Characterization of variation induced by low-energy N^+ and cloning of differentially expressed cDNA of a mutant in *Arabidopsis thaliana* *

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Abstract Using *Arabidopsis thaliana* as experimental materials, the variations induced by low-energy N^+ have been investigated. Germination rate of the treated seeds is lower than that of the control, and it decreases with the intensification of the radiation. The phenotypic variations have been observed in M_2 plants irradiated with higher doses, such as chlorisis, semilethality, plant morphology, and changes of blooming habit and fertility. In random amplified polymorphic DNA (RAPD) analysis on M_2 seedlings, some differences including band deletions or additions are found in treated plants compared to the control and the differences are associated with the radiation doses. One of the M_1 plants from the seeds irradiated with the dose of 80×10^{15} N^+/cm^2 is a dwarf variant. Its stable M_6 generation, mutant T80II, is used to construct subtractive cDNA library and to clone differentially expressed cDNA. A 721 bp cDNA fragment is partly homologous with *GRF7* gene.

Keywords: low energy ion beam, *Arabidopsis thaliana*, variation, cDNA, subtractive hybridization library.

Low-energy ion implantation, considered as a new mutation breeding technique, has caught more attention from researchers of China and Japan. The effects of ion implantation on plants and microorganisms have been well documented[1~5]. Yu et al. reported that low-energy ion implantation was able to etch away the cell coat and to dig paths to form some channels in seeds. Based on that, they thought that foreign gene could transfer into the embryo cells through the channel if it was wide enough[6]. By means of this technique, several transgenic plants were obtained in rice and wheat[6~8]. These results make low-energy ion implantation a possible way to introduce genes into plant tissues in addition to its use as a mutation source in breeding.

In the present study, the mutation effects of low energy N^+ implantation at different doses on *Arabidopsis thaliana* (L.) Heynh were investigated and a subtractive cDNA library specific to T80II, one of the mutants induced by low-energy N^+ with the dose of 80×10^{15} ion/cm^2, was constructed and some differentially expressed cDNA were cloned using the subtractive hybridization technique enhanced by PCR for the first time. Furthermore, one of the cDNA fragments was sequenced and analyzed.

1 Materials and methods

1.1 Irradiation of N^+ ion on *A. thaliana*

All dry seeds treated were derived from a single plant of a wild type of *Arabidopsis thaliana*, ecotype Columbia. The implantation system used in this study has been described previously[1]. The N^+ beam was generated by the equipment in the Ion Beam Bioengineering Center, Institute of Plasma Physics, Chinese Academy of Sciences. The N^+ ion had energy of 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 which were composed of 300 dry seeds each was about 1×10^{15} ions/cm^2. The treatments were 0 (used as the control), 40×10^{15}, 60×10^{15} and 80×10^{15} ions/cm^2.

1.2 Culture of the irradiated seeds

The irradiated seeds were grown in a greenhouse at 25/12 ℃ day/night with a photoperiod of 16 h light. A dwarf plant was selected in the plants from seeds treated with a dose of 80×10^{15} ions/cm^2. The dwarf plant became a stable mutant after 5 generations of self-pollination and was known as T80II (see Results). The wild type of *A. thaliana* and T80II

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were used to construct a subtractive cDNA library.

1.3 DNA isolation

Genomic DNA was isolated from the control and M_2 irradiated plants respectively according to Rogers et al.[9].

1.4 Random amplified polymorphic DNA (RAPD) assay

Taq DNA polymerase and dNTP were provided by Shanghai Sangon Ltd. The random primers (CYA1 ~ 20) were purchased from Beijing SBS Genetech Co., Ltd. Twenty-five microliters of amplification mixture was used in PCR analysis. Amplification was programmed at 94 ℃ for 5 min, followed by 35 cycles of 94 ℃ for 1 min, 45 ℃ for 1 min, and 72 ℃ for 2 min. A 72 ℃ incubation for 10 min was included as a final step. Amplification products were separated by electrophoresis on a 1.5% agarose gel.

1.5 Subtractive hybridization and cloning cDNA products

The methods of subtractive hybridization and preparation of the "tracer" cDNA primer were carried out as previously described with minor modification[10]. Four rounds of subtraction against "driver" cDNA from control plants were performed. Dot blotting technique, by the use of a DIG High Prime DNA Labeling and Detection Starter Kit I (Boehringer Mannheim, Germany), was applied to verify the subtractive hybridization efficiency. The original total "driver" cDNA was labeled and served as a probe. The subtraction cDNAs were ligated to pUC18 to construct a subtractive cDNA library following a published method[11]. The PCR technique was used to screen the cDNA fragments with pUC18 vector-specific primers. The PCR products with a proper cDNA size were analyzed by dot blotting.

