The analysis of proteome changes in sunflower seeds induced by N^+ implantation

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In this work, the proteomic changes induced by N^+ ion implantation were investigated using a sunflower seed model by a two-dimensional electrophoretic analysis. To further understand the changes of total protein irradiated with N^+ ion, a proteomic analysis of N^+ ion implantation seeds was developed. Among approximately 369 total protein spots displayed in 2-D gels, eight specific proteins were found in non-implanted seeds while four proteins were found in implanted seeds. Six proteins were used for MALDI-TOF MS analysis, of which only two had been reported before. The proteins designated as No. 29 showed 23.4% homology to MADS-box transcriptional factor HAM59, while No. 279 protein had 23.20% identity to homeobox-leucine zipper protein HAHB-4. The analysis of proteome changes induced by N^+ implantation could provid a new clue to studing mutation mechanism of ion implantation. To our knowledge, this is the first report about the analysis of proteome changes induced by N^+ implantation in sunflower seeds.

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1. Introduction

X-ray has been successfully used for breeding many improved varieties via induced genetic mutation (Muller 1927). Since then, others mutational sources, such as γ-rays, laser rays and neutrons, have been widely applied in crop mutation. Developing better mutagenic sources with higher mutation rate and wider mutational spectrum has always intrigued many researchers' interests. For example, in 1972, FAO/IAEA began to sponsor an international research program to develop new mutagenic sources. Reitz *et al* (1995) discussed biological effects of heavy ions from cosmic radiation, and Yu *et al* (1991, 1993) first applied low-energy ions to induce mutation in rice, thereby offering a new concept in developing new mutagenic sources. Ion beams have a higher linear energy transfer (LET) than do X-rays and gamma-rays, and the range of ion beams in the target materials can be controlled (Tanaka 1997; Yoshihiro *et al* 1999). The effects resulted from ion implantation may be generation of secondary electrons, characteristic X-ray and thermal spikes (Wei *et al* 1995; Song *et al* 1998).

For heavy ion, strand break production in plasmid DNA has been studied extensively by using different kinds of radiations covering a wide range in LET and specific energy for plasmids (Brons *et al* 2003). Various studies on cell inactivation induced by ion beams have been performed (Kraft 1987; Kiefer 1992), although only a limited number of such studies have focused on plants (Nishimura *et al* 1997; Vasilenko and Sidorenko 1995). In our previous report, DNA variation and the mutational 'hotspot' induced by low-energy ion implantation were identified by

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Abbreviations used: 2-DE, two-dimentional electrophoresis; DTT, dithiothreitol; LET, linear energy transfer; MS, mass spectrometer.

random amplified polymorphic DNA (RAPD) marker and DNA sequencing (Chang *et al* 2003). It showed that low energy ion beams had more effect on thymine than other bases. This mechanism is similar to that of γ-rays, but not UV (Bridges *et al* 1968; Charles and Alessandro 2002; Chang *et al* 2003). Furthermore, distinct changes in numbers, kinds and activities of total soluble proteins, esterase isozyme and peroxidase isozyme were observed after low-energy ion implantation (Wu *et al* 1996; Li *et al* 2001).

Even though the effects on esterase isozyme, peroxidase isozyme and DNA modification from low-energy ion implantation were validated in course of plant development, an in-depth research is expected for the proteomic 'domino' effects of low-energy ion implantation. Proteomeic analysis, consisting of highly reproducible two-dimensional electrophoresis (2-DE) coupled with mass spectrometer (MS) and database searches, has enabled us to identify and characterize proteins in the process of low-energy ion implantation (Wasinger *et al* 1995). In this paper, we investigated the effects of N^+ ion implantation in sunflower seeds proteome and the associated mechanisms regarding ion implantation were also explored.

