Hormones and tomato seed germination

Yongqing Liu

Promoters: **Dr. C.M. Karssen**
Professor of plant physiology
Dr. Zemin Luo
Professor of biochemistry, Hunan Agricultural University, P.R. China

Co-promoters: **Dr. R.J. Bino**
Head of the Department of Reproduction Technology, Centre for Plant Breeding and Reproduction Research (CPRO-DLO)
Dr. H.W.M. Hilhorst
The Department of Plant Physiology

Propositions (Stellingen)

1. Modern agriculture demands a fast, uniform and high germination of seed to get high-quality stand establishment of field seedlings. Studies on the mechanisms and regulation of germination are a essential for agricultural practice.
   - *This thesis*

2. In tomato seeds visible germination occurs when the potential of embryo growth exceeds the restraint imposed by the surrounding tissues.
   - *This thesis*

3. Seed dormancy is a consequence of the interaction between internal factors like plant hormones and external factors such as light, temperature and moisture.
   - *This thesis*

4. The recorded effects of priming on the storability of seeds are contradictory. One of the possible reasons for this contradiction is that the action-mechanism of priming is not well known.
   - *This thesis*

5. The arrest of cell cycle activities during seed development indicates that maturation has started, whereas the reactivation of the cell cycle upon imbibition can be considered as an indication of the breakage of dormancy and the induction of germination.
   - *This thesis*

6. The influences of gibberellins and abscisic acid on water relations of tomato seeds are complemented by weakening of the endosperm tissue rather than by cell wall loosening of the radicle.
   - *This thesis*

7. Partners can cooperate well when they need each other equally, otherwise a balancing mediator is required.

8. Nature is a wonderful teacher, and a seed is one of the simplest teaching aids.

- Award A. Khan, *Horticulture Science Review* 1991, 13: 131-181

9. In China there is a popular saying 'truth or false can only be told by practice'. In the Netherlands the phrase 'learning by doing' is often heard. Both proverbs show that experience is the most reliable and non-culture-based way to gain knowledge.

10. A logical imagination, an applicable protocol and hard work are necessary tools to acquire an advantageous position in scientific exploration.

11. Persons of similar intelligence, capability and interest behave like elements with similar physical and chemical properties which act antagonistically in a confined system.

12. Frustrations and adverse circumstances either put one downhearted or further initiate one's potential.

13. Success depends on the opportunity one receives (from the heaven), the method one uses (on the earth) and the personality one shows (to the people).

14. What is more important: money or reputation? the answer will distinguish the capitalist from the socialist.

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Hormones and tomato seed germination

Yongqing Liu

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Cover figure: Seed a dream (Chinese handwriting by the author)

Preface

Truly, this thesis could never have been completed without a full cooperation between the Department of Plant Physiology, Wageningen Agricultural University and the Department of Reproduction Technology, Centre for Plant Breeding and Reproduction Research (CPRO-DLO) in the Netherlands. Here, I would like to take this opportunity to express my sincere appreciation to all the people who helped me in one way or another. Firstly, I deeply thank my promotor, professor Cees M. Karssen for his kindly offering me such opportunity to do a PhD program under his supervision in the Netherlands. I will never forget our first meeting in his office in August 1992, where the subject of the thesis was determined. I very much appreciate that he still spent some time to give me in-time guidance at the key points during my study though his agenda had been always full since he became the rector magnificus of Wageningen Agricultural University in 1993.

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I am most deeply indebted to Dr. Raoul J. Bino. It is him that kindly accepted me as a visiting researcher in the Department of Reproduction Technology, Centre for Plant Breeding and Reproduction Research (CPRO-DLO) in October 1991, and appointed Ir. W.J. van der Burg as my first Dutch supervisor in the technology section, where I learnt some basic techniques for seed technological and physiological research and finished my first paper in collaboration with Mr. J.W. Aartse, Mr. R.A. van Zwol and Dr. H. Jalink. It is him that tried hard to find financial support for me in the Netherlands and arranged me in the physiology section to continue my investigations under his supervision in CPRO-DLO, resulting in three other papers in collaboration with Mr. J.H.W. Bergervoet, Dr. C.H. Ric de Vos, Dr. H.L. Kraak and Dr. S.P.C. Groot. No words could really express my gratitude for what he has done for me. I very much appreciate the way he thinks, the way he communicates and the way he integrates all things together. His intelligence and wisdom, his sophisticated words and behavior and his attitude to the people have deeply impressed me, and will considerably influence my future scientific career as well as my personal life.

I owe a great deal to Mr. Jaap G. van Pijlen, not only for his help in preparation of test materials, chemicals and facilities for my experiments but also for his humorous manner which always creates a pleasant atmosphere for the people working with him in the Department of Reproduction Technology, Centre for Plant Breeding and Reproduction Research (CPRO-DLO).

It is acknowledged that all my studies in the Netherlands were made possible both by the financial support from Wageningen Agricultural University and Hunan Agricultural University (a sandwich fellowship), the Netherlands Ministry of Agriculture, Nature and Fisheries (IAC fellowship), the Ministry of Education and Science (NUFFIC fellowship) and

the State Education Commission of The People's Republic of China (from October 1991 to August 1992), and by the supply of many facilities in the Department of Plant Physiology, Wageningen Agricultural University and in the Department of Reproduction Technology, Centre for Plant Breeding and Reproduction Research (CPRO-DLO).

I will always be grateful to my mother university, Hunan Agricultural University, for giving me the chance to study in the Netherlands and maintaining my position and salary in the university during my absence. My sincere thanks are also given to my Chinese supervisor, professor Zemin Luo, and my former adviser, professor Meijuan Shen, Hunan Agricultural University, P.R. China, for their continuous encouragement on my research work and critical discussions on my papers.

My final thanks certainly go to my parents, my brothers, particularly my dear wife Jinlian Tan, my lovely son Qian Liu, for their fully understanding, support and continuous encouragement. Their love companies me wherever forever.

Christmas eve 1995, Wageningen Yongqing Liu

Contents

Abstract

Using GA- and ABA-deficient mutants, exogenous gibberellins (GAs), abscisic acid (ABA) and osmoticum, we studied the roles of GAs and ABA in the induction of cell cycle activities, internal free space formation and changes in water relations during seed development and imbibition in tomato. First of all, it was demonstrated that endogenous ABA plays an important role in induction of primary dormancy, prevention of viviparous germination and arresting cell cycle activities of the radicle-tip cells in G_1 phase upon maturation. Secondly, the flow cytometric analysis of seeds of the three genotypes upon imbibition in water, GA_{4+7} or ABA solutions, revealed that GAs influence germination of intact seeds by acting upon the processes that precede cell cycle activation, or in other words, the weakening of the endosperm opposite radicle tip, induced by GAs, promotes cell growth and subsequently induces nuclear replication activity upon imbibition, while ABA affects growth by acting upon the processes that follow cell cycle activation. Studies with the controlled deterioration test (CD) show that, after osmotic priming, the advancement of germination of seeds, expressed as the ratio of 4C nuclei of radicle-tip cells, is positively related with the germination rate, and negatively related with the storability of the treated seeds. In addition, the determination of water relations illustrates that the absence of GAs and ABA during development and imbibition may alter water relations of both fruit and seed tissues, which certainly contributes to the mechanism preventing precocious and viviparous germination. Finally, it is concluded that exogenous GAs and ABA can relieve or strengthen the mechanical restraint imposed by the tissues surrounding the embryo, upon embryo water uptake, resulting in promoting or inhibiting germination by GAs and ABA respectively.

Abbreviations

ABA	abscisic acid
CD	controlled deterioration
C value	constant DNA value of a haploid genome
DAP	days after pollination
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
FP	fresh PEG priming
FP+G	fresh PEG plus GA priming
G_0	quiescent phase of cell-cycle
G_1	pre-synthesis phase of cell-cycle
G_2	post-synthesis phase of cell-cycle
GAs	gibberellins
GA_{4+7}	mixture of the gibberellins GA_4 and GA_7
gib-1	gibberellin-deficient mutant
LSD	the least significant differnece
mRNA	message ribonucleic acid
NP	normal PEG priming
NP+G	normal PEG plus GA priming
PEG	polyethylene glycol
RNA	ribonucleic acid
SE	standard error
sit^w	abscisic acid-deficient mutant
T_{80}-T_{20}	days between 20% and 80% of maximum germination
T_{50}	days of 50% maximum germination
wt	wild type
ψ	water potential
ψ_n	osmotic potential
ψ_p	pressure potential

Chapter 1

General introduction

Tomato seeds

Tomato (*Lycopersicon esculentum* Mill.) is one of the most important annual vegetables in the world. Many new varieties are created each year by intensive breeding efforts to meet the different requirements all over the world. Common tomato plants are diploid and reproduced by seeds. Newly bred varieties are almost all hybrids normally produced by artificial crossing. Therefore, high-quality seeds are required to match these high-cost hybrids to ensure their authentic value. Considerable efforts have been made to improve seed quality during development and after harvest (Heydecker and Coolbear 1977, Khan 1991). More profound knowledge on the physiological and biochemical changes and their interactions during development, pre-treatment and germination will certainly contribute to the improvement of seed quality (Khan 1991). A tomato seed contains a fully developed embryo that is enclosed by a considerable amount of endospermal tissue and a thin seed coat with seed hairs (Smith 1935). Compared with the seed of *Arabidopsis thaliana*, a model plant species for scientific research, a tomato seed has the advantage of a fairly large size which enables the study of the location of physiological and biochemical activities in different seed parts during development and germination (Groot 1987).

Embryogenesis

In tomato, the zygote starts to divide into an embryo proper and a suspensor at 5 days after pollination (DAP). Then the embryo proper grows very fast whereas suspensor cells almost cease to proliferate, resulting in a ball-shaped embryo at 10 DAP. The young embryo continuously grows and reaches the heart-shape stage at 15 DAP and the torpedo-shape or cotyledon-embryo stage at 20 DAP. The suspensor handle which anchors at the micropylar end, never disappears until the embryo has fully developed with long curved cotyledons at 30 DAP. At the same time, the fertilized central cell or the endosperm nucleus starts to divide and occupies the space where the nucellus tissue originally exists. Then, the developing seed starts to dehydrate. The dry weight of the developing seed continuously increases until 40 DAP when the milky endosperm becomes solid. At the same time, the seed gains its desiccation-tolerance and germinability. Thereafter, seed maturation continues until the acquisition of full germinability at 50 DAP or even later (Berry and Bewley 1991, Demir and Ellis 1992). However, under some climatic conditions, and in certain genotypes, tomato seeds may have primary dormancy at the end of the maturation period (Groot and Karssen 1992).

Dormancy

Dormancy is defined as the disability of seed to germinate under favourable environmental conditions. Dormancy is of ecological significance for seed species to survive in the wild (Hilhorst 1995). Although in seed physiology many terms are in use to classify dormancy, a simple, generally accepted classification factually distinguishes two types of dormancy: primary or secondary dormancy (Karssen 1982). This classification is meaningful because primary dormancy is essentially related to seed development and maturation whereas

secondary dormancy can only occur after the seed is dispersed and is often subject to the annual dormancy cycle in the seed bank and is reversible. When primary dormancy is relieved and suitable conditions are present, germination may occur. If germination does not occur, secondary dormancy may have been induced. Secondary dormancy can be relieved and induced again many times from year to year (Fig. 1). In the dormant state, seeds have low metabolism and are insensitive to the environment, thereby, prolonging the seed's life-span (Bewley and Black 1994).

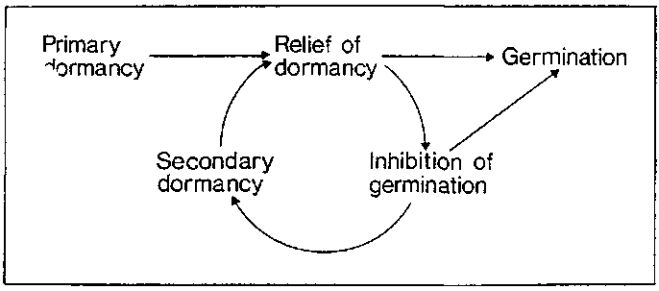

Figure 1. A schematic diagram of the cycle in which seed dormancy is induced and relieved. After Karssen (1982).

In different seed species, dormancy may be induced through different mechanisms (Hilhorst 1995). The mechanism of dormancy is not fully understood. General speaking, dormancy is a consequence of the action of the internal factors like plant hormones, interacting with external factors such as light, temperature, moisture and inorganic chemicals. The presence of the hormone ABA, as a general growth inhibitor, has long been associated with the induction of primary dormancy during seed development (Hilhorst and Karssen 1992). Characteristically, the ABA content increases during the first half of fruit development and declines at the later phase of maturation, when the seed water content decreases (Bewley and Black 1994). ABA is produced in leaves and transported into fruit tissues and seed parts (Goldbach and Goldbach 1977, Downey and McWha 1979). Apart from the induction of dormancy, ABA is thought to prevent precocious germination (Farrant et al. 1993, Finch-Savage et al. 1992). As an alternative hypothesis it was suggested that ABA may be indirectly involved in the prevention of viviparous germination by increasing the sensitivity of seeds to the osmotic environment (Groot and Karssen 1992).

In some cultivars like Moneymaker, tomato seeds may manifest primary dormancy after maturation under certain cultivation conditions (Groot 1987). The dormancy in tomato seeds is not a true dormancy (no germination at any set of environmental conditions), but a relative dormancy (only germinate at a restrict range of environmental conditions).

Studies with the ABA-deficient tomato mutant (*sit*^{w}) showed that ABA plays an important role in the induction of primary dormancy (Groot and Karssen 1992). This primary dormancy in wild type tomato seeds can be relieved by a dry storage period (Groot 1987). Since secondary dormancy in tomato seeds can be induced by far-red light and retained in the dark, this particular type of dormancy is related to phytochrome action (Lercari 1991). Dormancy in many seed species can be relieved or broken by these physiological treatments (Khan 1991).

Osmotic priming

After harvest, seed quality in many crops can be increased by physiological treatments that are based on seed hydration such as presoaking, wetting, humidification, osmotic priming, solid matrix priming and pre-germination (Khan 1991). Of all the physiological treatments, osmotic priming, i.e. seed hydration at a low water potential while preventing radicle protrusion, followed by dehydration, has been most intensively studied (Heydecker et al. 1973). Since tomato seeds are of a great interest to the vegetable seed industry, many efforts have been made to optimize the priming procedures (Alvarado and Bradford 1988, Argerich and Bradford 1989, Dahal and Bradford 1990) in combination with a low temperature (Coolbear et al. 1987), growth regulators (Finch-Savage and McQuistan 1991), irradiation and seed coating (Khan 1991). Many approaches have been used to reveal the 'priming' mechanism that allows the seed to achieve the beneficial changes that are consistent with the improvements of rate and uniformity of seedling emergence. One of the hypotheses to explain the positive effects of priming on germination performance is the induction of biochemical repair mechanisms during the pre-imbibition. This repair activation may relieve the damages to some macromolecules, which are acquired during storage (Rao et al. 1987). Unfortunately, these positive results may be followed by a reduction in storage life (Argerich and Bradford 1989). The recorded effects of priming on the storability of seeds are somewhat contradictory (Argerich and Bradford 1989). For instance, Georghiou et al. (1987) reported that in sweet pepper (*Capsicum annuum* L.), osmoconditioning in a 0.4 M mannitol solution for 4 d considerably delayed ageing and increased seed longevity. One of the possible reasons for this contradiction is that the mechanisms behind the priming procedure are far from well known. It is rational to hypothesize that the improvement of storability after priming should be related to the biochemical repair mechanism, while a decline of storability may be paralleled with the progression of the germination processes during the pre-imbibition period. Therefore, in fact, the priming mechanism(s) can not be fully understood without understanding the germination mechanism(s).

Germination

Upon maturation, the quiescent seed is a complex of old and new generations. The tissues from the fertilized cells (embryo and endosperm) are from the new generation and are entirely protected by the maternal tissues (seed coat and pericarp). After shedding, the

quiescent seeds start to germinate under the required environmental conditions. In many species, seed germination is determined by the maternal tissue. Seed manifests some sorts of sensors that can perceive and respond to the environmental changes (Bewley and Black 1994). The variation in response to the natural conditions within a seed population results in a wide range of germination times. However, modern agriculture demands a fast and uniform stand establishment with a high germination. Therefore, studies on the mechanisms and regulation of the germination response form an essential basis for agricultural practice.

Seed germination encompasses the initiation of growth of a previously quiescent or dormant embryo. For most seeds, germination begins with the imbibition of water and ends with radicle protrusion. Imbibition is generally a triphasic process, with an initial rapid water uptake, followed by a plateau phase with a little change in water content, and a subsequent increase in water content coincident with radicle growth (Bradford 1986). Together with imbibition, the seed metabolism is soon activated (Osborne 1983). At the beginning of imbibition, an amount of solutes leak out of the imbibing seed into the imbibition medium due to the disorganization of cell ultrastructure and the disruption of the cell membrane during its transition from a crystaline phase to a gel phase (Bewley and Black 1994). This disorganization or disruption may seriously deteriorate the seed (Bewley and Black 1994). However, the leachate that contains some germination inhibitors like ABA, may facilitate germination as well (Abdul-Baki and Stoner 1978). The first enzymatic activity which is provoked by the seed is directed towards the repair of damages which have accumulated during maturation, storage and imbibition (Osborne 1983). Gradually, the syntheses of proteins, RNAs and DNA, and nuclear replication or cell division in some seed species occur before visible germination (Bino et al. 1993, Elder and Osborne 1993, Weges 1987). All these metabolic activities in combination with water uptake by the seed, result in the growth of embryo. Visible germination occurs when the potential of the embryo growth exceeds the restraint as imposed by the surrounding tissues.

In tomato seeds, the completion of germination is considered to be dependent both upon the expansion force of the embryo and the occurrence of endosperm weakening (Bradford 1990, Karssen et al. 1989). The mechanical restraint of the endosperm layer opposite the radicle tip can be measured by an Instron 1122 Universal testing instrument (Groot and Karssen 1987). Using this method it was found that the endosperm is weakened prior to visible germination, which can be ascribed to the activities of a series of hydrolytic enzymes (Groot et al. 1988). The hydrolytic enzyme activity is stimulated by GAs excreted from the radicle tip The degree of reduction in the restricting force against the embryo growth, imposed by the endosperm, is the key for control of seed germination. However, Liptay and Schopfer (1983) suggested that the difference in germinability between two genotype lines resided in the germinating embryo, or in other words, the embryo growth mainly determines seed germination. Apparently, embryo growth and endosperm weakening may play different roles in germination, and their relationship needs further clarification.

Cell cycle

Seed germination is the start of the growth of a new plant. Growth simply depends upon cell division, enlargement and differentiation. The sequence of processes occurring during cell division is often referred to as the cell cycle. Within the cell cycle four different phases can be distinguished (Fig. 2). DNA replication occurs during interphase, known as S phase. Before and after the S phase, interphase cells are engaged in growth and metabolic activities. These two phases are respectively called the G_1 and G_2 phases of the interphase. G_2 is normally followed by a cell division or mitosis (metaphase or M phase). In diploid somatic cells at G_1 phase, the nuclei contain the 2C DNA value, whereas the nuclei in G_2 phase have 4C DNA value. Here, 'C' stands for the 'constant value' of the DNA content in the haploid tissue. After mitosis, divided cells re-enter the G_1 phase.

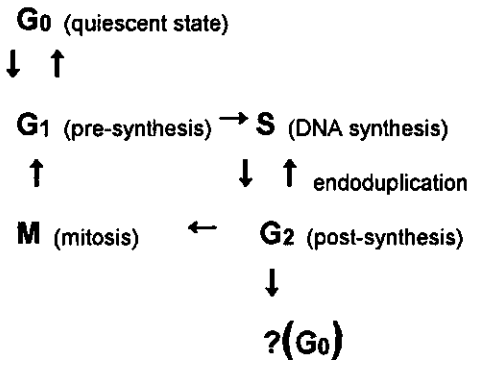

Figure 2. A schematic diagram of the cell cycle in higher plants

Little is known about cell cycle activities during seed formation, physiological priming and germination processes. In most orthodox seed species, after maturation or shedding, the water content of a dry seed is too low for the cell cycle to proceed (Bino et al. 1992). In dry seeds, nuclear replication stages have been identified using autoradiography and Feulgen staining, and it was found that embryos of some species contained both 2C and 4C nuclei, while others solely comprised 2C nuclei (Bewley and Black 1994). Flow cytometry with the use of double-strain DNA-specific fluorescent dyes has offered possibilities to quickly determine the DNA amount of large numbers of nuclei. Bino and his colleagues (1992, 1993) have used this technique to quantify the amounts of nuclear DNA with high accuracy in various seed species and seed parts. In dry tomato embryos, most nuclei were found to arrest in the G_1 (Bino et al. 1992).

The expression of phytohormone responsive genes

In recent years a great effort has been made in our knowledge on the functions of phytohormones at the molecular level. Indeed, many phytohormone response genes have

been identified. In particular, the expression of ABA and GAs responsive-genes has been intensively studied in respect to seed maturation and germination. During seed maturation, the expression of certain ABA response genes was found to be correlated with the development of desiccation tolerance. For instance, in maize (*Zea may* L.), viviparous (nondormant) mutant seeds, which lack ABA or do not respond to it, neither fully desiccate nor accumulate certain ABA response gene *lea* (late embryogenesis abundant) products (Kriz et al. 1990). Some ABA-responsive genes are also responsible to environmental stresses such as osmotic stress, high temperature and desiccation etc. As ABA levels increase in the tissue subjected to the stress, it is often considered that the expression of the ABA-responsive genes induced by the stress, is mediated by the increase of ABA (Henson 1984). During germination in cereal seeds, GA induces the expression of genes necessary for utilization of the stored seed reserves and for seedling growth. At this time the effects of ABA on gene expression are generally antagonistic to those of GA. For example, application of exogenous ABA to germinating cereal seeds sharply reduces levels of GA-responsive hydrolase mRNAs and proteins. This is presumably due to the ABA-mediated induction of factors that inhibit hydrolase transcription and/or translation (Nolan and Ho 1988, Rogers 1988). This inhibition may also occur at the level of hydrolase activity: in some cereals, ABA promotes the accumulation of a protein that inhibits germination-specific amylase isozymes (Leah and Mundy 1989). Recent studies have shown that some GA-responsive genes also respond to ABA (Hattori et al. 1992, Urao et al. 1993). The mutually antagonistic effects of these two hormones may be interpreted at the level of gene expression (Gubler et al. 1995).

Hormone-deficient mutants

Cytokinins, auxins, gibberellins, abscisic acid and ethylene all play important roles both in plant growth and development and in seed maturation and germination (Bewley and Black 1994). In the past decades, the majority of studies on hormonal regulation of seed development and germination were based on correlations between either the application of exogenous growth regulators, or the simultaneous occurrence of peak value of the endogenous hormone content, and the morphological, physiological and biochemical changes. In fact, these types of correlations neither excluded the possibility that the application of exogenous growth regulators artificially changed the normal growth and developmental processes, nor eliminated the possibility that hormones are accidentally involved in the specific aspects of seed development and germination. Therefore, to deal with the regulation of plant hormones in seed development and germination, two other approaches, are frequently adopted in recent years: (1) the use of hormonal synthesis inhibitors to reduce the hormone content *in situ*, and (2) the use of hormone deficient or responsiveness mutants. Particularly, the latter approach offers a deliberate means of elucidating the regulatory functions of plant hormones. This method has been successfully applied in studies on the roles of endogenous GAs and ABA in the development and germination of *Arabidopsis thaliana* and tomato seeds (Groot and Karssen 1987, Groot and

Karssen 1992, Karssen and Łaçka 1986, Ooms et al. 1993).

In recent years, seeds in several plant species were chemically mutated and some mutants which lack the capacity to synthesize, or are insensitive to specific hormone, were selected by screening the botanical traits specific to the hormone deficiency (Reid 1990). Using the mutants it was found that in seeds of *Arabidopsis thaliana* and tomato, GAs are absolutely required for the germination (Koornneef and Van der Veen 1980, Karssen and Łaçka 1986, Groot and Karssen 1987), while ABA is mainly responsible for the induction of primary dormancy and the prevention of vivipary (Groot and Karssen 1992, Ooms et al. 1993). Based on the germination of the GA and the GA/ABA double mutant, Karssen and Łaçcka (1986) formulated a revision of the hormone-balance theory of seed dormancy and germination, in which the role of ABA is restricted to the induction of dormancy during seed development, and in which it is postulated that GAs are active in the stimulation of germination.

In the background of the tomato cultivar (cv.) Moneymaker, the GA-deficient mutant (*gib-1*) and the ABA-deficient mutant (*sit*^{w}), were selected after treating the seeds with ethylmethanesulfonate (Koornneef et al. 1981).

GA-deficient *gib-1* seeds were selected in the M_2 population when they were not able to germinate in water but did germinate in 10 μM GA_{4+7}. GA-deficient plants are extreme dwarfs with dark green leaves, short internodes and abnormal sterile flowers. The application of GA_{4+7} promotes plant growth and restores the fertility resulting in fruit development and seed set (Groot and Karssen 1988). No significant GA-like activity was detected in extracts from *gib-1* immature fruits, though the plants were sprayed with exogenous GA_{4+7} before flowering, whereas high levels of GAs were found in the immature wild type (wt) fruits (Fig. 3) (Koornneef et al. 1990).

The *sit*^{w} genotype was selected for wilting in the M_2 population. In the turgid leaves, the ABA level of *sit*^{w} plants is about 10% of that of wild type plants (Cornish and Zeevaart 1985). In the developing seeds, ABA levels of *sit*^{w} are strongly reduced as compared to the wild type seeds. The peak of the ABA content in wild type seeds occurs around 30 DAP whereas no obvious changes in ABA levels in *sit*^{w} seed have been detected throughout development (Fig. 4). Significantly, even in the mature stage, ABA levels in seeds of the wild type are still 10-fold higher than in the *sit*^{w} genotype (Groot and Karssen 1992).

Present study

The roles of gibberellins and abscisic acid in the regulation of seed development, dormancy and germination in tomato, are studied in this thesis with the use of GA- and ABA-deficient mutants in combination with the application of exogenous GAs, ABA and osmoticum to the germination medium. Special attention is paid to the effects of GAs, ABA and osmoticum on the occurrence of free space, the induction of nuclear replication and the changes of water relations of different genotype seeds during maturation and imbibition. In Chapter 2, the internal morphological changes in seeds after osmopriming and

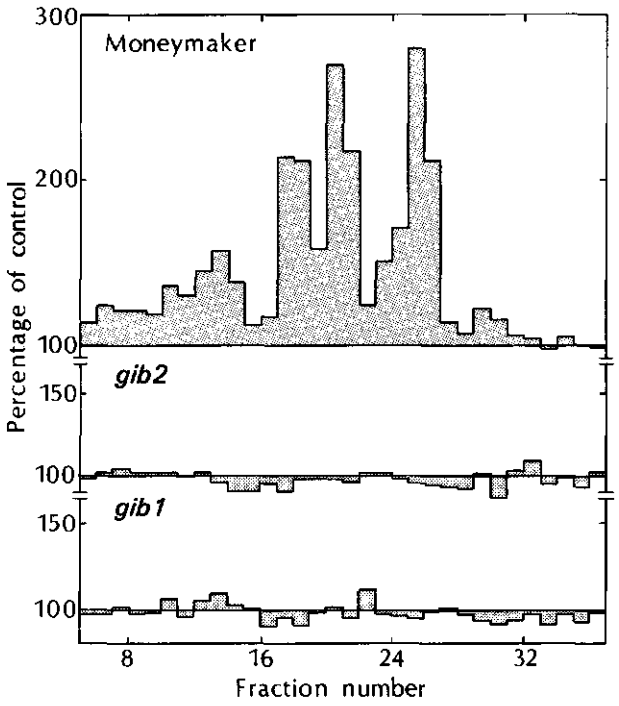

Figure 3. Response in the d-5 maize bioassay to the acidic extracts of immature wild type (cv. Moneymaker), *gib1* and *gib2* fruits. Each extract was obtained from 20 g of immature fruit. Growth of maize seedlings expressed as percentage of control (first + second leaf sheath = 41 mm). After Koornneef et al. (1990).

hydropriming are demonstrated. In Chapter 3, a newly developed osmotic priming technique, called 'fresh PEG priming', its effects on germination performance or storability, and the mechanisms in relation to the induction of cell cycle activities during priming, are presented. In Chapter 4, the regulation of the cell cycle in tomato seeds by GAs and ABA is studied during imbibition and subsequent germination. In Chapter 5, the causes of the induction of free space and nuclear replication activity are analyzed with special reference to the role of ABA in the induction of primary dormancy during maturation and the function of GAs in the weakening of the endosperm during osmotic priming. In Chapter 6, water relations of pericarp, locular tissue, intact seeds and embryos are shown during seed formation and maturation. In Chapter 7, the influences of GAs and ABA both on water relations and on water contents during imbibition of water or PEG solution with or without GAs or ABA are determined in relation to the germination performance. Finally, a model

for the regulation of the induction of dormancy and the germination progress by GAs and ABA is presented in the general discussion, Chapter 8, followed by a summary in English, Dutch and Chinese.

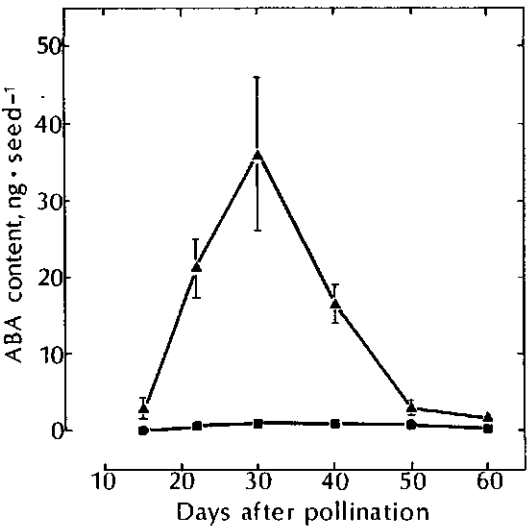

Figure 4. ABA contents of the wild type (▲) (cv. Moneymaker) and ABA-deficient mutant *sit*^{w} seeds (■) in different developing stages. Vertical bars indicates SD (n = 3). After Groot et al. (1991).

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Chapter 2

X-ray studies on changes in embryo and endosperm morphology during priming and imbibition of tomato seeds

In cooperation with W.J. van der Burg, J.W. Aartse, R.A. van Zwol, H. Jalink and R.J. Bino

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Chapter 2

ABSTRACT. Morphological changes in tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) seed during osmopriming and imbibition ('hydropriming') were followed using X-ray photographs. Embryo, endosperm and free space areas were measured. Both osmopriming and hydropriming resulted in free space development (+8.1 and +10.8% of the whole seed planar area respectively), about all at the cost of the endosperm area. Planar dimensions of whole seeds being relatively constant, measurements revealed that the dimension perpendicular to the planar surface, the thickness, could account for the volume increase of primed seeds reported in literature. In dead seeds only a small amount of free space developed while the planar area of the seed remained the same. In the imbibing viable seeds no deterioration of endosperm could be detected until the moment of root protrusion.

Seeds which were osmoprimed directly after harvest, i.e. in the fresh state, did not show the induction of any free space, while free space developed normally after dehydration and a second priming treatment. Apparently, a dehydration step prior to the priming treatments is a required for the occurrence of free space in osmo- or hydroprimed seed.

On X-ray photographs of hydroprimed seeds the radicle tip was found to adhere tightly to the endosperm cap. This results in various forms of damage to the root tips upon redrying. Priming did not introduce cotyledon abnormalities.

