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**A functional analysis of cell cycle
events in developing and germinating
tomato seeds**

Renato D. de Castro

A functional analysis of cell cycle events in developing and germinating tomato seeds

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Proefschrift

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op gezag van de rector magnificus
van de Landbouwniversiteit Wageningen,
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Cover: A confocal laser scanning micrograph showing DNA synthesis (green/FITC) in tomato seed embryonic nuclei.

BIBLIOTHEEK
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Propositions (Stellingen)

1. Developmental arrest is an imposed condition during seed maturation but is not required for the formation of a viable embryo (*this thesis*).
2. As in lower plants, seeds of the tomato ABA-deficient mutant do not undergo developmental arrest and metabolic quiescence (*this thesis*).
3. In tomato embryogenesis histo-differentiation is dependent on cell division, whereas the completion of morphogenesis is dependent on cell expansion (*this thesis*).
4. Cell division is not a prerequisite for the completion of germination by tomato seeds, but is required for normal germination (*this thesis*).
5. In tomato, primary dormancy is a condition imposed during seed development, which does not reflect the complete arrest of embryonic cell cycle activities, whereas quiescence does (*this thesis*).
6. One will reach the top of the mountain only if he/she is decided to face the difficulties of the walk to the top.
7. The terms 'semi-recalcitrant' and 'intermediate recalcitrance' fail to predict how a seed will behave upon drying. They only make sense when the drying-, storage- and rehydration conditions are indicated (*Ellis RH, Hong TD and Roberts EH, 1990. Journal of Experimental Botany 41: 1167-1174*).
8. Cooperation is one of the most sublime things in life, but interference is one of the most unpleasant.
9. Dormancy is an ignored aspect of crop seed quality (*Hilhorst and Toorop, Advances in Agronomy 61: 111-165*).
10. Brazilian and Dutch time-scales do not always comply.
11. The poor conditions of most developing countries should not be considered as the result of a lack of efficiency, but as the result of the demands of developed countries.

Propositions belonging to the Ph.D. thesis titled 'A functional analysis of cell cycle events in developing and germinating tomato seeds'.

Renato D. de Castro
Wageningen, 11 september 1998

Preface

All the work, which has been presented in this thesis, is part of a long lasting concept which have pushed me forward in life. This concept is based on the difficulties a person can face in life. The aim was, and still is, to learn from these difficulties and be able to be successful, even if perfection is not achieved. For that, the faith in God and in yourself, the sense of humor (whenever possible!), the capacity of recognizing and distinguishing what is wrong from what is right, the friendship, tolerance and cooperation, are all just some of the "items" which made this work possible and which certainly added to my experience in life.

In this perspective, I first of all should say, and I believe also on behalf of my wife, that we are very thankful to the Dutch people and The Netherlands, as we have had an impressively positive period in life here, in this country. After nearly five years, our relation with the Dutch is wonderful, as we, and the Dutch people could understand, tolerate and accept each other in the best way possible. That is the first major important aspect for anyone living abroad. That is what provides us with the energy required to overcome the difficulties of not being in your home country.

I would like to take this opportunity to express my sincere appreciation to all the colleagues at the Centre for Plant Breeding and Reproduction Research (CPRO-DLO). This has been the place where I have spent most of my time while in The Netherlands and, therefore, it is the place where I have met most of the Dutch people I know. I would like to thank everyone from CPRO who have helped me in one way or another. In particular, I would like to thank very much Jan HW Bergervoet for our friendly relationship through all these years and also for all his technical assistance in the laboratory and with the computer drawings in this thesis. For the same reasons, I am also thankful to Haichun Jing. I should say that he has done always greatly as a friend and as a guest worker at CPRO. I wish him all the success he deserves on his Ph.D. program and future work in Wageningen and in China. I am also thankful to Mariette Busser and José Spaans, respectively the former and the present secretary of the Department of Reproduction Technology, for their assistance and for the good moments we have had together.

My very special thanks go to Dr. Raoul J Bino, who, representing CPRO, has accepted me at the Department of Reproduction Technology under his own supervision. I greatly appreciate the way he manages to carry out so many tasks as head of the Department and still be efficient on what he does. I also appreciate his prompt support and belief in my work. I must say that all together it has greatly contributed to the success of the work we have produced, and that was initiated by him. My greetings of good luck and success in future life goes for his wife and children as well.

There are special things that become relevant in life. One of them was the development of an initially professional relationship into a final deep friendship. For that, I

am most deeply thankful to Dr. Henk WM Hilhorst, who has accepted me as a Ph.D. student at the Laboratory of Plant Physiology of the Wageningen Agricultural University (WAU). *My dear "sir", I am of the opinion that our relation overcame the limits of what human beings can imagine in terms of what is possible and what is impossible. I believe your supervising contribution to my work has come up to be "irrelevant" near to what we are able of doing together, as friends. I shall say that your contribution has never been irrelevant to me, and it will never be, but our friendship will always be the most important and relevant aspect to be considered by me. That, you can be sure, will always be my position.* For certain, he will be an example and will add in my scientific and personal lives. For his wife to know, *I wish that your children succeed in life as you both do together.*

My sincere thanks also go to the colleagues at the Lab. of Plant Physiology and the Lab. of Plant Cytology and Morphology, especially Dr. André A.M van Lammeren and Henk Kieft, for their invaluable help with the immunocytochemical studies, Dr. Norbert CA de Ruijter for his kind advise on the use of the confocal laser scanning microscope, and Dr. Peter Wittich for his help with the layout of this thesis. The people at the greenhouse were always very friendly and helpful with growing the tomato plants. I am also grateful to Boudewijn van Veen of MediaService for his kindness and help with the computer images.

Besides my co-promotors, Raoul and Henk, there is a person who is even busier as he has the task of being the rector magnificus of the Wageningen Agricultural University (WAU). I thank Prof. Cees M Karssen for being my promotor and being able to find some time and dedicate it to my Ph.D. research project. Your intelligence and critical view have certainly added to your capacity in evaluating my work along these years and giving your final approval to it. As we have discussed before, I am, through my Ph.D. project, an example of what has been established in terms of cooperation between WAU and DLO. I shall be thankful for the cooperation between Henk (WAU) and Raoul (CPRO-DLO) which has made the conclusion of my thesis possible. Furthermore, I would like to wish you, professor Karssen, all the success as one of the people leading and coordinating this cooperation. Besides, I also wish expansion on "your" cooperation with Brazil, not only in respect with the Lavras Federal University (Universidade Federal de Lavras, UFLA), but with Brazil as a whole. I think that The Netherlands, as a tiny and so developed country have a lot to contribute to an opposite "image", which is a country so big and still under development, as Brazil. I let you know that I am happy of being able to contribute with this cooperation through this thesis, which I dedicate to UFLA, WAU and CPRO-DLO, as the institutions involved in this cooperation between Brazil and The Netherlands.

In that respect, I will always be grateful to UFLA for supporting my Ph.D. program in The Netherlands through the cooperation established with WAU. My sincere thanks are also given to Prof. Silas C. Pereira, Prof. José da Cruz Machado, Prof. Maria das Graças GC Vieira, and to Prof. Fabiano R do Vale, all from UFLA, for their encouragement and for motivating me to come to Wageningen for my PhD. I also thank Dr. Cees Langerak, from CPRO-DLO, for helping me in the achievement of this objective.

I would not forget to acknowledge the Brazilian friends in The Netherlands. I am thankful to all of them who have given to me and to my wife the credibility of friends and the help in all respects. I would like to wish, not only those who are close friends, but to the whole Brazilian community in Wageningen or in the Netherlands good luck and success in their objectives and life while abroad.

It is also acknowledged that my studies in The Netherlands were made possible by the financial support from the Brazilian Ministry of Education (CAPES) and the Netherlands Ministry of Education and Science (NUFFIC).

I am greatly thankful to my mother, who, in the absence of my father, has bravely succeeded in life and became able of giving me the chances to have good education, enabling me to follow my professional career. *"Sou muito grato a minha mãe, quem, na ausência do meu pai, conseguiu com muito esforço ter sucesso na vida, me dando uma boa educação, permitindo com que eu seguisse minha carreira profissional"*. Besides, I have to be thankful to my loved father for enabling me to come to this world, and who always kept his protective eyes over me from the heavens, since I was six years old. I appreciate also the support of my sisters and brother by understanding that my life had to be far from theirs. I am thankful also to Dr. Otaviano J de Araújo and Sueli O de Araújo, respectively my uncle and aunt, who always motivated me in my life and also provided me with previous and precious experience in living abroad, which enabled me to speak English. These are all factors, which contributed to my life and studies in The Netherlands.

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*I dedicate this thesis to
Suely and Luca*

Renato

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Chapter

1

Cell cycle events in developing and germinating tomato seeds: introduction

R.D. de Castro

Seeds are the primary dispersal units of higher plants containing the complete genetic make up of the species. They are complex biological structures, which, in millions of years, have adapted to diverse and often austere environments. Seeds are able to withstand drought and extreme temperatures and may remain viable for prolonged periods of time that may extend to hundreds of years. These features make seeds the main contributor to the preservation of the plant world's genetic diversity, and are a fascinating system for biological research. Furthermore, seeds are of eminent importance as a source of food and nutrition for more than two thirds of the world population.

The seed consists of nutrient reserve storage tissue(s), an embryo and encapsulating structures designated for protection and that may also regulate germination. Seeds are unique, as their formation requires the involvement of two consecutive phases in the plant's life cycle, *i.e.* the sporophyte and the gametophyte, from which tissues with differing ploidy levels develop, *i.e.* the embryo and the endosperm. They retain also the unique characteristic of withstanding desiccation without losing the ability of immediate metabolic reactivation upon rehydration. Likewise, seeds may possess mechanisms to sense the environment, thus ensuring germination under favourable conditions.

Seeds are objects of many different kinds of research. The morphological and physiological processes that occur during development and germination have been studied and described extensively (Figure 1a,c). However, information about the regulatory

mechanisms controlling these processes has begun to emerge only after the introduction of genetic and molecular-biological technology to this field (reviewed by Bewley and Black, 1994; Galau et al., 1991; Goldberg et al., 1994; Harada, 1997; Hilhorst, 1995; Kermode, 1995; Koornneef and Karssen, 1994; Raghavan, 1997). Analyses of the changes in gene expression patterns that occur during seed development and postgerminative growth (Figure 1b) have contributed clues about the regulatory programs governing both periods (Chlan and Dure, 1983; Dure et al., 1981; Dure, 1985).

Although these modern molecular-genetic approaches, which have yielded extraordinary enhancement of the knowledge in plant developmental biology, attempt to explain and understand the functioning and the behaviour of seeds, most of this knowledge is often based on studies, which have chosen seeds as convenient objects of study. There is no doubt that over the last decades, our understanding of the processes involved in seed development and germination, including the control of dormancy has expanded. Notwithstanding these advances, very little is known about many subjects of primary importance in seed science. As discussed by Karssen (1993), a great deal of integrated, interdisciplinary study is still required for seed science *in sensu strictu*, in order to better understand seed function and behaviour.

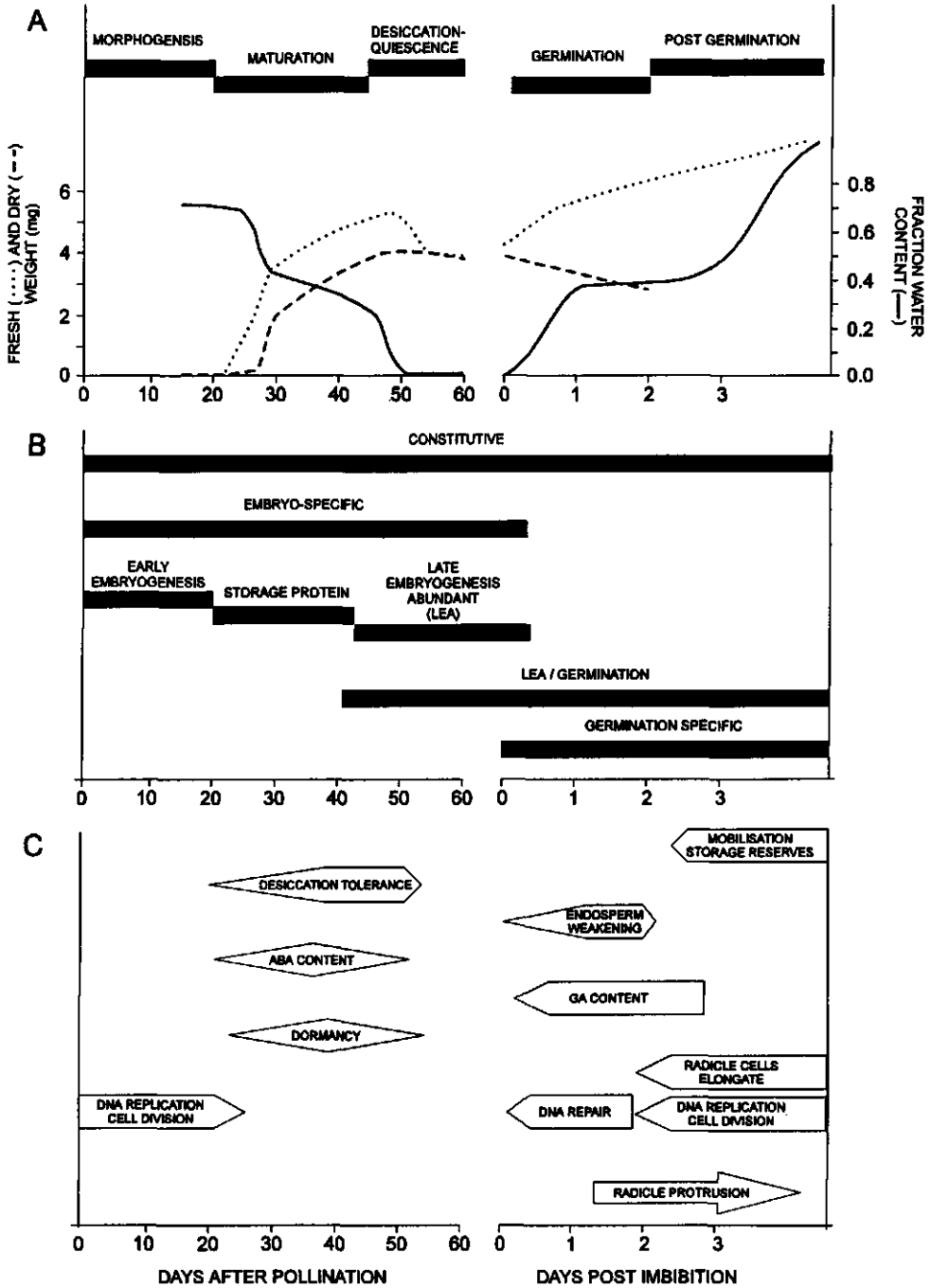
Phases of seed development and germination

Seed development can be divided in two conceptually distinct phases (Figure 1a). One is a period of morphogenesis during which the embryo's body plan is established through intensive cell divisions and the embryonic organs and tissues are formed (reviewed by Goldberg et al., 1994; Meinke, 1995; West and Harada, 1993).

Figure 1. *Seed development and germination.*

- (a) Physiological parameters divide seed development into distinct phases. Time course of changes in fresh and dry weights and in fraction water content of developing and germinating embryos, and postgerminative seedlings are shown. Bars indicate stages of seed development and germination. Data are from oilseed rape embryos and seedlings, taken from Comai and Harada (1990), reviewed by Harada (1997).
- (b) Gene expression patterns divide seed development into specific stages. A conceptual representation of the accumulation of seven mRNA sets that are present during seed development, germination and postgerminative growth. Bars indicate the period during which the indicated mRNA set is detected. For points of reference, mRNA accumulation patterns are fit to the time scale in (a). Adapted from Dure (1985) and Goldberg et al. (1989).
- (c) Time course of major events associated with seed development, germination and subsequent postgerminative growth. For points of reference, the physiological events are fit to the time scale in (a) and (b). Adapted from Bewley (1997) and Hilhorst et al. (1998).

Cell cycle events in developing and germinating tomato seeds: introduction



The other phase is a subsequent period of seed maturation. It includes the arrest of tissue and organ formation, the accumulation of nutrient reserves, changes in embryo size and in fresh and dry weights, the suppression of precocious germination, the acquisition of desiccation tolerance, dehydration and quiescence, and, in many species, the induction of dormancy (Koornneef and Karssen, 1994). Germination and postgerminative growth (Figure 1a) represent the phases during which metabolic and morphogenetic reactivation of the quiescent seed occurs (reviewed by Bewley, 1997; Harada et al., 1988, 1997).

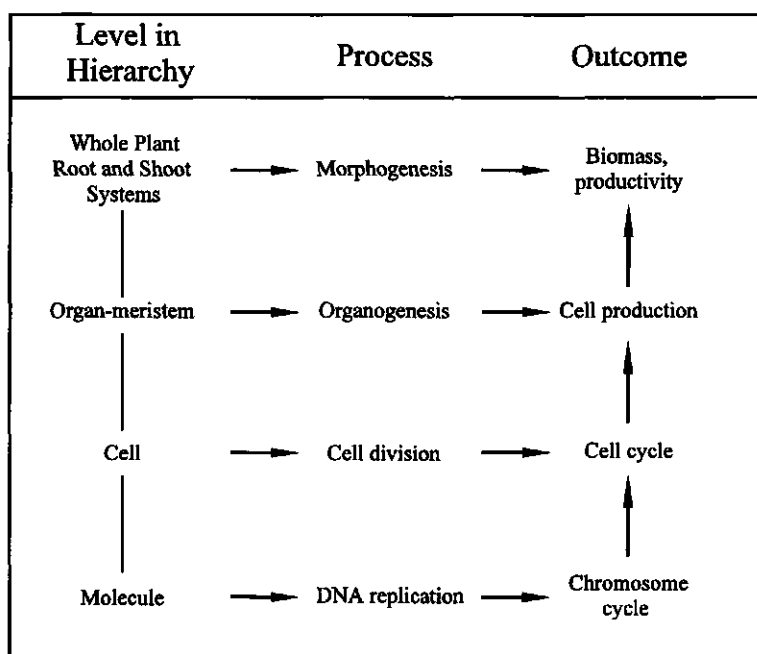


Figure 2. Scheme to show the relation between DNA replication, cell division and organogenesis in plants.

An autopoietic system (Varela et al., 1975) showing the sequential outcome of processes (horizontal arrows) in relation to the complexity of the system (upward-directed arrows). Adapted from Barlow (1993).

It has been argued that developmental arrest is an imposed condition during seed maturation that is not required for the formation of a viable embryo (Walbot, 1978).

Contrary to higher plants, seeds from lower plants for instance do not undergo developmental arrest and metabolic quiescence. The implication is that seed plants have incorporated processes related to seed maturation, dormancy and germination into the continuous mode of morphogenetic developmental characteristics of many lower plants. The regulatory programs controlling the arrest of growth and metabolism during development and their reactivation during germination are, therefore, of relevance and will certainly involve the arrest and reactivation of cell cycle related events.

Growth

Growth depends on cell division and elongation. The sequence of processes that occur during cell division is referred to as the cell cycle, which is dependent on DNA replication, and which leads to specific patterns of organogenesis and morphogenesis, *i.e.* cellular differentiation (Figure 2). At the 'deeper', cytological and molecular levels, the cell cycle involves a chromosome cycle in which DNA synthesis towards replication occurs during interphase, and a mitotic cycle which leads to cell division (Figure 3). The patterning of the cell division cycle resolves into recursive patterns of configurations of the cytoskeleton components, as the "sub cellular machinery" required for cytokinesis, *i.e.* cell division, and growth.

Microtubules are one main component of the cytoskeleton. They are unbranched cylinders of about 25 nm in external diameter, with an open central channel of about 15 nm, and are assembled from heterodimers containing one α -tubulin polypeptide and one β -tubulin polypeptide, each with a molecular weight around 50 to 55 kD and about 450 amino acids (Figure 4). Microtubules are related to the cell cycle through distinct reorganisational or configurational arrays, *i.e.* the interphase cortical arrays, and the preprophase bands, spindles, and phragmoplast mitotic arrays (Derksen, et al., 1990; Goddard et al., 1994) (Figure 3).

Synthesis and replication of DNA occurs during interphase and may last several hours. Therefore, interphase is a phase, which, within the cell cycle, lasts longer than mitosis. Within interphase there is a phase of quiescence (G_0) coupled to a phase of growth and pre-synthesis (G_1), during which the nuclei of diploid cells contain an arbitrary 2C DNA value, referring to the amount of DNA, and during which DNA repair may occur. Subsequently, synthesis towards replication of DNA occurs during the synthetic or S-phase. Finally, the G_2 phase, comprising nuclei with 4C DNA values, resolves interphase in preparation for mitosis. The mitotic phase comprises several distinct stages based on the microtubular arrays, *i.e.* (pre)prophase, metaphase, anaphase and telophase (Figure 3).

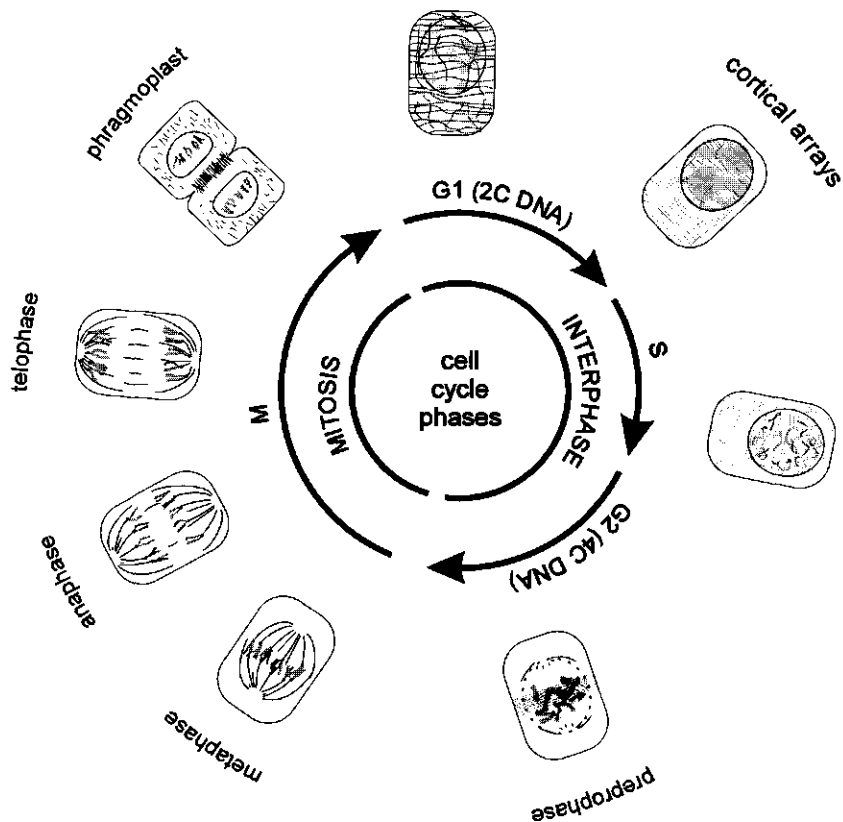


Figure 3. *Diagram of microtubular cytoskeleton arrays in relation to cell cycle phases.*

DNA replication (2C to 4C DNA) occurs in a period that lasts several hours, known as "S-phase" of the cell cycle. Mitosis ("M-phase") commences after a "gap" ("G₂-phase"). The "G₁-phase" follows mitosis, to complete the cycle. Changes in the microtubular cytoskeleton system are geared to these events. Cortical arrays are found during G₁- and S-phases. The preprophase band begins to form at the end of the S-phase and is fully condensed just before the nuclear envelope breaks at the end of prophase of mitosis. It is then supplanted by the mitotic microtubular cytoskeleton arrays of the spindle, which then turn to give way to the phragmoplast array. Finally cytokinesis occurs and cortical arrays are reinstated. Adapted from Gunning and Steer (1996).

Both DNA synthesis and the microtubular organisation, have been extensively studied in seeds, taking into consideration subjects such as cellular events in embryogenesis and endosperm formation during seed development, and in organogenesis during postgerminative growth (reviewed by Bershadsky and Vasiliev, 1988; Barlow, 1993; Clayton, 1985; Francis and Herbert, 1993; Raghavan, 1997; Xu, 1995). Yet, a great deal of knowledge is still required in order to understand the relationships among cell cycle events in seeds, and their contribution to seed functioning during development and germination.

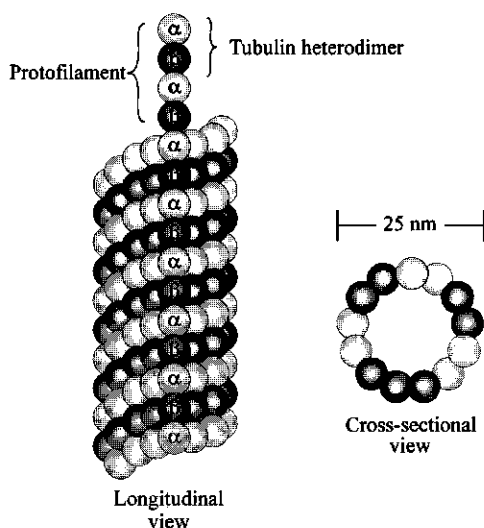


Figure 4. Schematic model of the microtubule.

The microtubule is a hollow cylinder whose wall is composed of 13 protofilaments. Each protofilament is constructed from alternating α -tubulin and β -tubulin molecules. Modified from Kleinsmith and Kish (1995).

Objectives

The tomato (*Lycopersicon esculentum* Mill.) seed is considered a convenient research object because of its relatively simple structure. The mature seed consists of a full-grown embryo embedded in a thick-walled endosperm and is surrounded by a thin seed coat. Its size, in the order of 2-5 mm, allows for easy manipulation and dissection. Therefore, tomato seeds have been used most extensively as a "model system" to study the physiology and biochemistry of seed development, germination and dormancy (Hihorst et al., 1998).

In this thesis, tomato seeds are used to study cell cycle related events, *i.e.* DNA synthesis and the microtubular cytoskeleton, during development and germination. The progression of growth, *i.e.* cell division and expansion, is analysed in relation with developmental events, such as increase in dry weight, germinability and achievement of

quiescence or dormancy. A similar analysis is made during release of dormancy and reactivation of growth during germination.

