

## Gene expression profiles in promoted-growth rice seedlings that germinated from the seeds implanted by low-energy N<sup>+</sup> beam

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The stimulation effect that some beneficial agronomic qualities have exhibited in present-generation plants have also been observed due to ion implantation on plants. However, there is relatively little knowledge regarding the molecular mechanism of the stimulation effects of ion-beam implantation. In order to extend our current knowledge about the functional genes related to this stimulation effect, we have reported a comprehensive microarray analysis of the transcriptome features of the promoted-growth rice seedlings germinating from seeds implanted by a low-energy N<sup>+</sup> beam. The results showed that 351 up-regulated transcripts and 470 down-regulated transcripts, including signaling proteins, kinases, plant hormones, transposable elements, transcription factors, non-coding protein RNA (including miRNA), secondary metabolites, resistance proteins, peroxidase and chromatin modification, are all involved in the stimulating effects of ion-beam implantation. The divergences of the functional catalog between the vacuum and ion implantation suggest that ion implantation is the principle cause of the ion-beam implantation biological effects, and revealed the complex molecular networks required to adapt to ion-beam implantation stress in plants, including enhanced transposition of transposable elements, promoted ABA biosynthesis and changes in chromatin modification. Our data will extend the current understanding of the molecular mechanisms and gene regulation of stimulation effects. Further research on the candidates reported in this study should provide new insights into the molecular mechanisms of biological effects induced by ion-beam implantation.

**Keywords:** implantation with low-energy ion beam; stimulation effect; analysis of microarray

### INTRODUCTION

Ion-beam technology as a new mutation method has been extensively applied to organisms for gene (genetic) transformation, mutation breeding, creation of aneuploids and analyses of organism structures by ion-beam etching [1–3]. Low-energy ion beam implantation is very different from other types of physical radiation because of its lower energy, mass deposition and charge transfer, unlike X-rays, for example, which have only energy, no mass deposition and a charge transfer [4].

In ion implantation experiments, a radiostimulation effect has been observed, conferring beneficial agronomic qualities to present-generation plants, such as the improvement of leaf production and the intrinsic composition in tobacco leaves and sweet-leaf chrysanthemums [5]. If ion energy

and fluence are controlled properly, the reproducibility of the promoted growth effects increases. No matter whether the stimulation effect is inherited or not, its significant value can be directly applied to the present generation. This application is worthy of further exploration, for example, in improvement of the germination speed and growth potential of grass seedlings and trees on waste mountains. This biological effect is partly ascribed to the etching process of low-energy ions on seeds, which can improve the permeability of the shell surface and facilitate the exchange of external substances (nutrients and water, etc.). Consequently, the germination rate is increased and the growth potential is enhanced and maintained [6]. In addition to the etching effect, it should be noted that the expression of some genes is activated and thus the types, structures and activities of the proteins determined by these expressions are

subsequently changed. Most previous research has focused on the family of proteins or genes responding to ion-beam implantation, such as the functional proteins that eliminate harmful free radicals in cells [7–9], and are responsible for signal pathways [10]. However, current findings are not enough to fully understand the molecular mechanisms behind the biological stimulation effects induced by ion-beam implantation, as there is very little knowledge of comprehensive gene expression profiles. Understanding the comprehensive characteristics of the transcriptome expression profiles is important in order to understand the conserved and diverse molecular mechanisms of response to ion-beam implantation. On one hand, it may cast light on what key gene networks in plants respond to ion-beam implantation stress. On the other hand, it may be helpful to understand the inherent factors in cells contributing to the mutations induced by ion-beam implantation.

Rice, one of the most important cereal crops, is a staple food for half of the world's population and has been used as an excellent model, though *Arabidopsis* is more commonly used as it has a relatively smaller genome, which has been completely sequenced [11]. Seeds of rice were often used as the implanted test materials for plant mutation breeding. To comprehensively understand the molecular regulation of the stimulation effect induced by ion-beam implantation and investigate the inherent factors in cells contributing to the radiomutation effect, we analyzed the transcriptome features of promoted growth rice seedlings germinating from seeds implanted by low-energy nitrogen ion beam, using Agilent Rice Gene Expression Microarray. These results will shed light on the overall characteristics of expression profiles associated with the radiostimulation effects induced by ion-beam implantation, which will help to improve plant mutation efficiency in low-energy ion-beam implantation.

## MATERIALS AND METHODS

### Plant materials

Dry seeds of rice cultivar Xindao-18 (*Oryza sativa* L. ssp. *japonica*) were used in ion implantation. After implantation with the ion beam, all seeds were planted on sterile medium with 0.8% agar (Sigma) in a climate chamber in the dark at 28°C. After the seeds had been incubated for 96 h, the seedlings were divided into two groups: one group was used for the RNA isolation, the other group was used in the investigation of the simplified seed vigor index from the 10-day-old seedlings grown in a climate chamber during a 12-h dark/12-h light cycle at 28°C.