Key words: Embryo, endosperm, imbibition, priming, seed, tomato, X-ray photo-graphy, radiography.

INTRODUCTION

Osmoconditioning or seed priming, *i.e.* pre-sowing in an osmotic solution, followed by dehydration, was first proposed as a seed treatment by Heydecker, Higgins and Gulliver (1973). This treatment was found to enhance seed germination speed and uniformity for many species, particularly for the annual vegetable crops including tomato (Brocklehurst and Dearman, 1983; Haigh and Barlow, 1987; Alvarado and Bradford, 1988; Argerich and Bradford, 1989).

Because of the moderate seed size and the positive response to osmotic priming, tomato seed has been frequently used as test material to reveal the mechanism of seed priming in a series of studies of physiology (Groot and Karssen, 1987; Haigh and Barlow, 1987; Dahal and Bradford, 1990), biochemistry (Berry and Bewley, 1991; Bino *et al.*, 1992), and anatomy (Argerich and Bradford, 1989). Some aspects however still remain unclear.

In some cases, seed size has been found to correlate with seed germination performance. It was reported that the volume of tomato seed was increased after priming as compared to the unprimed control (Barlow and Haigh, 1987; Argerich and Bradford, 1989). However, whether the change in volume of primed tomato seed should be attributed to the

enlargement of the embryo, the endosperm or both (Argerich and Bradford, 1989), is still unknown.

The endosperm and to some extent also the seed coat opposite the radicle tip of tomato seeds are considered to be a major mechanical restraint for radicle protrusion. Correspondingly, the factors and treatments which could increase or decrease this restricting force will affect germination (Groot and Karssen, 1987; Haigh and Barlow, 1987; Bradford, 1990; Dahal and Bradford, 1990). On the other hand, the embryo is enfolded in the endosperm and has its own effects on germination, not only because the endosperm weakening is related with the hormone secretion by the embryo (Groot and Karssen, 1987) but also by the protrusion force produced by the expansion of the embryo against the endosperm (Liptay and Schopfer, 1983).

Presently some newly developed non-destructive tests (Chen and Sun, 1991) for the evaluation of seed quality may be adopted to study the effects of seed priming. Especially the X-ray photographing technique offers the possibility for non-destructive observations (Simak *et al.*, 1989). It has been demonstrated that evaluation of internal tomato seed structures with X-ray photographs is possible and that the morphology of the embryo may relate well with seedling transplant morphology (Van der Burg *et al.*, in press).

The present experiments were designed to follow physical changes of tomato seed during priming and germination.

MATERIALS AND METHODS

Seed germination. Seeds of tomato (*Lycopersicon esculentum* Mill. cv Moneymaker) were extracted from red mature fruits setting on plants growing in the greenhouse in autumn 1991. The seed slurry was put into a 16% HCl solution of equal volume under room temperature for about 30 minutes, then rinsed in tap water for 5 minutes, followed by dehydration in a 25°C oven for 2 days and in a ventilated drying drum (23°C and 32% RH) for another 2 days. These dried seeds with about 7% moisture content (fresh weight basis) were sealed in aluminium seed bags and stored at 4°C for later use.

After X-ray photographing, seeds of each treatment were sown for germination in order to establish the relationship between the physical changes and the germination characteristics. Seeds were germinated and evaluated according to the International Rules for Seed Testing (ISTA, 1985), 20-30°C, 0.2% KNO_3, on filter paper in light.

Dead seeds were obtained by incubating the seeds contained in a closed test tube, in a 125°C autoclave for 15 minutes. These autoclave-killed seeds were equilibrated in the drying drum for two days. To confirm their inability to germinate, all dead seeds were germinated as well as checked with the topographical tetrazolium test after X-ray investigation (ISTA, 1985).

Seed priming. The seed priming treatment was carried out on one layer of filter paper moistened with 5 ml -1.0 MPa polyethylene glycol (PEG)-6000 solution (Michel and

Kaufmann, 1973) in a 9 cm diameter petri dish sealed in a polyethylene plastic bag. 50 seeds for each dish were incubated in a 20°C dark cabinet. After priming the primed seeds were rinsed in tap water for 5 minutes and dried in the drying drum for 2 days as described above.

In a separate experiment, tomato seeds were placed into the priming solution directly after fresh extraction from the fruit (*i.e.* directly after the Hcl treatment and rinsing, without drying). This procedure is further referred to as 'fresh priming'.

Seed imbibition and subsequent drying ('hydropriming'), was carried out under the same conditions as with the germination tests as described above. Hydropriming was performed with tap water. After imbibition the seeds were dehydrated in the drying drum for 2 days. In a second experiment seeds were imbibed for 40 hours and subsequently dried to equilibrium moisture content as above, after which image analysis measurements were carried out.

Seed measurements. For the measurement of seed size, 3 replicates of 30 seeds of each treatment were placed on a flat surface in a line touching each other, so that the average length of either longitudinal or transverse dimensions could be determined. Seed thickness was detected one by one for the same 90 seeds of each treatment using a dial gauge. Seed weight was measured with an electronic balance of 0.0001 g accuracy using 3 replicates of 100 seeds.

Of each X-ray photograph, the area of the whole seed, the embryo and endosperm were measured using a digital tablet and the computer program Sigma Scan™ (Jandel Scientific, 1988 version 1.10), and a low-power binocular microscope. In a separate experiment, more accurate measurements of the areas were performed in which radiographs were read into the computer with a video camera and measured with TCL-Image™ image analysis software (TNO Institute of Applied Physics, Delft, The Netherlands, 1990 version 4.6). In interactive mode, the areas of interest could be manually drawn and automatically analyzed.

X-ray photography. In order to obtain a clear X-ray photograph on both dry and wet seeds, a specially developed seed carrier was placed about 2 cm from the X-ray source window. The X-ray photograph was made at 10 KeV and 5 seconds exposure time for dry seed, and 7 seconds for wet seed, using a 43805N X-Ray System (Faxitron™ series, Hewlett Packard, USA). One replicate of 18-20 seeds was taken from each treatment for X-ray investigation.

RESULTS

Seed free space. Free space is a term that is used to describe the empty area between embryo and endosperm within a seed. It is visible on X-ray photographs after seed priming

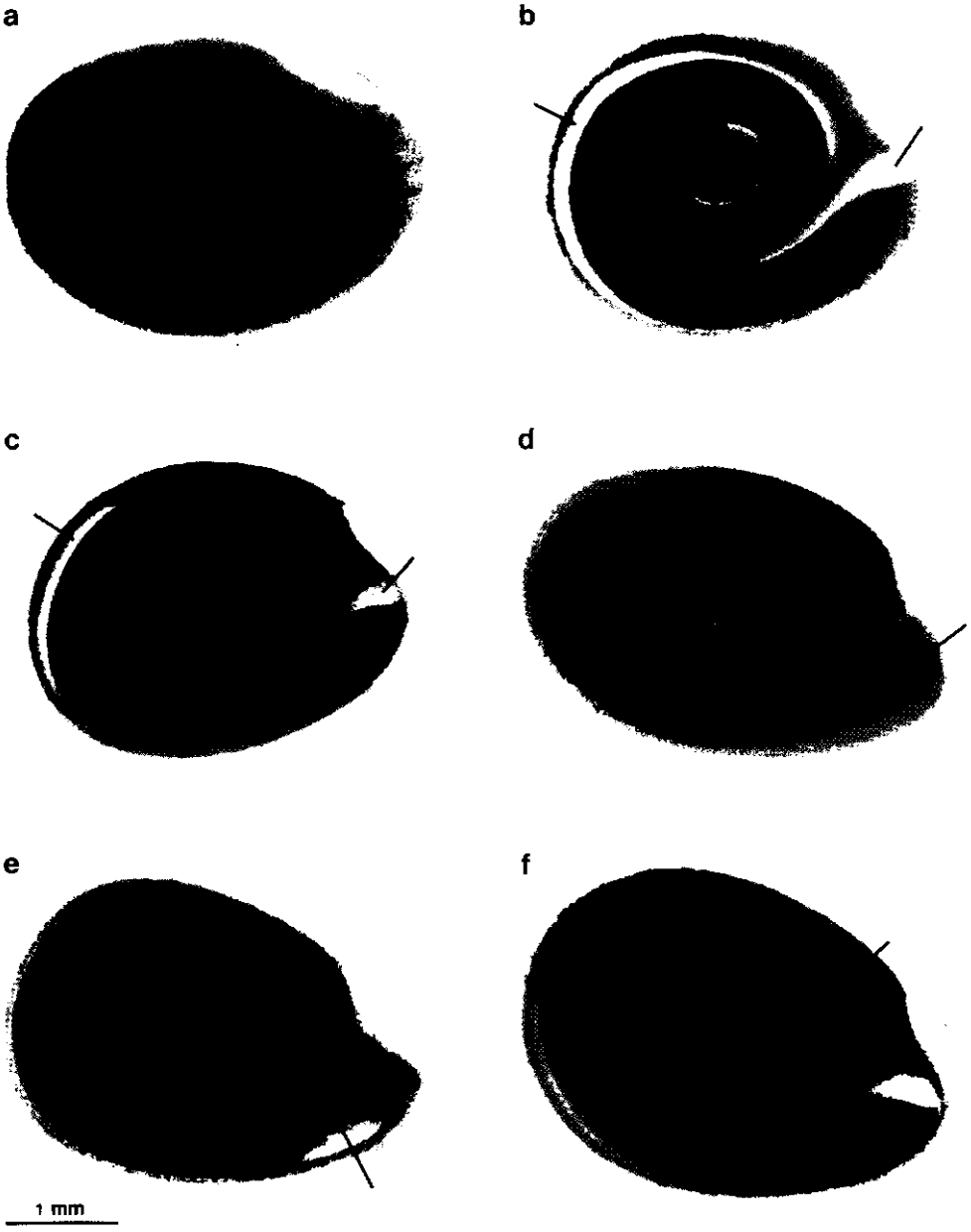

Figure 1. X-ray photographs showing the internal morphology of tomato seed. (a) Dry control seed without any free space, embryo and endosperm are filling the whole seed; (b) Osmoprimed seed with larger amount of free space after dehydration; (c) Hydroprimed seed with moderate free space after dehydration; (d) Blunt radicle tip of 60-hour hydroprimed dry seed; (e) Nicked radicle tip of 48-hour hydroprimed dry seed; (f) Disintegrating endosperm of 48-hour hydroprimed dry seed.

treatments (Argerich and Bradford, 1989). However, this free space does not only occur after osmotic priming treatments but was also found in control seeds, and after short time imbibition ('hydropriming') (Fig. 1b, 1c). The free space may occur in different parts of the seed. It is normally located around the embryo, most frequently around the radicle tip, and often around the cotyledons in the centre area of the seed. During PEG priming and subsequent drying, changes in the area and the position of free space were followed. Free space started to develop around the radicle tip. The area of free space increased during the first 4 days of priming with up to 20% of the whole seed area (Fig. 2). The frequency of seeds with free space increased gradually from the start of the osmopriming treatment to the 8th day, when the maximum value of 60% was reached. During hydropriming, free space appeared in about 70% of the seeds in the first 12 hours of hydropriming (Fig. 3) while the area of free space also increased, reaching the maximum value of 9% of the whole seed area at the same time (Fig. 4). Thereafter, the free space disappeared gradually in terms of both the number of seeds with free space and the area of free space. This process continued until 60 hours after sowing when some seeds (< 5%) had germinated.

In order to understand what causes the occurrence of free space, autoclave-killed and freshly-primed seeds were used. Autoclave-killed seeds, dead seeds, showed only a slight increase in free space both after osmopriming and hydropriming (Fig. 5).

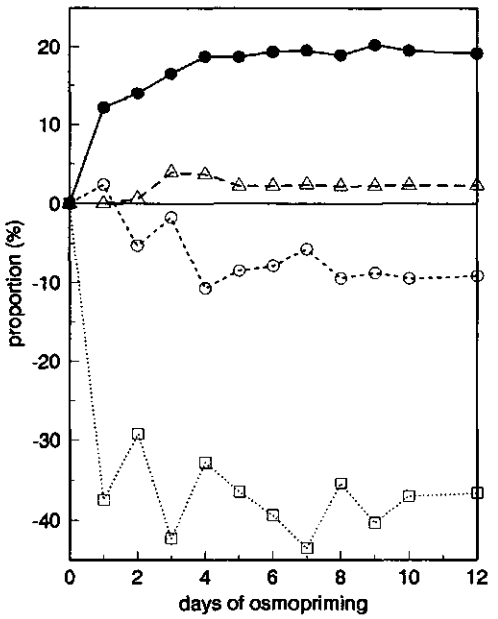

Figure 2. Proportional changes of embryo, endosperm and free space area in osmoprimed seed. Proportions were calculated by dividing the proportion of the different seed parts over that of the average value of the unprimed control lot. Values are averages of 18-20 seeds. The maximum standard error is 5.7893.

Seeds were also primed in PEG for 8 days directly after harvest, without drying, which we called 'fresh priming'. This did not result in any free space after drying, even though they show similar volume increases as the normally primed seeds (Table 1). When these freshly primed seeds were primed again after dehydration by the same PEG solution followed by redehydration, the free space did occur as with normally primed seeds.

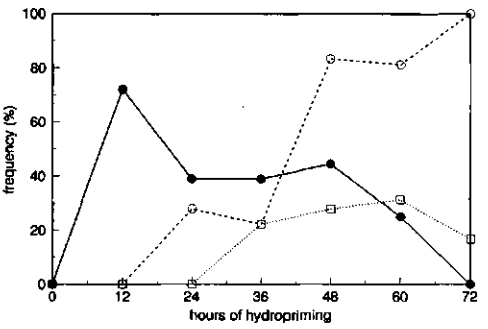

Figure 3. Frequency of hydroprimed seeds with free space, blunt and nicked radicle tips. A small number of imbibing seeds (less than 5%) had germinated at 60 hours after sowing.

Morphological changes in the embryo. An embryo within a control tomato seed is entirely enfolded by the surrounding endosperm, and occupies about 60% of the total seed area (the X-ray photographs are two-dimensional so that only planar surface parameters of tomato seed parts can be measured). This proportion decreased gradually with 10% or less during 4 days osmopriming followed by dehydration. Thereafter, there was no obvious change in the embryo area (Fig. 2). For the hydroprimed seeds, in contrast, the proportion of embryo to the total seed area, decreased sharply in the first 4 hours (-8%), then increased until 24 hours after sowing up to the point at which the proportion of the embryo became larger (+6%) than that of the untreated seed (Fig. 4). After that it remained constant until the moment of root protrusion.

During hydropriming and osmopriming, morphological changes in the radicle tips were observed. A normal tomato radicle tip is obtuse and has a round tip (Fig. 1a). However, when the seeds contained free space derived from either osmopriming or hydropriming followed by dehydration, the shape of radicle tips in these seeds became more pointed (Fig. 1b). In hydroprimed seeds, also blunt radicle tips were found, depending on the hydropriming period (Figs. 3, 1d). The tips of hydroprimed seeds may show various forms of aberrations and damage. Frequently the tips are indented, 'nicked' (Fig. 1e). Occasionally root tips are observed which are in close contact with the endosperm cap and show internal ruptures of the tissue resulting in free space within the root tip itself. It may even happen that the extreme part of a root tip breaks off, while the remainder of the root retreats. Germination tests indicated that seeds with blunt radicle tips germinated faster

and had longer root lengths. Seeds with nicks below the radicle tips, germinated more slowly and had shorter root lengths (data not shown).

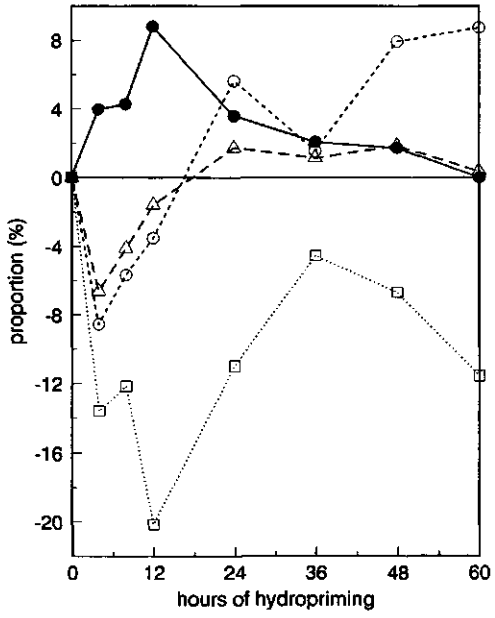

Figure 4. Proportional changes of embryo, endosperm and free space area in hydroprimed seed. Proportions were calculated by dividing the proportion of the seed parts by that of the average value of the unprimed control. Values are averages of 18-20 seeds. The maximum standard error is 6.4848.

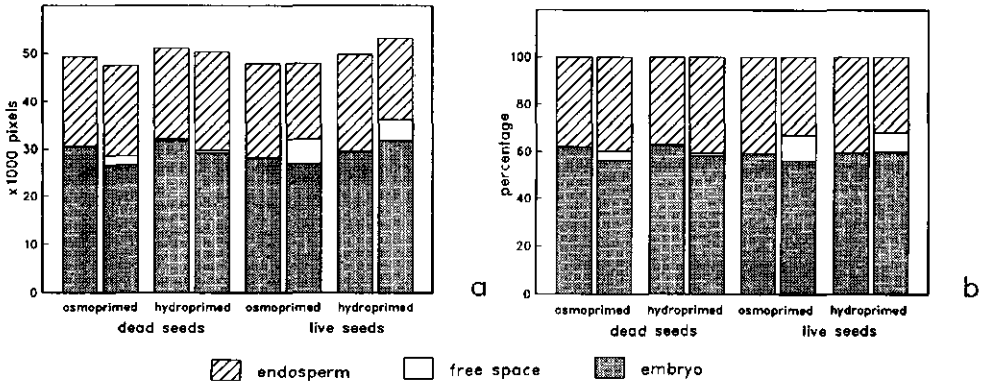

Figure 5. Development of free space in dead and live seeds during osmopriming or hydropriming. Left column of each pair represents the original situation before treatment, the right columns represent the distribution of embryo, free space and endosperm after the treatment. Each column represents the average of 10 measurements. The maximum standard error is 1.3%. a. actually measured values (number of pixels); b. areas expressed in percentages.

On the X-ray pictures of fresh seeds which were imbibed for 60 hours, the radicle tip is markedly dark which may be caused by the higher water content of the radicle tip compared with the other seed tissues and represents a region of intense swelling (Fig. 6). No morphological changes of the cotyledons were induced by the priming treatments.

Morphological changes in the endosperm. The endosperm occupies about 40% of the total seed area. After osmopriming, the planar area of the endosperm declined sharply in the first day (Fig. 2). During hydropriming, the endosperm area decreased in the first 12 hours of imbibition (Fig. 4), then returned to increase to some extent but never approached the original value. After 36 hours the proportion of endosperm declined again until 60 hours after sowing when some seeds germinated and most of other the seeds were close to germination (Fig. 4).

Irregular spots were found in the endosperm close to the cotyledons, and are recognized as darker spots on the X-ray photograph (Fig. 1f). The irregular spots may reflect differences in the water content of the endosperm caused by partial disintegration of the tissue. The number of spots remained unchanged during osmopriming and germination up to the moment of root protrusion. During root protrusion, the amount of disintegrating endosperm increased. Endosperm absorption continued until the tissue had entirely disappeared at the moment when the cotyledons separated from the seed coat.

Seed size and weight. Seed weight after PEG priming remained unchanged, whereas seed size in transverse, longitudinal and median dimensions increased significantly (Table 1). There was no statistical difference between the different PEG priming times (data not shown).

Table 1. Effects of priming on seed weight and seed size. The results of seed weight were based on 3x100 seeds, the results of seed size were obtained with 3x30 seeds. Duncan's multiple comparison was conducted as significance assessment index (P = 0.05). The same letter in each column means no statistical significance.

Seed treatments	seed weight (g/1000 seeds)	seed size (mm) thickness	transverse	longitudinal
control (unprimed)	3.502a	0.959b	2.725b	3.635b
osmoprimed	3.515a	1.101a	2.770a	3.770a
freshly-primed	3.504a	1.064a	2.810a	3.805a

During hydropriming, the transverse dimension decreased in the first 12 hours, and increased thereafter (Fig. 7). The change in thickness, *i.e.* the median dimension perpendicular to the planar surface, of hydroprimed seed followed another pattern. Seed thickness increased by about 10% in the first 12 hours and then over-contracted by about 10% as compared to the same seed before treatment (Fig. 7). This is coincides with the opposite dynamics observed in the planar surface of the seeds. The temporary increase in

seed thickness occurs simultaneously with a temporary decrease in the seed transverse dimension.

During hydropriming, seed weight declined slightly after 48 hours, in which some seeds germinated or were close to germination (Fig. 7). Although this change is not significant, it may reflect the digestion of nutrients and the disintegration of the endosperm.

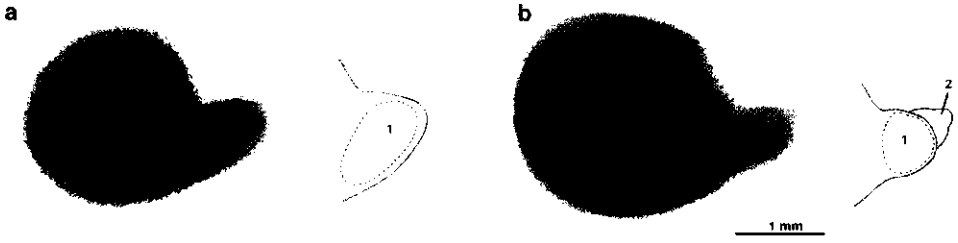

Figure 6. X-ray photographs of wet seeds after 60-hour hydropriming. (a) seed close to germination, showing region of intense swelling (1); and (b) seed directly after root protrusion, showing (1) region of intense swelling and (2) the emerging radicle tip.

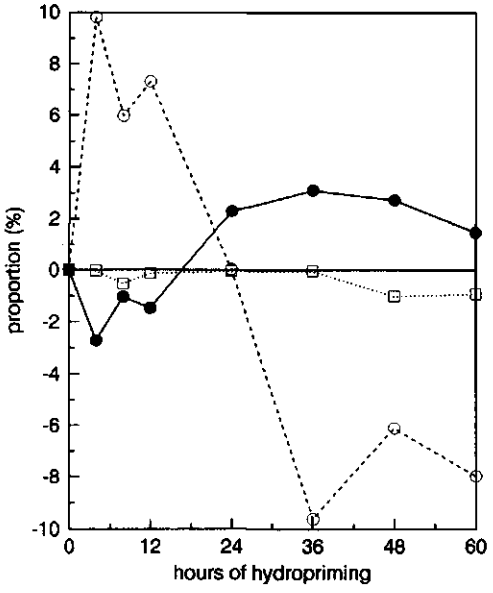

Figure 7. Dynamics of seed size changes (transverse dimension, seed thickness and seed weight) for different hydropriming periods. The proportions are ratios of the values of the individual seeds before and after the treatment. Values are averages of 3 replicates of 30 seeds.

DISCUSSION

The occurrence of free space in primed tomato seed was suggested to play a role in accelerating seed germination, possibly due to the facilitating of water uptake and it was hypothesized that the origin of free space might, in part, be attributed to the greater seed volume after priming (Argerich and Bradford, 1989). In the present studies, the X-ray images were quantified and the amount of free space was correlated with the area of the whole seed, the embryo and the endosperm.

Free space was not found when seeds were osmoprimed in the wet state, directly after harvest from the fruit, even though these seeds increased in volume too (Table 1). Free space however, was induced in these seeds by a second hydration and dehydration step. This demonstrates that the first desiccation process is a requirement for the occurrence of free space. Apparently the endosperm loses its flexibility upon dehydration. This loss of flexibility may also explain the rapid decline in endosperm area in primed seeds: the hydration results in a proportionally larger swelling of the embryo, and a compression or displacement of the endosperm in the process. The area of endosperm in the planar area becomes less (Fig. 5), but because the seeds increase in thickness (Table 1, Fig. 7), it is reasonable to assume that the endosperm gets displaced in a 'vertical' direction, perpendicular to the planar surface. This physical explanation is the more likely when considering that the greatest increase in free space occurs during the first few hours of imbibition already. Argerich and Bradford (1989) observed a 36% volume increase in primed seeds. If a seed is considered as an ellipsoid, and the volume of this ellipsoid is calculated using the dimensions of table 1, it can be calculated that the small changes in seed planar area combined with the changes in thickness, would cause volume increases of about 20%. The 10% higher estimates of the seed volume increases by Argerich and Bradford are possibly due to an over-estimation. They did not actually measure the seed volumes, but measured the length of a column packed with seeds. Because the seeds are more round after priming, they pack less well, resulting in more air between the seeds.

Size changes were also studied based on X-ray photographs (Fig. 5) and accurately measured with image analysis. In these measurements only the hydropriming treatment of live seeds resulted in a small increase in total seed area (Fig. 5a), all other seeds remained of the same size as compared to their original state. It must be remembered however, that the seed coat is not visible in these X-ray photographs, and that a possible change in testa thickness or looseness would not be detected here.

The measurements revealed that the free space increase in osmoprimed seed (+10.8% on average, Fig. 5a) was correlated with a large decrease in the endosperm area and a small decrease in embryo area (Fig. 5b). In hydroprimed seed, there was a smaller increase in free space (8.1%, Fig. 5a), combined with a reduction of only the endosperm area (Fig. 5b).

Some induction of free space was also found after osmopriming and hydropriming of some autoclave-killed seeds. These seeds were not viable anymore, as they did not

germinate and showed no colour reaction in the tetrazolium test. The occurrence of free space in these seeds may indicate that, although the seeds were not viable, some biophysical functions such as semi-permeability may still have continued.

The endosperm of tomato seed was reported to be a major restraint for radicle protrusion during germination (Argerich *et al.*, 1983; Liptay and Schopfer, 1983; Groot and Karssen, 1987; Haigh, 1988; Dahal *et al.*, 1990). This restraint can be seen very clearly on the X-ray photographs (Fig. 1d and 7a), even when the radicle had started to emerge from the endosperm and testa opposite the radicle (Fig. 7b), the testa is not being visible on the X-ray photographs. Radicle protrusion through the endosperm is the result from the turgor in the radicle tip (Bradford, 1990) and the weakening of endosperm (Groot and Karssen, 1987). Using X-ray photography, the process of radicle protrusion may be followed in time. Both the disintegration of the endosperm and differences in radicle turgor may result in changes in absorption of X-rays. For instance, on the X-ray pictures of seeds which were imbibed for 60-hours, the radicle tip is markedly dark which may be caused by the higher water content of the radicle tip compared with the other seed tissues (regions of intense swelling, Fig. 6). This is in agreement with similar observations made by Watkins et al. (1985) in pepper seed. In decoated seeds, they observed that the endosperm cap swelled outward before radicle emergence.

During hydropriming, the radicle that is enveloped in the endosperm, starts to grow against the opposite endosperm. This protruding expansion force may be visualized using X-ray photography of the hydrated seed (Fig. 7a). In contrast with osmoprimed seed, where the root tips withdraw and appear with slender tips (Fig. 1b), the root tips in hydroprimed seeds often appear as blunt tips (fig. 1d), i.e. they are still in the same position as in the fresh imbibed state before dehydration. The tips of hydroprimed seeds may show various forms of aberrations and damage. The tips may be indented, 'nicked' (Fig. 1e), which apparently is a result of the retreating of the root which only succeeded in part, because the rest of the tissue remained adhered to the endosperm. In some cases the root tip was internally ruptured. These seeds germinated more slowly, and their roots do not elongate as rapidly as normal ones.

At present we are further analyzing developmental processes during tomato seed germination in order to correlate the morphological characteristics inside the seed with the germination performance of the seedling.

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Chapter 3

Effects of osmotic priming on dormancy and storability of tomato (*Lycopersicon esculentum* Mill.) seeds

In cooperation with R.J. Bino, W.J. van der Burg and H.W.M. Hilhorst

(Seed Science Research, in press)

ABSTRACT. Freshly harvested tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) seeds were osmotically primed for 8 d in -1.0 MPa PEG-6000 solution and dried to about 6% water content for storage. Such so-called fresh PEG priming enhanced seed germination and improved seedling performance as compared with the untreated control. Fresh PEG priming neither alleviated seed dormancy nor promoted DNA replication as was the case when seeds were dried upon harvest and subsequently primed in PEG (normal PEG priming). However, the addition of 10 μM GA_{4+7} to the osmotic priming solution triggered replicative DNA synthesis of fresh priming seeds and further enhanced the germination process. After 5 months of storage in ambient temperature conditions, fresh PEG primed seeds maintained more positive effects gained from priming, whereas, normal PEG primed seeds had lost the promoting effects on germination. Normal PEG primed seeds were much more susceptible to controlled deterioration than fresh PEG primed seeds. It is suggested that the advancement of germination is negatively correlated with seed storability. The mechanisms of seed priming in relation to nuclear replication activities and physical changes are discussed.

Key words: DNA replication, dormancy, free space, fresh priming, tomato seed.

INTRODUCTION

Seed quality in many crops, including tomato (*Lycopersicon esculentum* Mill.), can be increased by osmotic priming, *i.e.* the imbibition by seed in an osmotic solution, followed by dehydration (Heydecker *et al.* 1973). The positive effects of osmotic priming such as the increase in rate and uniformity of germination are widely recognized (Heydecker and Coolbear 1977) and several hypotheses have been put forward to explain them. Priming may induce the repair of damage to nucleic acids and proteins, which has been acquired during storage (Rao *et al.* 1987). Priming may also trigger the actual germination processes which are exhibited as the induction of nuclear replication activity (Bino *et al.* 1992). The dehydration and rehydration steps of the priming procedure itself induce changes in the seed's internal morphological structure (Liu *et al.* 1993) and the state of the cell membranes (Khan *et al.* 1978).

The role of gibberellins in seed germination prior to root protrusion (Karssen *et al.* 1989) and in the mobilization of reserves in post-germination seeds (Jacobsen and Chandler 1987) has well been documented. The application of gibberellins for the termination of seed dormancy has been adopted in standard germination protocols. In general, osmotic priming does not affect the final germination percentage of many species. However, when gibberellins were added to the priming solution, an increase in the percentage of germination was found in *Primula acaulis* seeds (Finch-Savage *et al.* 1991).

In order to understand better the mechanisms of priming, we studied the influence of osmotic priming on dormancy, viability and storability of tomato seeds in relation to the

nuclear replication activities and internal morphological changes within the seed. The effects of the addition of gibberellins to the priming solution were also studied in combination with controlled deterioration.

MATERIALS AND METHODS

Seed material. Tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) plants were grown in a greenhouse with a temperature range of 21-31 °C both in 1991 (CPRO-DLO, the Netherlands) and in 1995 (Department of Plant Physiology, WAU, the Netherlands). Flowers were self-pollinated by vibration and labeled to obtain seeds of certain days (d) after pollination (DAP) in 1991. Seeds were extracted by mixing the seed slurry into a 3.2% HCl solution of equal volume for 3 hours (h) and were then rinsed with running tap water for 5 min. Seeds were directly sown for germination (wet sowing or WS) or dehydrated at 25 °C for 2 d and equilibrated in a ventilated drying drum (23 °C and 32% RH) for another 2 d. These dried seeds had a moisture content of 6.3% (fresh weight basis) and were stored in sealed aluminium foil bags at ambient temperature.

