

# Induction of Exogenous Molecule Transfer into Plant Cells by Ion Beam Bombardment

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**ABSTRACT** Although the technology of ion-beam-induced gene transfer into either plant or bacterial cells has been successfully established, relevant mechanisms have not been understood. This work aimed to study the process of induction and thus to develop applications of ion beam bioengineering. Cells of various plant tissues were bombarded in vacuum with argon and nitrogen ion beams at energies of 15-30 keV with fluences ranging from  $5 \times 10^{14} - 3 \times 10^{16}$  ions/cm<sup>2</sup>. The ion bombardment effects on tissue viability and neutral red dye molecule transfer into the cells through the cell envelope were investigated. The results showed that the characteristics of the tissue survival from the ion bombardment and penetration of the dye molecules into the cells through the cell envelope depended on ion species, energy and fluence. For 30-keV argon-ion bombardment at a fluence of  $2 \times 10^{15}$  ions/cm<sup>2</sup>, the dye molecules entered the cells without fatal injury, whereas under other conditions, the dye either did not enter the cells or stained the nuclei. On the cell envelope surface, ion-bombardment-induced crater-like structures were observed. Calculations indicated that exogenous molecule transfer into living plant cells can be achieved by ion beams with appropriate physical parameters such that the ion range and the radiation damage range lie within the solid cell wall thickness.

**KEYWORDS:** exogenous molecule transfer, ion beam bombardment, plant cells, cell envelope, cell wall.

**ABBREVIATIONS:** Ar, argon; Au, gold; BA, Benzyladenine; GA<sub>3</sub>, Gibberellic acid; MS, Murashige and Skoog; N, nitrogen; NAA,  $\alpha$ -Naphthaleneacetic acid; NR, Neutral Red; SEM, Scanning Electron Microscopy; TEM, Transmission Electron Microscopy; VW, Vacin and Went; W, tungsten.

## INTRODUCTION

Ion beam technology has been widely applied in the fields of physics and materials science. The technology is typified by ion bombardment, a physical process in which energetic charged particles are accelerated by an electric field and transported to a target into which they penetrate, introducing foreign atoms, electric charge, and radiation damage in the near surface region.<sup>1</sup> Heavy ion beams have recently been used to bombard biological materials for genetic modification purposes, particularly for the mutation of plants and bacteria<sup>2-6</sup>, in which the DNA structure is modified by relatively high energy ( $\sim 10^2 - 10^3$  keV) ion beam irradiation. More recently, attempts have been made to use relatively low energy (a few 10 keV) ion-beam bombardment for the direct transfer of exogenous macromolecules such as DNA and vital dye into biological cells. Yu *et al*<sup>7</sup> reported the successful

*GUS* and *CAT* gene transfer into the suspension cells and mature rice embryos following the 20-30-keV argon (Ar)-ion bombardment. Hase *et al*<sup>8</sup> developed tobacco-pollen-mediated gene transfer using carbon ion beam bombardment. We have described our experiments in transferring Trypan blue (a vital dye) into *Curcuma* embryos induced by bombardment with argon ion beam.<sup>9</sup> There is intrinsic difference between the irradiation and bombardment for DNA modification processes. In ion-beam mutagenesis a large number of cells are irradiated and DNA modifications are randomly induced in the nucleus, of which the desired ones are subsequently selected out.<sup>3</sup> In ion-beam-induced DNA transfer only the cell envelope is bombarded in order to allow a subsequent transfer of whole DNA into the internal cell region.<sup>7</sup> A recent report<sup>10</sup> described the interaction of energetic ions with bacterial cells, inducing direct DNA transfer into *E. coli*, indicating that ion beams with an energy such that the ion range is

approximately equal to the cell envelope thickness, at a certain range of fluence, are able to create suitable conditions for DNA transfer through the bacterial cell envelope without irreversible damage. Although the technology of ion-beam-induced gene transfer into either plant or bacterial cells has been successfully established, relevant mechanisms have not been understood. Some mechanisms have been proposed, such as pathways created by ion bombardment, enhanced permeability from ion beam etching, and charge exchange resulting from ion implantation<sup>7</sup>, but none of them has been well supported experimentally.

Here we report in more detail our systematic and comprehensive studies on the induction of exogenous molecule transfer into plant cells by heavy ion bombardment in order to explain how the induction occurs, and the mechanisms involved in creating passages or channels through the cell envelope and enhancing its permeability. A vital dye, neutral red (NR), was used not only as the cell-injury-testing signal but also as the exogenous molecules to be transferred. The information obtained from the dye molecule transfer and physical changes on the plant cell envelope resulting from ion-beam-cell-surface interaction provides a necessary basis for induction of DNA transfer by ion beams.

