Dormancy cycling in seeds: mechanisms and regulation

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Thesis

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Content

Chapter 1

General Introduction

The current study was aimed at further delineating the regulatory principles of dormancy. The main focus was on a biophysical approach to study cellular properties that may reflect the seed's dormancy status, including membrane properties, metabolic activity and dormancy-associated gene expression. We have employed Electron Paramagnetic Resonance (EPR) techniques and differential screening for gene expression. In this chapter, after a general physiological introduction to the dormancy phenomenon a short introduction will be given to EPR based research methods, as well as to differential gene expression. Finally, the objectives and scope of this thesis are described.

Dormancy and dormancy cycling

The life cycle of most plants starts, and often ends, at the seed stage. In most species mature seeds are shed and dispersed. At this stage of its life cycle the seed may be dormant and will, by definition, not germinate under favourable conditions (Bewley, 1997). Nikolaeva (2004) formulated a dormancy classification system reflecting the fact that dormancy is determined by morphological and physiological properties, as well as ecological and geographical peculiarities. Based on this system Baskin and Baskin (2004) have proposed a classification system which includes 5 classes of seed dormancy: physiological, morphological, morphophysiological, physical and combinational dormancy. Physiological dormancy is divided in three levels: deep, intermediate and non-deep. Seeds with deep physiological dormancy require a prolonged cold or warm incubation in the imbibed state (stratification) before germination can take place. Seeds with non-

deep physiological dormancy cannot germinate without either excision of the embryo, gibberellic acid, scarification, after-ripening, dry storage or a short warm or cold stratification. Morphological dormancy is characterised by an underdeveloped, but differentiated embryo. Embryos of morphologically dormant seeds need time and suitable temperatures to grow before germination can take place. Morphophysiological dormancy is characterised by an underdeveloped embryo that also has a physiological component to its dormancy. Physical dormancy is usually characterised by water-impermeable layers of palisade cells in the seed or fruit coat that control water uptake. Mechanical or chemical scarification can break physical dormancy. Combinational dormancy is characterised by both water impermeability and a physiological dormancy component.

Physiological dormancy is one of the most common forms of seed dormancy. In this thesis *Arabidopsis thaliana* and *Sisymbrium officinale* seeds were chosen, as their germination and dormancy behaviour is well described. *Arabidopsis thaliana* has well known advantages for molecular studies and in *Sisymbrium officinale* dormancy can be fully separated from the germination event (Hilhorst and Karssen, 1989).

Arabidopsis thaliana and *Sisymbrium officinale* seeds are classified as possessing non-deep physiological dormancy. Different states of physiological dormancy can be distinguished. Seeds may be primary dormant upon shedding, as at this stage the seeds are not sensitive to germination-inducing factors such as light or nitrate (Hilhorst, 1990 a, b). Primary dormancy is usually lost after (prolonged) dry storage; this is called after-ripening. Once this block of primary dormancy is removed, imbibed seeds are sub-dormant; they can start germination by exposure to light, or become secondary dormant if environmental conditions disfavour germination. Although secondary dormancy is similar in nature to primary dormancy, in *Sisymbrium officinale* there are differences, such as the sensitivity to dormancy breaking agents such as light, gibberellins and nitrate (Derkx and Karssen, 1993a; see below). In addition, Cadman *et al.* (2006) have found that in *Arabidopsis thaliana,* freshly imbibed primary dormant seeds showed a gene

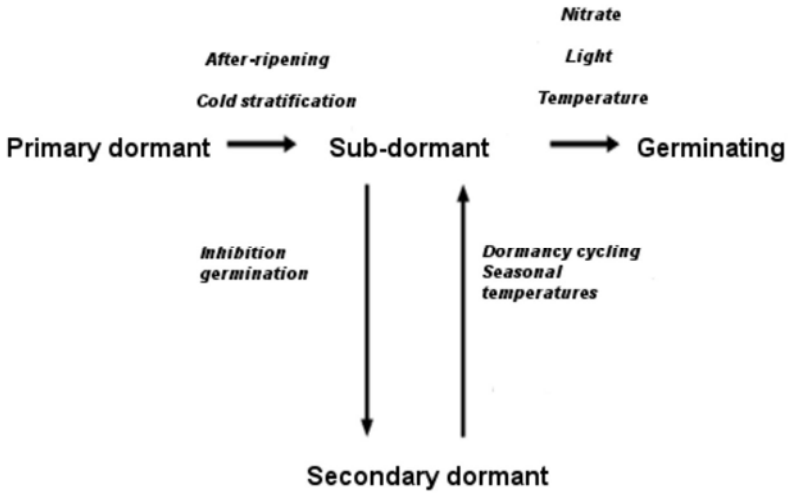

Figure 1: Seeds are primary dormant upon shedding and require after-ripening or cold stratification to becom sub-dormant. Sub-dormant seeds can germinate by exposure to light, nitrate and the right temperature, or become secondary dormant if environmental conditions disfavour germination. The cycling between sub-dormancy and secondary dormancy can occur several times

expression pattern that was rather similar to the gene expression pattern of secondary dormant seeds but had generally lower expression levels of the dormancy-associated genes. Like primary dormant seeds, secondary dormant seeds placed under favourable conditions will not germinate unless dormancy is broken first. Repeated breaking and inducing of dormancy is called ‘dormancy cycling (Figure 1) and may occur many times until environmental conditions are favourable for the induction of germination (Taylorson, 1972; Bouwmeester and Karssen, 1993). By keeping their metabolism low, dormant seeds are potentially capable of surviving years of cycling in and out of dormancy, without loss of viability and thus increase their chances of successful regeneration (Derkx et al., 1993; Chibani et al., 2006).

Temperature

Temperature is one of the main environmental factors controlling dormancy and germination (Hilhorst, 1998). For example, germination in the field is restricted to a limited period of time when the field temperature overlaps with the temperature range in which germination can take place (Vleeshouwers *et al*., 1995), the 'germination temperature window'. Sub-dormant seeds exposed to light exhibit a broad window and they can germinate over a wide temperature range. Dormant seeds (exposed to light) exhibit a narrow window and germination can only take place within a small range of temperatures (Karssen, 1982; Bouwmeester and Karssen 1992). The temperature required for breaking of dormancy may differ from the temperature that is optimal for germination. While cold/heat shocks can break dormancy, prolonged periods of time at sub-optimal temperatures and in darkness can induce (secondary) dormancy (Kępczyński and Bihun, 2002). The induction of dormancy by a sub-optimal temperature treatment is more rapid at higher temperatures than at lower temperatures.

Light and nitrate

Sisymbrium officinale seeds are not only dependent on the right temperature for germination, they are also dependent on the simultaneous presence of light (through phytochrome) and nitrate (Hilhorst *et al.,* 1986; Hilhorst, 1990a, b; Derkx and Karssen, 1993). In species such as *Arabidopsis thaliana* there is no need for the simultaneous presence of light and nitrate: its seeds can germinate in the presence of light only but the presence of nitrate may reduce the requirement for light (Batak, 2002). Dormancy cycling is dependent on the sensitivity to dormancy breaking factors, such as light and nitrate. Thus, this sensitivity may change over time (Hilhorst, 1990 a, b). The sensitivity/responsiveness to phytochrome and nitrate is thought to be regulated by the changes in the number of available phytochrome and nitrate receptors by variations in (seasonal) temperatures (Derkx and Karssen, 1993).

Phytochrome

One of the most important environmental sensors in plants are the phytochromes. Phytochromes are biliproteins that are synthesised in the inactive red light absorbing (Pr) form (Casal and Sanchez, 1998) and red light converts them into bioactive far-red absorbing (Pfr) absorbing isomers (Whitelam and Devlin, 1997). Pfr converts back into Pr in the dark. In lower plants phytochromes are probably rigid cytosolic, probably plasma membrane-associated. Hilhorst (1998) hypothesized that in higher plants the phytochrome and nitrate receptors may also be (temporarily) associated with membranes. But in higher plants there is a switch from primarily cytosolic towards a more dominating nuclear function (Nagy and Schäfer, 2000). Phytochromes are known to regulate GA synthesis, which promotes germination (Hilhorst and Karssen 1998; Yamaguchi et al., 1998, 2004; Ogawa et al., 2003). As temperature has been shown to influence endogenous GA concentration in seeds (Derkx, Vermeer and Karssen, 1994; Yamaguchi *et al*., 2004), and sensitivity to GA (Derkx and Karssen, 1993; Yamaguchi *et al*., 2004) phytochrome could also have a temperature dependent effect. The effect temperature has on phytochrome was further analysed by Donohue *et al.* (2007), who showed that phytochromes mediate dormancy and germination responses to seasonal cues that the seed experiences during maturation and after dispersal. Heschel *et al.* (2007) showed that in *Arabidopsis thaliana* 5 different phytochromes seem to be working at different temperatures, suggesting that phytochromes have a potential role in regulating seasonal timing of germination.

Gibberellins (GAs)

The need for light and nitrate in seed germination can be circumvented by application of gibberellins (GAs) to the seeds. It was hypothesized that nitrate can act as a cofactor to light and that light may induce the biosynthesis of gibberellins. Applying GAs can thus lead to completion of germination without application of light and nitrate (Hilhorst *et al*., 1986). It has indeed been shown that light

stimulates GA-biosynthesis, through a direct effect of phytochrome on the GA 3-oxidase gene (Yamauchi *et al*., 2004). GAs are suggested to stimulate germination by 2 different actions directed at (1) the embryo, by promoting the growth potential, and (2) the surrounding tissues, particularly the endosperm. Seed germination may be prevented or delayed by the mechanical constraint of the seed coat that the embryo has to overcome before it can take up water and nutrients (Chen and Bradford, 2000; Leubner-Metzger, 2001; McIntyre, 1996). The strength of this barrier can be reduced by stimulating endosperm degradation. In gibberellin-deficient seeds only exogenous GA_{4+7} or endosperm plus testa removal can induce germination, indicating that GA_{4+7} can induce endosperm weakening (Hilhorst and Karssen, 1992; Debeaujon and Koornneef, 2000). GA_{4+7} in the embryo is believed to migrate to the endosperm (Hilhorst and Karssen, 1992) where it induces expression of genes encoding enzymes that hydrolyze the endosperm cell walls (Debeaujon and Koornneef, 2000; Chen and Bradford 2000; Nonogaki *et al*., 2000; Manz *et al*., 2005). After endosperm weakening, the embryo can take up more water (Manz *et al.*, 2005), metabolic activity of the embryo is promoted and an additional degree of cell turgor required for the elongation of the radicle is acquired.

Membrane involvement in dormancy

For the past 3 decades, based on a wealth of circumstantial evidence, membranes have been suggested to be involved in the regulation of dormancy. Hendricks and Taylorson (1976, 1978 and 1979) have shown that dormancy induction by higher temperatures is accompanied by an increased leakage of amino acids. The increased leakage was suggested to be linked to the membrane transition temperature and this transition was considered the main limiting factor in germination over a wider temperature range. The membrane transition is accompanied by a disordering of the membrane and changes in membrane order may influence phytochrome activity, as the phytochrome receptor (or steps in its signal transduction pathway) may be membrane-associated (Hendricks and Taylorson, 1978; Nagy and Schäfer, 2000). These changes in membrane

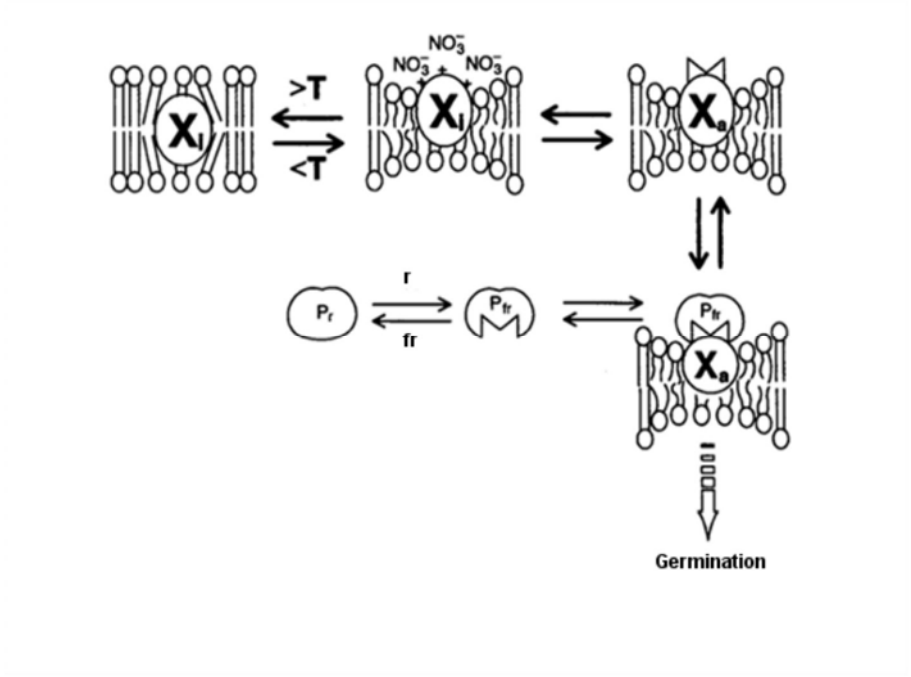

Figure 2: Model based on Hilhorst (1998). Nitrate receptors are suggested to occur in membranes. Under the influence of temperature and time the membrane fluidity changes, allowing the receptors to move to the membrane surface, where nitrate and phytochrome can bind and subsequently germination can take place. X_i and X_a are inactive and active (nitrate) receptors, respectively. P_r and P_{fr} are inactive and active forms of phytochrome, respectively. r is red light and fr is far red light.

organisation are a possible explanation for changes in responsiveness to light and nitrate. A hypothesis was suggested by Hilhorst (1998) (Figure 2), in which the changes in responsiveness to light and nitrate were explained. Phytochrome and nitrate receptors may be associated with membranes. Temperature has a profound influence on membrane fluidity which, on its turn, may determine the magnitude of movement of the receptors within the membranes. In one fluidity conformation the receptors will be at the surface, available for nitrate and phytochrome to bind whilst in the other conformation the receptors are within the membrane and therefore not available for binding of nitrate and phytochrome. One aspect of temperature-induced membrane changes is homeoviscous adaptation (Sinensky, 1974), the mechanism by which unsaturated fatty acids aid in maintaining membranes in a

fluid state, necessary for biological functioning (Cyril *et al*., 2002). A temperature induced increase or decrease in membrane fluidity can be counteracted by an increase in synthesis of *de novo* fatty acids or by desaturation of existing fatty acids (Sato and Murata, 1980), in order to maintain the fluidity.

Electron Paramagnetic Spectroscopy (EPR)

Spin label ESR is a non-destructive technique whereby a paramagnetic molecule (i.e. spin label) is used to tag macromolecules in specific regions. Using the EPR spectra, from the spin label, the type of environment in which the spin probe is located can be determined.

EPR can be very useful in studying seed dormancy behaviour as it can thus give detailed information about the structural and dynamic properties of the cytoplasm or lipid fraction, including membranes, of the seed sample (Marsh,1981). The rotational correlation time (τ_R) of the spin probe in its local environment can be measured by EPR. The τ_R is defined as the time it takes for the spin probe to rotate one radian (~60°) around its axis. In other words, the shorter the τ_R, the faster the rotational motion of the spin probe. The choice of spin probe is important. Depending on the polarity of the spin probe, it will partition into the a-polar oil phase, the polar aqueous cytoplasm, and/or in the membrane, making it a useful tool to study different cellular properties of dormancy and germination, e.g. membrane fluidity and cytoplasmic viscosity. By measuring oxidation-reduction rates of the probe, metabolic activity can be investigated with these spin probes. In this thesis 3 different spin probes were used: 4-Oxo-2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPONE; Figure 3A), 3- carboxy-proxyl (Figure 3B) and a methyl ester of 5-doxyl stearic acid (Figure 3C).

TEMPONE is a small (MW 168) molecule that is easily soluble in water but slightly apolar, making it also soluble in the lipid environment. Thus, TEMPONE can be found in both the aqueous cytoplasm and in lipid bodies. TEMPONE can give information based on the partitioning of this spin probe between the aqueous

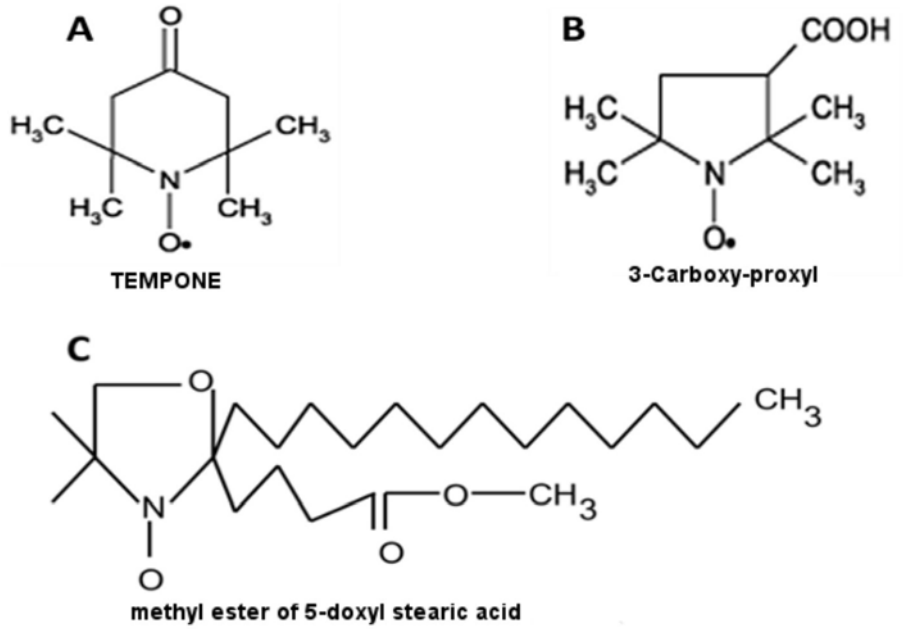

Figure 3A: Structure of TEMPONE spin probe
Figure 3B: Structure of 3-carboxy-proxyl spin probe
Figure 3C: Structure of methyl ester of 5-doxyl stearic acid spin probe

cytoplasm and oil bodies. TEMPONE can be used to study the cytoplasmic volume and cytoplasmic viscosity (Golovina and Hoekstra, 2002), but also the metabolic activity, as it can be reduced to non-paramagnetic species, depending on the reducing power of cells. This reduction can be used to study cellular metabolic rates (Jung *et al*., 1998).

3-Carboxy-proxyl (CP) is also a relatively small (MW 186) spin probe that is easily soluble in water. CP is more polar than TEMPONE due to the presence of an OH group. This group increases the probability of these molecules to form hydrogen bonds, making it particular suitable to study cytoplasmic viscosity.

5-Doxyl stearic acid is often used to study membrane fluidity (Benatti *et al*., 2001; Bianconi *et al*., 1988; Turchiello *et al*., 2000). The methyl ester of 5-doxyl stearic

acid is targeted to cellular membranes. This spin probe is weakly anchored in the head group area due to the high hydrophobicity of the methyl ester. As a result the methylated spin label is localized in a deeper position in the bilayer than its un-methylated counterpart (Sanson *et al*., 1976).

Seed dormancy and gene expression

Several methods have been used to analyse the transcriptional differences between primary dormant and long term dormant seeds. Micro-array analysis is a much used technique and has been employed to study dormancy transitions in *Arabidopsis thaliana* (Cadman *et al.,* 2006; Finch-Savage *et al.,* 2007). Here the cDNA subtraction library of *S. officinale* was chosen over micro-array analysis. With a cDNA subtraction library the cDNA of interest is tagged and the cDNA you want to compare this with is subtracted from the tagged cDNA. The rationale for a cDNA subtraction library over micro-array analysis was to prevent cross-species hybridization difficulties. A major issue with cross-species hybridization is the effect of sequence divergence on probe affinity, which is not only a function of phylogenetic distance. Due to differences in sequence divergence rates, such effects are not uniform across all genes. At present it is difficult to correct for such effects during the analysis of micro-array data (Bar-Or *et al*., 2007). Subtraction libraries have been used to identify key genes and pathways in plants and seedlings (de los Reyes, 2003). cDNA subtraction libraries, however, pose a whole new set of difficulties; a large number of clones needs to be sequenced in order to obtain an overall impression of the transcriptome of a developmental state of the seed. Gene expression of most genes needs to be verified, as some of the genes picked up may appear in both the forward and reversed libraries. Primary dormant seeds and long-term primary dormant seeds were used, as compared to the primary dormant seeds and secondary dormant seeds used in other chapters. Primary and secondary dormant seeds are imbibed in different media, making comparison difficult. Long-term primary dormant seeds are seeds imbibed in water in the dark for 10 days, making the comparison with primary dormant seeds easier.

Long-term primary dormant seeds do not differ from secondary dormant seeds in gene expression patterns (Cadman *et al.,* 2006).

Objectives

Over the past decades significant progress has been made in understanding seed dormancy. Although apparently similar in nature, some genuine differences have been found between primary and secondary dormancy, e.g. the sensitivity to dormancy breaking factors (Derkx and Karssen, 1993) or differences in expression intensity of dormancy related genes (Cadman *et al.,* 2006). These differences may reflect the differences in depth of dormancy. The general aim of this thesis was to not only study the differences and similarities between primary and secondary dormancy, but also sub-dormancy and germination in *Sisymbrium officinale* and *Arabidopsis thaliana* in order to enlarge our understanding of this topic. More specifically, the objectives of this thesis were:

To analyse membrane involvement in the regulation of dormancy;

To analyse changes in metabolic activity and in cytoplasmic viscosity in dormancy cycling;

To identify differences in gene expression between different dormancy states.

Scope of the thesis

Chapter 1: General Introduction

A short introduction to dormancy, dormancy cycling, and the membrane involvement in dormancy regulation is presented as well as the research approaches in this thesis.

Chapter 2: Changes in metabolism in* Sisymbrium officinale *seeds during the transition between dormant and non-dormant states, as measured by electron paramagnetic resonance spectroscopy.

The changes in dormancy and germination were linked to general seed metabolism in *Sisymbrium officinale* seeds. Low cytoplasmic volume and reduced metabolism were linked to dormancy, while germinating seeds exhibited a high cytoplasmic volume and metabolism. Sub-dormant seeds exhibited an intermediate metabolism.

***Chapter 3: Altered membrane properties are associated with dormancy transitions in seeds of* Sisymbrium officinale**

The changes in dormancy and germination were linked to changes in membrane properties in Sisymbrium officinale. At low and high temperatures membrane fluidity could be linked to dormancy, particularly primary dormancy. However, these changes did not seem to be caused by (changes in) fatty acid unsaturation.

***Chapter 4: Altered cytoplasmic properties are associated with dormancy transitions in seeds of* Sisymbrium officinale**

Cytoplasmic properties were studied using the spin probe 3-carboxyl-proxyl. The observed changes in cytoplasmic viscosity may be linked to changes in metabolism and the changes in vitrification temperature may be linked to changes in the content of high-molecular weight compounds.

Chapter 5: Desaturases are associated with dormancy transitions in* Arabidopsis thaliana *seeds

The effects of mutations in desaturases were assessed on the induction of dormancy and membrane fluidity in *Arabidopsis thaliana* seeds. The conversion of

linoleic acid (18:2) into linolenic acid (18:3), appeared to be the most important conversion associated with dormancy induction, especially in the fatty acid desaturases 3 (*fad3*) mutant. However, desaturases activity did not show any involvement in changes in membrane fluidity.

Chapter 6: Characterization of large scale differences in transcription between short term and long term primary dormant seeds

Using a cDNA subtraction library differences in gene expression between primary dormant and long term primary dormant seeds were studied in *Sisymbrium officinale* and compared to *Arabidopsis thaliana* seeds. This yielded a set of conserved dormancy related genes. Candidate genes involved in dormancy are genes involved in maintaining the stability and integrity of cell compartments and macromolecules.

Chapter 7: General Discussion

All the results are combined to obtain a general picture of the molecular and biophysical changes taking place during transitions among dormancy states.

CHAPTER 2

Changes in metabolic and redox activity in *Sisymbrium officinale* seeds during the transition between dormant and non-dormant states

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Abstract

Physiological dormancy is reversible and this enables seeds to cycle in and out of dormancy until the conditions are favourable for germination. In this way seeds can survive in the soil for extended periods of time. It has been argued that dormancy cycling must be an energy-efficient process to explain long-term survival. In *Sisymbrium officinale* seeds, storage lipids are the main source of energy.

Mobilisation of these lipids is expected to occur if energy is required for dormancy cycling. As yet, there is no evidence that changes in respiration rate, respiratory pathways or their enzymes are essentially linked to the regulation of dormancy. EPR results with a TEMPONE spin probe showed that high R-values (water over lipid content ratio) were associated with the breaking of dormancy, indicating high cytoplasmic volume. When germination was inhibited and secondary dormancy induced, R-values and metabolic activity were reduced. Germinating seeds displayed a quick and substantial chemical reduction of the spin probe, leading to a decay of the EPR signal. In sub-dormant seeds the signal decay rate was not as high as that of germinating seeds, but much higher than that of dormant seeds. The oxygen consumption of primary dormant and secondary dormant seeds was very low (less than 0.5% of total oxygen content in 63 hours), but slightly faster in primary dormant than in secondary dormant seeds. In conclusion, germination was linked to a higher cytoplasmic volume and higher metabolic activity or higher capacity for reduction. When germination was inhibited and dormancy induced the cytoplasmic volume and metabolic activity were reduced.

Introduction

Dormancy is the failure of an intact viable seed to germinate under favourable conditions (Bewley, 1997). One of the most common forms of seed dormancy is called ‘physiological dormancy’. It is distinguished from other dormancy types by its reversible nature, allowing dormancy cycling (Taylorson, 1972; Bouwmeester and Karssen, 1993). Physiological dormancy may occur as primary dormancy or as secondary dormancy. Primary dormancy is acquired on the mother plant during maturation, and is observed in seeds upon shedding. Primary dormancy can be lost during dry storage, which is commonly referred to as dry after-ripening. Once primary dormancy is lost, imbibed seeds are sub-dormant; they are capable to complete germination provided that a final dormancy releasing factor, usually light,

is available, or become secondary dormant if environmental conditions disfavour germination. Like primary dormant seeds, secondary dormant seeds placed under favourable conditions will not complete germination unless dormancy is broken first. Upon breaking of dormancy, the right environmental cues trigger germination. Seeds can cycle in and out of dormancy repeatedly, if environmental conditions allow dormancy to be broken but are not favourable for germination (Taylorson, 1972; Bouwmeester and Karssen, 1993). Although apparently similar in nature, genuine differences in primary and secondary dormancy have been observed. *S. officinale* seeds differ in their sensitivity to dormancy breaking factors such as gibberellic acid and nitrate (Derkx and Karssen, 1993a), with the lowest sensitivity in the seeds with secondary dormancy. This difference in sensitivity may reflect the difference in depth of dormancy.

Mature dry seeds usually contain 5-10% water on a fresh weight basis (Hilhorst and Karssen, 1992). Under these conditions metabolic activity is virtually reduced to zero. Metabolic activity increases upon the uptake of water, which is a triphasic process. Rapid initial uptake (I) is followed by a plateau phase (II) and a further uptake (III) once germination is completed and radicle protrusion begins (Bewley, 1997). One of the first changes upon imbibition is the resumption of respiratory activity. After a steep initial increase in oxygen consumption the rate declines until the radicle penetrates the surrounding structures. At this time, a second burst of respiratory activity occurs (Bewley, 1997). In dormant seeds it is likely that for successful survival the metabolic activity of the seeds is reduced to avoid untimely depletion of reserves. However, although reduction in O_2 uptake has been demonstrated after dormancy induction in Sisymbrium (Derkx *et al.,* 1993) and in lettuce (Powell *et al.,* 1983) there was no correlation between O_2 uptake and dormancy cycling (Derkx *et al.,* 1993). Proteomic analysis of *A. thaliana* has shown that, upon imbibition, two different sets of enzymes controlling metabolism can accumulate, one set is up-regulated in both dormant and non-dormant seeds, while the other set is only up-regulated in non-dormant seeds (Chibani *et al.,* 2006), implying that this additional metabolic activity, is only observed in non-dormant

seeds. Proteins up-regulated only in the non-dormant seeds include the neoglucogenesis enzymes 1,6-Fru bisphosphate aldolase and cytosolic isoforms of GAPDH, providing energy from stored lipids required for seedling establishment, and isocitrate lyase, an enzyme involved in storage lipid mobilization.

There is no evidence that changes in respiration rate, respiratory pathways, or their enzymes are essentially linked to the regulation of dormancy (Bewley, 1997). Upon release from secondary dormancy, there is an increase in respiration, but this is slower than in seeds emerging from primary dormancy (Powell *et al*., 1984). These observations raise the question as to how much or how little of the respiration measured in primary dormant seeds is really essential for its maintenance, and how much is excess, resulting in a high background that masks any subtle metabolic changes taking place (Bewley, 1997; Powell *et al*., 1984).

In this paper we tested the hypothesis that the metabolic rate of primary and secondary dormant seeds is lower than that of sub-dormant and germinating seeds. We used *S. officinale* (hedge mustard) as it has a well-described germination and dormancy behaviour, and the breaking of dormancy can be fully separated from the germination event (Hilhorst and Karssen, 1989). Electron paramagnetic resonance (EPR) was used to study changes in cytoplasmic volume and metabolic activity, measured as reducing power of cells, in dormant and sub-dormant seeds. The metabolic activity was further characterised by measuring single seed oxygen consumption.

Materials and Methods

Germination

Seeds of *Sisymbrium officinale* (L.) Scop. were collected in a field in the vicinity of Wageningen, The Netherlands in 2004. Seeds were cleaned, dried at 20ºC to 85 mg water/g dry seed, and stored at 5 ºC until use (2005-2008). Prior to

germination, seeds were surface sterilized in 1% sodium hypochlorite for 1 minute and rinsed with demineralized water for 5 minutes. Triplicates of 30 seeds were sown in 5-cm Petri dishes on two layers of filter paper (Schleicher & Schuell No 595), moistened with 1.5 ml of either water, 25 mM potassium nitrate (Fisher) or 100 µM GA_{4+7} (Sigma). Seeds were imbibed for 1 or 10d in the dark at 25°C after which they were irradiated with a saturating red light (620-700nm, Philips) pulse for 10 minutes, or kept in the dark (Hilhorst and Karssen, 1988). After irradiation, seeds were transferred back to the dark at 25ºC. Germination was scored every day after irradiation, for 1 month, under safe green light.

Germination tests in 96 wells plates, for concomitant oxygen measurements, were done in triplicate. To each well 2 filter papers were added (Schleicher & Schuell No 595), cut to the size of the wells. Filter papers were moistened with 20 µl H_2O, 25 mM KNO_3 or 100 µM GA_{4+7} before one seed per well was added. After 1 or 10d of imbibition in the dark at 25°C, seeds were irradiated for 10 minutes with a saturating red light (620-700 nm) pulse. Germination was scored every hour after irradiation.

Water content

Upon surface sterilization, imbibition and dark-incubation for 1 or 10 days, seeds were placed shortly in a 9-cm Petri dish filled with 2 filter papers and 3 ml H2O, to ensure that an equal amount of water was attached to the seeds' surface of each replicate, making the standard error of each measurement comparable. Water content was assessed of 15 replicates of 10 seeds, by weighing before and after drying at 103ºC for 17 hours on a 7-decimal balance. Water content was expressed on a dry weight basis, in g H2O/ g dry weight.

