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Root Hair Formation: F-Actin-Dependent Tip Growth Is Initiated by Local Assembly of Profilin-Supported F-Actin Meshworks Accumulated within Expansin-Enriched Bulges

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Plant root hair formation is initiated when specialized elongating root epidermis cells (trichoblasts) assemble distinct domains at the plasma membrane/cell wall cell periphery complexes facing the root surface. These localities show accumulation of expansin and progressively transform into tip-growing root hair apices. Experimentation showed that trichoblasts made devoid of microtubules (MTs) were unaffected in root hair formation, whereas those depleted of F-actin by the G-actin sequestering agent latrunculin B had their root hair formation blocked after the bulge formation stage. In accordance with this, MTs are naturally depleted from early outgrowing bulges in which dense F-actin meshworks accumulate. These F-actin caps remain associated with tips of emerging and growing root hairs. Constitutive expression of the GFP-mouse talin fusion protein in transgenic *Arabidopsis,* which visualizes all classes of F-actin in a noninvasive mode, allowed *in vivo* confirmation of the presence of distinct F-actin meshworks within outgrowing bulges and at tips of young root hairs. Profilin accumulates, at both the protein and the mRNA levels, within F-actin-enriched bulges and at tips of emerging hairs. ER-based calreticulin and HDEL proteins also accumulate within outgrowing bulges and remain enriched at tips of emerging hairs. All this suggests that installation of the actin-based tip growth machinery takes place only after expansin-associated bulge formation and requires assembly of profilin-supported dynamic F-actin meshworks.

Key Words: **actin; expansin; *in situ* hybridization; maize; profilin; root hair; tip growth, transgenic *Arabidopsis.***

INTRODUCTION

Only few higher plant cell types use localized tip growth for their expansion (see Fig. 1 in Kropf *et al.*, 1998). One of these types is the root hair—a rapidly growing tubular extension of a specialized superficial epidermal cell, the trichoblast (e.g., Bünning, 1951). During the early stages of this unique form of terminal cytodifferentiation, the

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outward-facing cell wall of the prospective epidermal trichoblast grows by means of accretion of cell wall polymers. The emergence and growth of the hair at a distinct domain along the outer epidermal wall occur later when the phase of diffuse cell growth draws to a close. While the polarity of diffuse growth is determined largely by arrays of cortical microtubules (MTs) (for maize roots, see Baluška *et al.*, 1992, 1995), tip growth polarity is dictated via the actin cytoskeleton (for pollen tubes, see Derksen *et al.*, 1995; Vidali and Hepler, 2000b; for root hairs see Miller *et al.*, 1999; Emons and de Ruijter, 2000). Actin filaments (AFs) support the uniquely polarized cytoplasmic architecture and unipolar extension of tip-growing cells in both lower and higher plants (Heath, 1990, 2000; Steer, 1990; Pierson and Cresti, 1992; Geitmann and Emons, 2000; Vidali and Hepler, 2000b).

Instrumental in the actin-mediated regulation of tip growth might be the two small actin-binding proteins, profilin and actin depolymerization factor (ADF), each of which has been shown to increase dynamicity of AFs in response to various exogenous and endogenous stimuli (Carlier, 1998; for plant cells, see Staiger *et al.*, 1997; Gibbon and Staiger, 2000; Kovar and Staiger, 2000). Didry *et al.* (1998) reported that profilin and ADF increase dynamicity of the F-actin via separate mechanisms and in a complementary fashion. Jiang *et al.* (1997) showed that ADF is redistributed toward tips during initiation of root hairs in maize root trichoblasts and remained accumulated there during tip growth of root hairs. Moreover, F-actin (Lloyd *et al.*, 1987) and spectrin-like proteins (de Ruijter *et al.*, 1998) were also reported to be abundant at the very tips of growing root hairs. In conflict with these findings, however, are recent reports claiming depletion of F-actin from extreme apices of growing root hairs (Miller *et al.*, 1999; de Ruijter *et al.*, 1999; Emons and de Ruijter, 2000). Similar depletions of F-actin have been repeatedly reported also for very apices of *in vitro* grown pollen tubes (Miller *et al.*, 1996; Vidali and Hepler, 2000b). In fact, this F-actin depletion has been interpreted as a prerequisite for unhindered vesicle fusions with the plasma membrane (Miller *et al.*, 1999; de Ruijter and Emons, 1999; Emons and de Ruijter, 2000). However, diverse tip-growing cells of lower plants are invariably equipped with dense F-actin meshworks throughout their apices (e.g., Steer, 1990; Heath, 1990, 2000). Also, both pollen tubes and root hairs immediately cease their tip growth when exposed to very low levels of F-actin drugs that do not affect the integrity of subapical F-actin bundles and cytoplasmic streaming (for root hairs, see Miller *et al.*, 1999; for pollen tubes, see Gibbon *et al.*, 1999; Vidali and Hepler, 2000a). All this suggests the existence of delicate F-actin population at extending tips which is essential for the tip growth of both pollen tubes and root hairs.

Here we report that tip-growing hair apices are equipped with dense F-actin meshworks which are assembled during the bulge outgrowth. Moreover, we show that profilin as well as its mRNA accumulate within outgrowing bulges and growing hair tips. This unique F-actin population, which is presumably extremely dynamic due to high amounts of ADF (Jiang *et al.*, 1997) and profilin (Braun *et al.*, 1999; this study), is suggested to be essential for installation of the tip-growth machinery. As part of this process, ER-derived elements positive for HDEL and calreticulin are recruited to support both outgrowing bulges and tip-growing root hair apices. In contrast, the bulge formation seems to proceed even in trichoblasts devoid of AFs and MTs and apparently involves expansin-mediated local cell wall loosening.

MATERIALS AND METHODS

Plant Material, Handling, Experimental Treatments, and Sample Embedding

Maize grains (*Zea mays* L., cv. Alarik) were obtained from Force Limagrain (Darmstadt, Germany), soaked for 6 h, and germinated in moistened rolls of filter paper for 4 days in darkness at 20°C. In order to disturb the cytoskeleton, roots were exposed to the MT depolymerizing drugs oryzalin, and colchicine (Sigma-Aldrich Chemie, Deisenhofen, Germany) and the F-actin depolymerizing drug latrunculin B (Calbiochem, Bad Soden, Germany) (oryzalin and latrunculin B were used at 10^{-5} M, colchicine at 10^{-3} M, for 2 h in each case). Photo-documentation of root apices was done using a stereo-macroscope (Wild, Heerbruggen, Switzerland).

Root tips were fixed either immediately after the drug treatments or following 12 h of growth in order to let MT-devoid and F-actin-devoid trichoblasts develop further. Fixation of selected apical root segments (0–7 mm) was done with 3.7% formaldehyde (made up in stabilizing buffer (SB): 50 mM pipes, 5 mM $MgSO_4$, and 5 mM EGTA, pH 6.9) and lasted for 1 h at room temperature. Following a brief rinse in SB, the root apices were dehydrated in a graded ethanol series diluted with phosphate-buffered saline (PBS) and embedded in low-melting-point (35°C) Steedman's wax (for details, see Baluška *et al.*, 1992). The 10 most longitudinal sections (7 μm thick) were allowed to expand, using drops of distilled water, on slides coated with Mayer's albumen (for further technical details, see Baluška *et al.*, 1992, and Vitha *et al.*, 2000a,b). Scanning electron microscopy of root surfaces was performed using a glycerol liquid substitution technique—for details see Šamaj *et al.*, (1998).

