RESEARCH PAPER

Nuclear dynamics during the simultaneous and sustained tip growth of multiple root hairs arising from a single root epidermal cell

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Abstract

Nuclear dynamics in root hairs, which depends upon the actin cytoskeleton, appears to be an important factor in root-hair tip growth. Previous evidence suggests that there is an absolute requirement for the nucleus to be a fixed distance from the growing root-hair tip for tip growth to proceed. To test this hypothesis, nuclear dynamics were examined in root-hair cells bearing multiple root hairs. The majority of root-hair cells of transgenic plants overexpressing the ROP2 GTPase (ROP2OX) bear multiple root hairs. Simultaneous and sustained fast tip growth occurred in multiple root hairs of ROP2OX, with the continual presence of tip-localized cytoplasm in these growing hairs. Nuclear dynamics were imaged in ROP2OX by co-expressing a transgene encoding a nuclear localization signal (NLS)–green fluorescent protein (GFP) fusion protein. The nucleus was in continual proximity to one of the growing root-hair tips, whilst the other tip elongated at a similar rate but in the absence of the nucleus from the shank of that root hair. To test whether this phenomenon was an artefact of ROP2 overexpression, nuclear dynamics were examined in wild-type and NLS-GFP transgenic plants. Multiple root hairs on the same cell underwent simultaneous and sustained fast tip growth, with the nucleus lying deep within the shank of only one of these hairs. The nucleus was also moved into the root-hair tip during the severe root-hair tip branching which is characteristic of ROP2OX transgenic plants. These results suggest that fast tip growth can proceed in some multiple root hairs at extreme distances from the nucleus.

Key words: Nucleus, multiple root hair, nuclear localization signal, ROP2 GTPase, tip growth.

Introduction

Root hairs are tip-growing cellular processes that arise from specific root epidermal cells, called trichoblasts (Dolan et al., 1994). A complex genetic network controls root-hair morphogenesis in this model plant cell (Parker et al., 2000; Grierson and Schiefelbein, 2002). The onset of tip growth in root hairs occurs immediately after the first visible stage of root-hair morphogenesis, localized swelling formation on the cell surface at the apical (i.e. closest to the root tip) end of the trichoblast (Dolan et al., 1994). Root-hair tip growth requires a tip-high intracellular calcium gradient (Ca\textsuperscript{2+}), and tip-growing root hairs show a tip-localized influx of extracellular Ca\textsuperscript{2+} (Ca\textsuperscript{2+}; Bibikova et al., 1997; Wymer et al., 1997). The actin cytoskeleton is required for tip growth in root hairs (Miller et al., 1999; Baluška et al., 2000), but microtubules, which are required for maintaining the direction of tip growth in root hairs, are not required for tip growth per se (Bibikova et al., 1999). In wild-type (WT) plants, root hairs that are actively tip growing can be detected by the presence of vesicle-rich cytoplasm in the root-hair tip (Miller et al., 1999). Root hairs that are not growing lack this tip-localized cytoplasm, but have a vacuole that extends into the root-hair tip (Miller et al., 1999).

Root-hair tip growth in WT plants is accompanied by the migration of the nucleus into the shank of the growing root hair (referred to hereafter as anterograde migration) and a change in nuclear shape from spheroidal to a more dynamic shape, which is usually elongated along the major axis of the root hair (Galway et al., 1997; Chytilova et al., 2000). Using bright-field microscopy, nuclei of root-hair

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cells are readily identifiable as a large subcellular structure, especially within the shank of the growing root hair (Ketelaar et al., 2002). Nuclei of transgenic plants expressing a nuclear localization signal–β-glucuronidase–green fluorescent protein (NLS-GUS-GFP) construct are brightly labelled with GFP (Chytílova et al., 2000). NLS-GUS-GFP expression does not appear to affect the root-hair cell adversely (Chytílova et al., 2000). Propidium iodide staining of DNA has confirmed that these subcellular structures are nuclei (Chytílova et al., 2000). Inhibitor studies have shown that nuclear dynamics in root hairs of NLS-GUS-GFP plants depends upon the actin cytoskeleton, and not microtubules (Chytílova et al., 2000; Ketelaar et al., 2002). A further study confirmed that this nuclear movement is energy-dependent (Śliwińska et al., 2002). Optical trapping of the nucleus was shown to inhibit tip growth in WT root hairs (Ketelaar et al., 2002). In the same study the nucleus in the cow1-2 root-hair branching mutant was found to undergo transient retrograde migration from one tip towards another tip on the same root hair-bearing cell. Periods of tip growth, with the characteristic tip-localized cytoplasm, alternated between these two tips dependent on the proximity of the nucleus. These results appear to show that nuclear location has an important role in allowing root-hair tip growth to proceed.