EPR

Seed coats of dry seeds were treated with sandpaper for 20 seconds resulting in a superficial bruising of the seed coat, before surface sterilization and imbibition, in order to facilitate entry of the spin probe into the seed. A parallel germination test showed no effect of this treatment on the germination behaviour. Seeds were imbibed for 1 or 10d in the dark. Seed samples were then incubated in perdeuterated 4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPONE) spin probe and 120 mM potassium ferricyanide. Due to paramagnetic interactions, ferricyanide causes broadening of the TEMPONE signal to apparent invisibility. Because ferricyanide does not pass the plasma membrane, the non-broadened narrow lines in EPR spectra originate from the intracellular location of TEMPONE molecules (Golovina *et al.*, 1997; Golovina *et al.*, 2001). After 10 minutes of incubation the seed samples were loaded into a 2-mm capillary for spectra recording. Ferricyanide was not used in the experiments where the reduction rate of spin probe was determined. All seeds were kept in the dark and measurements were done under dimmed white light. EPR spectra were recorded using an X-band EPR spectrometer (Bruker Elexis E500 CW_EPR , Rheinstetten, Germany). To prevent overmodulation and saturation of the EPR signal, microwave power was limited to 5 mW, the modulation amplitude was 0.3 Gauss (G) and the scan range was 100 G.

Oxygen uptake

Following surface sterilization seeds were pre-incubated in the dark for 1 or 10d. After pre-incubation, seeds were irradiated with red light (620-700 nm) for 10 minutes and kept in the dark for 5h, before transferring 64 seeds per treatment to a 96-well plate, one seed per well, containing 2 filter papers (Schleicher & Schuell No 595) moistened with 20 µl of solution. Oxygen uptake was measured using the Q2-test (ASTEC Inc.; www.astec-global.com), which is a non-invasive method whereby oxygen levels in closed wells containing a single seed are determined using

fluorescence life-time properties of an oxygen sensitive dye. An air-tight transparent foil with dots of the fluorescent oxygen sensitive dye was placed on top of the plates and sealed to close every well individually. Complete darkness could not be guaranteed for all plates, and therefore all plates received a light pulse. As a control, the seal on one of the empty wells was pierced; scans were taken every 30 min in the dark.

Up to 32 wells were left empty in the 96-well plate. The O2 measurements of these wells were used for calibration of the data. After calibration, data were smoothed using 9-point symmetrical smoothing analysis (with a start and finish ramp). The differences in respiration rate can be analyzed by measuring differences in the slopes of the oxygen uptake. The differences in the slopes were measured by synchronization analysis, setting the steepest point in the oxygen consumption (15% oxygen levels) for both treatments at 45h, followed by a subsequent t-test of the whole process of oxygen uptake.

Results

In *S.officinale* seeds the different physiological states could be clearly distinguished and were easy to manipulate. Seeds imbibed in H_2O that failed to germinate, both in the light and dark (Figure 1b, 1g), were considered primary dormant. Seeds imbibed in KNO_3 solution ('sub-dormant') only required a light pulse (620-700 nm) of at least 10 minutes, to release (sub) dormancy and to complete germination (Fig.1a, 1f). The light pulse was required within a certain time window; when delivered after 10d of dark imbibition on KNO_3, seeds had acquired secondary dormancy and the light pulse was not sufficient to induce germination (Fig. 1c). Seeds imbibed in 100 µm GA_{4+7} completed germination both in the light (Fig. 1d) and in the dark (Fig.1e), which indicates that the light requirement was bypassed. Seeds imbibed on GA_{4+7} in the light germinated faster than seeds imbibed on KNO_3 in the light and than GA treated seeds in the dark. We studied aspects of metabolic

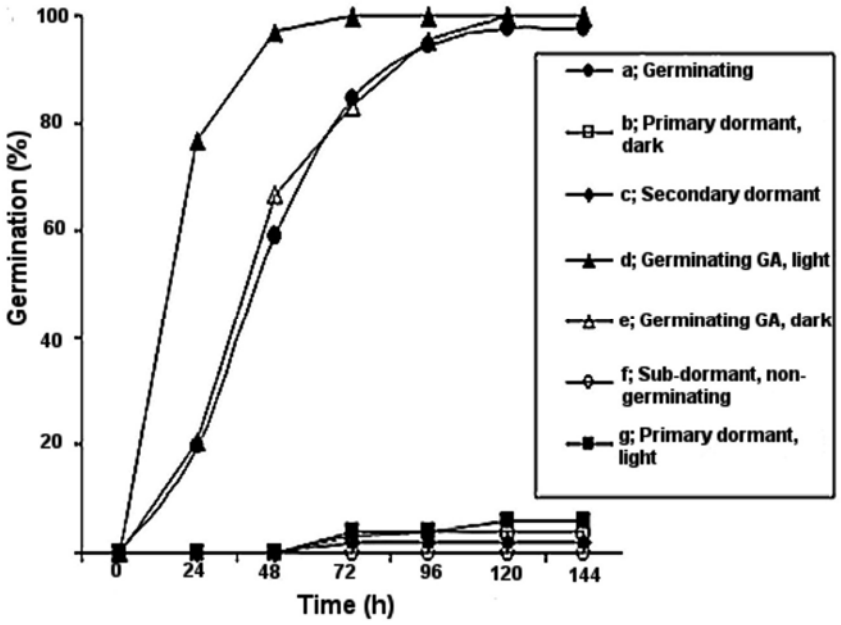

Figure 1: Germination vs time curves of *Sisymbrium officinale* seeds after the following pre-treatments:
a. 25mM KNO_3 for 1d + light pulse (●; germinating);
b. H_2O for 1d in darkness (□; primary dormant, dark);
c. 25 mM KNO_3 for 10d in darkness + light pulse (♦; secondary dormant);
d. 100 µM GA_{4+7} for 1d + light pulse (▲; germinating GA light);
e. 100 µM GA_{4+7} for 1d in darkness (Δ; germinating GA dark);
f. 25 mM KNO_3 for 1d in darkness (◊; sub-dormant, non-germinating).
g. H_2O for 1d + light pulse (■; primary dormant, light)
Where applicable, the light pulse was given after 8h of imbibition, after which seeds were placed back in the dark

activity in order to associate the different physiological states with different metabolic rates. To achieve this we used EPR spectroscopy to measure cytoplasmic and metabolic properties and the Q2-test to measure O_2 uptake.

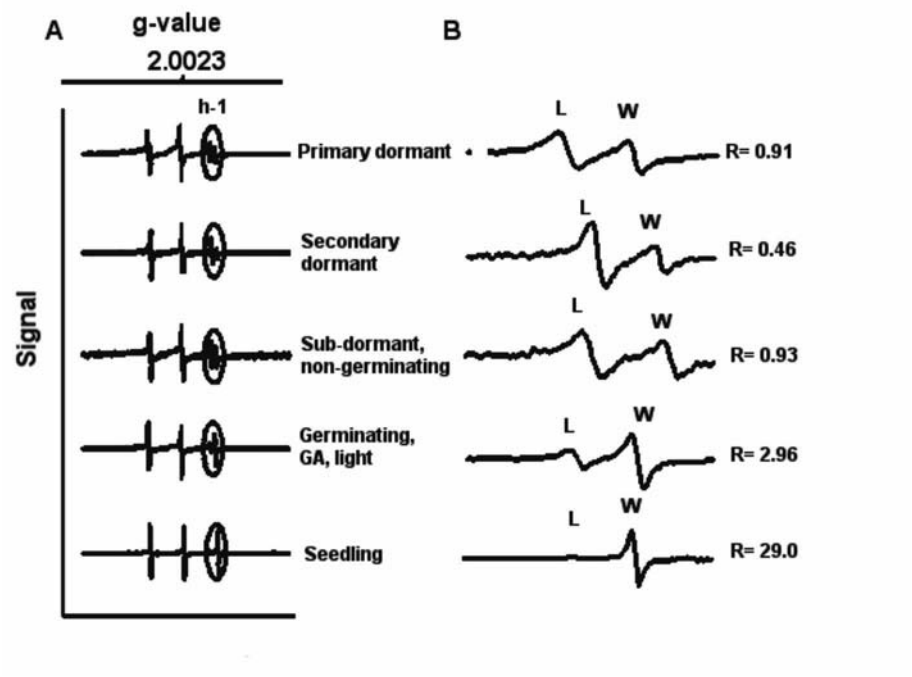

Figure 2. A. EPR spectra of 1 mM TEMPONE in seeds in different states of dormancy, in germinating Sisymbrium seeds and in seedlings. (Sub-dormant seeds were imbibed in 25mm KNO_3; Germinating seeds were imbibed in 100 µM GA_{4+7}). No radicle protrusion had occurred in any of the treatments, except of the seedlings. Seedlings were sampled after 7d of imbibition in GA_{4+7}.

Cytoplasmic volume changes and lipid mobilization in seeds of different dormancy states

The EPR spectrum of perdeuterated TEMPONE from seeds is the superposition of spectra coming from the aqueous cytoplasm, oil bodies and the seed coat (Golovina and Hoekstra, 2002). The relatively broad component of the spectrum originates from the spin probe located in the seed coat and is not analyzed in this study. The spectra of TEMPONE located in the aqueous cytoplasm and lipid bodies each have 3 narrow lines due to fast rotation of the spin probe molecules (Figure 2A). However, these narrow line spectra differ in the distance between the lines. These distance is called the isotropic hyperfine splitting constant and

depends on the polarity of the environment. The spectra from TEMPONE in oil bodies have a smaller distance between the lines in comparison with the spectra originating from TEMPONE in aqueous cytoplasm. These two kinds of spectra are resolved in the high field (right-hand) region of the spectrum because of the combined effects of the changes in the g-value and the isotropic hyperfine-splitting constant in the peak position of both components (Golovina et al., 1997; Golovina and Hoekstra, 2002). The g-value is the most characteristic value that describes an EPR spectrum, and is a unitless measurement of the intrinsic magnetic moment of the electron. The g-value can give information about the paramagnetic center's electronic structure. The g-value is strongly affected by the environment of the unpaired electron of the spin label. The g-value for a free electron, g_e, is 2.0023193. The value of g can vary, and can be calculated. The interaction of the unpaired electron with other electrons in the same atom is usually treated as a coupling of the unpaired electron spin with its orbital momentum. Such coupling produces a splitting of the signal into three separate transitions with characteristic g-values. The high-field region of the spectra consists of two peaks of which the left peak represents the lipid (L) component and the right peak (W) represents the water component (Figure 2A). At a first approximation the heights of the peaks are proportional to the number of TEMPONE molecules in each compartment, which, at a given partition coefficient depends on the volumes of these compartments. The changes in ratio (R) of W over L will indicate the changes in the cytoplasmic volume, or in lipid content, or both. Seedlings and germinating seeds imbibed in GA_{4+7} showed a high R-value (Figure 2B) in comparison with dormant and sub-dormant seeds. This is indicative of cellular expansion or oil mobilization, or both. Sub-dormant and primary dormant seeds showed comparable R values, 0.93 and 0.91, respectively, whereas secondary dormant seeds had a lower R value of 0.46. A low R value could also be an indication of water loss. However, the water content of the whole sub-dormant, primary dormant and secondary dormant seeds showed no significant differences among treatments (Table 1; P values of 0.07, 0.7 and 0.06, respectively).

Treatment	WC (g/g dry weight)	Standard deviation	Dry weight (g)	Standard deviation
GA_{4+7} 1d, germinating	2.28	0.14	0.00297	0.00032
KNO_3 1d, sub-dormant	2.10	0.14	0.00284	0.00016
H_2O 1d primary dormant	2.02	0.10	0.00297	0.00018
KNO_3 10d, secondary dormant	2.12	0.029	0.00285	0.00016

Table 1: Water content (WC) and dry weights of primary dormant (H_2O 1d), secondary dormant (KNO_3 10d), sub-dormant (KNO_3, 1d) and germinating seeds (GA_{4+7}, 1d). Number of observations per average is 10.

Reduction of the spin probe signal to measure rate of cellular metabolism

Although spin probes are stable free radicals, they can be reduced to non-paramagnetic species in living cells. Ferricyanide can rapidly reoxidize the reduced forms of spin labels even if the ferricyanide is located in the apoplast, due to fast exchange of spin probe molecules over the plasma membrane (Kaplan *et al*., 1973). This allows the observation of the stable in time EPR signal from living material. However, the reduction of spin probe moleucules can also be used as a tool to study the rate of cellular metabolism. IN this case spin probes are used without ferricyanide and the decay rate of the EPR signal is indicative of the concentration of reducing agents inside the cells. Redox activity increases within germinating seeds, as a response to the oxidative burst that occurs upon resumption of metabolic activity.The oxidative burst may impose stress to the seed, thus influencing germination (Wojtyla *et al.,* 2006). To scavenge the reactive oxygen species, antioxidants are produced. Antioxidants are necessary to re-establish the reducing intra-cellular redox environment to prevent inhibition of protein synthesis (Wojtyla *et al.,* 2006). Antioxidants, especially glutathione and ascorbic acid are very effective in reducing spin probes (Fuchs *et al*., 1997). Reduction of the spin probe can be also caused by activation of the electron

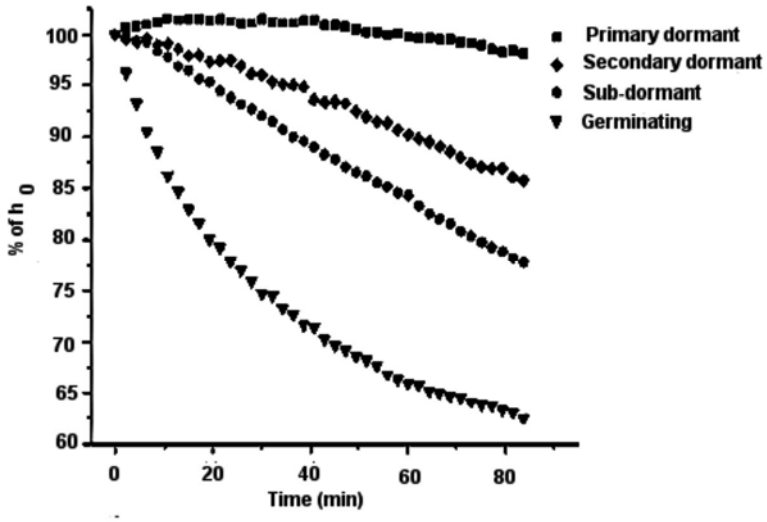

Figure 3. Decrease in the amplitude h_0 of the central spectral line of the ESR spectra of TEMPONE with time as an estimation of the rate of cellular metabolism. Primary dormant (■), secondary dormant (♦), sub-dormant non-germinating (●) and germinating (▼) Sisymbrium seeds were analyzed.

transport chain (Chapman *et al.,* 1985) The EPR signal intensity decay can therefore be used as a good measure of the redox activity and, hence, the metabolic activity in the seed (Jung *et al.,* 1998).

To study metabolic activity in *S. officinale* seeds in different physiological states, we labelled these in a solution of perdeuterated TEMPONE, without ferricyanide, and monitored the changes in the amplitude of the central spectral line in time. The height of the central component of the EPR spectra (h_0) in this case is a good estimation of the total number of paramagnetic species in the sample.

Germinating seeds displayed a 38% reduction of the spin probe in 90 minutes (Figure 3). In sub-dormant seeds primary dormancy is broken but light is still required to complete germination. The signal decay rate of these seeds was

not as high as that of germinating seeds, but much higher than that of primary dormant seeds. The signal decay rate of seeds with secondary dormancy was not as high as in sub-dormant seed, but much higher than that in seeds imbibed in H_2O for 1 day that possess primary dormancy.

Respiration

With differences in the rate of the spin probe reduction we also expected differences in respiration rates because of the possible involvement of the electron transport chain. Therefore, O_2 uptake was measured using the Q_2-test. This test is a non-invasive method whereby fluorescence life-time properties of an oxygen sensitive dye are recorded. In the Q_2-machine, single seed measurements were performed and a germination test under identical conditions was carried out alongside the Q_2-test. As complete darkness could not be guaranteed in the Q_2-machine, oxygen measurements were only done on primary dormant, secondary dormant, KNO_3-imbibed germinating and GA_{4+7}-imbibed germinating seeds.

Figure 4A shows O_2 uptake and germination percentages over time for all the different conditions. Both seeds imbibed in GA_{4+7} and in KNO_3 in the light, reached almost 100% germination within 48 hours. The t_{50} values for germination were 25 and 29h, respectively, showing that half maximal germination was reached 4h faster when seeds were imbibed in GA_{4+7}. The t_{50} values for O_2 uptake were 27h for seeds in GA_{4+7} and 34h for seeds imbibed in KNO_3, showing that half maximal O_2 uptake was reached 7h faster in seeds imbibed in GA_{4+7} as compared to seeds imbibed in KNO_3. Figure 4B shows that oxygen uptake started earlier ($P= 1.6*10^{-6}$) in GA_{4+7} imbibed seeds than in KNO_3 imbibed seeds. Synchronization analysis, setting the steepest point in the oxygen consumption (15% oxygen levels for both treatments) for both treatments at 45 h, and subsequent t-tests of the whole process of oxygen uptake (Figure 5A) showed that the process of oxygen uptake for these 2 treatments was significantly different, within the 1% significance level, with seeds imbibed in KNO_3 displaying slower O_2 uptake. Primary and secondary

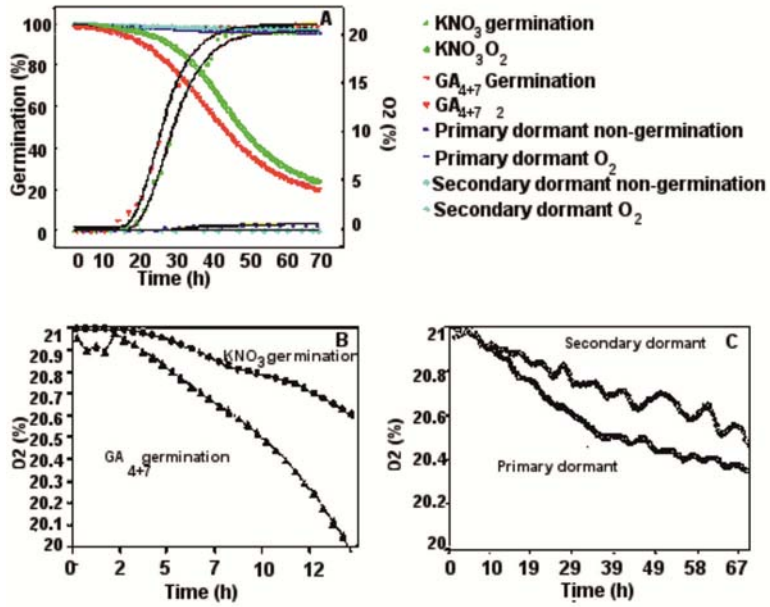

Figure 4. A. Maximum germination and O_2 consumption of seeds over time. Measurements were started after 8h (T 0 = 8), when seeds were transferred to the light. O_2 measurements and germination assays were done in parallel, under similar conditions. O_2 values are mean values as measured in 64 individual seeds.

B. Magnification of the first 12h of O_2 uptake of germinating seeds after an 8h pre-incubation, before the start of radicle protrusion. O_2 values are mean values as measured in 64 individual seeds.

C. Magnification of the O_2 uptake of primary and secondary dormant seeds after an 8h pre-incubation. O_2 values are mean values as measured in 64 individual seeds, excluding germinating seeds.

dormant seeds showed a background germination of less than 5%, but higher in primary dormant seeds. Both treatments, excluding the germinating seeds, exhibited a very slow oxygen uptake (total 0.55% for secondary dormant and 0.65% for primary dormant seeds in 63 hours) (Figure 4C) with a significantly higher rate in primary dormant, as compared to secondary dormant seeds between 19 and 49 hours (Figure 5B).

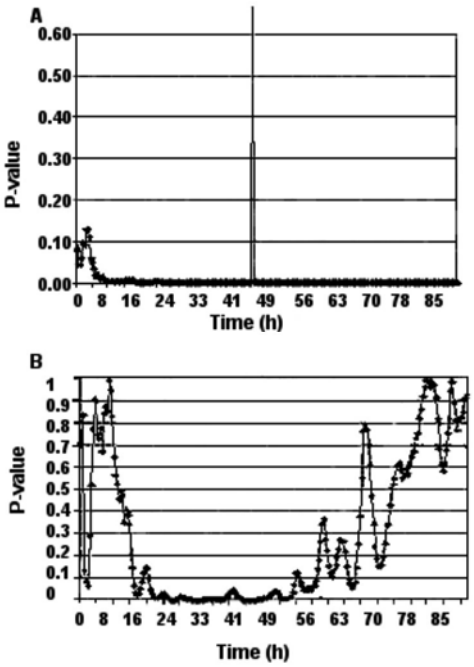

Figure 5. A. P-values of synchronized O_2 consumption measurements of germinating seeds imbibed in KNO_3 and in GA_{4+7}. Synchronizing the oxygen consumption at its steepest point allowed analyzing differences in the process of oxygen uptake. As a consequence of the synchronization at 45 h the P-value is approximately 1.

B. P-values of O_2 consumption measurements of primary and secondary dormant seeds imbibed in H_2O for 1 day and KNO_3 for 10 days. Oxygen consumption measurements were not synchronized, as there is not a steepest point.

In panels A and B, P-values below 0.01 are significantly different.

Discussion

Seeds imbibed in water for 1d (primary dormant) and seeds imbibed in KNO_3 for 10d (secondary dormant) did not complete germination in the light or dark, thus displaying a similar absence of the light response leading to visible germination (Figure 1). However, in the light, the sensitivity to the dormancy breaking factor nitrate differed, since primary dormant seeds responded to nitrate but secondary dormant seeds did not (Hilhorst, 1990b). Thus, secondary dormancy induced by prolonged darkness at the optimal germination temperature was different from primary dormancy in *S. officinale*. Dormancy induction was faster than in

Arabidopsis thaliana in which it took up to 80 days of dark incubation for seeds to lose their sensitivity to light and nitrate (Cadman *et al*., 2006).

Primary dormant and secondary dormant seeds displayed differences in the ratio between water and lipid components of the EPR spectra. Water content measurements (Table 1) showed that in both dormancy states amounts of water were not significantly different. Lipid mobilization is necessary for the transition from dormancy to germination and in seedling establishment in *Arabidopsis thaliana*, as was proven with the *comatose* mutants. However, lipid breakdown itself appeared not to be an important prerequisite for germination but rather functioned as a signal (Footitt *et al.*, 2002, 2006). Here the seed dry weight of the dry, primary dormant and secondary dormant seeds did not differ significantly (Table 1), indicating that no significant lipid mobilization, degradation and utilization in energy metabolism had occurred. However, the dry weight measurements may not have been precise enough to determine small differences. Also the low R-values for the dormant states suggest an absence of lipid mobilization and/or cell expansion (Figure 2). The differences in R-value could also be due to a change in lipid/water content locally. These small changes will be averaged out in the water content measurements as water content is measured of the whole seed.

For successful survival of seeds in the soil one would expect the metabolic activity of dormant states to be low, to prevent fast depletion of reserves. Metabolic activity, as measured by reducing capacity, of primary dormant seeds was hardly detectable (Figure 3), whereas metabolic activity but not oxygen consumption (Figure 4A, C) in the used measurement set-up, of the secondary dormant seeds was considerably higher. The primary dormant seeds had been imbibed in water whereas the secondary dormant seeds had been imbibed in nitrate. This suggests that nitrate had a stimulating effect on reducing activity without increasing respiration, even when the seeds were dormant. The dormant seed is known to have a restricted availability of metabolites and energy (Garciarrubio et al., 1997), due to presence of ABA. It is possible that nitrate treatment led to metabolic changes enabling the seed to overcome this inhibition (Matakiadis et al., 2009).

Although both types of seed were dormant the different imbibition medium could thus be responsible for changes in metabolism. The difference between primary and secondary dormancy in these seeds was very clear. However, as they had been imbibed in different media, comparisons are difficult to make. Comparing secondary dormant and sub-dormant nitrate-imbibed seeds is less complicated; both were imbibed in nitrate in the dark and differed only in incubation time. Secondary dormant seeds have passed through a phase of light-responsiveness that applies to the seeds in which dormancy is released by nitrate (Derkx & Karssen, 1993b). Metabolic activity (Figure 3) was considerably higher in sub-dormant non-germinating seeds than in secondary dormant seeds, which shows that the initially high metabolic activity slows down when germination is not initiated and secondary dormancy is induced. This is in contrast with earlier findings, reporting that dormant and sub-dormant seeds do not differ appreciably in their metabolic activity (reviewed by Bewley, 1997). Reduction in metabolic activity observed in these secondary dormant seeds may conserve energy and presumably serves to prevent depletion of seed reserves and reduced viability during dormancy cycling in the soil (Derkx and Karssen, 1993a). We hypothesize that the metabolic rate slows down even further after longer incubation, to a similar level as in primary dormant seeds that were not exposed to nitrate.

Energy metabolism and oxygen consumption are expected to be related, thus, increased oxygen consumption was anticipated for secondary dormant seeds, as compared to primary dormant seeds. The oxygen consumption of primary dormant and secondary dormant seeds was very low (less than 0.5% in 63 hours), but slightly slower in secondary dormant than in primary dormant seeds. This difference was significant between 16 and 50h of imbibition, and thus suggests that reducing capacity and oxygen consumption are not coupled. Dormant seeds have been shown to consume oxygen at very low rates (Derkx et al., 1993; Powell et al., 1984). In the study by Derkx et al (1993) on seeds of *S.officinale* it was shown that it took approximately 20d of induction of secondary dormancy at 24 °C to attain the minimum very low levels of oxygen consumption.

Thus, an alternative explanation for this discrepancy between oxygen consumption and metabolic activity is that secondary dormancy was not fully attained after 10d, despite the fact that germination did not occur.

Even though seeds imbibed both in nitrate and GA_{4+7} germinated to a high percentage in the light, seeds imbibed in GA_{4+7} circumvented the light requirement. It has been proposed that nitrate can act as a cofactor to light-induced biosynthesis of GAs and to subsequent completion of germination (Hilhorst *et al*., 1986). In Arabidopsis, light activates phytochrome via PHYB, an inducer of GA-3-oxidases (GA3OX1 and GA3OX2), which catalyse the final step in the synthesis of bioactive GAs, even if germination does not occur afterwards (Yamaguchi, *et al.,* 1998; Oh *et al.*, 2006). Thus, completion of germination can take place after imbibition in GA_{4+7} without light, but not on nitrate without light (Hilhorst *et al*., 1986). Light, however, may sensitize the seed to GA_{4+7}, facilitating the germination process and resulting in faster germination (Figure 1; Hilhorst *et al.*, 1986; Derkx and Karssen 1993b).

Upon imbibition, seeds start taking up water which is sufficient for metabolic activity and oxygen consumption to resume. However, after a steep initial increase the oxygen uptake rate declines until the radicle penetrates the surrounding structures (Bewley, 1997). Indeed, when seeds were non-dormant but had not received a light pulse ('sub-dormant'), their water content did not appear to be different from that of their dormant counterparts (Table 1). When seeds were induced to germinate in GA_{4+7} the R-value was very high (Figure 2B), which could indicate an increase in cytoplasmic volume due to cell enlargement and/or oil body degradation, prior to radicle protrusion, however, the water content measurement of germinating seeds did not show this (Table 1). The difference in R-value could be due to a change in lipid/water content locally, which is averaged out in the whole seed water content measurements.

Nitrate and GA_{4+7} can both stimulate the growth potential of the embryo (McIntyre, 1996; Debeaujon and Koornneef, 2000; Alboresi, 2005). A possible explanation for the differences in the cytoplasmic volume could be that the seed

coat acts as a mechanical constraint that the embryo has to overcome before it can take up further water and nutrients (Chen & Bradford, 2000; Leubner-Metzger, 2001; McIntyre, 1996). This barrier can be reduced in time by stimulating seed coat and endosperm degradation. For example, in gibberellin-deficient seeds of tomato or Arabidopsis only exogenous GA_{4+7} or endosperm and testa removal could induce germination (Hilhorst and Karssen, 1992; Debeaujon and Koornneef, 2000), indicating that GA_{4+7} can induce endosperm weakening. GA_{4+7} in the embryo is believed to migrate to the endosperm (Hilhorst and Karssen, 1992) where it induces expression of genes encoding for enzymes that hydrolyze the endosperm cell walls (Debeaujon and Koornneef, 2000; Chen and Bradford 2000; Nonogaki *et al*., 2000; Manz *et al*., 2005). Nitrate in combination with light can stimulate GA production which may result in seed coat degradation. However, when light is not supplied it cannot (Hilhorst *et al*., 1986). After endosperm weakening, the embryo can take up more water (Manz *et al*., 2005), metabolic activity of the embryo is promoted and an additional higher degree of cell turgor required for the elongation of the radicle is acquired. EPR results showed that seeds were metabolically active when imbibed in nitrate or GA_{4+7} (Figure 3), but the germinating seeds imbibed in GA_{4+7} were metabolically more active than the sub-dormant seeds imbibed in nitrate. Q_2-tests were performed after a light pulse was given; therefore both KNO_3- and GA_{4+7}-imbibed seeds readily completed germination. O_2 uptake was faster and started earlier in GA_{4+7} imbibed seeds, but this extra amount of O_2 uptake did not seem necessary for germination, as both germinated to a similar high percentage. However, it did result in faster germination.

In conclusion, germination was linked to a higher cytoplasmic volume locally and higher metabolic activity or higher capacity for reduction. When germination was prevented, dormancy was induced and the cytoplasmic volume and metabolic activity did not increase. The use of non-destructive spin-label EPR spectroscopy to measure changes in cytoplasmic volume and lipid mobilization has proven to be a unique tool to monitor germination related phenomena in time in living seeds

Chapter 3

Dormancy transitions in seeds of *Sisymbrium officinale* are associated with altered membrane properties

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Abstract

Temperature is the main environmental factor involved in the regulation of seed dormancy. As membranes are often considered the primary target for temperature perception they have been implicated in the regulation of dormancy. Membrane properties may be altered to adapt to the temperature. One way of achieving this is by homeoviscous adaptation, the mechanism by which unsaturated fatty acids aid in maintaining the membranes in a fluid state. Here we tested the hypothesis that changes in dormancy and germination concur with changes in some membrane properties. At low and high temperatures the membrane fluidity could indeed be linked to dormancy, especially primary dormancy. Breaking of dormancy induced the membranes to become more fluid, whereas membrane rigidity was partially restored in secondary dormant seeds. The changes in fluidity were not related with changes in glutathione levels. The changes in membrane fluidity did not appear to

be caused by changes in unsaturation. At temperatures that are physiologically relevant for germination the fluidity of membranes did not differ between different dormancy states.

Introduction

Temperature is the principal environmental factor involved in the (seasonal) control of dormancy and plays a decisive role in the regulation of both dormancy and germination (Hilhorst 1998). Seasonal changes in temperature determine the responsiveness of seeds in the soil seed bank to factors that further break dormancy and induce germination, including light and nitrate. In addition, the prevailing field temperature has to overlap with a permissive range of germination temperatures (determined by the dormancy status of the seeds) before germination can take place, which illustrates the dual role of temperature in seasonal flushes of seedling emergence (Karssen, 1982; Bouwmeester and Karssen, 1992).

Membranes have often been implicated in the regulation of dormancy in seeds, as these are considered the primary target of temperature perception at the cellular level (Minorsky, 1989; Murata and Los, 1997, Penfield 2008). However, the principles of temperature perception, as well as how thermal history is remembered are not yet understood. Membranes are capable of altering their properties in order to adapt to changes in environmental temperature. One aspect of these changes is homeoviscous adaptation (Sinensky, 1974), the mechanism by which unsaturated fatty acids aid in maintaining membranes in a fluid state necessary for biological functioning (Sato and Murata, 1980; de Vivrille *et al*., 2002). When the temperature increases, membranes become more fluid because of an increased rotational and lateral movement of membrane lipids. To counteract this increase in fluidity, the amount of unsaturated fatty acids is decreased by suppression of desaturation of fatty acids and acceleration of *de novo* synthesis of saturated fatty acids. When the temperature decreases, synthesis of saturated fatty

acids ceases and existing fatty acids are desaturated to counteract the decrease in membrane fluidity (Sato and Murata, 1980). Membrane fluidity influences general membrane properties, such as membrane permeability, for amino acids, and the movement or orientation of molecules associated with or incorporated in the membrane (Hilhorst, 1998).