2. Materials and methods

2.1 *Sunflower seeds and ion implantation treatment*

Homozygous sunflower seeds (named 1278) released from dormancy were obtained from our laboratory. Seed capsule were thoroughly stripped. The N^+ ion was implanted into the thoroughly stripped sunflower capsella (embryos, but not cotyledon) according to the method (Yu *et al* 1993) offered by ASIPP (Institute of Plasma Physics, Academia Sinica). The implantation system used a high-current ion source of the Oak Ridge National Laboratory (ORNL) type (heat cathode, reflective arc, double-plasma ion source). The target plate, which is thermostatic with a water cooling system, is located 2 m from the ion source, and the uniformity of ion distribution within an area of 15 cm diameter on the plate should be near 95%. The parameters used in the experiments were: 25 KeV of N^+, 6.26×10^{16} ions/cm^2 of pulse particle fluence, and 2.50×10^{18} ions/cm^2 of the total particle fluence. The pulse duration, interpulse interval and pulse dose are 5 s, 25 s with an ion density of 25 mA/cm at 3×10^{-2} Pa. Before implantation, air-dried seeds of the different strains were put on target plates with the embryo part facing the ion beam as described in the method. Every treatment was performed in triplicate with 80 seeds each. The temperature was kept below 50°C during the process. Seeds treated by alternative method and non-treated seeds were considered as controls.

2.2 *Imbibitions of seeds*

Seeds were sterilized by 70% alcohol for 30 s and 1.0% sodium hypochlorite (NaClO) for 10 min, and washed three times with sterile water. After 24 h imbibitions in sterile water, seeds were prepared for protein extraction.

2.3 *Protein extraction*

Seeds after imbibitions were chosen randomly. Cotyledon was removed and the remainders used for protein extraction. Tissue samples were ground in liquid nitrogen, suspended in 10% (w/v) trichloroacetic acid (TCA) in acetone with 0.07% (w/v) dithiothreitol (DTT) at –20°C for 1 h, and centrifuged again at 4°C. This step was repeated three times and then the pellets were lyophilized for 1 h. Proteins were solubilized with lysis buffer containing 8 M urea, 4% CHAPS, 10 mM DTT, 40 mM Tris base, pH 8.5 (Karine *et al* 2001). Protein concentration was estimated by Bradford protein assays, using bovine serum albumin as standard (Bradford *et al* 1976).

2.4 *2-DE, image analysis and protein identification*

Two-dimensional electrophoresis was carried out according to the reported method (Karine *et al* 2001). Briefly, isoelectrofocusing was performed in the Multiphor® system (Amersham Pharmacia Biotech, Uppsala, Sweden) with IPG dry strips forming an immobilized nonlinear gradient pH of 3 to 10 (Immobiline DryStrip, pH 3–10 NL, 18 cm; Amersham Pharmacia Biotech, Uppsala, Sweden). After equilibration of IPG strips, the second dimension was run on a Multiphor II unit using precast XL 12–14% gradient gels (Amersham Pharmacia Biotech, Uppsala, Sweden). Five hundreds μm of protein for each sample were used in electrophoresis analysis. For each condition analysed, 2-D gels were made in triplicate and from two independent protein extractions.

Image analysis was performed using ImageMaster 2-D Elite version 3.01 (Amersham Pharmacia Biotech, Uppsala, Sweden). After spot detection and background subtraction, gels were aligned and matched for the quantitative determination of the spot volumes. After image analysis, delayed extraction MALDI mass spectra were recorded on a REFLEX III reflectron time of flight mass spectrometer (Bruker-Daltonics, Bremen, Germany). Porcine trypsin autolysis products were used for the internal peptide mass calibration. Protein with the peptide mass maps was identified using the MASCOT search engine *(http://www.matrixscience.com/)*. Theoretical masses and pI were calculated using ExPASy tools *(http://www.expasy.ch)*.

