Changes in DNA base sequences in the mutant of *Arabidopsis thaliana* induced by low-energy N^+ implantation

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Abstract To reveal the mutation effect of low-energy ion implantation on *Arabidopsis thaliana in vivo*, T80II, a stable dwarf mutant, derived from the seeds irradiated by 30 keV N^+ with the dose of 80×10^{15} ions/cm^2 was used for Random Amplified Polymorphic DNA (RAPD) and base sequence analysis. The results indicated that among total 397 RAPD bands observed, 52 bands in T80II were different from those of wild type showing a variation frequency 13.1%. In comparison with the sequences of *A. thaliana* in GenBank, the RAPD fragments in T80II were changed greatly in base sequences with an average rate of one base change per 16.8 bases. The types of base changes included base transition, transversion, deletion and insertion. Among the 275 base changes detected, single base substitutions (97.09%) occurred more frequently than base deletions and insertions (2.91%). And the frequency of base transitions (66.55%) was higher than that of base transversions (30.55%). Adenine, thymine, guanine or cytosine could be replaced by any of other three bases in cloned DNA fragments in T80II. It seems that thymine was more sensitive to the irradiation than other bases. The flanking sequences of the base changes in RAPD fragments in T80II were analyzed and the mutational "hotspot" induced by low-energy ion implantation was discussed.

Keywords: low-energy N^+ beam, mutagenesis, *Arabidopsis thaliana*, RAPD, base changes, sequence analysis.

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Arabidopsis thaliana (L.) Heynh has many advantages for genome analysis, including a short generation time, small size, large number of offspring, and a relatively small nuclear genome in comparison to other angiosperms and contains a low proportion of repetitive DNA comparatively. Furthermore, the analysis of the completed genome sequence of *A. thaliana* has been reported[1].

Low-energy ion implantation has attracted more and more attention from researchers in China and Japan since recent studies have reported the complex biological effects of ion implantation, such as lower germination and survival rates, and genetic variations in plants and microorganisms[2—4]. The biological effects of low-energy ions are more complex than other mutagenic sources, such as X-rays, λ-rays, laser rays because it involves not only combination of energy deposition (similar to λ-ray radiation) but also mass deposition and charge exchange of energetic ions[3]. Studies with different biological materials suggested that ion irradiation caused chromo-

some aberration[3,4], single-strand and double-strand breaks of isolated DNA[5,6] and base changes of M13mp DNA[7]. However, few investigations focus on the DNA damages and base sequence changes induced by low-energy N^+ irradiation in higher plants *in vivo*.

Williams et al.[8] developed random amplified polymorphic DNA (RAPD) technique to detect genetic variations and suggested that the RAPD assay could, in some instances, detect single base changes in genomic DNA and other forms of polymorphism, including deletions or insertions of a primer site which make priming site too distant to support amplification, or insertions that change the size of a DNA segment without preventing its amplification. Therefore, the variation of RAPD markers may reflect the DNA base sequence changes in addition to DNA recombination.

In the present study, we adopt RAPD technique to detect the base changes in a mutant of *Arabidopsis thaliana* induced by low-energy N^+ implantation. Then some RAPD fragments of the mutant and control plants were isolated, cloned and sequenced for comparing their base sequences with the sequences of *A. thaliana* in the GenBank in order to characterize the mutagenesis induced by low-energy ion beam at DNA level.

1 Materials and methods

1.1 Plant materials

All dry seeds used in this study were derived from a single plant of wild type of *A. thaliana*, ecotype Columbia. The irradiated seeds were grown in a greenhouse at a temperature of about 25℃ in the daytime and 12℃ at night. A dwarf plant was found in the plants from the treated seeds. The dwarf plant became a stable mutant after 5 generations of self-cross and it was named T80II (see section 2). T80II and the wild type of *A. thaliana* were used to do RAPD analysis and sequence comparison.

