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Effects of Locally Targeted Heavy-ion and Laser Microbeam on Root Hydrotropism in *Arabidopsis thaliana*

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***Arabidopsis thaliana*/Heavy-ion microbeam/Root hydrotropism/Laser ablation/Radiosurgery.**

Classical studies on root hydrotropism have hypothesized the importance of columella cells as well as the *de novo* gene expression, such as auxin-inducible gene, at the elongation zone in hydrotropism; however, there has been no confirmation that columella cells or auxin-mediated signaling in the elongation zone are necessary for hydrotropism. We examined the role of root cap and elongation zone cells in root hydrotropism using heavy-ion and laser microbeam. Heavy-ion microbeam irradiation of the elongation zone, but not that of the columella cells, significantly and temporarily suppressed the development of hydrotropic curvature. However, laser ablation confirmed that columella cells are indispensable for hydrotropism. Systemic heavy-ion broad-beam irradiation suppressed *de novo* expression of *INDOLE ACETIC ACID 5* gene, but not *MIZU-KUSSEI1* gene. Our results indicate that both the root cap and elongation zone have indispensable and functionally distinct roles in root hydrotropism, and that *de novo* gene expression might be required for hydrotropism in the elongation zone, but not in columella cells.

INTRODUCTION

Roots respond to a number of environmental cues such as gravity, light, touch, and moisture gradients with gravitropism, phototropism, thigmotropism, and hydrotropism, respectively. Root hydrotropism is most likely to play an important role in the acquisition of water.[1)] Studies using seedling roots of pea, cucumber, and maize have shown that de-tipped roots of pea and maize seedlings display a remarkable reduction in hydrotropism, and that an endoxyloglucan glucosyl transferase gene from pea, *PsEXGT* and an auxin-responsive gene from cucumber, *CsIAA1*, are asymmetrically expressed in the elongation zone during the root hydrotropic response of pea and cucumber, respectively.[2–5)] More recently, we established an experimental system of hydrotropism using *Arabidopsis* seedling roots, and showed that the requirement of root cap cells as well as auxin response in root hydrotropism also seemed to be true for *Arabidopsis*,[6,7)] however, there has been no confirmation that columella cells or elongation zone are necessary for hydrotropism.

Cell ablation is one of the ways to determine the tissue(s) responsible for root hydrotropism. Laser ablation of a specific cell type has previously been used to prove the necessity of root cap cells in gravitropism.[8)] Although cell ablation is a powerful technique for elucidating the necessity of certain cells, difficulty arises in using this technique to determine the role of the elongation zone, because laser ablation of this tissue results in the root being snapped, and it becomes unsuitable for monitoring root curvature. On the other hand, high-linear energy transfer (LET) heavy-ion irradiation non-destructively inactivates cells through the induction of DNA double-strand breaks.[9)] Indeed, it has been reported that the effect of heavy-ion microbeam is significantly different from that of laser microbeam in silkworm eggs.[10,11)] Recently, a system of heavy-ion microbeam irradiation with high precision has been developed.[12,13)] In this context, Tanaka *et al.* used heavy-ion beam to determine the

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effect of irradiation on root gravitropism, and suggested that the positional effect of irradiation partly differs between heavy-ion beam and laser.[12] However, the interpretation of the effect of this type of treatment remains controversial, because there has been no report that examined gene expression of interest for determining whether heavy-ion irradiation inactivates plant cellular processes.

In this study, we investigated the role of root cap cells and elongation zone on root hydrotropism by positional irradiation of heavy-ion and laser microbeam. In addition, we examined the auxin-responsiveness by monitoring the induction of *de novo IAA5* expression after systemic irradiation of heavy-ion broad-beam.

