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BRIEF COMMUNICATION

Changes in AFLP and SSR DNA polymorphisms induced by short-term space flight of rice seeds

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Abstract

Differences of both amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) polymorphisms were compared between the 60-d-old rice (*Oryza sativa* L. cv. DH7) and F3 rice plants (SP3) derived from seed, which endured a 7-d-space flight in March 2002. Total leaf AFLP DNA bands amplified from 22 primer pairs were 537 in DH7, whereas 562 in SP3. From the total 267 SSR DNA bands generated by 267 primer pairs, 39 were polymorphic with 22 larger (56 %) or 17 smaller (44 %) fragment size bands. The greatest numbers of AFLP DNA bands were amplified by primer E1M1 in DH7 (33) and E3M1 in SP3 (35), whilst the least by E4M3 in DH7 (14) and E5M2 in SP3 (16).

Additional key words: fragment size, *Oryza sativa*, primer pairs.

The unique outer space environments, including high-energy cosmic radiation, micro-gravity, and ultra vacuum, are different from those on the Earth. In either short-term (hours to days) or long-term (months to years) experiments onboard spacecrafts had showed that plant growth and metabolic activities were directly or indirectly affected by space environments (Halstead and Dutcher 1987, Dutcher *et al.* 1994, Stankovic 2001, Bubenheim *et al*. 2003, Yu *et al*. 2007). Those growth and metabolic differences might reflect plant adaptive responses to space environments, and a synergy of space microgravity and radiation was probably the most significant (Stankovic 2001). However, effects of space environments on plants at the molecular level are largely unknown.

Four DNA markers [amplified fragment length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and simple sequence repeats (SSR)] have been widely used for detecting DNA polymorphisms in plants (Philips and Vasil 2001, Varshney *et al.* 2004, Ovesná *et al*. 2005, Joshi *et al*. 2007, Mo *et al.* 2008, Yao *et al.* 2008, Akriditis *et al.* 2009), including rice (Virk *et al.* 2000, Garris *et al.* 2005). For example, a 13.2 % leaf RAPD polymorphism was detected in the first-generation of sweet pepper from seeds endured a 5-d flight with a Chinese satellite (Liu *et al.* 1999). Recently, a 10.8 % RAPD polymorphism was observed in leaves from tomato seeds endured a 6-year flight in the Mir (Nechitailo *et al*. 2005). Among them, AFLP and SSR are more widely used than RAPD and RFLP for field crops (Powell *et al.* 1996, Russell *et al.* 1997, Philips and Vasil 2001, Ren *et al*. 2003, Varshney *et al.* 2004, Jeung *et al.* 2005). The AFLP markers are highly efficient to conduct background selection with the highest diversity index of genotypes, and both defined and undefined chromosomal segments are amplified with short time and low cost (Russell *et al.* 1997, Philips and Vasil 2001). In contrast, the SSR markers are often employed because of their reproducibility, multiallelic nature, codominant inheritance, relative abundance, good genome coverage and high frequency of polymorphism detection (Powell *et al.* 1996; McCouch *et al.* 2002), but the generated polymorphisms are limited to defined chromosomal segments. Thus AFLP and SSR markers effectively complement each other for polymorphism analyses.

Rice as a major food resource was among the most

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Abbreviations: AFLP - amplified fragment length polymorphism; SSR - simple sequence repeat.
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frequent space passengers (Liu *et al*. 2004, Chen *et al.* 2006). The objective of this study was to determine differences of both AFLP and SSR DNA polymorphisms between a doubled haploid rice (*Oryza sativa* cv. DH7) and a premature mutant SP3 obtained after three generations from the DH7 seeds that endured a 7-d-space flight in China's "Shenzhou-III" Spacecraft in March 2002. The spacecraft had a 96 hours flying by 108 orbits within 250 km for perigee and 350 km for apogee. The radiation ranged from 0.095 to 0.197 mGy and microgravity 10^{-3} to 10^{-6} g with 22 °C in the seed capsule during the flying. Both the original DH7 and the SP3 were then grown in a greenhouse at the Beijing Spaceflight Center, China (14-h photoperiod with irradiance of 165 μmol m^{-2} s^{-1}, day night temperature of 27/21 °C and relative humidity of 70 %). The seedlings were irrigated weekly with nutrient solution. Fully expanded fresh leaves from 20 individual plants (60-d-old) of both the DH7 and SP3 were collected, mixed and stored at -80 °C. Leaf tissue powder (2 g) was extracted by 15 cm^3 SDS buffer, precipitated by 15 cm^3 isopropyl alcohol according to McCouch *et al.* (1988), then washed with 70 % ethanol, re-suspended in 700 cm^3 of TE buffer (10 mM Tris-HCl + 1 mM EDTA, pH 8.0), and incubated with 10 cm^3 of RNase (20 mg cm^{-3}) at 37 °C for 30 min. After incubation, 0.7 cm^3 of chloroform/isoamyl alcohol (24:1, v/v) was added and then the mixture was centrifuged at 12 000 *g* for 10 min at 4 °C. The upper DNA-containing phase was transferred to a new Eppendorf tube. DNA was precipitated by 3 M NaAc (pH 5.2) and 100 % ethanol (1:2, v/v) and collected by centrifugation at 1 200 *g* for 5 min. The DNA pellet was air dried, and re-suspended in 0.05 cm^3 of TE buffer. The extracted DNA was then used for AFLP or SSR analysis.

