GERMINATION AND DORMANCY OF SINGLE TOMATO SEEDS

A study using non-invasive molecular and biophysical techniques

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Germination and dormancy of single tomato seeds:
A study using non-invasive molecular and biophysical techniques

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Stellingen

behorende bij het proefschrift: Germination and dormancy of single tomato seeds; A study using non-invasive molecular and biophysical techniques

1) De sterkte van de expressie van 35S::luciferase in individuele zaden is geen maat voor de kiemsnelheid van datzelfde zaad (*dit proefschrift*).

2) Niet de totale hoeveelheid ATP in een zaad is gerelateerd aan kieming , maar wel de lokale ophoping van ATP in de kiemwortel (*dit proefschrift).*

3) Fase III van wateropname in de kiemwortel van het embryo start al voor de zichtbare kieming . Dit is in tegenstelling met het algemeen aanvaarde 3 fasen model voor water opname van zaden, waarin fase III ná kieming plaatsvindt (*dit proefschrift*).

4) *Linker histones* spelen een belangrijke rol in down-regulation van genexpressie, door aanpassing van DNA-architectuur (Wolffe, Cell **77**, 13-16, 1994).

5) Een levensvatbaar niet gekiemd zaad, is niet noodzakelijkerwijs in kiemrust.

6) Een zaad van een hormoon-deficiënte mutant geimbibeerd in een oplossing van datzelfde hormoon, is fysiologisch niet gelijk aan het wild-type.

7) Het misstaat een plantenfysioloog niet om als dierfysioloog naar cellulaire processen te kijken.

8) Natuurontwikkeling is een *contradictio in terminis.*

Patrick Spoelstra
28 mei 2002

Voorwoord

Als kind zagen mijn ouders een echte bioloog in me, die op ontdekkingsreis gaat, de oerwouden in, op zoek naar planten en dieren. Nu komen dit soort voorspellingen veelal niet uit, anders was Nederland gezegend met een brandweerman of politie agent op elke hoek van de straat. Toch ben ik geheel intuïtief in Wageningen beland voor de studie biologie. Of was het vanwege de diavoorstelling over Pyreneeën-excursies tijdens een open dag? Al tijdens de eerste practica werd mijn interesse gewekt voor de planten. Het kon daarom ook niet uitblijven dat dit uiteindelijk de richting was waarin ik afstudeerde met afstudeervakken aan de toenmalige vakgroep Plantencytologie en –morfologie en de vakgroep Plantenfysiologie. Ik rolde op deze wijze bijna vanzelf het promotieonderzoek in. Toen begon de periode op de vakgroep met veel memorabele momenten.. Er zijn veel leuke, minder leuke, en gekke momenten gepasseerd (Wie fietste toch over de gang op de bovenste verdieping v/d Banaan?). Tijdens deze periode is de basis gelegd voor dit proefschrift. De eerste 2½ jaar heb ik daarbij veel ondersteuning gehad van Ronny. Onze jacht op de homozygoot eindigde na 2 jaar met een feestje in de kroeg. Dit was overigens geen uitzondering, want veel gezelligheid was te vinden in de naschoolse activiteiten en tijdens de pauzes in de illegale koffiehoek met diens inwoners (John, Jan Hendrik, Wessel e.a.). In 2000 zocht ik het ruime sop en koos voor een carrière in het bedrijfsleven, dit terwijl het onderzoek nog niet volledig was afgerond. Promoveren naast een fulltime baan is beslist geen sinecure. Hierbij ben ik veel dank verschuldigd aan Julia en Peter voor hun inzet bij het H1-verhaal. Tijdens mijn periode bij Roper Scientific B.V., heb ik veel baat gehad bij de flexibele opstelling van Wim van Dieren en in de periode daarna met het in bruikleen hebben van de Metamorph-sleutel.. Henk was als copromotor in alle fasen maar met name ook in de laatste twee jaar onmisbaar vanwege z'n optimisme en ondersteuning, al dan niet in de vorm van schrijfsessies, snel een manuscript nakijken of het slobber-bier van de Boni. Gelukkig is Linus toch altijd geduldig gebleven, en had hij vaak een heldere kijk op de zaken. Hierbij bedank ik iedereen die hierboven staan genoemd en ook de overige bewoners van de Banaan. Henk van As dank ik voor de samenwerking bij het NMR-werk. Michiel, je wilde in mijn voorwoord; bij deze. Hierbij bedank ik ook mijn ouders voor de stimulans en de geboden kansen. Edwin bedankt voor de opmerking in 1996: "Geil, dan krijgen we toch nog een doctor in de familie." De laatsten zullen de eersten zijn en dus bedank ik speciaal Jolanda voor haar engelengeduld in al die weekenden waarin aan het proefschrift gewerkt werd, al dan niet in gedachten of feitelijk achter de PC.

Patrick

Kudelstaart, 23-04-02.

Contents

1

General Introduction

General Introduction

The seed is the generative dispersal unit of higher plants. Its functions lie in dispersal of the plants' genetic material and 'living' through periods (or seasons) of less favourable conditions (e.g. drought, low temperatures, darkness) for vegetative survival. Germination is the event that preludes the emergence of a new plant.

This thesis is aimed at getting a better understanding of processes which underlie the germination of seeds, especially tomato seeds (*Solanum lycopersicum L.*). The study was focussed on the ability and inability (germinating vs. dormant seeds) of tomato seeds to germinate under optimal conditions (25 °C in the dark, with sufficient supply of water on filter paper in a petri-dish).

Germination: definitions

Seed germination is often different in agronomic terms as compared to a seed scientific view. In seed science germination *sensu stricto* is the process which starts with the uptake of water by the dry seeds and which ends with the protrusion of an embryo root (the radicle) through the surrounding tissues (e.g. endosperm and testa). Further processes including the formation of secondary roots and unfolding of cotyledons are regarded as part of subsequent growth processes (Bewley and Black, 1994). Germination in the agronomic view is the whole process of sowing a seed in the soil until emergence of a young seedling with cotyledons. The advantage of the scientific definition of germination is that it is a clear definition, which is true for all seeds and avoids confusion. The disadvantage is the risk, to be looking at processes during germination which might as well be related to post-germinative events.

Germination itself is projected at a small timescale compared with the larger timescale of subsequent growth of the radicle and cotyledons, which also are part of functions of the seed. In this thesis the scientific definition of germination is adopted.

Germination of tomato seeds

The physiology of tomato seeds has been extensively studied. They have a clearly distinguishable morphology and are of convenient size. Tomato seeds are therefore an attractive model system to study seed germination and dormancy .

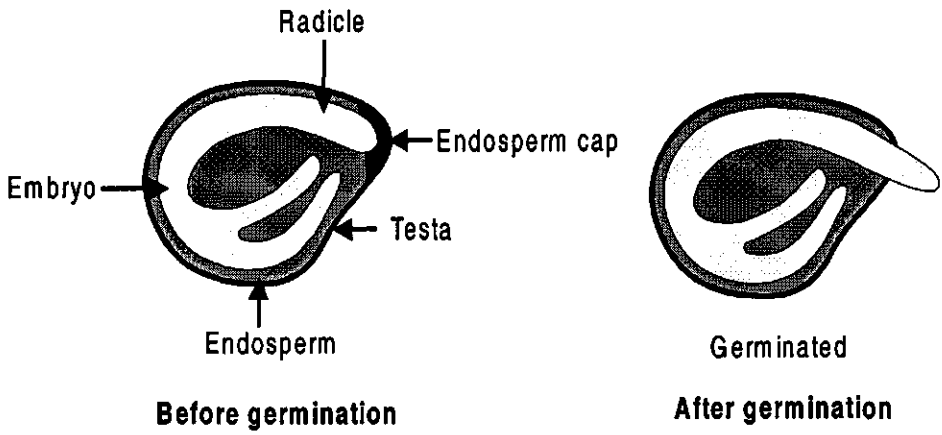


Figure 1: Schematic representation of the morphology of tomato seeds.

Germination of tomato seeds starts with imbibition of the dry seed with water and ends with protrusion of the radicle through the endosperm and testa (Figure 1). The embryo is surrounded by a rigid endosperm. The part of the endosperm opposite the radicle is called the endosperm cap. Radicle protrusion is dependent on weakening of this endosperm cap, thereby relieving the radicle from the mechanical barrier. In this respect, whether radicle protrusion occurs or not, radicle growth is the net result of

its growth potential (protruding force) and the opposing strength of the endosperm cap.

The restraint of the endosperm cap decreases during germination through the action of cell wall degrading enzymes of which endo-ß-mannanase (EC 3.2.1.78) has been most extensively studied (Groot and Karssen, 1987; Karssen *et al.*, 1989; Nomaguchi *et al.*, 1995; Nonogaki *et al.*, 1992; Nonogaki *et al.*, 1995; Nonogaki and Morohashi, 1996; Nonogaki *et al.*, 1998; Nonogaki *et al.*, 2000; Still and Bradford, 1997; Still *et al.*, 1997; Toorop *et al.*, 1996; Toorop *et al.*, 2000). Recently, other possible candidates contributing to endosperm cap weakening have been reported, including expansins which facilitate cell wall extension in the endosperm cap (Chen and Bradford, 2000) and polygalacturonase, another cell wall degrading enzyme (Sitrit *et al.*, 1999).

The growth potential of the radicle of fully imbibed seeds depends on the water potential (Ψ) of the cells within the radicle. The Ψ is composed of an osmotic potential (Ψ_π) and an opposite pressure potential (turgor, Ψp). During imbibition of tomato seeds Ψ of the embryo is approximately –1.5 MPa , while the Ψ of the whole seeds is in equilibrium with that of the imbibitional solution (Haigh and Barlow, 1987). Obviously, the radicle is prone to take up water. Uptake of water should result in elongation of cells in the radicle and emergence of the radicle through the endosperm cap. For cells to elongate, cells walls must become extensible. Expansins have been proposed to function as cell wall loosening factors by disrupting non-covalent linkages (e.g. hydrogen bonds) between cellulose and hemicellulose thereby facilitating cell elongation or expansion (Cosgrove, 1998). During germination of tomato an expansin (LeEXP8) is expressed in the radicle (Chen and Bradford, 2000). These findings indicate that endosperm cap weakening only is not sufficient for the

radicle to protrude, as was already hypothesised by Haigh and Barlow (1987). They proposed the existence of a genetically regulated, growth enabling, process, independent of hydration.

Dormancy: definitions

Dormancy can be defined as the inability of a viable seed to germinate under conditions favourable for germination. Dormancy can be divided into primary and secondary dormancy (Figure 2) (Crocker, 1916; Hilhorst, 1998; Karssen, 1982). During development a seed may acquire dormancy. This dormancy is called primary dormancy. A primarily dormant seed will not germinate upon imbibition by water. If primary dormancy is absent the seed will then germinate upon imbibition by water. Primary dormancy can be relieved (broken) in the dry state by after-ripening for periods of months to years which is accelerated by elevated temperatures. In the imbibed state primary dormancy can be relieved by cold stratification or chilling for several days or weeks at (non-freezing) low temperatures. If after breaking of primary dormancy, seeds encounter favourable conditions for germination (e.g. light, temperature), germination may be completed. If these conditions are not met, seeds may enter a new state of dormancy, called secondary dormancy. Secondary dormancy, again, can be relieved by cold stratification. In a seasonal pattern seeds may switch between states of dormancy and no dormancy, which is called dormancy cycling (Karssen, 1982).

Both types of dormancy are found in tomato seeds. Often freshly harvested seed batches contain a certain percentage of primarily dormant seeds. Cold stratification is most effective in breaking this dormancy. Secondary dormancy can be induced in non dormant tomato seeds by irradiation with farred light. Secondary

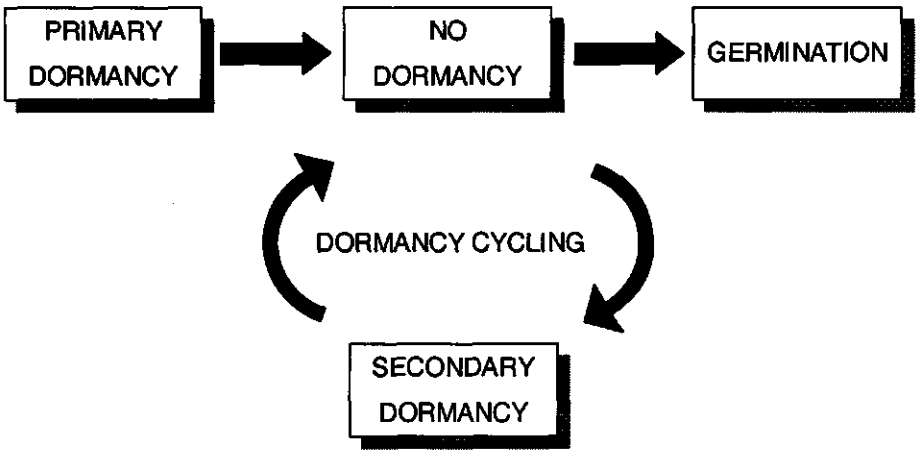


Figure 2: Schematic representation of changes in dormancy. Adapted from Hilhorst (1998).

dormancy in these seeds can also be relieved by cold stratification (de Castro *et al.*, 2001).

The study of dormancy in tomato may be hampered greatly by what can be referred to as *the dormancy paradox*: Dormant, non-dormant and dead seeds cannot be distinguished within the same seed batch. Only when a radicle protrudes from a seed, it was obviously alive and non-dormant (in Chapter 2 this paradox is addressed). The dormancy paradox not only applies to mixed seed batches with both non-dormant and primarily dormant seeds, but also applies to the induction of secondary dormancy. Not all seeds within in a seed batch are sensitive to farred light. A certain percentage escapes from secondary dormancy induction. Only prolonged periods of incubation may distinguish dormant from non-dormant seeds. Furthermore, dormancy is not an 'all or none' event. It may be argued that difference in germination rate is an expression of dormancy. In other words a seed batch may germinate for 100% but still possess a certain degree of dormancy.

Dormancy is an actively maintained arrest of development. Dormant seeds do not necessarily 'slumber' in a metabolically low maintained state. Dormant seeds

show active respiration and ATP levels which do not distinguish them from their germinating counterparts (Derkx *et al.*, 1994a). Dormant seeds also show a specific gene expression pattern (Anderberg and Walker-Simmons, 1992; Bradford *et al.*, 2000; Goldmark *et al.*, 1992; Li and Foley, 1995)

The role of the phytohormones abscisic acid and gibberellins during germination and dormancy

The effects of abscisic acid (ABA) and gibberellins (GAs) on the development of seeds and on germination and dormancy have been studied extensively. Despite the studies of physiological responses to exogenously applied GA or ABA and knowledge derived from hormone mutants, the pathways of ABA and GA action in seeds are far from elucidated. Effects of these hormones are also not always unambiguous. The application of GA may break dormancy in one species but can be totally ineffective in a second species. Notwithstanding the existence of possibly conflicting results, several generalisations can be made with respect to the effects of ABA and GA. ABA and GA often have counteracting effects: GA stimulates germination or the breaking of dormancy, whereas ABA often inhibits germination or induces dormancy. The ratio, rather than the absolute amounts of ABA and GA appears to be decisive in the developmental state of seeds. This was demonstrated by the suppression of the non-germinating phenotype of the Arabidopsis GA-deficient mutant, *ga1*, by crossing with the ABA deficient mutant *aba1* (Koornneef *et al.*, 1982). Similarly, in maize, the GA-deficient mutant d1 could suppress the mutant phenotype of the ABA deficient *vp5* mutant (White *et al.*, 2000). It has recently been shown that ABA and GA signal transduction pathways do interact in controlling the developmental state of seeds. The inhibition of GA biosynthesis could mimic ABA regulated accumulation of

maturation phase mRNA in cultured embryos of maize (White and Rivin, 2000). More convincingly, PKABA1, an ABA induced protein kinase, inhibits the induction by GA of the GAMyb transcription factor. GAMyb is involved in the activation of α-amylase by GA, the enzyme involved in storage food mobilisation in aleurone layers of barley (Gómez-Cadenas *et al.*, 2001). When the barley orthologue of SPY (responsible for the slender GA response mutant *spindly* in Arabidopsis) was transiently expressed in barley aleurone layers it was able to abolish α-amylase activity and activate an ABA inducible promoter. SPY is believed to be a negative regulator of GA signalling (Robertson *et al.*, 1998).

Our current knowledge of the functions of ABA and GA in tomato seeds has been mainly derived from studies with the ABA deficient *sit*^{w} mutant and the GA deficient *gib1* mutant. Both mutants have lesions in their hormone biosynthetic pathways. In the *gib1* mutant the activity of the two step conversion of geranylgeranylpyrophosphate (GGPP) to ent- kaurene is blocked (Bensen and Zeevaart, 1990). In the *sit*^{w} mutant the last step of ABA-biosynthesis via the oxidation of ABA-aldehyde to ABA is negatively affected (Sindhu and Walton, 1988). The *sit*^{w} mutant seed does not display primary dormancy and seeds may germinate viviparously in the ripe tomato fruit (Groot and Karssen, 1992). Endo-ß-mannanase activity was higher in endosperm of *sit*^{w} seeds compared to wild type seeds (Hilhorst and Downie, 1996) but the $\Delta\Psi$ of the *sit*^{w} embryo was comparable with that of wild type seeds (Liu, 1996) during germination. *gib1* seeds do not germinate in water. Exogenously applied GA is required to complete germination. Endo-ß-mannanase activity is absent during imbibtion of *gib1* seeds by water but application of GA will induce endo-ß-mannanase activity (Groot *et al.*, 1988). Also in the *gib1* mutant the $\Delta\Psi$ of the embryo during imbibition with water was unaffected (Liu, 1996).

These two mutants have been extensively used as controls for ABA and GA directed processes in tomato seeds. Imbibition of wild type seeds in ABA has also been used frequently as a control for ABA related processes. Wild type seeds imbibed in 10 μM ABA do not germinate but are not rendered dormant. As soon as seeds are transferred to water they resume germination. Only after prolonged incubation seeds may acquire secondary dormancy.

It is clear that GA is involved in germination. However a role for GA in the breaking of dormancy has not been convincingly established. GA was unable to relief all tomato seeds from primary or secondary dormancy (de Castro *et al.*, 2001). Sensitivity of seeds to ABA or GA is also an important factor. Non-dormant seeds may be insensitive to ABA and dormant seeds may be insensitive to GA. Cold stratification which can be used to break both primary and secondary dormancy apparently sensitises seeds to GA (Derkx *et al.*, 1994a; Hilhorst and Karssen, 1992). From the above it is clear that the action of ABA and GAs in tomato seeds is complex and results should be interpreted carefully.

Variation : the need for non-destructive methods aimed at the single seed

Variation in nature is omnipresent. Obviously this is also true for seeds. Seeds may vary in many aspects such as size, shape, colour, germination rate or depth of dormancy. Variation among single seeds is a mechanism to maximise the likelihood of survival of the plant's progeny (Benjamin, 1990). Variation can be determined genetically or may be caused by micro-environmental influences, which result in phenotypic variation.

Tomato is a self pollinating crop from which the different cultivars are highly isogenic. Nevertheless, single tomato seeds may vary greatly in many aspects. This

largely phenotypic variation may be due to positional effects of fruits along trusses, between trusses, or the position of a seed in a fruit (Demir and Ellis, 1992; Hatcher, 1940). Existence of large variations among single seeds is apparent first of all from the variation in germination rate of the individual seeds. Existence of variation in enzyme activity was demonstrated for endo-ß-mannanase which diffused from single endosperm caps during germination (Still and Bradford, 1997; Still *et al.*, 1997). Variation in endo-ß-mannanase activity was 4 orders of magnitude in extreme cases and 100 fold in most cases.

Inherent to most methods employed in the study of seed physiology is the destructive and averaging nature (seeds are pooled into a sample). Several pitfalls are apparent: 1) given the 4 orders of magnitude difference, it is likely that the average of the assessed parameter is largely composed of just a few extremes. 2) the direct link between the parameter studied and the timing of germination is lost in a destructive assay. The observation of germination is limited to germination rate constants such as the time point at which the first radicle starts to emerge, percentage of germination, the time point at which 50% of seeds have germinated (T_{50}) or the time point of maximum germination. This might hinder the interpretation of events e.g. whether they occur prior to or after radicle protrusion (germination related or growth related) 3) processes which are localised in a small region within the seed and which are essential for germination may be totally averaged out and, consequently, disregarded.

Techniques that aim at localising processes or metabolites within seeds or/and are non-destructive might contribute to our understanding of seed performance. Such methods, retain both population and single seed information and display a direct link to completion of germination of a single seed. Such methods may include

immunolocalisation, *in situ* hybridisation, monitoring of reporter gene activity and *in vivo* imaging of water distribution.

Outline of the thesis

Although the experimental work in this thesis differs greatly with respect to the employed techniques, all experiments are focussed on using single seed techniques in the study of dormancy or germination in tomato seeds. In this way it is shown how seed science can benefit from single seed assays.

Chapter 2 describes how variation between single tomato seeds was studied non-destructively with the use of a Cauliflower mosaic virus promoter (CaMV 35S) in combination with a firefly (*Photinus pyralis*) luciferase reporter gene. The dormancy paradox is addressed by using luciferase activity as a marker for primary and secondary dormancy

In Chapter 3 luciferase was used to visualise ATP distributions in cryosections of tomato seeds either during germination or during primary and secondary dormancy. Primarily dormant seeds were separated from germinating seeds by the use of the transgenic 35S::luciferase seeds, which were described in chapter 2.

A turbo spin echo NMR imaging technique was applied in chapter 4 to visualise water distributions during germination of single tomato seeds.

H1 linker histones are proteins in the nucleus which play a role in chromatin organisation, thereby influencing gene expression. In chapter 5 two functionally different linker histones were analysed for expression during germination and secondary dormancy. A model is proposed for the regulation of gene expression at the level of the chromatin in relation to germination or dormancy.

In chapter 6 all results are discussed in relation to each other and current opinions in seed physiology.

2

A cauliflower mosaic virus promoter-luciferase reporter gene construct as a non-destructive marker in the study of variation in timing of germination response to external stimuli and dormancy in single tomato seeds (*Solanum lycopersicum* L.)

P Spoelstra, R.V.L. Joosen and H W. M. Hilhorst

A combination of a luciferase (firefly) reporter gene and the 35S (CaMV) promoter was transfected to tomato (*Solanum lycopersicum* L.) in order to study the progress of single tomato seed germination in a non-destructive manner. Luciferase was mainly expressed in the endosperm cap; gene activity was essentially linked to the progress of germination and was used as a marker for variation among single seeds. Luciferin uptake was rate limiting in the embryo and not in the endosperm. Variation among single seeds was up to 150 fold but did not directly correlate to the germination rates of single seeds. Expression of the 35S::luciferase reporter gene could be manipulated in single seeds by ABA and GA. Single seeds revealed individual thresholds or sensitivities for ABA and GA and responded in a dose-response dependent manner. The expression of luciferase was absent in secondarily and primarily dormant seeds. This enabled us to non-destructively separate dormant from germinating seeds prior to emergence of the radicle from the seed. The use of the luciferase reporter gene in seeds is discussed.

Introduction

Genotypic and phenotypic variation is omnipresent in biology. Obviously this is also true for seeds. For example, single seeds from the same ecotype or originating from the same plant, vary in their rate of germination, thus spreading emergence over time. This seed to seed variation is a biological trait, which is commonly regarded as a mechanism to increase probability of survival of plant species through their offspring (Benjamin, 1990). Tomato is a self-pollinating crop from which the different cultivars are highly isogenic. Notwithstanding this, seed to seed variation in tomato is still present. This seed to seed variation in isogenic lines of tomato should be considered as largely phenotypic. Phenotypic seed to seed variation might be caused by positional effects of the seed within the fruit or the position of that fruit in a truss or the position of that truss on the plant (Demir and Ellis, 1992; Hatcher, 1940).