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β -Tubulin accumulation and DNA replication in imbibing tomato (*Lycopersicon esculentum* Mill.) seeds

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Summary. 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 G₁ phase of the cell cycle. In addition, β -tubulin was not detectable on western blots. Upon imbibition in water, the number of cells in G₂ started to increase after 24h, and a 55 kD β -tubulin signal was detected between 24 and 48h. 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 in water, compared with imbibed control seeds. By contrast, when aged seeds were 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 activated during seed imbibition.

Introduction

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 G₂ 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 rapidly be 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 (MPF) 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 at the protein and mRNA levels (Georgieva et al., 1994b). The expression pattern of these proteins was correlated with nuclear events. Possibly, the proto-oncogenes 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 these transformations into different arrays are dependent on the interactions between microtubules and MAPs (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 50kD. For several plant species, β -tubulin genes have been characterized (Guiltinger 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). Histo-immunological 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 was demonstrated that the progression of the cell cycle through G_1 phase was dependent on the turnover of 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 happens 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 in water (Heydecker and Coolbear, 1977). This may be due to activation of pre-germinative 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 on the expression of β -tubulin in relation to nuclear DNA replication and on the role of cell cycle activation in tomato seed germination. β -tubulin expression and the induction of DNA synthesis is studied in control, primed, and aged seeds. The goal of our studies is to gain a better understanding of the initial processes, which are activated during seed imbibition.

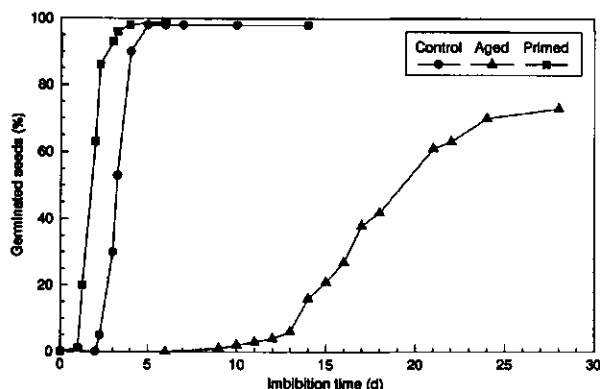
Results

Germination tests

The germination of the aged, primed, and control tomato seed lots were scored by counting the number of germinated seeds daily (Figure 1). The uniformity and germination performances of control seeds were high (Figure 1 and Table 1). Compared to the control seeds, priming improved germination performance by a significant decrease in the mean germination time (Figure 1 and Table 1). 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 1).

Figure 1. Germination of control, primed and aged tomato seeds.

Priming was performed in -1 MPa PEG-6000 at 20°C for 7d and aging was brought about by treatment at 60°C and 45% RH for 8d. After these pretreatments, seeds were dried back to their equilibrium moisture content prior to imbibition in water.



Nuclear replication activity

As was previously demonstrated (Bino et al., 1992), cells in the embryo root tips of dry and imbibed tomato seeds contain nuclei with either 2C values (G_1 phase of cell cycle) or 4C values (G_2 phase with replicated DNA). Therefore, the number of nuclei with 4C values, expressed as percentage of total number of nuclei (2C + 4C), was used to follow nuclear DNA replication activity upon seed imbibition (Figure 2). In root tips of control seeds, the percentage of 4C nuclei was low (5%) and increased rapidly between 24 and 48h 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).

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

Data are means (\pm S.D.) of 4 replicates of 50 seeds each. TG, total germination; NS, normal seedlings; t_{50} , mean germination time, time to 50% germination of total germinated seeds; $t_{75}-t_{25}$, germination uniformity, time between 25% and 75% germination of total germinated seeds.

Treatment	TG(%)	NS(%)	t_{50} (d)	$t_{75}-t_{25}$ (d)
Control	100	97 \pm 3	3.3 \pm 0.1	0.7 \pm 0.1
Priming	99 \pm 1	98 \pm 2	1.8 \pm 0.0**	0.7 \pm 0.1
Aging	74 \pm 3**	7 \pm 3**	17.5 \pm 0.8**	5.0 \pm 0.6**

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

Upon imbibition of these primed seeds, the number of 4C nuclei doubled within 8h to a similar value (40 to 50%) as in 2d imbibed control seeds. In contrast to control and primed seeds, aged seeds did not show an increase of 4C values during the first 5d of imbibition, *i.e.* the percentage of 4C nuclei fluctuated between 6 and 12%.

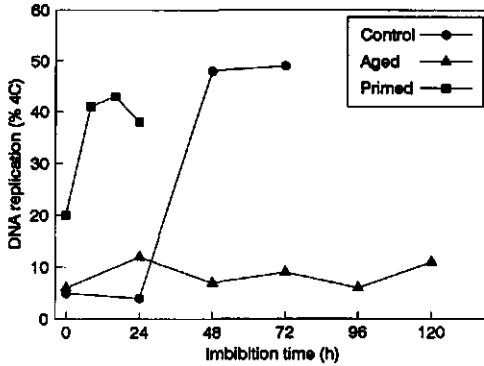


Figure 2. DNA replication activity in imbibing tomato seeds.

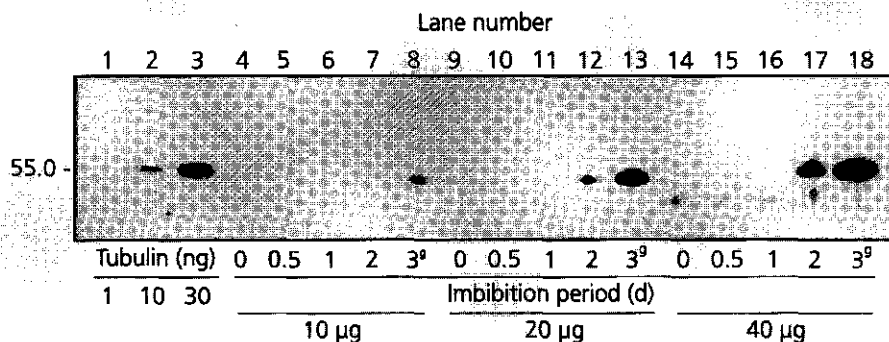
Nuclei were isolated from radicle tips of aged, control and primed seeds and analyzed by flow cytometry. DNA replication stage is expressed as the number of 4C nuclei, in the percentage of the total number of nuclei analyzed (2C + 4C).

Immunodetection of β -tubulin following one- and two-dimensional PAGE

In imbibed control seeds, a blot containing a range of three different amounts of root tip protein extract (10, 20 and 40 μ g, respectively) was immunodetected for β -tubulin at 5 different imbibition times. With all protein 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 μ g total protein extract were loaded (Figure 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 12 and 24h imbibed seeds (Figure 3, lanes 4, 5, 6, 9, 10, 11, 14, 15, and 16). A strong signal was detected after 48h of imbibition in the 20 μ g and 40 μ g concentration range. The intensity of the β -tubulin signal increased up to 72h of imbibition (germinated seeds), when the intensity of the signal could be compared to that of the pure tubulin at 10 to 30 ng (Figure 3, lanes 2 and 3).

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

To determine whether the signal at 55kD represented one single polypeptide or different isoforms, root tip proteins from 48h imbibed seeds were separated by two-dimensional PAGE and, subsequently, immunoblotted with the β -tubulin antibody (Figure



Western blot analysis of β -tubulin following SDS-PAGE of 3 different amounts of proteins extracted from embryo radicle tips of imbibing control tomato seeds, cv. LERICA. lanes 1 to 3: pure tubulin; lanes 4 to 8: 10 μ g protein range; lanes 9 to 13: 20 μ g protein range; lanes 14 to 18: 40 μ g protein range. The film was exposed for 7 min. ^a: germinated seeds.

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 simultaneously imbibed and protein samples were loaded on the same gel that was immunoblotted following one-dimensional SDS-PAGE (Figure 5). A clear β -tubulin signal with increasing intensity was observed in samples from control seeds imbibed for 48 and 72h (Figure 5, lanes 15 and 16), whereas this signal was not detected in aged seeds (Figure 5, lanes 4 to 11). For aged seeds, the β -tubulin signal was detected only after 8d of imbibition (data not shown); germination started 2d later. In seeds that were primed, *i.e.* incubated in -1.0 MPa PEG-6000 for 7d and then dried back to their equilibrium moisture content, the β -tubulin signal was already clearly present in the redried seed, before imbibition in water (Figure 5, lane 17). The intensity of the signal in dry primed seeds was somewhat higher compared to that in the control seeds imbibed for 48h (Figure 5, lanes 15 and 17). When primed seeds were subsequently imbibed in water, the intensity of the signal increased up to 12 and 24h of imbibition (Figure 5, lane 18 and 19), and reached its maximum at 48h of imbibition, when seeds were already germinated. Analysis of the β -tubulin signal during priming

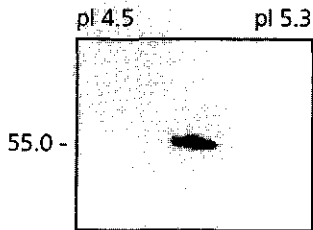


Figure 4. β -Tubulin isotypes in embryos of tomato seeds.

Two-dimensional PAGE and Western blot analysis of β -tubulin polypeptides extracted from embryo radicle tips of control seeds imbibed for 48h. Three spots can be observed at 55 kD around pI 4.9.

showed that the signal could be detected after 3 to 4d of priming (Figure 6, lanes 8 and 9). The intensity of the signal increased up to 5d and remained relatively constant during further priming (Figure 6, lanes 10 to 14).

Discussion

The expression of β -tubulin, a protein required for the passage through the cell cycle, was analyzed in imbibing tomato seeds. Using protein extracts from embryonic root tips, one β -tubulin signal of about 55kD could be immunodetected. 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 (Figure 4), at least three β -tubulin isotypes were found in the embryo root tip samples of control seeds after 48h of imbibition. This could be the result of a coevolution with cell type specific microtubule associated proteins (MAPs) (Fosket and Morejohn, 1992), since it is known that different tubulin isotypes are expressed in tissues of various plant species (Hussey et al., 1988; 1990; Kopzak et al., 1992; Snudstad 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, 12h and 24h imbibed control seeds (Figures 3 and 5), and in all aged seeds (Figure 5). The immunodetection limit of the system used was between 1 and 10 ng of pure bovine brain tubulin. Possibly, the level of β -tubulin in these 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 to membranes, required a treatment with 0.1M Na_2CO_3 at a pH greater than 11.5 so that the hydrophobic form was converted into a hydrophilic and extractable form.

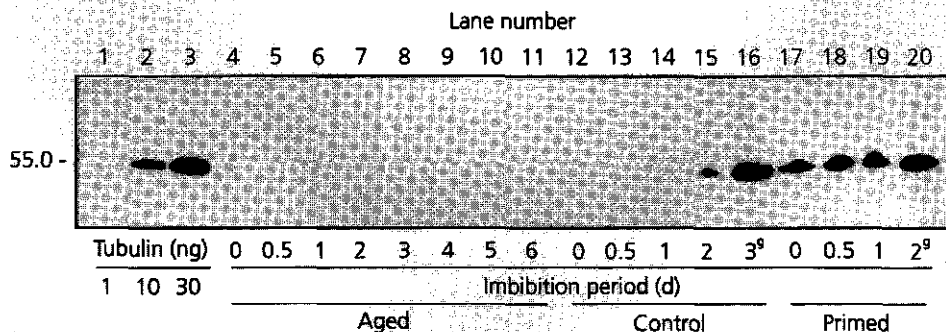


Figure 5. Effect of aging and priming on β -tubulin accumulation during imbibition of tomato seeds.

Aging and priming were carried out as described in Figure 1. Gel was loaded with a total of 20 μ g 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. *: germinated seeds.

The present results with tomato seeds show that the signal of β -tubulin increased in cells of the embryo root tip within 48h of imbibition (Figure 3). At this time also, DNA replication as judged by the 2C to 4C transition in root tip nuclei, was observed (Figure 2). In addition, during priming in PEG, the β -tubulin signal increased between 3 and 4d of treatment (Figure 6), concomitant with DNA replication activity (Bino et al., 1992). Once DNA replication was initiated, *i.e.*, after 24h of imbibition in water or after 2d 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 anti-microtubular reagents provided evidence that nuclear cell cycle events depend upon 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 S-phase of the cell cycle.

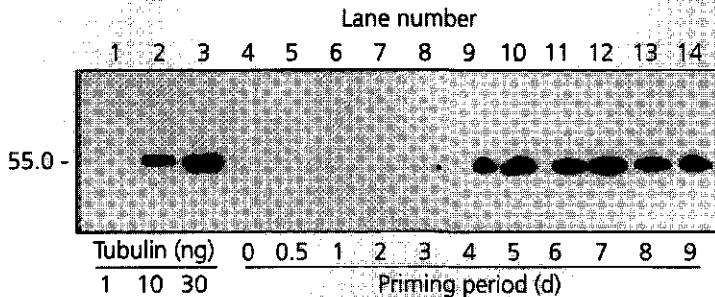


Figure 6. Effect of priming on the accumulation of β -tubulin in tomato seeds.

A total of 20 μ g of proteins extracted from the embryo root tips at 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.

The present results also indicate that the amount of β -tubulin was higher when visible germination was achieved (Figures. 3 and 5). This increase of the β -tubulin signal is probably related to the progression of the cell cycle through G_2 towards 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 days after visible germination, to decline till undetectable levels at 6 d after germination (Jongewaard et al., 1995).

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 in 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, it can be hypothesized 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 which are activated during imbibition of seeds.

Experimental procedures

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 days at 20°C and 32% RH (moisture content $6.3\% \pm 0.1$, 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 6h to a maximum of 7d 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 $7.1\% \pm 0.1$) for 8d. Treated seeds were then dried back to equilibrium moisture content over a saturated CaCl_2 solution for 3d.

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

Germination tests, moisture contents and statistical analysis. Both moisture content (MC) determinations (two replicates of 1g each) and germination tests (four replicates of 50 seeds each) were carried out according to the conditions recommended by ISTA (International Seed Testing Association, 1993). The germination characteristics are expressed as total germination (TG), number of normal seedlings (NS), mean germination time (t_{50} : time to 50% of total germinated seeds) and germination uniformity ($t_{75}-t_{25}$: time between 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 $P = 0.01$).

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 (DAPI) was added to the isolation buffer (Saxena and King, 1989). After chopping, the suspension was passed through a 25 μm nylon mesh and immediately analyzed. For each sample, 3 to 5 seeds were used and flow cytometric determinations were made in triplicate, using a PAS II flow cytometer (Partec GmbH, Münster, Germany) equipped with a HBO-100 mercury arc lamp, a TK-420 dichroic mirror, and a GC-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 in which the 1C value comprises the DNA content of the unreplicated haploid chromosome complement (Howard and Pelc, 1953). Using the signals obtained from tomato leaf tissue, the gain settings were adjusted so that the 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 priming and imbibition of the seeds 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 directly added 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) was 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 the micro-protein assay procedures (Bio-Rad), modified 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), 1, 10 and 30 ng, respectively, 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 (CHAPS), 2% (v/v) β -mercaptoethanol and 2% (v/v) 2-D Pharmalyte 3-10 (Pharmacia). Immobiline pH 4-7 gels and 8-18% 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 (PVDF) membrane (0.45 μ m, Amersham) using a Novablot Electrophoretic Transfer unit (Pharmacia), operating at 0.8 mA/cm² and 30 V at 4°C. The transfer buffer consisted of 25 mM Tris, 192 mM glycine and 10% (v/v) methanol (pH 8.7).

Chemiluminescence immunodetection of β -tubulin. All steps of the immunodetection 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 1h, and probed with 1 μ g/ml mouse monoclonal anti- β -tubulin antibody (Boehringer Mannheim, clone KMX-1), diluted in 0.5% (w/v) blocking solution for 1h. Membranes were then washed twice with large volumes (minimum of 30 ml for a 10 x 10 cm membrane) of TBST (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 mU/ml POD-conjugated secondary antibody diluted in 0.5% (w/v) blocking solution. Thereafter the membranes were washed again, for four times with large volumes of TBST for 15 min each, and further processed according to Leying et al. (1994). The immunoblot was incubated with a premixed detection solution, 125 μ l/cm², consisting of 100:1 mix 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 safe light, several sheets of photographic films (Hyperfilm-ECL, Amersham) were exposed for different periods of time, varying from 20 sec to 10 min, and developed according to the manufacture's protocol.

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Chapter

3

Detection of β -tubulin in tomato (*Lycopersicon esculentum* Mill.) seeds: optimisation of extraction and immunodetection procedures

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Summary. The SDS-PAGE profiles of SDS-denaturated total protein (MODIL) extracts of imbibing tomato seeds showed no large qualitative differences with those of water-soluble (HEPES) proteins. However, the protein concentrations of the HEPES samples were significantly lower than the MODIL ones. The presence of SDS and DTT in MODIL buffer at an alkaline pH was essential for high yields of β -tubulin. The absence of DTT in HEPES extraction buffer may have allowed oxidation and subsequent loss of β -tubulin extracted from radicle tips from germinated seeds. The optimization of the β -tubulin extraction procedure and improvements of the immunodetection system allowed us to obtain a good impression of the β -tubulin accumulation pattern in whole tomato seeds and in the different tissues. β -tubulin accumulation in seeds was shown to be tissue-dependent, the highest concentration being found in embryo radicle tip tissue.

Introduction

Tubulin polypeptides are components of both animal and plant microtubules. Monomers have a molecular weight of approximately 55kD each, and form a heterodimer by means of non-covalent, hydrophobic interactions (Sackett and Lippoldt, 1991; Fosket et al., 1993). During cell cycle and mitosis in higher plant cells, different cell-cycle-stage-dependent microtubules arrays are involved in a wide range of activities including chromosome segregation, cell plate and cell wall formation.

Existing knowledge on tubulins in cells concerns neurotubulins. Evidence of the conserved nature of the peptide between the Eukaryotes is provided by the cross-reactivity between neurotubulins antibodies and plant microtubules of *Leucojum* endosperm cells (Franke, et al., 1977), the successful higher plant tubulin polymerisation *in vitro* (Morejohn and Fosket, 1982), and the cross-reactivity of higher plant antibodies with intracellular microtubules in animals cells (Piquot and Lambert, 1988). Since these discoveries, immunocytochemical, in particular immunofluorescence techniques, have yielded numerous new findings on microtubules and tubulin properties and activities in plants (Wick et al., 1981; Clayton and Lloyd, 1994; Young et al., 1994; Binarova et al., 1993; Liu et al., 1993, 1994; Schmit et al., 1994). However, procedures have still to be developed and improved to allow the detection of the low tubulin content in plant tissue cells and to overcome its instability during extraction due to endogenous proteases (Piquot and Lambert, 1988). The improvement of extraction methods together with Western blot analysis of tubulins fractioned by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis has been an important step. An effective extraction and detection method of low plant tubulin content has been described using an autoradiographical immunoblotting method (Fukuda and Iwata, 1986). Several other extraction and immunodetection procedures for identification and/or quantification of higher plant tubulins are known, most of them from *in vitro* plant cell cultures and seedlings (Liu et al., 1993, 1994; Schmit et al., 1994; Fukuda and Iwata, 1986; Morejohn and Fosket, 1984; Mizuno et al., 1981; Mizuno, 1985; Laporte et al., 1993).

However, little is known about tubulin from seed tissues. Biochemical and immunological properties of tubulin from monocotyledon endosperm cells have been reported (Piquot and Lambert, 1988), and recently we reported on the pattern of β -tubulin accumulation, in relation to cell cycle activities in imbibing tomato (*Lycopersicon esculentum* M.) seeds (de Castro, et al., 1995). Here we discuss the extraction of β -tubulin from dry and imbibing tomato seeds, in combination with an improved immunochemiluminescence detection protocol, which allowed us to detect β -tubulin levels below 10 ng.

Results

Protein extraction

Two different protein extraction methods were compared: SDS-denaturated total proteins using a modified Laemmli (Laemmli, 1970) buffer, referred to as MODIL, and water-soluble proteins using HEPES buffer. The results in Table 1 show that significantly lower protein levels content were obtained at all four imbibition periods when using HEPES extraction buffer. Gel electrophoresis showed that there were no significant qualitative differences in polypeptide patterns between both types of extraction, either from whole seed or from radicle tip tissue. The only significant difference was observed in the polypeptides patterns of germinated radicle tip samples compared to all other samples, in that the radicle tip extracts contained only traces of reserve proteins (results not shown).

Immunodetection of β -tubulin

Whole seed and radicle tip protein extracts prepared with MODIL and HEPES buffers were used for immunodetection of β -tubulin at four different seed imbibition times. In all types of protein samples, the monoclonal anti- β -tubulin antibody recognised a protein band with a molecular weight of about 55kD, although not at all imbibition periods. In general, the signal was stronger in MODIL samples compared to HEPES samples, and in radicle tip samples compared to whole seed samples. In radicle tip samples the signal was detectable starting from 24h of imbibition and its intensity increased with the imbibition period, being higher after 48h of imbibition in germinated radicle tip samples, whereas specifically in the corresponding HEPES sample the intensity of the signal decreased drastically. This was thought to be a result of protease activity, which was tested by adding a cocktail of protease inhibitors to the HEPES buffer. However, the decrease in the intensity appeared to be due to the buffer composition itself. Later it was proven as being related to the absence of dithiothreitol (DTT) in the HEPES

Table 1. Amounts of protein extracted from imbibing (0, 24 or 48h) seeds using SDS-denaturated total protein (MODIL) or water-soluble protein (HEPES) extraction buffers.

Proteins were extracted from whole seed or radicle tip tissues of dry and imbibing seeds. Data represent the means of two replicates per imbibition period of samples collected from each tissue, i.e. 5 whole seeds or 20 to 30 isolated radicle tips.

Imbibition period (h)	Protein content ($\mu\text{g}/\mu\text{l}$)	
	MODIL	HEPES
0	12.3 ± 0.6	2.8 ± 0.8
24	10.1 ± 0.7	3.5 ± 0.5
48	10.2 ± 1.4	3.7 ± 1.3
48 ^g	9.1 ± 2.2	2.1 ± 0.6

^g Seeds germinated after 48h of imbibition

buffer as tested by adding DTT to the buffer. This problem was specific only to radicle tip samples prepared from germinated seeds (results not shown).

Effects of pH on β -tubulin extraction

To study the effect of pH on the extraction efficiency of β -tubulin from seed tissues, radicle tip protein extracts were prepared with MODIL buffer, not only at pH 6.8, but also at pH 7.5 and 9.0. A blot containing extracts of three different seed imbibition periods was immunodetected for β -tubulin (Figure 1). At pH 6.8, β -tubulin was detectable only in samples of 24h or longer imbibition periods. But at pH 7.5, a weak β -tubulin signal was also detectable in the dry, non-imbibed sample. At pH 9.0, the signal was even stronger in all samples.

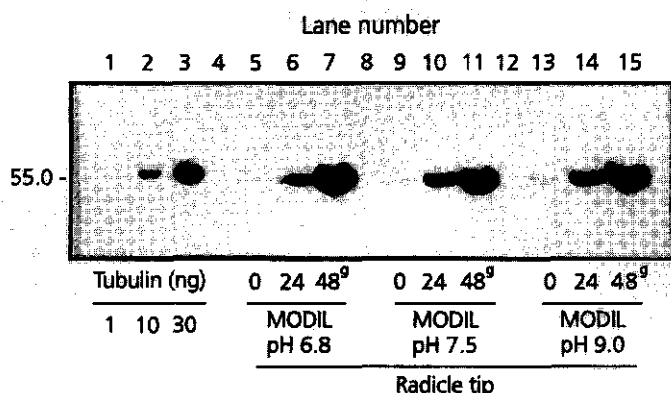


Figure 1. Effect of pH (MODIL) on the extraction of β -tubulin.

The gel was loaded with 20 μ g of proteins extracted from radicle tip tissue of dry and imbibing (0, 24 or 48h) tomato seeds. Lanes 1-3, pure tubulin; lanes 5-7, MODIL pH 6.8; lanes 9-11, MODIL pH 7.5; lanes 13-15, MODIL pH 9.0. (^g) germinated.

β -tubulin accumulation pattern in different seed tissues

A blot containing whole seed, seed coat plus endosperm, whole embryo, and radicle tip protein extracts prepared with MODIL buffer at pH 9.0 was immunodetected for β -tubulin to obtain its accumulation pattern in these different tomato seed tissues during imbibition (Figure 2). Compared to samples prepared with MODIL buffer at pH 6.8 (results not

shown), β -tubulin could now be detected in whole seed samples, but still only in 48h imbibed and germinated samples. Only very low and about equal levels could be detected in the seed coat plus endosperm from 48h imbibed and germinated seed samples. In whole embryo samples, β -tubulin was detected after 24h imbibition, its signal increased after 48h and was highest in embryos from seeds which had germinated after 48h imbibition. A similar pattern, but beginning with the dry, non-imbibed sample was detected in isolated radicle tips (Figure 2).

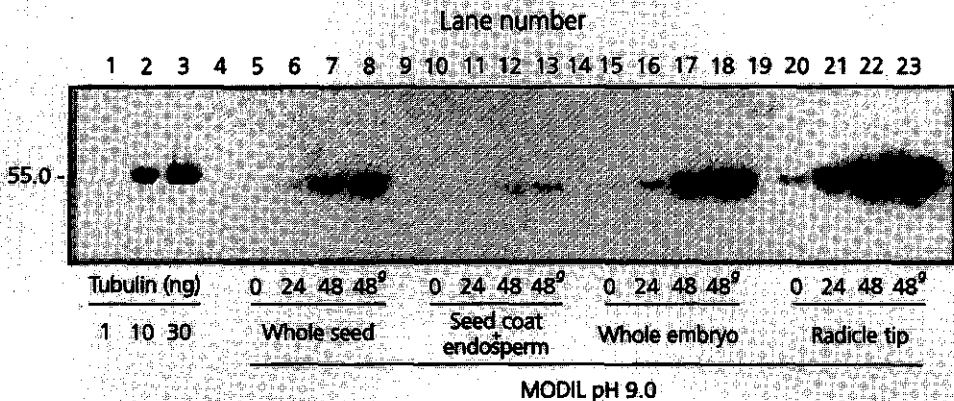


Figure 2. β -Tubulin accumulation (MODIL, pH 9.0) in different seed tissues.

