Roles of Phosphatidylinositol 3-Kinase in Root Hair Growth[1][OA]

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The root hair is a model system for understanding plant cell tip growth. As phosphatidylinositol 3-phosphate [PtdIns(3)P] has been shown in other plant cell types to regulate factors that affect root hair growth, including reactive oxygen species (ROS) levels, cytoskeleton, and endosomal movement, we hypothesized that PtdIns(3)P is also important for root hair elongation. The enzyme that generates PtdIns(3)P, phosphatidylinositol 3-kinase (PI3K), was expressed in root hair cells of transgenic plants containing the PI3K promoter:β-glucuronidase reporter construct. To obtain genetic evidence for the role of PtdIns(3)P in root hair elongation, we attempted to isolate Arabidopsis (*Arabidopsis thaliana*) mutant plants that did not express the gene *VPS34* encoding the PI3K enzyme. However, the homozygous mutant was lethal due to gametophytic defects, and heterozygous plants were not discernibly different from wild-type plants. Alternatively, we made transgenic plants expressing the PtdIns(3)P-binding FYVE domain in the root hair cell to block signal transduction downstream of PtdIns(3)P. These transgenic plants had shorter root hairs and a reduced hair growth rate compared with wild-type plants. In addition, LY294002, a PI3K-specific inhibitor, inhibited root hair elongation but not initiation. In LY294002-treated root hair cells, endocytosis at the stage of final fusion of the late endosomes to the tonoplast was inhibited and ROS level decreased in a dose-dependent manner. Surprisingly, the LY294002 effects on ROS and root hair elongation were similar in *rhd2* mutant plants, suggesting that RHD2 was not the major ROS generator in the PtdIns(3)P-mediated root hair elongation process. Collectively, these results suggest that PtdIns(3)P is required for maintenance of the processes essential for root hair cell elongation.

Root hairs are projections from the epidermal cells of the root that play critical roles in the uptake of water and nutrients and in anchoring the plant to the soil (Peterson and Farquhar, 1996). Root hairs are often used as a model system to uncover general principles underlying cell polarity and polar growth in plants. Studies on the root hair model have led to the identification of several crucial components of the tip growth machinery, such as the tip-focused cytoplasmic calcium ion gradient (Wymer et al., 1997), the cytoskeleton (Miller et al., 1999), reactive oxygen species (ROS; Foreman et al., 2003), and small G proteins (Jones et al., 2002; Preuss et al., 2004). In addition, phosphoinositides (Braun et al., 1999; Vincent et al., 2005) and phosphatidic acid (Ohashi et al., 2003) are also suggested to be involved in root hair growth. However, our understanding of the signaling network involved in root hair tip growth still remains incomplete. In particular, aspects of how and which lipids are involved in modulating root hair growth remain unknown.

Phosphatidylinositol 3-phosphate [PtdIns(3)P] is a phosphoinositide that is present at very low levels in plant cells (Brearley and Hanke, 1992). It is synthesized by phosphatidylinositol 3-kinase (PI3K), which in turn is encoded by the only PI3K gene reported in plants, *VPS34*. PtdIns(3)P and PI3K are essential for normal plant growth (Welters et al., 1994) and have been implicated in diverse physiological functions, including root nodule formation (Hong and Verma, 1994), auxin-induced production of ROS and root gravitropism (Joo et al., 2005), root hair curling and *Rhizobium* infection in *Medicago truncatula* (Peleg-Grossman et al., 2007), increased plasma membrane endocytosis and the intracellular production of ROS in the salt tolerance response (Leshem et al., 2007), and stomatal closing movement (Jung et al., 2002; Park et al., 2003).

The cellular and biochemical mechanisms of action of PtdIns(3)P are the focus of intense investigation. In yeast studies, PtdIns(3)P has been shown to be essential in vesicle-mediated delivery of vacuolar enzymes (Stack and Emr, 1994), vesicle docking/fusion at the endosome (Peterson et al., 1999), and endosome-to-Golgi retrograde transport (Burda et al., 2002). Roles for PtdIns(3)P in vesicle trafficking have been reported in plants as well. Overexpression of the PtdIns(3)P-binding protein in Arabidopsis (*Arabidopsis thaliana*) protoplasts inhibits trafficking of the vacuolar protein

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sporamin (Kim et al., 2001). A similar failure to deliver vacuolar proteins was reported in tobacco (*Nicotiana tabacum*) suspension cells treated with pharmacological agents that interfere with PtdIns(3)P synthesis (Matsuoka et al., 1995). A role for PtdIns(3)P in endocytosis has also been shown in plants. PI3K inhibition suppresses FM1-43 uptake into tobacco cells (Emans et al., 2002) and into Arabidopsis root cells treated with salt stress (Leshem et al., 2007). There are also indications that PtdIns(3)P plays a role in other essential processes. For example, PtdIns(3)P in plants activates kinases and their downstream signaling processes, similar to those of PtdIns(3,4,5)P_3 in animal cells (Deak et al., 1999). If indeed PtdIns(3)P is important for maintaining many basic functions of cellular activities, including endocytosis and trafficking to vacuoles, gross alterations in development and physiology are expected in PtdIns(3)P-depleted plants.

We hypothesized that PtdIns(3)P is important for tip growth of root hair cells because (1) ROS and the cytoskeleton, essential factors for root hair cell growth, are modulated by PtdIns(3)P in other cell types (Park et al., 2003; Joo et al., 2005; Leshem et al., 2007; Choi et al., 2008) and (2) PtdIns(3)P is essential for normal morphology and movement of endosomes in root hairs (Voigt et al., 2005). Using pharmacological and genetic tools, we found that the maintenance of a normal level of PtdIns(3)P is required for root hair elongation. Vesicle trafficking and ROS formation, processes found to be essential for the elongation of root hairs, were compromised in the presence of a PI3K inhibitor. These results establish PtdIns(3)P and PI3K as important factors for normal root hair growth.

RESULTS

VPS34 Is Expressed in Root Hair Cells

To determine the site of PI3K expression, we generated transgenic Arabidopsis plants harboring the *VPS34* promoter:GUS reporter construct. We cloned the upstream promoter region of *VPS34* (−1,391 to +125 in relation to ATG) and generated a translational fusion with the GUS coding sequence in the pBI121 vector. This construct was introduced into Arabidopsis by the floral dipping method (Clough and Bent, 1998), and the transformants were analyzed for GUS expression. Seven independent lines tested for GUS activity showed similar expression patterns. GUS activity was detected in almost all vegetative tissues (Fig. 1A), including root hairs (Fig. 1B). Expression of *VPS34* in root hair cells was confirmed by reverse transcription (RT)-PCR analysis using RNA extracted from root hair cells (Fig. 1D). The RNA template was specific for the root hair cells, as indicated by the amplification of root hair cell-specific *EXPANSINA7* transcript and no amplification of the non-hair cell-specific *GLABRA2* transcript. When whole root RNA was used as a template, *GLABRA2* was amplified.