3. Results

3.1 *The characterization of proteins related to ion implantation*

After treatment, implanted seeds and controls were selected to evaluate the proteomic changes in sunflower embryonic axes using 2-DE. Seeds treated with non-N^+ ion implantation showed identical 2-DE protein map with non-treated seeds. So, those two maps were stood for one map (figure 1). The 2-DE reference maps were shown in Figures 1 and 2 after staining. In those two maps, many proteins disappeared and others appeared in implanted seeds and controls. We were interested in proteins coded as 29, 62, 65, 191, 210, 221, 240 and 242 that emerged in controls (figure 1) but not in implanted seeds (figure 2), the experimental molecular mass of which ranged from 16 kDa to 34 kDa with experimental p*I* between 4.75 and 9.00. After 24 h imbibition, 4 proteins coded as 94, 199, 279 and 280 were identified in implanted seeds with molecular mass from 16 kDa to 26 kDa and p*I* 6.25–8.00. Furthermore, labelled proteins in controls except 29 were characterized as having low molecular weight (16 kDa–19 kDa) and relatively broad range of isoelectric point (4.75–9.00). Intriguingly, molecular mass of labelled proteins with narrow range of isoelectric point in implanted seeds distributes broader than that of proteins in controls (figures 1 and 2).

3.2 *The identification of proteins with MALDI-TOF MS and database*

Six proteins in controls and four proteins showing reversible behaviour implanted seeds were excised from preparative gels and analysed by MALDI-TOF MS. Those proteins were identified by tryptic fingerprinting with MALDI-TOF MS. In the following figure 3 represents MALDI-TOF mass fingerprinting of spot 29 and figure 4 stands for MALDI-TOF mass fingerprinting of spot 279.

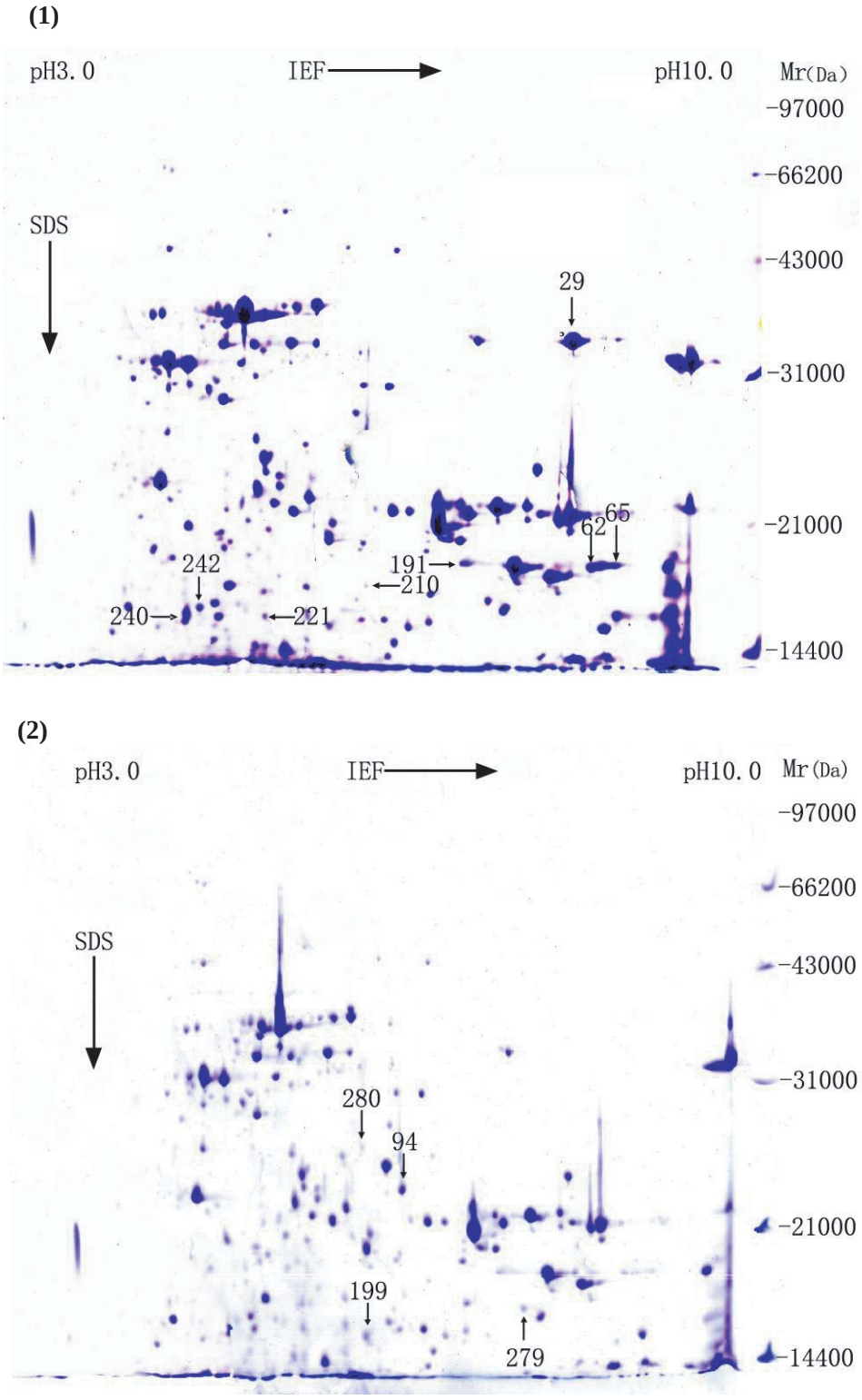
The peptide masses were compared against the SWISS-PROT databases using the PeptIdent *(http://www.expasy.ch/tools/pepident.html)*. Only two proteins coded as 29 and 279 have homologues within the SWISS-PROT databases. After searching SWISS-PROT databases, we found percentage of amino acids in spot 29 covered 23.48% with MADS-box transcriptional factor HAM59 (figure 5), and the MALDI-TOF coverage between spot 279 and homeobox-leucine zipper protein HAHB-4 was 23.20% (figure .6).