Based on a wealth of circumstantial evidence Hilhorst (1998) has proposed a model in which temperature alters membrane fluidity, which in turn would result in altered conformation of membrane proteins (e.g. receptors) and/or membrane permeability, and ultimately in a change in dormancy status (cf. chapter 1). The level of unsaturation of the membrane phospholipids would function as 'temperature memory'. Changes in dormancy coincide with changes in sensitivity and responsiveness to naturally occurring factors such as light and nitrate (Hilhorst, 1990a, b). The model suggests that the availability of the receptor sites for light and nitrate depends on their synthesis and accessibility. The accessibility of the receptor may be a function of membrane fluidity. With the increase of membrane fluidity the receptor protein moves to the membrane surface and becomes available for perception of phytochrome and nitrate signals.

Here we test the hypothesis that changes in dormancy and germination concur with changes in membrane properties. Membrane fluidity was studied with electron paramagnetic resonance (EPR) spectroscopy. This technique allows the study of the physical properties of an (membrane) environment where a spin probe is embedded. The motion characteristics of the probe derived from the shape of EPR spectra are used as a measure of the fluidity of the membrane.

Spin probe approach can also be used to determine the redox status of the environment. Nitroxide radicals can be reduced to non-paramagnetic hydroxylamines by reducing agents. Reduced forms of spin labels can be reoxidized to paramagnetic forms (Swartz, 1987). The observed intensity of the EPR spectrum relates to the equilibrium state between these reactions. Any shift in equilibrium between reduction of the spin probe molecules and re-oxidation of their reduced forms will change the spectral intensity. Redox status of the seed has

been suggested to play a role in dormancy and germination: not only does it change in response to biotic and abiotic stress but it can also interplay with hormonal signalling (Bailly *et al.,* 2008). Glutathione is an essential component of the redox status, and is capable of reducing spin probes (Bobko *et al.,* 2007). Here changes in the intensity of EPR spectra due to reduction of the spin probe were further analysed by measuring oxidised and reduced glutathione content of the seed. Desaturase involvement in membrane fluidity changes was characterised by NMR. *Sisymbrium officinale* (Hedge mustard) seeds were used, as this species has a well-described germination and dormancy behaviour, and the breaking of dormancy can be fully separated from the actual germination event (Hilhorst and Karssen, 1989).

Materials and Methods

Germination

Seeds of *Sisymbrium officinale* (L.) Scop. were collected in a field in the vicinity of Wageningen, The Netherlands in 2004. Seeds were cleaned, dried at 20ºC to 85 mg water/g dry seed, and hermetically stored at 5ºC until use (2005-2008). Prior to germination, seeds were surface sterilized in 1% sodium hypochlorite for 1 minute and rinsed with demineralized water for 5 minutes. Triplicates of 30 seeds were sown in 5-cm Petri dishes on two layers of filter paper (Schleicher & Schuell No 595), moistened with 1.5 ml of either demineralised water or 25 mM potassium nitrate (Fisher). Seeds were imbibed for 1 or 10 days in the dark at 25°C after which they were irradiated with a saturating red light (620-700nm, Phillips) pulse for 10 minutes or kept in the dark (Hilhorst and Karssen, 1989). After irradiation, seeds were transferred back to the dark at 25ºC. Germination was scored daily after irradiation, for 1 month under safe green light.

EPR

The methyl ester of 5 doxyl stearic acid (5-mDS(A)) was used as a spin probe. This spin probe is only weakly anchored in the phospholipid head group area due to the high hydrophobicity of the methyl ester. As a result, the methylated spin label is localized in a deeper position in the membrane bilayer than its unmethylated counterpart (5-DS(A)) (Sanson *et al*., 1976). 5-mDS(A) has often been used to study membrane fluidity (Benatti *et al*., 2001; Bianconi *et al.,* 1988; Turchiello *et al.*, 2000).

Seeds were imbibed in water or 25 mM potassium nitrate for 8 hours or 10d in the dark. Medium attached to the surface of the seed was removed with filter paper before seed coats were removed using tweezers, after which seeds were dried at room temperature. Dry seeds were placed in a 1mM solution of membrane spin probe in hexane. After 1d the spin probe solution was removed, seeds were washed twice with hexane and placed at 3% RH for 3-4 days to remove the remaining hexane from the seeds. Seeds were re-hydrated by humidification for 3 hours at 100% RH. EPR spectra were recorded with an X-band EPR spectrometer (Bruker model 300E Analytik, Rheinstetten, Germany). To prevent over-modulation and saturation of the EPR signal, microwave power was limited to 5 mW and modulation amplitude of 3G for solid-state and 1G for fluid type spectra was used. In the case of 2-component spectra the lowest modulation amplitude of 1G was used. Field scan widths of 100G were used.

Nuclear Magnetic Resonance Spectroscopy (NMR)

The NMR spectra were recorded on an Avance II spectrometer (Bruker, Rheinstetten, Germany), operating at 300.13 MHz for protons and at 75.47 MHz for carbons, and equipped with a solid-state magic angle spinning (MAS) probe. Dry *Sisymbrium officinale* samples with seed coats and without additional treatments were packed into a 7 mm Zirconia rotor and spun under a magic angle at a spinning speed of 5 kHz. ^{13}C MAS single-pulse excitation spectra were obtained

with 20-30 K scans, using a recycle delay of 2s, 200 kHz spectral width, 8k data points and under low-power decoupling. The ^{13}C 90°C pulse width was 5 µs. ^{13}C NMR chemical shifts were assigned according to the literature (Gunstone, 1993; Fan, 1996; Jie and Mustafa, 1997).

Single-pulse ^{13}C NMR MAS (magic angle spinning) was used for analyses of the fatty acid (FA) composition of lipids in seeds of different physiological states. By spinning the sample under the magic angle θ_m (ca. 54.74°, where $\cos^2\theta_m=1/3$) with respect to the direction of the magnetic field, the normally broad lines become narrower, increasing the resolution for better identification and analysis of the spectrum. Since magic angle sample spinning eliminates line broadening arising from differences in magnetic susceptibility, it significantly improves NMR spectroscopy of liquids that are found in an inhomogeneous environment. The use of this technique facilitates nondestructive measurements of oil composition in viable plant seeds (Rutar, 1989). The cellular components, which are in a solid state in dry seeds (proteins and carbohydrates) were present as very broad lines in the 13C spectra in our experiments and were treated as a base line.

The area between 127-132 ppm is derived from olefin carbon atoms of fatty acids (http://lipidlibrary.aocs.org/nmr/nmr.html; Table 1).

The fatty acids with one, two and three double bonds can be identified in ^{13}C NMR MAS spectra of all Sisymbrium samples (Figure 1). We attributed the shifts in the spectra to fatty acids commonly present in seeds: oleic acid (18:1), linoleic acid (18:2) and α-linolenic acid (18:3) (http://lipidlibrary.aocs.org/nmr/nmr.html; Table 1). The peak at ≈14 ppm is characteristic of the terminal methyl (CH_3) group in all types of fatty acids

Fatty acid	Double bonds	Chemical shift (ppm)
Oleic (18:1)	$-C^{9}=C^{10}-$	≈130 (9, 10)
linoleic acid (18:2)	$-C^{9}=C^{10}-C-C^{12}=C^{13}-$	≈130 (9, 13); ≈128(10, 12)
α-linolenic acid (18:3)	$C^{9}=C^{10}-C-C^{12}=C^{13}-C-C^{15}=C^{16}-$	≈130 (9); ≈128 (10, 12, 13); ≈127(15); ≈132(16)
All fatty acids	$-C^{18}H_3$	≈14(18)

Table 1:. NMR spectroscopy of fatty acids and their derivates. Results are shown for primary dormant, sub-dormant and germinating seeds of *Sisymbrium officinale.* For attributing shifts to specific fatty acids see table 1 and http://lipidlibrary.aocs.org/nmr/nmr.html

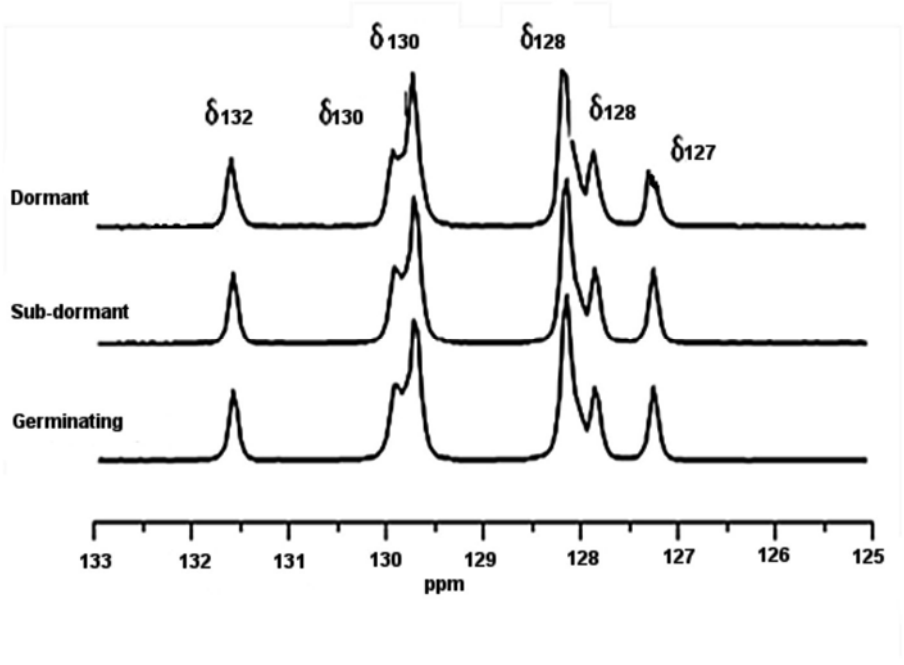

Figure 1. NMR spectroscopy of fatty acids and their derivates. Results are shown for primary dormant, sub-dormant and germinating seeds of *Sisymbrium officinale.*

The integrated area under the peak is proportional to the number of carbons participating in double bonds. Taking into account the data presented in Table 1, the fatty acid composition of seed lipids was calculated as follows:

$$(18:3) = \frac{I_{131.6}}{I_{14.1}}$$

$$(18:2) = \left[\frac{1}{2}\right]\left[\frac{I_{128}}{I_{14.1}} - 3\,(18:3)\right]$$

$$(18:1) = \left[\frac{1}{2}\right]\left[\frac{I_{129.7}}{I_{14.1}} - 2(18:2) - (18:3)\right]$$

I is the integrated area under a specific peak

FA content was also used to calculate a double bound index (DBI), which shows the average number of double bonds per fatty acid. The DBI was calculated by the formula: DBI = [1x(18:1) + 2x(18:2) + 3x(18:3)].

HPLC

Glutathione (GSH) and glutathione disulphide (GSSG) were extracted from dry seeds, seeds imbibed for 8h in the dark in water or KNO_3 and seeds imbibed in KNO_3 for 10d in the dark. GSH and GSSG were extracted on ice in 0.1 M HCl from freeze-dried, finely ground seed according to Kranner (1998). Briefly, this assay uses fluorescence labeling of thiols with monobromobimane (mBBR). Total glutathione was determined after reduction of disulfides by dithiothreitol [DTT (pH 8-8.3)]. For oxidized glutathione measurements, thiols were blocked with *N*-ethylmaleimide (NEM). After removal of NEM, the remaining disulfides were reduced with DTT and labeled with mBBR. All extractions were done from 6 biological replicates and the results were subjected to a t-test (p=0.001).

Results

Germination

In *S.officinale* the different dormancy states could be clearly distinguished and were easy to manipulate (see Chapter 2, Figure 1). Seeds that failed to germinate in H_2O were considered primary dormant. Seeds required a combination of KNO_3, to alleviate dormancy, and a light pulse (620-700 nm) of at least 10 minutes to complete germination. The light pulse was required within a certain time window; when delivered after 10d of imbibition on KNO_3, seeds had become secondary dormant and the light pulse was not sufficient to induce germination, and other methods were needed to break dormancy. The primary dormant, secondary dormant and sub-dormant states were used to study membrane fluidity by using the EPR spin probe technique.

Membrane fluidity

A methyl ester of 5 doxyl stearic acid (5-mDS(A)) was used as a spin probe. The location of the spin probe is relatively deep in the phospholipid bilayer, so that the spectra of 5-mDS(A) give information about the average fluidity of the membranes (Benatti *et al*., 2001; Bianconi *et al.,*1988; Turchiello *et al*., 2000). At 220K the 5-mDS(A) spectrum in hydrated Sisymbrium seed membranes is of the 'powder' type (Figure 2). A powder type spectrum originates from randomly orientated completely immobilized spin label molecules (Marsh, 1981). Increasing the temperature up to 260 K gradually increases the motional freedom of the spin probe without changing the anisotropic character of the spectra. This increase in motional freedom of spin probe within the ordered spectra is characterized by a decrease of the distance between the outermost extremes, $2A_{max}$, of the spectra, and narrowing of the spectral lines (figure 2). $2A_{max}$ can be used to characterize the degree of membrane ordering at low temperatures. An increase in this parameter

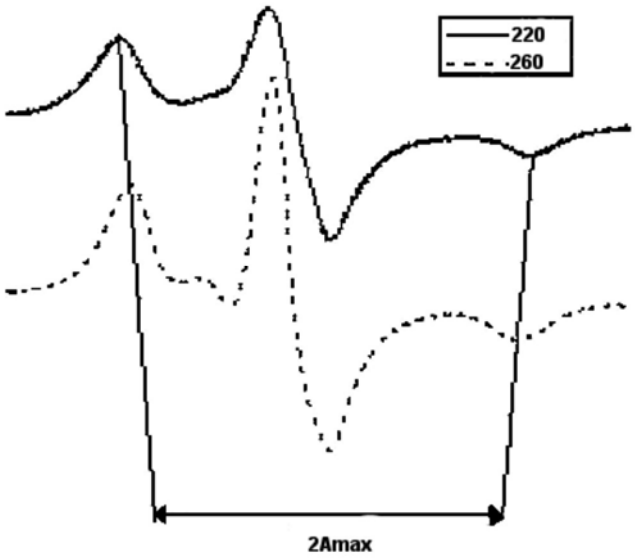

Figure 2. EPR spectra of 5-mDS(A) in primary dormant *Sisymbrium officinale* seeds. Spectra were recorded at 220K (top) and 260K (bottom). The distance between the outermost extremes, 2Amax, is indicated by the arrow. $2A_{max}$ decreases with increasing temperature.

corresponds to an increased ordering of spin label in the membrane environment (Golovina and Hoekstra, 2003). In Sisymbrium seeds $2A_{max}$ decreased with increasing temperature due to temperature induced membrane disordering (Figure 3). The decrease in $2A_{max}$ started later in primary dormant seeds than in secondary dormant and sub-dormant seeds. This indicates that spin probe molecules are more immobilized in membranes of primary dormant seeds.

The changes in the spectral shape at temperatures above 270K indicate the appearance of a mobile component along with the immobile component (Golovina and Hoekstra 2002, 2003; Figure 4A). $2A_{max}$ for the immobile component could not be determined precisely anymore. There was not much difference in temperature of appearance of the mobile component between the different dormancy states,

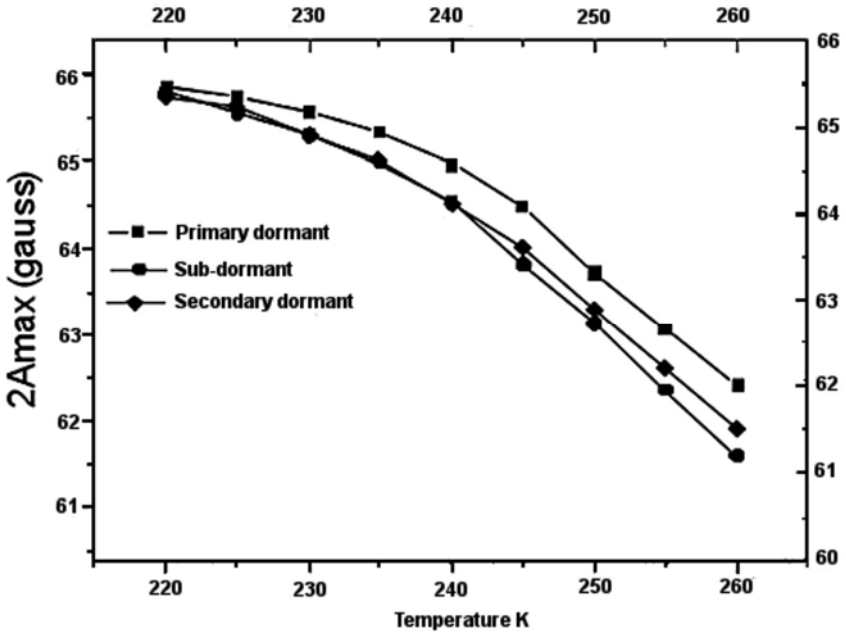

Figure 3. $2A_{max}$ for primary dormant (■), secondary dormant (♦) and sub-dormant (●) seeds of *Sisymbrium officinale* plotted against the temperature. The position of the Y-axis was not the same for all treatments, as the starting point was sometimes different; the scale, however, was the same.
The left Y-axis is for primary dormant seeds, the right Y-axis is for secondary and sub-dormant seeds

although the appearance seems more pronounced in primary and secondary dormant seeds at 290/295K, shown by the slightly larger mobile component, as compared to the immobile component. From 295K onwards, the mobile component is clearly higher than the immobile components (Figure 4B). The two components of the spectra indicate the presence of two types of domains within the membranes: a fluid (mobile) and a solid (immobile) one. Mobility of the spin probe in each domain continued to increase with temperature. Besides that, the population of spin probes in the solid domain gradually decreased.

At 305K the fluid component dominated the spectrum (Figure 5, insert). Although not apparent, there is still an immobile component present. The immobile component did not overlap with the low-field (h_{+1}) and the high-field (h_{-1}) lines of the

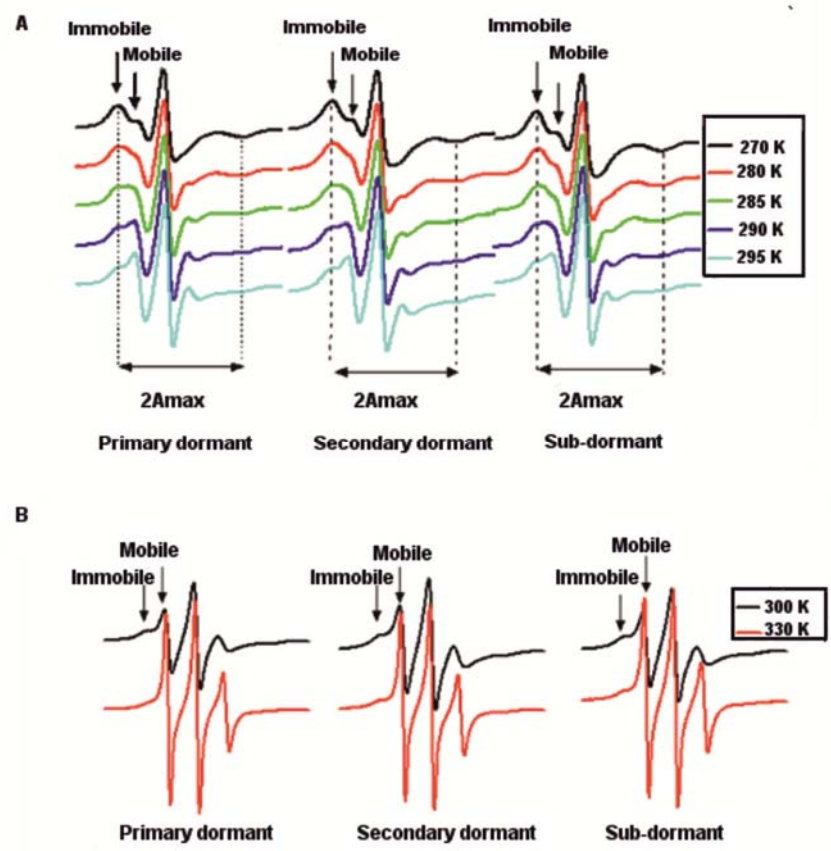

Figure 4 The changes of the spectral shape of the 5-mDS(A) spin probe with an increase in temperature. **A.** The transition from a one-component spectrum to a two-component spectrum occurred within the range of temperatures from 270K to 295 K.
B. Increasing the temperature above 295 K reduced the immobile component whereas the mobile component increased in the range between 300K and 330K.

Results are shown for primary dormant, sub-dormant and secondary dormant seeds of *Sisymbrium officinale***.**

mobile component, and the broadening of these lines, originating from rotational motion of the spin probe, is different for the low-field (h_{+1}) and the high-field (h_{-1}) lines. The high-field and low-field lines can be used to calculate the viscosity in the fluid domains in the membranes (Golovina and Hoekstra, 2002). For that we used the ratio between the low-field and high-field lines as the empirical parameter, which is proportional to viscosity (Kuznetzov *et al.,* 1971). The fluid domains in

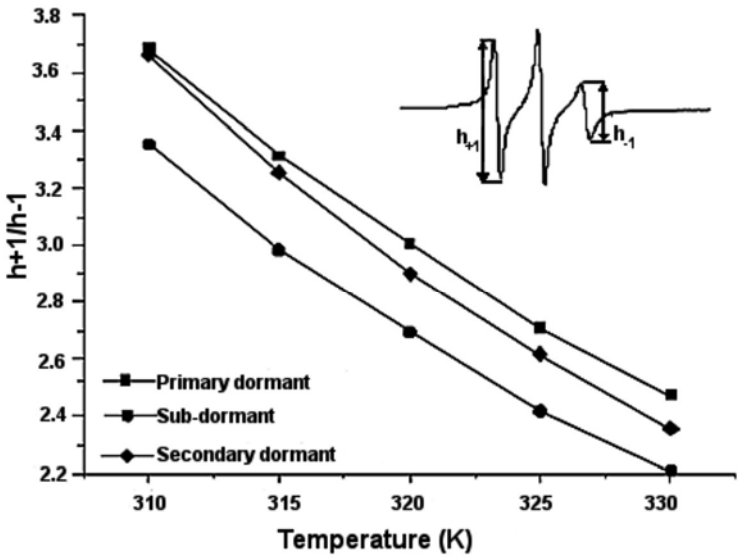

Figure 5: Changes in the ratio of the low-field (h_{+1}) and high-field (h_{-1}) signals (see insert, spectrum at 305K) of the mobile component of 5MESL spectra in seeds with temperature. This ratio is an indicator of the viscosity of the spin probe environment in membranes. Results are shown for primary dormant, sub-dormant and secondary dormant seeds of *Sisymbrium officinale.*

primary dormant seed membranes had the highest viscosity, while in sub-dormant seed membranes the viscosity was the lowest (Figure 5). The viscosity of the membranes of secondary dormant seeds was the same as that of primary dormant seeds at 310 K, however it decreased quicker with temperature but not to the same degree as that of sub-dormant seeds.

The spectra recorded from labelled seeds are the superposition of all spectra from different membranes of different cells within the seeds. If only a small amount of all the cellular membranes is involved in seed dormancy, the dynamic parameters obtained from EPR spectra will not show this because of averaging of the signal. The indirect approach to study the changes taking place in parts of the membrane is to analyze changes in the integrated intensity of the EPR spectrum. This will show the total number of paramagnetic centres responsible for the spectrum.

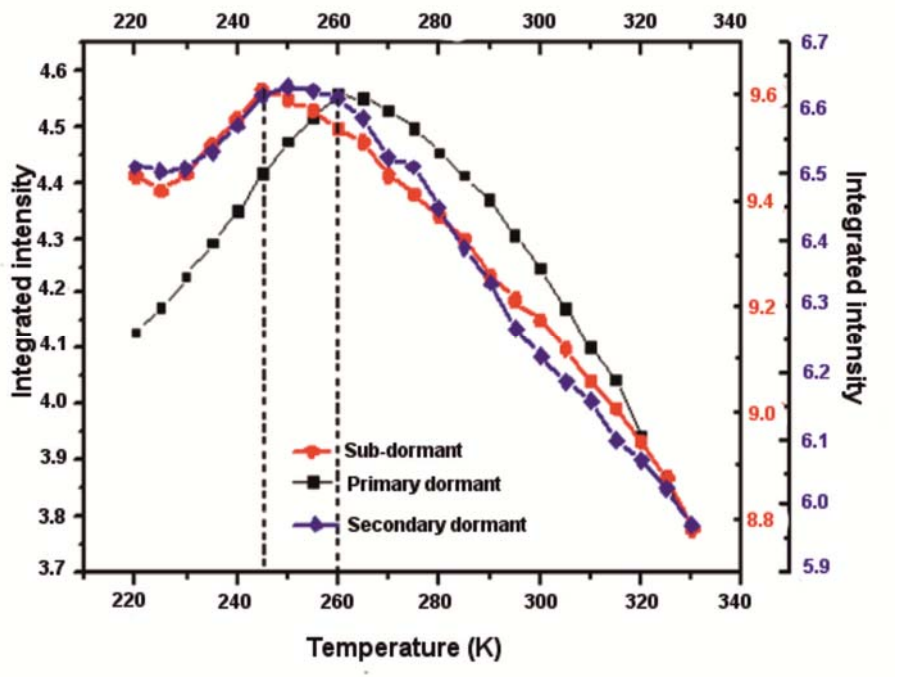

Figure 6: The integrated intensity of the EPR spectra plotted against the temperature. Note that the y-axes of all treatments are on a different scale for easy comparison of the curves.

Results are shown for primary dormant, sub-dormant and secondary dormant seeds of *Sisymbrium officinale.*

Integrated intensity of the spectrum is a cumulative parameter, which is sensitive to changes even in small amounts of cellular membranes (Golovina, *et al.,* 2010).

The intensity of the EPR spectrum is determined by the number of nitroxide radicals in the sample. The intensity of the spectrum can decrease or increase with temperature. The decrease of the signal is caused by chemical reduction of spin label and the increase of the signal is caused by re-oxidation of the reduced forms of spin labelled molecules. At lower temperatures the oxidation is the dominating reaction and at higher temperature the reduction is dominating (E. Golovina, personal communication). The oxidation of the spin label is possibly related to the diffusion of oxygen (Chen and Swartz, 1988). The reduction of the spin label depends on the presence of reducing molecules and on the molecular mobility in the system, which allows molecular diffusion. The integrated intensity of the EPR

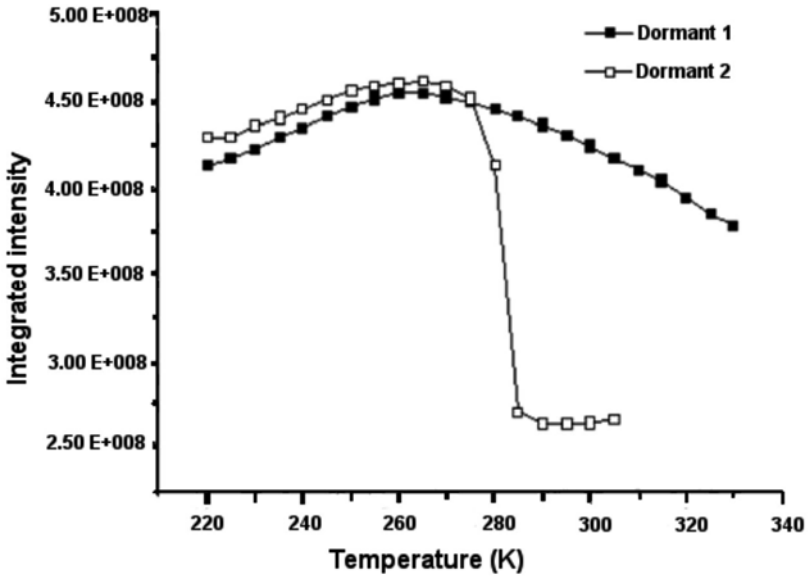

Figure 7: The integrated intensity of EPR spectra plotted against the temperature. Both samples represent primary dormant seeds but dormant 1 are partially hydrated seeds and dormant 2 are excessively hydrated seeds, showing that when bulk water is present there is a sharp decrease in the integrated intensity at 273 K

spectra was plotted against the temperature (Figure 6) and showed that the temperature at which domination of the oxidation of the spin label switched to

domination of the reduction of the spin label was at 245K in sub-dormant and 250K in secondary dormant seeds. However, in primary dormant seeds the switch occurred at a higher temperature (260K). Due to this shift the level of the spectral intensity at higher temperatures was higher for primary dormant seeds than for secondary dormant and sub-dormant seeds.

When bulk water is present a sharp decrease in integrated intensity can be observed at the temperature of water melting at around 273K (Figure 7), which is caused by the absorption of energy by free water present in the cavities and not by

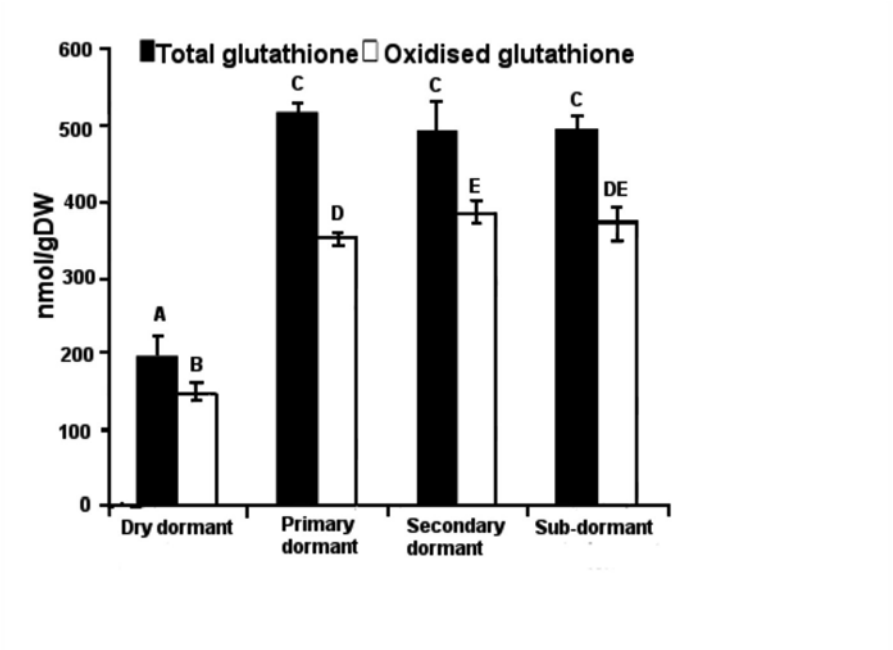

Figure 8: Contents (nmol/g dry weight) of oxidized and total glutathione in primary dormant, sub-dormant, secondary dormant seeds and dormant non pre-imbibed seeds (dry dormant), as measured with HLPC. Error bars indicate standard deviation (n=6). Letters above columns represent significance differences as calculated with t-test.

a sharp reduction of the spin probe. This relates to the deterioration of the Quality factor (Q-factor) of the cavity, which is related to the proportion of energy stored and dissipated within the cavity. We could not detect a sharp decrease in integrated intensity (Figure 6) and therefore we can conclude that there was no bulk water present in the seed samples.

