Seed Science Research (2001) **11**, 45–54 DOI: 10.1079/SSR200059

Depth of dormancy in tomato (*Lycopersicon esculentum* Mill.) seeds is related to the progression of the cell cycle prior to the induction of dormancy

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Abstract

Cell cycle activities are initiated following imbibition of non-dormant seeds. However, it is not known whether cell cycle related events other than DNA replication also remain suppressed in imbibed dormant seeds. The objective of this study was to demonstrate that the transitions between the non-dormant and dormant (both primary and secondary) states are reflected in cell cycle events, such as DNA replication and the changing patterns of the microtubular cytoskeleton involved in the processes of growth and development. The present studies were conducted on seeds from tomato (*Lycopersicon esculentum* cv. Moneymaker) that possessed primary dormancy or were manipulated to attain secondary dormancy. In addition, a non-dormant abscisic acid (ABA)-deficient mutant, *sit*^{w}, was used. DNA replication, as measured by flow cytometry, and β-tubulin accumulation, analysed by immunoblotting, were compared with immunocytological studies of active DNA synthesis and microtubular cytoskeleton formation. It is shown that the depth of dormancy, which distinguishes primary and secondary dormancy, may depend on the progression of the cell cycle prior to the induction of dormancy.

Keywords: β-tubulin, BrdU, cell cycle, cytoskeleton, DNA synthesis, dormancy, *Lycopersicon esculentum*

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Introduction

During maturation seeds may enter a state of primary dormancy. This type of dormancy is characterized by the inability of the mature seeds to germinate under favourable conditions. Dry storage, especially at elevated temperatures, or a chilling treatment in the imbibed state often remove dormancy. If the conditions for germination of the non-dormant seeds are not favourable, seeds may become dormant again. This state of dormancy is called secondary dormancy (Bewley and Black, 1994; Karssen, 1995).

Seed dormancy is an ill-understood phenomenon. The transitions between the dormant and non-dormant states are not well characterized (Cohn, 1996; Hilhorst, 1997). So far, the only clear difference demonstrated between these states is the sensitivity to environmental factors that stimulate germination, such as light, nitrate and the range of temperatures over which germination can occur (Hilhorst, 1990a, b). Non-dormant seeds are more responsive to these factors and germinate over a wider range of temperatures. The responsiveness to gibberellins may also increase upon relief of dormancy (Derkx and Karssen, 1993; Hilhorst, 1995). Yet, the transitions between states of dormancy are not reflected in any change in general metabolic activity, as measured by respiratory activity (Derkx and Karssen, 1993; Derkx *et al*., 1993). However, germination of non-dormant seeds is accompanied by an increase in respiratory and other metabolic activities, followed by the mobilization of food reserves.

The formation of the microtubular cytoskeleton and synthesis of embryonic DNA are required to enable cell expansion and division, which lead to germinative growth and radicle protrusion in tomato (*Lycopersicon esculentum* cv. Moneymaker) seeds (Jing *et al*., 1999; de Castro *et al*., 2000). These cell cycle events commence during imbibition of non-dormant

seeds. DNA replication appears to be suppressed in tomato seeds with secondary dormancy (Groot *et al*., 1997). In embryos from dormant lines of *Avena fatua*, slow but continuous DNA replacement (repair) was observed. It was suggested that this maintained genome integrity, to distinguish it from replicative DNA synthesis associated with germination and growth (Elder and Osborne, 1993). However, it is not known whether other cell cycle related events also remain suppressed in imbibed dormant seeds. The objective of this study was to demonstrate that transitions between the non-dormant and dormant (both primary and secondary) states are reflected in cell cycle events such as DNA synthesis and changes in appearance of the microtubular cytoskeleton.

For the present study we have used seeds from tomato (*Lycopersicon esculentum* cv. Moneymaker) which possessed primary dormancy, or which were manipulated to acquire secondary dormancy. In addition, the non-dormant, abscisic acid (ABA)-deficient, *sit*^{w} mutant was used. DNA replication, as measured by flow cytometry, and β-tubulin accumulation, as analysed by immunoblotting, were compared with immunocytological observations of DNA synthesis and microtubular cytoskeleton formation.

Material and methods

Seed material

Seeds of tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) with an initial moisture content of 6.0 ± 0.1% (fresh weight basis) were used. Seeds with primary dormancy were obtained from tomato plants grown in a greenhouse during the summer of 1996 under natural daylight at 25°C/20°C day/night average temperatures. After harvest seeds were removed from the fruits, dried over a saturated $CaCl_2$ solution for 2 d at 20°C and 32% relative humidity (de Castro *et al*., 1995) and stored at –20°C until use. Full primary dormancy was obtained by imbibing seeds in water at 25°C in darkness and keeping them incubated under the same conditions for 1 month. After this period approximately 20% of the seeds had germinated. They were removed and the remaining (dormant) seeds were used in the experiments and are referred to as seeds with primary dormancy. These seeds were incubated for a further 7 d under the conditions mentioned above. After this period, seeds were chilled at 10°C for 24 h, exposed to continuous white light, or incubated in GA_{4+7} (Plant Protection Ltd, Surrey, UK) in darkness to break dormancy, as described below, and scored for germination over 7 days. Seeds of the ABA-deficient *sit*^{w} mutant were used as fully non-dormant seeds.