### Implantation of low-energy N<sup>+</sup> ion beam and investigation of simplified seed vigor index

The low-energy ion beam implantation of seeds was performed with the Ion Beam Bioengineering Facility (UIL.0.512, TNV, Russia). For implantation, the germs of

seeds (62 days after harvesting) were positioned facing upward toward the incoming ion beam, while endosperms of seeds were downwardly immersed into the polyfoam that was fixed in the sample disks and then put into the facility's target chamber. When the vacuum of the target chamber was below 10<sup>-2</sup> Pa, the seeds in the sample disk were implanted with low-energy (40 KeV) N<sup>+</sup> with the ion fluences: 3 × 10<sup>17</sup> N<sup>+</sup>/cm<sup>2</sup>, 6 × 10<sup>17</sup> N<sup>+</sup>/cm<sup>2</sup>, 9 × 10<sup>17</sup> N<sup>+</sup>/cm<sup>2</sup>. Controls included seeds that were in normal surroundings and a vacuum without ion implantation for 264 min, the same as the implantation with fluence 6 × 10<sup>17</sup> N<sup>+</sup>/cm<sup>2</sup>. After implantation, all seeds were planted on sterile medium with 0.8% agar (Sigma) in a climate chamber under dark conditions at 28°C. For each fluence of ion-beam implantation, three independent biological replicates were used. Two hundred seeds were implanted in each replicate. The germination rate (from 100 seeds) was investigated from the 7-day-old seedlings, and all the seedlings that were used to investigate the germination rate (from 100 seeds) were oven dried at 80°C for 12 h to investigate the dry weight (g).

Germination rate = Number of the  
seedlings/total seeds × 100%

Simplified seed vigor index = Drought weight of the  
seedlings (g) × Germination rate × 100

Data were pooled from three independent experiments.

### RNA extraction

Total mixed RNAs from the promoted growth rice seedlings were isolated using RNA plant reagents (Tiangen Biotech) and purified by use of the RNeasy Plant Kit (Qiagen). The yield and purity of RNA were determined spectrophotometrically (Nanodrop ND1000). Thirty uniform rice seedlings were used to extract the total RNA to construct the RNA pool of a biological replicate. Therefore, at least 15 total RNA pools were constructed, including three control samples, three samples treated with a vacuum, and nine samples implanted by ion implantation.

### Agilent microarray hybridization and data analysis

The Agilent Microarray hybridization (Agilent-015241 Rice Gene Expression Microarray) and raw data analysis were carried out by the ShanghaiBio Company Ltd, including the procedures for cDNA and cRNA synthesis, cRNA Cy3 fluorescence labeling (GE healthcare PA13105), hybridization (Agilent G2545A), washing, scanning (Agilent G2565BA Microarray Scanner System), data collection and normalization. This experiment was performed three times, resulting in three biological replication samples for each ion fluence for the significant statistics.

To screen the differentially expressed transcripts between the implanted samples and the controls, we collected the

expression signals, and the present/absent calls from the raw data and analyzed them using the SBC Analysis System in a one-way ANOVA (<http://sas.ebioserivce.com>), and sorted the differentially expressed transcript data in Excel. Based on the statistics analysis, a transcript was considered significantly up- or down-regulated if it met all of the following criteria: (i) showed a statistically significant differential expression at the adjusted  $P$  value  $< 0.05$ ; (ii) had a cut-off value at a two-fold change; (iii) had 'present' calls on all of the three replicates samples for the controls and/or the implantation.

### Bioinformatics analysis

The functional cataloging of differentially expressed transcripts was carried out by the web software FunCat 2.1 ([http://mips.helmholtz-muenchen.de/proj/funecatDB/search\\_main\\_frame.html](http://mips.helmholtz-muenchen.de/proj/funecatDB/search_main_frame.html)). One transcript could be classified into more than one catalog group.

The annotation for the transcripts represented by the microarray was described according to the following databases: Agilent probe name (<http://www.ebi.ac.uk/microarray-as/aer/lob?name=adss&id=2375208716>); National Center for Biotechnology Information (NCBI, [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)); European Molecular Biology Laboratory (EMBL, <http://www.embl-heidelberg.de>); <http://aranet.mpimp-golm.mpg.de/ricenet>; <http://rice.plantbiology.msu.edu/>; <http://bioinformatics.cau.edu.cn/easygo/>; Shanghai Biotechnology Corporation Analysis System (SAS) web server; and the Rice Annotation Project (RAP) database (<http://rapdb.dna.affrc.go.jp/>).

## RESULTS

### Simplified vigor index

The results showed that the smaller-fluence implantation of the  $N^+$  ion beam ( $6 \times 10^{17} N^+/cm^2$ ) enhanced the vigor index ( $P < 0.05$ ) of the rice seedlings, and the larger-fluence implantation ( $9 \times 10^{17} N^+/cm^2$ ) damaged the rice seedlings because of the smaller simplified vigor index compared with the controls ( $P < 0.05$ ) (Table 1). In order to investigate the key molecules in encouraged rice seedlings responding to ion-beam implantation, we isolated the total mixed RNA from the samples that had undergone the ion-beam implantation with ion fluence:  $6 \times 10^{17} N^+/cm^2$  and exposure to the vacuum for microarray.

### Differentially expressed transcripts

We analyzed genome-wide gene expression profiles of three sample groups including the controls, the vacuum-treated and the ion-implanted, using the Agilent-015241 Rice Gene Expression Microarray, including probes to query 42 476 transcripts (the microarray data was deposited in gene expression omnibus [GEO] with the accession number GSE36152). Based on the analysis of the materials and methods, the resulting analysis revealed that there were great

**Table 1.** Simplified vigor index of implanted seeds

Samples	Germination rate $\times 100$ (mean $\pm$ SD)	Vigor index (mean $\pm$ SD)	$P$ -value for $t$ -test
Controls	81.85 $\pm$ 2.31	9.55 $\pm$ 1.69	
Vacuum	84.81 $\pm$ 2.21	12.14 $\pm$ 0.8	0.074
Fluence 1	86.67 $\pm$ 1.11	12.14 $\pm$ 1.02	0.085
Fluence 2	88.89 $\pm$ 0.00	14.52 $\pm$ 1.36	0.017
Fluence 3	51 $\pm$ 7.2	5.29 $\pm$ 0.99	0.02

Simplified vigor index was investigated using the 10-day rice seedlings in each independent experiment replicate. Fluence 1 refers to  $3 \times 10^{17} N^+/cm^2$ ; fluence 2 refers to  $6 \times 10^{17} N^+/cm^2$ ; fluence 3 refers to  $9 \times 10^{17} N^+/cm^2$ .

