RESEARCH PAPER

Synchronously developing collet hairs in *Arabidopsis thaliana* provide an easily accessible system for studying nuclear movement and endoreduplication

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Abstract

Early *Arabidopsis thaliana* seedling growth includes the highly synchronous development of hairs from every epidermal cell of the collet (the root–hypocotyl transition zone). The dynamics of collet hair growth, and accompanying nuclear movement and endoreduplication, were followed using a combination of different fluorescent probes for time-lapse imaging and flow cytometry. Using laser-scanning confocal microscopy on the double-transgenic *Arabidopsis* hybrid line NLS-GFP-GUS × YPM, there appeared to be a correlation between nuclear position and the cell tip during growth of the collet hair cells, as occurs in asynchronously developing root hairs. However, disruption of nuclear movement in the growing collet hairs using low concentrations of cytoskeletal inhibitors demonstrated that nuclear positioning close to the tip of the cell is not essential for tip-directed growth of the hair. Nuclear DNA content increases from 4C to 16C during development of the collet hairs. Following cessation of growth, nuclei moved to the base of the hairs and then their movement became asynchronous and limited. Co-visualization of RFP-highlighted prevacuolar vesicles and GFP-labelled nuclei showed that, whereas small vesicles allowed unimpeded nuclear movement within the hair, transient stops and directional reversals coincided with the presence of larger vesicles in close proximity to the nucleus. *Arabidopsis* collet hairs provide a robust, easily accessible, naturally synchronized population of single tip-growing cells that can be used as a model cell type for studying nuclear movement and endoreduplication.

Key words: confocal microscopy, cytoskeleton, DNA content, flow cytometry, fluorescent protein, nucleus migration, tip growth.

Introduction

The collet, the part of a seedling axis that is the transition zone between the root and hypocotyl (root–hypocotyl junction) has been recognized and so-named since 1849 (Compton, 1912). Epidermal hairs that are formed in the collet region anchor the seedling to the substratum and facilitate geotropic responses and water uptake well before the development of root hairs (Parsons, 2009). Collet hairs look very similar to root hairs and over time have been described in literature as ‘branch roots’ (in 1693 by Leeuwenhoek according to Sundberg, 2011), ‘collar rhizoids’ (Tillich, 1995; Sokoloff et al., 2008), ‘hypocotyl hairs’ (Parsons, 2009), and ‘root hairs’ (Scheres et al., 1994; Cheng et al., 1995; Schneider et al., 1997; Bernhardt and Tierney, 2000; Lin and Schielefbein, 2001; Molhøj et al., 2001; Wang et al., 2001; Diet et al., 2004; Galway, 2006; Derbyshire et al., 2007). Tiers of cells that form collet initials in *Arabidopsis* can be identified as early as the heart stage of embryo development (Scheres et al., 2002) and become clearly demarcated during seed germination. Whereas epidermal cells of the root hardly change in length until post-germination radicle extension, cells of the lower hypocotyl region elongate 1.5-fold and those of the collet nearly 2-fold (Sliwinska et al., 2009). The total number of epidermal cell files in the root–hypocotyl junction is intermediate between that of the root and the hypocotyl, and the junction has one more layer of ground tissue than the root
et al. lapse imaging of growing events during collet hair development by combining time-manner as part of their normal development. and characterizing cells that are readily accessible and that any living plant cell. The present study focused on identifying to and during endoreduplication has not been carried out for entirety, the study has been extended beyond the active growth period to show altered nuclear behaviour in mature hypocotyl (Lin and Schiefelbein, 2001). There is a different arrangement of the bundles of xylem and phloem in the collet compared with that in the root and hypocotyl (reviewed in Compton, 1912). The collet region also displays a different gene expression pattern compared to the root and the hypocotyl (Masucci and Schiefelbein, 1994; Freshour et al., 1996; Schneider et al., 1997; Lin and Schiefebein, 2001; Molhøj et al., 2001). Furthermore, position-dependent GLABRA2 expression, which occurs only in root epidermal cells that are in contact with two underlying cortical cells, is absent from collet cells (Masucci et al., 1996; Lin and Schiefelbein, 2001). The different expression pattern is consistent with the production of a hair from every collet epidermal cell. Although the collet region is both anatomically and molecularly distinct from other regions of the seedling, its presence is often ignored and a detailed description of collet epidermal hair development is not available.

In contrast to dividing cells, which progress through the sequence of G1, S, G2, and M phases, endocycling cells repeat rounds of the S phase without undergoing the M phase. This process is called endoreduplication and leads to increased nuclear DNA content/ploidy of the cell (Brauer et al., 2010). Endoreduplication is generally associated with growth and specialized differentiation of cells. A high frequency of endopolyploid nuclei occurs during endosperm and suspensor development, cotyledon expansion, hypocotyl elongation, xylem cell differentiation, and trichome, pavement cell, and trichoblast development (reviewed by Sugimoto-Shirasu and Roberts, 2003; Brauer et al., 2010). The number of endocycles and the time interval between endocycles in a particular cell is difficult to predict, even if anticipated based on the developmental stage of a tissue or organ. Consequently, even though a large number of studies have established the highly polysomatic nature of Arabidopsis plants, with nuclear DNA content ranging between 2C and 32C (Galbraith et al., 1991; Masubelele et al., 2005; Sliwinska et al., 2009), the visualization of sequential events leading up to and during endoreduplication has not been carried out for any living plant cell. The present study focused on identifying and characterizing cells that are readily accessible and that carry out endoreduplication synchronously in a predictable manner as part of their normal development.