ROP GTases are eukaryotic signalling switches that play fundamental roles in plant development (Li et al., 2001; Yang, 2002). It has been shown previously that transgenic plants overexpressing the ROP2 GTPase (ROP2 OX) have multiple swellings on each root hair-forming cell, often widely spaced along the cell length (Jones et al., 2002). Many, but not all, of these multiple swellings on ROP2 OX root hair-forming cells undergo the transition to tip growth, forming multiple root hairs on the same cell that grow longer than WT root hairs, and often branch at their tips (Jones et al., 2002). Investigations were made into the movement of the nucleus within ROP2 OX multiple root hair-bearing cells and within the rare multiple root hairs that form on WT root hair-bearing cells. The results show that the close proximity of the nucleus to the growing tip is not an absolute requirement for tip growth to proceed.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana seed were surface-sterilized for 4 min in 10% (v/v) household bleach, 4 min in ethanol:water:bleach mixture (7:2:1 by vol.), and rinsed 2×2 min in sterile water. Seeds were plated either on the surface of sterile semi-solid growth medium (Jones et al., 2002) containing 0.1% (w/v) phytogel, and grown in horizontal orientation or beneath the surface of semi-solid medium containing 0.5% (w/v) phytogel and grown in vertical orientation. Plated seeds were stratified for 48 h at 4 °C then transferred to growth cabinets at 20 °C with a 24 h light regime. For crossing experiments, seedlings were transferred to soil and grown at 20 °C under a 12 h light/12 h dark regime. The WT Columbia (Col-0) ecotype was used.

ROP2 OX transgenic plants (Li et al., 2001) were selected on medium supplemented with kanamycin (100 µg ml⁻¹). NLS-GUS-GFP transgenic plants were selected by screening for the characteristic punctate GFP fluorescence in root tissue (Chytílova et al., 2000).

Microscopy

Growing root hairs were identified by their tip-localized cytoplasm. Each experiment was replicated at least three times. Microscopy was performed using a Zeiss Axioshot microscope fitted with a Qimaging Retiga 1300 12-bit monochrome CCD camera linked to a computer running OpenLab v3.0.9 (Improvision). Zeiss filter set 10 (excitation 450–490 nm, emission 515–565 nm) was used for GFP detection. All UV exposure times were optimized to <1 s.

Results

Multiple root-hair formation in transgenic plants overexpressing the ROP2 GTPase

The multiple swellings on ROP2 OX root hair-bearing cells (Jones et al., 2002) form simultaneously, but only some of these undergo the transition to tip growth (Fig. 1A). The position of these swellings along the length of the ROP2 OX root hair-bearing cell is biased towards the apical end of the cell (Jones et al., 2002). Although most root hairs form at this apical end of the cell, it is not possible to predict whether an individual multiple swelling will undergo the transition to tip growth or whether any subsequent root hair will sustain tip growth thereafter. Indeed, some root hairs located in basal locations along the cell length continue to elongate, whilst other multiple root hairs located much closer to the apical end of the same cell stop growing (Fig. 1B).

Simultaneous and sustained tip growth of multiple root hairs in transgenic plants overexpressing the ROP2 GTPase without retrograde nuclear migration

Nuclear location and nuclear migration have previously been shown to influence tip growth in both WT and cow1-2 root hairs (Ketelaar et al., 2002), but it is not known whether these factors influence tip growth in ROP2 OX multiple root hairs. To investigate this, observations were made of the location of nuclei in ROP2 OX root hair-bearing cells. The nucleus, which is often clearly visible within the cell body during early root-hair morphogenesis in ROP2 OX multiple root hair-bearing cells (Fig. 1C), subsequently undergoes anterograde migration into the shank of one of the multiple root hairs. For instance, this is sometimes the most apical root hair (Fig. 1D) or the most-basal root hair (Fig. 1E, F) or other combinations, including the middle root hair in cells bearing more than two root hairs (data not shown). To investigate whether the presence or absence of a nucleus within the root-hair shank affected subsequent tip growth, extended periods of tip growth were observed in these ROP2 OX cells. Significantly, during both the early (slow) and later (fast) phases of tip growth (Dolan et al.,
sustained and simultaneous tip growth was observed in ROP2 OX multiple root hairs, supported by a nucleus situated deep within the shank of one of the root hairs (see supplementary Movies S1, S2 at JXB online; Fig. 2A, B).

