注入碳离子对甜菊的生物学效应

沈明山　蒋先志　徐金森　陈　亮　陈睦传*
(厦门大学细胞生物学及肿瘤细胞工程教育部重点实验室，厦门 361005)

摘要：　对甜菊(*Stevia rebaudianum* Bertoni)种子注入能量为 75 keV、剂量为 $10^{14}/cm^2$ 的碳离子，研究其种子萌发期产生的一系列生物学效应。注入碳离子的种子萌发率略高于对照组，但种苗的成活率比对照组低($P < 0.02$)。运用假设检验法分析，结果表明注入离子的种子出苗株高显著高于对照组($P < 0.01$)。萌发 4 d 的处理组，叶片细胞壁增厚，胞间连丝扩大，内有高电子密度的物质沉积，细胞质膜皱折，膜上有高电子密度的沉积颗粒，质膜向细胞壁方向突起，质膜上的颗粒通过胞间连丝转移或沉积于细胞壁上。上述现象可能与注入离子的过壁运输有关，也可能与胼胝质的积累有关。此外，实验组植物的叶绿体发达，片层结构明显多于对照组，两个质体发生中心同时存在于同一个叶绿体内；线粒体丰富，内嵴发达；微体内可见典型的晶格排列结构。这些结果表明，离子注入种子以后，种子萌发期的合成代谢和呼吸代谢能力明显强于对照组。

关键词：　甜菊；碳离子注入；细胞器；生物学效应
中图分类号：Q942　　文献标识码：A　　文章编号：0577-7496(2000)09-0892-06

Biological Effects of *Stevia rebaudianum* Induced by Carbon Ion Implantation

SHEN Ming-Shan, JIANG Xian-Zhi, XU Jin-Sen, CHEN Liang, CHEN Mu-Chuan*
(*The Key Laboratory of Ministry of Education for Cell Biology and Tumor Cell Engineering, Xiamen University*, Xiamen 361005, China)

Abstract:　The biological effects during seed germination were investigated after the dry seeds of *Stevia rebaudianum* Bertoni were implanted with carbon ion beam of 75 keV and 10^{14} ions/cm^2. The results showed that the germination rate of carbon ion implanted seeds was slightly higher than that of the control, but the survival rate of the treated seedlings, on the contrary, was lower than that of the control ($P < 0.02$), while the height of the treated seedlings was significantly higher than that of the control ($P < 0.01$). On the 4th day after germination, the leaf cell wall in the treated group was thick, some high electron-dense substance deposited in the enlarged plasmodesma; Cell membrane creased with high electron-dense granules deposited on it. The plasma membrane protruded towards cell wall, and the granules shifted via plasmodesma or deposited onto cell wall. These phenomena may be related to the conveyance of implanted ions across cell wall, or be related to the accumulation of callose. In addition, the implantation of carbon ions could increase the lamellae of the chloroplast and cause high development of the chloroplast which sometimes contained two plastid centers in an individual chloroplast. Also, the highly developed cristae, abundant mitochondria and typical crystalloid structure in microbody could be found. All these results indicated that the anabolic and catabolic activities in the seedlings implanted with carbon ions before germination were obviously more active than those in the controls.

Key words:　*Stevia rebaudianum*; carbon ion implantation; organelle; biology effects

Stevia rebaudianum Bertoni (Compositae), a herb with high economic and medicinal value, is the third biggest natural sugar source after sugarcane and sugar beet in the world. China is the first rank in the cultivation, processing and product exportation of *S. rebaudianum*[1]. Therefore, breeding high-yield stevia varieties with high glucoside content is the key to the sustained development of stevia industry. Ion beams with tens and thousands of keV energy, used in the method first developed in China, have been implanted into crop seeds to change their genic structure, which finally brings about the mutagenic breeding of crops and leading us to search for the needed improved strains. Many researches have found that ion implantation could make dozens of plant strains, including rice, cotton, wheat and maize to produce changes in the germination rate, growth speed and

Received: 1999-11-22　Accepted: 2000-03-31
Foundation item: The National Key Project of Interdiscipline (19890300).
* Author for correspondence. E-mail: <xianzhi@jingxian.xmu.edu.cn>. Tel.: 0592-2186630; Fax: 0592-2186630.

plant morph[2-4]. Shu *et al*[5] and Shen *et al*[6] reported that after implanting N^+ into stevia seeds with appropriate dose, the growth speed of the seedlings was accelerated; the individual plant had deep roots, luxuriant leaves, and increased yield; and the content of high-quality rebaudioside-A in total glucosides could be improved.