Indirect Immunofluorescence for Conventional Microscopy

Steedman's wax root sections were dewaxed (see Baluška *et al.*, 1992) and incubated with the following monoclonal antibodies: chicken brain anti-α-tubulin (Amersham International, Buckinghamshire, UK), chicken gizzard anti-actin (ICN, clone C4, from ICN, Costa Mesa, CA), and anti-HDEL antibody (Napier *et al.*, 1992; Baluška *et al.*, 1999). The following polyclonal antibodies were applied: *Arabidopsis* anti-myosin VIII (Reichelt *et al.*, 1999), bovine uterus anti-myosin (skeletal and smooth; from Sigma-Aldrich Chemie; M7648), maize anti-profilin ZmPRO4 (Gibbon *et al.*, 1998), maize anti-calreticulin (Napier *et al.*, 1995; Baluška *et al.*, 1999), and maize anti-expansin (generous gift from Daniel Cosgrove). All antibodies were diluted in PBS (1:200, actin and tubulin; 1:100, expansin, profilin, and the two myosins; 1:20, calreticulin; 1:10, HDEL) and applied for 60 min at room tempera-

ture. Specificity of all antibodies in immunofluorescence was confirmed by using preimmune sera (data not shown). Moreover, the same standardized protocol was used for all antibodies and resulted in the expected distribution patterns (e.g., actin and tubulin). In Western blots, all antibodies used recognized expected protein species (data not shown). Lyophilized fluorescent DNase I (Molecular Probes Europe BV, Leiden, Netherlands) was gently mixed with PBS (0.5 μM) and applied on dewaxed sections for 10 min. After another rinse in SB, those sections labeled with the two myosins, profilin, expansin, and calreticulin primary antibodies were stained with FITC-conjugated anti-rabbit IgGs raised in goat (Sigma-Aldrich Chemie) diluted 1:100 in PBS for 60 min at room temperature. Sections labeled with tubulin, actin, or HDEL were stained with FITC-conjugated anti-mouse IgGs raised in goat (Sigma-Aldrich Chemie) diluted 1:200 in PBS for 60 min at room temperature. A further rinse in PBS (10 min) preceded 10 min in 0.01% toluidine blue (diluted in PBS) which diminished autofluorescence of maize root tissues (Baluška *et al.*, 1992) and fluorescence was examined in an Axiovert 405M inverted microscope (Zeiss, Oberkochen, Germany) equipped with epifluorescence and standard FITC filters (BP 450–490, LP 520). Photographs were taken on Kodak T-Max 400 films.

Confocal Microscopy of Samples Prepared with the Freeze-Shattering Technique

The freeze-shattering procedure was modified after Braun and Wasteneys (1998). Young roots were split longitudinally into halves and fixed for 30 min with 1% formaldehyde and freshly prepared 1% glutaraldehyde (Grade I, stored at −20°C) in SB. After several rinses in SB, the buffer was gradually replaced with PBS, incubated three times with freshly prepared 1 mg/ml $NaBH_4$, washed again in PBS, placed in cold (−10°C) methanol for 5 min, rinsed with PBS containing 50 mM glycine, and transferred to PBS containing 0.05% pectolyase and 4 mM mannitol for 60 min. After permeabilization with PBS/glycine containing 1% Triton X-100, the half-roots were gently squashed between two polyethyleneimine-coated microscope slides and frozen in liquid nitrogen for 1 or 2 min. The frozen slides were ripped apart, put back together again, and then pressure was applied to fracture the frozen tissue. After thawing, the root fragments were incubated with the first antibodies, anti-α-tubulin (Sigma-Aldrich Chemie), anti-profilin ZmPRO3 (Karakesisoglou *et al.*, 1996), and anti-actin for 2 h or overnight at 37°C. After three rinses in the same buffer, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies for 2 h at 37°C. Stained cells were rinsed three times with PBS and mounted in 0.1% *para*-phenylene diamine and 50% glycerol to minimize fluorescence fading. Immunofluorescence images were collected using a Leica confocal microscope TCS4D (Leica, Heidelberg, Germany).

Confocal Microscopy of F-Actin in Trichoblasts and Root Hairs of GFP-Talin-Transformed Arabidopsis Seedlings

In order to assess the reliability of our fixation-based results, and considering the published data showing the absence of actin caps at root hair tips (Miller *et al.*, 1999; de Ruijter *et al.*, 1999), we applied confocal microscopy on living trichoblasts during root hair initiation and root hair growth in GFP-transformed *Arabidopsis* seedlings. GFP-mouse talin fusion protein can be constitutively expressed and specifically labels F-actin in all cells without having any apparent impact on plant development (Kost *et al.*, 1998, 2000).

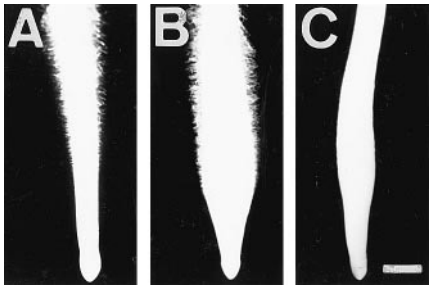

FIG. 1. Latrunculin B inhibits root hair formation. (A, B, C) Representative root apices grown for 12 h in filter paper rolls following 2 h submergence in water (A), oryzalin (10^{-5} M) (B), and latrunculin B (10^{-5} M) (C). Note the absence of hairs in the newly grown portion of latrunculin-treated root apices. Bar = 1020 μm.

In Situ Hybridization

For *in situ* hybridization experiments, the 3′ untranslated region of cDNA for ZmPRO4 was used. To prepare gene-specific probes, the region between nucleotide 608 and nucleotide 793 was amplified by PCR (Perkin-Elmer Cetus TC 480) using the following primers: 5′-TAAAAGTTC GTCATGTTCTG-3′ and 5′-AGTAA-AATGCACTCGTTCCC-3′ (Gibbon *et al.*, 1998). The full-length cDNA ZmPRO4 (generous gift of C. J. Staiger, Purdue University, West Lafayette, IN) was used as a template. The PCR fragment (approx 300 bp) digested with *Bam*HI/*Eco*RI was ligated into pSPT 18/19 vector (Boehringer Mannheim, Germany). Both antisense and sense RNA probes were synthesized with T7 or SP6 RNA polymerases from *Bam*HI- and *Eco*RI-linearized pSPT 18/19 vectors. For RNA labeling with digoxigenin-UTP by *in vitro* transcription the DIG RNA labeling kit (SP6/T7) was used (Boehringer Mannheim, Germany). Immunological detection of the hybridized probes was performed with alkaline phosphatase-conjugated anti-digoxigenin Fab fragment (Boehringer Mannheim) diluted 1:125, dehydrated through a graded ethanol series (last step in 100% ethanol:xylene, 1:1, v/v), and then mounted in Depex (Sigma-Aldrich Chemie).

