Dynamic Regulation of Endocytic Vesicle Recycling and PIN2 Localization in Arabidopsis Roots under Varying Light Qualities

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In many root tropic behaviors, auxin is the essential phytohormone to regulate a cell growth directing root development. It was reported that light promotes the translocation of auxin carrier proteins such as PINs (PIN-FORMED) providing a polarity for roots to complete negative phototropism. These PIN proteins are known to be translocated via endocytic vesicle recycling in root cells. However, an direct influence of light conditions on endocytic vesicle recycling mechanism controlling tropic behaviors in Arabidopsis root cells are not well assessed. In this study, we compared the activity of endocytic vesicle recycling and PIN2 localization in root cells at root transition zone grown under (1) light regime (16 h light / 8 h dark) for 5 d, (2) light regime for initial 4 d followed by 24-h of dark, and (3) continuous dark for 5 d. In the result, dark-grown seedlings showed lower rate of endocytotic activities in root transition zones, compared to the light-grown roots. Interestingly, light-promoted endocytic recycling activity was attenuated to the level equivalent to dark-grown roots after 24-h of dark treatment. PIN2-GFP was shown to accumulate in vacuoles both in dark-grown and 24-h dark treatment seedlings. Moreover, the PIN2-GFP signal found in 24-h dark-treated roots was stronger than in the dark-grown sample. Here we propose a model for dynamic regulation of PIN2 localization regulated by endocytic vesicle recycling in the transition of light circumstances, which might be important for roots to prepare for upcoming unfavorable light.

Keywords : Arabidopsis roots, Brefeldin A, endocytic vesicle recycling, light/dark adaptation, PIN2, skotomorphogenesis

INTRODUCTION

The sunshine is the most important energy resource to every land-dwelling creature. Plants receive the benefit of light to complete photosynthetic reaction. Since light is directly linked to energy availability, plants have evolved to regulate their growth and development depending on external light conditions. A mechanism that plant can recognize and change a direction of growth toward light is called phototropism. It is an essential movement for plants to effectively collect the energy in the form of photons. Unlike aerial part of plants such as leaves, root system in many plant species prefer to grow belowground, because roots have roles for anchoring their body and absorbing the necessary water and nutrients from soil. The behavior of roots towards light is completely opposite to that of aboveground half of plants.

As Darwins described in last century, root gravitropism, the ability that roots can sense gravity and grow vertically, is well recognized as one of root tropisms (Darwin, 1880). This tropism is necessary for roots to explore in patchy soil environment. In addition to gravity, recent studies in plant physiology have revealed that Arabidopsis thaliana expresses all photoreceptors not only in shoot but also in root portion, and therefore roots can sense the external light stimuli. Upon roots are illuminated (this is the sign for plant that its roots are above the soil), tips of roots start growing back into darkness as fast as possible (thus, seeking for the soil). This phenomenon is called negative phototropism (escaping phototropism), which is now regarded as one of important root tropisms.

In Arabidopsis thaliana, root growth is accelerated under continuous illuminated condition (with a light intensity in normal growth chamber ca. 120 μmol m⁻² s⁻¹ of white light) compared to dark grown roots. The light-enhance root growth might be representing the light-escape tropism. We have previously demonstrated that short-time blue light illumination to roots promoted the generation of reactive oxygen species (ROS) in root apex region (Yokawa et al., 2011; 2013; 2014). ROS is radical-oxygen molecules acting as important signaling molecules, which drive propagation of secondary cellular signaling molecules. It suggests that ROS generated upon illumination triggers the negative phototropism in the roots (fast root growth).

In the tropic behavior of roots, the major phytohormone, auxin (IAA; indole-3-aceticacid) plays a crucial role by regulating the growth of root cells. As one of auxin functions, it alters the rate of root growth depending on its concentration. During tropism, auxin molecule is transported asymmetrically from one side to the other side of root apex in order to develop a steep gradient in auxin concentration eventually changing the speed of growth. It
thus enables the root apex to bend.

