Plant Physiol. (1995) 109: 499–504

β-Tubulin Accumulation and DNA Replication in Imbibing Tomato Seeds[1]

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The activation of the cell cycle in embryo root tips of imbibing tomato (*Lycopersicon esculentum* Mill. cv Lerica) seeds was studied by flow cytometric analyses of the nuclear DNA content and by immunodetection of β-tubulin. With dry seeds, flow cytometric profiles indicated that the majority of the cells were arrested at the G_1 phase of the cell cycle. In addition, β-tubulin was not detectable on western blots. Upon imbibition of water, the number of cells in G_2 started to increase after 24 h, and a 55-kD β-tubulin signal was detected between 24 and 48 h. Two-dimensional immunoblots revealed at least three different β-tubulin isotypes. Thus, β-tubulin accumulation and DNA replication were induced during osmotic priming. These processes, as well as seed germination rate, were enhanced upon subsequent imbibition of water, compared with control seeds that imbibed but were not primed. By contrast, when aged seeds imbibed, DNA replication, β-tubulin accumulation, and germination were delayed. In all cases studied, both DNA replication and β-tubulin accumulation preceded visible germination. We suggest that activation of these cell-cycle-related processes is a prerequisite for tomato seed germination. Furthermore, β-tubulin expression can be used as a parameter for following the initial processes that are activated during seed imbibition.

In seeds of tomato (*Lycopersicon esculentum* Mill.) and pepper (*Capsicum annuum* L.), imbibition is coupled to initiation of DNA replication in cells of the embryo root tip, as was demonstrated by flow cytometric analysis of isolated nuclei (Bino et al., 1992; Lanteri et al., 1993). In these species, the embryonic cells progress through the S phase of the cell cycle into the G_2 phase before visible germination. Also, in embryos of maize (*Zea mays* L.), the cell cycle is activated during the first phases of germination (Georgieva et al., 1994a). Proteins involved in the cell cycle must either be present in the dry seed or be rapidly synthesized de novo upon imbibition prior to activation of the cell cycle. In frog eggs, it has been shown that both the entry into mitosis and the activation of maturation promoting factor require the synthesis of proteins involved in the cell cycle (Murray and Hunt, 1993). In maize embryos, proteins related to nuclear proto-oncogenes and mammalian tumor suppressor gene were detected, as were the corresponding mRNAs (Georgieva et al., 1994b). The expression pattern of these proteins was correlated with nuclear events. Possibly, the proto-oncogene products have a functional role as transcription activators during seed imbibition (Georgieva et al., 1994b).

As cells progress through division and differentiation, microtubules undergo continuous assembly, disassembly, and rearrangement into new configurations. All of these transformations into different arrays are dependent on the interactions between microtubules and microtubule-associated proteins (Goddard et al., 1994). Several distinct arrays of microtubules are formed transiently as plant cells proceed through the mitotic cell cycle: the most prominent are the interphase cortical, pre-prophase, spindle, and phragmoplast arrays (Fosket and Morejohn, 1992; Goddard et al., 1994). Microtubules are assembled from heterodimers containing one α-tubulin and one β-tubulin polypeptide, each with a molecular mass of approximately 50 kD. For several plant species, β-tubulin genes have been characterized (Guiltinan et al., 1987; Hussey et al., 1988, 1990; Rogers et al., 1993), and for *Arabidopsis thaliana* the entire α- and β-tubulin gene family has been described (Kopczak et al., 1992; Snustad et al., 1992). In carrot, it was shown that the expression of β-tubulin isotypes is dependent on the developmental stage of the tissue analyzed (Hussey et al., 1988). Histoimmunological studies have shown that several antibodies exhibit cross-reactivity with tubulins from a wide variety of divergent species (Silflow et al., 1987; Morejohn, 1991; Fosket and Morejohn, 1992). In dividing plant tissues, studies with tubulin antibodies indicate that the progression through the cell cycle is associated with changes in the specific organization of the microtubular cytoskeleton (Hussey et al., 1990; Traas et al., 1992), whereas in animal cells, the induction of S phase coincides with transient depolymerization of microtubules (Crossin and Carney, 1981; Thyberg, 1984). In maize roots, it has been demonstrated that the progression of the cell cycle through the G_1 phase is dependent on the turnover of

[1] R.D.d.C. was supported with a Ph.D. fellowship from Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior and the Brazilian Ministry of Education.