Presented here is an approach to understanding nuclear events during collet hair development by combining time-lapse imaging of growing Arabidopsis seedlings expressing a NLS-GFP-GUS fusion protein (Grebenok et al., 1997a,b) with flow cytometry of cells from the collet region. Subcellular events in the collet hair were characterized further using seedlings simultaneously expressing probes targeted to the nucleus and plasma membrane and to the nucleus and assorted endomembrane vesicles and prevacuoles. The salient findings of this study document the highly coordinated tip growth exhibited by collet hairs in synchrony with nuclear behaviour and an increase in DNA C-value. In characterizing collet hair development in its entirety, the study has been extended beyond the active growth period to show altered nuclear behaviour in mature hairs. In contrast to its synchronized movement during active growth, the nucleus moves erratically back-and-forth in the fully developed collet hair, close to its base. This restricted nuclear movement could result from spatial hindrance caused by numerous, large, prevacuolar vesicles and enlarging vacuoles. Further, using low concentrations of cytoskeleton-disrupting drugs that altered anterograde nuclear movement but not tip growth, it has been established that, contrary to the conclusions drawn from observations on growing root hair cells, tip growth of collet hairs is not intimately linked to nuclear positioning within the cell. Presented here are detailed live-imaging-based observations that establish the developing collet hair as an easily accessible model system that can be used to further dissect nuclear behaviour and endoreduplication in higher plants.

Materials and methods

Plant material

Seeds of Arabidopsis thaliana transgenic lines were used: (1) constitutively expressing the NLS-GFP-GUS chimeric protein (Grebenok et al., 1997a,b); (2) a hybrid between the NLS-GFP-GUS line and the LTI6b line expressing EYFP::PIP2A that localizes to the plasma membrane (Cutler et al., 2000); (3) a hybrid between the NLS-GFP-GUS line and the RFP::2xFYVE line that highlights nuclei, endosomes, prevacuolar vesicles, and small vacuoles (Voigt et al., 2005); (4) GFP-MAF4 (Mathur and Chua, 2000); and (5) GFP-Talin (Kost et al., 1998) and wild-type (WT) ecotype Columbia. The NLS-GFP-GUS line seeds germinated 8–10 h later than the other lines, probably because they had been in storage longer, which often delays germination (Black et al., 2006). Further seedling development was similar in all lines; collet hair elongation took about 20 h.

Confocal microscopy

Seeds were imbibed on wetted filter paper in 5.5-cm-diameter Petri dishes at 20 ± 1 °C in darkness. At about 6–8 hours after imbibition started (HAI) embryos were isolated, placed in a One-chamber Lab-Tek Chambered Coverglass (Fisher Scientific, Mississauga, ON, Canada), and covered with about 2 ml of 0.8% (w/v) agar (Fisher Scientific). The collet region of the axis was studied from just before hair emergence until the seedling was 7 days old (168 HAI). Videos were produced from confocal stacks collected using an inverted Leica DMR2 confocal microscope connected to a Leica TCS SP2 system (Leica Microsystems, Mannheim, Germany), with two different visible light lasers: Ar (50 mW: 458 nm, 488 nm, and 514 nm) and GreenHeNe (1.2 mW: 543 nm). Because of the large size of the picture files and the limited capacity of the computer program to collect data, only the Ar laser was used for long-term movies of the hybrid line NLS-GFP-GUS × YPM. This allowed detection of both GFP and YFP fluorescence, but only in one colour. The LAS AF program (Leica Microsystems) was used for 3D video processing and measurements of hair lengths and distances between the nucleus and the hair tip. The Imaris 6.4.0 (Bitplane Scientific Software) program was used to create 3D volume-rendered images of the nuclei.

Flow cytometry

For flow cytometry (FCM), collets from WT seedlings growing on wetted filter paper at 20 ± 1 °C in darkness were excised at 40, 48, 60, 68, 76, 86, and 96 HAI. Since collet hairs grow in all directions, to avoid damage to them during cutting, the lower part of the hypocotyl and upper part of the root had to be included. Nuclei
were isolated by chopping 20 axis parts in 1 ml of buffer (0.1 M TRIS, 2.5 mM MgCl₂·6H₂O, 85 mM NaCl, 0.1% v/v Triton X-100, pH 7.0), supplemented with propidium iodide (50 µg ml⁻¹) and RNase A (50 µg ml⁻¹). After chopping, the suspension was passed through a 50-µm mesh nylon filter and analysed using a CyFlow SL Green (Partec, Münster, Germany) flow cytometer, equipped with a high-grade solid-state laser with green light emission at 532 nm long-pass filter RG 590 E, DM 560 A, as well as with side (SSC) and forward (FSC) scatter detectors. For each sample, the DNA content of 1000–2000 nuclei was measured. Analyses were performed on four replicates, using logarithmic amplification. Histograms were evaluated using the FloMax program (Partec) and the percentage of nuclei with particular DNA contents and the mean C-value (Lemontey et al., 2000) were calculated before applying a one-way analysis of variance and a Duncan’s test.

Additionally, collet hairs were dissected from 50 seedlings at 76 and 96 HAI (as precisely as possible, but some root hairs and epidermal cells may also have been included) and DNA content was measured in about 200 nuclei using the same procedure as for axis fragments.

Only nuclei having a DNA content of at least 8C were considered to be endopolyploid, since it is not possible to distinguish by FCM analysis the 4C nuclei in cells that have just entered endoreduplication (i.e. being in the G₁ phase of the first endocycle) from those within cells in the G₂ phase of the mitotic cycle.