Manipulation of secondary dormancy

Secondary dormancy was induced in non-dormant seeds by a far-red light (>730 nm) treatment for 5 min at hourly intervals during the first 24 h of imbibition at 25°C, followed by 7 d at 25°C in darkness (Groot *et al*., 1997). Over 96% of the seeds treated with far-red irradiation did not germinate after the 7 d of incubation in the dark, and were therefore characterized as seeds with secondary dormancy. These seeds were then submitted to a chilling treatment at 10°C for 24 h, transferred to light or incubated in GA_{4+7} (pH 4), as for the seeds with primary dormancy.

Germination

Germination analysis was conducted on four replicates of 50 seeds placed on two layers of filter paper (Whatman No. 1) in 9 cm Petri dishes soaked with 6 ml distilled water or 10 μM GA_{4+7}, at 25 ± 1°C in darkness or under white fluorescent light (Philips TL 84) for 7 d. Germination was expressed as the percentage of seeds that exhibited approximately 1 mm radicle protrusion.

DNA replication and detection of β-tubulin

Two replicates of five whole embryos were used for the flow cytometric analysis of the nuclear DNA content according to Sacandé *et al*. (1997). Briefly, embryos were chopped with razor blades into 1 ml of nuclear isolation buffer (0.2 M mannitol, 10 mM MES, 10 mM NaCl, 10 mM KCl, 10 mM spermine tetrahydrochloride, 2.5 mM Na_2-EDTA, 2.5 mM dithiothreitol (DTT), 0.05% v/v Triton X-100, pH 5.8). The mixture was sieved through 88-μm nylon mesh, and to the filtrate 0.04 ml of a 1 mg ml^{-1} propidium iodide solution was added. The DNA content of isolated nuclei was measured 10 min after staining of the sample using a flow cytometer (Coulter Corp., Miami, FL, USA; model Epics XL-MCL). For all samples at least 10,000 nuclei were analysed.

Extraction and detection of β-tubulin by Western blotting were conducted as described previously (de Castro *et al*., 1995, 1998). Briefly, excised embryos were frozen immediately in liquid nitrogen and ground to a powder. Proteins were extracted in a buffer consisting of 62.5 mM Tris–HCl, 2% SDS, 15 mg ml^{-1} DTT and 7% glycerol, pH 9.0. The samples were incubated for 10 min at room temperature and centrifuged for 7 min at 17,000 *g*. Electrophoresis was performed with precast 8–18% polyacrylamide SDS gradient gels. After electroblotting, the blotting membranes (PVDF, 0.45 μm, Amersham, Buckinghamshire, UK) were incubated in a blocking solution and subsequently in a monoclonal mouse anti-β-tubulin antibody solution of

1 μg ml^{-1} (Roche Diagnostics, Almere, The Netherlands). After extensive washing, membranes were probed with a secondary peroxidase (POD)-conjugated anti-mouse antibody (50 mU ml^{-1}) (Roche Diagnostics). Membranes were exposed to photographic film (Hyperfilm-ECL, Amersham, Buckinghamshire, UK).

Immunohistochemical detection of BrdU and β-tubulin

After imbibition in water, seeds were immersed in a bromo-deoxyuridine (BrdU) solution (Amersham; dilution 1:500 v/v) at 25°C in the dark, either as sectioned seeds or isolated embryos. In order to avoid possible interference between labelling procedures for BrdU and β-tubulin, separate sections of the seed material were used (Rös and Wernicke, 1991). The cytotoxicity and induction of germination by various pulse lengths in BrdU solution were assessed by comparing the pattern of the flow cytometric profiles and the microtubular cytoskeleton with the normal pattern observed after immunolabelling of β-tubulin in the absence of BrdU (Rös and Wernicke, 1991). Preliminary analysis showed that 3 h was the minimum pulse length required for detecting DNA synthesis. Immunohistolabelling of β-tubulin was conducted on material not incubated with BrdU. Embryos were fixed in 4% paraformaldehyde for 4 h, rinsed, dehydrated and embedded in butyl-methylmetacrylate according to Baskin *et al*. (1992). Samples were sectioned and affixed on slides by carefully laying the section on top of a drop of water, stretching it by means of chloroform vapour and heating it on a hot plate at 60°C. Sections were further processed either for the immunofluorescence detection of DNA synthesis (incorporated BrdU) or for the visualization of the microtubular cytoskeleton (β-tubulin) according to Xu *et al*. (1998). Anti-β-tubulin (Amersham) was diluted 1:200 v/v; anti-BrdU (Amersham) was diluted 1:1 v/v. In both cases, the second antibody used was fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse (Amersham) diluted 1:200 (v/v). Nuclear DNA was counterstained with 1 mg ml^{-1} propidium iodide (PI) (Molecular Probes, Eugene, OR, USA). Omission of the first antibody and application of pre-immune serum served as controls and showed no fluorescence. Confocal laser scanning microscopy and photography were as described by Xu *et al*. (1998).