MATERIALS AND METHODS

Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* (ecotype Columbia) were sterilized with a solution of 5% (v/v) sodium hypochlorite and 0.05% (v/v) Tween 20 for 5 min, washed with distilled water, and germinated on 0.2% Gellan Gum (Sigma, St. Louis, MO) plates containing 1/2 Murashige and Skoog (MS) medium (Sigma), as described by Kaneyasu *et al.*[7] Upon germination, plates were set in a vertical position so that the seedlings grew straight along the surface of the medium. The seedlings were then placed in an incubator and grown at 23°C under continuous light. For all the experiments, we used seedlings with straight roots, 1.0 to 1.5 cm in length.

Irradiation with heavy-ion microbeam and broad-beam

The system of collimated heavy-ion microbeam and raster-scanned broad-beam was installed at Takasaki Ion Accelerators for Advanced Radiation Application (TIARA) facilities of Japan Atomic Energy Agency (JAEA), for which the setup and irradiation procedure have been described previously.[12–15] In this study, 180-μm diameter microbeam (135 keV/μm) and 25 cm^2 area broad-beam (160 keV/μm) of 18.3 MeV/u carbon ions were used. For microbeam irradiation, six seedlings were aligned on a microchamber composed of

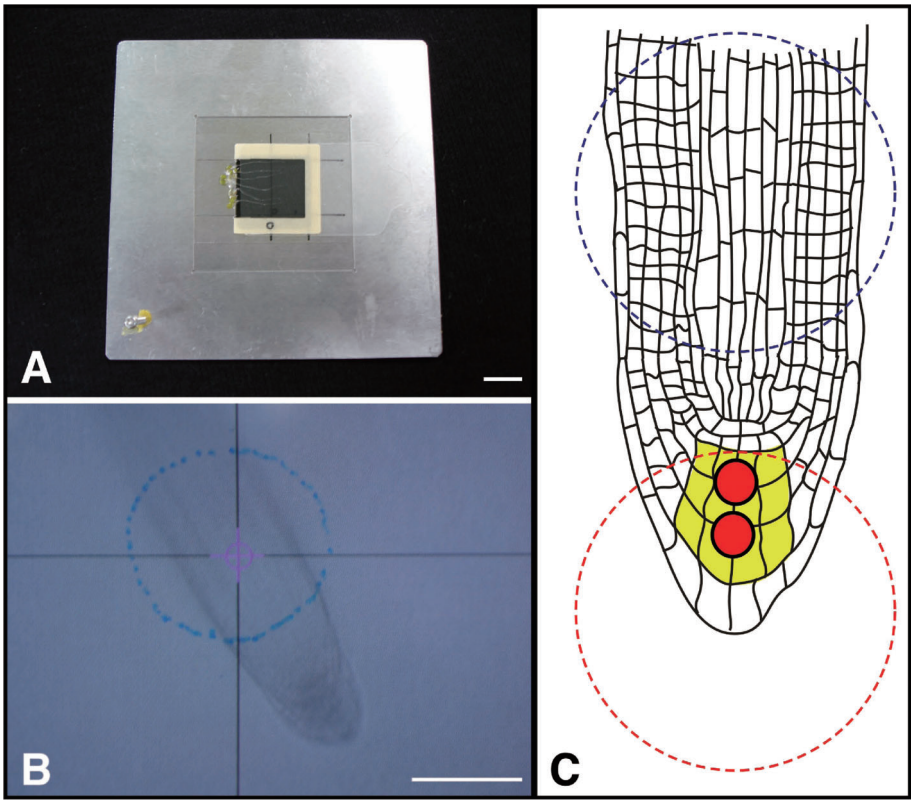

Fig. 1. Heavy-ion and laser microbeam irradiation of *Arabidopsis* seedlings. A: *Arabidopsis* seedlings aligned in a microchamber. B: Microscopic image of heavy-ion microbeam irradiated *Arabidopsis* seedling roots. Blue dotted circle at the elongation zone shows the approximate area of irradiation, and the pink circle with a cross indicates the center of the irradiated area. C: Schematic representation of the heavy ion and laser irradiation. Blue and red dotted circles indicate the sites of the heavy ion microbeam irradiations for elongation zone and columella cells, respectively. Small red circles indicate the sites of laser beam irradiation. Laser ablated columella cells were colored yellow. Scale bars in A and B represent 1cm and 100 μm, respectively.