In addition, theoretical molecular mass and theoretical p*I* were provided in ExPASy Molecular Biology Server *(http://cn.expasy.org/tools/pi_tool.html)* (table 1).

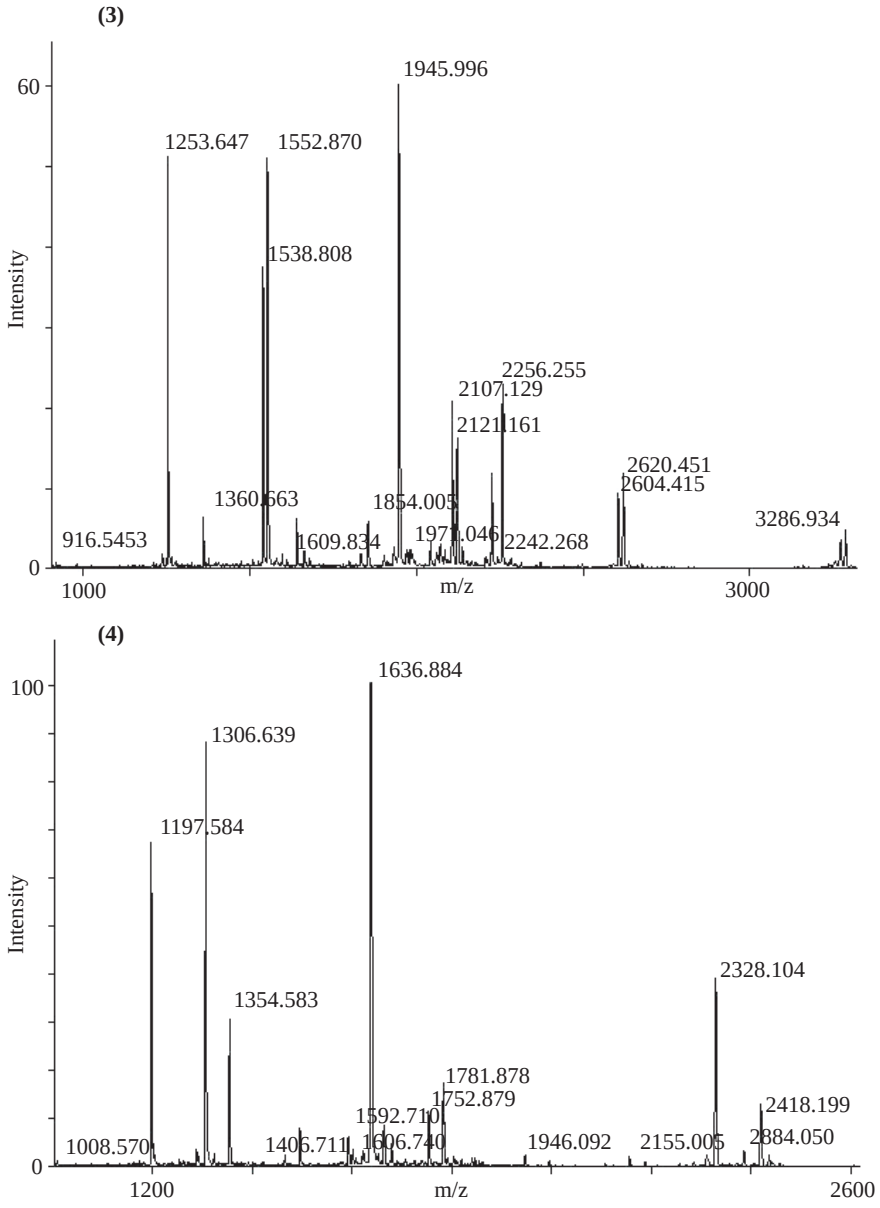
4. Discussion

Ion implantation technique had been considered as a new and effective mutational source (Yu *et al* 1991; Reitz *et al* 1995; Tanaka 1997). The biological effects induced by ion energy, momentum transferring and ion depositing process, were more complex than those made in other radiations (Yu *et al* 1991; Yu and Shao 1994; Reitz *et al* 1995; Yu 2000; Wu and Yu 2001). Heretofore ion beam is broad-spectrum, single ion implantation is the next object which is more sensitive than multiple ion beams. Cells were damaged by the momentum transfer and energy deposition of implantation ions (Yu *et al* 1993). Furthermore, it was reported that Cu^{2+} could cause changes in the contents of starch and protein in bean (Su *et al* 1996). Paula *et al* (2005) surveyed responses of maize to UV-B radiation using DIGE 2D gels and identified selected proteins by mass spectrometry, which could show the probable mechanisms of morphological and physiological responses in plants elicited by UV-B radiation. In this study, we employed proteomics technique to analyse the biological effects induced by N^+ in sunflower seeds. Approximately 369 proteins in implanted seeds gels and no-implanted seeds gels were identified, and obvious proteomic difference between implanted seeds and non-implanted seeds was observed.