Results

Non-dormant seeds

The quantification of replicated 4C DNA nuclei, analysis of β-tubulin accumulation, DNA synthesis activity and microtubular cytoskeleton formation in non-dormant seeds confirmed results of a previous study (de Castro *et al*., 2000). Briefly, non-dormant seeds completed germination in the dark within 72 h (Fig. 1). The flow cytometry data for dry seeds indicated a low level (<5%) of embryonic nuclei containing 4C DNA (Fig. 2), and there was no active DNA synthesis detected by BrdU incorporation (Fig. 3A); β-tubulin was not detectable in whole embryo extracts (Fig. 4, lane 4), and microtubular arrays were not observed (Fig. 5A,D). After 24 h of imbibition, incorporation of BrdU was observed (not shown), concomitantly with a significant ($P < 0.05$) increase in the relative number of 4C nuclei (Fig. 2), accumulation of β-tubulin (Fig. 4, lane 5), and appearance of cortical and mitotic microtubular cytoskeleton arrays in the embryonic radicle tip region (not shown). The higher intensity of cell cycle

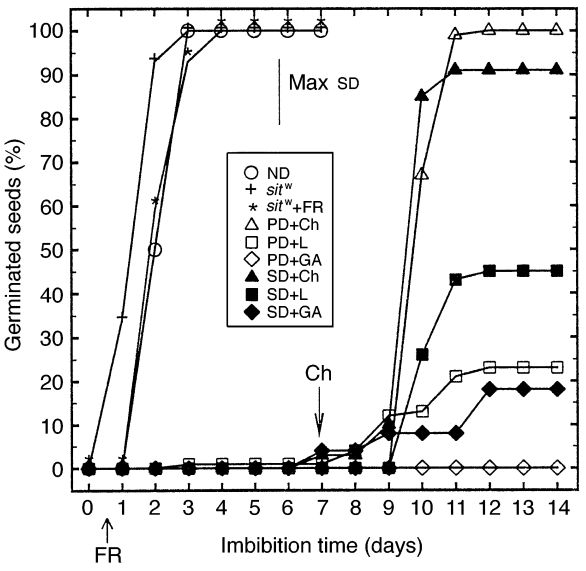


Figure 1. Germination of non-dormant and dormant tomato (cv. Moneymaker) seeds. Germination of the non-dormant (ND) wild-type control seeds and of the ABA-deficient (*sit*^{w}) seeds was scored during 7 d of incubation at 25°C in the dark. Germination was also scored for the ABA-deficient seeds when treated with far-red (*sit*^{w}+FR) light irradiation during the first 24 h of incubation. Seeds with primary dormancy (PD) and seeds with secondary dormancy (SD) were initially incubated for 7 d at 25°C in the dark. Then seeds were submitted to a chilling (+Ch) treatment and further incubated for another 7 d at 25°C in the dark, further incubated for 7 d under white light (+L), or further incubated for 7 d in 10 μM GA_{4+7} (+GA). Arrows indicate the moments during incubation at which the far-red (FR) or the chilling (Ch) treatments were applied. Seed were considered germinated when radicles had protruded at least 1 mm. Maximum standard deviation is indicated (vertical bar).

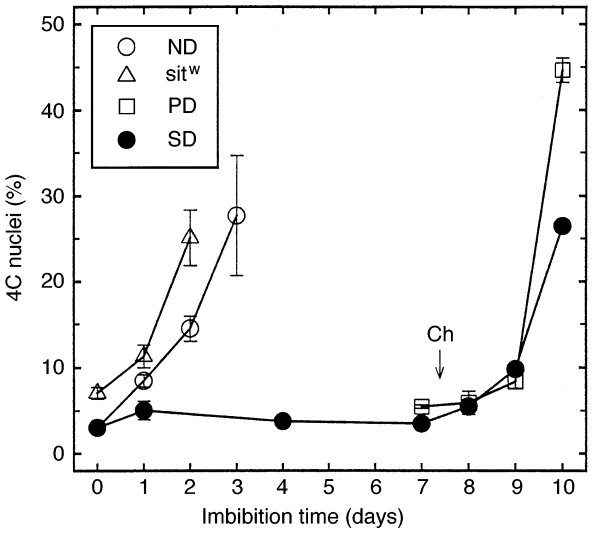


Figure 2. Nuclear 4C DNA in embryos of non-dormant and dormant tomato seeds. Frequencies of nuclei with 4C DNA contents are expressed as percentages of the total number of embryonic nuclei (2C+4C). Percentages of embryonic 4C nuclei are indicated for non-dormant (ND) wild-type control seeds and for ABA-deficient (*sit*^{w}) seeds during incubation at 25°C in the dark. 4C DNA counts for seeds with secondary dormancy (SD) were taken during the 7 d of incubation at 25°C in the dark and after subsequent chilling (Ch), whereas counts for seeds with primary dormancy (PD) were only taken after chilling. Arrow indicates the moment at which the chilling treatment was applied. Vertical bars represent the standard deviation (± SD) of the mean for two replicates of five embryos (not shown if smaller than the symbol). A minimum of 10,000 gated nuclei were counted for all samples.

events at 48 h of imbibition (Figs 2 and 4) preceded radicle protrusion, characterizing the completion of germination.