The gel was loaded with 20 μ g of proteins extracted from whole seed (lanes 5-8), seed coat plus endosperm (lanes 10-13), whole embryo (lanes 15-18) or radicle tip (lanes 20-23) tissues of dry and imbibing (0, 24 or 48h) tomato seeds. Lanes 1-3 were loaded with pure tubulin. (^g) germinated.

Effect of protein content loaded

To check the possibility of obtaining a β -tubulin accumulation pattern similar to the one of radicle tip samples, as in the latter experiment, a blot was produced from a gel loaded with 70 μ g in 40 μ l wells of MODIL pH 9.0 whole seed protein extracts, instead of 20 μ g in 20 μ l wells. The immunodetected blot now allowed us to obtain such a pattern, using whole seed extracts (Figure 3).

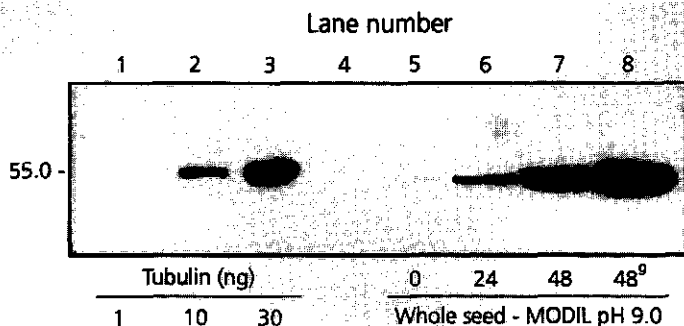


Figure 3. *β -Tubulin accumulation in whole seeds.*

The gel was loaded with 70 μ g of proteins extracted from whole dry and imbibing (0, 24 or 48h) tomato seeds. **Lanes 1-3**, pure tubulin; **lanes 5-8**, MODIL (pH 9.0) whole seed. (^g) germinated.

Discussion

It has been proven possible to detect tubulin in about 50 mg of fresh plant material (Fukuda and Iwata, 1986). However, seeds often imply much smaller amounts of material with even smaller tubulin contents.

Although we have recently reported on the immunochemiluminescence detection of β -tubulin in tomato seeds imbibed for 48h or more (de Castro, et al., 1995) improvement of the protein extraction and immunodetection procedures was required in order to detect lower β -tubulin contents. Since microtubules are present in all stages of a typical plant cell cycle (Goddard et al., 1994), a constitutive amount of β -tubulin was expected to exist in dry seeds and in seeds imbibed for shorter periods.

The loss of the β -tubulin signal in the HEPES germinated radicle tip extract (not shown) was unexpected, since the signal could be detected in earlier imbibition periods in a pattern similar to that observed for the MODIL samples, which allowed an effective detection of β -tubulin in germinated radicle tips. Moreover, germination requires cell expansion for which β -tubulin is a key component (de Castro, et al., 1995). Consequently, the loss of β -tubulin in the HEPES germinated radicle tip extract was considered an artifact which could be due, for instance, to activity of proteases (Piquot and Lambert, 1988) which

could be acting at the moment of radicle protrusion and seedling growth. However, we have shown that the problem was due to the lack of DTT in the HEPES buffer.

The cells of the embryonic radicle tips apparently become increasingly accessible to O_2 once the radicle has protruded through the seed coat (Goddard et al., 1994; Callis, 1995), therefore becoming highly susceptible to oxidation as well. Oxidation of β -tubulin sulfhydryl groups into disulfide bridges may have induced its loss during isolation and extraction, since the addition of DTT to the HEPES buffer allowed the recovery of the β -tubulin signal (not shown). Tubulin polypeptides contain a considerable number of sulfhydryl groups (Stephens, 1970; Lee et al., 1973; Mellon and Rebhuhn, 1976). Sulfhydryl groups were found to be important for *in vivo* polymerisation of tubulins and assembly of microtubules, being influenced by intracellular levels of reduced glutathione (Oliver et al., 1976; Liebmann et al., 1993). Glutathione is a cellular sulfhydryl component essential in reductive processes for synthesis and degradation of proteins (Meister and Anderson, 1983). Oxidation of glutathione has been reported in tomato seeds as a result of aging (de Vos et al., 1994). Furthermore, decreasing the number of tubulin free sulfhydryls in human cells inhibited tubulin polymerisation, but the process could be immediately reversed by the addition of DTT (Ikeda and Steiner, 1978). DTT is known to prevent oxidation of other proteins as well, by reduction of disulfide bridges (Sepaio and Meunier, 1995). It was also found that DTT should be included in the isolation buffer of tubulin from epicotyls of *Vigna angularis* (Mizuno et al., 1981). When using MODIL buffer without DTT, SDS alone could denature the polypeptides fast enough to promote some degree of β -tubulin solubilisation, but not as effective as when DTT was present in the buffer (not shown).

It may be concluded that the combination of SDS-denaturing and DTT-reducing effects of the MODIL buffer gave the best solubilising and preserving effect on β -tubulin extraction from tomato seeds. Nevertheless, the very low content of β -tubulin expected to exist in dry (non-imbibed) seed tissues was only extractable when the pH of the MODIL buffer was increased to the limit of its buffering capacity, pH 9.0. Alkaline pHs were shown to be more effective, not only in tubulin gel mobility (Piquot and Lambert, 1988) but also in its extraction when hydrophobically associated with membranes (Beltramò et al., 1994).

The modified procedure allowed us to detect less than 10 ng of tubulin in approximately 5 mg of seed material, *i.e.* 20 embryonic radicle tips. The relatively high β -tubulin content detected in the imbibing radicle tip region expressed strong correlation with the observed high cell cycle activities in this same region (Bino et al., 1992; 1993).

It may be assumed that tubulin from seed coat plus endosperm cells seemed to exist in a stable form not related to cell cycle activation since β -tubulin showed relatively very little or no expression during seed imbibition. The β -tubulin accumulation pattern observed

in radicle tips could only be reproduced in whole seeds when a much higher total protein content was loaded on the gel.

Summarising, we have been able to develop an efficient method for the extraction of β -tubulin from tomato seeds. Its accumulation pattern detected during seed imbibition and germination could be well defined. The method has also been applied successfully to seeds of other species such as neem (*Azadirachta indica* A. Juss) (Sacandé et al., 1997), cabbage (*Brassica oleracea* L.) and pea (*Pisum sativum* T.) (unpublished data). Tubulin accumulation in imbibing seeds appears to play an important role in seed germination and may therefore be considered as a potential marker of the progress of germination (de Castro, et al., 1995).

Experimental procedures

Seed material and imbibition conditions. Seeds of tomato (*Lycopersicon esculentum* M., cv Moneymaker), batch 1992, were dried over a saturated CaCl_2 solution for 2d at 20°C and 32% RH (seed moisture content $6.3 \pm 0.1\%$, fresh weight basis) (International Seed Testing Association, 1993) and stored in a moisture-proof container at 5°C until use. Seed imbibition took place with seeds placed on top of filter paper soaked with distilled water in a sealed Petri dish. Seeds were then kept in an incubator in continuous darkness and 25°C from 0h (dry seeds) up to 48h, when seeds started to germinate, presenting visible radicle protrusion with a approximately 1 mm.

Protein extraction methods. After seeds were allowed to imbibe for the appropriate time, samples consisting of 5 whole seeds, 5 seed coats plus endosperms, 5 whole embryos, or 20 to 30 radicle tips excised from the seeds, were used for protein extraction. The different tissues were put in separate ice-cold Eppendorf reaction assay tubes. After approximately 15 min, i.e. the average time required to isolate the tissue, the tubes were placed in liquid N_2 and subsequently the seed material was ground to a powder. Two protein extraction methods were used to prepare the protein samples, i.e. a) SDS-denaturated total protein extraction and b) water soluble protein extraction. In the former method, we added 160 μl (for whole seed, seed coat plus endosperm and whole embryo) or 40 μl (for radicle tips) of MODIL buffer (62.5 mM Tris-HCl, 2% (w/v) SDS, 15 mg/ml DTT and 7% (v/v) glycerol, pH 6.8 - 9.0), directly to the frozen powder which was further processed as described previously (Chapter 2). In the latter method, in the first step we added 80 μl for whole seeds or 20 μl for radicle tips of Hepes buffer (100 mM Hepes, pH 7.0) directly to the frozen powder. After mixing, the samples were incubated for 10 min at room temperature and centrifuged for 7 min at 17.000 g. In the second step, the supernatants were mixed with an equal amount of two times concentrated MODIL buffer, and boiled for 10 min. To this method we refer to here as HEPES buffer extraction method.

Protein quantitation. Protein concentrations of all supernatants were measured following micro-protein assay procedures (Bio-Rad), modified from the methods described previously (Lowry et al., 1951; Bradford, 1976) using BSA as standard.

Electrophoresis and Electroblothing. Electrophoresis was done on one-dimensional polyacrylamide gels with 20 or 70 μg of proteins samples loaded and separated on a precast 8-18% SDS gradient ExcelGel, according to the

manufacturer (Pharmacia). Three different amounts of pure bovine brain tubulin, 1, 10 and 30 ng, were also loaded as reference samples. After PAGE, proteins were electroblotted for immunodetection of β -tubulin, but with the transfer being done in only 2h, instead of overnight (Chapter 2).

Chemiluminescence Immunodetection of β -tubulin. Immunodetection of β -tubulin was done as described previously (Chapter 2), with some modifications. The incubation period of the immunoblots in blocking solution, as well as the incubation in the mouse monoclonal anti- β -tubulin antibody solution, was increased to 2h. All washing steps were done with large volumes of washing solution (approximately 100 ml for a 100 cm² membrane) to efficiently avoid unspecific binding of the secondary antibody and background on the blots. Films were exposed for different periods of time, varying from 30 sec to a maximum of 8 min. All other immunodetection steps were kept the same.

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Chapter

4

DNA synthesis and microtubular organization in developing tomato (*Lycopersicon esculentum* Mill) seeds are altered in gibberellin- and abscisic acid-deficient mutants

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Summary. Cell cycle events were studied in tomato seed tissues during development, *i.e.* between 21 and 70 days after pollination (DAP), by combining quantitative and cytological analysis of DNA synthesis and β -tubulin accumulation. Nuclear DNA amounts were assessed by flow cytometry and DNA synthesis was analysed by immunohistolabeling of bromodeoxyuridine (BrdU). β -tubulin accumulation was analysed by Western blotting and compared to the organization of the microtubular cytoskeleton by immunohistolabelling. Histodifferentiation of wild type embryos required both DNA synthesis and the microtubular cytoskeleton, whereas expansion growth only depended on the microtubular cytoskeleton, which degraded upon maturation. In the gibberellin-deficient *gib1* mutant, embryo histodifferentiation was uncoupled from expansion growth, indicating the involvement of GA in the synchronisation of both developmental events, possibly by acting on DNA synthesis activity. In the ABA-deficient *sir*^W mutant embryonic DNA synthesis activity was not suppressed upon completion of histodifferentiation and the microtubular cytoskeleton network was re-established during maturation. This suggests that ABA controls the arrest of DNA synthesis and indirectly the re-establishment of the microtubular cytoskeleton and subsequent embryo growth towards viviparous germination. Furthermore, endosperm expansion was characterised by DNA endoreduplication and absence of the

microtubular cytoskeleton, which was however present in the *sir*^{wt} endosperm. Degeneration of the seed coat occurred under a collapse of the DNA ploidy levels and breakdown of microtubules. Induction of full seed germinability, desiccation tolerance and dormancy was related to the completion of embryo histodifferentiation but was independent of the state of the microtubular cytoskeleton during expansion growth.

Introduction

During histodifferentiation of tomato seeds, the embryo is metabolically highly active, and the endosperm is milky. Thereafter, a period of seed growth occurs as the embryo and endosperm expand due to accumulation of reserves, *i.e.* the embryo becomes curled and the endosperm solid. This leads to a rapid increase in dry weight and a decrease in water content, (Berry and Bewley, 1991; Groot et al., 1987). However, the maturation phase occurs within the ripe fruit. Therefore, tomato seeds do not dry as in a typical orthodox pattern, but seed moisture content remains relatively high, at around 50% (fresh weight basis). Despite this, the embryo enters a metabolically quiescent state (Berry and Bewley, 1992; Liu et al., 1997).

Hormones are involved in the processes of seed development and germination (Bewley and Black, 1994). Absciscic acid (ABA) is usually active during embryo maturation (Ackerson, 1984; Black, 1992; Kermode, 1995) and in studies with the ABA-deficient tomato *sir*^w mutant, ABA was shown to be essential for the induction of dormancy and inhibition of precocious germination (Groot et al., 1987; Liu et al., 1997). In contrast to the wild type tomato (cv. Moneymaker), dormancy is not expressed in the *sir*^w mutant, *i.e.* the embryo does not enter a quiescent state during seed maturation. Therefore, seeds of this mutant as well as of ABA-deficient mutants of *Arabidopsis thaliana* (Koornneef et al., 1989; Ooms et al., 1993) and maize (*Zea mays*) (Robichaud et al., 1980) may germinate viviparously during development. Gibberellins (GA) normally act as germination promoters. Germination of mature tomato seeds depends on embryonic GA synthesis (Groot et al., 1987). Mature seeds of the GA-deficient *gib1* mutant of tomato and also of *Arabidopsis* do not germinate unless GA is applied to the imbibition medium (Koornneef and van der Veen, 1980; Groot et al., 1987; Liu et al., 1994). Yet, GA has not been reported to be essential for the germination of immature tomato seeds, which are able to germinate but are not able to produce viable seedlings as early as 25 DAP when removed from the fruit and imbibed in water (Berry and Bewley, 1991).

During seed development, programmed transitions occur from a stage of cell proliferation to that of quiescence in the meristematic tissues of the mature embryonic axis (Buddles et al., 1993). The relationship between these transitions, cell cycle events, and the action of hormones is not yet well understood. In previous studies, we observed an accumulation of β -tubulin in embryos, concomitant with an increase in 4C DNA amounts prior to the completion of germination (de Castro et al., 1995, 1998).

In the present study, DNA synthesis activity and β -tubulin accumulation were analysed both quantitatively and histochemically in developing wild type (WT) and hormone-deficient tomato seeds. By using the GA-deficient *gib1* and ABA-deficient *sir*^w

mutants, the role of GA and ABA in the regulation of histodifferentiation, expansion growth and maturation are demonstrated. Furthermore, by relating DNA synthesis and changes in the microtubular cytoskeleton (microtubular cytoskeleton) configurations to other developmental markers such as dry weight (DW), germinability, desiccation tolerance and dormancy, coordination of these events during tomato seed development is shown.

Results

Characterisation of seed development

Changes in seed morphology were related to the changes in seed DW (Black, 1992). Seed DW increased in all genotypes until 42-49 days after pollination (DAP) (Figure 1), accompanying a decline in moisture content (not shown). A rapid increase in DW was observed in WT and *sir*^w embryos and endosperms plus seed coat between 28 and 35 DAP. During this period, embryos developed from torpedo to curled shape whereas the endosperms developed from milky to firm and the color of the seed coats changed from green to brownish. The DW of *gib1* embryos and endosperm plus seed coats was significantly lower than that of WT and *sir*^w at 28 and 35 DAP ($P < 0.05$). This shows that the increase in DW of *gib1* seeds was slower and became comparable with those of WT and *sir*^w only at 42 DAP when *gib1* embryos also reached the curled shape. The DW of tissues in all genotypes were similar ($P > 0.05$) after 42 DAP. At 56 DAP some *sir*^w seeds started germinating within the fruit.

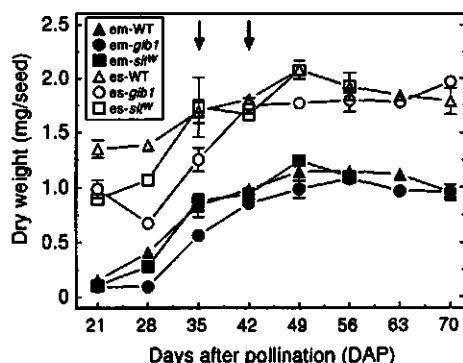


Figure 1. Development of seeds of wild type (WT) tomato and its GA-deficient (*gib1*) and ABA-deficient (*sir*^w) mutants.

The increase in dry weight (\pm SE) of embryos (em) and endosperm plus seed coat (es) in the different genotypes is shown between 21 and 70 DAP. Arrows (thin arrow for WT and *sir*^w; thick arrow for *gib1*) indicate the age at which embryos became curled, endosperm firm and seed coat brownish. Data were not obtained for *sir*^w seeds after 56 DAP because seeds of this genotype had germinated viviparously.

Germinability and ABA content

Germinability of developing seeds was determined to assess desiccation tolerance and dormancy in relation to nuclear DNA synthesis and β -tubulin accumulation. WT seeds could germinate from 21 DAP onwards, germinability increased to 98.7% at 35 DAP to decrease to 4% at 56 DAP (Figure 2a). Seeds which were ungerminable after 35 DAP did not respond to 10 μ M GA₄₊₇, but germinated after a chilling treatment (not shown). After 63 DAP, seeds regained germinability without the requirement of chilling (Figure 2a). *Gib1* seeds only germinated in the presence of 10 μ M GA₄₊₇ (Figure 2a); germination percentages increased from 10% at 35 DAP to 100% at the later stages. The germination percentages of *sit^w* seeds increased from 20% between 21 and 28 DAP to 90% at 35 DAP and reached 100% afterwards (Figure 2a).

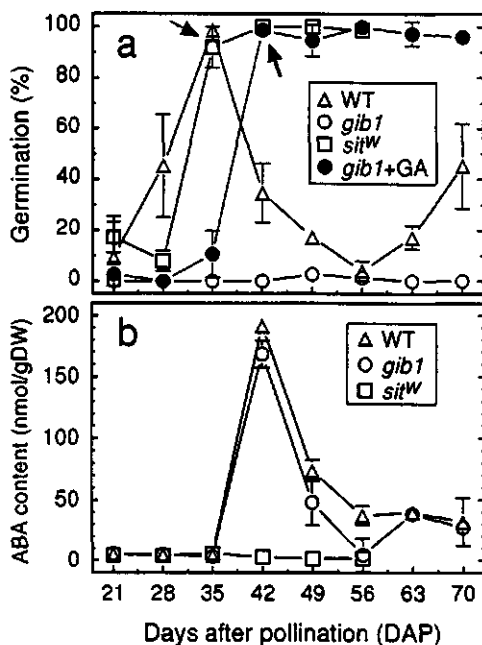
Seeds acquired desiccation tolerance at the same stages at which the highest levels of germinability were reached, e.g. 35 DAP for the WT and *sit^w* and 42 DAP for the *gib1*. Prior to the acquisition of desiccation tolerance, seeds germinated only through hypocotyl protrusion and did not produce viable seedlings (not shown).

Figure 2. Relation between germinability and ABA content of developing seeds.

(a) Germinability (\pm SE) of WT, *gib1* and *sit^w* is shown between 21 and 70 DAP. Germinability of WT seeds was reduced after 35 DAP due to dormancy induction. *Sit^w* seeds did not acquire any level of dormancy. *Gib1* seeds were germinable later than WT and *sit^w* seeds (only in the presence of 10 μ M GA₄₊₇). Arrows (thin arrow for WT and *sit^w*; thick arrow for *gib1*) indicate the age at which seeds became desiccation tolerant.

(b) ABA content (\pm SE) in seeds of all genotypes is shown between 21 and 70 DAP.

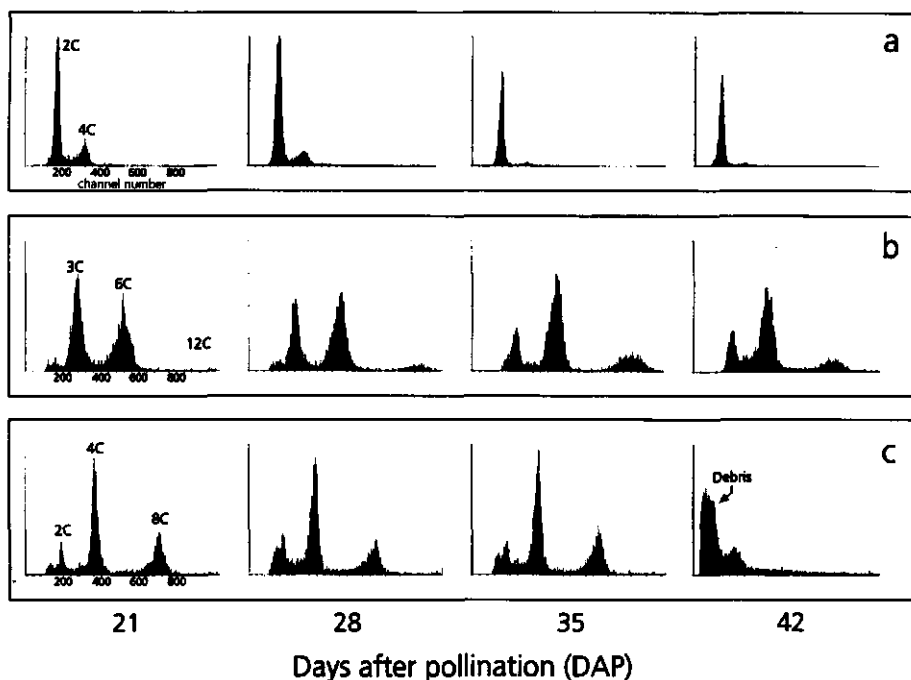
Germination and ABA content data were not obtained for *sit^w* seeds after 56 DAP because seeds of this genotype had germinated viviparously.



Until 35 DAP, all genotypes expressed low levels of ABA, below 10 nmol/gDW (Figure 2b). However, at 42 DAP a peak in ABA content above 150 nmol/gDW was observed in WT and *gib1* seeds, after which the levels declined to levels below 50 nmol/gDW from 56 DAP onwards. ABA content in the *sir*^w seeds was maintained at levels below 10 nmol/gDW throughout the stages of development analysed.

Patterns and distribution of nuclear DNA synthesis

Embryo. Flow cytometric profiles from diploid embryo nuclei of WT (Figure 3a), *gib1* and *sir*^w (not shown) showed one large peak (2C/G1) and a second small peak (4C/G2) with about twice the amount of fluorescence throughout development. The 4C DNA content in WT embryos decreased from 21 to 35 DAP, to remain at a constant low level afterwards (Figure 3a). Although the flow cytometry histograms of *gib1* embryos were similar to those of the WT (not shown), the total number of 4C nuclei at 21 and 28 DAP, expressed as a percentage of the total number of nuclei (2C plus 4C), was significantly lower (Figure 4). For the *sir*^w embryos, the percentage at earlier stages of development was similar to that of the WT but the relative amount of 4C nuclei increased from 35 DAP onwards (Figure 4).



DNA synthesis in the embryonic tissue was detected by immunohistochemical visualisation of bromodeoxyuridine (BrdU) incorporated into actively replicating DNA (S-phase) (Gratzner, 1982). The highest number of BrdU labeled nuclei was found at 21 DAP in embryos of all genotypes (Figure 5a,b), less at 28 DAP (Figure 5c,d) and none from 35 DAP onwards (Figure 5e). BrdU labelling was always distributed throughout the embryonic tissue. No differences were observed among the genotypes with respect to the number of nuclei with BrdU incorporated DNA.

Endosperm. The flow cytometric histograms of the triploid endosperm tissue of WT showed only two peaks at 21 DAP, which related to 3C and 6C DNA amounts, respectively (Figure 3b). As seed development progressed to 28 DAP, the amount of 3C DNA decreased concomitantly with an increase in 6C and appearance of a third peak related to 12C. The patterns and relative DNA amounts of *gib1* and *sir*^W endosperms (not shown) were comparable to those of WT and remained stable from 35 DAP onwards. BrdU immunolabelling of endosperm nuclei of all genotypes showed DNA synthesis throughout the tissue at 21 and 28 DAP (Figure 5f), but not at 35 DAP.

Figure 3. Flow cytometry profiles of DNA contents in seed tissues during development.

- (a) Flow cytometry profiles of WT diploid embryo nuclei showing a decrease in 4C DNA content until 35 DAP. The pattern was constant after 42 DAP.
- (b) Flow cytometry profiles of WT triploid endosperm nuclei showing occurrence of polyploidy (12C) after 28 DAP. The pattern was constant after 42 DAP.
- (c) Flow cytometry profiles of WT diploid seed coat nuclei showing a collapse of the DNA ploidy peaks after 35 DAP. The high amount of debris in the lower channels indicate cell death. There are no data available after 42 DAP because the seed coat died.

The flow cytometry profiles of *gib1* and *sir*^W (not shown) seed tissues were similar to those of WT.

A minimum of 10 000 gated nuclei counts were considered for analysis of all samples. This represented a minimum of 70% of the total embryonic nuclei counts, 40 to 60 % endosperm nuclei counts and a minimum of 75% seed coat nuclei counts. However, approximately only 6600 gated nuclei counts were possible for samples from seed coat tissue at the moment at which the ploidy peaks collapsed. This represented only 13% of the total counts.

Seed coat. Seed coat tissue of all genotypes showed 2C, 4C and 8C DNA peaks (Figure 3c). Ploidy levels and relative DNA amounts in the seed coat of all genotypes did not change between 21 and 35 DAP. However, when the seed coats became fully brown, i.e. at 42 DAP for wild type and *sir*^W, and at 49 DAP for *gib1*, all ploidy peaks collapsed and a high amount of debris appeared in the lower channels (Figure 3c). Thereafter, the seed coats became hard, and isolation of intact nuclei was not possible. DNA synthesis was observed by immunolabelling of BrdU at 21 and 28 DAP, showing that DNA synthesis occurred in nuclei throughout the seed coat tissue (Figure 5f).