The study of seed physiology often employs methods which involve pooling of seeds into larger samples, from which a physiological parameter is usually assessed in a destructive manner. By applying such methods, a direct link with germination of that particular sample of seeds is lost; germination can only be described by the germination percentage or germination rate of a seed sample in a parallel experiment under the same conditions. Both the germination data and the value of the assessed parameter are a population average. Population averages give no information about the response to a treatment of single seeds within that population. In terms of dormancy studies, by pooling seeds in larger samples an additional problem occurs; dormant and non-dormant seeds might be pooled within the same sample. In general, dormant and non-dormant seeds within a seed batch can only be distinguished after incubation under germination conditions. Only when a seed germinates and a radicle becomes visible, that single seed was obviously non-dormant. Viable seeds that do not

germinate within the boundaries of normal germination time for a certain species must then be considered dormant.

In any seed batch that is studied, seed to seed variation is imminent through the variation in germination time of the single seeds. Many biological processes and traits underlie this variation in germination time. Every single seed has it own set of biological processes and traits, such as, sensitivity to external and internal factors (i.e. threshold level), enzyme activities, ATP production and water content, which together comprise the processes that underlie germination.

Study of seed physiology could benefit from experiments which preserve data of these processes and combine both population and single seed data (population data being simply an average of single seed data). In order to accomplish this, germination needs to be studied non-destructively at the single seed level. Only few studies have been carried out on germination of single seeds (Jalink *et al.*, 1998; Still and Bradford, 1997). These studies have reported on seed to seed variation with respect to cell wall degrading enzyme activity of endo-ß-mannanase (4-5 orders of magnitude differences) and chlorophyll fluorescence as indicator of maturity of seeds (100 fold differences). In order to monitor single seeds during the progress of germination in a non-destructive manner, we transfected tomato plants with a luciferase reporter gene fused to a cauliflower mosaic virus promoter (CaMV 35S).

The CaMV 35S promoter is often used for expression studies of foreign genes in plants (Benfey *et al.*, 1989). The firefly luciferase gene encodes a protein which catalyses the oxidative decarboxylation of firefly luciferin, using oxygen, ATP and Mg^{2+}, thereby releasing a single photon at 562 nm (DeLuca and McElroy, 1974). Luciferin is an amphipathic molecule, which can easily penetrate into plant tissues. When germinating seeds or plants expressing a luciferase reporter gene are incubated

or sprayed with a luciferin solution, photons will be emitted by these seeds or plants (Ow *et al.*, 1986). These emitted photons can be spatially and temporally resolved with a deeply cooled or intensified CCD camera. In the present study we demonstrate the use of a CaMV 35S-luciferase reporter gene construct as a marker for tomato seed germination. Luciferase activity from single germinating seeds shows a pattern, which is associated with the progress of germination of a single seed. The expression of luciferase in seeds is discussed in relation to populations and single seeds that germinate or are in a state of dormancy.

Experimental procedures

Plant growth conditions and seed harvesting

Tomato plants were soil grown in growth chambers at a 16 h light (35 Wm^{-2}) - 25°C, 8 h dark - 19°C regime at a RH of 70 % or in a greenhouse. Tomato fruits were picked at the red stage and the seeds and locular tissue were removed from the ripe tomatoes by cutting. The locular tissue was digested by adding an equal volume of 2% (v/v) hypochloric acid and stirring for 2 hours. Seed were rinsed thoroughly under running tap water en transferred onto two layers of filter paper and dried for 3 d at 35% RH at 21°C. Dry seeds were either stored at room temperature or at 4°C.

Induction and of secondary dormancy

Secondary dormancy was induced in wild-type seeds by far-red ($\lambda > 730$ nm) irradiation for 5 min at hourly intervals during the first 24 h of imbibition at a temperature of 21°C followed by incubation in the dark at 25°C for 5 days (de Castro *et al.*, 2001). During this period less than 3% of the seeds germinated.

Reporter gene construct

The reporter gene construct was composed of the CaMV 35S promotor (–348 to +8 sequence; Benfey *et al.*, 1989; Gardner *et al.*, 1981) fused to the original luciferase coding sequence (de Wet *et al.*, 1985). In front of the luciferase coding sequence an N-terminal SV40 nuclear localisation signal was inserted (van der Krol

and Chua, 1991). This construct was ligated into a binary vector, pMON721 and transformed to *Agrobacterium tumefaciens* (Strain ABI).

Plant transformation

Transgenic tomato cv Moneymaker (MM) plants were obtained via *A. tumefaciens* mediated transformation of cotyledon explants, as follows:

Preparing the Feeder Layer

A 100 mL *Petunia albino cobache* suspension culture was maintained in 250 mL erlenmeyer flaks at 25°C under a 16 h light 8 h dark regime on a rotary shaker at 120 rpm in MS medium (pH 6.0; KOH adjusted) supplemented with 0.5 mg/L BA, 0.5 mg/L 2,4-D and 30 g/L sucrose. The suspension culture was subcultured every 10 days by resuspending 50 mL suspension culture into 50 mL of fresh medium. For feeder layers, 7-day old suspension cultures were used. Feeder layers were prepared by pipetting 2 mL suspension culture into 9 cm petri-dishes containing MS medium (pH 5.9; KOH adjusted) supplemented with 2 mg/L NAA, 1 mg/L BAP and 7 g/L plant tissue agar. The 2 mL of suspension culture was evenly spread over the medium by shaking and covered with a Whatman NR 2 filter paper. Feeder layer plates were subsequently incubated for 3 days at 25°C and a 16 h light 8 h dark regime.

Transformation Procedure

Moneymaker seeds were surface sterilised in a 15 % (v/v) sodium hypochlorite solution for 30 min, rinsed twice in sterile water and subsequently germinated in glass jars on MS medium containing 3% (w/v) sucrose under a 16 h light, 8 h dark light regime (25°C). Cotyledon explants were cut from 8 d old

seedlings and subsequently co-cultivated on feeder layers of *Petunia albino cobache* cells for 24 h at a 16 h light 8 h dark regime (25°C). After co-cultivation cotyledon explants were removed from the feeder layers with tweezers and shortly submerged in *A. tumefaciens* inoculum. The inoculum was prepared by culturing *A. tumefaciens* containing the transformation vector overnight at 28°C in 30 mL of LB medium containing 50 µg/mL kanamycin, 25 µg/mL chloramphenicol and 30 µg/mL spectomycine to an O.D. of 0.6-1.0. The *A. tumefaciens* culture was then centrifuged at 1600 g for 20 min. The supernatant was removed and the pellet was resuspended in 30 mL sterilised Millipore water. After inoculation cotyledon explants were blotted for 15 min on sterile filter paper and transferred back to the feeder layer and incubated for another 48 h at a 16 h light 8 h dark regime (25°C). The cotyledon explants were then transferred to an MS medium (pH 5.9; KOH adjusted) containing 20 g/L sucrose, 200 mg/L carbenicillin or cefotaxin, 200 mg/L vancomycin, 150 mg/L kanamycin, 2 mg/L zeatin and 1.8 g/L Phytagel (Sigma Aldrich) and incubated at 25°C at a 16 h light 8 h dark regime. After 3-6 weeks, callus was observed and also small shoots of 1-2 mm in length. Both callus and attached shoots were transferred to fresh medium. Shoots which reached a length of >1.5 cm were transferred to MS medium containing 20 g/L sucrose, 200 mg/L carbenicillin or cefotaxin, 200 mg/L vancomycin and 1.8 g/L phyta-gel (Sigma Aldrich). Shoots which formed roots were sprayed with 0.1 mM luciferin (Molecular Probes) solution containing 0.01% (v/v) Tween 80 and were tested for luciferase expression with an intensified CCD camera (Hamamatsu Photonics Corp., Japan). Transgenic plantlets (i.e. the primary transformant), expressing luciferase were transferred to soil. Seeds were harvested from the primary transformants (T_0). T_1 plants were analysed via southern blotting for the number of reporter genes inserted into the genome. Luciferase expression during germination

was also tested for the different transgenic seed populations. A transgenic line was selected with highest luciferase expression during germination, displaying two copies of the transgene on southern blots. A homozygous line was obtained in the fifth transgenic generation (T_5).

Measurement of luciferase expression in situ

In all experiments transgenic seeds were imbibed in 0.1 mM luciferin (Molecular Probes). Seeds were imbibed in 2.5 mL luciferin solution in 5 cm Ø plastic containers on 3 layers of filter paper. Alternatively seeds were imbibed in 10 mL luciferin solution in 12 cm glass petri-dishes on 3 layers of filter paper. To minimise reflection of emitted photons on the filter paper the top filter paper was a black coloured filter paper (Whatman NR 9). For *in situ* measurement of luciferase expression seeds were germinated in a transparent incubator at 25°C (S160 Total Visibility Incubator, Stuart Scientific Co. Ltd, UK). Alternatively seeds were imbibed in 2.5 mL 0.1 mM luciferin solution supplied with 10 µM ABA, GA_{4+7}, 500 $\mu g.ml^{-1}$ cordycepin or 400 $\mu g.ml^{-1}$ cycloheximide.

Photons emitted by seeds were spatially resolved with a liquid nitrogen cooled back-thinned CCD camera (Princeton Instruments Versarray™ 512B, Roper Scientific) operated via Metamorph 4.1 (Universal Imaging Corp.) software. Sixteen bit monochrome images were generated by on chip integration for 15 or 30 min of photons emitted by single seeds. Images of photon emission from seeds were analysed for average pixel value using Metamorph 4.1 (Universal Imaging Corp.). The pixel values within those images are a direct measurement of the activity of the luciferase protein present in seeds. Luciferase activity in all figures is expressed in arbitrary

units (AU). Time lapse measurements were performed by generating 1 or 2 images every hour during a 2-3 days period, depending on the nature of the experiment.

Luciferase extraction and in vitro assay

Ten seeds were frozen in liquid nitrogen and ground with 2 chrome-vanadium bullets (Ø 4 mm) in a 2.2 mL Eppendorf tube on a Braun Biotech Int. Mikro-Dismembrator U, at 1600 rpm for 3 minutes. The ground sample was suspended in 500 µL ice cold extraction buffer (0.25 mM Tris HCl pH 7.8, 2 mM EDTA, 10 % glycerol (v/v), 1 % (v/v) Triton x-100 and 2mM DTT). The extracts were then centrifuged at 16000 g for 5 min. The supernatant was removed (i.e. enzyme extract) and then immediately frozen into liquid nitrogen in 2 aliquots of 200 µL.

The protein content of the extracts was determined with a Pierce, BCA protein kit in triplicates of 10 µL samples of the extract mixed with 100 µL of the BCA mixture. Absorption was measured on a Mios Mercks, MR 7000 Dynatech multiwell reader at a wave length of 550 nm.

Luciferase activity of the protein extracts was determined with a bioluminescence assay and a Labsystems Luminoskan DS luminometer. The assay buffer (pH 7.8) consisted of 20 mM tricine, 5 mM $MgCl_2$, 0.1 mM EDTA, 3.3 mM DTT, 500 µM ATP (Boehringer) and 500 µM luciferin (Molecular Probes). Triplicates of 10 µL of the protein extract were pipetted into white microtiter plates (Solid White, 96-Well, Costar) and 50 µL of assay buffer was injected into individual wells automatically and photon emission measured for 10 sec.

RNA isolation, reverse transcriptase PCR and hybridisation

RNA isolation was modified from Wan and Wilkins, 1994. Ten seeds were frozen into liquid nitrogen and ground with 2 chrome-vanadium bullets (Ø 4 mm) in a 2.2 mL Eppendorf tube on a Braun Biotech Int. Mikro-Dismembrator U, at 1600 rpm for 3 minutes. The ground sample was suspended in 700 µL hot borate buffer (80°C) containing, 0.2 M sodium borate decahydrate, 30 mM EGTA, 1 % SDS (w/v), 1% sodium deoxycholate (w/v), 2% (w/v) PVP (M_r 44000) and 10 mM DTT. The sample was then transferred to a 2.2 mL Eppendorf tube, containing 0.35 mg proteinase K and incubated in a water bath at 42°C for 1.5 h. After adding 55 µL 2 M KCl and subsequent incubation on ice for 1 h, samples were centrifuged at 12000 g for 20 min at 4°C. The supernatant was transferred to a 15 mL tube and 270 µL 8 M LiCl was added, after which samples were incubated overnight at 4°C. Samples were then centrifuged at 12000 g for 20 min at 4°C and the pellet was resuspended in 1 mL ice cold 2 M LiCl and subsequently centrifuged at 10000 g for 10 min at 4°C (the latter 2 steps were performed twice). The pellet was then resuspended in 400 µL 10 mM Tris-HCl and remaining debris was spun down by centrifugation at 12000 g for 10 min at 4°C. The supernatant, containing the RNA was transferred to a 15 mL tube and 40 µL 2M KAc was added for incubation on ice for 15 min, followed by centrifugation at 12000 g for 10 min at 4°C. The RNA in the supernatant was precipitated with 1.3 mL ethanol and incubated overnight at –20°C, which was followed by centrifugation at 11000 g for 30 min at 4°C and a washing step with 70% ethanol and subsequent centrifugation at 11000 g for 30 min at 4°C. The remaining pellet was dried in a speed vacuum rotor and subsequently suspended in 40 µL RNase free water. The RNA amounts in the extracts were quantified in a Phamacia GeneQuant RNA/DNA Calculator (Pharmacia/ LKB Biochrom Ltd. model 80-2103-98) and stored at –80°C.

RNA samples were DNase treated with 2U DNase (Boehringer) and 20 U RNAsin (Gibco BRL Life Technologies). The remaining RNA was quantified with the GeneQuant and on a 1.5% agarose formaldehyde gel. First strand cDNA was synthesised from 2.5 µg RNA using reverse transcriptase with Oligo(dT) primers (Superscript^{tm} Preamplification System for First Strand cDNA Synthesis Gibco/BRL Life Technologies). PCR reactions were performed on cDNA samples with luciferase specific primers. Samples were taken after 26 and 30 cycles. Equal amounts of PCR samples were subsequently run on 1.5 % agarose gel and blotted onto nylon membrane followed by hybridisation according to the manufacturers instructions (GeneScreen Plus, Life Science Products). Blots were incubated on phosphor screens for several hours and scanned on a Molecular Dynamics Storm 840 phosphor imager (Molecular Dynamics Storm 840). The signal was quantified using Metamorph 4.1 (Universal Imaging Corp.) and intensities were corrected with intensities of ribosomal bands of the corresponding RNA samples on an ethidium bromide gel.

Results and discussion

Luciferase activity is located in the endosperm

The expression of the 35S::luciferase reporter gene was monitored during the course of germination of tomato seeds. Germination of tomato seeds is believed to be controlled by the endosperm cap opposing the radicle tip. In this respect germination is a result of the net force of radicle protrusion and endosperm cap restraint. The endosperm cap is anatomically different from the rest of the endosperm and shows tissue specific gene expression (Chen and Bradford, 2000). This is also true with respect to luciferase activity, which was strongest in the endosperm cap and of lower intensity in the lateral endosperm. For the experiments described in this paper we have chiefly utilised a transgenic 35S::luciferase tomato homozygous line with strong expression levels and with 2 segregating copies of the transgene present. Seeds which were imbibed in 0.1 mM luciferin and dissected into endosperm cap, lateral endosperm and embryo displayed a very low luciferase activity in the embryo and in the lateral endosperm (Figure 1A). Other lines originating from the same primary transformant were also tested. Single copy lines displayed a luciferase activity which was a factor 100 lower compared with double copy lines (possibly due to position effects). Seeds with 2 copies of the transgene which were still hemizygous, imbibed in 0.1 mM luciferin did display luciferase activity in the embryo when dissected and placed on a 0.1 mM luciferin solution. Dissecting the embryo from these seeds during imbibition in 0.1 mM luciferin and placing the embryo on water instead of luciferin showed that luciferin is a limiting factor for luciferase activity in the embryo (Figure 1B). It is known that the endosperm limits the uptake of water by the embryo (Haigh and Barlow, 1987); obviously this is also true for luciferin. In intact seeds activity in

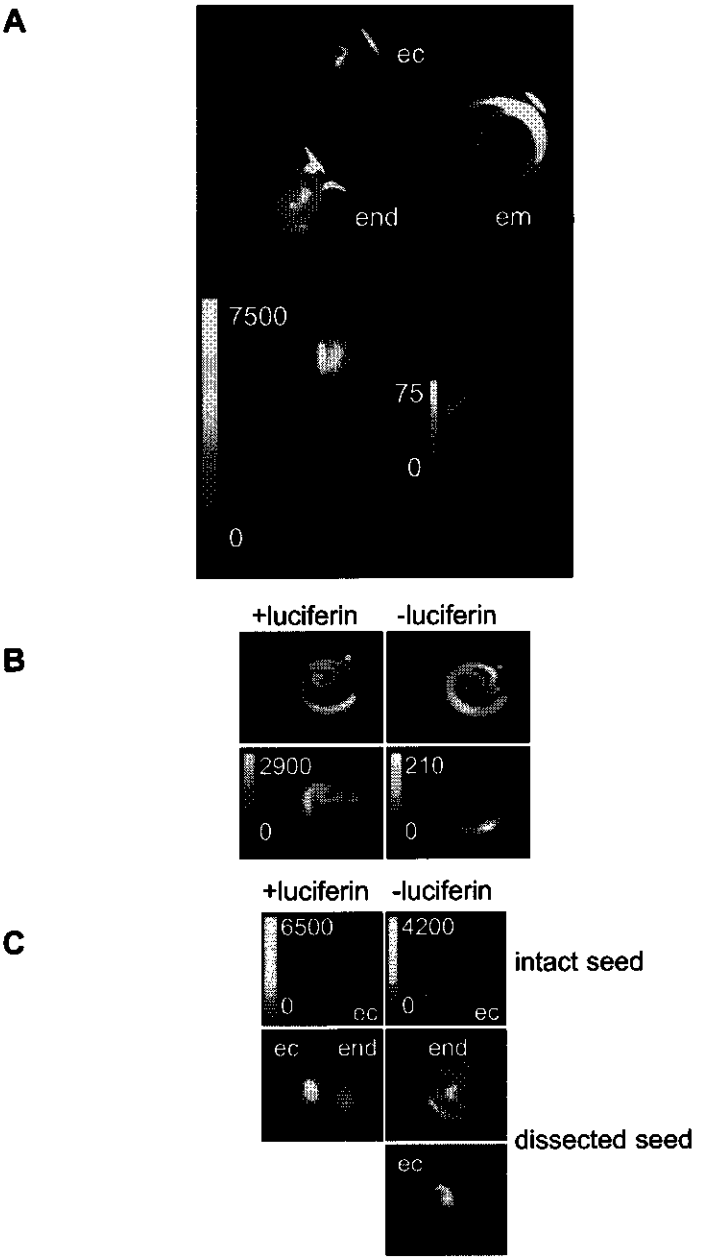


Figure 1: A) (lower panel) Luciferase activity in the lateral endosperm (end) the endosperm cap (ec) and the embryo (em) of homozygous 35S::luciferase tomato seeds imbibed in 0.1 mM luciferin. (upper panel) Corresponding bright field image of dissected tomato seed. B) (lower panel) Effect of luciferin supply to dissected embryo's of hemizygous 35S::luciferase tomato seeds. Tomato seeds were imbibed in 0.1 mM luciferin, dissected and placed either on water (-luciferin) or on 0.1 mM luciferin (+ luciferin). (lower panel) Corresponding bright field image of embryo's of the dissected tomato seed. C) Effect of luciferin and wound response on luciferase activity in lateral endosperm (end) and endosperm cap (ec) from dissected homozygous tomato seeds (lower panel) and intact tomato seed (upper panel). Tomato seeds were imbibed in 0.1 mM luciferin, dissected and placed either on water (-luciferin) or on 0.1 mM luciferin (+luciferin). See also Colour Pages.

the embryo was not detected (or was masked by activity from the endosperm). Luciferase activity in the endosperm was affected by dissection of seeds but this increase in activity was independent of luciferin (Figure 1C) and more likely a wound induced activity increase (van Leeuwen, 2001). Independent of copy number of the transgene, either present homozygously or hemizygously, all seeds displayed the highest luciferase activity in the endosperm cap with a similar increase during germination (see below).

The average luciferase activity of single seeds as shown in this paper is largely accounted for by activity that resides in the endosperm caps of single seeds. Given the regulating role of the endosperm cap in germination of tomato seeds and the strict relation between luciferase activity and germination (see below), the 35S::luciferase reporter gene is an excellent marker for germination.

Luciferase activity was not detected directly upon imbibition of transgenic 35S::luciferase seeds in 0.1 mM luciferin. We designated the period without activity as 'delay time'. The onset of luciferase activity marked the end of the delay time. Luciferase activity onset was determined for single seeds in time-lapse images by calculating pixel intensity S/N ratios. Seeds with pixel intensities higher than 1.5 times the background noise, in three subsequent time-lapse images, were considered luciferase activity-positive. With the progress of germination of a single seed, luciferase activity showed a sigmoid curve (R^2 = 0.99) during germination and reached a maximum at the point at which the radicle tip penetrated the endosperm cap and protruded (Figure 2). The shape of the luciferase activity curve during germination was similar among all germinating seeds. Nevertheless, the intensity of the luciferase activity originating from single seeds varied considerably (see below).

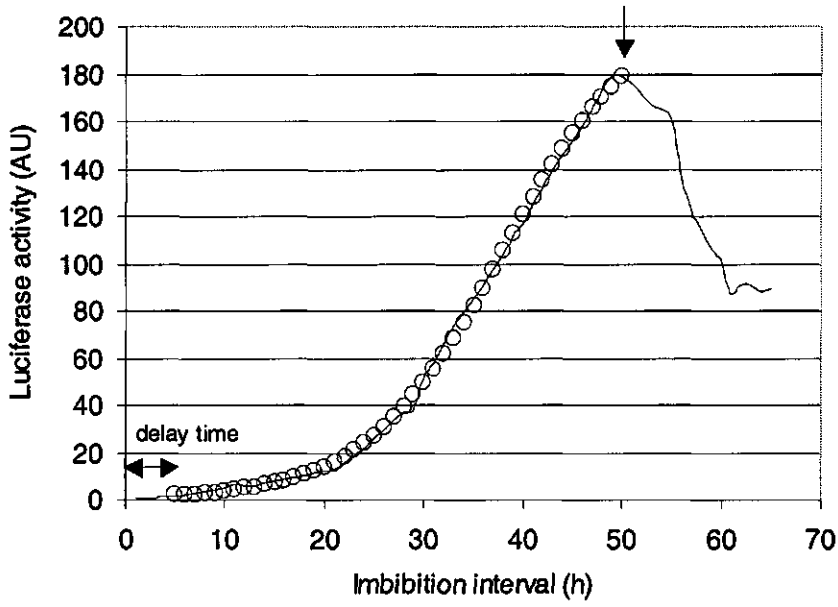


Figure 2: Typical luciferase activity curve during germination of a single tomato seed. Indicated is the delay time (= time between start of imbibition and start of luciferase activity) with a length of 6 h and the point at which the tomato seed completed germination (the arrow at maximum luciferase activity).