## MATERIALS AND METHODS

### Plant tissues

Table 1 shows a list of the plant species, mostly horticultural ones, and tissue culture-derived explants used in this study. The explants were rehydrated in sterile distilled water for 30 min, thereafter cultured onto artificial media [Murashige and Skoog (MS)<sup>11</sup> + NAA and kinetin each at 0.5 mg/l for items 1 and 16 (Table 1), White<sup>12</sup> for items 2 and 6-8, MS+NAA and kinetin each at 1.0 mg/l for items 3 and 4, MS + BA 1 mg/l for items 5 and 17, Vacin and Went (VW)<sup>13</sup> + 20% coconut water for items 9-14, and MS supplemented with BA, GA<sub>3</sub> and NAA at 1, 0.1 and 0.01 mg/l, respectively, for items 15, 18 and 19] kept at 28±1 °C under continuous light approximately at 13 μmol/m<sup>2</sup>/s for 15 days. The fresh onion outermost cell layer from its bulb scale (uncultured) was also used in this study. Pieces of fresh tissue specimens, about 2-4 mm in size, were fixed onto a petri dish using sterile autoclaved tape, and divided into two groups, one to be exposed to the ion beam for bombardment and the other, non-exposed group, as a vacuum-treated control. Some fresh tissues were also used as controls to compare the vacuum and low-temperature effects on the samples. Four tissue pieces of each species were employed in each treatment such that four replicates per fluence were applied. Each experiment was repeated at least three times.

### Ion beam bombardment

The ion bombardment was carried out using the 150-kV mass-analyzed heavy ion implantation facility at Chiang Mai University.<sup>14,15</sup> In this machine, ions were produced by a radio-frequency ion source, electrostatically extracted and accelerated, magnetically mass-analyzed and focused, and finally transported to the target chamber where a special bio-sample holder was installed (Fig 1). Ar and nitrogen (N) ions were used with energies of 15, 20 and 30 keV at fluences of 0.5, 1, 2, 4, 10, 15, and 30 × 10<sup>15</sup> ions/cm<sup>2</sup>. Because the term "dose" has different meanings within the ion-implantation and biological-irradiation communities, here we avoid the term completely and use "fluence" to refer to the ion-bombardment intensity.

The whole beam line including the target chamber (Fig 1a) and the sample holder (Fig 1b) was constructed from stainless steel. The sample holder was designed to hold a standard petri dish (9 mm in size) which could expose four different tissue targets to the ion beam. Pulsed beam modes were adopted with the beam periodically sweeping across the exposure holes of the sample holder. The beam fluence (*f*) was determined from the target beam current (*I*) and the bombardment time (*t*) as  $f \propto It$ . In order to measure the beam current correctly, an electron suppression tube was mounted in front of the holder shutter and biased to -200 V to suppress the emitted secondary electrons from the metal shutter surface due to ion beam bombardment. The beam current densities were varied from 3 to 10 μA/cm<sup>2</sup>. The fluence of each pulse irradiating the target was about 3-5 × 10<sup>12</sup> ions/cm<sup>2</sup>. During ion bombardment, the pressure in the target chamber was kept around 10<sup>-3</sup> Pa by a turbomolecular pump, and the temperature of the target in such an environment was estimated to be about 0°C. During each experiment, the tissue specimens were under these conditions for about 1.5-2 hours.

### Neutral Red dye transfer

After ion beam bombardment, the tissues were immediately rehydrated in sterile distilled water for 30 min. The NR vital dye with a molecular weight of 300 Da<sup>16</sup> was chosen to be introduced into the bombarded plant cells and also to rapidly determine the ion-bombardment effect on structural modifications of the cell wall and the cell survival.<sup>16</sup> The rehydrated tissues were placed on a glass slide, stained with the NR solution (1 mg/ml in phosphate buffer 50 mM, pH 7.5) and observed under a light microscope.