A total of 22 *Eco*RI and *Mse*I primer pairs (Table 1, 2) were used for AFLP analysis (Vos *et al.* 1995, Cho *et al.* 1996). Purified genomic DNA (250 ng) was digested with *Mse*I and *Eco*RI at 37 °C for 3 h and ligated with *Mse*I and *Eco*RI adapters at 37 °C for 10 h. Ligations were diluted 10-fold in sterilized water and used as templates in the pre-amplification PCR reaction. Pre-selective amplification was carried out by using primers with an additional base at the 3' end in order to reduce numbers of fragments. Conditions for pre-selective amplification were 2 min pre-denaturation at 95 °C and followed by 30 cycles of 30 s denaturations at 95 °C, 30 s annealing at 56 °C and 60 s extensions at 72 °C. Pre-amplified products (0.005 cm^3) were then run on electro-phoresis (1 % agarose gel) to confirm the size and purity of the PCR products. Selective amplification was performed with 20-fold diluted pre-selective products with primers (Table 1) for 2 min at 95 °C, 12 cycles of 50 s at 95 °C, 40 s at 65 °C, 60 s at 72 °C, including a 1 °C reduction per cycle; then 31 cycles of 50 s at 95 °C, 40 s at 56 °C, 60 s at 72 °C, and for 5 min at 72 °C for final extension. Products from the selective amplification (0.006 cm^3) were electrophoresed in 6 % denaturing gel with 7 M urea. AFLP bands were visualized following autoradiography (*Kodak*™ *Biomax*™ MR film). Bands in the gel were analyzed by the *Bandscan 4.0* (*Glyko*, Novato, CA, USA).

Table 1. Serial numbers and sequences of primer used in pre-amplification and amplification in AFLP analyses. E_0 and M_0 indicate pre-amplification primers.

Primer	Sequence (5'---3')
E_0	GAC TGC GTA CCA ATT CA
E1	GAC TGC GTA CCA ATT CA AAC
E2	GAC TGC GTA CCA ATT CA AAG
E3	GAC TGC GTA CCA ATT CA ACA
E4	GAC TGC GTA CCA ATT CA ACC
E5	GAC TGC GTA CCA ATT CA ACG
E6	GAC TGC GTA CCA ATT CA ACT
E7	GAC TGC GTA CCA ATT CA AGC
E8	GAC TGC GTA CCA ATT CA AGG
M_0	GAT GAG TCC TGA GTA AC
M1	GAT GAG TCC TGA GTA AC CAA
M2	GAT GAG TCC TGA GTA AC CAC
M3	GAT GAG TCC TGA GTA AC CAG

Table 2. Number of AFLP bands generated with 22 AFLP primer pairs in the control DH7 and the SP3 plant. Means are significantly different at $P \leq 0.01$ (*t*-test).

Primer pair	Number of bands DH7	SP3	total	Polymorphic bands DH7	SP3
E1M1	31	32	34	2	3
E2M1	33	31	35	4	2
E3M1	27	35	35	0	8
E4M1	24	24	27	3	3
E5M1	26	27	29	2	3
E6M1	28	27	29	2	1
E7M1	25	32	32	0	7
E8M1	29	28	34	6	5
E1M2	19	17	20	3	1
E2M2	26	30	32	2	6
E3M2	28	27	33	6	5
E4M2	20	18	21	3	1
E5M2	18	16	26	10	8
E6M2	19	21	23	2	4
E7M2	30	29	32	3	2
E8M2	23	22	25	3	2
E1M3	20	25	26	1	6
E2M3	28	28	30	2	2
E3M3	19	22	23	1	4
E4M3	14	24	24	0	10
E5M3	27	26	27	1	0
E6M3	23	21	25	4	2
Mean±SE	24.4±1.0	25.6±1.0	28.3±1.0	2.7±0.5	3.9±0.6

A total of 267 SSR primer pairs (*Sangon Biological Engineering Technology & Service Co*., Shanghai, China; sequences are available at http://mtm.cshl.org/microsat/ssr.html) were used for SSR analysis (Panaud *et al.* 1996, Temnykh *et al.* 2000). Genomic DNA concentration was

80 ng per sample. The amplified program was first performed by 5 min pre-denaturation at 94 °C, followed by 1 min denaturation at 94 °C, 1 min annealing at 55 °C, then 2.5 min extension at 72 °C for 35 cycles, and final extension for 10 min at 72 °C. The amplification products were loaded into a 4 % agarose gel for electrophoresis in 1× TBE buffer. Gels were stained with ethidium bromide and photographed by *Alpha Imager*^{TM} *System 2200* (*Alpha Innotech Corporation*, San Leandro, CA, USA).