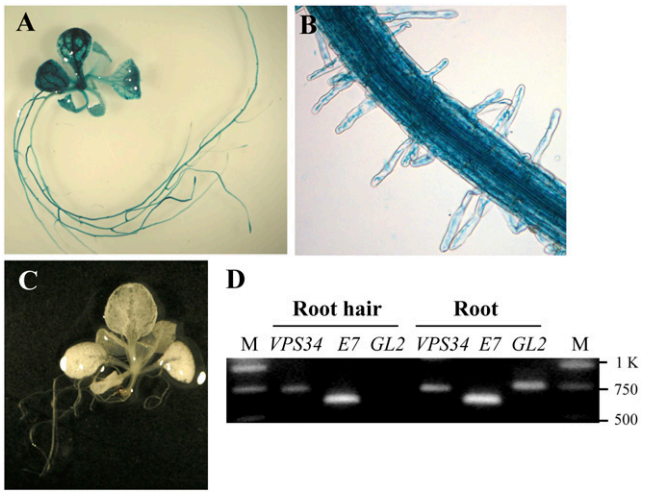

Figure 1. *VPS34* is expressed in Arabidopsis root hairs. A and B, GUS activity (indicated by blue) of transgenic plants harboring the *VPS34* promoter:GUS reporter construct in whole plants (A) and root and root hairs (B). C, GUS activity of transgenic plants harboring an empty vector construct. D, RT-PCR analysis of RNA from Arabidopsis root hair cells (Root hair) or whole root tissues (Root). *EXPANSINA7* (*E7*) was used as a positive control for hair cell-specific amplification; *GLABRA2* (*GL2*) was used as a control for non-hair cell-specific amplification. Gene-specific primer sets were designed to include intron(s) to discriminate amplification from cDNAs versus genomic DNA.

VPS34/vps34 Mutant Plants Showed Root Hair Growth Similar to Wild-Type Plants

We attempted to isolate homozygous T-DNA insertional knockout-PI3K plants (SALK-007281 and GABI-418H02), but no homozygous mutant line could be found. The self-fertilized heterozygous plants produced progeny that segregated 1:1 for wild-type and heterozygous plants but no homozygous knockout plants, due to defects in the development of male gametophyte carrying the *vps34* allele (Lee et al., 2008). To test whether heterozygous plants showed any defects in root hair growth, we grew progeny of *VPS34/vps34* plants on half-strength Murashige and Skoog (MS) plates for 5 d and measured the root hair length of each plant. The plants were then allowed to grow for 2 more weeks, and their genotypes were identified by genomic DNA PCR. *VPS34/vps34* plants did not differ from *VPS34/VPS34* plants in root hair length ($P > 0.1$ by Student's t test; Fig. 2): mature root hair length was 466.5 ± 8.7 μm and 452.1 ± 6.5 μm in *VPS34/VPS34* and *VPS34/vps34*, respectively.

Expression of the PtdIns(3)P-Binding Domain GFP:2xFYVE Inhibited Root Hair Growth

Because homozygous *vps34* plants could not be produced, we examined the roles of PtdIns(3)P on root hair growth using complementary methods. To block signal transduction downstream of PtdIns(3)P,

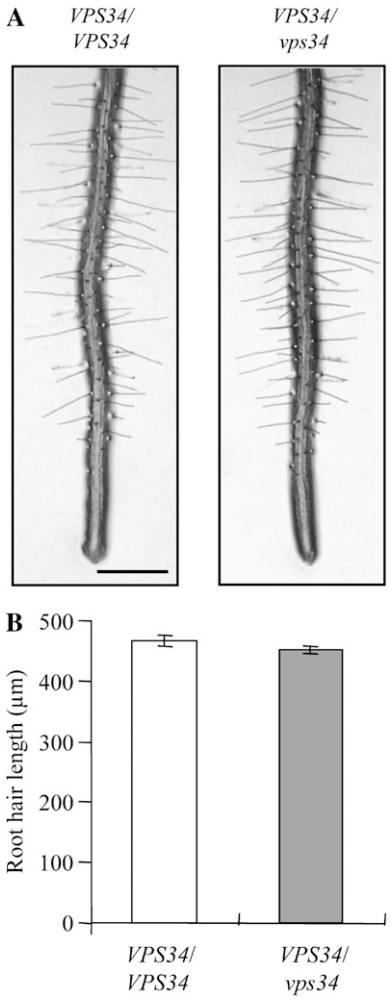

Figure 2. The *VPS34*/*vps34* mutant does not differ from the wild type in mature root hair length. A, Root hairs of the wild type and a heterozygous knockout mutant, *VPS34*/*vps34* (GABI_418H01), grown on half-strength MS plates for 5 d. T-DNA insertion at the *VPS34* locus was confirmed for each plant using PCR-based genotyping. Bar = 0.5 mm. B, Root hair length of wild-type and *VPS34*/*vps34* plants. Values represent means ± SE from 180 to 190 root hairs.

we made mutant plants expressing a PtdIns(3)P-binding protein, GFP:2xFYVE (for Fab1, YOTB, Vac1, and EEA1) under the control of the promoter of a root hair-specific gene, *EXPANSINA7* (Cho and Cosgrove, 2002; Kim et al., 2006; Fig. 3A). FYVE has been reported to bind specifically and with high affinity to PtdIns(3)P in yeast, mammals, and plants (Gillooly et al., 2000; Voigt et al., 2005; Vermeer et al., 2006).

First, we examined the distribution of GFP:2xFYVE in root hair cells and observed that it localized to vesicles of various sizes (Fig. 3B), similar to the localization pattern of yellow fluorescent protein:2xFYVE reported previously in Arabidopsis root hair cells (Vermeer et al., 2006). In contrast, free GFP spread throughout the cytoplasm (Fig. 3C). We then compared the mature root hair length of transgenic plants expressing GFP:2xFYVE with that of wild-type plants. Mature root hairs of 5-d-old seedlings of three independent transgenic lines expressing GFP:2xFYVE were reduced in length compared with those of wild-type plants (Fig. 3D; FYVE-1, 431.7 ± 7.4 μm; FYVE-2, 485.3 ± 7.5 μm; FYVE-3, 504.8 ± 7.6 μm; wild type, 536.1 ± 5.9 μm; $P < 0.01$). Transgenic plants expressing GFP alone showed no difference in root hair length compared with wild-type plants (data not shown). The growth rate of root hairs in mutant plants was also reduced compared with those of wild-type plants (Fig. 3E; FYVE-1, 0.6 ± 0.1 μm min^{-1}; FYVE-2, 0.8 ± 0.1 μm min^{-1}; FYVE-3, 0.9 ± 0.1 μm min^{-1}; wild type, 1.2 ± 0.1 μm min^{-1}; $P < 0.01$).

Because the extent of growth inhibition exhibited by the three independent FYVE lines was various, we tested whether or not they correlated with the expression level of FYVE by observing the fluorescence intensity of GFP:2xFYVE. The fluorescence in root hairs of 5-d-old mutant plants was photographed under the same conditions and compared. The fluorescence intensity was strongest in FYVE-1 plants and weakest in FYVE-3 plants (Fig. 3F; $P < 0.01$). Virtually no fluorescence was detected in wild-type plants. Therefore, the level of expression of GFP:2xFYVE seemed to correlate with the extent of inhibition of root hair growth in the transgenic plants.

LY294002, a PI3K-Specific Inhibitor, Inhibited Root Hair Growth

To observe short-term, rapid effects of PtdIns(3)P reduction on root hair elongation, we performed pharmacological experiments using the PI3K-specific inhibitor LY294002 (Jung et al., 2002). Arabidopsis seedlings were grown on half-strength MS plates for 4 d, transferred to the same plates containing various concentrations of LY294002, and then grown for an additional 1 d. The root hairs of plants on plates containing LY294002 were shorter than those on control plates (Fig. 4, A and B), and this inhibitory effect of LY294002 on root hair length was concentration dependent between 2 and 30 μM ($P < 0.01$; Fig. 4C). The effect of LY294002 on root hairs was specific for tip elongation: LY294002 did not affect cell viability or root hair initiation. Bulges (≤40 μm) were normal in shape (Fig. 4D), and the number of the total root hairs was not altered ($P > 0.1$; Fig. 4E). The site of root hair initiation did not change (Fig. 4D), nor was the occasional branching of the hair cells affected by the drug.