### Scanning and transmission electron microscopy observation of the cell wall

The ion-bombarded and control specimens were observed by scanning electron microscopy (SEM) and

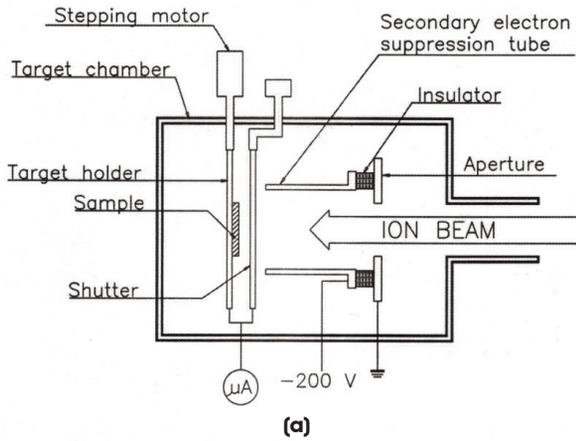


Fig 1. (a) Schematic of the ion-beam target chamber. (b) Photograph of the sample holders (in two different sizes).

transmission electron microscopy (TEM) using the JEOL 840 A scanning electron microscope and JEOL 1200 EX II transmission electron microscope respectively.

**RESULTS AND DISCUSSION**

**Vacuum effect on cell survival**

Since the ion beam treatment under high vacuum condition caused water loss and created a low-temperature environment for the tissues, the effect on the cell survival due to these harsh conditions was therefore first tested separately from the ion bombardment effects. Before the ion bombardment experiment, the effect due to pressure of about  $10^{-3}$  Pa (which led to a specimen temperature of about  $0^{\circ}\text{C}$  or lower) on the tissues was tested. SEM micrographs in Fig 2 show the difference in shape between the fresh control (Fig 2a) and vacuum treated cells (Fig 2b). The significant shrinking of the cells in vacuum demonstrated the loss of water in the cells. However, the vacuum-conditioned cells were found to survive after they were returned to the natural environment. The cell turgor caused by vacuum was restored in cell appearance after an incubation of 30 minutes in sterile distilled water (Fig 3, a-c). Furthermore, growing tests for the plant species and tissues subjected to appropriate ion bombardment in vacuum showed growths or survival steady states for almost all of the plants, as shown in Table 1. These facts indicated that the vacuum and low

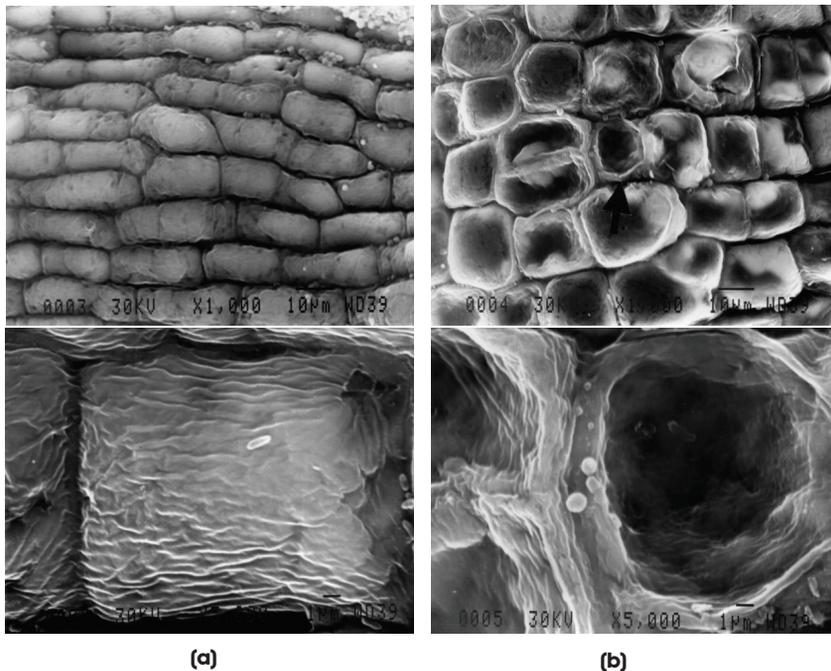


Fig 2. SEM photographs of an example of the vacuum effect of water loss from *Curcuma* embryo cells: (a) fresh control and (b) vacuum treated ( $10^{-3}$  Pa, 2 hrs), in different magnifications. Scale: width of each photograph in the upper row is  $110\ \mu\text{m}$ , and that in the lower row is  $22\ \mu\text{m}$ . The arrow in (b) indicates the cell being magnified.

**Table 1.** Plant species and tissues used in the study, and their survival and *in vitro* growth states 15 days after Ar-ion bombardment at 30 keV to a fluence of  $2 \times 10^{15}$  ions/cm<sup>2</sup> in vacuum (pressure of  $10^{-3}$  Pa).

