Novel Plant Imaging and Analysis

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Water, Elements and Gas, Utilizing Radiation and Radioisotopes

Springer

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ISBN 978-981-33-4991-9 ISBN 978-981-33-4992-6 (eBook)
https://doi.org/10.1007/978-981-33-4992-6

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

This book summarizes what kind of research I have been pursuing throughout my career as a researcher. Originating as a radiochemist, I jumped into the plant physiological field, which was filled with riddles. As I proceeded further in this field, I noticed that most of the questions I found are about the fundamental activity of the plant, for example, how ions are actually moving in the solution within a plant or the balance of intake and outgo. Although we could determine some mechanisms, such as the roles of transporters or channels, there was no explanation of the total movement of water or ions throughout a whole plant, rather than at specific sites of the cells. In addition, there are many questions, such as how the concentration of each ion changes with the movement of water. From a chemical point of view, diffusion and osmotic pressure, sometimes including Brownian motion, are the fundamental explanations of movement. However, it seemed that these were not the main theme to incorporate into the discussion. What regulates the movement of ions or water in each tissue, balancing with the other tissues? The research to determine these actual movements of water or ions could be slightly different from the research to determine the role or mechanism of transporters or channels. This research is to know the whole plant activity itself. Therefore, before observation at the microscopic level, the overall activity of the plant was investigated. In addition, some of our observations are now progressing to the microscopic level, including gene expression.

Another point I noticed was that by the most effective utilization of radiation or radioisotopes (RIs), the fundamental activity of the plant could be analyzed from different perspectives, which might lead us to develop an original research field. In recent decades, the number of researchers utilizing radiation or RIs has been drastically reduced. Most of researchers seem to avoid using RIs or radiation now. However, without utilizing these tools, we cannot study or determine many basic activities of plants. Since water and inorganic ions are essential for plant growth, I focused on these fundamental matters, developing nondestructive tools, mostly imaging methods.

Many questions about plant activities arose from our findings. For example, what is the chemical state of ions or water, which roots are absorbing from soil? Through neutron beam imaging, we found that there is always a space adjacent to the surface of the root, indicating that the root is not touching the solution in the soil. This is because of the root tip movement, circumnutation, which was confirmed by using a Super-HARP camera. The root was always pushing the soil aside to guide the orientation of the root development. The next question was whether the root was absorbing water solution or water vapor, and the same question could be asked for ions in soil. Further questions addressed metals. Is the root absorbing metal vapor in soil? I could not yet determine the answer to these questions. Another riddle is that, no one had ever discussed the circulation of water in the internode within a plant. When trace amount of water was measured by labeling water with RI, a tremendous amount of water was found to leak horizontally from xylem tissue and push the water already present in the internode into the xylem to travel upward via xylem tissue. In approximately 20 min, half of the water present in the internode was calculated to be replaced. Was there any previous measurement of these movements? Photosynthesis activity presented yet another basic riddle. It was amazing to determine that the transfer orientation of photosynthate was different according to the tissue where the photosynthate was produced. The motive force affecting this phloem flow orientation is not known. This phenomenon was found by imaging the $^{14}CO_2$ gas fixation process by a real-time RI imaging system we developed. The real-time imaging of RI movement provided another exciting method to analyze the routes of xylem flow and phloem flow.

To pursue original research, we developed our own measurement and imaging systems. Except for the neutron source, which is a research reactor, all the devices or systems presented in this book are our original work. My goal is to show how the utilization of radiation or RIs is an indispensable tool for plant research; therefore, this book mainly focuses on new imaging or measuring methods with the results obtained utilizing the tools presented, and most of the further research results we pursued are omitted. However, fundamental questions encountered in our research are described in many parts of this book.

Since we are sure that there are hardly any other comparable systems developed by other people now, including systems for the measurement of the actual movements of water and ions, the author sincerely hopes that this book will attract research interest in the utilization of radiation and RIs.