There was no alternation in the rate of tip growth between these root hairs, no reduction in tip-localized cytoplasm (arrowhead). The arrows indicate the non-growing root hairs. (C) ROP2 OX root-hair cell bearing four multiple hairs undergoing the transition to tip growth. Note the position of the nucleus (arrowhead) close to the base of one of these hairs. (D–F) The nucleus is located in various positions within a root-hair cell bearing multiple root hairs. (D) Both root hairs are tip growing, but the nucleus (arrowhead) is moving into the shank of the most basal root hair. (E) Both root hairs are tip growing (basal root hair is out of the plane of focus) but the nucleus (arrowhead) is moving into the shank of the most apical root hair. (F) Only the most apical root hair is tip growing as the basal root hair lacks tip-localized cytoplasm, but the nucleus (arrowhead) lies within the shank of this non-tip-growing root hair. Scale bar in A=75 μm, in B=20 μm, for B, D, E, and F, and in C=20 μm.

Nuclear dynamics during multiple root-hair tip growth

1994) sustained and simultaneous tip growth was observed in ROP2 OX multiple root hairs, supported by a nucleus situated deep within the shank of one of the root hairs (see supplementary Movies S1, S2 at JXB online; Fig. 2A, B). There was no alternation in the rate of tip growth between these root hairs, no reduction in tip-localized cytoplasm in either root hair (Miller et al., 1999), nor any significant retrograde movement of the nucleus. These results suggest that, at least in these ROP2 OX multiple root hairs, tip growth does not specifically require the presence of a nucleus within the shank of the growing root hair or retrograde migration of the nucleus from one tip towards another.

To confirm there was no nuclear material in other parts of these root hair-bearing cells, ROP2 OX plants were crossed with plants overexpressing the NLS-GUS-GFP construct (Chytilova et al., 2000). These NLS-GUS-GFP plants have a root-hair phenotype indistinguishable from that of WT plants (Fig. 3A). From the segregating F2 populations derived from this cross individual plants were selected with the normal homozygous ROP2 OX root-hair phenotype (Jones et al., 2002) and the brightest GFP fluorescence (Fig. 3B). These plants were self-fertilized to produce lines homozygous at both the ROP2 OX and the NLS-GUS-GFP loci. In all ROP2 OX NLS-GUS-GFP plants tested a single nucleus was observed within each root-hair cell (data not shown). Although very occasionally transient and localized fluorescent processes were seen to extend from the main body of the nucleus, as previously reported (Chytilova et al., 2000), these subnuclear structures were less pronounced than those described previously (data not shown).

It has been suggested previously that these subnuclear structures are artefacts of NLS-GUS-GFP overexpression (Ketelaar et al., 2002). Previous observations of NLS-GUS-GFP expression in root hairs have only been made during early root-hair morphogenesis and in fully elongated mature root hairs (Chytılóva et al., 2000). Here observations were made during the intermediate stage of sustained tip growth in ROP2 OX NLS-GUS-GFP multiple root hairs, where one multiple root hair lacked any visible GFP fluorescence and the other contained a bright fluorescent body corresponding to the size and shape of the nucleus observed under the light microscope (see supplementary Movies S3, S4 at JXB online; Fig. 3C). Sustained and simultaneous tip growth was observed in both types of multiple root hair (Fig. 3D). Growth rates were within the range reported previously for...
the fast phase of root-hair tip growth (1.0–2.5 μm min\(^{-1}\); Dolan et al., 1994) in both nuclei-containing and nuclei-free root hairs (1.0–1.4 μm min\(^{-1}\)). This fast rate of tip growth was maintained in both nuclei-containing and nuclei-free root hairs until root-hair lengths were over 500 μm, when observations ceased (data not shown). ROP2 OX root hairs begin to undergo tip branching when they are approximately 500–600 μm long (Jones et al., 2002). Consistent with findings in WT root hairs (Ketelaar et al., 2002), the nuclei observed in ROP2 OX multiple root hairs remained at a relatively constant distance (50–100 μm) from the growing tip of the root hair within which they were located and at an

