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RESEARCH PAPER

Phosphatidylinositol 3-kinase activity and asymmetrical accumulation of F-actin are necessary for establishment of cell polarity in the early development of monospores from the marine red alga *Porphyra yezoensis*

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Abstract

The polarized distribution of F-actin is important in providing the driving force for directional migration in mammalian leukocytes and *Dictyostelium* cells, in which compartmentation of phosphatidylinositol 3-kinase (PI3K) and phosphatidylinositol phosphatase is critical for the establishment of cell polarity. Since monospores from the red alga *Porphyra yezoensis* are a real example of migrating plant cells, the involvement of the cytoskeleton and PI3K was investigated during their early development. Our results indicate that the asymmetrical localization of F-actin at the leading edge is fixed by the establishment of the anterior–posterior axis in migrating monospores, which is PI3K-dependent and protein synthesis-independent. After migration, monospores adhere to the substratum and then become upright, developing into multicellular thalli via the establishment of the apical–basal axis. In this process, F-actin usually accumulates at the bottom of the basal cell and development after migration requires new protein synthesis. These findings suggest that the establishment of anterior–posterior and apical–basal axes are differentially regulated during the early development of monospores. Our results also indicate that PI3K-dependent F-actin asymmetry is evolutionally conserved in relation to the establishment of cell polarity in migrating eukaryotic cells.

Key words: Cell polarity, cytoskeleton, F-actin, monospore, phosphatidylinositol 3-kinase, *Porphyra yezoensis*.

Introduction

The establishment of cellular and subcellular asymmetries, which are directed by an oriented axis referred to as cell polarity, is critical for growth and development (Cove, 2000). When *Dictyostelium* cells and leukocytes respond to external impulses such as cAMP and cytokinins, they rapidly form a leading edge on the side exposed to the highest concentration of chemoattractant, with a trailing edge appearing on the opposite side (Firtel and Chung, 2000; Van Haastert and Devreotes, 2004). Formation of the leading edge occurs in parallel with the polarized localization of F-actin, whereas assembled myosin II is enriched at the trailing edge. Thus, the polarized distribution of cytoskeletal components provides the driving and contractile forces required for directional cell migration during chemotaxis (Affolter and Weijer, 2005). The involvement of the cytoskeleton in the establishment of cell polarity has also been reported in land plants (Staiger, 2000; Hepler *et al*., 2001; Smith, 2003; Finka *et al*., 2007). F-actin and microtubules (MTs) have also been shown to play important roles in the establishment of polarity during tip growth of pollen tubes and root hairs (Fu *et al*., 2001; Sieberer *et al*., 2005). These findings show that the polarized accumulation of

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Abbreviations: AUX1, AUXIN RESISTANT 1; BDM, 2, 3-butanedione monoxime; Cyt B, Cytochalasin B; ESL, enriched sea life; FYVE, Fab1, YOTB, Vac1, and EEA1; Lat B, Latrunculin B; MT, microtubule; Noc, Nocodazole; PI, phosphatidylinositol; PI3K, phosphatidylinositol 3-kinase; PI(3)P, phosphatidylinositol-3-phosphate; $PI(3,4)P_2$, phosphatidylinositol-3,4-bisphosphate; $PI(3,5)P_2$, phosphatidylinositol-3,5-bisphosphate; $PI(4,5)P_2$, phosphatidylinositol-4,5-bisphosphate; $PI(3,4,5)P_3$, phosphatidylinositol-3,4,5-trisphosphate; PIN2, PIN-FORMED 2; PLC, phospholipase C; PTEN, phosphatase and Tensin homologue; SHIP1, Src homology 2 domain-containing inositol-5-phosphatase 1.

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cytoskeletal elements, especially F-actin, is important for the establishment of cell polarity in both animals and plants.

The molecular mechanisms regulating the asymmetrical distribution of F-actin have been studied extensively in *Dictyostelium* cells and leukocytes. One of the first asymmetrical responses to chemoattractants is the localized accumulation of phosphatidylinositol (PI)-3,4,5-trisphosphate [PI(3,4,5)P_3], the product of phosphatidylinositol 3-kinase (PI3K) (Merlot and Firtel, 2003; Dormann *et al*., 2004). In a new leading edge of *Dictyostelium* cells, preferential activation of PI3K at the side facing the chemoattractant gradient is necessary for polarized F-actin localization and directional movement (Parent and Devreotes, 1999; Firtel and Chung, 2000; Bourne and Weiner, 2002). By contrast, the phosphatase and Tensin homologue (PTEN), which dephosphorylates PI(3,4,5)P_3, is localated on the trailing edge where it acts as a negative regulator of PI3K signalling in *Dictyostelium* cells (Iijima *et al*., 2002). In addition, Nishio *et al*. (2007) argued that the Src homology 2 domain-containing inositol-5-phosphatase 1 (SHIP1), not PTEN, is the key regulator of neutrophil migration. The localized distributions of PI3K and PI(3,4,5)P_3 phosphatase therefore help cells define their polarity by organizing the polarized localization of F-actin (Charest and Firtel, 2006).

In plants, polar auxin transport contributes to the formation of cell and tissue polarity. For example, the auxin influx carrier AUXIN RESISTANT 1 (AUX1) and the auxin efflux carrier PIN-FORMED 2 (PIN2) play important roles in auxin transport from the root tip to differentiated tissues during the gravitropic response (Rashotte *et al*., 2001). The functions of AUX1 and PIN2 require clathrin-mediated vesicle trafficking and are regulated by the activities of the small GTPase and phospholipase Dζ2 (Fischer *et al*., 2006; Kleine-Vehn *et al*., 2006; Li and Xue, 2007). The involvement of the small GTPase and PI signalling has also been demonstrated during polarized tip growth of pollen tubes and root hairs (Kost, 2008). Rho GTPases belonging to the plant-specific Rac-Rop subfamily are also thought to play a role in tip growth by controlling F-actin organization and vesicle trafficking at the plasma membranes of the apex region (Kost *et al*., 1999; Li *et al*., 1999; Molendijk *et al*., 2001). Similarly, PI-4,5-bisphosphate, PI(4,5)P_2, is restricted to the apex of the elongation region in pollen tubes and root hairs (Kost *et al*., 1999; Helling *et al*., 2006) by co-localization with PI phosphate 5-kinase, which synthesizes PI(4,5)P_2 from PI(4)P (Kusano *et al*., 2008; Stenzel *et al*., 2008). Moreover, the tip localization of PI(4,5)P_2 is maintained by the absence of phospholipase C (PLC) at the apex and its presence at the flanking region of the tip, which results in catalysis of PI(4,5)P_2 into diacylglycerol and inositol-1,4,5-triphosphate (Dowd *et al*., 2006; Helling *et al*., 2006). Since PI(4,5)P_2 activates Rho GTPases (Fauré *et al*., 1999; Kost *et al*., 1999), the functions of the small GTPases and PI signalling are interrelated. The above findings therefore suggest that there are similarities in the functions of PIs and their negative regulators in animals and plants, that is, PI(3,4,5)P_3 and PI phosphatases in animals seem functionally analogous to PI(4,5)P_2 and PLC in plants.