Spin probes can be reduced by different metabolites, including glutathione (Bobko *et al.,* 2007), which is an essential component of the redox status of living cells. Upon imbibition, total and oxidised glutathione contents rose significantly (Figure 8). Primary dormant, sub-dormant and secondary dormant seeds contained similar total glutathione contents. However, oxidised glutathione levels increased from

Sisymbrium	18:1	18:2	18:3	DBI
Primary dormant	32.0	28.3	39.7	2.03
Sub-dormant	34.6	24.5	40.9	2.01
Germinating	34.0	25.2	40.8	2.05

Table 2: Relative content (percentage of total) of unsaturated fatty acid and double bond index (DBI) for Sisymbrium officinale seeds in different physiological states, as calculated from 13C NMR MAS spectra

primary dormant (350 nmol/g dry weight (DW)) to sub-dormant (370 nmol/g DW) to secondary dormant (384 nmol/g DW) seeds, with a significant difference between primary dormant and secondary dormant seeds (p= 0.001).

Fatty acid composition

Membrane fluidity can be adjusted by introducing or reducing the number of double bonds in the fatty-acyl chains of the membrane lipids, making the membrane more or less fluid (Los and Murata, 2004; Sato and Murata, 1980). The unsaturated fatty acid percentage and the double bond index of total fatty acids of primary dormant, sub-dormant and germinating seeds were measured by NMR and calculated. The percentage of 18:1 appeared slightly reduced in primary dormant seeds, while the percentage of 18:2 was slightly elevated when compared with sub-dormant and germinating seeds (Table 2). Percentages 18:3 and DBI did not differ among dormancy states. Because these data are obtained for bulk lipids, i.e. including oil, care has to be taken when membrane fluidity is discussed. Membranes might have fatty acid composition different from oil (Sheffer *et al.,* 1986; Millar *et al.,* 2000; Voelker, 2001). Membrane fluidity can be also modified by sterols and other compounds or membrane-editing by corresponding enzymes (Millar *et al.,* 2000).

Discussion

Membranes have been suggested to be the primary target for temperature perception (Murata and Los, 1997) and membranes and membrane fluidity have often been implicated in the regulation of dormancy and germination (Hilhorst, 1998). Temperature induced changes in membrane fluidity have been proposed to be the primary temperature sensing event (Murata and Los, 1997). However, the non-specific effects of membrane-altering agents used in experiments testing this, coupled with a lack of any evidence for defects in temperature-regulated gene expression in Arabidopsis desaturase mutants, have lead to skepticism that this could be a possible temperature sensing system in plants (Penfield, 2008; Somerville and Browse, 1996).

We determined changes in membrane fluidity in the different physiological states of *S.officinale* seeds. Seeds of this species require a combination of light and nitrate for germination to take place. If the light pulse is not given within a certain time window, seeds become secondary dormant, and will not germinate. The light and nitrate pulses alone cannot break dormancy and induce germination; the seed will need these triggers within a certain temperature window (Karssen, 1982). The present EPR measurements clearly show that primary dormant seeds contained less-fluid membranes than secondary dormant and sub-dormant seeds at low temperatures (Figure 3). When the temperature was increased to 270 K a relatively mobile component appeared in the EPR spectrum, together with the already present immobile component (Figure 4A). In this temperature range membrane fluidity can be estimated by the proportion of mobile and immobile components. The characteristics of the mobile component cannot be properly calculated due to overlapping of the spectra. This proportion seems to be similar for all dormancy types, indicating that at physiological temperatures that are suitable for germination, the membranes for all dormancy types are equally fluid. At temperatures above 300K (Figure 4B) the mobile component becomes the main component in the ESR spectrum. Although these high temperatures are not

physiologically relevant for the seed they might show underlying differences between the dormancy states. At T>300K membrane fluidity can be calculated from the shape of the narrow-line spectrum. (Figure 5, inset). The ratio between the low-field peak and the high-field peak was calculated as a parameter, which relates to the viscosity of the spin label environment. The lesser values of this ratio in the whole range of temperatures above room temperature in sub-dormant seeds suggests that during the transition from the primary dormant to the sub-dormant state, viscosity decreased and, hence, membrane fluidity increased notably. When secondary dormancy was induced membranes became more rigid again at high temperatures. At 310K primary dormant and secondary dormant seeds showed a similar fluidity but at higher temperatures the membranes of secondary dormant seeds remained more fluid than those of the primary dormant seeds. Primary dormant seeds had been imbibed on water for 1d in the dark, while sub-dormant seeds had been imbibed in nitrate for 1d in the dark. The difference in membrane fluidity could thus be an effect of the imbibition medium rather than the different dormancy states. However, secondary dormant seeds appeared to have a less fluid membrane than sub-dormant seeds, while both had been imbibed on nitrate. Secondary dormant seeds have been sub-dormant before becoming secondary dormant due to absence of a light pulse within the required time (Derkx & Karssen, 1993a). This suggests that secondary dormant seeds had a more fluid membrane after 1d when seeds were sub-dormant, but with induction of secondary dormancy the membranes became more rigid again. These results support the membrane hypothesis, which suggests that the changing responsiveness to naturally occurring factors like light and nitrate (Hilhorst, 1990a,b) may indeed be a function of membrane fluidity (Hilhorst, 1998).

The changes in integrated intensity with temperature (Figure 6) give more information about the changes of the spin label within the seeds with temperature that are not visible in the shape of the EPR spectra. The spin label reduction in primary dormant seeds started at a higher temperature than in sub-dormant and secondary dormant seeds. Although the temperatures at which the changes take

place are not physiologically relevant for dormancy and germination they do show the possible differences between the dormancy states.

The reducing reaction depends on the presence and mobility of reducing molecules. Glutathione is assumed to be the main redox compound for most living cells (Gilbert, 1995). A central role for oxidized glutathione in overcoming seed dormancy stages was suggested (Kranner and Grill 1996), as oxidized glutathione accumulation could serve in protecting thiol groups from desiccation induced oxidative injury; desiccation is one of the later developmental stages and may be associated with dormancy. However, the role for glutathione in dormancy alleviation has not been confirmed (Bahin *et al.,* 2011).

Differences in oxidized glutathione levels were small but significant between primary dormant and secondary dormant seeds and could be a sign of a local effect. There were no differences between primary dormant and sub-dormant seeds, indicating that glutathione was not the (only) cause of the reducing reaction within the EPR spectra. This is in contrast with results of Wang and Faust (1994) and Fontain *et al.* (1995) who found that the breaking of dormancy in barley seeds and apple buds was closely related to an increase in reduced glutathione levels; other people, however, were also not able to confirm a role for glutathione in dormancy alleviation (Bahin et al., 2011). Glutathione levels were all measured at room temperature, which is above the temperature where the switch from oxidation to reduction of spin label takes place in primary dormant, as well as secondary dormant and sub-dormant seeds. The location of glutathione (cytoplasm) compared to the location of the 5-mDS(A) spin probe (membrane core) makes glutathione a very unlikely candidate for the reducing reaction. In addition to that, in a dry system with high molecular immobilization the reaction between the two is hardly possible, and there is little to no metabolism at this temperature. Glutathione, or other reducing factors, could still be present in the same amounts in primary dormant, secondary dormant and sub-dormant seeds but in a more immobile state and thus less accessible at a higher temperature in dormant seeds.

These data coincide with the lesser mobility of the spin probe molecules in membranes in dormant seeds at higher temperatures (Figure 5).

Desaturases have often been implicated in membrane fluidity (Sato and Murata, 1980; Cyril *et al.,* 2002) Based on ^{13}C NMR spectra, we found slight differences in 18:1 and 18:2 fatty acids content of lipids between dormant and sub-dormant/germinating seeds. It is possible that the total signal masks the differences in fatty acid composition and double bond index of membrane lipids. The desaturation of fatty acids counteracts the decrease in membrane fluidity (Sato and Murata, 1980). According to the spin-label data, sub-dormant seeds have more fluid membranes in comparison with primary dormant seeds, and therefore an increased DBI of membrane lipids should be expected for these seeds. However, the small differences found in 13C NMR spectra do not support this expectation. Two reasons of such discrepancy can be mentioned. First, the FA composition of (some) seed membranes might be different from that determined for bulk lipids from ^{13}C NMR spectra (Sheffer et al., 1986). Second, other than DBI factors might modify membrane fluidity, such as sterols, flavanoids and other amphiphiles.

In conclusion, particularly primary dormancy was linked to more rigid membranes at low and high temperatures. Membranes became more fluid upon breaking of dormancy. When secondary dormancy was induced, membranes became more rigid at high temperatures but the membrane rigidity of primary dormant seeds was not restored completely. At temperatures relevant for germination the fluidity of membranes does not differ between different physiological states. Desaturation of fatty acids of membrane lipids could be involved in membrane fluidity but changes in desaturation of bulk lipids were so small that they cannot prove this mechanism. The increased fluidity of membranes of sub-dormant seeds may allow the receptors for nitrate and light to become available for binding and allow germination to take place (Hilhorst, 1998), however, at physiological optimal temperatures for germination no major differences in membrane fluidity were

found. As some changes in membrane fluidity are seen at temperatures not relevant for germination the membrane hypothesis described in chapter 1 cannot be completely discarded, however, it is not likely to be the main temperature sensing event that leads to germination. A suggestion how temperature sensing works and eventually leads to germination could be the circadian clock (Penfield, 2008). The circadian clock consists of at least three interlocking transcriptional feedback loops. One of the key features of the circadian clock is their ability to perform robustly across a wide range of temperature regimes. Several genes involved in the circadian clock have also been shown to play a role in promotion of seed germination (Penfield and Hall, 2009).

Chapter 4

Dormancy transitions correlate with altered cytoplasmic properties in seeds of *Sisymbrium officinale*

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Abstract

The regulation of seed dormancy is tightly linked with abiotic stress factors from the environment. Seeds may anticipate a period of adverse conditions by shutting down their metabolism and engaging a stress response in order to protect cellular constituents from irreversible damage. The regulation of dormancy and survival of seeds under conditions that mimic storage in the soil are largely dependent on the composition of the cytoplasm. In this study the colligative properties of cytoplasmic water were attempted to be linked to physiological dormancy states of *Sisymbrium officinale* seeds. Cytoplasmic properties were studied using the spin probe 3-carboxyl-proxyl. Tests on PEG, an osmoticum, showed that primary and secondary dormant embryos did not grow as well as sub-dormant and germinating embryos on higher concentrations of PEG and were, thus, less tolerant to osmotic stress.

Therefore, we conclude that dormancy of *S. officinale* seeds is also, at least partially, located in the embryo.

The 'ease' at which vitrified water melts and the viscosity of the cytoplasm show that dormant and sub-dormant seeds possessed a higher viscosity than the germinating seeds. The viscosity of secondary seeds appeared to be intermediate, but the 'ease' at which the vitrified water melted was similar to that of primary dormant seeds.The higher viscosity of the dormant and non-dormant seed may be a way of the seed to survive prolonged periods in the hydrated state. As a result of the changes in viscosity the temperature of vitrified water melting was lower (265K) for germinating seeds than for primary, sub- and secondary dormant seeds (280K). In conclusion, we have shown, for the first time, that cytoplasmic viscosity may increase upon the induction of dormancy. The observed changes in cytoplasmic viscosity may be linked to changes in metabolism and the changes in vitrification temperature may be linked to changes in the content of high-molecular weight compounds.

Introduction

For a seed to germinate under a range of conditions, or withstand extended periods of drought, excessive heat and frost, it needs to be able to adapt quickly to the environment. Seeds that have already initiated germination cannot stop this process anymore. Therefore, these seeds need to be able to continue germination during changes in temperature, water availability, salinity, etc. For example, a seed can only cope with changes in osmotic potential of the environment if it maintains its ability to take up water and nutrients from the environment. In order to take up water from an environment with low osmotic potential, the water potential of seed cells and tissues has to be even lower (more negative).

For a dormant seed the main environmental changes that take place when buried in the soil are changes in temperature and changes in its water content

(hydration/dehydration cycles). Changes in temperature have a profound influence on the lipid and cytoplasmic properties of the (dry) seed. In general, higher temperature will increase molecular mobility and, therefore, also the 2 or 3 dimensional diffusion of compounds in both membranes and the cytoplasm, at least of the dry seed (Buitink *et al.,* 2000a/b; Chapter 3).

By seasonal sensing, seeds are known to anticipate a stressful period by shutting down their metabolism and engaging a stress response. One of the possible stress responses in seeds is the loss of water from dormant tissue (Pnueli *et al.*, 2002). At reduced water content, many reactions that may have had the potential to damage cells and cell constituents under such adverse physical conditions are suppressed. The stress response, e.g. dehydration, may induce the synthesis of compounds that protect the cells against dehydration damage, such as late embryogenesis-abundant (LEA) proteins (Ingram and Bartels, 1996) and anti-oxidants. The LEA proteins are implicated in stabilizing and protecting cellular structures (Shinozaki et al., 1999) and may also protect cells during the course of de/rehydration (Pneuli *et al*., 2002). The mechanism of dehydration and concomitant synthesis of protective compounds to cope with adverse conditions has been described for buds (Rohde and Bhalerao, 2007), but has been studied mainly in micro-organisms, such as bacteria (Potts, 1994) and yeast (Hohmann, 2002), as well as invertebrates, such as tardigrades and rotifers (Danks, 2000). The link between dormancy and stress has hardly been studied in seeds, although Cadman et al. (2006) have shown that the dormancy and stress responses in seeds of Arabidopsis thaliana are intimately associated at the level of gene expression.

Although intermediate water content may reduce seed longevity (Roberts and Ellis, 1989) another possible survival strategy under frost conditions could be not the loss of water, but vitrification, as opposed to crystallisation of water components (Langis and Steponkus 1990). Vitrification of living tissue, or intracellular glass formation, is generally induced by rapid cooling of cells. However, in seeds intracellular glasses can be formed simply by drying to 10% moisture content, or less (Leopold et al., 1994). It is not clear if we can compare these two forms of

vitrification, as one is a 'water'-glass and the other is a 'non-water'-glass. The complex chemical matrix of seeds prevents crystallization. Raffinose oligosaccharides and late embryogenesis abundant (LEA) proteins appear to be decisive for the occurrence of glass formation rather than crystallization and play a role in glass stabilization (Buitink *et al*., 2000b). A high viscosity glass can inhibit protein unfolding and denaturation and provide protection against heat stress by counteracting the effect of elevated temperature on diffusion in the cytoplasm and protein unfolding (Klimov and Thirumalai; 1997). Vitrification may be necessary but is not sufficient to protect the biological system during desiccation as the glass transition behaviour appears to be identical in desiccation tolerant and desiccation sensitive seeds and pollen and it is assumed that other factors are involved (Sun *et al.*, 1994; Buitink *et al.*, 1996; Sun and Leopold 1997). So far, it is not clear if vitrification is important for the regulation of dormancy.

In this study we have attempted to monitor the physical properties of the cytoplasm in hydrated seeds to link these to the physiological dormancy states. *Sisymbrium officinale* seeds were used, as their dormancy states are clearly distinguishable and easy to manipulate (Hilhorst and Karssen, 1989). The cytoplasmic viscosity and vitrification temperatures of the different dormancy states of the seeds were analysed using Electron Paramagnetic Resonance (EPR). 3-Carboxyl-proxyl (CP) was used as spin probe. CP is a polar molecule which resides mainly in the aqueous environment.

Materials and Methods

Germination

Seeds from *Sisymbrium officinale* were collected in a field in the vicinity of Wageningen, The Netherlands in 2004. Seeds were cleaned, dried at 20ºC to 85 mg water/g dry seed, and stored at 5ºC. Prior to germination, seeds were surface

sterilized in 1% sodium hypochlorite for 1 minute and rinsed with demineralized water for 5 minutes. Triplicates of 30 seeds were sown in 5-cm Petri dishes on two layers of filter paper (Schleicher & Schuell No 595), moistened with 1.5 ml of either water, 25 mM KNO_3 ,or 100 µM GA_{4+7} (Sigma-Aldrich, Zwijndrecht, The Netherlands). Seeds were imbibed for 1 or 10d in the dark at 25°C after which they were irradiated with a saturating red light (620-700nm, Phillips) pulse for 10 minutes or kept in the dark (Hilhorst and Karssen, 1988). After irradiation, seeds were transferred back to the dark at 25ºC. Germination was scored every day after irradiation, for 1 month under green 'safe' light.

Germination on PEG

Seeds were sown on Petri dishes on two layers of filter paper (Schleicher & Schuell No 595), moistened with 1.5 ml of either water, 25 mM KNO_3, or 100 µM GA_{4+7} (Sigma-Aldrich, Zwijndrecht, The Netherlands). Seeds were imbibed for 1 or 10 days in the dark at 25°C. After this pre-incubation the seed coat and endosperm were removed with tweezers and intact embryos were imbibed in different concentrations of polyethylene glycol (PEG) in triplicate, at 25°C with an 8/16h light/dark regime. The growth potential was assessed by measuring the length of isolated embryos daily for 10d.

EPR

Seeds were imbibed in water, 25 mM KNO_3, or 100 µM GA_{4+7} (Sigma-Aldrich, Zwijndrecht, The Netherlands), for 1 or 10 d in the dark. Seed coats were removed and seeds/embryos were then dried at 25°C. Embryo samples were then incubated in 1 mM perdeuterated 3-carboxy-proxyl (CP) spin probe, with ferricyanide unless measuring changes in signal intensity, for 10 minutes, hydrating the seeds completely. After 10 minutes of incubation the embryo samples were removed from the solution, blotted with filter paper and loaded into a 2-mm capillary for spectra recording. EPR spectra were recorded using an X-band EPR spectrometer (Bruker

E500 Elexys CW, Rheinstetten, Germany). To prevent overmodulation and saturation of the EPR signal, microwave power was limited to 5 mW, the modulation amplitude was less than the width of the narrowest lines in the spectra, and the scan range was 100 G. The first spectrum was recorded at room temperature (not shown), after which the CP samples were cooled to 220 K. From then onwards, the temperature was slowly increased. The spectra were recorded at temperatures between 220 and 305 K (+/- 1K) at increments of 5 degrees.

Results

Germination

In *S.officinale* the different dormancy states could be clearly distinguished and were easy to manipulate (see Chapter 2, Figure 1). Seeds imbibed in H_2O failed to germinate both in the light and dark, indicating that these seeds were primary dormant. Seeds required KNO_3 to become sub-dormant, and a light pulse (620-700 nm) of at least 10 minutes to complete germination. The light pulse was required within a certain time window; when applied after 10 days of imbibition in KNO_3, seeds had become secondary dormant and the light pulse was not sufficient to induce germination. Seeds imbibed in 100 µM GA_{4+7} completed germination both in the light and in the dark, which indicates that the light requirement was bypassed. The cytoplasmic properties of seeds of all four dormancy states were studied by EPR, with 3-carboxy-proxyl (CP) as probe.

Germination on PEG

Decoated embryos of primary dormant, secondary dormant, sub-dormant and germinating seeds all showed the same growth in water (Figure 1). However, growth of these embryos on different concentrations of PEG revealed that at more negative osmotic potentials of PEG, primary dormant and secondary dormant

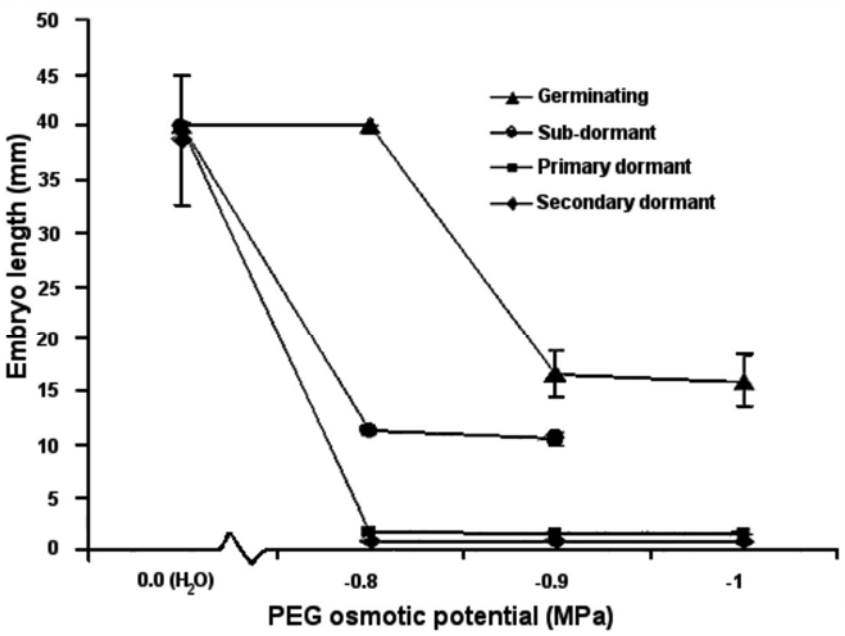

Figure 1: Length (mm) of embryos after incubation for 144h in PEG solutions of different osmotic strength. Seeds were pre-incubated in the dark on 25mM KNO_3 for 1 day (●)(sub-dormant), H_2O for 1 day (■)(primary dormant), 25 mM KNO_3 for 10 days (♦)(secondary dormant) and GA_{4+7} for 1 day (▲)(germinating), before seed coats were removed and seeds were transferred to the PEG medium. Error bars represent standard deviation.

seeds were less resistant to the inhibitory action of PEG than germinating and sub-dormant seeds (Figure 1).

EPR Measurements

3-Carboxy-proxyl spectra

For characterization of the water fraction in hydrated seeds in different physiological states, seeds were labelled with perdeuterated 3-carboxyl-proxyl (CP). CP is a relatively small (MW 186) molecule that is easily soluble in water. It is a polar molecule, due to its OH group. This group increases the probability to form hydrogen bonds and, thus, CP resides mainly in the aqueous cytoplasm. All seed

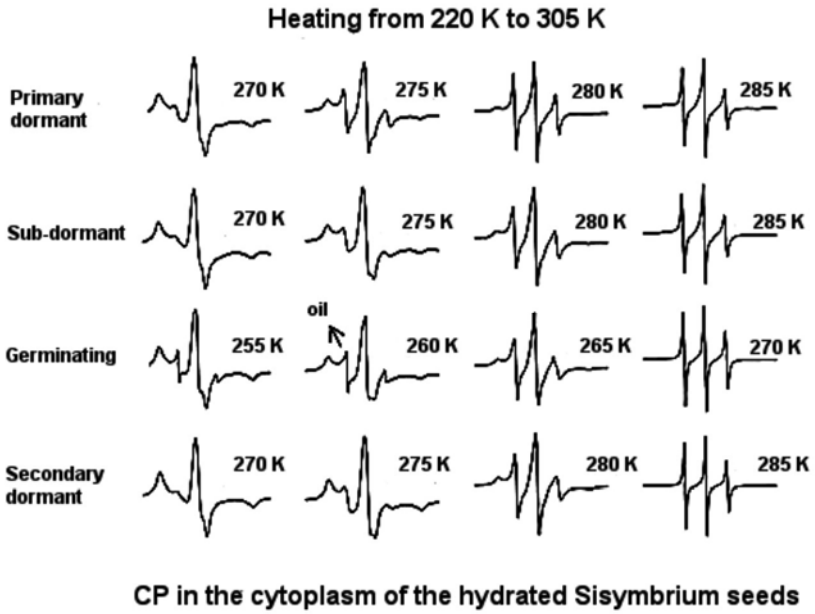

Figure 2: CP Spectra of *Sisymbrium officinale* seeds in different dormancy states. Seed samples are slowly heated from 220 K to 300K. The melting of vitrified water occurred at 280 K in dormant, seco. ndary dormant and sub-dormant seeds. Vitrified water melted at 265 K in germinating seeds

samples were cooled to 200 K and then slowly heated. The spectra were recorded from 220 K to 305 K with 5-degree steps.

At low temperatures all the spectra showed vitrified water. Above 260 K (germinating seeds) or 275 K (dormant and sub-dormant seeds) part of the water appears in a liquid state as can be seen by the appearance of narrow-line spectra (Figure 2). Complete melting of vitrified water occurred at about 280 K, in dormant, secondary dormant and sub-dormant seeds; in germinating seeds melting of vitrified water occurred at around 265 K. The narrow component in the spectra of germinating seeds below 265 K originates from CP in oil (Figure 2). This can be concluded from the distance between narrow lines, which are typical for hydrophobic environment.

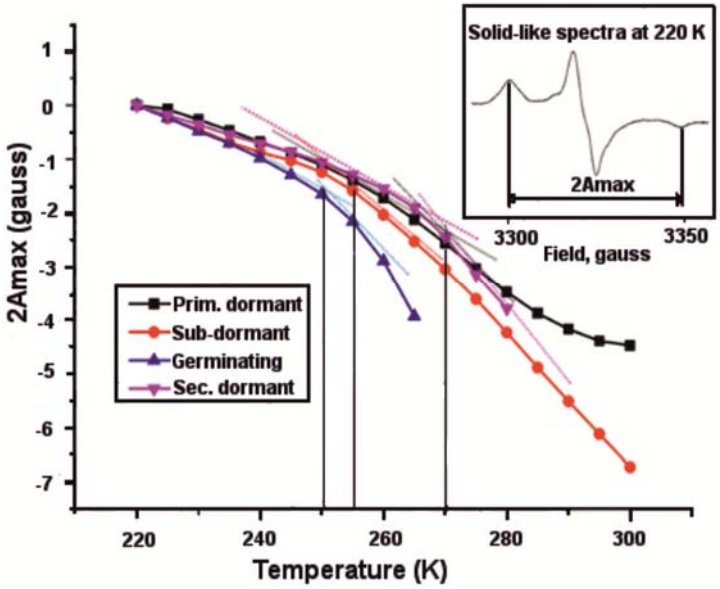

Figure 3: Changes of the distance between the outermost extremes, $2A_{max,}$ of the immobile (solid-like) component of CP spectra in *Sisymbrium officinale* seeds in different dormancy states, with temperature. Breaking points of curves are indicated by the dotted lines.

Motional freedom, within the solid-like spectra, as measured by the distance between the outermost extremes ($2A_{max}$) can be used as a tool to measure the 'ease' at which vitrified water melts (Kumler and Boyer, 1976) ($2A_{max}$ is determined only for solid-like spectra; see chapter 3). Plotting $2A_{max}$ against temperature will show a linear temperature dependency of $2A_{max}$ due to increase of the motional freedom. However, at a certain temperature, there is a break in the temperature dependency, as from this break the rate of changes of the motional freedom with temperature increases (Kumler and Boyer, 1976; Buitink *et al,* 1998; Buitink *et al*, 2000). The break occurred at 270K for primary and secondary dormant seeds, at 255Kfor sub-dormant seeds and at 250K for germinating seeds. Figure 3 shows that vitrified water melted much easier in germinating seeds than in any other seeds.

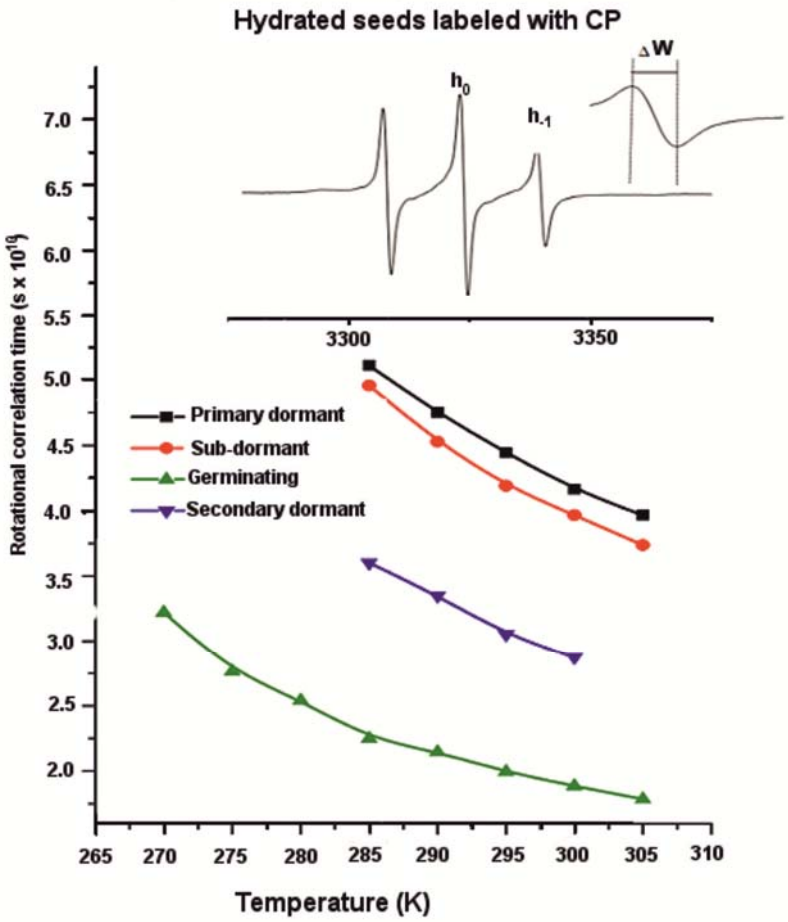

Figure 4: Rotational correlation time of CP in the cytoplasm of *Sisymbrium officinale* seeds in different dormancy states at different temperatures

The narrow line spectra of CP in aqueous cytoplasm can be used to calculate the rotational correlation time for CP molecules according to the formula:

$T_R = 6.5 \times 10^{-10} \Delta W_0 ((h_0/h_{-1})^{1/2}-1)$

in which h_0 and h_{-1} are the heights of the central and high-field lines (Figure 4A) and ΔW_0 is the peak-to-peak width of the central line. The rotational correlation time is proportional to the viscosity (Keith and Snipes, 1974). In Figure 4 the rotational correlation time of CP in the cytoplasm at temperatures above the melting temperature for all 4 seed samples is shown. The τ_R and, thus, cytoplasmic viscosity were high in primary dormant and sub-dormant seeds, and low in germinating seeds. In the secondary dormant seeds the viscosity had intermediate values.

Discussion

3-Carboxy-proxyl (CP) was used in an EPR study to investigate the colligative properties of cytoplasm water in seeds in different dormancy states, as it can give information about how seeds adapt their cytoplasmic properties to periods of dormancy. *Sisymbrium officinale* seeds were used for this, as their dormancy states are clearly distinguished and easy to manipulate (Hilhorst and Karssen, 1989). Dormancy can be broken by supplying the seeds with nitrate and a light pulse within a certain time frame. When the light pulse is suspended, seeds become secondary dormant and will not germinate. When the seed coat (and endosperm) is removed, embryos will grow on water, even when pre-incubated to be dormant. This could be an indication that *Sisymbrium officinale* seeds have a seed coat/endosperm imposed dormancy (Bewley, 1997). However, tests on PEG, an osmoticum, showed that primary and secondary dormant embryos did not grow as well as sub-dormant and germinating embryos on higher concentrations of PEG (Figure 1) and were, thus, less tolerant to osmotic stress. Therefore, we conclude that dormancy of *S. officinale* seeds is also, at least partially, located in the embryo. This is the result of differences in the base water potential for germination, which is higher (less negative) in dormant seeds, as compared to non-dormant

seeds (Bradford, 2005). This implies that the seed coat/endosperm imposes a restriction on embryo growth, and the dormant embryo itself cannot gain sufficient force to overcome this restriction. To enable the seed to germinate and grow in an environment with a low osmotic potential (more negative) it needs to enhance the water potential of lots of cells to be able to take up water. It was suggested by Nabors and Lang (1971) that germination stimulating factors cause an increase of the osmotic potential of the embryonic axis, and therefore increase the water-absorbing capacity of the seed. The osmotic potential of the embryonic axis could decrease by an increase in the concentration of low-molecular weight substances. The low molecular weight substances will decrease the viscosity of the cytoplasm and the temperature of water vitrification. Here we studied the viscosity of the cytoplasm and the vitrification temperature of the seed, at different states of physiological dormancy.