In order to achieve cell-to-cell transport of auxin, several auxin carrier proteins must be aligned on one side of cells in response to a direction of external input of information (such as light stimulus). In root negative phototropism, it was reported that the re-location of auxin efflux carrier (PIN-FORMED) 1, 2 and 3 proteins were involved in asymmetric auxin distribution and negative phototropism in roots mediated by a Brefeldin-A (BFA) sensitive-trafficking pathway (Geldner et al., 2003; Wan et al., 2012; Zhang et al., 2013; 2014). PIN2 proteins (PIN-FORMED 2; auxin efflux carrier) in root cells change their distribution upon response to environmental light (Laxmi et al., 2008). In addition, the re-location of PIN2 in root cells upon illumination was reported to be completed within 30 min (Wan et al., 2012). Wan et al. (2012) further demonstrated that blue light promotes the basipetal (shootward) polar auxin transport facilitated by PIN2, which regulates the negative phototropism in roots. Arabidopsis roots grown in partially darkened-Petri dish system (only root part is protected from light) showed weak PIN2 accumulation in cross walls in root meristematic cells. Taken together, based on these mechanisms roots can respond to incoming light and escape from light. In addition to PIN-related reaction, Dyachok et al. (2011) reported that light-activated COP1, E3 ubiquitin ligase, enhances actin polymerization and F-actin bundling in root cells, resulting in fast root growth under light growth conditions. It was also reported that light regulates F-actin bundling in maize coleoptiles (Waller and Nick, 1997), suggesting that both aerial and under-ground portions of plants share the same mechanism for light-driven reorganization of cellular skeletons, possible in the course of cellular axis formation. Through self-referencing regulatory circuits between polar auxin transport and auxin induced actin reorganization, self-amplification of auxin transport which is central element to auxin-dependent patterning is achieved (Nick et al., 2009). The interplay between F-actin and polar auxin transport is mediated by endocytic vesicle recycling in the transition zone of root apex, and it controls the root tropisms (Baluška et al., 1996; 2004; 2005; 2010; Baluška and Mancuso, 2013).

It is well studied that a re-localization of auxin carrier proteins such as PINs, requires the endocytic vesicle recycling mechanisms (including endocytosis and exocytosis), these are very fundamental and important cellular machineries for transporting mainly membrane-associated proteins or compounds. Therefore, it is thought that endocytic vesicle recycling is essential to almost all tropic behaviors of plants. However, an impact of light on this endocytic vesicle recycling has not been documented in details, although effect of light (especially blue light) on PIN re-localization or actin reformation was reported (Zhang et al., 2013; 2014). In this study, we report the dynamic control of endocytic vesicle recycling modulated in response to different light conditions for root growth.

MATERIALS AND METHODS

Plant growth condition

At first, seeds of Arabidopsis thaliana, ecotype Col-0 and PIN2::PIN2-GFP were sterilized by 2% of sodium hypochlorite (ROTH, Karlsruhe, Germany) in the presence of 0.1% of Triton-X (ROTH, Karlsruhe, Germany) for 5 min. Secondly, these seeds were rinsed out by water for 4 times. These seeds were then planted on solidified 0.4% (w/v) phytagel plates (Sigma, Steinheim, Germany) containing half-strength of Murashige-Skoog nutrient mixture (Duchefa, Haarlem, The Netherlands) and 1% (w/v) sucrose (pH 5.8 adjusted with KOH). These Petri dishes were incubated at 4°C in dark for 1 d for imbibition and placed vertically at 23-25°C in the light (under light regime of 16 h light/8 h dark with white light from fluorescent lamp, 120 μmol m⁻² s⁻¹) or in the dark. For dark adaptation experiment, 4 day-old seedlings of light grown (16 h light/8 h dark) seedlings were transferred in the dark for 24 h.

Confocal microscopy

Seedlings of Col-0 (wild type) were stained with 4 μM FM4-64, membrane-staining fluorescence probe (Sigma, Steinheim, Germany) for 10 min. FM4-64 was prepared from a stock solution at 2000 times higher concentration dissolved in dimethyl sulfoxide (DMSO, Sigma, Steinheim, Germany). The seedlings were then incubated in 1/2 MS medium containing 35 μM BFA (Sigma, Steinheim, Germany). BFA was made from stock solution at 1000 times higher concentration dissolved in DMSO. All the images of FM-stained BFA-compartment of PIN2::GFP were taken though a confocal laser microscopy (Fluoview FV1000, Olympus, Tokyo, Japan). Both FM 4-64 and GFP were excited by 488 nm blue light emitted by Argon laser. Fluorescence emissions by FM 4-64 were taken between 630 and 700 nm. Fluorescence emissions by GFP were collected between 500 and 600 nm. Total areas of BFA-compartments were calculated by ImageJ software (ver. 1.43u for Mac OSX, http://imagej.nih.gov/ij/).

RESULTS AND DISCUSSION

Light promotes endocytic vesicle recycling in roots

In this study, we monitored the endocytic vesicle recycling process in root cells under different lighting conditions to elucidate the events reflecting the negative phototropic response in roots. A rate of endocytic vesicle recycling can be estimated with Brefeldin-A (BFA) treatment, which blocks a formation of vesicles from endoplasmic reticulum, thus reversibly preventing the transport of secretory proteins to the Golgi apparatus, resulting in the formation of round-shaped aggregated structure called BFA-compartment in cytosolic space. As Fig. 1(A) shows, BFA-compartments visualized with fluorescence probe, FM4-64 in red color, present in epidermal cells in roots. Size and number of BFA-compartments indicate a speed of endocytic vesicle recycling reflecting root tropic response. If the size of observed compartments were very small, it might indicate that roots are active in tropic...
movements. In Fig. 1(A)-a, big compartments were detected in the roots grown under light condition for 5 d, whereas roots grown under continuous dark condition showed smaller and less number of compartments (Fig. 1(A)-c). Although it was known that blue light-induced root negative phototropic curvature is BFA sensitive (Wan et al., 2012; Zhang et al., 2013), the result observed here suggests that roots always maintain high endocytic vesicle recycling activity for negative phototropism under continuous light-exposed condition.