Treatments. Seeds of different maturities harvested in 1991 and fully mature seeds (>60 DAP) harvested in 1995 were osmotically primed on one layer of filter paper wetted with 5 mL of -1.0 MPa PEG-6000 solution (Michel and Kaufmann, 1973). Osmotic priming of fresh seeds in PEG or PEG plus 10 μM GA_{4+7} solution directly after harvest was called fresh PEG priming (FP) or fresh PEG plus GA priming (FP + G). Priming of seeds that were first dried upon harvest, in PEG or PEG plus 10 μM GA_{4+7}, was called normal PEG priming (NP) or normal PEG plus GA priming (NP + G). The seeds, in 9-cm petri dishes (50 seeds to each dish) sealed with a polyethylene bag, were incubated in a dark cabinet at 20 °C for 8 d. After priming, these seeds were rinsed with running tap water for 5 min and dried at room temperature for 2 d, then equilibrated in the drying drum as described above for another 2 d.

Storage and controlled deterioration. Primed and control seeds were sown without storage or stored in sealed aluminum foil bags at an ambient room temperature (between 17 and 25 °C) for 5 months and then sown. For controlled deterioration, seeds were first equilibrated at 20 °C and 45% RH for 3 d, at which time the seeds reached a moisture content of about 7.1%, and immediately sealed in aluminium foil bags. These seeds were deteriorated in an oven at 60 °C for 4 d and then dried back to their original moisture content.

X-ray photography. For X-ray analysis, one replicate of 20 seeds from each treatment was placed 25 cm from the X-ray source window. The X-ray photograph was made at 10 keV and 3.5 min exposure time, using a 43805N X-ray System (Faxitron™ series, Hewlett Packard, USA).

Flow cytometry. Preparation of nuclear samples, flow cytometric performance and data processing were all conducted as previously described (Bino *et al.* 1993). The PAS II flow cytometer (Partec GmbH, Munster, Germany), equipped with a HBO-100 mercury-arc lamp, a TK-420 dichroic mirror and a GG-435 long-pass filter, was employed to measure 3-5 replicates of 5 embryo root tips of each treatment. All analyses were performed using peak-height detection and logarithmic amplification (Bino *et al.* 1993). The DNA amount 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. Using the signals obtained from tomato leaf tissue, the gain settings were adjusted so that signals of all intact nuclei were registered within the channel range.

Germination. Seed germination was performed under ISTA conditions (1993), at an alternating temperature of 20/30 °C with 16-h dark and 8-h white light, respectively, on one layer of filter paper moistured with 0.2% KNO_3 solution. Rotten seeds were removed daily. Visible root protrusion was recognized as germination. At the end of the germination tests, ungerminated seeds were inspected by longitudinally cutting them into two halves. Those seeds in which the embryo and endosperm were firm and had a bright white colour, were recognized as living dormant seeds. Shoot height and root length of seedlings were measured at 10 and 14 d after sowing for the unaged and the controlled deteriorated seed lots, respectively. Parameters for the evaluation of seed germination were the percentages of germination (i.e. all germinated seeds), normal seedlings, and dormant seeds, the germination time (T_{50}, days to reach 50% maximum germination), and the uniformity (T_{80}-T_{20}, the days between 20% and 80% of maximum germination).

Statistical analysis. Data were ANOVA analysed as a randomized block design with three replicates of 50 seeds. Comparison between different treatments was expressed as average value. Percentages were analysed after angular transformation, but untransformed values are shown in the tables to facilitate comparison.

RESULTS

Seed maturation and dormancy. At about 45 DAP, seeds started to gain germinability. Seed germinability reached a maximum value in the period 50-55 DAP, and declined afterwards (Fig. 1A). More dormant seeds were observed in the over-matured seed lots (60-65 DAP) (Fig. 1E). When seeds were stored for 5 months, this situation was reversed, i.e. more dormant seeds were found in the less-matured seed lots (<50 DAP) (Fig. 1F). As compared with the desiccated control, the wet sown seeds germinated faster, more uniformly, produced more normal seedlings and with greater root length (Table 1). However, in the less-matured seed lots more dormant seeds were found than in the desiccated control (Fig. 1E)).

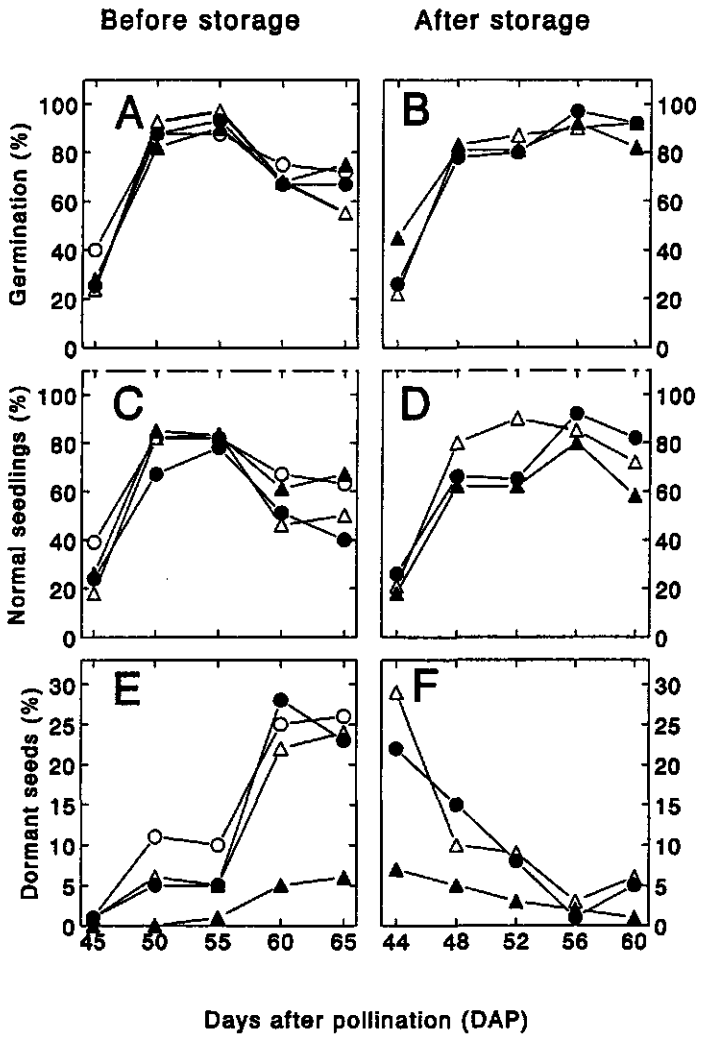

Figure 1. Effects of maturation and priming treatments on seed germination and seedling development before or after storage for 5 months at ambient temperature conditions. ● = control, ○ = wet sowing, ▲ = normal PEG priming, △ = fresh PEG priming.

Table 1. Germination performance of tomato seeds harvested at 55 DAP in 1991 before and after 5 months of storage under ambient temperature conditions

Treatment	Germination (%)	Normal Seedlings (%)	Dormant seeds (%)	Germination rate (T_{50})	Uniformity (T_{80}-T_{20})	Root length (cm)
Before storage						
CTRL	90.0	78.0	5.3	6.2	3.1	4.80
WS	87.3	82.0	10.0	5.8	1.8	5.96
FP	94.7	83.0	5.3	4.5	1.8	4.65
NP	90.0	82.7	0.7	3.3	1.5	6.05
$LSD_{0.05}$	5.22	5.69	4.70	1.24	0.49	0.55
After storage						
CTRL	97.3	90.0	2.0	3.6	1.5	4.12
FP	90.0	85.3	6.0	2.6	1.3	4.60
NP	92.0	80.0	0.7	3.7	1.6	4.68
$LSD_{0.05}$	4.22	5.65	3.89	0.56	0.44	0.84

Effects of osmotic priming on germination. In the seeds harvested in 1991, fresh PEG priming enhanced germination and increased uniformity before storage as compared with the untreated control. Normal PEG priming improved germination performance in all parameters except germination percentage and normal seedlings compared with the control and with the wet sown seeds (Table 1). In addition, normal PEG priming reduced dormancy to some extent while fresh PEG priming gave no alleviation of dormancy (Fig. 1E, Table 1).

After 5 months of storage, all seed lots except normal PEG-primed seeds showed a slight reduction in root length, but all had a much lower germination time and uniformity than before storage (Table 1). After storage, the germination percentage and the number of normal seedlings of normal PEG-primed seeds were significantly lower than in the control. Nevertheless, the positive effect on dormancy breaking by normal PEG priming was maintained (Fig. 1F, Table 1). In contrast, fresh PEG primed seeds retained almost all advantages of osmopriming, i.e. the seeds germinated significantly faster and more uniformly though not significantly than the control, and gave a relatively higher number of normal seedlings than the normal PEG primed seeds (Fig. 1D, Table 1).

In the seed lot harvested in 1995, all priming treatments enhanced seed germination as far as germination time was concerned (Table 2). Besides fresh PEG plus GA treatments, other priming treatments also improved seedling performance (Table 2). Normal PEG priming was better than fresh PEG priming whereas the treatments with PEG plus 10 μM GA_{4+7} were better than those with PEG alone. Controlled deterioration greatly

reduced the qualities of all seed lots (Table 2). Compared with the untreated control, normal PEG primed seeds were much more susceptible to deterioration than fresh PEG-primed seeds. The addition of GAs to the PEG solution reduced even further the resistance of the treated seeds to deterioration (Table 2).

Table 2. Germination performance of mature tomato seeds harvested in 1995 before and after controlled deterioration (ageing) at 60 °C, 45% RH for 4 d

Treatments	Germination (%)	Normal seedlings (%)	Germination rate (T_{50})	Uniformity (T_{80}-T_{20})	Shoot height (cm)	Root length (cm)
Before ageing						
CTRL	100.0	97.8	4.8	1.4	2.60	7.52
FP	97.8	94.4	3.7	1.0	2.95	8.66
FP + G	100.0	98.9	3.5	1.4	3.69	7.89
NP	100.0	97.8	3.1	0.8	3.23	8.97
NP + G	100.0	98.9	2.5	1.2	4.33	9.37
$LSD_{0.05}$	2.45	3.85	0.49	0.41	0.340	1.015
After ageing						
CTRL	100.0	95.6	8.9	1.9	3.28	8.94
FP	100.0	91.7	9.3	2.8	3.18	7.87
FP + G	74.4	72.2	10.3	2.7	2.75	6.98
NP	83.3	82.2	10.8	3.0	2.36	6.98
NP + G	14.4	1.1	11.8	2.2	0	0.51
$LSD_{0.05}$	6.71	5.16	1.21	0.82	1.063	1.434

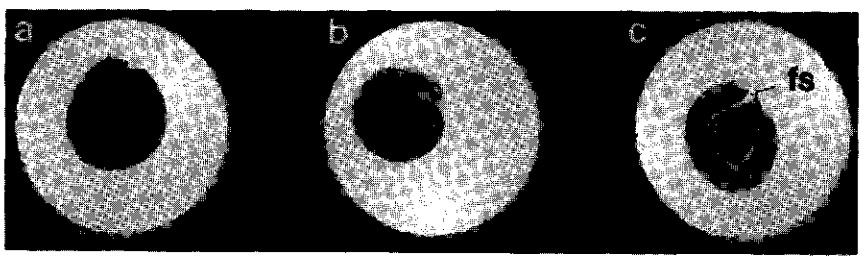

Figure 2. X-ray photographs of the control (a), freshly primed (b) and normally primed seeds (c). Note the free space (fs) in the normally primed seeds.

Morphological changes of normally primed seeds. X-ray photographs of control seeds demonstrated that the endosperm enveloped the curved embryo in the seed. After osmotic priming, both normal and fresh PEG primed seeds showed a similar increase in seed size (data not shown), but the normal PEG priming gave a large induction of free space between embryo and endosperm. The freshly primed seeds showed no changes in internal morphological structures (Fig. 2).

DNA replication during osmotic priming. The cells in the embryo root tips started replication of nuclear DNA after 2 d of normal PEG priming. Thereafter the ratio of 4C to 2C increased rapidly until day 6, then gradually until day 14 (Fig. 3). No nuclear replication activity was found during fresh PEG priming (Figs 3, 4) but, the addition of GA_{4+7} to the priming solution induced DNA synthesis of radicle-tip cells during this treatment (Fig. 4). No mitosis occurred in seeds during osmotic priming treatments (data not shown).

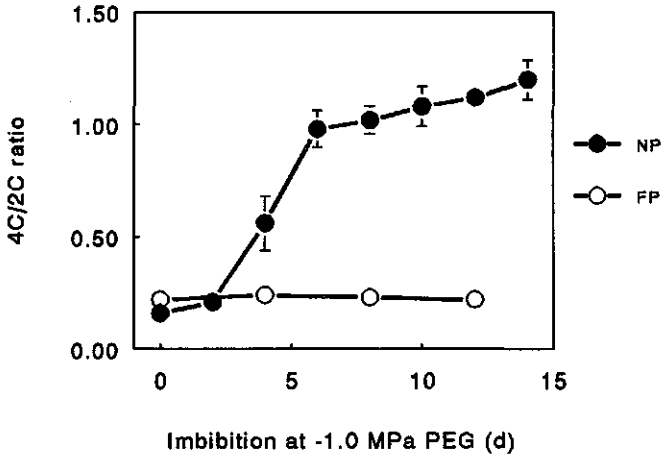

Figure 3. Changes in 4C/2C ratio in embryo radicle-tip cells of tomato seeds during osmotic priming. NP = Normal PEG priming, FP = Fresh PEG priming.

DISCUSSION

Each tomato cultivar and each seed lot may have a specific degree of dormancy (Odland 1938, Hilhorst 1995). The cultivar Moneymaker, which is used in the present study, produces dormant seeds after harvest, but this dormancy soon disappears after short-term dry storage (Groot and Karssen 1992). Penaloza and Eira (1993) reported that after 45 d of dry storage under normal laboratory conditions, seed germination of 3 tomato cultivars increased by 5, 36 and 59%, respectively. Within one cultivar the degree of dormancy

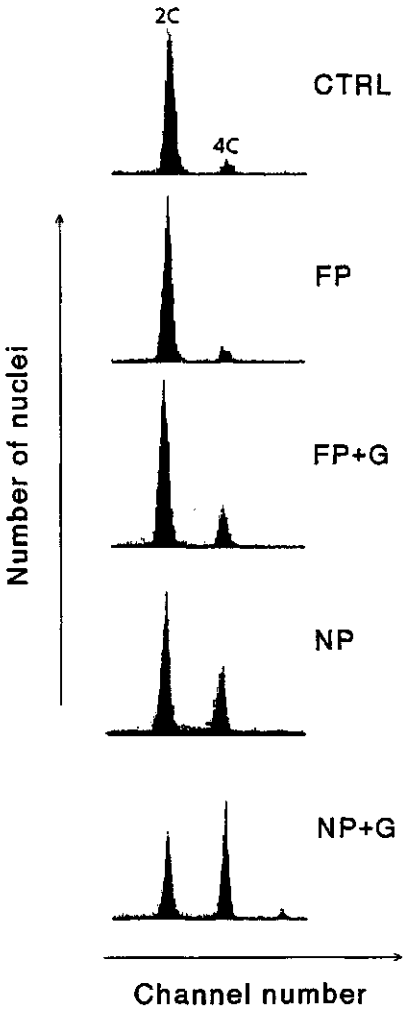

Figure 4. Histograms of flow-cytometric analysis of nuclei from tomato embryo radicle-tip cells of dry control seeds (CTRL) and seeds freshly or normally primed in -1.0 MPa PEG-6000 with (FP + G or NP + G) or without addition of 10 μM GA_{4+7} for 8 d (FP or NP).

may differ under different growing conditions or with different management methods (Groot 1987). The external factor(s) inducing such primary dormancy are yet unknown. We did not find any dormant seeds in the lot harvested in 1995, but in the lots harvested in 1991, primary dormancy was observed, especially at the post-maturation stage (60-65 DAP). Demir and Ellis (1992) found that between 35 and 45 d after anthesis there was

some dormancy in the incompletely filled, developing tomato seeds. The discrepancy in time scale of dormancy induction is difficult to interprete, since the causes of this reduction in germination response are still unknown. Compared with the untreated control and the freshly primed seeds, normal PEG priming alleviated dormancy. Using the same cultivar Moneymaker, Finch-Savage and McQuistan (1991) found that normal PEG priming significantly increased the total percentage of germination. For some other tomato cultivars, an increase in germination was also observed after short-term hydration (12-24 h imbibition in water) and dehydration (Penaloza and Eira 1993). This may be caused by the leaching out of germination inhibitor(s) from the seeds (Abdul-Baki and Stoner 1978).

Gibberellin-deficient tomato seeds exhibit high level dormancy, as they do not germinate under normal conditions. This dormancy is possibly imposed by the endosperm, as it can be broken either by the removal of the endosperm cap over the radicle tip or by the induction of endosperm weakening by the application of gibberellins to the germination medium (Groot and Karssen 1987). The start of germination of gibberellin-deficient seeds, induced by exogenous gibberellins, is coincident with the induction of DNA replication activity in the radicle-tip cells (Liu *et al.* 1994). It appears that DNA replication activity of radicle-tip cells is correlated with the breaking of dormancy in gibberellin-deficient seeds. Likewise, 8 d of fresh PEG priming did not induce nuclear replication of radicle-tip cells whereas the addition of 10 μM GA_{4+7} to the osmotic priming solution, did trigger replicative DNA synthesis (Figs 3, 4). Seeds which had been freshly primed with GAs, germinated faster but had a reduced storability than seeds primed without GAs. Apparently, the germination performance of the seeds was positively correlated with the induction of DNA synthesis during priming (Fig. 4, Table 2). Therefore, DNA replication in embryo root tips was suggested as a physiological parameter for measuring germination advancement of the primed seeds (Lanteri *et al.* 1994). On the other hand, we found a negative correlation between germination performance after storage and the amount of DNA synthesis in aged seeds (Fig. 4, Table 2). This indicates that the advancement of germination is negatively correlated with seed storability.

The recorded effects of priming treatments on the storability of seeds are somewhat contradictory. For instance, Georghiou *et al.* (1987) reported that in sweet pepper (*Capsicum annuum* L.), osmoconditioning in a 0.4 M mannitol solution for 4 d considerably delayed ageing and increased seed longevity. However, a reduction in seed storage life was observed by other investigators for some species like tomato (Argerich and Bradford 1989b), rice (*Oryza sativa* L.) (Basu and Pal 1979) and lettuce (*Lactuca sativa* L.) (Weges 1987, Tarquis and Bradford 1992). Seed storability is determined by at least two factors: (i) the amount of energy which is available for germination, and (ii) the amount of damage which accumulates during development and storage and which has to be repaired before germination (Osborne 1983). Before root protrusion, the low molecular weight substances available for respiration can not increase considerably, since the mobilization of the main storage reserves in the seed is almost completely a post-germination event (Bewley and Black 1985). The advancement of the germination processes during priming continuously

consume these substances, and consequently, may shorten seed longevity. However, the repair of DNA damage will increas longevity (Osborne 1983). As suggested by Osborne (1983), upon imbibition of seeds, replicative DNA synthesis is almost a final biochemical activity which occurs much later than the synthesis of proteins, RNAs and unscheduled DNA. This may indicate, that when endoduplication of nuclear DNA in the radicle tip has started, the germination process has progressed to a point where the seed can not stay at this stage without a reduction in storability.

The occurrence of free space in normal PEG-primed seeds has been suggested to play a role in accelerating the rate of germination, possibly by facilitating the uptake of water (Argerich and Bradford 1989a). Free space, which was not found in fresh PEG-primed seeds, was induced by the second hydration and dehydration step as the endosperm lost its flexibility upon dehydration (Liu *et al.* 1993). During normal PEG priming, the embryo expands and compresses the endosperm tissue, particularly at the location opposite to the radicle tip (Liptay and Zariffa 1993). Both the compression force of the embryo and the hydrolytic activities on the endosperm cell walls may deform the tissues and will facilitate the protrusion of the root upon rehydration. However, the physical damage which is induced by the expansion upon imbibition and the shrinkage upon desiccation may also reduce seed storability.

In newly harvested seeds, the positive effects of fresh PEG priming, though less than normal PEG priming, should be ascribed to the advancement of germination. As we did not find free space and nuclear replication activity in fresh PEG primed seeds as compared with normal PEG primed seeds, this advancement of germination is apparently due to the activation of some other process which occurred before nuclear replication. One possibility is that fresh priming induces hydrolytic enzyme activity which allows endosperm weakening (Groot and Karssen 1987). The reason why fresh priming was not able to trigger DNA synthesis in embryo root tips is unknown. It may be that the embryos can not produce sufficient GAs, or are insensitive to endogenous GAs, because addition of GAs did induce DNA replication (Fig. 4). Possibly, the drying of seeds upon harvest induces GA production upon subsequent imbibition or/and increases the sensitivity to endogenous GAs upon rehydration.

The biochemical reactivation of seed upon imbibition is understood to be programmed in a certain sequence, of which DNA synthesis is one of the final steps (Osborne 1983). Each biochemical activity has a certain contribution to endosperm weakening and to the growth of the embryo. The cascade of activities finally leads to the protrusion of the radicle through the seed. In our future studies we will further concentrate on the interaction between dormancy and storability and the hormonal regulation of cell cycle processes in the tomato seed.

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Chapter 4

Nuclear replication activities during imbibition of gibberellin- and abscisic acid-deficient tomato (*Lycopersicon esculentum* Mill.) mutant seeds

In cooperation with Jan H.W. Bergervoet, C.H. Ric De Vos, Henk W.M. Hilhorst, H. Lieke Kraak, Cees M. Karssen and Raoul J. Bino

(Planta 1994, 194, 368-373)

ABSTRACT. The role of ABA and GA in the induction of cell cycle activities during imbibition and subsequent germination of tomato seeds has been studied. Using flow cytometry, nuclear replication activity in embryo root tips isolated from seeds of the abscisic acid (ABA)-deficient mutant *sit*^{w}, the gibberellic acid (GA)-deficient mutant *gib-1*, and the wild type (MM) tomato (*Lycopersicon esculentum* Mill., cv Moneymaker) upon imbibition in water, 10 μM GA_{4+7}, 5 μM ABA or 5 μM ABA + 10 μM GA_{4+7} was investigated. The nuclei of fully matured dry MM, *sit*^{w} and *gib-1* seeds predominantly revealed 2C DNA signals, indicating that most root tip cells arrested their cell cycle activity at the G_1 phase of nuclear division. However, ABA-deficient *sit*^{w} seeds contained a significant higher amount of G_2 cells (4C DNA) compared with the other genotypes, suggesting that during maturation cell cycle activity in *sit*^{w} seeds is less efficiently arrested in G_1. Upon imbibition in water, an induction of the 4C signal, indicating nuclear replication, was observed in the root tip cells of both MM and *sit*^{w} embryos. The augmentation in the 4C signal occurred before visible germination. *Gib-1* seeds did not show cell cycle activity and did not germinate in water. Upon imbibition in GA, both cell cycle activity and subsequent germination were enhanced in MM and *sit*^{w} seeds, and were induced in *gib-1*. In ABA, the germination of MM and *sit*^{w} seeds was inhibited while nuclear replication of these seeds was not affected. It is concluded that GA influences germination by acting upon processes that precede cell cycle activation, while ABA affects growth by acting upon processes that ensue cell cycle activation.

Key words: Abscisic acid, Cell cycle, DNA synthesis, Germination, Gibberellic acid, Seed, Tomato.

INTRODUCTION

Both abscisic acid (ABA) and gibberellic acids (GA) play important roles in the development, maturation and germination of seeds (Bewley and Black 1985). ABA is essential for the induction of seed dormancy, as was demonstrated in experiments with seeds derived from the ABA-deficient tomato mutant *sit*^{w} (Karssen et al. 1983). Seeds of the ABA-insensitive mutants of *Arabidopsis thaliana* showed no dormancy, while desiccation tolerance and longevity were reduced (Ooms et al. 1993). Studies with the GA-deficient tomato mutant *gib-1* have shown that GA is essential for seed germination (Groot et al. 1987). A major action of GA appeared to be the induction of enzymatic degradation of the mannan-rich walls of endosperm cells opposing the root tip region, prior to radicle protrusion (Groot et al. 1988).

Using flow cytometry, Bino et al. (1992), Lanteri et al. (1993) and Georgieva et al. (1994) showed that DNA synthesis precedes visible germination of tomato, pepper (*Capsicum annuum*) and maize (*Zea mays*) seeds. DNA levels in embryos of fully mature

dry tomato and pepper seeds revealed large amounts of 2C DNA signals, indicating that most cells had arrested their cell cycle at the presynthetic G_1 phase of nuclear division. After 1 day of imbibition in water, an augmentation of the 4C DNA signal in the embryonic root tip region was observed. This increase in 4C DNA could be ascribed to cells that had entered the post-synthetic G_2 phase of nuclear division. Little is known about the molecular basis of cell cycle control in higher plants, in contrast to that in animal cells and fungi where the mechanisms underlying progression through the cell cycle have been studied in considerable detail (Draetta 1990; Traas et al. 1992). In animal cells it appears that a number of highly conserved key proteins control the progress through the cell cycle via a phosphorylation cascade. Some of these proteins have also been reported for plant cells (Traas et al. 1992). It may be postulated that the cell cycle in plants is regulated by hormones. In cultured cells of various plant species and in hypocotyl segments of watermelon (*Citrullus lanatus*) seedlings, GA induces cells in G_1 phase to enter the DNA synthetic phase of the cell cycle (Jacqmard 1969; Edelman and Loy 1987). With regard to seeds, it was demonstrated that ABA inhibited DNA synthesis, as measured by the incorporation of ^{3}H-thymidine, in embryos dissected from mature *Haplopappus gracilis* seeds (Galli et al. 1979) and during early germination of *Avena fatua* seeds (Elder and Osborne 1993). In addition, Levi et al. (1993) using flow cytometry, observed that in cultured pea (*Pisum sativum*) embryos, 100 μM ABA almost completely inhibited the transition from G_1 to G_2 phase.

The aim of the present study was to test the effects of ABA and GA on replicative DNA synthesis during tomato seed imbibition. The experiments were performed with the ABA-deficient (*sit*^{w}) and GA-deficient (*gib-1*) mutants of cv Moneymaker, isolated and phenotypically characterized by Koornneef et al. (1985). Developing *sit*^{w} seeds contain about 3% of the ABA level of wild-type seeds (Groot et al. 1991). This strong reduction in ABA has no significant effect on seed development, although, viviparous germination may occur in ripe fruits (Groot and Karssen 1992). Endogenous GA has an important role both in development and germination of seeds. *Pisum sativum* seeds with a decreased GA level manifest a reduced likelihood to develop to maturity compared with wild type seeds (Swain et al. 1993), seeds from the tomato *gib-1* mutant show a normal development but require applied GA to germinate (Groot 1987).

Using flow cytometry, we studied nuclear replication activity in *sit*^{w}, *gib-1* and wild type seeds upon imbibition in water and in GA, ABA or ABA + GA. In this way we tested the role of ABA and GA in the induction of cell cycle activities during imbibition and subsequent germination of tomato seeds.

MATERIALS AND METHODS

Plant material. Tomato (*Lycopersicon esculentum* Mill.) seeds of the homozygous *sit*^{w} and *gib-1* mutants and their wild type (MM) isogenic parent (cultivar Moneymaker) were harvested in 1988 under conditions described by Groot and Karssen (1987) and stored at

4°C and 7% seed water content (fresh weight basis). At these conditions *sit*^{w} seeds can be stored without a total loss of germination capacity.

Germination tests and pretreatments. Four replicates of 20 seeds of each genotype were placed on 2 filter papers in a 5 cm Petri dish moistened with 1.5 mL of distilled water, 10 μM GA_{4+7}, 5 μM ABA, or 10 μM GA_{4+7} + 5 μM ABA solutions. The dishes were sealed with parafilm and incubated in the dark at 25°C. Seeds were considered germinated when radicle protrusion was clearly visible. During the first 2 days, seed germination was counted twice a day, thereafter germination was scored daily till day 5.

Preparation of nuclear samples. For flow cytometry, seeds were removed from the dishes at 12 h time intervals and subsequently dried in a ventilated drum (23°C, 32% RH) for 4 d, and stored at 4°C for later use. Embryo root tips were dissected from the seeds and chopped with a razor blade in a modified Saxena and King (1989) nucleus isolation buffer (0.2 M mannitol, 10 mM 2(N-morpholino)ethanesulfonic acid, 10 mM NaCl, 10 mM KCl, 10 mM spermine tetrahydrochloride, 2.5 mM ethylenediaminetetraacetic acid, 2.5 mM dithiothreitol, 0.05% Triton X-100 (v/v) and 0.05% (w/v) Na-azide at pH 5.8), as described previously (Bino et al. 1993). Root tips were used as this tissue optimally reflected the effects of imbibition on replicative DNA synthesis during tomato seed germination (Bino et al. 1992). To detect DNA, 10 $mg.l^{-1}$ of the fluorescent dye 4',6-diamidino-2-phenylindole (DAPI) was added to the isolation buffer. 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. Statistical analyses of data were performed with the Tukey-Kramer multiple comparisons test.

Flow cytometry. The PAS II flow cytometer (Partec GmbH, Münster, Germany) was 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 fluorescent signals are presented as frequency distribution histograms over 500 channels, starting from channel number 20. Histograms were stored on disk and processed using the software package FLOW 1.0 (CPRO-DLO). The DNA amount 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. Using the signals obtained from tomato leaf tissue, the gain settings were adjusted so that signals of all intact nuclei were registered within the channel range.

RESULTS

Effects of ABA and GA on germination. In water (Fig. 1A), MM seeds started to germinate

after 36 h of imbibition and the final germination was 98%. The ABA-deficient *sit*^{w} seed lot started to germinate after only 24 h of imbibition and the final germination was 72%, the GA-deficient *gib-1* seeds did not germinate in water within 120 h of imbibition. In ABA (Fig. 1B), both MM and *gib-1* seeds did not germinate within 120 h of imbibition, while some germination (12%) was observed for the *sit*^{w} seeds. In GA (Fig. 1C), both the sit^{w} and *gib-1* seeds started to germinate after 24 h of imbibition and the germination after 120 h was 72%; MM seed germinated slightly faster in GA than in water. A combination of GA and ABA (Fig. 1D) considerably reduced seed germination as compared with the response in water or GA alone. After 120 h, *sit*^{w} seeds showed only 6% germination, whereas MM and *gib-1* seeds started to germinate after 72 h and the germination after 120 h was 43% (*gib-1*) and 25% (MM).

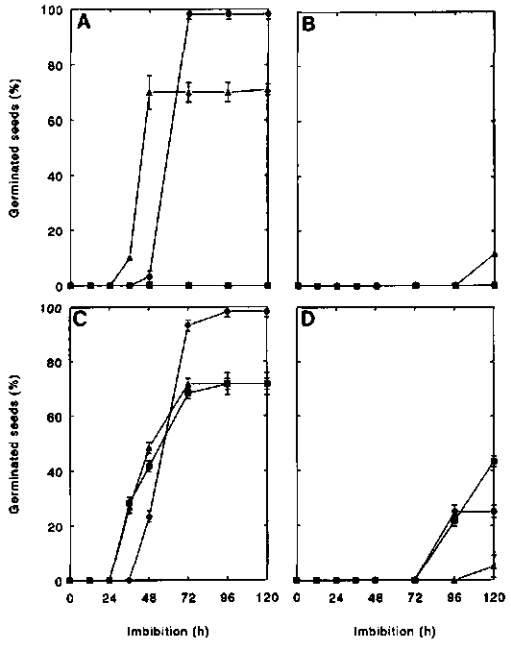

Figure 1. Germination percentages (mean ± SE) of wild type MM (•), GA-deficient *gib-1* (■) and ABA-deficient *sit*^{w} (▲) tomato seeds during imbibition in water (A), 5 μM ABA (B), 10 μM GA_{4+7} (C) or 5 μM ABA + 10 μM GA_{4+7} (D).