Although PI(3)P, PI(3,4)P_2, and PI(3,5)P_2, but not PI(3,4,5)P_3, can be found in plant cells (Mueller-Roeber and Pical, 2002), little is known about the function of D3-phosphorylated PIs in the determination of plant cell polarity. For example, the functional significance of monophosphorylated PI(3)P remains confused. Helling *et al*. (2006) demonstrated that overexpression of the FYVE (Fab1, YOTB, Vac1, and EEA1) domain, which specifically binds to PI(3)P (Gillooly *et al*., 2000), had no effect on the polarized localization of PI(4,5)P_2 in pollen tubes, indicating that PI(3)P is not involved in the regulation of polarized tip growth in plants. By contrast, the functional involvement of PI(3)P during tip growth in pollen tubes was presented by Lee *et al*. (2008) using the same technique. At present, the functional significance of D3-phosphorylated PIs in polarity determination in plants cannot be ruled out.

To address this possibility, we focused on the migration and becoming upright (standing for further development) of monospores from the marine red alga *Porphyra yezoensis*. Monospores, which are produced in monosporangia at the marginal region of the thallus (Miura, 1985), are an example of moving plant cells (Guiry, 1990; Pickett-Heaps *et al*., 2001; Ackland *et al*., 2007). The motility of spores released from red algae is thought to be important for adhesion and further development after becoming upright, since unsettled spores either form a callus or die (Nakazawa, 1958; Imada *et al*., 1971; Polne-Fuller *et al*., 1984). Recently, it was reported that F-actin and myosin are important in the migration of monospores from the red alga *P. pulchella* (Ackland *et al*., 2007); however, the roles of cytoskeletal elements in the determination of cell polarity have not yet been examined. In the present study, the close relationship between asymmetrical accumulation of F-actin and the determination of cell polarity in the early development of monospores from *P. yezoensis* is demonstrated. Moreover, evidence is also presented of the involvement of PI3K in the establishment of the asymmetrical localization of F-actin in monospores. These results suggest that PI3K-dependent F-actin asymmetry in migrating cells is evolutionally conserved among red algae, slime moulds, and mammals.

Materials and methods

Discharge of monospores

The cultivation of gametophytic blades of *P. yezoensis* strain TU-1 was performed as described by Fukuda *et al*. (2008). The medium (enriched sea life; ESL) was renewed weekly until gametophytes

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were 1–2 cm long, and monosporangia had formed. To induce the discharge of a large number of monospores, thalli with monosporangia were given a mild osmotic shock in deionized water for 10–15 s as described by Ackland *et al*. (2007), then transferred into a 90×15 mm Petri dish containing ESL medium and incubated for 0.5–1.0 h at 15 °C under 60 μmol m^{-2} s^{-1} light. After removal of the thalli, monospores released into the medium were transferred and cultured on 20×20 mm cover glasses in 6-well plates (Iwaki Sci Tech Div., Asahi Techno Glass, Japan) with ESL medium, for the observation of both motility and the staining of F-actin.

Treatment of monospores with cytoskeleton inhibitors

Working solutions of 0.2 mM for Cytochalasin B (Cyt B; MP Biomedicals, France), 25 μM for Latrunculin B (Lat B; Wako Pure Chemical Industries, Japan), and 3 μM for Nocodazole (Noc; MP Biomedicals) were prepared in ESL medium by the dilution of stock solutions resolved in dimethyl sulphoxide (DMSO; Wako Pure Chemical Industries) and stored at –30 °C. DMSO did not exceed 1% in each dilution. A working concentration of 10 mM for 2, 3-butanedione monoxime (BDM; Sigma, USA) was freshly prepared by resolving in ESL medium. It is well known that Cyt B and Lat B cleave actin filaments into pieces and impair the polymerization of G-actin, respectively (Hartwig and Stossel, 1979; Spector *et al*., 1989), while Noc and BDM had an inhibitory effect on the MTs and myosin (Kropf *et al*., 1990; Poulsen *et al*., 1999). For the evaluation of the effects of these inhibitors on the early development of monospores, 6- or 24-well plates (Iwaki Sci Tech Div., Asahi Techno Glass) were used for the treatment of monospores with chemicals at the working concentrations indicated above. The percentage of migrating monospores or germlings per well was determined after 3 h and 48 h incubation in 6- and 24-well plates, respectively, with *c*. 50 monospores per examination and three repetitions per experiment. To identify the renewed cell wall, 0.01% Fluorescent Brightener 28 (Sigma) was used to stain monospores directly after 48 h incubation on a cover glass filled with ESL medium with or without inhibitors in 6-well plates.

Treatment of monospores with PI3K and PTEN inhibitors

Accumulation of F-actin at the leading edge has also been observed during PI(3,4,5)P_3-dependent migration of *Dictyostelium* cells and leukocytes (Firtel and Chung, 2000; Bourne and Weiner, 2002). Thus, the effects of PI3K and PTEN inhibitors on monospore development were examined. The PI3K inhibitor LY294002 (Promega, USA) and its analogue LY303511 (Calbiochem, USA) were used to treat monospores at 1, 2.5, 5, 10, or 15 μM prepared from 50 mM stock solutions in DMSO stored at –30 °C. After incubation for 3 h and 24 h in ESL medium with LY294002 or LY303511, the percentage of migrating monospores and germlings was determined as described above.

