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Procedures for chemical fixation in immunohistochemical analyses of PIN proteins regulating polar auxin transport: Relevance to spaceflight experiments

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ABSTRACT

The mechanism by which gravity controls the polar transport of auxin, a plant hormone regulating multiple physiological processes in higher plants, remains unclear, although an important role of PIN proteins as efflux carriers/facilitators in polar auxin transport is suggested. We are going to study the effect of microgravity on the polar transport of auxin, focusing on the cellular localization of its efflux carrier, PsPIN1 in etiolated pea seedlings and ZmPIN1a in etiolated maize seedlings grown under microgravity conditions on the International Space Station (ISS) using immunohistochemical analyses according to space experimental plans (Ueda, 2016). To obtain adequate results regarding the cellular localization of functional proteins, prolonged chemical fixation processes as well as chemical fixatives should be well-matched to the properties of functional proteins as antigens since experimental analyses will be performed on the ground after keeping samples for a long duration on the ISS. As a result of ground verification, clear detection of the cellular localization of PsPIN1 and ZmPIN1a immunohistochemically was successful based on the results of several kinds of chemical fixation tested, even when etiolated pea and maize seedlings were fixed by immersion in chemical fixative for a long duration. The addition of 0.1% (w/v) Nonidet P-40 to chemical fixative composed of 50% (v/v) ethanol and 5% (v/v) acetic acid or that of 50% (v/v) methanol and 5% (v/v) acetic acid has led to a significant improvement in the immunohistochemical detection of PsPIN1 or ZmPIN1a. These chemical fixatives were also shown to be storage-stable for a long time before use. In this study, adequate chemical fixatives and fixation protocols were developed, which can be used to detect localization of PsPIN1 and ZmPIN1a proteins in young etiolated pea and maize seedlings, respectively, using anti PsPIN1 and ZmPIN1a antibodies. These protocols can be used in spaceflight experiments to investigate the effects of the microgravity environment on the ISS on PIN protein localization in pea and maize seedlings.

1. Introduction

Space experiments on the International Space Station (ISS) have unique restrictions compared to ground experiments. In some cases, it takes long duration from the launch until the start of a space experiment on the ISS, and/or from completing the space experiment until the start of analyses on the ground with returned sample from the ISS (Kiss et al., 2007; Kiss et al., 2014; Kiss, 2015). The former duration reduces the quality of plant materials and chemical materials for the space experiments such as decrease in germination rate and denaturation of chemicals. The latter duration results in the deterioration of space-experimented plant materials, such as the decomposition of RNA, proteins, etc. To avoid these restrictions, some space experiments with GFP-reporter gene expression in roots and hypocotyls of transgenic line of *Arabidopsis* were performed by a confocal fluorescence microscope for observation and with a quantitative real time PCR thermal cycler for gene expression on the ISS (Ferl and Paul, 2016; Parra et al., 2017; Soga et al., 2018). However, for biological samples that cannot produce gene-modified organisms and are not suitable for confocal microscopic observation due to large size or transparency of

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tissues, detail analyses on the ground after returning of biological samples from the ISS will be required.

Plant hormone auxins, among which indole-3-acetic acid (IAA) is the most predominant, play a crucial role in regulating multiple physiological processes in plant growth and development such as cell elongation, tropic movement, vascular patterning, apical dominance and root initiation (Berleth and Sachs, 2001; Ueda et al., 2014). Auxin mainly biosynthesized in the shoot apex is transported between cells in a rootward direction via a combination of membrane diffusion and carrier-mediated transport system in the plant axis, generating auxin maxima and gradient within tissues that are instrumental in the diverse regulation of various plant developmental processes (Muday and Murphy, 2002; Robert and Friml, 2009; Baskin et al., 2010). This unique directional transport is called polar auxin transport, and considered to be regulated by several functional proteins, influx and efflux carrier proteins, located in plasma membrane (Friml and Palme, 2002; Muday and Murphy, 2002; Benjamins et al., 2005; Bandyopadhyay et al., 2007; Miyamoto et al., 2011). Studies relating to one of the floral mutants of *Arabidopsis thaliana, pin-formed (pin*) mutant showing diminished-polar auxin transport in its inflorescence axis (Okada et al., 1991), resulted in the identification of the *AtPIN1* gene encoding a 67-kDa protein similar to bacterial and eukaryotic efflux carrier proteins. It was demonstrated that AtPIN1 protein is localized on the rootward end of plasma membrane facilitating rootward transport of auxin in vascular tissue (Gälweiler et al., 1998; Friml and Palme, 2002). Thus, AtPIN1 is considered an essential component that participates directly in auxin transport or assists in the assembly of other proteins with efflux activity (Friml and Palme, 2002).