Nuclear replication stages in dry seeds. As shown by the flow cytometric histograms (Fig. 2), root tips of dry MM seeds revealed one large peak at channel 100 and a small peak around channel 185, the latter representing 4.8% of the total DNA signals (Fig. 2A). As deduced from leaf tissue of the same diploid tomato genotype (data not shown), the first peak around channel 100 corresponded with the 2C DNA level from diploid nuclei at the pre-replication stage of nuclear division (G_1 stage), whereas the peak at channel 185 represented diploid nuclei with 4C DNA (G_2 stage). Thus, the DNA of dry MM seeds was

mainly in the 2C pre-replication stage. The flow cytometric profile of embryo root tips from dry *gib-1* seeds (Fig. 2C) similarly showed a large 2C peak, whereas 6.5% of the nuclei yielded 4C signals. The 4C peak in dry *sit*^{w} seed root tips (Fig. 2E) represented 14.2% of the total DNA signals, and this value was significantly higher ($P < 0.05$) than the 4C signals from MM and *gib-1*.

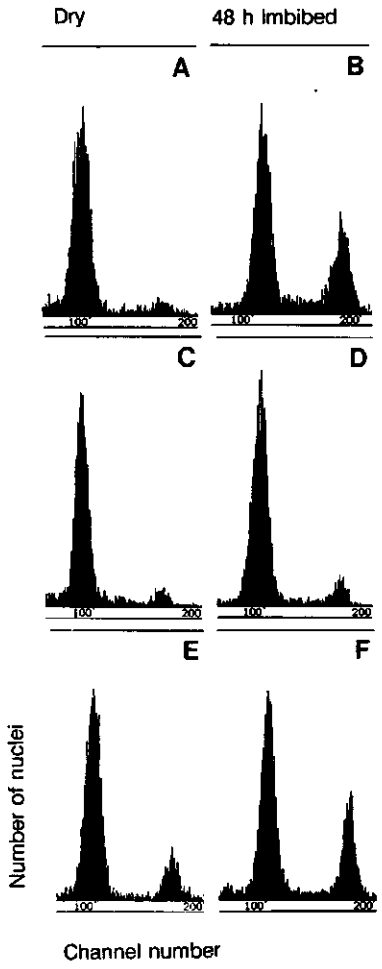

Figure 2. Histograms of flow cytometric analysis of nuclei from tomato embryo root tip cells of dry and 48 h water imbibed seeds; 4,650 nuclei from dry (A) and 5,636 nuclei from water imbibed (B) ungerminated wild type MM seeds; 3,841 nuclei from dry (C) and 4,239 nuclei from water imbibed (D) GA-deficient *gib-1* seeds; 4,460 nuclei from dry (E) and 5,048 nuclei from water imbibed (F) ABA-deficient *sit*^{w} seeds. Peaks at channel 100 and 185 represent 2C and 4C DNA, respectively.

Effects of ABA and GA on nuclear replication stages during imbibition. Between 12 and 36 h imbibition in water, the percentage of 4C signals in embryo root tip cells from both

MM and *sit*^{w} seeds increased significantly (Fig. 3A). After 48 h, ca 37% of cells from MM seeds and 23% of the *sit*^{w} seeds had entered the G_2 phase (Figs 2B and 2D). No increase in DNA replication was observed in *gib-1* seeds during 48 h imbibition in water: at all stages ca 7% of the cells manifested 4C signals (Figs. 2F and 3A). During imbibition of MM and *sit*^{w} seeds in ABA (Fig. 3B), 4C levels increased significantly more than after 48 h of imbibition in water (Fig. 3A). Imbibition in GA induced a considerable increase in 4C levels in *gib-1* root tips (Fig. 3C). The increase in 4C signals of *sit*^{w} and MM seeds was comparable as with incubation in water. As was found for GA alone (Fig. 3C), imbibition in GA + ABA (Fig. 3D) also induced an increase of 4C signals in MM, *sit*^{w} and *gib-1* seeds.

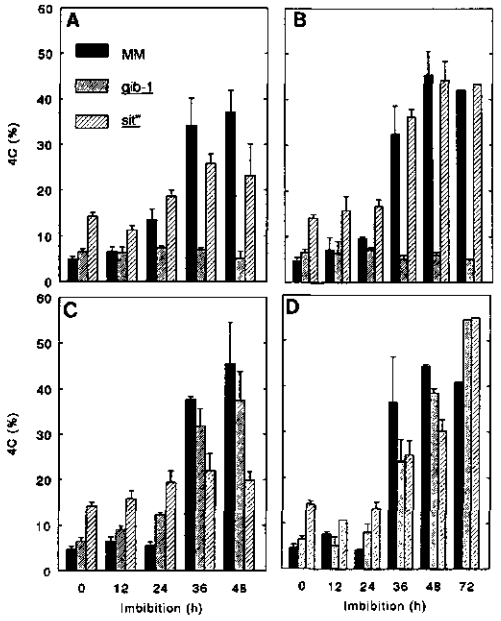

Figure 3. Percentages of 4C nuclei (mean ± SE) in tomato root tips of wild type MM, GA-deficient *gib-1* and ABA-deficient *sit*^{w} seeds imbibed in water (A), 5 μM ABA (B), 10 μM GA_{4+7} (C) or 5 μM ABA + 10 μM GA_{4+7} (D).

DISCUSSION

Nuclear replication stages in dry tomato seeds. In dry MM, *sit*^{w} and *gib-1* seeds most embryo root tip cells have arrested cell cycle activity at the G_1 phase of nuclear division. However, ABA deficient *sit*^{w} seeds contained a significantly higher amount of G_2 cells compared with the other genotypes. ABA has been shown to be an inhibitor of cell division (Evens 1984) and may play an important role in the arrest of cell cycle activities during seed maturation (Bouvier-Durand et al. 1989). During wild type seed development, the

endogenous ABA concentration peaks at about 50 d after pollination (Berry and Bewley 1992). Apparently, nuclear replication activity in maturing *sit*^{w} seeds is less efficiently arrested in G_1 compared with the wild type and *gib-1* mutant. Possibly, the control of ABA over nuclear replication activity may be associated with the induction of dormancy and desiccation tolerance.

Nuclear replication activity during imbibition in water. Upon imbibition in water an induction of the 4C signal, indicating nuclear replication, was observed in the root tip cells of MM seeds. The increase in 4C signals in *sit*^{w} seeds was less prominent than that found in the MM seeds. This difference may be caused by the reduced dormancy of the *sit*^{w} seeds, which prompted the cells to enter mitosis and the seeds to germinate at an earlier stage of imbibition. During germination, mitosis will proceed causing 4C cells to re-enter 2C stage. This will lead to a lower percentage of 4C cells in comparison with seed lots that germinate at later stages of imbibition. Indeed, *sit*^{w} seeds germinated about 12 h earlier than wild type seeds. In addition, the difference in the augmentation of 4C signals may be related to the low germination percentage of the *sit*^{w} seeds (72%) compared with MM seeds (98%). Seeds from the GA deficient *gib-1* mutant did not germinate and did not manifest an induction of nuclear activity upon imbibition in water.

Nuclear replication activity during imbibition in ABA and GA. Abscisic acid delays germination, whereas GA promotes the germination rate upon imbibition of mature seeds (Karssen et al. 1983, 1987). In the present study, the addition of ABA to the imbibition medium delayed the germination of both MM and *sit*^{w} seeds for more than 48 h, while GA advanced it. The germination of *gib-1* seeds was completely dependent on the presence of GA. Using the same genetic material and similar hormonal concentrations, Ni and Bradford (1993) found less pronounced effects on the germination rates. This variation might be explained by differences in experimental conditions or by seed lot effects.

Although the MM and *sit*^{w} seeds did not germinate within 72 h of imbibition in ABA (Fig. 1B), a strong induction of nuclear replication activity was indicated by the considerable increase in 4C signals (Fig. 3B). This induction of DNA synthesis in the presence of ABA is in contrast to the results of Levi et al. (1993) and Elder and Osborne (1993). However, these authors used 100 μM ABA to inhibit germination, while we used only 5 μM. The responses of tomato seed germination to ABA have been quantitatively characterized using a time course population-based threshold model (Ni and Bradford 1992). In tomato, the primary site of action of ABA is probably the endosperm cap, where it may inhibit the expression of cell wall hydrolyzing enzymes that are responsible for cell wall weakening (Liptay and Schopfer 1983; Groot 1987). Ni and Bradford (1992) suppose that low levels of ABA may only delay the activity of such enzymes, whereas higher concentrations would completely inhibit enzyme activity. Possibly, high ABA levels inhibit the induction of DNA synthesis and therefore completely block the initiation of the germination processes, whereas low ABA levels reduce the expression of hydrolyzing enzymes but do not inhibit

the induction of DNA synthesis. Interestingly, in tomato seeds, a pre-imbibition in ABA reduced the time to 50% germination from 14 to 4 d and reduced the spread in times to germination after subsequent imbibition in water (Finch-Savage and McQuistan 1991). Apparently, the pre-imbibition in ABA initiates the induction of nuclear replication activity and other pre-germinative processes but prevents visible germination. Possibly, this induction of pre-germinative processes sustains a rapid inception of cellular activities upon subsequent imbibition in water which results in both an accelerated germination rate and an improved seedling uniformity.

Seeds of *gib-1* showed no induction of nuclear replication activity upon imbibition in water, while imbibition in GA induced an increase in 4C signals which preceded seed germination. Apparently, GA is necessary to initiate DNA synthesis. This is in agreement with several studies using meristematic tissues of various plant species, in which GA promoted cell division by inducing cells in G_1 to enter the DNA synthetic phase (Edelman and Loy 1987). In tomato seeds, GA has also been shown to induce the enzymatic degradation of the endosperm prior to radicle protrusion (Groot et al. 1987). There are several possibilities for the relation between replicative DNA synthesis and endosperm weakening in tomato seeds. Firstly, it might be hypothesized that GA induces DNA synthesis which initiates expression of various enzymes that are responsible for endosperm weakening (e.g. endo-β-mannanase). Secondly, the absence of endosperm weakening opposite the embryo radicle might limit cell growth and hamper cell cycle activities upon imbibition in water. Indeed, when the endosperm and testa of the *gib-1* seeds were removed, embryos slowly germinated without the addition of GA and grew into dwarf plants (Groot 1987). The third explanation is that the two phenomena, nuclear replication and endosperm weakening, are parallel events without any causal relation. This latter explanation is more likely since imbibition in GA reduced the time to germination of MM and *sit*^{w} seeds, but did not reduce the time to activation of nuclear replication, compared to imbibition in water. Moreover, when ABA was supplemented to the GA imbibition medium, the induction rate of G_2 phase in *gib-1* seeds was not changed while the time to germination was considerably delayed.

Seed functioning is accompanied by programmed transitions from cell proliferation to quiescence upon maturation and from quiescence to reinitiation of cellular metabolism upon imbibition. In the present study, the latter transition was monitored using flow cytometry. The induction of cell cycle activity during imbibition was manifested by a shift from 2C to 4C DNA level in cells of the embryo root tip. In all seeds, DNA replication preceded germination. In the GA-deficient mutant, both germination and DNA replication depended upon the presence of GA, which indicated that GA action preceded cell cycle activation. ABA, on the other hand, inhibited germination of all seeds but did not affect DNA replication. Obviously, ABA acted upon processes that ensued cell cycle activation. However, despite the obvious importance of these control mechanisms, very little information is available at the molecular level concerning those elements that regulate the cell cycle in plants (Buddles et al. 1993). Studies on the relation between the induction of

cell cycle activity and the expression of β-tubulin, one of the main proteins involved in nuclear division, during seed imbibition, are in progress.

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Chapter 5

Nuclear replication activity and free space induction during maturation and imbibition of gibberellin- and abscisic acid-deficient tomato (*Lycopersicon esculentum* Mill.) mutant seeds

In cooperation with Henk W.M. Hilhorst, Steven P.C. Groot and Raoul J. Bino

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ABSTRACT. Using X-ray photography and flow cytometry, the internal morphology and nuclear replication activity of wild type (wt), GA- (*gib-1*) and ABA-deficient (*sit*^{w}) tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) mutant seeds were studied. During seed formation, cell cycle activities occur in the radicle tips of the embryos until 45 days after pollination (DAP) when the endosperm becomes solid and seed starts to gain desiccation-tolerance and germinability in all three genotypes. Upon maturation (45 DAP onwards), most cells in the seeds of three genotypes arrest the cell-cycle in the G_1 phase. However, a relatively high amount of 4C signal was observed in the radicle-tip cells of *sit*^{w} compared with wild type and *gib-1*. At the well-matured stage (60 DAP), there were about 2% of seeds with free space in wild type and *gib-1*, and about 13% in *sit*^{w}. At the over-matured stage (75 DAP), even more seeds with free space were found in *sit*^{w}, whereas no increase in the proportion of the seeds with free space was detected in the other two genotypes. In -1.0 MPa PEG-6000 with or without 10 μM GA_{4+7}, no germination occurred of well-matured wild type and *gib-1* seeds, whether or not they were dried after harvest. However, *sit*^{w} seeds were able to germinate both in over-mature fruit and in -1.0 MPa PEG-6000. Priming of dried seeds in -1.0 MPa PEG induced a large amount of free space in almost all seeds of the three genotypes, and DNA replication activity in the radicle-tip cells of wild type and *sit*^{w} seeds. However, PEG priming of fresh (non-dried) seeds had no effect on the amount of free space and DNA replication activity in wild type or *gib-1* seeds, but did induce free space in about 20-25% of *sit*^{w} seeds and provoked DNA replication in *sit*^{w} seeds. Removal of the endosperm and testa opposite the radicle tip of seeds resulted in root protrusion, free space and DNA replication activity of the seed. It is concluded that ABA is crucial for the efficient arrest of tomato embryo radicle-tip cells in G_1 phase upon maturation, whereas GAs play an important role in re-initiating replicative DNA synthesis activity upon germination.
Key words: DNA replication; dormancy; flow cytometry; free space; *Lycopersicon esculentum*; maturation; priming; seed; tomato

INTRODUCTION

Both abscisic acid (ABA) and gibberellins (GAs) play important roles in seed development and germination (Bewley and Black 1994). ABA is essential for the induction of seed dormancy, as was demonstrated in experiments with the ABA-deficient tomato (*Lycopersicon esculentum* Mill.) mutant *sit*^{w} (Groot and Karssen 1992) and ABA-insensitive mutants of *Arabidopsis thaliana* (Karssen et al. 1983, Ooms et al. 1993). GA-deficient mutants of *Arabidopsis* and tomato require GAs for seed germination (Koornneef and van der Veen 1980, Groot and Karssen 1987). The absence of ABA or GAs during tomato seed

development may alter water relations of both fruit and seed tissue, which could contribute to the mechanism preventing precocious germination (Liu et al. 1996). Hormone deficient mutants have also been used to investigate the roles of GAs and ABA in the development of fruits (Groot et al. 1987) and flowers (Nester and Zeevaart 1988).

During maturation or upon germination important morphological changes occur in seed tissues. X-radiography was introduced as a non-destructive method for analyzing internal structures in the relative large-size seeds of forest trees (Simak and Gustafsson, 1953). Currently, X-radiography is used for routine testing of horticultural and forest tree seed quality (Simak, 1991). Argerich and Bradford (1989) have used this method to study embryo and endosperm development in tomato seeds. Van der Burg et al. (1993) have developed X-ray analysis to link aberrations in tomato embryo with seedling abnormalities. We used the same method to investigate the internal morphological changes of tomato seeds upon desiccation, rehydration and redehydration after a pre-sowing imbibition treatment in water (hydropriming) or an osmoticum like polyethylene glycol (PEG) (osmopriming) (Liu et al. 1993). One of the changes observed is the induction of free space, a term which is used to describe the empty area between the embryo and endosperm within a seed (Argerich and Bradford 1989). It was found that the induction of free space by osmopriming was related with the first desiccation of seed upon harvest. However, some seeds also present free space directly after harvest. Possibly, this free space is related to the seed maturation stage, the environmental condition in which the seed develops, or to the genotype.

Using flow cytometry and immunodetection of proteins on Western blots, Bino et al. (1992) and De Castro et al. (1995) showed that DNA replication and ß-tubulin accumulation in radicle-tip cells of tomato embryos, indicating cell cycle activities, preceded visible germination upon imbibition in water or PEG. We have found that fresh PEG priming, where tomato seeds were primed in PEG directly after harvest without desiccation, did neither promote DNA synthesis nor free space, but still enhanced germination without an obvious loss of storability (Liu et al. 1996). However, the addition of exogenous GAs to the priming solution triggered replicative DNA synthesis of fresh primed seeds, enhanced the germination performance, and reduced the storability (Liu et al. 1996). Seed *gib1* did not show DNA synthesis and did not germinate in water (Liu et al. 1994). In the *gib-1* seeds, DNA replication activity in the radicle-tip cells as well as germination could be provoked by the addition of exogenous GAs (Liu et al. 1994). The responses of tomato seed germination to water stress, ABA and GAs have been quantitatively characterized by using a time-course population based threshold model (Ni and Bradford 1993). A major action of GAs appeared to be the induction of the enzymatic degradation of mannan-rich walls of endosperm cells opposite the radicle tip region, prior to radicle protrusion (Groot et al. 1988). The relationship between DNA replication activity in the radicle-tip cells and the weakening of the endosperm opposite the radicle tip, is not yet known (Liu et al. 1994).

Using X-ray photography and flow cytometry, we studied the changes of seed free

space and nuclear replication activity in wild type, *gib-1* and *sit*^{w} tomato seeds upon maturation and after osmotic priming. In this way, we tried to find the causes of the induction of free space and attempted to clarify the regulative role of the endosperm tissue opposite the radicle tip on nuclear replication activity of radicle-tip cells.

MATERIALS AND METHODS

Plant materials. Tomato (*Lycopersicon esculentum* Mill.) seeds of the homozygous GA-(*gib-1*), ABA-deficient (*sit*^{w}) mutants and their wild type (wt) isogenic parent (cv. Moneymaker) were grown in a green house with a temperature range of 21-31 °C (Department of Plant Physiology, Wageningen Agricultural University, The Netherlands). Flowers were self-pollinated by vibration and labelled to obtain seeds of certain days after pollination (DAP). Seeds were harvested, isolated and stored under conditions described by Groot and Karssen (1987). In most cases, for each genotype, two seed lots harvested at 60 DAP (well-matured) or 75 DAP (over-matured) were used.

Treatments. Well-matured seeds (60 DAP) of wild type, *gib-1* and *sit*^{w} were osmotically primed on one layer of filter paper wetted with 5 mL of -1.0 MPa polyethylene glycol-6000 (PEG) solution (Michel and Kaufmann, 1973). Osmotic priming of fresh, non-desiccated seeds in PEG or PEG plus 10 μM GA_{4+7} solution directly after harvest was called fresh PEG priming (FP) or fresh PEG plus GA priming (FP + G). Priming of seeds that were first dried upon harvest, in PEG or PEG plus 10 μM GA_{4+7}, was called normal PEG priming (NP) or normal PEG plus GA priming (NP + G). Fifty seeds were placed in 9-cm petri dishes, sealed within a polyethylene plastic bag and incubated in a dark cabinet at 20 °C. Wild type and *gib-1* seeds were treated for 8 days (d) whereas *sit*^{w} seeds were only treated for 4 d in order to avoid visible germination. After priming, seeds were rinsed with running tap water for 5 minutes (min) and dried at room temperature for 1 d, then equilibrated in a drying drum (23 °C and 32% RH) for at least 2 d. After imbibition of water or -1.0 MPa PEG-6000 for 1 d, about 50 seeds of the well-matured lots of each genotype were detipped by carefully removing the endosperm and testa tissues opposite the radicle tip, and immediately dried for X-ray photography, or further incubated for another 1 d, and then directly used for flow cytometry.

X-ray photography. For X-ray analysis, 1-3 replicates of 50 seeds of each treatment were placed 25 cm from the X-ray source window. The X-ray photograph was made at 10 keV and 3.5 min exposure time, using a 43805N X-ray System (Faxitron™ series, Hewlett Packard, USA).

Flow cytometry. Preparation of nuclear samples, flow cytometric performance and data processing were all conducted as previously described (Bino et al. 1993). Briefly, the PAS II flow cytometer (Partec GmbH, Münster, Germany), equipped with a HBO-100

mercury-arc lamp, a TK-420 dichroic mirror and a GG-435 long-pass filter, was employed to measure each treatment with 2-3 replicates of 5 embryo radicle tips. All analyses were performed using peak-height detection and logarithmic amplification (Bino et al. 1993). The DNA amount 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. Using the signals obtained from tomato leaf tissue, the gained settings were adjusted so that signals of all intact nuclei were registered within the channel range.

Germination. Seed germination was performed under International Seed Testing Association (ISTA) conditions (1993), at an alternating temperature regime of 20/30 °C with 16 h dark and 8 h light, respectively, on one layer of filter paper moistened with distilled water. Root protrusion of 1 mm was recognized as germination. Seedlings were evaluated as normal or abnormal according to ISTA rules (ISTA 1993). At the end of germination tests, ungerminated seeds were inspected by longitudinally cutting them into two halves. Those seeds, in which the embryo and endosperm were firm and showed a bright white colour, were recognized as alive dormant seeds. Parameters for the evaluation of seed germination were the percentage germination (all germinated seeds), normal seedlings (%), dormant seeds (%) and germination rate (T_{50}, days of 50% maximum germination).

Statistical analysis. Germination data were ANOVA analyzed as a randomizing block design with three replicates of 50 seeds. Comparison between different treatments was expressed as the average value plus a standard error. Percentage was analyzed after angular transformation, but untransformed values are shown in the table to facilitate comparison.

RESULTS

Effects of maturation, GAs and osmotic priming on germination. During maturation no viviparous germination was observed in wild type, *gib-1*, and well-matured *sit*^{w} seeds, but 10% *sit*^{w} seeds had viviparously germinated in the over-matured fruits. Besides these viviparous seeds, it was found by X-radiography that the embryonic root tips of about 8% *sit*^{w} seeds had just protruded through the testa. Some of these germinated seeds were damaged upon extraction and subsequent desiccation. Therefore, although 90.7% of over-matured *sit*^{w} seeds germinated, only 72% of them became normal seedlings (Table 1). In water, wild type seeds of both well-matured and over-matured lots started to germinate after 4 d of imbibition and the final germination was 98% and 91.3% respectively (Fig. 1A). Seeds of *gib-1* did not germinate in water (Fig. 1B). ABA-deficient *sit*^{w} seeds of both well-matured and over-matured lots started to germinate in water after 2 d and 1 d of imbibition with a final germination of 100% and 90.7% respectively (Fig. 1C). In GA_{4+7}, wild type seeds germinated slightly faster than in water, and a small increase in

germination percentage was found in over-matured wild type seeds (Fig. 1A). GA-deficient *gib-1* seeds of both well-matured and over-matured lots were induced to germinate after 3 d of imbibition in 10 μM GA_{4+7} and with a final germination of about 85% (Fig. 1B). At the over-matured stage, when imbibed in water, compared to the well-matured stage a

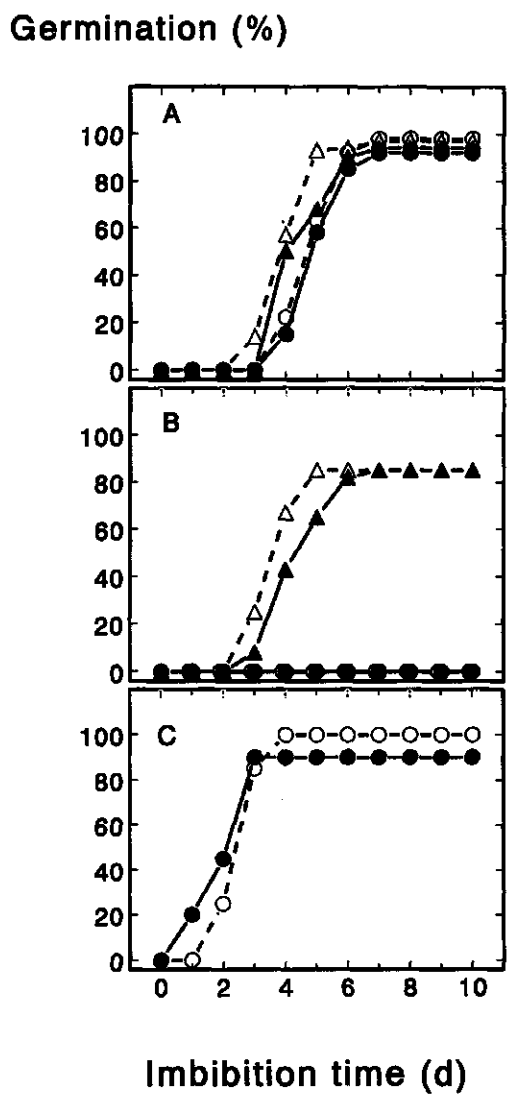

Figure 1. Time course of germination of well-matured (open symbols) and over-matured (solid symbols) wild type (A), *gib-1* (B) and *sit*^{w} (C) seeds upon imbibition in water (○,●) and in 10 μM GA_{4+7} solution (△,▲).

reduction in germination and normal seedlings was found in wild type and *sit*^{w} seed lots, and an induction of dormancy was found in wild type and *gib-1* seed lots (Table 1). During either normal or fresh PEG priming, germination of wild type and *gib-1* seeds was completely prevented within the observed period by -1.0 MPa PEG with or without 10 μM GA_{4+7}. But *sit*^{w} seeds started to germinate after 5 d (fresh PEG priming) or 6 d (normal PEG priming) of imbibition in -1.0 MPa PEG and reached 61% (fresh PEG priming) or 40% (normal PEG priming) of germination after 10 d of imbibition.

Effects of maturation, osmotic priming and detipping on the formation of free space. In most seeds, the testa and endosperm tissues fully enclose the embryo, without any free space. However, free space is visible on the X-ray photographs of some seeds in all genotypes upon harvest and subsequent drying (Fig. 2). At the well-matured stage, about 1-3% of wild type and *gib-1* seeds had free space, and no increase was observed throughout seed maturation (Fig. 2). However, more seeds with free space were found in *sit*^{w} than in wild type and *gib-1* (Fig. 2 and 3). In *sit*^{w}, the number of the seeds with free space increased with seed maturation (Fig. 2). In all three genotypes, normal PEG or PEG plus GA priming and subsequent drying induced free space in more than 95% of the seeds (Fig. 3). A small induction of free space was also found in *sit*^{w} seeds which were freshly primed in PEG or PEG plus GAs (Fig. 3). In contrast, fresh PEG or PEG plus GA priming had no effect on the induction of free space in wild type and *gib-1* (Fig. 3). Almost all viviparously germinated seed in *sit*^{w} lots had free space upon desiccation. An induction of free space was also found when seeds were detipped.

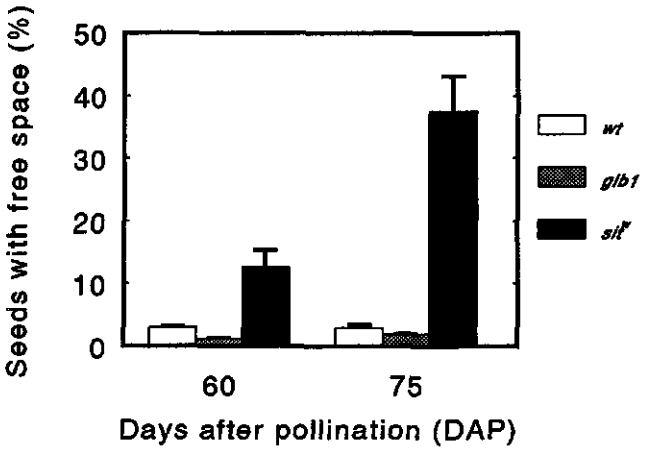

Figure 2. Percentages of wild type, *gib-1* and *sit*^{w} seeds with free space upon harvest at 60 and 75 DAP.

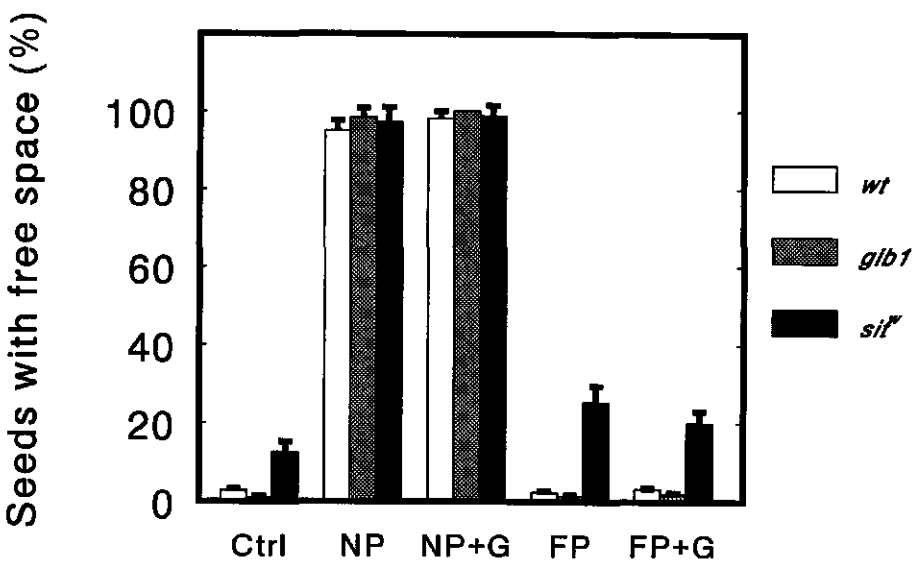

Figure 3. Percentages of wild type, *gib-1* and *sit*^{w} seeds with free space upon harvest at 60 DAP (CTRL), and after normal PEG priming (NP), normal PEG plus 10 μM GA_{4+7} priming (NP + G).

Effects of maturation, osmotic priming and detipping on nuclear replication activity. In the radicle-tip cells of wild type, *gib-1* and *sit*^{w} seeds, upon maturation (45 DAP) one large peak at channel 100 and a small peak around channel 185 were observed on the flow cytometric histograms (Fig. 4). Besides these two peaks, however, at 30 DAP, there were a large peak around channel 250 (Fig. 4). As deduced from leaf tissue of the same diploid tomato genotype, the first peak corresponded with the 2C DNA level from diploid nuclei at the pre-replication stage of nuclear division (G_1 phase), whereas the peaks around channel 185 and 250 represented the diploid nuclei with 4C DNA or endoduplicated nuclei with 8C (G_2 phase), respectively. During seed formation (30 DAP), the cell cycle activities are quite obvious until 45 DAP when the endosperm becomes solid and seed starts to gain desiccation tolerance and germinability in all three genotypes. Upon maturation (45 DAP), cell cycle activities became quiescent with arrest of most cells in G_1 and a small amount of cells in G_2 (Fig. 4). This flow-cytometric profile of radicle-tip cells in wild type and *gib-1* dry seeds did not change throughout the maturation stages (Fig. 4). However, the 4C peak in the radicle tips of dry *sit*^{w} seed increased with maturation, and showed a significantly higher value both in well-matured and in over-matured seeds compared with that in dry wild type and *gib-1* seeds (Fig. 4 and Table 2).