1.6 Northern blot analysis

A method of Northern blot analysis, using a labeled clone of T80IISH43 as a probe, was followed as described by Sambrook et al.[11]. The probe labeling was carried out according to the technical manuals of Random Primer DNA Labeling Kit (TaKaRa, Biomedicals, Kusatsu, Japan).

1.7 Sequence analysis

Escherichia coli (DH5α) containing plasmid with the differentially expressed cDNA was analyzed for cDNA sequence of the insert by Shanghai Sangon Ltd. The sequencing primers are M13 (+) and M13 (−). The cDNA sequences were compared with their homologies in GenBank via NCBI using BLAST2 program (http://www.ncbi.nlm.nih.gov/BLAST/).

2 Results

2.1 Effects of ion irradiation on the developmental and phenotypic variations of *A. thaliana*

The seed germination and seedling formation rates of *A. thaliana* irradiated by N^+ were lower than those of control plants, which decreased with the intensification of the radiation doses. The germination rate and seedling formation rate of control seeds were 89.7% and 77.0% respectively. However, the germination and seedling formation rates of seeds irradiated with doses of 40×10^{15}, 60×10^{15} and 80×10^{15} N^+/cm^2 were 74.7% and 66.5%, 45.3% and 38.2%, 23.3% and 21.4% respectively. Among the M_1 plants from the treated seeds, no variants were found except a dwarf variant with a lower growth potential, shorter siliques (Fig. 1), which came from the seeds irradiated with the dose of 80×10^{15} N^+/cm^2. We obtained the M_2 generation of the dwarf variant by self-pollination. The ratio of dwarf plant to a high one was about 3∶1. The plants from this variant were selected for additional 4 self-pollination to obtain a genetically stable dwarf mutant T80II in M_6, which was used as the plant material to construct a subtractive cDNA library. In M_2 generation, all the 222 seedlings from seeds irradiated with the dose of 40×10^{15} N^+/cm^2 grew normally. There was no discernable phenotypic abnormality in comparison

Fig. 1. Control plants of *A. thaliana* (a) with the height of more than 11 cm and M_1 dwarf variant (b) with the height of about 3 cm.

with the control. Among 76 seedlings that originated from seeds irradiated with the dose of $60 \cdot 10^{15}$ N^+/cm^2, 3 seedlings were found to have no flower buds in the upper part of inflorescence. There were some distinct phenotypic variations in the seedlings irradiated with the dose of 80×10^{15} N^+/cm^2, including chlorisis, semilethality, morphological variations (i.e. leaf and flower shape variations, plant height variation, etc.) and changes in blooming habit and fertility. Some phenotypic variations of flowers in plants irradiated with the different doses of N^+ are illustrated in Fig. 2. In addition, the percentage of abnormal flowers increased with the intensification of radiation doses (data not shown).

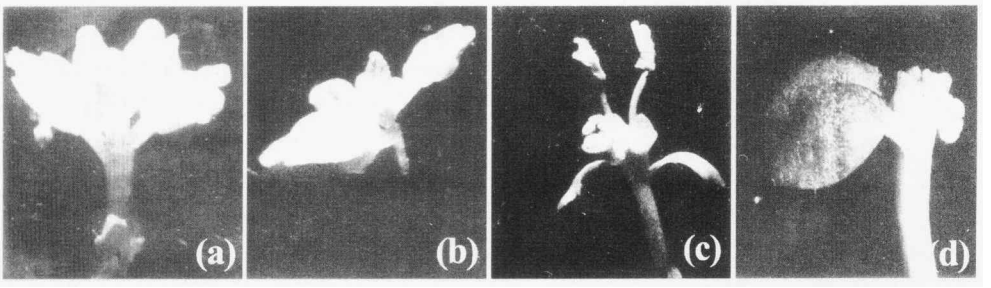

Fig 2. Some phenotypic variations of flowers in M_2 plants irradiated with different doses of N^+. (a) Control; (b) the plants irradiated with $40 \cdot 10^{15}$ N^+/cm^2, showing the normal flower bud and inflorescence differentiation normally; (c) some plants irradiated with $60 \cdot 10^{15}$ N^+/cm^2 gave birth to some flower buds without floral shoots and the inflorescences of these flower buds were short; (d) some plants irradiated with $80 \cdot 10^{15}$ N^+/cm^2 produced the flower buds without floral shoots and there were some petals on these flowers but no normal stamens and pistils.