Nuclear size measurements

To estimate the ploidy of the collet cells, the surface area of individual nuclei in Supplementary Videos S1–S3 (available at JXB online) was measured using ImageJ software (NIH). The measurements were performed at bulge formation, when the collet hairs reached half and full length, and after the nuclei moved back to the base of the hair and established back-and-forth movement for a short distance (n = 40 for each time point). As a basis for relating the size of a nucleus to its ploidy, the size of the nuclei in the root tip was measured directly before and after mitosis, which are considered as having 4C and 2C DNA, respectively (n = 18).

Cytoskeleton-disrupting drug treatments

Seeds were imbibed on wetted filter paper in 5.5-cm-diameter Petri dishes at 20 ± 1 ℃ in darkness. At about 24 HAI they were placed in a One-chamber Lab-Tek Chambered Coverglass and covered with 0.8% (w/v) agar supplemented with 200 nM oryzalin (Crescent Chemical Co, Singapore) or 50 nM latrunculin B (Calbiochem, San Diego, USA). The time-lapse videos were produced as described above.

**Results and discussion**

**Nuclear movement is highly synchronized during coordinated collet hair development**

The NLS-GFP-GUS Arabidopsis line (Grebenok et al., 1997a,b) was selected for the present study because the fusion protein exclusively accumulates within the nucleoplasm and thus enables nuclear movement to be followed using fluorescence microscopy. This line was used previously to study cell division, nuclear shape, and movement in different tissues (Chytilova et al., 1999, 2000). The initial question posed was: to what extent do the dynamics of nuclear migration in collet hairs resemble those of the more extensively studied root hairs (Lloyd et al., 1987; Sato et al., 1995; Chytilova et al., 1999, 2000; Ketelaar et al., 2002; van Bruaene et al., 2003), which are physiologically distinct in that they grow asynchronously as the seedling develops? Use of time-lapse confocal microscopy allowed collet cells to be viewed before the initiation of tip growth (Fig. 1). It is reported that the collet region in Arabidopsis consists of 4–7-cell tiers

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**Fig. 1.** Nuclear movement in the collet epidermal cells of the GFP-transformed Arabidopsis NLS-GFP-GUS line. (A) At 50 hours after imbibition started (HAI), prior to the extension of the collet hairs, nuclei appear round. (B) At 56 HAI, elongated nuclei have migrated into the expanding collet hairs: the location of the nuclei indicates that hairs emerged simultaneously from each of the 4 tiers of collet epidermal cells. (C) At 58 HAI, nuclei continue an outward directed movement. Bar = 100 µm. Collet hairs of this line emerged about 8–10 h later than those of other studied lines. cl, Collet; h, hypocotyl; r, root. Snapshots were obtained from time-lapse Supplementary Video S1 (this figure is available in colour at JXB online).
(Cheng et al., 1995; Lin and Schiefelbein, 2001) but here only 3–5-cell tiers were observed to produce collet hairs. At about 50 HAI, the nuclei in four tiers of cells situated above the root cap (the root cap nuclei can be easily identified by their brighter GFP fluorescence) started to display short, erratic movements. Another 4 h later, nuclei started to move away from the base of the cell and this coincided with the initiation of hair tip growth. In contrast to the epidermis of the root, which in Arabidopsis is differentiated into files of trichoblasts (H-cell position) and atrichoblasts (N-cell position), of which only H-cells produce hairs (Cormack, 1947; Grierson and Schiefelbein, 2002), all collet epidermal cells developed hairs. This phenomenon was previously mentioned by Lin and Schiefelbein (2001), but not demonstrated visually. The nuclei continued to move acropetally in synchrony, until at about 74 HAI they reversed their movement and retreated towards the base of the hair. This basipetal movement was consistent but less synchronized; nearly all nuclei reached the base of the hair in about 10 h (Fig. 2, Supplementary Video S2). Thereafter, most nuclei did not return to the hair tip, but moved back-and-forth over short distances close to the base of the collet hair in an unsynchronized manner. This movement did not change even in collet hairs of 7-day-old seedlings. Similar nuclear basipetal movement has been observed in mature non-growing root hairs of Raphanus sativus (Sato et al., 1995). In Arabidopsis, the migration of nuclei to a random position has been reported in fully grown root hairs (Ketelaar et al., 2002), while Chytilova et al. (2000) and van Bruaene et al. (2003) have reported bidirectional nuclear movement similar to that observed in the collet hairs.

In addition to the difference in their position, another feature that distinguishes between the two types of hairs is that root hairs develop progressively along the root during its growth, starting in a region of the post-mitotic growth zone distal to the growing root-tip, whereas the collet hairs all develop almost simultaneously in a narrow band. As in the Hydatellaceae (Sokoloff et al., 2008), there is an interval between the development of collet hairs and root hairs in Arabidopsis seedlings. Therefore, the extent to which growth rates and extension might be comparable between them was investigated. According to previous observations, root hairs grow slowly at first (0.3–2.5 μm min⁻¹) and more quickly after they reach 20–40 μm (1.1–2.5 μm min⁻¹); towards the end of their growth, the rate slows again (Dolan et al., 1994; Wymer et al., 1997). The growth rate of the collet hairs is also slower at the beginning of their elongation (0.35 μm min⁻¹), increasing to about 1 μm min⁻¹ when the hairs reach 200 μm, and then decreasing again, to 0.5–0.6 μm min⁻¹, shortly before growth arrest (Fig. 3; n = 15). The length of the collet hairs was measured as being slightly