RESULTS

MTs Are Dispensable for Root Hair Formation in Maize While F-Actin Is Essential

In order to probe the importance of MTs and AFs for root hair formation, root apices were exposed to effective and specific drugs. Root cells were made devoid of MTs by exposing the root apices to oryzalin (10^{-5} M) and colchicine (10^{-3} M) for 2 h. These particular treatments were shown earlier to be effective in depolymerizing all MTs in maize root cells for at least 12 h following removal from the respective solutions (Baluška *et al.*, 1995) and this was confirmed also in the present study (for oryzalin see Figs. 4A and 4B). Despite the absence of MTs, abundant root hairs covered the newly formed swollen portion of the root apex (see Fig. 1B for oryzalin treatment), resembling the abundant root hairs on the control roots (Fig. 1A). On the

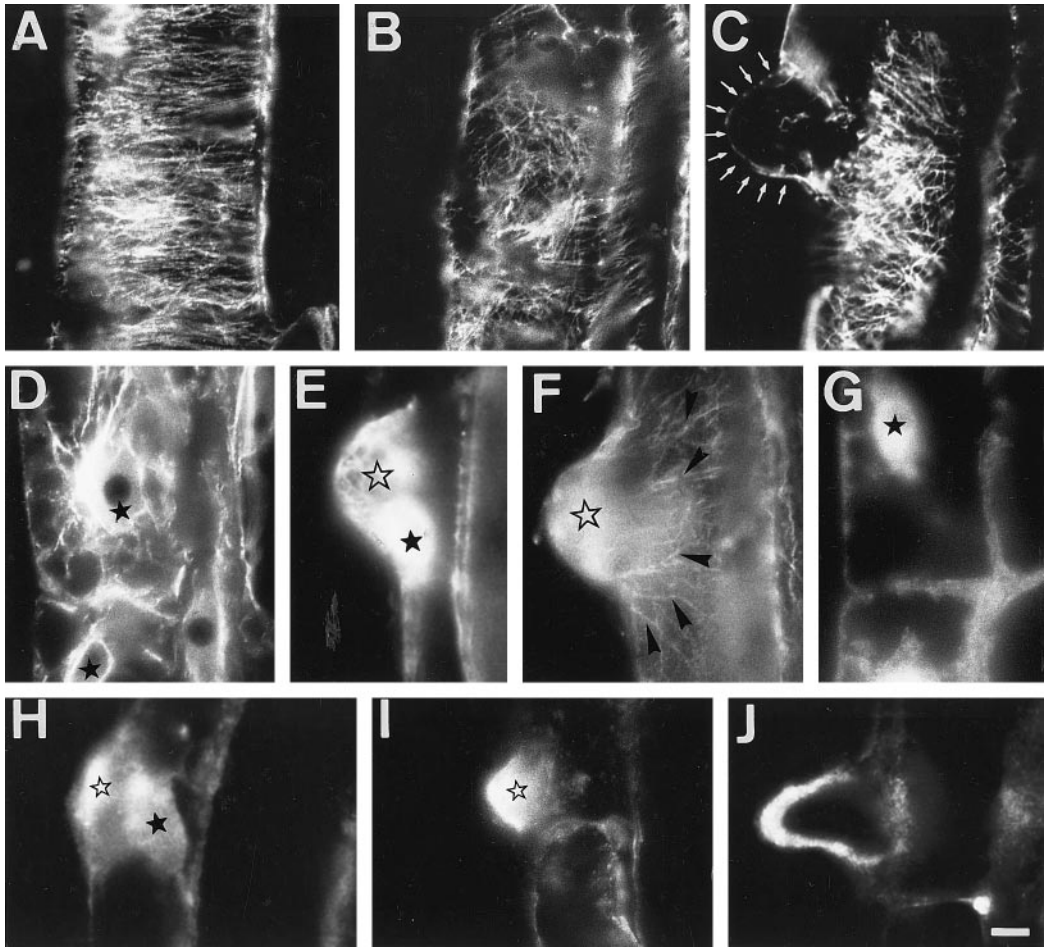

FIG. 2. Redistribution of cytoskeletal elements during root hair formation. (A, B, C) Distribution of cortical MTs in trichoblasts initiating root hairs. (B, C) Maximal randomization of cortical MT arrays occurs during bulge outgrowth (MT-depleted bulging domain is indicated with arrows in C). (D) AFs are organized as prominent perinuclear bundles in trichoblasts commencing root hair initiation. (E, F) Accumulation of actin within the bulging domain during root hair initiation. Note that AFs focus toward the outgrowing bulge (arrowheads in F). (G) Latrunculin B-treated roots (2 h exposure to 10^{-5} M followed by 12 h of root growth in filter paper rolls) exhibit only diffuse actin fluorescence which accumulates preferentially within nuclei, as shown here on an example of epidermis cells. (H, I, J) Accumulation of ZmPRO4-reactive profilins at the bulge and at the apex of an emerging root hair. Asterisks indicate the positions of nuclei, and empty asterisks mark the outgrowing bulges. Bar = 8 μm, except in H, I, J where it represents 12 μm.

other hand, trichoblasts devoid of F-actin after immersing roots into latrunculin B solution (10^{-5} M, 2 h) failed to repolymerize F-actin during the subsequent 12 h (compare Figs. 4C and 4D) and no root hairs formed on the newly grown portion of root apex (Fig. 1C).

MTs in Trichoblasts Initiating Root Hairs: Randomization and Depletion of Cortical Arrays from Outgrowing Bulges

Trichoblasts are equipped with dense arrays of transversely aligned cortical MTs (Fig. 2A). As outgrowth of the bulge proceeds, cortical MTs become depleted and randomized (Fig. 2B), especially in the vicinity of bulged domains (indicated with arrows in Fig. 2C) which are distinctly depleted of MTs (Fig. 2C). Nuclei move toward the bulge and are associated with increasing numbers of radiating endoplasmic MTs (data not shown).

Redistribution of F-Actin and Profilin in Trichoblasts Initiating Root Hairs

Prospective trichoblasts were characterized by a prominent actin-based cytoskeleton which was organized in the form of numerous longitudinal bundles of AFs surrounding the centrally located nuclei and preferentially contacting the end walls (Fig. 2D). After distinct bulges had appeared, a global reorganization of the actin cytoskeleton took place:

there was a prominent accumulation of actin fluorescence within the bulged subcellular domain (Fig. 2E) and AFs became progressively focussed into the bulge (arrowheads in Fig. 2F). This strong actin fluorescence within the bulge appeared diffuse at the level of resolution provided by either conventional or confocal microscopy (see, respectively, Figs. 2E and 2F, and Figs. 8A–D for images taken from GFP-transformed *Arabidopsis* seedlings). Trichoblasts of roots exposed to latrunculin B (10^{-5} M) for 20 min showed only diffuse fluorescence of G-actin-latrunculin B complexes, which accumulated throughout the cytoplasm but localized preferentially within the nuclei (Fig. 4C). Importantly, latrunculin B-treated roots failed to repolymerize F-actin in their cells when kept for a further 12 h in the latrunculin-free environment of moist filter paper rolls (Fig. 4D). No accumulation of actin and profilin fluorescence could be recorded within any root epidermis cell of latrunculin B-treated roots (data not shown).

Outgrowing bulges and tips of emerging root hairs were found to be enriched also with profilin (Figs. 2H–J), an actin-binding protein associated with the dynamic actin cytoskeleton (Gibbon and Staiger, 2000). Early outgrowing bulges were enriched with profilins reactive to antibody raised against the ZmPRO4 profilin isoform of maize (Figs. 2H and 2I). Profilin continued to accumulate at this location in emerging hairs (Fig. 2J). The aberrant bulges of latrunculin-treated trichoblasts do not accumulate profilins (not shown).

F-Actin-Depleted Trichoblasts Still Perform Site Selection Followed by Bulge Formation and Accumulate Expansin within Their Loosened Cell Walls

Although root epidermal cells devoid of F-actin fail to form hairs, closer inspection of their surfaces using a scanning EM technique revealed that the process of bulge formation continued (Figs. 3A and 3B). Nevertheless, when compared with bulges at surfaces of untreated roots, F-actin-devoid bulges are clearly wider and mechanically unstable, as evidenced by their typical bursting or shrinking during the sample preparation for scanning EM (arrowheads in Fig. 3B). This might be attributed not only to the general absence of F-actin networks (Fig. 2D) but also to a more loosened type of cell wall due to local accumulations of expansins. Figures 4E and 4F show expansin at the bulge and hair apex in control roots. Aberrant trichoblast bulges made devoid of F-actin showed local accumulation of expansins (Figs. 4G and 4H). In Fig. 4H, the portion of the aberrant wide bulge which had become depleted of expansin (thin arrow in Fig. 4H) ceased in its outgrowth, whereas a small, expansin-positive domain in the bulge (thick arrow in Fig. 4H) continued to grow.