Item	Plant species	Tissue	Survival and growth state
1	<i>Dendranthema</i> Hybrids	leaf	grown/developed
2	<i>Zea mays</i>	leaf, embryo*	grown/developed
3	<i>Euryclis amboinensis</i>	leaf base	grown/developed
4	<i>Hippeastrum</i> Hybrids	leaf base	steady state
5	<i>Gladiolus</i> Hybrids	leaf base	dead
6	<i>Cucurbita moschata</i>	embryo	steady state
7	<i>Curcuma</i> sp.	embryo*	steady state
8	<i>Zingiber</i> sp.	embryo*	steady state
9	<i>Cymbidium tracyanum</i>	protocorm	grown/developed
10	<i>Dendrobium cruentum</i>	protocorm	steady state
11	<i>D. albosanguineum</i>	protocorm	grown/developed
12	<i>Ascocentrum curvifolium</i>	protocorm	grown/developed
13	<i>Paphiopedilum</i> sp.	protocorm	grown/developed
14	<i>Gnetum gnemon</i>	lateral bud	grown/developed
15	<i>Fragaria vesca</i>	lateral bud	grown/developed
16	<i>Tacca</i> sp.	lateral bud	steady state
17	<i>Globba</i> sp.	lateral bud	grown/developed
18	<i>Broussonetia papyrifera</i>	stem	grown/developed
19	<i>Maesa ramentacea</i>	stem	steady state

\*: whole naked embryos were used.

temperatures for the operating period (1.5-2 hours) did not affect the viability of the cells. Thus the vacuum effect was negligible and final results would be only attributed to ion bombardment.

## Ion bombardment effects

### Survival of plant cells

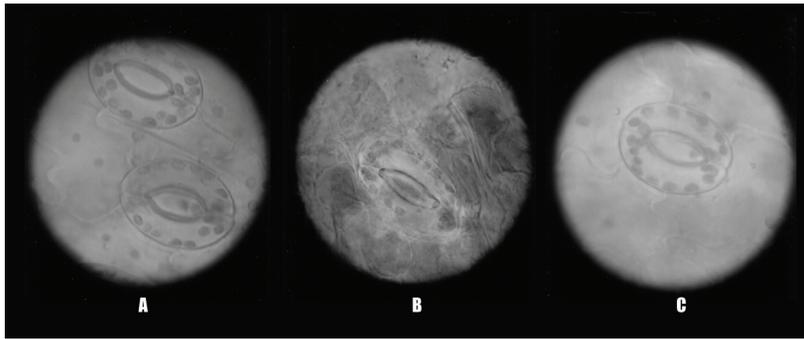
As shown in Table 1, after Ar-ion bombardment, almost all of the species and tissues could grow or survived at a steady state, except for *Gladiolus*. Ion beam effects on embryo germination and growth of the embryos bombarded under different conditions, *ie* ion species and fluence, were compared with the vacuum-treated control.<sup>9</sup> The naked corn embryos in tissue culture could still germinate after 30-keV Ar- or 15-keV N-ion bombardment at fluences of  $5 \times 10^{14}$ ,  $1 \times 10^{15}$  and  $2 \times 10^{15}$  ions/cm<sup>2</sup>, but with the growth retarded by up to about 50% (Table 2). Germination and growth did not occur for fluences higher than  $1 \times 10^{16}$  ions/cm<sup>2</sup> at the above-mentioned energies of the ions, or for any fluences at energies higher than 20 keV when N ions were used (data not shown). The results indicated that under appropriate ion bombardment conditions (for a certain ion species, with proper energy and fluences), ion bombardment did not affect survival of the treated plant cells.