Seeds with primary dormancy

After prolonged dark incubation of over 1 month, dormant seeds hardly showed any sign of cell cycle activity. DNA synthesis did not occur, as the flow cytometric analysis did not detect any increase in the number of 4C nuclei (Fig. 2), and the incorporation of BrdU could not be detected (not shown). Accumulation of β-tubulin (Fig. 4) or formation of microtubular cytoskeleton (not shown) could also not be detected. After a 1 d chilling treatment, dormancy was efficiently released, as seeds germinated fully within 72 h (Fig. 1). DNA synthesis could still not be detected after the 1 d chilling treatment (Fig. 2). However, a trace amount of β-tubulin became detectable (Fig. 4) and some cortical microtubules became apparent, notably in cells of the radicle tip region (Fig. 5B,E). DNA synthesis activity was only detected 1 d after seeds were transferred back to 25°C in the dark but well before radicle protrusion. A significant ($P < 0.05$) increase in the number of replicated 4C DNA nuclei was detected (Fig. 2), as well as BrdU incorporation (Fig. 3B). At this stage, the level of β-tubulin had increased (Fig. 4), correlating with the appearance of an elaborate microtubular cytoskeleton, which was composed not only of cortical arrays, but also of mitotic arrays, such as pre-prophase bands, spindles and phragmoplasts (Fig. 5C, F–H). After 2 d at 25°C in darkness, the number of 4C nuclei (Fig. 2), the level of BrdU incorporation (Fig. 3C) and the level of β-tubulin (Fig. 4), as well as the appearance of cortical and mitotic microtubular arrays, had all intensified (not shown), coincident with the completion of germination (Fig. 1), as in the non-dormant seeds. Imbibition in GA_{4+7} did not induce germination, whereas under white light some seeds germinated (Fig. 1). The activation of DNA synthesis (not shown), the accumulation of β-tubulin (Fig. 4), and the formation of the microtubular cytoskeleton in seeds under these conditions were detectable only after a subsequent chilling treatment, which released seeds from dormancy (not shown).

Induction and release of secondary dormancy

Far-red irradiation was very effective in inducing secondary dormancy. During a subsequent dark incubation of 7 d, germination was virtually absent (<4%) (Fig. 1). Transferring the seeds to continuous white light at day 7 induced 45% germination within 72–96 h (Fig. 1). A 1-day chilling treatment proved more effective and resulted in 85% germination within 48 h in the dark. GA_{4+7} was hardly effective in the breakage of dormancy, as only a few seeds responded to the hormone (Fig. 1). A subsequent chilling treatment appeared to have an additive effect to that of GA_{4+7} and stimulated germination up to 95% (not shown).

After the far-red treatment and subsequent 7 d incubation in the dark, the number of embryonic 4C DNA nuclei remained ($P > 0.05$) at the dry non-dormant seed levels (Fig. 2). However, at the end of this period, at day 7, some nuclei with BrdU incorporation (Fig. 3D), trace amounts of β-tubulin (Fig. 4), and a moderate amount of cortical microtubules (Fig. 5I), which were not detected in dry seeds, became detectable in the embryonic tissues. After the 1-day chilling treatment, β-tubulin was not detectable (Fig. 4) and cortical microtubules became less apparent, although some cells in the central cylinder of the hypocotyl were still intensely labelled (Fig. 5J). BrdU incorporation could still be detected after the chilling at levels comparable to that before