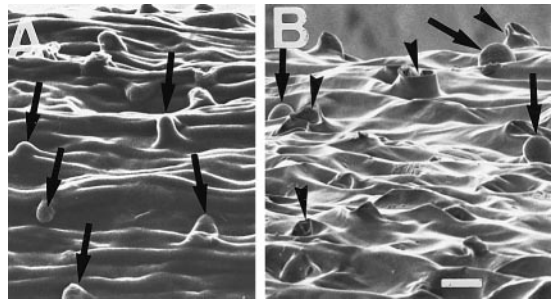

FIG. 3. F-actin-devoid bulges are mechanically unstable. (A) Scanning EM images depicting bulges (arrows) distributed upon control root surfaces. (B) Roots devoid of F-actin in their cells still produce bulges (arrows), but they have more rounded shapes and are mechanically unstable (arrowheads). Bar = 36 μm for A and 22 μm for B.

In Situ Hybridization Reveals That mRNA of ZmPRO4 Profilin Isoform Accumulates within Outgrowing Bulges

In order to analyze further the enrichments of F-actin and profilin within outgrowing bulges and emerging root hair tips, we have performed *in situ* hybridization with an anti-sense RNA probe prepared against the maize profilin isoform, ZmPRO4. ZmPRO4 mRNAs accumulated selectively within the outgrowing bulges (for three different stages of bulge formation see Figs. 5A, 5C, and 5E; and for corresponding controls with sense RNA probe see Figs. 5B, 5D, and 5F). Such mRNA enrichments continued to be preserved also at tips of growing root hairs but, importantly, were absent from epidermis cells in latrunculin B-treated roots (data not shown) indicating that intact F-actin is required for profilin mRNA targeting.

G-Actin and Myosins Do Not Accumulate within Bulges during Root Hair Formation

Neither G-actin pools (Figs. 6A and 6B) visualized with FITC-coupled DNase I (Hitchcock, 1980) nor plant myosins (Fig. 6C for myosin-like protein, Figs. 6D and 6E for the plant myosin VIII) showed any distinct fluorescent capping of the outgrowing bulges and of the tips of emerging hairs. This indicates, as already evidenced by the depletion of tubulin signal within outgrowing bulges, that F-actin and profilin caps do not result from local cytoplasmic accumulations but represent real subcellular accumulations of these proteins and of profilin mRNA.

Rearrangement of ER-Derived Elements in Maize Trichoblasts Initiating Root Hairs

In order to probe distributions of ER-derived elements, cells were labeled with specific monoclonal (HDEL peptide) and polyclonal (maize calreticulin) antibodies raised

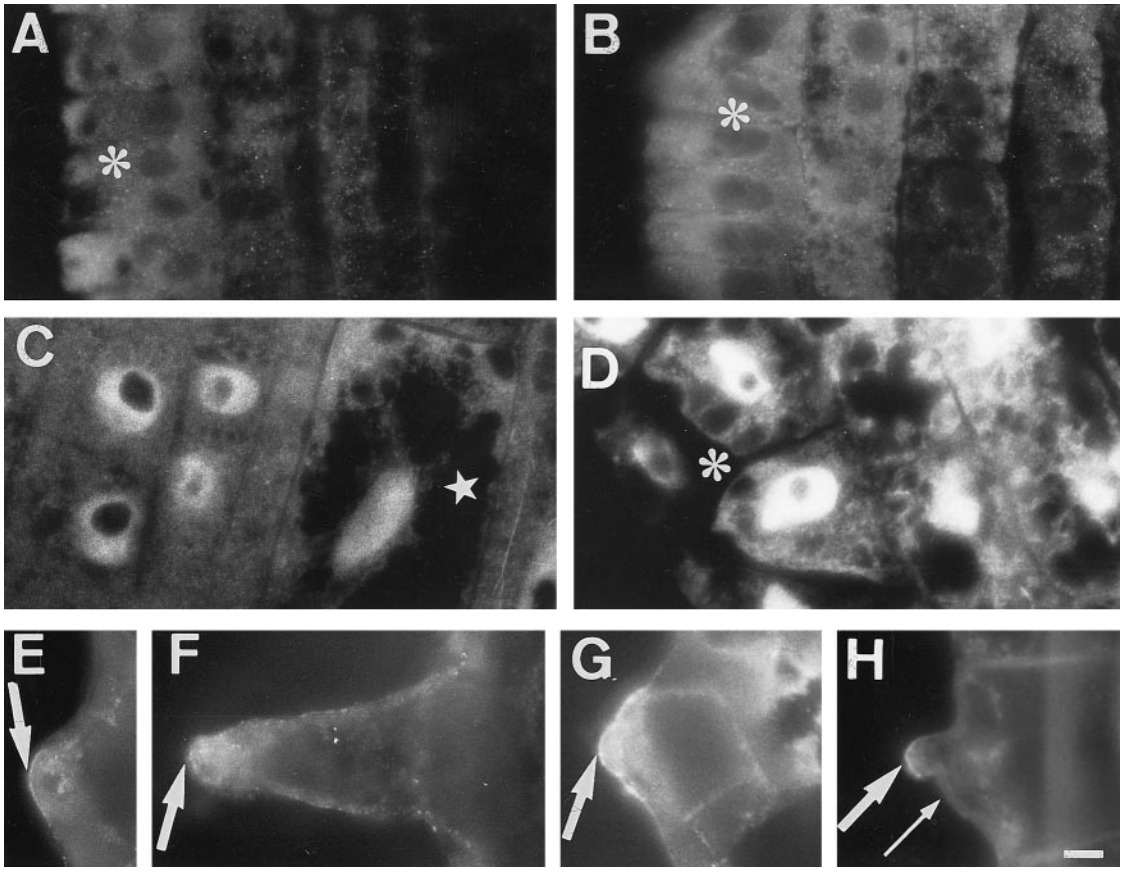

FIG. 4. Effectivity of cytoskeletal drugs and cytoskeleton-independent accumulation of expansin in cell walls of trichoblasts devoid of F-actin. (A) Depolymerization of all microtubules after treatment of maize root apices with oryzalin (10^{-5} M, 2 h). (B) Oryzalin-exposed cells fail to repolymerize microtubules during a subsequent 12 h in the hair-inducing, drug-free environment of filter paper rolls. (C) Depolymerization of all actin filaments after treatment with latrunculin B (10^{-5} M, 2 h). Star indicates metaxylem element. (D) Maize root apices so treated fail to repolymerize actin filaments during the subsequent 12 h in the hair-inducing, drug-free environment of filter paper rolls. Asterisk marks chaotically divided epidermis cells. (E, F) Accumulations of expansin at an outgrowing bulge and at an apex of young root hairs. (G, H) Expansin locally accumulates also in cell walls of aberrant bulges of latrunculin B-treated trichoblasts (thick arrow in G), while bulge portions ceasing their outgrowth show expansin depletion in cell walls (thin arrow in H). In contrast, expansin accumulates further at the growing portion of an aberrant, F-actin-depleted bulge (thick arrow in H). Bar = 8 μm.

against ER antigens. Anti-HDEL antibody highlighted the prospective trichoblasts on account of their extremely abundant and densely arranged ER elements (Figs. 6F and 6F′). An early event during root hair formation was the assembly of an HDEL-enriched domain at the plasma membrane (Fig. 6G). The formation of the bulge was associated with further accumulation of the HDEL signal (Figs. 6H and 6I) and the signal continued to show preferential localization to the hair tips during their tip growth (Fig. 6J). A similar sequence of events characterized the redistributions of maize calreticulin during hair initiation and subsequent growth (Figs. 6K, 6L, and 6M). F-actin-devoid trichoblasts failed to assemble ER-enriched subcellular domains, and the aberrant bulges formed in latrunculin-treated trichoblasts did not accumulate the ER-derived protein elements that are positive for HDEL and calreticulin antibody reactions (for HDEL proteins see Figs. 6N–P).

