorphic loci; *PPL* = percentage of polymorphic loci. SM = space-mutagenized plants; OS = ground-based controls.

SRAP analysis

A total of 126 amplified bands were generated with 12 selected primers across the 120 plants in the SM and OS groups with an average of 10.5 bands per primer pair. Among these bands, 116 were polymorphic (P = 92.06%). The number of polymorphic bands generated by each primer ranged from 7 (em6/me6, em9/me11, and em12/me11) to 16 (em4/me11) with an average of 9.7 per primer (Table 2).

At the group level, *P* and *PPL* in the SM group were 115 and 91.27%, respectively, which was higher than that of the OS group (98 and 77.78%, respectively). N_O for the SM group was 1.9127 ± 0.2834, which differed significantly from that of the OS group (1.7778 ± 0.4174) (Table 4). Moreover, N_E, *h* (Nei, 1973), and *I* were also used to estimate the level of

genetic diversity. At the group level, the SM group showed higher N_E, h, and I values (1.4887 ± 0.3302, 0.2930 ± 0.1631, and 0.4452 ± 0.2177, respectively) than the OS group (1.4570 ± 0.3597, 0.2688 ± 0.1862, and 0.4031 ± 0.2596, respectively) (Table 4).

Table 4. Genetic variations of *Robinia pseudoacacia* based on SRAP marker.

Group	Sample size	N_O	N_E	h	I	P	PPL (%)
SM	60	1.9127 ± 0.2834*	1.4887 ± 0.3302	0.2930 ± 0.1631	0.4452 ± 0.2177	115	91.27
OS	60	1.7778 ± 0.4174	1.4570 ± 0.3597	0.2688 ± 0.1862	0.4031 ± 0.2596	98	77.78

N_O = observed number of alleles; N_E = effective number of alleles; h = Nei's gene diversity; I = Shannon's information index; P = number of polymorphic loci; PPL = percentage of polymorphic loci; SM = space-mutagenized plants; OS = ground-based controls. *Indicate significant difference compared to OS at the 0.05 probability level.

Morphology analysis

The CV values for 10 of the characteristics exhibited a high level of variation (>30%) across both groups. These included NB, TPL, TPMW, BSTL, BSTBW, BSTMW, LPL, LVA, TLPL, and TLVA (Table 5). Comparing the 22 characteristics, the number of branches showed the highest degree of variation (CV = 50.82% in the SM group and 49.67% in the OS group), and the number of leaflets of pinnate fronds showed the least variation (CV = 15.61% in the SM group and 15.24% in the OS group; Table 5). As a whole, the SM group showed slightly higher CV values in comparison with the OS group. The analysis of variance (mean square) shown in Table 5 revealed that some characteristics were significantly different between the SM and OS groups. These included PH (P = 0.038), BD (P = 0.000), NB (P = 0.004), BSTL (P = 0.001), BSTMW (P = 0.014), LVA (P = 0.000), and TLVA (P = 0.037). Other characteristics did not show significant differences between the 2 groups (P > 0.05).

Table 5. Morphological classification of characters and basic statistical data of samples.