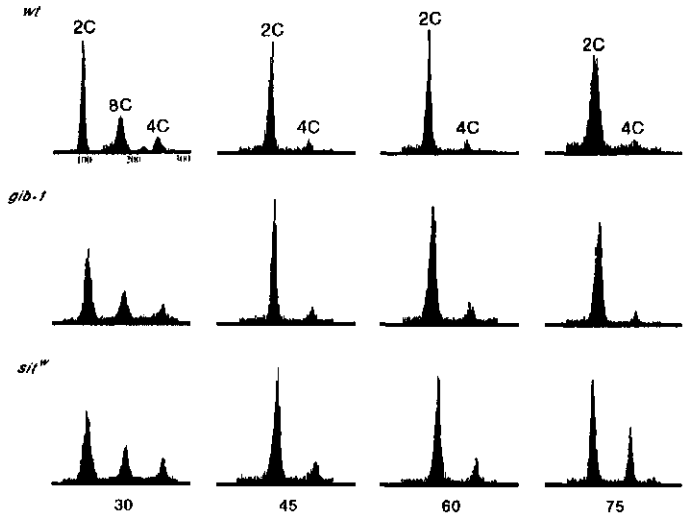

Figure 4. Flow-cytometric histograms of nuclei from embryo radicle tip cells of wild type, *gib-1* and *sit*^{w} seeds, extracted at 30, 45, 60, 75 DAP.

Table 1. Effects of fruit maturation on seed germinability and dormancy. The germination tests were performed in distilled water for wild type and *sit*^{w} seeds or in 10 μM 10 GA_{4+7} solution for *gib-1* seeds.

Genotype, DAP	GP (%)	NS (%)	DS (%)	T_{50} (d)
wild type				
60	98.0	98.0	0	5.19
75	91.3	91.3	3.3	5.28
gib-1				
60	85.3	85.3	8.0	3.92
75	84.7	82.0	12.7	4.55
sit^{w}				
60	100.0	98.7	0	2.90
75	90.7	72.0	0	2.28
$LSD_{0.05}$	4.62	5.18	4.10	0.415

Note:

GP = germination percentage; NS = normal seedlings; DS = dormant seeds; T_{50} = time when 50% of the total number of germinated seeds have germinated.

Upon normal PEG priming (NP), an induction of 4C signal, indicating nuclear replication, was observed in the radicle-tip cells of wild type and *sit*^{w} seeds, but not in *gib-1* seeds (Table 2). With the addition of 10 μM GA_{4+7} to the PEG solution (NP + G), an increase in 4C signal was induced in the radicle-tip cells of *gib-1* seeds prior to visible root protrusion

(Table 2). Upon fresh PEG priming (FP), no increase in 4C signal was found in the radicle-tip cells of both wild type and *gib-1* seeds, but an induction of 4C signals was observed in *sit*^{w} seeds (Table 2). The addition of 10 μM GA_{4+7} to the PEG solution promoted nuclear replication in wild type and *gib-1* seeds upon fresh PEG priming (Table 2). Imbibition of detipped seeds of all genotypes in water or in -1.0 MPa PEG, induced a high 4C signal (Table 2).

Table 2. The percentage of 4C nuclei from embryo radicle-tip cells of dry control seeds of wild type, *gib-1* and *sit*^{w} harvested at 60 DAP (CTRL) and seeds which have been freshly primed (FP) or normally primed in -1.0 MPa PEG-6000 (NP) without GAs or with 10 μM GA_{4+7} (FP+G or NP+G), or detipped after imbibition in water. The values in the table are expressed as the average of 2-3 replicates plus or minus a standard error.

Treatments	*wt*	*gib-1*	*sit*^{w}
CTRL	10.3 ± 1.08	13.2 ± 0.30	18.3 ± 0.94
FP	11.4 ± 1.15	12.6 ± 0.55	29.0 ± 3.57
FP+G	22.2 ± 2.22	30.2 ± 1.03	39.7 ± 3.12
NP	20.5 ± 1.86	14.9 ± 1.21	41.8 ± 3.57
NP+G	45.8 ± 6.25	49.6 ± 1.69	48.6 ± 3.07
Detipped	35.5 ± 1.33	44.9 ± 0.60	43.8 ± 1.65

DISCUSSION

At 30 DAP when the tomato fruit is green and the endosperm in the seed is in the milky state, the embryo is already fully developed. At 45 DAP, the fruit colour starts to turn yellow and the endosperm becomes solid. At the same time, the seeds have gained maximum dry weight, have become desiccation tolerant, and show high germinability. At 60 DAP, the fruit becomes red and seeds have gained maximum germinability (Berry and Bewley 1991, Demir and Ellis 1992, Fig. 1). At the over-mature (75 DAP) stage, a decrease in seed germinability was observed in wild type and ABA deficient *sit*^{w} seeds, which was mainly due to the reduction of seed viability, and partly related with the induction of seed dormancy (Table 1). In the GA-deficient *gib-1* seeds, fruit over-maturation had no effect on germination performance.

In primed tomato seeds, free space is induced by the desiccation upon harvest, and the subsequent hydration and redehydration steps of the priming procedure (Liu et al. 1993). Upon the imbibition of dry seeds, the embryo expands and the endosperm tissue is compressed. Upon redehydration, free space is formed by shrinkage of the embryo and loss of the endospermal flexibility (Liu et al. 1993). Possibly, in these cases, the

endosperm is partly digested, leaving a small amount of empty area, the free space. In freshly harvested seeds, more seeds with free space are found in *sit*^{w} lots compared to the other two genotypes. The number of the seeds with free space is positively related to the occurrence of viviparous germination. Apparently, during viviparous development, the endosperm is degraded and free space is induced.

Between 30 and 45 DAP, the cell cycle activities of the radicle-tip cells in all three genotypes changed markedly. Upon maturation at 45 DAP, the cell cycle of most cells entered a quiescent stage. At the same time, seed started to gain desiccation-tolerance and germinability. Apparently, an arrest of most cells in G_1 phase of nuclear replication marks the end of the active growth of the tomato seed and the gain of desiccation tolerance. At 60 and 75 DAP, most embryo radicle-tip cells of wild type, gib-1 and *sit*^{w} seeds, had arrested cell cycle activity at the G_1 phase of nuclear division. However, ABA-deficient *sit*^{w} seeds contained a significantly higher amount of G_2 cells compared to the other two genotypes. Abscisic acid has been shown to be an inhibitor of cell division (Evans 1984) and may play an important role in the arrest of cell cycle activities during seed maturation (Bouvier-Durand et al. 1989). During development of wild type seed, the endogenous ABA induces a kind of dormancy, arresting the germination of the seeds within the wet fruit, whereas such dormancy is not induced in the ABA deficient *sit*^{w} seeds (Groot and Karssen 1992). The present experiments show that in wild type seeds, nuclear replication activity becomes arrested at 45 DAP. Apparently, ABA is needed for this, since the arrest is only temporally manifested in developing *sit*^{w} seeds.

Upon normal PEG priming or imbibition in water, the DNA amount in the nuclei of radicle-tip cells of wild type seeds duplicates prior to visible germination (Bino et al. 1992). Cell cycle activity is considered to be one of the preconditions for embryonic root protrusion (De Castro et al. 1995). The ratio of 4C/2C in radicle-tip cells has been found to be positively related to the speed of germination, but negatively related to the storability of seeds of tomato (Liu et al. 1996) and pepper (*Capsicum annuum* L.) (Saracco et al. 1994). In wild type seeds, fresh PEG priming did not induce DNA replication. This indicates that the first desiccation upon harvest is necessary to alleviate the dormant state of wild type seeds. However, in *sit*^{w} seeds, fresh priming induced further DNA replication and germination. Apparently, endogenous ABA is responsible for the dormant state of the mature wild type seed and the proper arrest of DNA replication. GA-deficient *gib-1* seeds showed no induction of nuclear replication activity upon imbibition in PEG alone, whereas the addition of GA_{4+7} induced an increase in 4C signals (Table 2). Apparently, GAs are necessary to initiate DNA replication in intact seeds. This is also in agreement with studies using meristematic tissues of various plant species, in which GAs promote cell division by inducing cells in G_1 to enter the DNA synthetic phase (Edelman and Loy 1987).

In *gib-1* seeds, removal of the endosperm and the testa opposite the radicle tip region induced nuclear replication activity upon imbibition either in water in the absence of GAs. This indicates that GAs act indirectly, and that the absence of endosperm weakening opposite the embryo radicle might limit cell growth and cell cycle activities upon imbibition.

An alternative hypothesis is that nuclear replication activity in radicle tips is regulated by water uptake. The mechanical restraint imposed by the tissues surrounding the embryo obviously limits the flow of water into the embryo (Haigh 1988, Dahal and Bradford 1990). What factors are required to initiate the cell cycle in higher plants are far from known. The increase in cell size as an initiator for cell cycle has been found in yeast and animal. It is possible that the initiation of cell cycle also corresponds to the cell growth in seed tissues. Cell growth of seed tissues is characterized by water uptake upon imbibition. In this case, it may be hypothesized that the cell cycle is initiated only when the water uptake by seed tissues has reached a certain level.

In seed, the transitions from cell proliferation to quiescence upon maturation and from quiescence to re-initiation of cellular metabolism upon imbibition are tightly programmed. In many orthodox seed species, these two transitions are separated by a desiccation step when the fruit matures. Hormones, especially ABA, appear to play a crucial role in the arrest of seed development in a quiescence state, and help to provide that the seeds properly respond to the water stress condition (Berry and Bewley 1992). In ABA-deficient tomato seeds, cell cycle activities are only temporally arrested during maturation and the seed may germinate inside the fruit. Apparently, the programming of the induction of dormancy during maturation and the relieve of dormancy upon imbibition are regulated both by hormones and water stress. From our results, it is clear that the programming of the dormancy state coincides with the arrest of the cell cycle, while the release of dormancy during imbibition corresponds with the induction of cell cycle activities. Cell cycle activities may therefore give a valuable marker for determining the physiological status of the seed.

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Chapter 6

Water relations of GA- and ABA-deficient tomato mutants during seed and fruit development and their influence on germination

In cooperation with Raoul J. Bino, Cees M. Karssen and Henk W.M. Hilhorst

(Physiologia Plantarum 1996, 96, 425-432)

ABSTRACT. In order to explain the differing germination behaviour of seeds from wild type, the gibberellin-deficient (*gib1*), or abscisic acid-deficient (*sit*^{w}) mutants of tomato (*Lycopersicon esculentum* cv. Moneymaker), growth and water relations of fruit tissues, seeds and embryos were determined during development. It was the objective to determine whether the hormones influenced water relations of fruit and seed tissues and as a consequence germinability. Despite the up to 70% lower fruit weight of the mutants, the development of fresh and dry seed weight was similar to that of the wild type. Water relations of pericarp and locular tissues in wild type and *gib1* fruits were characterized by a slowly increasing Ψ_π that reached the constant value of Ψ of - 0.60 MPa at 35-40 days after anthesis, resulting in a complete loss of turgor. However, *sit*^{w} fruit tissues maintained their turgor throughout development, mainly as a result of considerably lower Ψ_π values but similar Ψ values compared with the other genotypes. The Ψ of wild type and *gib1* seeds decreased from - 0.50 to -0.80 MPa and Ψ_π from - 0.80 to - 1.00 MPa between day 30 and 40. From day 40 onwards Ψ_p was similar in both genotypes, approximately 0.20 MPa. As in the fruit tissues, *sit*^{w} seed water relations were also characterized by higher turgor values than the other genotypes. Up to day 40 Ψ_p was around 0.40 MPa, dropped temporarily to zero, and increased again to approximately 0.40 MPa at day 50. Embryo water relations of both mutants deviated from the wild type in that Ψ_p of the *gib1* and *sit*^{w} embryos remained at 3.5 MPa and 2.5 MPa, respectively, from day 40 onward, whereas in wild type embryos Ψ_p decreased from 3.0 MPa at day 35 to approximately 1.0 MPa at 50 days after anthesis. This was mainly due to an increasing Ψ_π which was absent in the mutants. Throughout development there was equilibrium between Ψ of pericarp, locular tissues and seeds but between embryo and seed or fruit tissues Ψ gradients of up to 1.5 MPa were calculated in the wild type, and up to 1.0 MPa in the mutants. Thus, precocious germination is prevented by the action of the fruit osmotic environment and ABA on the seed tissues surrounding the embryo and not the embryo itself. Embryos have a Ψ which is low enough to overcome the solute potential of the fruit tissues throughout development.

Key words: Abscisic acid, fruit development, germination, gibberellins, hormone mutants, *Lycopersicon esculentum*, precocious germination, seed development, tomato, water relations.

INTRODUCTION

During development of seeds of many species the fruit tissues desiccate simultaneously with the seed. Seed water content decreases to 5-15 % and the vascular connection with the parent plant is lost. However, in fleshy fruits, seeds mature in a moist environment and

the final seed water content remains high, e.g. 40% in tomato (Berry and Bewley 1991). When tomato seeds are removed from the fruit at an early stage of development they germinate readily on water. This suggests that, within the fruit, seed germination is suppressed. It has been proposed that the osmotic environment of the fruit tissues plays an important role in the suppression of precocious germination (Berry and Bewley, (1991). Abscisic acid (ABA) has been implied in the induction of dormancy during seed development and in the prevention of precocious germination. In overripe fruits of the ABA-deficient *sit*^{w} mutant of tomato viviparous germination is a common phenomenon and the mature seeds are never dormant Seeds from the *sit*^{w} mutant contain only trace amounts of ABA throughout development whereas in wild type seeds ABA content peaks between mid and late maturation. Mature wild type seeds also contain very low amounts of ABA but may still be dormant (Groot and Karssen, 1992). Apparently, ABA influences developmental processes which still affect germination at maturity when ABA contents have dropped to negligible levels.

Gibberellins (GAs) are the natural antagonists of ABA action and stimulate germination of many species (Bewley and Black, 1994). Mature seeds from the gibberellin-deficient *gib1* tomato mutant and the *ga1* mutant from *Arabidopsis thaliana* do not germinate in the absence of exogenous gibberellins, indicating that GAs play a keyrole in germination (Karssen et al. 1989). Developing seeds contain large amounts of GAs (Bewley and Black, 1994), yet GAs are not absolutely required for seed development. Apart from a requirement for flower induction and fertilization, GAs are not involved in the control of seed development in tomato. Seeds of the *gib1* mutant develop normally with respect to seed weight and protein content (Groot et al. 1987). Germination growth is accompanied by water uptake and thus requires a disequilibrium in water potential between the seed and its environment (Haigh and Barlow 1987, Bradford 1992). For *Brassica napus* embryos it has been shown that ABA inhibits germination in a similar way as a low water potential of the environment (Schopfer and Plachy 1985). Oppositely, it may be hypothesized that GA lowers the seed water potential, and may thus promote germination.

There is a lack of understanding if and how plant hormone action may influence water relations and, consequently, the germination performance of seeds. In the present study the *sit*^{w} and *gib1* mutants of tomato are used to determine whether absence of these hormones during seed development influences the water relations of the fruit and seed. This may provide information about the germination behaviour of these mutants, both during development, *in vitro* and *in vivo*, and at maturity.

MATERIALS AND METHODS

Plant material. Tomato (*Lycopersicon esculentum* L. cv. Moneymaker) plants of the homozygous GA-deficient *gib1* (line W335)) and ABA-deficient *sitiens* (*sit*^{w}) mutants and their wild type isogenic parent were grown in pots in the greenhouse at a minimum temperature of 22°C during the photoperiod and 18°C during the night in the summer of

1993. The photoperiod was maintained at 16 h by additional irradiation from high-pressure mercury halide lamps (Philips HPI/T) at 18 Wm^{-2}. Once a week, seedlings and plants of *gib1* and *sit*^{w} were sprayed with approximately 2 ml of 10 μM GA_{4+7} (Berelex GA4/7, ICI Holland, Rotterdam, The Netherlands) or 10 μM ABA (racemic mixture, Sigma, St Louis, MO, USA), respectively, on the tops of the plants only, to allow normal flower development on *gib1* plants or to prevent wilting of *sit*^{w} plants. A higher dose of GA in the spraying solution (50-100 μM) could restore normal growth. The dose used in the present experiments was just sufficient to induce normal flowering, thus minimizing possible uptake of exogenous GAs by fruits and seeds (S. P. C. Groot 1987. Thesis Wageningen Agric. Univ., Wageningen, The Netherlands). Fully-opened flowers were labelled and then self-pollinated by vibration.

Determination of fruit weight. Three fruits of representative size among the population of each maturation stage were used for determination of fruit fresh weight.

Determination of water content. Three replicates of 20 seeds removed from freshly harvested fruits followed by brief blotting with filter paper tissue, were weighed to determine fresh weight. Subsequently, seeds were dried at 130 °C for 1 h and reweighed to determine dry weight.

Measurement of water- and osmotic potential. The water potential (Ψ) of tomato fruit tissues, whole seeds and seed parts were detected by thermocouple psychrometry. A C-52 sample chamber (Wescor Inc., Logan, UT, USA) was adapted for connection to an NT-3 Nanovoltmeter (Cecaquon, Pullman, WA, USA). The sample chamber was placed in an airtight glove box kept at 95-100% relative humidity by a stream of water-saturated air at a constant temperature of 25 ± 1 °C. All manipulations were carried out under these conditions. At least three replicates of three seeds, five embryos, and pieces of pericarp and locular (sheath) tissue, each using a different fruit, were measured. Before measurement of whole seeds the locular tissue was carefully removed with forceps. Water potential measurements of embryos were not taken prior to 25 days post anthesis because at this early stage of development embryos could not be isolated without damaging them to an unacceptable extent. Equilibration time was 30 min and cooling time 30 s. All measured samples were immediately put into liquid nitrogen after which the osmotic potential (Ψ_π) of the thawed material was determined. The psychrometer was calibrated weekly with fresh, standard PEG 6000 (Serva, Heidelberg, Germany) solutions of different osmotic potential as calculated according to Money (1989). The pressure potential (Ψ_p) was calculated from the equation: $\Psi_p = \Psi - \Psi_\pi$. Water potential gradients were calculated by subtracting the measured Ψ values of the different tissues.

Germination. Triplicates of 20 locular free seeds, each using a different fruit, were sown in a 5-cm Petri dish on two layers of filter paper (Schleicher & Schull, no. 595) in 1.5 ml

distilled water or 100 μM GA_{4+7} (ICI, UK). The Petri dishes were sealed with Parafilm and placed in an incubator at 25 ± 1 °C in darkness. Germination was considered completed when radicle protrusion was visible. Germination was scored daily for 10 days.

RESULTS

Fruit and seed growth. The *gib1* and *sit*^{w} mutant plants showed retarded growth. The regular spraying of the *gib1* plants with 10 μM GA_{4+7} still resulted in some dwarf growth but flower development was normal. Growth of the *sit*^{w} plants was also retarded because of evident drought stress.

The ABA- and GA-deficient plants produced considerably smaller fruits than wild type plants (Fig. 1a). Fresh weight of wild type fruits was three to four times that of the mutant fruits at 60 days after anthesis. Relative water content of the fruit tissues did not differ between genotypes and remained at 97% throughout development (results not shown). From 35-40 days after anthesis onwards, wild type fruits maintained growth whereas both *sit*^{w} and *gib1* fruit growth was reduced or arrested, respectively. At 60 days after anthesis *sit*^{w} fruit weight was 40% higher than *gib1*. As judged from color development, fruit ripening was temporally similar among the three genotypes (data not shown).

The increase of seed dry weight and water content was independent of fruit size and did not vary among the three genotypes throughout the developmental period (Fig. 1b,c). Main decrease (85 down to 55%) in seed water content of all genotypes occurred between 30 and 40 days post anthesis.

Fruit and Seed Water Relations.

Fruit tissues. Pericarp tissue of wild type fruits showed a slowly increasing (less negative) Ψ_π until it approached the constant value of the Ψ of - 0.60 MPa at around 20 days after anthesis, resulting in a complete loss of turgor of the ripe pericarp tissue (Fig. 2a). Between 30 and 50 days after anthesis both Ψ and Ψ_π decreased to a value around - 0.75 MPa. Wild type locular tissue displayed a similar pattern as pericarp tissue except that at 20 days post anthesis Ψ_π of the locular tissue was substantially more negative (Fig. 3a). Values of Ψ and Ψ_π of locular tissue from the *gib1* mutant showed essentially similar patterns as found for the wild type (Fig. 3b). However, the increase of Ψ_π of *gib1* pericarp tissue was delayed as compared with the wild type and did not commence until 20 days post anthesis (Fig. 2b). Water relations of *sit*^{w} pericarp tissue development deviated considerably from the wild type pattern (Fig. 2c). Whereas Ψ values were comparable with those of the wild type pericarp, Ψ_π values were considerably lower (more negative), especially from day 35 onwards when *sit*^{w} Ψ_π decreased sharply. As a result of this, the Ψ_p of pericarp tissue from *sit*^{w} fruits increased from the constant value of approximately 0.35 MPa between day 10 and 35 to 0.60 MPa at 50 days after anthesis whereas turgor of wild type and *gib1* pericarp was zero. Water relations of locular tissue during development of *sit*^{w} fruits also deviated substantially from those of the wild type.

However, contrary to the pericarp, the Ψ_p of the *sit*^{w} locular tissue dropped from approximately 0.80 MPa to 0.20 MPa between days 40 and 50 post anthesis, due to a larger rise in Ψ_π during that part of development.

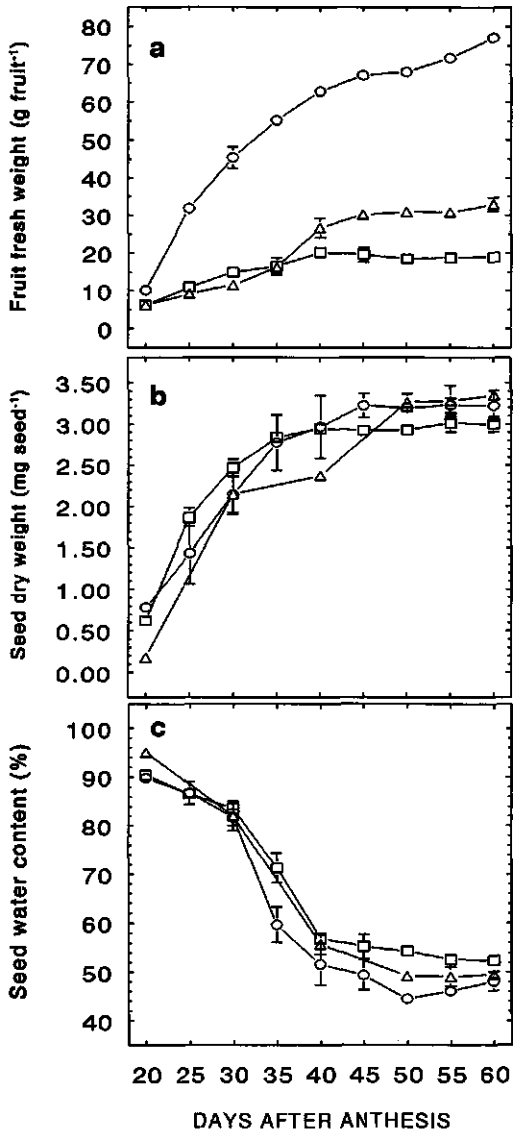

Figure 1. Fruit fresh weight (a), seed dry weight (b) and seed water content on fresh weight basis (c) during development of wild type (○), *gib1* (□) and *sit*^{w} (△) fruits and seeds. Bars are SE of means.

Seeds. Following the loss of seed water between days 30 and 40 post anthesis, Ψ of wild type and *gib1* seeds decreased from - 0.50 to - 0.80 MPA and Ψ_π from - 0.80 to - 1.00 MPa (Fig. 4a,b). Apart from a considerably lower Ψ_π at day 10 of wild type seeds, wild type and *gib1* seed Ψ and Ψ_π were approximately similar over the developmental period. From 40 days post anthesis onwards, Ψ_p was similar in both genotypes and was approximately 0.20 MPa. Again, the pattern of Ψ and Ψ_π of *sit*^{w} seeds deviated from that of wild type and *gib1* seeds (Fig. 4c). The Ψ_π of the *sit*^{w} seeds slowly decreased from - 0.90 MPa at day 10 to - 1.20 MPa at 50 days after anthesis. These values are only slightly lower than those measured in wild type and *gib1* seeds. Up to 35 days post anthesis the *sit*^{w} Ψ remained close to values around - 0.40 MPa as was also found in the other genotypes. However, between day 35 and 40 DAA a characteristic drop in Ψ from - 0.40 to - 1.10 MPa occurred, followed by a steep increase to - 0.80 MPa between 40 and 50 days after anthesis. This 'dip' in Ψ was also found in the locular tissues (Fig. 3c) but, in contrast to the seeds, in these tissues the drop in Ψ was accompanied by an even greater one in Ψ_π, resulting in an increase of Ψ_p. This was also observed in the pericarp but to a lesser extent (Fig. 2c).

Embryos. Embryo water relations in seeds of wild type plants are characterized by a steep decrease of Ψ_π from -2.0 to -5.0 MPa between day 30 and 35 after anthesis and a less pronounced decrease in Ψ from - 1.2 to - 2.5 MPa between 30 and 40 days post anthesis (Fig. 5a). From 35 DAA onwards Ψ_π increased to approximately -3.5 MPa at day 50, whereas Ψ remained constant between day 40 and 50. Final Ψ_p was 1.2 MPa. Between 30 and 40 days after anthesis Ψ_π of *gib1* and *sit*^{w} embryos decreased to values between - 4.0 and -5.0 MPa and remained constant thereafter (Figs. 5 b, c). Both genotypes also displayed relatively high Ψ after 30 days post anthesis as compared with wild type, resulting in high Ψ_p at maturity, 3.5 MPa in *gib1* and 2.5 MPa in *sit*^{w}.

Water potential gradients between embryo, seed and fruit tissues. Ψ gradients between embryo and seed, seed and locular tissue and locular tissue and pericarp were calculated from the data presented in Figs 2-5 (Fig. 6). In all three genotypes Ψ gradients between seed and locular tissue and between locular and pericarp tissues were practically absent. However, Ψ gradients between embryo and seed were substantial in all genotypes. In wild type seeds this gradient decreased from approximately - 0.70 MPa at day 30 post anthesis to - 1.70 MPa at day 40-50. (Fig. 6a). In *gib1* seeds this decrease was largely absent leaving gradient values at - 0. 60 to - 0.90 MPa throughout development (Fig. 6b). The same was true for *sit*^{w} seeds but gradient values were comparatively low up to 30 DAA (- 0.40 MPa) followed by a small decrease to - 1.10 MPa at 50 DAA (Fig. 6c).

Seed germination. Germinability of wild type and *sit*^{w} seeds started to increase between 25 and 35 days after anthesis to approximately 90% around day 50 (Fig. 7). Seeds of the *gib1* mutant were non-germinable throughout the developmental period. However, on 100

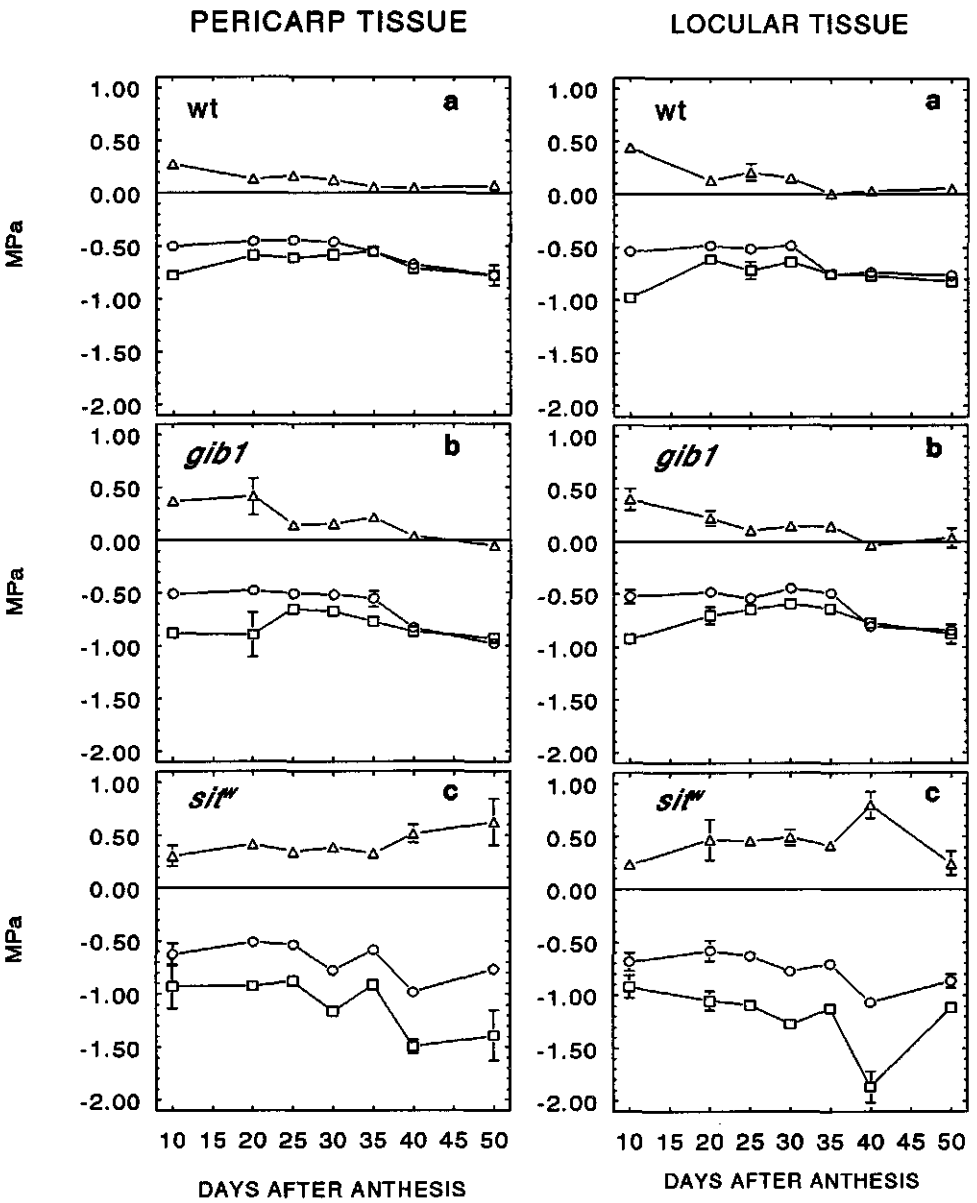

Figure 2 (left). Water potential (○), osmotic potential (□) and pressure potential (△) of wild type (a), *gib1* (b) and *sit*^{w} (c) pericarp tissue during development. Pressure potentials were calculated by subtracting water- and osmotic potentials. Bars are SE of means.

Figure 3 (right). Water potential (○), osmotic potential (□) and pressure potential (△) of wild type (a), *gib1* (b) and *sit*^{w} (c) locular tissue during development. Pressure potentials were calculated by subtracting water- and osmotic potentials. Bars are SE of means.

