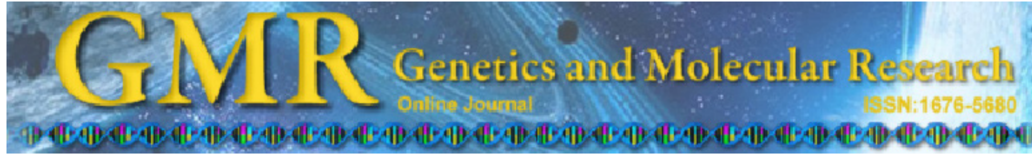

SRAP analysis of DNA base sequence changes in lotus mutants induced by Fe^{+} implantation

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ABSTRACT. Ion implantation, a new biophysically mutagenic technique, has shown great potential for crop breeding. To reveal the mutation effect of low-energy ion implantation on Baiyangdian red lotus, sequence-related amplified polymorphism markers were used to amplify and detect the DNA sequence differences in mutants induced by Fe^{+} ion implantation. A total of 121 primer combinations were tested in 6 mutants and a control. Seven primer combinations (me1 + em3, me1 + em14, me9 + em3, me8 + em2, me6 + em1, me11 + em5, and me6 + em5) generated clear bands with high polymorphism and good repeatability. The results showed that among 15,317 bases cloned, 146 bases in 6 mutants were different from those of the wild type, showing a variation frequency of 0.95%. The types of base changes included deletion, insertion, transversion, and transition. Adenine was more sensitive to the irradiation than were the other bases. The results suggested that mutational "hotspots" probably exists in lotus and are induced by low-energy ion implantation.

Key words: Baiyangdian red lotus; Ion implantation; SRAP marker; Specific fragment; Sequence analysis

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INTRODUCTION

Lotus, also known as water lily, is one of the top 10 traditional garden flowers and an economically valuable aquatic plant belonging to the genus *Nelumbo*, family Nelumbonaceae. In China, *Nelumbo* is the aquatic crop with the largest planting area and richest varieties. Lotus is rich in nutrition and widely used; every part of lotus is valuable. Lotus has great potential for use as food, ornamentals, medicine, and packaging (Zhou et al., 2002). In recent years, ornamental lotus has become popular in flower market consumption for its capability for beautifying cities, decking gardens, and dressing up courtyards. Therefore, broad market incentives exist for improving the genetic quality of the lotus and breeding new varieties with high ornamental value - e.g., multicolor, multi-flowering, long flowering period.

As a new and developing mutation breeding method, ion beam implantation is characterized by a low damage rate, high mutation rate, and wide mutation spectrum. Breeding technology using mutations induced by ion implantation has been applied in crops, ornamental plants, medicinal plants, and microorganisms (Wang et al., 2008). In 2005, new lotus mutants were generated using an ion implantation method. After 3 generations of observation, 6 lotus mutants with different floral designs, flower colors, and flowering periods have been identified. According to a popular theory, the mutation mechanism occurs through energy absorption, mass deposition, and the charging of energetic ions in plant seeds, which result in sequential bio-effects. To date, few investigations focused on the base sequence changes induced by low-energy ion implantation in higher plants.

In the current investigation, sequence-related amplified polymorphism (SRAP) DNA markers were used to amplify the genomic DNA of lotus mutants and controls. Then, SRAP fragments of the mutants and the controls in radiation-sensitive sites were cloned and sequenced for base sequence comparison.

MATERIAL AND METHODS

Plant material

Fe^{+} ions were used to bombard the seeds of Baiyangdian red lotus under vacuum conditions at 2 MeV with a fluence of 1 x 10^{12} ions/cm^2. The corresponding untreated species were regarded as controls. A total of 200 seeds, including half of the treated seeds and half of the controls, were soaked for approximately 1 month and then transplanted to tanks. All seeds used in the experiment were harvested from the same homozygote plant. The fine variable plants had been screened in 2006. The mutants - including lotus 1, lotus 2, lotus 3, lotus 4, lotus 5, and lotus 6 - and the controls (provided by Prof. Ying Su from Beijing Normal University) were chosen as the experimental materials in 2009 (Figure 1).

DNA extraction

Total genomic DNA was isolated from leaf tissues of the 6 mutants (F_3 generation) and the controls using an improved cetyltrimethylammonium bromide method according to a procedure described by Guo et al. (2004). The integrity of the purified genomic DNA was evaluated using 0.8% agarose gel electrophoresis.

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Figure 1. Materials used in this study. Lotus 1-6 = mutants 1-6.