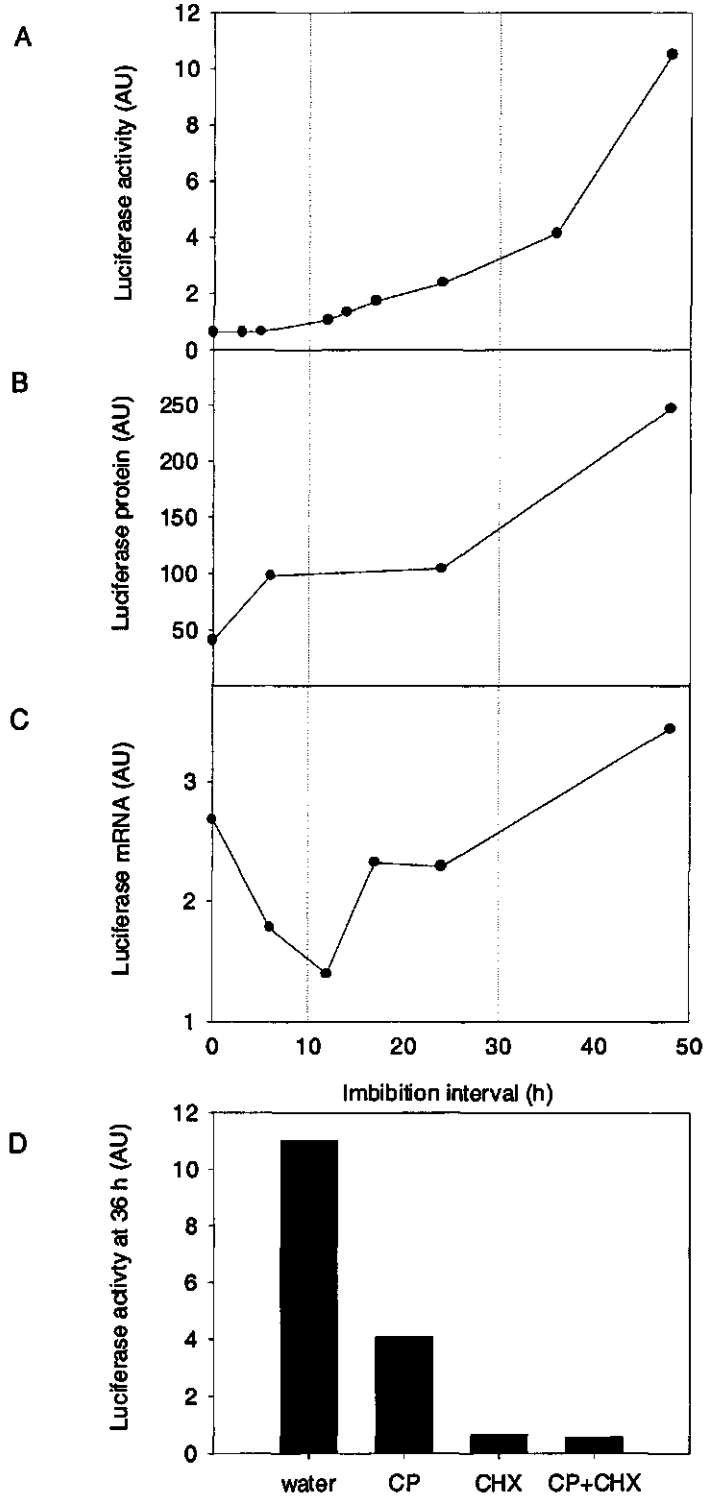
Luciferase activity depends on transcription and translation

The changes in *in vivo* luciferase activity correlated well with luciferase mRNA accumulation, determined via RNA extraction and RT-PCR with luciferase specific primers and with the increase in extractable luciferase protein levels. Luciferase mRNA and protein were extracted from populations of 10-25 seeds after 0, 6, 12, 17, 24 and 48 h of imbibition. Luciferase protein could be extracted in small amounts from dry seeds. At 6 h of imbibition the extractable luciferase protein levels had increased slightly. Both extracted and *in situ* luciferase activity increased during the remaining time of imbibition. (Figure 3B). mRNA accumulation also increased during imbibition; however the levels decreased during the first 10-12 h. (Figure 3C) of imbibition. Possibly, luciferase mRNA was conserved during late maturation and

desiccation. Apparently luciferase mRNA is part of a class of residual RNA's which are preserved during desiccation but are degraded during early imbibition (Bewley and Black, 1994). With *de novo* synthesis of mRNA's during imbibition luciferase mRNA re-appeared in the seed. Luciferase activity could be strongly reduced by inhibiting transcription with 500 $\mu g.mL^{-1}$ cordycepin and translation with 400 $\mu g.mL^{-1}$ cycloheximide (Figure 3D) during imbibition. It can be concluded that luciferase activity depended both on transcription of the luciferase gene and translation of luciferase mRNA.

The start of *in vivo* luciferase expression, mRNA accumulation and increase in extractable luciferase protein, corresponded approximately with the time point at which tubulin protein accumulation was first detected during germination of tomato seeds (12h of imbibition; de Castro, 1998). At this time point seeds have reached their full water potential and metabolic activity (Dahal *et al.*, 1996; Haigh and Barlow, 1987). In this respect this period might also reflect the uptake of luciferin by the endosperm cap. This period in which catabolic processes start in the imbibing seeds varied in length between seed batches and single seeds (see next section).

Figure 3: Comparison between *in vivo* luciferase activity (A), extractable luciferase protein (B) and luciferase mRNA levels (C) from populations of tomato seeds during germination.
D) Effect of inhibition of transcription with 500 $\mu g.mL^{-1}$ cordycepin (CP) and translation with 400 $\mu g.mL^{-1}$ cycloheximide (CHX) on luciferase activity at 36 h of imbibition.


A
Luciferase activity (AU)
12
10
8
6
4
2
0
B
Luciferase protein (AU)
250
200
150
100
50
C
Luciferase mRNA (AU)
3
2
1
0
10
20
30
40
50
Imbibition interval (h)
D
Luciferase activty at 36 h (AU)
12
10
8
6
4
2
0
water
CP
CHX
CP+CHX

Variation in the length of the delay time

All single seeds within a seed batch showed a delay in the onset of luciferase activity. This might indicate that full start of metabolic activity varied among germinating seeds. A logical assumption would be, that seeds with an early start of metabolic activity (i.e. a short delay time), might have progressed faster through germination, compared to seeds with a later start of gene expression. In order to test this hypothesis the delay time of seed batches and single seeds were compared with the time to radicle protrusion.

Seeds from greenhouse grown plants harvested in 1998 were used to determine the variation in length of this delay time. The delay time of 244 single seeds was determined. The average delay time in this seed batch was 8.5 h. The delay time of single seeds within a seed batch showed a log-normal distribution (Figure 4). This frequency distribution diagram was used to separate the population into 3 sub-populations; seeds with a delay time shorter than (μ-σ) (average minus standard deviation), seeds with a delay time between (μ-σ) and (μ+σ), and seeds with a delay time longer than (μ+σ). Seeds from these 3 sub populations did not differ significantly in average time to germination (Tg, Figure 4). Imbibition of seeds in 10 μM ABA or GA_{4+7} to respectively inhibit or promote germination did not have an effect on the average delay time (data not shown).

Distribution of luciferase activity among single seeds of a population imbibed in water, ABA or GA

Single seeds varied considerably in their *in vivo* luciferase activity during germination. The variation of *in vivo* luciferase activity between single seeds within

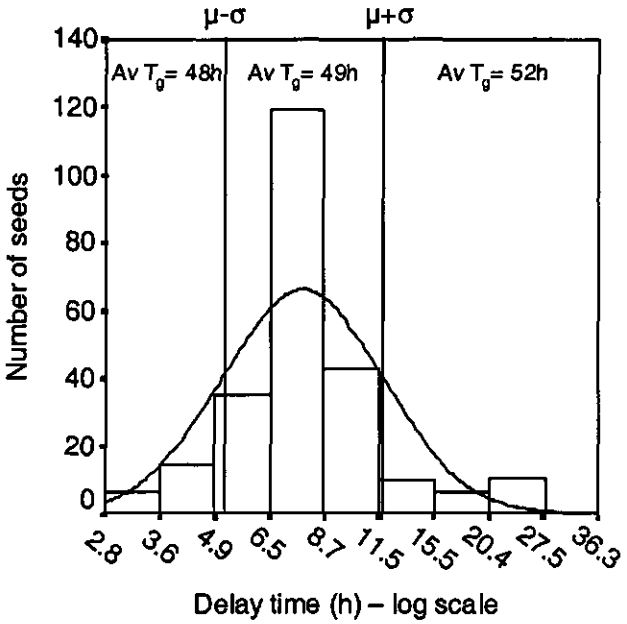


Figure 4: Frequency distribution of delay time of 244 single tomato seeds plotted on a log time scale. The total population is divided into 3 sub-populations: tomato seeds with an delay time below (μ-σ), between (μ-σ) and (μ+σ) and above (μ+σ). The average time to germination (Av Tg) is indicated for these 3 sub-populations.

the same seed batch was 3 to 150 fold at different time points of imbibition. Nevertheless this large variation did not results in aberrant averages of luciferase activity of populations of seeds due to a few seeds with extreme high luciferase activity (thus skewing the average), as was hypothesised by Still and Bradford (1997). This was also illustrated by the fact that luciferase activity in a population of seeds showed a log normal distribution (Figure 5), thus seeds with extreme high luciferase activity are present within the distribution at low frequencies. Still and Bradford (1997) detected an even larger variation between single seeds with respect to endo-ß-mannanase activities (100 to 10.000 fold). Seeds showing high *in vivo* luciferase activity might represent that part of the seed population which has furthest progressed in germination (e.g. luciferase expression increases with progress of germination) as compared to seeds with low activity. In other words, luciferase activity of a single

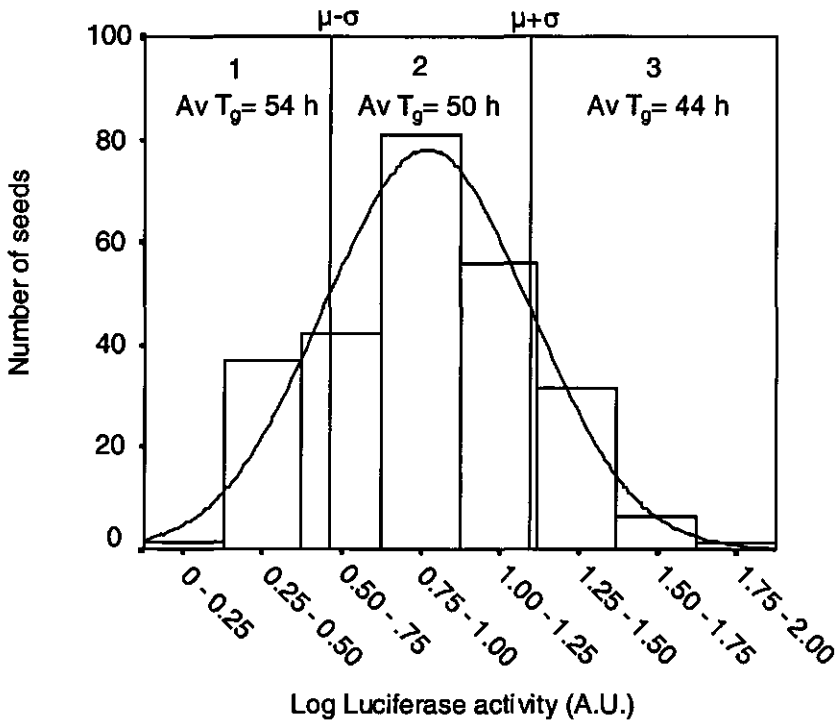


Figure 5: Log-frequency distribution of luciferase activity of 255 tomato seeds at 24 h of imbibition. The total population is divided into 3 sub-populations: Tomato seeds with an luciferase activity below (μ-σ), between (μ-σ) and (μ+σ) and above (μ+σ). The average time to germination (Av Tg) is indicated for these 3 sub-populations

seed might be a reflection of the speed of germination of that single seed. If so, plotting luciferase activity of single seeds on a biotime scale (Bradford and Trewavas, 1994) is expected to even out the observed variation. Variation in luciferase activity might also be an intrinsic variation between single seeds in their gene expression. Plotting luciferase activity of single seeds during germination on both a biotime scale (normalized time scale for each single seed in which time point of germination equals 1 and start of imbibition equals zero) and a relative luciferase scale showed that, besides timing differences, single seeds also had an intrinsic variation in luciferase activity independent of germination (Figure 6B). The lack of a simple correlation

between luciferase activity of a single seed and time point of germination (Tg) is consistent with data of Still and Bradford (1997) who did not find a correlation between endo-ß-mannanase in single seed endosperm caps and germination. However, the authors applied a destructive method in their study. It is interesting to note that preliminary results have shown that no relation existed between luciferase activity in endosperm caps and endo-ß-mannanase activity within that same endosperm cap (Spoelstra, unpublished results). Seeds with high luciferase activity

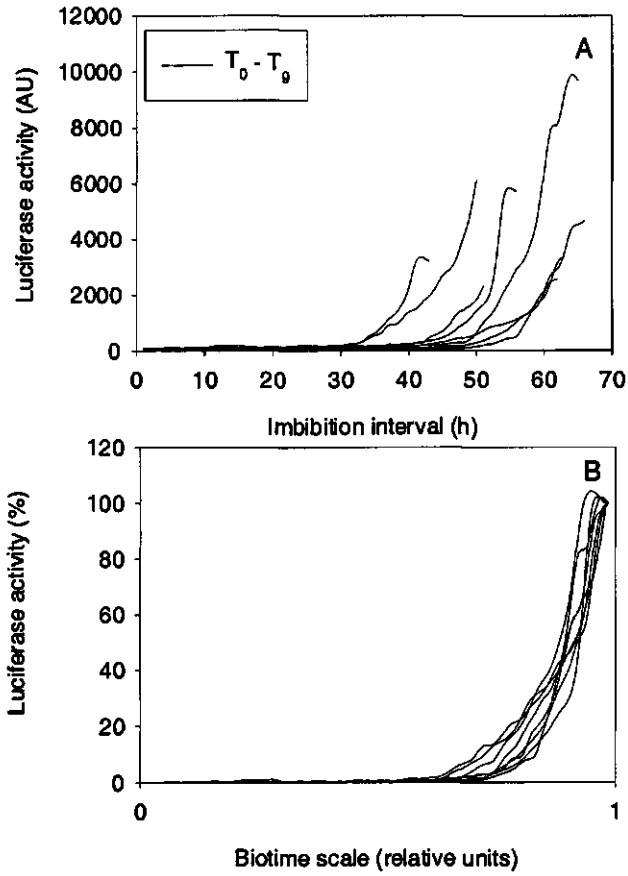


Figure 6: Luciferase activity of single tomato seeds from start of imbibition to time point of germination (T_0- T_g) plotted on a luciferase activity scale (A) and on both a normalized luciferase activity scale and biotime scale (B) (normalized time scale for each single tomato seed in which time point of germination equals 1 and start of imbibition equals zero).

did not necessarily have high endo-ß-mannanase activity and *vice versa*.

Possibly due to the combination of timing and intrinsic variation in luciferase activity, only a correlation between luciferase activity and speed of germination existed when considering population extremes (i.e. extremely low or high luciferase activity). A frequency diagram of luciferase activity at 24 h (at least 10 h before the first seeds had completed germination), was plotted (Figure 5). The frequency distribution diagram was used to divide the whole tested population of seeds into 3 sub-populations (see previous section). The average time to germination of the outer extreme populations differed 10 h, which was tested significantly different with a *student's-t-test* ($p < 0.05$; confidential interval 5%). The average Tg of the population of seeds with luciferase activity between $(\mu-\sigma)$ and $(\mu+\sigma)$, did not differ significantly from the other 2 sub-populations (Figure 6). These results were comparable to the sorting of single Brassica seeds into 3 sub-populations based on their individual chlorophyll fluorescence. Seeds with low and intermediate chlorophyll fluorescence did not differ in germination rate, but seeds with extremly high fluorescence showed an average germination rate which was significantly lower (Jalink *et al.*, 1998).

Germination of tomato seeds can be stimulated by GA_{4+7} and inhibited by ABA. We tested the effect of 10 µM ABA or GA_{4+7} in the imbibition solution, on the luciferase activity during germination. 10 µM ABA fully inhibited germination and also lowered luciferase activity and mRNA accumulation (Figure 7). The stimulating effect of 10 µM GA_{4+7} on T_{50} of a population of 150 seeds (Time to 50% germination, 45 h vs. 52 h in water) coincided with an increased luciferase activity and luciferase mRNA accumulation (Figure 7). The fact that the average luciferase activity increased or decreased through exogenously added GA or ABA does not provide information on how single seeds respond to GA or ABA. According to Bradford and Trewavas

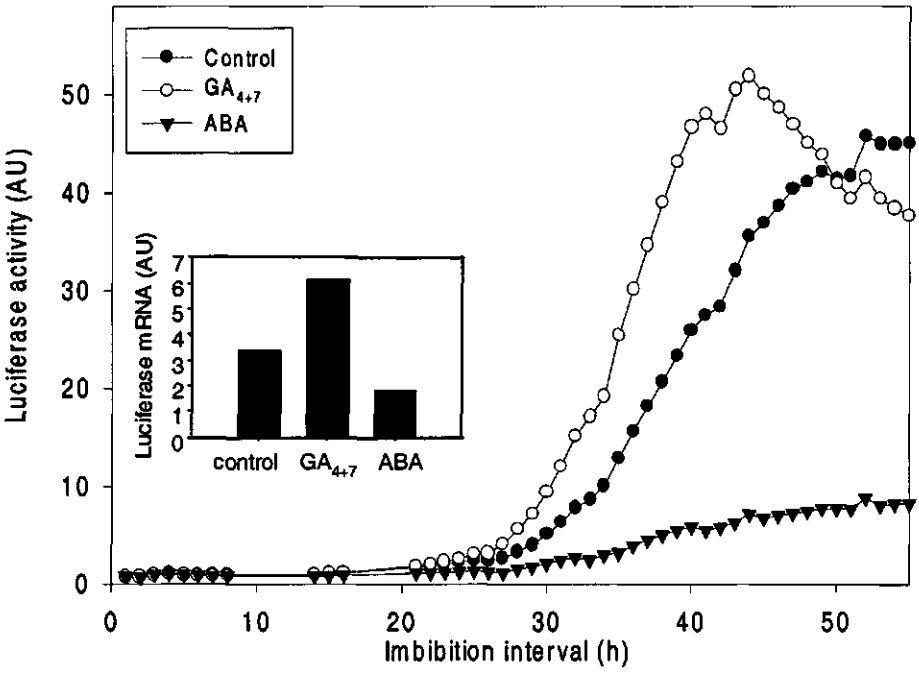


Figure 7: The effect of 10 μM GA_{4+7} or ABA on the luciferase activity of a population of 150 tomato seeds during imbibition. GA_{4+7} stimulated both germination and the luciferase activity. ABA fully inhibited germination and lowered the luciferase activity. Insert shows corresponding mRNA accumulation at 48 h determined through RT- PCR with luciferase specific primers.

(1994), single seeds have different individual threshold levels (i.e. sensitivities) to hormones such as GA. With increasing concentrations of GA more seeds will respond. Once stimulated above the threshold level a single seed might respond linearly to GA over a limited concentration range. Thus increase in response of single seeds to GA and recruitment of additional seeds compose the dose response curve (Bradford and Trewavas, 1994). We have tested how single seeds respond to an increasing concentration of GA. A population of 25 seeds showed a dose response dependent increase in luciferase activity with increasing GA concentrations (Figure 8A). A frequency diagram was plotted for 150 single seeds which were imbibed in water or 10 μM GA_{4+7} (Figure 9). In the presence of 10 μM GA_{4+7} the distribution

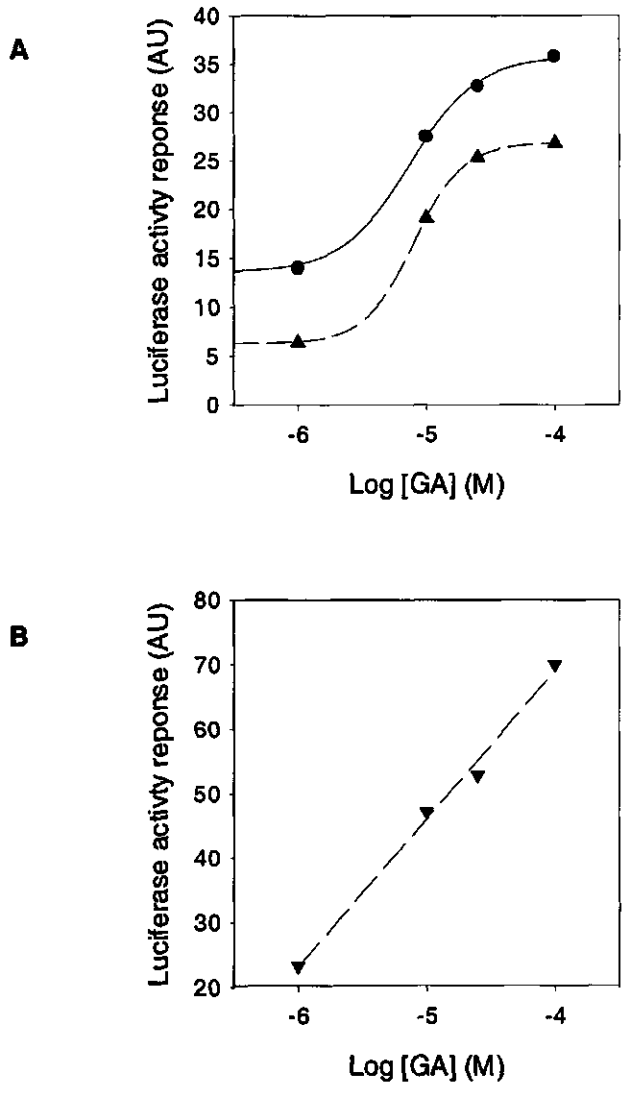


Figure 8: A) (●) Dose response curve of luciferase activity of tomato seeds at various GA_{4+7} concentrations (1, 10, 25 & 100 μM). (▲) Dose response curve of luciferase activity of tomato seeds which had a below average luciferase activity. B) (▼) Linear dose response curve of luciferase activity of tomato seeds which had an above average luciferase activity.

became skewed towards higher luciferase activities. At a concentration of 10 μM GA_{4+7} the response in luciferase activity was not maximal (Figure 8A). This indicates that not all seeds responded to this concentration of GA (i.e. the threshold in sensitivity was not exceeded for all seeds) and does explain the skewed frequency

distribution. Seeds with a threshold sensitivity below 10 μM GA_{4+7}, in theory, have an increased luciferase activity in contrast with the remaining part of the population which threshold sensitivities lie above 10 μM. We therefore hypothesise that seeds which have a lower sensitivity threshold should have higher luciferase activities in presence of 10 μM GA_{4+7} in contrast with seeds with thresholds in excess of 10 μM GA_{4+7}. Seeds with a below population average showed a sigmoid response to increasing GA_{4+7} concentration (Figure 8A). Seeds with an above average luciferase activity responded in a linear fashion to increasing GA_{4+7} concentrations (Figure 8B). These observations were consistent with the theory of Bradford and Trewavas (1994). The dose response curve of luciferase activity is composed of both the linear increase in activity of responsive seeds and the growing number of seeds which become responsive.

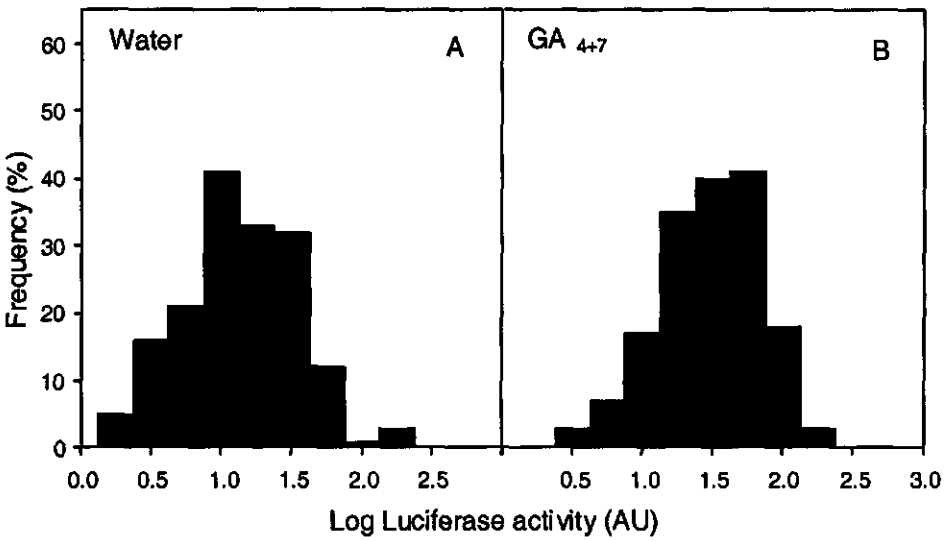


Figure 9: *Frequency distribution diagrams of luciferase activity of 150 tomato seeds at 36 h of imbibition in water (A) and 10 μM GA_{4+7} (B).*

Luciferase activity is absent in seeds with primary and secondary dormancy.