Confocal Microscopy of F-Actin and MTs in Trichoblasts Initiating Root Hairs

In order to support the above immunofluorescent images obtained using Steedman's wax, immunofluorescence analysis was performed using the freeze-shattering method combined with confocal microscopy (Braun and Wasteneys, 1998). This allowed whole trichoblasts to be viewed revealing further relevant details on cytoskeletal rearrangements during root hair formation. The developmental sequence of progressive AF bundle rearrangements during root hair formation is documented in Figs. 7A–C. As one of the earliest features, longitudinal AFs

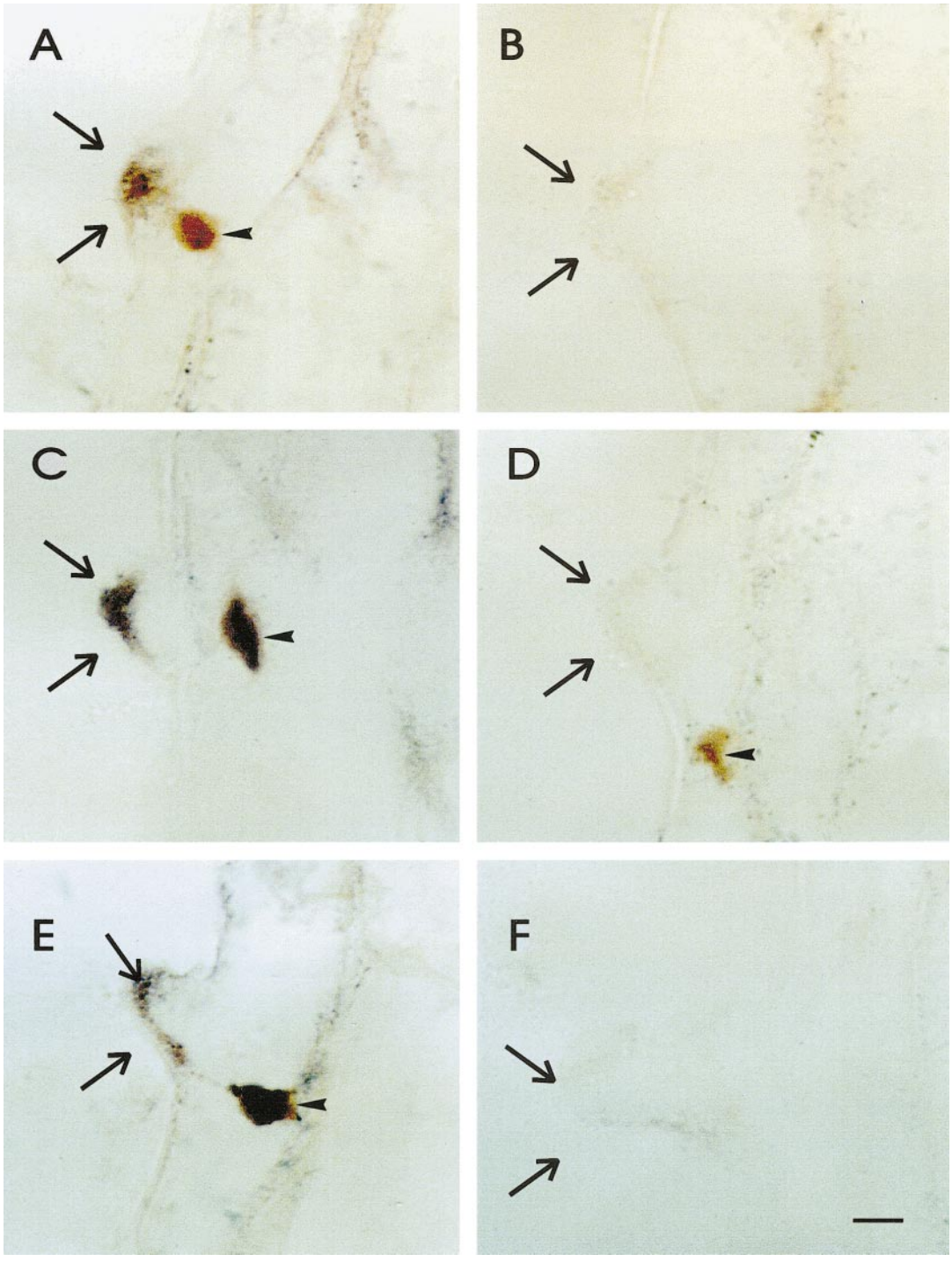

FIG. 5. Redistribution of ZmPRO4 profilin mRNA during root hair formation. (A, C, E) Profilin mRNA hybridized with an anti-sense RNA probe accumulate within tips of outgrowing bulges throughout root hair formation (indicated by arrows). (B, D, F) Controls with sense RNA probe show no signal within bulges (see arrows) of similar developmental stages as shown in A, C, and E. Nuclei are indicated by arrowheads. Bar = 12 μm.

were seen to run between cross-walls and start to contact the prospective bulging site (Fig. 7A). Later, diffuse actin fluorescence selectively accumulated within the bulge toward which transvacuolar bundles of AFs selectively focussed (Fig. 7B) and remained there during the later stage of root hair emergence (Fig. 7C). In contrast, ran-