It has been established that PTEN and SHIP1 are negative regulators of PI(3,4,5)P_3 production by PI3K in *Dictyostelium* cells and leukocytes (Iijima *et al*., 2002; Nishio *et al*., 2007). Although the possible existence of SHIP1-like activity in monospores could not be examined, PTEN-like activity was tested using bpv(pic), a PTEN-specific inhibitor (Schmid *et al*., 2004). The bpv(pic) (Calbiochem) was used at concentrations of 0.01, 0.1, 1, 10, or 100 μM after resolving in ESL medium. The percentage of migrating monospores and germlings was also determined after 3 h and 24 h incubation as described above. Assays were repeated three times using *c*. 50 monospores per examination.

Treatment of monospores with protein synthesis inhibitor

Freshly released and developing monospores were treated with cycloheximide (Calbiochem) at a concentration of 18 μM, which was made from 44 mM stock solution stored at –30 °C in DMSO. The assay was repeated three times using *c*. 50 monospores per experiment.

Visualization of F-actin in freshly released monospores

F-actin in freshly released monospores was visualized with the fluorescent probe Alex Flour 488 phalloidin (Molecular Probes, USA) at a concentration of 5 U ml^{-1}. The probe was made by diluting methanol-resolving stock solution (200 U ml^{-1}) with actin buffer (100 mM PIPES, 10 mM EGTA, 5 mM $MgSO_4$, and 0.3 M mannitol, pH 6.9) containing 2% (v/v) glycerol to improve visualization (Olyslaegers and Verbelen, 1998). After incubation in 1.5 ml plastic tubes containing ESL medium with or without cytoskeleton inhibitors, monospores were collected by centrifugation at 80 *g* for 1 min and then resuspended in the phalloidin solution. After incubation for 10 min at room temperature in the dark, monospores were gently rinsed with phosphate buffered saline (PBS) by centrifugation at 80 *g* to remove the phalloidin, and mounted on a slide with 4% *n*-propyl gallate resolved in 90% glycerol and 10% PBS (Lovy-Wheeler *et al*., 2005). Actin filaments were observed and photographed using a Leica DM 5000 B fluorescence microscope equipped with a Leica DFC 300 FX camera. All images were obtained using a ×100 oil immersion objective. Excitation (480/40 nm) and emission wavelengths (527/30 nm) were made using filters (Medical Agent Co., Japan) for the Alex Flour 488 phalloidin. Photomicrographs were taken using a Leica DFC 300 FX camera system and images were collected and processed into plates using the Adobe Photoshop 7.0 software package.

Staining F-actin in monospores

Monospores were incubated on cover glasses in 6-welled plates containing ESL medium at 15 °C under 60 mmol m^{-2} s^{-1} light. When monospores were migrating or becoming upright, the cover glasses were transferred into PBS containing 3.7% paraformaldehyde and incubated for 1–5 min at room temperature. The length of fixation was determined by the stage of development. Next, the cover glasses were rinsed three times with PBS, incubated in PBS containing 0.1% Triton X-100 for 1–5 min, and then with 5 U ml^{-1} Alex Flour 488 phalloidin dilution for 20–30 min. Actin filaments were observed and photographed as described above.

Results

Migration of monospores

It was first found that monospores of *P. yezoensis* are motile at two different stages during their early development. After the formation of monosporangia (Fig. 1A, B), somatic cells located at the edge of the thallus changed shape and started to be released (Fig. 1C, D). The shape of the released cells, that is, monospores, immediately became rounded (Fig. 1E). This was followed by retractile and amoeboid movement during the second morphological change at which point they had a tapered tail (Fig. 1F, G). After adhering to the substratum, they became upright and the bottom of these cells became elongated (Fig. 1H, I). The first asymmetrical cell division occurred perpendicular to the apical–basal axis (Fig. 1J). In the following, the focus is mainly on the second period of movement after release.

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Organization of F-actin in the early development of monospores

To clarify the relationship between F-actin and development, the organization of F-actin was observed in monospores during migration and becoming upright. By staining freshly released monospores with Alex Flour 488 phalloidin, actin filaments were observed as bundles in the cell (Fig. 2A). Once monospores moved, F-actin became densely assembled at the leading edge (Fig. 2B–D). When they became upright, it then accumulated at the bottom of the monospores (Fig. 2E, F); this was also observed during further growth (Fig. 2G, H). After the first asymmetrical division, F-actin covered two cells equally, whereas localized F-actin was still observed at the bottom of the germlings (Fig. 2I). Although occurrence of the second cell division was random (Fig. 2J, K), F-actin commonly assembled at the cleavage site prior to division. Pre-positioning of F-actin at the cleavage site was also observed in the third and fourth cell divisions (Fig. 2L, M), and during cell divisions on gametophytic blades (Fig. 2N). Taken together, these findings suggest that F-actin plays roles in the establishment and maintenance of cell polarity by accumulating at the leading edge during movement and at the bottom of germlings, and by

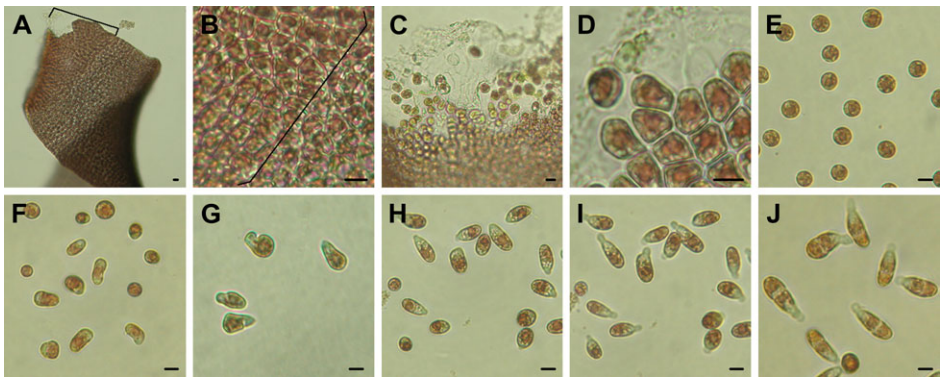

Fig. 1. Discharge, movement, and becoming upright of monospores from *P. yezoensis*. (A) Thallus of *P. yezoensis* (strain TU-1). The square bracket indicates monosporangia formed at the edge of the thallus. (B) Magnified view of monosporangia and vegetative cells. Left of the bracket shows cells in monosporangia. (C, D) Release of monospores. When somatic cells are released from the thallus, they move by changing in shape. (E) Re-rounding of freshly released monospores in ESL medium. (F, G) Retractile and amoeboid movement of monospores observed 3 h after release. They mainly showed a tapered tail. (H–J) Side views of monospores becoming upright. (H) Monospores attached to substratum observed 5 h after release; at this stage, monospores start to become upright. (I) Growth of monospores observed 8 h after release. (J) First asymmetrical division observed 24 h after release. Scale bars=10 μm.