Tomato seeds, which are freshly harvested often exhibit dormancy, which is called primary dormancy (Crocker, 1916; Karssen, 1982). This type of dormancy can be overcome by cold-stratification in the imbibed state, but also by after-ripening over a period of months in the dry state. Dormancy can be reinduced in non-dormant tomato seeds, by applying irradiation with far-red light. This reinducible dormancy is called secondary dormancy, which can be broken by cold-stratification (de Castro *et al.*, 2001). A freshly harvested seed batch can be a mixture of primarily dormant seeds and non-dormant seeds. Luciferase activity was monitored in such seed batches. Primarily dormant seed could be distinguished from germinating seeds, by absence of luciferase activity at 48 h of imbibition. Seeds that showed activity all germinated. Also secondarily dormant seeds did not reveal any luciferase activity. Absence of luciferase activity in dormant seeds corresponded to very low levels of extractable

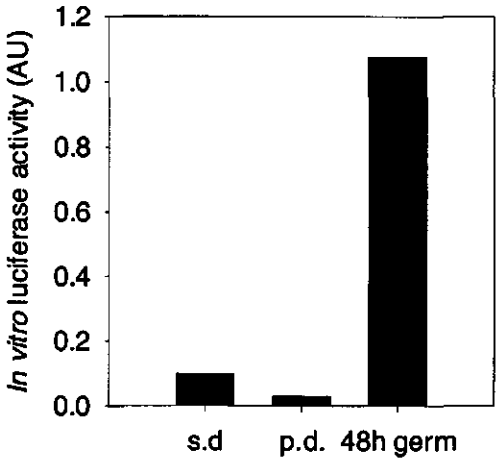


Figure 10: Comparison between extractable luciferase protein levels in secondarily dormant (s.d.), primarily dormant (p.d.) and germinating tomato seeds (48 h germ). Dormant tomato seeds were extracted 48 h after start of imbibition.

luciferase protein, compared to germinating seeds at 48 h of imbibition (Figure 10).

The non-destructive detection of luciferase activity has enabled us to distinguish between dormant and germinating seeds in a seed batch prior to visible radicle protrusion of the germinating seeds. To our knowledge, this has not been reported in literature before. Only with NMR imaging of water distribution differences between non-germinating and germinating seeds were detected non-destructively (Hou *et al.*, 1997). Dormant and germinating seeds, selected through Detection of luciferase activity revealed physiological differences in further investigations (see Chapter 3 of this thesis).

Concluding remarks

We have demonstrated the use of a non-destructive technique to monitor the germination of single tomato seeds. The 35S::luciferase was primarily expressed in the endosperm cap. Variation of luciferase activity among single seeds was large and only showed a weak relationship with germination performance of a single seed. It may be expected that the study of a single parameter in relation to germination performance will not reveal such a possible relationship. Many other parameters are involved and intrinsic variation combined with variation due to timing differences will obscure the (possible) relationship. Also the length of the delay time of a single seed (i.e. start of luciferase activity) did not relate to germination performance of a single seed. This indicates that the time point of full commencement of metabolism in single seeds is independent of the time point of germination.

In terms of usage of luciferase as a reporter gene coupled to seed or plant specific promoters, limited uptake of luciferin by the embryo is likely to interfere with correct interpretation of the results. It is therefore concluded that luciferase as a

reporter gene has only a limited application in the study of gene expression in seeds due to limitation of luciferin uptake by the embryo.

We have demonstrated that single tomato seeds most likely display individual responses to stimuli, such as hormones. These individual responses consist of a unique threshold and linear dose response action on addition of GA.

The monitoring of luciferase activity in partly dormant seed batches will be useful in studies in which dormant and germinating seeds need to be separated prior to radicle protrusion. In chapter 3 of this thesis, this method is employed in the study of distribution of ATP in dormant and germinating seeds.

3

Dormant and germinating tomato seeds (*Solanum lycopersicum* L.) show a different distribution of ATP in the embryo

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The distribution of ATP in tomato seeds was visualised using firefly luciferase and an intensified CCD camera. The distribution of ATP was imaged in germinating tomato seeds at intervals of 3, 6, 17, 24 or 48 h and in seeds in state of primary or secondary dormancy. Germinating seeds showed a distribution of ATP with highest ATP concentration located in the radicle. In contrast to germinating seeds, ATP was distributed more evenly in dormant seeds. It was shown that total ATP concentrations in seeds may not be related to the occurrence of germination. Distribution within the seed can be related to the occurrence of germination.

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Introduction

ATP is the main energy source for biological processes, including seed germination. In the quiescent dry seed, the adenosine phosphate pool is mainly composed of AMP and ADP (Bewley and Black, 1994). ATP is synthesised rapidly upon water uptake and resumption of metabolic activity in the seed. ATP is essential for germination and is used in anabolic processes such as RNA and protein synthesis (Coolbear *et al.*, 1990; de Castro *et al.*, 1995).

Dormancy is often observed in seeds of wild species but despite extensive breeding, dormancy can also occur in cultivated species, such as tomato (Benjamin, 1990; Still *et al.*, 1997). Obviously, mechanisms which control seed dormancy are still present in cultivated species. The complexity of factors that impose or underlie seed dormancy has led to the fact that there is no unambiguous definition or classification of seed dormancy. Many authors have adopted the classification into primary and secondary dormancy (Karssen, 1982). Seeds may acquire primary dormancy towards the end of development and start of desiccation on the mother plant. Seeds that disperse from the mother plant can either be non-dormant or primarily dormant. Freshly harvested seed batches of tomato may contain seeds in a state of primary dormancy (de Castro, 1998; Still and Bradford, 1997). Such a batch is therefore a mixture of dormant and non-dormant seeds. This primary dormancy disappears within months of after ripening in the dry state, or can be relieved by cold stratification of seeds in the imbibed state. If non-dormant imbibed seeds receive external signals, which inhibit germination, these seeds may acquire secondary dormancy. Cold stratification is also efficient for relieving seeds from secondary dormancy. Secondary dormancy can be induced in tomato by far red light irradiation (de Castro *et al.*, 2001).

Dormant seeds show active gene expression, protein synthesis and ATP accumulation (Goldmark *et al.*, 1992; Li and Foley, 1995). This clearly indicates that dormancy is an actively maintained physiological state in seeds. Dormancy may be considered as a mechanism to survive prolonged periods of unfavourable conditions. Evidently energy expenditure in the dormant state is expected to be low. The extent to which dormant seeds synthesise ATP, has been subject of several studies. Changes in dormancy do not necessarily coincide with changes in respiratory activity or ATP synthesis (Derkx *et al.*, 1994a).

The firefly luciferase-luciferin (*Photinus pyralis*) system has proven to be a helpful tool in plant and animal science (Aflalo, 1999). Luciferase catalyses the decarboxylation of luciferin, it thereby consumes ATP and oxygen and generates a photon (562 nm). The number of emitted photons is a direct measurement of the number of ATP molecules that were converted to AMP and PP_i. The luciferase-luciferin system has been used in plant and animal research in three ways: 1) It has been used to measure ATP in extracts of plant tissues. 2) It has been used as a reporter gene for studying expression of native genes in plants and animals (van der Krol and Chua, 1991; van Leeuwen *et al.*, 2000). 3) Walenta and co-workers (1990) devised a method based on the luciferase-luciferin system to spatially resolve ATP distributions in cryosections of tumour spheroids. In the present research we utilised the luciferase-luciferin system in two ways. First we have non-destructively monitored the expression of the CaMV 35S promoter-luciferase reporter gene to distinguish between dormant and non-dormant seeds of tomato (Chapter 2) and, subsequently, spatially resolved ATP distributions in cryosections of these seeds.

Material and methods

Seed material

Wild-type, cv. Moneymaker and transgenic 35S::luciferase seeds (Chapter 2) were used for ATP imaging. Wild-type tomatoes were harvested in 1991 and transgenic tomatoes in 1999. Seeds were extracted from ripe tomatoes and stirred in 1% (v/v) HCl for 2 h to remove locular tissue, dried and stored at 5°C. For germination experiments 25 seeds were imbibed in 1 mL distilled water in 50 mm Petri-dishes on 1 layer of filter paper (Schleicher & Schuell no. 595). Seeds were imbibed in water for 3, 6, 17, 24 or 48 h. During imbibition seeds were kept in the dark at a temperature of 25°C.

Induction and relief of secondary dormancy

Secondary dormancy was induced in wild-type seeds by far-red ($\lambda > 730$ nm) irradiation for 5 min at hourly intervals during the first 24 h of imbibition at a temperature of 21°C and subsequent incubation in the dark at 25°C for 5 days (De Castro, 2001). During this period less than 3% of the seeds germinated. In order to break secondary dormancy, seeds were given a cold treatment of 3 days at 4°C, after which the seeds were transferred to 25°C in the dark and left to germinate for 24 h.

Measurement of luciferase expression in seeds

25 Transgenic seeds containing the CaMV 35S-luciferase construct were imbibed in 2.5 mL of a 0.1 mM luciferin solution (Molecular Probes) in 5 cm Ø plastic containers on 3 layers of filter paper. Photons emitted by seeds were spatially

resolved with a liquid nitrogen cooled CCD camera (Roper Scientific, Princeton Instruments -Versarray-512B) operated via Metamorph 4.1 (Universal Imaging Inc.) software. At 48 h of imbibition seeds with primary dormancy were selected by absence of luciferase activity.

Imaging of ATP distribution in cryosections of tomato seeds

Visualisation of ATP is based upon the reaction of firefly luciferase and luciferin with ATP, O_2 and Mg^{2+}. The reaction generates oxyluciferin and photons with a wavelength of 562 nm at a pH of 7-8. The number of emitted photons is directly related to the number of ATP molecules converted into AMP and PPi (stoichiometry of 1:1).

Imbibing seeds were frozen in liquid nitrogen and 20 µm median sections from 10 seeds per imbibition interval were cut on a cryostat (Microm CR50 H, bio-med, Heidelberg, Germany) at -20 °C with a steel knife. Sections were collected on glass slides with a frozen film of 50 µL of buffered gel containing luciferase and luciferin. This gel (see also Walenta *et al.*, 1990) consisted of 6% gelatin (w/v) and 300 mM glycerol, 200 mM Hepes (pH 7.75), 100 mM disodium hydrogen arsenate, 3% polyvinylpyrolidone (M_r 44000; w/v), 10 mM $MgCl_2$, 1 mM luciferin and 2 U/L luciferase (Boehringer, Mannheim, Germany) . The glass slides with the cryosections were kept frozen until measurement. At the beginning of the measurement cryosections were thawed within a few seconds. ATP molecules from the thawed cryosection reacted with luciferin and luciferase in the gel. The patterns of emitted photons were captured with an intensified 16 bit CCD camera, with a 50 mm lens (Nikon) and 70 mm c-mount extension tubes (Argus-50/2D-luminometer, Hamamatshu), in slice mode at a gain of 9.8. Although the reaction continued for a

period of about 20 min, emitted photons were only integrated during the first 3 min. Prolonged measurements lead to a distorted image of ATP distribution due to diffusion of ATP through the gel.

Calibration and data analysis

The pixel intensities in 16 bit monochrome images of the bioluminescence reaction were measured with the use of Metamorph 4.0 (Univeral Imaging Inc.). The intensity of the bioluminescence reaction was calibrated to ATP concentrations, using frozen filter paper discs (Ø 2.5 mm) with 1µL of ATP solutions ranging from 25 to 450 mM. The discs were placed on top of the reaction gel (Figure 1). Photon emission was integrated during the first 3 min and the bioluminescence intensity was quantified in the radicle and whole sections.

Statistical analysis of the data was performed via a paired sample *student's t-test* (comparison of ATP distribution differences within seeds) or independent sample *student's t-test* (comparison of ATP distribution differences between seeds of different imbibition intervals) at a confidence level of 95% with the SPSS 7.5.2 program.

Extraction of ATP

The extraction of ATP was performed with a trichloric acetic acid extraction procedure modified from Saglio and Pradet (1980). Triplicates of 10 wild-type seeds were frozen into liquid nitrogen. The seeds were ground with 2 iron bullets (Ø 4 mm) in a 2.2 mL Eppendorf tube on a Braun Biotech Int. Mikro-Dismembrator U, at 1600 rpm for 3 minutes. 100 µL of a 0.6 M trichloric acetic acid solution in diethyl-ether stored at -20° C was added to the ground seeds. Samples were then homogenized in 2

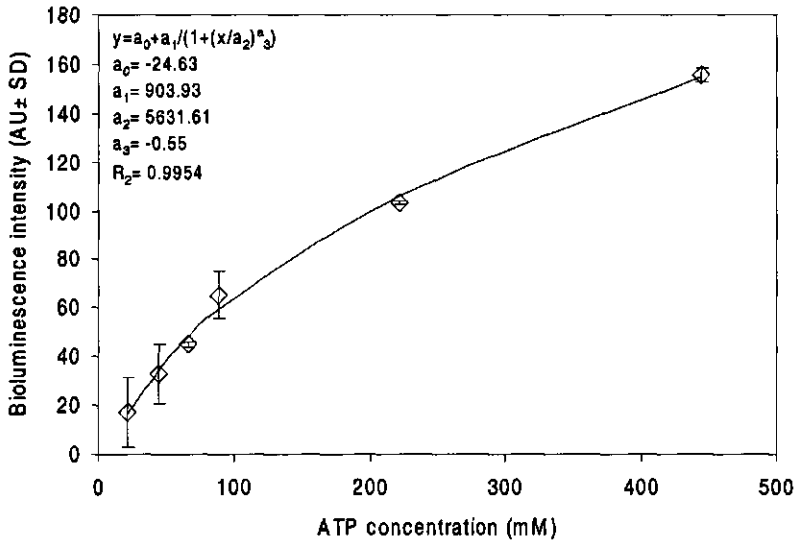


Figure 1: Calibration curve (a sigmoid regression formula, which is shown in upper left corner) of bioluminescence intensity expressed in arbitrary units (AU) plotted against the ATP concentration expressed in mM. Bars indicate standard deviations.

times 250 µL and 1 time 500 µL of 0.6 M aqueous trichloric acetic acid. Subsequently, extracts were centrifuged for 10 min at 16000 g. The supernatant was transferred to 12 mL tubes and trichloric acetic acid was removed by extraction with 3 volumes of diethyl-ether. All previous steps were performed at 0-4 °C. Remaining traces of diethyl-ether were eliminated by placing samples in a speed vacuum rotor for 10 min.

ATP levels in extracts were assayed by means of a luciferin-luciferase assay (Boehringer, Mannheim, Germany) ATP-detection-kit CLSII using a Labsystems Luminoskan DS luminometer for 96 multiwell plates. ATP extracts were diluted 3 times and 10 µL of the diluted extract was added to 100 µL of 0.2 mM tricine buffer (pH 7.6). The CLSII assay mix was injected at a volume of 50 µL. 10 Sec. after injection, photon emission was measured for 10 sec.

Results

ATP visualisation and ATP extraction yield similar results when studying total ATP levels during germination

Visualising ATP can yield data about total amounts of ATP present in a tissue, but also reveal tissue specific distribution of ATP. At each imbibition interval shown, 3-4 sections per seed were cut from a total of 10 seeds. ATP levels in single seeds were calculated by averaging the ATP concentrations as measured in those 3-4 sections. ATP concentrations at different imbibition intervals were calculated by averaging ATP concentrations found in single seeds. The average ATP concentrations found in these single seeds varied over a 2.5 to 5 fold range between seeds of the same genotype and imbibition interval (data not shown). In order to establish if determining ATP concentrations in our system (seeds) is a reliable method, a trichloric acid ATP extraction was performed (Saglio and Pradet, 1980) at the same imbibition intervals with the same seed batch and ATP concentrations as assessed with both methods were compared. Both techniques yielded similar results when the pattern of ATP accumulation during imbibition was compared. ATP could already be detected at 3 h of imbibition, after which a fast increase until 17 h was observed. ATP concentrations did not increase further between 17 and 48 h of imbibition (Figure 2A, B). At 48 h of imbibition, 50% of the seeds had completed germination (i.e. showed radicle protrusion). Both germinated and non-germinated seeds were pooled in the case of ATP extraction. ATP levels in sections of germinated and not yet germinated seeds did not differ significantly ($p>0.05$).

ATP was visualised in cryosections of seeds at 3, 6, 17, 24 and 48 h of imbibition. Cutting cryosections of dry seeds or seeds at very early stages of

imbibition (i.e. prior to 3 h. of imbibition), was not successful, due to insufficient hydration of the seeds. Both the embryo and endosperm contained ATP during germination. However the ATP concentration in the embryo was higher compared to the endosperm (average of 1.5 times higher at 24 h of imbibition). Within the embryo the highest concentrations of ATP was located in the radicle at all stages of imbibition (Table 1; Figure 3A-E). For statistical testing, the ATP concentration in the radicle was compared to the average ATP concentration in the cotyledons. The ATP concentration in the radicle at the different imbibition intervals was 1.3 to 2.0 ($p<0.05$) times higher than the ATP concentration in the cotyledons (Table 1).

Table 1: ATP concentrations in radicle and cotyledons in mM, the corresponding standard errors of mean (SEM), the ratio between ATP concentration in radicle and cotyledons and the significance of differences between radicles and cotyledons during germination and for primarily or secondarily dormant seeds and for secondarily seeds which received a cold treatment for 3 days at 4°C with subsequent germination of 24 h at 25°C.

	radicle	SEM	cotyledons	SEM	ratio	**significance**
3 h germination	28.0	2.1	19.9	1.5	1.4	$p < 0.05$
6 h germination	31.6	2.3	23.9	1.1	1.3	$p < 0.05$
17 h germination	73.5	5.4	35.9	2.0	2.0	$p < 0.05$
24 h germination	52.3	4.0	35.9	2.6	1.5	$p < 0.05$
48 h germination	50.4	5.2	32.3	2.1	1.6	$p < 0.05$
primarily dormant	45.6	3.2	42.3	1.3	1.1	$p > 0.05$
secondarily dormant	63.9	5.3	60.3	4.6	1.1	$p > 0.05$
secondarily dormant + cold treatment + 24 h germination	112.7	10.3	69.3	6.3	1.6	$p < 0.05$

The ATP distribution in dormant seeds differs from that in germinating seeds

Freshly harvested seeds batches can contain percentages of seeds in state of primary dormancy . Non-dormant transgenic 35S::luciferase seeds, showed luciferase activity during germination, while dormant seeds did not show this luciferase activity (Chapter 2). This observation was used to distinguish dormant and non-dormant seeds

in freshly harvested seed batches. Seeds were imbibed for 48 h in 0.1 mM luciferin and screened for luciferase activity. Cryosections from these seeds with primary dormancy were cut and ATP distributions were imaged. The ATP was distributed more evenly in seeds with primary dormancy than in germinating wild-type seeds. The ATP concentration in the radicle was comparable to that in the cotyledons (Table 1; Figure 3H), in contrast with the situation in germinating seeds (Figure 3A-E).

Secondary dormancy in tomato seeds was induced by far red light irradiation (de Castro *et al.*, 2001). ATP was visualised in seeds in state of secondary dormancy. As in primarily dormant seeds, the secondarily dormant seeds showed an ATP distribution which was different from the germinating seeds (Table 1; Fig. 3F). The ATP concentration in the radicle was again comparable to the ATP concentration in the cotyledons, as in seeds with primary dormancy.

A cold treatment was used to break the secondary dormancy, which resulted in 100% germination within 72 hours of incubation after transferring the seeds to 25°C. ATP distributions were imaged in cryosections at 24 h after transferring these seeds from 5 to 25 °C. The earlier observed pattern of ATP distribution in germinating seeds, with highest concentration located in the radicle was restored (Table 1, Figure 3G) The ATP concentration in the radicle was 1.6 times higher compared to the average ATP concentration in the cotyledons ($p<0.05$, Table 1).

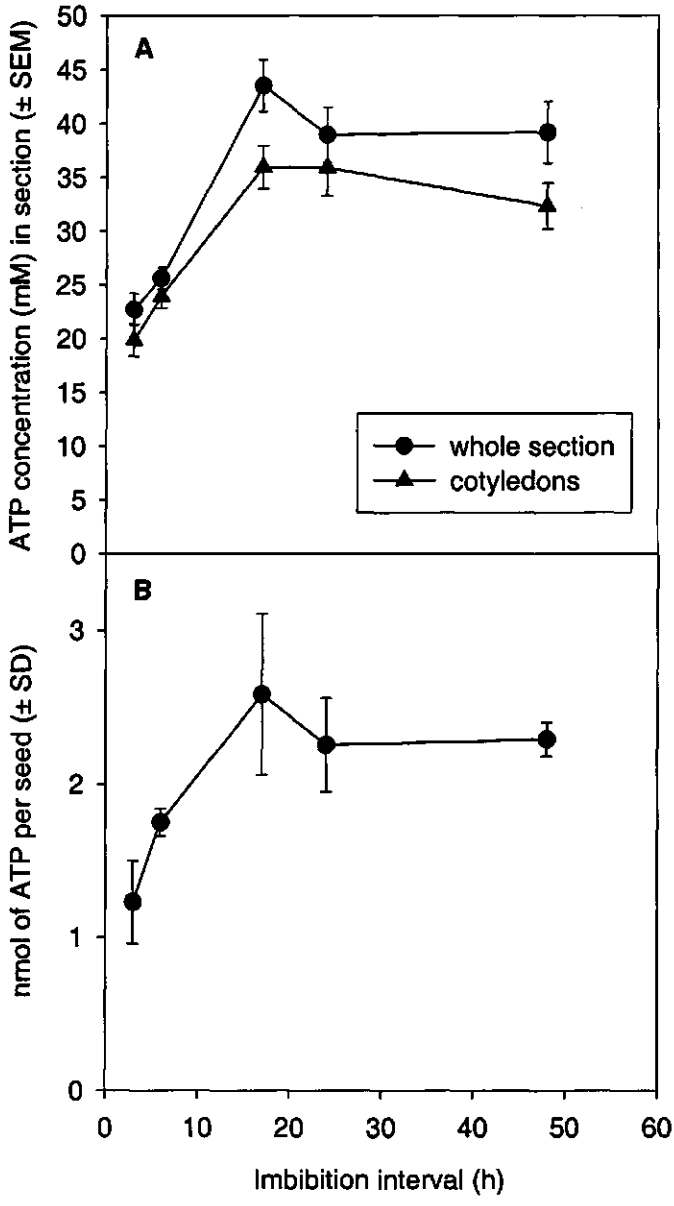


Figure 2: A) The levels of ATP during germination of wild-type seeds as assessed by ATP imaging with luciferase and luciferin. Bars indicate the standard error of mean. B) The levels of ATP during germination of wild-type seeds as assessed through ATP extraction. Bars indicate standard deviations.