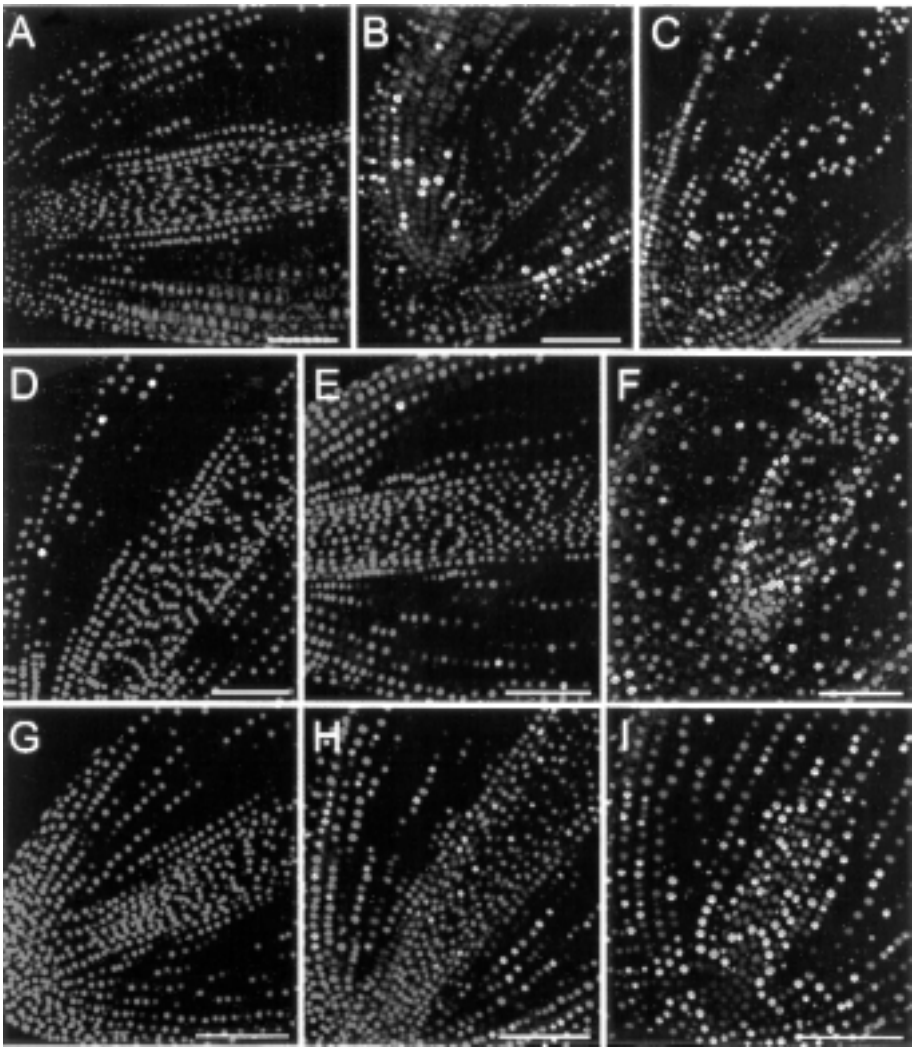


Figure 3. Activation of DNA synthesis in embryos of non-dormant and dormant tomato seeds. Fluorescence micrographs present patterns of longitudinal sections of embryos from non-dormant wild-type control and *sit*^{w} seeds, and from seeds with primary and secondary dormancy. Most nuclei show red fluorescence as a result of staining with propidium iodide (PI). Nuclei which show green fluorescence are labelled with anti-BrdU and goat anti-mouse/FITC, indicative of BrdU incorporation into actively replicating DNA (S phase). Bars = 100 μm. (A) Embryonic radicle tip region of dry non-dormant control seeds. (B,C) Radicle tips of seeds after release of primary dormancy by a chilling treatment; (B) ungerminated seeds 1 d after the chilling treatment and pulse labelled for 3 h; (C) germinated seeds 2 d after the chilling treatment. (D–F) Radicle tips of seeds upon induction of secondary dormancy by a far-red irradiation and subsequent release of dormancy by a chilling treatment; (D) some nuclei are labelled with BrdU, showing active DNA synthesis during the far-red treatment; (E) DNA synthesis immediately after the chilling treatment; (F) higher levels of DNA synthesis 1 d after the chilling treatment. (G–I) Radicle tips of the fully non-dormant *sit*^{w} seeds; (G) dry seeds; (H) ungerminated seeds after 1 d of imbibition; (I) germinated seeds after 2 d of imbibition.

chilling (Fig. 3E), and also the number of 4C DNA nuclei remained unchanged (Fig. 2). One day after the transferring back to 25°C, a substantial increase in β-tubulin accumulation was detected (Fig. 4). As expected, this occurred in parallel with a recovery of the microtubular cytoskeleton, which became more intensely labelled, showing cortical and mitotic arrays (Fig. 5K). At this time, the number of 4C DNA nuclei had significantly increased ($P < 0.05$) (Fig. 2), reaching a level comparable to that of the non-dormant seeds at 24 h of imbibition and correlated to a more intense detection of BrdU incorporation (Fig. 3F).