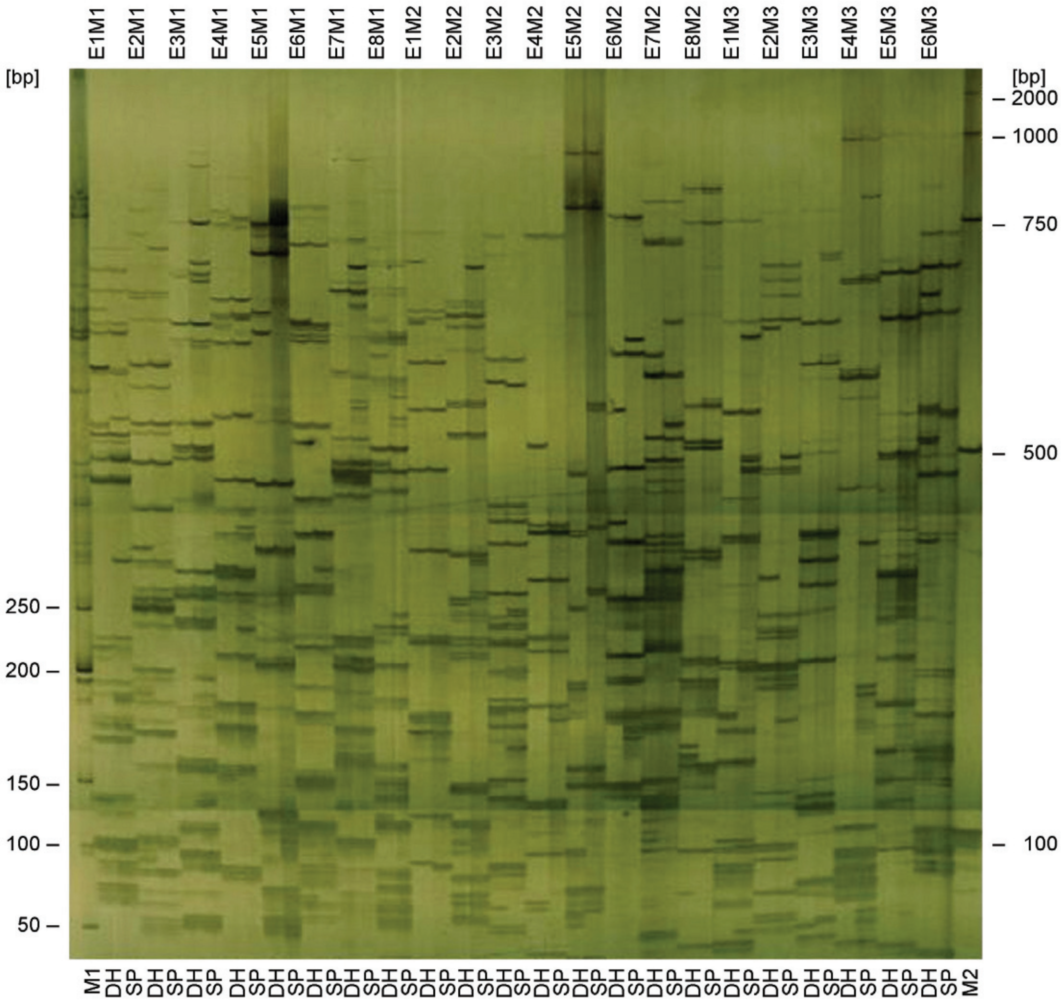

Fig. 1. AFLP amplification using leaf genomic DNAs as templates. EaMb represented combination of amplification primers of *Eco*RI and *Mse*I digest enzymes (see Table 1 for primer sequences). M1 and M2 - 100 bp DNA ladder, DH - control DH7 plant; SP- SP3 plant.