1.2 Seed irradiation

The implantation system used in the present study had been described previously by Yu et al.[9]. The equipment in the Ion Beam Bioengineering Center, Institute of Plasma Physics, Chinese Academy of Sciences supplied the N^+ beam. The N^+ ion used had energy 30 keV with the current 25 mA/cm and a pulse of 5 s at an interval of 25 s. The fluency of each pulse to the samples was about 1×10^{15} ions/cm^2. The dose was 0 (used as control) and 80×10^{15} ions/cm^2. For each dose, 300 dry seeds were treated.

1.3 DNA isolation and RAPD assay

Genomic DNA was isolated from 10 plants of mutant and the control respectively according to Rogers et al.[10]. The gross DNA solution was incubated for 30 min at room temperature with RNase A to remove the RNA during DNA isolation processes.

Random primers, Taq DNA polymerase and dNTP were purchased from Shanghai Sangon Ltd. (Shanghai, China). The amplification reactions contained 10 mmol/L Tris-HCl, pH 9.0, 10 mmol/L KCl, 8 mmol/L $(NH_4)_2SO_4$, 2 mmol/L $MgCl_2$, 0.08% NP-40, 200 μmol/L of each dNTP,

0.27 μmol/L primers, 50 ng of genomic DNA. Fifty microliter system containing 2.5 units of Ampl Taq DNA polymerase was adopted. Amplification was performed in Whatman Biometra® TGRADIENT thermocycler. Amplification parameters were 92℃ for 5 min followed by 45 cycles at 95℃ for 30 s, 34℃ for 15 s, and 72℃ for 1 min. A final extension was performed at 72℃ for 10 min. Amplification products were separated on a 1.0% agarose gel.

1.4 Recovery and clone for amplified polymorphic DNA fragments

The desired amplified fragments were recovered from agarose gel using the Wizard™ PCR Preps DNA Purification System (Promega) and then cloned using the pGEM®-T Easy Vector System (Promega) according to the instructions of the manufacturer. The recovered DNA was amplified again following a program of 95℃ for 4 min, 35 cycles of 95℃ for 15 s, 52℃ for 45 s, and 72℃ for 1.5 min. A final extension was performed at 72℃ for 8 min.

1.5 DNA sequencing

The *Escherichia coli* (DH5α) harboring recombinant plasmid was sent to sequence on both strands and assembled by Shanghai Sangon Ltd. The sequencing primers are T7/SP6.

T7 Sequencing Primer, 5′ d (TAATACGACTCACTATAGGGCGA) 3′

SP6 Sequencing Primer, 5′ d (CAAGCTATTTAGGTGACACTATAG) 3′

2 Results

2.1 The radiation effects of low-energy N^+ and identification of a dwarf mutant, T80II

The seed germination and seedling formation rates of *A. thaliana* irradiated by N^+ with the dose of 80×10^{15} ions/cm^2 were lower than those of the control plants. The seed germination and seedling formation rates of the control were 89.7% and 77.0%, but the treated seeds, 23.3% and 21.4% respectively. The time for seed germination of treated seeds delayed about one week compared to the control plants. There were few obvious variations in M_1 plants from the treated seeds. However, a dwarf variant with normal flowers and shorter siliques was found among the M_1 plants. We obtained the M_2 generations of the dwarf variant by self-cross. 65 dwarf plants and 21 normal plants were found in its M_2 generations and the segregation ratio for dwarf and normal plant height was 3∶1. Chi-square analysis showed a 3∶1 Mendel segregation at a level of $p < 0.05$. It suggested that the dwarf character was probably caused by a dominant mutation on a single locus. The dwarf plants were chosen to do 4 consecutive self-crosses and the hereditarily stable dwarf mutant, T80II (fig. 1(b)) selected in M_6 was used as the plant material for subsequent experiments. In addition, some phenotypic variations were found in the M_2 generations originated from the treated seeds. The types of phenotypic variations included chlorisis, semilethality, morphological variations (such as leaf and flower shape variations, plant height variation) and changes in blooming habit and fertility.