**Table 2.** Differences of gene expression profiles between the samples exposed to vacuum and ion implantation

Treatment	Up-regulation		Down-regulation	
	Number	FC score	Number	FC score
Vacuum	165	2–23.44	117	2–40.51
Ion implantation	351	2–21.62	470	2–176.89

FC = fold change; 351 up-regulated transcripts and 470 down-regulated transcripts (including five microRNA probe sets) showed statistically significant differential regulation in the promoted growth rice seedlings germinating from the seeds with exposure to  $N^+$  beam implantation fluence:  $6 \times 10^{17} N^+/cm^2$  as compared with the control samples. There are 119 more down-regulation transcripts than up-regulation transcripts. The fold changes of the down-regulated transcripts were from 2–176.89, meanwhile the fold changes of the up-regulated transcripts were from 2–21.62. However, 165 up-regulated transcripts and 117 down-regulated transcripts showed statistically significant differential regulation in the samples exposed to a vacuum. The fold changes of the down-regulated transcripts were from 2–40.51, meanwhile the fold changes of the up-regulated transcripts were from 2–23.44.

differences (Table 2) in gene expression profiles between the vacuum-treated samples and the ion-implanted samples. More transcripts were differentially expressed underlying the ion implantation than the vacuum exposure, and fold changes of gene expression in ion-implanted samples were more distinctive than in the vacuum-treated samples.

### Functional catalog of differentially expressed transcripts underlying vacuum and ion-beam implantation

In order to have a clear functional categorization of the differentially expressed genes, FunCat was used. FunCat has been established as a robust table annotation scheme that offers both meaningful and manageable functional

classification. One transcript can be clustered into more than one catalog group. The differentially expressed transcripts in the vacuum-treated samples and the ion-implanted samples were classified into 19 groups. In addition, there was one classification not yet clear-cut in which the genes had putative functional information but could not be classified clearly into one of the above 19 groups, and one unclassified group in which the genes had no homology to known proteins (Table 3). The functional catalog showed that the functional features of the two datasets were strikingly different in the aspects below. First, the number of the differentially expressed genes in vacuum-treated samples was less than that in the ion-implanted samples in each function group. Second, there were no up-regulated genes in the ion-implantation samples that were clustered into function groups with differentiation, but there were five up-regulated genes in the vacuum-treated samples. Third, it

is interesting to note that hardly any of the transcripts associated with transposable elements were significantly differentially expressed underlying the vacuum, but 14 up-regulated genes were significantly differentially expressed underlying the ion implantation. Only up- and down-regulated genes related to energy, and up-regulated genes related to interaction with the environment were equally distributed.

### Statistically significant pathways for the differentially expressed transcripts underlying the ion implantation

Twenty-five down-regulated transcripts were involved in 52 pathways in the Kyoto Encyclopedia of Genes and Genomes (KEEG) database. Meanwhile 20 up-regulated transcripts were involved in 30 pathways in the KEEG

**Table 3.** Functional grouping of differentially expressed transcripts underlying vacuum and ion beam implantation

Functional category	Up-regulation		Down-regulation	
	Vacuum	Ion implantation	Vacuum	Ion implantation
Metabolism	56	100	23	120
Energy	1	10	1	7
Storage protein	5	6	2	2
Cell cycle and DNA procession	10	29	7	43
Transcription	9	43	14	69
Protein synthesis	0	8	2	7
Protein fate	19	44	12	33
Binding or cofactor	86	165	44	199
Regulation protein function	12	23	4	21
Transport	27	40	16	52
Signal transduction	20	41	9	61
Cell rescue	49	62	23	89
Interaction with environment	51	59	22	107
Transposable elements	0	14	0	1
Cell fate	15	24	4	17
Development	4	22	6	32
Cellular components	19	39	14	44
Differentiation	5	0	3	16
Subcellular localization	80	161	51	197
Classification not yet clear-cut	31	36	23	117
Unclassified proteins	5	44	8	21

A total of 351 up-regulated transcripts and 470 down-regulated transcripts exposed to ion implantation and 165 up-regulated transcripts and 117 down-regulated transcripts exposed to a vacuum were grouped based on FunCat 2.1. One transcript may be clustered into more than one group.

**Table 4.** Statistically significant pathways in KEEG for the up-regulated transcripts

Pathway name	Hits	Total	Percent	Enrichment test <i>P</i> -value
Flavone and flavonol biosynthesis	1	3	33.33	0.0381
Flavonoid biosynthesis	2	10	20.0	0.0058
Nitrogen metabolism	2	29	6.9	0.0359
Pyrimidine metabolism	3	82	3.66	0.0493

Hits refer to the number of up-regulated transcripts clustered into certain pathways; the numbers in the Total column refer to the number of genes in certain pathways; percent refers to the number of hits/number of total genes in certain pathways.