The inhibitory effect of LY294002 on root hair growth was confirmed at the single cell level. Plants grown on half-strength MS plates for 5 d were transferred to slide glass chambers, and the growth rate of root hairs was measured after 4 h of incubation for stabilization. The inhibitory effect of LY294002 was detectable within minutes (Fig. 5A). The root hair growth rate in plants treated with 30 μM LY294002 (0.2 ± 0.1 μm min^{-1}) was severely reduced compared with that of control plants (1.3 ± 0.1 μm min^{-1}; Fig. 5B).

LY294002 also altered the cytoplasmic architecture of the root hair cell. Growing root hairs reportedly

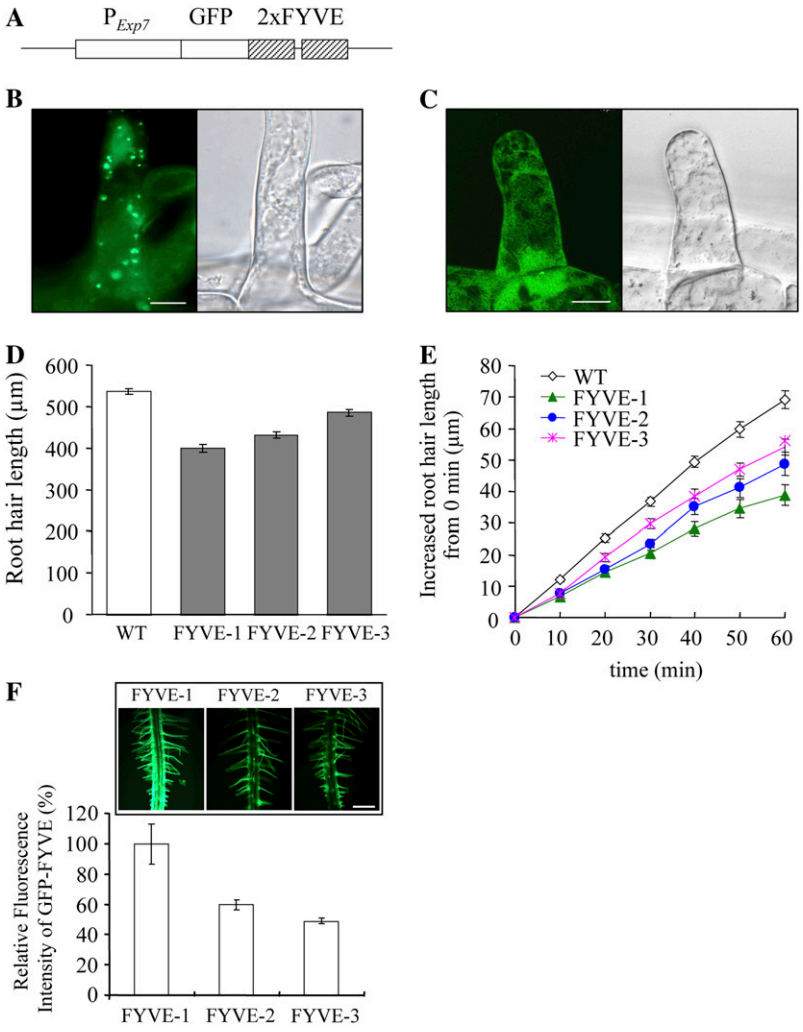

Figure 3. Overexpression of GFP:2xFYVE inhibits root hair growth. A, Schematic representation of the GFP:2xFYVE construct under the control of the root hair-specific promoter of *EXPANSINA7* (P_{Exp7}). B, Fluorescence and differential interference contrast (DIC) images of root hairs in transgenic plants expressing GFP:2xFYVE. Bar = 10 μm. C, Fluorescence and DIC images of root hairs in transgenic plants expressing free GFP. Bar = 10 μm. D, Mature root hair length of wild-type (WT) and transgenic plants expressing GFP:2xFYVE. The 10 longest hairs were measured at 2.5 to 3.5 mm from the tip of each primary root. Values represent means ± SE from 160 to 200 root hairs. E, Growth rate of root hairs of wild-type and transgenic plants expressing GFP:2xFYVE. Values represent means ± SE from eight to 19 root hairs. F, Fluorescence intensity of root hairs of transgenic plants expressing GFP:2xFYVE. The fluorescence in root hairs of 5-d-old mutant plants was photographed under the same conditions, and the average peak pixel intensities of the root hairs were compared. Values represent means ± SE from 39 to 50 root hairs. The inset at the top of the graph shows fluorescence images of the transgenic lines. Bar = 200 μm.

display a polarized organization of the cytoplasm, in which organelles necessary for the formation of the new cell wall accumulate in the tip. Thus, the apical region of growing root hairs, known as the clear zone, lacks large organelles or vacuoles (Ovečka et al., 2005). When tip growth is terminated, this polarized organization of cytoplasm gradually disappears, large organelles and vacuoles invade the tip, and finally, the root hair becomes surrounded by only a thin cytoplasmic layer. We confirmed the polar organization in control root hairs that continued to grow: they maintained the clear zone filled with dynamic vesicles at the apical region (Fig. 6A). Within several minutes after treatment with LY294002, the clear zone at the tip of the root hair retracted and was invaded by large vacuoles (Fig. 6B), indicating that the hair cell had changed from growing to nongrowing status.

LY294002 Inhibits Endocytosis in Root Hair Cells

Endocytosis occurs actively in growing root hairs (Ovečka et al., 2005) and helps recycle the plasma membrane and other components of trafficking. To test whether PI3K plays a role in endocytosis in root hair cells, we labeled root hairs with FM1-43, a dye widely used for studying plasma membrane recycling in plants (Emans et al., 2002; Ovečka et al., 2005; Leshem et al., 2007). Seedling roots were incubated for 5 min in an FM1-43-containing liquid medium, washed, and transferred into normal growth medium without FM1-43. For treatment with LY294002, seedlings were preincubated for 30 min in a medium containing 30 μM LY294002, which remained in the medium for subsequent staining with FM1-43. Incubation with FM1-43 resulted in strong fluorescence signals at the plasma membrane, followed by a gradual transfer of the fluorescence to the cytoplasm, which was likely due to the development of many speckles of fluorescent endosome-like vesicles (Fig. 7A). The size of these fluorescent vesicles increased over time, as reported previously (Ovečka et al., 2005). These vesicles were also detected in the root hairs treated with LY294002 (Fig. 7B), and there was no noticeable difference in the size or fluorescence intensity of these vesicles compared with those in control hair cells until 1.5 h after drug treatment. The effect of LY294002 became apparent after 3 h of incu-