The ‘ease’ at which vitrified water melts (Figure 3) and the viscosity of the cytoplasm (Figure 4) show that dormant and sub-dormant seeds possesed a higher viscosity than the germinating seeds. The viscosity of secondary seeds appeared to be intermediate, but the ‘ease’ at which the vitrified water melted was similar to that of primary dormant seeds. This difference in cytoplasmic viscosity could be caused by a change in presence of high-molecular weight substances. Cytoplasmic viscosity could be of importance in the dormancy of the seed. At high viscosity the seed is protected against protein unfolding (Klimov and Thirumalai, 1997) and heat stress due to reduced diffusion within the cytoplasm. The higher viscosity of the dormant and non-dormant seed may be a way of the seed to survive prolonged periods in the hydrated state. The high viscosity in the dormant state was also found by Dijksterhuis et al. (2007), who studied fungal spores. However, they came to the conclusion that the higher viscosity was not mainly caused by a high concentration of solutes but by the high proportion of ordered spin probe molecules in the vicinity of macro-structures. Sun (2000) linked reduced metabolism in red oak seeds to an increased viscosity. In Chapter 2 we found a

reduction in metabolic activity for dormant and non-dormant seeds, as compared to germinating seeds, which may be linked to the increased viscosity found here.

As a result of the changes in viscosity the temperature of vitrified water melting was lower (265K) for germinating seeds than for primary, sub- and secondary dormant seeds (280K) (Figure 2). The lower vitrification and melting temperatures for germinating seeds could be due to the presence of higher concentrations of low-molecular weight substances, as these are known to lower the vitrification temperature (Wolfe and Bryant 1999). The low-molecular weight substances could originate from the mobilisation of storage metabolites. While in non-dormant and dormant seeds the higher molecular weight substances (e.g. raffinose family of oligosacharides, sucrose, trehalose etc.) are more dominant (Kou et al, 1988; Downie and Bewley, 2000) in germinating seeds high molecular weight substances are hydrolysed into more low-molecular weight substances (e.g. glucose, fructose) (Downie and Bewley, 2000). These low-molecular weight substances increase the osmotic potential (make it more negative) and, therefore, the water absorbing capacity of the seed (Nabors and Lang, 1971).

In conclusion, we have shown, for the first time, that cytoplasmic viscosity may increase upon the induction of dormancy. The better water absorbing capacity of germinating seeds may be caused by hydrolysis of storage metabolites into low molecular weight compounds which will then cause a decrease in vitrification temperature. Even though it is not clear if this is the main cause for a change in viscosity of the cytoplasm, the increased cytoplasmic viscosity in the dormant state may contribute to a long-term maintenance of seed viability. However, it is not clear yet if the increase in cytoplasmic viscosity is the result or the cause of reduced metabolic activity within the seed.

Chapter 5

Involvement of desaturases in membrane fluidity and dormancy cycling in seeds of *Arabidopsis thaliana*

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Abstract

Dormancy cycling in seeds depends on changes in sensitivity or responsiveness to naturally occurring factors that are known to break dormancy or stimulate germination. Receptors for these factors are likely to occur in membranes and based on that, a model was proposed in which regulation of dormancy depends on accessibility of membrane-bound receptors. Accessibility of membrane-bound receptors can change by changing the membrane fluidity. Membrane fluidity is largely determined by composition of unsaturated fatty acids in the membrane phospholipids, which is a function of temperature and activity of desaturases. Here we tested the hypothesis that unsaturated fatty acids and desaturase gene activity

are involved in the induction of secondary dormancy by changing membrane fluidity. To test this hypothesis Arabidopsis thaliana desaturase mutants *fad3, fad7, fad8, ads1* and *ads2* and wild type seeds were used. All mutants were characterised in their dormancy phenotype. Membrane fluidity and fatty acid composition were analysed when enough seeds where present, whilst the expression of desaturase genes was analysed in wild type seeds.

Whilst ADS2 seems to have an influence on membrane fluidity and is expressed in non-dormant seeds, it does not show any changes in dormancy phenotype; this may be due to functional redundancy. FAD3 activity shows changes in relation to dormancy and germination but it is not involved in changes in membrane fluidity. Although desaturase activity may change the membrane fluidity or influence the germination/dormancy phenotype, the two are not linked, unless the effects of these enzymes are very localised within the seeds.

Introduction

Dormancy cycling in seeds of *Arabidopsis thaliana* and *Sisymbrium officinale* depends on changes in sensitivity or responsiveness to naturally occurring factors that are known to break dormancy or stimulate germination, such as light and nitrate (Hilhorst, 1990 a/b). *Sisymbrium officinale* seeds have an absolute dependency on a combination of light and nitrate to terminate dormancy and initiate germination (Hilhorst *et al.,* 1986, Hilhorst, 1990a, b). In *Arabidopsis thaliana* light, but not nitrate, is an absolute requirement for germination (Derkx and Karssen, 1994). There are several possible mechanisms that explain how responsiveness to light and nitrate can change; two possible explanations suggest that (1) either the amount of active receptors or (2) the accessibility of their ligands changes with the induction and relief of dormancy (Raison *et al*., 1980; Di Nola and Mayer, 1986; Hilhorst, 1998). Receptors for light (through phytochrome) and nitrate are likely to occur in membranes and with that in mind Hilhorst (1998) proposed a

model in which regulation of dormancy cycling depends on accessibility of membrane-bound receptors (chapter 1). In this model the magnitude of movement of receptor proteins within the membrane depends on membrane fluidity. A higher fluidity may cause the receptor to move to the membrane surface, where it then becomes accessible for nitrate and phytochrome. Membrane fluidity is largely determined by the content of unsaturated fatty acids in the membrane phospholipids, which on its turn is a function of temperature and activity of desaturases. Thus, the breaking and inducing of dormancy may be achieved by changes in temperature that induce an increase or decrease of fatty acid unsaturation.

A downward shift in temperature results in a decrease in membrane fluidity, whereas an upward shift may cause fluidization of the membrane (Los and Murata, 2004; Vigh, 1998). Decreased/increased membrane fluidity can cause extensive damage to the membranes; therefore membranes are able to adjust to the new conditions to maintain their fluidity. This is called homeoviscous adaptation (Sinensky, 1974). Membrane fluidity can be adjusted at low temperature by introducing double bonds into the fatty-acyl chains of membrane lipids, making the fatty acids more unsaturated and, hence, the membrane more fluid (Los and Muratta, 2004). The introduction of double bonds in fatty acids is mediated by desaturases. Following an upward shift in temperature, the degree of unsaturation of fatty acids of membrane phospholipids may again decrease, by suppression of the desaturation of fatty acids and acceleration of *de novo* synthesis of saturated fatty acids, thereby diluting the unsaturated fatty acids (Sato and Murata, 1980).

There are three types of desaturases: Acyl-CoA desaturases, Acyl-ACP desaturases and Acyl-lipid desaturases (Murata and Wada, 1995). Acyl-CoA desaturases introduce double bonds into fatty acids that are bound to Coenzyme A, and are only found in animal, fungal and yeast cells. Acyl-ACP desaturases can introduce the first double bond into plant fatty acids that are bound to an Acyl-carrier protein (ACP), a reaction which occurs in the plastids of plant cells (Murata and Wada, 1995). All double bonds can be introduced by Acyl-lipid-desaturases

into fatty acids that have been esterified to glycerolipids, bound to the endoplasmatic reticulum or chloroplast membrane (Murata and Wada, 1995). Since the Acyl-lipid desaturases introduce double bonds directly into the fatty acids of membrane lipids they are most efficient in regulating unsaturation in response to temperature changes (Murata and Wada, 1995). Acyl-lipid desaturases introduce double bonds at specific sites within the carbon chains. The order in which these desaturases operate is very strictly determined: the first double bond is introduced by the ∆9 desaturase (an Acyl-ACP- or Acyl-lipid-desaturase), introducing a double bond in the 9th position of a $C_{18,}$ fatty acid converting stearic acid (18:0) into oleic acid (18:1) (Murata and Wada, 1995). The second double bond is introduced by a ∆12 or ∆6 desaturase (Acyl-lipid-desaturases), introducing a double bond in the 12th position of oleic acid (18:1) converting it into linoleic acid (18:2). This double bond is only introduced into fatty acids that have a double bond at the ∆9 position. The third and last double bond is introduced by an ω3 or ∆15 desaturase (Acyl-lipid-desaturases), converting linoleic acid (18:2) into linolenic acid (18:3) (Matos *et al*., 2007). This desaturase only introduces double bonds into fatty acids that have a double bond at the ∆12 position (Hagashi and Murata, 1993).

The aim of the present work was to assess the effects of mutations in desaturase genes on the induction of secondary dormancy. To test the hypothesis that membrane desaturases are involved in the induction of secondary dormancy, we used *Arabidopsis thaliana* fatty acid desaturase (FAD) mutants and wild type seeds as control. Here we used the *fad3*, *fad7*, *fad8*, *ads1* and *ads2* mutants. The *fad3, fad7* and *fad8* mutants are mutated in the ω3 desaturase genes (Murata and Wada, 1995), whereas *ads1* and *ads2* are mutated in a Δ9 desaturase gene (Murata and Wada, 1995). The *fad3*, *fad7*, *fad8*, *ads1* and *ads2* mutants were characterised in their dormancy phenotype whilst the expression of desaturase genes was analysed in wild type seeds.

Materials and Methods

Seed germination and plant growth to obtain higher quantity of seeds

Seeds were obtained from the Arabidopsis Biological Resource Centre (ABRC). Wild-type and *fad3*, *fad7*, *fad8*, *ads1*, *ads2* mutants were all from the Columbia genetic background. Of all seeds 2 different mutant lines in the same gene were ordered, if available. Seeds were sterilized for 1h using a gas sterilizer containing bleach and 1.2% HCl. Seeds were sown on 0.5 MS medium containing 2.3 g /l vitamins, 0.8 % purified agar (Duchefa) pH = 6.0, 1.95 g/l MES hydrate (Sigma) and 10 mg/l kanamycin. Petri dishes containing medium with seeds were kept at 4°C for 3 days. After 3 days dishes were transferred to 24°C under a 16h/8h light/dark regime. After seeds had germinated and plants were big enough they were transferred to the ARASYSTEM (www.arasystem.com) and kept at 24°C (16h/8h, light/dark).

Seed germination

Freshly harvested wild type and mutant seeds were stratified at 4°C for 3 days to break any dormancy present. After stratification seeds were irradiated with red light for two hours and transferred back to the dark, at 5, 15, 20 and 25°C. Secondary dormancy was induced after stratification at 4°C by incubating the seeds in the dark for 10 days at a range of temperatures, before the light pulse was given and seeds were germinated at the same temperature.

Isolation of genomic DNA

100-200 mg young leaves were collected in an Eppendorf tube and frozen in liquid nitrogen. Leaf material was ground to a fine powder. DNA extraction buffer (0.3 M

NaCl, 50 mM Tris (pH 7.5), 20 mM EDTA, 2% Sarkosyl, 0.5% SDS, 5 M Urea and 5% (v/v) Phenol pH 7.5) was added and samples were refrozen and ground again. A phenol/chloroform extraction was performed; samples were mixed gently and spun at 12.000 rpm for 5 minutes. DNA was precipitated with iso-propanol and kept at room temperature for 5 minutes, then washed with 70% ethanol and dried briefly. DNA pellets were dissolved in TE buffer containing 10 µg/ml RNAse and stored at 4°C.

RNA isolation from mutant seed material and gene expression analysis for detection of homozygous plants

30 mg of seed material was collected per sample and RNA was isolated using the SV Total RNA isolation system (Promega) according to the manufacturer's instructions. cDNA was synthesized, using iScript^{tm} (Biorad), from 1 µg total RNA, in a mixture containing RNase H^+ iScript reverse transcriptase, RNase inhibitor, oligo (dT) and random hexamers (used as random primers). The reaction protocol was 5 min at 25ºC, 30 min at 42ºC followed by 5 min at 85ºC and hold at 4ºC. For detection of homozygous mutant plants and gene expression analysis of mutant plants quantitative real time PCR (QRT-PCR) was performed using the Bio-Rad system (iCycler iQ Real Time PCR detection device). The iQ Sybr Green Supermix (BioRad) was used, containing SYBR green I dye, hot-start iTaq DNA polymerase, buffer and dNTPs qualified for quantitative PCR. The Supermix also contained fluorescein used for the correction of the readings and 5 µl of cDNA. Primers used are listed in Table 1 (for detection of homozygous plants) and Table 2 (for expression analysis). The standard thermal profile used was 3 min at 95ºC, followed by 45 cycles of 15s at 95ºC, 1s at 60ºC and 2 min at 72ºC.

Gene symbo	AGI ID	T-DNA line	Forward (5'→3')
			Reverse (3'→ 5')
FAD3	AT2G29980	SALK_042036	AATTATACCAGTCGTGGCCG
			TTTTTCTTTTATTGCGAGTTTGTC
FAD7	AT3G11170	SALK_049635	AACAGTGGAGCAAGAGTCAGG
			ACAGAACTCACCACCAGAACC
		SALK_096415	CAAAGACGATAGCGACGTCT
			TGGT CAACTAGTTGGGTTTG
FAD8	AT5G05580	SALK_137876	GGAAGGTTTAGCACACCATATTG
			AGAATGACGAATCATGGCATC
		SALK_093590	GTAGGCAGATGAAGCATGAGG
			GACCAAAGAGAGCAACCCTTC
ADS1	AT1G06080	SALK_044895	TTCAAAGGGACATCGTCAATC
			GGCAGTTGGTTCAGGTACCTC
		SALK_069299	CCACATTTGTCCTTCTTCCAC
			TTAAGAGTCGCCCTCATTGTC
ADS2	AT2G31360	SALK_016783	AGTGTGGAAGAAGAGCAAACG
			TTTCTTCTTTTATCGTAAACGACG
		SALK_079963	GAGCTGTCTCCAATTCATGTG
			TCATTCTCTTGCTCTCTTGGC

Table 1: Primers used for detection of genomic DNA

Gene symbol	Forward (5'→ 3')
	Reverse (3'→ 5')
FAD3	AAGAAGAAAGGTTTGATCCGAGTGC
	TGGCCCAGAAAAGTGTTCCTTGG
FAD7	TGAACAGTGTGGTCGGTCAT
	GCATCACGAGAGGCAGTGTA
FAD8	CTGAGAGGAGGGCTCACAAC
	GAAGTGGCAGAGGTCCAGAG
ADS1	GGAGAATAACAAGAAAATGGCAGCG
	TTCGGTGGTAAGAGACGGTGATACC
ADS2	TTCATT TCTTGCTCTCTTGGCTCC
	TCAATCGGATCTCCCTGAATAGCG

Table 2: Primers used for gene expression analysis

RNA isolation from wild type seeds for analysis of fatty acid desaturase gene expression

Following treatment, seeds were stored in 2-ml tubes at -80°C. Total RNA was extracted using a hot borate method (modified from Wan and Wilkins, 1994). Seeds were ground with mortar and pestle, submerging the mortar and pestle in liquid nitrogen before grinding. Ground seeds were kept submerged in liquid nitrogen prior to RNA extraction. Ground seeds were re-suspended in 700 µl extraction buffer [(0.2M sodium tetraborate decahydrate, 30 mM EGTA, 1% w/v SDS, 1% w/v sodium deoxycholate, pH 9.0) containing 14 mg polyvinylpyrolidone and 1.1 mg dithiothreitol], which had been heated to 80°C. The seed suspension was added to 0.35 mg proteinase K in a 2 ml round-bottomed tube and incubated at 42°C for 90 min. 56µl 2M KCL was then added and samples incubated on ice for 1h. Samples were centrifuged at 16000 g at 4°C, after which the supernatant was transferred to a new tube and its volume determined. A one-third volume of 8M LiCl (to give a final 2M LiCl concentration) was added and samples were incubated overnight at 4°C. Samples were centrifuged, and pellets washed by vortexing in 2M LiCl until the supernatant remained colourless. Pellets were re-suspended in 300 µl diethylpolycarbonate (DEPC) treated water, and RNA precipitated by the addition of 30 µl 2M potassium acetate and 990 µl ice-cold 100% ethanol, and incubation overnight at -80°C. RNA was pelleted by centrifugation at 16000 g for 30 min at 4°C, followed by a wash with 70% ethanol. Partially dried pellets were re-suspended in 50µl DEPC-treated water. RNA concentration and quality were determined using a WPA lightwave s2000 UV/Vis spectrophotometer.

QRT-PCR of wild type seeds

Total RNA from 3 biological replicates per treatment were used. cDNA was synthesized using a transcriptor first strand cDNA synthesis kit (Roche), according to the manufacturer's instructions. Primers were used as described in Table 2. PCR reactions were performed using LightCycler FastStart DNA master SYBR

green I (Roche), in a mixture containing 5 µl cDNA in a volume of 25 µl. The thermal profile used was 10 min at 95ºC, followed by 45 cycles of 1 min at 95ºC, 2 min at 60ºC and 1 min at 72ºC. Comparative Ct values were calculated and ANOVA tests were used to statistically analyse the data.

Nuclear Magnetic Resonance Spectroscopy (NMR)

The NMR spectra were recorded on an Avance II spectrometer (Bruker, Rheinstetten, Germany), as described in chapter 3. Dry *Arabidopsis thaliana* seeds with seed coats and without additional treatments were packed into a 7 mm Zirconia rotor and spun under a magic angle at spinning speed of 5 kHz. ^{1}H MAS spectra were recorded at room temperature with a spectral width of 6 kHz applying single-pulse sequences with a 10 µs rf pulse and 5 s repetition delay between scans. Time domain size of the spectra was 8 k with a number of accumulations of 32. The narrow range of proton chemical shifts makes it difficult to identify individual fatty acids.

^{13}C MAS single-pulse excitation spectra were obtained and analysed as described in chapter 3, and was used for analyses of the fatty acid (FA) composition of the mutants and wild type.

Electron Paramagnetic Resonance

The methyl ester of 5 doxyl stearic acid (5-mDS(A)) was used as a spin probe. This spin probe is weakly anchored in the phospholipid head group area due to the high hydrophobicity of the methyl ester. As a result, methylated spin label is localized in a deeper position in the membrane bilayer than its un-methylated counterpart (5-DS(A)) (Sanson *et al*., 1976). 5-mDS(A) is often used to study membrane fluidity (Benatti *et al*., 2001; Bianconi *et al.,* 1988; Turchiello *et al*., 2000; chapter 3). Arabidopsis seeds were imbibed in water for 8h in the dark. Medium attached to the surface of the seed was removed with filter paper before seed coats were

removed using tweezers, after which seeds were dried at room temperature. Dry seeds were placed in a 1mM solution of membrane spin probe in hexane. After 1d the spin probe solution was removed, seeds were washed twice with hexane and placed at 30% RH for 24 hours to remove the remaining hexane from the seeds. Seeds were re-hydrated by humidification for 3h at 100% RH.

EPR spectra were recorded with an X-band EPR spectrometer (Bruker E500 Elexys CW, Rheinstetten, Germany). To prevent over-modulation and saturation of the EPR signal, microwave power was limited to 5 mW, the modulation amplitude 3G for solid-state and 1G for fluid type spectra. In the case of 2-component spectra the lowest modulation amplitude of 1G was used. Field scan widths of 100G were used.

Results

Identification of homozygous T-DNA insertion mutants

Nine SALK T-DNA lines (Alonso *et al*., 2003) representing T-DNA insertions in 5 desaturase genes were obtained from the ABRC (Table 1). Seeds from the homozygous mutant plants were tested for expression of the desaturase genes using qRT-PCR, and none showed significant expression (data not shown).

Phenotypic analysis

Phenotypic analysis did not show appreciable differences between wild type and most mutant plants. However, the fatty acid desaturase 7 mutants (*fad7*; Salk_096415) showed slow growth. Plants were lagging 2 weeks behind in development and flowering. No differences in seed and seed coat structure could be observed.

Arabidopsis thaliana	**18:1 %**	**18:2 %**	**18:3 %**	**DBI**
Wild type	39,3	41,3	19,4	1,68
ads1	43,9	36,1	20,1	1,73
ads2	38,1	43,9	18,0	1,67
fad3	41,6	51,9	6,5	1,51

Table 3: Relative unsaturated fatty acid composition and double bond index for Arabidopsis thaliana wild type and mutant seeds as calculated from ^{13}C NMR MAS spectra

Fatty acid composition

The fatty acid composition (of the seed oil) of wild type and of the *ads2* and *ads1* mutants was comparable (Table 3). However, the *fad3* mutants [lacking the ω3 desaturase, responsible for converting linoleic acid (18:2) into linolenic acid (18:3)] showed a reduced 18:3 percentage and, likely as a consequence, an increased 18:2 content, resulting in a reduced double bond index of *fad3*. Fatty acid contents of *fad7* and *fad8* mutants could not be established due to seed shortage.

Membrane fluidity

A methyl ester of 5 doxyl stearic acid (5MESL) was used as a spin probe to give information about the average fluidity of the membranes (Benatti *et al*., 2001; Bianconi et al., 1988; Turchiello *et al.*, 2000). The 5-mDS(A) spectra in Arabidopsis, at lower temperature (T<270 K), showed that the membranes were in the solid state. The mobility of the label in the solid membrane matrix can be characterized by $2A_{max}$ (Chapter 3, this thesis). The decrease of $2A_{max}$ with increasing temperature is caused by the increase of label mobility (Figure 1). Wild type seeds and *fad3* mutant seeds showed the same temperature dependence of $2A_{max}$ despite the fact that the fatty acid composition differed most between these two samples. Membrane fluidity of ads 1 was the highest and of ads 2 was the lowest in spite of roughly similar to the wild type FA composition and DBI (Table 3).

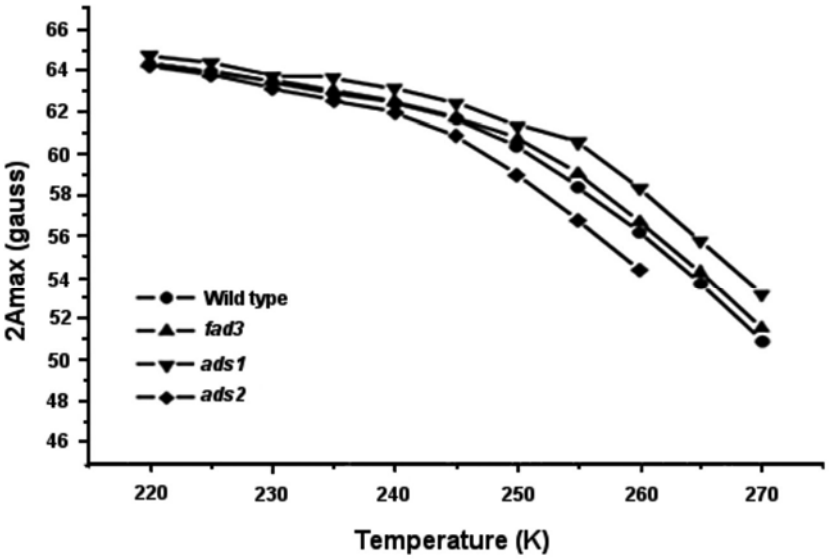

Figure 1: The temperature dependence of 2Amax values of 5-mDS(A)spectra from hydrated de-coated wild type, ads1, ads2 and fad3 seeds. 2Amax is a measure for membrane ordering. With increasing temperature the membrane ordering will be less and, hence, 2Amax becomes lower. ads1 mutant shows the highest membrane ordering and ads2 mutant shows the least membrane ordering. The degrees of membrane ordering in wild type and fad3 mutant are intermediate and do not significantly differ from each other.

At higher temperatures the ratio between h_0 and h_{-1} can be used as an estimate of the membrane viscosity (Golovina and Hoekstra, 2002; insert Figure 2). Here we observed again that wild type and *fad3* mutant seeds had a comparable viscosity, even though the fatty acid composition differed. The *ads2* mutant seeds had a much lower viscosity than all other seeds (Figure 2). The *ads1* mutant seeds showed an intermediate viscosity to wild type and *ads2* mutant seeds. The *fad7* and *fad8* mutants were not analysed by EPR due to shortage of seeds.

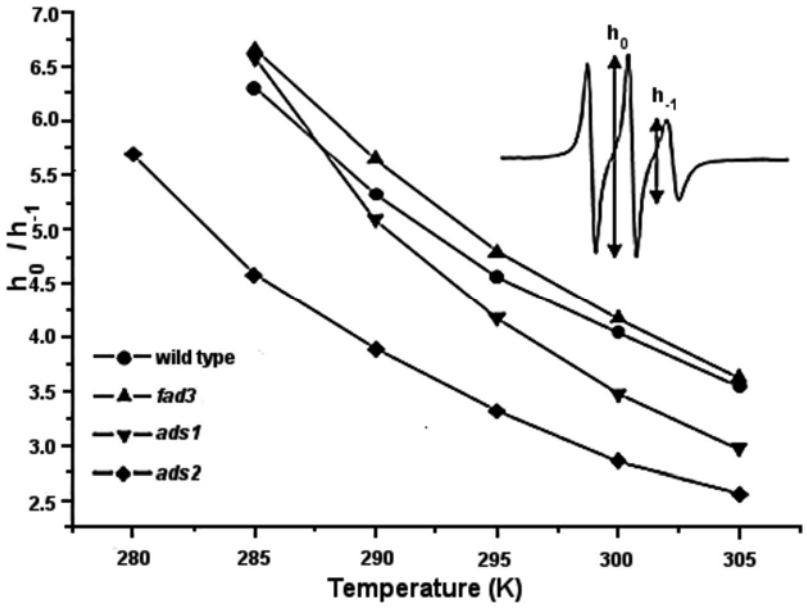

Figure 2: The temperature induced changes in membrane fluidity of hydrated wild type and mutants ads1, ads2 and fad3. Membrane fluidity is expressed as the ratio of the central line height (h_0) to the low-field (h_{-1}) line height of 5-mDS(A) spectra (insert). The *fad3* and wild type seeds show a comparable viscosity, *ads2* mutants show a much lower viscosity, while *ads1* mutant seeds show an intermediate viscosity

Germination

Freshly harvested wild type Columbia seeds were pre-incubated at 4°C for 3 days to break any dormancy present. After pre-incubation seeds were irradiated with red light for two hours and transferred back to the dark, at 5, 15, 20 and 25°C. Wild type seeds germinated up to 100% (Figure 3A) at all temperatures. From the t_{50} values (Figure 3B) it can be seen that seeds reached half maximum germination significantly earlier, as confirmed by t-test, at 20 and 25°C than at 5 and 15 °C (p-value: 0.0000008 and 0.009), implying that 20 and 25°C are the more optimal temperatures for germination than 5 and 15°C for this Arabidopsis accession. At 5°C maximum germination was achieved only after 24d, at the other temperatures

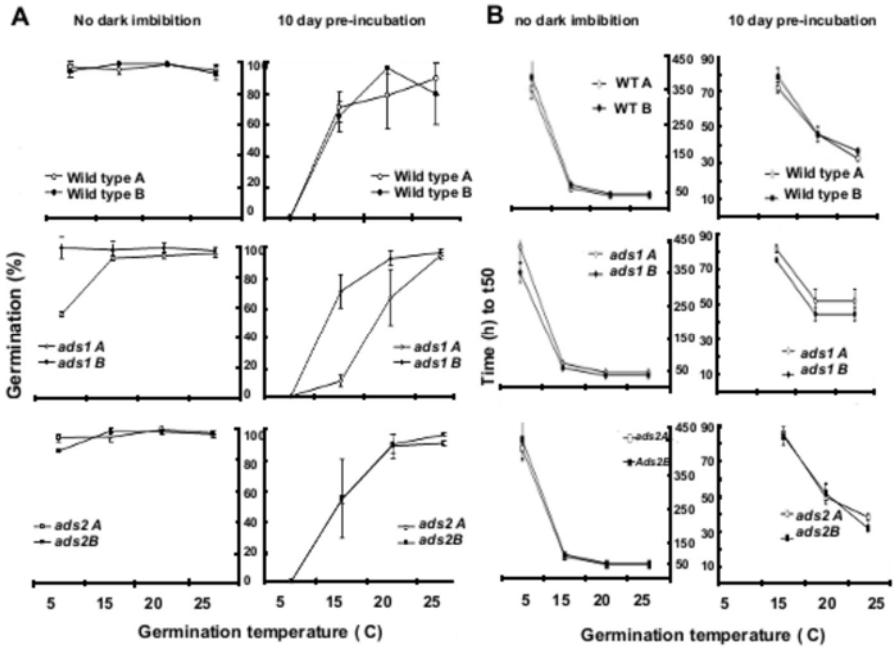

Figure 3: A. Total germination and **B.** time to half-maximal germination (t_{50}) of wild type, *ads1* and *ads2* seeds. A and B in mutant names indicate different sites of the mutation in the same gene. A and B in wild type indicate replicates coming from different seed batches. Seeds were imbibed in water at 4°C for 3d before being transferred to the light (left) or kept in the dark for 10 d (right) before being transferred to the light. 3 replicates were used. Vertical bars represent standard deviation

maximum germination was achieved within 4d. All mutants reached similar levels of germination (Figures 3 and 4), apart from the *ads1A* mutants. The *ads1A* mutant germinated only for 55% at 5°C; however, the *ads1B* mutant germinated for 100% at 5°C. The difference between A and B is caused by a different site of the mutation in the same gene.

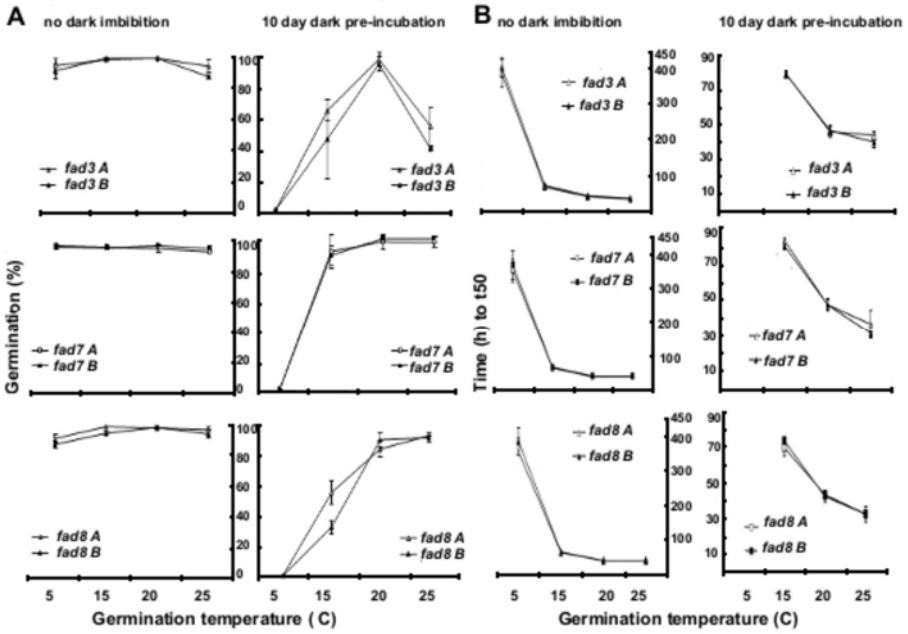

Figure 4: A. Total germination and **B.** time to half-maximal germination (t_{50}) of *fad3, fad7* and *fad8* seeds. A and B in mutant names indicate different sites of the mutation in the same gene. Seeds were imbibed in water at 4°C for 3d before being transferred to the light (left) or kept in the dark for 10 d (right) before being transferred to the light. 3 replicates were used. Vertical bars represent standard deviation

Induction of secondary dormancy

After pre-incubation at 4°C, seeds were incubated in the dark for 10 days at a range of temperatures to induce secondary dormancy, before the light pulse was given and seeds were germinated at the same temperature. Germination of wild type seeds at 20 and 25°C reached 80% or more, at 15°C germination only attained 67% (Figure 3A). The t_{50} values differed significantly among all these three temperatures, as tested by t-test, (Figure 3B; p-values, 0.00000047, 0.000000016, and 0.0008), showing that seeds incubated at 25°C reached their optimum germination faster than seeds imbibed at a lower temperature. In none of the genotypes incubated at 5°C was germination initiated within 24d. At 20°C all the mutants germinated to a level very close to wild type seeds, with the exception of

the *ads1A* mutant which germinated for only 60%. The greatest variation in germination was observed at 15°C. Only the *ads1B* mutant germinated to the same percentage as the wild type (65%). Of the other genotypes *fad7A, fad7B* germinated at 15 C to a higher level (90%) than the wild type, whereas the rest showed germination below that of the wild type of which the *ads1A* mutant showed only 10% germination. The *fad3* mutants showed wild type germination levels at 20°C, but was the only genotype that displayed lower germination (50%) at 25°C. The t_{50} for all mutants was comparable with the wild type, with only the *fad7* mutants showing a somewhat lower t_{50} (70 vs 80h at 15°C).