β -tubulin and microtubular cytoskeleton

Embryo. β -tubulin accumulation was detected by Western blotting in embryo extracts of developing seeds of the three genotypes (Figure 6). The level of soluble β -tubulin was highest at 21-28 DAP. Thereafter, the levels of β -tubulin decreased and reached undetectable levels in WT embryos after 42 DAP. In *gib1* embryos the levels were still

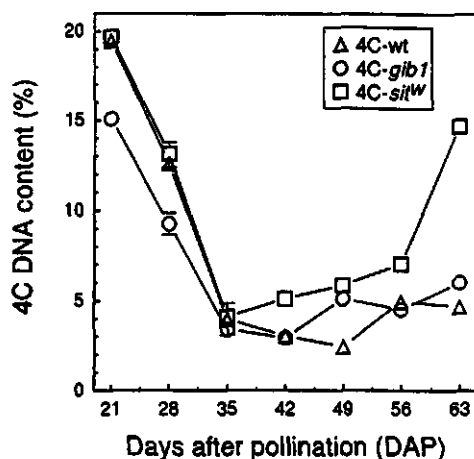


Figure 4. Total 4C DNA contents in developing embryos.

Number of nuclei with 4C DNA contents (\pm SE), expressed as percentage of the total number of nuclei ($2C + 4C$) determined by flow cytometry, are shown for WT, *gib1* and *sir^W* embryos between 21 and 63 DAP. The number of 4C nuclei is lower in *gib1* embryos until 35 DAP, whereas in *sir^W* embryos it increases after 35 DAP.

Figure 5. DNA synthesis in seed tissues during development.

Fluorescence micrographs of longitudinal sections of WT embryos and endosperm plus seed coat are shown. All nuclei are fluorescent in red by staining with propidium iodide (PI). Some nuclei are fluorescent in green showing labelling with fluorescein isothiocyanate (FITC) indicating BrdU incorporation into replicating DNA (S-phase). Bars indicate 50 μ m.

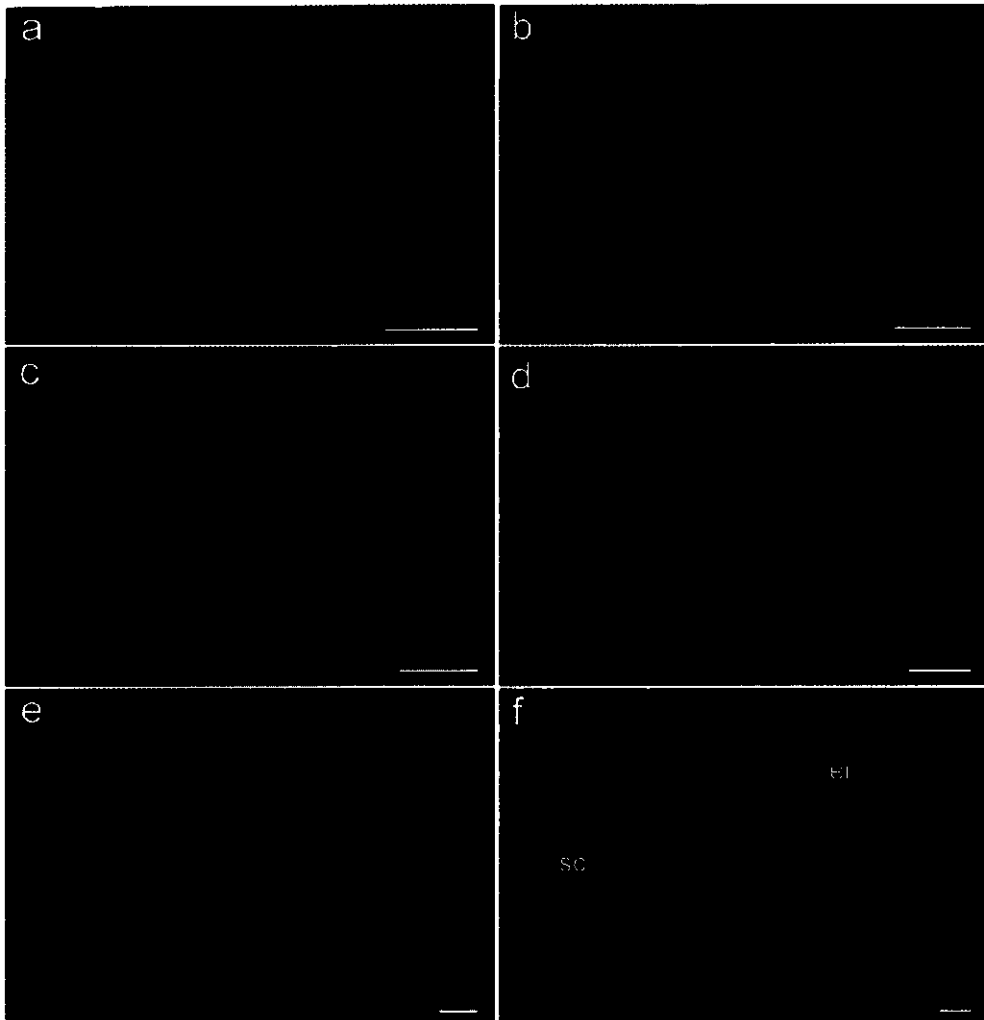
- (a,b) Radicle (a) and shoot meristem plus cotyledonary (b) regions at 21 DAP showing more nuclei labelled with BrdU than at 28 DAP (c,d).
- (c,d) Radicle (c) and shoot meristem plus cotyledonary (d) regions at 28 DAP showing more nuclei labelled with BrdU than at 35 DAP (e).
- (e) Radicle region at 35 DAP showing the absence of BrdU incorporation. The same occurred in the shoot meristem plus cotyledonary region.
- (f) Overview of endosperm (en) plus seed coat (sc) at 21 DAP showing nuclei of both tissues labelled with BrdU.

There appeared to be no specific region within each seed tissue containing higher or lower number of nuclei incorporated with BrdU. From 35 DAP onwards BrdU incorporation was neither observed in embryo nor in endosperm plus seed coat nuclei.

The detection of BrdU incorporation in *gib1* and *sir^W* tissues during seed development was similar to that of WT.

relatively high at 42 DAP and became undetectable only after 49 DAP. In *sir*^w embryos β -tubulin was detected at all stages of development. A transient decline occurred until 49 DAP and increasing levels were detected thereafter.

Immunolabelling of sections revealed that β -tubulin was either present in cytoskeletal arrays or in granules (Figure 7). At 21 and 28 DAP, embryos of all genotypes showed a well developed microtubular cytoskeleton which consisted of both cortical microtubules and mitotic arrays, such as preprophase bands, spindles and phragmoplasts,



distributed along the embryo (Figure 7a,b). The mitotic arrays were more abundant at 21 DAP than at 28 DAP. At 35 DAP, only cortical microtubules were present (Figure 7c,d). At 42 DAP, cortical microtubules were found in the embryonic shoot meristem, hypocotyl and radicle region, but were less abundant in the cotyledons (Figure 7e,f). At 49 DAP, microtubules were virtually absent from the cotyledons of all genotypes whereas in the shoot meristem, hypocotyl and radicle regions, a large amount of fluorescent granules was observed in WT and *gib1* embryos (Figure 7g). By 63 DAP, the microtubular cytoskeleton had been completely broken down. (Figure 7i,j). In contrast, *sit^w* embryos still showed high numbers of microtubules, well organised in cortical arrays in the hypocotyl and radicle region at 49 DAP (Figure 7h), and an elaborate microtubular cytoskeleton was reconstituted as development progressed from 56 to 63 DAP (Figure 7k,l), which was just prior to viviparous germination.

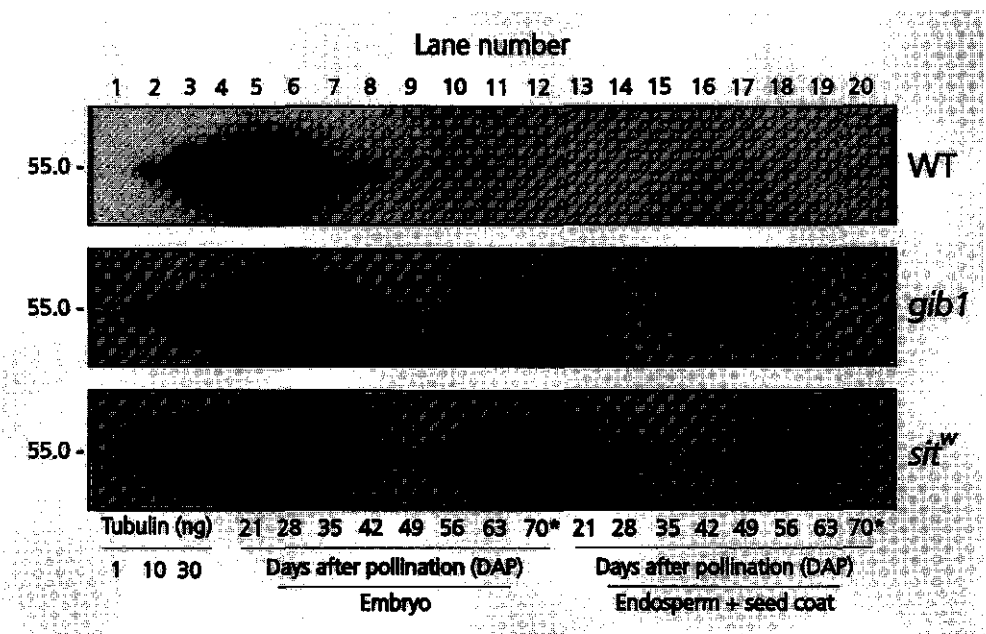


Figure 6. β -Tubulin accumulation in seed tissues during development.
 β -Tubulin levels in embryos (lanes 5-12) and endosperm plus seed coat (lanes 13-20) of WT, *gib1* and *sit^w* seeds are shown between 21 and 70 DAP. Note the slower decline in β -tubulin levels in *gib1* embryos and the transient decline in *sit^w* embryos. Genotypes are indicated on the right hand side. Total protein loaded per lane was 30 μ g. Lanes 1 to 3 were loaded with 1, 10 and 30 ng pure bovine brain tubulin, respectively. Molecular weight (kD) is indicated on the left hand side. The films were exposed for a maximum of 1 min. There was no protein sample from the *sit^w* mutant at 70 DAP (*, lanes 12 and 20) because at that stage all seeds were germinated viviparously. Lane 4 was left empty.

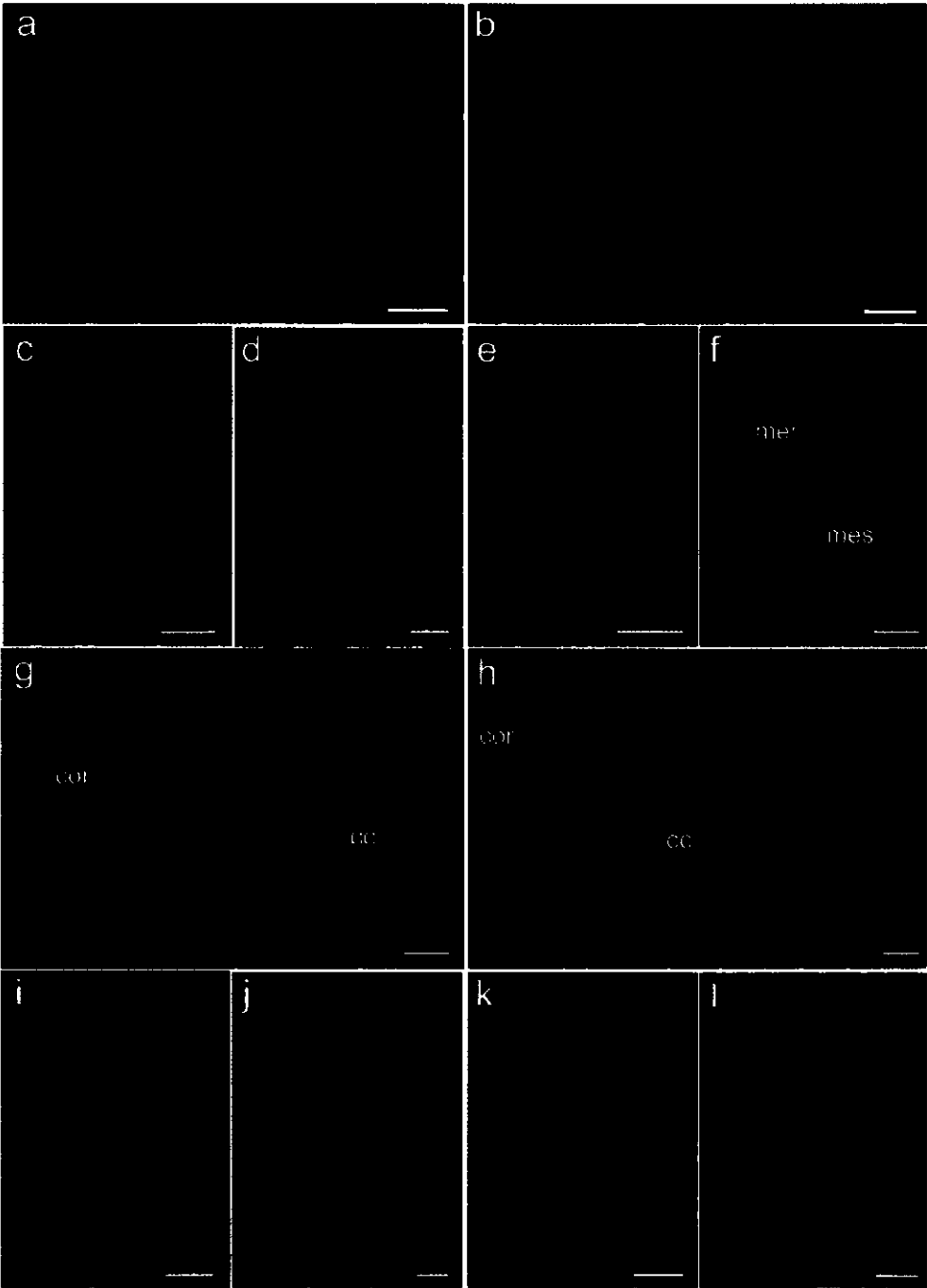
Endosperm and seed coat. β -tubulin was not detected by Western blotting in extracts from WT and *gib1* endosperm plus seed coat throughout the stages of development analysed, and only trace amounts were detected in the *sit^w* mutant (Figure 6). The immunohistochemical analysis of β -tubulin at 21 and 28 DAP showed the presence of microtubules and fluorescent granules in endosperms and seed coat of WT and *gib1* at 21 and 28 DAP. Instead, in the *sit^w* endosperm the microtubular cytoskeleton could still be observed at these stages, but not anymore at 35 DAP (Figure 7m,n).

Discussion

Developmental arrest of the embryo is characterised not only by cessation of DNA synthesis but also by a complete breakdown of the microtubular cytoskeleton

Embryogenesis of tomato seeds was studied by analysis of the mechanism of DNA synthesis coupled with β -tubulin accumulation. The decrease in 4C DNA amounts observed in the embryos of WT, *gib1* and *sit^w* seeds between 21 and 35 DAP is in agreement with previous observations in isolated embryonic radicle tips (Liu et al., 1997). This is an indication of a reduction in DNA synthesis activity, which was confirmed by a decrease in the number of embryonic BrdU labelled nuclei, whereas the high amounts of β -tubulin detected between 21 and 28 DAP corresponded well with an elaborate microtubular cytoskeleton. Apart from cortical microtubules, also mitotic arrays were observed, but only during earlier development and throughout the embryonic tissue. The cessation of DNA synthesis and mitotic events between 28 and 35 DAP in all three genotypes indicated the completion of histodifferentiation.

In WT, the sharp increase in DW between 28 and 35 DAP occurred concomitantly with the growth of the embryos into the final curled shape which still contained an abundant network of cortical microtubules, albeit at lower β -tubulin levels. The lower amounts of β -tubulin at 35 DAP may have been a dilution effect caused by reduced density of the microtubular cytoskeleton as a result of embryo growth. The further decline in β -tubulin amounts after 35 DAP coincided with the depolymerization of microtubules, which became first apparent in the cotyledons, to proceed in time towards the shoot meristem, hypocotyl and radicle regions of the mature embryo. The fluorescent granules probably result from the breakdown of the microtubular cytoskeleton and might precede the degradation of β -tubulin. The transition from 42 to 49 DAP was marked by the attainment of maximum dry weight and minimal moisture content, indicating the achievement of physiological maturity.



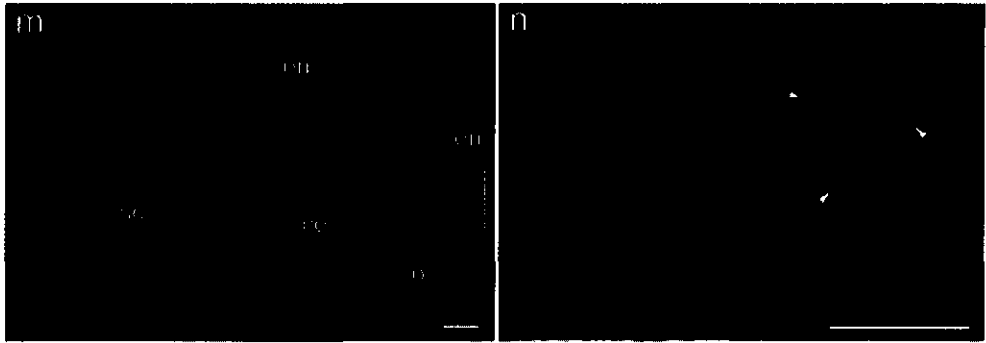


Figure 7. Microtubular cytoskeleton organisation in tomato seeds during development.

Fluorescence micrographs present patterns of longitudinal sections of WT and *sit^m* embryos (a-l, bars indicate 10 µm) and *sit^m* seeds (m,n, bars indicate 100 µm) labelled with anti- β -tubulin/FITC. 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) Radicle region of WT embryo at 21 DAP showing intense labelling of microtubules in cortical and mitotic arrays. Other mitotic arrays, such as preprophase bands and phragmoplasts, were also observed.
- (b) Cotyledonary region of WT embryo at 28 DAP showing intensive microtubular labelling as at 21 DAP (a). However, mitotic arrays were less at 28 DAP.
- (c,d) Radicle tip (c) and cotyledonary (d) regions of WT embryos at 35 DAP showing still intense labelling of microtubules but only in cortical arrays. Note the criss-cross pattern of cortical microtubules in the meristematic root tip (c), and presence of cortical microtubules accompanying the differentiating vascular cells (d).
- (e,f) Radicle (e) and cotyledonary (f) regions of WT embryos at 42 DAP showing less labelling of microtubules relative to 35 DAP (c,d). Albeit in lower amount, cortical microtubules are still present in the radicle (e) region. The cotyledons (f) contain even less microtubules than the radicle. Microtubules are present mainly in the meristeme (mer) and absent in the mesophyll (mes).
- (g) Overview of the cortex and stelar region of the radicle of WT embryos at 49 DAP, showing microtubules still present in the stele (central cylinder, cc) and fluorescent granules especially in the cortex (cor) near the central cylinder.
- (h) Overview of the cortex (cor) and central cylinder (cc) region in the radicle of *sit^m* embryos at 49 DAP (same as for WT in (g)) showing a marked persistence of microtubules and absence of fluorescent granules.
- (i,j) Radicle tip (i) and cortex plus central cylinder (j) regions in the radicle of WT embryos at 63 DAP. Note the presence of only remnants of microtubules in the radicle tip (i). Even less microtubules are present in the central cylinder (j) than at 49 DAP (h), but again fluorescent granules were present in the cortex region above the radicle tip (j).
- (k,l) Radicle tip (k) and cortex plus central cylinder (l) regions in the radicle of *sit^m* embryos at 63 DAP (same as for WT in (i,j)). Contrary to the WT (i,j), there is an overall increase in presence of cortical microtubules just prior to the occurrence of viviparous germination.
- (m) Overview of *sit^m* seed at 21 DAP showing an intense labelling of the microtubular cytoskeleton in the cotyledons (co), some in the outer layers of the seed coat (sc), and very low amount of cortical microtubules in the endosperm (en), only in (n). Note how abundant the microtubular cytoskeleton is at this stage in the embryo relative to the other seed tissues.
- (n) Close view of the region between the right side cotyledon and endosperm which is within the rectangle in (m) showing the microtubular cytoskeleton delineating the *sit^m* endosperm cells (arrows), at 21 DAP. At this same stage, no microtubules were detected in the WT and *gib1* endosperms (not shown).

The microtubular organisation pattern in developing *gib1* seeds was in general similar to that of the WT seeds, although it persisted for longer time in the embryo (not shown).

Apparently, the cessation of developmental growth started in the cotyledonary and shoot apical region of the embryo and preceded that of the hypocotyl and radicle basal region. Once physiological maturity has been achieved and seeds enter the maturation phase, expansion growth is arrested leading to a cessation of the structural rearrangements (Clayton, 1985). Therefore, at this stage the microtubular cytoskeleton was no longer required and the cell cycle arrested, despite further changes in germinability (see below).

Histodifferentiation is uncoupled from expansion growth in gib1 embryos

Gib1 seeds developed at a slower rate than WT and *sit^u* seeds. This was reflected in the slower decline in β -tubulin levels, in the later achievement of the curled embryo shape and in a slower increase in DW. The slower development might have resulted from the lower DNA synthesis activity until 35 DAP, as detected by flow cytometry. Yet, the arrest of DNA synthesis and cell division occurred simultaneously between 28 and 35 DAP in all genotypes. This suggests that in the *gib1* embryos, histodifferentiation was uncoupled from expansion growth. It suggests that cell division is restricted to histodifferentiation in the completion of embryogenesis *sensu strictu* (Monnier, 1988), whereas growth is a function of cell and tissue expansion, although both events may occur concomitantly. In WT and *sit^u* seeds, there was an overlap between both events. The development of *Arabidopsis* embryos into the curved ('walking-stick') shape is considered to be the result of cell division and expansion (Mansfield and Briarty, 1992; West and Harada, 1993). As in tomato, this might also be a result of an overlap between both events. The uncoupling of cell division and expansion in *gib1* embryogenesis delayed the increase in DW and embryo growth. This suggests that GA is involved in the synchronization of cell differentiation and expansion, possibly through its action on DNA synthesis activity. Recently, Swain et al. (1998) reported that GA-deficient pea (*Pisum sativum*) seeds grew slower than WT seeds. They concluded that the slower growth was only indirectly influenced by the distribution of assimilates to the developing pea seeds and that the role of GA was to promote some process(es) directed to embryo growth.

DNA synthesis and re-establishment of the MC is not suppressed in sit^u seeds during maturation

The relative number of 4C DNA nuclei was higher in the *sit^u* embryos than in WT and *gib1* after 35 DAP, with a sharp increase at 63 DAP. This indicated an increase in DNA

synthesis activity after the completion of histodifferentiation. *Sir*^{wt} embryos showed an increase in β -tubulin levels after 49 DAP and as a clear result the re-establishment of the microtubular cytoskeleton network. Apparently, the ABA deficiency prevented the arrest of cell cycle activities. This, taken together with the moist environment of the seeds and the osmotic conditions inside the fruit (Liu et al., 1996) may have lead to the re-activation of both DNA synthesis and microtubule accumulation, precluding the occurrence of viviparous germination after 56 DAP. Indeed, nuclear 4C DNA amounts increase and β -tubulin accumulates in embryos during germination when mature tomato seeds are imbibed in water but do not show radicle protrusion yet (de Castro et al., 1995; 1998). However, viviparous germination is a slow process, with a duration of over two weeks, *i.e.* from 49 to 63 DAP, whereas germination in water takes only 48 to 72h (Liu et al., 1994; de Castro et al., 1995; 1998). The viviparous process may then result in cell cycles with very slow DNA synthesis. This may have been the reason why DNA synthesis could not be detected in *sir*^{wt} embryos through immunohistolabelling of BrdU from 35 DAP onwards. Apparently, the presence of ABA was essential for the suppression of DNA synthesis activity and subsequent re-establishment of the microtubular cytoskeleton during the transition to maturity, *i.e.* attainment of maximum DW between 42 to 49 DAP. Consequently, dormancy was absent in the *sir*^{wt} seeds. The absence of the ABA peak in *sir*^{wt} seeds, at 42 DAP, correlated with the increase in embryonic DNA synthesis. It is tempting to speculate that the ABA action is required to block DNA synthesis.

Induction of germinability, desiccation tolerance and dormancy requires completion of histodifferentiation, but is independent of the MC organization during embryo growth

Although unable to produce viable seedlings, some WT and *sir*^{wt} seeds could germinate during histodifferentiation, *i.e.* between 21 and 28 DAP, when torpedo shaped embryos were not yet fully differentiated and still underwent intense cell cycle activity, at low dry weight and high moisture content. Apparently, the switch from a developmental mode to a germinative mode does not require the completion of histodifferentiation. Berry and Bewley (1991) concluded that the inability of producing viable seedlings at these early stages of development was due to the lack of sufficient reserves in the still liquid tomato seed endosperm, because young embryos isolated from these seeds grew into viable seedlings when placed on a nutrient medium.

The drop in germinability of WT seeds observed immediately after 35 DAP resulted from the induction of dormancy as seeds did germinate after a chilling treatment (Karssen, 1995). The acquisition of dormancy may have been caused by the peak in the seed ABA

content at 42 DAP, which in tomato is known to result mainly from ABA produced in the embryonic tissue (Berry and Bewley, 1991; Hoche et al. 1991). The involvement of ABA in inducing dormancy has been confirmed in a number of species (Bewley and Black, 1994, Black, 1992). However, chilling did not have any promoting effect on the germinability of *gib1* seeds, in which ABA peaked at 42 DAP as well. On the other hand, dormant WT seeds were not sensitive to exogenous GA and were only able to germinate after chilling. These results suggest that not only ABA but also GA may be required in seed tissues for dormancy induction during tomato seed development. This contrasts earlier conclusions that a GA-ABA-balance is not needed for dormancy induction (Karssen and Groot, 1987; Karssen and Lacka, 1986).

Furthermore, the acquisition of desiccation tolerance in tomato seeds, from 35 DAP onwards, appeared to have no relation with the peak in ABA content, unless the sensitivity to ABA was very high, as shown in studies with ABA-insensitive mutants of *Arabidopsis* (Koornneef et al., 1989). ABA is often linked to acquisition of desiccation tolerance (Bewley and Black, 1994; Kermode, 1995 and Vertucci and Farrant, 1995). In conclusion, induction of germinability, desiccation tolerance or dormancy requires the completion of histodifferentiation, but appears to have no relationship with the presence and organization of the microtubular cytoskeleton.