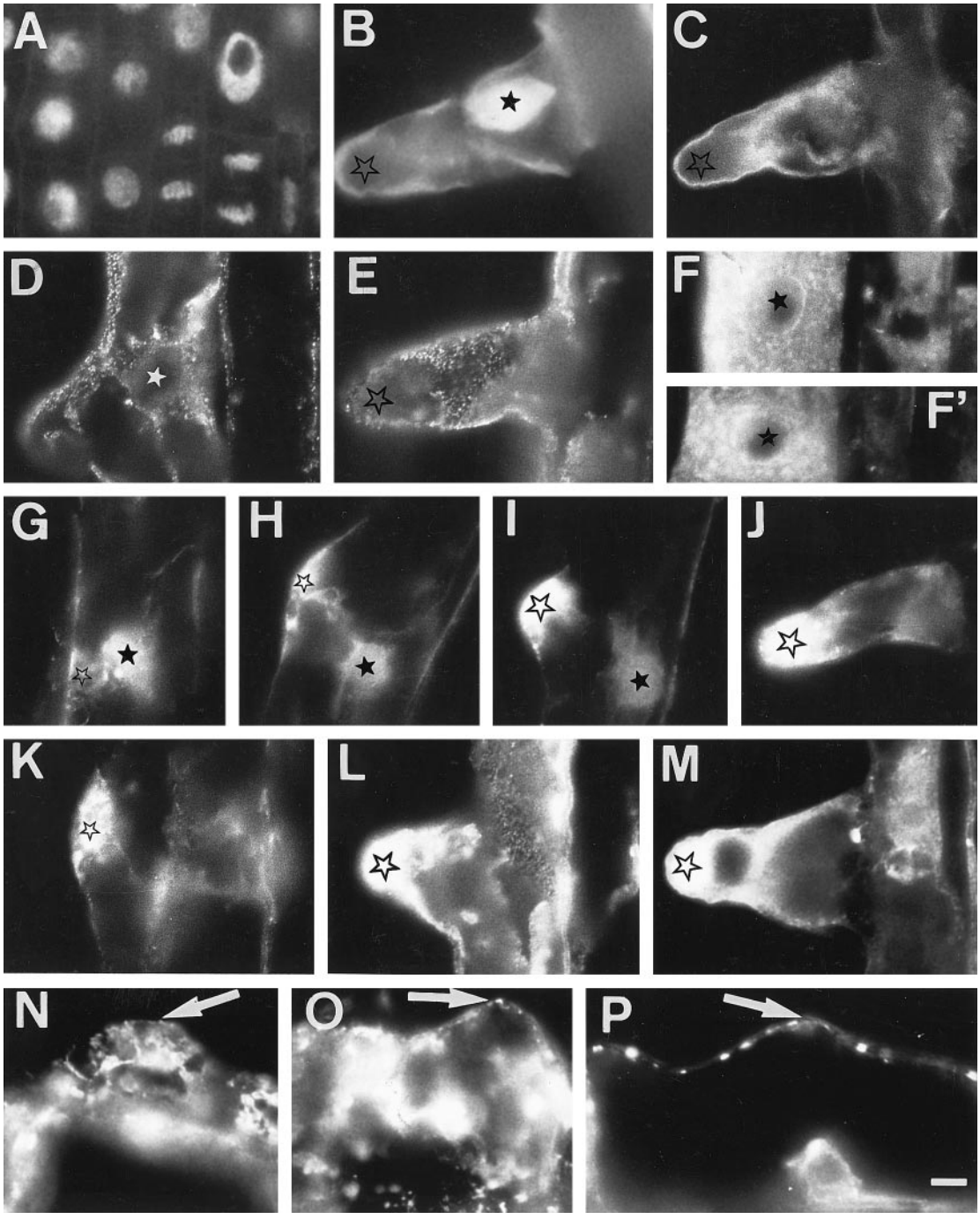

FIG. 6. Distribution of G-actin, myosins, and ER elements in trichoblasts and emerging root hairs. (A) Fluorescent DNase I-positive nuclei within the meristem. (B) Fluorescent DNase I reveals homogeneous G-actin distribution within the emerging root hair. (C) Myosin-like protein is also distributed homogeneously throughout the emerging root hair. (D, E) Unconventional plant myosin VIII in young root hairs cut either medially (D) or tangentially (E) to include the plasma membrane within the section. (E, F′) Strong HDEL fluorescence in trichoblasts located within the basal part of the transition zone (F) and within the apical part of the root elongation region (F′). (G, H, I) Accumulation of HDEL signal at the site of a prospective (G) and an outgrowing (H, I) bulge. (J) Preferential enrichment of the HDEL signal at the growing root hair tip. (K, L) Prominent calreticulin accumulations at the outgrowing bulge (K) and at the tip of an emerging root hair (L). (M) Strong calreticulin signal remains at the tip of a growing root hair. (N, O, P) ER retention HDEL signal does not accumulate within bulges devoid of F-actin (arrows in O and P), although some loose accumulation is visible in a few bulges (arrow in N). Positions of nuclei are indicated with asterisks, whereas empty asterisks mark the outgrowing bulges. Bar = 8 μm.

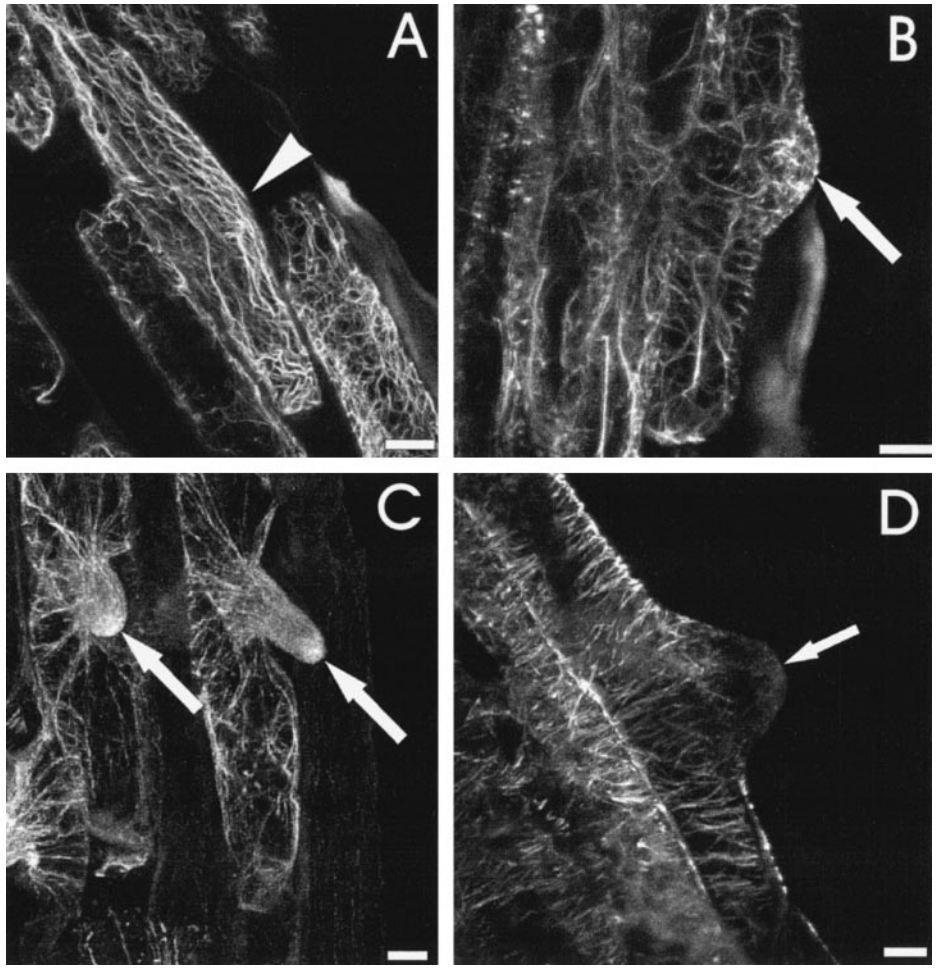

FIG. 7. Confocal microscopy of AFs and MTs visualized with a freeze-shattering technique. (A, B, C) Confocal images of the actin cytoskeleton in cells of maize root epidermis. (A) Predominantly longitudinal actin AFs contacting the presumptive domain (arrowhead) at the outer tangential cell wall initiating bulge formation. (B, C) Later in the process of root hair formation, AFs randomized and accumulate within the outgrowing bulge (B) and at emerging root hair tips (arrowheads in C). (D) Tubulin fluorescence reveals that MTs are depleted within the outgrowing bulge (arrowhead). Bar = 10 μm.

domized arrays of cortical MTs were depleted throughout the outgrowing bulges (Fig. 7D).

Confocal Microscopy of Abundant F-Actin within Bulges and at Tips of Emerging Root Hairs in GFP-Talin-Transformed Arabidopsis Seedlings

The complete *in vivo* sequence of F-actin accumulations during root hair formation was recorded using a confocal microscope applied to trichoblasts of living GFP-talin-transformed *Arabidopsis* seedlings in which all their cellular F-actin was made fluorescent (Kost *et al.*, 1998, 1999, 2000; Mathur *et al.*, 1999). Four images document the bulge outgrowth (Figs. 8A and 8B), young recently emerged hairs, and rapidly growing root hairs (Fig. 8C). In this later phase of root hair formation, accumulation of F-actin toward actively growing root hair tips was at its maximum; distinct F-actin caps were present throughout the growth phase of root hairs (for more mature root hairs, see Fig. 8D).