a coverslip (Matsunami Glass, Osaka, Japan) and a seal (TaKaRa Slide seal for *in situ* PCR, Takara Bio, Shiga, Japan). The microchamber was filled with the low-melting agar, which is 1/2 MS medium and 0.25% low-melting agarose (SeaPlaque, FMC BioProducts, Rockland, ME), to prevent drying of the seedlings during irradiation. The microchamber (Fig. 1A) was set on a stage which was set on the irradiation apparatus.[13] The location of the microbeam irradiation target was determined using a microscope (TMD300, Nikon, Kanagawa, Japan) and Autoscope software, as described.[14] During irradiation, the target samples were kept under microscopic observation (Fig. 1B). For broad-beam irradiation, 20–24 seedlings were placed in a 6 cm plastic dish (Asahi Techno Glass Corp., Chiba, Japan) without contacting each other, and moistened with the low-melting agar to avoid drying. The dish was covered with 8-μm thick Kapton polyimide film (DuPont-Toray, Tokyo, Japan), and the samples were systemically irradiated, as described.[15]

Laser ablation of columella cells

Seedlings were aligned on a microchamber, as described above. Ablation of columella cells were observed in real time on the video monitor, and laser light from a pulsed dye laser (440 nm) was delivered through a MicroPoint laser interface system (Photonic Instruments Inc., St. Charles, IL). The intensity of the laser beam was adjusted so that it was possible to make a hole in the coverslip. 5 pulses at 50 ms intervals were delivered in each irradiation.

Hydrostimulation of Arabidopsis seedlings

Immediately after microbeam irradiation or laser ablation, seedlings were recovered from the microchamber and placed on medium containing 1% agar and 1/2 MS medium. The seedlings were maintained in a vertical position on the agar medium for 15 min to remove excess liquid, and then subjected to an agar-based hydrotropism assay, according to the method of Kaneyasu *et al.*,[7] except that the agar plates were supplemented with 1/2 MS medium. All plates were photographed using a digital camera (model EOS20D: Canon Inc., Tokyo, Japan). Root growth and hydrotropic curvature were measured using NIH Image software. Statistical analysis was done using the Student's two-tailed *t* test.

Induction of auxin-responsive gene expression

After systemic heavy-ion irradiation, seedlings were transferred into liquid 1/2 MS medium containing 1 μM indole-3-acetic acid (IAA). For mock-treated cultures, an equivalent concentration of the vehicle, dimethylsulfoxide, was added to the cultures. Then, the seedlings were incubated in a growth chamber in the dark at 23°C. 1 h after incubation, seedlings were transferred into RNAlater (Ambion, Austin, TX) at 4°C until RNA isolation.

RNA isolation and quantitative reverse transcription PCR

Total RNA from whole seedlings was extracted using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), according to the manufacture's instruction. Following DNase treatment, first-strand cDNA synthesis was performed using a ReverTra Ace kit (Toyobo, Osaka, Japan), with 1 μg of total RNA as the template. After heat inactivation of the reverse transcriptase at 99°C for 5 min, samples were diluted with 80 μl of 10 mM Tris-HCl (pH 8.0) and stored at –20°C until use. Quantitative reverse transcription PCR was performed using MyiQ and iQ SYBR