Using optical microscopic technique, Shen *et al*[7] and Cheng *et al*[8] reported the effect of low-energy ion implantation on the stevia and cotton cell division exponent, nuclear and chromosomal aberration pattern. But so far, little work has been done on the ultrastructural changes. Song *et al*[9] investigated the action of low energy ion beam on microorganisms by scanning electron microscope (SEM). After statistical analysis of the biological effects of carbon ion implantation with different energy and dosage on *S. rebaudianum*, we found the treatment with 75 keV × 10^{14} carbon ions/cm^2 to be optimum. In order to elucidate the cellular and molecular mechanism of low energy ion implantation, we report here the changes of biological characteristic induced by the low energy ions in *S. rebaudianum*.

1 Materials and Methods

1.1 Materials

Dry seeds of *Stevia rebaudianum* Bertoni (Yunbin strain) were obtained from Prof. SHU S-Z in the Institute of Crop Resources, Chinese Academic of Agricultural Sciences and were implanted with carbon ion beam in the Institute of Low Energy Nuclear Physics at Beijing Normal University.

1.2 Methods

1.2.1 Implanting energy and dosage The seeds of *S. rebaudianum* were irradiated continuously for 3 min. Carbon ion with energy of 75 keV and dosage of 10^{14} ions/cm^2 was implanted, abbreviated as 75 keV × 10^{14} ions/cm^2, at a dosage rate of 5.5×10^{11} ions·cm^{-2}·s^{-1}. The implanting conditions were monitored and controlled by the Institute of Low Energy Nuclear Physics at Beijing Normal University.

1.2.2 Evaluation of effects on growth and development of seedlings All seeds sterilized with alcohol and mercuric chloride were germinated in disinfected nourishing soil, on 4-layered sterilized wet filter paper or on MS culture medium as 50 seeds per group and were controlled in anti-insect condition. Measurement and statistical analysis were made for the germination rate on the 10th day, for the number and percentage of seedlings with 4 leaves on the 20th day, and for the heights of seedlings in soil on the 30th day.

1.2.3 Preparation of ultrastructural sections *S. rebaudianum* leaves were quickly cut into 1 mm × 2 mm sized pieces on the 4th day of germination and were fixed in 2.5% glutaraldehyde in 0.1 mol/L sodium phosphate, pH 7.2 at 0—4 ℃ for 5 h and postfixed in 2% osmium tetroxide for 2 h after rinsed with the same buffer. Samples were dehydrated in alcohol series and imbedded in Epon 618. Sections were prepared by microtomy (LKB-8800 III) and photographed with a JEM-100 CX II after doubled-stained with uranyl chloride and lead citrate[10].

1.2.4 The hypothesis testing method[11] was adopted for the analysis of the height difference of seedlings.

2 Results

2.1 Effects of carbon ion implantation on seed germination rate, and seedling growth and development

The germination rate of seeds implanted with given energy and dosage of carbon ions was slightly higher than that of the controls, but some germinating seeds died thus decreasing the seedling survival rate ($P < 0.02$). None the less, growth of the treated seedlings was superior to the controls. The mean height of the survived seedlings was higher than that of the controls by 27.2%; some seedlings died, with a death rate amounted to 24.4% higher than that of the control (Table 1). The leaf shape changed obviously, and the number of seedlings with dented leaves increased by 5.7%.