### Microscopic modification of cell envelope

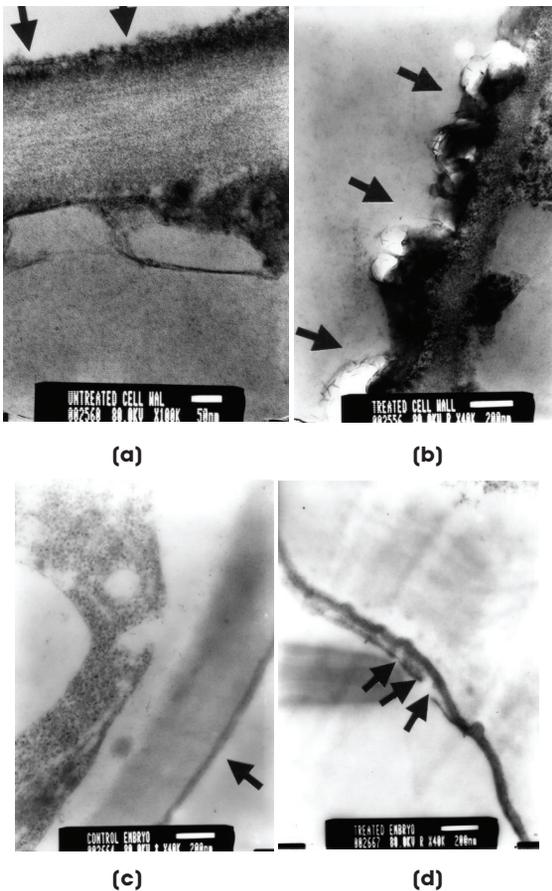
TEM photographs (Fig 4) show that at fairly high fluences, ion bombardment caused severe and extensive damage to the cell envelope (Fig 4b) and death of the cell, as demonstrated by the dye staining of the nucleus<sup>9</sup> (also see Fig 6d). Suitably low fluence ion bombardment created modification of the outer layer of the cell envelope without complete damage (Fig 4d). The partial damage and thinning of the cell envelope are due to ion sputtering and etching, and the extent of this kind of damage has been found to depend on ion energy and fluence. Generally, bombardment with high-energy and high-fluence ion beam resulted in extensive damages to the cell envelope. A comparison between the cell envelope surfaces of the vacuum-treated control and the ion-bombarded specimens is shown in Fig 5. The ion-bombarded cell envelope surface was featured by blistering, exfoliation and cavity formation (Fig 5a), whereas the vacuum-treated control surface was very smooth (Fig 5b). Close examination of the damaged surface revealed some dispersed craters, which were large and deep. The results indicated that ion bombardment modified the cell envelope structure and was able to create appropriate damage under certain conditions.

**Table 2.** Average heights (in cm) of the seedling grown from the naked corn embryos in tissue culture 7 days after ion bombardment.

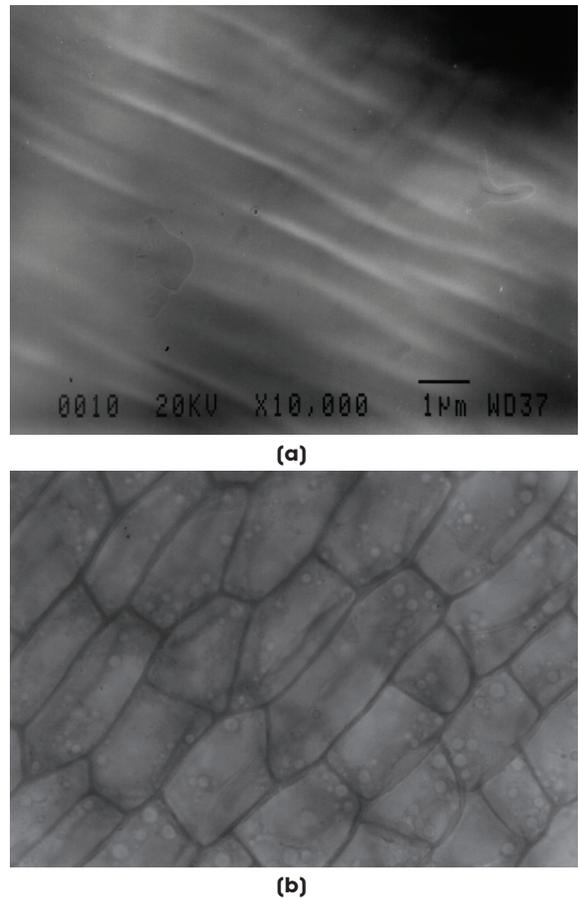
Fresh control	Vacuum-treated control	Ion-beam bombardment	Ion Fluence (ions/cm <sup>2</sup> )			
			$5 \times 10^{14}$	$1 \times 10^{15}$	$2 \times 10^{15}$	$1 \times 10^{16}$
7.0	6.7	15-keV N ions	3.5	2.9	3.0	1.2
		30-keV Ar ions	2.9	3.0	2.9	1.0