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Abbreviations: C, DNA content of the unreplicated haploid chromosome complement; TBS, Tris-buffered saline.

the microtubular cytoskeleton (Baluška and Barlow, 1993). However, the relation between synthesis of tubulins and cell-cycle activity is not yet fully elucidated.

Imbibing seeds, in which the cell cycle is activated, may be a suitable way to study the relationship between tubulin synthesis and DNA replication. In imbibing tomato seeds, the cell cycle progresses to G_2 prior to germination (Bino et al., 1992). This accumulation of cells in G_2 takes place in tomato because mitosis and cell division do not finish prior to protrusion of the root tip through the seed coat (Argerich and Bradford, 1989). Moreover, by preconditioning seeds in an osmotic solution (priming) followed by redrying, tomato seeds can be stably arrested in G_2 (Bino et al., 1992). Priming may improve both the rate and the uniformity of seed germination upon subsequent imbibition of water (Heydecker and Coolbear, 1977). This may be due to activation of pregerminative processes, including cell-cycle activation. Aging, on the other hand, is known to reduce seed viability and results in a decreased germination rate.

In this paper, we report the expression of β-tubulin in relation to nuclear DNA replication and the role of cell-cycle activation in tomato seed germination. β-Tubulin expression and the induction of DNA synthesis was studied in control, primed, and aged seeds. The goal of our studies was to gain a better understanding of the initial processes that are activated during seed imbibition.

MATERIALS AND METHODS

Seed Material

Seeds of tomato (*Lycopersicon esculentum* Mill. cv Lerica) were obtained from Zaadunie (Enkhuizen, The Netherlands). Seeds were dried over a saturated $CaCl_2$ solution for 2 d at 20°C and 32% RH (moisture content 6.3 ± 0.1%, fresh weight basis) and stored in a moisture-proof container at 5°C until use.

Priming, Aging, and Imbibition Conditions

Osmotic priming was carried out on top of filter paper soaked with 30% (w/v) PEG-6000 (−1.0 MPa) in a sealed Petri dish from 6 h to a maximum of 7 d at 20°C in darkness (Bino et al., 1992). After priming, seeds were washed for 5 min under running tap water to remove PEG from the seed coat. Aging of seeds was performed by controlled deterioration at 60°C and 45% RH (moisture content 7.1 ± 0.1%) for 8 d. Treated seeds were then dried back to equilibrium moisture content over a saturated $CaCl_2$ solution for 3 d.

Imbibition by seeds for the β-tubulin assay took place with seeds placed on top of filter paper soaked with either priming solution or distilled water in a sealed Petri dish. The seeds were then kept in an incubator in continuous darkness and 20°C from 0 d (dry seed) up to 9 d, depending on the treatment.

Germination Tests, Moisture Contents, and Statistical Analysis

Both moisture content determinations (two replicates of 1 g each) and germination tests (four replicates of 50 seeds each) were carried out according to the conditions recommended by the International Seed Testing Association (1993). The germination characteristics are expressed as total germination, number of normal seedlings, mean germination time (time to germination of 50% of total germinated seeds), and germination uniformity (time between germination of 25 and 75% of total germinated seeds). Seeds were scored as germinated when the root tip protruded through the seed coat. Student's t test was used to analyze differences between the treatments (significant at $\alpha = 0.01$).

Flow Cytometry of Nuclei

Embryo root tips were dissected from the seeds and incubated in nucleus isolation buffer as described previously (Bino et al., 1993). To detect DNA, 10 mg/L of the fluorescent dye 4′,6-diamidino-2-phenylindole were added to the isolation buffer (Saxena and King, 1989). After the root tips were chopped, the suspension was passed through a 25-μm nylon mesh and immediately analyzed. For each sample, three to five seeds were used and flow cytometric determinations were made in triplicate, using a PAS II flow cytometer (Partec GmbH, Münster, Germany) equipped with an HBO-100 mercury arc lamp, a TK-420 dichroic mirror, and a GG-435 long-pass filter. All analyses were performed using peak height detection and logarithmic amplification (Bino et al., 1993). The amount of DNA in the nuclei is proportional to the fluorescent signal and is expressed as arbitrary C values (Howard and Pelc, 1953). The signals obtained from tomato leaf tissue were used, and the gain settings were adjusted so that signals of all intact nuclei were registered within the channel range.