Endosperm does not require maintenance of cell architecture during expansion growth

The synthesis of DNA detected in the triploid endosperm cells at 21 and 28 DAP by immunohistolabelling of incorporated BrdU, probably occurred as DNA endoreduplication, as could be deduced from the polyploid flow cytometric profiles. The overall absence of karyokinesis and cytokinesis after 21 DAP may have enabled DNA endoreduplication in endosperms of all genotypes (Murray and Hunt, 1993). Endoreduplication of DNA is known to occur in maize endosperm (Doehlert et al., 1994), as well as in many other species, after the cessation of mitotic events and differentiation (D'Amato, 1984), correlating with cell expansion and/or initiation of reserve accumulation (Lopes and Larkins, 1993; DeMason, 1997). This was observed also in the present study. β -tubulin was not detected in WT and *gib1* endosperms throughout the stages of development analysed, correlating with the absence of microtubular cytoskeleton. However, trace amounts of β -tubulin as well as "partial" microtubular cytoskeleton were detected in the ABA-deficient *sit*⁺ mutant at the earlier stages of development, which corresponded to the period of accumulation of reserves. This suggests the involvement of ABA in the endospermic microtubular cytoskeleton organization. However, the absence of an integrated cortical

microtubular cytoskeleton in WT and *gib1* endosperms implies that the expansion of the tomato endosperm and development into a firm consistency might occur randomly, apparently with no structural control, limited by the seed coat tissues only.

The breakdown of DNA and microtubular cytoskeleton in the seed coat precede cell death upon maturation

As in the endosperm, the lack of mitotic events and/or microtubules in the diploid seed coat tissue from 21 DAP onwards, enabled DNA synthesis through endoreduplication of DNA, as detected by flow cytometry and immunolabelling of BrdU between 21 and 28 DAP. In the endosperm, however, the flow cytometric profiles showed that all DNA ploidy peaks were stable from 35 DAP onwards, whereas in the seed coat the peaks collapsed when the tissue changed color and degenerated. The fragmentation of DNA resulted in high amounts of debris in the lower, subdiploid flow cytometric channels. This was also observed in apoptotic cells of *Nicotiana* protoplasts (O'Brien et al., 1998). In the tomato seed coat, fragmentation of DNA occurred concomitantly with the breakdown of the microtubular cytoskeleton. Breakdown of microtubules is known as a characteristic feature of cell death by apoptosis (Endersen et al., 1995; Pittman et al., 1994). Programmed cell death may also be the case in the seed coat tissue, in which the whole tissue dies after the hairs have been formed and the tissue is modeled into a protective barrier around the embryo and endosperm (Pennel and Lamb, 1997).

A combined quantitative and immunocytochemical analysis of DNA synthesis and tubulin accumulation provided means for a better understanding of the relation between seed development and cell cycle-related events. This approach is of importance for the identification of genes, which control developmental processes that occur at the level of seed formation. Based on the level of DNA synthesis and tubulin accumulation we are now able to divide the seed developmental program in various clearly defined steps. This may help in the analysis and isolation of the genes, which are regulating the developmental switches. A similar approach can be used to define the time sequence of DNA synthesis and tubulin accumulation during seed germination (Chapter 5). This will give insights on how cell cycle related processes are reactivated upon imbibition at the cellular level.

Experimental procedures

Plant Material. Tomato plants from WT cultivar Moneymaker and its GA- and ABA-deficient mutants, *gib1* and *sir⁺*, were grown simultaneously in a greenhouse under natural daylight at 25°C/20°C day/night average

temperatures. Plants of the *gib1* mutant were sprayed once a week with a 10 μ M GA₄₊₇ (Plant Protection LTD., Surrey, UK) solution to allow normal flowering, whereas plants of the *sir*^w mutant were sprayed once a week with a 10 μ M ABA (ACROS, Geel, B) solution to reduce wilting (Groot et al., 1987, Liu et al., 1996). Sufficient flowers of the three genotypes were self-pollinated, allowing a minimum of 5 WT and 15 *gib1* or *sir*^w fruits to be harvested at the same time at different DAP. After excision, all tissues were either immediately used for analysis or frozen in liquid nitrogen and stored at -70°C. Water content (fresh weight basis) of 3 replicates of 20 embryos or endosperms plus seed coat was determined by measuring the weights of the fresh tissues and the tissues after drying at 130°C for 1h.

Germination. Germination analysis was conducted on 3 replicates of 25 seeds placed on top of 2 layers of filter paper soaked with 6 ml distilled water or 10 μ M GA₄₊₇ or 10 μ M ABA, at 25 \pm 1°C in darkness. Germinability was expressed as the percentage of total seeds that germinated and scored daily for 7 days. Normal seedlings were scored after 7d of imbibition. The effect of a chilling treatment was tested on 42-56 DAP non-germinated WT seeds at 10°C for 24h and then bringing them back to 25°C for another 7d.

ABA Extraction and Quantification. The ABA extraction from whole seeds was based on the procedure of Raikhel et al. (1987) modified by Berry and Bewley (1992). Two replicates of 25 fresh seeds were analyzed and ABA was quantified using a Phytodetek ABA immunoassay kit, based on a monoclonal antibody ELISA method (Sigma/Idetek, St. Louis, MO, USA), using (+/-)-ABA as a standard.

Flow Cytometry. Depending on the stage of development, two replicates of 10 (21 to 28 DAP) or 5 seeds (35 DAP onwards) were used for flow cytometry measurements. Embryos, endosperms or seed coats were freshly excised. Extraction of nuclei, flow cytometry and measurements of DNA contents were performed as previously reported (Sacandé et al., 1997).

Detection of β -tubulin. Total protein extraction from embryo or endosperm plus seed coat tissues, as well as protein concentration determinations, electrophoresis, Western blotting and immunochemiluminescence detection of β -tubulin were conducted as described previously (Chapters 2 and 3).

Immunohistochemical detection of BrdU and β -tubulin. A 3h pulse length BrdU (Amersham, Buckinghamshire, UK) incorporation was found to be the optimal to detect DNA synthesis when isolated embryos or sectioned seeds were immersed in BrdU (1:500 v/v) solution, at 25°C in darkness. The cytotoxicity (Rös and Wernicke, 1991) and induction of germination by various pulse lengths in BrdU solution were assessed in each sample and for each treatment, 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 throughout development. Immunohistolabelling of β -tubulin was conducted on material not incubated with BrdU, because BrdU incubation proved cytotoxic (Rös and Wernicke, 1991) as it was harmful to the microtubular cytoskeleton, especially in the young 21 to 35 DAP embryos. After isolation for β -tubulin analysis or after incorporation with BrdU, the sampled materials were fixed, embedded in butylmethylmetacrylate, sectioned, and affixed on slides according to Baskin et al. (1992). In the BrdU and β -tubulin immunolabeling, respectively, the use of anti-BrdU (1:1 v/v) and anti- β -tubulin (1:200 v/v) as first antibodies (Amersham, Buckinghamshire, UK), FITC conjugated goat anti-mouse (1:200 v/v) as second antibody (Amersham, Buckinghamshire, UK), as well as microscopy were according to Xu et al. (1998). Nuclear DNA was counterstained with 1 mg/ml propidium iodide (PI) (Molecular probes, Eugene, OR, USA) in BrdU incorporated sections. In all sections, omission of the first antibody and application of preimmune serum served as controls and showed no fluorescence.

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Chapter

5

DNA synthesis and appearance of microtubular cytoskeleton during tomato (*Lycopersicon esculentum* Mill.) seed imbibition results in cell division prior to radicle protrusion

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Summary. Cell cycle events were studied in embryos of tomato (*Lycopersicon esculentum* Mill, cv. Moneymaker) seeds during imbibition in water and osmotic priming by combining quantitative and cytological analysis of DNA synthesis and β -tubulin accumulation. Nuclear DNA amounts were assessed by flow cytometry, and DNA synthesis was analysed by immunohistolabeling of bromodeoxyuridine (BrdU). β -tubulin accumulation was analysed by Western blotting and related to the organisation of the microtubular cytoskeleton, visualised by immunohistolabeling. Most embryonic nuclei of dry untreated control seeds were arrested in the G₁ phase of the cell cycle. This indicated absence of DNA synthesis, *i.e.* S-phase, as confirmed by the absence of BrdU incorporation. In addition, β -tubulin was not detected on Western blots and microtubules were not present. Upon imbibition in water and completion of germination, DNA synthesis was activated in the radicle tip, spreading towards the cotyledons. This resulted in an increase in the number of nuclei in G₂. Concomitantly, β -tubulin accumulated and, as a result, β -tubulin assembled into increasing numbers of microtubules forming elaborate cytoskeleton networks, which also advanced from the radicle tip towards the cotyledons. Thus, DNA synthesis and the formation of the microtubular cytoskeleton were activated during imbibition, leading to embryonic cell expansion and division, which preceded the protrusion of the radicle through the seed coat. The activation of DNA synthesis and formation of the microtubular

cytoskeleton was also observed throughout the embryo when seeds were osmoprimed, but essentially without mitosis, *i.e.* the cell cycle was arrested in the G₂ phase. The activation and progression of the cell cycle correlated with the enhanced germination performance of primed seeds as compared with control seeds.

Introduction

Embryos of maturing seeds exhibit a programmed transition of a stage of cell proliferation to that of quiescence (Buddles et al. 1993). The transition in maturing tomato seeds is characterised by the arrest of most embryonic cells in the G_1 phase of the cell cycle (Liu et al. 1997). In chapter 4, the pattern of DNA synthesis activity was analyzed during tomato seed development, through flow cytometry and immunohistochemical detection of Bromodeoxyuridine (BrdU) incorporation, and related to the accumulation of β -tubulin and configuration of the microtubular cytoskeleton. That study showed that DNA synthesis accompanied mitotic events throughout the embryonic tissue during the phase of histodifferentiation. Further growth of the embryo was merely through cell expansion and was characterised by the breakdown of the microtubular cytoskeleton and decreasing levels of β -tubulin.

The transition from quiescence to that of cell proliferation occurs during imbibition and germination of tomato seeds, and is characterized by increasing numbers of radicle tip cells that are in the G_2 phase of the cell cycle (Bino et al. 1992). This increase in cells with 4C DNA levels is accompanied by accumulation of β -tubulin, not only during seed imbibition, *i.e.* prior to radicle protrusion, but also during seed priming in PEG-6000 (Chapters 2 and 3). Both the relative number of cells in G_2 and the level of β -tubulin observed at the end of the priming treatment were correlated with the effectiveness of the priming treatment (Chapter 2).

During imbibition of tomato seeds, DNA synthesis and β -tubulin accumulation apparently concentrated in the embryonic radicle tip. Despite all the recent efforts, however, the relation between DNA replication and β -tubulin accumulation during seed germination is not yet understood. Questions as to whether the increase in 4C DNA is causally related to the accumulation of β -tubulin, whether it is restricted to the radicle tip, and whether it leads to cell division prior to radicle protrusion still need to be clarified. We address these questions through an immunohistochemical analysis of DNA synthesis activity and organization of the microtubular cytoskeleton and relate them to the quantitative analysis of DNA replication and β -tubulin accumulation.

Results

Germination

Germinability of control and primed seeds was determined to assess its relation with embryonic nuclear DNA synthesis and β -tubulin accumulation. Most primed seeds germinated within 24h of imbibition and control seeds within 48h (Figure 1). Germination was completed within 48h and 72h for primed and control seeds, respectively. Thus, primed seeds germinated approximately 24h earlier than the untreated ones.

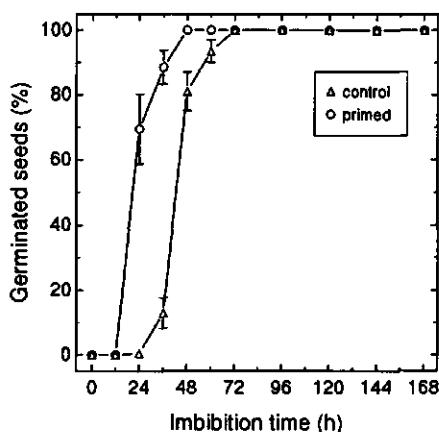


Figure 1. Germination of control and primed tomato seeds.

Priming was performed in -1MPa PEG-6000 at 25°C for 7d. After the pretreatment, seeds were dried back to their equilibrium moisture content prior to imbibition in water. Germination (\pm SE) was scored for 7d.

Amounts and distribution of nuclear DNA synthesis

Flow cytometric histograms from embryonic nuclei of dry control seeds showed one large peak corresponding to the 2C DNA content (G_1 phase of the cell cycle), and a second smaller peak with about twice the amount of fluorescence, corresponding to nuclei with replicated 4C DNA content (G_2 phase) (not shown). During imbibition in water, the relative portion of 4C nuclei significantly increased (Figure 2), indicating nuclear DNA replication activity. An increase in the frequency of embryonic 4C nuclei was also observed after 7d of priming in PEG. The frequency of 4C nuclei in dried primed (7%) seeds was higher ($P < 0.05$) compared to that of dry control

seeds (3%), and comparable ($P > 0.05$) to that of control seeds (8%) imbibed for 24h (Figure 2). Upon renewed imbibition in water, the frequency of 4C nuclei increased further, as in control seeds. However, in primed seeds the increase was faster. At 24h of imbibition, the number of 4C nuclei in ungerminated primed seeds was comparable ($P > 0.05$) to that in ungerminated control seeds at 48h. Furthermore, the frequency of 4C nuclei in

germinated primed seeds (41%) was higher ($P < 0.05$) compared to that in control seeds (28%), which germinated after 48h (not shown).

DNA replication, as detected by flow cytometry, was compared with the analysis of DNA synthesis visualized by immunohistochemical detection of bromodeoxyuridine (BrdU) incorporated into actively replicating DNA (Ellward and Dörner 1985; Gratzner 1982)(Figure 3). BrdU incorporation was not observed in embryonic nuclei from dry control seeds (Figure 3a), but was observed in increasing levels from 12h of imbibition (Figure 3b-d) onwards. Initially, most of the BrdU labeling occurred in the radicle tip. However, at 48h BrdU labeling was also observed in the hypocotyl, shoot meristem and cotyledons (Figure 3d-g and h). BrdU labeling was also detected in embryonic nuclei of primed seeds, at levels, which were similar before and after re-drying (Figure 3h). As in control seeds, most BrdU labeling in embryos of primed seeds occurred in nuclei of the radicle tip region but in lower numbers than in embryo radicle tips of 12h imbibed control

seeds (Figure 3b and h). Upon renewed imbibition in water, the number of labeled nuclei in primed embryos increased until completion of germination, in a pattern similar to that observed in embryos of control seeds (not shown). These observations were based on replicate samples, which gave closely similar data.

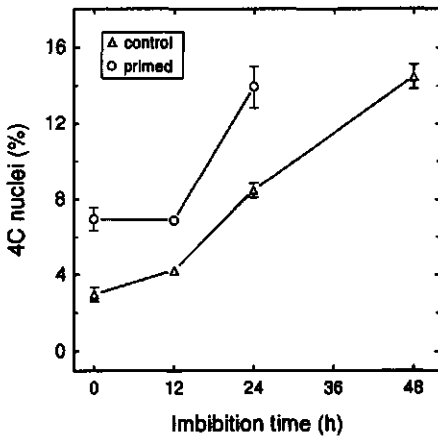


Figure 2. Nuclear 4C DNA in embryos of tomato seeds during imbibition.

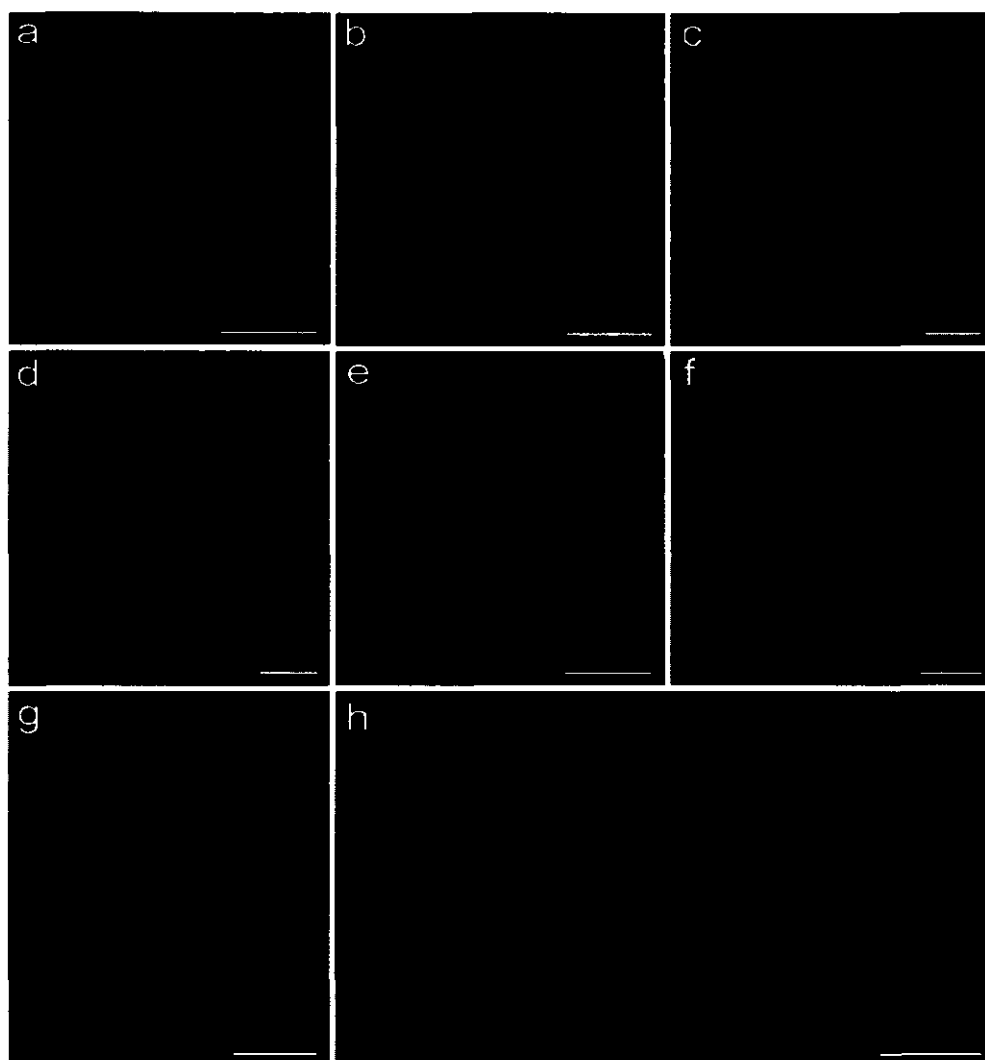
Frequency of nuclei with 4C DNA contents (\pm SE), expressed as percentage of the total number of nuclei (2C+4C) from embryos of control or primed seeds during imbibition. With all samples, a minimum of 10 000 nuclei was analysed.

β -tubulin and microtubular cytoskeleton

The level of soluble β -tubulin in embryos of control seeds increased during imbibition. β -tubulin was not detected in embryos of dry control seeds, but increasing levels were detected from 12h of imbibition onwards, being highest at 48h in embryos of germinating seeds (Figure 4). β -tubulin had accumulated in embryos also after

the osmotic priming of the seeds. However, the level of soluble β -tubulin in primed embryos after seed re-drying appeared lower than before drying. During renewed imbibition of the primed seeds in water, β -tubulin levels increased further reaching maximum levels at 24h of imbibition in embryos of germinating seeds (Figure 4).

The pattern of β -tubulin accumulation, detectable on Western blots, was compared with its microtubular pattern detected by immunohistochemistry (Figure 5). Analysis of



sectioned embryos from dry seeds, or seeds isolated during imbibition, showed that labeling of β -tubulin was found either in the form of fluorescent granules or assembled in microtubular cytoskeletal arrays.

Typically, embryos of dry control seeds did not contain a microtubular cytoskeleton but contained fluorescent granules, present in cells of the stele in the hypocotyl (not shown) and meristele in the cotyledons (Figure 5a-c). However, during seed imbibition, β -tubulin labeling showed an increasing appearance of microtubules, while the fluorescent granules disappeared. Microtubules appeared at 12h of imbibition, first in the radicle tip region where the formation of an integrated cortical microtubular cytoskeleton was observed (Figure 5d-f). From 12h of imbibition onwards, the appearance of the cortical microtubular configurations advanced towards the hypocotyl, the shoot meristem and finally towards the cotyledons (Figure 5d-l). The accumulation of microtubules in the hypocotyl and cotyledons was initiated in the cells of the central cylinder (stele) and meristele, respectively, where fluorescent granules were observed (Figure 5f, i and l). Mitotic microtubular arrays were also observed. They first appeared in the radicle tip region after 24h of imbibition, and functioned in cell division before the radicle protruded (Figure 5g). Cell divisions were confirmed by counterstaining of the nuclear DNA with PI (not shown). When the radicle protruded, at 48h of imbibition, the cortical cytoskeleton was apparent in cells throughout the embryonic tissues, while at the same moment the number of mitotic arrays and divisions had increased in the radicle tip and progressed also towards the hypocotyl (Figure 5j-l).

Figure 3. *Development of DNA synthesis in tomato embryos during seed germination.*

Fluorescent micrographs present patterns of longitudinal sections of embryos from untreated control seeds during germination (a-g), as well as of embryos from dried primed seeds (h). Most nuclei show red fluorescence as a result of staining with propidium iodide (PI). Nuclei which show green fluorescence are labelled with fluorescein isothiocyanate (FITC) indicative for BrdU incorporation into actively replicating DNA (S-phase). Bars indicate 100 μ m (a-e, g, h) or 25 μ m (f).

- (a) Embryonic radicle tip region of dry control seeds showing the absence of BrdU incorporation after a 3h pulse labeling indicating absence of DNA synthesis.
- (b) Radicle tip of control seeds imbibed for 12h and pulse labelled for 3h showing nuclei labelled with BrdU indicating the start of nuclear DNA synthesis in the radicle tip.
- (c) Radicle tip of control seeds imbibed for 24h. Note that there are more nuclei in the radicle labelled with BrdU than at 12h (b), indicating higher DNA synthesis activity in the radicle at this stage.
- (d-g) Radicle tip (d), shoot meristem (e,f) and cotyledons (g) of germinated control seeds at 48h of imbibition. At this stage, DNA synthesis activity in the radicle tip was highest, and had also started in the shoot meristem and cotyledons.

In the close view of the shoot meristem (f) unsynchronized cells containing nuclei with various levels of BrdU/FITC labeling, showing early and late stages of S-phase.

Although with less labeling, the labeling pattern in embryos of ungerminated seeds at 48h of imbibition was comparable to that of germinated seeds.

- (h) Embryonic radicle tip region of dried primed seeds. DNA synthesis was induced during priming as BrdU labelled nuclei were detected in embryos after 7d of seed osmopriming (not shown) as well as in embryos of dried primed seeds (h). In part, this suggests that the actively replicating nuclear DNA was not affected by drying.

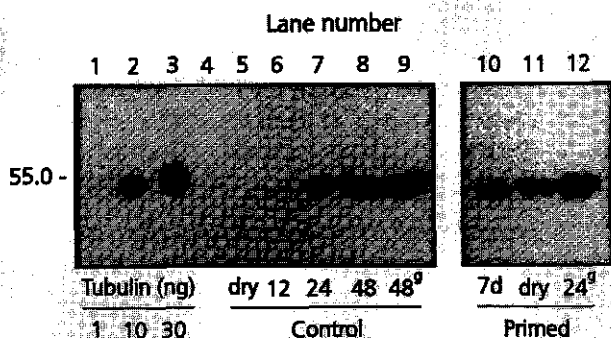


Figure 4. β -Tubulin accumulation in embryos of tomato seed during germination.

β -Tubulin levels are shown for embryos of untreated control seeds during imbibition in water (lanes 5-9), as well as for those of seeds after 7d of osmopriming, after re-drying, and during subsequent imbibition in water (lanes 10-12).

Total protein loaded per lane was 30 μ g. Lanes 1 to 3 were loaded with 1, 10 and 30 ng pure bovine brain tubulin, respectively. Molecular weight (kD) is indicated on the left hand side. The films were exposed for a maximum of 1 min.

(^a) Embryos of seeds that had germinated, i.e. approximately with 1mm radicle protrusion.

Microtubules accumulated in embryos also during seed priming. After 7d of osmotic priming, the radicle tip, hypocotyl and shoot meristem contained cells with a clear and well established cortical microtubular network, whereas in the cotyledons most microtubules were found in the meristele cells (Figure 5m-o). Mitotic arrays were, however, not observed in the primed embryos. The distribution pattern of cortical microtubules in primed embryos was comparable before and after re-drying. However, a significant number of small fluorescent granules was observed after re-drying, not only in the radicle tip region, but also in cells of the cortex, in the hypocotyl, and shoot meristem. These are the same regions where the cytoskeleton had mostly accumulated during priming (Figure 5p, q and t). The fluorescent granules observed in the cotyledons were observed since seeds were in a dry control state (Figure 5c, o and r), but those in the radicle appeared only when seeds were re-dried after priming. During subsequent imbibition in water, the granules disappeared while the microtubular cytoskeleton reconstituted and appeared

throughout all embryonic regions (not shown), in a pattern comparable to control seeds. However, mitotic arrays started to appear 12h earlier, also resulting in cell divisions prior to the onset of radicle protrusion. At 24h of imbibition, the protruded radicles of the primed seeds contained a relatively larger number of mitotic arrays compared to the protruded radicles from control seeds at 48h of imbibition (not shown).