Fig. 2. Movement of nuclei with the collet hair tips during growth and their return to the base of the cells of the GFP-transformed Arabidopsis NLS-GFP-GUS line. (A) At 72 HAI, collet hair growth and movement of nuclei with the tip almost completed. (B) At 80 HAI, nuclei move towards the base of the collet hairs. (C) At 94 HAI, nuclear movement towards the base completed; most nuclei now move asynchronously within a short distance of the basal region. Bar = 100 μm. The arrowheads follow the nuclear movement in individual hairs in A–C. The arrows indicate the direction of nuclear movement (↓ acropetal, ↑ basipetal, † back-and-forth). Snapshots were obtained from time-lapse Supplementary Video S2 (this figure is available in colour at JXB online).
movement in root hairs (77 or 63 μm; Grierson and Schiefelbein, 2002; Ketelaar et al., 2002, respectively). Collet hair nuclei moved at a similar velocity as tip elongation (mean for 8 h of hair growth 0.6 ± 0.1 μm min⁻¹, n = 10; Supplementary Videos 1 and 3). After growth of the collet hairs ceased some of the nuclei moved closer to the tip, or even moved back-and-forth a little, before starting to regress towards the hair base, presumably when the subcellular barrier (the vesicle-rich zone; Sieberer et al., 2002) that kept them at a nearly fixed distance from the tip was no longer present. The intense fluorescence of the collet hair tip suggested an accumulation of plasma-membrane-component-containing vesicles during hair elongation (Supplementary Fig. S1, Supplementary Video S4). As in mature root hairs (Galway et al., 1997), this characteristic vesicle-rich zone at the tip was no longer apparent when the hair had achieved its full length and the concentration of cellular membranes became even along the whole length of the hair (Fig. 5, Supplementary Video S5). No change in shape or length of the collet hairs was observed after hair maturity during 7-day seedling growth, with most of the nuclei moving back-and-forth close to the hair base and only a few nuclei reaching the tip.

The cytoskeleton is involved in nuclear movement and positioning in collet hairs

It has been suggested that in root hairs the position of the nucleus is important for continued tip growth; the cytoskeleton has been implicated (Lloyd et al., 1987; Chytilova et al., 2000; Ketelaar et al., 2002; Sieberer et al., 2002). According to the literature, positioning of the nucleus during root hair extension and its basipetal movement after cessation of its growth depends upon the F-actin cytoskeleton; microtubules are suggested not to be involved. The question arises as to whether the link between tip growth and nuclear position in collet hairs is essential for their elongation. To answer this, cytoskeleton-disrupting drugs, oryzalin and latrunculin B were applied to the double-transgenic NLS-GFP-GUS × YPM hybrid line. The concentration of the drugs (200 and 50 nM, respectively) was optimized using two transformed Arabidopsis lines, GFP-MAP4 (Mathur and Chua, 2000) and GFP-Talin (Kost et al., 1998), which target microtubules and filamentous (F-)actin strands, respectively (Supplementary Figs. S2, S3) to depolymerize the cytoskeleton but not cause inhibition of collet hair growth. Unlike previous studies on root hairs, where the cytoskeleton-disrupting drugs were applied for a short time after the nucleus had already entered the hair (Lloyd et al., 1987; Chytilova et al., 2000; Ketelaar et al., 2002; Sieberer et al., 2002), here the seeds were placed on growth medium containing the drug during germination (at 24 HAI) and maintained on it thereafter. This allowed observation of the effects of microtubule or actin depolymerization on the development of collet hairs and on nuclear movement. Even at the low concentrations, both drugs changed the appearance of hair cells and disturbed nuclear movement (Table 1). As in Arabidopsis

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**Fig. 3.** Collet hair length, distance between the nucleus and hair tip, and rate of hair growth in the seedlings of the double-transgenic Arabidopsis hybrid line NLS-GFP-GUS × YPM from the time when the nuclei enter the hairs until the latter reach their full length (thereafter, the nuclei start to move back towards the base of the cell; not shown). Values correspond to Fig. 4, Supplementary Fig. S1 and Supplementary Video S3. Error bars represent standard deviation (n = 15).

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Over 1200 μm at 64 HAI (Fig. 3), and at full length could reach 1500–1600 μm. By comparison, maximum root hair lengths are shorter and usually range about 600–1000 μm (Galway et al., 1997; Ryan et al., 2001; Grierson and Schiefelbein, 2002; Diet et al., 2004). In the only previous report on collet hair length, although shorter than recorded here, they were still longer than the root hairs (Diet et al., 2004). The diameter of a collet hair is about 7–10 μm (8.3 ± 1.0 μm; n = 60), similar to that of a root hair.

Next it was asked whether nuclear movement in collet hairs is distinct from that in root hairs. To determine this, the NLS-GFP-GUS line was crossed with the LTI-6B line expressing EYFP::PiP2A (hereafter called YPM) that localizes to the plasma membrane (Cutler et al., 2000). The hybrid NLS-GFP-GUS × YPM allowed the boundaries of the cells to be viewed as well as the nuclei (Fig. 4, Supplementary Video S3). At the start of root hair formation, the initial bulging of the outer cell wall is always towards the root apex (lower) end of the trichoblast (Ryan et al., 2001). In contrast, it has been suggested that collet hairs emerge closer to the centre of the cell (Lin and Schiefelbein, 2001). Here, collet hair formation from different surface regions of the epidermal cell initial was observed, and these did not coincide with the angle of subsequent hair growth, which could be towards the hypocotyl, root, or perpendicularly to the collet body. The diameter of the bulge on the collet hair cell surface was 30.7 ± 3.6 μm; n = 11). The nucleus entered the collet hair about 1.5 h (84 ± 39 min; n = 10) after bulge formation, when the hair was about 30 μm long (32.8 ± 9.6 μm; n = 30), and then followed the growing tip at an almost constant distance behind of 50–60 μm (Figs. 3 and 4, Supplementary Video S3); this is similar to measurements of nuclear movement in root hairs (77 or 63 μm; Grierson and Schiefelbein, 2002; Ketelaar et al., 2002, respectively). Collet hair nuclei moved at a similar velocity as tip elongation (mean for 8 h of hair growth 0.6 ± 0.1 μm min⁻¹, n = 10; Supplementary Videos 1 and 3). After growth of the collet hairs ceased some of the nuclei moved closer to the tip, or even moved back-and-forth a little, before starting to regress towards the hair base, presumably when the subcellular barrier (the vesicle-rich zone; Sieberer et al., 2002) that kept them at a nearly fixed distance from the tip was no longer present. The intense fluorescence of the collet hair tip suggested an accumulation of plasma-membrane-component-containing vesicles during hair elongation (Supplementary Fig. S1, Supplementary Video S4). As in mature root hairs (Galway et al., 1997), this characteristic vesicle-rich zone at the tip was no longer apparent when the hair had achieved its full length and the concentration of cellular membranes became even along the whole length of the hair (Fig. 5, Supplementary Video S5). No change in shape or length of the collet hairs was observed after hair maturity during 7-day seedling growth, with most of the nuclei moving back-and-forth close to the hair base and only a few nuclei reaching the tip.

