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

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Abstract

The completion of germination of seeds of *Arabidopsis thaliana* is marked by the appearance of the radicle through the surrounding endosperm and testa. Using confocal microscopy and green fluorescent protein (GFP)-transformed embryos to highlight the epidermal cell walls it has been possible to conduct time-lapse photography of individual embryos during their germination. This reveals that the elongation of embryo cells to effect completion of germination does not occur within the radicle itself, but rather within a discrete region that is immediately proximal to the radicle. This region, identifiable as the lower hypocotyl and hypocotyl–radicle transition zone, is also definable by accumulation of carbohydrate-containing bodies during germination, and distinct GFP expression of GAL4–GFP in enhancer trap lines. Flow cytometric studies show that there is an increase in the proportion of 4C nuclei in the axis which coincides with a considerable increase in length of the hypocotyl, and the occurrence of endopolyploid (8C and 16C) nuclei accompanies the 2-fold increase in mean cell size in the region of elongation, the lower hypocotyl, and hypocotyl–radicle transition zone. Thus the observed cell elongation during germination is accompanied by an increase in nuclear DNA content, and the resultant elongation of the axis to effect radicle emergence is due to cell expansion, not to cell division. When studying the molecular events involved in the completion of germination, therefore, it may be prudent to focus on this region of elongation.

Key words: *Arabidopsis thaliana*, cell cycle, cell elongation, DNA content, endoreduplication, germination, green fluorescent protein, hypocotyl, radicle, transition zone.

Introduction

The embryo of dicotyledonous seeds, the next generation of plant, is composed of two cotyledons and an axial region. In many species, such as *Arabidopsis thaliana* L. Heynh, this region includes a plumule, which becomes the shoot apex, separated by a hypocotyl from the radicle, which contains the root meristem. There has been some debate as to where the hypocotyl ends and the radicle begins, but detailed anatomical (Esau, 1965) and molecular (Lin and Schiefelbein, 2001) studies of young seedlings indicate that the basal hypocotyl–radicle junction (transition zone) coincides with the last root cap cell, and that the proximal root hair is the landmark for the lower limit of the hypocotyl. Germination involves those events which occur between imbibition of a dry seed and emergence of the radicle through the enclosing structures (Bewley and Black, 1978), in *Arabidopsis* these being the thin endosperm and testa. It has been widely assumed that since it is the radicle that emerges from the seed this is the structure that elongates to effect the completion of germination (‘visible germination’). There are suggestions in the literature, however, that it is the hypocotyl that extends to push out the radicle [e.g. in *Arabidopsis*, lettuce (*Lactuca sativa*), French bean (*Phaseolus vulgaris*), and broad bean (*Vicia faba*); Srivastava and Paulson, 1968; Musatenko *et al.*, 1981; Obroucheva *et al.*,]
1995; Yamaguchi et al., 2001; Antipova et al., 2003). However, most of the supporting data were obtained from micrographs of fixed and sectioned material, and hence different embryos were used for each time point of germination studied. Since it is not possible to predict which embryo is going to germinate, and when, the data can be regarded only as suggestive.

Cell elongation is often accompanied by an increase in nuclear DNA content due to endoreduplication (DNA replication without subsequent mitosis; Melaragno et al., 1993; Gendreau et al., 1998; Kato and Lam, 2003; Barow, 2006). Genes related to DNA synthesis, but not cell division, are induced in a gibberellin (GA)-dependent fashion during the early stages [9 hours after imbibition (HAI)] of Arabidopsis seed germination (Ogawa et al., 2003). Endoreduplication in imbibed Arabidopsis seeds has been detected just before, or at the time of, radicle protrusion although it was suggested that it is restricted to cells of the cotyledons (Barrôco et al., 2005). Thus the coincidence between endoreduplication and germination in Arabidopsis is not clear. Using confocal microscopy combined with time-lapse photography, it has been possible to follow individual Arabidopsis embryos with visibly fluorescent cell walls throughout germination, and to define precisely which cells elongate before the time of radicle emergence. Flow cytometric analysis of the cell cycle in different parts of the embryo during imbibition has allowed verification of where, and at which phase of germination, DNA synthesis occurs. Germination is poorly understood from a molecular standpoint, and defining which cells are specifically involved in the completion of this phenomenon provides an important target for such studies.