**Table 5.** Statistically significant pathways for the down-regulated transcripts

Pathway name	Hits	Total	Percent	Enrichment test <i>P</i> -value
$\beta$ -Alanine metabolism	2	20	10.0	0.0377
Glycolysis/gluconeogenesis	4	89	4.49	0.0421

Hits refer to the number of down-regulated transcripts clustered into certain pathways; the numbers in the Total column refer to the number of genes in certain pathways; percent refers to the number of hits/number of total genes in certain pathways.

database. The statistically significant pathways for the differentially expressed transcripts are listed in Tables 4 and 5.

Flavonoid biosynthesis pathways, including flavone and flavonol, are promoted in the rice seedlings 96 h after germination of the seeds implanted by the nitrogen-ion beam. Two up-regulated transcripts, including anthocyanidin reductase and flavonoid 3'-monooxygenase, were involved in these pathways. Flavonoids are synthesized by the phenylpropanoid metabolic pathway [12]. Flavonoids are the secondary metabolites involved in several aspects of plant development and defense. They color fruits and flowers, favoring seed and pollen dispersal, and contribute to the adaptation of plants in varying environmental conditions such as cold or UV stress [13], and pathogen attacks [14]. The up-regulated flavonoids and flavonol pathways indicate that they are important in responding to the ion-implantation implantation and contributing to the stimulation effect of the implantation with the low-energy ion beam.

Pyrimidine metabolism is one of the important specialized pathways of nucleic acids, including cytosine, uracil, thymine and their derivatives, which are the components of genetic materials. The promoted pyrimidine metabolism implies that it contributes to the promoted growth of the rice seedlings from the ion-implanted seeds because more pyrimidine and their derivatives are beneficial to the cell division, activity, growth and enhancement of stress tolerance.

$\beta$ -Alanine metabolism and glycolysis/gluconeogenesis pathways (Table 5) are down-regulation. In plants,  $\beta$ -Alanine may play a role in regulating growth and contributing to cell defense.  $\beta$ -Alanine increases in response to high temperature, drought and low oxidative stress. It prevents protein aggregation and reactivates the thermally

denatured enzyme in vitro [15]. The role of the down-regulated  $\beta$ -Alanine metabolism in the process of responding to ion-beam implantation is a topic that should be studied further.

Glycolysis is the process of converting glucose into pyruvate and generating small amounts of adenosine triphosphate (ATP, energy) and nicotinamide adenine dinucleotide (NADH, reducing power). Oxidative stress is impaired glycolysis [16]; in accordance with the down-regulated  $\beta$ -alanine metabolism, these findings implied that implantation with an ion beam can promote the seeds to take up oxygen during germination and enhance the growth of the seedlings germinating from ion-implanted seeds.

### The up- and down-regulated transcripts with greater than 10-fold changes underlying the ion implantation

In order to present a rich source of target genes for further study into the molecular functions that govern the response to ion implantation, here we show the differentially expressed transcripts with tremendous changes (fold change greater than 10). Four tremendously up-regulated transcripts were annotated as peroxidase, including three transcripts associated with haem peroxidase. Haem peroxidases (or heme peroxidases) catalyze a number of oxidative reactions. Haem peroxidases in plants were thought to provide protection to cells under oxidative stress, protection against toxic peroxides, hydrogen peroxide removal in chloroplasts, biosynthesis of the cell wall, defense responses against wounding, indole-3-acetic acid (IAA) catabolism and ethylene biosynthesis [17]. This finding implies that ion implantation on plant seeds can induce harmful oxygen free radicals (OFR), and haem peroxidases

are important in protecting cells from OFRs induced by ion-beam implantation.

Two transcripts related to eggshell proteins show greater than 10-fold changes. Little information on eggshell proteins in plants could be found in the literature, except for on the seed coat, so we did not infer the likely function of the up-regulated eggshell protein transcripts in response to the physical ion implantation.

One transcript related to ubiquitin showed greater than 10-fold changes. Post-translational protein modifications play a critical role in most cellular processes through their unique ability to rapidly and reversibly alter the functions of synthesized proteins, multi-protein complexes and intracellular structures. In eukaryotes, such modifications frequently occur by attaching a small polypeptide to the target protein. Ubiquitin and small ubiquitin-related modifiers (SUMO) are among those polypeptides [18]. Regulation of protein degradation by ubiquitination is important in many plant processes, from embryogenesis to floral organ production, through its central role in many hormone pathways. More recent evidence provides molecular mechanisms for hormonal cross-talk and links the ubiquitin system to biotic defense responses [19]. These up-regulated transcripts related to ubiquitin suggest a role for these genes in regulating the development and germination of the seeds implanted by ion beams.

Three up-regulated transcripts with greater than 10-fold changes annotated as no hits, are represented as the candidate genes for further study of the responses to implantation with low-energy ion beams.

The down-regulated transcripts with greater than 10-fold changes are in more dispersed functional categorizations than the up-regulated transcripts. They are clustered into transcription factors (two), signaling transduction intermediates (five), catalytic activity (three) and unknown function family proteins (three). With regard to the polypeptide signals, rapid alkalization factor (RALF) was considered as a repressor to arrest root growth and development [26]. These down-regulations should contribute to the promoted growth of the rice seedlings from the seeds implanted by the nitrogen ion beam.