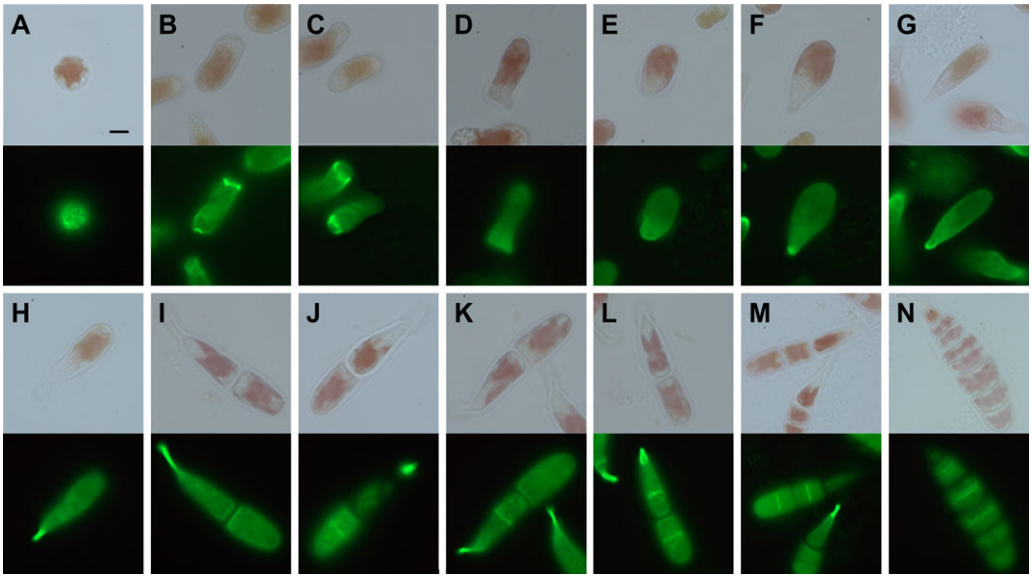

Fig. 2. F-actin distribution during the early development of monospores. F-actin was stained with Alex Flour 488 phalloidin. Upper and lower photographs in each panel show bright-field and fluorescent images, respectively. (A) Freshly released monospores. (B, C) Migrating monospores. (D–F) Adhering monospores. Accumulation of F-actin was observed at the attachment point. (G, H) Elongating monospores. (I) First asymmetric cell division. (J–N) Pre-position of F-actin at the cleavage site before cell division during growth of germlings. Scale bar=5 μm.

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pre-localizing at the division site during the early development of monospores.

Effects of cytoskeleton inhibitors on the early development of monospores

To observe the effects of defects in the cytoskeleton on the motility and becoming upright of monospores, freshly released monospores were collected immediately and incubated with different concentrations of the cytoskeleton inhibitors Cyt B, Lat B, Noc, and BDM. After 3 h incubation, more than 50% of the monospores started migrating and formed the tapered tail in medium without inhibitors (Fig. 3Aa, upper); in these cells F-actin was localized at the leading edge (Fig. 3Aa, lower). However, monospores treated with cytoskeleton inhibitors maintained a spherical shape (Fig.3Ab–e). In these experiments, since