Protein Extraction and Concentration Determinations

Proteins were extracted from embryo root tips because the increase in cell-cycle activities during seed imbibition, as measured by DNA replication activity, is predominantly higher in this region than in other embryo and seed tissues (Bino et al., 1992, 1993). After the seeds were primed and allowed to imbibe for the appropriate time, 40 to 60 root tips, excised from the isolated embryos, were pooled and transferred into an Eppendorf reaction assay tube, frozen in liquid N_2, and subsequently ground to a powder. For one-dimensional PAGE, 100 μL of modified Laemmli (1970) lysis buffer, consisting of 62.5 mM Tris-HCl, 2% (w/v) SDS, 15 mg/mL DDT, and 7% (v/v) glycerol, pH 6.8, were added directly to the frozen powder. After mixing, the samples were boiled for 10 min and centrifuged for 7 min at 17,000g. For two-dimensional PAGE, 100 μL of ice-cold Hepes buffer (100 mM, pH 7.0) were added to the frozen powder, mixed, incubated for 10 min, and centrifuged for 7 min at 4°C and 17,000g.

Protein concentration of the supernatant was measured following micro-protein assay procedures (Bio-Rad), mod-

ified from the methods described by Lowry et al. (1951) and Bradford (1976), using BSA as a standard.

Electrophoresis and Electroblotting

For one-dimensional PAGE, protein samples were loaded and separated on a precast 7.5% SDS homogeneous ExcelGel (Pharmacia). Three different concentrations of pure bovine brain tubulin (Molecular Probes, Eugene, OR), 1, 10, and 30 pg, were used as reference samples. For two-dimensional PAGE, proteins in the supernatant were precipitated with TCA-acetone, vacuum dried, and resuspended in a lysis buffer containing 9 M urea, 0.5% (w/v) 3-([3-cholamidopropyl]dimethylammonio)-1-propane sulfonate, 2% (v/v) β-mercaptoethanol, and 2% (v/v) 2-D Pharmalyte 3–10 (Pharmacia). Immobiline pH 4 to 7 gels and 8 to 18% SDS gradient Excel gels (Pharmacia) were used in the first and second dimensions, respectively.

After PAGE, proteins were electrotransferred overnight from the gel to a Hybond-polyvinylidene difluoride membrane (0.45 μm, Amersham) using a Novablot electrophoretic transfer unit (Pharmacia) operating at 0.8 mA/cm^2 and 30 V at 4°C. The transfer buffer consisted of 25 mM Tris, 192 mM Gly, and 10% (v/v) methanol (pH 8.7).

Chemiluminescence Immunodetection of β-Tubulin

All steps of the immunodetection procedure were performed at room temperature with gentle agitation on a roller incubator. After blotting and subsequent washing in TBS (pH 7.5), membranes were blocked in 1% (w/v) blocking solution (Boehringer Mannheim) for 1 h and probed with 1 μg/mL mouse monoclonal anti-β-tubulin antibody (Boehringer Mannheim, clone KMX-1), diluted in 0.5% (w/v) blocking solution for 1 h. Membranes were then washed twice with large volumes (minimum of 30 mL for a 10- × 10-cm membrane) of TBS with 0.5% (v/v) Tween-20, pH 7.5, for 10 min each, then washed twice with 0.5% (w/v) blocking solution for 10 min each, and probed with 50 milliunits/mL peroxidase-conjugated secondary antibody diluted in 0.5% (w/v) blocking solution. Thereafter, the membranes were washed again (four times with large volumes of TBS with 0.5% (v/v) Tween-20 for 15 min each) and further processed according to the method of Leying et al. (1994). The immunoblot was incubated with a premixed detection solution, 125 μL/cm^2, consisting of a 100:1 mixture of prewarmed substrate solution A and starting solution B (Boehringer Mannheim), for 1 min, and fitted between two pieces of overhead sheets into a film cassette. Then, in a dark room under a safelight, several sheets of photographic film (Hyperfilm-ECL, Amersham) were exposed for different periods, varying from 20 s to 10 min, and developed according to the manufacturer's protocol.