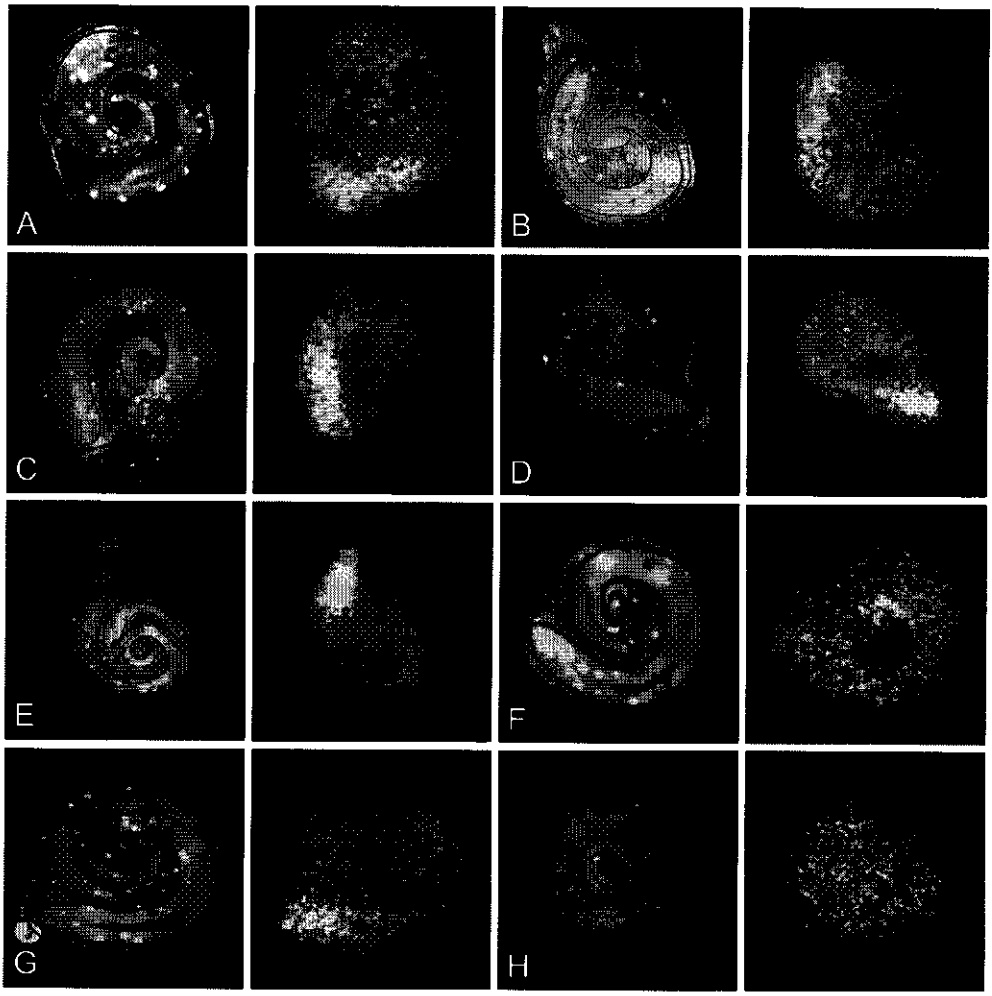


Figure 3: Images of ATP distributions in cryosections of tomato seeds. Wild-type seeds at 3 h (A), 6 h (B), 17 h (C), 24 h (D) and 48 h germinated (E). Secondarily dormant wild-type seed after 5 days of incubation (F). Wild-type seed after secondary dormancy induction and cold treatment for 3 d and 24 h at 25°C (G). Seeds in state of primary dormancy at 48h of imbibition (H). See also Colour Pages.

Discussion

ATP visualisation and ATP extraction yield comparable results when studying total ATP levels during germination

In the present study we demonstrated the usefulness of the firefly luciferase-luciferin system to image the ATP distribution in tomato seeds. This technique was modified after an earlier study of ATP imaging in tumour spheroids (Walenta *et al.*, 1990). ATP concentrations varied over a 2.5 to 5 fold range between single seeds of the same genotype and treatment. Seed to seed variation studied with the use of a single seed assay has been demonstrated before by Still and Bradford (1997). They observed a variation of 4-5 orders of magnitude in endo-ß-mannanase activity in single endosperm caps of tomato seeds. Clearly, seed to seed variation can be found for a wide range of metabolite levels and enzyme activities.

The pattern of ATP accumulation during germination of wild-type seeds as assessed through imaging was comparable to the accumulation of ATP determined through the classical method of ATP extraction. The accumulated level of ATP in seeds cannot be correlated to germination. The seeds with secondary dormancy tested, showed a higher concentration of ATP compared to the germinating wild-type. These seeds originated from different seed batches harvested 7 years apart. Comparing dormant seeds with seeds from the same batch which received a cold-treatment to break dormancy, showed higher concentrations in the latter. Thus absolute ATP levels do not necessarily correlate with germination; ATP concentrations may also vary with genotype and seed lot.

Germination of tomato seeds correlates with ATP distribution but not with the extractable ATP pool per se

Earlier studies on the accumulated levels of ATP in correlation with dormancy or germination performance have yielded conflicting results (i.e. not always a correlation was found). These studies dealt with a large number of plant species, in which different biological processes, such as dormancy, ageing and seed vigour were correlated to ATP levels (Ching, 1973; Jain *et al.*, 1983; Lunn and Madsen, 1981; Siegenthaler and Douet-Orhant ., 1994). Several causes for these contradictory results were suggested (Mazor *et al.*, 1984; Perl, 1986). The rate of ATP turnover in cells is high. Based upon respiration data of Dahal *et al.*, (1996) we can estimate that a single tomato seed at 24 h of imbibition at 25°C, produces approximately 7 nmoles of ATP per min. Combined with our data at 24 h of imbibition at 25°C, which showed an extractable pool of ATP of 2.25 nmoles per seed, this suggests that the whole ATP pool within a seed is turned over within minutes. This suggests that accumulated ATP concentrations and changes therein are negligible compared to actual levels of ATP synthesised and turned over. ATP pools do not always reflect metabolic rates in a tissue, since the ATP pool is a result of the balance between processes of synthesis and utilisation (Perl, 1986).

However, the present results show that not the ATP concentration in whole seeds *per se* but the distribution of ATP within a seed is correlated with germination. Germinating wild-type seeds showed a typical distribution of ATP with the highest levels localised in the radicle (ATP concentration in the radicle was 1.3 to 2.0 times higher compared to the cotyledons). Dormant wild-type seeds showed a more even distribution of ATP with equal levels in radicle and cotyledons. Germination processes that utilise energy in the form of ATP, such as DNA replication (de Castro

et al., 2000; Liu *et al.*, 1994), cell division, microtubuli assembly (de Castro *et al.*, 2000) and synthesis of endo-ß-mannanase (Toorop *et al.*, 1996) are known to start in the radicle during germination of wild-type seeds but are absent in dormant seeds. In this respect, higher ATP levels in the radicle might reflect higher metabolic activity in the radicle of germinating seeds opposed to absence of these processes in dormant seeds.

Conclusion

From the present study it is clear that the distribution of ATP within tomato seeds is related to germination rather than the concentration within whole seeds. Data presented here would not have been obtained through extraction of ATP from whole seeds; a higher ATP level in the radicle is not necessarily reflected in a higher extractable pool of total ATP. Clearly the study of seed physiology can benefit from techniques which give detailed information on the *in vivo* and spatial distribution of metabolites within seeds.

4

Imaging of water distribution in germinating tomato seeds

(*Solanum lycopersicum* L.)

P. Spoelstra, A. Heemskerk, H. van As and H.W.M. Hilhorst

1H-NMR Turbo Spin Echo Imaging was used to visualise water distribution in germinating tomato (*Solanum lycopersicum* L. cv. Moneymaker) seeds. Water uptake by the embryo was triphasic, whereas the endosperm only showed two phases in water uptake. Water uptake by the embryo was limited through restriction of water uptake by the endosperm. This resulted in a lower water content of the embryo compared to the endosperm. Within the embryo, the radicle had the lowest amount of water. Prior to visible germination, the radicle started to take up extra water. A swelling of the radicle was observed which did not directly result in endosperm cap rupture but did result in outward swelling of the endosperm cap. Only after radicle protrusion did the rest of the embryo take up extra water.

Introduction

As a final stage in seed maturation seeds of many plant species desiccate, thereby facilitating seed dispersal and survival. Seed germination is initiated upon reimbibition with water and ends with the protrusion of the radicle through the surrounding tissues. Water uptake during germination of seeds is triphasic (Bewley and Black, 1994). Phase I of water uptake shows a sharp increase in water content of the seed, which is due to a large water potential ($\Delta\Psi$) gradient between the seed and the environment. Phase II is a period of variable duration in which little or no change in water content of the seeds is observed. During phase III, the radicle starts to take up extra water as it protrudes through the surrounding tissues, such as endosperm or pericarp.

In tomato seeds, the embryo is surrounded by a rigid endosperm. Water uptake of the embryo is inhibited by the endosperm, possibly via restriction of embryo swelling, which results in lower water content compared to the endosperm and a $\Delta\Psi$ of –1.5 MPa or below during phase II , whereas the whole seed is in equilibrium with that of the imbibing solution (Haigh and Barlow, 1987; Liu, 1996). For the seeds to germinate the radicle has to protrude through the part of endosperm opposing it: the endosperm cap. It is well established that the endosperm cap is weakened by hydrolytic enzyme activity during germination of tomato seeds (Haigh and Barlow, 1987; Karssen *et al.*, 1989; Toorop *et al.*, 1996). Weakening of the endosperm cap facilitates protrusion of the radicle. For protrusion of the radicle extra water uptake is needed while cells in the radicle elongate. Possibly, the weakening of the endosperm cap also facilitates this uptake of extra water by the radicle (Haigh and Barlow, 1987; Liu, 1996)

^{1}H NMR imaging has been used to study water distributions and physical properties like viscosity of water, membrane water permeability and compartment sizes in plant tissues (Chudek and Hunter, 1997; Ishida *et al.*, 2000; McFall and van As, 1996). Imaging of water in seeds was demonstrated by several authors (Fountain *et al.*, 1998; Ishida *et al.*, 1995; Jenner *et al.*, 1988). NMR imaging is a non-destructive technique, which can reveal possible relations between water uptake and germination of a single seed, which cannot be obtained, by destructive techniques.

We have investigated the distribution and uptake of water by the different tissues of tomato seeds during germination with the use of 3D turbo-spin-echo (TSE) NMR imaging (Scheenen *et al.*, 2000; van der Toorn *et al.*, 2000), in order to delineate the spatial and temporal distribution of water uptake by the different seed tissues and to determine whether an extra uptake of water is required for radicle protrusion through the endosperm cap.

Results

Water content and 3D TSE imaging

Water uptake by seeds during germination has often been studied by weighing the imbibed seed and expressing the water content on a dry weight or fresh weight basis. Haigh and Barlow (1985) and Liu (1996) presented these data for tomato seeds. TSE imaging can also be used to study water uptake or water contents in plant material (Scheenen *et al.*, 2000). In order to verify the accuracy of data on water uptake as assessed by our TSE imaging experiments we compared data acquired with both methods. Fresh weights of imbibing seeds were determined prior to radicle protrusion at 5, 17, 30 and 42 h of imbibition. Proton densities of 6 slices containing all data of a single seed were measured and averaged for an integral 'water content' of a single seed during germination (i.e. distribution of water among different tissues is disregarded). Figures 1A and 1B show the results of this comparison. Both methods revealed a similar pattern of water uptake during germination, with a rapid water uptake during phase I, which lasted approximately 12 h and a stationary or slow increase in water content during phase II. These observations were consistent with the data presented by Haigh and Barlow (1985) and Liu (1996).

3D TSE imaging of water content and distribution during germination of seeds

Figure 2 shows 4 slices of a single seed during the first 12 h (phase I) of imbibition. The first image was acquired at 2 h of imbibition. Free spaces between the embryo and endosperm were observed (Figure 2A), which appear similar to the free spaces detected by x-ray imaging of tomato seeds as reported by Liu (1996). The free

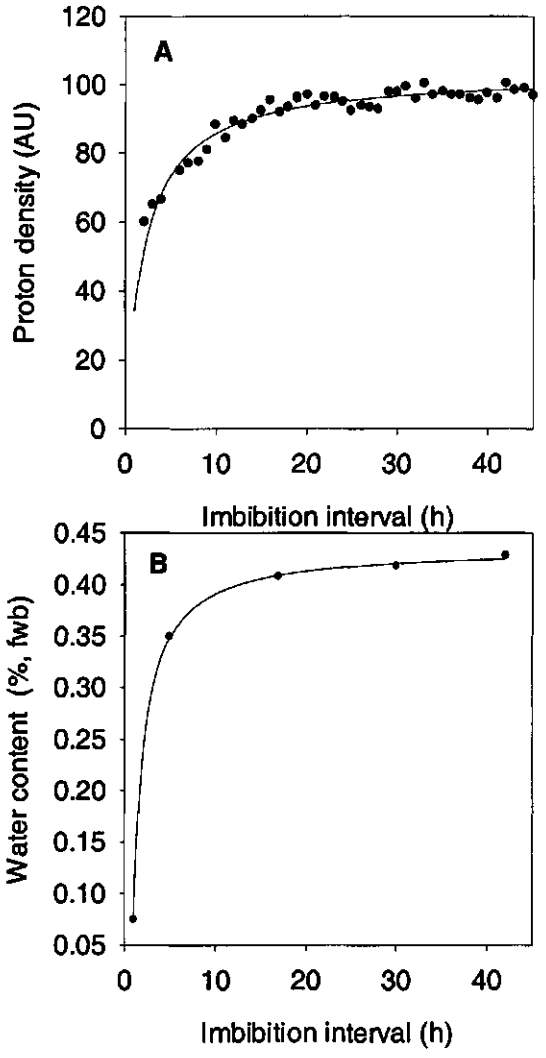


Figure 1: A) Average proton density (AU) in a total of 6 slices, of a single tomato seed during germination as assessed through 3D TSE NMR imaging. B) Average water content (% of FW) of 15 tomato seeds during germination, determined by weighing single seeds.

spaces in the seed disappeared during the course of imbibition, presumably due to swelling of the embryo. With the progress of imbibition of seeds, image contrast changed. The endosperm remained clearly distinguishable. However details of the embryo became obscured due to swelling of the embryo and disappearance of free spaces between the embryo and endosperm (Figure 2B).

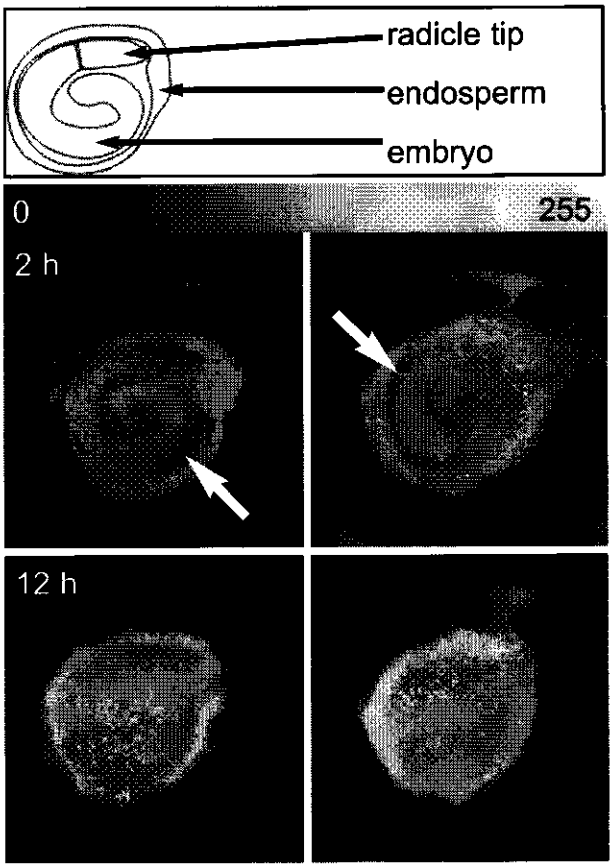


Figure 2: Proton density images of 2 subsequent slices of the same seed acquired through 3D TSE NMR imaging at 2 h and 12 h of imbibition of a single seed. The arrow indicates free space in the seed which disappeared during the first 12 h of imbibition. See also Colour Pages.

The endosperm, the embryo (minus radicle tip) and the radicle tip were separately analysed for the average greyscale values (i.e. proton density). Figure 3 shows the analysis of 2 single seeds during imbibition, of which one germinated during the course of the TSE imaging experiment (Figure 3A). An increase in moisture content was detected in all seed parts during phase I. The length of phase I in the individual seed parts was 10-15 h. Phase II was observed in all seed parts and showed only a slight increase or no increase in water content. Water was distributed in a reproducible fashion during imbibition: the endosperm displayed the highest amount of water compared to the embryo and radicle tip. The radicle tip contained the lowest amount of water (Figure 3).

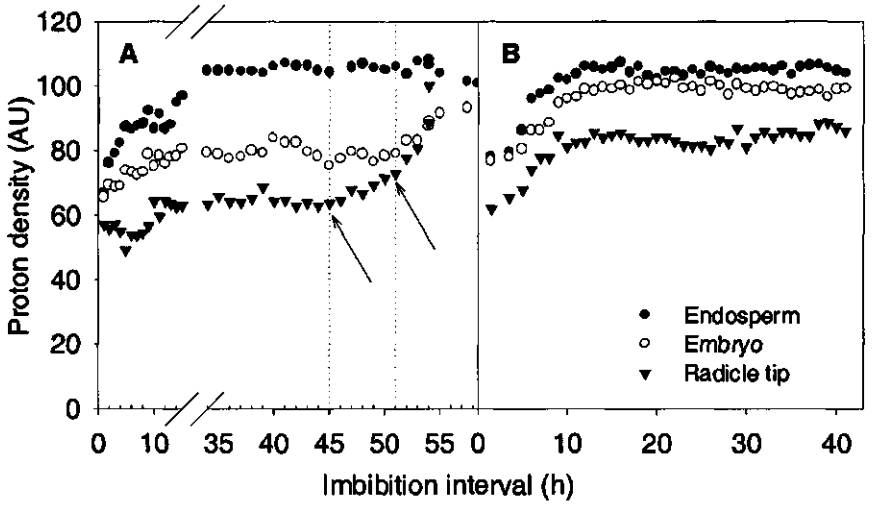


Figure 3: Average proton densities, representing water content, during germination in the endosperm, radicle tip and embryo in a single slice of 2 individual seeds acquired through 3D TSE NMR imaging: A) germinating seed; B) non-germinated seed. In panel A the first arrow (45 h) indicates start of water uptake by the radicle and the second arrow (52 h) indicates the time point of radicle

Phase III of water uptake is believed to be initiated upon germination of the seed, which is marked by protrusion of the radicle through the endosperm and testa (Bewley and Black, 1994). Prior to protrusion of the radicle through the endosperm,

an outward swelling of the endosperm cap was observed (Figure 4). The protruding radicle appeared to cause the outward swelling of the endosperm cap. During this protrusion an extra water uptake by the radicle tip was detected (Figure 3A, from 45 h onward). It has been suggested that weakening of the endosperm cap (Haigh and Barlow, 1987) facilitates the uptake of water by the radicle. This would imply that water has to diffuse through the endosperm cap into the radicle thereby generating an increased local proton density in NMR measurements. Such an increase in moisture content of the endosperm (endosperm cap) opposite the radicle was detected prior to radicle protrusion at 45h and onward (Figure 5).

After radicle protrusion through the endosperm cap (Figure 4; 52/57 h) the growing radicle tip continued to take up water. The rest of the embryo only displayed extra uptake of water after the radicle had protruded through the endosperm (i.e. after completion of germination). Clearly the radicle entered phase III of water uptake shortly before actual protrusion through the endosperm and the rest of the embryo entered phase III of water uptake after this event. The endosperm seemed not to enter phase III of water uptake; the water content was nearly unchanged prior to and after completion of germination (Figure 3A).

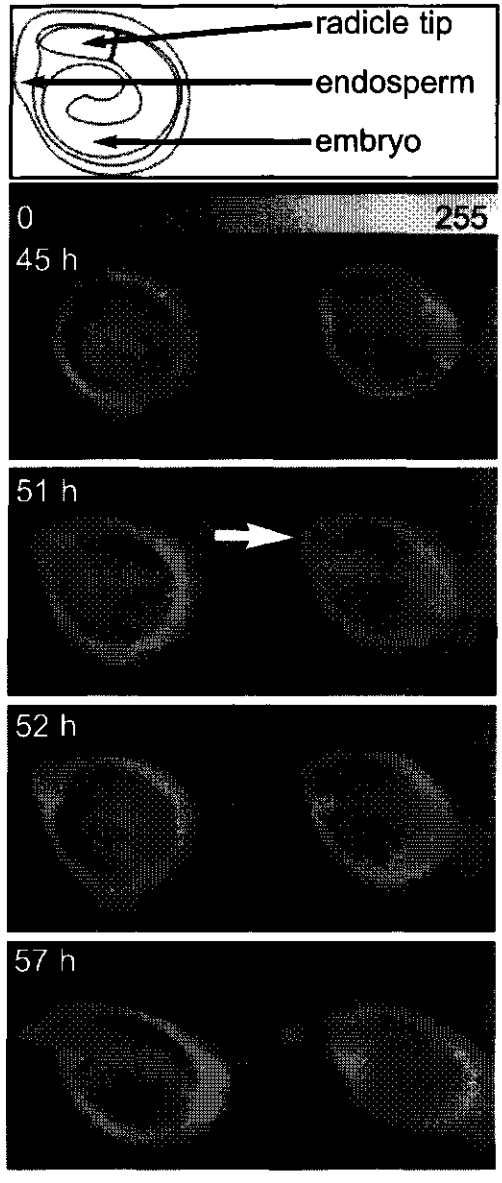


Figure 4: Water distribution in 2 slices of a single seed around the the time point of radicle protrusion. Arrow (51h) indicates outward swelling of the endosperm cap (compare to 45 h), prior to radicle protrusion, Radicle has protruded at 52h of imbibition which can be clearly seen at 57 h. See also Colour Pages.

germination event. However, de Castro *et al.* de Castro *et al.*(2000), found mitosis in the radicle prior to radicle protrusion, just like we observed phase III water uptake prior radicle protrusion. The renewed water uptake by the radicle prior to protrusion of the radicle through the endosperm cap coincided with an increase in water content of the endosperm opposite the radicle tip. This might indicate a facilitated water uptake due to weakening of the endosperm cap. Weakening might have two effects: 1) Water is able to pass through ruptures between cells in the endosperm cap; 2) The radicle is able to swell due to increased elasticity of cell walls in the endosperm cap. However, given the current knowledge of tomato seed germination it is unlikely that the endosperm is a simple physical barrier obstructing the radicle from water uptake and protrusion. The puncture force, the measure of endosperm cap weakening (Chen and Bradford, 2000; Toorop *et al.*, 2000), declines continuously during germination. If endosperm cap weakening is responsible for water uptake by the radicle, a gradual uptake of water during germination might be expected (not the sudden uptake as we observed). Thus the growth potential of the radicle or embryo must be another important factor in germination (Ni and Bradford, 1993) to explain the sudden water uptake. The strength of this growth potential was demonstrated by the outward swelling of the endosperm cap prior to actual breakage of the endosperm cap. Moreover, when tomato embryos were dissected from fully imbibed seeds and placed on water, an instant increase in fresh weight was observed but actual outgrowth of the radicle of the dissected embryo was not observed until 30 h of imbibition, only a few hours before the intact seed would show radicle protrusion (Haigh and Barlow, 1987). This indicates the existence of an internal process which controls outgrowth of the embryo during imbibition. Cell elongation is needed for the radicle to protrude. For cells to elongate, the cell wall should become extensible. Expansins have been

proposed to modify non-covalent bindings in cell walls between cellulose and hemi-cellulose micro fibrils thereby facilitating cell elongation or expansion (Cosgrove, 1998). A tomato expansin (LeEXP8) has been reported to be expressed in the radicle of germinating seeds prior to radicle protrusion (Chen and Bradford, 2000). Once the cell walls in the radicle have become extensible and the restraint in the endosperm cap has been weakened enough, the radicle will protrude through the endosperm thereby taking up water.

After radicle protrusion the rest of the embryo started to take up water as well. This might be due to the relieved constraint by the endosperm or a directed transport of water from radicle towards the cotyledons. The endosperm did not enter a phase III of water uptake. The endosperm was already fully imbibed during phase II. This seems consistent with the function of the endosperm; control of germination and storage of food reserves.

