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CHINA NUCLEAR SCIENCE & TECHNOLOGY REPORT

中国离子注入生物效应研究

STUDYING OF ION IMPLANTATION EFFECT ON THE BIOLOGY IN CHINA

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摘　要

自从低能离子生物效应在中国发现以来，离子注入生物效应研究受到人们广泛的关注。低能离子束，作为新的诱变源已成功地应用于农作物和微生物改良；作为细胞加工的手段，已成为有效的转基因工具。文内简述了离子注入引起的生物效应的一些基本的现象；报道在群体、细胞和分子等不同层次上的分析结果；给出离子注入生物效应研究若干应用的实例；最后讨论这一研究领域要解决的一些问题和今后发展的方向。

STUDYING OF ION IMPLANTATION EFFECT ON THE BIOLOGY IN CHINA

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ABSTRACT

Since low energy ion effect on the biology was observed, the ion implantation as a new mutagenic source has been widely used in improving crops and modifying microbes in China. The basic phenomenon of ion implantation effect on the biology and analytical results are reported, and the examples of its application and its further development are shown.

INTRODUCTION

As we have known the studying of the interaction between low energy ions and solid materials was started thirty years ago. Since ion injection of semiconductors and ion beam modification of materials took place in the early 1960's and in the late end 1970's, respectively, the science of ion implantation materials has been quickly developed in the world. For a long time, however, people almost does not pay attention to low energy ion effect on the biology. In the middle of 1980's the biological effect induced by ion implanting into the seeds was observed in our laboratory[1]. A program of mutation breeding for rice by low energy ion beam then was performed in Institute of Plasma Physics, collaborating with Institute of Rice in 1986[2]. Through exploring the mutagenic effect for years, it has been found that this approach possesses higher mutation rate and wider mutational spectrum with higher survival rate[3]. Since then the ion implantation, as a new mutagenic source, has been widely used in improving crops in agriculture[4,5]. Up to now, there has been more than one hundred groups taking part in this project in China[6]. In 1989 the author observed that the cellular walls could be etched by ion beam, and suggested to study the interaction between the cells and low energy ions and tried to find its application for the bioengineering[7]. As a result, Gus and CAT gene were successfully transferred into the intact cells and ripe embryo of rice etched by ion beam, and the activities of the foreign gene materials were detected in these samples[8].

In fact, research on the ion implantation effect on the biology in some areas is different from traditional irradiation effects. Our main research interest is to explore what happened in a thin layer of the biology facing ion beam only due to short ionic range, but the irradiation actions originate in the entire body of the biology because the irradiation field is not resolving power in the space. In the layer there are three kinds of original physical process, i. e. energy exchanging, ion beam sputtering and slowed-down ions depositing[9]. It is well known that the development of the irradiation biology has already lasted for seventy years, and many achievements have been obtained. However, low energy ion effect on the biology only was recognized in a few years ago. It is obviously that the basic mechanism of irradiation effects has been well understood at cellular level, but the low energy ion effects are less known at the same level. Therefore, it is necessary to study on the biological effects induced by low energy ions.

This paper will report some phenomenon observed by ion implanting into the biological samples of crop seeds, cells and microbes, and describe the analytic results at cellular and DNA level. Finally, the paper will exhibit the application examples of ion implantation in crop improvement and bioengineering, and discuss its possible development of low energy ion biology.

1 BASIC PHENOMENON

When crop seeds were injected by the ions, a proper dose could promote their germination, and a over-load dose would result in damage. This is a universal phenomena as the same as the irradiation effect. With a careful designed experiment, it has been found that the survival rate with the doses could be revealed in a special pattern for the various corps implanted by ions, involving the microbes(Fig. 1). It means that the survival rate is decreased in exponential law with the dose increasing at the first dose stage; the rate will be afterward risen at the second stage; and then it is again lowered at the final stage(see Fig. 1). In order to simulate the pattern of the survival rate related to dose mathematically, an experiment has been designed, in which the ultraviolet activities of the tyrosine is detected after the sample was injected by N^+ at dose ranging 1×10^{15} to 30×10^{15} ions/cm^2. By considering both sides of the energy exchange and the slowed-down ions deposition, dose response for the ultraviolet activities at the tyrosine is reduced with Poisson distribution[10]:

$$S = 1\text{-}A[1 - (e^{-kD} + Bk_1 De^{-k_1 D} + Ck_2^2 D^2 e^{-k_2 D})]$$

where S and D represent the survival rate and dose respectively; k, k_1, k_2 are irradiation coefficients; B, C are experiment parameters; and A (equals to N_0/N) represents the proportion of the injected tyrosine number to the total number. This equation fits well in experiment results(Fig. 2). Although this equation can not completely reflect the dose response for the survival rate of the corp seeds after ion implanting, it will, mathematically, simulate the curve of the dose response during studying ion implantation effect on various corp seeds and microbes as long as the coefficients are carefully selected.