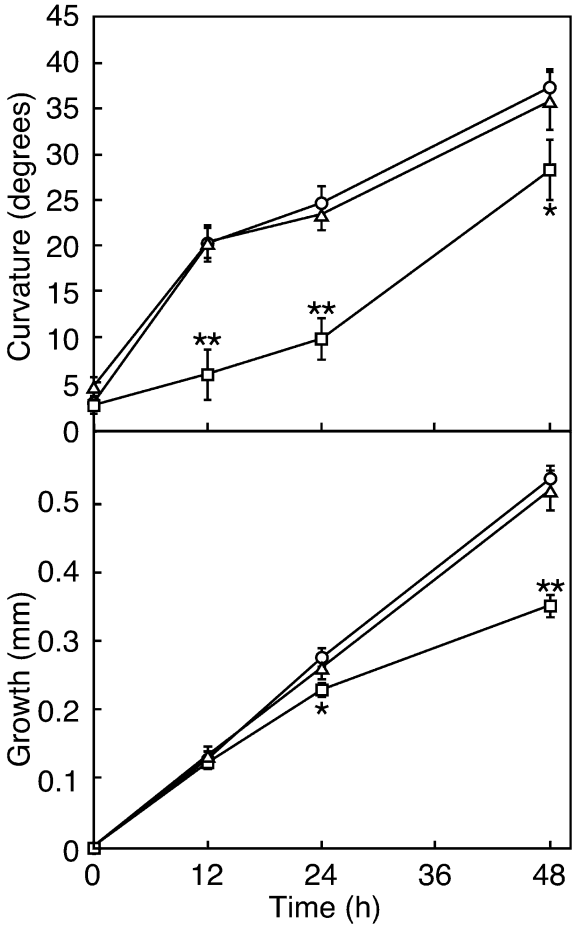

Fig. 2. Effect of local heavy-ion microbeam irradiation on root hydrotropism. Cells residing in the columella root cap and elongation zone were locally irradiated with carbon ions at a dose of 500 Gy and compared to non-irradiated control seedling roots. Changes in hydrotropic root curvature (top) and root growth (bottom) are shown. Open circles, control seedling roots; open triangles, roots with irradiated columella cells; open squares, roots with irradiated elongation zone cells. Data represents the means ± SE of 30 individuals from two independent experiments. Asterisks indicate statistically significant differences, as determined by the Student's two-tailed *t*-test (*, $P < 0.05$; **, $P < 0.01$).

Green Supermix (Bio-Rad Laboratories, Hercules, CA), according to the manufacture's instruction. The sequences of the upstream and downstream oligonucleotide primers were as follows: 5'-CCGGAGAAAGAACAGTCTCG-3' and 5'-CCAAGGAACATTTCCCAAGG-3' for *INDOLE ACETIC ACID 5* (*IAA5;* At1g15580), 5'-TGACTTCTCCGGCGCGTAGC-3' and 5'-CCAAGCTCCGTTCCACTACC-3' for *MIZU-KUSSEI1* (*MIZ1;* At2g41660), and 5'-TCAATCTCATCTTCTTCCGC-3' and 5'-CAATCGTGATGACTTGCCCA-3' for *ACTIN2* (*ACT2;* At3g18780), respectively. Primer annealing and extension reactions were carried out at 58°C for 20 sec and 72°C for 20 sec, respectively, to amplify *IAA5 and ACT2*; and at 55°C for 20 sec and 72°C for 40 sec, respectively, to amplify *MIZ1*. Quantification was performed using a series of dilutions of plasmids containing the cDNAs of the respective genes being examined.

RESULTS

Effect of heavy-ion and laser microbeam on root hydrotropic response

It has been reported that both root cap cells and elongation zone play important roles in root curvature during hydrotropism.[16] However, to date, there has been no functional evidence showing that these two regions are important for the hydrotropism. To investigate the role of columella cells and elongation zone in the root hydrotropic response of *Arabidopsis* seedlings, columella root cap and elongation zone proximal to the root tip were locally irradiated with 500 Gy of 180-μm-diamter carbon-ion microbeam (Fig. 1B, C). Control *Arabidopsis* seedling roots responded to a water potential gradient and developed orthotropic curvature (Fig. 2). In contrast, when cells of the elongation zone proximal