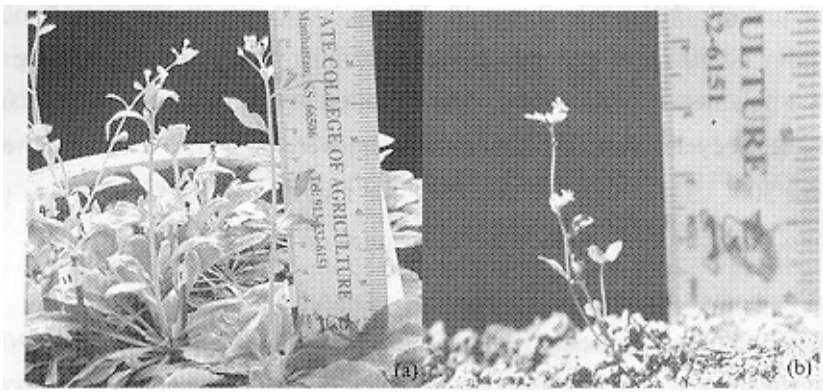

Fig. 1. The height comparison between the control plants and the mutant T80II. (a) Control plants with plant height over 11 cm; (b) the dwarf mutant T80II derived from a seed irradiated by low-energy N^+ with the dose of 80×10^{15} ions/cm^2 and it was about 3 cm tall. The photographs were taken after about 55 days of seeding.

2.2 RAPD pattern changes of the mutant T80II induced by ion implantation

The plants of the mutant T80II and wild type were collected to isolate genomic DNA and carry out the RAPD assay. All the 40 random primers produced stable products in RAPD reactions. Although most of the bands in RAPD profiles were common between control and T80II, the RAPD products coming from 27 random primers exhibited differences, including at least one DNA fragment deletion or fragment addition. Some RAPD polymorphic products were chosen to show the results (fig. 2). 52 bands in T80II were different from those of wild type showing a variation frequency of 13.1% in observed total 397 RAPD bands in T80II and control plant.

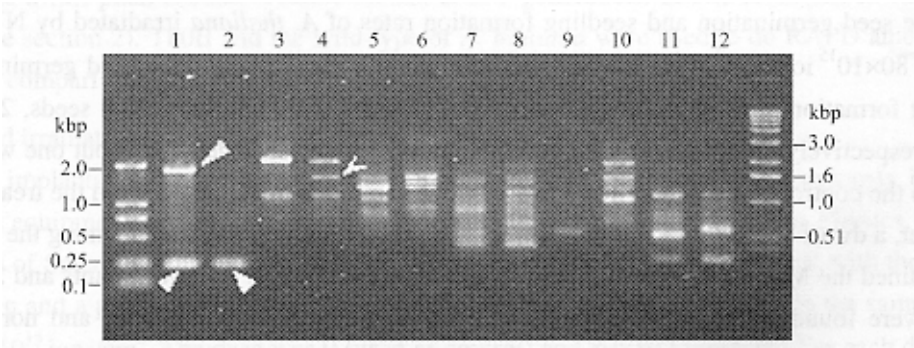

Fig. 2. A profile of RAPD products with obvious differences between the control and T80II. Lanes 1 and 2, RAPD products of control and T80II with the primer 5′-CTGGGGCTGA-3′ respectively. Upper arrow indicates CK173-1 cloned and lower arrows indicates CK173-2/T80II173-2 cloned. Lanes 3 and 4, with the primer 5′-TCATCCGAGG-3′. Arrow indicates T80II175 cloned. Lanes 5 and 6, with the primer 5′-TCTCCGCCCT-3′. Lanes 7 and 8, with the primer 5′-GGTGGTGATG-3′. Lanes 9 and 10, with the primer 5′-AATGCGGGAG-3′. Lanes 11 and 12, with the primer 5′-AAAGTGCGGC-3′.