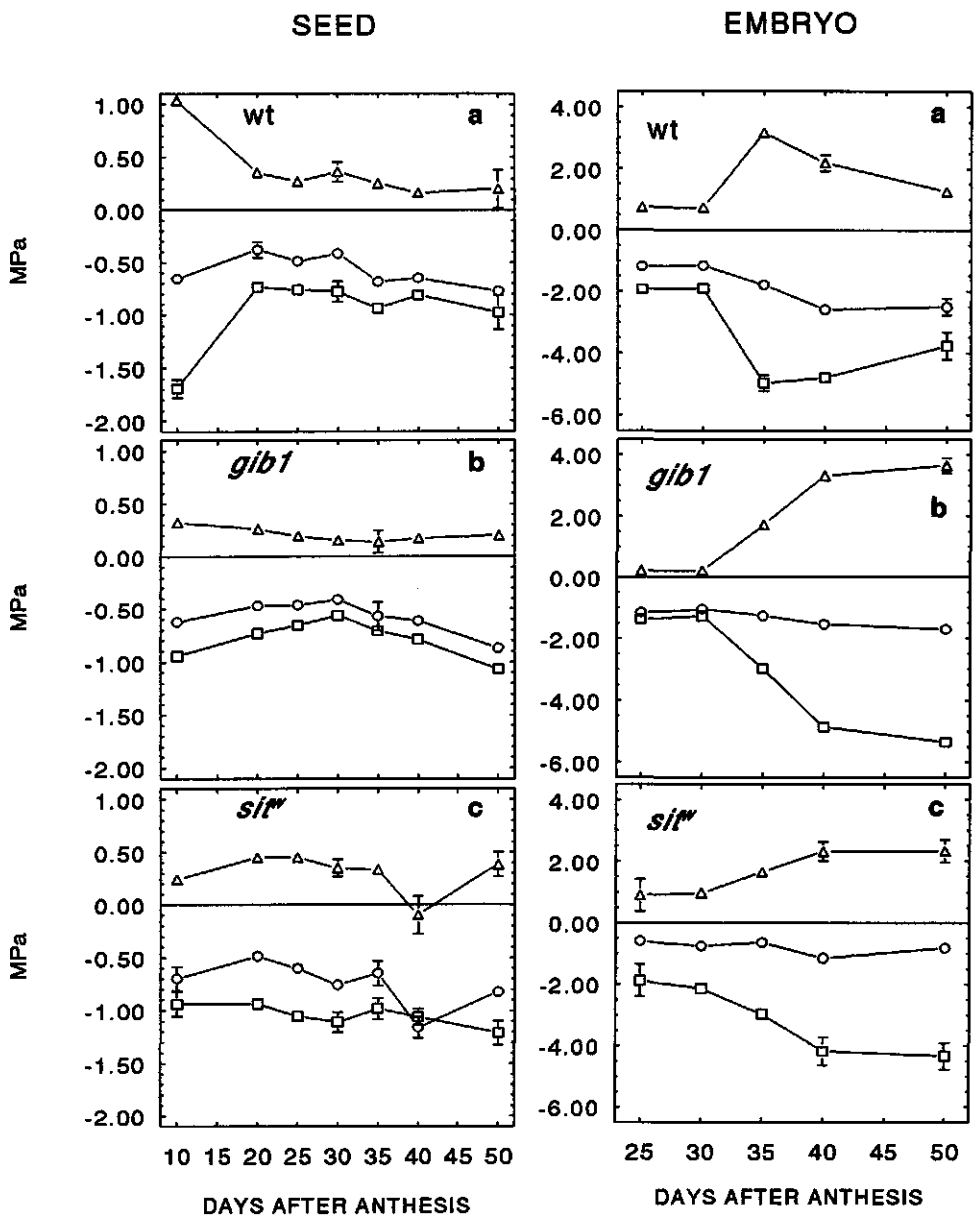

Figure 4 (left). Water potential (○), osmotic potential (□) and pressure potential (△) of wild type (a), *gib1* (b) and *sit*^{w} (c) seeds during development. Pressure potentials were calculated by subtracting water- and osmotic potentials. Bars are SE of means.

Figure 5 (right). Water potential (○), osmotic potential (□) and pressure potential (△) of wild type (a), *gib1* (b) and *sit*^{w} (c) embryos during development. Pressure potentials were calculated by subtracting water- and osmotic potentials. Bars are SE of means.

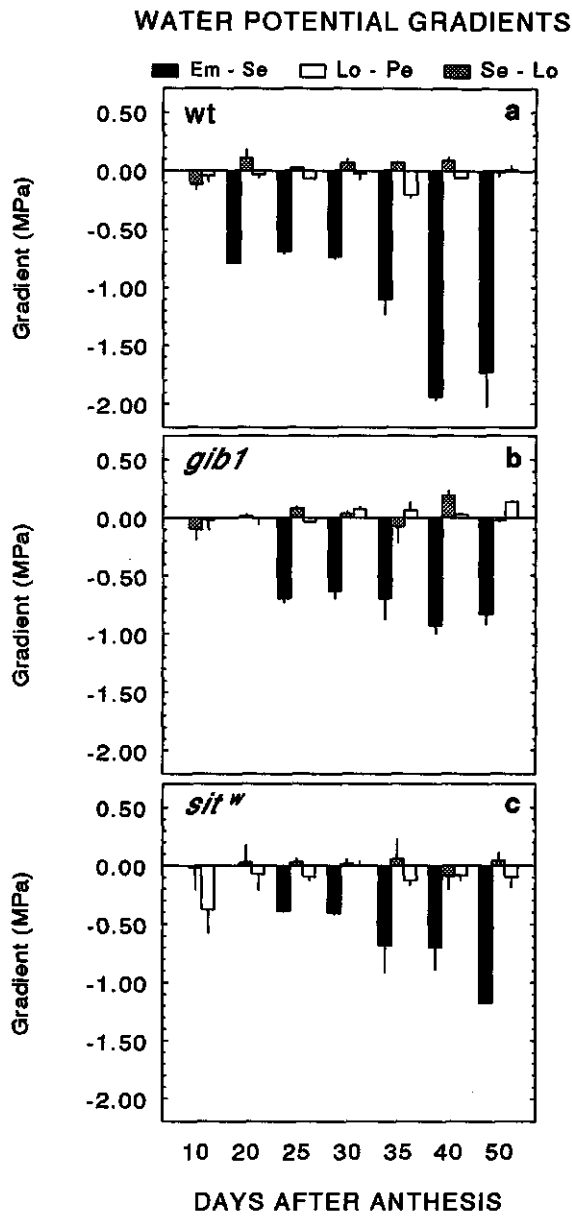

Figure 6. Water potential gradients between fruit and seed tissues. Gradients were calculated from water potentials of embryos and seeds (Em-Se), seeds and locular tissue (Se-Lo), and locular and pericarp tissue (Lo-Pe) of wild type, *gib1* and *sit*^{w}. Error bars are SE of means.

μM GA_{4+7} germinability of these seeds during development was similar to the other genotypes.

DISCUSSION

Growth of both the GA- and ABA-deficient tomato mutants was retarded under the present conditions. The major reason for impaired fruit growth on *sit*^{w} and *gib1* plants has been shown to be reduced pollen production and hence a lower number of seeds per fruit (Groot et al. 1987, 1991). The number of seeds per fruit strongly influences fruit growth in tomato (Varga and Bruinsma 1976). In the present experiments, the number of seeds per fruit of *sit*^{w} and *gib1* plants was approximately half of that of the wild type (results not shown), which is reflected in a comparable increase in fruit fresh weight up to 35 days post anthesis. The water relations measured during development of wild type and *gib1* Moneymaker fruit tissues, resulting in a complete loss of turgor at maturity are in agreement with results reported for the locular tissue of the tomato cultivar Caruso (Berry and Bewley 1992) and the pericarp of muskmelon fruit (Welbaum and Bradford 1988). The loss of turgor is very likely the result of the loss of cell integrity (softening) during ripening of the fruit. The turgor of the *sit*^{w} fruit tissues was not lost at 35-40 days after anthesis as in the other genotypes. These positive turgor values were mainly the result of lower (more negative) Ψ_{π}. Apparently, the pericarp and locular tissue of the *sit*^{w} fruits contained higher amounts of osmotically active solutes than wild type and *gib1* fruits. This is reflected in the *sit*^{w} fruit weight which exceeded that of *gib1* fruits from days 35-40 onwards, possibly resulting from a higher sink strength. In a study with tomato ripening mutants it was shown that the maximum ABA content of the pericarp tissue of the very

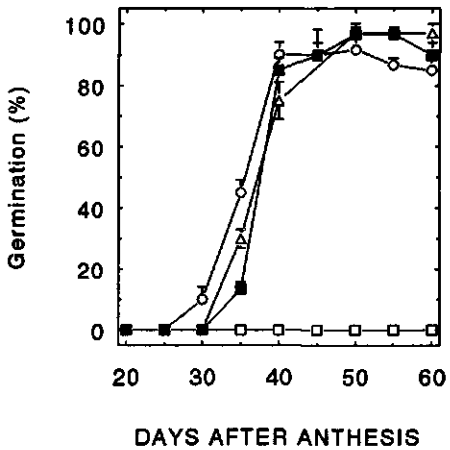

Figure 7. Germinability of intact seeds taken from the fruits at several stages of development. Germination of wild type (○), *gib1* (□) and *sit*^{w} (△) seeds was tested on water (open symbols) or on 100 μM GA_{4+7} (*gib1*, ■). Bars are SE of means.

slow softening *nor* mutant was approximately a third of that of normal cv. Rutgers fruits. ABA levels peaked around 70 days after anthesis in *nor* fruits, whereas in the wild type maximum ABA content was measured at day 50 (McGlasson and Adato 1976). Thus a role for ABA in tomato fruit ripening is very likely although its site of action remains obscure.

Fresh and dry seed weights were similar in the three genotypes throughout development and thus independent of fruit growth. Fresh and dry weights of mature *sit*^{w} seeds have been shown to be similar to those of wild type seeds (Groot et al. 1991). However, our results with the *gib1* mutant contradict earlier observations that GA-deficient seeds contained less water throughout development and underwent dehydration at an earlier stage than wild type seeds, whereas dry weights were similar (Groot et al. 1987). In the present study we did not find notable differences in seed water content among the genotypes. The cause of this discrepancy is not known. The main decrease in seed water content of all genotypes between days 30 and 40 coincides with the formation of a firm endosperm and the loss of water from the integuments, resulting in the formation of the testa (Smith, 1935, Groot et al. 1991, Berry and Bewley, 1991).

At 10 days after anthesis Ψ_{π} of wild type seeds was lower than that of *gib1* seeds but during further development water relations were similar between both genotypes. This indicates that the absence of GAs during seed development did not affect the water relations of the seeds. Water relations of *sit*^{w} seeds deviated from the wild type pattern, especially at around day 40 when Ψ_{p} dropped to zero in the *sit*^{w} seeds. This temporary loss of turgor of the *sit*^{w} seeds coincides with the period of testa formation from the collapsing integument after initiation of the formation of trichomes from the epidermis of the integument (Smith 1935). Recently, it was shown that the testa of ripe *sit*^{w} seeds consists of only one cell layer whereas wild type testa are five cells thick (Hilhorst and Downie 1996). Conceivably, the much thinner layer of cells between endosperm and locular tissue allows leakage of cell contents from the endosperm to the surrounding tissues during testa formation. It would explain the drop in Ψ and Ψ_{p} with the Ψ_{π} remaining constant in the seeds and the concomittant decrease of Ψ and Ψ_{π} and increase in Ψ_{p} of the locular tissue.

Embryo water relations of all genotypes were characterized by a steep decrease of Ψ_{π} between 30 and 35 (wild type) or between 30 and 40 days (mutants) after anthesis. This is indicative of the accumulation of osmotically active compounds during this phase of development. In wild type embryos the lowest (most negative) Ψ_{π} value was attained at day 35, five days earlier than in *gib1* and *sit*^{w} embryos. It is possible that the decrease in Ψ_{π} reflects the increasing pool of imported assimilates that are to be converted into polymeric storage compounds. When accumulation of storage compounds exceeds the import of assimilates, an increase (less negative) of Ψ_{π} may be anticipated. This increase was only observed in wild type embryos, suggesting that both the absence of GAs and ABA impaired reserve food accumulation. There are some indications that GA and ABA are involved in storage of reserve food. The ABA-deficient and -insensitive *aba,abi3* double mutant of *Arabidopsis thaliana* is impaired in the synthesis of long-chain fatty acids (DeBruijn et al. 1993). As a result, assimilates imported into the seeds were to a much

larger extent converted into starch. Furthermore, ABA- deficient seeds from *A. thaliana* and *gib1* and *sit*^{w} embryos from tomato contain lower amounts of total protein but considerably higher levels of soluble carbohydrates than the respective wild-types (Groot et al. 1987, 1991, Meurs et al. 1992, DeBruijn et al. 1993, Hilhorst and Downie 1996).

Water uptake by the seeds and subsequent germination can only occur when the Ψ of the seed is more negative than the Ψ of the environment. In the cultivar used here, we did not find appreciable Ψ gradients between seeds and surrounding locular tissue or pericarp in all three genotypes. However, substantial Ψ gradients existed between embryo and whole seed in wild type and both mutants. This strongly indicates that water uptake by the embryo is prevented by the mechanical restraint exerted by endosperm and testa. Haigh and Barlow (1987) could not detect any lowering of embryo Ψ_{π} or buildup of Ψ_{p} in mature tomato seeds prior to radicle protrusion. They concluded that radicle emergence is dependent upon reduction in the endosperm constraint rather than upon the increase of embryo Ψ_{p}. It has indeed been shown that endosperm weakening occurs prior to radicle protrusion in mature tomato seeds and that this process is stimulated by GA and inhibited by ABA (Groot et al. 1988). Ni and Bradford (1993) found good correspondence between the effects of GA and ABA on the radicle emergence of tomato seeds in osmotic solutions and the effects of these hormones on endosperm softening. They also concluded that only endosperm weakening was required to facilitate water uptake by the embryo. Based on measurements of water content and water relations of the different tissues in muskmelon (*Cucumis melo* L.) seeds Welbaum and Bradford (1990) reached a similar conclusion. Thus it appears that in mature tomato and muskmelon seeds the regulation of germination resides in the endosperm and that ABA and GA are involved. In a series of experiments with developing tomato seeds Berry and Bewley (1992) demonstrated that the germination response of isolated seeds to osmoticum more closely resembled their response to fruit tissues than did their response to ABA. They proposed that the osmotic environment plays a greater role than endogenous ABA in the prevention of precocious germination. Because of the steep gradients in Ψ between embryo and fruit tissues found in the present study we can only conclude that the osmotic environment of the seeds within the fruits inhibits processes that contribute to endosperm weakening. ABA in fruit and seed tissues may have an additional role in the prevention of endosperm weakening. The osmotic environment may act by reducing the activity of hydrolytic enzymes that are involved in endosperm weakening. It has been shown that the activity of the enzyme endo-ß-mannanase in the endosperms of fenugreek (Spyropolous and Reid, 1988) as well as tomato (Hilhorst and Downie, 1996) is negatively influenced by osmotic stress. This enzyme is associated with endosperm degradation in a number of species (Reid, 1985).

We hypothesize that in wild type tomato seeds the combined action of ABA and solute potential prevents endosperm weakening and hence germination. In the *sit*^{w} mutant ABA levels are negligible and would allow (slow) endosperm degradation and, ultimately, radicle protrusion in overripe fruits. In the absence of GAs, as in the *gib1* mutant, endosperm softening does not occur since it is under absolute control of GA (Groot and Karssen,

1987). In this respect the formation of a firm endosperm between 20 and 30 days after anthesis (Berry and Bewley, 1991), prior to the induction of germinability, may be of great significance.

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Chapter 7

Influence of GA and ABA on water relations of tomato (*Lycopersicon esculentum* Mill.) seed during germination and osmotic priming

In cooperation with Zemin Luo, Henk W.M. Hilhorst, Cees M. Karssen and Raoul J. Bino

ABSTRACT. The effects of gibberellins and abscisic acid on water uptake of tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) seeds were studied during germination and osmotic priming. Using thermocouple psychrometry, the water relations of seeds and embryos from the GA-deficient mutant (*gib-1*), the ABA-deficient mutant (*sit*^{w}), and wild type tomato were analyzed upon imbibition in water, 10 μM GA_{4+7}, 10 μM ABA, -1.0 MPa PEG-6000, -1.0 MPa PEG plus 10 μM GA_{4+7} or 10 μM ABA solutions. *Sit*^{w} seeds germinated faster than wild type seeds both in water and in 10 μM GA_{4+7} solution whereas *gib-1* seeds did not germinate in water. Thus, the water potential (Ψ) and osmotic potential (Ψ_{π}) of wild type and *sit*^{w} seeds were triphasic in response to the water uptake whereas that of *gib-1* seeds expressed a biphasic curve upon imbibition in water. No difference in water relations of intact seeds upon imbibition of water was found among the three genotypes. However, when fully imbibed in water (24 h of imbibition), the three genotypes differed in the water relations and water contents of intact embryos. It appeared that germination performance was negatively related to the water content of whole intact seed whereas positively related to that of the intact embryo. The mechanical restraint imposed by the tissue surrounding the embryo obviously limited the water flow into the embryo by restricting its expansion. Exogenous GAs and ABA might relieve or strengthen this restraint upon embryo water uptake, resulting in promoting or inhibiting germination by GAs and ABA respectively. A corresponding effect of GAs or ABA on dissected embryo water uptake was not found. Therefore, it is concluded that the influences of GA and ABA on water relations of tomato seeds are complemented by modifying the endosperm weakening rather than by the cell wall loosening in radicle.

Key words: ABA; GA; germination; osmotic priming; tomato seeds; water relations

INTRODUCTION

In tomato (*Lycopersicon esculentum* Mill.) seeds, the completion of germination is considered to be dependent both upon the expansion force of the embryo and upon the occurrence of endosperm weakening (Bradford 1990, Karssen *et al.* 1989). Directly measuring the mechanical restraint of the endosperm layer opposite the radicle tip upon imbibition of tomato seeds, Groot and Karssen (1987) found an obvious endosperm weakening prior to visible germination which they attributed to the activities of a series of hydrolytic enzymes. These enzymes were stimulated by gibberellins (GAs) excreted from the radicle tip (Groot *et al.* 1988). Since then, focusing on the endosperm weakening of tomato seeds, attempts have been made to determine the differences both in germinability between genotypes (Karssen *et al.* 1989, Leviatov, Shoseyov and Wolf 1994) and between the seed lots imbibing in different conditions, such as low temperature (Leviatov,

Shoseyov and Wolf 1995), far red light (Nomaguchi, Nonogaki and Morohashi 1995), water stress or abscisic acid (Ni and Bradford 1993). It was concluded that the degree of reduction in the restricting force against the embryo growth, imposed by the endosperm, is the key to control seed germination. Thus, it appears that the radicle growth potential, i.e. potential to expand (reversible) and elongate (not reversible) upon imbibition, only plays a minor role in the regulation of tomato seed germination since many factors which may influence germination could not change the radicle growth potential. However, upon finding that the removal of endosperm opposite the radicle tip did not make seeds of a cold sensitive line germinate like a cold tolerant line at 10 °C, Liptay and Schopfer (1983) suggested that the difference in germinability between two genotype lines resided in the germinating embryo. Using X-ray analysis technique it was found that the expansion of the embryo of intact seed upon imbibition was longitudinally orientated to the direction of radicle elongation (Liu *et al.* 1993). This expansion was a result of the increase in the size of radicle cells as mitosis was not observed before protrusion (Liu *et al.* 1995). Exogenous GAs induce DNA replication in tomato radicle tips (Liu *et al.* 1994) and abscisic acid inhibits the water uptake of intact embryos of *Brassica napus* seeds (Schopfer and Plachy 1984). These phenomena indicate that gibberellins and abscisic acid might be involved in changing the embryo growth potential. It is of interest to know how exogenous and endogenous gibberellins and abscisic acid act on embryo growth and what are their relations to the endosperm weakening. Here, we have used seeds of gibberellin- and abscisic acid-deficient tomato mutants to study the influences of GA or ABA upon the water relations during germination and osmotic priming.

METHODS AND MATERIALS

Materials. Seeds of GA- (*gib-1*) and ABA-deficient (*sit*^{w}) mutants and wild type (*wild type*) were harvested in 1988. Seeds were isolated from mature fruits and incubated in 1% HCl for 1 h to remove the remnants of the mucilaginous locular issue. Thereafter the seeds were rinsed with tap water, dried at room temperature and stored in closed containers in a refrigerator at 7 °C until use. Comparisons between genotypes were always made with seed lots from the same harvest date.

Germination tests. Four replicates of 20 seeds of each genotype were placed in a 5-cm Petri dish on two filter papers (Schleicher & Schull no. 595) wetted with 1.5 mL of distilled water, 10 μM GA_{4+7}, 10 μM ABA, -1.0 MPa PEG-6000 (Michel and Kaufman 1973) with or without 10 μM GA_{4+7} solutions. The dishes were sealed with Parafilm and incubated in the dark at 25 °C. Visible radicle protrusion was recognized as germination. Germinated seeds were counted every 12 hours in the first 3 days and then daily until 10 d of imbibition.

Measurement of water uptake. Upon imbibition at 20 °C in distilled water (H_2O) or 10 μM

GA_{4+7} (GA), 10 μM ABA (ABA), -1.0 MPa PEG-6000 (P), -1.0 MPa PEG plus 10 μM GA_{4+7} (P+G) or plus 10 μM ABA (P+A) solutions respectively, 3 replicates of 5 intact seeds, intact embryos dissected by the time of observation ('intact embryo'), and the embryos dissected at 1 h after imbibition ('dissected embryo'), were withdrawn from imbibition at intervals and blotted between two layers of filter paper by gently pressing, and immediately weighed. Dry weight was determined by weighing after drying at 132 °C for 1 h.

Determination of water potential (Ψ) and osmotic potential (Ψ_π). The water potential of whole seeds and seed parts was detected by thermocouple psychrometry, a C-52 sample chamber (Wescor, Logan, USA) connected to a NT-3 Nanovoltmeter (Cecaquon, Pullman, USA). All manipulations were carried out as previously described (Liu *et al.* 1995). At least 2-3 replicates of 3 seeds or five embryos were measured for each treatment. The psychrometer was calibrated weekly with fresh standard PEG-6000 solutions of different osmotic potentials as calculated according to Money (1989). The pressure potential was calculated from the equation: $\Psi_p = \Psi - \Psi_\pi$.

Statistic analysis. Data were ANOVA analyzed as a randomized block design with 4 replicates of 20 seeds for germination tests, 3 replicates of 5 seeds or seed parts for water uptake determinations and 2-3 replicates of 3 seeds or 5 embryos for the measurements of water relations. All average values are presented with standard error bars in the figures.

RESULTS

Effects of GA, ABA and osmotic stress on germination. In water, wild type seeds started to germinate after 36 h of imbibition and the final germination was 98%. *Sit*^{w} seeds started to germinate after only 24 h of imbibition with final germination of 72%. *Gib-1* seeds did not germinate throughout imbibition in water. In GA_{4+7}, both *sit*^{w} and *gib-1* seeds started to germinate after 24 h of imbibition and germination after 120 h was 72%. Wild type seeds germinated slightly faster in GA_{4+7} than in water. In ABA, seeds of all three genotypes failed to germinate within the observed period (10 d) (results not shown). Germination of wild type and *gib-1* seeds was completely inhibited by -1.0 MPa PEG-6000, with or without 10 μM GA_{4+7}. But *sit*^{w} seeds started to germinate at 6 or 7 d of imbibition in -1.0 MPa PEG-6000 alone or -1.0 MPa PEG-6000 with GA_{4+7}, and reached 31% or 42% of germination at 10 d of imbibition, respectively (Figure 1).

Effects of GA and ABA on water uptake of seeds and seed parts. Wild type and *sit*^{w} seeds presented a triphasic curve of water uptake in water and differed significantly in water content between 48 and 72 h whereas *gib-1* seeds had no third phase as they did not germinate throughout imbibition (Figure 2). The dissected embryos took up water very quickly so that they finished the first phase in 2 hours upon imbibition in water (data not

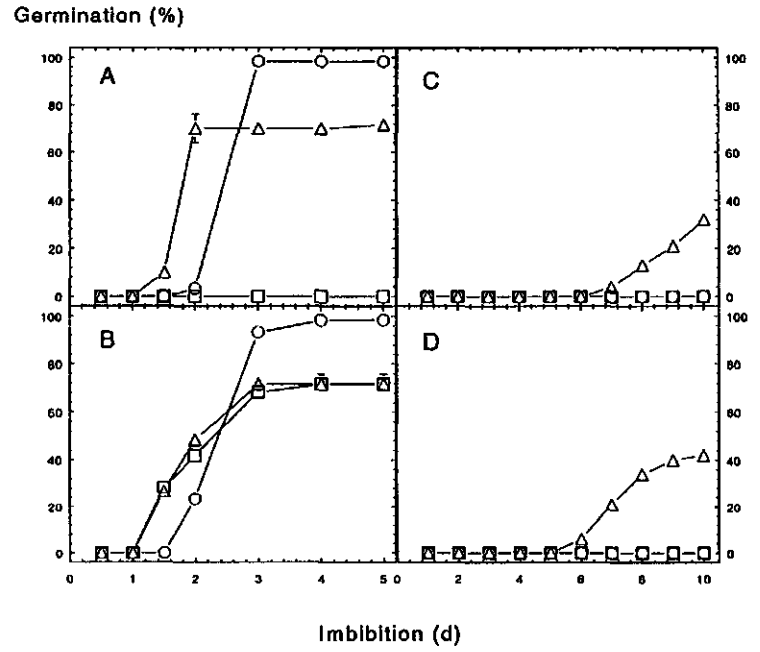

Figure 1. Germination percentage of *wild type* (○), *gib-1* (□) and *sit*^{w} (▵) seeds upon imbibition (A) in water; (B) in 10 μM GA_{4+7} solution; (C) in -1.0 MPa PEG solution and (D) in -1.0 MPa PEG plus 10 μM GA_{4+7} solution.

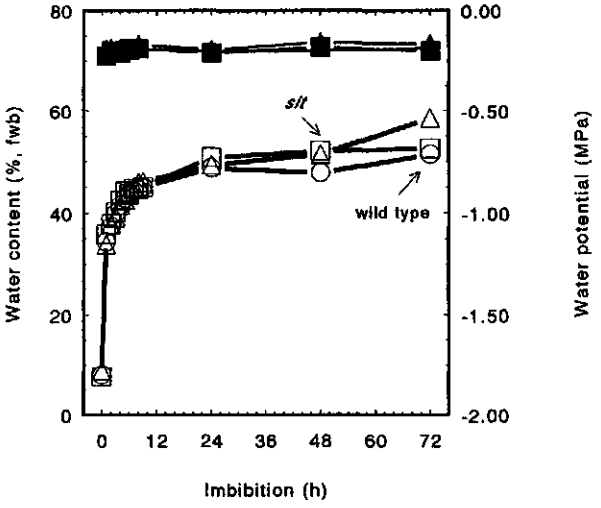

Figure 2. Changes in water contents (open symbols) and water potential (solid symbols) of *wild type* (○, ●), *gib-1* (□, ■) and *sit*^{w} (▵, ▲) seeds upon imbibition in water.

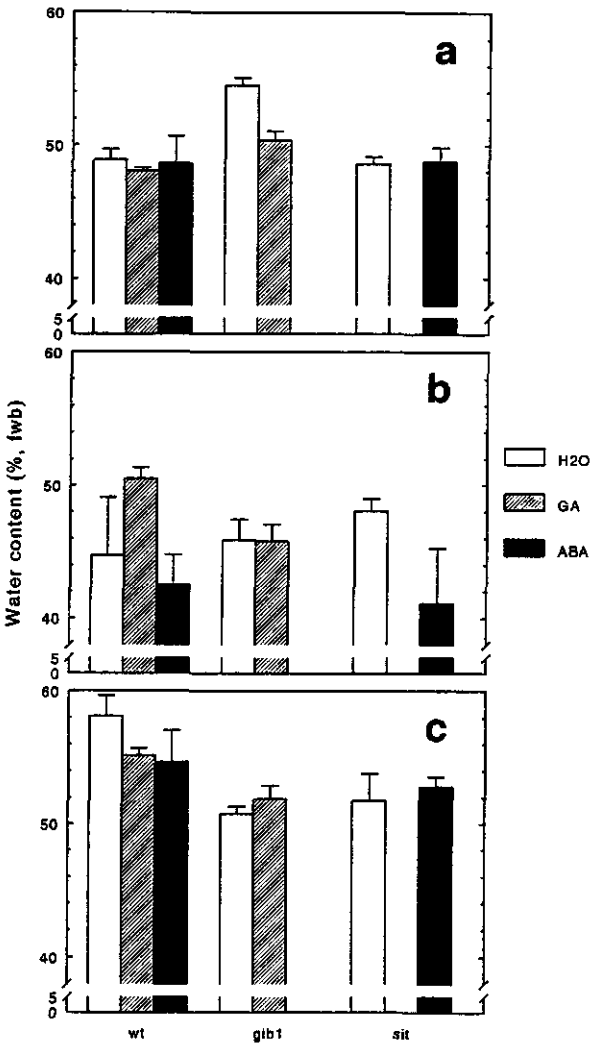

Figure 3. Influence of 10 μM GA_{4+7} and 10 μM ABA on the water uptake of (a) intact seeds, (b) intact embryos, and (c) dissected embryos in wild type, *gib-1* and *sit*^{w} at 24 h of imbibition (%, fresh weight basis).

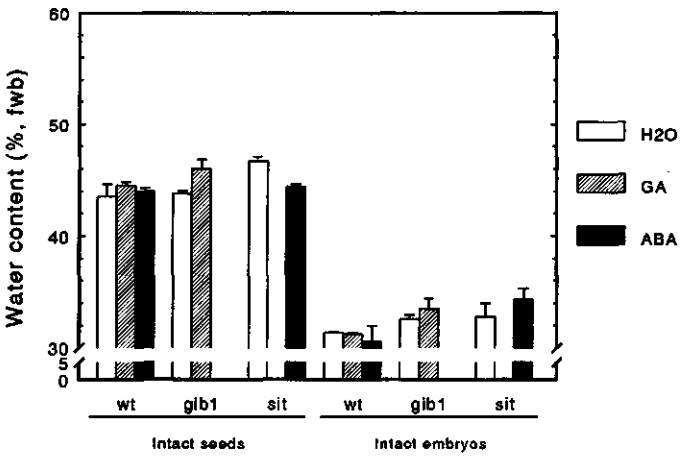

Figure 4. Influence of water stress with and without 10 μM GA_{4+7} or ABA on water uptake of both intact seeds and intact embryos in different genotypes at 4 d of imbibition of -1.0 MPa PEG-6000 solution (%, fresh weigh basis).

shown). However, the intact seeds required about 12 hours to get fully imbibed. At fully imbibed status (at 24 h of imbibition), the water content of *gib-1* seeds was significantly higher than the other two genotypes (Figure 3a). By contrast, in intact embryos, *sit*^{w} had higher water content than wild type and *gib-1* embryos (Figure 3b). The water content of dissected embryos of all genotypes both in water and in 10 μM GA_{4+7} and ABA solutions were much higher than that of intact embryos (Figure 3b, c). Exogenous growth regulators had no significant effect on water uptake of dissected embryos of *gib-1* and *sit*^{w} and some inhibiting effect in wild type (Figure 3c). Upon imbibition in 10 μM GA_{4+7}, the water content of wild type seeds showed no change whereas that of *gib-1* seeds was significantly lower (Figure 3a). By contrast, wild type intact embryos took up much more water in GA_{4+7} than in water (Figure 3b). However, the water contents of *gib-1* intact embryos remained at similar values both in GA_{4+7} and in water (Figure 3b). In 10 μM ABA, the water contents of both wild type and *sit*^{w} embryos were obviously lower than in water (Figure 3b) though the seed water contents in both genotypes remained constant (Figure 3a). Upon imbibition in -1.0 MPa PEG solution for 4 days, the water contents of all genotypes was about 10-15% lower for seeds and 30-32% lower for embryos than in water for 1 day (Figure 3a, b, Figure 4). However, *sit*^{w} seeds had slightly higher water content than the other two genotypes. It appeared that the combination of GAs or ABA with PEG solution had no effects on water content of intact embryo as compared with PEG solution alone (Figure 4). Furthermore, the difference between intact seed and intact embryo was significantly smaller in water than in -1.0 MPa PEG solution (Figure 3).