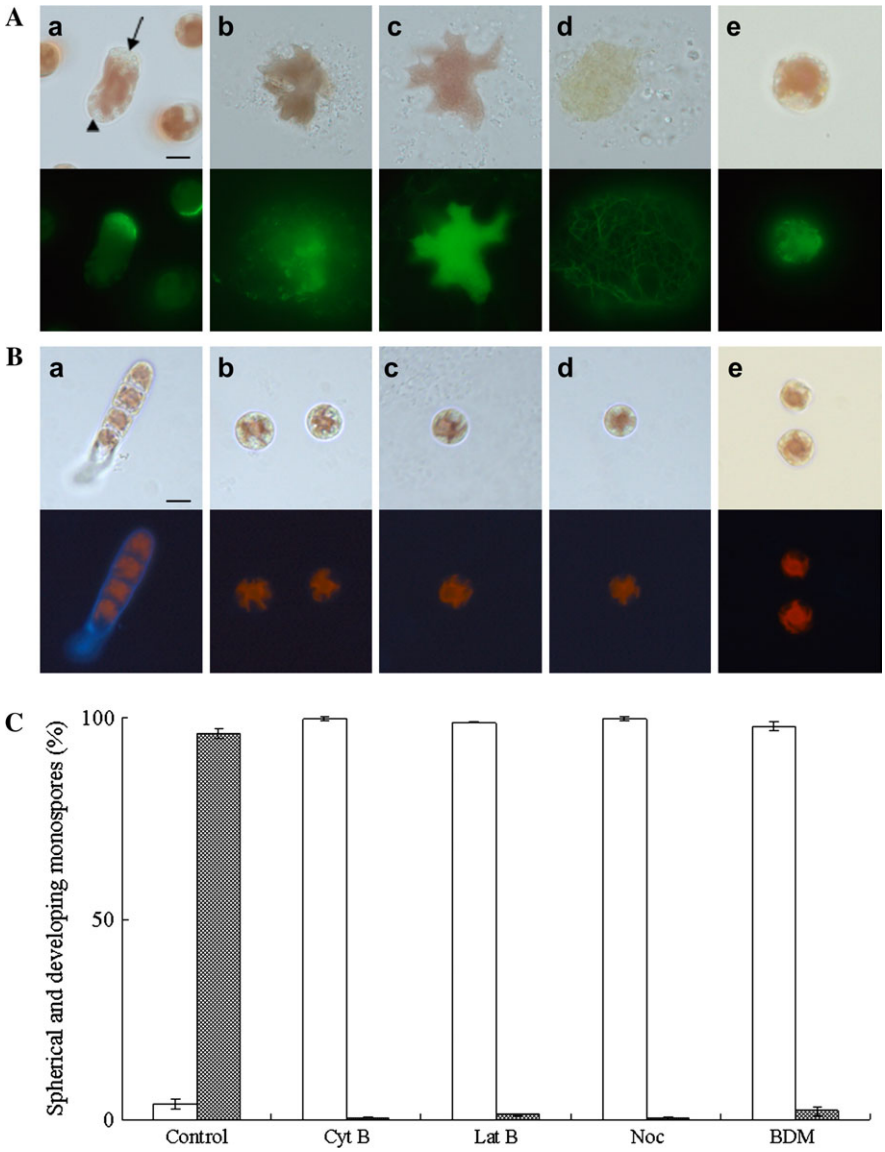

Fig. 3. Effects of cytoskeleton inhibitors on the early development of monospores. (A) Effects of cytoskeleton inhibitors on polarized F-actin accumulation. F-actin was stained with Alex Flour 488 phalloidin after incubation with or without cytoskeleton inhibitors for 3 h. Upper and lower photographs in each panel show bright-field and fluorescent images, respectively. (a) Monospores incubated with ESL medium containing 0.67% DMSO as a control. Leading and trailing edges are indicated by an arrow and an arrowhead, respectively. (b) Monospores treated with 0.2 mM Cyt B. (c) Monospores treated with 25 μM Lat B. (d) Monospores treated with 3 μM Noc. (e) Monospores treated with 10 mM BDM. Scale bar = 5 μm. (B) Effect of cytoskeleton inhibitors on cell wall synthesis. Monospores were treated with cytoskeleton inhibitors for 48 h. Upper and lower photographs in each panel show bright-field and fluorescent images, respectively. (a) Germling grown in ESL medium with 0.67% DMSO as a control. (b) Monospores treated with 0.2 mM Cyt B. (c) Monospores treated with 25 μM Lat B. (d) Monospores treated with 3 μM Noc. (e) Monospores treated with 10 mM BDM. Scale bar = 10 μm. (C) Comparison of the effects of cytoskeleton inhibitors on motility and development of monospores. White and grey bars show the rate of non-development and development of monospores, respectively, after treatment of freshly released monospores with cytoskeleton inhibitors for 48 h (concentrations as in A and B). Data are presented as mean ±SD (n=3).

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inhibitor-treated cells became too brittle to bear the weight of cover-glass, these cells lost their original spherical shapes and were crushed into the flat shape, which increased the autofluorescence of the chloroplasts especially in the case for the treatment with actin inhibitors (Fig. 3Ab, c). As shown in Fig. 3A, cytoskeleton inhibitors affected the distribution of F-actin. Treatment with 0.2 mM Cyt B and 25 μM Lat B disrupted the organization of F-actin (Fig. 3Ab, c). When monospores were treated with 3 μM Noc, the organization of F-actin was weakened (Fig. 3Ad), although F-actin was cleaved into short fragments by treatment with 30 μM Noc (data not shown). However, F-actin was retained by treatment with 10 mM BDM as in the freshly released monospores (Fig. 3Ae). Moreover, as shown in Fig. 3B and C, after 48 h incubation in control culture 95% of the monospores became upright and developed to the 4-celled stage with a thick cell wall. By contrast, inhibitor-treated monospores were not able to germinate and maintained their round shape without cell wall development after 48 h incubation. These results indicate that F-actin, MTs, and myosin are necessary for movement and becoming upright of monospores, suggesting that the cytoskeleton plays critical roles in the early development of monospores. It was further supported by the observation showing the reversible effects of inhibitors after removal of the drugs by washing (data not shown).

Involvement of PI3K in the migration of monospores

As show in Fig. 3Aa, migrating monospores formed a tapered shape as in *Dictyostelium* cells and leukocytes, which prompted us to examine the possible involvement of PI3K and PI(3,4,5)P_3-phosphatase in the movement of *P. yezoensis* monospores. First, monospores were treated with a gradually increasing concentration of LY294002 and LY303511, the specific inhibitor of PI3K and its analogue, respectively. After 3 h incubation, the polarized localization of F-actin was gradually prevented with an increasing concentration of LY294002 along with morphological changes, whereas about 84% of the monospores started movement in the control medium (Fig. 4A). In parallel with this, the migration of monospores and the formation of germlings decreased in a dose-dependent manner during treatment with LY294002 for 3 h and 24 h (Fig. 4Ba, c). By contrast, LY303511 had no effect