The following is a summary of each chapter in this book.

Bunkyo-ku, Tokyo, Japan Tomoko M. Nakanishi

Acknowledgement

The studies presented in this book were mostly proceeded in my laboratory, named "Radio-Plant Physiology", Faculty of Agricultural and Life Sciences, The University of Tokyo, since I joined this faculty about 30 years ago from radiochemistry field and I found this name fits to my laboratory. To introduce the application of radiation and radioisotopes, I sincerely thank for the people who participated at each category of my work in the laboratory.

First of all, I thank to the people who are now in the Radio-Plant Physiology Laboratory, Prof. Keitaro Tanoi, Associate Prof. Natsuko I. Kobayashi, Assistant Prof. Ryosuke Sugita and the people who once worked with us and now in other organizations, Associate Prof. Satomi Kanno at Nagoya University, Associate Prof. Naoto Nihei at Fukushima University, Associate Prof. Atsushi Hirose at Hoshi University, Associate Prof. Jun Furukawa at Tsukuba University, Dr. Tomoyuki Ohya at QST (National Institutes for Quantum and Radiological Science and Technology), Associate Prof. Hiroki Rai at Akita Prefectural University, Dr. Masato Yamawaki at AIST (National Institute of Advanced Industrial Science and Technology). And I am much obliged to all the people who worked at the laboratory, as secretaries, Ms. Utako Shinohara and Ms. Megumi Anzai, technicians, students, graduate students.

I am also thankful to the people at JAEA (Japan Atomic Energy Association) who arranged the use of an atomic reactor or an accelerator with very useful discussions, Drs. Masato Matsubayashi, Hiroshi Iikura and other staffs and I would like to express my gratitude to Drs. Kazutoshi Suzuki, Kotaro Nagatsu, Noriko Nishioka and other staffs at QST who arranged for us to produce ^{18}F, ^{15}O as well as ^{28}Mg. I especially thank to Dr. Ren Iwata who is a Prof. Emeritus at Tohoku University now to provide us the chemical separation method of ^{28}Mg from the target. About phosphate imaging, I would like to thank deeply to Dr. Laurent Nussaume at CEA, in France, for the corporative work using RRIS (Real-time Radioisotope Imaging System).

Introduction and Executive Summary

Currently, powerful methods derived from molecular genetics have resulted in a tendency to focus research on the molecular aspects of biology and tend to leave behind important aspects of the activity of intact plants. However, the intact plant itself has high potential to integrate functions and to respond to diverse environmental conditions. To study the activity and development of living plants, nondestructive techniques are basic and essential. The imaging method is a particularly important tool. Fluorescent imaging is rapidly developing and has become overwhelmingly common in most biological studies. However, imaging utilizing radiation or radioisotopes (RI) has a definite advantage compared to fluorescent imaging from the perspective of quantitative analysis and indifference to lighting conditions. All of the data presented here introduce our work in water- and element-specific imaging and measurement in plants. However, a very limited number of people have utilized radiation or radioisotopes (RIs) for physiological research in living plants. Therefore, I would like to present my experience showing how imaging using radiation or RI holds promise to open new fields of plant research.

Although water and elements are essential for plant growth, kinetic studies of these two materials in situ are not progressing well.

In the case of water, for example, when we examine a representative activity of the plant, photosynthesis, where it is well known that sugar is produced from water and carbon dioxide gas in the air, we soon notice that there is very little research on water. There have been many studies on the mechanism of photosynthesis, the chemicals produced after carbon fixation, or the effect of carbon dioxide gas. However, in most cases, it is taken for granted that there is sufficient water already present in plant tissue for the chemical reaction. Though we could determine the kinds of chemicals produced by photosynthesis, we do not know how much water is needed or moving within the plant.

Water

Our first target was water, namely, how to obtain a water-specific image nondestructively. Neutron beam imaging was applied to provide water-specific images. Using a neutron beam, we could visualize water-specific images of plants, including roots and flowers, which were never shown before. Each image suggested the plant-specific activity related to water.