Since the beginning of observing ion implantation effect on rice, men have devoted themselves to the research on genetic variation induced by low energy ions. It is generally recognized that higher mutation rate and wider mutational spectrum could be obtained with greater survival rate of the seeds modified by ion implantation. Of all mutational types, the chlorophyll damage was easily observed. Table 1 show the survival rate in M1 generation and the chlorophyll mutations in M2 for some varieties of rice modified by ion implantation, as compared with that of treatment with γ-rays[11]. It could be seen from Table 1 that the frequencies of the chlorophyll mutation induced by ion implantation are higher than that of treatment with γ-rays. The chlorophyll mutational spectrums induced by ion implantation and γ-rays are different. The proportion of albina, xantha and striata is 24.9%, 28.6% and 46.5% for ion implantation mutation, and 60.5%, 9.5% and 30.0% for γ-rays respectively.

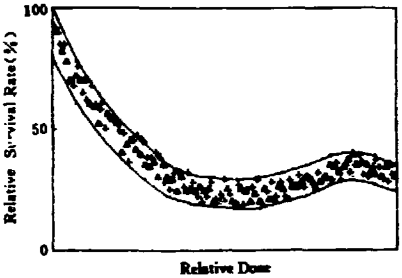
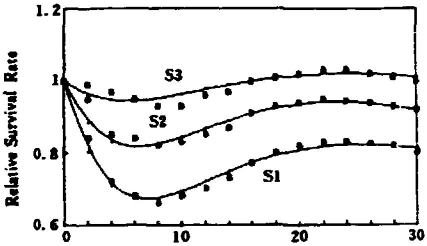


Fig. 1 Response Dose of the Survival Rate of Crops and Microbes Implanted by Ions



Dose($\times 10^{15}$N/cm²)

Fig. 2 Response Dose for UV-absorption activities of Tyrosine

* ——experimental data;

— — ——theoretical curve

S1, S2 and S3——samples with different thickness.

Table 1 Effects of ion implantation and γ-rays on the survival rate in M1 and chlorophyll mutation rate in M2 for rice

Varieties	Survival rate(%)			Mutation rate(%)		Albina		Xantha		Striata	
	CK	I. B. *	γ-ray**	I. B.	γ-ray	I. B.	γ-ray	I. B.	γ-ray	I. B.	γ-ray
02428	88.9	79.0	69.3	0.73	0.32	24.82	63.64	64.96	5.45	10.22	30.2
Fu8-1	90.0	84.8	75.1	0.38	0.34	26.02	58.02	11.38	13.85	62.6	28.4
8619	92.0	85.0	76.5	1.22	0.78	23.60	59.20	9.55	9.20	66.8	31.6
average	90.3	82.9	73.6	0.78	0.48	24.9	60.5	28.6	9.5	46.5	30.0

* I. B. ; Ion implantation mutation, 7×10^{16}ions/cm², N^+ with 35 keV

* * γ-ray, dose is 300Gy.

It is well known that albina belongs the lethal damage due to no the photosynthesis. The proportion of the albina for rice induced by ion implantation is greatly lower than that of the albina induced by γ-rays, it means that the damage caused by ion implantation is lighter. This can be seen from Fig. 3, there are more seedlings with Xantha and Striata, but there is only an albina seedling in Fig. 3(a).

It is interesting that a chlorophyll damaged plant of rice was selected only from

M1. Its leaves were yellow at planting stage and could begin to change the color to green at the flowering stage, and its plant height was decreased from 96 cm of CK to 40 cm. These characteristics have been repeated in the posterities(Fig. 3(b)).