Water relations of the intact seed and embryo during imbibition. Because of the limitations of the psychrometer and the strong matric potential, the Ψ of dry seeds could not be detected. Upon imbibition in water, the Ψ of seeds in all genotypes increased so rapidly that it had already reached about -0.20 MPa within 1 h (Figure 2). Afterward, the Ψ of imbibing seeds remained constant both in pre- and in post-germination. No difference in water relations of intact seeds was found among the genotypes (Figure 2). The Ψ of intact embryos increased much slower than that of the intact seed (Figure 2, 5). This increase in the embryo Ψ of all genotypes did not finish until 24 h of imbibition in water. When seeds started to germinate the Ψ of the germinated seeds showed little change (Figure 2), as compared with a small but significant increase in the intact embryo Ψ of the germinated seeds in both wild type and *sit*^{w} types (Figure 5). Since *gib-1* seeds did not germinate the embryo Ψ kept at about -1.2 MPa level and the Ψ_π gradually increased resulting in a corresponding reduction of the Ψ_p throughout imbibition in water except the initial period (< 24 h) (Figure 6). When fully imbibed (24 h of imbibition), intact wild type embryos had the highest and *gib-1* had the lowest turgor pressure (Figure 5, 6).

Upon imbibition in -1.0 MPa PEG-6000 solution, the Ψ of wild type intact embryos declined slightly as the $\Psi\pi$ had little change throughout the imbibition, resulting in a decrease of the Ψ_p (Figure 7). In *sit*^{w} seeds, a significant rise in both Ψ and Ψ_π of embryos resulted in a corresponding decline in Ψ_p prior to the visible germination at 6 d of imbibition (Figure 8).

DISCUSSION

Radicle protrusion of tomato seed is determined by two counteracting physical factors, i.e. the growth potential of the radicle against the surrounding tissues and the restricting strength on radicle elongation imposed by the surrounding tissues (Bradford 1986). Germination occurs when the growth potential of the radicle is greater than the restricting strength. Radicle growth may be characterized by an increase in cell number (mitosis) and cell size (expansion and elongation). Radicle expansion and elongation mainly depend on water uptake upon imbibition. Endosperm weakening, or the enzymatic degradation of the surrounding tissues opposite the radicle tip, occurs during imbibition prior to the root protrusion in lettuce (Georghiou, Psaras and Mitrakos 1983, Pavlista and Valdovinos 1978), pepper (Wakins and Cantliffe 1983) and tomato (Groot and Karssen 1987). This weakening is related to the activities of cell-wall hydrolytic enzymes which may be manipulated by gibberellins and abscisic acid (Groot *et al.* 1988, Karssen *et al.* 1989). Groot and Karssen (1987) found that the mechanical restraint imposed by endosperm plus testa never fully disappeared prior to visible germination of tomato seeds. After degradation to a great extent, the remaining restraint of the surrounding tissues must be overcome by embryo growth. So far it has been difficult to measure the actual growth potential of the radicle (Karssen *et al.* 1989). However, it is known that no cell division in tomato radicle takes place before visible germination (Liu *et al.* 1995).

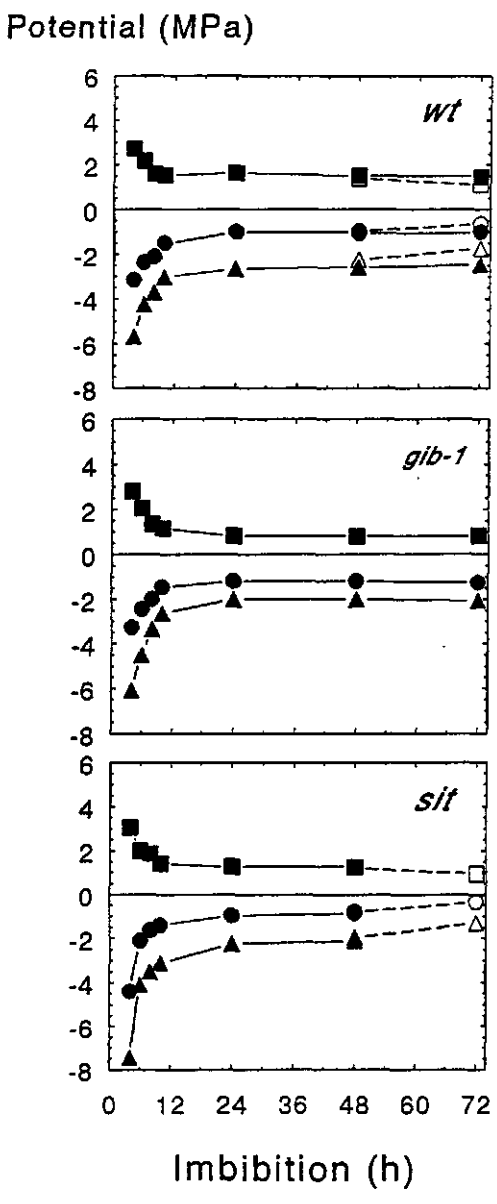

Figure 5. Water potential (●, ○), osmotic potential (▲, △) and pressure potential (■, □) of intact embryos within either not germinated (solid symbols) or germinated (open symbols) seeds of *wild type* and *sit*^{w}.

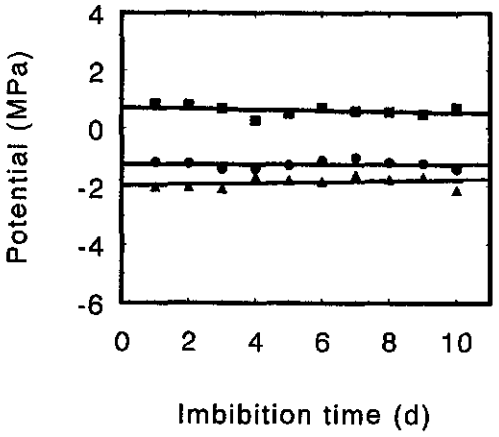

Figure 6. Changes in water potential (●), osmotic potential (▲) and pressure potential (■) of *gib-1* intact embryos throughout imbibition in water.

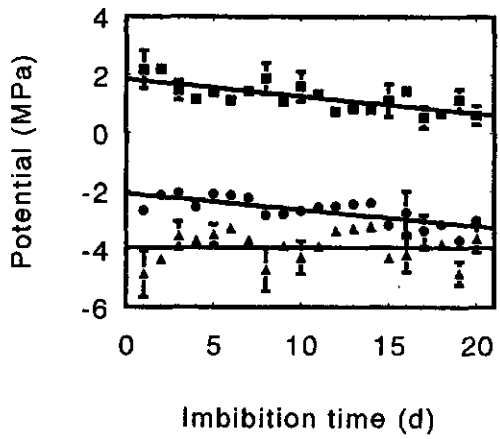

Figure 7. Changes in water potential (●), osmotic potential (▲) and pressure potential (■) of *wild type* intact embryos during imbibition in -1.0 MPa PEG-6000 solution.

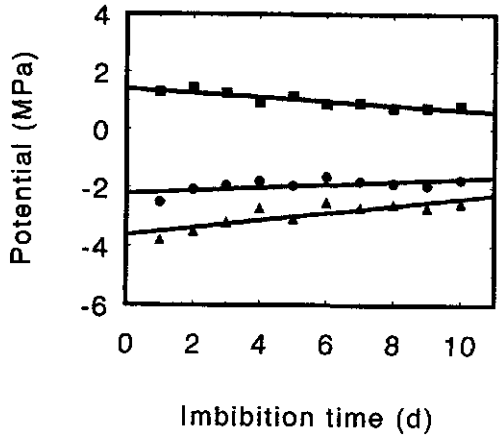

Figure 8. Changes in water potential (•), osmotic potential (▲) and pressure potential (■) of *sit*^{w} intact embryos upon imbibition in -1.0 MPa PEG-6000 solution.

In the present study, we did not find any actual increase of Ψ_p in the embryos prior to germination (Figure 5) as suggested by Bradford (1986). In the non-germinating *gib-1* embryos, Ψ and Ψ_π remained constant over an imbibition period of 10 d (Figure 6), indicating again that in these seeds reserve food mobilization did not occur. Upon imbibition in -1.0 MPa PEG solution, a constant values of Ψ in the embryo should have been expected if the medium osmotic potential is constant. However, a slight decline in Ψ in the wild type embryo was observed during the imbibition in PEG (Figure 7), probably because of a slow evaporation during this period, leading to a corresponding reduction in the medium Ψ. The Ψ_p of the wild type embryo should, therefore, also decline through imbibition in PEG if the Ψ is adjusted to a consistent level. This actual increase in embryo Ψ_π for the wild type upon imbibition in -1.0 MPa PEG solution, indicates a reduction of some osmotica, most of which are low weight compounds, possibly serving as the respiration substrate when the metabolic reactivation for the damage repair (Ashraf and Bray 1993) and germination progress (Bino *et al.* 1992) are undergoing upon imbibition. Endosperm weakening occurs during PEG imbibition of wild type (Karssen *et al.* 1989). The embryo then relaxates to some extent, resulting in a decrease in Ψ. A small amount of sugars may be produced and transported to the embryo. As small amount, it was not visible in Ψ_π (Hilhorst and Downie 1996). However, no net accumulation of osmotica was found in the embryos throughout imbibition. This finding copes with the conclusions made by Dahal and Bradford in tomato (1991) and by Weges in lettuce (1987), but contradicts what Tabeka found in lettuce (1980).

The ability of tomato seeds to germinate is genetically inherited (Cannon *et al.* 1973).

The genotypes we used here, differ in the capacity to germinate in water, i.e. *sit*$^{-}$ seeds germinate much faster than wild type seeds whereas *gib-1* seeds do not germinate without an addition of exogenous GA (Karssen *et al.* 1989). This difference is considered to be located in the embryo because it remains after detipping (Karssen *et al.* 1989). Using two tomato lines differing in the capacity to germinate at low temperature (15 °C), Liptay and Schopfer (1983) also found that removal of endosperm opposite radicle tip did not eliminate this difference. Therefore, they concluded that, upon imbibition of tomato seeds, the ability in lowering Ψ_p (Bradford 1986, Liptay and Schopfer 1983) and/or increasing cell wall extensibility (Schopfer and Plachy 1985) of the radicle determines the seed germinability of different genotypes. However, using the same cold-resistant seed line PI341988 as Liptay and Schopfer used (1983), Leviatov *et al.* (1994) reported that all lines exhibited similar radicle elongation and the removal of the seed coat and endosperm layer in front of the radicle tip abolished the difference in germination rate under low temperature, indicating that the difference in germinability is situated in endosperm and/or seed coat.

Generally speaking, the water content or water potential of the dissected embryos reflects the embryo growth potential, whereas the difference in water content or water potential between the intact and dissected embryos corresponds with the intensity of the restraint imposed by the surrounding tissues. So far, there was no direct correlation of the germinability with the water content or water potential of either the intact seeds, or the intact or dissected embryos. Thus, it appears that the differences in seed germinability among genotypes originate from both the embryo and surrounding tissues. Nevertheless, the difference of the water contents between intact seeds and intact embryos in the reported imbibition conditions was well related to the germination performance, i.e. the smaller the difference was the faster the seeds germinated (Figure 3a, b). Therefore, we hypothesize that the value of this difference positively reflects the sum of the embryo growth potential and the restricting force of the surrounding tissues on the water uptake by the intact embryo.

As an integrity tissue, the endosperm has been interpreted as a limitation of water supply available to the embryo, by restricting embryo expansion upon imbibition, though it is permeable to water (Dahal and Bradford 1991, Haigh and Barlow 1987). In mature tomato seeds, the dissected embryos of which the mechanical constraint of the endosperm is excluded, had much lower Ψ_p than both endosperm and seed coat (Berry and Bewley 1992). This lower Ψ_p may account for the higher water content of the dissected embryo (Figure 3b, c). Removal of the endosperm and seed coat has been proven to facilitate seed germination, but may or may not eliminate the differences among the genotypes differing in capacities to germinate (Liptay and Schopfer 1983). We also found that the differences in water content of the dissected embryos still remained (Figure 3c), which may also contribute to the germination performance. Using the same genotypes as we used here, Ni and Bradford (1993) found that the differences in the Ψ_p of the intact embryos at the plateau of imbibition in water were far too small to account for the differences in the

threshold Ψ for seed germination. Therefore, in tomato seeds, it is evident so far that the endosperm and seed coat, rather than the embryo itself, still play a key role in regulating seed germination in tomato (Groot and Karssen 1987, Ni and Bradford 1993, Hilhorst and Downie 1996) and all external regulating factors may stimulate or inhibit the germination by influencing endosperm weakening.

It has long been found that GA promotes seed germination whereas ABA delays or inhibits this process (Bewley and Black 1985). ABA inhibits embryo water uptake in rape seed (*Brassica napus* L.) possibly by the prevention of cell wall loosening in the radicle (Schopfer and Plachy 1985). However, when tomato embryos were dissected from the intact seed after 1 h of imbibition and put into water or 10 μM GA_{4+7} or ABA solution, we did not find any corresponding promoting or inhibiting effects of these two plant growth regulators upon the dissected embryo water uptake (Figure 3c). This indicates that GA and ABA do not influence water uptake by changing cell wall aspects (cell wall loosening) or the metabolic process (increase in Ψ_π) in embryo, which may contribute to the increase or decrease in the embryo Ψ_p. But interestingly, exogenous GAs and ABA did increase or decrease the difference in water content between intact seeds and intact embryos (Figure 3a, b), which, as previously hypothesized, may result in promoting or inhibiting germination. We believe that the endosperm weakening upon imbibition, which are stimulated or inhibited by GA and ABA respectively, may relieve the mechanical constraint on the embryo growth and allow the Ψ of embryo to become lower to absorb more water from the surrounding environment (Bradford 1994). Therefore, it is neither necessary nor possible to build up an efficient Ψ_p of the radicle to overcome the mechanical constraint imposed by the endosperm prior to root protrusion upon imbibition (Haigh and Barlow 1987, Ni and Bradford 1993).

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Chapter 8

General discussion

Introduction

During the past decade, significant progress has been made in our knowledge on the physiology of seed development, dormancy, priming and germination in several plant species. In particular, the hormonal regulation of dormancy induction and germination in both *Arabidopsis thaliana* and tomato (*Lycopersicon esculentum* Mill.) seeds have been extensively studied. With a few exceptions, no clear-cut distinction between dormancy breakage and germination induction is recognized. Therefore, it is logical to seek a 'common place' or a 'key factor' for the breakage of dormancy and the induction of germination. In this chapter, a general discussion on the mechanism of seed dormancy and germination in tomato is presented on the basis of the findings from this thesis and the results from other studies. Special attention is paid to the regulation by gibberellins (GAs) and abscisic acid (ABA) and by osmotic stress of seed desiccation tolerance, dormancy induction and germination performance. At the end of this discussion, a model is presented to summarize the main findings from this thesis and to demonstrate the possible links between them.

Desiccation tolerance

One of the most important morphological changes during tomato seed development is the formation of a firm endosperm. The change from milky to firm endosperm takes place around 35 ± 5 days after pollination (DAP) when the water content of the seeds drops from >85% to <55% (Chapter 6). Meanwhile, a large quantity of osmotically active solutes accumulates in the embryos and decreases ψ_π from -2.0 to -5.0 MPa (Chapter 6). At the same stage of seed development, the cell cycle becomes arrested in most cells of the seed tissues (Chapter 5). From that stage on, seeds are physiologically mature and have gained desiccation-tolerance and germinability (Demir and Ellis 1991, Berry and Bewley 1991, Chapter 3). Osmotic stress tolerance involves changes in cellular chemistry. For instance, glycophyte plants respond to high osmoticum by changes in the composition of cell wall polysaccharides and proteins (Iraki et al. 1989). These changes appear to be the result of the accumulation of solutes and proteins that can be induced by osmotic stress (Rhodes 1987). A number of ABA-responsive genes are expressed in higher plants in response to an increase of the osmotic stress (see review by Skriver and Mundy 1990). One of these ABA-responsive genes, called the *GAB* gene, is also expressed during natural desiccation of developing seeds of chick-pea (*Cicer arietinum* L.) (Colorado et al. 1995). In tomato seeds, ABA levels peak shortly before or after the onset of seed desiccation (Berry and Bewley 1992, Groot and Karssen 1992). Therefore, it has been suggested that ABA plays an important role in developing seeds in the induction of seed desiccation tolerance in combination with osmotic stress (Kermode 1990). However, seeds of the ABA-deficient mutant *sit*^{w} develop normally. Therefore, it appears that the establishment of desiccation tolerance is independent of the increase in the ABA levels during maturation, but responds to the decrease in the ψ of the surrounding tissues of the developing seeds (Chapter 6). This finding was confirmed by molecular biological studies in which some

ABA-induced genes such as *Osmotin* and *TAS14*, in leaves from tobacco (*Nicotian tobaccum* L.) and tomato plants, were also found to be induced by osmotic stress (Godoy et al. 1990). Studies with the ABA-deficient and -insensitive double mutant (*aba-1, abi3-1*) seeds of *Arabidopsis thaliana* also show that either ABA or osmotic stress and related gene expression meets the minimal requirements for acquisition of desiccation tolerance (Ooms et al. 1994). In other words, the function of endogenous ABA on the development of desiccation tolerance during development of both tomato and *Arabidopsis thaliana* seeds may be partly or fully replaced by osmotic stress within the fruit tissues.

DNA replication and water relations

In the cascade of germination, the activation of DNA synthesis is almost the final step leading to visible germination (Osborne 1983). In tomato, upon imbibition no cell cycle activities were found in dormant seeds (Chapter 4, Chapter 5). Seeds will only complete germination when dormancy is broken, and DNA synthesis is initiated in the radicle tips prior to visible germination. Therefore, it was concluded that the activation of cell cycle in seeds upon imbibition can be considered as an indication of the breakage of dormancy and the induction of germination. The cell division in the radicle tips does not take place until 24 h after visible germination (Liu and Bino 1995). This indicates that DNA replication may not be the cause for the cell growth of the radicle tips, but as mentioned later, may be the consequence of the cell growth. Gibberellin-deficient *gib-1* seeds do not show the initiation of DNA replication activity and do not germinate in water (Chapter 4) or in PEG solution (Chapter 5). When exogenous GAs are added to the imbibition solution, or when the endosperm layer opposite the radicle tip is removed, DNA replication activity in the radicle tips of *gib-1* seeds is started (Chapter 4, Chapter 5), and, subsequently, the seeds germinate in water (Chapter 4). Therefore, it appears that the regulation by GAs on DNA replication activity of radicle tips is mediated by the weakening of the endosperm tissue opposite the radicle tip (Chapter 5). The mechanical restraint imposed by the tissues surrounding the embryo obviously limits the flow of water into the embryo (Haigh 1988, Dahal and Bradford 1990, Chapter 7). What factors are required to initiate the cell cycle in higher plants is far from known. In yeast and animal, the increase in cell size might be the initiator for cell cycle. It is possible that the initiation of the cell cycle also corresponds to the cell growth in seed tissues. Water uptake by seed upon imbibition causes cell growth of the seed tissues. In this case, it may be hypothesized that the cell cycle is initiated only when the water uptake by the seed tissues has reached a certain level. X-ray radiography of imbibing tomato seeds has shown that upon imbibition in water the embryo expands to a large extent along the axis orientation (Chapter 2). From the X-ray photographs it is clear that at the moment of radicle protrusion the density of the X-ray absorption is significantly higher in the radicle tip than in the other parts of the seed. The density of X-ray absorption positively reflects the water content of the tissues or tissue parts. This indicates that the cells in the radicle tip absorb more water and expand more than those in the other parts, therefore resulting in an initiation of the cell cycle. An

analysis of the correlation between the water content of the seeds and the DNA replication activity of the radicle-tip cells showed that no DNA replication is initiated when the water content of the whole seed is below 35% (S.P.C. Groot and J.G. van Pijlen, personal communication). GAs stimulate seed germination by increasing water content of intact embryos (Chapter 7) whereas ABA acts in the reverse way. Little effect of GAs and ABA has been found on the growth potential of the embryo in dissected seeds (Chapter 7). Therefore, the influence of these two hormones on water relations of tomato seeds is complemented by the endospermal weakening.

Dormancy and germination

In some tomato cultivars like Moneymaker, seeds may manifest symptoms of primary dormancy at harvest (Groot 1987, Chapter 3). ABA-deficient mutant *sit*^{w} seeds do not show such symptoms under any growth condition, whereas GA-deficient mutant *gib-1* seeds always behave dormant. The germination of tomato seeds is controlled by phytochrome since it can be stimulated by red light and inhibited by far-red radiation. Many internal and external factors are supposed to be involved in the regulation of dormancy. Notwithstanding that, the removal of the endosperm layer plus testa in the area opposite the radicle tip always causes germination. From this, it is concluded that the dormancy is imposed by the endosperm layer opposite the radicle tip. The endosperm weakening in tomato has been found to be induced by GAs (Groot and Karssen 1987). Therefore, it is hypothesized that all internal and external factors involved in the induction and the relief of dormancy in tomato seeds are related to the *de novo* synthesis of GA or/and to the enhanced sensitivity of the seed parts to GAs.

Osmotic stress: Unlike many other orthodox species whose seeds desiccate when they are physiologically matured, tomato seeds reside in a fully hydrated environment throughout development. This environment is characterized by a low water potential (around -0.85 MPa) of the pericarp and the locular tissues within the fruit (Chapter 6). Possibly, this particular water potential keeps seeds in the developmental mode. Berry and Bewley (1991) found that immature tomato seeds (30-35 DAP, cv. Caruso) germinated precociously when removed from the fruit and imbibed in water. The same authors further confirmed that the development of these immature seeds was maintained or promoted by ex situ fruit tissue with which they were in contact (Berry and Bewley 1992). It was found that when the mature seeds isolated from wild type (cv. Moneymaker) tomato fruits, were directly incubated in a -1.0 MPa PEG-6000 solution (fresh PEG priming), the DNA replication of radicle-tip cells was not provoked and the seeds did not germinate (Chapter 3). By contrast, when the matured seeds were directly incubated in water, the DNA replication of radicle-tip cells was triggered and the seeds subsequently germinated (Chapter 3, Chapter 5). Apparently, in tomato seeds, a relief of the osmotic stress terminates the development and induces the germination process.

Light and temperature: The stimulating effect of light on seed germination of many plant species has been known for a long time. The effect of red light can be reversed by far-red treatments. It has generally been agreed that red/far-red reversibility proves the involvement of the plant pigment phytochrome, which appears in an active form P_{fr} and an inactive form P_r. It is likely that the first step of the transduction chain that leads to germination is the binding of P_{fr} to a specific receptor X, resulting in a phytochrome independent intermediate $P_{fr}X$. There is no evidence for the nature of this P_{fr} binding component, but it is generally assumed to be located in a membrane. Two main models, the monomeric (Duke et al. 1977) and the dimeric model (VanDerWoude 1985), have been hypothesized to explain the response of seed germination to light.

Temperature is probably the most important external factor regulating seed dormancy and germination. Many seeds require a chilling pre-treatment or alternating temperature to break dormancy (Bewley and Black 1994). Several examples can be found where temperature affects the rate of breakage and the induction of dormancy (Cone and Spruit 1983). In tomato seeds (cv. Moneymaker), the primary dormancy can be induced by a temperature >25 °C at the late development stage, and can be maintained under the same temperature for at least 6 months (Y. Liu, unpublished results). This primary dormancy can be broken by a pre-treatment at a temperature between 5 and 20 °C for only one day (Y. Liu, unpublished results). In chick-pea seeds, Colorado et al. (1994) found that an ABA-induced protein GAB was induced by a high temperature (30 °C) but not by a low temperature (8 °C). This ABA-induced protein may be related to the inhibition of germination (Colorado et al. 1991).

ABA and GAs: Abscisic acid and gibberellins are the most important phytohormones in the manipulation of seed dormancy and germination (Khan 1977). The antagonistic effects of these two hormones are the main basis of the hormone-balance theory (Karssen and Łaçcka 1986). Endogenous ABA is responsible for the induction of primary dormancy in tomato seeds during development (Groot and Karssen 1992). This primary dormancy may be related to a developmental arrest. For instance, the cell cycle activities in *sit*^{w} embryonic tip was found to be less efficiently arrested in G_1 as compared with that in wild type (Chapter 5). Using ABA-deficient (*aba*), ABA-insensitive (*abi*) and reduced dormancy (*rdo*) mutants, Koornneef and his coworkers also found that the *RDO1* gene is involved in the seed maturation process that leads to a developmental arrest in *Arabidopsis* (Lèon-Kloosterziel et al. 1996). Primary dormancy in tomato seeds can be relieved by a pre-sowing treatment in water (Y. Liu, unpublished results) or by osmotic priming in PEG solution at low or favourable temperatures (Chapter 3). The first desiccation after harvest plays a role in terminating the development of primary dormancy, since it was not relieved by PEG priming without a preceding desiccation. The possible cause for the maintenance of primary dormancy may be that the freshly primed seeds show no decrease of their endogenous ABA content. Groot and Karssen (1992) found that the ABA content of wild type seeds reduced from 0.8 to 0.1 ng.seed^{-1} during the first 18 h of imbibition. Many

components including ABA may leach out due to the abrupt phase transition of membranes of the imbibing seeds (Abdul-Baki and Stoner 1978).

The endosperm weakening results from the degradation of the mannan-rich cell walls, which is catalyzed by a series of hydrolytic enzymes (Groot et al. 1988). The synthesis or activation of these enzyme proteins is positively associated with the GAs which are either synthesized endogenously or gained exogenously from the germination medium during imbibition. Exogenous GAs generally induce the germination of dormant seeds. Moreover, the addition of exogenous GAs to the osmotic solution relieves the dormant state of the freshly osmoprimed seeds (Chapter 5). Therefore, the induction of germination of tomato seeds is ultimately determined by the weakening of the endosperm layer opposite the radicle tip (Groot and Karssen 1987). The relief of dormancy is also determined by the endosperm weakening.

ABA inhibits seed germination by counteracting the action of GAs. For instance, using gibberellin-deficient *gib-1* seeds, Groot (1987) found that 0.2 μM GA_{4+7} was able to induce endosperm weakening after 24 h of imbibition whereas a complete inhibition was achieved by simultaneous incubation in 1 μM ABA. There is no evidence that ABA-induced products directly inhibit seed germination (Skriver and Mundy 1990). For instance, application of exogenous ABA to germinating cereal seeds sharply reduces levels of GA-responsive hydrolase mRNAs and proteins. This is presumably due to the induction of factors that inhibit hydrolase transcription and/or translation (Nolan and Ho 1988, Rogers 1988). It is likely, therefore, that ABA influences the germination progress either by inhibiting the processes that stimulate GAs synthesis, or by the inactivation of GA-specific mRNA transcription and protein translation, or both. With regard to the first hypothesis, it is possible that an ABA-induced protein competitively binds to P_{fr}, leading to an inactive complex which can not induce GA synthesis. For the second hypothesis, it is possible that ABA competes with GAs to bind to the GA-specific receptor(s), resulting in a null complex, subsequently decreasing the rate of GA-specific mRNA and protein synthesis. Working with GA-deficient mutant *gib-1* seeds, Toorop, Bewley and Hilhorst (personal communication) found that ABA did not inhibit endo-β-mannanase activity which is GA-induced and is considered to be the key hydrolytic enzyme for endosperm weakening (Groot and Karssen 1987). Recent studies have also shown some evidences to support the second hypothesis. For instance, the *Atmyb2* gene of *Arabidopsis thaliana* (Urao et al. 1993), the *C1* gene of maize (*Zea may* L.) (Hattori et al. 1992) and the *Mybs* gene of barley (*Hordeum vulgare* L.) (Gubler et al. 1995), whose expressions are induced by GA, are also under the control of ABA (Gubler et al. 1995).

A model

The results presented in this thesis and in the present discussion can be summarized in a descriptive model (Fig. 1). In this model, the release of dormancy and induction of germination is based on the weakening of the endosperm tissue. ABA and GAs play antagonistic roles in the manipulation of the endosperm weakening at the level of gene

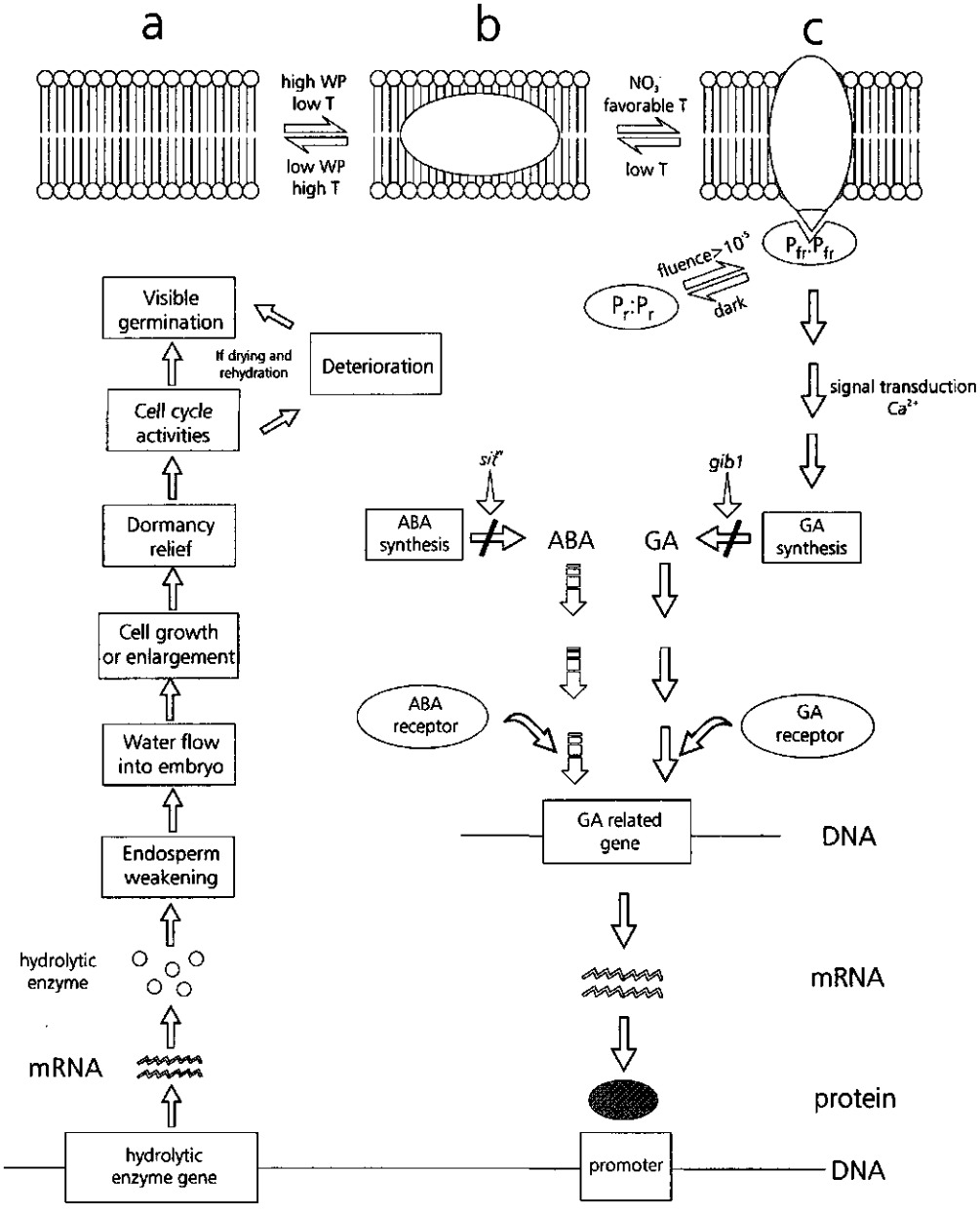

Figure 1. A model describing the regulation of dormancy breakage and germination induction by GA and ABA in tomato seeds

expression. Other external environmental factors such as water, temperature, nitrate, calcium etc., mediate the whole process leading to *de novo* GA synthesis either by mutually acting with phytohormones on the gene expression, or by modifying the properties of the receptors to which phytochrome or phytohormones will bind. All receptors are located in the membrane.