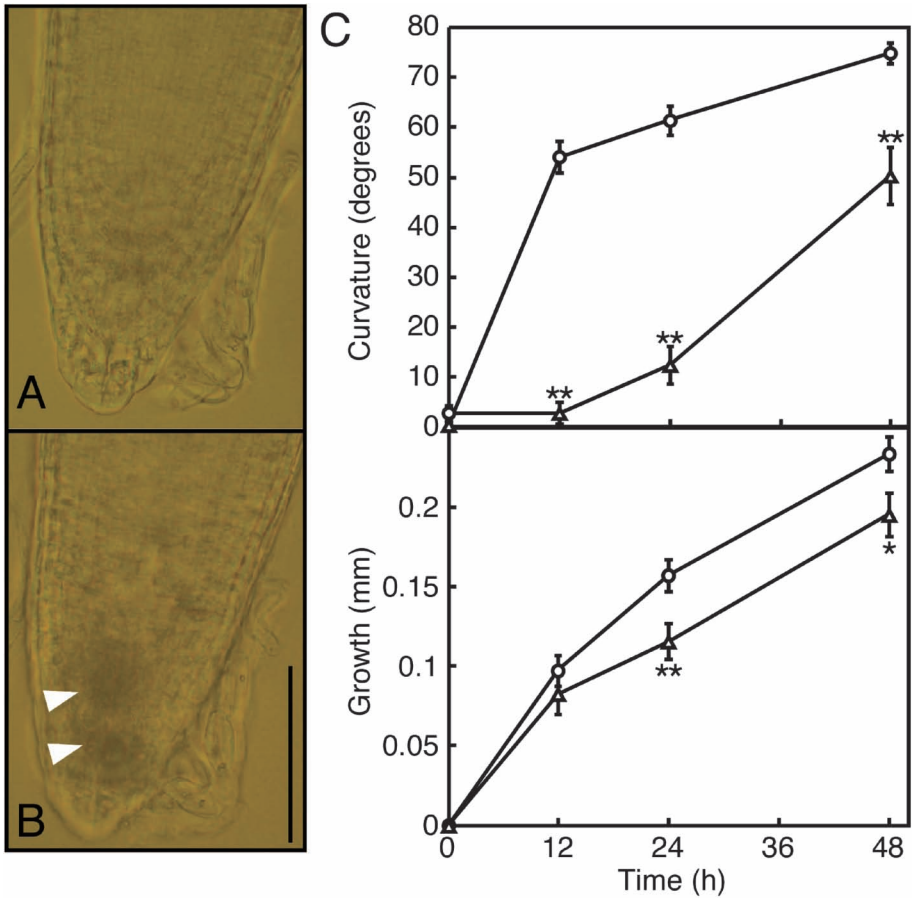

Fig. 3. Hydrotropic response of columella cell-ablated roots. A, B: Micrographs of columella cells before (A) and after (B) laser beam ablation. Arrowheads indicate the sites of ablation. Scale bar represents 100 μm. C: Changes in hydrotropic root curvature (top) and root growth (bottom) after laser beam irradiation are shown. Open circles, control; open triangles, samples with ablated columella cells. Data represents the means ± SE of 21 (control) and 28 (laser ablated) samples from two independent experiments. Asterisks indicate statistically significant differences, as determined by the Student's two-tailed *t*-test (*, $P < 0.05$; **, $P < 0.01$).

to the root tip were exposed to carbon-ion microbeam irradiation, the hydrotropic response was significantly repressed, particularly in the early phase. The overall root growth during the corresponding period was less affected by irradiation. Unexpectedly, localized carbon-ion irradiation of the root cap cells did not affect the development of hydrotropic curvature or root growth (Fig. 2). Because this result was somewhat contradictory to our previous results that suggested the necessity of root cap cells for the hydrotropic response,[6] we further investigated the functional role of the columella cells by laser ablation. As shown in Fig. 3, laser ablation of stories 1 and 2 (the first and second proximal cell layers, respectively) of the columella cells severely repressed the development of hydrotropic curvature for as long as 12 h post-irradiation, indicating that columella cells play an important role in the early phase of the hydrotropic response. Laser ablation of root cap cells did not affect the overall root growth during this period.