2.2 RAPD assay

Among 20 random primers we used, 16 were able to produce RAPD products. The product size ranged from 200 bp to 1200 bp. The primers CYA-10 and CYA-19, two primers which had good amplification stability were chosen to carry out the RAPD assay for control and 47 M_2 plants irradiated with the doses of 40×10^{15}, 60×10^{15} and 80×10^{15} N^+/cm^2, respectively. The RAPD results showed that there was no difference between 10 control plants. But the RAPD of M_2 plants irradiated with N^+ showed some differences, including amplified fragment deletions or additions compared to the control and the differences were associated with the radiation dose. Some RAPD results are illustrated in Fig. 3.

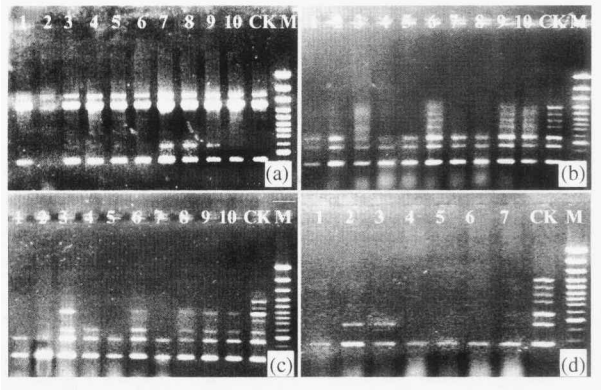

Fig. 3. Some RAPD profiles of the control and treated plants with the primers CYA-10 and CYA-19. (a) The profile of control and the plants irradiated with $60 \cdot 10^{15}$ N^+/cm^2 using the primer CYA-19; (b), (c) and (d) the profiles of the control and plants irradiated with the doses of $40 \cdot 10^{15}$, $60 \cdot 10^{15}$ and $80 \cdot 10^{15}$ N^+/cm^2 using the primer CYA-10 respectively CK, control; 1 ~ 10, the generations treated with different doses; M, DNA marker.

2.3 Cloning and analysis of differentially expressed cDNA of the mutant induced by N^+

In order to obtain the differentially expressed cDNA of the mutant T80II, we constructed a subtractive cDNA library using T80II as a "tracer" and the control plants as a "driver". After 4 rounds of subtractive hybridization, dot blotting was performed to determine the subtraction efficiency using the original total "driver" cDNA as a probe to hybridize with the subtraction cDNA produced after each round (Fig. 4 (a)). The results showed that the hybridization signals became fainter and fainter after each

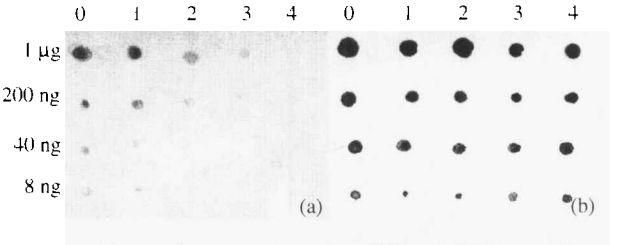

Fig. 4. The results using original total "driver" cDNA as probe (a) and original total "tracer" cDNA as proble (b) to hybridize with the subtraction cDNA produced after each subtraction round. 0, total "tracer" cDNA; 1 ~ 4, the subtraction cDNA after the first, second, third and fourth subtraction round respectively.

round. After the 4th round of subtractive hybridization, no hybridization signal appeared in the driver cDNA. Furthermore, we used the original total "tracer" cDNA as a probe to hybridize with the subtraction cDNA after each round (See Fig. 4 (b)). There were strong hybridization signals in each hybridization. This revealed that the subtraction cDNA was derived from the mutant T80II. The results of PCR amplification and dot blotting for the cDNA fragments in positive clones confirmed that the inserted fragments were from T80II (data not shown). It indicated that the construction of the subtractive cDNA library was successful.

2.4 Sequence analysis of inserted fragments in the recombinant clones

Some inserted fragments were sequenced and they were compared with their homologies in GenBank via NCBI using BLAST2 program. The sequence of T80IISH43 (No. 43 clone) is shown in Fig. 5. The comparison of T80IISH43 with homologous sequence indicated that part sequence which was from 273 bp to 461 bp in T80IISH43 showed homology to the sequence from 3514 bp to 3702 bp in *Arabidopsis* 14-3-3 protein GF14 nu (GRF7) gene with the frequency of 98%. It suggests that the protein encoded by T80IISH43 probably belongs to 14-3-3 proteins.