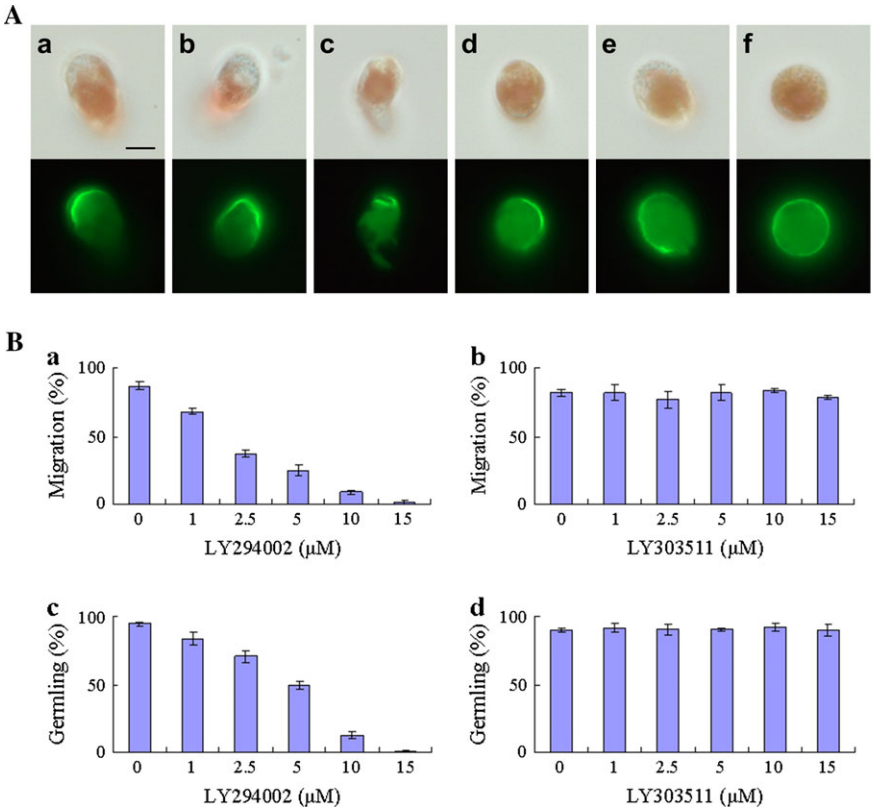

Fig. 4. Effects of the PI3K inhibitor on the motility and becoming upright of monospores. (A) Effect of LY294002 on morphology and asymmetrical F-actin accumulation. Freshly released monospores were treated with LY294002 for 3 h. F-actin was stained with Alex Flour 488 phalloidin. Upper and lower photographs in each panel show bright-field and fluorescent images, respectively. (a) Migrating monospores grown in ESL medium containing 0.3% DMSO. (b–f) Monospores incubated with an increasing concentration of LY294002 (b, 0.5 μM; c, 2.5 μM; d, 5 μM; e, 10 μM; f, 15 μM). Scale bar = 5 μm. (B) Effects of LY294002 and its analogue LY303511 on the early development of monospores. Freshly released monospores were treated with an increasing concentration of LY294002 (a, c) and LY303511(b, d) for 3 h (a, b) and 24 h (c, d). (a, b) Effects on migration. (c, d) Effects on formation of germlings. Data are presented as mean ±SD (n=3).

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(Fig. 4Bb, d). Moreover, the effect of LY294002 was reversible after washing the monospores (data not shown). These results indicate that PI3K activity is involved in migration through the formation of the anterior–posterior axis and the regulation of asymmetrical F-actin accumulation at leading edges. However, PI3K does not play a role in adhesion and becoming upright of monospores, since migrating monospores developed to germlings in the presence of LY294002 (Fig. 4Bc).

When monospores were incubated with an increasing concentration of bpv(pic) (0.01, 0.1, 1, 10, and 100 μM) for 3 h or 24 h, no effect was observed (data not shown). Thus, PTEN-like activity does not function during directional migration of monospores, although it is still unclear whether *P. yezoensis* cells have a PTEN-like activity.

Effect of inhibition of protein synthesis on the early development of monospores

Finally, the effects of the inhibition of protein synthesis on the establishment of cell polarity were examined. For this purpose, freshly released monospores, migrating monospores, monospores becoming upright, and germlings were treated with 18 μM cycloheximide. The fate of cycloheximide-treated monospores and germlings was observed after 24 h and 48 h, respectively. When cycloheximide was added to freshly released monospores, they were able to start migrating, but motility was lost after 24 h treatment (Fig. 5A, E, I). In the case of treated migrating monospores, migration was not inhibited by 24 h treatment (Fig. 5B, F, J). It is also notable that they were able to adhere to the substratum, although becoming upright was completely inhibited. Consistent with these findings, asymmetrical localization of F-actin was observed in cycloheximide-treated migrating monospores, but not in freshly released monospores treated with cycloheximide for 6 h and 24 h (Fig. 5M-T). Thus, new protein synthesis is necessary for the maintenance of the asymmetrical distribution of F-actin in freshly released monospores (Fig. 5M, N, Q, R). It was also found that cycloheximide completely inhibited cell division and development in monospores and germlings (Fig. 5C, D, G, H, K, L). In this case, F-actin was not observed in these cells after 6 h treatment (data not shown).

Taken together, these findings indicate that protein synthesis is not important for the establishment of the anterior–posterior axis, but is indispensable for the maintenance of asymmetrical distribution of F-actin, development of germlings after attachment to the substratum, and cell division during growth.

Discussion

In the present study, it has been demonstrated that motility of *P. yezoensis* monospores is dependent on the organization of actin, myosin, and MTs (Figs 1–3). Moreover, F-actin was shown to accumulate asymmetrically at the leading edge in migrating monospores having a tapered tail and at the bottom of germlings (Figs 2, 3A, 4A). Since MTs and F-actin are generally involved in the arrangement of the cytoskeleton, which is crucial for the establishment and maintenance of cell polarity (Samaj *et al*., 2000; Jedd and Chua, 2002), the localized accumulation of F-actin is thought to play roles in the establishment and/or maintenance of anterior–posterior and apical–basal axes in monospores. In animal cells, the polymerization of actin at the leading edge pushes the plasma membrane forward, resulting in the formation of pseudopodial protrusions (Affolter and Weijer, 2005). As shown in Figs 2–4, the polarized localization of F-actin near the leading edge was observed in migrating monospores, although actin filaments were randomly observed in freshly released monospores (Fig. 2). It was found that motility of monospores was completely inhibited by the disruption of local F-actin accumulation by Cyt B and Lat B (Fig. 3). These findings support the notion that F-actin plays a role in generating the force for protrusion and directed migration in monospores. Indeed, the importance of polarized F-actin localization in the establishment of cell polarity has also been demonstrated during the directional migration of *Dictyostelium* cells and mammalian leukocytes (Parent and Devreotes, 1999; Firtel and Chung, 2000; Bourne and Weiner, 2002). It is therefore postulated that the polarized accumulation of F-actin is closely related to the establishment of the anterior–posterior axis during the migration of monospores.

As shown in Figs 2, 3A, and 4A, the organization of F-actin in monospores was analysed using the fluorescent probe phalloidin. Although phalloidin does not penetrate cell walls (Cooper, 1987), it was permeable to freshly released monospores without requiring formaldehyde fixation, which is consistent with the lack of a cell wall in monospores immediately after their release from gametophytes (Hawkes, 1980). During migration, the cell wall of the monospores is gradually formed and completely synthesized before adhesion to the substratum (data not shown). Previously, the critical role of the cell wall was demonstrated in *Fucus* embryos, in which it is required for fixation, but not formation, of the axis (Kropf *et al*., 1988). Thus, it is possible that cell wall synthesis is important for the fixation of the anterior–posterior axis during migration in *P. yezoensis* monospores. Elucidation of the collaboration between the cell wall and cytoskeleton via a transmembrane bridge, which was proposed in *Fucus* embryos (Kropf *et al*., 1989), will be essential for further understanding of the role of the cell wall in the maintenance of cell polarity.