RESULTS

Germination Tests

The germination of the aged, primed, and control tomato seed lots was scored by counting daily the number of germinated seeds (Fig. 1). The uniformity and germination performance of control seeds were high (Fig. 1; Table I). Germination performance was improved in primed seeds, compared to the control seeds, as shown by a significant decrease in the mean germination time (Fig. 1; Table I). Aging, on the other hand, resulted in a marked loss of seed quality, as indicated by a significant increase in the mean germination time, as well as by a significant decrease in the uniformity of germination and the percentage of normal seedlings (Table I).

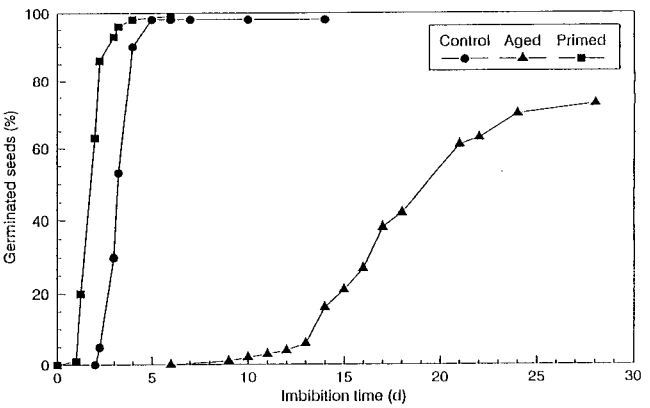

Figure 1. Germination of control, primed, and aged tomato seeds, cv Lerica, upon imbibition of water. Priming was performed in −1 MPa PEG-6000 at 20°C for 7 d, and aging was brought about by treatment at 60°C and 45% RH for 8 d. After these pretreatments, seeds were dried back to their equilibrium moisture content prior to use.

Nuclear Replication Activity

As was previously demonstrated (Bino et al., 1992), cells in the embryo root tips of dry tomato seeds and tomato seeds that had imbibed contained nuclei with either 2C values (G_1 phase of the cell cycle) or 4C values (G_2 phase with replicated DNA). Therefore, the number of nuclei with 4C values, expressed as a percentage of the total number of nuclei (2C plus 4C), was used to follow nuclear DNA replication activity upon seed imbibition (Fig. 2). In root tips of control seeds, the percentage of 4C nuclei was low (5%) and increased rapidly between 24 and 48 h of imbibition. Compared with the controls, primed seeds contained significantly higher numbers of nuclei in 4C before imbibition (20%), indicating that DNA replication was initiated during the priming treatment (Bino et al., 1992). Upon imbibition of these primed seeds, the number of 4C nuclei doubled within 8 h to a value (40–50%) similar to that of control seeds that had imbibed for 2 d. In contrast to control and primed seeds, aged seeds did not show an increase in 4C values during the first 5 d of imbibition: the percentage of 4C nuclei fluctuated between 6 and 12%.

Immunodetection of β-Tubulin following One- and Two-Dimensional PAGE

In control seeds that had imbibed, a blot containing a range of three different amounts of root tip protein extract (10, 20, and 40 μg) was processed for immunodetection of β-tubulin at five different imbibition times. With all protein

Table I. *Effects of priming (−1 MPa PEG-6000, 20°C, 7 d) and aging (60°C, 45% RH, 8 d) treatments on germination performance of tomato seeds, cv Lerica*

Data are means ± SD of four replicates of 50 seeds each. TG, Total germination; NS, normal seedlings; t_{50}, mean germination time, time to germination of 50% of total germinated seeds; t_{75}–t_{25}, germination uniformity, time between germination of 25 and 75% of total germinated seeds.