Fatty acid desaturase gene expression in wild type Arabidopsis thaliana seeds

Desaturases were further analysed by studying their transcript abundance in wild type seeds that were imbibed for 1 or 10 days at 15 or 25°C, to characterize optimal and suboptimal conditions for germination. Although germination reached comparable levels at both temperatures there is a difference in the t_{50} between the two temperatures (figure 3).Gene expression analysis was done on seeds that were pre-incubated for 1 day at 4 °C and were subsequently incubated in the dark or in the light; seeds imbibed for 10 days were incubated for 10 days in the dark before transferring them to either the light or keeping them in the dark for 8 hours. Seeds imbibed in the dark do not germinate at all, seeds imbibed for 10 days in the dark before transferring them to the light, do germinate (Figure 3), however, to a lesser value than seeds not imbibed in the dark for 10 days. Gene expression was analysed by the comparative C_t method and further analysed by t-test (p=0.05).

FAD3 gene expression (Figure 5) was significantly higher in the least dormant seeds at 15 and 25°C. When seeds were light irradiated during imbibition for 1 day, gene expression was higher than when kept in the dark for 10 days at optimal germination temperatures. There was no effect of the temperature on transcript abundance which corresponds with comparable germination at 15 and 25°C, albeit

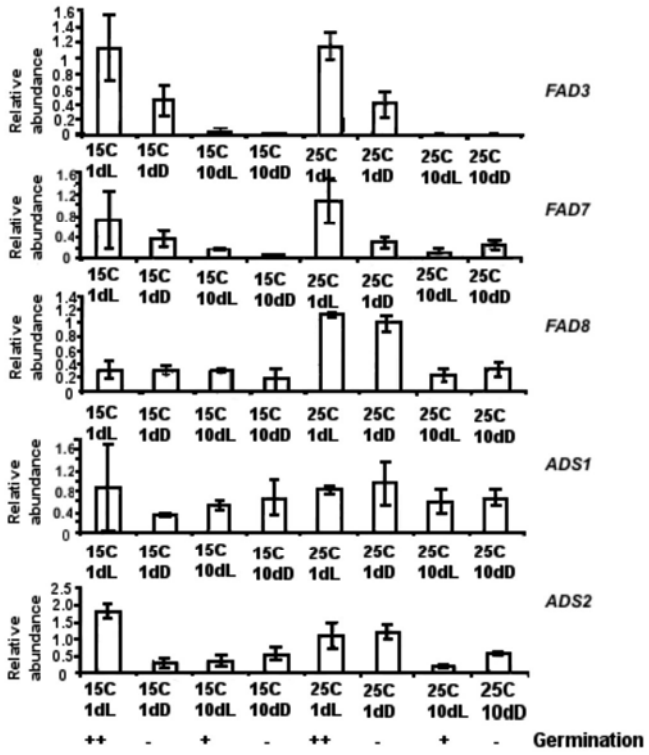

Figure 5: Relative transcript abundance of *FAD3, FAD7, FAD8, ADS1* and *ADS2* genes in wild type seeds. Seeds were imbibed at 15 or 25°C, for 1 (1d) or 10 (10d) days, after which they were transferred to the light (L) or kept in the dark (D). 5 replicates were used per sample. Gene expression was analysed with the comparative C_t method and further analysed with ANOVA, standard deviations are given. Germination status of the seeds is given, - stands for no germination; + for some germination; ++ for up to 100% germination.

with different t_{50}. Although without prolonged dark imbibition fad3 mutants showed 100 % germination, they showed the most dormant phenotype after 10 days dark pre-incubation at suboptimal temperatures (Figure 4A) suggesting that *FAD3* gene expression may be associated with optimal conditions for breaking dormancy and

inducing germination. *FAD7* gene expression (Figure 5) was significantly higher in seeds imbibed in the light for 1 day, at 15 and 25°C than in all other treatments, so *FAD7* seems to be associated with induction of germination too. *FAD8* gene expression (Figure 5) was significantly higher for 1 day imbibed seeds at 25°C as compared with all other treatments. Both these 1-d imbibed seeds and 1-d imbibed seeds at 15°C had their dormancy broken, however only 1-d imbibed seeds at 25°C showed higher *FAD8* gene expression. *ADS1* and ADS2 gene expression (Figure 5) did not show a pattern that could be clearly related to dormancy, although the abundance of *ADS1* and *ADS2* transcripts was significantly different between the different treatments (e.g. ADS2 gene expression being significantly higher for 1-d imbibed seeds in the light than for 10-d imbibed seeds

Discussion

The aim of this work was to determine the relation between desaturase activity and seed dormancy. *Arabidopsis thaliana* Columbia wild type plants and *fad3*, *fad7, fad8, ads1* and *ads2* mutant plants in a Columbia back ground were used. The *ads1* and *ads2* mutants are mutated in a Δ9 desaturase. The Δ9 desaturases introduce a double bond in the 9th position of the $C_{18,}$ converting stearic acid to oleic acid (Murata and Wada, 1995).The *fad3, fad7* and *fad8* mutants are mutated in ω3 desaturase and therefore are not capable of forming double bonds at the Δ15 position of the C_{18} acids (Murata and Wada, 1995), converting linoleic acid (18:2) to linolenic acid (18:3) (Matos et al., 2007).

NMR data showed that the FA composition of *ads1* and *ads2* mutants did not differ much from wild type seeds.

ads1 and ads2 Mutant and wild type seeds both are able to germinate up to 100% in the light at temperatures ranging from 5 to 25 °C after a short cold imbibition at 4 °C (Figure 3), showing that both wild type and mutant seeds exhibited similar vigour. A shift from 4°C to higher temperatures, such as 25°C

generally increases membrane fluidity. As *ads1* and *ads2* mutants did not show any differences in FA composition, no differences in germination pattern were expected. Imbibition of WT seeds for 10 days in the dark before they are transferred to the light should induce secondary dormancy as in e.g. *Sisymbrium officinale* seeds (Derkx *et al*., 1993; Hilhorst, 1990a/b; Chapter 2 of this thesis). However, in Arabidopsis seeds induction of secondary dormancy has been shown to take longer in the Cvi ecotype (Cadman *et al*., 2006). In Figure 3A we see that after 10 day pre-incubation wild type seeds did not longer germinate at 5°C, but still germinated up to a high percentage at 20-25°C, at 15 C germination percentage was somewhat less than at 20 – 25 C (Figure 3A). After 10 days of dark incubation, seeds completed germination faster when imbibed at 25°C (Figure 3B), making this the more optimal temperature for germination. Apparently, the 10 day pre-incubation had made the germination temperature window narrower, and germination was reduced outside this window. Thus, Arabidopsis seeds acquire secondary dormancy at low temperatures, which underlines the winter annual properties of the Columbia accession (Cadman *et al*., 2006).

The germination pattern was similar for *ads1B* and *ads2A* and *B* mutants but the *ads1A* mutant showed significantly reduced germination at 5 °C (without dark imbibition) and at 15°C (after 10 days of dark pre-incubation) as compared with wild type (Figure 3A). The abundance of *ADS1* and *ADS2* transcripts was significantly different between the different treatments. *ADS2* gene expression was significantly higher for 1-d imbibed seeds in the light than for 10-d imbibed seeds. The fatty acid composition of *ads1* and *ads2* mutants was comparable to wild type seeds but the membranes of *ads2* mutants were more fluid and of lower viscosity than that of *ads1* and wild type seeds (Figs. 2, 3). Thus, although *ads1A* mutants show a different germination pattern than wild type seeds, the germination pattern results of the *ads1B* combined with the membrane fluidity, FA composition and gene expression levels lead to the conclusion that *ADS1* genes are not involved in dormancy and germination. ADS2 gene activity did not seem to show a clear relation with germination and dormancy (Figure 5; compare the results for the *ads2*

mutants), nor with the fatty acid composition, but it had the most profoundly changed membrane fluidity and viscosity. ADS1 and ADS2 are both endoplasmatic reticulum based desaturases that catalyze the formation of the first double bond in the FA, and if this double bond is not formed no other double bonds are formed (Murata and Wada, 1995). Thus, it is very likely that these mutants show functional redundancy since membranes cannot exist if only saturated fatty acids are present (Stubbs and Smith, 1984; Los and Murata, 1998). ADS2, however, has been shown to be able to change membrane fluidity whereas ADS1 is not (Fukuchi-Muzutani *et al.,* 1998). This is in agreement with our results. So while some functions of ADS1 and ADS2 might be interchangeable, not all functions are. The fatty acid content obtained here is the content for bulk lipids, i.e. oil and membranes. Membranes might have fatty acid composition different from oil (Sheffer *et al.,* 1986; Millar *et al.,* 2000; Voelker, 2001). Therefore changes found in membrane fluidity but not in fatty acid content might not mean that fatty acids and desaturases are not the cause of the change in fluidity. However, membrane fluidity can be also modified by sterols, flavonoids and other compounds. Testing of ads1/ads2 double mutants would be a next step in analyzing the functions; however, ADS1, ADS2 and ADS3 are known to be able to switch regiospecificity (Heillman *et al.,* 2004) making analysis more difficult.

NMR data confirmed that the *fad3* mutant is impaired in the conversion of linoleic acid to linolenic acid, although still one third of the linolenic acid is present (Table 3). Mutant and wild type seeds both germinated up to 100% at temperatures ranging from 5 to 25 °C after a short cold imbibition (Figure 3A, 4A), showing that both wild type and mutant seeds exhibited similar vigour. A shift from 4°C to higher temperatures, such as 25°C generally increases membrane fluidity. The fluidity is maintained by decreasing the degree of unsaturation of fatty acids of membrane lipids, which is achieved by acceleration of *de novo* synthesis of saturated fatty acids, as this dilutes the unsaturated fatty acids (Sato and Murata, 1980). As this process does not involve the action of desaturases itself, mutants were not expected to exhibit a germination pattern different from wild type seeds.

The *fad3* mutants showed increased dormancy after 10 day pre-incubation at 25°C, as compared to wild type seeds, with clearly narrowed germination temperature window. However, at 20°C, the germination percentages of *fad3* mutants and wild type seeds are high and both up to 100 %. At 25°C wild type seeds showed a high percentage of germination but this was not seen in the *fad3* mutant seeds where germination was reduced to 50%. At 15°C the germination of fad3 mutants seems slightly reduced after 10 day pre-incubation compared to wild type seeds, however, this is not significant.

Even though desaturases are known to introduce double bonds at low temperatures and not at high temperatures (Los and Murata, 2004), *FAD3* was expressed to similar levels at both 25 and 15°C (Figure 5). In *fad3* seeds 18:3 production is reduced (Table 3; Nishida and Murata, 1996; Horiguchi *et al*., 2000; Matos *et al*., 2007), thus an increase in 18:3 level, mediated by FAD3, could be necessary to keep the membranes fluid enough for dormancy breaking (Hilhorst, 1998). Nevertheless, EPR measurements did not show appreciable differences in membrane fluidity between wild type and *fad3* mutants (Figure 1 and 2). However, measurements were done after 1d, when there is also no difference in germination between the two treatments. The expectation would be that after a 10-d pre-incubation, when there is a clear difference in dormancy and germination pattern between genotypes, there would be a difference in membrane fluidity; unfortunately this could not be measured here, due to a shortage of seeds. Previously, we have found that secondary dormancy induction is accompanied by membranes becoming less fluid (This thesis, Chapter 3). In *fad3* mutant seeds membranes can only become slightly more fluid and at higher temperatures (10d at 25°C) a much higher fluidity might be needed to keep seeds non-dormant. The lower fluidity of *fad3* mutants compared to wild type seeds at higher temperatures might be the reason for partial induction of secondary dormancy at 25°C which did not occur in wild type seeds. The *FAD3* gene expression pattern found here is similar to that found by Finch-Savage *et al.* (2007) in the Cvi accession of *A. thaliana*. The present results combined with those of Finch-Savage *et al.* (2007)

show that expression of *FAD3* is low in dormant seeds and higher when dormant seeds are exposed to cold or light. It is interesting to see that in wild type seeds that germinated after a 10-d pre-incubation, there was no *FAD3* expression. *FAD3* is post-transcriptionally controlled by temperature (Collados *et al*., 2006) but transcriptionally by light (Collados *et al.,* 2006). The light induced transcription combined with the reduced sensitivity to light (Hilhorst 1990a/b) after prolonged incubation might explain why there is no *FAD3* expression after 10 days.

The *fad8* mutant seeds showed a similar germination pattern as wild type seeds. However, at 15°C, germination only reached a maximum of 55% after a 10 day pre-incubation (Figure 3, 4), suggesting that the germination window had narrowed. Gibson *et al* (1994) reported that *FAD8* transcript levels may increase at low temperature, making the membrane more fluid. This could be the cause of changes in germination induction at low temperature as seen here. However, *FAD8* gene expression increases when dormancy is broken and/or germination is induced, but only at 25°C (Figure 5). This is in disagreement with results in the eFP browser that showed higher FAD8 expression levels when dormancy is broken and germination is induced and where no differences in expression between cold imbibition or imbibition at 22°C were found. The reason for the discrepancy between Gibson et al (1994) and eFP browser is unknown.

FAD7 is a chloroplast based light-induced desaturase (Los and Murata, 1998). The gene expression results (Figure 5) show a light dependency for 1-d imbibed seeds. After 10d of pre-incubation, however, the *FAD7* gene was not up-regulated by light anymore. After secondary dormancy is induced seeds become less responsive to light (Hilhorst, 1990a/b) and this coincides with *FAD7* not being up-regulated. Although the expression pattern suggests *FAD7* to be involved in germination, the mutant germination pattern did not confirm this. This could be due to functional redundancy, as there are more ω3 desaturases and *fad7* mutants only show the mutant phenotype under certain conditions, as other ω3 desaturases may take over its role (Gibson *et al*., 1994). As FAD7 protein is mainly present in the thylakoid membrane of chloroplasts (Poghosyan *et al*., 1999) gene expression

will not lead to functional proteins within the seed and therefore FAD7 is unlikely to be involved in germination itself.

Some but not all desaturases are shown here to change in relation to dormancy and germination. FAD3, FAD7 and FAD8 all catalyze the conversion of linoleic acid (18:2) to linolenic acid (18:3) (Matos *et al.*, 2007). From the present results we can conclude that this is an important conversion and is likely to be involved in dormancy and germination. Although all three FADs catalyze the same conversion, their functions in dormancy and germination appear to be different. Especially FAD3 is shown to have a relation with dormancy and germination. However, FAD3 involvement was not supported by measurements of membrane fluidity. This could indicate that desaturases may influence the germination/dormancy phenotype of Arabidopsis seeds but, possibly not via changes in membrane fluidity unless effects of these enzymes are very localized within the seed, e.g. in the radicle.

Therefore the question remains how in seed germination responsiveness to light and nitrate can change; this probably cannot be explained by changes of membrane fluidity due to desaturase activity leading to changes in accessibility of ligands.

Chapter 6

Characterization of dormancy related genes in *Sisymbrium officinale* (L.) Scop. using a cDNA subtraction library

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Abstract

Dormancy depth of imbibed seeds changes continuously in response to the environment. These changes in dormancy depth might be the result of differences in sensitivity to dormancy breaking factors. *Sisymbrium officinale* seeds show reduced sensitivity to nitrate after long imbibition in the dark. Transcriptional differences between primary and long-term primary dormant seeds (seeds imbibed for 1 and 10 days in water, respectively, before application of light) of *S. officinale* were analysed using two cDNA subtraction libraries and compared to *Arabidopsis thaliana*. Several genes were differentially expressed between dormancy states, in *S. officinale* as well as *A. thaliana*, however, a large amount of genes found in *S. officinale* were differently expressed in *A. thaliana* in similar dormancy states. The

genes found in the subtraction libraries were classified using FunCat and TAGGIT classification. A large set of genes found were stress related genes which might be the seeds strategy to cope with the adverse conditions met during dormancy. However, the exact role of these genes in the regulation of primary and long term primary dormancy can only be verified by functional analysis, e.g. by reverse genetics.

Introduction

Seeds are equipped to survive extended periods of unfavourable conditions before germination and plant establishment take place under more favourable conditions. The absence of germination under favourable conditions for germination is called dormancy. One of the most widely occurring types of seed dormancy is physiological dormancy. Physiological dormancy acquired during maturation on the mother plant, is called primary dormancy and is observed in seeds upon shedding. Primary dormancy in Arabidopsis can be removed during dry storage (after-ripening) and/or cold stratification (pre-chilling). After removal of these blocks to germination seeds are capable of completing germination if environmental conditions are favourable; if environmental conditions are not favourable for germination, seeds may become secondary dormant. Seeds can cycle in and out of dormancy repeatedly; this is called dormancy cycling (Taylorson, 1972; Bouwmeester and Karssen, 1993).

The depth of the dormancy of imbibed seeds changes continuously in response to the environment (Finch-Savage *et al*., 2007). After-ripened or pre-chilled seeds may respond to dormancy breaking factors such as light and nitrate and start germination. However, when primary dormant seeds are imbibed for a long time (long term primary dormant) or when seeds are secondary dormant, they become less responsive to these dormancy breaking factors (Derkx and Karssen, 1993a), especially nitrate. The difference in sensitivity may reflect the difference in

depth of dormancy. Several methods have been used to analyse the transcriptional differences between primary dormant and long-term primary dormant seeds. Micro-array analysis of dormancy states in *Arabidopsis thaliana* has shown that genes expressed in long-term primary dormant seeds group away from genes expressed in primary dormant seeds in a principal component analysis (Cadman *et al.,* 2006). It was confirmed that long term primary dormancy is a deeper dormancy than primary dormancy. To analyse transcriptional differences between the primary dormant and long term primary dormant seeds of *Sisymbrium officinale*, cDNA subtraction libraries were generated. Although cDNA subtraction library analysis is very labour intensive it was chosen over micro-array analysis to prevent cross-species hybridization difficulties. Genes found in the libraries were compared to gene expression in *Arabidopsis thaliana* using the eFP browser and classified using FunCat and TAGGIT classification systems. A stress response is thought to be the seeds way of coping with adverse conditions met during dormancy; dormancy and stress responses are strongly linked and largely overlapping (Cadman *et al.,* 2006; Hilhorst, 1995), therefore the genes found in the subtraction libraries were compared to known stress related genes.

Material and Methods

Plant material, germination conditions

Seeds of *Sisymbrium officinale* (L.) Scop. were collected in a field in the vicinity of Wageningen, The Netherlands in 2004. Seeds were cleaned, dried at 20ºC to 85 mg water/g dry seed, and stored at 5ºC until use (2005-2008). Prior to germination, seeds were surface sterilized in 1% sodium hypochlorite for 1 minute and rinsed with demineralized water for 5 minutes. Triplicates of 30 seeds were sown in 5-cm Petri dishes on two layers of filter paper (Schleicher & Schuell No 595), moistened with 1.5 ml of either demineralised water or 25 mM potassium nitrate (Fisher). Seeds were imbibed for 1 (primary dormant, water; non-dormant, potassium

nitrate) or 10 (long term primary dormant, water; secondary dormant, potassium nitrate)) days in the dark at 25°C after which they were irradiated with a saturating red light (620-700nm, Phillips) pulse for 10 minutes or kept in the dark (Hilhorst and Karssen, 1989). After irradiation, seeds were transferred back to the dark at 25ºC. Germination was scored every day after irradiation, for 1 month under safe green light.

Total RNA isolation from seeds

Following treatment, seeds were frozen in liquid nitrogen and stored in 2-ml tubes at -80°C. Total RNA was extracted using a hot borate method (modified from Wan and Wilkins, 1994). Seeds were ground with mortar and pestle, submerging the mortar and pestle in liquid nitrogen before grinding. Ground seeds were kept submerged in liquid nitrogen prior to RNA extraction. Ground seeds were re-suspended in 700 µl extraction buffer [(0.2 M sodium tetraborate decahydrate, 30 mM EDTA, 1% w/v SDS, 1% w/v sodium deoxycholate, pH 9.0) containing 14 mg polyvinylpyrrolidone and 1.1 mg dithiothreitol], which had been heated to 80°C. The seed suspension was added to 0.35 mg proteinase K in a 2 ml round-bottomed tube and incubated at 42°C for 90 minutes. 56µl 2M KCL was then added and samples incubated on ice for 1h. Samples were centrifuged at 16000 g at 4°C, after which the supernatant was moved to a new tube and its volume determined. A one-third volume of 8M LiCl (to give a final 2M LiCl concentration) was added and samples were incubated at 4°C overnight. Samples were centrifuged, and pellets washed by vortexing in 2M LiCl until the supernatant remained colourless. Pellets were re-suspended in 300 µl diethylpolycarbonate (DEPC) treated water to inactivate RNAses, and RNA precipitated by the addition of 30 µl 2M potassium acetate and 990 µl ice-cold 100% ethanol, and incubation at -80°C overnight. RNA was pelleted by centrifugation at 16000 g for 30 min at 4°C, followed by a wash with 70% ethanol. Dried pellets were re-suspended in 50-µl DEPC-treated water.

Gene	Forward (5'--> 3)
	Reverse (3'--> 5)
XERO1	AGAAGAAGGGAATTACGGAGAAAA
	CCGGGCAGGTACGCAAACAAGT
LEA	CCTGGTGGGATCGCCGGTTCA
	CTT CCCGTCGCACCCGTCAAAAC
AVP1	TGGCAAAGAAGGGAGCGAAGA
	ACTGGTGGTGCCTGGGACAAC
ANACO60	GCGGCCGAGGTTTCTAGGGATACA
	GGGGATACTTCTTACCACGAGCAC
FAD2	TTGATAATCGGAAAAGGCGTGGTG
	CGCTCAATCCCTCGCTCTTTCC
RIN2	AGGTGCTGGTGGAAGGA
	GAGAGCAATGAACAGAAGC

Table 1: Primers used for expression analysis of Dehydrin XERO1 (XERO1), Late embryogenesis abundant domain containing protein (LEA), Vacuolar-type H+-pumping pyrophosphatase (AVP1), NAC domain containing protein 60 (ANACO60), Delta-12-fatty acid dehydrogenasse (FAD2), and RPM1 interacting protein 2 (RIN2) genes

RNA concentration was determined using a WPA light wave s2000 UV/Vis spectrophotometer.

cDNA subtraction libraries

The cDNA subtraction libraries were constructed using the PCR-select cDNA subtraction kit (CLONTECH). cDNAs of 1-day H_2O imbibed seeds (primary dormant) and of 10-day H_2O imbibed seeds (long-term primary dormant) were used as tester and driver. Fragments of the forward and reverse subtraction libraries were cloned into E. coli competent cells (Promega) and plated on LB agar plates supplemented with ampicillin (0.1 mg/ml) and grown overnight at 37°C. Plasmids were isolated using the High Pure Plasmid Isolation Kit (Roche). A

minimum insert size of 200bp was set as criterion for further investigation, to avoid difficulties with finding homologue genes in the NCBI database. Sequencing was performed at the Wolfson Institute for Biomedical Research (UCL) using AB sequencing technology.

qRT PCR validation of subtraction library data

The expression of selected genes was quantified by qRT-PCR. Total RNA from 3 biological replicates per treatment was used. cDNA was synthesized using a transcriptor first strand cDNA synthesis kit (Roche), according to the manufacturer's instructions. Primers for real-time PCR were generated using DNAstar software PrimerSelect 5.01 with the sequences obtained in the subtraction library. Gene specific primer sequences are given in Table 1. Genes chosen for validation were normalized with 18S rRNA. PCR reactions were performed using LightCycler FastStart DNA master SYBR green I (Roche). Reactions were performed in triplicate for each biological replicate, in a mixture containing 5µl of first-strand cDNA in a volume of 20µl. The standard thermal profile used was 10 min at 95 °C followed by 45 cycles of 10 sec at 95C°, 20 sec at 60°C and 10 sec at 72 °C. Comparative Ct values were calculated and ANOVA tests were used to statistically analyse the data.

Results and Discussion

Germination

In *S. officinale* different dormancy levels can be distinguished which are easy to manipulate. After-ripened seeds imbibed in H_2O failed to germinate in the dark and

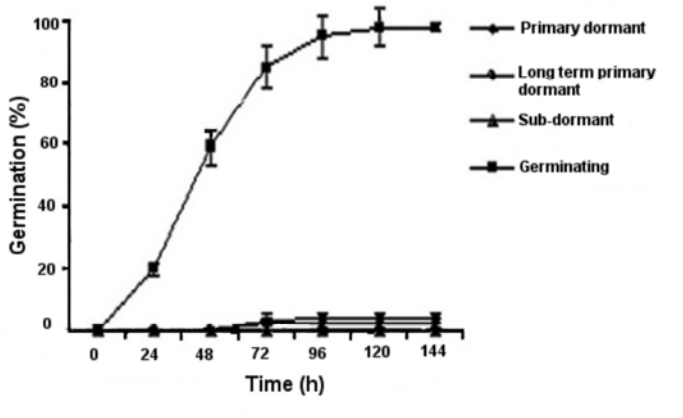


Figure 1: Germination of Sisymbrium officinale seeds after pre-treatment in 25mM KNO_3 for 1 day, germinating (light) (■), H_2O for 1 day, primary dormant (♦), H_2O for 10 days, long term primary dormant (●), 25 mM KNO_3 without a subsequent light-pulse, sub-dormant (dark) (▲). A light pulse was given after 8 hours of imbibition, except for treatments labeled 'dark', and seeds were placed back in the dark.

in the light and were, thus, considered primary dormant (Figure 1). Seeds required both KNO_3 and light to break dormancy and initiate germination. When H_2O imbibed seeds are not given KNO_3 and light within a certain time frame (10 days) seeds will not respond to KNO_3 and light anymore because they have become long- term primary dormant. Other factors e.g. chilling or nitrate/light at higher concentrations/intensities may be required to relieve dormancy, making long-term primary dormancy a deeper dormancy than primary dormancy. To find possible differences between primary dormant and long-term primary dormant seeds, a cDNA subtraction library of primary dormant vs. long-term primary dormant seeds was constructed. In previous chapters of this thesis differences between primary and secondary dormancy were sought after. However, the primary and secondary

dormancy phenotypes not only differed in time of imbibition, but also in imbibition medium (water versus nitrate). If these were used for the subtraction library, not only differences in dept of dormancy would be found but also differences in response to water and nitrate. Therefore primary dormant and long-term primary dormant seeds were used here, as these only differed in imbibition time (1d vs. 10d).

cDNA subtraction library construction and sequence selection

Two reciprocal cDNA subtraction libraries were constructed, one enriched for rare messages in primary dormant seeds; and the other one enriched for rare messages in long term primary dormant seeds. One tenth of the subtraction library was cloned into *E. coli*. Fifty percent of the colonies contained plasmid with a single insert of sufficient size (above 200bp) for sequencing and analysis. Of each library, 50 genes were sequenced. We found at least 12 sequences per library appearing more than once in, mainly, identical fragments. All sequences showed *Arabidopsis thaliana* orthologs with BLASTN, only e-values of 2E-09 or better were accepted. In Table 2 the gene lists are given for all the genes that were differentially expressed in the subtraction library enriched for primary dormant (Table 2a) and long term primary dormant (Table 2b) seeds. The Arabidopsis thaliana orthologs, functional description, cDNA length, query coverage, e-value and FunCat MIPS classification, expression levels as found on the eFP browser and the ratio between the expression levels are given. Sequences that were found more than once are only mentioned once.

Table 2: List of genes found in the primary dormant (2A) and long term primary dormant (2B) libraries. The primary dormant library is the subtraction library enriched for primary dormant genes while the long term primary dormant library is enriched for long term primary dormant genes. The *Arabidopsis thaliana* orthologs, functional description, cDNA length, query coverage, e-value and FunCat MIPS classification, expression values, as found with the eFP browser, and the ratio between the expression values are given. Sequences that were found more than once are only mentioned once. Comparison with the eFP browser was done by using 24 hour water imbibed *Arabidopsis thaliana* seeds as a comparison for primary dormant seeds in *Sisymbrium officinale* (PD24h) and 30 day water imbibed *Arabidopsis thaliana* seeds were used as a comparison for long term primary dormant seeds in *Sisymbrium officinale* (PD30d).