2.2 Effects of carbon ion implantation on growth of *S. rebaudianum* seedlings

In order to access the significant difference between the growth of treated seeds and that of the control, the seedling heights were measured on the 30th day after sowing (Table 2). As there was no significant difference in the variance of data between the treated group and the control ($F = 1.336$), the mean values of the two groups were compared by t-test which showed that the heights of the treated plants were significantly higher than those in the control ($t_{(121)} = 9.76$, $P < 0.01$). This indicated that implantation on dry seeds with carbon ions of 75 keV and 10^{14}/cm^2 has a pronounced promotive effect on the growth of stevia. Based on the data in Table 2, it could be shown by calculation that the difference among individuals in the treated group was greater than that in the control group.

2.3 Effects of carbon ion implantation on the ultrastructure of the leaf cell wall and cell membrane

As shown in Figs. 1, 2, the cell walls and cell membranes in the controlled leaf cells after 4 days of germination were smooth with no electron-dense deposits over the middle lamella and the plasmodesma could be viewed with narrow passage, whereas, in the ion beam treated samples, cell walls expanded with deposits located on the wall of the enlarged plasmodesma and high electron-dense particles precipitated on the uneven, irregular cell membranes. It was also detected that deposits of different density in adjacent cells, were released towards the cell wall through the projecting membrane and were transported through the plasmodesma (Fig. 3). All these findings were not detected as the treated seedlings resumed natural development (Fig. 9).

Table 1 The germination rate and seedling growth under different cultural conditions

Groups	Test				Control			
	T1	T2	T3	Mean±SD	C1	C2	C3	Mean±SD
Germination rate on wet filter paper (%)	84	78	82	80.7±3.4	80	76	78	78.0±2.0
Germination rate on MS culture medium (%)	92	88	88	98.3±2.3	86	84	86	84.7±1.2
Germination rate in soil (%)	62	60	64	62.0±2.0	54	58	60	57.3±3.1
No. of 4-leaf seedlings/ No. of total seedlings 1) (in soil)	0.70	0.85	0.81	0.79±0.08	0.71	0.66	0.67	0.68±0.04
No. of seedlings/No. of germination seeds (in soil) 2)	0.71	0.77	0.73	0.74±0.03	0.85	0.93	0.88	0.89±0.04
Mean height after 30 days (in soil, cm)	4.15	4.42	4.06	4.21±0.22	3.52	3.19	3.23	3.31±0.18

1) Total seedlings were seedlings with 4 or less leaves. 2) No. of seedlings on 20th day/ No. of seedlings on 10th day.