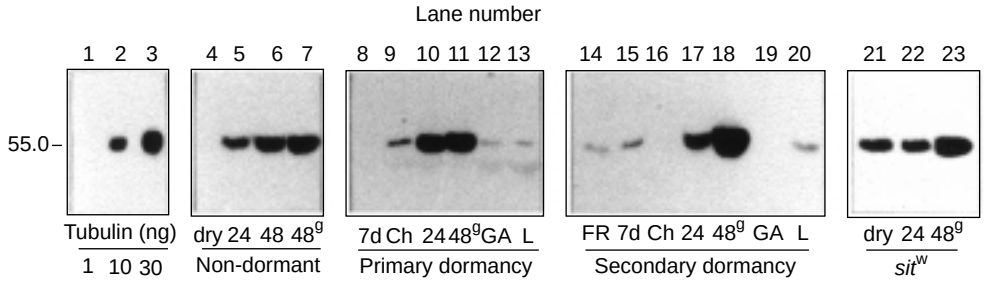
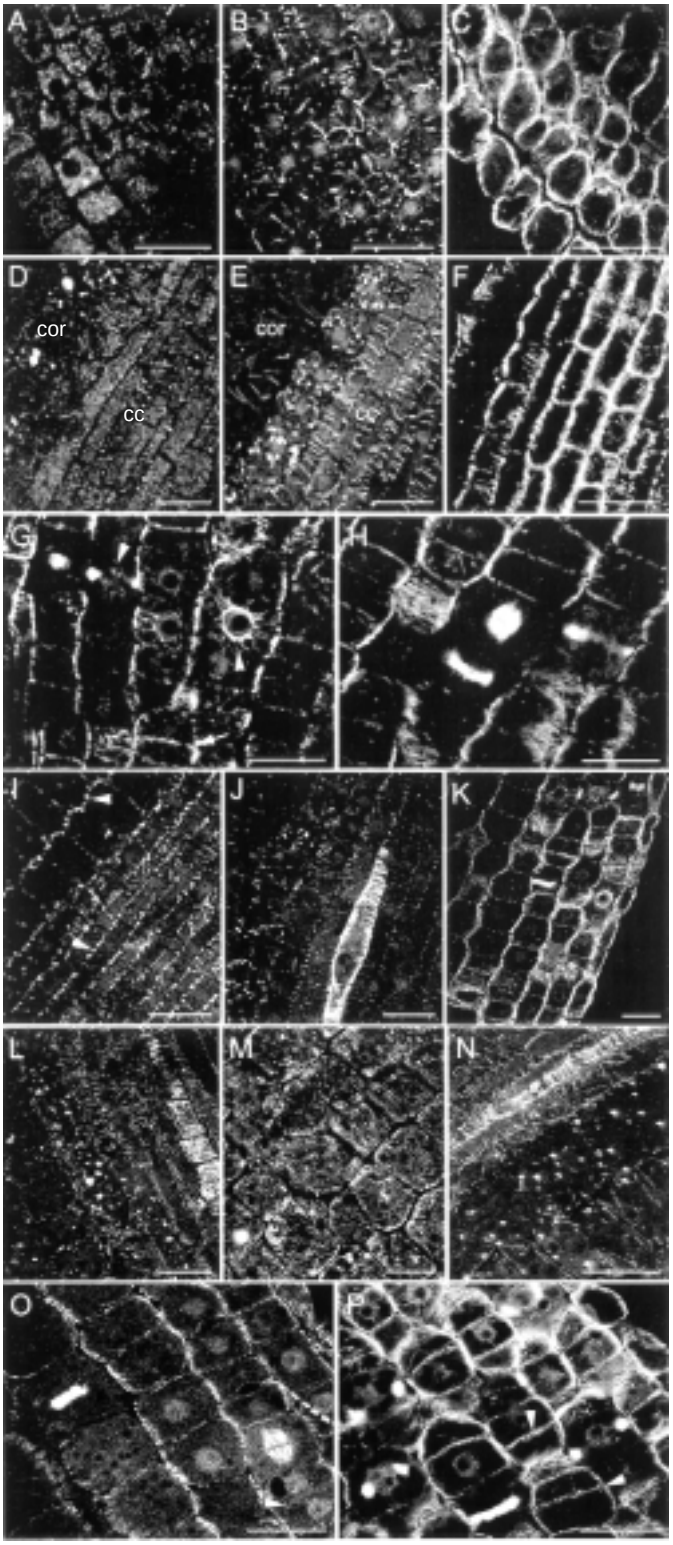


Figure 4. β-Tubulin accumulation in embryos of non-dormant and dormant tomato seeds. Lanes 1–3 were loaded with 1, 10 and 30 ng pure bovine brain tubulin, respectively. Lanes 4–23 were loaded with 30 μg total protein for each sample. Molecular weight (kDa) is indicated on the left-hand side. The films were exposed for a maximum of 1 min. (g) Embryos of seeds that had germinated, i.e. with approximately 1 mm radicle protrusion. (Lanes 4–7) β-tubulin levels are shown for embryos of dry non-dormant control seeds, and after 24 and 48 h of imbibition. (Lanes 8–13) β-tubulin levels in embryos of seeds with primary dormancy; (8) after an incubation period of 1 month plus 7 d at 25°C in dark; (9) at the end of the chilling (Ch) treatment; (10, 11) 1 d and 2 d after the chilling treatment, respectively; (12, 13) seeds incubated for 7 d at 25°C in the dark and then subsequently incubated for another 7 d in 10 μM GA_{4+7} or under white light, respectively. (Lanes 14–20) β-tubulin levels in embryos of seeds with secondary dormancy; (14) after the far-red light irradiation treatment; (15) after a subsequent 7 d incubation at 25°C in dark; (16) immediately after the chilling treatment; (17, 18) 1 d and 2 d after the chilling, respectively; (19, 20) seeds incubated for 7 d at 25°C in the dark and then subsequently incubated for another 7 d in 10 μM GA_{4+7} or under white light, respectively. (Lanes 21–23) β-tubulin levels in embryos of dry *sit*^{w} seeds, and after 24 and 48 h of imbibition, respectively.