Arabidopsis thaliana orthologue	Gene description	cDNA length (bp)	% query coverage	e- value	MIPS classification Class	MIPS subclass	PD24h	PD30d	Ratio
AT1G01930	Zinc finger protein-related, mRNA	749	64	0.0E+00	99		860.02	196.65	4.4
AT1G07920	Elongation factor 1-alpha/EF-1-alpha	776	76	1.0E-67	12, 16, 70	16.01 70.16	4328.92	2525.2	1.7
AT1G13690	ATPase E1; nucleic acid binding (ATE1)	235	83	1.0E-113	16	16.03	317.37	522.61	0.6
AT1G30880	Unknown protein, mRNA	855	42	4.0E-142	70	70.16	1221.25	2372.9	0.5
AT1G35190	Oxidoreductase, 2OG-Fe(II) oxygenase family protein mRNA, complete cds	628	58	3.0E-80	1	1.20	604.95	725.49	0.8
AT1G51200	Zinc finger (AN1-like) family protien	587	78	3.0E-112	16	16.03 16.17	1844.52	3978.8	0.5
AT1G68790	LINC3 (Little nuclei13)(LINC3) mRNA	673	57	0.0E+00	99		300.37	314.96	1
AT1G79920	Heat shock protein 70, putative/HSP70	558	94	8.0E-69	16	16.19	251.8	468.97	0.5
AT2G16590	FIP15.3 mFRNA	214	81	6.0E-137	Not found		Not present	Not present	-
AT2G36460	Fructose-biphosphate aldolase	275	94	4.0E-63	1, 2, 70	1.05 70.16	1419.65	1419.4	1
AT2G38530	LIPID TRANSFER PROTEIN 2 (LTP2); lipid binding	380	85	2.0E-129	16, 20 42	16.09	6279.95	3188.1	2
AT3G04120	Glyceraldehyde-3-phosphate dehydrogenase C subunit (GAPC)	366	91	2.0E-78	1, 2, 32, 34, 70	1.05, 32.01 70.03, 70.16	477.22	195.95	2.4
AT3G12120	Delta-12-fatty acid dehydrogenase (FAD2) mRNA	241	94	3.0E-144	1, 70	1.06 70.07	291.6	370.04	0.8
AT3G22490	Late embryogenesis abundant protein, putative LEA protein, putative mRNA	636	71	2.0E-121	41		2725	2648.6	1
AT4G22640	Cupin family protein mRNA	617	83	6.0E-149	4		7250.82	641.32	11.3
AT3G41768	Cytosolic small ribosomal subunit	335	89	0.0E+00	12, 16, 70	70.03	Not present	Not present	-
AT3G44110	DnaJ homologue 3 (ATJ3)	541	79	1.0E-129	14, 16	16.01	1376.67	1788.9	0.8
AT3G44290	NAC domain containing protein 60; transcription factor (ANACO60) mRNA	549	70	0.0E+00	11, 41		Not present	Not present	-
AT3G54860	Vacuolar protein sorting 33; protein transporter (ATVPS33) mRNA	612	95	8.0E-65	14, 20, 42		209.3	289.56	0.7
AT4G00810	60S acidic ribosomal protein P1 (RPP1B)	300	75	2.0E-100	12, 70	70.03	481.37	199.22	2.4
AT4G13200	Unknown protein mRNA	411	78	9.0E-50	99		484.37	135.34	3.6
AT4G16150	Calmodulin-binding protein mRNA	718	24	1.0E-179	11, 16	16.01	Not present	Not present	-
AT4G25230	RPM1 interacting protein 2; proteinbinding/ zinc ion binding (RIN2) mRNA	534	93	2.0E-126	14, 16, 40, 70	16.01,16.17 16.19,70.02	161.57	141.22	1.1
AT4G28520	Cruciferin 3; nutrient reservoir (CRU3)	470	95	9.0E-82	4, 41		7119.2	6988.3	1
AT4G34660	AME3; kinase (AME3) mRNA	247	86	2.0E-57	14, 70	70.16	49.3	44.3	1.1
AT4G40030	Histone H3.2 mRNA	195	80	4.0E-116	16, 42, 70	16.03,70.10 70.26	2487	3888.7	0.6
AT5G22290	NAC domain containing protein 89; transcription factor (ANACO89) mRNA	549	75	1.0E-139	11, 41		283.37	335.57	0.8
AT5G24350	Unknown protein mRNA	374	92		99		185.12	214.37	0.9

Arabidopsis thaliana orthologue	Gene description	cDNA length (bp)	% query coverage	e- value	MIPS classification class	MIPS Sub-class	PD24h	PD30d	ratio
AT1G08060	Maintenance of Methylation (MOM)	262	77	3.0E-18	1, 10, 11, 42, 70	70.10	142.7	232.7	0.6
AT1G08420	Kelch repeat-containing protein/ serine/threonine phosphoesterase family protein	352	87	1.0E-106	14		118.2	152.6	0.8
AT1G15280	Glycine-rich protein	214	87	4.0E-28	99		175.9	302.3	0.6
AT1G15690	AVP1 (vacuolar-type H+-pumping pyrophospahtase	535	82	0.0E+00	1, 14, 20, 32, 34, 70	1.04,32.01, 70.02,70.16, 70.22	1491	1469.7	1
AT1G23290	Ribosomal protein L27A	529	38	2.0E-76	12, 14, 70	70.03	1662.3	657.7	2.5
AT1G67430	60S ribosomal protein L17	684	38	0.0E+00	12, 14, 70	70.03	2027.2	709.1	2.9
AT1G75830	Low-molecular-weight cysteine-rich 67 (LCR67/PDF1.1) mRNA	371	72	1.0E-57	32, 70	32.05,70.011	5035.3	4517.1	1.1
AT1G79260	Unknown protein mRNA	183	87	8.0E-37	99		30.8	20.2	1.5
AT2G04270	Glycoside hydrolase starch-binding domain containing protein mRNA	359	80	1.0E-121	1, 11, 70	1.03, 1.05, 70.03,70.16,7 0.26	54.9	76.7	0.7
AT2G23110	Unknown protein mRNA	125	88	2.0E-09	99		975.7	1032.3	0.9
AT3G01650	RING domain ligase1 protein binding, zinc ion binding (RGLG1) mRNA	214	64	2.0E-65	14		209.5	374.4	0.6
AT3G11830	Chaperonin, putative mRNA	397	81	1.0E-12	14, 16	16.01,16.19	380.8	1104.2	0.3
AT3G13330	Binding mRNA	289	49	1.0E-100	99		289.7	281.8	1
AT3G43520	Unknown protein	372	86	3.0E-109	70	70.26	945	787.4	1.2
AT3G50980	Dehydrin XERO1 mRNA	410	91	2.0E-75	32, 34	32.01	4399.6	3708.7	1.2
AT3G55010	Phosphoribosylformylglycinamidinecyc-loligase (ATPURM/PUR5) mRNA	397	72	4.0E-95	1, 70	1.03,70.03, 70.26	123.3	131.3	0.9
AT3G56340	40S ribosomal protein S26	559	90	8.0E-24	12, 16, 70	70.03	469.8	162.8	2.9
AT3G56350	Superoxide dismutase (Mn), putative/manganese superoxide dismutase, putative mRNA	450	37	5.0E-165	32, 70	70.16	2273.1	1697.4	1.3
AT4G21020	Late embryogenesis abundant domain containing protein/ LEA domain-containing protein mRNA	615	90	4.0E-54	41, 70	70.16	1454.9	2019.1	0.7
AT4G24690	Ubiquitin-associated (UBA)/TS-N domain-containing protein/octicosapeptide/Phox/Bemp1 mRNA	374	52	1.0E-95	16, 70	16.17, 70.03	13.4	36.4	0.4
AT5G11710	(EPSIN1); binding mRNA	475	87	3.0E-161	14, 16, 20, 70	16, 70.04, 70.08	102.5	58.6	1.8
AT5G35180	Unknown protein mRNA	576	89	0.0E+00	99		142.7	232.7	0.6
AT5G35430	Binding mRNA	211	92	6.0E-45	99		118.2	152.6	0.8
AT5G35440	MOK9_2 mRNA	211	88	7.0E-45	70		175.9	302.3	0.6
AT5G44430	PDF1.2c	291	84	5.0E-73	32		1491	1469.7	1
AT5G60980	Nuclear transport factor 2 (NTF2) (RRM)-containing protein	306	84	5.0E-99	16, 20	16.03	1662.3	657.7	2.5
AT5G64840	General control non-repressible 5 (ATGCN5)	310	87	2.0E-117	20, 34, 70	70.26	2027.2	709.1	2.9
AT5G66120	3-Dehydroquinate synthase, putative	241	89	2.0E-70	1, 70	1.01, 1.04 1.05, 1.20 70.16,70.26	5035.3	4517.1	1.1

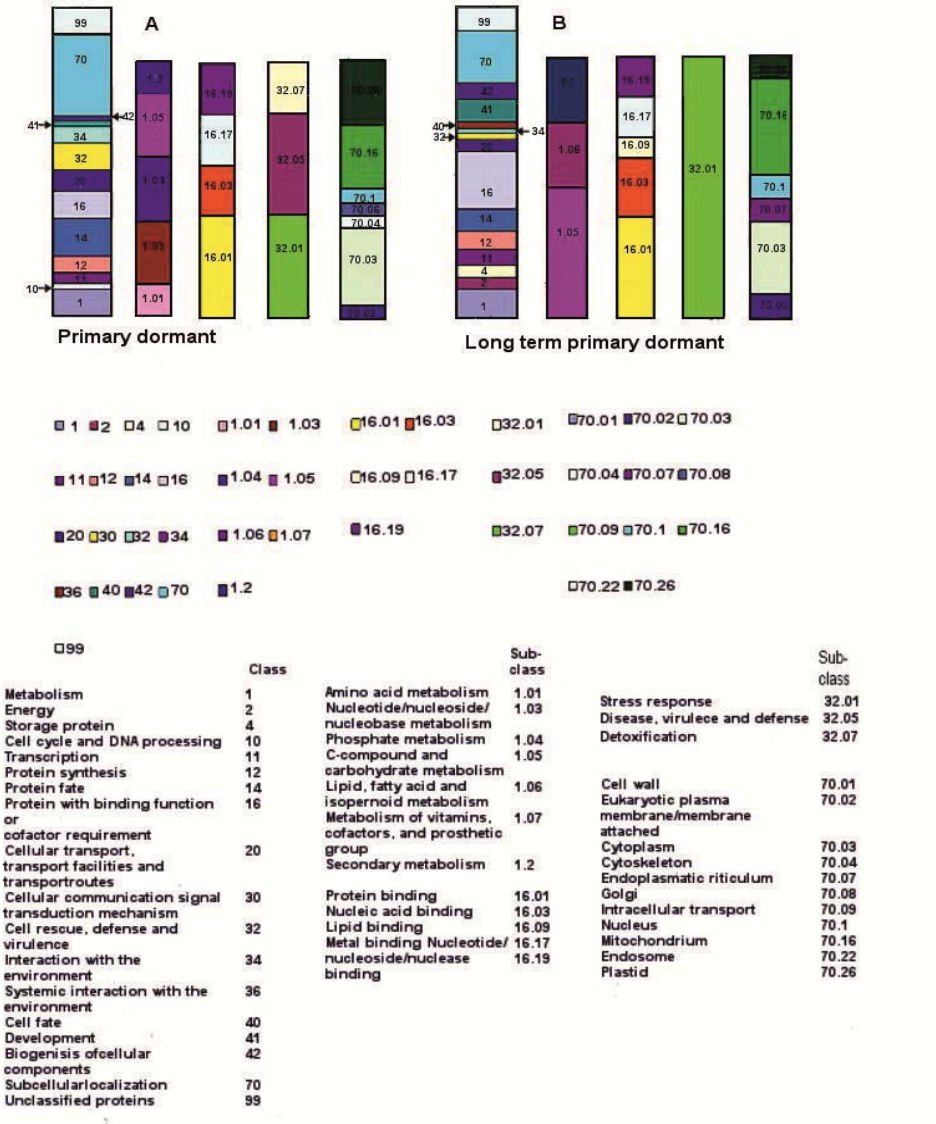
eFP browser

Both the primary dormant and the long term primary dormant libraries were compared with (microarray) expression data from Arabidopsis, using the eFP browser (www.bar.utoronto.ca; Geisler-Lee *et al*., 2007; Winter *et al.*, 2007) (Table 2). The PD24h and PD30d treatments were used for comparison; PD24h are Arabidopsis seeds imbibed for 24 hours in water in the dark, these seeds require light for germination. PD30d are Arabidopsis seeds imbibed for 30 days in water in the dark. These seeds also require light for germination but have been shown to possess a deeper dormancy (Cadman *et al.,* 2006), and this is the best comparable treatment to long term primary dormant Sisymbrium seeds. Normalized expression levels are given in Table 2. The ratios between PD24h and PD30d are expected to be above 1 in the primary dormant Sisymbrium library and below 1 in the long-term primary dormant library. Of the primary dormant library (Table 2a) 43% showed the expected gene expression pattern of up-regulated genes of PD24h as compared to PD30d. 46% showed a gene expression pattern completely opposite to what we expected and for 11% the PD24h and PD30d treatments did not differ in gene expression. Of the long term primary dormant library (Table 2b) 46% showed a gene expression pattern with up-regulated expression for PD30d as compared to PD24h, as expected; 37% showed a completely opposite expression pattern from what was expected and for 17% expression of both PD24h and PD30d did not differ. The 4 genes that did not appear in the eFP browser are disregarded here. The subtraction library was generated from *Sisymbrium officinale* seeds whereas the Cadman (2006) and Finch-Savage (2007) gene sets and the eFP browser gene sets were all generated from *Arabidopsis thaliana.* The genes that were expressed in a similar fashion in seeds from both species are the more conserved genes. In Arabidopsis genes related to metabolism and energy are more highly expressed during early imbibition of primary dormant seeds, as compared with the long-term dormant states (Cadman *et al*., 2006). In contrast, the long-term dormancy states contain a greater representation in the transcription, cell cycle and DNA processing

functional groups (Cadman *et al*., 2006). The gene expression differences between the Sisymbrium subtraction library and the analysed Arabidopsis gene sets could suggest that the subtraction library was not 100% accurate. However, since two different species are compared, the genes found differentially expressed between subtraction library and Arabidopsis gene set could also be species-specific, dormancy-associated genes.

Functional Classification (FunCat)

Arabidopsis thaliana orthologs were classified according to the Functional Catalogue (FunCat) of the Munich Information Centre for Protein Sequences (MIPS) (Figure 2, Table 2). FunCat enables the assignment of multiple categories to a single gene (Ruepp *et al*., 2004). Figure 2 shows a chart with the functional classification of genes differentially expressed in the primary dormant (A) and long-term primary dormant (B) subtraction libraries. Classification categories for both dormancy types were very different, most notable differences were in protein with binding function or co-factor requirement (16), that had a larger representation in the long term primary dormant category (19% vs. 9%), while the cell rescue, defence and virulence category (32) displayed a greater representation of genes in primary dormant (8.7%) than long term primary dormant library (1.9%). Also the sub-cellular localization functional category (70) displayed a greater representation of genes in the primary dormant (26%) than in the long term primary dormant library (17%). These classes were further sub-classified to display the differences between the two libraries in more detail (Figure 2 A, B). In class 16 5 genes were found in the primary dormant library compared to 10 in the long-term primary dormant library (Table 2A/2B). 3 genes of the long term primary dormant library classified in more than 1 sub-class. One gene of the long term primary dormant library was classified in the lipid binding (16.09) sub-class, compared to 0 in the primary dormant library. The cell rescue, defence and virulence (32) class showed 5 genes in the primary dormant library compared to 1 in the long term primary



Class		Sub-class		Sub-class	
Metabolism	1	Amino acid metabolism	1.01	Stress response	32.01
Energy	2	Nucleotide/nucleoside/ nucleobase metabolism	1.03	Disease, virulece and defense	32.05
Storage protein	4	Phosphate metabolism	1.04	Detoxification	32.07
Cell cycle and DNA processing	10	C-compound and carbohydrate metabolism	1.05		
Transcription	11	Lipid, fatty acid and isopernoid metabolism	1.06	Cell wall	70.01
Protein synthesis	12	Metabolism of vitamins, cofactors, and prosthetic group	1.07	Eukaryotic plasma membrane/membrane attached	70.02
Protein fate	14	Secondary metabolism	1.2	Cytoplasm	70.03
Protein with binding function or cofactor requirement	16	Protein binding	16.01	Cytoskeleton	70.04
Cellular transport, transport facilities and transportroutes	20	Nucleic acid binding	16.03	Endoplasmatic riticulum	70.07
Cellular communication signal transduction mechanism	30	Lipid binding	16.09	Golgi	70.08
Cell rescue, defense and virulence	32	Metal binding Nucleotide/ nucleoside/nuclease binding	16.17 16.19	Intracellular transport	70.09
Interaction with the environment	34			Nucleus	70.1
Systemic interaction with the environment	36			Mitochondrium	70.16
Cell fate	40			Endosome	70.22
Development	41			Plastid	70.26
Biogenisis ofcellular components	42				
Subcellularlocalization	70				
Unclassified proteins	99				

Figure 2: MIPS classification of genes found in primary dormant and long-term dormant subtraction libraries. The main MIPS classifications are shown by the larger bars. The metabolism class (1), the protein with binding function or cofactor requirement (16), the cell rescue, defence and virulence category (32) and the sub-cellular localization class (70) are sub-divided and shown on the sides

dormant library (Table 2A/2B). For the subcellular localization class (70) 16 genes were found in the primary dormant library while 10 were found in the long term primary dormant library (Table 2A/2B). Sub-dividing this class shows that genes present in both libraries classify for more than one sub-class. The primary dormant library had a greater representation in cytoplasm (70.03) (6 versus 3) and plastids (70.26) (5 versus 1).

Cadman *et al*. (2006) have shown that the metabolism class (1) exhibited major differences between primary dormant and long term primary dormant seeds, which is in contrast to what was found here. The metabolism class was further subdivided (Figure 2) to display possible differences between the two dormancy types. Even though both libraries show almost the same number of genes in the metabolism class (i.e. 5 and 4, see Table 2A and 2B), subdividing this class shows that genes present in the primary dormant library classify in more than one sub-class, while genes present in the long term primary dormant library are only present in one subdivision each (Figure 2). Primary dormant seeds show more genes in nucleotide/nucleoside/nucleobase metabolism (2 versus 0; cat. 1.03) and phosphate metabolism (2 versus 0; cat. 1.04) (Figure 2). The long-term primary dormant library shows one gene present in the sub-class of lipid/fatty acid/isoprenoid metabolism (cat. 1.06) where no genes of the primary dormant library are present. Only one tenth of the subtraction library was analysed, this should be an appropriate amount to give a rough estimate of the genes represented in each class and subclass; however, analysing more genes will give a much more representable classification.

TAGGIT classification

MIPS classification can be unsatisfactory because the classification system does not reflect specific processes previously shown to be important for seed biology (Carrera *et al*., 2007). To distinguish genes encoding proteins related to seed

dormancy, development and germination, we analysed the *Arabidopsis thaliana* orthologs of genes differentially expressed in the subtraction libraries with the TAGGIT workflow. The TAGGIT workflow re-annotates gene lists according to defined functions and provides more useful information related to seed biology (Carrera *et al*., 2007), giving an indication of the developmental status of the seed. However, only 4 genes of each subtraction library could be assigned to a category in the TAGGIT classification. This could be due to the still small number of genespresent in the TAGGIT workflow, the small number of genes present in the subtraction libraries or to the fact that the genes present in the subtraction libraries may not be seed specific. The newly assigned genes were found in the dormancy category (AT3G50980, a dehydrin, in the primary dormant library), the translation category (AT1G23290 and AT1G67430, both encoding for ribosomal proteins, in the primary dormant library and AT1G07920 and AT4G00810, an elongation factor and a ribosomal protein, respectively, in the long term primary dormant library) and in the seed storage proteins/late embryogenesis abundant category (AT4G21020, a LEA protein in the primary dormant and AT3G22490 and AT4G28520, a LEA protein and cruciferin, respectively, in the long term primary dormant library

Stress related gene expression

In many organisms the regulation of dormancy is associated with abiotic stress responses. The combination of metabolic/developmental arrest and stress resistance/tolerance is a powerful strategy for the (long-term) survival of organisms under adverse conditions (Lubzens *et al.,* 2010). At the level of gene expression, dormancy and stress responses are strongly linked and largely overlapping (Cadman *et al*., 2006; Hilhorst, 1995). In general, competent organisms anticipate a stressful period by shutting down their metabolism and engaging in a stress response. One key method to resist stress is the expelling of water from dormant tissue (Pnueli *et al*., 2002), as water has a potential to damage cells under adverse physical conditions, e.g. freezing. Thus, the stress response may include

dehydration, but also the generation of protective compounds such as late embryogenesis-abundant (LEA) proteins and anti-oxidants (Ingram and Bartels, 1996; Raynal, 1999; Singh, 2007). Other stress responses are engaged to harden the seed against cold, salinity stress or pathogen attacks. In the subtraction library a large group of stress related genes was found (Table 2A,B); in the primary dormant library (Table 2A) one *LEA* gene was found (ortholog to AT4G21020), a water and, consequently, freezing related stress gene *Dehydrin XERO1* (ortholog to AT3G50980), a salinity stress related gene, *AVP1* (ortholog to AT1G15690) and two pathogen related genes (orthologs to AT1G75830, *LCR67/PDF1.1* and AT5G44430, *PDF1.2c*). In the long term primary dormant library (Table 2B) one *LEA* (ortholog to AT3G22490), one cold stress related gene *FAD2* (ortholog to AT3G12120), 2 heat-shock related genes (orthologs to AT1G07920, an elongation factor, and AT3G44110, a DnaJ homologue 3/heat shock protein) and one pathogen attack related *RIN2* (ortholog to AT4G25230) were found.

Expression of randomly chosen stress related genes was analysed by qRT-PCR under different dormancy conditions. Gene expression was analysed for primary dormant seeds in the dark and in the light, long term primary dormant seeds in the dark, secondary dormant seeds in the dark, sub-dormant seeds in the dark and in the light. Secondary dormancy was included here, to analyse differences between two varieties of deep dormancy; both are induced by a long imbibition time, but for secondary dormant seeds nitrate was the imbibition medium. This would disclose the effect that nitrate may have on gene expression associated with germination and dormancy. Dormancy conditions were obtained in the same seed batch as in which the subtraction library was made, apart from primary dormant 1dH(D) and long term-primary dormant 10dH(D).

The LEA proteins are implicated in stabilizing and protecting cellular structures (Shinozaki *et al.*, 1999) and may also protect cells during the course of de/rehydration (Pnueli *et al*., 2002). *LEA* are found in the primary dormant and long-term primary dormant library and therefore are an interesting gene to analyse.The *LEA* (ortholog to AT4G21020) found in the primary dormant

subtraction library and expressed higher in PD30d seeds of Arabidopsis, (http://bar.utoronto.ca) with a ratio of PD24h vs. PD30d of 0.7 (Table 2A), appeared to be constitutively expressed at low levels in Sisymbrium and did not show significant expression differences between treatments (Figure 3). The water and freezing stress tolerance related gene dehydrin *XERO1* (Roberton and Chandler, 1992) (ortholog to AT3G50980) has been proposed to contribute to freezing tolerance by its protective effect on membranes (Puhakainen *et al.*, 2004). Dehydration related genes are also expressed during late embryogenesis when plants naturally lose water, and ABA is thought to be involved in the expression of deydrins (Roberton and Chandler, 1992). *XERO1* was found in the primary dormant library and qRT-PCR analysis showed that seeds imbibed in water for 1 day, light or dark (1dH (L) and 1dH(D)), exhibited higher transcript abundance than the long term primary dormant and non-dormant seeds (10A, 10dN(D), 10dH(D) and 1dN(L), 1dN(D)) (Figure 3). This could indicate that XERO1 expression levels drop with increasing depth of dormancy or disappearance of dormancy. However, the transcript abundance of 1A is not significantly different from 10A, as standard errors are too big, showing there is no clear link between dormancy depth and XERO1 expression levels. In Arabidopsis the ratio between PD24h and PD30d was 1.2.

The salinity stress related gene *AVP1* (ortholog to AT1G15690) is a vacuolar proton pump that increases salt and drought tolerance of higher plants, by increasing cation uptake and retaining water (Gaxiola *et al*., 2001). Salt and drought tolerance are important for non-dormant and germinating seeds when seedlings have to establish themselves under stress conditions. Expression of this gene was reported to increase sharply in 10-d old seedlings of Arabidopsis when exposed to various kinds of stress, including cold, osmotic, salt and heat, as well as to ABA (Zeller *et al.,* 2009). Thus, it is not surprising that, in anticipation of transition to the seedling stage, the increased expression of AVP1 in primary dormant seeds increased even further in non-dormant/germinating seeds, and

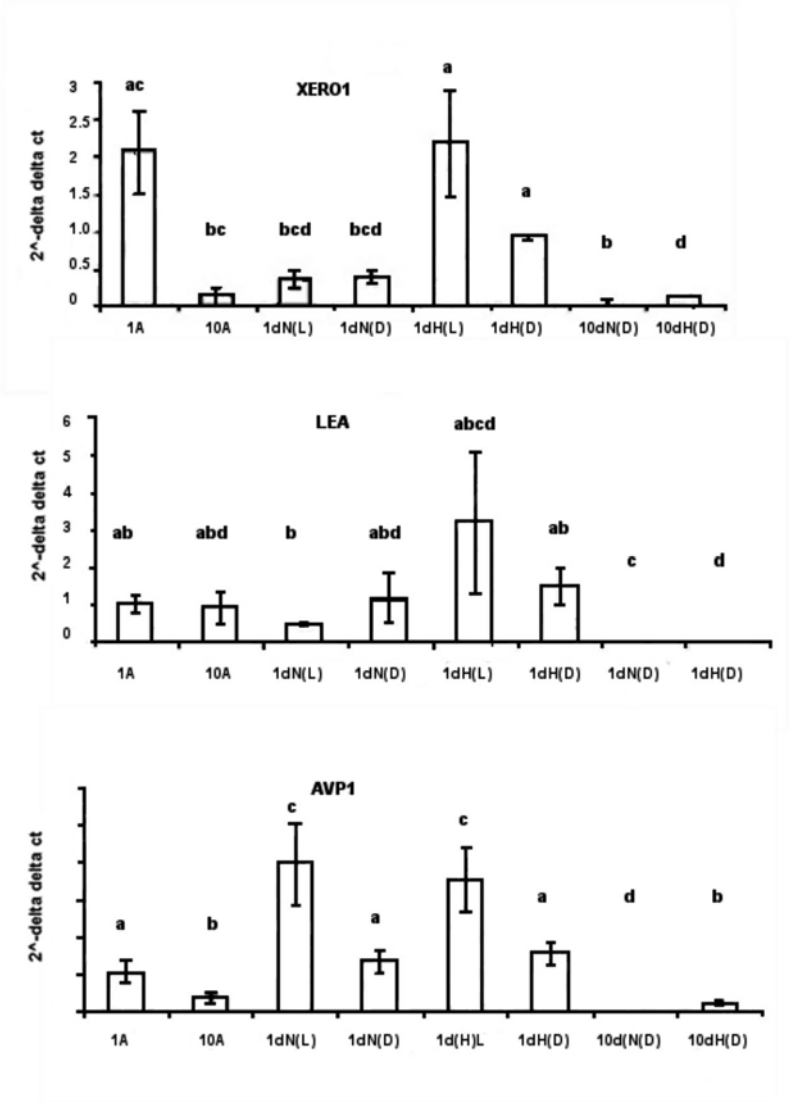


Figure 3: Expression of 3 genes selected from the primary-dormant subtraction library: Dehydrin XERO1 (XERO1) and Late embryogenesis abundant domain containing protein (LEA) and Vacuolar-type H+-pumping pyrophosphatase (AVP1), Eight treatments are shown: 1A: primary dormant seeds used in library, 10A: long-term dormant seeds used in library, 1d N(L): seeds imbibed for 1 day in KNO3 in the light (germinate),1dN (D): seeds imbibed for 1 day in KNO3 in the dark (do not germinate; need light), 1dH (L): seeds imbibed in water for 1 day in the light (do not germinate; need nitrate), 1dH (D): seeds imbibed in water for 1 day in the dark (do not germinate; need light and nitrate), 10dN (D): seeds imbibed in KNO3 for 10 days in the dark (do not germinate), 10dH (D): seeds imbibed in water for 10 days in the dark (do not germinate). Error bars represent standard deviations. a, b, c and d represent significant differences

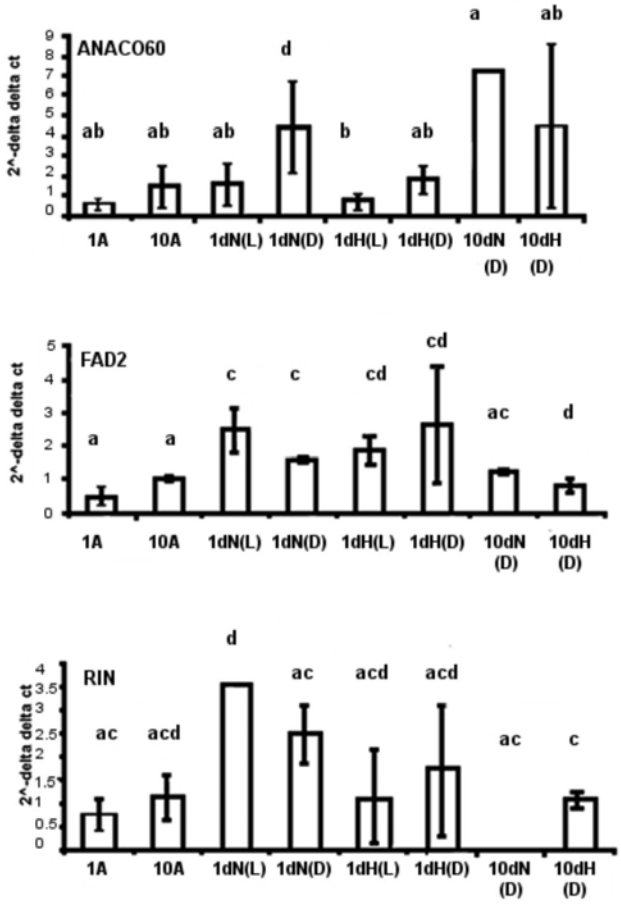


Figure 4: Gene expression of 3 genes selected from the long-term primary dormant subtraction library: NAC domain containing protein 60 (*ANACO60*), Delta-12-fatty acid dehydrogenasse (*FAD2*), and RPM1 interacting protein 2 (*RIN2*). Treatments as in Fig. 3. Error bars represent standard deviations. a, b, c, and d represent the significant differences.

appeared to be under the control of light (Fig. 3). The ratio between 1A and 10A was 2.95 and significant. Cadman *et al*. (2006) found a ratio of 1 for Arabidopsis.

Gene expression of some of the stress–associated genes in the long-term primary dormant library was also analysed. Fatty acid desaturases aid in adjusting membrane fluidity in response to temperature changes (Los and Murata, 1998; Chapter 5). The ortholog to the *FAD2* (AT3G12120) gene is involved in cold stress

tolerance in plants (Matos *et al.*, 2007). *FAD2* is a fatty acid desaturase 2 involved the conversion of 18:1 to 18:2 fatty acids, allowing changes in temperature.

Although the Arabidopsis gene sets of Cadman et al., (2006) and Finch-Savage et al., (2007) showed that PD30d seeds exhibitied a higher expression of FAD2 than PD24h seeds (Table 2b) with a ratio of 0.8, qRT-PCR analysis showed no differences in the FAD2 expression pattern between short and long term incubation (Fig. 4) with a non-significant ratio between 1A and 10A of 0.57, however, it is a similar type of response. Apart from 1A, expression seemed to be somewhat higher in the 1d treatments but this was not significant. Interestingly 1A -1dH(D) and 10A -10dH(D) have received a similar treatment, however expression of FAD2 is not comparable. Dormancy states have been obtained using different seed batches for these treatments, and this might explain the difference in expression levels. Standard deviation for 1dH(D) is very large and makes the results difficult to interpret.

RPM1 interacting protein RIN2 (ortholog to AT4G25230) is involved in the hypersensitive response to pathogens e.g. *Pseudomonas syringae*. *RIN2* is predominantly located in the plasma membrane where it contributes to the *RPM1*

hypersensitive response (Kawasaki *et al.,* 2005). *RIN2* was found in the long-term primary dormant library. The analysed gene sets did not show differences in expression of *RIN2* between short and long term dormancy states (Figure 4). The ratio between 1A and 10A was 0.67, and was not significant. The ratio found for Arabidopsis was 1.1 (http://bar.utoronto.ca; table 2B). In the long-term primary dormant library AT3G44290, a NAC domain containing protein 60, *ANACO60*, was found. *ANACO60* encodes for a plant-specific transcriptional regulator, expressed in developmental stages (Ooka *et al.*, 2003). qRT-PCR analysis (Figure 4) showed that *ANACO60* was not significantly differentially expressed between primary dormant and long term primary dormant seeds, displaying a ratio of 0.4 (not significant). This gene showed high expression in 4-d old seedlings of 5 out of 34 Arabidopsis accessions and may be related to their specific stress phenotypes (Lempe *et al.,* 2005). The qRT-PCR results showed that although stress responses

are involved in dormancy control, the genes selected from the subtraction library were often not differentially expressed between treatments.

Conclusions

The cDNA subtraction library was chosen, over micro-array analysis, as the preferred method to find transcriptional differences between primary dormant and long term primary dormant seeds of *Sisymbrium officinale*. The rationale for this choice was the prevention of cross-species hybridization difficulties. A major issue with cross-species microarray hybridization is the effect of sequence divergence on probe affinity, which is not only a function of phylogenetic distance. Due to differences in sequence divergence rates, such effects are not uniform across all genes. At present it is difficult to correct for such effects during the analysis of microarray data (Bar-Or *et al.,* 2007).