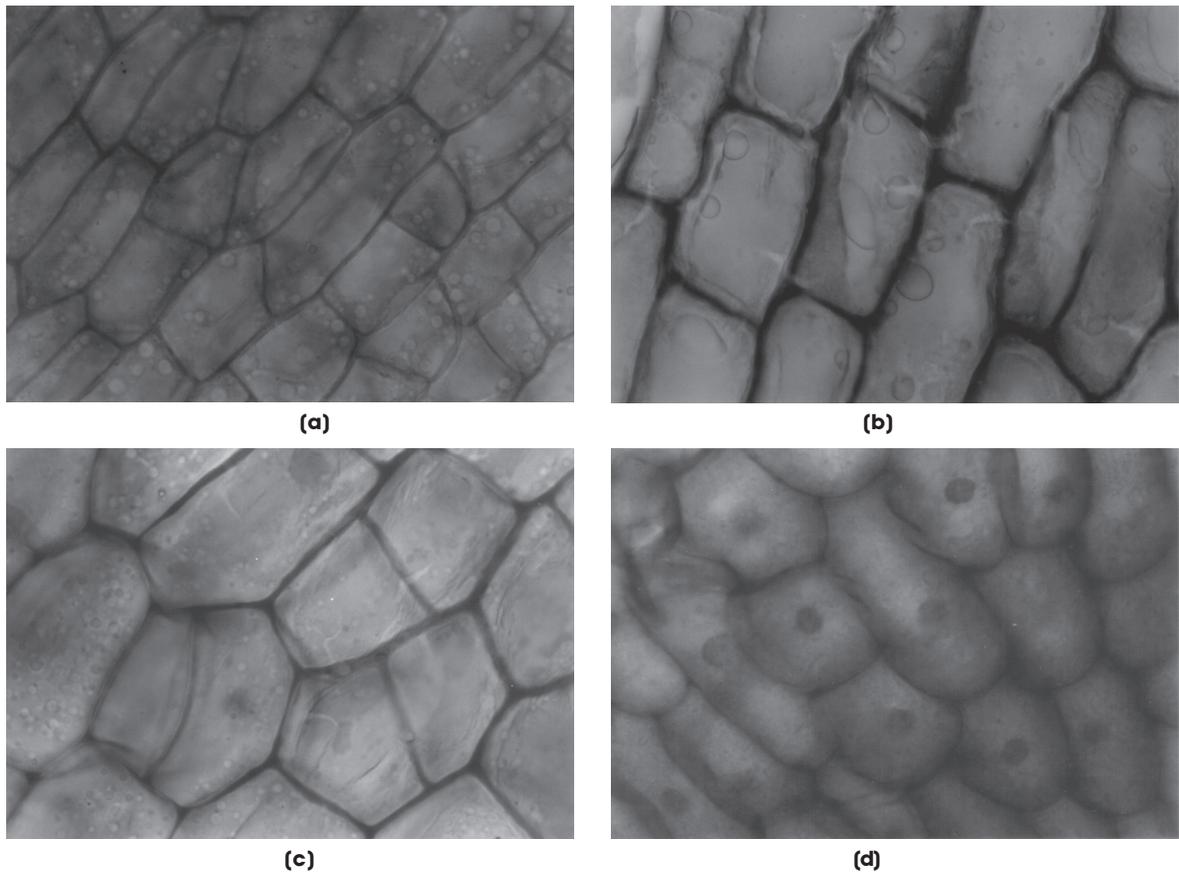
**Fig 3.** Light-microscopic photographs of an example of the effect of vacuum on guard cells from *Dendranthema* leaf: (a) fresh control, (b) vacuum-treated control (at  $10^{-3}$  Pa for 2 hrs), and (c) the vacuum-treated control after 30 min of rehydration in distilled water. Scale: width of each photograph is 60  $\mu$ m.



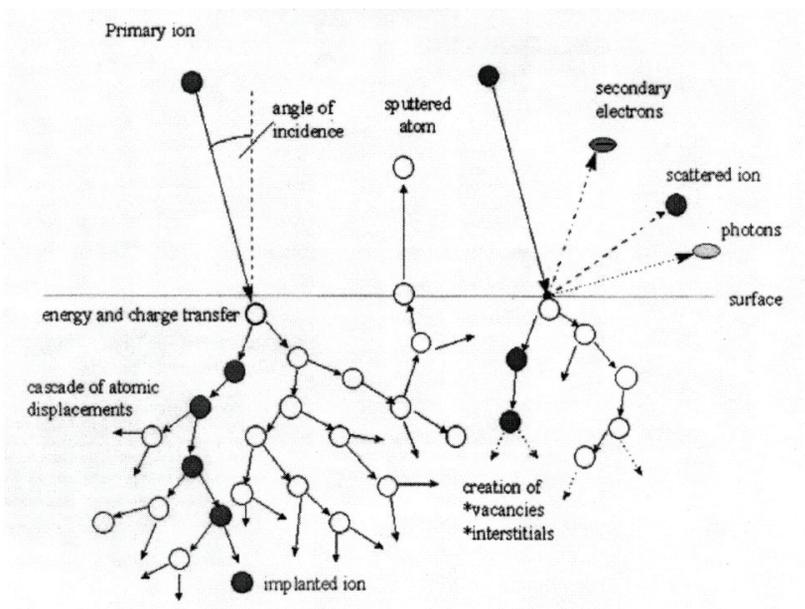
**Fig 4.** TEM photographs of the *Curcuma* embryo cell envelopes of (a) the vacuum-treated control, (b) the Ar-ion-bombarded ( $30\text{ keV}$ ,  $2 \times 10^{15}$  ions/cm<sup>2</sup>) cell, (c) the vacuum-treated control, and (d) the Ar-ion-bombarded ( $30\text{ keV}$ ,  $1 \times 10^{15}$  ions/cm<sup>2</sup>) cell. The arrows indicate the outside of the cell envelope. The scales are as indicated by the bars in the photographs