Polar rootward auxin transport is demonstrated experimentally using the through the agar-block technique as follows: one cut surface of a subapical stem segment is placed in contact with a donor block containing IAA, and the other cut surface is placed in contact with a receiver block containing no IAA, revealing that IAA moves independently of the segment's orientation relative to gravity when IAA is applied to the shootward cut surface. However, when IAA is applied to the rootward cut surface, no IAA can be found in the receiver block (Mohr and Schopfer, 1995). This result led us to study whether polar auxin transport is controlled under gravity on earth. For this study, experiments under microgravity conditions in space are extremely valuable. Therefore, we conducted a space experiment on the Space Shuttle in 1998 (STS-95) which clearly showed that a close gravity-controlled relationship exists between polar auxin transport and morphogenesis in etiolated pea seedlings (Ueda et al., 1999; Ueda et al., 2014). Results obtained from microgravity-simulated experiments using a 3-D clinostat were almost the same (Miyamoto et al., 2005; Hoshino et al., 2006, Hoshino et al., 2007; Ueda et al., 2014). To clarify the mechanism by which gravity controls polar auxin transport at molecular biological levels using dicotyledonous pea and monocotyledonous maize seedlings, we plan to conduct further space experiments on the ISS, focusing on the localization of efflux carrier proteins and their gene expression (Ueda, 2016). In this space experimental plan, the etiolated pea and maize seedlings grown under microgravity conditions on the ISS will be treated by chemical fixatives and recovered on the ground while maintaining the chemically fixed conditions. And then localization of PIN proteins responsible for polar auxin transport will be analyzed by immunohistochemical methods. In our space experiment, the chambers setting pea and maize seeds were brought to the ISS on February 2017 by SpaceX-10 spaceship. On the ISS, after supplying distilled water, the chambers were placed in the Cell Biology Experiment Facility (CBEF), which is an incubator unit consisting of a microgravity compartment and a centrifuge compartment (Yano et al., 2012). At the end of incubation, etiolated pea and maize seedlings were fixed in the Chemical Fixation Bag (CFB) consisting of a triple-sealed bag (Space Life Sciences Flight Experiments Information Package, 2014). The space experiments were conducted on March 2017. The samples were returned to the ground on March 2017 by SpaceX-10. Detail procedures of space experiment will be reported elsewhere.

Some chemical fixatives such as RNAlater and formaldehyde have been shown to be capable of preventing the degradation of mRNAs and proteins, and maintaining morphological shape expressed and/or appearing in the space environment (microgravity conditions) after appropriate returning to a ground laboratory (Kamada et al., 2000; Takahashi et al., 2000; Paul and Ferl, 2011; Yamazaki et al., 2016; Morohashi et al., 2017). For immunohistochemical analyses, however, the chemical fixatives are generally freshly prepared before use, and the duration of fixation is a few hours to days. In the case of a space experiment, chemical fixatives are prepared and sealed in special chemical fixation equipment such as Kennedy Space Center Fixation Tubes (KFT) and CFB before the launch (Paul et al., 2005; Space Life Sciences Flight Experiments Information Package, 2014). Also, in space experiments on the ISS, there are restrictions on available chemicals and their concentrations due to the safety rules and compatibility with various pieces of equipment. Furthermore, in our plant experiment, plant samples will be immersed in chemical fixatives until immunohistochemical analyses on the ground. However, little is known whether immunohistochemical analyses can be performed on samples after prolonged immersion in stored chemical fixatives. To obtain adequate results regarding the cellular localization of functional proteins using immunohistochemical analyses, chemical fixation processes as well as chemical fixatives should be well-matched to the properties of functional proteins as antigens.

In order to carry out this study, it is essential to produce or to obtain antibodies of auxin efflux carrier of pea and maize plants. We have already reported the production of an antibody of auxin efflux carrier of PsPIN1 (Kamada et al., 2018). However, until now we did not have the antibody of ZmPIN1a, a representative PIN protein in maize, thus tried to produce it based on the data of Carraro et al. (2006). We also propose chemical fixatives suitable for immunohistochemistry of PsPIN1 of etiolated pea seedlings and ZmPIN1a of etiolated maize seedlings grown in space from the aspects of interference with antigen-antibody reaction and maintenance of the cell shapes even in a long-term storage assumed before chemical fixation and long-term immersion in chemical fixatives. These preliminary investigations on the ground will provide important techniques to achieve beneficial space experiments.

2. Materials and methods

2.1. Plant materials and growth conditions

Plant materials and growth conditions of etiolated pea (*Pisum sativum* L. cv. Alaska) seedlings were carried out according to the method reported by Kamada et al. (2018).

Seeds of maize (*Zea Mays*. cv. Golden Cross Bantam) were kindly provided by Koukaen (Yoichi, Hokkaido, Japan). As the seed bed, rockwool blocks (width 82 mm × depth 54 mm × height 32 mm) cut from a large sheet of rockwool (Culture Mat, Nippon Rockwool Co., Ltd., Tokyo, Japan) were individually placed in acrylic resin boxes (W 82 mm × D 54 mm × H 144 mm) of a precise fitting size. For ventilation, each box had twelve holes (10 mm in diameter) on the sides, which were covered with hydrophobic fluoropore membrane (MilliSeal; Millipore, Merck, Tokyo, Japan). Twenty seeds were set so as to be completely buried beneath the block surface, with the seed embryo set perpendicular to the block. After supplying 120 mL of distilled water, each box was kept for 4 days at 25 °C in the dark. The plant materials were immediately fixed with fixative solutions for immunohistochemical analyses, or frozen until use for western blotting analyses.