### Transcription factors and chromatin modification underlying the ion implantation

A total of 20 out of 351 up-regulated transcripts were clustered into the gene ontology (GO) term of transcription factor (TF) activity (GO: 0003700). Meanwhile, 53 out of 470 down-regulated transcripts were clustered into the GO term of transcription factor activity. These differentially expressed transcripts were categorized into 12 distinct TF families (Table 6). While the divergent enrichment feature between the up and down-TF group was distinct, AP2/EREBP (11), zinc finger (8), HB (8), MYB (5), WRKY (5), bHLH (4) and SRS (2) transcription factors were

**Table 6.** Family distribution of differentially expressed transcription factors

TF family	Down-regulation		Up-regulation	
	No.	Percent (No/470*100%)	No.	Percent (No/351*100%)
ABI3/VP1	1	1.89	0	0
AP2/EREBP	11	20.75	4	20.0
AUX/IAA	1	1.89	0	0
bHLH	4	7.55	3	15.0
bZIP	1	1.89	2	10.0
Zinc finger (C2C2-CO-like)	2	3.77	1	5.0
Zinc finger (C2C2-Dof)	2	3.77	0	0
Zinc finger (C2C2-GATA)	2	3.77	0	0
Zinc finger (C2H2)	2	3.77	1	5
EIL	1	1.89	0	0
GARP-G2-like	1	1.89	0	0
HB	8	15.09	0	0
HSF	0	0.00	3	15.0
MADS	1	1.89	0	0
MYB and MYB-related	5	9.43	1	5
NAC	1	1.89	0	0
SRS	2	3.77	0	0
TAZ	0	0.00	2	10.0
TCP	1	1.89	0	0
Trihelix	1	1.89	0	0
WRKY	5	9.43	0	0
ZF-HD	1	1.89	0	0

Matching using the SAS web server and DRTF (<http://drtf.cbi.pku.edu.cn/>) was performed to explore the differentially expressed transcripts to cluster into each transcription factor (TF) family. The percent refers to the ratio of up- or down-transcription factors clustered into each TF family, comprising the total 351 up- or 470 down-regulated transcription factors. Raw data for the cluster are not shown.

prominently down-regulated. Meanwhile, AP2/EREBP (4), bHLH (3), HSF (3), bZIP (2) and TAZ (2) transcription factors were prominently up-regulated. There are fewer up-regulated transcription factors than down-regulated. This divergent enrichment feature suggests that events responding to implantation with ion beams in rice seedlings may require different TFs and/or a combination of TFs from

distinct families. These TFs affect the growth and development of rice seedlings through alterations in metabolism and gene expression.

Many transcription factors contain a function to modify the chromatin structure, such as histone acetylation and deacetylase, among others. Histone modifications have the potential to influence many fundamental biological processes, some of which may be epigenetically inherited [20]. In order to investigate the likely epigenetic molecular basis involved in responding to the ion beam implantation, we explored the differentially expressed transcripts related to chromatin modification, not taking into account the transcription factors. Transcripts described as histone deacetylase (HDAC1), centromere protein-like proteins, a methyl-CpG binding domain (MBD) containing proteins, WD-40 proteins, Zn-finger and RING domain-containing proteins had enhanced expression. This finding implied that epigenetic regulation is involved in the biological effects induced by ion-beam implantation, and is even likely to contribute to the promoted growth of rice seedlings from the ion-implanted seeds.

Our data showed that only transcripts related to Zn-finger and RING domain-containing proteins are down-regulated, which indicates the special regulation of this protein family. The details of these proteins are discussed in the following.

### **Transposable elements underlying the ion implantation**

Fifteen differentially expressed transcripts related to transposable elements (TEs) included 1 down-regulation transcript and 14 up-regulation transcripts. Transposons (TEs) are segments of DNA that can move around to different positions in the genome of a single cell. There are three distinct types: DNA transposons (Class II), miniature inverted-repeats transposable elements (MITEs; Class III) and retrotransposons (Class I) [21]. Each group of TEs contains autonomous and non-autonomous elements. Autonomous elements have open reading frames (ORFs) that encode the products required for transposition. Non-autonomous elements that are able to transpose have no significant coding capacity, but retain the cis-sequences necessary for transposition [22]. Our data showed that most of the differentially expressed transcripts associated with TEs were up-regulation or reactive, which implicates that increasing transposition events in cells may occur after implantation with the ion beam.

### **Non-protein coding transcript underlying the ion implantation**

Nine down-regulated and 10 up-regulated transcripts were annotated as non-protein coding RNA. A non-coding RNA (ncRNA) or non-protein-coding RNA (npcRNA) is a functional RNA molecule that is not translated into a protein. Non-coding RNAs belong to several groups and are

involved in many cellular processes including translation, RNA splicing, gene regulation, genome defense and chromosome structure [23]. From the limited information available on these differentially expressed transcripts related to non-protein coding RNAs, we can not infer their likely role in responding to the stress of implantation with an ion beam, but they represent the target candidates for further study.

### **Defense/stress response-related transcripts underlying ion implantation**

A total of 91 down-regulated transcripts were clustered into GO: 0006950 (response to stress), comprising 19.4% (91/470) of total down-regulated transcripts. Meanwhile, 53 up-regulated transcripts were clustered into GO: 0006950, comprising 15.1% (53/351) of total up-regulated transcripts. These differentially expressed transcripts associated with responses/defenses to stress were largely abiotic/biotic stress-response proteins, such as disease-resistant, pathogenesis-related proteins, kinases and other stress-response proteins. It is interesting that heat shock-related proteins (five), disease resistance proteins (NB-ARC; four), nucleotide-binding site leucine-rich repeat proteins (NBS-LRRs; four), receptor protein kinases (two), dehydration responsive element binding proteins (DREB; two), multidrug resistance protein (one), physical impedance induced protein (one), abscisic stress ripening protein (one) and delta 1-pyrroline-5-carboxylate synthetase (P5CS; one) were only up-regulated, while universal stress proteins (six), late embryogenesis abundant proteins (three), hypoxia-induced protein conserved region family proteins (three), auxin-induced proteins (two), thiamine pyrophosphokinase family protein (one), harpin-induced 1 domain containing protein (one) were only down-regulated. Other stress proteins, such as leucine-rich repeat proteins, kinases and pathogenesis-related proteins were expressed in both up-regulation and down-regulation by different transcripts. This finding indicates that rice cells respond to the implantation of the nitrogen-ion beam through promoting or weakening some responsive pathway or system.