Table 2 Heights of *Stevia rebaudianum* on 30th day of sowing in soil *

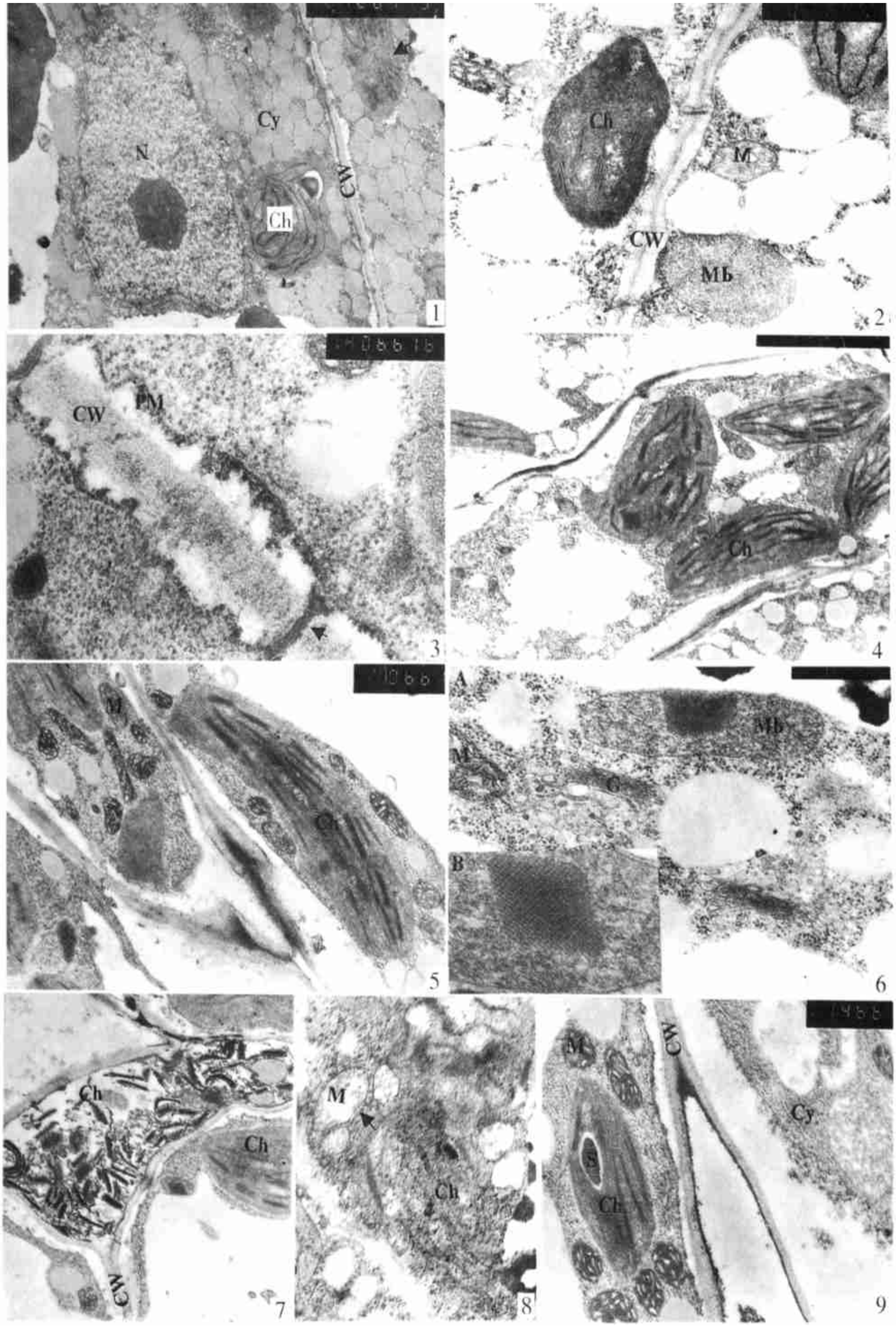
Groups	Plant height (cm)											Mean±SD (cm)
Carbon ion	4.5	4.4	5.0	4.7	4.8	5.2	3.4	4.3	4.6	4.1	3.8	
implanted	3.7	4.5	2.8	4.6	4.9	4.5	4.7	3.6	4.4	4.5	4.6	
(n_1= 53)	4.3	4.9	4.4	3.9	4.7	3.8	4.0	4.3	4.6	4.4	4.2	4.19±0.57
	3.9	4.5	3.8	3.7	4.2	4.3	3.2	4.0	4.3	4.6	3.1	
	2.9	2.7	3.9	3.8	4.5	4.4	4.7	4.8	3.9			
Control	4.0	3.7	3.3	3.6	2.9	3.9	3.8	3.5	3.7	3.6	2.6	
(n_2= 70)	3.9	2.9	3.5	3.7	3.0	3.5	2.6	3.5	3.8	3.1	3.5	3.26±0.49
	2.8	3.6	3.1	3.1	3.9	3.8	3.3	3.0	3.6	3.3	3.6	
	4.1	3.2	3.4	3.6	3.5	4.0	2.4	3.7	3.8	3.1	2.8	
	2.8	2.7	3.0	3.1	3.4	3.5	3.4	3.7	3.6	3.0	2.9	
	3.0	3.4	3.5	3.2	3.3	2.9	2.8	2.5	2.6	2.5	2.4	
	2.9	2.2	2.2	2.1								

* Data were collected for each stevia plant growing on the 30th day after sowing in the soil.