SRAP

Polymerase chain reaction (PCR) primer pairs were synthesized by Shanghai Sangon (Shanghai, China) (Table 1) in a deoxyribonucleotide triphosphate mixture, Mg^{2+}, and *Taq* DNA polymerase (TaKaRa, Dalian, China). PCR was carried out in a 25-μL reaction mixture consisting of 2.5 μL 10X PCR buffer (Mg^{2+} free), 1.5 μL (2.5 mM of each) deoxyribonucleotide triphosphate, 0.5 μL 10 μM primers, 1.8 μL 25 mM Mg^{2+}, 1.5 U *Taq* polymerase, and 40-80 ng template DNA. Amplification was carried out with the following PCR program: 1 cycle at 94°C for 5 min, 5 cycles at 94°C for 1 min, 35°C for 1 min, and 72°C for 1 min, and then 35 cycles for which the annealing temperature was increased to 50°C. A final 8-min extension at 75°C was also performed. PCR products were stored at 4°C.

Cloning and sequencing of special DNA fragments

The putative special band amplified in the SRAP experiments was excised from 1.8% agarose gel using a UNIQ-10 DNA gel extraction kit (Shanghai Sangon). The purified target DNA fragments were cloned using pMD19-T vector (TaKaRa) according to manufacturer in-

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structions. The chimeric plasmid was transferred to *Escherichia coli* strain DH5α via chemical transformation. Cloned fragments were sent to Shanghai Sangon for sequencing. Sequences were comparably analyzed with the DNAMAN software.

RESULTS

Screening specific elements of 6 mutants using SRAP

A total of 121 primer combinations were tested in 6 mutants and a control. Seven primer combinations generated clear bands with high polymorphism and good repeatability (Figure 2). The 7 primer combinations were me1 + em3, me1 + em14, me9 + em3, me8 + em2, me6 + em1, me11 + em5, and me6 + em5 (for primer sequences, see Table 1). To avoid contamination and genetic heterogeneity, a negative PCR control with no template DNA was carried out simultaneously, and all experiments were repeated at least 3 times. The specific DNA fragments were cloned and sequenced.

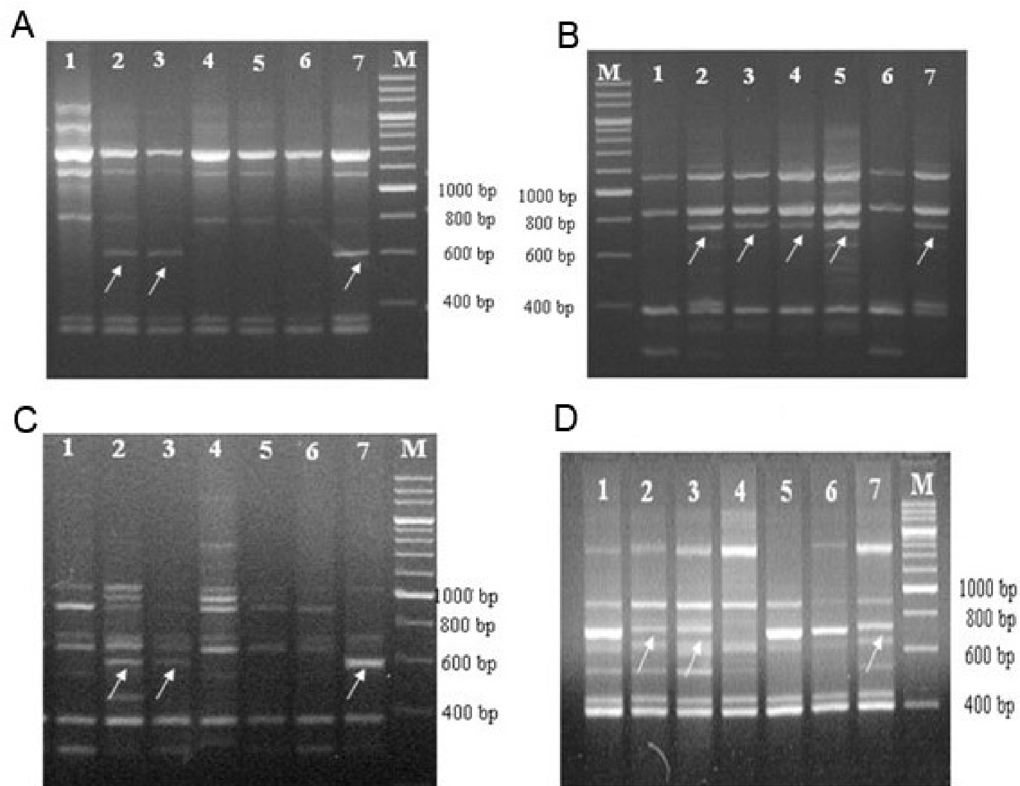

Figure 2. SRAP pattern of 6 mutants and the control. **A.** Amplified result of primer me11 + em5. **B.** Amplified result of primer me6 + em5. **C.** Amplified result of primer me1 + em6. **D.** Amplified result of primer me2 + em8. *Lane 1* = Lotus 1; *lane 2* = lotus 2; *lane 3* = lotus 3; *lane 4* = lotus 4; *lane 5* = lotus 5; *lane 6* = lotus 6; *lane 7* = control. Arrows show target bands cloned.