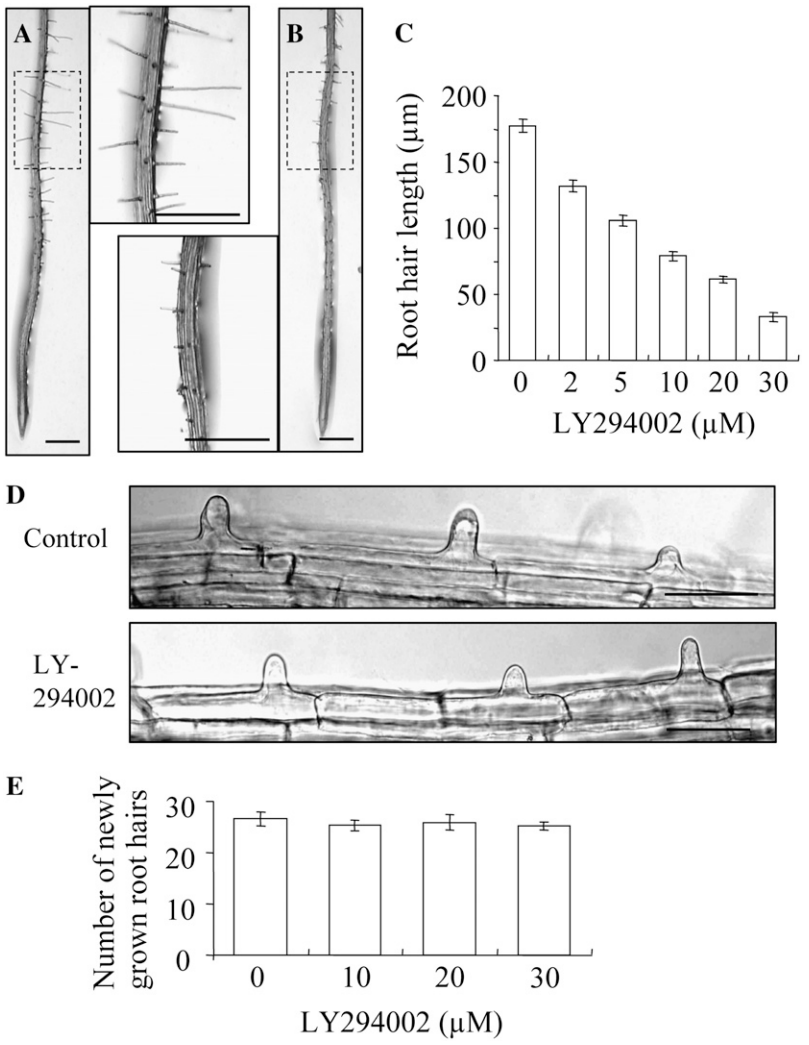

Figure 4. LY294002, a PI3K-specific inhibitor, inhibits root hair growth. A and B, Root hairs of a seedling grown on half-strength MS plates for 4 d, transferred to a plate containing DMSO (solvent control; A) or 30 μM LY294002 (B), and then grown for an additional 1 d. Magnified images of the rectangular areas are attached to the top right (A) and bottom left (B) of the images of the roots. Bars = 0.5 mm. C, Dose-dependent effects of LY294002 on root hair growth. The length of root hairs after transfer was measured. Values represent means ± SE from 162 to 231 root hairs. D, No effect of LY294002 on bulge shape. Bulges remained shorter than 40 μm in LY294002-treated roots, but their shapes were normal. Bars = 50 μm. Note also that the number of bulges was not altered by LY294002. E, No effect of LY294002 on total number of root hairs including bulges. The number of root hairs (including bulges) was counted at 1 d after transfer. Values represent means ± SE from 12 to 21 plants.

bation, when the fluorescent vesicles of control cells had fused to the tonoplast (Fig. 7C; $n = 29$). In root hairs treated with LY294002, the fluorescence of FM1-43 did not incorporate into the tonoplast but remained in the large endosomes (Fig. 7D; $n = 29$), suggesting that LY294002 inhibits the last stage of endocytosis, the fusion of late endosomes with tonoplast.

LY294002 Decreases ROS Levels in Root Hair Cells

Because ROS are known to be crucial components of the tip growth machinery in root hairs (Foreman et al., 2003), we tested whether LY294002 affected intracellular ROS levels in root hairs. Five-day-old seedlings were preincubated in an LY294002-containing medium for 30 min, and ROS levels in the root hairs were estimated using the dye 2′,7′-dichlorodihydrofluorescin-diacetate (DCF-DA; Lee et al., 1999). The intracellular ROS level of root hairs treated with 10 μM LY294002 decreased to 70.4% ± 4.4% of the control, and it was further decreased to 54.6% ± 3.4% when the concentration of LY294002 was increased to 20 μM (Fig. 8, A and B).

Recently, ROS were detected in vesicles of root cells exposed to high NaCl (Leshem et al., 2006, 2007). To test whether the ROS of root hairs are also detected in such vesicles, we observed the fluorescence of DCF using confocal microscopy. Optically sectioned root hair cells revealed that the fluorescence of DCF occurred mainly in a dotted pattern (Fig. 8C). These fluorescent dots were highly mobile and overlapped with the fluorescence of the styryl membrane dye FM4-64, which stains endosomes and vacuoles in the cell, as indicated by the yellow color of the dots in the merged image (Fig. 8C, top). In the magnified view of the image, the green fluorescence of DCF was found encapsulated in the enlarged endosome membranes stained red with FM4-64 (Fig. 8C, bottom).

LY294002 Decreases Growth and Reduces ROS Levels in *rhd2* Root Hair Cells

AtrbohC/RHD2, a NADPH oxidase, has been reported as a major source of ROS generated in root hairs (Foreman et al., 2003). Therefore, we were curious

whether the inhibitory effect of LY294002 on the ROS level is due to its inhibition of AtrbohC/RHD2 activity. To examine this possibility, we tested whether LY294002 reduced ROS levels in the root hairs of the SALK_071801 T-DNA insertional mutant of AtrbohC/RHD2.

Four-day-old seedlings of *rhd2* mutants grown on half-strength MS plates were transferred to plates with or without 30 μM LY294002 and were grown for an additional 1 d. Root hairs of *rhd2* mutants were very short or burst on plates of pH 5 (31 $\pm$ 1 μm; Fig. 9A), but they grew almost normally on plates of pH 6, although they were slightly shorter than those of the wild type (225 $\pm$ 5 μm and 261 $\pm$ 5 μm for *rhd2* mutants and the wild type, respectively; $P < 0.01$; Fig. 9B). On pH 6 plates, treatment with LY294002 further inhibited the growth of *rhd2* mutants to 20% of control untreated levels (44 $\pm$ 2 μm; Fig. 9B), and the extent of inhibition was similar to that found in wild-type plants (23% of untreated control levels; 59 $\pm$ 2 μm). Next, intracellular ROS of the *rhd2* mutant were detected using DCF-DA. ROS levels were below the detection limit in the root hair cells of *rhd2* plants at pH 5 (Fig. 9C). At pH 6, the ROS level of *rhd2* mutants was 77.0% $\pm$ 3.8% compared with that of wild-type plants under control conditions. When treated with 30 μM LY294002, the ROS level of *rhd2* mutants was further decreased to 55.9% $\pm$ 2.5% of the wild-type level under control conditions, which was only slightly smaller than the wild-type level under the same treatment (62.6% $\pm$ 2.0%; Fig. 9D). The pattern of ROS in root hair cells of *rhd2* plants growing at pH 6 (Fig. 9E) did not differ from that in wild-type plants (Fig. 8C), showing a dotted pattern.

DISCUSSION

PtdIns(3)P has been suggested to be important for root hair growth (Voigt et al., 2005; Peleg-Grossman et al., 2007), although it has never been directly assayed. Here, we characterized in detail the effects of PtdIns(3)P on the tip growth of root hairs by blocking signal transduction downstream of PtdIns(3)P or by inhibiting PI3K activity. Transgenic plants expressing the PtdIns(3)P-binding FYVE domain had short root hairs and reduced hair growth rate, and LY294002, a specific inhibitor of PI3K (Jung et al., 2002), rapidly inhibited the tip growth of root hairs in a concentration-dependent manner. Two major target sites of inhibition were identified: the inhibition of the fusion of late endosomes with tonoplast (Fig. 7) and ROS generation (Fig. 8).