However, only a small number of people employ water-specific imaging produced by neutron beams for water-related studies in plants. Therefore, we briefly present how to acquire the image and what kind of water image is taken by neutron beam irradiation. We present a variety of plant samples, such as flowers, seeds, and wood disks. It was noted that neutrons could visualize the roots imbedded in soil without uprooting. When a spatial image of the root imbedded in soil was created from many projection images, the water profile around the root was analyzed. Then, fundamental questions were raised, such as whether plants are absorbing water solution or water vapor from the soil, because there was always a space adjacent to the root surface and hardly any water solution was visualized there. The roots are in constant motion during growth, known as circumnutation, and it is natural that the root tip is always pushing the soil aside to produce space for the root to grow. If the roots are absorbing water vapor, then the next question is about metals. Are the roots absorbing metal vapor? Since we tended to employ water culture to study the physiological activity of plants, the physiological study of the plants growing in soil was somewhat neglected. Later, when we could develop a system to visualize the movement of element absorption in a plant, there was a clear difference in element absorption between water culture and soil culture.

The next approach to research on water was to measure the small amount of water actually moving within a plant. The best method is to utilize radioisotope (RI)-labeled water and measure the radiation from outside of the plant. However, it is rather difficult to label water, since there are only limited kinds of RI for tracing water. The first trial utilized ^{18}F because trace amounts of this nuclide are produced when water is irradiated with a helium beam. It is well known that trace amounts of RI exhibit radiocolloidal behavior and move with larger amounts of chemicals. When utilizing ^{18}F to trace water movement, another fundamental question to consider was the features that characterize drought-tolerant and drought-sensitive plants. It is natural to suppose that drought-tolerant plants have strong water absorption; therefore, by analyzing the water absorption mechanism of tolerant plants and by introducing this function to sensitive plants, it might be possible to make the sensitive plants more tolerant.

However, when water uptake was studied in naturally developed drought-tolerant and drought-sensitive cowpea, selected from 2000 cowpea plants grown in the field of Africa, the result was unexpected. Under normal conditions, the amount of water absorbed by the drought-tolerant strain was much lower than that absorbed by the sensitive strain, as if showing the low capability of water absorption. On the other

hand, much higher amount of water was absorbed by the sensitive sample. However, this water absorption amount drastically changed after drying treatment. The tolerant strain began to absorb much more water than usual, whereas the sensitive strain could not absorb as much water as before the treatment. That is, the drought-tolerant plant required only a small amount of water under normal conditions, but when a drought condition was introduced, some mechanism was activated to absorb much more water. This result provided us with an important lesson. Analyzing the mechanism of drought tolerance only by comparing the water absorption of tolerant and sensitive plants might not readily reveal the reason for drought tolerance. The features of the naturally produced plants showed us different mechanisms that might not match our expectations developed in the laboratory.

Next, we performed water measurements using ^{15}O-labeled water, which has an extremely short half-life of 2 min. Here, we found another astonishing result, which was "water circulation" in the plant internode. A tremendous amount of water was always leaking from xylem cells, which had been regarded as a mere pipe to transfer water from the root to the aboveground parts. Although nondestructive measurements could not be performed, ^{3}H-labeled water was also employed to verify the phenomenon of horizontal water leakage from xylem cells. In another subsequent study, it was shown that the water flowing out from the xylem was pushing out the water already present in the stem and then returning to the xylem again to move upward. The water velocity in the internode was kept constant, and through simulation, it took less than 20 min to exchange the water already present in the stem with newly absorbed water. How does this happen? Is the water already present in the internode different from newly absorbed water? Since the half-life of ^{15}O is extremely short, the measuring system we developed needed special devices to determine the small amount of water actually moving in the stem.