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**Fig. 3.** Nuclear dynamics during multiple root-hair tip growth in ROP2 OX NLS-GUS-GFP transgenic plants. (A) The root-hair phenotype of NLS-GUS-GFP transgenic plants is indistinguishable from that of WT plants. Upper panel: bright-field image showing the root-hair phenotype. Lower panel: fluorescence image showing the characteristic punctate GFP expression pattern within the nuclei. Note that the fluorescence signal is less easily detected within the shank of tip-growing root hairs. (B) ROP2 OX NLS-GUS-GFP transgenic plants were identified by their root-hair phenotype (in the upper panel, note the inset showing detail of multiple root-hair formation) and by their characteristic punctate GFP expression pattern within nuclei (lower panel). (C, D) Sustained and simultaneous tip growth of ROP2 OX NLS-GUS-GFP multiple root hairs. (C) Upper panel: bright-field image showing multiple root hairs arising from the same cell (inset shows detail of the common site of swelling formation of these root hairs). Lower panel: fluorescence image showing that the upper root hair lacks any visible GFP fluorescence, whilst the lower root hair contains a brightly fluorescent body corresponding to the size and shape of the nucleus seen in the bright-field image. See supplementary Movie S3 at JXB online, which shows the absence of any visible alternation in the rate of tip growth between these root hairs and the continual presence of tip-localized cytoplasm in both root hairs, and Movie S4, which confirms the progressively anterograde movement of the nucleus (lying within the lower root hair). (D) Graph showing the sustained increase in the length of both root hairs over a period of 90 min during the phase of fast tip growth. Scale bars: A=150 μm; B=500 μm; C=20 μm.
increasing distance from the base of the root hair. However, the tip of the nuclei-free multiple root hair was at a correspondingly increasing distance from the nucleus. These results suggest that, at least in ROP2 OX multiple root hairs, the proximity of the nucleus to the growing tip is not an absolute requirement for sustained fast tip growth.

Simultaneous and sustained tip growth of multiple root hairs without retrograde nuclear migration occurs independently of ROP2 overexpression

It is possible that this long-distance support of tip growth by a nucleus lying within another root hair is a phenomenon specific to ROP2 overexpression. To investigate this possibility multiple root-hair formation was examined in plants lacking the ROP2 OX construct. WT root hair-bearing cells can, under certain growth conditions, produce rare twin root hairs from the same root hair-forming site (Jones et al., 2002). As these rare twin root hairs also occur in NLS-GUS-GFP plants, which have WT root-hair development (Fig. 3A), the position of the nucleus within these root hairs during tip growth was examined. As in ROP2 OX plants, sustained and simultaneous fast tip growth also occurred in NLS-GUS-GFP multiple root hairs where the single nucleus was located deep within the shank of one of these root hairs (see supplementary Movies S5–S7 at JXB online; Fig. 4A–C), and within the range of growth rates previously reported (2.1–2.5 μm min⁻¹; Dolan et al., 1994). Nuclei remained at a similar distance (55–100 μm) from the growing tips of NLS-GUS-GFP hairs to those observed in ROP2 OX root hairs. Similar sustained and simultaneous tip growth was observed in the rare twin root hairs in WT plants (data not shown). These results show that the observations made in ROP2 OX plants are not a specific effect of ROP2 overexpression.

The nucleus undergoes further anterograde migration into the root-hair tip during tip branching in ROP2 OX NLS-GUS-GFP root hairs

ROP2 OX root hairs branch at their tips after root-hair elongation is complete (Jones et al., 2002). It is not clear whether the normal cessation of tip growth observed in WT root hairs (Dolan et al., 1994) occurs in ROP2 OX root hairs. In ROP2 OX root hairs the number of new root-hair tips appears to increase as root hairs grow older, with increasingly elaborate branches being formed (Fig. 5A). As nuclei undergo rapid long-distance movement inside the shank of mature NLS-GUS-GFP root hairs with no apparent co-ordination between hairs (Chytilova et al., 2000), investigations were made into nuclear movements during the morphogenesis of ROP2 OX-induced tip branching. In ROP2 OX NLS-GUS-GFP root hairs the nucleus often undergoes further anterograde migration at the onset of tip branching, which is preceded by a characteristic swelling of the root-hair tip (Movie S8; Fig. 5B, C). The nucleus also appears to migrate into the root-hair apex for the duration of this tip branching (Movies S9, S10; Fig. 5D). Elaborate tip branching develops slowly over the course of days so it was not possible to track nuclear movements in a single root hair from the start of tip branching until elaborate branching had formed. However, in older elaborately branched hairs the nucleus was located in a wide range of locations, with many remaining close to the most extreme root-hair apex (Fig. 5E) and others lying deep within the root-hair shank (data not shown).