Treatment	TG	NS	t_{50}	t_{75}–t_{25}
	%		d	
Control	100	97 ± 3	3.3 ± 0.1	0.7 ± 0.1
Priming	99 ± 1	98 ± 2	1.8 ± 0.0[a]	0.7 ± 0.1
Aging	74 ± 3[a]	7 ± 3[a]	17.5 ± 0.8[a]	5.0 ± 0.6[a]

[a] Significantly different from control (Student's *t* test, $P < 0.01$).

concentrations used, the monoclonal anti-β-tubulin antibody recognized a protein band with a molecular mass of about 55 kD, but stronger signals were obtained when 20 or 40 μg of total protein extract were loaded (Fig. 3, lanes 12, 13, 17, and 18). At an intermediate exposure time (7 min) this β-tubulin signal was not detectable in root tips of dry seeds or in seeds that had imbibed for 12 or 24 h (Fig. 3, lanes 4–6, 9–11, and 14–16). A strong signal was detected after 48 h of imbibition in the 20-μg concentration range. The intensity of the β-tubulin signal increased up to 72 h of imbibition (germinated seeds), when the intensity of the signal could be compared to that of the pure tubulin at 10 to 30 pg (Fig. 3, lanes 2 and 3).

When the photographic film was exposed for longer times (more than 10 min), a weak β-tubulin signal could be observed in the 24-h sample in the higher concentration range (40 μg). However, at this longer exposure time, greater background was obtained as well (data not shown). Thus, 20 μg of protein extract was used routinely in the experiments.

To determine whether the signal at 55 kD represented one, single polypeptide or different isoforms, the proteins from root tips of seeds that had imbibed for 48 h were separated by two-dimensional PAGE and subsequently immunoblotted with the β-tubulin antibody (Fig. 4). The two-dimensional immunoblots of these seeds revealed three different 55-kD polypeptides with pI values of about 4.9, indicating the presence of at least three β-tubulin isotypes.

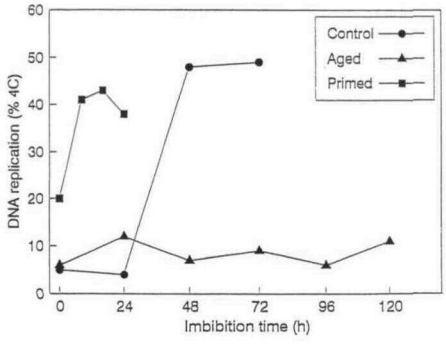

Figure 2. DNA replication activity in imbibing tomato seeds, cv Lerica. Nuclei were isolated from root tips of aged, control, and primed seeds and analyzed by flow cytometry. DNA replication stage is expressed as the percentage of 4C nuclei in the total number of nuclei analyzed (2C plus 4C).

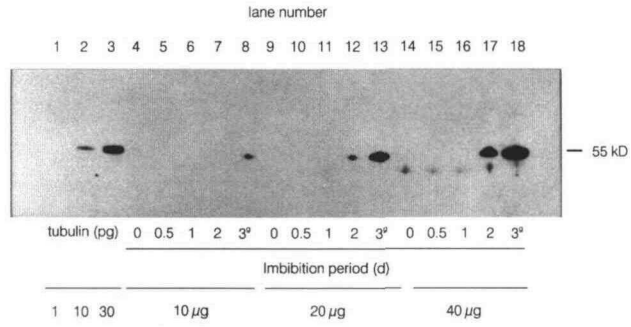

Figure 3. Western blot analysis of β-tubulin following SDS-PAGE of three different amounts of proteins extracted from embryo root tips of imbibing control tomato seeds, cv Lerica. Lanes 1 to 3, Pure tubulin; lanes 4 to 8, 10 μg of protein; lanes 9 to 13, 20 μg of protein; lanes 14 to 18, 40 μg of protein. The film was exposed for 7 min. Superscript g, Germinated seeds.