The research of the mutation induced by low energy ion was widely carried out at various levels, i. e. in population genetics, single character and single gene levels. Table 2 shows an example of the mutation rate in population genetics obtained early from rice modified by ion implantation. For studies on the mutation of crops with a single character, a kind of wheat with blue bran and tomato with the tomentose cane were injected by N^+. In M2 generation, it was found that the frequency of the wheat bran color from blue turning to normal was 14.7%, the frequency of turning grayish white was 27.5%; but the mutation rate for tomato from tomentose cane turning to un-tomentose was 25.0%. In order to research exactly on the mutation rate of a single gene, the plasmid PUC 19 was selected as the label genes. It is well known that the plasmid PUC 19 consists of two genes, it means that E. Coli with the plasmid PUC 19 could produce a resistibility for penicillin, and its coenobia would be stained to be blue when the substrate is composed of IPTG and X-Gal.

(a) (b)

Fig. 3 Chlorophyll Mutation for Rice Modified by Ion Implantation Dose $6\sim8\times10^{16}N^+/cm^2$ with 35 keV

(a)Albina, Xantha and Striata in M2 generation;

(b)To breed a yellow-plant line selected from chlorophyll mutations of M1 generation of rice modified by ion implantation. Green plants are controlled group.

Table 2 Mutation rate of M2 for rice in % [3]

Varieties	Chlorophyll	Ripe stage	Plant height	Fertility	Other	Total
Luwuhong	0.21	3.4	3.4	--	1.7	8.6
CO12	0.39	--	--	2.1	--	2.4
02428	0.59	5.9	0.4	--	7.5	14.4
Average	0.39	3.1	1.3	0.7	3.1	8.5

The experiments were done in two situations: (A) the uncovered PUC 19 was injected by ions, and then transferred into E. Coli; (B) the PUC 19 was firstly transferred into E. Coli; and E. Coli with the plasmid PUC 19 was then injected by ions. In case A the mutation rate of the stained gene was more than 30%. In case B the mutation rate of the resistant and the stained gene were 9.8% and 0.2% respectively. It has been also found that the color of a coenobia began to turn into blue after seven days. It seems that the regulator gene in E. Coli could be changed by ion implantation.

2 PRIMARY ANALYSIS

In order to study the biological effects induced by low energy ions, some of the analytical works have been made at cellular and molecular levels.

(a) (b)

Fig. 4 Photographs of Rice Suspension Cells Etched by 1.5×10^{15} Ar^+ ions/cm^2
(a) without Ar^+ implantation;
(b) Ar^+ implantation with 30 keV.

The surface features of cells injected by ion beam would be observed by using scanning electron microscopy (Fig. 4). The surface of the un-injected cell was smooth, but there were many small holes in the rougher cell walls facing ion beam after ion implantation. When the dose increased the holes would become large and large, finally resulting in breaking the cell wall. On the other hand, the negative of the modified cells was electrically changed because of the positive ion implantation (see Ref. 8). The electrophoretic mobility of the cells was quickly decreased under a low dose stage, then slowly reduced to 0. 5[V/s]/[V/m] comparing with 2. 0[V/s]/[V/m] of the control group. The changes of the feature and negative of cell surfaces would influence them at the biological effects. Typical effects were that the growth was delayed and the survival rate was decreased.

The chromosomal aberration was observed at the root cells of cotton during karyomitosis. Types of the chromosomal aberration were micronuclei, bridges and fragments, etc. (see Fig. 5). From Fig. 5 it shows that there are single, double and multi-micronuclei and multi-bridges. Of these types, the frequencies of the micronuclei were higher than that of other aberration, and would be increased when increasing the injected dose (see Fig. 6). The dose with the micronuclei's frequencies has a profile as the same as that of free radical content in dry cotton seeds implanted by ions. The relationship between the micronuclei and free radical is unclear right now. The free radical was only in a thin layer and its thickness was about two hundred micrometers from the injected surface[12]. If the composition of the thin layer was analyzed by spectrographic method, the content of the injected ions could be increased. Fig. 7 shows XPS spectrum of thymine before or after implanting N^+. From integrating N_{1s} spectrum of the implanted N^+ sample the content of nitrogen would be about 30% higher than the original content. The N_{1s} peak of the injected sample is superposed by multi-peaks of various nitride. Binding energy of N_{1s} is in the range from 398. 60 eV to 400. 99 eV, and it drifts apart from the main peak of the un-injected sample in which binding energy of N_{1s} is 400 eV. This fact shows that the injecting N^+ could combine with other molecule to form new constructions. This point has been naturally observed by using other methods, like ultraviolet, laser, mass-spectrum, during analyzing the tyrosine injected by ions.