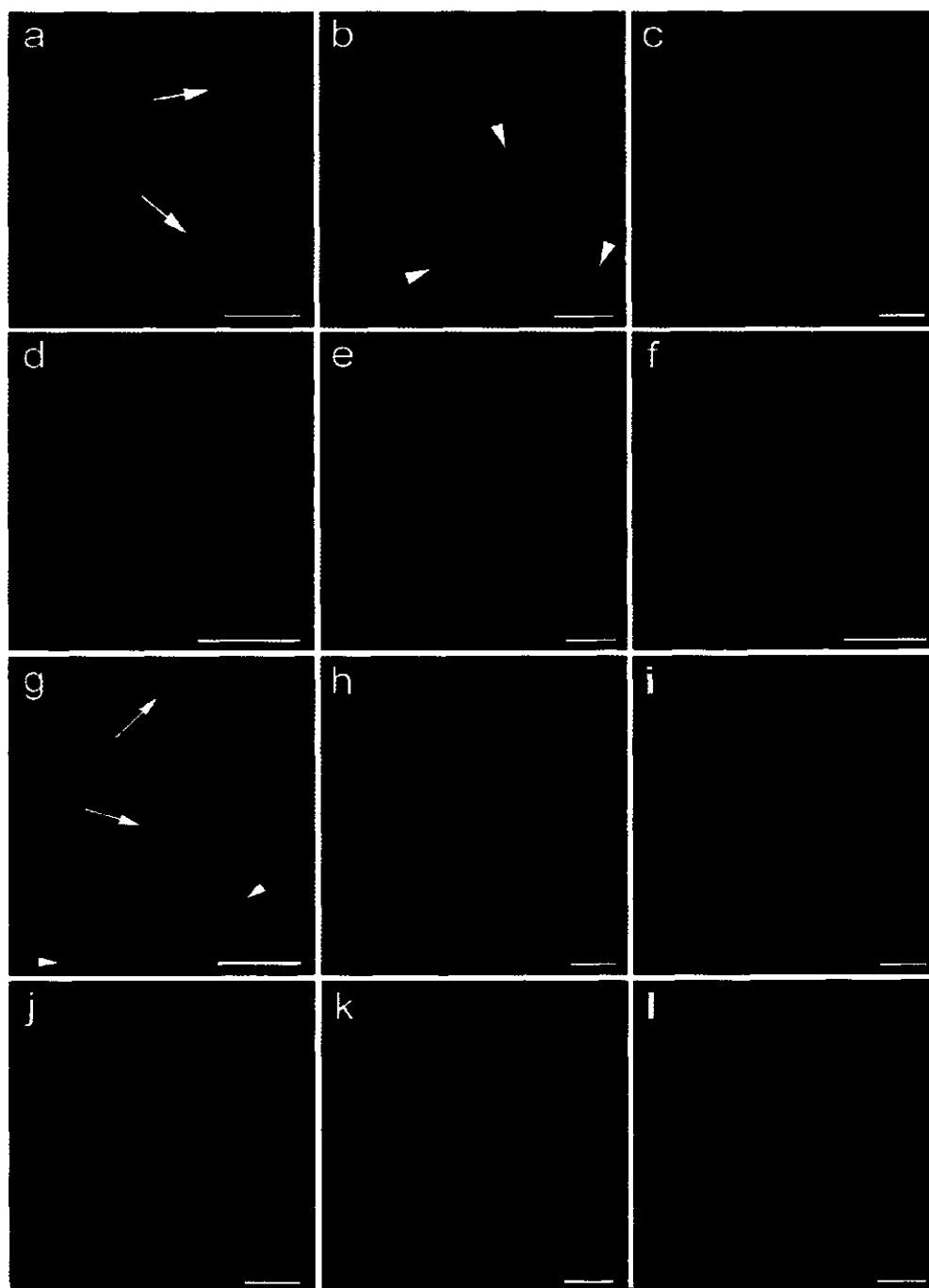
Discussion

During seed imbibition, DNA synthesis and appearance of microtubular cytoskeleton progress from the embryonic radicle tip region towards the cotyledons and result in cell division prior to radicle protrusion

As observed in previous studies (Bino et al. 1992, 1993; de Castro et al. 1995, 1998; Liu et al. 1994, 1997), and also in chapters 2 and 3, the levels of 4C DNA and β -tubulin were low in embryos of dry control tomato seeds. This reflects the pattern of seeds during late maturation when most embryonic cells were arrested at the G_1 phase of the cell cycle and β -tubulin was undetectable (Chapter 4). In the dry state most metabolic activities in the mature seed are suppressed (Roberts and Ellis 1989; Osborne and Boubriak, 1997) and contribute to the maintenance of the arrest of cell cycle in the G_1 phase.

The immunocytological analysis of DNA synthesis through BrdU incorporation and of β -tubulin in the microtubular cytoskeleton, allowed us to better understand the relationship between both cell cycle events during tomato seed development. The absence of BrdU incorporation into embryonic nuclear DNA from dry control seeds showed the lack of DNA synthesis, whereas the absence of a microtubular cytoskeleton, which is degraded during maturation (Chapter 4), reflected the lack of β -tubulin.

Seeds overcame the quiescent state upon imbibition. The initial accumulation of β -tubulin at 12h of seed imbibition occurred concomitantly with the initial assembly of the cortical microtubular cytoskeleton and DNA synthesis in the radicle. β -Tubulin apparently was synthesized *de novo*, and as a component of microtubules, a prerequisite for cell expansion. As expansion of cells coincided with S-phases we suggest a relation between the occurrence of high amounts of β -tubulin and the occurrence of DNA synthesis. Thus the synthesis of β -tubulin and assembly into cortical microtubules in meristematic cells might be a prerequisite for entrance to the S-phase, as observed also in wheat root tips (Gunning and Sammut, 1990). Similarly, the initiation of cell cycle events in maize roots requires a turnover of the microtubular cytoskeleton (Baluška and Barlow 1993).



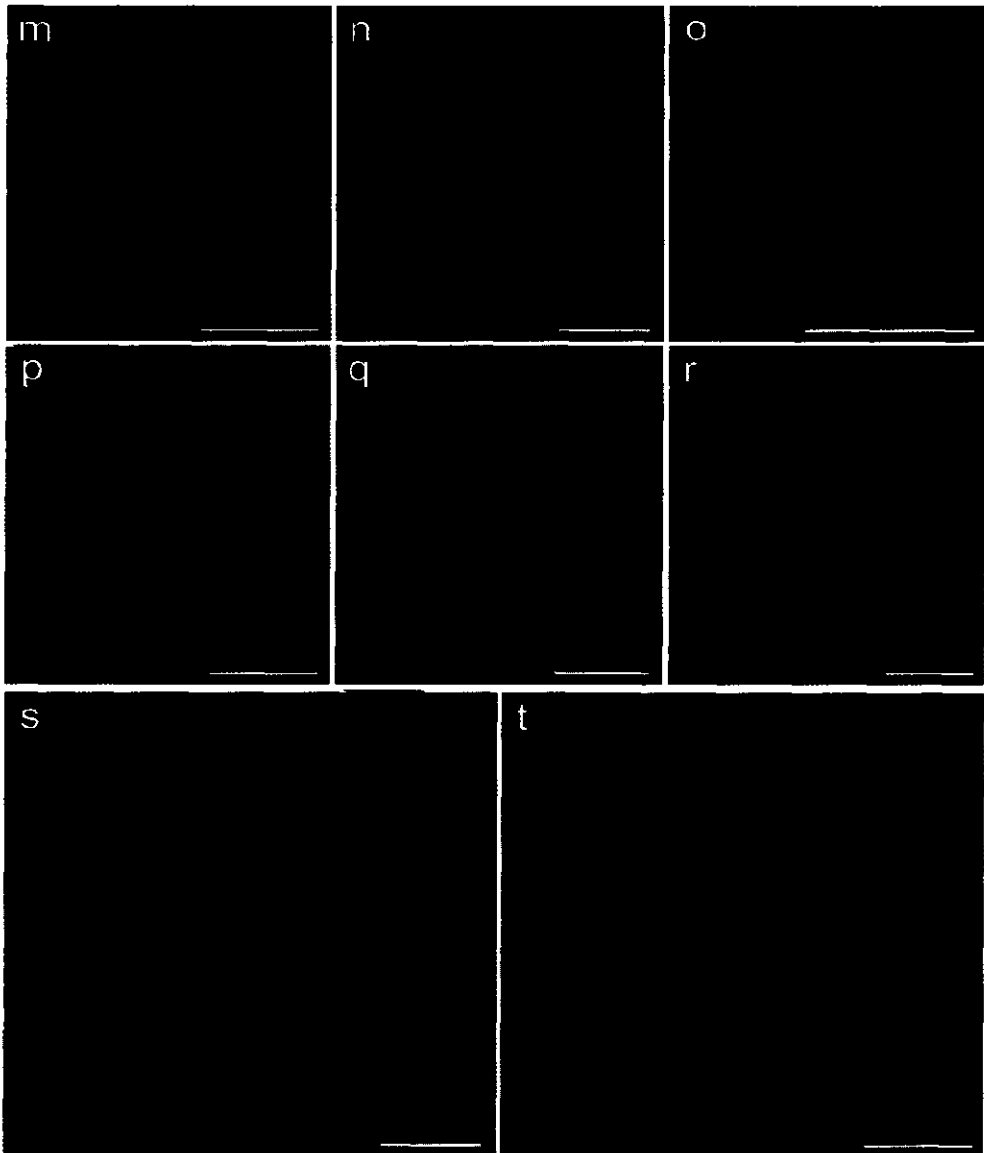


Figure 5. *Development of microtubular cytoskeleton in embryos during tomato seed germination.*

Fluorescent micrographs present patterns of longitudinal sections of embryos from untreated control seeds during germination (a-l), as well as of embryos from primed seeds before and after drying (m-t) labelled with anti- β -tubulin/FITC. The latter images (primed) are all in "confocal Z-series" projections. Bars indicate 20 μ m.

Because the sections are relatively thin (4 μ m) with respect to the diameter of the cells only a few cells have their cortical cytoplasm with microtubules in the plane of the section.

(Osborne and Boubriak, 1997; Boubriak et al., 1997). Microtubules, however, were likely to be sensitive to drying, as they were observed partly depolymerized after drying, *i.e.* depolymerization characterised by the presence of granules or clusters of tubulin. The fact that the amount of soluble β -tubulin detected after drying was still relatively high, may be explained by the fact that microtubules are dynamic structures and may exist in an equilibrium between soluble tubulin subunits and the polymerized microtubules (Bartolo and Carter 1991b). Depolymerization of microtubules was observed by Bartolo and Carter (1991a) in mesophyll cells of spinach (*Spinacia oleracea* L.) also as a characteristic response to dehydration. Moreover, they observed a recovery of microtubules upon rehydration, as observed here in embryos of primed tomato seeds upon subsequent imbibition in water.

Although the frequency of 4C nuclei after the priming treatment was comparable to that of untreated seeds imbibed in water for 24h, lower numbers of BrdU labeled nuclei were detected in primed embryos. This may be the result of a slow process of DNA synthesis, which, under the controlled osmotic hydration, required 7d in order to result in a comparable number of 4C nuclei. DNA replication is known to be retarded when seed hydration is limited (Bino et al. 1993; Saracco et al. 1995). Contrary to the imbibition in water, imbibition in -1.0 MPa PEG-6000 did not lead to mitosis. This implies that during priming the cell cycle was arrested allowing the synchronisation of cells in G₂. Apparently, this is a checkpoint controlled by the osmoticum and explains why the number of 4C or G₂ nuclei becomes invariable after 7d of osmopriming (van Pijlen et al. 1996). Nevertheless, mitotic events and also cell divisions occurred earlier in embryos of primed seeds upon subsequent imbibition in water and in higher numbers relative to the control seeds. The pre-activation of the cell cycle, required for growth, apparently was related to the higher frequency of 4C nuclei and mitotic divisions in embryos of germinated primed seeds, relative to those of untreated seeds. This may explain why pre-treated tomato seed lots dispose of a better germination performance over untreated seeds (Heydecker and Coolbear 1977; Argerich et al. 1989; Argerich and Bradford 1989).

In conclusion, the combined quantitative and immunocytochemical analysis of DNA synthesis and β -tubulin accumulation provided means for a better understanding of the reactivation of the cell cycle during seed germination. The re-initiation of cellular activity that occurs during tomato seed imbibition includes both cell expansion and division, precluding the completion of germination. This implies the activation of metabolism, which may also occur in imbibing dormant seeds (Bewley and Black, 1994). The present approach is being considered in Chapter 6 in the analysis of dormancy in tomato seeds in relation to the suppression and/or reactivation of the cell cycle.

Experimental procedures

Seed Material and Priming Conditions. Seeds of tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) with a moisture content of $6.0 \pm 0.1\%$, were used in the present study. Seed drying, storage and osmopriming in -1.0 MPa PEG-6000 at 25°C for 7d were as previously described (Chapters 2 and 3).

Germination. Germination analysis was conducted on 4 replicates of 50 seeds placed on top of 2 layers of filter paper soaked with 6 ml distilled water at $25 \pm 1^\circ\text{C}$ in darkness for 7d. Germinability was expressed as the percentage of total seeds that germinated, i.e. when they had approximately 1mm radicle protrusion.

Flow Cytometry and Detection of β -tubulin. Two replicates of 5 whole embryos were used for flow cytometric analysis of nuclear DNA contents according to Sacandé et al. (1997). Extraction and detection of β -tubulin by Western blotting were conducted as described previously (Chapters 2 and 3).

Immunohistochemical detection of BrdU and β -tubulin. Seeds, which were imbibed in the PEG-6000 solution, or in water, were subsequently immersed in a BrdU solution (Amersham, Buckinghamshire, UK; dilution 1:500 v/v) at 25°C in the dark, either as sectioned seeds or isolated embryos. The cytotoxicity of the BrdU solution (Rös and Wernicke 1991) was assessed at various pulse lengths by comparing the pattern of the flow cytometric profiles and the microtubular cytoskeleton with the normal patterns observed in the absence of BrdU. A 3h pulse length was found to be optimal because it allowed detection of BrdU incorporation without cytotoxicity, as observed after longer periods of incubation. Yet the microtubular cytoskeleton was only investigated in material not incubated with BrdU in order to avoid any negative effect of immersion in BrdU containing solutions (Chapter 4). Embryos were fixed in 4% paraformaldehyde, dehydrated and embedded in butylmethacrylate according to Baskin et al. (1992). Samples were sectioned, affixed on slides and processed either for the detection of incorporated BrdU or for microtubular cytoskeleton. The labeling of β -tubulin and BrdU were according to Xu et al. (1998). Anti β -tubulin (Amersham, Buckinghamshire, UK) was diluted 1:200 v/v; BrdU (Amersham, Buckinghamshire, UK) was diluted 1:1 v/v. FITC conjugated goat anti-mouse (1:200 v/v) was the second antibody (Amersham, Buckinghamshire, UK). Nuclear DNA was counterstained with 1 mg/ml 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).

Acknowledgments

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

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Summary. Cell cycle activities are initiated upon imbibition of non-dormant seeds. However, it is not known whether cell cycle related events also remain suppressed in imbibed dormant seeds. The objective of our study was to demonstrate that transition between the non-dormant and dormant (both primary and secondary) states is reflected in cell cycle events such as DNA replication, and changing patterns of the microtubular cytoskeleton. We have conducted the present studies on seeds from tomato (*Lycopersicon esculentum* cv. Moneymaker) which possessed primary dormancy or which were manipulated to become secondarily dormant. In addition, a non-dormant ABA-deficient mutant, *sit*^w was used. DNA replication, as measured by flow cytometry, and β -tubulin accumulation, analyzed by Western blotting, were compared with immunocytological studies of DNA synthesis and microtubular cytoskeleton formation. In summary, we have 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. 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.

Introduction

During maturation seeds may enter a state of dormancy, called primary dormancy. This type of dormancy is characterized by the inability of the mature seeds to germinate under favorable conditions. Dry storage, especially at elevated temperatures or a chilling treatment in the imbibed state often removes dormancy. If the conditions for germination of the non-dormant seeds are not favorable, 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 transition between the dormant and non-dormant states are not characterized well (Hilhorst, 1997, 1998). So far, the only clear difference demonstrated between these states is the sensitivity to environmental factors that stimulate germination, such as light and 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. Occasionally, the responsiveness to gibberellins increases upon relief of dormancy (Hilhorst, 1995). Yet, the transitions between states of dormancy are not reflected in any change in general metabolic activity (Derks and Karssen, 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 embryonic DNA synthesis are required to enable cell expansion and division, which lead to germinative growth and radicle protrusion (Chapter 5). These cell cycle events commence upon imbibition of non-dormant seeds. DNA replication appears to be suppressed in tomato seeds with secondary dormancy (Groot et al., 1997). However, it is not known whether other cell cycle related events also remain suppressed in imbibed dormant seeds. The objective of our study was to demonstrate that transition between the non-dormant and dormant (both primary and secondary) states is reflected in cell cycle events such as DNA replication, and changing patterns of the microtubular cytoskeleton.

For our studies we have used seeds from tomato (*Lycopersicon esculentum* cv. Moneymaker) which possessed primary dormancy or which were manipulated to become secondarily dormant. In addition, a non-dormant ABA-deficient mutant, *sir*^w was used. DNA replication, as measured by flow cytometry, and β -tubulin accumulation, analyzed by Western blotting, were compared with immunocytological studies of DNA synthesis and cytoskeleton formation.

Results

Non-dormant seeds

The analysis of DNA synthesis activity, *i.e.* by flow cytometry and by incorporation of BrdU, and the analysis of β -tubulin accumulation and of microtubular cytoskeleton formation in non-dormant wild type "control" seeds largely confirmed our previous studies (Chapter 5). Briefly, non-dormant seeds completed germination in the dark within 72h (Figure 1). The flow cytometry data for dry seeds indicated a low level (< 5%) of embryonic nuclei containing 4C DNA amounts (Figure 2), and there was no active DNA synthesis detected by BrdU incorporation (Figure 3a); β -tubulin was not detectable in whole embryo extracts (Figure 4, lane 4), and microtubular arrays were not observed (Figure 5a,d). After 24h of imbibition an active incorporation of BrdU was observed (not shown), concomitantly with a significant ($P < 0.05$) increase in the relative number of 4C nuclei (Figure 2), accumulation of β -tubulin (Figure 4, lanes 5-7), and appearance of cortical and mitotic microtubular cytoskeleton arrays in the embryonic radicle tip region (not shown). The higher intensity of cell cycle events at 48h of imbibition (Figures 2 and 4) preluded radicle protrusion, characterizing the completion of germination.

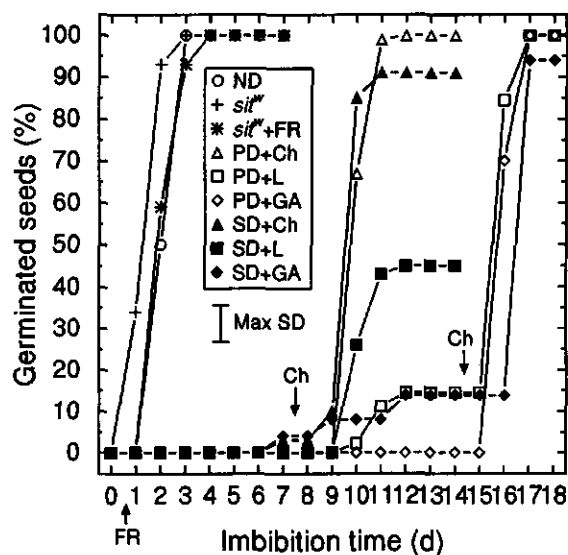


Figure 1. Germination of non-dormant and dormant tomato (*cv. Moneymaker*) seeds.

Germination of the non-dormant (ND) wild type control seeds and of the ABA-deficient (*sit*) seeds was scored during 7d of incubation at 25°C in the dark.

Germination was also scored for the ABA-deficient seeds when treated with far-red (*sit*+FR) light irradiation during the first 24h of incubation.

Seeds with primary dormancy (PD) and seeds with secondary dormancy (SD) were incubated for 7d at 25°C in the dark and then submitted to a chilling (+Ch) treatment. Subsequently, the seeds were transferred to white light (+L), or incubated in 10 μ M GA₄₊₇ (+GA) for another 7d of germination scoring.

Seeds with primary or secondary dormancy, which did not germinate under white light or in GA after 7d, were then also submitted to a chilling treatment.

Arrows indicate the moment during incubation at which the far-red (FR) or the chilling (Ch) treatments were applied. Maximum standard deviation is indicated (bar).

Seeds with primary dormancy

After prolonged dark incubation of over one month, the 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 (Figure 2), and the incorporation of BrdU could not be detected (not shown). Accumulation of β -tubulin (Figure 4) or formation of microtubular cytoskeleton (not shown) was also not detected. After a 1d chilling

Figure 2. Nuclear 4C DNA in embryos of non-dormant and dormant tomato seeds.

Frequencies of nuclei with 4C DNA contents (\pm SD) are expressed as percentage 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 (*sir*⁺) seeds during imbibition and completion of germination at 25°C in the dark.

Percentages of embryonic 4C nuclei are indicated for dormant seeds either during the 7d of incubation at 25°C in the dark (for seeds with secondary dormancy, SD), or after the 7d incubation only (for seeds with primary dormancy, PD), after chilling (Ch), and during subsequent completion of germination.

Arrow indicates the moment during incubation at which the chilling (Ch) treatment was applied.

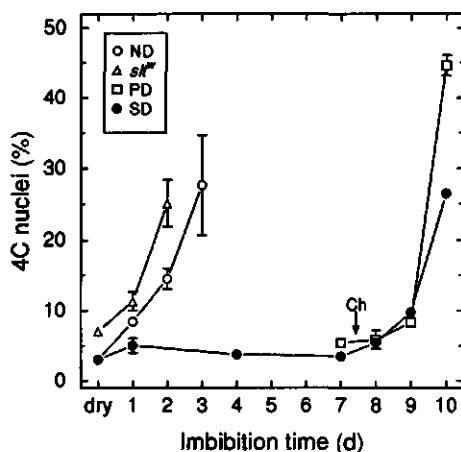


Figure 3. Activation of DNA synthesis in embryos of non-dormant and dormant tomato seeds.

Fluorescence micrographs present patterns of longitudinal sections of embryos from non-dormant wild type control and *sir*⁺ 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 fluorescein isothiocyanate (FITC), indicative for BrdU incorporation into actively replicating DNA (S-phase). Bars indicate 100 μ m.

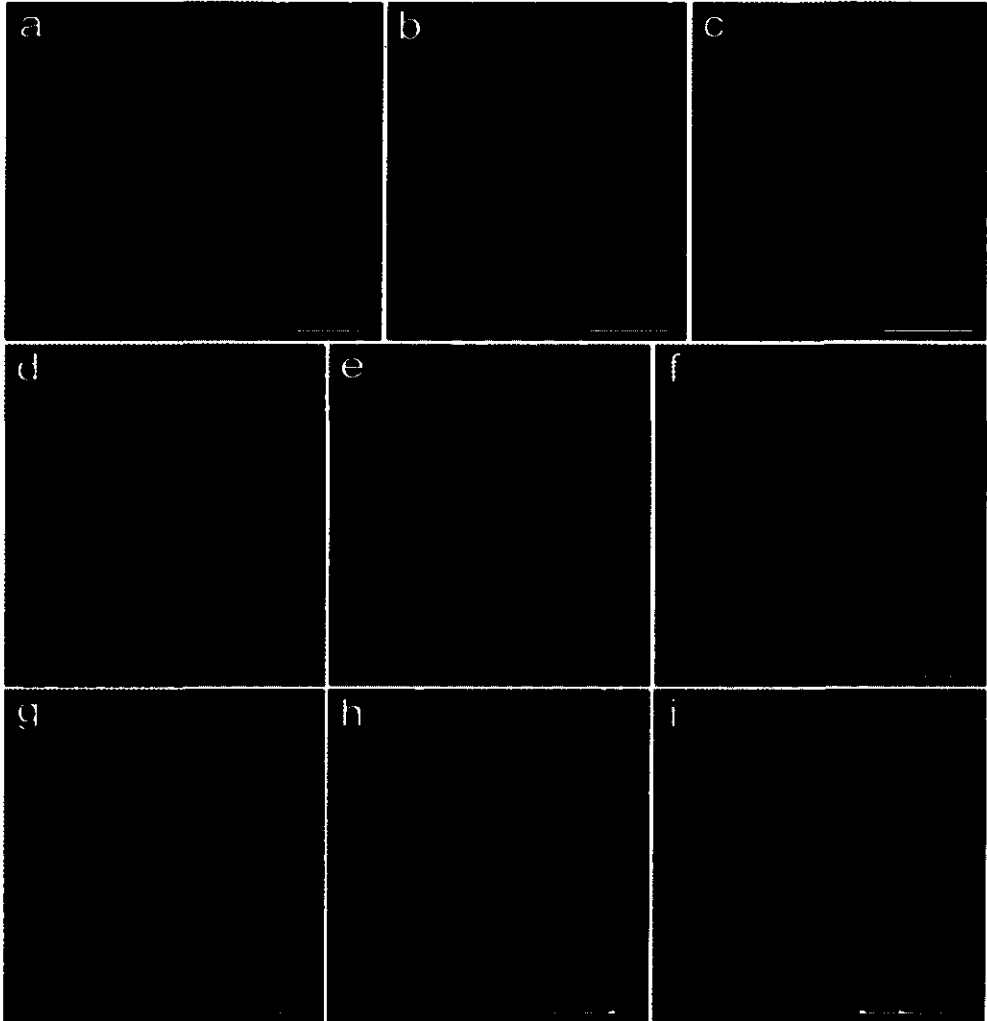
Embryonic radicle tip region of dry non-dormant control seeds showing the absence of BrdU incorporation after a 3h pulse labelling indicating absence of DNA synthesis.

(b,c) Radicle tips of seeds after release of primary dormancy by a chilling treatment; (b) ungerminated seeds 1d after the chilling treatment and pulse labelled for 3h showing nuclei labelled with BrdU indicating activation of nuclear DNA synthesis; (c) germinated seeds 2d after the chilling treatment.

(d-f) Radicle tips of seeds upon induction of secondary dormancy by a far-red irradiation treatment and subsequent release of dormancy by a chilling treatment; (d) some nuclei are labeled with BrdU showing DNA synthesis during the far-red treatment; (e) detection of DNA synthesis immediately after the chilling treatment in levels comparable to that after the far-red treatment and subsequent 7d of incubation at 25°C in the dark (not shown); (f) higher levels of DNA synthesis 1d after the chilling treatment indicating release of secondary dormancy.