To find out the base sequence changes in the specific bands, 11 RAPD fragments were cloned and sequenced. Among them 4 were specific for T80II, 3 were specific for the control and remaining 4 were shared by both T80II and the control.

2.3 Sequence analysis of *Arabidopsis* genomic DNA variations induced by ion implantation

To reveal the changes in DNA base sequence induced by low-energy ion implantation on T80II, the sequences of cloned RAPD fragments were compared with their homologies in GenBank via NCBI using BLAST2 program (http://www.ncbi.nlm.nih.gov/BLAST/). The serial numbers of cloned fragment, fragment sizes, sequence homologies, mutation frequencies and GenBank accession numbers for the fragments are listed in table 1.

As shown in table 1, the cloned fragments from the control plants (CK23, CK173-1, CK179, CK173-2 and CK162) exhibit no or few base modifications. There were 5 base changes in total 4080 base pairs of the largest fragment sequences recognized by GenBank and showed a mutation frequency of 0.12%. It fell into the range of experimental errors. However, one or more base changes were detected in the fragments cloned from T80II and a mutation frequency of one base mutation per 16.8 bases in average exhibited. In the fragments specific for T80II, i.e. T80II60, T80II121, T80II168 and T80II175, base mutation frequencies of 7.97%, 20.24%, 0.58% and 14.23% appeared respectively which were all obviously higher than that of the control fragments cloned. The homology between T80II173-2 and CK173-2, as well as between T80II162 and CK162 was all around 99% compared with the software DNAMAN2.0. It meant that the two sequences in each pair were homologous. However, the mutation frequency of 0.42% and 0.93% was exhibited in T80II173-2 and T80II162 respectively. They were higher than those of their counterpoints, CK173-2 and CK162. The results showed that base changes with higher mutation frequencies occurred in both polymorphic and unpolymorphic fragments in T80II in comparison with the control.

Table 1 Base sequence comparisons of amplified polymorphic fragments with their homologous sequences in GenBank

Serial numbers of cloned DNA fragments [a)]	Cloned fragment sizes/bp	The largest fragments (bp) recognized by GenBank	The largest homologous fragments/bp	Number of base changes /bp	Mutation frequency (%)[b)]	GenBank accession numbers
CK23	337	332	332	0	0	–
CK173-1	1864	1862	1862	0	0	–
CK179	584	581	579	2	0.34	–
T80II60	521	514	473	41	7.97	AF544037
T80II121	847	242	193	49	20.24	AF544036
T80II168	1395	1391	1383	8	0.58	AF544040
T80II175	1567	1166	1000	166	14.23	AF544038
CK173-2	239	236	236	0	0	–
T80II173-2	239	236	235	1	0.42	AF544041
CK162	1071	1069	1066	3	0.28	–
T80II162	1070	1070	1060	10	0.93	AF544039

a) CK23, CK173-1 and CK179 were cloned fragments specific for the control plants. T80II60, T80II121, T80II168, and T80II175 were cloned fragments specific for the dwarf mutant T80II. CK173-2/T80II173-2 and CK162/T80II162 were cloned fragments shared by the control plants and T80II. b) Mutation frequency (%) = number of base changes (bp)/ the largest fragment (bp) recognized by GenBank.

The base changes in a primer site or the break and rearrangement of DNA strands all can cause DNA polymorphisms. In the sequence of fragment T80II168, we found at least one T/A→C/G transition occurred at the 5′ primer site (the origin sequence TGCC***T***GGTGA was turned to TGCC***C***GGTGA). The primer that attached completely on this site in T80II genomic DNA made the sequence amplified in the RAPD reaction successfully. However, the primer could not attach on the site in the control DNA that caused the sequence not to be amplified successfully in the RAPD reaction. It caused the polymorphic band to appear in T80II.