Most of the disease resistance genes (R genes) in plants encode NBS-LRR proteins and are involved in the detection of diverse pathogens, including bacteria, viruses, fungi, nematodes, insects and oomycetes [24]. In order to detect these diverse biotic challenges, it is up to the up-regulated NBS-LRRs and NB-ARCs after the ion-beam implantation. However, universal stress proteins were down-regulation, for which gene-encoding proteins are known to provide bacteria, archaea, fungi, protozoa and plants with the ability to respond to a plethora of environmental stresses, like nutrient starvation, drought, high salinity, extreme temperatures and exposure to toxic chemicals [25]. As a kind of physical stress, implantation with the ion beam repressed the expression of genes related to universal stress proteins,

which implied a unique feature in biological effects induced by ion-beam implantation on plant seeds.

Heat shock proteins and the signaling proteins are involved in responding to UV irradiation, desiccation and ionizing radiation [26]. The highly conserved heat shock proteins (HSPs) are constitutively expressed and function as molecular chaperones that facilitate the synthesis and folding of proteins. Under stressful conditions, such as heat shock, pH shift or hypoxia, the increased expression of HSPs protects the cell by stabilizing unfolded proteins, giving the cell time to repair or re-synthesize damaged proteins [27]. Implantation can induce high temperatures, so the up-regulated HSPs and HSFs were thought to have the most important role in protecting the cell by stabilizing unfolded proteins after implantation of low-energy ion beams.

### **Up-regulated transcripts related to signal transduction/kinases/transporters underlying ion implantation**

Signals play important roles in bio-effects induced by low-energy ion implantation [28]. MAPK and Akt signaling pathways are involved in responding to carbon ion irradiation in human cells [29]. Not taking into account the TFs, approximately 32 out of the 351 up-regulated transcripts encoded proteins that were related to signaling, such as calcium-related proteins and kinases. Calcium sensor proteins, such as calmodulin (CaM), play important roles in gene regulation [30]. The plasma membrane  $\text{Ca}^{2+}$  ATPase (PMCA) is vital for regulating the amount of  $\text{Ca}^{2+}$  within cells. CaM is recognized as a major  $\text{Ca}^{2+}$  sensor and orchestrator of regulatory events through its interaction with a diverse group of cellular proteins [31]. As expected, calcium-related proteins are up-regulated in rice seedlings growing from the ion-implanted seeds, as they are important regulators of the response to the ion-beam implantation. At least, 16 up-regulated transcripts encoded kinase domain-containing proteins; some examples are receptor kinases/receptor-like kinases, calcium signal-related kinases, phospholipid signal-related kinases and unclassified kinase. Kinases are used extensively to transmit signals and control complex processes in cells. One of the largest groups of kinases is protein kinases, which act on and modify the activity of specific proteins [32]. Other up-regulated transcripts associated with signaling transductions were the encoded proteins involved in special signaling pathways such as WNT and WNK. These up-regulated special signal proteins, including arm-repeat proteins and the CONSTANS (CO) protein, may be involved in ion-implantation diverse signal transduction to the nucleus and responding to ion-beam implantation stress.

Twenty-one out of the 351 up-regulated transcripts encoded the transport proteins, including drug transporters, transmembrane transporters and substrate-specific transporter activity, while also including those predicted to encode

amino acid transporters, metal transporters (including  $\text{Ca}^{2+}$ ,  $\text{K}^+$ ) and phosphate transporters, which played important roles in signal transduction. These up-regulated transporters suggested that many signaling pathways are involved in regulation of gene expression after implantation with low-energy ion beams.

### **Plant hormone underlying the ion implantation**

Ten out of the 351 up-regulated transcripts were associated with abscisic synthesis (four), abscisic signaling (four), gibberellin synthesis (one), ethylene signaling (one), all of which indicate the importance of abscisic signaling in rice seedlings in responding to the implantation of the nitrogen ion beam. However, most (eight) of the 10 out of 470 down-regulated transcripts were involved in auxin signaling (three) and ethylene signaling (five). This difference in expression pattern suggests that abscisic may be implicated mainly in rice seedlings in response to the implantation of the nitrogen ion beam (details in discussion).

## **DISCUSSION**

Our analysis of the gene expression profiles for the stimulated biological effects of ion-beam implantation revealed comprehensive characteristics of transcriptomes during the germination of seeds with exposure to a low-energy ion beam. This suggests that the differentially expressed transcripts clustered into many groups were associated with the requirements of different events while adapting to the ion implantation. These data provide information on a large number of candidate genes for further elucidation regarding the molecular mechanisms of the stimulated biological effects of ion-beam implantation and its use for improvement of corn.