(g-i) Radicle tips of the fully non-dormant *sir*⁺ seeds; (g) dry seeds showing absence of DNA synthesis; (h) ungerminated seeds after 1d of imbibition showing activation of DNA synthesis; (i) germinated seeds after 2d of imbibition.

treatment, dormancy was efficiently released as seeds germinated fully within 72h (Figure 1). DNA synthesis could still not be detected after the 1d chilling treatment (Figure 2). However, a trace amount of β -tubulin became detectable (Figure 4) and some cortical microtubules became apparent, notably in cells of the radicle tip region (Figure 5b,e). DNA synthesis was only detected 1d after seeds were transferred back to 25°C in the dark. A significant ($P < 0.05$) increase in the number of 4C nuclei was detected (Fig 2), as well as



BrdU incorporation (Figure 3b). At this stage, the level of β -tubulin had increased (Figure 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 (Figure 5c,f,g,h). After 2d under these conditions, the number of 4C nuclei (Figure 2), the level of BrdU incorporation (Figure 3c), the level of β -tubulin (Figure 4), and the appearance of cortical and mitotic microtubular arrays had all intensified (not shown), preluding the completion of germination of most seeds (Figure 1), as in the non-dormant wilde type (Chapter 5). Imbibition in GA did not induce germination, whereas under white light some seeds germinated (Figure 1). The activation of DNA synthesis (not shown), the accumulation of β -tubulin (Figure 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 7d germination was virtually absent ($< 4\%$) (Figure 1). Transferring the seeds to continuous white light induced germination of 45% of the seeds within 48-72h (Figure 1). A 1d chilling treatment proved more effective and resulted in 85% germination within 48h in the dark. GA was hardly effective in the breakage of dormancy, as only a few seeds responded to the hormone (Figure 1). A subsequent chilling treatment appeared to have an additive effect to that of GA and stimulated germination up to 95% (Figure 1).

After the far-red treatment and subsequent 7d incubation in the dark, the number of embryonic 4C DNA nuclei remained ($P > 0.05$) at the dry non-dormant seed levels (Figure 2). However, during this period some nuclei with BrdU incorporation (Figure 3d), as well as trace amounts of β -tubulin (Figure 4), and a moderate amount of cortical microtubules (Figure 5i), which were not detected in dry seeds, became detectable in the embryonic tissues. After the 1d chilling treatment, β -tubulin was not detectable (Figure 4) and cortical microtubules became less apparent, although some cell files in the central cylinder of the hypocotyl were still intensely labeled (Figure 5j). BrdU incorporation could still be detected after the chilling, at levels comparable to that before chilling (Figure 3e), whereas the number of 4C DNA nuclei remained unchanged (Figure 2). One day after the transferring back to 25°C a substantial increase in β -tubulin accumulation was detected (Figure 4). As expected, this occurred in parallel with a recovery of the microtubular cytoskeleton, which became more intensely labeled, showing cortical and mitotic arrays (Figure 5k). At this time, the number of 4C DNA nuclei had significantly increased ($P < 0.05$) (Figure 2),

reaching a level comparable to that of the non-dormant seeds at 24h of imbibition, and correlated to a more intense detection of BrdU incorporation (Figure 3f).

The escape experiment was performed to determine the time of imbibition at which the seeds could still be sensitive to the far-red treatment. After 24 to 36h of imbibition, approximately half of the seeds could still become dormant (Figure 6). From those seeds which escaped from the far-red inhibition after 36h of imbibition, approximately half produced abnormal seedlings, whereas before 36h all seedlings were normal. Abnormalities mainly appeared as necrotic areas on roots and cotyledons (not shown). Far-red irradiation was most effective at early stages of imbibition, in that the subsequent chilling treatment was less effective, indicating a deeper dormancy than found at later stages of imbibition.

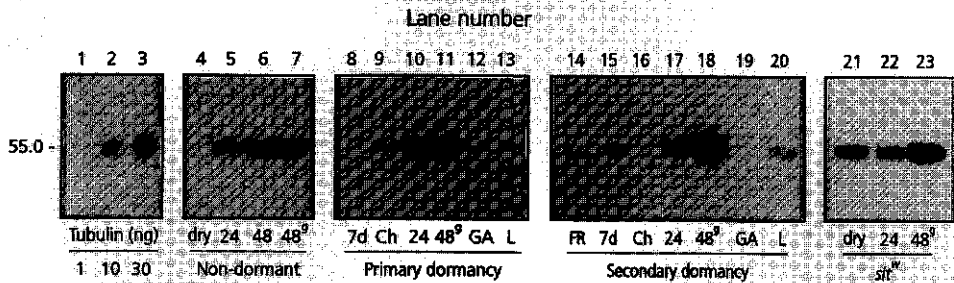
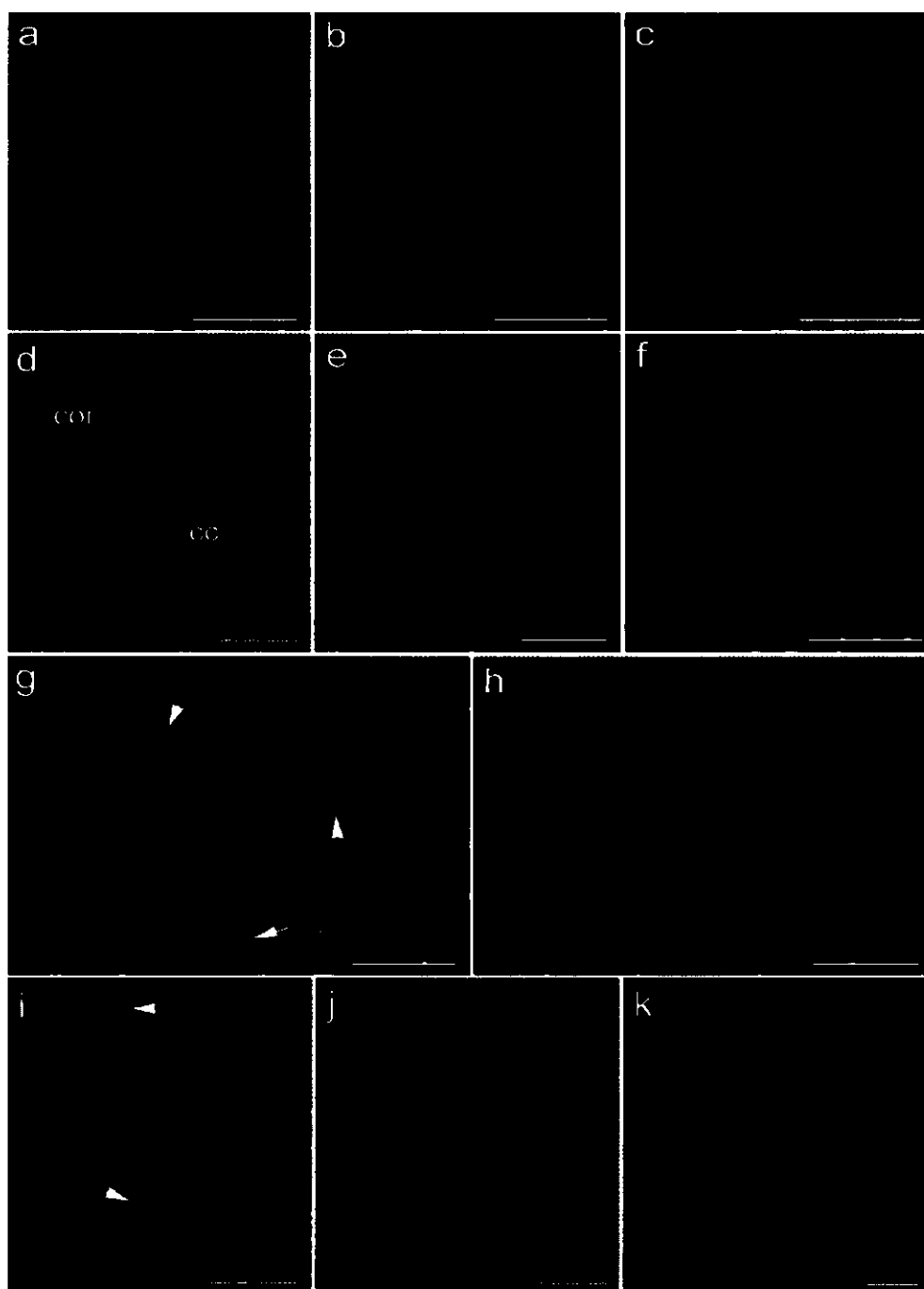


Figure 4. β -Tubulin accumulation in embryos of non-dormant and dormant tomato seeds.

Lanes 1-3 were loaded with 1, 10 and 30 ng pure bovine brain tubulin, respectively.

- (lanes 4-7) β -tubulin levels are shown for embryos of dry non-dormant control seeds, and after 24 and 48h of imbibition.
- (lanes 8-13) β -tubulin levels in embryos of seeds with primary dormancy; (8) after an incubation period of 1 month plus 7d at 25°C in dark; (9) at the end of the chilling (Ch) treatment; (10, 11) 1d and 2d after the chilling treatment, respectively; (12, 13) seeds incubated for 7d at 25°C in the dark and then subsequently incubated for another 7d in 10 μ M GA₄₊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 7d incubation at 25°C in dark; (16) immediately after the chilling treatment; (17, 18) 1d and 2d after the chilling, respectively; (19, 20); seeds incubated for 7d at 25°C in the dark and then subsequently incubated for another 7d in 10 μ M GA₄₊₇ or under white light, respectively.
- (lanes 21-23) β -tubulin levels in embryos of dry *sit*⁺ seeds, and after 24 and 48h of imbibition.

Note that β -tubulin accumulated in embryos upon imbibition of non-dormant seeds, as well as after release of primary and secondary dormancy by the chilling treatment. Total protein loaded per lane was 30 μ g. Molecular weight (kD) is indicated on the left hand side. The films were exposed for a maximum of 1 min. (⁸) Embryos of seeds that had germinated, i.e. with approximately 1mm radicle protrusion.



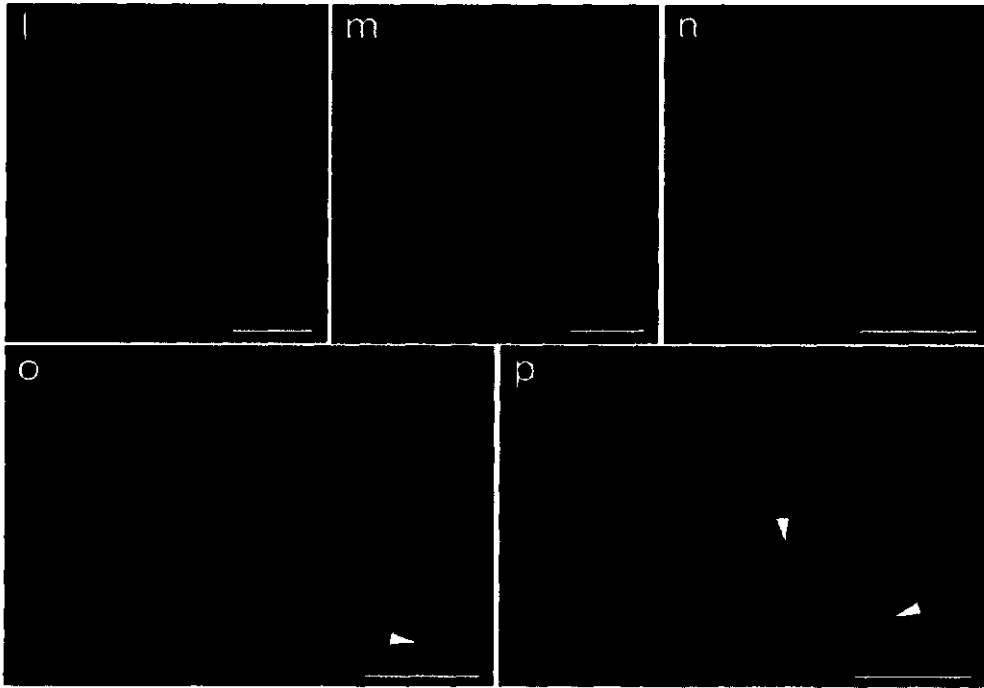


Figure 5. Appearance of microtubular cytoskeleton in embryos of non-dormant and dormant tomato seeds.

Fluorescence micrographs present patterns of longitudinal sections of embryos from non-dormant wild type control and *sir*⁺ seeds, and from seeds with primary and secondary dormancy labelled with anti- β -tubulin and goat anti-mouse/FITC. Bars indicate 25 μ m.

Because the sections are relatively thin (4 μ m) 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,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) 1d after the chilling treatment; (g,h) mitotic (arrowheads) and cytokinetic (arrow) arrays in cells of the radicle also 1d 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 in general were less apparent after the chilling than after the far-red treatment (i) and subsequent 7d incubation at 25°C in dark (not shown); (k) The microtubular network was recovered 1d after the chilling treatment, showing cortical and mitotic arrays and divisions.
- (l-p) Embryos from *sir*⁺ 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 depolymerised as observed through the large numbers of tubulin granules; (o) recovery of microtubules after 1d 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 2d of imbibition.

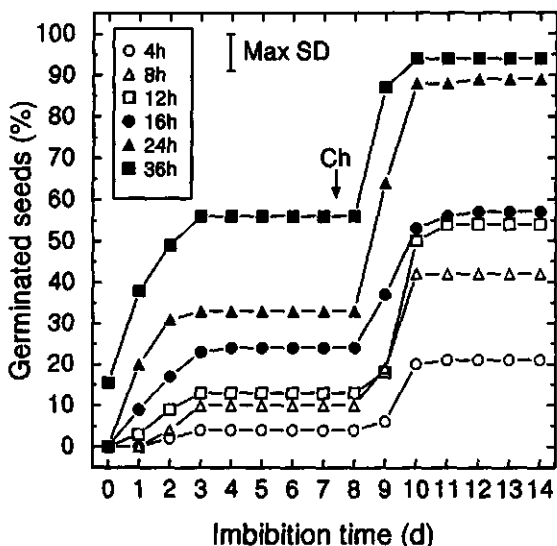
The *sir*^w mutant

Over 90% of the *sir*^w seeds completed germination in the dark within 48h (Figure 1). The number of 4C nuclei in embryos of dry seeds was significantly higher ($P < 0.05$) than that of the dry control seeds (Figure 2). However, BrdU incorporation was not detectable after three hours of pulse labeling (Figure 3g). Yet, the amount of β -tubulin in embryos of the dry *sir*^w seeds was high (Figure 4) compared to dry non-dormant control seeds, in which β -tubulin was undetectable. Also in contrast with dry control seeds, the embryos of dry *sir*^w seeds contained an intense cortical microtubular cytoskeleton network, visualised not only in the radicle tip region, but also in other parts of the embryo, although its microtubules appeared highly depolymerised (Figure 5l-n). Upon imbibition, 4C DNA (Figure 2) and BrdU labeled nuclei (Figure 3h,i) were detected in increasing numbers. In parallel, the level of β -tubulin increased further (Figure 4), and the microtubular cytoskeleton reconstituted into an elaborate cortical cytoskeleton in cells throughout the embryo, and also mitotic arrays were observed in the radicle tip region (Figure 5o,p). As in control seeds (Chapter 5), the intensification of the cell cycle events preluded the completion of germination in *sir*^w.

Figure 6. Determination of the moment during seed imbibition at which seeds escape the induction of secondary dormancy by far-red irradiation.

Non-dormant seeds were first incubated in water for 4, 8, 12, 16, 24 or 36h at 25°C in the dark and then submitted to a far-red irradiation treatment. Germination was scored before the far-red treatment (not shown), as well as after the far-red treatment, during a period of 7d incubation, after a chilling treatment, and during another 7d of incubation. Normal seedlings were scored after the first 7d of incubation and after the total period of 14d of incubation following the far-red treatment (not shown).

Arrow indicates the moment during incubation at which the chilling (Ch) treatment was applied. Maximum standard deviation is indicated (bar).



Discussion

Seeds with primary dormancy did not show any detectable cell cycle activity in the embryos when incubated in dark, not even after prolonged incubation. GA and white light were not, or only slightly, effective in inducing germination. This insensitivity was also reflected in the absence of relevant cell cycle activity. The chilling treatment increased the sensitivity to light and GA which resulted in almost full germination after transfer to 25°C. This supports the hypothesis that dormancy can be defined by a lack of sensitivity to germination stimulants (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 microtubular cytoskeleton was observed. However, there was no detectable synthesis of DNA. This indicates that the formation of the microtubular cytoskeleton is associated with the dormancy-breaking process *sensu strictu*. Transferring the seeds to 25°C after chilling resulted in DNA synthesis and formation of the microtubular cytoskeleton, which is clearly associated with the germination process (Chapter 5).

The situation in seeds in which secondary dormancy was induced by the far-red treatment appeared to be different. 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. As previously observed (Chapter 5), DNA synthesis is initiated in the embryonic radicle tip of non-dormant tomato seeds within the first 12h of imbibition. This occurs concomitantly with the accumulation of β -tubulin and the establishment of cortical microtubular cytoskeleton. Apparently, the far-red irradiation induced the secondary dormancy when the cell cycle had been already activated during the first hours of seed imbibition. This suggests that secondary dormancy may be induced while the germination process is in progress. The results of the escape experiment indicate that the far-red irradiation may induce secondary dormancy when applied before 36h of imbibition. At that phase of the germination process DNA synthesis and formation of microtubular cytoskeleton may have progressed to such a level that arrest of the cell cycle is no longer possible. The necrotic regions observed in the abnormal seedlings from seeds which escaped the inhibition by far-red irradiation after 36h of imbibition, may also be related to the progression of the cell cycle, as all seeds which were imbibed for less than 36h produced normal seedlings. However, further investigations are needed to address this aspect. Furthermore, in the ABA-deficient *sir*^w mutant, germination follows seed development within the fruit without intervening developmental arrest (Liu et al, 1997;

Chapter 4). Consequently, at harvest *sir*⁺ embryos contain amounts of 4C DNA nuclei and microtubular cytoskeleton that are comparable to those of germinating wild type control seeds at an imbibition interval of 24 hours (Chapter 5). Indeed, far-red irradiation only had a marginal effect on the germination of *sir*⁺ seeds (Figure 1). Its effect was expressed in a slight delay of the start of germination.

The seeds with secondary dormancy also 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 primarily dormant seeds, in the secondarily dormant seeds the chilling treatment gave rise to a partial degradation of the microtubular cytoskeleton and BrdU incorporation could still be detected. Primary dormancy is induced during seed development in the absence of DNA synthesis activity, but in the presence of an elaborate cortical microtubular cytoskeleton network (Chapter 4). In contrast, secondary dormancy is induced under conditions where DNA synthesis is active, as well as the microtubular cytoskeleton. From our results 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 secondarily dormant seeds to GA and light is higher than in primarily dormant seeds indicates that the difference between primary and secondary dormancy in tomato is largely quantitative. Apparently the initiation of the cell cycle, *i.e.* specially the initiation of DNA synthesis activity, contributes to this response. The escape experiment shows that the longer the seeds are imbibed before far-red irradiation, the less dormant they become. This is expressed in the effectiveness of the chilling treatment. Nevertheless, there seems to exist a "window" in which the far-red treatment is most effective. When the far-red irradiation is applied from the beginning of imbibition, the chilling treatment is more effective in breaking dormancy, than at 4h of imbibition. This indicates a more "shallow" dormancy.

The fact that the microtubular cytoskeleton appeared partly degraded after the chilling treatment in seeds with secondary dormancy, as also observed in the dry *sir*⁺ seeds, suggest that microtubules in tomato seeds are also sensitive to cold and drought stresses (Bartolo and Carter, 1991; Okamura et al., 1993; Pihakaski et al. 1995). Microtubules may be recovered after cold or drought induced depolymerization (Bartolo and Carter, 1991; Murata and Wata, 1991), as was observed when seeds were returned to 25°C, after the chilling treatment. The detection of similar numbers of nuclei incorporating BrdU throughout induction and release of secondary dormancy may reflect an ability of S-phase nuclei to tolerate conditions that might block the cell cycle (*e.g.* far-red), once DNA-repair has occurred previously. For example, S-phase nuclei may tolerate desiccation when DNA repair has taken place during the initial phases of the germination process (Osborne and Boubriak, 1997; Boubriak et al., 1997).

In summary, we have 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. 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.

Experimental procedures

Seed Material. Seeds of tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) with a moisture content of $6.0 \pm 0.1\%$, were used. Seeds with primary dormancy were obtained from tomato plants grown in a greenhouse during the summer of 1996, under natural daylight at $25^\circ\text{C}/20^\circ\text{C}$ day/night average temperatures. The seeds were dried as previously described (Chapter 2) and stored at -20°C until use. A seed batch possessing full primary dormancy was obtained by allowing the seeds to imbibe in water at 25°C in darkness, and keeping them incubated under the same conditions during the course of one month. After this period approximately 20% of the seeds 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 kept for a further 7d incubated under the conditions mentioned above. After this period, seeds were submitted to a chilling treatment by exposing the seeds to a temperature of 10°C , transferred to light or incubated in GA for dormancy release, and scored for germination. Seeds of the ABA-deficient *sid*⁺ mutant were used as fully non-dormant seeds.

Manipulation of secondary dormancy. Secondary dormancy was induced by applying irradiation with far-red light ($> 730\text{ nm}$) for 5 min at hourly intervals during the first 24h of imbibition at 25°C , followed by 7d of incubation at 25°C in the darkness (Groot et al., 1997). Over 96% of the total amount of seeds treated with far-red irradiation did not germinate after the 7d of incubation in the dark and were, therefore, characterized as seeds with secondary dormancy. These seeds were then submitted to a chilling treatment, transferred to light or incubated in GA for dormancy release, as done for the primarily dormant seeds.

To determine the phase when the far-red irradiation would become ineffective during germination ("escape experiment"), the far-red irradiation was started 4, 8, 12, 16, 24, and 36h after imbibition. Germination was scored prior to the far-red treatment and during the subsequent 7d of incubation at 25°C in the darkness.

Germination. Germination analysis was conducted on 4 replicates of 50 seeds placed on top of 2 layers of filter paper soaked with 6 ml distilled water or $10\text{ }\mu\text{M}$ GA₄₊₇, at $25 \pm 1^\circ\text{C}$ in darkness or under white fluorescent light (Philips TL 84) for 7d. Germinability was expressed as the percentage of total seeds that germinated, i.e. when they had approximately 1mm radicle protrusion.

Flow Cytometry and Detection of β -tubulin. Two replicates of 5 whole embryos each were used for the flow cytometric analysis of the nuclear DNA contents according to Sacandé et al. (1997). Extraction and detection of β -tubulin by Western blotting were conducted as described previously (de Castro et al. 1995, 1998; Chapters 2 and 3).

Immunohistochemical detection of BrdU and β -tubulin. Seeds, which were imbibed in water were subsequently immersed in a BrdU solution (Amersham, Buckinghamshire, UK; dilution 1:500 v/v) at 25°C in the dark, either as sectioned seeds or isolated embryos. The cytotoxicity of the BrdU solution (Rös and Wernicke

1991) was assessed at various pulse lengths by comparing the pattern of the flow cytometric profiles and the configuration of microtubular cytoskeleton with the normal patterns observed in the absence of BrdU. A 3h pulse length was found to be optimal because it allowed detection of BrdU incorporation without cytotoxicity, as observed after longer periods of incubation. Yet the microtubular cytoskeleton was only investigated in material not incubated with BrdU in order to avoid any negative effect of immersion in BrdU containing solutions (Chapter 4). Embryos were fixed in 4% paraformaldehyde for 4h, rinsed, dehydrated and embedded in butylmethacrylate according to Baskin et al. (1992). Samples were sectioned, affixed on slides and processed either for the detection of incorporated BrdU or for the visualization of the microtubular cytoskeleton. The labeling of β -tubulin and BrdU were according to Xu et al. (1998). Anti β -tubulin (Amersham, Buckinghamshire, UK) was diluted 1:200 v/v; BrdU (Amersham, Buckinghamshire, UK) was diluted 1:1 v/v. FITC conjugated goat anti-mouse (1:200 v/v) was the second antibody (Amersham, Buckinghamshire, UK). Nuclear DNA was counterstained with 1 mg/ml 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).

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Chapter

7

Cell cycle events in developing and germinating tomato seeds: general discussion

RD de Castro

Completion of development in tomato seeds is characterised by an arrest of most embryonic cells with nuclei in the G₁ phase of the cell cycle, comprising 2C DNA values or "amounts" (Liu et al., 1997). During imbibition and completion of germination, the cell cycle is reactivated and results in DNA synthesis towards duplication of the nuclear genome, as increasing numbers of cells contain nuclei in the G₂ phase of the cell cycle, comprising 4C DNA amounts (Bino et al., 1992; Liu et al., 1994). The precise quantitation of nuclear DNA amounts in seed tissues has been made possible after the introduction of flow cytometry into seed biology studies (Bino et al., 1992). The use of flow cytometry has greatly facilitated the analysis of cell cycle in seed biology studies and has provided much information about the patterning of DNA synthesis and cell cycle progression in relation to the physiological events that occur during seed development and germination (Bino et al., 1992, 1993; Lanteri, et al., 1993, 1996; Liu et al., 1994, 1996, 1997; Saracco, 1995; van Pijlen, 1996). Yet, more is needed in order to understand DNA replication in relation to other cell cycle events in seed development and germination.

Our approach included the analysis of DNA synthesis in relation to β -tubulin accumulation and of the formation of the microtubular cytoskeleton during the progression of the cell cycle, by means of quantitative and immunocytochemical analyses, as well as by physiological analyses. This approach proved efficient to better understand the complex

biological system of seed function and behaviour, as discussed by Karssen (1993). The present study has provided evidence that lead us to modify the current model of tomato seed development and germination (Figure 1). Using the tomato seed as a "model system", the present results may contribute to a better understanding of the physiological and biochemical processes involved in the development, germination and dormancy of seeds (Figure 1, Chapter 1), as discussed further in this chapter.