Sequence comparisons of fragments specific for T80II with their homologous sequences in GenBank revealed that low-energy ion irradiation probably caused the T80II genomic DNA strand breaks. For example, the cloned sequence size of T80II121 was 847 base pairs, but the largest fragment recognized by GenBank was only 242 base pairs (from base number 154 to number 394), which was located on chromosome I with the base number from 11792507 to 11792747. The sequences with the base number from 438 to 490, 516 to 553 of T80II121 were also located on chromosome I with the base number from 11792791 to 11792843 and 11792869 to 11792906, respectively. However, the sequence with the base number from 574 to 718 of T80II121 was located on chromosome III with the base number from 10579669 to 10579525 (fig. 3). In the sequences of T80II60 and T80II175, DNA strand breaks were also observed (data not shown). However, we could find the homologous sequences with almost full lengths of the polymorphic DNA fragments appeared in control RAPD assay in GenBank. So we deduced that low-energy ion irradiation resulted in the T80II genomic DNA strand breaks and rearrangements in the reparation process.

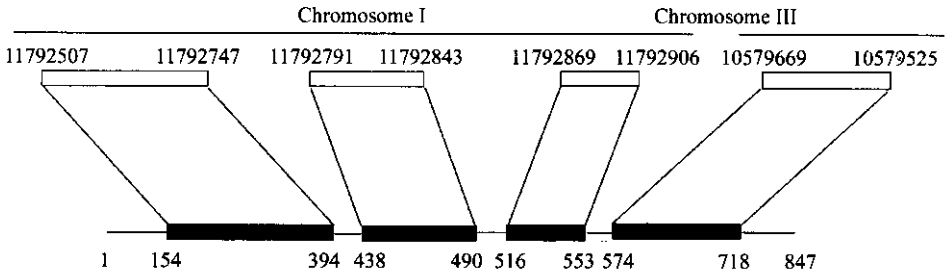

Fig. 3. The distributions of T80II121 fragment on the chromosomes in *A. thaliana*. The upper figures were homologous fragments of T80II121 on *A. thaliana* chromosomes, and the lower figures were cloned sequence of T80II121.

2.4 Analysis of base change types and characteristics of T80II genomic DNA variations induced by ion implantation

In the sequences of cloned DNA fragments in T80II, only considering the largest fragments recognized by GenBank, there were 275 base changes occurring. If considering the small fragments recognized by GenBank, the amount of base changes would be more than 275. The types of mutation included base transitions, base transversions, base deletions and base insertions. Fig. 4 shows the original data searched from GenBank. In addition, as shown in table 2, the frequency of

base substitutions (97.09%) was much higher than that of base deletions and base additions (2.91%). As for the 267 base substitutions, the frequency of base transitions (66.55%) was higher than that of the base transversions (30.55%) by more than twofold.

Table 2 Types of mutation in cloned T80II fragments induced by low-energy N^+ implantation

Types of mutation	Number of occurrence	Frequency (%)
Base substitutions	267	97.09
Transition	183	66.55
A→G	46	16.73
G→A	48	17.45
C→T	25	9.09
T→C	64	23.27
Transversion	84	30.55
C→G	6	2.18
G→C	10	3.64
A→C	10	3.64
C→A	13	4.73
A→T	11	4.00
T→A	8	2.91
G→T	12	4.36
T→G	14	5.09
Deletion	5	1.82
–A	1	0.36
–G	2	0.73
–T	2	0.73
Insertion	3	1.09
+A	3	1.09
Total	275	100

The results in table 2 also showed that changes in adenine (A), thymine (T), guanine (G) and cytosine (C) all could be induced by ion irradiation and each of them could be replaced by one of the other three bases. Adenine (A) changes occurred 71 times (including 1 deletion and 3 insertions) and guanine (G) changes occurred 72 times (including 2 deletions). Cytosine (C) changes occurred 44 times and thymine (T), 88 times (including 2 deletions). It suggested the base thymine (T) had the largest radiosensitivity than the other three bases.