5

Expression of two linker histones variants during dormancy and germination of tomato seeds *(Solanum lycopersicum* L.*)*;

Regulation By Abscisic Acid And Gibberellins?

P. Spoelstra, R.V.L. Joosen, J. Buitink, P.E. Toorop and H.W.M. Hilhorst

The expression of two functionally different linker histones has been studied during germination and secondary dormancy in tomato *Solanum lycopersicum* L. Expression of the *his1-s* gene was linked with secondary dormancy induction and secondary dormancy. Gene expression of *his1-s* was overall low during germination of tomato seeds. *leH1* gene expression increased during germination and was absent during secondary dormancy of tomato seeds. Chilling of secondarily dormant seeds resulted in induction of germination and in the induction of *leH1* expression. ABA was unable, when applied exogenously, to stimulate *his1-s* expression. *leH1* was under control of GA in the embryo of germinating seeds. The role of ABA and GA is discussed in relation to dormancy and germination. It is hypothesised that linker histones play a regulatory role in modulating gene expression and act as a switch between the dormant and germinating state of seeds.

Introduction

Histones are highly conserved proteins that are associated with the DNA of eukaryotes and play an important role in the organisation of chromatin. The basic subunit of chromatin is the nucleosome which consists of 200 bp duplex DNA wrapped around 2 copies of each structural histone, H2A, H2B, H3 and H4. The higher order organisation of the nucleosome is facilitated by binding of the nucleosome to a fifth type of histone, the linker histone or H1. The organisation of the DNA in the histone complex has a considerable effect on compacting DNA strands into chromatin, thus enabling the eukaryotic cell to organise DNA of over a meter long into a single cell nucleus (for review see: Ramakrishnan, 1997; Zyprian, 1994). Condensation of DNA by histones is an important mechanism involved in the regulation of gene expression. The linker histone H1 is responsible for reorganisation of the chromatin into a higher order structure. Without such a reorganisation, genes are inaccessible for the transcriptional machinery and thus gene expression is repressed (Wolffe, 1994).

H1 histones are subject to regulation both in a quantitative and qualitative way. They are known to be post-transcriptionally regulated through phosphorylation in a cell cycle dependent manner (Bradbury, 1992; Roth and Allis, 1992). Most organisms possess several highly polymorphic H1 histones of which the expression can be under control of or can be associated with different developmental and regulatory processes. This suggests repression of expression of certain classes of genes depending on the developmental program in a cell (Bouvet *et al.*, 1994; Shen and Gorovsky, 1996).

In tomato three different linker histone variants have been cloned and described. The first H1 histone described was designated le20 and *his1-s* in later

publications (Bray *et al.*, 1999; Cohen and Bray, 1990). *his1-s* expression in leaves was higher than in roots. High accumulation of mRNA was observed in wilted leaves, with exception of the ABA deficient mutant *flacca*, but also during development of seeds and green fruits. The *his1-s* gene is believed to be stress related and induced by ABA or under diurnal control (Corlett *et al.*, 1998). The *his1-s* gene has greatest similarity with H1-D from *L. pennellii* (Wei and O'Connell, 1996) and H1-3 from Arabidopsis (Ascenzi and Gannt, 1997). The *his1-s* gene shows only distant similarity with the two other linker histone in tomato which share a high homology. Jayawardene and Riggs (1994) reported on a second H1 histone sequence which was found to be expressed highest in meristematic tissues or tissues with a large proportion of cells actively engaged in cell cycle. The third linker histone was cloned by van den Heuvel *et al.* (1999), *leH1*. This histone was also expressed in meristematic tissues or tissues with a large proportion of cells actively engaged in cell cycle. The second and third linker histone genes share a significant sequence homology with the H1-1 and H1-2 genes from Arabidopsis (Gantt and Lenvik, 1991) and H1 from tobacco (Szekeres *et al.*, 1995).

Differential screening of cDNA libraries from germinating seeds, dormant seeds and seeds imbibed in ABA have suggested a possible role for the *his1-s* linker histone during dormancy of seeds (Spoelstra, unpublished results). Linker histones have been reported to both decrease or increase during germination (Dicorato *et al.*, 1995; Grellet *et al.*, 1977; Szekeres *et al.*, 1995). It is possible that these studies have dealt with functionally different sub-classes of H1 histones.

The role of ABA and GA in germination of seeds has been subject of extensive studies. Both linker histones have been suggested to be regulated by either ABA or GA (Cohen and Bray, 1990; Heuvel *et al.*, 1999), which makes them possible

candidates to play a role in the control of germination at the level of gene expression. In order to study the possible role of the linker histones *his1-s* and *leH1* in the control of germination and dormancy of tomato seeds, we studied the expression patterns of both genes during the induction and breaking of dormancy and the possible role of ABA and GA in the regulation of their expression. Moreover *his1-s* and *leH1* seem excellent tools to assess the operation of a GA/ABA balance in tomato seeds.

Material and Methods

Seed material and plant growth conditions

Wild-type seeds

Moneymaker tomato plants were soil grown in a greenhouse in 1991. Tomato fruits were picked at the red stage and the seeds and locular tissue were removed from tomatoes by cutting. The locular tissue was digested by adding an equal volume of 2% hypochloric acid and stirring for 2 hours. Seeds were rinsed thoroughly under running tap water en transferred onto two layers of filter paper and dried for 3 d at 21°C. Dried seeds were stored at 4°C in plastic containers.

gib1 mutant seeds

gib1 mutant plants were grown on soil in a greenhouse in 1997 and were weekly sprayed with a solution of 10 μM GA_{4+7} to enable flower induction (Groot *et al.*, 1987). Red fruits were picked and seeds were harvested and stored as described for wild-type seeds.

Transgenic seeds:

Transgenic Money Maker tomato plants, containing 2 homozygous copies of a 35S::luciferase construct (see Chapter 2; line B5, 6^{th} generation transgenic, second truss: T6B5-II) were grown in a greenhouse in 1999. Red fruits were picked and seeds were harvested, stored and germinated as described for wild-type seeds.

Germination conditions

General conditions

Triplicates of 25 seeds were imbibed in 1 ml of imbibition solution, on filter paper in 4.5 cm plastic petri dishes, sealed with Parafilm, at 25°C in constant darkness. Seeds were dissected into embryo and endosperm, frozen in liquid nitrogen and stored at –80°C until RNA extraction.

Wild-type and gib1 seeds

Wild-type seeds were imbibed in demineralised water for a period of 4, 12, 18, 24, 36, 42, 48, 57 and 67 h. Alternatively, wild-type seeds were imbibed in 10 μM of ABA for a period of 24 and 48 h. *gib1* mutant seeds were imbibed in either demineralised water or a solution of 10 μM of GA_{4+7} for a period of 24 and 34 h.

Induction and breaking of secondary dormancy

Secondary dormancy was induced in transgenic 35S::luciferase seeds (T6B5-II) by far-red ($\lambda > 730$ nm) irradiation for 15 min at hourly intervals during the first 48 h of imbibition at a temperature of 21°C and subsequent incubation in the dark at 25°C for 5 days (adapted from de Castro et al. 2001). After this period seeds were tested for germination and for luciferase activity by on chip integration of light emitted by these seeds (e.g. dormant seeds do not emit light) for 30 min on a Roper Scientific Princeton Instruments Versarray™ 512B liquid nitrogen cooled CCD camera operated at -90°C via Metamorph 4.1. (Universal Imaging Corp.) Seeds which revealed luciferase activity (non-dormant seeds; Chapter 2) were discarded. At this stage luciferase negative seeds are considered to be in a state of secondary dormancy. In order to break secondary dormancy, seeds were given a cold treatment for 2 days at

4°C, after which the seeds were transferred to 25°C in the dark and left to germinate. Seeds were dissected into endosperm and embryo at the different stages according to Table 1 and subsequently frozen in liquid nitrogen and stored at -80°C .

Table 1. Specification of samples taken at different stages during dormancy induction and breaking

treatment	far red irradiation	dormancy	breaking	germination
FR	48h/15 min.h^{-1}	-	-	-
SD0	48h/15 min.h^{-1}	5d 25°C	-	-
SD1	48h/15 min.h^{-1}	6d 25°C	-	-
SD2	48h/15 min.h^{-1}	7d 25°C	-	-
SD3	48h/15 min.h^{-1}	8d 25°C	-	-
SD4	48h/15 min.h^{-1}	8d 8h 25°C	-	-
C1	48h/15 min.h^{-1}	5d 25°C	24h 3°C	-
C2	48h/15 min.h^{-1}	5d 25°C	48h 3°C	-
G10	48h/15 min.h^{-1}	5d 25°C	48h 3°C	10h 25°C
G24	48h/15 min.h^{-1}	5d 25°C	48h 3°C	24h 25°C
G32	48h/15 min.h^{-1}	5d 25°C	48h 3°C	32h 25°C

RNA isolation, Reverse Transcriptase PCR and Hybridisation

RNA isolation was modified from Wan and Wilkins (1994). Twenty embryo's or endosperms were ground with 2 chrome-vanadium bullets (Ø 4 mm) in a 2.2 mL Eppendorf tube on a Braun Biotech Int. Mikro-Dismembrator U, at 1600 rpm for 3 minutes. The ground sample was suspended in 700 µL hot borate buffer (80°C) containing, 0.2 M sodium borate decahydrate, 30 mM EGTA, 1 % SDS (w/v), 1% sodium deoxycholate (w/v), 2% (w/v) PVP (M_r 44000) and 10 mM DTT. The sample

was then transferred to a 2.2 mL Eppendorf tube, containing 0.35 mg proteinase K and incubated in a water bath at 42°C for 1.5 h. After adding 55 µL 2 M KCl and subsequent incubation on ice for 1 h, samples were centrifuged at 12000 g for 20 min at 4°C. The supernatant was transferred to a 15 mL tube and 270 µL 8 M LiCl was added, after which samples were incubated overnight at 4°C. Samples were then centrifuged at 12000 g for 20 min at 4°C and the pellet was resuspended in 1 mL ice cold 2 M LiCl and subsequently centrifuged at 10000 g for 10 min at 4°C (the last 2 steps were performed twice). The pellet was then resuspended in 400 µL 10 mM Tris-HCl and remaining debris was spun down by centrifugation at 12000 g for 10 min at 4°C. The supernatant, containing the RNA was transferred to a 15 mL tube and 40 µL 2 M KAc was added for incubation on ice for 15 min, followed by a centrifugation at 12000 g for 10 min at 4°C. The RNA in the supernatant was precipitated with 1.3 mL ethanol and overnight incubation at –20°C, which was followed by centrifugation at 11000 g for 30 min at 4°C and a washing step with 70% ethanol and subsequent centrifugation at 11000 g for 30 min at 4°C. The remaining pellet was dried in a speed vacuum rotor and subsequently suspended in 40 µL RNase free water. RNA samples were DNase treated with 2U DNase (Boehringer) and 20 U RNAsin (Gibco BRL Life Technologies). The remaining RNA was quantified with the GeneQuant and on a 1.5% agarose formaldehyde gel. First strand cDNA was synthesised from equal amounts of RNA (2.5 µg) using reverse transcriptase with Oligo(dT) primers (Superscript^{tm} Preamplification System for First Strand cDNA Synthesis Gibco/BRL Life Technologies). RT-PCR was performed with *leH1* and *his1-s* specific primers (*his1-s* forward: ATC TGC CAA GGC TGT TAC TCA TCC, *his1-s* reverse: TGG CGT CGC TTT TGC TTT CT, *leH1* forward: CAA AGC CGA AGC CAA AA, *leH1* reverse: CGG CCG CAG ATA CAA ACC AC) according to the manufacturers instructions (Superscript^{tm}

Gibco/BRL Life Technologies). 10 µL was sampled at various PCR cycles and subsequently run on 1.5 % agarose gel and blotted onto nylon membrane followed by hybridisation according to the manufacturers instructions (GeneScreen Plus, Life Science Products). A DNA probe for *leH1* was kindly provided by Prof. G.J. Wullems (Dept. of Experimental Botany, Catholic University Nijmegen, the Netherlands). The *his1-s* probe was a 400 bp cDNA fragment which was identified during differential screening of cDNA libraries of tomato seeds imbibed in water or 10 µM ABA or secondarily dormant seeds (Spoelstra, unpublished). The 400 bp fragment was identified via sequence comparison in the NCBI BLAST search as the *Lycopersicon esculentum his1-s* gene (97% , e^{-171} homology NCBI accession number Z11842). Blots were incubated on phosphor screens for 12 hours and scanned on a Molecular Dynamics Storm 840 phosphor-imager.

Results

leH1 and his1-s are differentially expressed during germination of tomato seeds

The expression patterns of *leH1* and *his1-s* were studied during germination of wild-type tomato seeds cv. Moneymaker. The germination curve is shown in Figure 1A. The first seeds germinated at 42 h of imbibition. At 67 h 100% of the seeds had completed germination. At 57 h radicles which had protruded showed an average length of 1 mm and at 67 h protruded radicles had a average length of 3.5 mm. Germinated and ungerminated seeds at 42, 48, 57 and 67 h of imbibition were pooled for RNA extraction.

Both linker histones appeared to be differentially expressed in embryo and endosperm. *his1-s* expression in the endosperm was constantly low during germination (Figure 2). *his1-s* expression in the embryo was high at the start of imbibition and strongly declined after 4 h of imbibition, after which expression remained at lower levels (Figure 2). *leH1* showed a different expression pattern during germination with a decrease in expression in the endosperm after 18 h of germination and an increase in expression in the embryo during imbibition and prior to radicle protrusion. A peak in expression of *leH1* was observed at 57 h of imbibition at which time 80% of the seed population had completed germination. At 100% germination (67 h) expression of *leH1* had decreased substantially.

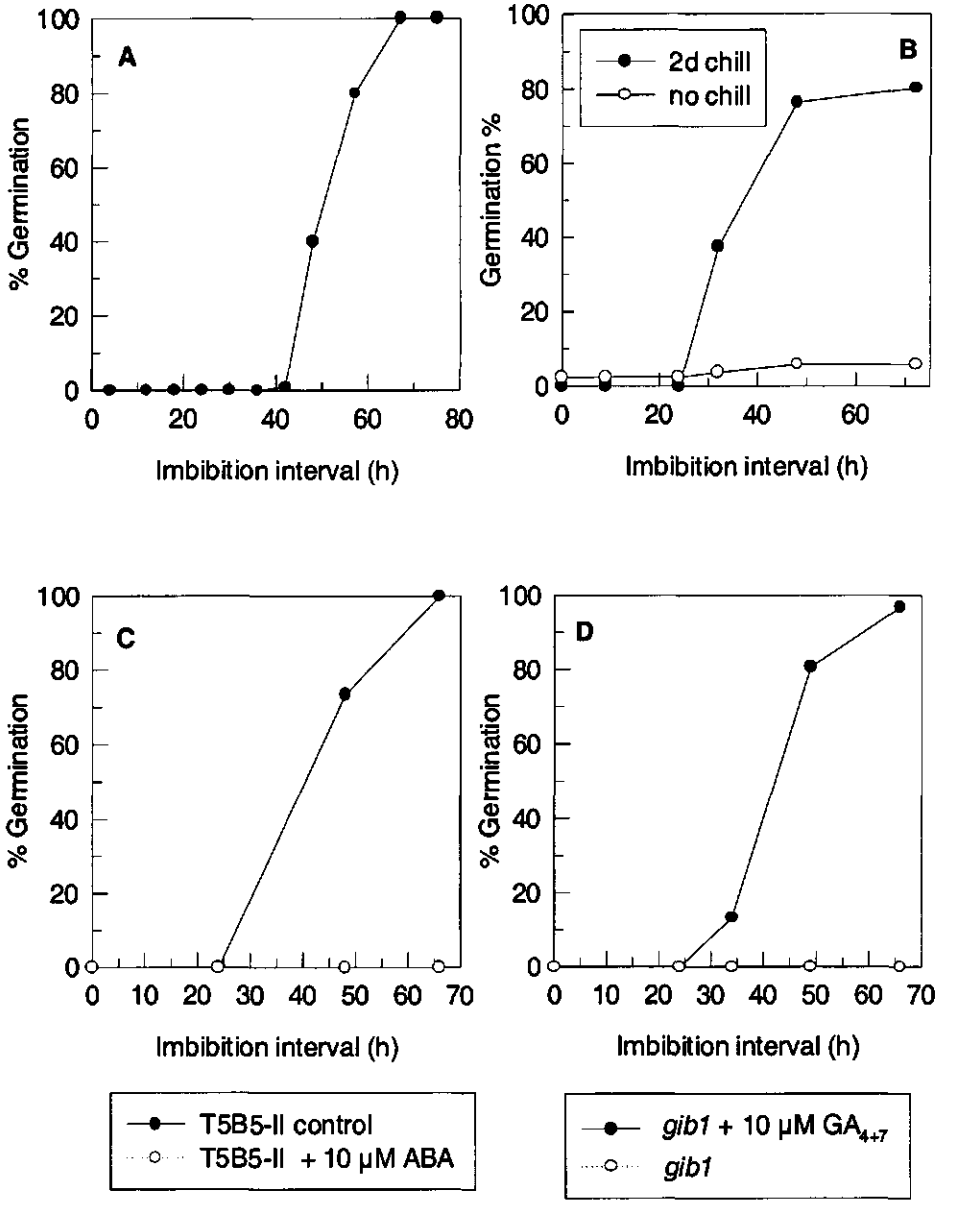


Figure 1: A) Germination curve of wild-type seeds in water. B) Germination curve of secondarily dormant T6B5-II seeds with (closed symbols) or without (open symbols) a 2d chilling treatment .C) Germination curve of non-dormant T6B5-II seeds in water (closed symbols) and 10 µM ABA (open symbols). D) Germination curve of *gib1* seeds in water (open symbols) or 10 µM GA_{4+7} (closed symbols).

his1-s expression is associated with secondary dormancy whereas leH1 expression is associated with germination

The expression profiles of *leH1* and *his1-s* were studied during the induction of secondary dormancy, during a subsequent period of secondary dormancy, during chilling and during subsequent germination at 25°C.During induction of dormancy by far red light irradiation expression of *his1-s* was strong, especially in the endosperm. During the first two days of secondary dormancy (SD0 and SD1; Figure 3) *his1-s* expression in the endosperm was maintained at a comparable level after which expression levels decreased (SD3 and SD4; Figure 3). Concomitantly with the decrease of expression of *his1-s* in the endosperm, expression during induction of secondary dormancy increased transiently in the embryo. During the breaking of dormancy by chilling the expression of *his1-s* was down regulated (C1 and C2; Figure 3). Chilling of the *secondarily dormant* seeds for 2 days induced germination of 80% of the seed population (Figure 1B). From secondarily dormant seeds which were not chilled but left at 25°C in the dark, only 6% completed germination. During germination (G10, G24 and G32; Figure 3) *his1-s* expression in both endosperm and embryo increased compared to the second day of chilling, but was still lower compared to peak levels during dormancy. The latter results were comparable with the results of figure 2, showing that *his1-s* expression was low during germination of wild-type seeds, with exception of expression in the embryo at 4 h of imbibition.

Whereas *his1-s* is expressed strongly during dormancy induction and during secondary dormancy, *leH1* expression was low (Figure 3). During breaking of dormancy by chilling of the seeds at 4°C no induction was detected of *leH1* expression. During the first 10 h after transfer of the seeds to 25° C, expression of *leH1* was strongly induced in the endosperm.

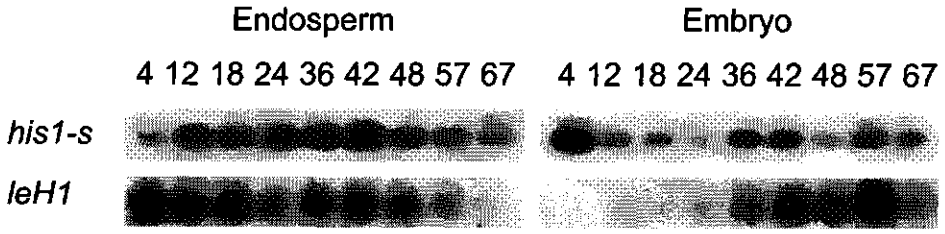


Figure 2: *his1-s* and *leH1* mRNA levels, as determined through RT-PCR of cDNA samples with specific primers in embryos and endosperm of T6B5-II seeds at different intervals (h) during germination.

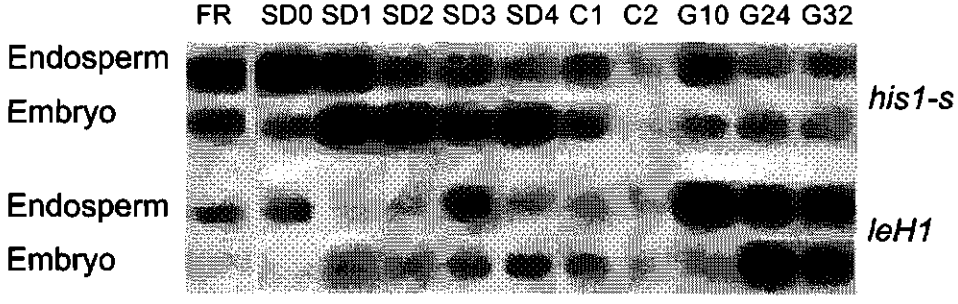


Figure 3: *his1-s* and *leH1* mRNA levels, as determined through RT-PCR of cDNA samples with specific primers, in endosperm and embryos of T6B5-II seeds during far red light irradiation (FR), secondary dormancy (SD0-SD4), chilling (C1, C2) and subsequent germination (G10, G24, G32). See table 1 for details on different stages.

At 24 and 32 h after transfer of the chilled seeds to 25°C expression of *leH1* in the endosperm decreased again. This decrease of *leH1* expression in the endosperm during germination was also observed during normal germination (Figure 2). Expression of *leH1* in the embryo increased during the first 10-24 h after transfer of the chilled seeds to 25°C (Figure 3), when germination starts (Figure 1B) corresponding with the results during normal germination (Figure 2).

***his1-s* expression in tomato seeds is not induced by exogenously applied ABA**

his1-s expression has been reported to require ABA for expression (Bray *et al.*, 1999) or is diurnally regulated (Corlett *et al.*, 1998). It has been known for years that ABA plays an important role in seed development and dormancy (Hilhorst and Karssen, 1992). Tomato seeds which were imbibed in 10 µM ABA solution did not germinate (Figure 1 C). The expression of the *his1-s* gene was assessed in endosperms and embryos at 24 and 48 h of imbibition of seeds in 10 µM ABA. Levels of expression in both the embryo and endosperm in ABA imbibed seeds were comparable with levels found in germinating seeds (Figure 4). Only at 24 h of imbibition in ABA the endosperm showed a somewhat higher *his1-s* expression compared to 24 h of imbibition in water.

leH1 expression is not necessarily under control of GA in gib1 mutant seeds

leH1 has been cloned after differential screening for genes in the GA deficient *gib1* mutant which are upregulated by GAs. *leH1* was found to be more abundant in leaves from GA treated *gib1* plants compared to non-treated *gib1* plants (van den Heuvel *et al.*, 1999). We have tested whether the *leH1* gene is regulated by GA_{4+7} in *gib1* tomato seeds. *gib1* seeds were imbibed in either water or a solution of 10 µM

GA_{4+7}. *gib1* seeds in water did not germinate (Figure 1D), whereas seeds imbibed in 10 μM GA_{4+7} did germinate. Radicle protrusion started around 34 h and at 67 h 97% of the *gib1* seeds had completed germination (Figure 1D). *leH1* was expressed at 24 h in the endosperm of *gib1* seeds imbibed in water at levels comparable with expression in endosperm at 24 h of GA imbibed *gib1* seeds. At 34 h of imbibition in GA_{4+7} the level of *leH1* expression in the endosperm of *gib1* seeds had decreased whereas without GA this decrease did not occur (Figure 5). This could be expected, since *leH1* expression in the endosperm also declined during germination of wild type seeds (Figure 2). Expression of *leH1* was low in the embryo of *gib1* seeds imbibed in water but was induced at 34 h of imbibition in GA_{4+7}. This induction was comparable to the increase in expression of *leH1* in germinating wild-type seeds (Figure 2)

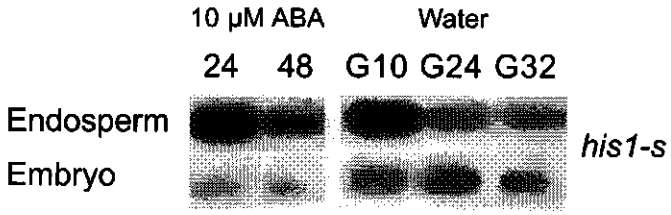


Figure 4: *his1-s* mRNA levels, as determined through RT-PCR with specific primers of cDNA samples in endosperm and embryos of T6B5-II seeds imbibed in water and 10 µM ABA for 24 h and 48 h and at 10, 24 and 32 h (G10, G24, G32) of germination (after cold treatment).