Materials and methods

Experimental materials

Seeds of A. thaliana line Q5 expressing a green fluorescent protein (GFP) fusion to the targeting signal for the vacuolar membrane protein δ-TIP (Cutler et al., 2000) and enhancer trap lines E230 and E1628 were provided by the Arabidopsis Biological Resource Center (ABRC, USA).

Confocal microscopy and staining of sections

Seeds were imbibed on humid filter paper in a 5.5 cm diameter Petri dish at 20 ± 1°C in darkness. At 16–18 HAI embryos were isolated, placed in a One-chamber Lab-Tek Chambered Coverglass (Fisher Scientific, Mississauga, ON, Canada), and covered with 0.8% (w/v) agar and a coverslip. Videos (using four different embryos) were produced from confocal stacks collected using a Leica Multiphoton TCS-SP5 (Leica Microsystems GmbH, Mannheim, Germany) confocal microscope with an argon laser. Cell length along the hypocotyl–radicle axis was measured at 10 different time points during germination and early seedling growth using the LAS AF program (Leica Microsystems). The cells are numbered with the most distal cell of the root cap as the starting point. The files obtained by confocal microscopy were converted to QuickTime videos.

Whole-embryo periodic acid (PA)–propidium iodide (PI) staining, imaging, and data analyses were performed as described by Truernit et al. (2008) using the Q5 GFP line (Cutler et al., 2000). Embryos were dissected from their seed coats using a scalpel and fixed in 50% (v/v) methanol and 10% (v/v) acetic acid overnight at 4°C. Tissues were then rinsed in water and incubated with 1% (w/v) PA for 40 min followed by rinsing in water. Staining was performed for 30 min in 100 mM sodium metabisulphite, 0.15 M HCl, to which PI was freshly added to a final concentration of 100 μg ml⁻¹. Embryos were then thoroughly rinsed in water before being cleared overnight in chloral hydrate (4 g of chloral hydrate, 1 ml of glycerol, and 2 ml of water). Samples were transferred to slides and mounted using Hoyer’s solution (30 g of gum arabic, 200 g of chloral hydrate, 20 g of glycerol, and 50 ml of water). GFP was imaged in the embryos following dissection and mounting in 70% (v/v) glycerol on a microscope slide.

PA–PI and enhancer trap GFP fluorescence images were acquired using a Zeiss LSM 510 META with an argon laser (Carl Zeiss, Jena, Germany).

Flow cytometry

For flow cytometry, embryos were isolated from dry seeds of the Q5 GFP line and at 8, 16, 24, 32, 40, and 48 HAI, and dissected into cotyledons and hypocotyl-radicle axes. Nuclei released from 20 seed parts constituted a sample, from which nuclei were isolated (Sliwinska, 2003) and stained using PI (50 μg ml⁻¹). For each sample, 5–7 × 10³ nuclei were analysed using a BD FACSCalibur (BD Biosciences, San Jose, CA, USA) flow cytometer. Analyses were performed on four replicates, using a logarithmic scale. Histograms were evaluated using the CELL Quest Pro Software program (BD Biosciences), and the percentage of nuclei with particular DNA contents, the (4C+8C+16C)/2C ratio, and the mean C-value (Lemontey et al., 2000) were calculated before applying one-way analysis of variance (ANOVA) and a Duncan’s test (Supplementary Tables S1–S4 available at JXB online).

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

Results and discussion

Analysis of embryo axis extension

The defined regions in the Arabidopsis embryo are marked in Fig. 1 as: cells 1–24, hypocotyl; 25–29, transition zone; 30–37, radicle.

A GFP-transformed line was used to determine the region of elongation in the embryo that resulted in
emergence of the radicle. Confocal microscopy allowed changes to be followed in real time, by collecting 40 Z-sections (step size 1 μm) every 15 min, from initial swelling of the embryo until production of the first root hairs (Supplementary Video S1 at JXB online, a time-lapse video from which stills are taken for Fig. 1A–J). Under the conditions to which the embryos were subjected during the confocal microscopy studies, the corresponding stages to events in the intact seed were: (i) the beginning of the testa rupture, 38 HAI (Fig. 1C); (ii) the beginning of the endosperm rupture, 46 HAI (Fig. 1E); and (iii) the emerged hypocotyl–radicle axis reaching 1–3 mm in length, 54 HAI (Fig. 1G). The timing of germination of an intact seed for stages (i), (ii) and (iii) was: 24, 32, and 40 HAI, respectively (see legend of Fig. 4).