Discussion

In both ROP2 OX multiple root hairs and in rare multiple hairs with WT root-hair development, a single nucleus can support sustained and simultaneous fast tip growth of at least two root hairs. This phenomenon occurs in the absence of noticeable retrograde nuclear migration or any periodic loss of tip-localized cytoplasm, which shows that tip growth is not being interrupted and re-established. A previous study indicated that nuclear proximity to the growing root-hair tip was a requirement for tip growth to proceed (Ketelaar et al., 2002). The results presented here show that, at least for some root hairs on multiple root hair-bearing cells, there is no requirement for the nucleus to be in close proximity to the growing tip.
It has been shown previously that root-hair tip growth in the cow1-2 root-hair branching mutant is dependent on the nucleus being located at a fixed distance from the actively growing tip (Ketelaar et al., 2002). COW1 has recently been cloned and found to encode a phosphatidyl inositol transfer protein-like protein (Böhme et al., 2004). In these plants, the nucleus migrates from one root-hair tip to the other, with periods of tip-localized cytoplasm and associated tip growth alternating between these tips. However, unlike the branched root hairs of cow1-2 plants, ROP2 OX multiple root hairs can be widely separated along the length of the same cell. In ROP2 OX plants there was no alternation of nuclear migration or alternation of tip growth between different multiple root hairs. In light of these results, it would be interesting to investigate whether the retrograde nuclear migration phenomenon reported in Ketelaar et al. (2002) is peculiar to the cow1-2 mutant or whether it is a feature of other mutants with branched root hairs, for example tip1 (Ryan et al., 1998). Simultaneous and sustained fast tip growth was also observed in rare multiple root hairs in WT and NLS-GUS-GFP plants, showing that this phenomenon occurs independently of ROP2 OX.

Unusually, nuclear dynamics in NLS-GUS-GFP root cells and root-hair cells is mediated by the actin cytoskeleton, and not microtubules (Chytílova et al., 2000), a finding confirmed by inhibitor and antibody studies in WT root-hair cells (Ketelaar et al., 2002). Although, it is not known what effect ROP2 OX has on F-actin organization in root hairs, expression of constitutively active and dominant negative ROP2 transgenes in root hairs disrupts normal fine F-actin organization in the root-hair tip (Jones et al., 2002), strongly supporting a role for ROP2 in regulating the formation of fine F-actin in the root-hair tip. Furthermore, overexpression of the Arabidopsis ROP1 GTPase in tobacco pollen tubes induced a network of F-actin filaments in the pollen tube tip and a transverse actin band behind the tip (Fu et al., 2001). If, as might be predicted, ROP2 OX enhances fine F-actin formation at the root-hair tip, it is possible that the distance between the nucleus and the root-hair tip would be greater in ROP2 OX root-hair cells than in WT root-hair cells. However, there was no obvious difference in this distance between these two genotypes. On the contrary, the nucleus moves closer to the tip at the onset of tip branching and that is characteristic of ROP2 OX root-hair morphogenesis. This is interesting, as microinjection of the lily villin protein into WT Arabidopsis root hairs resulted in actin unbundling and movement of the nucleus closer to the root-hair tip (Ketelaar et al., 2002). This anterograde nuclear movement in ROP2 OX root-hair cells could also be the result of an indirect effect on actin-unbundling proteins mediated by changes in ion gradients (e.g. Ca$^{2+}$) during the onset of tip branching.

Taken together, these results show that in these multiple root hairs the position of the nucleus at a fixed distance from the root-hair tip is not an absolute requirement for fast tip growth to proceed. Although the reasons for spontaneous branching in WT, ROP2 OX, and other mutants may differ, this does not invalidate the conclusion that growth can continue if the nucleus is not positioned behind the tip.

**Supplementary material**

The movies can be found as Supplementary material at *JXB* online.

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References