Analysis of flanking sequences of 275 base changes (data not shown) suggested that the changed bases were not related with any specific flanking sequences. According to the results, we did not find mutational "hotspot" in the cloned T80II fragments. Because of limited observation in our experiments, the question of mutational "hotspot" should be investigated further.

3 Discussion

Some researchers investigated mutation effects of low-energy ion implantation on naked DNA and found that it can cause single-strand breaks (SSB) and double-strand breaks (DSB) of

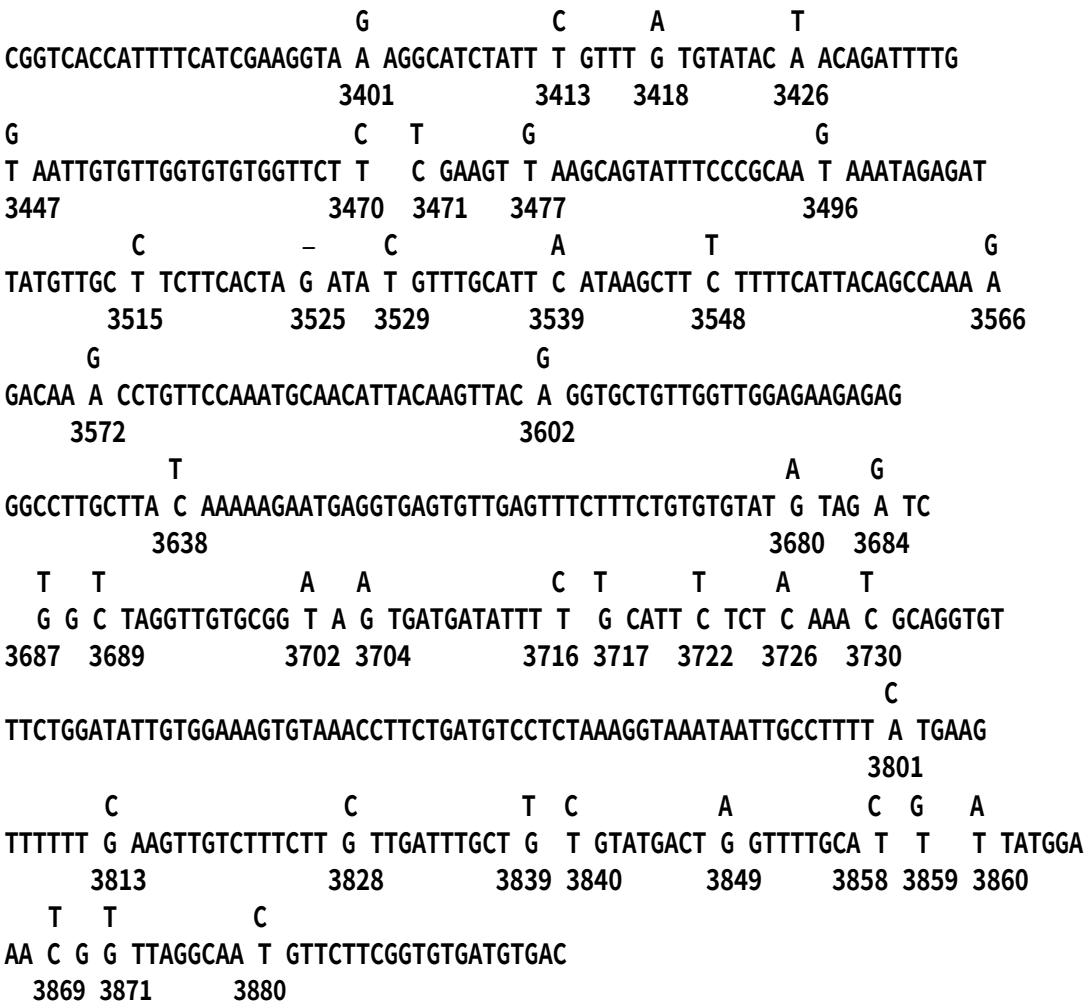

Fig. 4. Homologous comparison of the sequence of T80II60 with the sequences in GenBank. Base changes in T80II60 were shown above the wild type sequence in capital. Deleted bases were indicated by (–). The figures below the wild type sequence showed the sites of changed bases on chromosome V.