→

Figs. 1－9. **1.** Electron micrograph of a controlled stevia leaf cell, showing chloroplasts which develop slowly, normal nucleus and cytoplasm with high electron density. Note the plastid occurring center in chloroplast (arrow head), ×6 700. **2.** Showing chloroplast, mitochondrion and microbody developing slowly in the controlled stevia leaf cell. No high electron-dense substance can be detected in the plasmodesma and on the cell wall, cell membrane is even and smooth, ×20 000. **3.** In the ion treated stevia leaf cell, high electron-dense particles are found to deposit in the enlarged plasmodesma and on the cell wall, cell membrane is irregular. Note the high electron-dense particles being delivered through plasmodesma (arrow head), ×40 000. **4.** Chloroplasts in the treated cell appear as strips and abundant grana lamellae can be viewed, ×8 000. **5.** Mitochondria with highly developed cristae are found as strips in the ion treated cell, ×10 000. **6A.** Showing abundant Golgi apparatus and highly developed microbody with crystalloid structure in the ion treated cell, ×27 000; **6B.** Higher magnification of a portion in Fig. 6A, showing typical crystalloid structure, ×50 000. **7.** Showing the decomposed chloroplasts in some ion treated leaf cells, ×8 000. **8.** Showing the decomposed mitochondria (arrow head) which become vacuolate in the ion treated stevia leaf cell, ×14 000. **9.** The ultrastructures in the ion treated stevia leaf cell resume to be normalized on the 30th day of germination, ×14 000.

Abbreviations: Cy, cytoplasm; N, nucleus; G, Golgi apparatus; M, mitochondrion; PM, plasma membrane; Ch, chloroplast; Mb, microbody; CW, cell wall; S, starch grain.


N
Cy
Ch
CW
1
Ch
M
CW
Mb
2
CW
PM
3
Ch
4
M
Ch
5
A
Mb
M
B
6
Ch
Ch
CW
7
M
Ch
8
M
CW
S
Ch
Cy
9

2.4 Effects of carbon ion implantation on the ultrastructures of chloroplast, mitochondrion and microbody

Chloroplast in the controlled stevia leaf cells after 4 days of germination developed slower, most of them were highly electron-dense, in which few stroma and grana lamellae could be observed. Plastid centers could be detected in some chloroplasts, from which grana lamellae were radially arrayed (Figs. 1, 2). Chloroplasts in carbon ion treated leaves were found to be large and elliptical or striped and less electron-dense. Plastid centers and abundant grana lamellae were also visualized. All these indicated that they developed faster than those of the controls (Fig. 4). Mitochondria, with much more developed cristae, were rich and orderly arranged (Fig. 5). The most striking features of the treated samples were the abundant and developed Golgi bodies and the microbodies that developed in advance with typical crystalloid region, which occupied a quarter to half of the total sectional area of the microbody (Fig. 6).

During germination from the carbon ion treated seeds, sometimes, the chloroplasts and mitochondria might decompose and break down after they had developed to a certain extent (Figs. 7, 8). 5.27% of the 2 000 cells examined in the central area of the sections of the treated samples were detected with decomposed organellae, while only 0.23% were found in the controls. The decomposed organellae could not be found in the leaf cells on the 30th day of the seedlings grown from the carbon ion treated seeds.

3 Discussion

The present results showed that implantation of carbon ion of 75 keV$\times 10^{14}$ ions/cm^2 has a promotive effects on the growth and development of *S. rebaudianum*. Shen *et al*[6,7] have reported that different biological effects were induced by N^+ implantation at different energy levels and the effects of N^+ implantation at 30 keV$\times 10^{14}$ ions/cm^2 on *S. rebaudianum*, which seems to be the appropriate energy and dosage combination, involved the increase in the seed germination rate, promotion of plant growth and development and improvement in the quality and quantity of products. Luo *et al*[12] have reported the inhibitory effects of implanting carbon ion of 100 keV$\times 10^{15}$ ions/cm^2 on the seed germination rate, growth and development of seedlings and a decrease in the percentage of seedlings. Meanwhile, the changes in the ultrastructures of cells were also different to those described in this paper. Obviously, it is important to consider the ion type and its appropriate amount of energy and dosage when implanting ion into stevia seeds.