Code	Morphological traits	SM group					OS group				
		Mean	Max.	Min.	SD	CV (%)	Mean	Max.	Min.	SD	CV (%)
PH	Plant height (cm)*	354.50	568.00	161.00	81.70	23.05	379.70	587.00	192.00	92.11	24.26
BD	Basal diameter (mm)**	40.23	71.12	15.05	12.00	29.83	47.36	90.00	12.95	13.07	27.60
NB	Number of branches**	8.54	25.00	1.00	4.34	50.82	10.45	27.00	2.00	5.19	49.67
KS	Knot spacing (cm)	3.06	4.70	1.50	0.64	20.92	3.06	5.55	0.83	0.76	24.84
TPL	Trunk prickles length (mm)	11.21	30.59	3.18	4.99	44.51	12.70	22.95	3.96	4.04	31.81
TPBW	Trunk prickles base width (mm)	6.87	13.23	2.48	1.77	25.76	7.16	10.59	2.55	1.84	25.70
TPMW	Trunk prickles middle width (mm)	2.31	8.54	0.72	0.89	38.53	2.40	6.70	0.99	0.77	32.08
BSTL	Branch stipular thorn length (mm)**	10.46	22.97	1.98	4.69	44.84	12.08	24.19	2.73	4.64	38.41
BSTBW	Branch stipular thorn base width (mm)	4.19	9.65	1.05	1.66	39.62	4.85	10.36	1.89	1.96	40.41
BSTMW	Branch stipular thorn middle width (mm)*	1.88	5.54	0.44	0.72	38.30	2.08	4.33	0.67	0.80	38.46
NLPF	Number of leaflet of pinnate fronds	17.74	25.00	11.00	2.77	15.61	17.65	24.00	13.00	2.69	15.24
PFPL	Pinnate fronds petiole length (mm)	17.47	51.06	7.60	6.26	35.83	18.59	31.28	7.63	5.32	28.62
LL	Leaflet length (mm)	27.73	61.67	6.65	10.60	38.23	31.15	49.49	10.22	8.46	27.16
LW	Leaflet width (mm)	13.20	34.99	4.27	4.53	34.32	14.46	38.68	6.44	3.66	25.31
LPL	Leaflet petiole length (mm)	2.22	5.98	0.81	0.77	34.68	2.59	5.54	0.64	0.87	33.59
LVA	Leaflet vertex angle (°)**	136.07	303.09	54.98	43.70	32.12	113.47	245.56	49.96	39.67	34.96
LBA	Leaflet base angle (°)	124.44	186.60	60.83	19.30	15.51	114.11	176.31	61.89	23.51	20.60
TLL	Tippy leaf length (mm)	28.91	62.49	5.58	10.30	35.63	31.05	53.32	14.35	8.20	26.41
TLW	Tippy leaf width (mm)	16.53	37.67	7.24	5.42	32.79	16.59	27.48	7.32	4.36	26.28
TLPL	Tippy leaf petiole length (mm)	9.17	22.55	3.14	3.61	39.37	9.28	15.92	2.60	2.83	30.50
TLVA	Tippy leaf vertex angle (°)*	176.46	300.96	59.53	67.20	38.08	144.27	288.25	58.69	57.97	40.18
TLBA	Tippy leaf base angle (°)	99.47	141.57	68.22	15.70	15.78	93.59	137.75	52.86	18.15	19.39

SM = space-mutagenized plants; OS = ground-based controls; Max. = maximum; Min. = minimum; SD = standard deviation; CV = coefficient of variation. *Significantly different compared to OS at the 0.05 probability level. **Significantly different compared to OS at the 0.01 probability level.

DISCUSSION

In this study, genetic diversity between SM and OS *R. pseudoacacia* was analyzed by the SSR and SRAP marker systems combined with phenotypic traits.