PI3K Activity Is Closely Correlated with Root Hair Elongation

To provide direct genetic evidence for the role of PI3K and its product PtdIns(3)P on root hair growth, we attempted to isolate homozygous T-DNA insertional knockout-PI3K plants (SALK-007281 and GABI-418H02), but no homozygous mutant line could be identified. The self-fertilized heterozygous plants produced no homozygous PI3K-null seeds, because they were unable to produce viable male gametophytes carrying the *pi3k* allele (unpublished data). Heterozygous plants had no differences in their root hair growth compared with wild-type plants (Fig. 2). We

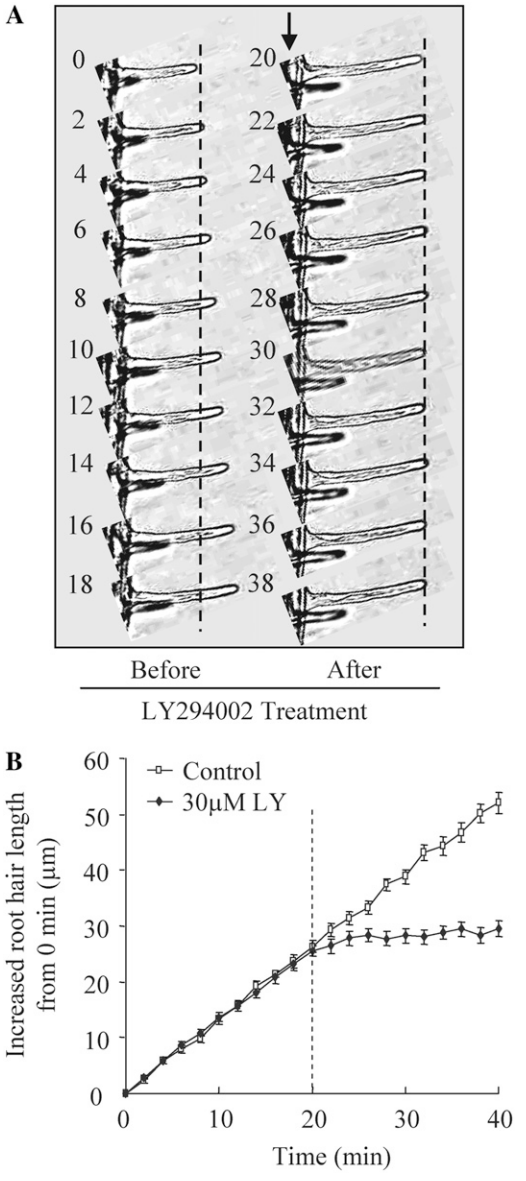

Figure 5. The effect of LY294002 on a single root hair. A, Five-day-old seedlings were transferred to a slide glass chamber, incubated for stabilization during 4 h, and their root hairs were photographed every 2 min. LY294002 was added into the medium at 20 min after initiation of the experiment (arrow). B, Growth rates of root hairs treated with LY294002 or DMSO (solvent control) were analyzed. LY294002 or DMSO was added to the medium at 20 min after initiation of the experiment (dotted line). Root hairs stopped growing by 2 min after treatment with 30 μM LY294002 (diamonds; $n = 28$), whereas they continued to grow after application of DMSO only (squares; $n = 19$).

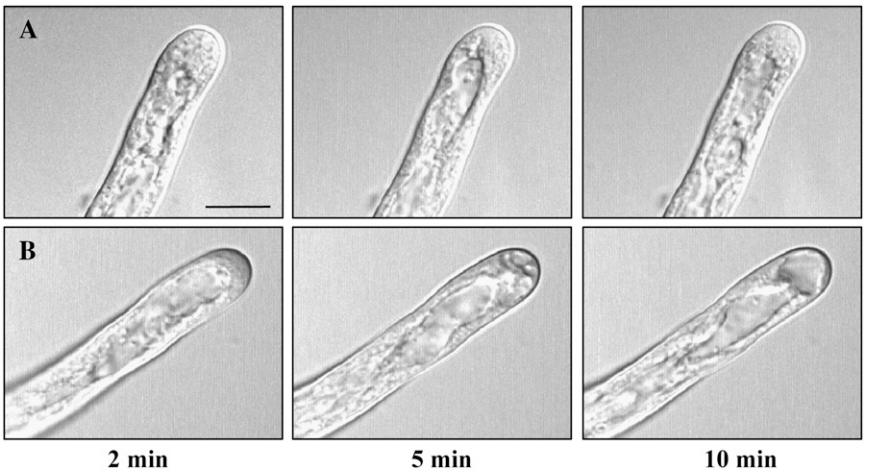

Figure 6. Treatment with LY294002 alters the cytoplasmic architecture of root hairs. A, Control growing root hair. The root hair tip was filled with dense cytoplasm. Bar = 10 μm. B, LY294002-induced (30 μM) protrusion of the vacuole into the root tip region.

also attempted to reduce the *PI3K* level using an RNA interference method, but only lines with a slight change in expression levels survived and these did not show any phenotypes (data not shown). Finally, we stably expressed a PtdIns(3)P-binding domain (FYVE) in Arabidopsis and were able to show that it reduced root hair growth in an expression level-dependent manner (Fig. 3). The FYVE domain we used specifically binds to PtdIns(3)P and has been used previously as a specific biosensor of this lipid (Gillooly et al., 2000; Voigt et al., 2005; Vermeer et al., 2006) as well as a blocker of signal transduction downstream of PtdIns(3)P by interfering with the normal interactions of PtdIns(3)P with other molecules. In the absence of viable mutants with reduced levels of PI3K, FYVE-expressing plants provided the second best genetic evidence for the involvement of PI3K in root hair growth. This conclusion was further supported by the results obtained using LY294002, a PI3K inhibitor (Fig. 4).

Root hair growth was severely inhibited by LY294002. The effect of LY294002 on root hairs was specific to elongation at the root hair tip (Fig. 4). Neither the root hair initiation site nor the number of root hairs was affected by treatment with LY294002 for 1 d. The effect of LY294002 on tip growth was not only very strong but also very rapid. Root hair growth was abolished within minutes after treatment with LY294002 (Fig. 5). This very rapid effect of the inhibition of PI3K was also reported by Vermeer et al. (2006); in that study, the yellow fluorescent protein:2xFYVE label disappeared from the vesicles and simultaneously appeared in the cytosol and nuclear area within minutes after treatment with wortmannin, another inhibitor of PI3K. The rapid effect of LY294002 was also apparent in cytoplasmic architecture: we could detect the protrusion of the vacuole into the root tip region within minutes of treatment with LY294002 (Fig. 6). This rapid disintegration of the clear zone and vacuolization of the root hair tip may have been mediated by the alteration of cytosolic Ca^{2+} oscillation and/or actin organization. A tip-focused gradient of cytosolic Ca^{2+} (Felle and Hepler, 1997; Wymer et al., 1997) and polarized actin organization (Miller et al., 1999) provide the spatial information for maintaining the clear zone, and LY294002 interferes with both Ca^{2+} oscillations and actin dynamics in guard cells (Jung et al., 2002; Choi et al., 2008).