**Fig 5.** SEM photographs of examples of ion bombardment effect on the cell envelope morphology: (a) vacuum-treated control of the onion monolayer cell envelope surface, and (b) onion monolayer cell envelope surface bombarded by  $30\text{-keV}$  Ar ions to a fluence of  $2 \times 10^{15}$  ions/cm<sup>2</sup>. The scale is as indicated by the bars in the photographs.



**Fig 6.** Light-microscopic photographs of the Neutral Red vital dye staining of 30-keV Ar-ion bombarded *Curcuma* embryo cells: (a) vacuum-treated control, (b) with a fluence of  $1 \times 10^{15}$  ions/cm<sup>2</sup>, (c) with  $2 \times 10^{15}$  ions/cm<sup>2</sup>, and (d) with  $4 \times 10^{15}$  ions/cm<sup>2</sup>. Scale: width of each photograph is 80  $\mu$ m. In (c), tiny dye particles were initially moving around, then gradually aggregating into bigger spheres. Finally these spheres gathered into rather large dark areas, which were thought to be vacuoles.



**Fig 7.** Schematic of basic interactions between incident energetic ions and a solid.

### Dye penetration

Fig 6 shows that the 30-keV Ar-ion bombardment induced NR penetration in *Curcuma* embryo cells. Normally, intact or uninjured plant cells can prevent the vital dye from entering the cells, while injured but still alive cells accumulate the dye in their vacuoles and then exhaust the exogenous molecules out of the cells via the process called exocytosis. Dead cells, on the other hand, will be stained by the dye.<sup>16</sup> Here we observed that the NR dye could not enter the cells of the non-bombarded embryo (Fig 6a), it could enter the cell wall and accumulated in the apoplast of the cells that were bombarded with 30-keV Ar-ion beam at a fluence level of  $1 \times 10^{15}$  ions/cm<sup>2</sup> (Fig 6b). When the fluence was increased to  $2 \times 10^{15}$  ions/cm<sup>2</sup>, the dye could enter inside the cells, where they streamed and circulated in the cytoplasm (Fig 6c), implying that the cells were functional and alive. At a higher fluence of  $4 \times 10^{15}$  ions/cm<sup>2</sup>, the cell envelope surface was severely damaged and the dye accumulated in the nuclear areas (Fig 6d), indicating that the cells did not survive. Ion bombardment using different ion species and dyes, such as N-ion and Trypan blue dye (molecular weight of 1000 Da<sup>17</sup>) was found to have the same effect on the dye molecule transferring into the cells, *ie* the penetration of the exogenous molecules is closely related to the ion energy and fluence.<sup>9</sup> For example, N-ion bombardment at 15 keV with fluences of  $1 \times 10^{15}$  and  $2 \times 10^{15}$  ions/cm<sup>2</sup> was found to be ineffective to the dye penetration into the cells, but caused cell death at 30 keV with the same fluences. The experimental results suggested that after appropriate ion bombardment the dye could enter the plant cells without irreversible damage.

### Mechanisms for Neutral Red transfer into the plant cells

The ion bombardment is thought to create "entrance" through the cell envelope for the penetration of the dye molecules into the cells. Both previous literatures<sup>18,19</sup> and our experiments have demonstrated that the energy of the ion beams should be suitably low in order to obtain positive results. Low-energy ions naturally have short ranges.<sup>1</sup> For example, the mean projected range of 30-keV Ar ions in water is calculated to be 63 nm.<sup>20</sup> However, the thickness of common cell walls varies from about 100 nm to many micrometers.<sup>21</sup> How can penetration of low-energy ions through the cell wall occur?