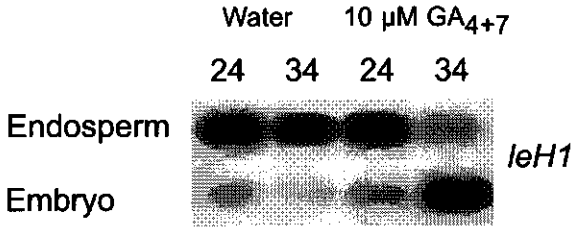


Figure 5: *leH1* mRNA levels, as determined through PCR with specific primers of cDNA samples in endosperm and embryos of *gib1* seeds imbibed in water or 10 µM GA_{4+7} at 24 and 34 h of imbibition.

Discussion

The expression profiles of the two linker histones, *leH1* and *his1-s* during germination and dormancy have confirmed the existence of different members of a class of linker histones, which are under control of distinct developmental processes.

It has been reported that linker histones are involved in chromatin organisation, thereby resulting in repression and expression of different classes of genes (Wolffe *et al.*, 1997). Different sub types of linker histones are involved in regulation of different classes of genes (Bouvet *et al.*, 1994; Shen and Gorovsky, 1996). Given the role of both ABA and GA in the control of development, germination and dormancy of seeds, an ABA and a GA regulated linker histone might fulfil such a role. We have shown that *leH1* and *his1-s* were expressed during dormancy and germination of tomato seed. High expression of *his1-s* was linked with secondary dormancy in seeds, whereas *leH1* was expressed at high levels in germinating seeds. This leads to the hypothesis that *his1-s* histone represses expression of genes which are involved in germination and the *leH1* histone represses gene expression which is related to dormancy. Concomitantly, the *his1-s* histone would induce ABA related gene expression and the *leH1* histones GA regulated gene expression. Expression of these subtypes of linker histones may be part of a developmental switch between the dormant and the germinating state of seeds. Earlier reports on H1 histones in seeds have also shown two types of histones during germination. Histones were cell cycle regulated and showed increasing levels during germination of seeds (Dicorato *et al.*, 1995; Szekeres *et al.*, 1995) or decreased in germinating seeds (Grellet *et al.*, 1977).

H1 histones are known to be post-transcriptionaly regulated through phosphorylation (Bradbury, 1992; Roth and Allis, 1992). Localisation and detection of both linker histone proteins, should therefore be necessary for a more detailed picture of the function of the two linker histones during germination and dormancy of tomato seeds.

It has been shown by immunolocalisation that H1-S protein accumulated in the nucleus and was associated with chromatin in tomato leaves which were subjected to water stress (Scippa *et al.*, 2000). The accumulation of the H1-S protein occurred several hours later than the induction of expression of the gene (Scippa *et al.*, 2000). This might indicate that although *his1-s* mRNA levels decline during the (4 days of) secondary dormancy in tomato seeds, H1-S protein levels might still be present in the nucleus.

his1-s has been reported to be ABA regulated (Bray *et al.*, 1999; Imai *et al.*, 1995). *his1-s* expression was indeed accompanied by raised ABA levels in wilted leaves, but also during seed development (Bray *et al.*, 1999). This evidence is not conclusive. *his1-s* has also been reported to be expressed in leaves in a diurnal manner, independent of ABA levels (Corlett *et al.*, 1998). In non-dormant tomato seeds subjected to 10 μM of ABA, *his1-s* mRNA levels were comparable to germinating seeds in water. ABA was unable to induce strong expression of *his1-s*; such a strong induction was only observed during secondary dormancy and secondary dormancy induction by far red light. The application of ABA to non-dormant tomato seeds inhibits germination but does not induce dormancy of the seeds. When ABA imbibed seeds are transferred back to water, seeds complete germination. ABA is also unable to inhibit the activity of cell wall degrading enzymes such as endo-ß-mannanase (Toorop *et al.*, 1996; Toorop *et al.*, 2000) or expansins (Chen and

Bradford, 2000) which are expressed in relation to germination. Thus, exogenous application of ABA to non-dormant seeds does not always seem to be the correct control for ABA related gene expression in tomato seeds during induction of dormancy or absence of germination. *his1-s* expression might occur during several processes in which ABA action is involved including the induction of primary dormancy during seed development (Bray *et al.*, 1999; Groot and Karssen, 1992; Hilhorst, 1995) or water deficit (Bray, 1997). Yet this does not exclude other possible ways of induction of *his1-s* expression without the action of ABA , such as far red light and the diurnal cycle (Corlett *et al.*, 1998). It is not clear whether ABA is involved in induction and maintenance of secondary dormancy (Berrie and Robertson, 1976; Bewley, 1980), although far red light irradiation did not induce dormancy in the ABA deficient *sit*^{w} mutant seeds (de Castro *et al.*, 2001). It is likely that *his1-s* expression is not under direct control of ABA (Corlett *et al.*, 1998). Interesting to note in this respect is the induction by ABA and salt stress of an mRNA (rd22) in vegetative organs of Arabidopsis, while it was not induced in seeds of Arabidopsis by ABA (Yamaguchi-Shinozaki and Shinozaki, 1993). Preliminary results in our laboratory showed that also *his1-s* may be induced in tomato seeds by imbibition under restriction of water uptake by –1.0 MPa PEG (data not shown).

leH1 was cloned by differential screening for GA regulated genes from cDNA populations from the *gib1* mutant in the presence and absence of GAs (van den Heuvel *et al.*, 1999). *leH1* expression was considerably lower in plant tissues of *gib1* plants compared to *gib1* plants which were grown under application of GA_3 to restore the wild type phenotype. This suggests a regulatory role for GA's in *leH1* expression. *gib1* seeds do not germinate without addition of GA to the imbibition medium (Groot *et al.*, 1988). *leH1* was highly expressed in the endosperm of *gib1* imbibed in water

while expression of *leH1* in the embryo of *gib1* seeds imbibed in water was low. Imbibition of *gib1* seeds in a solution of 10 μM GA_{4+7} restored the wild type phenotype in both endosperm (a decrease in *leH1* expression) and embryo (an increase in *leH1* expression). These results suggest that *leH1* expression is under control of GAs but is not necessarily linked to the occurrence of GAs in tomato seeds. *leH1* expression occurs during GA regulated processes such as germination but GA apparently does not directly regulate *leH1* expression in the endosperm of tomato seeds, as *leH1* expression is high in the endosperm of non-GA treated *gib1* seeds. However, the *leH1* mRNA levels might be a remainder of the expression of *leH1* in the endosperm during development. In our experiments *gib1* plants were weekly sprayed with a solution of GA_{4+7} to stimulate seed setting and fruit development (Groot *et al.*, 1987). Breakdown of certain classes of pre-existing mRNA during germination (Bewley and Black, 1994) may not occur in *gib1* seeds imbibed in water. Addition of GA to the imbibition medium restored the wild type germination phenotype and mRNA breakdown may have occurred during the first hours of germination (Bewley and Black, 1994).

In tomato three different linker histones have been described. *leH1* has a high homology with a related H1 linker histone described by Jayawardene and Riggs (1994). These two linker histones are expressed in tissues which show an active cell cycle. During tomato seed germination the cell cycle is initiated in the embryo (de Castro *et al.*, 2000; Liu, 1996) and during development the cell cycle is active in both embryo and endosperm in tomato seeds (Liu, 1996). The expression of *leH1* shows a close correlation with the activity of the cell cycle. Cell cycle activity was absent upon secondary dormancy induction and was induced again after dormancy breaking by chilling (de Castro *et al.*, 2001). Imbibition of wild type seeds in ABA did not inhibit

cell cycle activity but even stimulated cell cycle activity (Liu, 1996) which was reflected in higher *leH1* expression in ABA imbibed seeds (data not shown).

We have provided evidence that linker histones in tomato may play a regulating role during germination and dormancy. The involvement of ABA and GA in modifying the expression of *his1-s* and *leH1* is not fully understood and needs further investigation.

6

General Discussion

General discussion

This thesis describes studies on germination and dormancy of tomato seeds through the use of physiological, biophysical and molecular techniques. These different techniques have enabled us to study the expression of a CaMV 35S::luciferase construct as a marker for dormancy, germination and variation; the distribution of ATP within the seed was visualised with the luciferase-luciferin system; the uptake and distribution of water during germination were visualised by 3D TSE NMR-imaging. Finally, the expression of two linker histones was studied through RNA extraction and reverse-transciptase-PCR.

Dormancy and germination in tomato seeds

Germination and dormancy of tomato seeds are predominantly regulated by the endosperm. The endosperm limits outgrowth and water uptake of the embryo. For this reason dormancy in tomato is called 'coat-imposed' (Hilhorst and Karssen, 1992). Therefore, the ability of the embryo to protrude the radicle with a force that is high enough to overcome the mechanical restraint of endosperm and testa and the control of this process is important in the control of dormancy and germination. Browsing through literature available on germination of tomato seeds leads easily to the conclusion that the interaction between the embryo and the endosperm is complex. It seems hard to grasp the control of dormancy and germination in a few simple hallmarks. Several factors have been described to influence germination of tomato seeds, including light, hormones and temperature.

Light

Non-dormant tomato seeds do not require light to germinate. Nevertheless tomato seeds are sensitive to red light and far red light. Red light leads to conversion of phytochrome from the inactive form Pr to the active Pfr, while far red light causes the opposite conversion. Secondary dormancy can be induced in tomato seeds by far red light irradiation. A far red light pulse of 10 min at an intensity of 2.1 $mW.cm^{-2}$ at 24 h of imbibition inhibited germination and also inhibited 35S::luciferase expression in transgenic seeds (Chapter 2). A red light pulse of equal intensity of 10 min applied at 24 h was able to stimulate both 35S::luciferase expression and germination. Seeds which have received intermittent far red light pulses at 2.1 $mW.cm^{-2}$ for 48 h are rendered secondarily dormant. 35S::luciferase expression is then abolished and the ATP concentration in the radicle is lowered (Chapter 3). *his1-s* linker histone expression is strongly induced upon induction of secondary dormancy through far red light irradiation. The induction of *his1-s* expression was first detected in the endosperm but was delayed in the embryo (Chapter 5). Light perception in relation to germination within seeds by phytochrome is believed to be located in the embryonic axis (Bewley and Black, 1994; Frankland and Taylorson, 1983). Far red light inhibited 35S::luciferase expression which is mainly expressed in the endosperm cap (Chapter 2). Far red light irradiation also inhibited endo-ß-mannanase activity in the endosperm cap (Nonogaki *et al.*, 1995). This observation is supportive for the hypothesis that a diffusible factor, most likely GA, from the embryo stimulates endo-ß-mannanase activity in the endosperm cap (Groot and Karssen, 1987). Synthesis of GA in the embryo and diffusion of GA from the embryo may be inhibited by far red light irradiation, thus preventing endo-ß-mannanase activity in the endosperm cap.

The development of primary dormancy in tomato seeds with respect to light conditions is not well understood. Not all seeds are primarily dormant upon imbibition with water. The primarily dormant seeds could be distinguished by absence of luciferase activity (Chapter 2). Similar to secondarily dormant seeds primarily dormant seeds have a lowered ATP accumulation in the radicle compared to non-dormant seeds. It would be interesting to study how primary dormancy is acquired (is phytochrome involved?) and why not all seeds are primarily dormant upon imbibition. Growth conditions for tomato plants such as light composition (FR/R ratio) and temperature have not lead to an unambiguous rule of thumb of how primary dormancy may be induced (Hilhorst, personal communication).

Hormones

It has been well established that ABA and GA are involved in germination and dormancy of tomato seeds. GA and ABA influence the expression of 35S::luciferase when applied exogenously in a dose responsive manner. ABA inhibits both germination and corresponding luciferase activity but does not render seeds dormant. The high ATP accumulation in the radicle in germinating seeds was not influenced by application of 10 μM ABA (Spoelstra, unpublished results). This is also true for endo-ß-mannanase activity (Toorop *et al.*, 1996) and expansin expression (Chen and Bradford, 2000) in the endosperm cap. *his1-s* expression was not induced in non-dormant seeds which were imbibed in 10 μM ABA. Inhibition of germination by exogenously added ABA is probably based on the decrease in growth potential of the embryo (Bewley, 1997; Nomaguchi *et al.*, 1995), although the water potential of the embryo is unaffected by ABA (Liu, 1996). The decrease in growth potential is likely to be caused by the inability of cell walls in the radicle to elongate in the presence of

ABA (Schopfer and Plachy, 1985). NMR imaging showed that prior to actual emergence of the radicle the endosperm cap is pushed outward (Chapter 4). This is facilitated by the fact that cell walls have weakened under influence of hydrolysing enzymes. It has been proposed that ABA acts via inhibition of the second phase of endosperm weakening (Toorop *et al.*, 2000). This second phase might be related to the further undermining of endosperm cap restraint by the outward push caused by the protruding radicle. The fact that this second phase is absent in the presence of exogenously applied ABA is then a result of the absence of radicle protrusion.

Whereas ABA inhibits germination, it is stimulated by GA. 35S::luciferase expression was positively influenced by exogenously applied GA. Luciferase activity showed a sigmoid response to GA (Chapter 2). At a concentration of 10 μM GA_{4+7} still not all seeds were stimulated: apparently some seeds have sensitivity thresholds higher than this concentration. Luciferase activity appeared to be related to this threshold. Seeds with an above population average luciferase activity had lower threshold levels compared to seeds with luciferase activity below population average (Chapter 2). Seeds which were treated with GA concentrations higher than this threshold showed a linear response with increasing GA concentration. This is in accordance with the threshold theory postulated by Bradford and Trevawas (1994).

During tomato seed germination, GA is involved in regulation of endo-ß-mannanase activity (Groot and Karssen, 1987; Groot *et al.*, 1988) and expression of expansins (Chen and Bradford, 2000) and it stimulates cell cycle activity (de Castro, 1998; Liu, 1996). These processes are often absent in *gib1* seeds but can be restored by application of GA_{4+7}. *leH1* expression was partly influenced by GA in *gib1* seeds. *leH1* expression in the endosperm of *gib1* seeds was independent of exogenous GA, whereas GA had a stimulating effect on *leH1* expression in the embryo. The data

presented in this thesis have helped to better understand the variation in the response to GA and how ABA and GA are involved in the regulation of dormancy and germination of tomato seeds.

Temperature

Temperature can have a profound effect on germination and dormancy of tomato seeds. In this thesis we have used a low temperature treatment (chilling /cold-stratification) to break primary and secondary dormancy. How chilling affects the seeds in switching to a germination mode is hardly understood. Chilling of dormant tomato seeds may sensitise them for GA and light (de Castro *et al.*, 2001; Derkx *et al.*, 1994b). Both primarily and secondarily dormant seeds are relatively insensitive to treatments with GA and light prior to chilling. Chilling has also been suggested to act by delaying thermal revision of pre-existing Pfr to Pr and it induces membrane changes that may affect availability or sensitivity of receptor sites (Frankland and Taylorson, 1983). Involvement of the membrane may also occur through fluidity changes due to temperature changes. As a result membrane permeability may change with effects on intracellular pH and signal transduction (Hilhorst, 1998), leading to a altered gene expression pattern. Specific cold acclimation related gene expression, protein phosphorylation and release of Ca^{2+} in plants have been reported and recently reviewed (Thomashow, 2001). In Arabidopsis a family of transcriptional activators CBF/DREB1 play an important role in the plant response to low, non-freezing temperatures. It is not known whether or how such transcriptional activators may play a role during dormancy breaking by chilling. There is evidence for synthesis of rRNA

during low temperature presowing treatment (10°C) (Coolbear *et al.*, 1990) in tomato seeds.

Plants also respond to cold stress by solute accumulation, such as sugars. This solute accumulation may play a role in lowering the base water potential of the embryo thus increasing germinability (see also: Bradford, 1995; Bradford, 1996). Gene expression of *his1-s* was strongly down regulated during chilling at 4°C (Chapter 5). During chilling 'dormant' transgenic seeds did not show luciferase activity or 35S::luciferase expression. Upon transferring chilled seeds to germinating temperatures 35S::luciferase expression was initiated and the distribution of ATP displayed highest accumulation in the radicle (Chapter 3), a situation comparable with seeds which were initially non-dormant. *leH1* expression in both embryo and endosperm is also induced within 10 h after transferring chilled seeds to 25 °C (Chapter 5). Synthesis of rRNA during chilling may lead to an increased capability for translation and the *leH1* linker histone may then be involved in the increased gene expression by remodelling DNA architecture in favour of genes involved of germination processes.

Variation among single seeds and the use of single seed assays

In this thesis the degree and types of variation among single seeds have been demonstrated through imaging of ATP and imaging of changes in luciferase transgene expression. Single seeds varied a factor 2.5 to 5 with respect to ATP concentration. It is not known what the origin of this variation is (Chapter 3). Whether a higher ATP concentration results in a faster germination could not be determined due to the destructive nature of the technique albeit that this is unlikely given the lack of relation between luciferase activity in transgenic seeds and germination performance.

Luciferase activity varied among single seeds over a 150 fold range in extreme cases. This variation was partly related to the individual progress of germination of single seeds (luciferase activity increases during germination) but a strong correlation was possibly masked by intrinsic variation in activity between single seeds. In this respect a physiological process related to germination may be better expressed on a biotime scale basis (Bradford and Trewavas, 1994; Bradford, 1996), but even then variation is not fully evened out (Chapter 2). Seeds also varied in the length of the period from the start of imbibition until full commencement of metabolic activity, which was indicated by variation in delay time of luciferase activity. This period was independent of the final time point of germination of a single seed. Single seeds displayed also individual sensitivity thresholds for GA (Chapter 2). The apparently large scale variation between single seeds has raised the question whether the population average is determined by only a few extreme values (Bradford, 1996; pitfall 1; see Chapter 1 for description of the pitfalls connected with the use of pooled samples is seed physiology studies). This problem did not occur with respect to luciferase activity of single seeds in non-dormant seed batches. If necessary, this pitfall can be easily avoided by using a large number of single seeds and generating frequency distributions.

The direct link between a studied parameter to germination is only retained with non-destructive methods (pitfall 2). This was convincingly demonstrated by 3D TSE NMR imaging of water uptake in single germinating seeds. The uptake of water by the radicle prior to actual protrusion through the endosperm would never have been detected in a destructive method. Until recently uptake of water by the radicle was considered a post germination event (Chapter 4; Bewley and Black, 1994). By the use of ATP imaging and 3D TSE NMR imaging it was clearly demonstrated that localised

changes may be overlooked when more classical methods such as weighing seeds and extracting ATP are applied. The localised water uptake by the radicle, most likely by water 'travelling' through the endosperm cap and the high ATP accumulation in the radicle would never have been detected with the classical methods (pitfall 3).

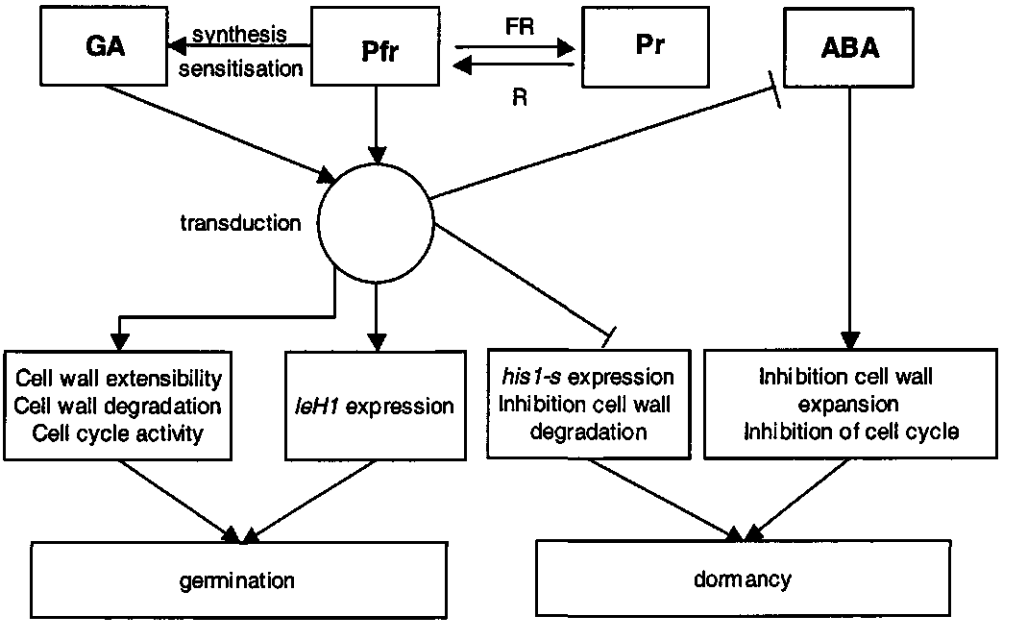
The use of the luciferase reporter gene to study gene expression in seeds

Within our laboratory luciferase has been extensively used as a reporter gene. Luciferase has proven to be a highly dynamical reporter with protein half life of 15 min in the presence of luciferin. Luciferin should easily penetrate in most plant tissues (Aflalo, 1999). Until recently luciferin supply was expected not to be rate limiting (van Leeuwen *et al.*, 2000). However it has been recently reported that luciferin uptake can be rate limiting for the luciferase reaction in leaves (Nass and Scheel, 2001). Experiments in this thesis have shown that in imbibing seeds the luciferin supply to the embryo was also limited. This is most likely due to the restriction of water uptake of the embryo by the endosperm, as was clearly demonstrated by 3D TSE NMR imaging. Furthermore, there is some preliminary evidence that the low water potential of the embryo is also limiting expression or activity of luciferase. Seeds imbibed in –1.0 MPa PEG displayed hardly any luciferase activity which was also reflected in low amounts of extractable luciferase protein. The luciferase signal originating from the endosperm was shown to be related to expression of the 35S::luciferase construct and translation of the luciferase mRNA. The low activity of luciferase in the embryo was partly caused by the limitation of luciferin uptake but in homozygous lines with 2 copies of the luciferase transgene it might also be related to gene silencing phenomena or positional effects (see also: van Leeuwen *et al.*, 2001). Transgene silencing might be avoided by the use of single copy lines (Finnegan and

McElroy, 1994). Unfortunately the single copy line which we obtained through transformation and selective breeding had a very limited luciferase expression in seeds which was hardly detectable, even with the most sensitive camera available.