Table 1. SRAP primers used in this study.

Forward primers	Sequences (5'-3')	Reverse primers	Sequences (5'-3')
me1	5'-TGAGTCCAAACCGGATA-3'	em1	5'-GACTGCGTACGAATTAAT-3'
me2	5'-TGAGTCCAAACCGGAGC-3'	em2	5'-GACTGCGTACGAATTTGC-3'
me3	5'-TGAGTCCAAACCGGAAT-3'	em3	5'-GACTGCGTACGAATTGAC-3'
me4	5'-TGAGTCCAAACCGGACC-3'	em5	5'-GACTGCGTACGAATTAAC-3'
me5	5'-TGAGTCCAAACCGGAAG-3'	em6	5'-GACTGCGTACGAATTGCA-3'
me6	5'-TGAGTCCAAACCGGTAA-3'	em7	5'-GACTGCGTACGAATTCAA-3'
me7	5'-TGAGTCCAAACCGGTCC-3'	em8	5'-GACTGCGTACGAATTCTG-3'
me8	5'-TGAGTCCAAACCGGTGC-3'	em9	5'-GACTGCGTACGAATTCGA-3'
me9	5'-TGAGTCCAAACCGGATG-3'	em12	5'-GACTGCGTACGAATTATG-3'
me10	5'-TGAGTCCAAACCGGGAT-3'	em14	5'-GACTGCGTACGAATTACG-3'
me11	5'-TGAGTCCAAACCGGGCT-3'	em15	5'-GACTGCGTACGAATTGAT-3'

Sequence analysis

To reveal the changes in DNA base sequence induced by low-energy Fe^+ ion implantation in Baiyangdian red lotus, we compared the sequences of cloned SRAP fragments with their homologs using DNAMAN. The serial numbers of the mutation sites, types, and frequencies are listed in Tables 2-8. We detected 146 base changes in 15,317 bases and calculated a mutation frequency of 0.95%. In the fragments specific for lotus 1, lotus 2, lotus 3, lotus 4, lotus 5, and lotus 6, base mutation frequencies of 1.41, 0.92, 0.53, 0.78, 1.50, and 0.93% appeared, respectively.

Table 2. Result of sequence alignment of primer me1 + em3.

Mutants	17th site	50th site	174th site	309th site	488th site	513th site	585th site	646th site
Control (754 bp)	C	T	A	A	A	G	G	C
Lotus 1	T	C	A	A	T	A	G	C
Lotus 3	T	C	A	A	T	A	G	C
Lotus 4	T	C	A	G	T	A	G	C
Lotus 6	T	C	G	A	T	A	A	T

Table 3. Result of sequence alignment of primer me11 + em5.

Mutants	491th site	570th site
Control	A	G
Lotus 2	-	G
Lotus 3	A	A

Table 4. Result of sequence alignment of primer me6 + em1.

Mutants	475th site
Control	C
Lotus 2	T
Lotus 3	T

Table 5. Result of sequence alignment of primer me8 + em2.

Mutants	475th site
Control	C
Lotus 2	T
Lotus 3	T

Table 6. Result of sequence alignment of primer me6 + em5.

Mutants	43th site	74th site	79th site
Control	A	A	T
Lotus 2	A	A	T
Lotus 3	A	A	T
Lotus 4	G	G	C
Lotus 5	A	A	T

Table 7. Result of sequence alignment of primer me1 + em14.

Mutants	29th site	81th site	94th site	173th site	182th site	248-249th site	254th site	264th site	292th site	423th site	485th site	501-502th site	503th site	666th site	681th site	690th site	711th site
Control	T	A	T	C	G	CA	G	G	T	C	A	-	G	T	G	A	C
Lotus 3	A	G	C	T	A	GC	A	G	C	T	G	CC	A	C	A	T	T
Lotus 6	T	G	C	T	A	GC	A	A	C	T	G	CC	A	C	A	T	T

Table 8. Result of sequence alignment of primer me9 + em3.