It has been reported that high electron-dense deposits are located over the expanded cell wall and the membrane of leaf cell during the germination of carbon ion implanted seeds, which may be caused by the precipitation of ions on the external surface of cell wall.[2,4] Our research suggests that the implanted ions may participate in the cell activities of material exchange and delivery. This suggestion is supported by the findings of the projections and high electron-dense deposits on the cell membranes, the density difference of deposits between adjacent cells and the precipitates in the expanded plasmodesma. In addition, the chloroplasts and mitochondria developing in advance indicate the enhancement of the anabolic and catabolic activities of the cells. Furthermore, microbodies, in which crystalloid regions could be detected to occupy 1/4—1/2 of the total area, developed in advance, and the synthesis of catalase shown to be improved[10,13]. This is also supported by our biochemical analyses (presented in another paper). It has been reported that catalase is an important enzyme participating in the activities of eliminating free radical and synthesizing callose in the cell[14]. The synthesis of callose, which is delivered to cell walls and make them thicker to resist the stimuli from the external environment, is promoted by the enhancement of catalase activity. This may be the reason that the ultrastructures of treated cell walls and membranes are obviously different from those of the controls. In conclusion, the carbon ions implanted at 75 keV $\times 10^{14}$ ions/cm^2 may participate in the cell activities of material exchange and delivery, improve organism's metabolic activities to eliminate harmful effects induced by ions implantation, and enhance organism's resistance to the external environment. All these are the cytological bases for the growth and development of the implanted stevia seedlings and can partially explain why the carbon ions implanted into seeds only in μm level by drilling a hole can make the treated plants be superior to the controls.

Although it has been found that there was no effect of carbon ion implantation at 75 keV$\times 10^{14}$ ions/cm^2 on seed germination rate, yet some seedlings died later which affected the percentage of seedlings in the treated samples being lower than that of controls. It may be related to the different damage to seeds during ion implanting[9]. The higher energy and dosage the implanted ions were, the more cells with damage on chloroplasts and mitochondria could be detected (about 5%)[12]. However, when carbon ion implantation at 75 keV $\times 10^{14}$ ions/cm^2, only 0.527% of the totally observed cells were found to be damaged. The works on N^+ implantation into cotton by Cheng *et al*[8] showed that the implantation would induce chromosomal aberration and micronuclei. Yang *et al*[15] investigated the effect of ion beam irradiation on the *lac Z* gene and found basic changes induced by ion implantation. Using RAPD technique, Ding *et al*[16] found that stevia DNA produced polymorphic amplification after N^+ implantation. All these findings indicate that when DNA, especially the naked chloroplast DNA, are damaged, the gene expression pattern will be changed. Therefore, the main reason for the dying of some seedlings after their germination may result from the damage induced by ion implantation, it also may account for the phenomenon that the individual difference of heights in ion-implanted samples is greater than that in the controls.

Acknowledgements: We thank Prof. SHU Shi-Zhen, from the Institute of Crop Resources, Chinese Academic of Agricultural Sciences for providing the *S. rebaudianum* seeds and Prof. LU Ting, of the Institute of Low Energy Physics, Beijing Normal University for the help in preparing carbon ion implanted samples. We also thank Prof. CHENG Xiao-Lin for his reviewing the manuscript and suggestions.

References:

[1] Shu S-Z(舒世珍), Chen M-C(陈睦传), Chen S-Q(陈绍裘), Chen S-P(陈绍潘), Zhao S-W(赵树文), Zhao P-X(赵鹏翔), Zhang X-C(张学才), Li Qin(李钦). Cultivation and Application Techniques of *Stevia rebaudianum* Bertoni in China. Beijing: Agriculture Press, 1994. 1—17. (in Chinese)

[2] Yu Z-L(余增亮). Low energy ion biology. *Science*(Shanghai)(科学, 上海), 1993, (4):36—38. (in Chinese)

[3] Lu T(陆挺), Xie L-Q(谢立青), Zhu F-S(朱风绥), Wei J-Z(卫俊智). Biological effects of ion implantation into plant seed. *Nucl Tech* (核技术), 1994, **17**(4):443—448. (in Chinese)

[4] Lu T, Cao H-M, Zhang R-W. Observing the effects of nitrogen ion implantation in seeds of lima Bean on positron annihilation. *Mat Sci Forum*, 1995, 175—178, 447—448.