Effects of Aging and Priming on β-Tubulin Expression

For comparison of the effects of aging and priming on β-tubulin signals during the imbibition periods, seeds from the aged, control, and primed seed lots were allowed to imbibe simultaneously, and protein samples were loaded on the same gel that was immunoblotted following one-dimensional SDS-PAGE (Fig. 5). A clear β-tubulin signal with increasing intensity was observed in samples from control seeds that had imbibed for 48 and 72 h (Fig. 5, lanes 15 and 16), whereas this signal was not detected in aged seeds (Fig. 5, lanes 4–11). For aged seeds, the β-tubulin signal was detected only after 8 d of imbibition (data not shown); germination started 2 d later. In seeds that were primed, i.e. incubated in −1.0 MPa PEG-6000 for 7 d and then dried back to their equilibrium moisture content, the β-tubulin signal was already clearly present in the redried seed, before imbibition of water (Fig. 5, lane 17). The intensity of the signal in dry primed seeds was somewhat higher compared to that in the control seeds that had imbibed for 48 h (Fig. 5, lanes 15 and 17). When primed

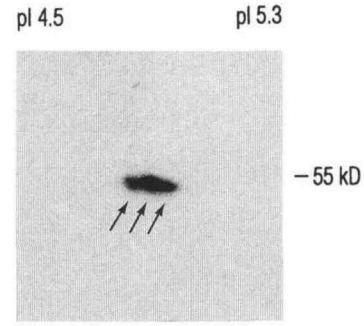

Figure 4. Two-dimensional PAGE and western blot analysis of β-tubulin polypeptides extracted from embryo root tips of control tomato seeds, cv Lerica, that had imbibed for 48 h. Three spots can be observed at 55 kD and approximately pI 4.9.

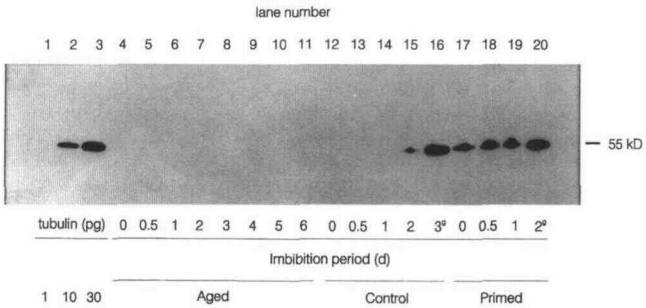

Figure 5. Effect of aging and priming on β-tubulin expression during imbibition by tomato seeds, cv Lerica. Aging and priming were carried out as described in Figure 1. The gel was loaded with 20 μg of proteins extracted from the root tips of dry and imbibing seeds. Lanes 1 to 3, Pure tubulin; lanes 4 to 11, aged seeds; lanes 12 to 16, control seeds; lanes 17 to 20, primed seeds. The film was exposed for 7 min. Superscript g, Germinated seeds.

seeds subsequently imbibed water, the intensity of the signal increased up to 12 and 24 h of imbibition (Fig. 5, lanes 18 and 19) and reached its maximum at 48 h of imbibition, when seeds were already germinated. Analysis of the β-tubulin signal during priming showed that the signal could be detected after 3 to 4 d of priming (Fig. 6, lanes 8 and 9). The intensity of the signal increased up to 5 d and remained relatively constant during further priming (Fig. 6, lanes 10–14).

DISCUSSION

The expression of β-tubulin, a protein required for passage through the cell cycle, was analyzed in imbibing tomato seeds. One β-tubulin signal of about 55 kD could be immunodetected when protein extracts from embryonic root tips were used. This molecular mass corresponded with that reported for tubulins in other plant tissues (Hussey et al., 1988; Kerr and Carter, 1990; Koontz and Choi, 1993). On the two-dimensional immunoblots (Fig. 4), at least three β-tubulin isotypes were found in the embryo root tip samples of control seeds after 48 h of imbibition. This could be the result of a co-evolution with cell-type-specific microtubule-associated proteins (Fosket and Morejohn, 1992), because it is known that different tubulin isotypes are expressed in tissues of various plant species (Hussey et al., 1988, 1990; Kopczak et al., 1992; Snustad et al., 1992; Rogers et al., 1993).