Mutants	109 site	177 site	183-197 site	219 site	234 site	397 site	579 site	618 site	726 site	732 site	780 site	849 site	897 site	901 site	922 site	929-930 site
Control	-	A	AGAAGAAGAAGAAGA	A	G	T	T	T	A	T	A	G	T	G	G	CC
Lotus 1	A	G	-	G	A	C	T	C	C	C	A	G	C	A	C	-
Lotus 2	A	A	-	G	A	T	A	C	C	T	A	A	T	A	C	-
Lotus 3	A	A	AGAAGAAGAAGAAGA	A	G	T	T	T	A	T	A	G	T	G	G	CC
Lotus 4	A	A	AGAAGAAGAAGAAGA	A	G	T	T	T	A	T	A	G	T	G	G	CC
Lotus 5	A	A	-	G	A	T	T	C	C	T	T	G	T	A	C	-

Analysis of base change types and characteristics of genomic DNA variations of mutants

Compared with the control sequences, cloned fragments of the mutants showed 146 base changes. As shown in Table 9, the mutations included transitions, transversions, deletions, and insertions. The frequency of base substitutions (58.22%) was higher than that of base deletions and base additions (41.78%). Of the base substitutions, the frequency of base transitions (45.21%) was 3.5 times of that of the base transversions (13.01%).

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Table 9. Types of mutation in 6 mutants induced by Fe^{+} implantation.

Types of mutation	Number of occurrence	Frequency (%)
Base substitutions	85	58.22
Transition	66	45.21
T C	17	11.64
C T	15	10.27
G A	22	15.07
A G	12	8.22
Transversion	19	13.01
T A	2	1.37
A T	7	4.79
G C	3	2.05
C G	2	1.37
A C	5	3.42
Deletion	52	35.62
A	31	21.23
G	15	10.27
C	6	4.12
Insertion	9	6.16
+A	5	3.42
+C	4	2.74
Total	146	100

The results showed that adenine, thymine, guanine, or cytosine could be replaced by other bases (see Table 9): adenine changes occurred 60 times (including 31 deletions and 5 insertions); guanine changes occurred 39 times (including 15 deletions); cytosine changes occurred 27 times (including 6 deletions and 4 insertions), and thymine changes occurred 19 times (no deletions and insertions). Therefore, adenine was the most sensitive to irradiation.

DISCUSSION

In recent years, the study of materials irradiated by ion implantation at the molecular level has increased. Molecular biology research on mutants induced by ion implantation, such as *Medicago sativa* L. (Chen et al., 2001), *Cucumis melo* L. (Chen et al., 2002), *Buchloë dactyloides* (Nutt.) Engelm. (Xiao et al., 2004), and *Dahlia pinnata* Cav. (Yu et al., 2008), have been carried out using RAPD markers. However, with the exception of studies in *Nicotiana tabacum* (Zhang et al., 1998) and *Arabidopsis thaliana* (Chang et al., 2003; Li et al., 2007), few investigations have focused on the base sequence changes induced by low-energy ion implantation in higher plants.

In this study, we identified 6 lotus mutants implanted with Fe^{+} at a dose of 1 x 10^{12} ions/cm^2. To reveal the molecular basis of low-energy Fe^{+}-induced changes in genomic DNA, we subjected the mutants to SRAP and base sequence analysis. SRAP is new DNA marker invented by Li and Quiros (2001). SRAP has been used to construct genetic maps (Pan, 2005; Yu et al., 2007; Liu et al., 2011; Zhang et al., 2011) and perform genetic polymorphism research (Ferriol et al., 2004; Li et al., 2010; Wang et al., 2010; Yildiz et al., 2011). Of 121 primer combinations used in the present study for SRAP assay, 7 showed differences in SRAP patterns such as DNA fragment deletions or additions between the 6 mutants and the control plants. In the cloned DNA fragments from the mutants, an average mutation rate of one base change per 104.9 bases was detected. The base changes included transitions, transversions, deletions, and insertions. According to Yu (2000), the implanted ions punch atoms of molecules in double-

stranded DNA chains and force them to shift from their original positions. The shifted atoms might interact with other elements in the DNA molecule and form additional new molecules, or they might shift completely and leave empty space at their original positions. The former leads to genetic effects such as base substitutions; the latter cause deletions and insertions of a single base or a small DNA fragment or even chromosome breakage and translocation. SRAP DNA marker can easily be used to detect these changes in DNA base sequences between the mutants induced by ion implantation and controls. SRAP analysis also showed that adenine was the most sensitive to irradiation. These results were the same as those reported by Zhang et al. (1998) and suggest that mutational “hotspots” induced by low-energy ion implantation probably exists in lotus.

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