### **Ion implantation is the principal physical stress contributing to ion-beam implantation biological effects**

A total of 821 differentially expressed transcripts were induced by the ion implantation; however, only 282 differentially expressed transcripts were induced by vacuum. There were 539 more differentially expressed genes induced by ion implantation than the vacuum, comprising 65.6% of 821 differentially expressed transcripts. At the same time, functional catalog results showed that the functional features of the two datasets were strikingly different. These findings suggest that ion implantation is the principal cause of ion-beam implantation biological effects, but the vacuum is the second cause.

### **The differentially expressed transcripts were induced by the compound physical and chemical factors of low-energy $\text{N}^+$ beam**

A biological organism is a complex system, and temporal and spatial changes in the biological structure greatly

complicate the studies of the interactions between energetic ions and the living biological targets. In order to explore the mutation mechanisms in rice seeds induced by ion implantation, a hypothesis that incorporates a combination of energy absorption, mass deposition and the charge transfer of energetic ions in seeds has been proposed [1]. When the penetration depth of the incident (primary) ions is smaller than the thickness of the target, these ions are stopped and implanted inside the sample. Through recombination, a large number of new molecules can be formed per single incident ion. When the primary ions slow down to the energy range in which chemical reactions can occur, a new chemical species that contains the incident ions can also be formed with a maximum yield of one molecule per incoming ion. From a biological standpoint, biomolecule damage, such as the disintegration and fragmentation of amino acids and nucleobases, and the radiolysis of water molecules in the surrounding regime of structural molecules give rise to DNA damage, chromosome aberrations and other biological effects. It has been reported that one-third of the damage in the genome of a living cell is directly caused by ionizing radiation, while two-thirds of the damage is caused indirectly. The direct effects include direct energy deposition and ionizing reactions in the biomolecules, whereas indirect damage is caused by radiation-induced reactive species (mainly radicals) in the medium (mainly water) surrounding the biomolecules. From this point of view, ion implantation acts on organisms like a compound mutagen with both physical and chemical mutagenicity, and its mutation efficiency is generally higher than a single mutagen [4]. In  $N^+$  ion-beam implantation experiments, these compound factors should contribute to the stimulation effects. This stimulation effect is partly also ascribed to the etching process of low-energy ions, which can improve the permeability of the shell surface and facilitate the exchange of external substances (nutrients and water, etc.). Consequently, the germination rate is increased and the growth potential is enhanced and maintained. In our ion-beam experiment, the temperature on the ion-implanted seeds was higher than in the vacuum-treated samples and the control. Our early work with ion-beam implantation on wheat seeds agrees with our microarray data showing statistically significant up-regulation of the transcripts related to HSP and HSFs. We did not show the temperature of the test samples in the present article due to a lack of further investigation.

### **The complex molecular components were involved in the stimulation effect induced by implantation of low-energy ion beam**

Genomic gene expression investigations of seeds implanted by ion beams have been neglected in the past. Most of the studies on ion-beam implantation in molecular biology have been focused on biological processes, such as DNA damage, repair and recombination, harmful free radicals

and peroxidase. However, our data showed that 821 differentially expressed transcripts were involved in the complex gene interaction. These transcripts encoded transcription factors, transporter proteins, kinases, enzymes, transposable elements functional proteins, proteins associated with signaling transduction and plant hormones, HSPs, proteins associated with resistance and tolerance, cell growth and division, DNA repair and recombination, and even non-protein encoding. This especially includes the plant hormones, kinases, transporters, transcription factors and secondary metabolites that may make up a coordinated molecular network to contribute to the stimulatory effect. So these findings imply that many complex molecular components are involved in the promoted growth of seedlings stimulated by the implantation of low-energy ion beams, not the independent pathways or molecular components. This coordinated functional network represents the candidate for further study.

### **Transposable elements may contribute to mutation effect of the implantation with low-energy ion beam**

TEs are major components of most eukaryotic genomes and are particularly abundant in plants, representing 80% of maize, 90% of wheat and 35% of rice genomes. TEs play an important role in genome and gene evolution. TE insertion can disrupt genes and mediate chromosome rearrangements, and can provide alternative promoters, exons, terminators and splice junctions [33]. TEs change the expression of some genes due to the transcription of ncRNA from the transposon promoters, which contribute to the epigenetic regulation of neighboring genes through mechanisms such as RNAi. It had the high potential impact of the TE transcription on the expression of the nearby genes [34]. For this reason, TE transcription was severely repressed in plants and only activated under certain precise circumstances. For example, pathogen infections, physical injuries or different abiotic stresses [35]. Here, functional proteins related to transposition, including four transcripts in class I and nine transcripts in class II, were up-regulated and encoded transposase, integrase and polyprotein. Six out of these 13 up-regulated transcripts were re-activated. These findings suggest that ion-beam implantation can promote and re-activate the transcriptional activity of TEs and sequentially offers the potential for insertion events. TEs insertion can disrupt genes and mediate chromosome rearrangements; their transcription may change the expression of their flanking genes. Re-activation of a silenced minimal *Mutator* TE system in maize [36], increasing divergent genetic polymorphisms of retrotransposon *Tto1* [37] in tobacco, and promotion of the transcriptional activity in wheat [8] underlying ion-beam implantation have been observed. This suggests that TEs may contribute to the mutation effect of ion-beam implantation. Our results showed

that a large number of genes related to transposition were up-regulated or re-activated, and also provided fundamental data to elucidate the roles of the TEs in stimulation effects induced by ion-beam implantation.