The fact is that the plant cell wall, which composes the main part of the plant cell envelope, is not in a continuous structure but consists of bundles of cellulose microfibrils.<sup>22</sup> From the primary structure of cellulose<sup>21</sup>, its chemical formula is C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>. A cellulose microfibril is about 3.5 nm in diameter in most higher plants, and

the microfibrils are cross-linked in a net style with about a 5-nm space in between.<sup>21</sup> So, it is deduced that only about  $3.5/(3.5+5)=7/17$  of the thickness of the cell wall is packed with structural material (this fully-solid cell wall is termed the compressed cell wall). From electron microphotography, the thickness of the cell wall of the experimented tissues was estimated to vary from 250 to 400 nm (Fig 4a). Hence, the thickness of the compressed cell wall was about 100-165 nm. When the cell was placed in the vacuum chamber, the fluid among the microfibrils in the cell wall was pumped out and the cell wall shrank (Fig 2). Therefore, during ion bombardment, ions only interacted with atoms of the solid structural materials.

Based on the data above, simulations of the interactions between the ions and the cell wall material (Fig 7) using PROFILE<sup>20</sup> and TRIM<sup>23</sup> programs were performed to predict the ion and radiation damage ranges in cellulose. The mean ranges of 30-keV Ar ions at a fluence of  $1 \times 10^{15}$ /cm<sup>2</sup> and the induced radiation damage in the plant cell wall material are around 50-60 nm. The ions and the damage basically distribute within the compressed cell wall region (about 100 nm), mostly near the top surface of the cell wall. This indicates that no damage occurs to the plasma membrane, and consequently there are no effects on the cell viability. As the fluence increases, surface sputtering should be taken into account. According to the special structure of the cell wall mentioned above, the sputtering effect is extremely heterogeneous at the cell wall surface. This is confirmed by the TEM (Fig 4) that at some locations the cell wall surface is etched more severely by sputtering. Because the sputtering yield is linearly proportional to the ion fluence<sup>24</sup>, at a fluence of  $2 \times 10^{15}$  ions/cm<sup>2</sup> NR could enter inside the cell wall and keep the cell alive. At a higher fluence of  $4 \times 10^{15}$  ions/cm<sup>2</sup>, ions might completely penetrate the cell wall that had been thinned by sputtering and hence cause fatal damage to the cell wall, plasma membrane and organelles inside the cell, resulting in the staining of the nuclei.

Thus, ion beams of energies and fluences, which have maximum ranges of ions and radiation damage around the thickness of the compressed cell wall (which means no significant damage to the plasma membrane) and produce surface sputtering effects and inner atomic collision cascades for solids (Fig 7), could affect the porous biological tissues in a similar way. The cellulose-pectin skeleton that constitutes the cell wall can be weakened and even pierced at some significantly weakened locations by extensive damage due to atomic collision cascades, and thus forming the crater-like structures. Because there has been experimental evidence of penetration of exogenous macromolecules (eg Trypan Blue dye with the molecular weight of 1000

Da<sup>9</sup> and DNA with 3.3 kb<sup>10</sup>) into cells induced by ion beams, it is inferred that the crater size should be sufficiently great for the movement of those exogenous molecules. This has been supported by our microscopic observation as described in the part of ion bombardment effect on the cell wall structure. The crater-like structures therefore constitute new molecule-exchange channels for exogenous macromolecule (such as DNA) transfer.

Gene transfer into cells has been induced by microparticle bombardment.<sup>25</sup> The technique, termed biolistics, uses DNA-covered heavy-metal (eg Au or W) microparticles, 1-4 μm in diameter, shot from a pressured-air gun to bombard the target cell at an ultrasonic velocity (eg 430 m/s). Thus, the induction mechanisms are completely different between the techniques of microparticle bombardment and ion beam bombardment. The former transfers gene by directly introducing the gene attached on the particles which now penetrate inside the cell and locate in either cytoplasm, or organelles, or nucleus. The latter transfers gene by first creating pathways on the cell envelope that is bombarded by ion beams and subsequently incubating the cell in a gene medium. The energy of a microparticle (eg Au, 1 μm in diameter, at a velocity of 430 m/s) is around an order of 10<sup>-8</sup> J, whereas the energy of an ion (eg Ar, at 30 keV) is in the range of 10<sup>-15</sup>-10<sup>-14</sup> J. As a result, the microparticle pierces the cell envelope and enters inside the cell, but the ion basically only interacts with the cell envelope. Therefore, ion bombardment is thought to be safer for the cell survival due to the controllable ion species, energy and fluence so as to control the interaction extent, and gene is transferred more naturally.

## CONCLUSION

Induction of exogenous molecule transfer into the living plant cells by ion beam bombardment in vacuum occurs when physical damage in the cell envelope is created by appropriate ion bombardment such that the ion range and radiation damage range lie within the compressed cell wall thickness. Certain types of damage structures can form channels for the exogenous molecules to transfer through the cell envelope.

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