(a) (b) (c)

Fig. 5 Chromosomal Aberration of Cotton Root Cells. Dry Seeds were Implanted by N^+ at doses $2 \sim 8 \times 10^{16}$ ions/cm^2 with 35 keV (a)Micronuclei; (b)Double Bridges; (c)Fragments.

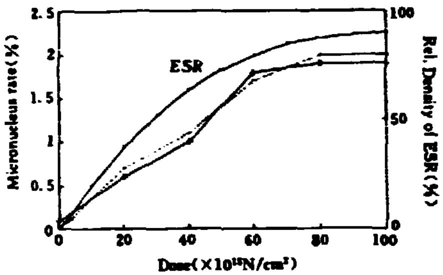


Fig. 6 Curves of Frequencies of Micronuclei and Density of Free Radical Signal with Dose

Sample: dry seeds of cotton.

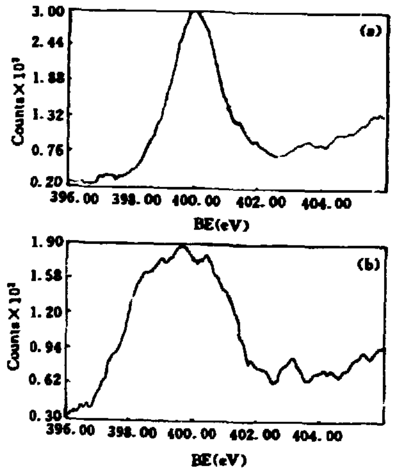


Fig. 7 XPS Spectrum of Thymine With or Without Ion Implantation.

(a)N_{1s} spectrum of CK;

(b)With ion implantation at dose 3×10^{15}ions/cm^2.

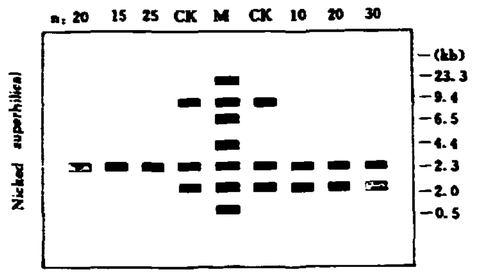


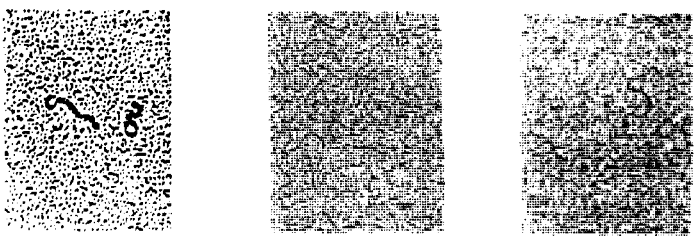
Fig. 8 Electrophoresis Photo of Plasmid PBR322 DNA Implanted by N^+ with 20 keV
left: un-covered DNA; right: covered DNA of E. Coli's
M: molcular standard of M. λ/Hind, CK: control groups;
dose: $n \times 10^{15}$ ions/cm^2 with 30 keV.

Low energy ion effect at DNA level were also detected. The plasmid PBR322 DNA, which is extracted from E. Coli with the ion injection, and the un-covered plasmid PBR322 DNA with ion implantation were analyzed by agarose gel electrophoresis (see Fig. 8)[5][13]. The un-covered DNA becomes nicked circular DNA from the superhelical circular after ion implantation. When dose increased further the nicked circular DNA would become linear DNA. The superhelical circular DNA in E. Coli implanted by ions also partly became nicked circular DNA, but there still was a little superhelical circular DNA. The reason, was that the cell wall and memberane in E. Coli shielded the DNA inside of E. Coli during ion implantation. Therefore, dose injected into DNA in E. Coli was less than in un-covered DNA when doses in both situations were the same. Fig. 9 shows a group of PUC19 DNA photos taken by the electron microscopy. Superhelical circular DNA became nicked circular DNA only at dose 3×10^{15} ions/cm^2 and became linear DNA only at dose 5×10^{15} ions/cm^2. Even though the injected dose was increased up to 3×10^{16} ions/cm^2 the PBR332 in E. Coli could not completely become nicked circular DNA [see Fig. 8].