Elements

Since the elements absorbed from roots are moving with water, studying element-specific movement within a plant is another theme of this book. For the first stage of the study of the elements, the distribution of the element within the plant tissue was presented employing neutron activation analysis (NAA). Since NAA allows nondestructive analysis of the elements in the sample, this is the only method to measure the absolute amount of elements in the sample. With the extremely high sensitivity to heavy elements and allowing multielement analysis, NAA provided the content of the many elements in each plant tissue.

The results showed that the element-specific profile varied throughout the whole plant, and this distribution tendency remained similar throughout developmental stage. There were many junctions of element-specific concentrations between the tissues, suggesting barriers to the movement of the elements. Generally, heavy elements tended to accumulate in roots, except for Mn and Cr. Even in a single leaf, there existed an element-specific concentration gradient. Of the elements measured,

Ca and Mg showed changes in concentration with the circadian rhythm. Since the amount of the element in a plant reflects the features of the soil where the plant grows, multielement analysis of the plant could specify the site of the agricultural products produced.

Before addressing the development of a real-time RI imaging system (RRIS), the production of RIs for essential elements for plant nutrition, ^{28}Mg and ^{42}K, is presented. The reason why concentrating on RIs is because when we examine the history of plant research, physiological research on the elements without available radioisotopes has not been well developed. For example, the boron (B) transporter was recently found, whereas there have been many transporter studies on phosphate (P), which has available radionuclides, ^{32}P and ^{33}P. Since there are no available radioisotopes for B for use in experiments, the study of B in plants is far behind compared to the other elements. Although it was not possible to produce radioactive nuclides for B, efforts were made to image other important elements for plant nutrition. Therefore, we developed a preparation method for elements whose available RIs were not previously employed in plant research. ^{28}Mg and ^{42}K are the radioisotopes we prepared; ^{28}Mg was produced using an accelerator, followed by chemical separation of the nuclide from the target. A root absorption study using ^{28}Mg as a tracer is presented as an example. It was found that the orientation of Mg transfer was different according to the site of the root where Mg was absorbed.

Mg and Ca are in the same group in the periodical table, have similar chemical properties, and are stained simultaneously by fluorescence probes; therefore, it was difficult to distinguish one from the other. Ca research is much further advanced than Mg research because of the high contribution of the available radioactive nuclide ^{45}Ca. Developing research on Ca has induced the development of fluorescent staining methods. However, since the overwhelming amount of Ca makes it difficult to distinguish Mg and Ca by imaging, the specific role of Mg has not yet been clarified.

Real-Time RI Imaging of Elements

Then, we proceeded to develop an imaging method utilizing the available RIs. We developed two types of real-time RI imaging systems (RRIS), one for macroscopic imaging and the other for microscopic imaging. The principle of visualization was the same, converting the radiation to light by a Cs(Tl)I scintillator deposited on a fiber optic plate (FOS). Many nuclides were employed, including ^{14}C, ^{18}F, ^{22}Na, ^{28}Mg, ^{32}P, ^{33}P, ^{35}S, ^{42}K, ^{45}Ca, ^{48}V, ^{54}Mn, ^{55}Fe, ^{59}Fe, ^{65}Zn, ^{86}Rb, ^{109}Cd, and ^{137}Cs. In the case of macro-RRIS, the steps to develop the imaging system were as follows. In the first generation, the plant sample and all the imaging devices had to be kept in the dark. In the second generation, a sealed plant box was prepared where only the aboveground part of the plant was able to undergo light irradiation to protect the highly sensitive CCD camera. Then, an on/off switch to irradiate the sample was

introduced into the imaging box, and a plastic scintillator was tested for imaging a large-scale plant.

Since radiation can penetrate the soil as well as water, the difference between soil culture and water culture was visualized. The plants grew much faster in water culture but with a low yield of grain. These culturing methods were applied to show the difference in ^{137}Cs absorption by the rice plant. ^{137}Cs was hardly absorbed by rice roots growing in soil, whereas water culture showed high absorption, which could provide some reassurance after the Fukushima Nuclear Accident and could indicate an important role of soil in firmly adsorbing the radioactive cesium.