Our results also indicate the critical involvement of PI3K activity in migration via the establishment of cell

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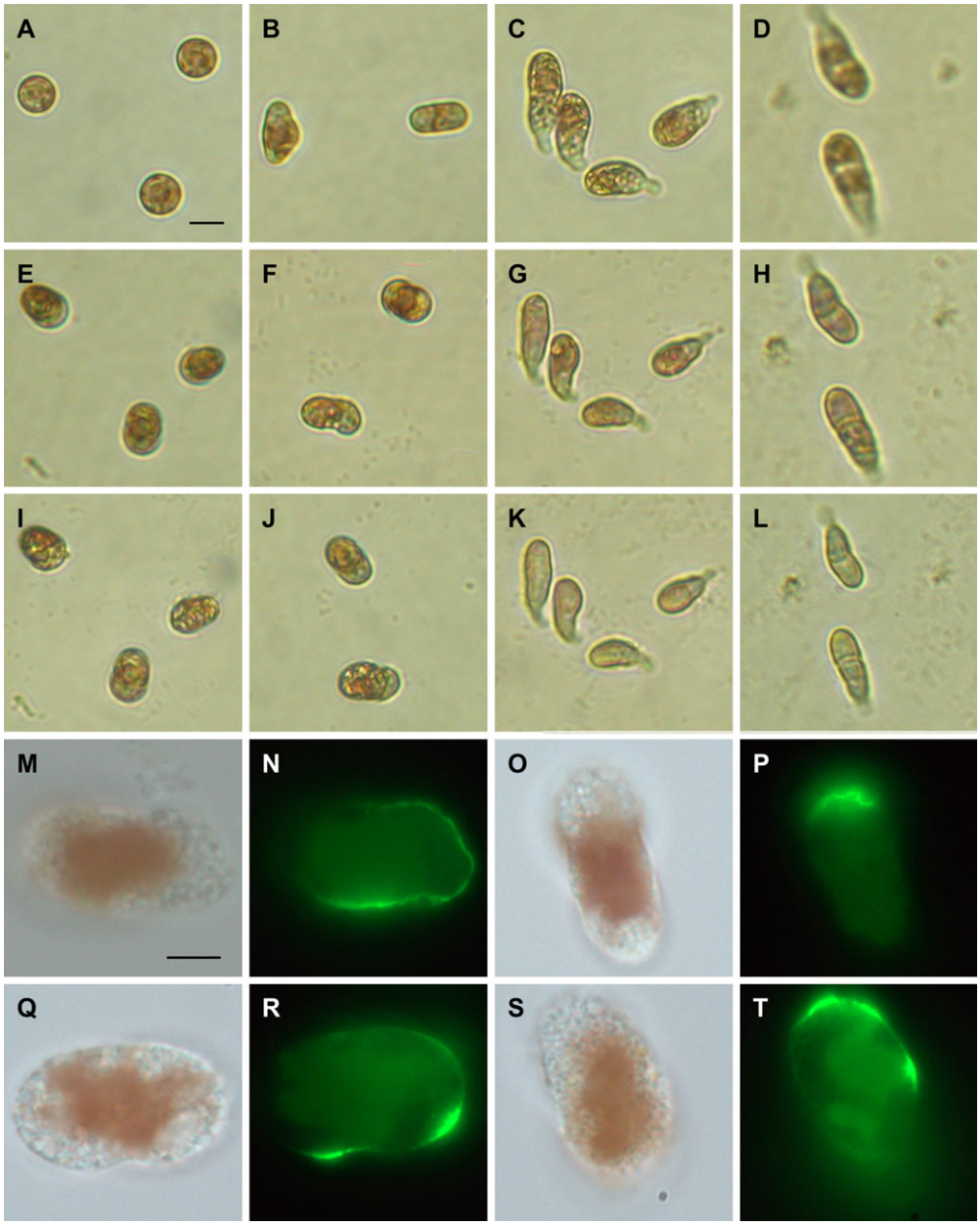

Fig. 5. Effects of a protein synthesis inhibitor on migration, becoming upright and cell division during the early development of monospores. Monospores were treated with 18 μM cycloheximide at various developmental stages: freshly released monospores (A, E, I), migrating monospores (B, F, J), monospores becoming upright (C, G, K), and 2-celled germlings (D, H, L). F-actin was stained with Alex Flour 488 phalloidin. (A–D) Monospores before treatment; (E–H) monospores 24 h after treatment; (I–L) monospores 48 h after treatment. (M–T) Effects of cycloheximide on polarized accumulation of F-actin. M, O, Q, S and N, P, R, T show bright-field and fluorescent images, respectively. Abnormal distribution of F-actin when freshly released monospores were treated with cycloheximide for 6 h (M, N). Normal polarized accumulation of F-actin when migrating monospores were treated with cycloheximide for 6 h (O, P). Organization of F-actin was partly weakened after treatment with cycloheximide for 24 h (Q, R). Abnormal distribution of F-actin when migrating monospores were treated with cycloheximide for 24 h (S, T). Scale bars: (A–L) 10 μm; (M–T) 5 μm.

polarity (Fig. 4). In *Dictyostelium* and leukocyte cells, the preferential activation of PI3K at the side of the cells facing the chemoattractant is important for establishing a new leading edge, which results in the local production of $PI(3,4,5)P_3$ and the subsequent regulation of cell polarization and directional movement (Parent and Devreotes, 1999; Firtel and Chung, 2000; Bourne and Weiner, 2002). Here, it is demonstrated that the PI3K inhibitor LY294002, but not its analogue LY303511, inactivates the motility of monospores and disorganizes F-actin localization in a dose-dependent manner (Fig. 4A). Thus, although $PI(3,4,5)P_3$ has not yet been observed in

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plants (Mueller-Roeber and Pical, 2002), it is hypothesized that D3-phosphorylated PIs such as PI(3)P, PI(3,4)P_2, and PI(3,5)P_2 have pivotal roles in axis formation in monospores. To identify the D3-phosphorylated PI(s) required for the migration of monospores, synthetic PIs, Di-C8-Ptdlns(3)P, Di-C8-Ptdlns(3,4)P_2, Di-C8-Ptdlns(3,5)P_2, and Di-C8-Ptdlns(3,4,5)P_3 (Echelon) were examined to rescue the ability of movement in LY294002-treated monospores. However, recovery of motility was not observed with any of the above synthetic PIs (data not shown). It is possible that the length of the fatty acids in these synthetic PIs was too short to observe an effect in *P. yezoensis* in which membrane phospholipids and galactolipids usually carry very long-chain unsaturated fatty acids such as arachidonic acid (20:4) and eicosapentaenoic acid (20:5) (Araki *et al.*, 1987).