GCGGGGGGATGGAGTTGTGGTCGCCGGTGGCCGAGATAATCCGGAGATCTAGTGAACCGT
CTCAATGTCTGGTCCAGTCCGGCGATGGAGCGAGGCAACAAGGCATGTGCGGCGAGATC
GTTCTGGCAATGCACCGTTTATGTGAGGTCTTACTAATCCGGGCTCCACTGTGTTATCTTTA
ATCGATTAGTAGCCTGATGTTCATTGGTTAGCTCGGGAAAAGCTCTAGCCTTGCTGGTTTG
CCTTGTATCATTGGTCGGGTCAGTCGGATATATGGTCCGCTTTTGGTTTAAGACCGAAACA
ACGAAAGCCGGGGATTTTGAGCCTTTCTGATGATGCGCCTCCGGGCGGTAATGCTATTGG
GACATGGATTACATTCTGATCAATAGGGATTAATAGTATGGATTTACTTTGTACTTCAGACT
AGGTTTTGGTCTCTTCCGTTTTAGTAGTTGTTCTCGTGTCGCTTAGAGTCTGTGTAGGTGG
TCTAAGAGATGATAATATTCTCATTTGTGGAGTATTATCTATCTCATTGTGGTTTAAAAATGC
AATGGTTTCTCGATCAGGTAGCGCTGGATCCGATAGTGTTTCAGGGTGATACTAGATCAAA
CCATTGCATTTGGGGTCCAATTTGATAGTGGGGAATGTTATGTACCTTCAATTGGATGTAAT
CATTTGGTAAATGAAAGTTGATGTTGAACCAAAAAAAAAA

Fig. 5. Sequence of cloned T80IISH43.

2.5 Northern blot analysis of T80IISH43

The result of Northern blot hybridization showed that T80IISH43 was strongly hybridized with total RNA of T80II (Fig. 6). However, no signal was observed when it was hybridized with total RNA of control plants (Fig. 6). This indicated that T80IISH43 was a differentially expressed cDNA of T80II.

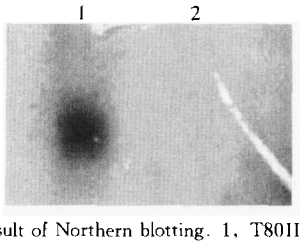

Fig. 6. Result of Northern blotting. 1, T80II; 2, control.

3 Discussion

The effects of low-energy N^+ at different doses on plant development and phenotypic variations reported before[1~3, 5] were confirmed by our experiments in *Arabidopsis thaliana*. Furthermore, we used one stable dwarf mutant T80II to construct a cDNA library using the subtractive hybridization technique enhanced by PCR and cloned some differentially expressed cDNA of T80II induced by low-energy N^+ implantation successfully. It is a new extension for the applications of low-energy ion implantation technique.

Low-energy ion implantation influenced the seed germination of *A. thaliana*. The ions with higher doses obviously inhibited seed germination and the germination rate decreased with the intensification of radiation doses. Most seeds were not able to germinate when the radiation dose was as high as 80×10^{15} N^+/cm^2. These results agreed with those reported by Zhang et al.[3] who used tobacco seeds as low-energy N^+ implantation targets.

Some distinct phenotypic variations were observed among the plants irradiated with the N^+ dose of 80×10^{15} and their RAPD profiles also showed obvious differences from those of control (Fig. 3 (d)). The RAPD differences reflect the genetic changes in DNA resulted from low energy N^+ ion implantation.

The analysis of the subtractive cDNA and inserted fragments in recombinant clones showed that it was successful to construct the subtractive cDNA library which contained the differentially expressed cDNA of T80II. It could be a way to use low-energy ion induced mutants as materials to clone the differentially expressed cDNA using the subtractive hybridization technique enhanced by PCR.

The Northern blotting analysis, using T80IISH43 as a probe, proves that T80IISH43 is a cDNA fragment specific to the mutant T80II, which is homologous to the part of 14-3-3 protein GF14 nu (GRF7) gene sequence. The genes encoding 14-3-3 proteins are a multigene family. It is generally accepted that the 14-3-3 proteins may function as kinase regulators in signal transduction/phosphorylation mechanisms[12]. Brandt et al. suggested that the 14-3-3 proteins in plant might act as regulators of phosphorylation processes in response to different kinds of stress[13]. The cDNA fragment T80IISH43, differentially expressed in T80II, was likely to be associated with the variations of characteristics that occurred in the mutant. Further studies are necessary to discover the full-length cDNA sequence of T80IISH43 and its potential functions.

In conclusion, the low-energy ion implantation resulted in a variety of variations in irradiated *A. thaliana* plants and their offspring. These variations are inherited and segregated. The establishment of subtractive cDNA library and cloning of differentially expressed cDNA from the mutant induced by low-energy ion implantation present a new possibility to apply low-energy ion implantation in biology, especially in molecular biology.

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