DISCUSSION

Bulge Site Selection and Bulge Outgrowth

We propose that formation of root hairs by the trichoblasts of the epidermis involves first the selection and isolation of a small site beneath the epidermal outer cell wall, and then the installation at this site of a special cytoskeletal framework which organizes the onset of tip growth. Our data document that both site selection and early bulge formation are cytoskeleton independent. In this respect, root hairs differ from hairs of lower plants

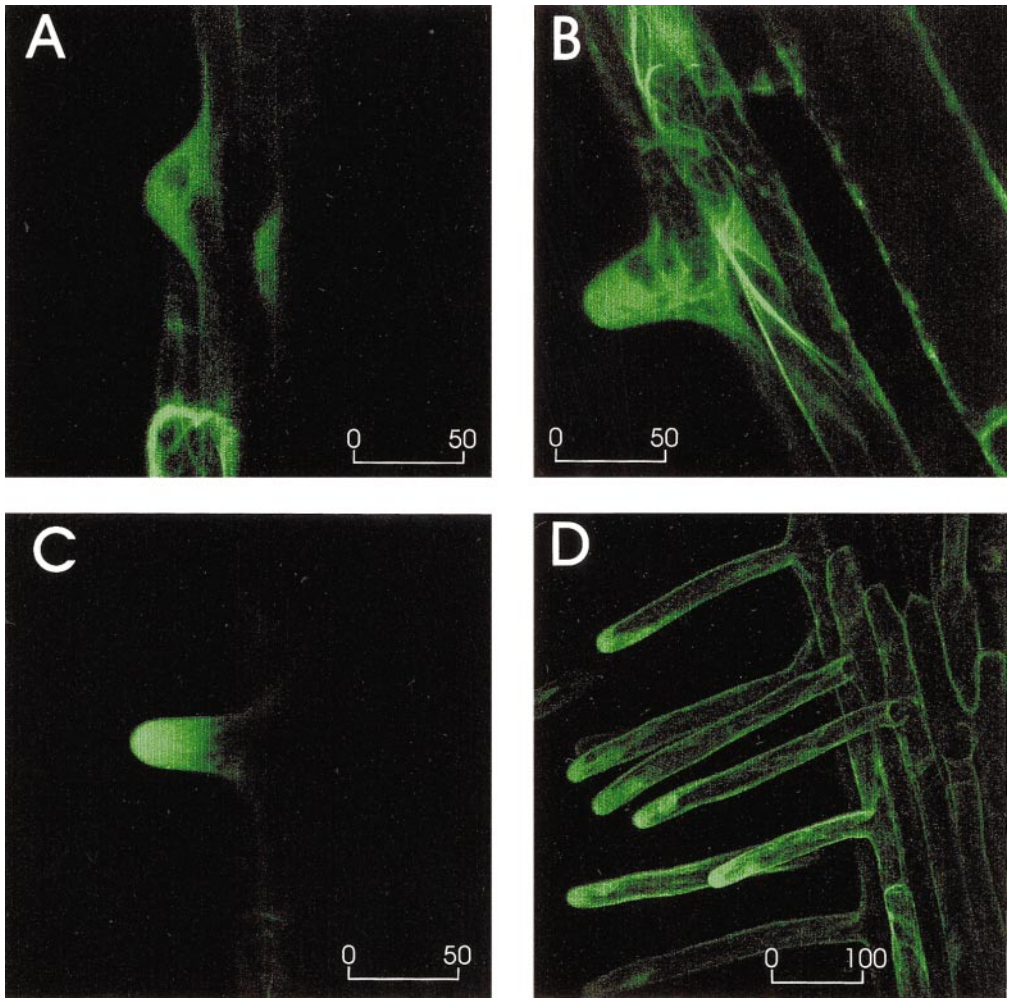

FIG. 8. *In vivo* confocal microscopy of F-actin in GFP-talin-transformed Arabidopsis. Developmental *in vivo* sequence of F-actin rearrangements and its accumulation within the outgrowing bulge (A) and at tips of emerging (B) and growing (C, D) root hairs of GFP-talin-transformed *Arabidopsis* roots. Bars = 50 μm for A, B, C, and 100 μm for D.

where F-actin patches or arrays mark the sites destined to initiate tip growth (Bachewich and Heath, 1998; Alessa and Kropf, 1999; Hable and Kropf, 1998, 2000). However, the dense F-actin meshworks enriched with profilin assemble within outgrowing bulges and they remain associated with extending tips of growing root hairs. These F-actin meshworks appear to be essential for both the onset and the maintenance tip growth, a situation which now corresponds well with the findings from lower plants (Heath *et al.*, 1990, 2000). Bulges formed in latrunculin B-treated roots often burst or show aberrant shapes, indicating that submembraneous F-actin meshworks are critical for stabilization of the plasma membrane within these subcellular domains. Exposure of growing root hairs to latrunculin B also results in an immediate malformation of their tips (Ovecka *et al.*, 2000). Similar findings were reported for bulges and early root hairs of *Vicia sativa* formed in the presence of cytochalasin D (see Fig. 6d in Miller *et al.*, 1999).

Rearrangement of MTs and Their Depletion from Bulge

MTs within growing hairs are distributed throughout the cytoplasm, running longitudinally and interconnecting the subapical nuclei with growing hair tips (Lloyd *et al.*, 1987; Emons, 1987; Ridge, 1988). This contrasts with the transverse (with respect to the root growth axis) dense arrays of cortical MTs which are typical for cells showing diffuse growth and which direct their own growth polarities (for maize roots, see Baluška *et al.*, 1992). The initiation of root hairs is associated with randomization and depletion of cortical MT arrays within the trichoblast, together with an

increase in the number of nucleus-associated MTs. Outgrowing bulges are initially depleted in MTs. Later, however, nucleus-associated MTs invade the emerging hairs and become involved in the polarization of the MT-nucleus-based plant cell body (Baluška *et al.*, 1997, 1998, 2000) leading to nuclear movements behind the extending hair tips (Lloyd *et al.*, 1987; Sato *et al.*, 1995).

Does Expansin-Mediated Cell Wall Loosening Facilitate the Bulge Outgrowth?

Cell walls of bulged domains are enriched with expansins, indicating that cell-wall-loosening factors might be involved in defining the growth of certain cell wall domains. Other possible cell-wall-loosening factors are loosely defined osmiophilic particles (Šamajová *et al.*, 1998) and arabinogalactan proteins (Šamaj *et al.*, 1999), both of which accumulate at the bulges and tips of growing hair apices. In addition to increased amounts of expansins secreted into cell walls, a further possibility is their local activation by means of acidification (McQueen-Mason *et al.*, 1992; Cosgrove, 1996). Such local cell wall acidification was reported by Bibikova *et al.* (1998) as the first recognizable event clearly preceding the bulge outgrowth. In fact, low pH dramatically stimulates root hair formation (Inoue *et al.*, 2000) while high pH inhibits root hair growth and formation (Ewens and Leigh, 1985). The loosened cell periphery domains then bulge out due to the decreased resistance against high internal turgor pressure which might even rise due to activation of potassium channels via apoplast acidification (Hoth *et al.*, 1997). In this respect, the root hair initiation resembles leaf primordium initiation which can be induced via localized upregulation of expansin genes (Fleming *et al.*, 1999).

Local Enrichments of F-Actin and Actin-Binding Proteins: F-Actin-Dependent Switch from the Bulge Outgrowth and Tip Growth

Outgrowing bulges and tips of emerging root hairs are invariably enriched with diffuse actin fluorescence while G-actin pools visualized with fluorescent DNase I do not accumulate to such an extent at these domains. This indicates that the seemingly diffuse actin fluorescence represents dense but delicate meshworks composed of oligomeric F-actin rather than an accumulation of G-actin. These F-actin meshworks typically associate with membranes and are critical for dynamic processes such as exocytosis, endocytosis, and signaling (reviewed by Volkmann and Baluška, 1999; Staiger, 2000). F-actin accumulation at growing root hair tips was reported also by Lloyd *et al.* (1987) using the rhodamine-phalloidin staining. But the most definitive proof for the existence of dense F-actin meshworks within bulges initiating root hairs and at emerging root hair tips is provided by *in vivo* analysis (present study) of GFP-talin-transformed seedlings of *Arabidopsis* in which all F-actin is rendered fluorescent (Kost *et al.*, 1998, 1999, 2000; Mathur *et al.*, 1999).