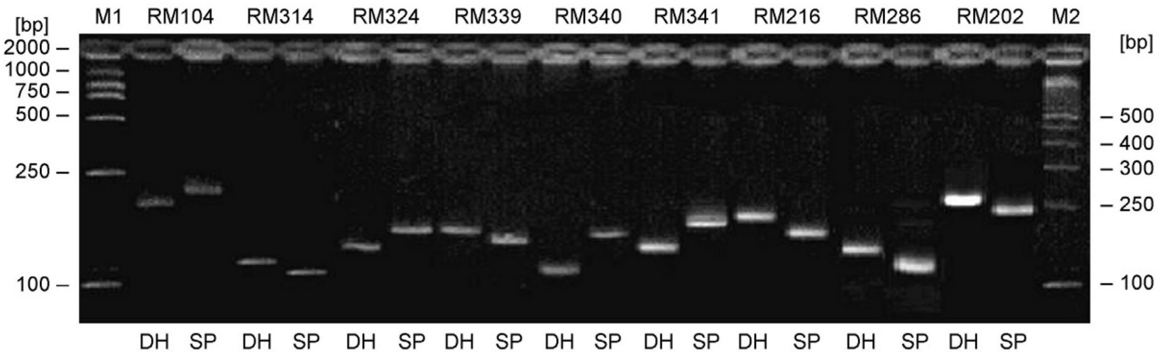

Fig. 2. SSR patterns amplified by SSR primers of RM104, RM314, RM324, RM339, RM340, RM341, RM216, RM286, RM202. M1 and M2 - 100 bp DNA ladder, DH - control DH7 plant, SP - SP3 plant.

For the 22 AFLP primer pairs used, both the total and the average DNA bands were greater in SP3 (562, 25.6 ± 1.0 %) than in DH7 (537, 24.4 ± 1.0 %) (Table 2, Fig. 1). Among the total 622 DNA bands amplified, 477 were presented in both DH7 and SP3 plants, and 145 bands were polymorphic (23.3 %). Of those 60 (9.6 ± 1.8 %) were exclusively displayed in DH7 and 85 (13.7 ± 2.1 %) in SP3 only. The greatest numbers of AFLP DNA bands were amplified by the primer E1M1 in DH7 (33) and E3M1 in the SP3 (35), while the least by E4M3 in DH7 (14) and E5M2 in SP3 (16) (Table 2).

A total of SSR 267 bands were generated by 267 SSR primers in both the DH7 and the SP3, and 39 (14.6 %) were exclusively polymorphic (the whole 306 SSR amplifications were not shown). Of those 39 polymorphic, the SSR fragment size was larger in 22 (56 %) and smaller in 17 (44 %). Compared to the DH7, PCR amplification fragments in the SP3 were increased from the primers of RM104, RM324, RM340 and RM341, but decreased from RM202, RM216, RM286, RM314 and RM339 (Fig. 2).

DH7 was one of 127 widely-used stable lines in China from a total of 133 DH lines by anther culturing of a F1 hybrid between Zhaiyeqing 8 (an *indica* variety) and Jingxi 17 (a *japonica* variety). Any line of those 127 lines is homozygous and identification of their quantitative trait locus controlling grain yield and quality had been reported (Lu *et al.* 1996, He *et al.* 2001). The third (Yu *et al*. 2007) and nine generation (Li *et al*. 2007) of rice mutants had been used to study effects of spaceflight environments on rice mutations. The third generation SP3 from the short-term 7-d-space-flown DH7 seeds was therefore chosen in this study, instead of the first generation of space-flown plants as usual counterparts.

Our results showed differences at the molecular level by both AFLP and SSR assays between the DH7 and the SP3 plant. We first performed AFLP analyses (Table 2, Fig. 1), which reflect difference of non-repetitive sequences (Cho *et al.* 1996, Vos *et al.* 1995). Our study showed 9.6 ± 1.8 % and 13.7 ± 2.1 % AFLP polymorphisms in the DH7 and in the SP3. Effects of spaceflight exposure on 11 rice cultivars from the same 7-d "Shenzhou-III" mission found a range of 1.6 to 10.8 % AFLP polymorphisms among the 10 japonica rice but 10.8 to 31.4 % in the indica rice genotypes (Yu *et al*. 2007). Another AFLP study found that 59.6 % was polymorphic in 17 rice genotypes, including three japonica mutants obtained by 15-d space flight with a recoverable satellite (Li *et al*. 2007). The 23.3 % total polymorphism in the present study was thus comparable as the SP3 was from a third generation between indica and japonica. We then performed SSR analyses (Table 2, Fig. 2), which reflect differences in repetitive sequences (Philips and Vasil 2001, Varshney *et al.* 2004). With a 14.6 % polymorphism, 85.4 % SSR bands were overlapped between the SP3 and the control DH7 in our study. Those genomic DNA differences demonstrated by both AFLP and SSR assays in the SP3 might result from the effect of short-term space-flight on the original seeds or reflect their adaptive response to space environments after grown on Earth (Stankovic 2001). It has been proposed that space-flight exposure to seeds or plants could lead to screen mutants for crop improvement (Dennis and Ding 2002, Liu *et al*. 2004), though the mechanisms of such space-induced mutagenesis are largely unknown. More investigations on performance of next (SP4) or further generation from those SP3 rice are thus warranted.

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