Development

Based on the patterning of embryo growth, seed development can be divided in two conceptually distinct phases (Figure 1a, Chapter 1, reviewed by Harada, 1997). The first phase is considered to be a period of morphogenesis during which the embryo's body plan is established through intensive cell divisions and the embryonic organs and tissues are formed (reviewed by Goldberg et al., 1994; Meinke, 1995; West and Harada, 1993). The second phase is considered to be a subsequent period of seed maturation, which includes the arrest of tissue and organ formation, the accumulation of nutrient reserves, changes in embryo size and in fresh and dry weights, the suppression of precocious germination, the acquisition of desiccation tolerance, dehydration and quiescence, and, in many species, the induction of dormancy (Koornneef and Karssen, 1994).

Although several developmental studies have been conducted on tomato seeds (Berry and Bewley, 1991; Demir and Ellis, 1992; Groot et al., 1987; Hocher, et al., 1991; Liu et al., 1996), there has been, in general, little attention paid to define these phases in tomato seed development. The patterning of DNA synthesis and microtubular cytoskeleton configuration during embryogenesis (Chapter 4), lead us to the conclusion that three distinct phases (Figure 1), comparable to that used in the studies of Hocher et al. (1991), must be considered as a concept to describe the development of the tomato seed. Compared to the general concept of seed development discussed in Chapter 1, the first developmental phase in tomato seeds is indeed the period of establishment of the embryo's body plan and formation of the embryonic organs and tissues. This is clearly established, as a period marked by intensive DNA synthesis activity, an abundant cortical and mitotic microtubular cytoskeletal network, and intensive cell divisions. Yet, the completion of morphogenesis is achieved only in a second phase, when the accumulation of nutrient reserves accompanies changes in embryo size and shape, and the main changes in fresh and dry weights takes place. Therefore, this period is mainly marked by the detection of an abundant cortical microtubular cytoskeleton network which, in the absence of DNA synthesis and cell divisions, leads to cell expansion growth, and the embryo reaches its final curled shape, coinciding with the attainment of maximum dry weight and physiological maturity.

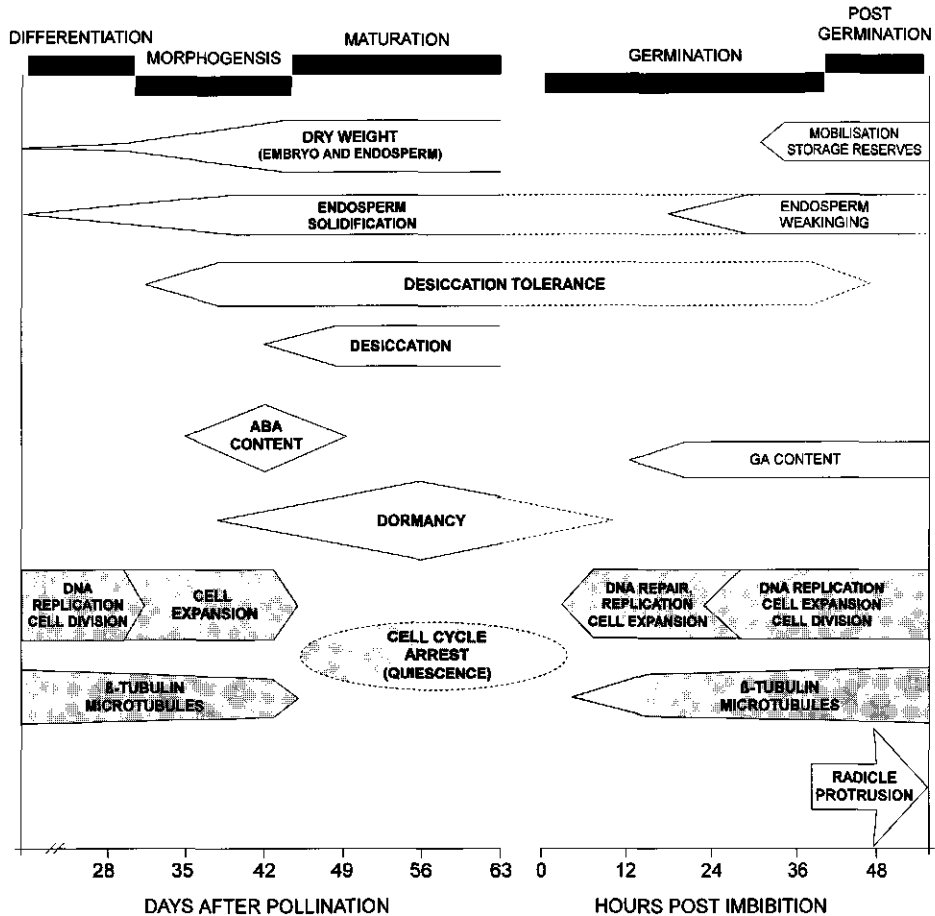


Figure 1. Tomato seed development and germination.

A model describing the development and germination of tomato seeds (cv. Moneymaker) based on the progression of cell cycle events (highlighted in gray), and the relation with physiological events that are identified at different stages of development and germination: modified from Hilhorst et al. (1998). Diverging lines indicate increasing and converging lines decreasing activities. The circled dotted line indicates quiescence as the "complete" arrest of cell cycle activities which leads to growth.

Because of this, we suggest that morphogenesis not necessarily includes cell division and histodifferentiation, but can also occur as a result of cell expansion only. Finally, the third phase, maturation, is marked by the complete degradation of the microtubular cytoskeleton, which is no longer visible in embryos of fully mature tomato seeds. Suppression of precocious germination, dehydration and quiescence takes place during this phase.

Interestingly, acquisition of desiccation tolerance is initiated during the second phase of growth and morphogenesis (Figure 1). The fact that an abundant microtubular cytoskeleton network is required during this phase implies that acquisition of desiccation tolerance may have no relation with the cortical microtubular cytoskeleton organization which, during this phase, appears to be exclusively linked to cell expansion growth. Furthermore, acquisition of desiccation tolerance also appeared not to have any relationship with the transient rise in ABA content, as it was induced before this rise occurred. Also seeds of the ABA-deficient *sit^w* mutant are desiccation tolerant. In a different situation, developing seeds of cv. Caruso were desiccation intolerant during the period associated with high ABA concentration (Berry and Bewley, 1992), confirming the absence of a relation between desiccation tolerance and ABA in tomato seeds. Nevertheless, the acquisition of desiccation tolerance seems to be linked, or requires, the cessation of DNA synthesis and mitotic events, as it occurs only after the completion of histodifferentiation. At this stage, seeds also become fully germinable and able to produce normal seedlings when isolated from the fruit and incubated in water. This is an aspect which appears to be linked not only with the completion of histodifferentiation but also with the solidification of the endosperm and hardening of the seed coat tissues as a growth restricting and protective barrier for the still growing embryo (Hilhorst and Downie, 1995). Before this period, some seeds are able to germinate, but mostly through hypocotyl protrusion and do not produce normal seedlings. As concluded before, this may be the result of the lack of sufficient reserves in the still liquid endosperm (Berry and Bewley, 1991). The absence of an integrated microtubular cytoskeleton in the endosperm during the stages of development analyzed, implies that the expansion of the tomato endosperm and development into a firm consistency might occur randomly, apparently with no structural or morphogenetic control, limited by the seed coat tissues only.

Primary dormancy (Karssen, 1995) was also initiated during the second phase of embryo growth and morphogenesis (Figure 1). Hence, as with the acquisition of desiccation tolerance, primary dormancy may not have a relation with the microtubular cytoskeleton organization. This shows that dormancy and quiescence are distinct events during seed development. Quiescence is always induced during the maturation phase, and is characterized by the arrest of cell cycle events, as there is no DNA synthesis activity and the microtubular cytoskeleton is completely degraded. Quiescence appears to be directly

related to the seed's or embryo's hydration level, which is lowest during maturation. However, primary dormancy may be associated with the endogenous ABA content, as it is induced immediately after the transient rise in ABA content, during the second phase of growth and morphogenesis. In several other developmental studies with tomato seeds (Groot et al., 1987; Berry and Bewley, 1992; Hoche et al., 1991), the peak in ABA content was detected at comparable stages of development. However, there was no dormancy induction, which means that primary dormancy may require other factor(s) for its induction. When dormancy is not induced, developing tomato seeds may germinate when isolated and transferred to water, even during a developmental stage associated with the highest ABA content (Berry and Bewley, 1992.).

The role of GA and ABA in seed development

The general features that govern embryogenesis are considered to be similar in higher plants (reviewed by Yadegary and Goldberg, 1997). Both DNA synthesis and the microtubular cytoskeleton configuration appeared to function similarly among the wild type, GA-deficient (*gib1*) and ABA-deficient (*sir*^w) tomato seed lines (Chapter 4). However, the timing of events that lead to cell differentiation, morphogenesis and maturation appeared to be influenced by GA and ABA. Embryo histodifferentiation appeared to be uncoupled from morphogenesis during the expansion growth of the slowly growing *gib1* embryos (Chapter 4). This implies that GA may play a role in embryogenesis, possibly by acting on the synchronisation of nuclear DNA synthesis and dry matter accumulation. GA-deficient pea (*Pisum sativum*) seeds also grow slower than wild type seeds, as an indirect result of the GA-deficiency in the distribution of assimilates (Swain et al., 1998) which, as in tomato, may be directly influencing DNA synthesis. The uncoupling of cell differentiation and morphogenesis has also been demonstrated with the use of *Arabidopsis* embryo pattern mutants, which indeed suggests that these processes are regulated independently (Yadegary and Goldberg, 1997). Suppression of precocious germination and induction of quiescence, which takes place during maturation of wild type seeds, do not occur in the ABA-deficient *sir*^w seeds. The maturation phase in *sir*^w seeds is, therefore, marked by the reactivation of DNA synthesis and recovery of the microtubular cytoskeleton network in the embryonic radicle tip, precluding the occurrence of viviparous germination. ABA-deficient mutants are often related to as non-dormant and, because of that, they may germinate viviparously. We may conclude that this is doubtful. In tomato, we would rather say that the *sir*^w mutant seeds are non-dormant because of the ABA deficiency *throughout* development. As we have discussed above, ABA appears to be required in interaction with

one or more other factors, so that dormancy can be induced. We assume that *sir*^w seeds may germinate within the fruit as a result of the osmotic conditions of the fruit during maturation, and of the structural characteristics of this mutant seed. *Sir*^w seeds have a thinner seed coat (Hilhorst and Downie, 1995). As a thinner seed coat imposes less mechanical resistance to embryo growth, the prevailing osmotic conditions in the locular tissues may allow water uptake by the embryo, reactivation of DNA synthesis activity and recovery of the microtubular cytoskeleton, preluding the viviparous germination.

Germination

Germination and postgerminative growth represent the phases during which metabolic and morphogenetic reactivation of the quiescent seed occurs (Harada et al., 1988). The reactivation of cell cycle events occurs as the “mirror image” of the developmental arrest (Figure 1). The first signs of cell cycle reactivation occurs within 12h of imbibition, as DNA synthesis activity and the appearance of cortical microtubular cytoskeleton is detected in the embryonic radicle tip cells of non-dormant seeds. Whether this DNA synthesis is related to repair or replication of the genome is unclear. As nuclei were in general completely labelled by BrdU, it is unlikely that this is exclusively the result of DNA repair, which is known to occur at early imbibition (Boubriak et al., 1997). DNA synthesis, towards replication of the genome in the first cell cycle, appears to take place during the next 12h of imbibition while the embryonic radicle tip cells expand before the first 4C nuclei and cell divisions can be detected after 24h of imbibition. Thus, the first 12h of imbibition appear to mark the re-start of growth in germinating tomato seeds.

Whether completion of germination (radicle protrusion) is dependent only on cell expansion or also on cell division is unclear (Bewley and Black, 1994). Current consensus is that cell division is largely a postgermination phenomenon. It has been demonstrated in tomato seeds that cell cycle activities are indeed initiated in the radicle tips cells and that embryonic DNA replication can be used to follow the progression of germination (Bino et al., 1992). Furthermore, the process by which the radicle can protrude through the seed coat is now shown to be dependent also on the microtubular cytoskeleton, acting not only on expansion of radicle tips cells, but also on cell division and expansion of the hypocotyl cells prior to radicle protrusion. Nevertheless, cell division may not be an absolute prerequisite for radicle protrusion, as seeds are able of completing germination when imbibed in a cell division inhibitor (hydroxyurea). However, under such conditions radicle protrusion is delayed and further growth of the seedling is severely retarded. This suggests that cell division is indeed required prior to radicle protrusion for the “normal” completion of

germination and subsequent seedling development.

When visible germination is inhibited by an osmoticum (priming), the cell cycle is arrested in G2 phase, not allowing subsequent cell division. This allows the synchronisation of the radicle tip cells, which appears to be related to the enhanced germination performance of the primed seeds (Heydecker and Coolbear, 1977; Argerich and Bradford, 1989). Furthermore, the appearance of the cortical microtubular cytoskeleton in primed embryos reflects the occurrence of cell elongation during priming, as observed in lettuce seeds (Cantliffe et al., 1984). Repair of DNA, rRNA and protein synthesis plays a role in the embryo of leek during priming (Bray et al., 1989; Davison and Bray, 1991). These processes may all play a role also in priming of tomato seeds, in addition to weakening of the endosperm cap (Toorop, 1998). The detection of BrdU incorporation in dry primed seeds after a 3h pulse labeling, reflects the tolerance of S-phase nuclei to desiccation (Osborne and Boudriak, 1997; Boubriak et al, 1997) as DNA repair has occurred during priming.

Cell cycle related processes are suppressed in dormant seeds. However, minor differences are observed. The depth of dormancy, which distinguishes primary and secondary dormancy, may depend on the progression of DNA synthesis activity prior to the induction of dormancy. Primary dormancy is induced during development (Chapter 4) at a phase when DNA synthesis activity is no longer detectable. However, in tomato it was induced in the presence of an elaborate cortical microtubular cytoskeleton (Chapter 6). Secondary dormancy, which is more "shallow", is imposed by far-red irradiation of seeds that may show DNA synthesis and cortical microtubules during imbibition. This indicates that DNA synthesis activity is a determinant of the depth of dormancy. Secondary dormancy may be induced after the germination process has proceeded to a certain extent. The point of no return appears to be determined by the progression of the cell cycle. This was clearly demonstrated with the escape experiment, as well as with the *sit*^w mutant, which was almost completely insensitive to the far-red inhibition (Chapter 6).

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Summary

Seeds are complex biological structures and the primary dispersal units of higher plants. They consist of nutrient reserve storage tissue(s), an embryo and encapsulating structures designated for protection and that may also regulate germination. Seeds have developed mechanisms of withstanding desiccation without losing the ability of immediate reactivation of embryo growth upon rehydration.

The arrest and reactivation of cell cycle related events appear to be intimately linked to the arrest of growth during seed development and reactivation of growth during germination. In this thesis, DNA synthesis, β -tubulin accumulation and appearance of microtubular cytoskeleton are studied in relation to morphological and physiological events that are involved in seed development and germination. Biochemical techniques are used to study the relation between DNA replication and β -tubulin accumulation in embryonic cells of germinating seeds (Chapters 2 and 3). Then, immunocytochemical techniques are applied for the detection and visualization of DNA synthesis activity and appearance of microtubular cytoskeleton in seed tissues during development and in embryos during germination (Chapters 4, 5 and 6).

The results obtained in this study show a general pattern of tomato seed development and germination based on the quantitation and distribution of nuclear DNA synthesis activity, β -tubulin accumulation and microtubular cytoskeleton appearance in cells of the seed tissues. The pattern in embryos during seed germination appeared as a "mirror image" of that during development (Chapters 4 and 5). Cell cycle activities are intense during early stages of seed development and then arrest as seeds become mature. Embryonic DNA synthesis activity, together with the presence of mitotic microtubular cytoskeleton arrays and cell divisions define the period of embryo histodifferentiation. Thereafter, the presence of cortical microtubular cytoskeleton and absence of DNA synthesis activity defines the phase of embryo growth and completion of morphogenesis. Finally, the microtubular cytoskeleton is degraded, as seeds become quiescent, defining the maturation phase. The roles of GA and ABA in seed development are evaluated in the differing developmental patterns observed for the GA-deficient (*gib1*) and ABA-deficient (*sir^{wt}*) mutants. Upon imbibition in water, the cell cycle is reactivated and becomes intense as germination is completed. Initially, the cortical microtubular cytoskeleton appears and DNA synthesis is initiated in meristematic cells of embryonic radicle tip region. Thereafter, replicated nuclear DNA is detected together with mitotic microtubular cytoskeleton arrays and cell divisions, prior to radicle protrusion through the seed coat. Both primary and

secondary dormancy is characterized by a very low cell cycle activity. The depth of dormancy appears to be related to the progression of the cell cycle prior to the induction of dormancy (Chapter 6).

Finally, in the form of a descriptive model, it is proposed to modify the current notion of the different phases in tomato seed development and germination.

Samenvatting

Zaden zijn complexe biologische structuren die de primaire verspreidingseenheid vormen van de hogere plant. Zij bestaan uit opslagweefsels met reservevoedsel, een embryo, alsmede omhullende weefsels ter bescherming van het embryo en regulatie van de kieming. Zaden hebben mechanismen ontwikkeld waarmee zij bestand zijn tegen uitdroging zonder verlies van het vermogen om na hydratatie onmiddellijk actief te worden.

De de- en reactivatie van processen die samenhangen met de celcyclus lijken nauw verbonden te zijn met de stopzetting van groei tijdens de zaadontwikkeling en hervatting van de groei tijdens de kieming. In dit proefschrift wordt de activiteit van DNA synthese, accumulatie van β -tubuline en voorkomen van microtubulair cytoskelet onderzocht in relatie tot morfologische en fysiologische gebeurtenissen die betrokken zijn bij de zaadontwikkeling- en kieming. Biochemische technieken worden gebruikt om de relatie tussen DNA-replicatie en de accumulatie van β -tubuline in embryocellen van kiemende zaden te bestuderen (Hoofdstukken 2 en 3). Vervolgens worden immunocytochemische technieken aangewend voor de detectie en het zichtbaar maken van DNA synthese en voorkomen van microtubulair cytoskelet in de zaadweefsels tijdens de ontwikkeling, en in embryo's tijdens de kieming (Hoofdstukken 4, 5 en 6).

De verkregen resultaten laten een algemeen patroon van de ontwikkeling en kieming van tomatenzaad zien, gebaseerd op de kwantificering en verdeling van DNA synthese activiteit in de celkernen, β -tubuline accumulatie, en het voorkomen van microtubulair cytoskelet in de cellen van de diverse zaadweefsels. Het patroon in embryo's tijdens de kieming is een 'spiegelbeeld' van het patroon tijdens de ontwikkeling (Hoofdstukken 4 en 5). De activiteit van de celcyclus is hoog tijdens de eerste fasen van zaadontwikkeling maar verdwijnt wanneer het zaad rijp wordt. De periode van embryonale histodifferentiatie wordt gekenmerkt door DNA synthese, de aanwezigheid van mitotische configuraties van het microtubulaire cytoskelet en celdelingsactiviteit. Daarna geven de aanwezigheid van corticale microtubulair cytoskelet en de afwezigheid van DNA synthese de fase aan van embryogroei en de vervolmaking van de morfogenese. Uiteindelijk wordt het microtubulaire cytoskelet afgebroken als het zaad in rust gaat en in de rijpingsfase terechtkomt. De rol van GA en ABA in de zaadontwikkeling kan worden afgeleid uit de verschillen in ontwikkelingspatronen die worden waargenomen in de GA-deficiente (*gib1*) en ABA-deficiente (*sir*^m) mutanten.

Na imbibitie in water wordt de celcyclus gereactiveerd en wordt intenser naarmate het moment van zichtbare kieming dichterbij komt. Aanvankelijk wordt het corticale

microtubulair cytoskelet zichtbaar en wordt DNA synthese geïnitieerd in meristematische cellen van het embryonale wortelpuntje. Daarna wordt gerepliceerd DNA gedetecteerd, samen met mitotische configuraties van het microtubulaire cytoskelet en celdelingen in het gehele embryo, alvorens het kiemworteltje door de zaadhuid naar buiten komt. Zowel primaire als secundaire kiemrust worden gekenmerkt door een zeer lage activiteit van de celcyclus. De diepte van de kiemrust lijkt te zijn gerelateerd aan de mate van progressie van de celcyclus voorafgaand aan de inductie van kiemrust (Hoofdstuk 6).

Tenslotte wordt, in de vorm van een beschrijvend model, voorgesteld het heersende beeld van de verschillende fasen in de zaadontwikkeling en -kieming aan te passen.

Sumário

Nesta tese, eventos do ciclo celular são analisados funcionalmente em relação a eventos fisiológicos identificados durante o desenvolvimento e germinação de sementes. O estudo foi conduzido em sementes de tomate, um "sistema modelo" para estudos de fisiologia em sementes.

Sementes são estruturas complexas e compõem a principal unidade de dispersão das plantas superiores, sendo geralmente constituídas de tecido(s) para reserva de nutrientes, um embrião e estruturas de revestimento designadas para proteção do embrião, e que também podem regular a germinação. O mecanismo de tolerância a dessecação foi desenvolvido pelas sementes, sem que houvesse perda da capacidade de reativação imediata do crescimento do embrião durante a embebição em água. A inativação e reativação de eventos relacionados ao ciclo celular parece estar intimamente ligado a interrupção do crescimento durante o desenvolvimento da semente, e reativação do crescimento durante a germinação.

Nesta tese, a síntese de DNA, o acúmulo de β -tubulina e o surgimento do esqueleto celular, ou cito-esqueleto, são estudados em relação a processos morfológicos e fisiológicos envolvidos no desenvolvimento e germinação de sementes. Técnicas biomoleculares são utilizadas na análise da duplicação do DNA em relação ao acúmulo de β -tubulina em células embrionárias durante a germinação (Capítulos 2 e 3). E técnicas imuno-citoquímicas são utilizadas na detecção e visualização da síntese de DNA e formação do cito-esqueleto nos vários tecidos da semente durante o desenvolvimento, e no embrião durante a germinação (Capítulos 4, 5 e 6).

Os resultados obtidos em semente de tomate, mostram um padrão específico de desenvolvimento e germinação, baseado em quantificação e distribuição das atividades de síntese de DNA, acúmulo de β -tubulina e formação do cito-esqueleto em células dos diferentes tecidos da semente. O padrão no embrião, observado durante a germinação (Capítulos 2, 3 e 5), reflete o oposto do padrão observado durante o desenvolvimento da semente (Capítulo 4). As atividades do ciclo celular, são intensas em fase inicial do desenvolvimento da semente, mas em seguida há uma redução e um bloqueio completo destas atividades em fase final de maturação. A detecção de atividade de síntese de DNA nas células do embrião, juntamente a presença de cito-esqueleto em forma de fusos mitóticos e divisões celulares, definem o período inicial de histo-diferenciação embrionária. Posteriormente, o cito-esqueleto é detectado em sua forma cortical somente, sem atividade

de síntese de DNA e divisões celulares. Desta forma, durante esta fase, o crescimento do embrião parece acontecer exclusivamente por expansão celular, quando então são finalizadas as mudaças morfogênicas que levam a formação completa do embrião, concluindo-se o processo de embriogênese. Finalmente, o cito-esqueleto é degradado e a semente entra o estado de repouso, ou quiescência, definindo a fase de maturação da semente.

O presente estudo também mostra a influência de giberelinas (GA) e ácido abscísico (ABA) sobre o desenvolvimento da semente de tomate, em relação às atividades do ciclo celular. Para isto, foram analisados os padrões de desenvolvimento de sementes de dois mutantes, um deficiente em giberelina (*gib1*) e outro em ácido abscísico (*si^w*). Os resultados mostram um padrão de desenvolvimento alterado em sementes destes mutantes.

Durante a embebição da semente, o ciclo celular é reativado, tornando-se intenso com o progresso e ocorrência da germinação, ou protrusão da radícula. Inicialmente, é detectado a formação de cito-esqueleto cortical e síntese de DNA nas células embrionárias da ponta da radícula. Posteriormente, é possível detectar DNA em sua forma duplicada, e cito-esqueleto em forma de fusos mitóticos, caracterizando ocorrência de divisão celular, antes da protrusão da radícula através do tegumento. Considerando-se dormência, tanto a dormência primária, quanto a secundária são caracterizadas em termos de uma baixa atividade de ciclo celular. Contudo, os resultados mostram que o "grau" de dormência parece estar relacionado à ocorrência e progresso das atividades do ciclo celular, principalmente síntese e duplicação de DNA, antes que a dormência seja induzida (Capítulo 6).

Finalmente, um modelo descritivo é proposto para modificação da atual noção das diferentes fases de desenvolvimento e germinação em sementes de tomate.

Curriculum vitae

R.D. de Castro

Renato Delmondez de Castro was born on October 13, 1965 in Brasília, which is the capital of Brazil and is situated in the geographically central point of the country. In December 1983 he obtained his certificate for the secondary level education in Brasília, and started in January 1985 to study Agronomy at the Universidade Federal de Lavras (UFLA), in Lavras. As a reference, Lavras is a small town, about 1000 Km from Brasília, located in the south of the state of Minas Gerais, south east region of Brazil, neighbouring the states of Rio de Janeiro and São Paulo. There he graduated in December 1989, and initiated in the following year his postgraduate program at the same University, receiving his Master degree of Agricultural Sciences, subject area Seed Science, in May 1992. In Lavras, he also found the person who would follow him on his journey in life, Suely Ribeiro de Carvalho, to whom he married.

In November 1993 he started with his Ph.D. research on seed physiology, involving the analysis of cell cycle events in seeds, but at this time much further away from his original home town, across the Atlantic in the northern hemisphere, at the Wageningen Agricultural University (WAU), in Wageningen, The Netherlands. His project involved the cooperation between the Laboratory of Plant Physiology - WAU and the Department of Reproduction Technology of the Centre for Plant Breeding and Reproduction Research (CPRO-DLO). His research project was embedded in the Graduate School of Experimental Sciences, and the results are discussed in this thesis. Also as a "result" of the living in Wageningen together with Suely, but not as part of the research project, Luca Carvalho de Castro was born.

Back in Brazil, Renato's plan is to submit a project proposal to the Brazilian government's research funding agencies. In this project he intends to continue working on the analysis of cell cycle related events in relation to seed physiology. He intends to carry out this project in cooperation with UFLA and the Dutch institutions, during a period of 2 to 3 years. In the mean while, Renato will be searching for job opportunities.

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