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root hairs (Bibikova et al., 1999; Ketelaar et al., 2002), cytoskeleton disruption shortened the collet hairs and caused a change in their shape (they became wavy or irregular), compared to those of untreated seedlings (Table 1, Figs. 6, 7, Supplementary Videos S6, S7). It also affected nuclear movement. Microtubule disruption by oryzalin completely disturbed the synchrony of nuclear behaviour. Interestingly, at 58 HAI when the collet hairs were already 200–300 μm long (Fig. 6E), more than half of the nuclei had not entered them, and when most of the hairs

Fig. 4. Bulge formation and hair growth in the collet of the double-transgenic Arabidopsis hybrid line NLS-GFP-GUS × YPM. (A–C) At 40–43 HAI, the initial bulging is from different regions of all the epidermal cells and is not related to the subsequent direction of hair growth, which can be up, down, or perpendicular to the axis. (D) At 44 HAI, nuclei have entered the growing hairs. (E, F) At 46–48 HAI, the nuclei move synchronously at the fixed distance from the hair tip. Bar = 100 μm. The arrowheads in A and B indicate the bulge (bg) and in C–F indicate the growing hair tip (t) and the nucleus (n) maintaining its distance from the tip. Snapshots were obtained from time-lapse Supplementary Video S3 (this figure is available in colour at JXB online).
had achieved their full length (66 HAI; Supplementary Video S6), about 40% of the nuclei still remained in the basal region. Thus, in contrast to previous suggestions (Morris, 2000; Ketelaar et al., 2002), the presence of the nucleus close to the tip is not necessary to maintain tip growth. This is supported by the observation of Jones and Smirnoff (2006), who showed multiple root hair formation from the same cell, which underwent simultaneous and sustained tip growth even though the nucleus was present close to the tip of only one of them. Here, those nuclei that entered the elongating hair usually were reaching no more than half way along its length; their distance from the tip varied from 50 to 250 μm (mean for the first 8h of hair growth 129.4 ± 60.7 μm, n = 20; Fig. 6, Supplementary Video 6). On the other hand, when actin was depolymerized by latrunculin B, the nuclei entered the elongating hairs but did not maintain either a fixed distance from the tip or the constant acropetal movement; the nucleus to tip distance varied between about 20 and 120 μm (mean for the first 8h of hair growth 48.4 ± 16.6 μm, n = 33; Fig. 7, Supplementary Video 7). Similar to untreated seedlings, in the fully grown collet hairs after oryzalin and latrunculin B treatment, most of the nuclei moved back-and-forth only for a short distance close to the hair base. Conclusions on the role of the cytoskeleton in nuclear movement drawn from the studies on root hairs have been ambiguous (probably because of how and when the inhibitors were applied), and they usually emphasize the importance of actin over microtubules (Lloyd et al., 1987; Chytilova et al., 2000; Ketelaar et al., 2002). In contrast, the present study revealed that both microtubules and actin are important to maintain synchronous nuclear movement in the collet hairs at a fixed distance from the growing tip. Moreover, since the direction of growth in the collet hair was usually disturbed, it can be suggested that the cytoskeleton is involved more in the maintenance of spatial coordination between the nucleus and other enlarging cytoplasmic compartments such as the vacuole. This possible relationship was explored further in subsequent experiments.

Fig. 5. Movement of the nuclei in the distal region of collet hairs of the double-transgenic Arabidopsis hybrid line NLS-GFP-GUS × YPM (the region close to the collet cell base is not included in the Z-stack) at 110 HAI (A) and 168 HAI (B). Each collet hair remains at its maximum length; only a few nuclei move erratically in this region of the hair while most of them move back-and-forth close to the basal portion of the hair; some nuclei look ‘fragmented’ (see text and Fig. 8 for explanation). Bar = 250 μm. The arrowheads follow the asynchronous nuclear movement in individual hairs (n1–n3). Snapshots were obtained from time-lapse Supplementary Video S5 (this figure is available in colour at JXB online).