Tomato seed germination and dormancy; A model

This tentative model aims to explain the relations between the effects of ABA, GA and light in the induction of dormancy and the germination of tomato seeds. Red light irradiation causes the conversion of the inactive phytochrome form Pr to the active form Pfr. The influence of Pfr on GA metabolism is two fold: through increased synthesis of GA (Toyomasu *et al.*, 1998; Yamaguchi *et al.*, 1998) and through interaction of the signal transduction pathway of GA and phytochrome which 'intersect' down stream. This may lead to sensitisation for GA (Weller *et al.*, 1994). Furthermore Pfr stimulates the catabolisation of ABA (Kraepiel and Rousselin, 1994). The expression of *leH1*, in this model, is under control of phytochrome (Chapter 5) but may be enhanced through GA via the overlap in signal transduction pathway. *leH1* causes the necessary DNA architectural changes to inhibit dormancy related gene expression. GA itself positively affects germination by enhancing cell cycle activity, the expression of cell wall degrading enzymes in the endosperm cap such as endo-ß-mannase, expansins which increase cell wall extensibility in the radicle. The time point of germination is determined by the interaction of the radicle growth potential or cell wall extensibility and the diminishing resistance of the endosperm cap. The radicle protrudes by uptake of extra water through the endosperm. It thereby causes an outward swelling of the endosperm cap which will rupture and allow the radicle to protrude (Chapter 4).



Far red light irradiation causes the phytochrome conversion of the active form Pfr to the inactive form Pr. This conversion may lead to an increased sensitivity for ABA or absence of the inhibiting effects of Pfr-phytochrome signalling on ABA levels (see also: Weatherwax *et al.*, 1998). ABA inhibits germination related gene expression like that of expansins and thus the growth potential of the embryo (Schopfer and Plachy, 1985). The conversion of the active form Pfr to the inactive form Pr also leads to inhibition of GA transduction by recruiting elements of the GA signal transduction pathway (Weller *et al.*, 1994). As a consequence, the expression of *his1-s* increases, which causes the necessary architectural DNA changes to inhibit germination related gene expression, while cell cycle activity and the expression of cell wall degrading enzymes like endo-ß-mannanase is inhibited. The combined effects of absence of Pfr-phytochrome signalling and the ABA signal transduction induce dormancy in the tomato seed.

The presented model can also explain why exogenously applied ABA does not induce dormancy in a non-dormant imbibed tomato seed and does not enhance *his1-s*

expression. For dormancy induction and *his1-s* expression the absence of the Pfr-phytochrome signal transduction is needed. The model may also explain why GA is often not capable to overcome dormancy in tomato seeds when applied exogenously, while dormancy can be overcome by combination of light and GA: the combined action of Pfr-phytochrome and GA is necessary to break dormancy.

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Summary

Formation, germination and dormancy of seeds are important steps in the life cycle of higher plants. The seed is the generative dispersal unit, which enables plants to spread and survive through periods or seasons of less favourable conditions. In agriculture tomato is an important crop and seed companies go through big efforts to deliver uniformly germinating seed batches. Uniform germination of a seed lot does not often come naturally. Seed to seed variation in timing of germination and also dormancy cause non-uniform germination of seed batches. This variation and dormancy of tomato seeds is the subject of the experimental work presented in this thesis. Several molecular and biophysical techniques have been used to expand our knowledge of tomato seed physiology.

The firefly luciferase-luciferin system has been used in two distinct techniques to study single tomato seeds. A reporter gene construct consisting of a CaMV 35S promoter and the luciferase gene was introduced in tomato by *Agrobacterium* mediated transformation (Chapter 2). Transgenic seeds were obtained and imbibed in 0.1 mM luciferin solutions. The expression of the luciferase gene was linked with photon emission from the seeds during germination. Luciferase was expressed in a developmental pattern during germination in all germinating seeds. Luciferase expression increased during germination. Although the expression pattern of luciferase was intrinsically linked with the completion of germination, the luciferase activity of a single seed could not be used as a prediction of the time point of visible germination or of the germination rate of a single seed. This was due to the

combination of both a time component and an intrinsic variation in the level of expression.

Both primarily and secondarily dormant tomato seeds did not show luciferase activity. This enabled us to distinguish, non destructively, dormant from germinating tomato seeds prior to radicle protrusion and, hence, separation of those seeds for future experiments.

Luciferase was also used to visualize distribution of ATP in sections of tomato seeds during dormancy and germination (Chapter 3). It was shown that not the overall ATP level or concentration of a seed was related to germination or dormancy *per se*, but merely the localised increase of ATP levels in the radicle. Dormant tomato seeds did not show an increase in the level of ATP in the radicle.

Germination of seeds starts with the uptake of water and finishes by water uptake by the radicle at the initiation of seedling growth. Water uptake by tomato seeds was studied with the use of NMR-imaging (Chapter 4) . Water uptake resulted in an uneven distribution of water over the seed tissues. The endosperm had higher water content during germination. Radicle protrusion was accompanied by an uptake of extra water, thereby stretching the endosperm outward which resulted in rupture of the endosperm cap, which marked the end of germination. In contrast with the commonly adopted model in which seeds take up extra water only after germination, tomato seeds showed this extra water uptake prior to germination.

Linker histones play an important role in the regulation of gene expression by remodelling DNA architecture. Distinct linker histones are thereby under control of different developmental processes in plants. With this in mind we have studied the expression of two different linker histones in tomato, which were originally believed to be under control of either GA or ABA, by the use of reverse-transciptase PCR.

ABA and GA are antagonists in the regulation of seed germination and this makes both linker histones excellent candidates to play a role in the regulation of germination and dormancy of tomato seeds (Chapter 5). It was shown that the two different linker histones were differentially expressed in seeds, in relation with dormancy or germination. The linker histones also appeared not to be necessarily under direct control of either GA or ABA. A model is presented in which dormancy and germination are controlled by the linker histones, which, on their turn, are under direct control of phytochrome signal transduction. Expression of the histones may be stimulated or accompanied by ABA or GA.

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Samenvatting

In de levenscyclus van hogere planten spelen zaden een belangrijke rol. Zaden dienen voor verspreiding van de soort en het individu maar ook om periodes en seizoenen te overleven welke ongunstig zijn voor groei. Daarnaast zijn zaden belangrijk in landbouwkundig opzicht, voor zowel pootgoed als consumptie. Aan zaden als uitgangsmateriaal voor gewassen worden hoge kwaliteitseisen gesteld met betrekking tot uniforme kieming, afwezigheid van kiemrust en houdbaarheid. Het is dus van belang om de processen te kennen die een belangrijke rol spelen in de variatie in kiemsnelheid tussen de individuele zaden in een populatie en in de regulatie van kiemrust. In deze dissertatie is hiernaar onderzoek verricht via meerdere moleculaire en biofysische technieken.

In hoofdstuk 2 staat beschreven hoe, op een non-destructieve wijze, door middel van transformatie van tomaat met een luciferase-gen uit het vuurvliegje en de 35 CaMV promotor de kieming van individuele tomatenzaden is geanalyseerd. Transgene zaden zijn daartoe gekiemd in een oplossing van luciferine. De fotonenemissie van deze zaden was direct proportioneel aan de expressie van het luciferase-gen. Deze expressie nam continue toe tijdens kieming en was afwezig in zaden welke in kiemrust verkeerden. De hoeveelheid luciferase activiteit was geen directe maat voor de snelheid van kieming van een individueel zaad. De variatie tussen zaden m.b.t. luciferase-activiteit bestond uit zowel intrinsieke (biologische) variatie als een tijdscomponent.

Luciferase-activiteit was volledig afwezig in zaden die in een staat van primaire of secundaire kiemrust verkeerden. Dit gegeven is gebruikt om op non-

destructieve wijze scheiding te kunnen aanbrengen tussen kiemende zaden en zaden in kiemrust geruime tijd voor het doorbreken van het kiemworteltje.

In hoofdstuk 3 is luciferase gebruikt om ATP te visualiseren in zowel kiemende zaden als zaden in kiemrust. Daarbij is gebleken dat niet de totale hoeveelheid ATP in een zaad gerelateerd is aan kieming of kiemrust, maar wel de locale ophoping van ATP in het kiemworteltje van het embryo. Deze locale ophoping van ATP in het kiemworteltje was niet aanwezig in zaden welke kiemrust vertoonden. Kieming van zaden in het algemeen begint met de opname van water en eindigt met een hernieuwde opname van water door het kiemworteltje als aanvang van de groei. De opname van water door endosperm en embryo van tomatenzaden werd gevisualiseerd door middel van *NMR-imaging* (hoofdstuk 4). Daarbij is gebleken dat de hoeveelheid water in het endosperm hoger is dan in het embryo. Vlak voor kieming begint het kiemworteltje extra water op te nemen waarbij het strekt en het endosperm naar buiten drukt, alvorens door het endosperm heen te breken. Dit proces van extra water opname door het embryo werd tot dusver beschouwd als een proces dat na kieming plaatsvindt.

De zogenaamde *linker histones* spelen mogelijk een belangrijke rol bij de regulatie van gen expressie door middel van herstructurering van de DNA-architectuur. Er bestaan daarbij verschillende vormen van deze histonen die elk onder controle staan van verschillende stadia en ontwikkelingsprocessen in de plant. In tomaat zijn deze histonen ook aanwezig. Aangenomen werd dat deze gecontroleerd worden door de plantenhormonen abscisinezuur (ABA) en gibberelline (GA). Twee van dergelijke histonen zijn bestudeerd in hoofdstuk 5. Hierbij bleek dat de twee verschillende histonen ieder een verschillende rol spelen in kieming en kiemrust van tomatenzaden. Er werd daarbij ook aangetoond dat regulatie door ABA of GA daarin

niet strikt noodzakelijk is. Dit heeft geleid tot een model waarin kiemrust en kieming van tomatenzaden gereguleerd wordt door deze *linker histones* en waarbij de fytochroom signaaltransductie een direct aansturende werking heeft die ondersteund wordt of gepaard gaat met de aanwezigheid van ABA of GA.

Curriculum Vitae

Patrick Spoelstra werd geboren op 3 juni 1972 te Glanerbrug. Op 1 jarige leeftijd verhuisde hij naar Almelo. Van 1984 tot 1991 was hij leerling van het RSG Erasmus te Almelo, waar hij in 1989 z'n HAVO diploma behaalde en in 1991 zijn VWO diploma. In 1991 begon hij aan een studie biologie aan de Landbouw Universiteit Wageningen. Voor een praktijk stage werd daarbij 5 maanden doorgebracht aan de Purdue University, West Lafayette in de V.S. Het ingenieurs-diploma werd behaald in april 1996 waarna hij aangesteld werd als onderzoeker in opleiding aan de vakgroep Plantenfsyiologie aan de Landbouw Universiteit Wageningen. Vanaf februari 2000 was hij werkzaam als Biological Application Specialist bij Roper Scientific B.V. te Vianen, waarna hij werd aangesteld als waarnemend Research Manager bij SBW International B.V. te Roelofarendsveen. Op 1 mei 2002 trad hij in dienst van Syngenta in Enkhuizen als Program Leader Seed Technology.

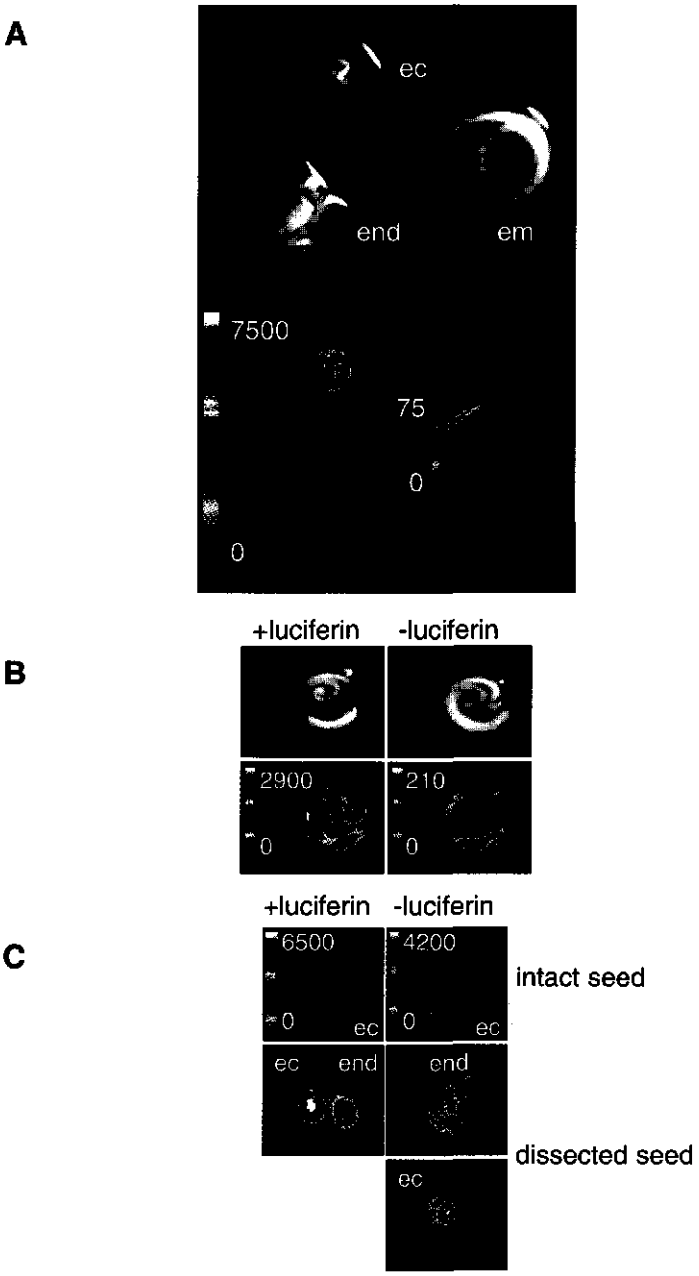


Figure 1: A) (lower panel) Luciferase activity in the lateral endosperm (end) the endosperm cap (ec) and the embryo (em) of homozygous 35S::luciferase tomato seeds imbibed in 0.1 mM luciferin. (upper panel) Corresponding bright field image of dissected tomato seed. B) (lower panel) Effect of luciferin supply to dissected embryo's of hemizygous 35S::luciferase tomato seeds. Tomato seeds were imbibed in 0.1 mM luciferin, dissected and placed either on water (-luciferin) or on 0.1 mM luciferin (+ luciferin). (lower panel) Corresponding bright field image of embryo's of the dissected tomato seed. C) Effect of luciferin and wound response on luciferase activity in lateral endosperm (end) and endosperm cap (ec) from dissected homozygous tomato seeds (lower panel) and intact tomato seed (upper panel). Tomato seeds were imbibed in 0.1 mM luciferin, dissected and placed either on water (-luciferin) or on 0.1 mM luciferin (+luciferin).

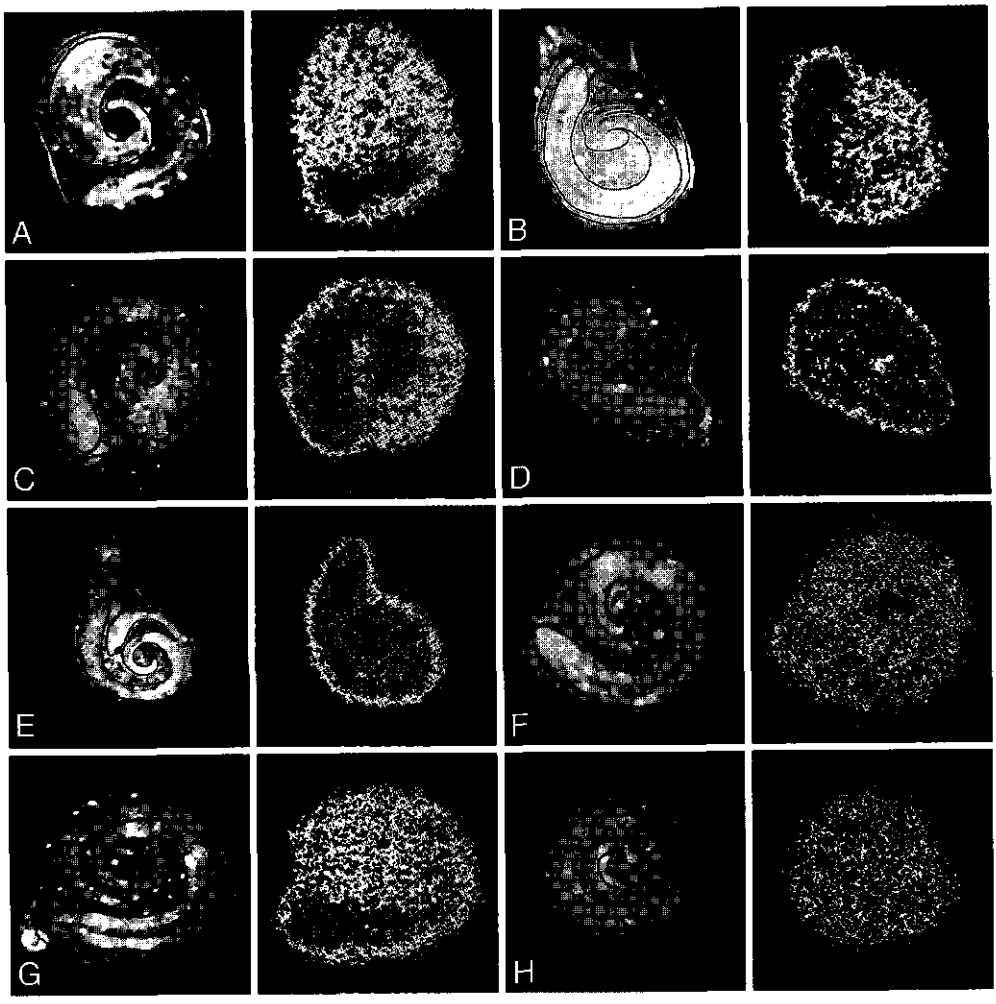


Figure 3: Images of ATP distributions in cryosections of tomato seeds. Wild-type seeds at 3 h (A), 6 h (B), 17 h (C), 24 h (D) and 48 h germinated (E). Secondarily dormant wild-type seed after 5 days of incubation (F). Wild-type seed after secondary dormancy induction and cold treatment for 3 d and 24 h at 25°C (G). Seeds in state of primary dormancy at 48h of imbibition (H).

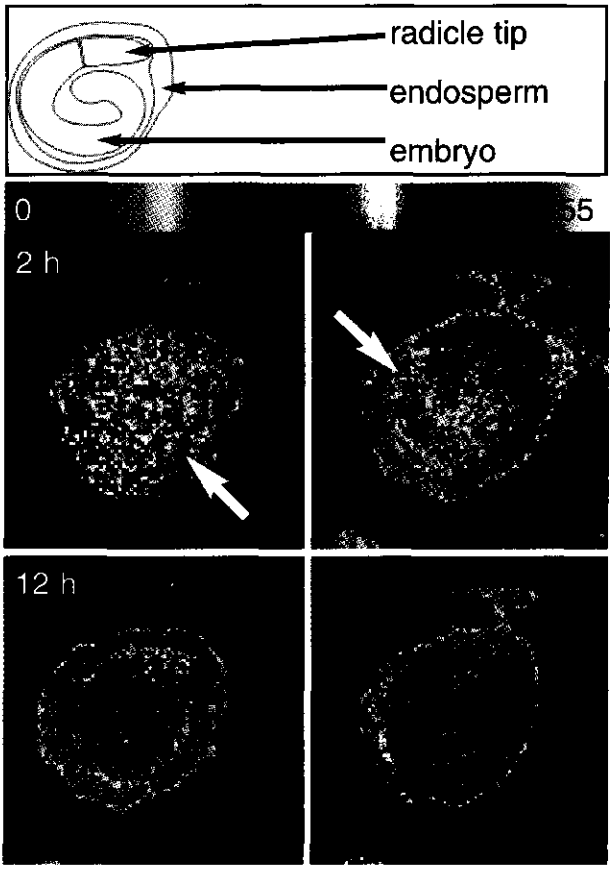


Figure 2: Proton density images of 2 subsequent slices of the same seed acquired through 3D TSE NMR imaging at 2 h and 12 h of imbibition of a single seed. The arrow indicates free space in the seed which disappeared during the first 12 h of imbibition.

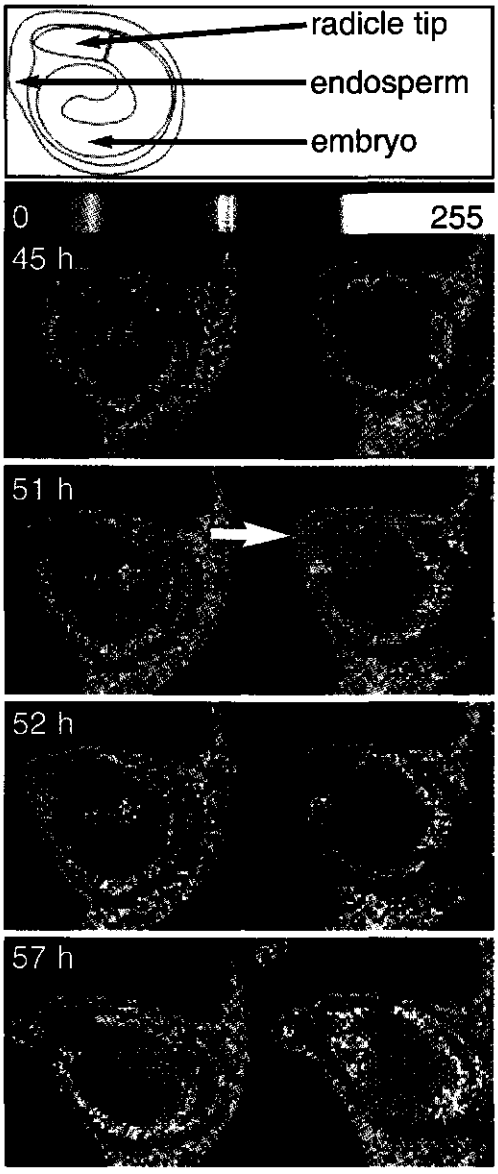
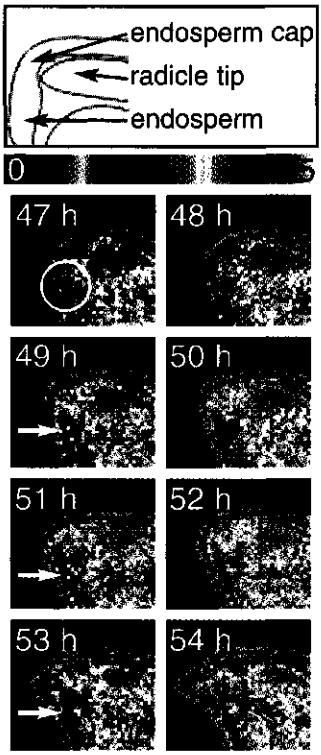


Figure 4: Water distribution in 2 slices of a single seed around the the time point of radicle protrusion. Arrow (51h) indicates outward swelling of the endosperm cap (compare to 45 h), prior to radicle protrusion, Radicle has protruded at 52h of imbibition which can be clearly seen at 57 h.

A



B

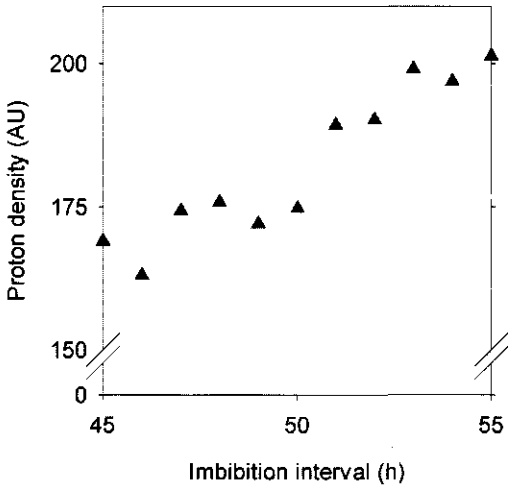


Figure 5: A) Localised uptake of water (single slice) through the endosperm just below the endosperm cap (region of interest) with increase in water content, represented by increase in proton density (arrow) starting at 47 h and continuing at 48 h, 49h, 50h, 51h, 52h, 53h and 54h. Radicle protruded at 51/52 h (see also Figure 4). B) Increase in proton density representing the uptake of water within the region of interest in panel A, between 45 and 55 h.