It is reported that low energy ion implantation could lead to variation of germplasm (Yu *et al* 1993; Song *et al* 1998; Yu 2000) due to the possible changes in genome structure and gene expression as shown in our previous study (Chang *et al* 2003). Furthermore, the variations of germ plasm were finally reflected in the changes of proteins (Wu *et al* 1996). Recently, a number of mutants such as dwarfing mutant and mutant without petals (Yang *et al* 1997) have been obtained using ion implantation. In this experiment, we employed N^+ ion to implant sunflower seeds regularly and obtained sunflower mutation with ligulate flower and no tubular floret (data not shown). In the meantime, we employed 2-DE and MALDI-TOF MS and discovered that two proteins changed obviously, which might possibly correlate with the sunflower mutation we found. Those mutations probably resulted from the ion indirect effects, such as X-rays (Wei *et al* 1995). In addition, the MADS-box genes act as transcription factors and are characterized by their conserved MADS domain, which functions in DNA binding, dimerization, and accessory-factor interactions. The MADS-box transcription factor HAM59 accommodates MADS-box genes to regulate generating tissue and the characteristics of floral organ (Shore and Sharrocks 1995). From the above results, we presumed that ion beam affected development of floral pattern which maybe useful in plant breeding, especially ornamental breeding. Homeobox-leucine zipper protein HAHB-4 belongs to an important transcription factor family in plants. It is involved in the expression and