Nuclear movement after cessation of tip growth depends upon interactions with prevacuoles and expanding vacuoles

Initially, nuclei moved only acropetally in the growing collet hairs, but with a few exceptions were unable to display similar behaviour after the cessation of tip growth. Instead their movement became erratic and non-synchronous. It was hypothesized that it was due to hindrance by some other cytoplasmic component. An RFP::2xFYVE line (hereafter called REndo) that highlights endosomes and

Table 1. Comparison of collet hair and nuclei characteristics in non-treated Arabidopsis seedlings and those treated with oryzalin and latrunculin B

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Treatment</th>
<th>None</th>
<th>200 nM oryzalin</th>
<th>50 nM latrunculin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hair emergence</td>
<td></td>
<td>At about 40 HAI</td>
<td>At about 46 HAI</td>
<td>At about 44 HAI</td>
</tr>
<tr>
<td>Hair length and shape</td>
<td></td>
<td>1200–1600 μm, regular</td>
<td>400–600 μm, wavy</td>
<td>150–700 μm, irregular</td>
</tr>
<tr>
<td>Hair diameter</td>
<td></td>
<td>8.3 ± 1.0 μm</td>
<td>10.3 ± 1.0 μm</td>
<td>8.3 ± 1.0 μm</td>
</tr>
<tr>
<td>Movement of nuclei during tip growth</td>
<td>Nuclei follow tip growth at a fixed distance of about 50–60 μm</td>
<td>Some nuclei never enter the hair; those which do not approach the tip (distance from the tip 50–250 μm)</td>
<td>Nuclei enter the hair but move at a variable rate; they do not maintain their distance from the tip (20–120 μm)</td>
<td></td>
</tr>
<tr>
<td>Movement of all collet hair nuclei is synchronized</td>
<td>Nuclei move continuously acropetally</td>
<td>Nuclei can move towards tip and back</td>
<td>Nuclei can move towards tip and back</td>
<td></td>
</tr>
<tr>
<td>Movement of nuclei in fully grown hairs</td>
<td>Most nuclei move back-and-forth close to the hair base</td>
<td>No synchrony of movement</td>
<td>Synchrony of movement is disturbed</td>
<td></td>
</tr>
</tbody>
</table>

HAI, hours after imbibition started.
prevacuolar vesicles (Voigt et al., 2005) was crossed with the GFP-NLS-GUS line. The hybrid line allowed observation of the cytoplasmic region surrounding the nucleus during its movement. During their growth, collet hairs contained many small fast-moving vesicles as well as large ones, the prevacuoles (Supplementary Video 8). Similar small motile vesicles and their large aggregates have also been noted in growing and full-length root hairs (Ovečka et al., 2005; Voigt et al., 2005). In mature collet hairs in the hybrid NLS-GFP-GUS × REndo line large prevacuolar vesicles appeared to impede the free movement of nuclei (Fig. 8, Supplementary Video S9). As long as the vesicles

**Fig. 6.** Bulge formation and hair growth in the collet of the double-transgenic Arabidopsis hybrid line NLS-GFP-GUS × YPM treated with 200 nM oryzalin. (A–C) At 48–52 HAI, bulge formation and early collet hair growth. (D–F) At 54–60 HAI, growth of hairs is not synchronized, hairs are shortened and wavy, and the nucleus does not follow the tip during hair elongation. Bar = 100 μm. The asterisks in B–F indicate nuclei that did not enter collet hairs. The arrowheads in D–F indicate the hair tip (t) and the nucleus (n). Snapshots were obtained from time-lapse Supplementary Video S6 (this figure is available in colour at JXB online).
were small and flowed in the narrow cytoplasmic stream along the hair’s longitudinal axis there was sufficient space for the nucleus to pass (Fig. 8C, Supplementary Videos S8, S9). However, as the collet hair aged, and after the nucleus had regressed towards the cell base, the large prevacuolar vesicles prevented its movement back towards the tip (Fig. 8B, Supplementary Video S9). In a few instances, after several failures to pass each other, the vesicles changed configuration, as did the shape of the nucleus, and thus the nucleus was able to continue its acropetal movement (Supplementary Video S9, nucleus n2 in Fig. 8A). Consequently, the nucleus was observed close to the

Fig. 7. Bulge formation and hair growth in the collet of the double-transgenic Arabidopsis hybrid line NLS-GFP-GUS x YPM treated with 25 nM latrunculin B. (A, B) At 46–48 HAI, bulge formation and early collet hair growth. (C–F) At 50–60 HAI, the growth of hairs is not synchronized, hairs are shortened and of irregular shape, and the nucleus follows the tip during hair elongation, but not at a fixed distance and at a changeable rate, sometimes approaching closely the tip. Bar ≈ 100 μm. The arrowheads in C–F indicate the hair tip (t) and the nucleus (n). Snapshots were obtained from time-lapse Supplementary Video S7 (this figure is available in colour at JXB online).
tip in only a limited number of hairs. An obvious obstacle to nuclear movement, other than the vesicles, is the large vacuole that occupies the elongated collet hair. This was not directly visible in the NLS-GFP-GUS × REndo line, but its shape could be distinguished from that of the nucleus as the latter exhibited its restricted movement in the narrow space between the vacuole and hair wall. Sometimes the nucleus appeared to undergo ‘fragmentation’ (Chytilova et al., 2000) but in fact it was elongating irregularly (Fig. 9), including the formation of long tubular extensions. When the Z-sections of the confocal scanning microscope caught such fragments of the nucleus (marked with broken lines in Fig. 9B), they gave a false impression of discontinuity.