The SSR and SRAP analyses showed that the SM group had a higher degree of genetic diversity (P = 115, PPL = 91.27%, N_O = 1.9127 ± 0.2834, N_E = 1.4887 ± 0.3302, h = 0.2930 ± 0.1631, I = 0.4452 ± 0.2177 with SRAP marker system; P = 51, PPL = 89.47%, h = 0.2534 ± 0.1533, I = 0.3980 ± 0.2069 with the SSR marker system) than the OS group (P = 98, PPL = 77.78%, N_O = 1.7778 ± 0.4174, N_E = 1.4570 ± 0.3597, h = 0.2688 ± 0.1862, I = 0.4031 ± 0.2596 with the SRAP marker system; P = 46, PPL = 80.70%, h = 0.2240 ± 0.1743, I = 0.3501 ± 0.2412 with the SSR marker system). For morphological traits, there was also some variation between the SM and OS groups. This indicated that there are some variations in the SM population that may have been caused by spaceflight. It has been proposed that spaceflight exposure in seeds or plants could lead to mutants for crop improvement (Normile and Yimin, 2002; Liu et al., 2004), although the mechanisms of space-induced mutagenesis are largely unknown. In this study, the 2 groups of seeds (SM and OS) came from and were cultivated in the same source. The only difference between the 2 groups was that the SM group was exposed to the space environment for 15 days, while the OS group was kept on Earth. There are profound differences between spaceflight conditions (weightlessness, high charge, high radiation, high energy particles, and other space factors) and those on Earth, and the unique environment of space may cause DNA variations in seeds of *R. pseudoacacia*. Thus, we suspect that the higher genetic diversity and morphologic variation observed in the SM *R. pseudoacacia* were caused by short-term spaceflight of the original seeds or reflect their adaptive response to space environments after growth on Earth. Similar results were reported by Wu et al. (2011) using ISSR markers to study genetic diversity of *Cistanche deserticola* after spaceflight. Their results also showed that the genetic diversity of the spaceflight specimens was higher than that of the ordinary specimens. Nechitailo et al. (2005) conducted an amplification analysis on the genomic DNA of tomato seedlings grown from seeds carried in the Russian MIR space station using RAPD technology. The results showed that, compared with the controls, plants from seeds carried in the space station generated different band patterns of amplified fragments. The rates of DNA mutations in the 5 plants from seeds carried in the space station were 8.4, 3.2, 2.8, 6.0, and 9.2%, and the rates of DNA mutation in controls were 0 and 0.4%.

The degree of polymorphism exhibited by the SSR and SRAP banding patterns in our study was relatively high (approximately 85 and 90%, respectively). Seedlings from 23 seed sources representing the natural range of black locust (*R. pseudoacacia* L.) were electrophoretically analyzed at 40 structural loci representing 18 enzyme systems by Surles et al. (1989), and an average of 71% polymorphism was found. Sun et al. (2009) observed a 93.41% rate of polymorphism in 10 populations of *R. pseudoacacia* in China using 10 selected primers for ISSR. Both of these studies indicated that *R. pseudoacacia* maintains very high levels of genetic diversity. Thus, we attributed the high degree of polymorphism as measured by SSR and SRAP banding patterns in our study to the higher level of genetic diversity of *R. pseudoacacia*.

The results of the present study demonstrate that the higher level of genetic diversity and morphologic variation in the SM group compared to the OS group might be caused by short-term spaceflight of the original seeds or could reflect their adaptive response to space environments after growth on Earth.

CONCLUSION

The present results showed molecular and morphological differences between the SM and OS groups. This suggests that the SSR and SRAP markers are effective for studying mutations and genetic diversity of *R. pseudoacacia*. These results indicate that genetic variation in *R. pseudoacacia* populations increases to some extent after spaceflight.

This is the first report to demonstrate the applicability of 2 molecular marker methods combined with morphological traits for evaluating the extent of mutation after spaceflight in forest trees. In addition, to the best of our knowledge, this is the first demonstration that spaceflight causes mutations in forest trees and increases genetic variation at the population level.

However, our study only identified differences between the SM and OS groups. For a better understanding of the mechanism of mutations (e.g., mutation rate and types) induced by spaceflight, further experiments will be necessary. Specifically, it will be important to select variants and clone and sequence the mutated fragments. This further study is currently in progress.

Our study investigated the molecular and morphologic effects of spaceflight on *R. pseudoacacia* seedlings obtained from seeds that endured a 15-day flight. Our results may provide valuable molecular evidence for the effects of the space environment on forest tree species and may contribute to future space breeding programs of forest trees.

ACKNOWLEDGMENTS

Research supported by the Special Fund Project for the Scientific Research of the Forest Public Welfare Industry (#201104013), the National Science Foundation of China (#31170629, #30640036), and the Chinese Academy of Agricultural Sciences (#2006HT100130). We also thank Dr. Gan Siming and Li Zhouqi for their kind help with revision of the manuscript and for valuable discussions. We also thank Professor Li Genqian for his enlightening discussions.

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