Root hair initiation in maize roots was reported to be associated with a redistribution of actin depolymerizing factor from trichoblasts toward the apices of emerging root hair tips (Jiang *et al.*, 1997). We have shown that maize profilins, recognized by antibodies raised against maize profilin isoforms ZmPRO3 and ZmPRO4 (Karakesisoglou *et al.*, 1996; Gibbon *et al.*, 1998), accumulate even earlier, within the bulging domain (see also Braun *et al.*, 1999). *In vitro* assay revealed that ADF synergizes by 125-fold the turnover rates of profilin-supported dynamic actin filaments (Didry *et al.*, 1998). All this strongly suggests that the tip growth machinery of root hairs requires a dynamic actin cytoskeleton supported by synergistic actions of ADF and profilin (Didry *et al.*, 1998). Intriguingly, maize profilins can alter actin organization independent of cytosolic calcium concentration (Kovar *et al.*, 2000).

Selective Recruitment of Profilin mRNA into F-Actin-Enriched Bulges and Tips of Root Hairs

There are at least two possibilities for how a small molecule like profilin can selectively accumulate within a distinct subcellular domain. First, profilin mRNA could be targeted toward the domain; site-specific translation then selectively enriches the protein. This is exactly what we have found in trichoblasts initiating root hairs. F-actin-dependent polarized targeting of profilin mRNA is in accordance with data published for other mRNAs (e.g., Jansen, 1999). Secondly, presumptive profilin poly-L-proline partners (e.g., Reinhard *et al.*, 1995) could also contribute to recruitment of profilin into distinct domains, as is known for animal cells (Kang *et al.*, 1997).

ER-Based HDEL Proteins and Calreticulin Accumulate within Bulges Initiating Root Hairs

In different tip-growing systems, ER elements have been reported to accumulate within the dome of the growing tip from where they extend toward the subapical nucleus (for pollen tubes, see, e.g., Derksen *et al.*, 1995). Although growing root hairs have not been analyzed as comprehensively, our data on subcellular localization of ER-resident proteins containing HDEL retention sequences indicate that ER elements are abundant in prospective trichoblasts. Initially, they are distributed throughout the trichoblast cytoplasm. Later, during bulge outgrowth, ER elements accumulate at the domain at the cell periphery which progressively transforms into the emerging hair. Strong ER signals, revealed by two different antibodies recognizing ER-based proteins, are closely associated with growing root hair tips. Our immunofluorescence data strongly suggest that dynamic F-actin meshworks of outgrowing bulges and growing root hair apices, supported by high amounts of profilin (this study) and ADF (Jiang *et al.*, 1997), effectively recruit endomembranes toward these cell periphery domains where they may buffer high cytoplasmic calcium levels (e.g., Wymer *et al.*, 1997).

Do Dense F-Actin Meshworks Only Stabilize Rapidly Extending Tips of Root Hairs?

The dense F-actin meshworks of root hair tips appear to be crucial for both tip growth and morphogenesis of root hairs. They are supposed not only to mechanically stabilize the plasma membrane under a loosened cell wall but also to maintain the integrity of the vesicle-rich "clear zone" needed for tip growth. Ovecka *et al.* (2000) showed that even the shortest exposures to latrunculin B resulted in an immediate disintegration of the cytoplasmic-rich clear zone and vacuolization of the root hair tip. Mature root hairs terminating their tip growth, or younger root hairs interrupted in their tip growth by challenges from cytochalasin D or *Rhizobium* Nod factor, disassemble both their actin and profilin caps (Braun *et al.,* 1999) and lose the clear zone (Miller *et al.,* 1999), whereupon AF bundles invade the vacuolized tips (Miller *et al.,* 1999; de Ruijter *et al.,* 1999). Moreover, cytochalasin D induces branchings of root hair apices (Ridge, 1990; Ovecka *et al.,* 2000). All this conforms with the hypothesis of Steer (1990), that submembranous F-actin mechanically stabilizes growing plant cells.

Another possibility is that actin polymerization supports tip growth more directly, by exerting a pushing force on the plasma membrane (e.g., Borisy and Svitkina, 2000). In support of this, our preliminary data indicate that putative plant homologues of Arp2/3 proteins (Klahre and Chua, 1999), which are crucial components of the F-actin-based nanomachine (Machesky and Gould, 1999; Borisy and Svitkina, 2000; Blanchoin *et al.,* 2000), are accumulated at growing maize root hair apices (F. Baluška, M. von Witsch, and D. Volkmann, in preparation). This suggests that the most dynamic F-actin population, organized perhaps in the form of dedritic meshworks, may exert a pushing force on the plasma membrane (Borisy and Svitkina, 2000; Blanchoin *et al.,* 2000). Borisy and Svitkina (2000) have been proposing that pollen tube tip growth is a form of cell movement in plants and that F-actin polymerization at the tube tip might be a motive force. Indeed, latrunculin B action on tip-growing cells revealed that the most dynamic F-actin population is critical for tip growth but is not required for cytoplasmic streaming (Gibbon *et al.,* 1999; Miller *et al.,* 1999; Vidali and Hepler, 2000b). In fact, this attractive scenario was proposed to be operating in pollen tubes several years ago (Sanders and Lord, 1989; Lord and Sanders, 1992). Numerous studies from lower plants also suggest that tip growth represents a plant-specific form of the actin-driven protrusive cell growth (Pickett-Heaps and Klein, 1998; Heath and Steinberg, 1999; Baluška *et al.,* 2000). Recently, the inherently mobile nature of wall-less higher plant cells was elegantly shown for post-telophase *Haemanthus* endosperm cells. After their extrusion from the embryo sac, these cells extend filopodia-like and lamellipodia-like processes from their surfaces (Bajer and Smirnova, 1999).

Conclusions and Outlook

Both site selection and bulge formation appear to proceed in a cytoskeleton-independent manner and result from local cell wall loosening, an event probably mediated by expansins (Cosgrove, 1996). Mechanically loosened cell wall domains are immediately challenged by high turgor pressure as the acidic apoplastic environment, which is typical of the domains initiating the bulge outgrowth (Bibikova *et al.,* 1998), is known to activate potassium channels (Hoth *et al.,* 1997). Local bulging out of the outer tangential cell walls may recruit actin-binding proteins, like profilin, ADF, and ARPs, which could then enhance the dynamism of F-actin within the bulges and emerging tips of the root hairs. Drug studies suggest that this highly dynamic F-actin population is critical for the onset and continuity of the tip growth of root hairs. Vectorial recruitment of exocytotic vesicles and secretory organelles is one possible scenario of how a polarized actin cytoskeleton can support tip growth. Other plausible roles for the dynamic F-actin caps at the tips, such as the attraction and polarization of MTs-nucleus-based cell bodies (Baluška *et al.,* 1997, 1998, 2000) and the exertion of pushing forces via submembranous actin-based nanomachines (Borisy and Svitkina, 2000; Blanchoin *et al.,* 2000), await further substantiation.

The following testable hypothesis emerges for the root hair initiation and formation: (a) local activation of plasma membrane H^+-ATPases results in acidification of a distinct cell wall domain; (b) this activates and recruits expansins resulting in local cell wall loosening while cortical MTs disassemble; (c) acidification of the apoplast activates potassium channels, thus increasing the turgor pressure; (d) the weakened cell periphery complex fails to resist the high internal turgor pressure and the plasma membrane stretches; (e) activation of putative stretch-activated calcium channels then induces a local increase of cytoplasmic calcium levels which, via modulating the actin cytoskeleton as well as other downstream effectors, culminates in the installation of the tip growth machinery.

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