PtdIns(3)P-Mediated Vesicle Trafficking Is Important for Root Hair Growth

The strong and rapid effect of LY294002 may be mediated by its role in vesicle trafficking, which is essential for establishing and maintaining polarity in root hairs. Interestingly, the drug altered only the late stage and not the initial stage of endocytosis; LY294002-treated root hairs had normal uptake of FM1-43 into endosomes, but they were compromised in the final fusion of the late endosomes to the tonoplast (Fig. 7). Inhibition of the fusion of late endosomes with tonoplast may inhibit the normal functions of vacuoles, such as maintenance of turgor, regulation of cytoplasmic ion levels and pH, and storage of amino acids and sugars. The maintenance of normal vacuolar functions requires many vacuolar resident proteins, including transporters and channels. Many of these proteins are delivered by vesicle trafficking from the endosomes to the vacuole (Vida et al., 1993; Marty, 1999; Wurmser et al., 1999), and the lack of endosomal fusion with the vacuole may alter the composition of lipids and proteins inside the vacuole as well as in the tonoplast and, thus, impair normal vacuolar functions. In particular, failure to maintain normal turgor pressure can cause reduction in the elongation of root hair cells.

This effect of the PI3K inhibitor on the late stage of endocytosis in root hairs is different from the recently published data showing that wortmannin inhibits the initial uptake of FM1-43 in Arabidopsis roots under salt stress (Leshem et al., 2007). The difference may be due to the difference in the drugs used (LY294002 versus wortmannin) or in the cell types observed (root hair cells versus other root cells). LY294002 specifically inhibits PI3K, whereas wortmannin inhibits not only

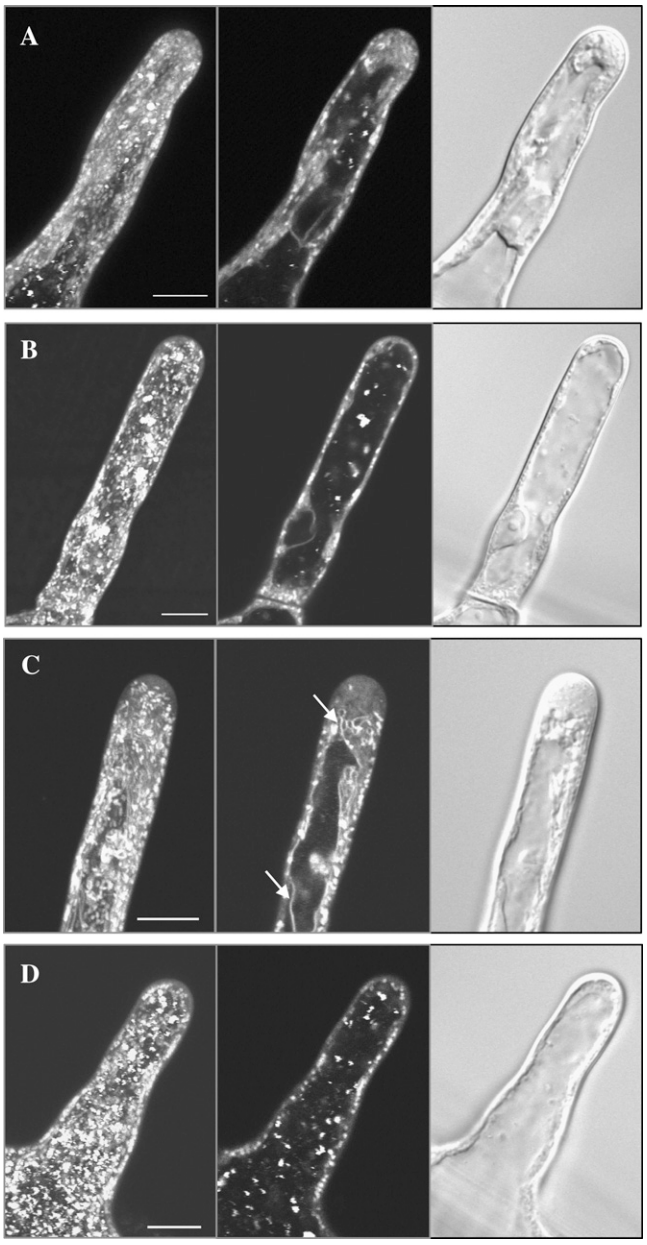

Figure 7. Treatment with LY294002 inhibits the final stage of endocytosis in root hair. Endocytosis was analyzed by pulse treatment of root hairs with 5 μM FM1-43 for 5 min followed by washing. Bars = 10 μm. A and B, Images of control root hairs (A) or root hairs preincubated in 30 μM LY294002 for 1 h (B; left, Z-stack image; middle, single layer of the middle plane; right, DIC image) at 30 min after washing. Staining of endosomes with FM1-43 is similar in control and LY294002-treated root hairs. C and D, Images of control root hairs (C) or root hairs preincubated in 30 μM LY294002 for 1 h (D) at 3 h after washing. The tonoplast was stained with FM1-43 in control root hairs (arrows) but not in root hairs treated with LY294002.

PI3K but also PI4K and PIPK (Jung et al., 2002). PI3K may also have different roles depending on the cell type. For example, in animal cells, PI3K has been reported to play various roles in vesicle trafficking, and PI3K inhibition has multiple phenotypes, including the inhibition of clathrin-dependent endocytosis (Martys et al., 1996; Spiro et al., 1996), receptor sorting in the early endocytic pathway (Siddhanta et al., 1998) and endosomal fusion (Jones and Clague, 1995; Li et al., 1995), the formation of enlarged late endosomes (Reaves et al., 1996; Fernandez-Borja et al., 1999), and inhibition of the delivery of procathepsin D from the trans-Golgi network to the lysosomal compartment (Brown et al., 1995; Davidson, 1995). These multiple functions of PI3K are possible because its product PtdIns(3)P interacts with diverse partner molecules, forming different complexes under different conditions or cell types. PtdIns(3)P is specifically recognized by proteins containing the Cys-rich zinc finger FYVE domain. This domain is found in many proteins implicated in membrane trafficking, including EEA1, Fab1, Vac1, Hrs, and Rab5 (Stenmark and Aasland, 1999; Nielsen et al., 2000), which are likely responsible for the diverse effects of PI3K activation. In Arabidopsis, there are many proteins containing FYVE domains, but their roles have not been well studied. Identification of the molecular complex and interacting partners of PI3K and PtdIns(3)P will be essential to fully understand the roles of PI3K in plant cells.

PtdIns(3)P-Mediated ROS Production Is Important for Root Hair Growth

Tip-localized ROS have been reported to be important signal mediators in growing root hairs, and NADPH oxidase has been suggested to be responsible for ROS generation (Foreman et al., 2003). In animal cells, PtdIns(3)P stimulates ROS generation via binding to the PX domain of $p40^{phox}$ (Ellson et al., 2001), a soluble factor of the NADPH oxidase complex. $p40^{phox}$ localizes to endosomes by binding to PtdIns(3)P (Zhan et al., 2002). ES cell lines possessing mutant $p40^{phoxR58A}$, in which PtdIns(3)P binding to the PX domain of $p40^{phox}$ has been prevented, show large reductions in intracellular ROS production in response to infection by *Staphylococcus aureus* (Ellson et al., 2006). In this cell type, inhibition of PtdIns(3)P accumulation by wortmannin correlates well with the inhibition of *S. aureus*-induced intracellular ROS production, suggesting that the two events may be causally linked. In plants, neither cytosolic factors of the NADPH oxidase complex nor intramembrane ROS generation mediated by NADPH oxidase has been shown. An in-gel NADPH oxidase activity assay using plasma membrane fractions of tobacco leaves indicates that Rboh proteins are localized in the plasma membrane and can produce O_2^- in the absence of additional cytosolic components (Sagi and Fluhr, 2001). However, this result does not exclude intracellular localization of some Rbohs because NADPH oxidase activity was not tested using other fractions of the cell. Recently, Leshem et al. (2007) reported that salt stress triggers PI3K-dependent plasma membrane internalization and ROS production within the endosomes of root cells. Intracellular ROS were encapsulated by endosomal membrane in