isolated DNA[5,6] and base changes of M13mp DNA[7]. Few investigations, however, focus on the DNA damages and base sequence changes induced by low-energy N^+ irradiation in higher plants *in vivo* except Zhang et al.[11] reported the changes in DNA polymorphism in tobacco M_1 plants irradiated with low-energy N^+ ion.

In this study we identified a dwarf mutant from the populations of the plants irradiated by N^+ with the dose of 80×10^{15} ions/cm^2. In order to reveal the molecular basis of low-energy N^+ on the genomic DNA, the dwarf mutant, T80II, was subjected to RAPD and base sequence analysis. Out of 40 random primers used for RAPD assay, 27 showed differences in RAPD patterns between T80II and the control plants, such as DNA fragment deletions or additions. And in the cloned

fragments in T80II, an average mutation rate of one base change per 16.8 bases was detected. It suggested that many changes occurred in the DNA sequences in T80II induced by low-energy ion implantation.

The great changes in genomic DNA polymorphisms in T80II may be caused by base changes as well as DSB. If base changes occurred at a primer site, it would result in the appearance of DNA polymorphisms. In our results, for instance, at least one base change (T/A→C/G) occurred at the 5′ primer site in the sequence of T80II168, which caused the specific fragment to appear. In addition to the base changes, the DSB and rearrangements of small fragments were found based on the unique sequence distribution of T80II121 (fig. 3), which could be thought as another reason for the variation in the RAPD pattern of the mutant.

No large deletions (ranging from several hundred base pairs to megabasepairs) were detected in T80II although DSB and rearrangements of small fragments were found in our experiments. One possible explanation might be that it is difficult to find them with RAPD method.

Our results agreed with Yang et al.[7] who used double strand M13mp18 DNA (RF1) as the low-energy ion irradiation target. We observed high frequency of A/T-G/C and C/G-A/T in our experiment. Traditional ionizing radiation (such as X ray and γ ray) or nonionizing radiation (such as UV) can cause A/T-G/C and C/G-A/T substitutions and these two types of mutation were correlative to group release (such as apurinic-apyrimidinic site and deamination). In a study on low-energy ion effects on nucleotides, Shao and Yu.[12] reported that inorganic phosphate and base were released from 5′-dTMP. So the group release probably was an important reason for base changes induced by ionizing radiation, including low-energy N^+.

The high frequency of G/C-A/T transition was also found in our experiment. It was similar with the results obtained by Miller[13] using ultraviolet light as mutagen to induce mutations in the *lacI* gene of *Escherichia coli* and by Tindall et al.[14] using lambda phage and prophage as targets to be irradiated by γ-ray. The simplest explanation for G/C-A/T transition is mispairing of adenine with a deaminated radiation product of cytosine during DNA replication. About half of the radiation products of cytosine were deaminated, and these might code thymine[14].

The sites of base changes in T80II induced by low-energy ions implantation were remarkably discrete (data not shown). It was difficult to find the mutational "hotspot" in the disposed conditions. Using M13mp18 DNA (RF1) as low energy ion irradiation target, Yang et al.[7] reported that the bases, around which were TG and CT in *lacZ* gene, inclined to change. It was the relatively centralizing site of base changes and it suggested that "hotspot" probably exists. So whether mutational "hotspot" induced by low-energy ion irradiation on *A. thaliana* exists or not could be investigated further.

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