The sit^{w} *mutant*

Over 90% of the *sit*^{w} seeds completed germination in the dark within 48 h (Fig. 1). The number of 4C nuclei in embryos of dry *sit*^{w} seeds was significantly higher ($P < 0.05$) than that of the dry wild-type seeds (Fig. 2). However, BrdU incorporation was not detectable after 3 hours of pulse labelling (Fig. 3G). Yet, the amount of β-tubulin in embryos of the dry *sit*^{w} seeds was high (Fig. 4) compared to dry non-dormant wild-type seeds, in which β-tubulin was undetectable. Also, in contrast with dry wild-type seeds, the embryos of dry *sit*^{w} seeds contained an intense cortical microtubular cytoskeleton network, visualized not only in the radicle tip region, but also in other parts of the embryo, although its microtubules appeared highly depolymerized (Fig. 5L–N). Upon imbibition, 4C DNA (Fig. 2) and BrdU-labelled nuclei (Fig. 3H,I) were detected in increasing numbers. In parallel, the level of β-tubulin increased further (Fig. 4), the microtubular cytoskeleton reconstituted into an elaborate cortical cytoskeleton in cells throughout the embryo, and mitotic arrays were observed in the radicle tip region (Fig. 5O,P). As in wild-type seeds, the intensification of the cell cycle events preceded visible germination.

Figure 5. (*Opposite*) Appearance of microtubular cytoskeleton in embryos of non-dormant and dormant tomato seeds. Fluorescence micrographs present patterns of longitudinal sections of embryos from non-dormant wild-type control and *sit*^{w} seeds, and from seeds with primary and secondary dormancy labelled with anti-β-tubulin and goat anti-mouse/FITC. Bars = 25 μm. Because the sections are relatively thin (4 μm) with respect to the diameter of the cells, only a few cells have their cortical cytoplasm with microtubules in the plane of the section. (A, D) Radicle tip region of embryos from non-dormant dry control seeds; (A) absence of microtubules in cells of the tip; (D) absence of microtubules in cells of the central cylinder (cc) and presence of granules of tubulin in cells of the cortex (cor). (B, C and E–H) Radicle tip region of embryos from seeds with primary dormancy; (B, E) microtubules appearing in cells of the tip (B), and in cells of the cortex and central cylinder (E) after a chilling treatment; (C, F) elaborate cortical microtubular cytoskeleton network in cells of the tip (C) and of central cylinder (F) 1 d after the chilling treatment; (G, H) mitotic (arrowheads) and cytokinetic (arrow) arrays in cells of the radicle also 1 d after the chilling treatment. (I–K) Radicle tip region of embryos from seeds with secondary dormancy; (I) appearance of cortical microtubules (arrowheads) after the far-red light irradiation treatment; (J) although some central cylinder cell files still contained intense cortical microtubules, they were, in general, less apparent after the chilling than after the far-red treatment (I) and subsequent 7 d incubation at 25°C in dark (not shown); (K) the microtubular network was recovered 1 d after the chilling treatment, showing cortical and mitotic arrays and divisions. (L–P) Embryos from *sit*^{w} seeds; (L–N) cortical microtubules in embryos of dry seeds, apparent in cells of the central cylinder (L) and of radicle tip (M), and also in cells of the cotyledons (N), but intensely depolymerized as observed through the large numbers of tubulin granules; (O) recovery of microtubules after 1 d of imbibition, showing cortical and mitotic arrays and divisions (arrowhead); (P) cortical and mitotic arrays and divisions (arrowheads) are more intense upon completion of germination after 2 d of incubation.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Discussion

Seeds with primary dormancy did not show any detectable cell cycle activity in the embryos when incubated in the dark, not even after prolonged incubation of over 1 month. GA and white light were not generally effective in inducing germination. This insensitivity was also reflected in the absence of cell cycle activity. The chilling treatment increased the sensitivity to light and GA which resulted in almost full germination after transfer to 25°C (not shown). This supports the hypothesis that dormancy can be defined by a lack of sensitivity to germination stimulants which can be restored by a dormancy-breaking treatment, such as chilling (Hilhorst and Karssen, 1992; Derkx and Karssen, 1993; Derkx *et al*., 1994). After the chilling treatment, but prior to transfer to 25°C, an increase in the levels of β-tubulin and cortical microtubular cytoskeleton was observed. However, there was no detectable DNA synthesis activity. The initial accumulation of tubulins and subsequent formation of microtubular cytoskeleton are prerequisites for the initiation of cell cycle events in embryonic tissue during seed imbibition and germination (de Castro *et al*., 1995, 1998, 2000; Jing *et al*., 1999), as well as during root growth (Gunning and Sammut, 1990; Baluška and Barlow, 1993). This indicates in the present study that the initial formation of the microtubular cytoskeleton is associated with the dormancy-breaking process *sensu strictu*, since it is assumed that germination is not induced or substantially delayed at the chilling temperature. Transferring the seeds to 25°C after chilling resulted in a sequential increase in the amount of β-tubulin, number of microtubules, and initiation of DNA synthesis which is resumed upon full DNA replication prior to mitosis, evidently associated with the germination process (de Castro *et al*., 1995, 1998, 2000; Jing *et al*., 1999).