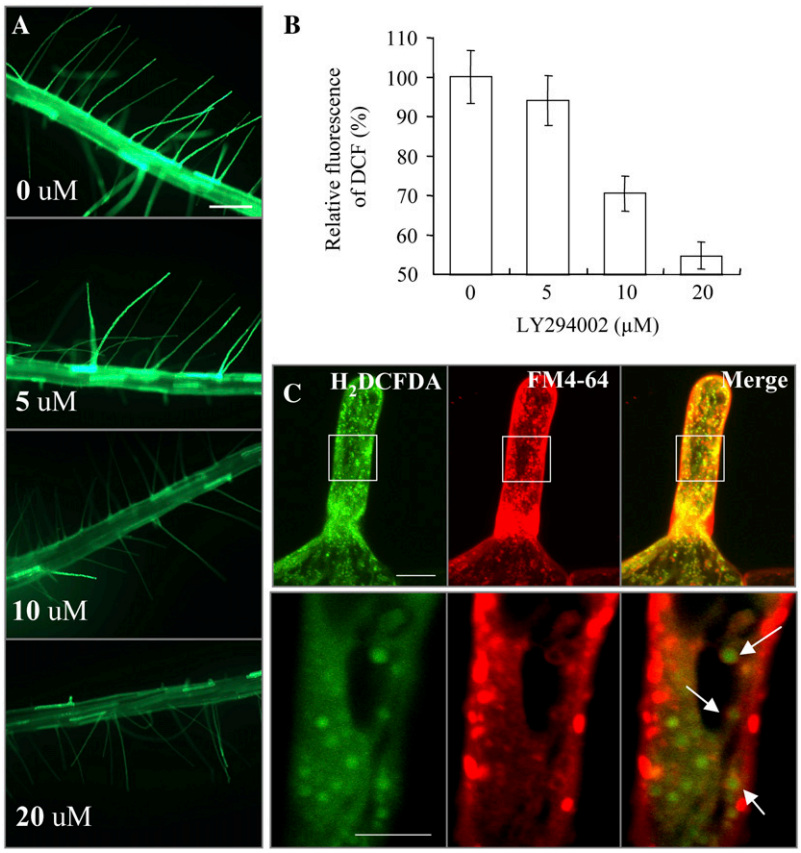

Figure 8. Treatment with LY294002 decreases the intracellular ROS level. A, Fluorescence images of root hairs pretreated with or without LY294002 and then loaded with DCF-DA solution. ROS level decreased in response to LY294002 in a dose-dependent manner. Bar = 200 μm and applies to all photographs. B, ROS level was analyzed by fluorescence intensity of DCF-DA dye. Values represent means ± SE from 47 to 60 root hairs. C, Top, fluorescence image of a root hair double stained with DCF-DA (green) and FM4-64 (red). Bar = 10 μm. Bottom, magnified views of the rectangular areas in the top images. Arrows (bottom) indicate ROS (green color) surrounded by endosomal membrane (red color). Bar = 5 μm.

root cells and were interpreted as the product of NADPH oxidase internalized from the plasma membrane in response to salt stress. In root hair cells, we also found ROS inside endosomes, and the level of ROS in these organelles was reduced after treatment with LY294002 (Fig. 8). This result suggests that endosomes may be a major source of ROS in growing root hairs, and PtdIns(3)P may stimulate ROS generation at the endosomal membrane. It would be interesting to test whether ROS are generated inside endosomes in other cases in which the importance of PI3K in ROS generation has been suggested: for instance, in guard cells during stomatal movement, during root hair curling, and during the gravity response of roots (Park et al., 2003; Joo et al., 2005; Peleg-Grossman et al., 2007).

To elucidate the molecular mechanisms of ROS stimulation by PI3K in root hair cells, we must first understand the molecular identities of NADPH oxidases. AtrbohC/RHD2 encoding a NADPH oxidase is considered a major source of ROS generated in root hairs because *rhd2* mutant plants, which are impaired in ROS production in their root hairs, can only develop root hairs up to the bulge stage and cannot elongate the cells longer (Foreman et al., 2003). However, we were able to rescue this severe phenotype of the *rhd2* mutant by elevating the pH of the growth medium (Fig. 9), as was also shown in a recent article by Monshausen et al. (2007). Our data from *rhd2* mutant plants suggests that ROS generators other than RHD2 are also involved in intracellular ROS generation during root hair tip growth. In the root hairs of *rhd2* mutants grown at pH 6, intracellular ROS content was as high as 77.0% ± 3.8% compared with the wild type. This ROS level in *rhd2* mutants was further reduced by LY294002 similarly as in the wild-type plants, and as a result, the ROS level was only slightly lower in the mutant than in the wild type in the presence of the PI3K inhibitor (Fig. 9D). The fact that LY294002 inhibited root hair elongation and reduced ROS levels to similar extents in the wild-type and mutant plants that did not express *RHD2* indicates that AtrbohC/RHD2 is not the most important ROS generator in root hair during the elongation stage.

Arabidopsis has a family of 10 NADPH oxidase genes, named Atrboh (for Arabidopsis respiratory burst oxidase homologues; Keller et al., 1998; Foreman et al., 2003), and according to the microarray data of Genevestigator, all genes except *AtrbocD*, *-H*, and *-J* are expressed in the root hair zone (http://www.genevestigator.ethz.ch). The necessity for ROS generators in addition to RHD2 for root hair cell elongation was suggested by Monshausen et al. (2007), based on the similarity of ROS patterns in the extracellular space of *rhd2* mutant and wild-type plants. Our observation was focused on endosomes rather than on extracellular space, but they led to the same conclusion that additional NADPH