^{28}Mg and ^{42}K, whose production methods were presented, were applied for RRIS to visualize the absorption image from the roots. In addition to ^{28}Mg and ^{42}K, many nuclides were applied to image absorption in the roots. Each element showed a specific absorption speed and accumulation pattern. There were three types of movement velocity: fast, medium, and slow. The image analysis of the absorption of Mg is presented as an example. Through successive images of the element absorption, phloem flow in the aboveground part of the plant was analyzed. The element absorption was visualized not only in the roots but also in the leaves, a basic study of foliar fertilization.

In the case of the microscopic imaging system, a fluorescence microscope was modified to acquire three images at the same time: a light image, fluorescent image, and radiation image. Although the resolution of the image was estimated to be approximately 50 μm, superposition showed the expression site of the transporter gene and the actual ^{32}P-phosphate absorption site to be the same in Arabidopsis roots.

Imaging of $^{14}CO_2$ Gas Fixation

We targeted not only the elements we can supply to the nutrient solution but also carbon dioxide gas to visualize the fixation process and the movement of assimilated carbon in a plant. This is another highlight of our study using RRIS. The interesting result was that the route of assimilated carbon was different depending on where the fixation took place. In Arabidopsis, most of the metabolites after photosynthesis were transferred to the tip of the main internode and roots when $^{14}CO_2$ gas was fixed and photosynthates were produced at rosette leaves, whereas most of the metabolites moved to the tip of the branch internode and hardly moved down to the roots when $^{14}CO_2$ gas was supplied to the aboveground parts of the plant other than rosette leaves. However, the route was also dependent on the developmental stage of the plant. Interestingly, it was possible to visualize and trace which tissue performed the fixation of $^{14}CO_2$ gas, i.e. carbon could be traced from the fixation site in tissue to tissue formation. However, especially in the case of ^{14}C imaging, image analysis should be carefully performed because of the self-absorption of the β-rays in tissue. To image $^{14}CO_2$ gas fixation in larger samples, approximately 50 cm in height, a

plastic scintillator was introduced, and the assimilation process of the gas was visualized for rice and maize.

3D Image Construction and MAR

Finally, spatial (3D) image construction from imaging plate (IP) images and the development of the microautography (MAR) method were presented. A rice grain was sliced every 5 μm, IP images were taken for successive slices, and the series of 2D images acquired by an IP were used to construct 3D images. In the case of ^{109}Cd and ^{137}Cs, the spatial distributions in the grain showed that the concentrations increased at the surface of the grain during the maturing process.

MAR was developed to increase the resolution of the image for the sliced plant sample. This revised MAR method showed the detailed distribution of ^{137}Cs accumulation in embryos, which indicated that the plumule and radicle, which grow as a meristem of a root or shoot, were protected from the accumulation of heavy elements. Because of daguerreotype imaging, MAR is now hardly used, and the film emulsion is not available. Here, this method was essentially recreated with a revised processing method for both sample preparation and imaging process.

Contents

About the Author

Tomoko M. Nakanishi The author is Prof. Emeritus and Project Prof., The University of Tokyo as well as President of Hoshi University in Tokyo. She majored in radiochemistry and received Ph.D. from The University of Tokyo, where she had set up her laboratory named radio-plant physiology. She was interested in role of water and elements in plants and found new aspects of plant physiology never expected before utilizing radiation and radioisotopes. After Fukushima nuclear accident, her group has vigorously studied the agricultural consequences of radioactive contamination. She received George Hevesy Medal Award, Ordre national du Mérite from French president and elected as a Foreign Member of the Royal Swedish Academy of Engineering Sciences and the Royal Society of Arts and Sciences in Gothenburg. She received an honorary Doctor of Chalmers University of Technology in Sweden.