The initial state of the membrane is that of a relatively hydrated seed (a). In the presence of GAs when the external water potential (ψ) is relatively high (fully hydrated) and the temperature is relatively low, a phytochrome receptor is synthesized and transported to the membrane (b). The synthesis and the transportation of this receptor is inhibited by the presence of ABA, or/and when the external water potential (ψ) is relatively low (osmotic stress), or/and when the temperature is relatively high. After changing the temperature to the optimal regime for germination, and upon the binding of nitrate or other compounds, the receptor undergoes a conformational change which allows the binding of P_{fr}:P_{fr} dimers to an activated receptor (c). These phytochrome dimers are formed at a fluence of more than 10^{-6} mol. s^{-1}. m^{-2} (VanDerWoude 1985). Only when the P_{fr}:P_{fr} dimers are bound to the receptors, might the P_{fr}:P_{fr}-X complexes generate a signal that leads to GA synthesis. The nature of this signal is unknown and these phytochrome receptors have not been identified. It has been suggested by DePetter et al. (1985) that GA increases sensitivity to light or phytochrome in *Kalanchoë blossfeldiana* seeds. There are some evidences that GA can bind to a receptor that is located in a membrane (Hooley et al. 1991, 1992, Smith et al. 1993), or in more detail, the plasma membrane (Gilroy and Jones 1994). As mentioned earlier, this receptor may also be competitively occupied by ABA. Based on the model that has been formulated to explain how GA induces a high-pI α-amylase in barley aleurone cells (Gobler et al. 1995), it is hypothesized here, that GA first binds to a receptor, presumably on the plasma membrane, and activates a signal transduction pathway that triggers the first-step in GA-responsive gene expression. Calcium may mediate this signal transduction (Rincon and Boss 1987). The newly synthesized GA-related proteins then activate the expression of a series of second-step GA-response genes. In this way, some corresponding proteins including the hydrolytic enzymes such as endo-ß-mannanase are synthesized, and endosperm weakening is started. With the decrease in the restraint of the endosperm, the turgor of the radicle relieves and more water is allowed to flow into the radicle. This results in a further growth and DNA replication in cells of the radicle tip, finally leading to visible germination.

This model is speculative and many details remain unclear. It is, for example, unknown whether and how the phytohormone activates the phytochrome receptor. No information is available to fill the gap between the P_{fr}:P_{fr}X complex and GA synthesis in this model. The activation of those catalytic enzymes involved in GA biosynthesis, may also be regulated by ABA and other external factors. The relationship between the phytochrome receptor and the GA-receptor has not been clarified, though both receptors are identified to be located in a membrane, most probably the plasma membrane. Probably, they are the same or similar proteins.

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Summary

Summary

The internal morphology of seeds from GA-deficient mutant *gib-1*, ABA-deficient mutant *sit*^{w} and wild type tomato plants was studied by X-ray photography. On the X-ray photographs an empty area between the embryo and the endosperm was found. This, so called 'free space', was present in dried well-matured seeds (Chapter 2, Chapter 5). Free space existed in about 2% of the wild type and in 13% of the *sit*^{w} (Chapter 5). At the over-matured stage, more seeds with free space were found in *sit*^{w}, whereas no increase was detected in the other two genotypes (Chapter 5). Osmotic priming of dried seeds induced a large amount of free space in almost all seeds of the three genotypes (Chapter 2, Chapter 5). Short-term (< 12 h) imbibition in water induced free space as well but to a less extent as compared with the osmotically primed seeds (Chapter 2). Priming of fresh (non-dried after harvest) seeds had no effect on the induction of free space in wild type and *gib-1* (Chapter 2, Chapter 5), but did induce free space in about 20-25% of the *sit*^{w} seeds (Chapter 5). Apparently, a dehydration step prior to the priming treatments is a prerequisite to induce free space (Chapter 2). The changes in free space area in wild type seeds were quantitatively analyzed during osmotic priming. Upon the imbibition of dry seeds, the embryo expands and the endosperm tissue is compressed. Upon redehydration, free space is formed by the shrinkage of the embryo and by the loss of the endospermal flexibility (Chapter 2). As compared with the unprimed control, the size of an osmotically primed wild type seed increased much more in the longitudinal dimension than in the transverse dimension (Chapter 2). This may indicate that the embryo mostly expands along the axis orientation. In addition, from the X-ray photographs it was clear that at the moment of radicle protrusion, the density of the absorption of X-rays was significantly higher in the radicle tip than in the other parts. Apparently, cells in the radicle tip absorbed more water (Chapter 2).

The water relations of fruit tissues, seeds and embryos were determined with a thermocouple psychrometer during development. Effects of GAs and ABA on water uptake and water relations of intact seeds or seed parts were also studied during osmotic priming and germination. The water contents of the fruit tissues of all genotypes little changed throughout the development (Chapter 6). In wild type and *gib-1*, the changes in water potential (ψ), osmotic potential (ψ_π) and pressure potential (ψ_p) of the pericarp and the locular tissues followed a similar pattern, i.e. the ψ remained constant until 30 DAP (wild type) or 35 DAP (*gib-1*). Thereafter, the cells of the fruit tissues lysed upon maturation. The ψ of fruit tissues dropped to the same levels as the ψ_π, resulting in a complete loss of turgor (Chapter 6). By contrast, the cells of *sit*^{w} fruit tissues did not degrade, their ψ, therefore, was not as low as the ψ_π. This resulted in a pressure potential much higher than those of wild type and *gib-1* (Chapter 6). The ψ_p values in *sit*^{w} seed were higher than in the other genotypes (Chapter 6). During maturation, the developing seeds of all genotypes started to loss water around 30 DAP. Upon maturation the final ψ of the embryo was lower than that of the whole seed, whereas the ψ of whole seed had been equilibrated with that of the locular or the pericarp tissues (Chapter 6). This indicates that the flow of

water between seed parts is not passively based on the ψ gradients, but is driven by an active mechanism. During osmotic priming of wild type seeds, the ψ of the embryos was constant, while the ψ_n slowly increased. This lead to a corresponding reduction of the ψ_p (Chapter 7). Apparently, the osmotically active solutes in the embryo did not accumulate, but were consumed by the metabolic activation during osmotic priming. Similarly, the ψ_p of *gib-1* embryos was also found to decline as the ψ_n elevated during imbibition in water (Chapter 7). No significant difference in water relations of intact seeds was found among the three genotypes upon imbibition in water (Chapter 7). The ψ, ψ_n and ψ_p of the intact embryos differed among the three genotypes (Chapter 7). In general, the growth potential of the embryo is determined by the water uptake by the dissected embryo. However, it appeared that the germination performance was positively related to the water content of intact embryos. The mechanical restraint imposed by the tissues surrounding the embryo obviously limited the water flow into the embryo by restricting its expansion (Chapter 7). Exogenous GAs and ABA might relieve or strengthen this restraint upon embryo water uptake. A corresponding effects of GAs and ABA on water uptake by the dissected embryo was not found. Therefore, the influences of GAs and ABA on water relations of intact embryo are realized by a modification in the endosperm weakening rather than by the cell wall loosening in the radicle (Chapter 7).

Nuclear replication activity in embryo root tips was analyzed using a flow cytometer. Like wild type, *gib-1* and *sit*^{w} seeds developed normally as far as their morphology is concerned (Chapter 6). However, unlike wild type and *gib-1* seeds, *sit*^{w} seeds did not express dormancy and could viviparously germinate upon maturation (Chapter 5). During seed formation, cell cycle in the cells of the radicle tips were active until 45 DAP when the endosperm has become solid and seed has gained desiccation-tolerance and germinability in all three genotypes (Chapter 5). Upon maturation (45 DAP onwards), the nuclei of wild type, *gib-1* and *sit*^{w} seeds predominantly showed 2C DNA signals, indicating that the cell cycle activity of most root-tip cells had arrested at the G_1 phase of nuclear division (Chapter 5). This flow-cytometric profile of radicle-tip cells in wild type and *gib-1* dry seeds did not change throughout the subsequent maturation stages (Chapter 5). However, the 4C peak in the radicle tips of dry *sit*^{w} seeds increased with maturation (Chapter 5). ABA-deficient *sit*^{w} showed a significantly higher 4C value both in the well-matured and in the over-matured seeds as compared with that in dry wild type and *gib-1* seeds (Chapter 4, Chapter 5). Upon imbibition in water, an induction of 4C signal, indicating nuclear replication, was observed in the radicle tip cells of both wild type and *sit*^{w} seeds. The augmentation in the 4C signal occurred before visible germination. GA-deficient *gib-1* seeds did not show nuclear replication activity and did not germinate without an addition of exogenous GAs. Upon imbibition in 10 μM GA_{4+7}, both nuclear replication activity and subsequent germination were enhanced in wild type and *sit*^{w} seeds, and were induced in *gib-1* (Chapter 4). In 5 μM ABA, the germination of wild type and *sit*^{w} seeds was inhibited while nuclear replication of these seeds was not affected (Chapter 4). It is concluded that GAs influences germination of intact seeds by acting upon processes that precede cell

cycle activation, while ABA affects growth by acting upon the processes that follow cell cycle activation (Chapter 4).

Normal PEG priming induced DNA replication activity in the radicle-tip cells of wild type and *sit*^{w} seeds, and consequently enhanced seed germination (Chapter 3). Fresh PEG priming is a newly developed priming method, i.e. fresh seeds after harvest are directly primed with PEG solution. Fresh PEG priming can enhance seed germination and improve seedling performance as compared with the untreated control. However, fresh PEG priming neither alleviated seed dormancy nor induced DNA replication activity in wild type or *gib-1* seeds (Chapter 3). The controlled deterioration tests showed that normally primed seeds were more susceptible to the controlled deterioration than freshly primed seeds (Chapter 3). The addition of exogenous GAs to the PEG solution triggered replicative DNA synthesis of freshly primed seeds and further enhanced the germination (Chapter 3). A removal of the endosperm and testa opposite the radicle tip also induced DNA replication activity of the *gib-1* seeds pre-imbibed both in osmoticum and in water (Chapter 5). It is concluded that the advancement of germination, expressed as the ratio of 4C nuclei of radicle-tip cells, is positively related with the germination rate, and negatively related with the storability of the treated seeds (Chapter 5). The weakening of the endosperm opposite radicle tip promoted the cell growth of the radicle. It is concluded that the cell growth may induce nuclear replication activity in the radicle tip upon imbibition (Chapter 5).

Finally, a general discussion on the mechanism of dormancy and germination in tomato seeds is presented on the basis of the findings from this thesis and the results from other studies (Chapter 8). Special attention is given to the physiological regulation of seed desiccation tolerance, dormancy and germination by GA and ABA, and by osmotic stress. A descriptive model is proposed to summarize the main findings from this thesis and to demonstrate the possible links between them. In this model, the induction of dormancy and germination is based on the same principle: the regulation of the weakening of the endospermal tissue opposite the radicle tip. ABA and GAs play antagonistic roles in the manipulation of the endospermal weakening at the level of gene expression. Other external environmental factors such as water, temperature, nitrate, calcium etc., mediate the whole process leading to GA synthesis either by mutually acting with phytohormones on gene expression, or by modifying the properties of the receptors which may be bound by phytochrome or phytohormones.

Samenvatting

Samenvatting

Met röntgenfotografie werd de interne morfologie van tomatenzaden van de GA-deficiënte mutant *gib-1*, de ABA-deficiënte mutant *sit*^{w} en het wildtype bestudeerd. Op de röntgenfoto's was een lege ruimte zichtbaar tussen het embryo en het endosperm. Deze zogenoemde 'vrije ruimte' was aanwezig in een deel van de gedroogde zaden die volledig aan de plant afgerijpt waren (Hoofdstukken 2 en 5). Het aantal zaden met vrije ruimte was 2% in het wildtype en 13% in *sit*^{w} (Hoofdstuk 5). In het overrijpe stadium bleek dat het aantal zaden met vrije ruimte in *sit*^{w} nog hoger was, terwijl geen stijging werd gevonden in de twee andere genotypen (Hoofdstuk 5). Een osmotische voorbehandeling induceerde een grote hoeveelheid vrije ruimte in bijna alle zaden van de drie genotypen (Hoofdstukken 2 en 5). Ook een korte (< 12 uur) imbibitie in water induceerde de vorming van vrije ruimte, maar de toename was minder dan na een osmotische voorbehandeling (Hoofdstuk 2). Osmotische voorbehandeling van verse (na oogst niet-gedroogde) zaden had geen invloed op de hoeveelheid vrije ruimte in het wildtype en *gib-1* (Hoofdstukken 2 en 5), maar gaf wel een inductie van vrije ruimte in 20-25% van de *sit*^{w} zaden (Hoofdstuk 5). Dit geeft aan dat een droogstap voorafgaande aan de voorbehandeling een voorwaarde is voor de inductie van vrije ruimte (Hoofdstuk 2). Het effect van een osmotische voorbehandeling op de hoeveelheid vrije ruimte in zaden van het wildtype werd kwantitatief geanalyseerd. Tijdens de wateropname van droge zaden, neemt het embryo in volume toe, waardoor het endospermweefsel wordt samengedrukt. Tijdens de droging na de voorbehandeling wordt vrije ruimte gevormd door de krimp van het embryo en door verlies van flexibiliteit van het endospermweefsel (Hoofdstuk 2). Vergeleken met de niet-behandelde controle is de afmeting van het voorbehandelde wildtype zaad veel meer in de lengterichting toegenomen dan in de dwarsrichting (Hoofdstuk 2). Dit geeft aan dat het embryo vooral langs de hoofdas in grootte toeneemt. Bovendien bleek uit de röntgenfoto's dat op het moment dat het worteltopje uit de zaadhuid verschijnt, de absorptie van de röntgenstraling in het worteltopje duidelijk hoger was dan in de andere onderdelen van het zaad. Dit geeft aan dat de cellen in het worteltopje meer water hebben opgenomen (Hoofdstuk 2).

Tijdens ontwikkeling werden de waterrelaties van de vrucht, de zaden en de embryo's bepaald met behulp van een thermokoppel psychrometer. Tijdens osmotische voorbehandeling en kieming werd het effect van GAs en ABA op de opname van water en de waterrelaties van intacte zaden of zaaddelen bestudeerd. De watergehaltes van de vruchtweefsels van alle genotypen veranderden nauwelijks tijdens de gehele ontwikkeling (Hoofdstuk 6). In vruchten van het wildtype en de *gib-1* mutant volgden de veranderingen in de waterpotentiaal (ψ), de osmotische potentiaal (ψ_{π}) en de drukpotentiaal (ψ_{p}) eenzelfde patroon. In beide genotypes bleef de waterpotentiaal lange tijd constant, tot 30 DAP (days after pollination = dagen na bestuiving) in het wildtype en tot 35 DAP in de *gib-1* mutant. Door het lyseren van de cellen van het vruchtweefsel tijdens rijping van de vrucht daalde de waterpotentiaal tot de waarde van de osmotische potentiaal wat resulteerde in een compleet verlies van de turgor (Hoofdstuk 6). De cellen van de vruchtweefsels van de *sit*^{w} mutant gingen daarentegen niet kapot en daardoor bleef de waarde van ψ hoger

(Hoofdstuk 6). In sitw zaden waren de waardes voor ψ_p hoger dan in de twee andere genotypen (Hoofdstuk 6). Tijdens afrijping begonnen de zaden van alle genotypen water te verliezen vanaf 30 DAP. Bij rijpheid was het uiteindelijke ψ van het embryo lager dan dat van het gehele zaad, terwijl de ψ van het gehele zaad gelijk was aan dat van de tomatenvrucht (Hoofdstuk 6). Dit geeft aan dat de verplaatsing van water tussen zaaddelen niet passief is gebaseerd op een gradiënt in ψ, maar een actief proces is. Tijdens de osmotische voorbehandeling van wildtype zaad bleef de ψ van de embryo's constant, terwijl de ψ_π langzaam in waarde steeg, wat resulteerde in een verlies aan ψ_p (Hoofdstuk 7). Blijkbaar vond geen ophoging plaats van osmotisch actieve stoffen maar werden juist verbruikt tijdens de osmotische voorbehandeling. Ook de ψ_p van *gib-1* embryo's daalde wanneer de ψ_π steeg tijdens opname van water (Hoofdstuk 7). Wat betreft de waterrelaties van het complete zaad tijdens opname van water werden tussen de drie genotypen geen belangrijke verschillen gevonden (Hoofdstuk 7). Daarentegen werden wel verschillen gevonden tussen de genotypen wat betreft de ψ, ψ_π en ψ_p van de intacte embryo's (Hoofdstuk 7). In het algemeen wordt de groeipotentie van embryo's bepaald door het meten van de wateropname van embryo's die uit het zaad zijn geprepareerd. De kieming van zaden bleek echter positief gerelateerd te zijn aan het watergehalte van de intacte embryo's. De mechanische weerstand van de weefsels rond het embryo beperkten kennelijk de expansie mogelijkheden en daarmee de wateropname van het embryo (Hoofdstuk 7). Exogene toediening van GAs en ABA kan deze weerstand opheffen of versterken wat resulteert in het bevorderen of tegenwerken van kieming. Een effect van GAs en ABA op de wateropname werd niet gevonden wanneer de embryo's uit het zaad werden geprepareerd. Hieruit bleek dat de invloeden van GAs en ABA op de waterrelaties in intacte zaden bepaald worden door een verandering van de verzwakking van het endosperm, en niet door een vermindering van de stevigheid van de celwanden van het worteltopje (Hoofdstuk 7).

De activiteit van de kerndeling in de worteltoppen van tomatenzaden werd bestudeerd met behulp van een doorstroomcytometer. De morfologische ontwikkeling van *gib-1* en *sit*^{w} zaden vond normaal plaats zoals in het wildtype (Hoofdstuk 6). Echter, in tegenstelling tot wildtype en *gib-1* zaden, vertoonden *sit*^{w} zaden geen kiemrust en konden ze tijdens afrijping in de vrucht kiemen (Hoofdstuk 5). Tijdens zaadontwikkeling bleef de celcyclus in de cellen van de worteltopjes actief tot 45 DAP. In alle drie de genotypen wordt op dat moment het endosperm vast en beginnen de zaden uitdrogingstolerant en kiemkrachtig te worden (Hoofdstuk 5). Bij afrijping (vanaf 45 DAP) laten de kernen van het wildtype, *gib-1* en *sit*^{w} zaden hoofdzakelijk 2C DNA signalen zien, wat aangeeft dat de activiteit van de celcyclus in de meeste cellen van het worteltopje gestopt is in het G1 stadium van de kerndeling (Hoofdstuk 5). De cellen van de worteltoppen van droge zaden van het wildtype en *gib-1* gaven geen verandering in het flowcytometrisch beeld tijdens de verdere afrijpingsstadia (Hoofdstuk 5). Echter, de hoogte van de 4C piek in de worteltopjes van *sit*^{w} zaden steeg tijdens de verdere rijping (Hoofdstuk 5). In de rijpe en overrijpe zaden was de 4C piek duidelijk hoger dan in zaden van het wildtype en de *gib-1* mutant (Hoofdstukken

4 en 5). In de worteltopcellen van na oogst gedroogde wildtype en sitw zaden werd een verhoging van het 4C signaal gevonden na opname van water. Deze toename in het 4C signaal geeft kerndelingsactiviteit aan en trad op voordat er sprake was van zichtbare kieming. *gib-1* zaden lieten geen kerndelingsactiviteit zien en kiemden niet zonder toevoeging van GAs. Door imbibitie in 10 uM GA4 + 7 werd de activiteit van de kerndeling en de daaropvolgende kieming bevorderd in zaden van het wildtype en *sit*^{w} en geactiveerd in *gib-1* zaden (Hoofdstuk 4). Imbibitie in 5 uM ABA verhinderde de kieming van het wildtype en *sit*^{w} zaden maar had geen effect op de activiteit van de kerndeling (Hoofdstuk 4). Uit de resultaten werd geconcludeerd dat GAs de kieming van intacte zaden beïnvloeden door processen te bevorderen die voorafgaan aan de activatie van de celcyclus en dat ABA de groei beïnvloedt door aan te grijpen op processen die volgen op de activatie van de celcyclus (Hoofdstuk 4).

Een normale voorbehandeling in PEG induceerde DNA replicatie in de worteltopcellen van wildtype en *sit*^{w} zaden en gaf daarmee een verbetering van kieming (Hoofdstuk 3). Een zogenaamde verse PEG voorbehandeling is een nieuw ontwikkelde voorbehandelingsmethode waarbij de 'verse' zaden direct na de oogst in PEG worden voorbehandeld. Een verse PEG voorbehandeling kan zaadkieming en zaailingkwaliteit verbeteren in vergelijking met de niet-behandelde controle. Echter, verse PEG voorbehandeling brak de kiemrust niet en gaf geen inductie van DNA replicatie activiteit in wildtype of *gib-1* zaden (Hoofdstuk 3). In een gecontroleerde stress-test bleek dat de normaal voorbehandelde zaden gevoeliger waren voor veroudering dan de vers voorbehandelde zaden (Hoofdstuk 3). Toevoeging van GAs aan de PEG oplossing tijdens een verse voorbehandeling, bracht DNA synthese op gang en gaf een verdere verbetering van de kieming (Hoofdstuk 3). Ook verwijdering van het endosperm- en het testaweefsel tegenover het worteltopje induceerde DNA replicatie in de *gib-1* zaden zowel tijdens imbibitie in een osmoticum als in water (Hoofdstuk 5). Uit de resultaten werd geconcludeerd dat het opgang komen van kieming, uitgedrukt als de ratio van het aantal 4C kernen in de worteltopcellen, positief is gerelateerd met de kiemsnelheid en negatief is gerelateerd met de bewaarbaarheid van de behandelde zaden (Hoofdstuk 5). Het verzwakken van het endosperm tegenover de worteltop zoals dat wordt geïnduceerd door GAs, zet de wortel tot groei aan. De groei van de cellen induceert tenslotte kerndeling.

In de algemene discussie wordt het mechanisme van kiemrust en kieming in tomatenzaden gepresenteerd op basis van de resultaten uit dit proefschrift en van andere studies (Hoofdstuk 8). Speciale aandacht wordt gegeven aan de fysiologische regulatie door GA en ABA en door osmotische stress op uitdroogtolerantie, de inductie van kiemrust en de kiemkwaliteit van zaden. Een verklarend model wordt voorgesteld dat de belangrijkste vondsten uit dit proefschrift samenvat en de mogelijke verbanden tussen de resultaten laat zien. In dit model is de inductie van kiemrust en kieming gebaseerd op hetzelfde principe: de regulatie van de verzwakking van het endospermweefsel tegenover de worteltop. ABA en GAs spelen een antagonistische rol bij de verzwakking van het endosperm op het niveau van de genexpressie. Andere externe omstandigheden zoals licht,

water, temperatuur, nitraat en calcium dragen bij aan het proces dat leidt tot de synthese van GA, hetzij door beïnvloeding van de genexpressie in samenspel met de fytohormonen, hetzij door aan te grijpen op de eigenschappen van receptoren, die gebonden kunnen worden door fytochroom of door de fytohormonen.

Zhong wen zhai yao

植物激素与番茄种子发芽

（摘　要）

本论文以GA(*gib1*)和ABA(sit^{W})两种缺陷型和相应野生型番茄(品种:Moneymaker)种子为材料，结合使用外源植物激素和渗透调节，着重研究GAs和ABA在调控种子发育、处理以及发芽过程中的内部形态、水分关系和胚根尖细胞核DNA含量变化以及这种变化在诱导种子休眠和萌发过程中的生理作用机制.

利用软X-光摄影技术检测成熟干燥种子内部形态时发现，绝大多数种子内部结构清晰完整，只有极少数种子表现出胚乳残缺和自由空隙．与其他两个基因型相比，更多的sit^{W}种子具有自由空隙(第五章)．自由空隙是指种子内部种胚与胚乳之间可以被X-光检测出来的空隙．这种空隙是由于干燥种子吸水时种胚膨胀、胚乳被挤压，然后干燥脱水时种胚过分收缩而胚乳又不能恢复原状所致(第二章)．经聚乙二醇(PEG)渗透调节处理后，几乎所有处理种子都有了自由空隙(第五章)．所以这种自由空隙可以用来检测渗透调节处理效果．短时间(<12小时)浸种处理也能诱导自由空隙(第二章)．但如果浸种时间过长，种胚由原来的伸长(可逆的)过渡到生长(不可逆的)，即使种子再度干燥到原含水量，种胚也不能恢复原位，自由空隙也不会产生．当种胚萌动到生长阶段，再度干燥将会明显地机械损伤种胚(第二章)．在X-光片上可以清晰地看到吸水种胚沿胚轴方向伸长，种子内部不同器官的含水量差异较大，在种子接近发芽时，胚根尖含水最多(第二章)．GAs对种子自由空间的形成没有影响，而内源ABA可以通过降低胎萌种子数来减少具自由空隙种子数(第五章)．

利用热偶湿度计分析发育、处理以及发芽过程中果实和种子水分关系时发现，发育过程中所有基因型果肉和种子胶囊的含水量保持相当稳定，野生型和*gib1*果肉和种子胶囊的水分关系比较一致，当果实成熟时它们的膨压变为零，而sit^{W}果肉和种子胶囊膨压一直较高(第六章)．种子生理成熟时开始脱水．在生理脱水过程中，种胚水势低于整个种子，而整个种子水势则与种子胶囊或果肉组织基本平衡(第六章)．这说明发育种胚的生理脱水并未按水势梯度进行，而是一个主动过程．果实内环境以及ABA是抑制发育种子未熟发芽和胎萌的基本原因，而不是种胚本身(第六章)．在渗透调节处理过程中，野生型种胚水势保持恒定，而渗透势逐渐上升，从而导致膨压下降(第七章)．这说明处理过程中种胚溶质不仅没有积累反而有所消耗．同样地，在浸种过程中*gib1*种胚膨压也随着渗透压的上升而有所下降(第七章)．浸种过程中不同基因型之间完整种子水分关系的变化无明显差异(第七章)．种子的最终发芽取决于种胚的生长力大于胚乳的机械阻力，而这种生长力又取决于解剖种胚的吸水力(含水量)．但是由于胚乳对种胚吸水有明显阻力，所以种子最终发芽只于完整种子种胚含水量有一定正相关(第七章)．通过渗透调节处理降低种胚渗透势来增加种胚生长势的设想是不太现实的，但渗透调节处理可以诱导胚根尖所对应胚乳层发生弱

化，从而有利于种胚生长．发芽过程中GAs和ABA可以通过增强或减轻胚乳弱化来促进或抑制种子发芽(第七章)．

与野生型一样，*gib1*和*sit*^{w}种子形态发育十分正常(第六章)．不象其它两个基因型，*sit*^{w}种子在任何情况下都无休眠，而胚根尖细胞核DNA含量明显高于其它两个基因型，如果果实过熟，滞留种子将发生胎萌(第五章)．通常地，野生型种子比*sit*^{w}种子发芽慢，而*gib1*种子则不能发芽除非加入外源GAs．利用细胞流检仪分析胚根尖细胞核DNA合成结果表明，浸种过程中野生型和*sit*^{w}种子在发芽前12小时胚根尖细胞核DNA增加十分明显，而*gib1*种子则无此变化(第四章)．加入外源GAs可以促使*gib1*胚根尖细胞核DNA合成和诱发种子发芽；加入外源ABA可以明显抑制野生型和*sit*^{w}种子发芽，但不影响胚根尖细胞核DNA合成(第四章)．因此，内源ABA在诱导种子休眠、防止胎萌和将胚根尖细胞周期休止在G1期起有重要作用．我们的实验说明，GAs和ABA调控种子发芽在时间顺序上是有差异的，GAs作用于胚根尖细胞核DNA合成之前，而ABA作用于胚根尖细胞核DNA合成之后(第四章)．

普通渗透调节处理可以促进野生型和*sit*^{w}种子胚根尖细胞核DNA合成、促使种子发芽快、发芽齐，但往往降低种子耐贮性(第三章)．番茄种子鲜湿处理是一种新发明的方法，即将刚收获的新鲜种子不经干燥而直接进行渗透调节处理(第三章)．该处理不引发野生型和*gib1*种子胚根尖细胞核DNA合成(第三章、第五章)．与普通渗透调节处理相比，鲜湿处理促进种子发芽的效果较差，但不影响种子耐贮性(第三章)．在鲜湿处理过程中加入GAs可以诱发种子胚根尖细胞核DNA合成、增强处理对促进种子萌发的效果，但同时降低种子耐贮性(第三章)．有趣的是，切除胚根尖所对应的胚乳层也可以诱发*gib1*种子胚根尖细胞核DNA合成(第五章)．这说明由GAs所引发的胚乳弱化可以诱发种子胚根尖细胞核DNA合成、促进种胚生长、进而促进种子萌发．因此，渗透调节处理所引起的种子胚根尖细胞核DNA合成比例(4C/2C)与促进种子萌发的效果呈正相关，与种子的耐贮性呈反相关(第五章)．

最后，作者根据本论文研究成果建立了一个基本模型．该模型认为番茄种子休眠的诱导、解除以及最终发芽的实现取决于胚根尖所对应胚乳机械阻力的减弱程度．而胚乳机械阻力的减弱又取决于种子内源GA的产生和种子对GA的敏感性．植物激素ABA可以通过控制GA基因的表达来抑制胚乳机械阻力的减弱进程，从而实现对种子发芽的生理调控．其它外界环境条件如温、光、水、硝酸根、钙离子等都是通过协调上述生理过程以促进或抑制种子内源GA的生化合成来影响种子的休眠和发芽．

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Curriculum vitae

Yongqing Liu was born in Shaoyang, a small town in the Hunan province of the People's Republic of China on the first of October in 1958. He initiated his basic education in a primary school in 1965, and terminated his teenage study in a secondary school in 1975. After working in the countryside as a farmer for two years, he was accepted to study at the beginning of 1978 in the Horticultural Department of Hunan Agricultural University in Changsha, the capital city of the Hunan province. Four years later, he graduated with a bachelor degree in the Agriculture Science, and was immediately employed by the same university. In 1983, he started an 'on duty' postgraduate study program at the Hunan Agricultural University, and received his Master degree of Plant Breeding in 1987. He became a lecturer in 1988, and was promoted to the title of associate professor in 1993. In September 1991, he came to the Netherlands to study in the Department of Reproduction Technology, Centre for Plant Breeding and Reproduction Research (CPRO-DLO) in Wageningen, and was accepted for a sandwich PhD program at the Department of Plant Physiology, from the Wageningen Agricultural University. Since then, he had been working with his PhD program both in China and in the Netherlands until the completion of this thesis.