### **Up-regulated transcripts associated with chromatin modification and non-coding transcripts may cause the epigenetic inheritance and phenotypic variation**

Low-energy ion-beam implantation is characterized as a wide spectrum mutation with limited physiological damage in present generations, and part of the phenotypic variation in the present generation can be inherited by offspring. However, many phenotypic variations did not genetically occur in the following generation, such as the  $M_4$  and  $M_5$  generations. As a review [38] showed, ion-beam implantation can induce DNA methylation, normal chromosome segregation and early segregation. So we suggest that low-energy ion-beam implantation can induce epigenetic inheritance and phenotypic variation in implanted present generations ( $M_1$ ). However, little is known about the likely molecular mechanisms for epigenetic inheritance caused by implantation with ion beams.

A fundamental characteristic of epigenetics is that the same genome can show alternative phenotypes, which are based on different epigenetic states. Several aspects of epigenetics are strongly linked to non-coding RNAs, especially small RNAs that can direct cytosine methylation, and histone modifications that are implicated in gene expression regulations in complex organisms [39]. Our data show that miRNAs are involved in regulating gene expression; miRNAs are post-transcriptional regulators, usually resulting in translational repression or target degradation and gene silencing [40]. So the differentially expressed ncRNAs including miRNAs may play a regulatory role in controlling adaptation to ion implantation stress resulting in abnormal development patterns.

Our data showed an enhanced epigenetic chromatin modification indicated by the up-regulated or down-regulated transcripts associated with HDAC1, centromere proteins, MBD-containing protein, WD-40 proteins, RING-finger proteins and non-coding proteins RNAs. HDACs catalyze the removal of acetyl groups from lysine residues in histones and non-histone proteins, resulting in transcriptional repression. Histone deacetylation gives a tag for epigenetic repression and plays an important role in transcriptional regulation [41]. Centromere proteins, histone H3-like centromeric proteins, are a group of proteins that form and/or mediate the function of centromeres, and may serve as an epigenetic marker that propagates centromere identity through replication and cell division [42]. An MBD-containing protein plays a role in gene silencing, as a transcriptional regulator that binds CpG islands in promoters where the DNA is methylated at position 5 of cytosine within CpG dinucleotides. It may associate with HDACs,

requiring nucleolar dominance that consists of silencing rRNA genes inherited from one progenitor in genetic hybrids [43]. WD-40 proteins participate in complex chromatin metabolism and gene expression and have been reported to be transcriptional repressors that interact either with co-repressors or in a complex with histone deacetylases, to regulate spermatogenesis, and to function as mitotic checkpoints to ensure accurate chromosome segregation [44]. RING-finger proteins are involved in mediating protein-protein interactions that have been implicated in a range of diverse biological processes, increasing the markers of heterochromatin and repressing gene expression [45]. The down-regulated RING-finger protein may get rid of heterochromatin markers to promote some gene expression. These differentially expressed transcripts associated with chromatin modifications may change the structure of chromatin and cause expression changes of certain genes to enhance the adaptation to ion-beam implantation stress.

For that which is described above, the genes related to epigenetic regulation may contribute to the biological effects of implantation with a low-energy ion beam. Abundant plant variations have been observed in the present generation after implantation into seeds with a low-energy ion beam. However, only a limited fraction of these variations is inherited by offspring. So, epigenetic inheritance must be considered in the application of ion-beam mutational plant breeding. Our analysis suggests that mutant scanning should be carried out on the  $M_3$  generation, not the present generation ( $M_1$ ).

### **ABA signaling system may play an important role in regulating the encouraged stress resistance**

The plant hormone abscisic acid (ABA) plays a major role in seed maturation and germination, as well as in adaptation to abiotic environmental stresses. As with most signal transduction pathways, ABA response eventually leads to changes in gene expression. The ABA-induced genes are enriched for those encoding proteins involved in stress tolerance, such as dehydrins and enzymes that detoxify reactive oxygen species, enzymes of compatible solute metabolism, a variety of transporters, regulatory proteins such as transcription factors, protein kinases and phosphatases, and enzymes involved in phospholipid signaling [46]. Consistent with the effects on gene expression predicted to affect metabolism, recent metabolome analyses have identified ABA-dependent drought-induced changes in synthesis of glucose, branched-chain amino acids, saccharopine, proline and polyamines [47]. In addition to effects on known protein-coding genes, a recent transcriptome analysis using the *Arabidopsis* genome tiling arrays has shown that nearly 8000 unannotated transcriptional units are present in the 'intergenic' regions, and 5–10% of these units are also regulated by ABA. ABA synthesis was consistent with the ABA signaling transduction, both of which were distinctly

up-regulated in our data, which implicates its important function in regulation of gene expression and metabolism in adaptation to ion-beam implantation. Our data analysis also showed the up-regulated transcripts related to dehydration responsive element bindings (DREBs), abscisic stress ripening protein, delta 1-pyrroline-5-carboxylate synthetase, glutamate dehydrogenase, and so on, which were regulated by ABA. We cannot describe the details of the action of the ABA regulation molecular network on gene expression in the present study, but these should be the further targets of the molecular regulation network in responding to ion-beam implantation into seeds.

Ethylene-responsive element binding proteins (EREBPs) encode a transcription factor and are responsible in part for mediating the response in plants to the plant hormone ethylene [48]. As well as the down-regulated EREBPs, two transcripts related to auxin (IAA) signaling protein were down-regulated in promoted growth rice seedlings germinating from the seeds implanted by a low-energy ion beam. This finding suggests that the coordinated regulation of auxin and ethylene is important in responding to implantation with low-energy ion beams; these data appear to be compatible with the interacting model of auxin and ethylene. This finding also suggests that implantation with ion beams is likely to change the development model of seedlings during germination through the regulation of plant hormones.

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