Before elongation of the embryo commenced, at 22 HAI (Fig. 1A), the length of the hypocotyl was almost 350 μm, the transition zone 60 μm, and the radicle 85 μm (Fig. 2, Table 1). It has been contended that germination of epigeous seeds (i.e. those in which the cotyledons emerge above the soil during seedling growth) begins with cell elongation in the radicle (resulting in its emergence through the surrounding structures), followed by hypocotyl growth (Obroucheva et al., 1995); however, in Arabidopsis embryos, no substantial increase in the length of the radicle during germination was observed. At the time immediately prior to emergence of the embryo through the endosperm at 46 HAI (Fig. 1E) it had elongated only by 10 μm (12%), while the hypocotyl was ~30% longer (by 200 μm), and the transition zone between these two regions had elongated by 44% (by almost 30 μm; Fig. 2, Table 1). By the time the radicle had emerged at 54 HAI (Fig. 1G), the radicle still had elongated by only 15%, whereas the other two regions were 52% and 108% longer, respectively. After this time, geotropic curvature of the radicle (already visible in Fig. 1H–J) precluded measurements of its length. Root hair formation occurred after germination (starting at 58 HAI) in cells 25 and 26 (Figs 1J, 2; Supplementary Video S1 at JXB online).

The first cells to elongate by ~10 μm (at 42–46 HAI; Fig. 1D, E; blue zone in Fig. 2) were the basal hypocotyl cells (nos 20–24), accompanied by the upper cells of the hypocotyl–radicle junction (25–27). (These measurements using confocal microscopy were repeated for three other germinating embryos, all of which showed the same pattern of change in cell lengths.) In addition, some individual cells of the hypocotyl, usually close to the junction with the cotyledons, underwent a substantial increase in length (Fig. 2, marked as blue). Thus elongation of the hypocotyl was accompanied by the gradual increase in the space between it and cotyledons, and further expansion of the junction cells is probably involved in the change in plane of the cotyledons as the seedling grows. When the hypocotyl–radicle axis was long enough to cause radicle protrusion (at 54 HAI), the elongation zone expanded upwards in the hypocotyl and downwards in the transition zone, but in the majority of cases only the cells numbered 19–27 elongated.

Fig. 1. Germination and early seedling growth of an isolated embryo of Arabidopsis. (A) 22 HAI, (B) 27 HAI, (C) 38 HAI, (D) 42 HAI, (E) 46 HAI, (F) 50 HAI, (G) 54 HAI, (H) 58 HAI, (I) 62 HAI, (J) 66 HAI. Timing of events related to germination and early seedling growth: 38 HAI, beginning of testa rupture; 46 HAI, beginning of endosperm rupture; 54 HAI, hypocotyl–radicle axis of 1–3 mm in length; 58 HAI, beginning of root hair formation. The scale at the bottom of each embryo marks the cell number, from the point of attachment of the cotyledon to the axis to the radicle tip.
Fig. 2. Length of the cells in the hypocotyl–radicle axis in an isolated Arabidopsis embryo during germination. Cell numbers correspond to those in Fig. 1. Cell nos 1–24: hypocotyl; 25–29: transition zone; 30–38: radicle. For timing of germination see legend of Fig. 1.

Table 1. Length of axial regions and mean cell length in isolated Arabidopsis embryos during germination and early seedling growth

Mean value of seven cell files. Timing of germination, corresponding to intact seed: 38 HAI, beginning of testa rupture; 46 HAI, beginning of endosperm rupture; 54 HAI, radicle emergence.

<table>
<thead>
<tr>
<th>HAI</th>
<th>Length (μm ± SD)</th>
<th>Mean cell length (μm ± SD)</th>
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<tbody>
<tr>
<td></td>
<td>Hypocotyl</td>
<td>Transition zone</td>
</tr>
<tr>
<td>22</td>
<td>347±30</td>
<td>59±13</td>
</tr>
<tr>
<td>27</td>
<td>361±34</td>
<td>61±12</td>
</tr>
<tr>
<td>38</td>
<td>398±24</td>
<td>68±13</td>
</tr>
<tr>
<td>42</td>
<td>425±30</td>
<td>75±16</td>
</tr>
<tr>
<td>46</td>
<td>453±34</td>
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<tr>
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<td>166±27</td>
</tr>
<tr>
<td>66</td>
<td>617±36</td>
<td>185±30</td>
</tr>
</tbody>
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* Includes data of cell measurements of intact seeds measured in PA–Pl-stained optical sections using Osirix software (Truernit et al., 2008); radicle measurements could not be made at 58–66 HAI following completion of germination (see Figs 1 and 2).

by >10 μm, as they also did during the early seedling growth. At this time, the most elongated cells (by >20 μm; green zone in Fig. 2) were almost exclusively in the transition zone.