Systemic heavy-ion irradiation suppresses de novo expression of auxin-responsive gene

We further examined changes in auxin-responsive gene expression in the presence of exogenously applied auxin to determine whether systemic heavy-ion irradiation with broad-beam inactivates irradiated cells, so as to confirm the effectiveness of heavy-ions on suppression of *de novo* gene expression. We chose *IAA5* gene as a probe, for its expression is highly sensitive to exogenously applied auxin among the *IAA* genes.[17] In the following experiments, we used seedlings that did not receive irradiation (cold-run seedlings) as a control, to take into account possible effects of the experimental procedure on gene expression levels. When control *Arabidopsis* seedlings were treated with 1 μM IAA for 1 h, there was a 43.3-fold increase in the mRNA of the auxin-responsive gene *IAA5* compared to mock-treated cultures (Fig. 4). In contrast, systemic irradiation of *Arabidopsis* seedlings with 500 Gy of carbon-ion broad-beam caused a 8.3-fold increase in the level of *IAA5* mRNA, which was significantly low when compared to control cultures. We also monitored *MIZ1* gene whose product is responsible for root hydrotropism in *Arabidopsis*. In contrast to the *IAA5*, 500 Gy of carbon beam irradiation caused neither increase nor decrease in *MIZ1* mRNA level.

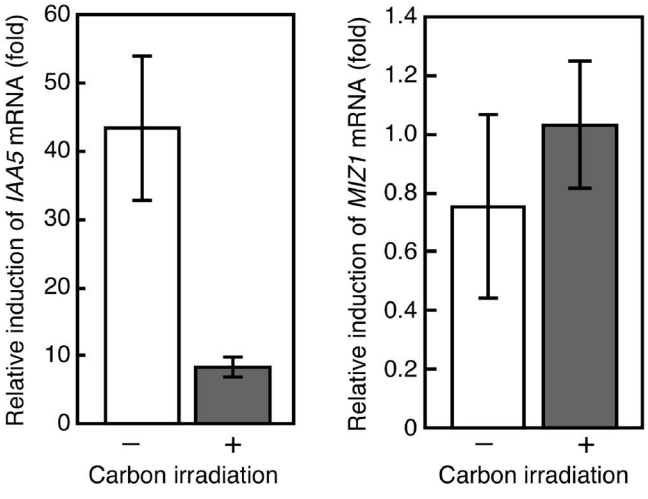

Fig. 4. Effect of heavy-ion beam irradiation on auxin responsiveness. Total RNA was extracted from either broad-beam-irradiated or non-irradiated samples and subjected to quantitative reverse transcription PCR. The levels of *IAA5* and *MIZ1* mRNA was normalized to that of *ACT2*, and is expressed relative to normalized transcript levels in non-treated samples, which was set as one. Open bars represent non-irradiated samples, grey bars represent irradiated samples. Data represents the means and standard error (± SE) of three independent experiments. A statistically significant difference was observed in *IAA5* expression levels as determined using the Student's two-tailed *t*-test ($P < 0.05$).

DISCUSSION

Generally, a tropic response consists of signal perception, signal transduction, and differential growth. Hydrostimulation leads to the rapid degradation of starch inside columella cells, and we recently showed that *MIZ1*, an essential gene for root hydrotropism, is expressed predominantly in root cap cells, particularly in columella cells.[6,18] The distal elongation zone resides in the region of the elongation zone that is proximal to the root cap. It has been reported that the distal elongation zone is the site of post-mitotic isodiametric growth, where rapid differential growth occurs during gravitropism.[19,20] It is possible that this tissue also plays an important role in root curvature during hydrotropism, as the region of differential growth during hydrotropism appears to be similar to that of gravitropism. In this study, we focused on the two above-mentioned tissues for monitoring the effect of local irradiation of heavy-ion and laser microbeams.