The situation in seeds in which secondary dormancy was induced by the far-red treatment appeared to be different from primary dormancy. Although germination was completely prevented by the far-red irradiation, some β-tubulin accumulated and some microtubular cytoskeleton was observed, as after the chilling treatment in seeds with primary dormancy. But, again, there was no increase in the number of 4C nuclei. However, the far-red irradiation allowed some BrdU incorporation. Apparently, the far-red irradiation induced the secondary dormancy when the cell cycle had been already activated during the first hours of seed imbibition. DNA synthesis is initiated in the embryonic radicle tip of non-dormant seeds within the first 12 h of imbibition. This occurs concomitantly with the accumulation of β-tubulin and the establishment of cortical microtubular cytoskeleton. This suggests that secondary dormancy may be induced while the germination process is in progress.

In the ABA-deficient *sit*^{w} mutant, germination follows seed development within the fruit without intervening developmental arrest (Liu *et al*., 1997; de Castro, 1998). Consequently, embryos from mature *sit*^{w} seeds contained amounts of 4C DNA nuclei and microtubular cytoskeleton that were comparable to those of germinating wild-type seeds at an imbibition interval of 24 hours. Far-red irradiation had a marginal influence on *sit*^{w} seeds (Fig. 1) and was expressed as a slight delay of the start of germination. Thus, apart from the progression of the cell cycle, the absence of ABA also appears to influence the inability of *sit*^{w} seeds to develop secondary dormancy.

Seeds with secondary dormancy hardly responded to GA, but there was a moderate response to light. The chilling treatment alone was effective enough to induce almost full germination without requirement for light. However, contrary to the seeds with primary dormancy, the chilling treatment of seeds with secondary dormancy gave rise to a partial degradation of the microtubular cytoskeleton while BrdU incorporation could still be detected. While secondary dormancy can be induced under conditions of active DNA synthesis and the presence of a microtubular cytoskeleton, it is apparent that the cell cycle is blocked by the far-red irradiation treatment and is resumed after the chilling. The fact that the germination response of seeds with secondary dormancy to GA and light is greater than in seeds with primary dormancy indicates that the difference between primary and secondary dormancy in tomato is largely quantitative. Apparently, the initiation of the cell cycle contributes to this response.

The fact that the microtubular cytoskeleton appeared partly degraded after the chilling treatment in wild-type seeds with secondary dormancy, as well as in dry *sit*^{w} seeds, suggests that microtubules in tomato embryos are sensitive to cold stress as well as to desiccation. This has been observed in other plant tissues, including leaves (Bartolo and Carter, 1991; Okamura *et al*., 1993; Pihakaski-Maunsbach and Puhakainen, 1995; Wallin and Strömberg, 1995). Microtubules may be reformed after cold- or drought-induced depolymerization (Bartolo and Carter, 1991; Murata and Wada, 1991). This was observed when seeds were returned to 25°C after the chilling treatment. The detection of similar numbers of nuclei incorporating BrdU during induction and subsequent release of secondary dormancy by chilling may reflect an ability of S-phase nuclei to tolerate conditions that might block the cell cycle (e.g. far-red) once DNA synthesis is taking place. S-phase nuclei may tolerate desiccation when DNA repair has taken place during the initial phases of the germination process (Boubriak *et al*., 1997; Osborne and Boubriak, 1997).

From the BrdU incorporation experiments it is not possible to distinguish repair synthesis of DNA from replicative synthesis in preparation for growth. Thus, it is possible that part of the BrdU incorporation at the beginning of imbibition or termination of dormancy is due to DNA repair (Elder and Osborne, 1993).

In summary, this study has shown that the depth of dormancy, which distinguishes primary and secondary dormancy in tomato, may depend on the progression of the cell cycle prior to the induction of dormancy. Primary dormancy is induced during development, at a moment when DNA synthesis activity appears to be arrested, whereas secondary dormancy may be induced when there is DNA synthesis activity.

Acknowledgements

The Brazilian Ministry of Education, through CAPES, and the Netherlands Organization for International Cooperation in Higher Education (NUFFIC) are acknowledged for financially supporting the PhD studies of R.D. de Castro.

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Received 18 May 2000
accepted after revision 6 November 2000