Because microtubules are present at all stages of a typical plant cell cycle (Goddard et al., 1994), a constitutive level of β-tubulin was expected in all tomato seed extracts. However, the present results showed no β-tubulin signal in dry control seeds that had imbibed for 12 h and 24 h (Figs. 3 and 5) or in any aged seeds (Fig. 5). The immunodetection limit of the system used was between 1 and 10 pg of pure bovine brain tubulin. Possibly, the level of β-tubulin in the control and aged seeds was below this limit. Another possibility could be the loss of β-tubulin during protein extraction due to any level of protease activity. However, this seems unlikely because the proteins from all seeds were extracted in a buffer containing SDS, which solubilizes all proteins and minimizes protease activity. A third possibility is that in the control and aged seeds β-tubulin is difficult to extract. Beltramo and co-workers (1994) reported that the extraction of tubulin, when associated with membranes, required a treatment with 0.1 M Na_2CO_3 at a pH greater than 11.5 so that the hydrophobic form was converted into a hydrophilic and extractable form.

The present results with tomato seeds show that the signal of β-tubulin increased in cells of the embryo root tip within 48 h of imbibition (Fig. 3). At this time also, DNA replication as judged by the 2C-to-4C transition in root tip nuclei was observed (Fig. 2). In addition, during priming in PEG, the β-tubulin signal increased between 3 and 4 d of treatment (Fig. 6), concomitantly with DNA replication activity (Bino et al., 1992). Once DNA replication was initiated, i.e. after 24 h of imbibition of water or after 2 d of priming, the intensity of the β-tubulin signal increased. Thus, in imbibing tomato seeds, the accumulation of β-tubulin apparently coincides with the replication of DNA. Studies with maize roots using antimicrotubular reagents provided evidence that nuclear cell-cycle events depend on the turnover of the microtubular cytoskeleton (Baluška and Barlow, 1993). In imbibing tomato seeds, DNA replication activity may correlate in a synchronized way with β-tubulin accumulation. However, it is not known whether this is a causal relation or whether it is due to the sensitivity of the immunodetection method used. Although β-tubulin, as a component of microtubules, is required for passage through the cell cycle, it is unknown whether de novo synthesis of this protein is a prerequisite for entering the S phase of the cell cycle.

The present results indicate that the amount of β-tubulin was higher when visible germination was achieved (Figs. 3 and 5). This increase in the β-tubulin signal is probably related to the progression of the cell cycle through G_2 toward mitosis and cell division, which might occur during seedling development following visible germination. β-Tubulin expression was not followed during subsequent seedling growth. However, in root tips of soybean seedlings, β-tubulin was found to be temporally expressed, with the transcripts of the gene being most abundant in the first few d after visible germination and declining to undetectable levels 6 d after germination (Jongewaard et al., 1995).

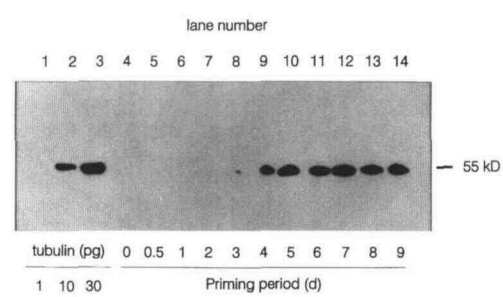

Figure 6. Effect of priming on the induction of β-tubulin synthesis in tomato seeds, cv Lerica. Twenty micrograms of proteins extracted from the embryo root tips at the given times of priming were loaded on the gel. Lanes 1 to 3, Pure tubulin; lanes 4 to 14, priming period. The film was exposed for 7 min.

When the time courses of DNA replication and β-tubulin accumulation in tomato seeds are compared with the germination data, it is obvious that in all cases activation of both cell-cycle-related events preceded visible germination. Priming induced both DNA replication and β-tubulin accumulation and accelerated the germination rate upon subsequent imbibition of water. On the other hand, aging of seeds considerably delayed both DNA cell-cycle-related events and germination. Based on this relation between seed germination and cell-cycle activity, we can hypothesize that cell-cycle-related processes play an important role in tomato seed germination and that β-tubulin expression can be a parameter for following the initial processes that are activated during imbibition by seeds.

ACKNOWLEDGMENTS

H.W.M. Hilhorst, S.P.C. Groot, M.M. van Lookeren Campagne, and M.J.M. Smulders are kindly acknowledged for critically reading the manuscript. We thank J.G. van Pijlen for carrying out the germination experiments.

Received March 20, 1995; accepted July 5, 1995.

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