[5] Shu S-Z(舒世珍), Wang C-L(王彩莲), Chen Q-F(陈秋方), Shen M(慎玫), Lu T(陆挺). A study on biological effect of ion implanted into sweetening chrysanthemum (*Stevia rebaudianum*) seeds Ⅰ: effect on botanical characteristics of sweetening chrysanthemum with low energy ion implanted. *Sugar Crops Chin* (中国糖料), 1998, (1):14—17. (in Chinese)

[6] Shen M(慎玫), Wang C-L(王彩莲), Chen Q-F(陈秋方), Shu S-Z(舒世珍), Lu T(陆挺). A study on biological effect of ion implanted into sweetening chrysanthemum seeds Ⅱ: analysis of the composition and yield of total stevioside. *Sugar Crops Chin* (中国糖料), 1998, (4):6—9. (in Chinese)

[7] Shen M(慎玫), Wang C-L(王彩莲), Chen Q-F(陈秋方), Lu T(陆挺), Shu S-Z(舒世珍). Cytological effect of nitrogen ion implantation into stevia. *Acta Agri Nucl Sin* (核农学报), 1997, **11**:141—144. (in Chinese)

[8] Cheng B-J(程备久), Li Z(李展), Wang G-M(王公明), Lin Y(林毅), Yu Z-L(余增亮), He J-J(何建军). The mutagenic effects of nitrogen ion implantation in cotton seed. *Acta Agri Nucl Sin* (核农学报), 1993, **7**:73—80. (in Chinese)

[9] Song D-J(宋道军), Yu X(余讯), Yu Z-L(余增亮). Study on the direct and indirect action of N^+ ion implantation on *Deinococcus radiodurans* and *E. coli*. *High Tech Lett*(高技术通讯), 1999, **9**(1):47—50. (in Chinese)

[10] Chen M-C(陈睦传), Hong W-L(洪维廉), Wang D-Y(汪德耀). Ultrastructural study on microbody of cells in leaves and root-tips of *Stevia rebaudianum* Bertoni. *Acta Bot Sin* (植物学报), 1988, **30**:96—98. (in Chinese).

[11] Xu D-Z(徐端正). Applications of Biostatistics in Pharmacology. Beijing: Science Press, 1986. 44—63. (in Chinese)

[12] Luo M-C(罗茂春), Shen M-S(沈明山), Xu J-S(徐金森), Jiang X-Z(蒋先志), Shao H-J(邵寒娟), Chen M-C(陈睦传), Lu T(陆挺), Shu S-Z(舒世珍). Effect of carbon ion implantation on the growth and development of chloroplast of *Stevia rebaudianum*. *J Xiamen Univ* (Natural Sci.)(厦门大学学报, 自然科学版), 2000, **39**:96—101. (in Chinese)

[13] Liu D(刘丹), Chen M-C(陈睦传), Hong W-L(洪维廉), Wang D-Y(汪德耀). The study on the configuration of the microbody crystalloid of *Stevia rebaudianum* Bertoni. *Acta Biophys Sin* (生物物理学报), 1991, **7**:18—24. (in Chinese).

[14] Zhu Y-X(朱玉贤), Li Y(李毅). Modern Molecular Biology. Beijing: High Education Press, 1997. 271—285. (in Chinese)

[15] Yang J-B, Wu L-J, Li L, Wu J-D, Yu Z-I, Xu Z-H. Sequence analysis of lac Z-mutations induced by ion beam irradiation in double-stranded M13 mp18 DNA. *Sci Chin* (Series C), 1997, **40**:107—112.

[16] Ding L(丁亮), Chen M-C(陈睦传), Shen M-S(沈明山), Xu J-S(徐金森), Jiang X-Z(蒋先志), Chen L(陈亮), Lu T(陆挺), Shu S-Z(舒世珍). RAPD analysis of seedling genomic DNA variation induced by N^+ ions implantation in stevia seeds. *Acta Biophys Sin*(生物物理学报), 1999, **15**:798—803. (in Chinese)

(责任编辑: 王 葳)