Previous report showed that 100 Gy irradiation of carbon microbeam effectively suppress root gravitropism.[12] However, our preliminary experiment showed that microbeam irradiation below 100 Gy was not effective to suppress root hydrotropism in *Arabidopsis* (data not shown), which led us to use more massive irradiation. Thus we adopted 500 Gy as a dose of microbeam and broad-beam irradiation for further study. The overall root growth during the corresponding period was less affected by irradiation. Upon incubation for longer periods of time, seedlings grew towards the area of high water potential, whereas overall root growth was severely repressed. Previously, it has been reported that the root elongation zone is spatially modulated by water stress.[21] Our results indicate that the region of cells that regulate root elongation in overall root growth differs from that of differential elongation.

Significant suppression of the development of hydrotropic curvature by localized carbon-ion irradiation of the elongation zone indicated that this region was necessary for root

hydrotropism (Fig. 2). Previously, we reported that asymmetric expression of an auxin inducible *CsIAA1* occurred during the development of hydrotropic curvature in elongation zone of cucumber seedling roots.[5] Moreover, we demonstrated that auxin response is necessary for root hydrotropism in *Arabidopsis*.[7] Together with the fact that *IAA5* gene is systemically expressed, and that the *de novo* expression of *IAA5* gene is suppressed by systemic heavy-ion beam, it is probable that auxin-responsiveness is also suppressed by localized heavy-ion beam irradiation. On the other hand, auxin-responsiveness of *MIZ1* gene expression was not altered by heavy-ion beam irradiation. Considering that *MIZ1* is predominantly expressed in columella cells, and that *MIZ1* is not an auxin-responsive gene, our present result suggests that the decrease in root hydrotropic curvature by heavy-ion beam irradiation is not due to the decrease of *MIZ1* mRNA level, rather *de novo* expressions of auxin-responsive *IAA* genes at elongation zone might be involved. However, our present data do not completely explain the necessity of *de novo* gene expression at the elongation zone during root hydrotropism, because heavy-ion beam irradiation could not inactivate the entire auxin-responsiveness of *IAA5* gene. It is possible that the effect of heavy-ion beam on gene expression differs gene by gene and thus the suppression of root bending cannot be explained by the fluctuation of *IAA5* gene expression alone. Also, the heavy-ion beam irradiation affects cellular activities other than gene expressions. So far, not a gene whose expression fluctuates during root hydrotropism has been identified. In future studies, efforts should be made to verify gene expression profiles of several genes to explain the above-mentioned issue.

Interestingly, the effect of irradiation to root cap cells on root hydrotropic response differed between heavy-ion microbeam and laser; the former less affected, but the latter significantly suppressed the hydrotropic curvature. Similarly, it was reported that the effects of irradiation of heavy-ion microbeam on silkworm eggs differed from those of laser microbeam.[10,11] This apparent contradiction might be explained by the difference of the primal target of the beams, namely laser affects not only nuclei but also proteins and membranes, while the primary target of heavy-ions is nuclei.[11] If so, it is likely that in columella cells, biological process that occurs inside does not require the existence of proper cell nuclei, such as *de novo* gene expression, rather the cellular component, which was already existed before irradiation, is sufficient for hydrotropism.

In summary, we examined the effect of heavy-ion microbeam irradiation as a potential tool for testing the functional roles of columella cells and cells of the elongation zone in the root hydrotropic response. We found that local irradiation of the elongation zone significantly suppressed the development of hydrotropic curvature, whereas local irradiation of the columella cells did not. Using laser ablation, we corroborated previous results showing that columella cells are necessary for the root hydrotropic response. Moreover, we demonstrated that heavy-ion irradiation suppressed *de novo IAA5* gene expression. By comparing the effects of heavy-ion irradiation with laser ablation, we showed that during hydrotropism, biological processes of the elongation zone and columella cells might differ each other.

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