Unique aspects of the transition zone

Evidence for an activation of metabolic processes within the transition zone during germination is shown in Fig. 3A–C.
Optical sections, using confocal microscopy, were taken of germinating embryos dissected from intact seeds, and stained using the PA–PI procedure (Truernit et al., 2008). This fluorescent carbohydrate staining procedure identified the presence of discrete intracellular bodies that first appeared by 18 HAI, prior to testa rupture (Fig. 3A,B), and increased in abundance in the lower hypocotyl region at 24 HAI, the time of testa rupture (Fig. 3C). The nature of these carbohydrate-containing bodies in this region is unknown; they may be starch, as suggested for celery embryos (Jacobsen and Pressman, 1979), or other complex glucans. Those in the root cap are probably statoliths, involved in post-germinative geotropic curvature of the root (Sæther and Iverson, 1991).

That there are localized patterns of gene expression in the lower hypocotyl transition region is supported by enhancer trap reporter patterns (Fig. 3D, E). GAL4–GFP enhancer trap line E230 (www.enhancertraps.bio.upenn.edu) shows distinct GFP expression within this region of elongation during germination at 18 HAI, prior to testa rupture (Fig. 3D). Enhancer trap line E1628 displays a similar pattern at the same time point (Fig. 3E). These localized patterns demonstrate a specific activation of gene expression during germination within the region of elongation, similar to that previously described for GA3oxidase1 in Arabidopsis (Yamaguchi et al., 2001) and the expansin (LeEXP8) gene in tomato (Chen et al., 2001).

Cell cycle changes in the embryo during germination

To determine if cell elongation, to effect the completion of germination, is accompanied by an increase in nuclear DNA content, flow cytometric analysis was performed on nuclei isolated from regions of the germinating and germinated embryo. The relationship between the cell size and ploidy has been described for Arabidopsis leaf, stem, and hypocotyl (Melaragno et al., 1993; Gendreau et al., 1998; Kato and Lam, 2003; Barow, 2006), but not for all of the embryo regions, and mostly in seedlings. Here, in the cotyledons, only the cells containing 2C and 4C DNA (C=DNA content of a holoploid genome with chromosome number n) were present during germination and early seedling growth (Fig. 4; Supplementary Fig. S1 at JXB online). There was no DNA replication in the cotyledons.

Fig. 3. PA–PI-positive intracellular bodies appear in the elongation zone of Arabidopsis embryos during germination. Optical sections (0.5 μM) of embryos (line Q5) stained with PA–PI (A–C). (A) 7 HAI. (B) 18 HAI, before testa rupture. (C) 24 HAI, beginning of testa rupture. (D) Enhanced fluorescence in the elongation zone prior to testa rupture at 18 HAI in GAL4–GFP enhancer trap lines E230 and E, E1628.
until endosperm rupture (containing ~95% of 2C and 5% of 4C nuclei), when the proportion of the 4C nuclei started to increase greatly, reaching >40% in seedlings producing the first root hairs (48 HAI). However, contrary to the contention of Barroêco et al. (2005), who analysed whole Arabidopsis seeds by flow cytometry, no endopolyploid nuclei were detected in the cotyledons. A different pattern of DNA replication occurred in the hypocotyl–radicle axis region, where a considerable increase in 4C nuclei had already occurred by the time of testa rupture. At the beginning of endosperm rupture (and before germination was completed) the axis consisted of 60% 2C, 39% 4C, and 1% 8C nuclei, and at 48 HAI (post-germination) a few nuclei with 16C DNA appeared. In previous reports, analyses of the whole seed/embryo of Arabidopsis during germination and early seedling growth did not detect any 16C nuclei (Barroêco et al., 2005; Masubelele et al., 2005), probably because they were masked by the larger number of nuclei with 2C, 4C, and 8C DNA, including those in the non-axial tissue. In this study, the mean C-value and the (4C+8C+16C)/2C ratio also significantly increased, marking the late phase of germination (Fig. 4). At corresponding times in isolated embryos the increase in proportion of 4C nuclei in the axis coincided with a considerable increase in length of the hypocotyl, and the occurrence of endopolyploid (8C and 16C) nuclei accompanied the 2-fold increase in mean cell size in the elongation zone (Fig. 4, Table 1). This confirms that the observed cell elongation during germination is accompanied by an increase in nuclear DNA content, and that the expansion of the axis to effect radicle emergence from the Arabidopsis seed is due to cell expansion, not cell division, and is consistent with previous gene expression analyses (Ogawa et al., 2003). Although the physiological significance of endoreduplication is still not well understood, it has been suggested that since the endocycles do not require reorganization of the cytoskeleton, it may allow for faster growth of a tissue than by cell proliferation (Kondorosi and Kondorosi, 2004), which might be of value during embryo germination.