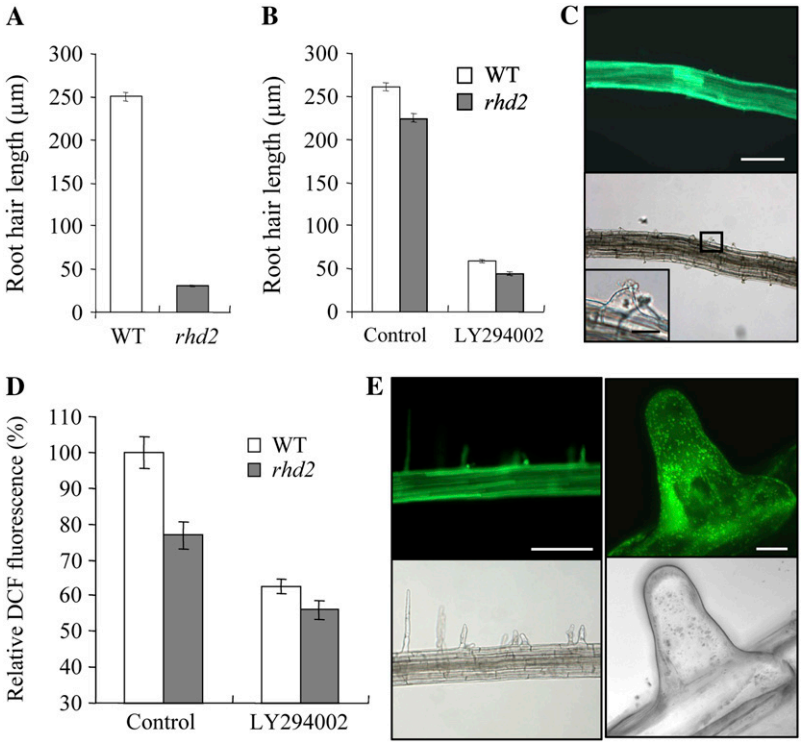

Figure 9. Treatment with LY294002 inhibits root hair growth and decreases intracellular ROS level of *rhd2* plants. A, Mature root hair length of wild-type and *rhd2* mutant plants grown on half-strength MS plates at pH 5. Values represent means ± SE from 230 to 250 root hairs. B, LY294002 effect on the growth of wild-type and *rhd2* mutant root hairs at pH 6. Seedlings were grown on half-strength MS plates for 4 d, transferred to plates containing 30 μM LY294002, and then grown for an additional 1 d. Values represent means ± SE from 320 root hairs. C, Fluorescence and DIC images of root hairs of *rhd2* mutant plants grown at pH 5 and loaded with DCF-DA. Bar = 200 μm. The inset shows a magnified image of the square area with a burst root hair. Bar = 20 μm. D, LY294002 effect on ROS level of *rhd2* mutant root hairs grown at pH 6. ROS level was analyzed by fluorescence intensity of DCF-DA dye. Values represent means ± SE from 120 to 160 root hairs. E, Fluorescence and DIC images of root hairs of a *rhd2* mutant plant grown at pH 6 and loaded with DCF-DA solution. Bar = 200 μm. Bar in magnified image = 10 μm.

oxidases are necessary for normal root hair elongation; we observed that endosomes of both wild-type and *rhd2* mutant root hairs contained bright fluorescence of ROS (Figs. 8C and 9E), which indicated that the ROS in these endosomal membranes were unlikely to be generated by RHD2. It is likely that AtrbohC/RHD2 plays a major role in ROS generation at the initial stage of root hair elongation, whereas at the later stage of elongation other ROS generators take over the role.

In summary, we have shown that PI3K is essential for the polarized elongation of root hair cells, as it is necessary for ROS generation inside endosomes and for the final stage of endocytosis in tip-growing root hair cells. The profound and rapid effects of PI3K inhibition on cytoplasmic architecture and root hair growth suggest that PI3K is also important for other signaling components in root hair cells, such as actin dynamics and the activities of small G proteins, in addition to vesicle trafficking and ROS formation. Interactions with diverse molecules are likely to be necessary for PI3K and PtdIns(3)P to perform such diverse functions; therefore, identifying the molecular partners of these molecules will improve our understanding of the mechanisms underlying root hair tip growth.

MATERIALS AND METHODS

Plant Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) plants were grown vertically on half-strength MS agar plates supplemented with 1.5% Suc in a controlled growth room with 16-h-light and 8-h-dark cycles at 22°C ± 2°C.

Measurement of Root Hair Length

Mature root hairs were observed in 5-d-old seedlings grown on half-strength MS agar plates. The 10 longest root hairs were selected at 2.5 to 3.5 mm from the tip of each primary root. To test the effect of LY294002 (A.G. Scientific) on root hairs, 4-d-old seedlings were transferred to drug-containing plates, grown for an additional 1 d, and newly grown root hairs were analyzed. Root hairs were photographed using a DMI 300-D (Nikon) camera, and their length was measured from photographs using the Interactive Measurement software package AxioVision 3.0.6 (Carl Zeiss). The *P* values were determined by Student's *t* test.

Assays for ROS Detection

ROS were detected using the dye DCF-DA (Molecular Probes) as described previously (Foreman et al., 2003). Five-day-old seedlings were incubated for 60 min at 4°C in 20 μM DCF-DA dissolved in DMSO, then washed with 0.1 mM KCl and 0.1 mM $CaCl_2$ (pH 6.0) and incubated for 60 min at 22°C before observation.

Assay for Endocytosis

Internalization of the plasma membrane was assayed using the styryl dyes FM1-43 [*N*-(3-triethylammoniumpropyl)-4-(4-[dibutylamino]styryl)pyridinium dibromide; Molecular Probes] and FM4-64 [*N*-(3-triethylammoniumpropyl)-4-(8-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide; Molecular Probes] at final concentrations of 5 or 10 μM in culture medium. Seedlings were either observed immediately without washing of the dye, or, for pulse labeling, the dyes were removed after 5 min of incubation by washing with plain culture medium. Root hairs were observed after washing for 10 min using a confocal microscope, Fluoview FV1000 (Olympus).

RT-PCR for Detection of *VPS34* Expression in Root Hair Cells

Arabidopsis root hairs were isolated as described previously (Lauter et al., 1996; Bucher et al., 1997), with some modifications. Roots of Arabidopsis seedlings were frozen and stirred in liquid nitrogen, which allows roots and

root hairs to break apart. Root hairs were separated by filtration through a 250-μm mesh. RNA was isolated from the root hairs using the PicoPure RNA isolation kit (Arcturus Bioscience) according to the manufacturer's instructions. Whole root RNA was prepared by grinding the seedling roots in a 2-mL plastic tube. The cDNA was synthesized by the Im-Prom2 reverse transcriptase kit (Promega), and PCR was conducted. Forty PCR cycles were performed with the root hair cell-specific cDNA template and 30 cycles with the whole root cDNA template. The gene-specific primer sets for PCR were 5′-CTTTAGAGATGAAGCTCGTCGGC-3′ and 5′-TGTGACTTGTCATCAGCAATCTTCGAT-3′ for *GLABRA2*, 5′-GTATCATCCCAGTTGCATACCGAA-3′ and 5′-GTTTGGTAGGGCAAAAGCCTAG-3′ for *EXPANSINA7* (Lee and Cho, 2006), and 5′-GAGAGGACGCTATAGTTAAGCGGCCAGTGA-3′ and 5′-TCTAAACGCTCTAGCTCTCCACGCTCATGC-3′ for *VPS34*.

Reporter Gene Constructs

For the *VPS34* promoter:GUS construct, the promoter region of *VPS34* (−1,391 to +125 in relation to ATG) was amplified from the genomic DNA of wild-type plants with specific primers (forward, 5′-GGATCCGTATGAAATTTAGACGATCAAAATTAGGGC-3′; reverse, 5′-AAGCTTCCGATCGTTATATGAAGATATAATCGGTGC-3′). The 35S promoter was eliminated from the pBI121 vector using the restriction enzymes *Hin*dIII and *Bam*HI. The promoter region of *VPS34* and the pBI121 vector were then ligated, and the resulting construct was transformed into Arabidopsis by the floral dipping method (Clough and Bent, 1998). The transformed seeds were selected on half-strength MS agar plates containing 30 μg μL^{-1} kanamycin. For the GUS reporter assay, the selected plants grown on half-strength MS plates were incubated in GUS solution with 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside at 37°C. Chlorophyll was extracted in 70% ethanol solution at 65°C for 50 min.

For the *EXPANSINA7* promoter:GFP-2xFYVE construct, a double FYVE finger of EEA1, which consists of residues 1,325 to 1,411 in duplicate with the linker GSGN, was prepared as described previously (Gillooly et al., 2000) and linked to the enhanced GFPs. The construct was driven by the *EXPANSINA7* promoter, which was isolated using PCR. The primers for the *EXPANSINA7* promoter were 5′-AAGCTTAATTAGGGTCCAAGGTTTGTTC-3′ and 5′-TCTAGATCTAGCCTCTTTTTCTTTATTC-3′. For quantitative analysis of the GFP:2xFYVE fusion protein in the *EXPANSINA7* promoter:GFP-2xFYVE transgenic lines, fluorescence in root hairs of 5-d-old mutant plants was photographed with a CCD camera (AxioCam; Zeiss). Individual root hair regions were delineated from these photographs, and the mean intensity of green fluorescence was measured using the Adobe Photoshop 7.0 software package (Adobe Systems).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number U10669.

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