Fig. 8. (A) Movement of the nuclei and vesicles in the collet hairs of the double-transgenic Arabidopsis hybrid line NLS-GFP-GUS × REndo, from 73 to 75.5 HAI. The cytoplasmic organization blocks the movement of the nucleus (n1) on the left towards the hair tip (located above the top of the picture) and, unless the configuration of the vesicle aggregation (v) changes, after a few failures to pass it (0–70 min) the nucleus recedes towards the base of the hair (120–150 min) (located below the bottom of the picture). In the other collet hair, vesicles are small enough to allow the nucleus (n2) to continue its undisturbed movement, first from near the tip (out of picture) (70–120 min) and then back (from 150 min). Bar = 25 μm. The arrowheads indicate the direction of the movement of the nuclei (n1 and n2). Snapshots were obtained from time-lapse Supplementary Video S9. (B) Digital 3D volume-rendering reconstruction of the nucleus blocked by a large vesicle (prevacuole). (C) Digital 3D volume-rendering reconstruction of the nucleus of the collet hair surrounded by small vesicles. The nucleus can continue its movement. The shape of the nucleus, elongated when seen from the top, is actually irregular, which allows it to squeeze through the narrow space between other organelles and the hair wall.
Nuclear DNA content and size increase during collet hair development

Endoreduplication often occurs in the cells of tissues that have high metabolic activity or in specialized cells such as root trichoblasts (Cutter and Feldman, 1970; Dosier and Rippel, 1978). Since all of the collet epidermal cells are trichoblasts and elongate before they form hairs (Sliwinska et al., 2009), it is likely therefore that they also undergo endoreduplication. This phenomenon has not been measured directly in collet hairs of any species. The size and fragility of these cells make it extremely difficult to apply the most commonly used method, FCM, since this requires isolation of a considerable number of nuclei. Here, flow cytometric analysis of nuclear DNA content revealed that the highest ploidy in the Arabidopsis collet region and collet hairs was 16C (Table 2, Supplementary Fig. S4). This is in agreement with the previous estimation of endopolyploidy in this species; in most organs it was not higher than 16C, only sometimes reaching 32C (Galbraith et al., 1991; Melaragno et al., 1993; Gendreau et al., 1997, 1998; Beemster et al., 2002; Schnittger et al., 2002; Masubele et al., 2005; Sugimoto-Shirasu et al., 2005; Jovtchev et al., 2006; Sliwinska et al., 2009).

It is well established that there is a linear relationship between nuclear size and DNA content (Silcock et al., 1990; Sugimoto-Shirasu and Roberts, 2003; Jovtchev et al., 2006), although it is not absolute. Therefore, the results presented here of the measurements of the size of collet nuclei are regarded only as supplementary to the FCM data. Based on previous measurements of collet cell size and nuclear DNA content (Sliwinska et al., 2009), as well as on the present measurements of nuclear size and flow cytometric analyses (Table 2, Fig. 10, Supplementary Videos S1–S3), it is evident that the nuclei of the collet epidermis undergo endoreduplication and this progresses simultaneously in the hairs (the polysomaty in a sample is due to the presence of nuclei from various collet tissues/cell types in addition to the hairs). It is likely that they were mostly 4C after the completion of germination, but already at the time of bulge formation the size of the nuclei considerably increased suggesting that they had undergone an endocycle and now possessed an 8C DNA content (n = 40; Fig. 10, Supplementary Videos S1, S3). This is supported by the increase in the mean C-value and the proportion of 8C nuclei in the collet region shortly after hair emergence (48 HAI; Table 2).

During collet hair development, a further increase in nuclear size was observed, and before the hairs achieved their full length, the DNA content in most of the nuclei was 16C (n = 40; Fig. 10, Supplementary Videos S1, S3). Also FCM revealed that when the growth of the hairs ceased (60 HAI; Table 2), an increase occurred in the proportion of 16C nuclei, accompanied by a decrease in 8C nuclei, which suggests that a second endocycle took place in some nuclei. The proportion of 8C nuclei did not change until 96 HAI; at that time, the mean C-value was the highest (over 6, compared to 4 before hair emergence). FCM analysis of the samples prepared from dissected collet hairs revealed the presence of a higher proportion of 16C nuclei than in the whole collet region: 8% at 76 HAI and 13% at 96 HAI. This suggests that at least part of the highest endopolyploidized nuclei detected among all collet nuclei were indeed those of

### Table 2. Flow cytometric analysis of the proportions of nuclei with different DNA content in the collet region of the seedlings of Arabidopsis thaliana

<table>
<thead>
<tr>
<th>HAI</th>
<th>Nuclei with DNA content of (%)</th>
<th>Mean C-value (± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2C</td>
<td>4C</td>
<td>8C</td>
</tr>
<tr>
<td>40</td>
<td>37.8</td>
<td>42.4</td>
</tr>
<tr>
<td>48</td>
<td>31.2</td>
<td>33.3</td>
</tr>
<tr>
<td>60</td>
<td>31.3</td>
<td>33.9</td>
</tr>
<tr>
<td>68</td>
<td>22.1</td>
<td>30.1</td>
</tr>
<tr>
<td>76</td>
<td>22.4</td>
<td>29.2</td>
</tr>
<tr>
<td>86</td>
<td>18.9</td>
<td>30.6</td>
</tr>
<tr>
<td>96</td>
<td>18.4</td>
<td>26.1</td>
</tr>
</tbody>
</table>

<sup>a–d</sup>, mean C-values followed by the same letter are not significantly different at p = 0.05 (Duncan’s test).

Stages of development: 40 hours after imbibition started (HAI), before collet hair emergence (sample included the root); 48 HAI, after collet hair emergence (hairs not full length); 60 HAI, collet hairs full length.

**Fig. 9.** Digital 3D volume-rendering reconstruction of two nuclei in the collet hairs of the Arabidopsis NLS-GFP-GUS line, as viewed through the confocal microscope (A) and after 90° rotation (B). Part of the right-hand-side nucleus (above surface X) is not included in the Z-stack. The shape and brightness of the nucleus in the confocal image depends on the position of the nucleus in the Z-stack and cannot be a base for measurement of size. The broken lines in (B) mark the possible plane of scanning that would result in an image of a ‘fragmented’ nucleus (this figure is available in colour at JXB online).