Dark treatment attenuates the endocytic vesicle recycling

As previously described, response of roots to incoming light are extremely quick (Yokawa et al., 2011). Light-exposed roots promote their elongation by seeking for light; however, which is known as negative phototropism (Yokawa et al., 2011). Silva-Navas et al. (2015) have recently proposed an improved Arabidopsis root growth system ‘D-Root’ that allows growth of only the root portion kept in darkness. Interestingly, they reported that root illumination shortens root length and promotes early emergence of lateral roots. Xu et al. (2013) also reported another version of improved-Petri dish system for the proper root growth and showed different PIN2 localization between light and dark grown roots. It is important in these new experimental systems allowing restricted exposure of plants only in the areal parts. However, there could be concerns that light sources set in these systems should not be close enough causing localized increase in temperature and unexpected reflection of light should be avoided.

Zhang et al. (2013) demonstrated that 30 min of unilateral blue light illumination changes the distribution of PIN3 (PIN-FORMED 3; auxin efflux carrier) on illuminated side versus shaded side in columella cells in Arabidopsis (Zhang et al., 2013). How can roots set a cellular situation back to normal growth condition, namely, under darkness? As shown in Fig. 1 (A-c) Laxmi et al. (2008) showed that accumulated fluorescence signal from enhanced GFP-fused PIN2 (PIN2-GFP) on plasma membrane in light grown roots was reduced to 62% of the initial level by 12 h after dark treatment.

In the present study, the fate of BFA-compartments in root cells during the light-to-dark transition phase (dark adaptation) were also assessed. As shown in Fig. 1(A)-b, small BFA-compartments were observed in roots with light-to-dark history, in which plant root were grown under light condition for 4 d, followed by 24 h of darkness. Interestingly, within 24 h in darkness, endocytic vesicle recycling completely went back to a steady state as shown by dark-grown roots (Fig. 1(A)-c).

Light-grown PIN2 localization in dark treatment

It was shown that PIN2 is re-localized from plasma membrane to vacuole when roots were transferred from light to dark condition (Laxmi et al., 2008). In this study, the re-location of PIN2-GFP signal from plasma membrane to vacuoles in the dark-grown and of 4 d light/24 h dark-treated seedlings were also observed as showed in Fig. 1(C)-a, b and c. Intriguingly, the PIN2-GFP signal found in vacuoles and vacuole-like small compartments of 24-h dark-treated roots was stronger than in dark-grown ones. Loss of fluorescence signal reflecting the presence of PIN2 in cytosolic region in 5 d dark-grown roots (Fig. 1(C)-c) might be attributed to the induced degradation of PIN2 proteins in the lytic vacuoles (Kleine-Vehn et al., 2008). However, what is interesting is that PIN2 proteins were still maintained in the vacuolar-like compartments for at least 24 h after the shift to dark condition (Fig. 1(C)-b). As summarize in Fig. 2, these findings might suggest the ability of roots that enables to prepare for upcoming unfavorable light situation, in order to respond as quickly as possible with a minimum cost, by stocking (buffering) certain amount of PIN2 proteins on such multi-vesiculate body (MVB) or pre-vacuolar compartments (PVC) for short period (Wan et al., 2012). In fact, this characteristic can be regarded as a “buffering memory” of plant root cells. Once light comes again, endocytic vesicle recycling in root cells...
is immediately activated (Fig. 1(A)-a), by which PIN2 can be pushed back to the cross walls (as shown in Fig. 1(C)-a) again to drive a negative phototropism.

In contrast, after experiencing several days under the dark condition, PIN2 will then be transported to vacuoles and degraded by lytic action (Fig. 1(C)-c). In a normal dark condition, PIN2 proteins will then be transported to vacuoles again to drive a negative phototropism.

In laboratories, *Arabidopsis* plants are normally propagated under 16 h light/8 h dark photoperiod, conferring a circadian rhythm to the plants. As we observed in this study, plant roots regulate a rate of endocytic vesicle recycling or indirect association of such a circadian rhythm with transduction in response to external light stimuli. A direct or indirect association of such a circadian rhythm with transduction in response to external light stimuli. A direct or indirect association of such a circadian rhythm with transduction in response to external light stimuli.

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