3 EXAMPLES OF APPLICATION

Ion implantation as a new mutagenic source has been used in China for years. The main objectives of the program are to improve crops in agriculture and to modify microbes in industry. Though various kinds of mutagenic source, such as γ-rays, laser rays, neutron, chemomor phosis etc. have been developed, people still insist on trying to find better one with higher mutation rate, wider mutational spectrum and easier control. As it has mentioned above, the mutation induced by ion implantation possesses high mutation rate and wide mutational spectrum with lower damage(i. e. higher survival rate). Therefore, breeders could expect to increase the breeding efficiencies or to reduce the breeding period by the induced mutation of ion implantation. Based on the objectives of breeding, breeders have selected a great quantity of mutants and some new genetype materials, some of them have been bred to new varieties, and have been directly used in production, some of them have been indirectly used in breeding. Sometimes a new genetype is more important than a new variety for breeding. For example, a new rice material with high frequency of apomixis HDAR001 obtained by the mutation of ion implantation is universally considered to be a new superior apomictic rice material[4]. It is of momentous current significance for hybrid vigor fixation and breeding of rice.

The mutation breeding of rice induced by ion implantation has made considerable headway. Double-cropping rice, one is Hsien rice named S_{9024} and the other is Keng rice D_{9055}, have already been popularized in province Anhui. Both of these varieties possess higher grain yield, broader disease resistance and better qualities. It is well known that the quality of Hsien rice is becoming generally worse, but the quality of S_{9042} is better, its five of eight items in quality index can reach the national standard of quality of rice. The D_{9055} has been assessed at the high quality rice by Anhui Government based on the national standard. Up to now, S_{9024} and D_{9055} have been already planted in two thousand hectares in eleven stations of double-cropping rice areas. Grain yields of both these varieties are in the range of 6. 5～8. 0 tons per hectare respectively, and increase by about 10% compared with that of the older varieties.



(a) (b) (c)

Fig. 9 Microscopy Photo of PUC19 DNA Implanted by N^+ with 15 keV, at doses: (a)CK, (b)3×10^{15} ions/cm^2, (c)5×10^{15} ions/cm^2.

In genetic engineering, the transferring of genes is an important technique. For the plant cells, the cell wall significantly obstructs penetration of foreign genetic materials when they are introduced into a cell through the wall. From section 3 it is noted that low energy ion beam like a scalpel could etch the cell wall. In this way people has transferred foreign gene into the intact cells of rice. Reference has reported the expression of 'foreign' gene Gus in living samples modified by low energy ion beam. The fluorescence intensities of the cells and ripe embryo with Gus gene are $3 \sim 12$ times higher than that of the un-modified receptors[8]. Recently, the authors successfully transferred the foreign gene PBI_{222} into ripe rice-embryo modified by low energy ion beam and obtained test-tube seedlings with the 'foreign' gene. The repeated experiments showed that two to three wound tissues could be obtained from per hundred embryos modified by low energy ion beam. The result will be published elsewhere.

4 DISCUSSION AND CONCLUSION

In this paper author reported the part of the progress of studies on low energy ion effects at mutation breeding and cellular level. Till now, further understanding for mutagenic mechanism is not clear, but a lot of people in China has been insisting on exploring it. Because the plant seed is a complex mosaic, it is worth devoting our attention to the research on ion implantation effect at cellular and microbe level. Although some parameters have already been collected and some phenomenon could be roughly explained, there are many problems still confronting nowadays. To solve these problems needs co-operating of the physicist and biologist.

Although studies on the effect of low energy ion on the biology induced by ion implantation were tentative, the job will bring broad prospects for its application in production and science. Besides above examples mentioned, low energy ions may be used in measuring the construction of cells at atomic and molecular levels. If various kinds of ions are injected into two sides of a cellular membrane by controlling range that results in a change of physical field across the membrane, people could obtain many messages of biological process, such as energy and mass transporting, gene regulating and message communicating. This would be a very significant work. These jobs demand perhaps an international co-operation.

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