**Fig. 10.** Ploidy levels of nuclei of the Arabidopsis NLS-GFP-GUS line. 2C, After mitosis in root tip cells; 4C, before mitosis in root tip cell; 8C, at the bulge formation in collet epidermal cell, 16C in growing collet hair. Bar = 10 μm.
the collet hairs. However, because of difficulties in obtaining by dissection a pure fraction of the fragile collet hairs, the isolated nuclei also may have contained some from the root hairs and cell layers other than the epidermis. Similarly to collet hairs, 16C nuclei were detected in the root hairs of *Arabidopsis* (Sugimoto-Shirasu et al., 2005).

A further increase in the size of the collet nuclei was observed when they returned to the base of the hairs and moved only back-and-forth for a short distance (Supplementary Video S2), although the nuclei with DNA content higher than 16C were not detected by FCM (Supplementary Fig. S4). It is possible, however, that they did not form a clear peak on the FCM histograms because of their low proportion in a sample. The results of measurements of nuclear size based on their fluorescence on 3D-processed confocal pictures should be treated with caution, because of the irregular size of the nuclei, their different positioning in the cell, and the possibility of capturing only a part of them during laser scanning (Fig. 9). It is also possible that to some extent the increased size of the nuclei was due to chromatin condensation rather than ploidy change, as has been reported to occur in germinating *Arabidopsis* seeds (van Zanten et al., 2011).

Nevertheless, the collet cells are a useful system in which to study simultaneous DNA synthesis/endoreduplication. The present study confirms that endopolyploidy is connected with cell growth and differentiation. However, it does not increase in proportion to cell size, suggesting that the maximal number of endocycles is genetically programmed. Similar conclusion was drawn from the studies on endoreduplication in sugar beet (Sliwinska and Lukaszewska, 2005; Lukaszewska and Sliwinska, 2007). Further studies on the collet hairs, using constructs that target fluorescent proteins to different cell/nuclear structures could provide more details on the functional significance of endoreduplication.

In conclusion, it has been demonstrated that hairs develop simultaneously from all epidermal cells of the collet, well before the root hairs, in a distinctly different region of the seedling axis. During tip growth, both microtubules and actin are required to maintain the movement of the nucleus close to the growing tip, although hair elongation proceeds even in the absence of the nucleus in this region. The initial synchronized acropetal movement of nuclei along with tip growth and their subsequent basipetal movement later becomes disturbed and the nuclei remain close to the base of majority of the collet hairs. This coincides with the presence of large vesicles, prevacuoles, and the vacuole. The ploidy of the collet hairs becomes disturbed and the nuclei remain close to the base of the root hairs, in a distinctly different region of the seedling axis. During tip growth, both microtubules and actin are simultaneously from all epidermal cells of the collet, well before the root hairs, in a distinctly different region of the seedling axis. During tip growth, both microtubules and actin are required to maintain the movement of the nucleus close to the growing tip, although hair elongation proceeds even in the absence of the nucleus in this region.

### Supplementary data

Supplementary data are available at JXB online.

**Supplementary Video S1.** Nuclear movement in the collet epidermal cells of the GFP-transformed *Arabidopsis* NLS-GFP-GUS line, from 40 to 62 HAI

**Supplementary Video S2.** Nuclear movement in the collet hairs of the GFP-transformed *Arabidopsis* NLS-GFP-GUS line, from 64 to 98 HAI

**Supplementary Video S3.** Bulge formation and hair growth in the collet region of the GFP- and YFP-transformed *Arabidopsis* hybrid line NLS-GFP-GUS × YPM, from 38 to 49 HAI

**Supplementary Video S4.** Early growth of the collet hairs of the GFP- and YFP-transformed *Arabidopsis* hybrid line NLS-GFP-GUS × YPM, from 44 to 50 HAI

**Supplementary Video S5.** Nuclear movement in the collet hairs of the GFP- and YFP-transformed *Arabidopsis* hybrid line NLS-GFP-GUS × YPM, from 108 to 168 HAI

**Supplementary Video S6.** Nuclear movement in the collet hairs of the double-transgenic *Arabidopsis* hybrid line NLS-GFP-GUS × YPM treated with 200 nM oryzalin, from 48 to 72 HAI

**Supplementary Video S7.** Nuclear movement in the collet hairs of the double-transgenic *Arabidopsis* hybrid line NLS-GFP-GUS × YPM, from 46 to 68 HAI

**Supplementary Video S8.** Dynamics of nuclei and vesicles in the growing collet hairs (at 50 HAI) of a GFP- and RFP-transformed *Arabidopsis* hybrid line NLS-GFP-GUS × REndo

**Supplementary Video S9.** Dynamics of the nuclei and vesicles in the mature collet hairs of a GFP- and RFP-transformed *Arabidopsis* hybrid line NLS-GFP-GUS × REndo, from 69 to 76 HAI

**Supplementary Fig. S1.** Collet hairs of the double-transgenic *Arabidopsis* hybrid line NLS-GFP-GUS × YPM during their early growth (44 HAI)

**Supplementary Fig. S2.** Effect of oryzalin on microtubules in the collet/root region of a seedling of the *Arabidopsis* GFP-MAP4 line

**Supplementary Fig. S3.** Effect of latrunculin B on actin in the collet/root region of a seedling of the *Arabidopsis* GFP-Talin line

**Supplementary Fig. S4.** Selected flow cytometric histograms of a nuclear preparation from the collet region of the *Arabidopsis* WT ecotype Columbia after staining with propidium iodide

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References


Sliwinska E, Bassel GW, Bewley JD. 2009. Germination of Arabidopsis thaliana seeds is not completed as a result of elongation of the radicle but of the adjacent transition zone and lower hypocotyl. Journal of Experimental Botany 60, 3587–3594.