Figures 1 and 2. **(1)** DE reference map for seeds protein controls. **(2)** 2-DE reference map of implanted seeds proteins. Mr, Molecular weight marker; IEF, isoelectric focusing; SDS, sodium dodecyl sulphate polyacrylamide gel electrophoresis. The numbers on the map are the codes of specific proteins indicated by arrow in controls.



Figures 3 and 4. MALDI-TOF mass fingerprinting of spot 29 **(3)** and spot 279 **(4)**. The peak value stands for mass charge ratio of corresponding peptide.

1 MSFPNESGEM SPQRKLGRGK IEIKRIENTT NRQVTFCKRR NGLLKKAYEL SVLCDAEVAL
61 IVFSSRGRLY EYANNSVKGT IDRYKKACLD PPSSGSVAEA NAQFYQQEAA KLRQQIANLQ
121 NQNRQFYRNI MGESLGNMPA KDLKNLEGKL EKGISRIRSK KNELLFAEIE YMPKRENELH
181 NNNQFLRAKI AENERSQQQH MSLMPGSSDY DLVPPHQPFD GRNYLQVNDL QPNNSYSCQD
241 QTPLQLV

Figure 5. Sequence coverage of MADS-box transcriptional factor HAM59 by MALDI-TOF data of spot 29. The 247 letters stand for amino acid sequence of MADS-box transcriptional factor HAM59.The underlines stand for the partial peptides of spot 29 matched with MADS-box transcriptional factor HAM59 amino acids.

1 MSLQQVPTTE TTTRKNRNEG RKRFTDKQIS FLEYMFETQS RPELRMKHQL AHKLGLHPRQ
61 VAIWFQNKRA RSKSRQIEQE YNALKHNYET LASKSESLKK ENQALLNQLE VLRNVAEKHQ
121 EKTSSSGSGE ESDDRFTNSP DVMFGQEMNV PFCDGFAYFE EGNSLLEIEE QLPDPQKWWE
181 F

Figure 6. Sequence coverage of homeobox-leucine zipper protein HAHB-4 by MALDI-TOF data of spot 279. The 181 letters stand for amino acid sequence of homeobox-leucine zipper protein HAHB-4.The underlines stand for the partial peptides of spot 279 matched with the amino acids of homeobox-leucine zipper protein HAHB-4.

Table 1. Identification of sunflower seed protein responding to N^+ ion implantation using MALDI-TOF MS.

Code	Protein name	Exp. Mol. Mass (kDa)[a]	Exp. p*I* [b]	Coverage (%)	Theor. Mol. Mass (kDa)[a,d]	Theor. p*I* [b,d]	Accession[c]
No. 29	MADS-box transcriptional factor HAM59	34.07	8.50	23.48	28.35	9.27	27657747
No. 279	Homeobox-leucine zipper protein HAHB-4	17.44	6.54	23.20	21.34	6.24	13447045

[a] Exp. Mol.Mass, Experimental molecular mass; Theor. Mol. Mass, theoretical molecular mass.

[b] Exp. p*I*, experimental p*I*; Theor. p*I*, theoretical p*I*.

[c] The accession number code refers to SWISS-PROT.

[d] Theoretical masses and pI were predicted by entering the sequence at ExPASy Molecular Biology Server (http://cn.expasy.org/tools/pi_tool.html)

modulation of genes in higher plants and correlates with the expression of storage genes in seeds, photomorphogenesis, and the growth of developmental organ (Schmidt *et al* 1987, 1990). In short, this study demonstrates that N^+ ion induced dramatic proteome changes, which in turn probably lead to mutations of plant phenotype. At the protein level, we tested that N^+ ion implant could be a very efficient way to generate many mutants.

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