A cDNA subtraction library, however, poses a whole new set of difficulties; a large number of clones needs to be sequenced in order to obtain an overall impression of the transcriptome of a certain developmental state of the seed. Gene expression of most genes needs to be verified, as some of the genes picked up may appear in both the forward and reversed libraries. Not all genes picked up in one library were found to be expressed differentially by qRT-PCR. This could be due to the sensitivity of the method, or the genes may have been selected erroneously.

Despite these methodological problems, several genes (in the primary dormant subtraction library orthologs AT1G75830, AT1G79260, AT3G43520, AT3G60980, AT3G56340, AT3G56350, AT5G11710, AT5G60980, AT5G64840 and AT5G66120, in the long term primary dormant subtraction library orthologs AT1G13690, AT1G30880, AT1G35190, AT1G51200, AT1G79920, AT3G12120, AT3G44290, AT3G54860, AT4G40030, AT5G22290 and AT5G24350) were identified that are similarly expressed in Sisymbrium and Arabidopsis, although not verified by PCR. The exact role of these genes in the regulation of primary and

long term primary dormancy can only be verified by functional analysis, e.g. by reverse genetics.

Chapter 7

Summarizing Discussion

Dormancy, diapause, rest and quiescence are all terms that are in use to indicate a state of (temporary) lowered metabolic activity ('hypometabolic state') in living organisms. Dormancy occurs in many organisms from all kingdoms, ranging from bears to mice and frogs, from fish to buds and seeds, and from crustaceans to yeast and bacteria (Lubzens *et al*., 2010). Dormancy and stress responses are closely associated. Many of the above organisms become dormant before they are exposed to stress conditions. The timing of this may depend on developmental phase (e.g. sexual versus asexual), seasonal rhythms, environmental cues and food supply, among others. The common denominators appear to be controlled reduction of metabolic activity, arrest of the cell cycle and the initiation of protective mechanisms.

However, whether the underlying mechanisms are similar between different organisms is a question yet to be answered. The first comparisons indicate remarkable similarities (Hilhorst, 2008). Some of the similarities are metabolic control mediated by AMP-activated protein kinase and the induction of protective late embryogenesis abundant (LEA) and heat-shock (HSP) proteins (Hilhorst, 2008).

Seasonal dormancy cycling is a characteristic, so far, only found in seeds. Being able to cycle in and out of dormancy allows the seed to survive decades or even centuries in the soil, allowing germination to be spread over time but only when optimal conditions are available, not only for germination but also for seedling establishment. The ecological significance of dormancy cycling has long been established (Karssen, 1982; Bouwmeester, 1990) but the physiological and molecular mechanisms behind it have only been studied in recent years. In this

thesis we have attempted to further elucidate the mechanisms behind dormancy, germination and dormancy cycling in seeds.

Germination stimulatory factors: light, nitrate and gibberellic acid (GA)

Sisymbrium officinale seeds do not complete germination after imibibition in water or nitrate when no light pulse has been given. The seeds need nitrate to become non-dormant and light to start germination (Chapters 2, 3, 5, 6). Nitrate acts in part by reducing the abscisic acid levels (Matakiadis et al., 2009), which at high levels promote dormancy. If the light pulse is not given within a certain time frame, seeds will become secondary dormant and the seed is not sensitive to light and nitrate anymore. Other methods are then needed to break the dormancy (Derkx and Karssen, 1993).

GAs can be used to circumvent the light and nitrate requirement for germination (Chapter 6). GA biosynthesis has been suggested to be mediated by light and nitrate as a cofactor (Hilhorst et al., 1986). Light both induces Phytochrome B (PHYB) expression and activates the resulting PhyB protein. The active PHYB protein reduces phytochrome-interacting factor 3-like 5 (PIL5) protein levels. PIL5 suppresses the *AtGA3ox1* and *AtGA3ox2* and activates *AtGA2ox2*. Reduction of PIL5 allows up-regulation of *AtGA3ox1* and *AtGA3ox2* and down-regulation of *AtGA2ox2* (Yamaguchi, 2008), although PIL5 does not bind *AtGA3ox1*, *AtGA3ox2* or *AtGA2ox2* genes (Yamaguchi, 2008), suggesting that additional components are required. ATGA3OX1 and ATGA3OX2 catalyse the final step in the production of bioactive GAs, even without germination taking place afterwards (Yamaguchi *et al*., 1998; Oh *et al*., 2006). *Sisymbrium officinale* seeds are thought to possess seed coat imposed dormancy but we found that embryos of dormant seeds lack growth force to overcome these restrictions (Chapter 3). It was therefore concluded that dormancy of this species is also located in the embryo.

GAs are capable of inducing enzymes that hydrolyze the endosperm cell walls (Debeaujon and Koornneef, 2000; Chen and Bradford, 2000; Nonogaki et al., 2000; Manz et al., 2005) and in this way GAs could be involved in overcoming restrictions imposed by the seed coat. On the other hand, GAs may also increase the embryo growth potential.

Metabolism and respiratory activity during changes in dormancy

For successful survival of the dormant seed, metabolic activity is reduced (see above), to avoid rapid depletion of reserves. The metabolic state of the seed was measured using electron paramagnetic resonance (EPR), with TEMPONE as a spin probe, and the respiratory activity was measured with the Q2-test (Chapter 2). Primary dormant seeds of *Sisymbrium officinale* showed hardly any metabolic or respiratory activity, but this was increased considerably when dormancy was broken by nitrate. However, when the light pulse was not given and the seeds had become secondary dormant the metabolic activity slowed down. The reduction of metabolism and respiratory activity in secondary dormant seeds did not reach the same low activity of metabolism of primary dormant seeds. However, we propose that after longer induction of secondary dormancy the metabolic activity and respiratory activity will slow down even further, eventually likely to a similar level as in primary dormant seeds.

Cytoplasmic changes during dormancy cycling

Dormancy has often been linked to the survival of the seed during periods of abiotic stress. In order to survive the stress periods seeds may adapt their cytoplasmic properties to the changing environment (Chapter 4). We tested this by EPR, using carboxyl-proxyl (CP) spin probe. Vitrification temperatures for cytoplasmic water were higher for dormant than for germinating seeds. The

vitrification temperature depends on the composition of the cytoplasm. In germinating seeds the storage reserve mobilization has started, allowing more low-molecular weight compounds in the cytoplasm. This causes a lower vitrification temperature. Also, the germinating seeds had a less viscous cytoplasm than the dormant seeds. The dormant seeds possess higher quantities of high-molecular weight compounds in their cytoplasm than germinating seeds in which these have been metabolized. The high molecular weight compounds have a greater influence on the viscosity than the low-molecular weight compounds. However, the differences in quantity of high and low-molecular weight compounds might not be the main cause for differences in viscosity. Dijksterhuis (et al., 2007) also found changes in cytoplasmic viscosity, in their research on fungal spores but they came to the conclusion that the higher viscosity was not mainly caused by a high concentration of solutes but by the high proportion of ordered PDT molecules in the vicinity of macro-structures. Sun (2000) linked increased viscosity to reduced metabolism of red oak seeds. We also found reduced metabolism in dormant seeds (Chapter 2). A more viscous cytoplasm could protect dormant seed, making them capable of surviving longer periods under abiotic stress, e.g. by protecting cellular structures (Shinozaki *et al*, 1999) and preventing de/rehydration (Pneuli et al, 2002).

The membrane hypothesis analyzed

Membranes have been implicated in dormancy over the past decades, as membranes are the primary target for temperature perception (Hilhorst, 1998). A hypothesis was put forward in which changes in responsiveness to naturally occurring dormancy breaking factors like light and nitrate was a function of membrane fluidity (Hilhorst, 1998). In Chapter 3 we showed that dormancy is indeed a function of membrane fluidity. Primary dormant seeds of *Sisymbrium officinale* appeared to have very rigid membranes, whereas breaking dormancy increased membrane fluidity considerably. However, when non-dormant seeds

became secondary dormant membrane fluidity decreased again, but not to the rigidity seen in primary dormant seeds. A more viscous membrane could affect the functions of membrane proteins and could either be a response to stress, e.g. because of the lipid-/protein-mediated activation of a signal pathway, or play a role in cell injury, e.g. because some proteins such as respiratory and transport proteins only function in a fluid membrane (Beney and Gervais, 2001).

Membranes are known to be able to adapt to changes in temperature by homeoviscous adaptation (Sinesky, 1974), the mechanism whereby unsaturated fatty acids aid in maintaining the membranes in a fluid state necessary for biological functioning (Cyril *et al*., 2002). The introduction of double bonds into the fatty acids is catalyzed by desaturases (Los and Murata, 1998). Desaturase involvement in changes in membrane fluidity due to changes in dormancy was tested in Chapter 3 (using *Sisymbrium officinale*) and Chapter 5 (using *Arabidopsis thaliana*). Although FAD3, involved in the conversion of linoleic acid to linolenic acid (Matos *et al*., 2005) could be related to dormancy and germination (Chapter 5), it was not found to be related to membrane fluidity in *Arabidopsis thaliana* (Chapter 5). The ADS2 mutant, normally involved in the conversion of stearic acid into oleic acid (Murata and Wada, 1995), on the other hand, did not show changes in fatty acid composition, or a different germination phenotype, but it did show a gene expression pattern with high expression in non-dormant seeds, enhanced by light, and increased membrane fluidity (Chapter 5). In *Sisymbrium officinale* there was no difference in fatty acid composition between different dormancy types, indicating no involvement of desaturases in changes in membrane fluidity. Although desaturase mutants seem to change germination behaviour and/or membrane fluidity, desaturases are not the cause of these changes in wild type seeds. Likely candidates for changing membrane fluidity are changes in the acyl chain length (Ohlrogge and Browse, 1995) or lipid-protein interactions (Yeagle, 1989). The transition from the dormant to the non-dormant state is marked by changes in the composition of membrane-associated proteins, mainly in the plasma membrane enriched fractions (DiNola et al., 1989).

'Old' model, new perspective

In the Membrane hypothesis, as formulated by Hilhorst (1998), phytochrome receptors may be (temporarily) associated with membranes. Temperature has a profound influence on membrane fluidity which, on its turn, may determine the magnitude of movement of receptors within the membranes. In one conformation the receptors will be at the surface, available for nitrate and phytochrome to bind whilst in the other conformation the receptors are within the membrane and therefore not available for binding of nitrate and phytochrome. Homeoviscous adaptation might be responsible for the changes in membrane fluidity (Chapter 1). However, continuing research on various aspects of this hypothesis has altered this view. Below the new findings will be discussed and a new hypothesis will be put forward.

The phytochromes that are thought to be involved in germination are PhyA, PhyB and PhyE (Nagy, 2000). PhyB is active over a range of temperatures and is induced by red/far red light (Nagy, 2000), which makes it the most likely candidate in our model. However, there is no evidence of membrane association for phytochrome. Studies have shown that on photoconversion of Pr to Pfr the phytochromes are translocated from the cytoplasm into the nucleus where they form intranuclear bodies of various sizes in light quality dependent and light quantity dependent fashion (Yamaguchi, 1999; Kircher, 1999/2002). However, a significant portion of the phytochrome remains cytosolic (Nagy, 2000). The active phytochrome protein reduces phytochrome-interacting factor 3-like 5 (PIL5) protein levels. Reduction of PIL5 allows up-regulation of *GIBBERELLIN 3-OXIDASE 1, AtGA3ox1,* and *GIBBERELLIN 2-OXIDASE 2, AtGA3ox2* and down-regulation of *AtGA2ox2* (Yamaguchi, 2008). ATGA3OX1 and ATGA3OX2 catalyse the final step in the production of bioactive GAs, even without germination taking place afterwards, whereas *AtGA2ox2* is a key gene in the degradation of GAs (Yamaguchi *et al*., 1998; Oh *et al*., 2006).

In Arabidopsis high affinity nitrate transporter 2.7 (NRT2.7) has been found to be the primary nitrate transporter (Chopin et al., 2007) and the dual affinity

nitrate transporter CHL1 has been reported to act as a sensor for nitrate (Ho et al., 2009). Within the seed nitrate enhances the degradation of ABA via the cytocrhome P450 8'-hydroxylase for ABA (CYP707A2: Matakiadis et al, 2009). The nitrate and GA pathways are loosely linked by ABA. ABA and GA metabolism are linked during seed germination, as seen by the increased GA biosynthesis in the *aba2-2* mutant and the suppression of GA biosynthesis by ABA during seed germination (Seo et al., 2006). The reduction of ABA by nitrate allows the biosynthesis of GA.

In Chapter 3 we found that membrane fluidity is indeed linked to dormancy and germination, however, not via the saturation and un-saturation of fatty acids.

These data together lead to the conclusion that the membrane hypothesis, as it exists, needs to be revised. Although membrane fluidity is involved in the dormancy/germination process, phytochrome light receptors are not located within the membrane. The nitrate transporter is located in the membrane, and changes in membrane fluidity could thus alter the accessibility of the nitrate transporter. However, as nitrate is not only a signal in the breaking of dormancy but also a nutrient for plant growth and development (Crawford, 1995) this is an unlikely option. So nitrate and light probably affect germination without a direct involvement of membranes.

Other factors related (changes in) cell membranes might be involved that lead to changes in dormancy and germination. Another gene involved in dormancy regulation and containing transmembranes domain is *DELAY OF GERMINATION (DOG1)* (Bentsink 2006). DOG1 accounts for genetic variation in natural populations and has been characterized at the molecular level. *DOG1*, encodes a novel plant-specific protein of unknown mode of action.*DOG1* is functionally related to primary dormancy in Arabidopsis Cvi. Its expression decreased with after-ripening and during imbibition, which led to the conclusion that the gene is related to dormancy induction during seed development (Bentsink, 2006). However, *DOG1* expression may increase again upon induction of secondary dormancy, as was shown in Arabidopsis Cvi (Finch-Savage et al., 2007). Because of the presence of

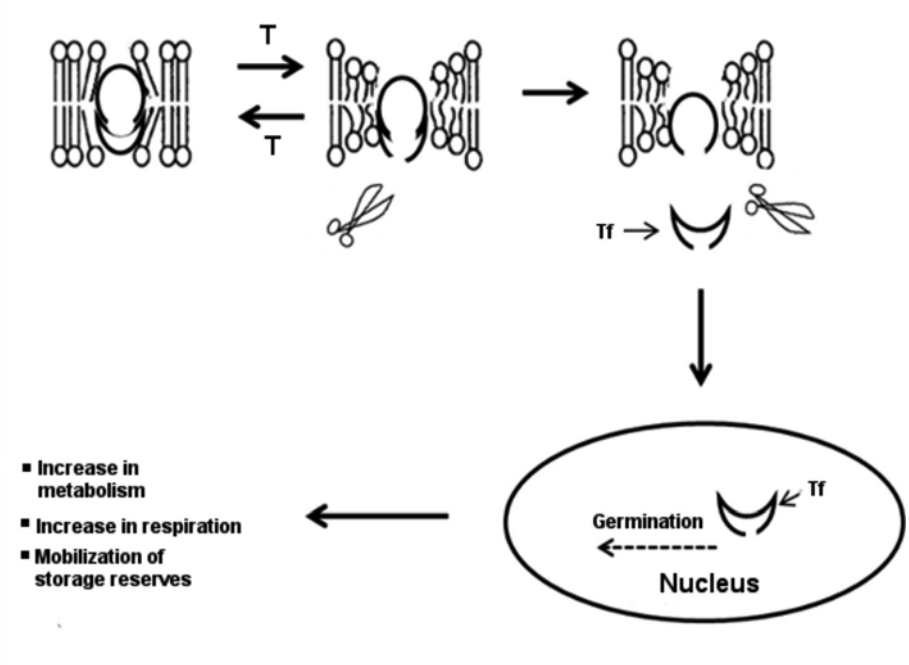


Figure 1: Model for the induction of germination. Under the influence of temperature (and with changes in dormancy) the membrane fluidity changes. The changes in membrane fluidity allow the (proteolytical) activation of a membrane anchored dormancy related transcription factor. The transcription factor (Tf) is transported to the nucleus where it activates the germination process. The germination process starts with an increase in metabolism, respiration and the mobilization of storage reserves. The effects of nitrate and light on dormancy and germination take place without membrane involvement and therefore are not included in this model

a transmembrane domain. *DOG1* might be the link between the changes in dormancy and membrane fluidity we found in this thesis. *DOG1* could be proteolytically activated by remodeling the membrane fluidity.

Seo et al. (2010) proposed a model whereby the membrane bound transcription factor NTL6 is proteolytically activated by remodeling of the membrane fluidity. NTL6 plays a role in cold-induced pathogen resistance. They found that the regulation by NTL6 changes with changes in membrane fluidity and their suggestion is that a cold-regulated membrane-associated metalloprotease may serve as a primary regulator for NTL6-mediated cold signaling. The

transcriptionally active NTL6 form is translocated from the plasma membrane to the nucleus where it triggers a pathogen resistance response.

Our suggestion is that a similar process may be present here (Figure 1). A membrane anchored dormancy related protein/transcription factor, e. g. *DOG1*, is activated by increasing the membrane fluidity. The activation of the transcription factor can be proteolytcally or by releasing the whole transcription factor from the membrane. In our findings the membrane fluidity was increased at extreme temperatures (chapter 3) which makes proteolytical activation less likely than activation by releasing the whole transcription factor. The activated form is transported to the nucleus where it activates the germination process. The germination process includes the increase in metabolism (Chapter 2) and respiration (Chapter 2) and the mobilization of storage reserves (Chapter 2). Mobilization of storage reserves leads to a decrease in cytoplasmic viscosity as found here in Chapter 4.

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Summary

The life cycle of most plants starts, and ends, at the seed stage. In most species mature seeds are shed and dispersed on the ground. At this stage of its life cycle the seed may be dormant and will, by definition, not germinate under favourable conditions (Bewley, 1997).

Seasonal dormancy cycling is a characteristic found in plant seeds. Being able to cycle in and out of dormancy allows the seed to survive decades or even centuries, allowing germination to be spread over time, but only when optimal conditions are available, not only for germination but especially for seedling establishment. In this thesis we have attempted to further elucidate the mechanisms behind dormancy, germination and dormancy cycling.

Sisymbrium officinale seeds need nitrate and light to start germination (Chapter 2, 3, 4, 6). Nitrate acts in part by reducing the abscisic acid (ABA) levels (a plant hormone that elevates dormancy levels). The action of light and nitrate can also be reached by applying gibberellins (GAs) to the seeds (Chapter 2, 3, 4, 6). GAs are capable of inducing enzymes that hydrolyze the ensdosperm walls (Debeaujon and Koornneef, 2000; Chen and Bradford, 2000; Nonogaki et al., 2000; Manz et al., 2005) In this way GAs could be involved in lowering the physical restrictions imposed by the resistance of the seed coat and the endosperm. On the other hand, GAs may also increase the embryo growth potential.

For successful survival of the dormant seed, metabolic activity is reduced to avoid rapid depletion of reserves. The metabolic activity of the seed was measured using electron paramagnetic resonance (EPR), with TEMPONE as a spin probe, and the respiratory activity was measured with the Q2-test (Chapter 2).We showed that primary dormancy was accompanied by hardly any metabolic or respiratory activity, and this increased considerably when dormancy was broken by nitrate. However,

when the light pulse was not given and the seeds had become secondary dormant the metabolic activity slowed down.

Regulation of dormancy is tightly linked with abiotic stress factors from the environment. The regulation and survival of the seed under stress conditions is largely dependent on the composition of the cytoplasm. We tested this by EPR, using carboxyl-proxyl (CP) spin probe (Chapter 4). The primary dormant and sub-dormant seeds possessed a higher viscosity than the germinating seeds. The viscosity of secondary dormant seeds appeared intermediate; however, the ease at which the vitrified water melted was similar to that of primary dormant seeds. As a result of the differences in viscosity, the temperature of vitrified water melting differed between the different dormancy states. The changes in cytoplasmic viscosity and vitrified water melting may be linked to changes in metabolism and the content of high molecular weight compounds.

As membranes are the primary target for temperature perception, they are often implicated in regulating dormancy. Therefore, Hilhorst (1998) put forward a hypothesis in which changes in responsiveness to dormancy breaking factors like nitrate and light was a function of cellular membrane fluidity. In Chapter 3 we indeed showed that dormancy is a function of membrane fluidity. Primary dormant seeds of *Sisymbrium officinale* appeared to have very rigid membranes, whereas breaking dormancy increased membrane fluidity considerably. However, when sub-dormant seeds became secondary dormant membrane fluidity decreased again, but not to the rigidity seen in primary dormant seeds. One of the most common ways in which cells control membrane fluidity is by homeoviscous adaptation with the help of desaturases. Desaturase involvement in changes in membrane fluidity due to changes in dormancy was tested in Chapter 3 (using *Sisymbrium officinale*) and Chapter 5 (using *Arabidopsis thaliana*). Here we found that although desaturase activity may change the membrane fluidity or influence the germination/dormancy phenotype, the two are not linked, unless the effects of these enzymes are very local within the seed. Finally, in Chapter 7, we presented a new model in which a membrane anchored dormancy related protein/transcription

factor is activated by changes in membrane fluidity. The activated form is transported to the nucleus, where it starts the germination process, which includes changes in metabolism and mobilization of storage reserves.

Samenvatting

De levenscyclus van de meeste planten begint en eindigt als zaad. Meestal vallen de rijpe zaden van de moederplant en komen dan verspreid op en in de grond terecht. In dit stadium van de levens-cyclus kan het zaad dormant zijn en zal dan - ook onder gunstige omstandigheden - niet kiemen(Bewley, 1997).

De seizoengebonden dormantie-cyclus is eigen aan planten. Het in en uit kiemrust kunnen komen zorgt er voor dat het zaad decennia- of zelfs eeuwenlang kan overleven, waardoor kieming zich over de tijd kan spreiden en alleen dan optreden als de optimale condities - voor kieming maar vooral ook voor overleving van de zaailing - zich voordoen. In dit proefschrift hebben we het dormantiemechanisme en de dormantie-cyclus verder onderzocht.

Sisymbrium officinale zaden hebben nitraat en licht nodig om te kiemen (Hoofdstuk 2, 3, 4, 6). Nitraat zorgt, gedeeltelijk, voor een vermindering van het gehalte aan abscisinezuur (ABA), een plantenhormoon dat de kiemrust bevordert. Het effect van licht en nitraat kan ook worden bewerkstelligd door aan zaden gibberellines (GAs) toe te voegen (Hoofdstuk 2, 3, 4,6). GAs kunnen enzymen induceren die de celwanden van het endosperm hydrolyseren (Debeaujon and Koornneef, 2000; Chen and Bradford, 2000; Nonogaki et al., 2000; Manz et al., 2005). Op deze manier zouden GAs betrokken kunnen zijn bij het verminderen van de fysieke beperkingen die worden veroorzaakt door de weerstand van zaadhuid en endosperm. Daarnaast kunnen GAs ook de groeipotentiaal van het embryo verhogen.

Voor succesvol overleven van het dormante zaad moet metabolische activiteit worden gereduceerd om ervoor te zorgen dat reserves niet worden uitgeput. Metabolische activiteit van het zaad is gemeten met de elektron paramagnetisch resonantie techniek (EPR), met TEMPONE als spin probe. De ademhalingsactiviteit is gemeten met de Q2 –test (Hoofdstuk 2). We hebben laten

zien dat primair dormante zaden bijna geen metabolische- en ademhalings-activiteit vertonen. De metabolische- en ademhalings-activiteit nam aanzienlijk toe als dormantie werd gebroken met nitraat. Zonder een lichtpuls werden de zaden echter secundair dormant en nam de metabolische activiteit af.

De regulatie van kiemrust is nauw verbonden met stressfactoren uit de omgeving. Regulatie en overleving van het zaad onder stress condities hangt in belangrijke mate af van de compositie van het cytoplasma. We hebben dit getest met behulp van EPR, met carboxyl-proxyl (CP) als spin probe (Hoofdstuk 4). De primair dormante en sub-dormante zaden vertonen een hogere viscositeit dan de kiemende zaden. De viscositeit van secundair dormante zaden leek intermediair. Het gevitrificeerde water smolt echter op ongeveer dezelfde wijze als bij primair dormante zaden. Als gevolg van het verschil in viscositeit smolt het gevitrificeerde water in de verschillende kiemruststadia bij verschillende temperaturen. De veranderingen in cytoplasmatische viscositeit en de verschillende temperaturen waarbij gevitrificeerd water smolt kon worden gekoppeld aan veranderingen in metabolisme en aan het gehalte van verbindingen met een hoog moleculair gewicht.

Aangezien membranen de eerste barrière zijn voor het “waarnemen” van temperatuur, wordt verwacht dat ze betrokken zijn bij de regulatie van kiemrust. In dat kader heeft Hilhorst (1998) een hypothese opgesteld waarin de veranderingen in reactie op kiemrustbrekende factoren, zoals nitraat en licht, afhangen van de vloeibaarheid van cellulaire membranen. In Hoofdstuk 3 hebben we laten zien dat dormantie inderdaad afhangt van de vloeibaarheid van het membraan. Primair dormante zaden van *Sisymbrium officinale* hadden erg rigide membranen, terwijl het breken van dormantie de membraanvloeibaarheid aanzienlijk verhoogde. Echter, als sub-dormante zaden secundair dormant werden, nam de vloeibaarheid weer af, maar niet tot dezelfde stijfheid als bij primair dormante zaden. Een van de meest voorkomende manieren om membraanvloeibaarheid te veranderen is door homeovisceuze adaptatie met behulp van desaturases. De rol van desaturases in veranderingen van membraanvloeibaarheid hebben we getest in Hoofstuk 3

(*Sisymbrium officinale*) en Hoofdstuk 5 (*Arabidopsis thaliana*). Hier hebben we gevonden dat, alhoewel desaturase activiteit membraanvloeibaarheid kan veranderen of betrokken kan zijn bij veranderingen in het kiemings/dormantie fenotype, de twee niet aan elkaar gekoppeld zijn, behalve als het enzym een erg lokaal effect zou hebben in het zaad.

In hoofdstuk 7 presenteren we een nieuw model, waarin een - in het membraan verankerde dormantie gerelateerd eiwit/transcriptie factor - wordt geactiveerd door veranderingen in membraanvloeibaarheid. De geactiveerde vorm wordt naar de kern getransporteerd en start daar het kiemproces, en daarmee de veranderingen in het metabolisme en de mobilisatie van opslagreserves.

Dankwoord

In 2004 verwachtte ik niet dat mijn promotie zoveel tijd – en vooral doorlooptijd – zou vergen. Nu is het dan eindelijk zo ver; het proefschrift is af. Terugkijkend was het een boeiende en leerzame tijd. Sommige zaken duurden wat langer dan gepland en dan bedoel ik niet alleen de schrijfperiode maar bijvoorbeeld ook het werken aan de subtraction library en de anderhalf jaar die ik in Wakehurst Place heb gewoond.

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In Wakehurst Place there was a large group of (PhD) students with whom I share many happy memories. Laura, Christiane, Chelo, Estrella, Aurelie, Elly, "The Australians", Rowena, Thomas, Honying, Carlos, Anderson, Bruna, Rose and many more. I lived with most of you and really enjoyed the meals, swims, BBQ's and late nights in front of the TV and in the library. Thanks also to all others at Wakehurst Place and Plant Physiology, I really enjoyed your friendliness.

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Chloe, jij betekend zo veel meer voor me dan ik kan beschrijven

Curriculum Vitae

Susanne Claessens was born on the 11th of November 1977 in Asten, The Netherlands. In 1995 she obtained her HAVO- and in 1997 her VWO-diploma at the, then, College Asten-Someren. In August 1997 she started her studies of Biology at the University of Utrecht. During the doctoral-phase, she undertook two internships. The first internship was at the Phytopathology group, where, under supervision of Dr. Ir Corné Pieterse, she conducted research into "*The interaction between two defense mechanisms in Arabidopsis thaliana"*. The second was at the Plant Genetics group, where she worked under the supervision of Dr. Léonie Bentsink and worked on *"Characterisation and mapping of dormant mutants in Arabidopsis thaliana".* In September 2002 she obtained her master degree and in 2004 started as PhD-student of the Plant Physiology group of Wageningen University and at the Royal Botanical Gardens Kew in Wakehurst Place. There the research described in this thesis was performed.

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: **Susanne Claessens**
Date: **20 February 2011**
Group: **Plant Physiology, Wageningen University & Research centre**

1) Start-up phase	*date*
► **First presentation of your project**	
Research meeting, Wakehurst place	Sep 17, 2004
► **Writing or rewriting a project proposal**	
The project propsal has been expended with Oxygen measurements and TAM	Aug 2004
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*2,5 credits**
2) Scientific Exposure	*date*
► **EPS PhD Student Days**	
PhD Day Wakehurst (UK)	Oct 22, 2004
PhD Day Kew (UK)	Feb 16, 2006
Wageningen University, The Netherlands	Sep 19, 2006
► **EPS Theme Symposia**	
Theme 3 Symposium 'Metabolism and Adaptation', Wageningen University	Oct 25, 2004
Theme 3 Symposium 'Metabolism and Adaptation', Wageningen University	Nov 06, 2007
► **NWO Lunteren days and other National Platforms**	
Meeting on experimental plant sciences, Lunteren	Apr 02-03, 2007
► **Seminars (series), workshops and symposia**	
Seminar series at Wakehurst Place monthly	2004 - 2008
An introduction to the morphology of fruits,Wakehuirst place	Jun 2004
Prof. P. de Wit Symposium on Systems Biology	Nov 04, 2004
► **Seminar plus**	
► **International symposia and congresses**	
ISSS seed meeting, Brisbane Australia;	May 08-13, 2005
ISSS meeting on molecular aspects of seed dormanc and germination, Salamanca Spain;	Jul 01-04, 2007
Sleeping Beauties-dormancy and resistance in harsh environments-Molecular, proteomic and metabolomic aspects, Berlin, Germany	May 18-20, 2008
► **Presentations**	
PhD day Kew, Wakehurst Place (poster)	Oct 22, 2004
ISSS seed meeting, Brisbane Australia (poster)	May 08-13, 2005
PhD day Kew, Wakehurst Place (poster)	Feb 16, 2006
Seminar series presentation, Wakehurst Place (oral)	Mar 22, 2006
ISSS meeting on molecular aspects of seed dormancy and germination (oral)	Jul 01-04, 2007
► **IAB interview**	Sep 18, 2006
► **Excursions**	
Subtotal Scientific Exposure	*14,0 credits**
3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Proteomics, EPS	Feb 27-Mar 02, 2006
Environmental signaling: Arabidopsis as a model, Summer School EPS	Aug 27-29, 2007
Molecular Phylogenies: Reconstruction & interpretation EPS	Oct 15-19, 2007
► **Journal club**	
Every 2 weeks at Wakehurst place	2004-2008
► **Individual research training**	
O2, Leiden, The Netherlands	2005
TAM, University of Londen; Proteomics, University of Sussex, UK	2006
Subtotal In-Depth Studies	*9,6 credits**
4) Personal development	*date*
► **Skill training courses**	
Presentation skills, Wakehurst place	Jul 2004; May 22-23, 2006
Scientific writing course, Wakehurst place	Jul 2004; May 31, 2006
Light cycler training	Jul 07-08, 2005
► **Organisation of PhD students day, course or conference**	
Journal club, Wakehurst Place	Jul 03, 2006
► **Membership of Board, Committee or PhD council**	
Student steering comittee, Wakehurst Place	2006
Subtotal Personal Development	*4,6 credits**
TOTAL NUMBER OF CREDIT POINTS*	**30.7**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*