The radicle has been generally considered to be the region of the embryo where the increase in 4C nuclei predominantly occurs during germination. Therefore, it has been recommended that changes in the radicle be monitored by flow cytometry to follow the advancement of germination and/or priming (Bino et al., 1992, 1996). However, as shown here, the basal part of the hypocotyl and the hypocotyl–radicle transition zone are where elongation occurs, accompanied by an increase in cell DNA content, following imbibition but prior to the completion of germination. Hence it is also

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**Fig. 4.** Changes in the cell cycle activity during germination of intact Arabidopsis seeds and early seedling growth. (A) Mean C-value. (B) (4C+8C+16C)/2C ratio. (C) Proportion of nuclei with different DNA content in cotyledons and the hypocotyl–radicle axis. Error bars are the SD (n=4). Values for the certain parameter marked with the same letter are not significantly different at P=0.05 (Duncan’s test); lower case letters, cotyledons; upper case letters, hypocotyl–radicle axis. Timing of germination: 24 HAI, beginning of testa rupture; 32 HAI, beginning of endosperm rupture; 40 HAI, hypocotyl–radicle axis of 1–3 mm in length; 48 HAI, seedling with the first root hairs. In a Petri dish, germination was faster than under the conditions used for confocal microscopy because for the latter it was necessary to limit geotropic curvature of isolated embryos by placing them between glass coverslips; this probably resulted in mild hypoxia, resulting in a slower completion of germination.
these regions of the embryo, and not just the radicle, which must be analysed to provide clearer information on germination advancement, which is important in seed production for predicting seed quality (Sliwinska, 2009).

Concluding remarks
By using confocal microscopy and time-lapse photography of individual Arabidopsis embryos, it has been possible to show that the completion of germination, which in intact seeds results in the emergence of the radicle (visible germination), is not the result of expansion of its own cells. Rather, cell expansion occurs in a discrete region that is proximal to the radicle, one which is also defined by unique gene expression, as shown by enhancer trap patterns, and metabolic activity that results in the deposition of bodies detected by carbohydrate staining. Completion of germination is the result of cell expansion, not cell division within the hypocotyl–radicle region, with the observed increase in DNA taking place by endoreduplication. Despite much research, the events that are essential for radicle emergence are unknown; identification of a discrete region responsible for the final stage of germination, i.e. cell elongation, suggests that a small number of cells are involved. Thus the usual practice of analysing whole embryos or even embryo axes could mask the changes taking place within them. This has implications both in research to determine the fundamental molecular and metabolic processes involved in germination, and for such agronomically important practices as the monitoring of seeds to predict quality.

Supplementary data
Supplementary data are available at JXB online.

Fig. S1. Selected DNA histograms of nuclear preparations from GFP-transformed Arabidopsis dry and imbibed seeds. (A) Cotyledons. (B–D) Hypocotyl–radicle axis. (A, B) Dry seeds. (C) 32 HAI (beginning of endosperm rupture). (D) 48 HAI (seedling with first root hairs).

Video S1. Changes in epidermal cell length during embryonic germination and early growth of GFP-transformed Arabidopsis. Time lapse interval: 15 min. 40 Z-sections, step size 1 μm.

Table S1. Analysis of variance of the mean C-value in the nuclei of the cotyledons.

Table S2. Analysis of variance of the mean C-value in the nuclei of the hypocotyl–radicle axis.

Table S3. Analysis of variance of the (4C+8C+16C)/2C ratio in the cotyledons.

Table S4. Analysis of variance of the (4C+8C+16C)/2C ratio in the hypocotyl–radicle axis.

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References