Other lines of evidence indicate that PI3K is involved in a variety of physiological phenomena in plants such as root hair growth, production of reactive oxygen species induced by auxin and salt stress, stomatal closure, and vesicle trafficking (Welters *et al.*, 1994; Matsuoka *et al.*, 1995; Jung *et al.*, 2002; Joo *et al.*, 2005; Leshem *et al.*, 2007). Recently, the regulation of actin organization by PI3K was reported in ABA-induced stomatal closure (Choi *et al.*, 2008). Moreover, the functional significance of PI(3)P was recently examined through the ectopic expression of the FYVE domain, which specifically binds PI(3)P (Gillooly *et al.*, 2000), in *Arabidopsis thaliana* (Helling *et al.*, 2006; Vermeer *et al.*, 2006; Lee *et al.*, 2008). Involvement of PI(3)P in the growth of root hairs was denied by Helling *et al.* (2006), which is not consistent with Lee *et al.* (2008) who reported the significance of PI(3)P in tip growth of root hairs. In fact, no expression system of any fluorescent protein has yet been established in multicellular red algae, and thus, it was not possible to monitor subcellular localization using fluorescent proteins in *P. yezoensis* cells. Development of fluorescent proteins and a system for genetic transformation in *P. yezoensis* is therefore necessary, although recently a transient gene expression system for the β-glucoronidase reporter gene has been established (Fukuda *et al.*, 2008).

In the present study, it is also demonstrated that new protein synthesis was not required for starting the migration of monospores, whereas the monospores becoming upright was prevented by cycloheximide treatment (Fig. 5). Thus, it is postulated that the establishment of anterior–posterior and apical–basal axes in monospores are differentially regulated in terms of the requirement of new protein synthesis. However, in *Dictyostelium* cells, inhibition of protein synthesis by the addition of cycloheximide resulted in the prevention of migration via a morphological change to a round shape by retraction of their pseudopodia; localization of actin polymerization

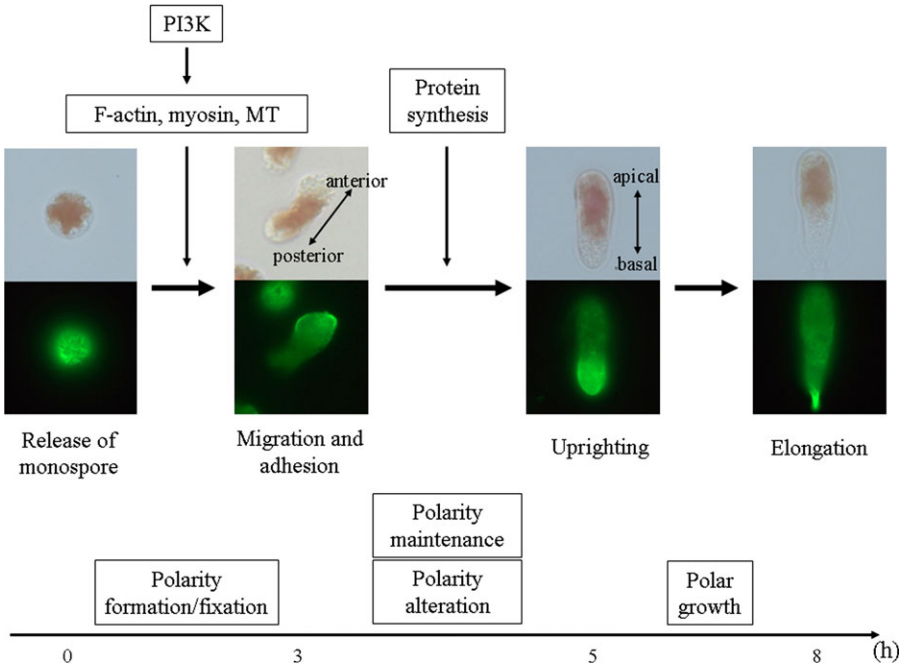

Fig. 6. A model showing the involvement of the cytoskeleton, PI3K, and protein synthesis in the early development of monospores from *P. yezoensis*. PI3K is thought to regulate the organization of F-actin, myosin, and MTs, providing the force for migration via the establishment of the anterior–posterior axis. F-actin is asymmetrically localized at the leading edge of migrating monospores. After adherence to the substratum, new protein synthesis is required for becoming upright of the monospores via the establishment of the apical–basal axis. Local accumulation of F-actin at the bottom of the monospores is important for the maintenance of apical–basal polarity.

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and PI3K was not influenced (Clotworthy and Traynor, 2006). Since, these results are not consistent with our findings whereby monospores were able to continue migration despite morphological changes in the presence of cycloheximide (Fig. 5), it is necessary to determine what kinds of pre-existing factors collaborate with PI3K and the cytoskeleton in *P. yezoensis* monospores.

Using the results obtained, a model is proposed for the early development of monospores (Fig. 6). The start of migration requires the formation of the anterior–posterior axis via PI3K-dependent organization of the cytoskeleton, which may be regulated by pre-existing proteins via post-translational modifications. After attachment to the substratum, monospores become upright and develop via the establishment and maintenance of the apical–basal axis and cell division with new protein synthesis. To confirm this model, subcellular localization of PI3K and D3-phosphorylated PIs must be determined and the molecular bases of the relationship between PI3K activity and the polarized localization of F-actin must also be elucidated. In addition, it is necessary to identify newly synthesized proteins required for the establishment of the apical–basal axis. Furthermore, it is important to analyse how monospores recognize cues for the establishment of cell polarity during their early development. In animals, the PAR-aPKC system is a type of molecular machinery that converts initial polarity cues into cellular events, such as the regulation of actin assembly, for the establishment of polarity axis (Suzuki and Ohno, 2006). In plants, $PI(3,4,5)P_3$ has not yet been observed (Mueller-Roeber and Pical, 2002) and no gene encoding factors composing a PAR-aPKC system have been found in the whole genome data of *A. thaliana* and rice (data not shown), although homologues of the *PI3K* gene have been found in terrestrial plants (Welters *et al*., 1994; Das *et al*., 2005) and *P. yezoensis* (data not shown). Thus, it is possible that cell polarity is established by uncharacterized machinery in migrating plant cells. Identification of the factors activating PI3K and elucidation of the function of D3-phosphorylated PI(s) in the transduction of signals from developmental cues in *P. yezoensis* monospores could provide a new insight into the molecular mechanisms required for the establishment of cell polarity in plants.

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