Regulation of seed dormancy and germination in *Arabidopsis thaliana*

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Abstract

Seed germination is one of the most important developmental steps in the life cycle of a higher plant. Because of this, seed producing plants have evolved mechanisms, such as dormancy, that time germination based on environmental cues. The present study uses *Arabidopsis thaliana* as a model to address questions about seed dormancy and germination. Three different lines of investigation were followed. The first involved an investigation of how light quality regulates phytohormones in order to control germination. This identified a light-dependent mechanism that differentially regulates expression of the *ALLENE OXIDE SYNTHASE* and *OXOPHYTODIENOATE-REDUCTASE 3* genes resulting in accumulation of *cis*-12-oxo-phytodienoic acid (*cis*-OPDA) and repression of seed germination under FR conditions. The second line of investigation involved a re-examination of the role of the ABSCISIC ACID INSENSITIVE (ABI) 5 and ABI4 transcription factors in regulating seed germination and oil mobilization respectively. The study found that abscisic acid (ABA) is able to block testa rupture in nicked seeds and this involves the ABI5, but not the ABI4, transcription factor. Furthermore, it was found that ABI4 is involved in the repression of ABA and *cis*-OPDA biosynthesis in a light-dependent manner, but has only a minor role in regulating oil mobilization in seeds. The third line of investigation focused on the regulation of dormancy during after-ripening and found that changes in phytohormone levels over an extended period can account for changes in dormancy state.

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Author’s Declaration

I hereby declare that I am the sole author of the work presented in this Thesis, unless otherwise acknowledged in the text. All sources are acknowledged as References. No part of this thesis has been previously submitted for any other degree, at this or any other university.

Chapter 1 - General Introduction

1.1 Seed Germination: a Brief Overview

1.1.1 The biological and economical importance of germination

The emergence of seeds in the course of higher plant evolution represented a major advance for the protection and dispersal of progeny (Linkies et al., 2010). When fully matured, seeds may be diverse in their shape and size among different species but all contain an embryo, which is the next generation of the plant. The embryo is enclosed by a protective structure, the seed coat, and in some species an alternative storage tissue may be present (endosperm, perisperm or megagametophyte), usually when the nutritive reserves are not stored within the embryo (Bewley et al., 2013; Baskin and Baskin, 2014).

Seed germination is a very complex and important phenomenon that marks the beginning of the life cycle of a higher plant. It represents the transition from a quiescent/protected to an active/susceptible state in orthodox seeds (i.e. seeds that are dry when mature). The completion of germination is followed by seedling establishment, under which a germinated seed becomes a photosynthetic autotrophic plant through utilization of the stored reserves. The major seed reserves are carbohydrates, oils, and proteins, and these are produced in crops as the basis of agriculture and are very important for human and animal diets (Bewley et al., 2013). Although the majority of the reserves are mobilized after seeds have germinated, this event is initiated prior to the completion of germination (Bewley et al., 2013; Baskin and Baskin, 2014).

In agriculture, seed vigor (i.e. the efficiency that a seed has to successfully germinate and establish (Perry, 1978; Perry, 1980)) is an important trait when crop stand uniformity is required (Rajjou et al., 2012; Finch-Savage and Bassel, 2016). Understanding the physiological and genetic mechanisms underlying seed germination and seedling establishment have applications for improving crops by manipulating these processes

either through the use of marker assisted selection and molecular breeding or genetic engineering.

1.1.2 Germination: how it is measured and what are the requirements

Germination starts with water uptake by the seeds, a process called imbibition, and ends when the embryonic axis, usually the radicle, emerges through the surrounding tissues (i.e. radicle protrusion) (Bewley et al., 2013; Baskin and Baskin, 2014). Thus, seed germination is measured by counting how many seeds have their radicle protruded within a population in a defined period of time.

Among all the environmental factors required for different species in order to complete their germination, water and oxygen are indispensable. Water uptake by seeds is a fundamental initial step and occurs in a triphasic manner when water is optimally available. Each phase reflects a different physical and metabolic step that drives water movement into the seeds. Phase I is marked by imbibition and imbibitional damage (e.g. disruption of cell walls and membranes); Phase II, also known as lag phase due to the temporary stop in imbibition, is marked by the reactivation of the metabolism; and only seeds that are able to complete germination may enter Phase III, which is marked by a restart in water uptake, embryo growth and seedling establishment (Leubner, 2006; Bewley et al, 2013; Baskin and Baskin, 2014).

Different elements drive the movement of water from the surroundings into a seed. Water potential (Ψ) is the unit used in plant physiology in order to express the free energy status and measure the net movement of water, which occurs from a high to a low Ψ (Taiz and Zeiger, 2010; Bewley et al, 2013). Ultimately, comparing a moist soil with a dry seed, the Ψ in the seeds are at least 5 orders of magnitude lower compared with the soil and therefore there is an intense water uptake into the seed upon imbibition. Phase I is very short and depends only on the matrix forces of the soil (e.g. capillarity of soil particles and

water binding to surfaces) and the seed (e.g. coat permeability and solutes in the cell), regardless of the dormancy levels (explained in section 1.2) or viability. Because of this rapid water uptake during Phase I, imbibition damage and leakage (e.g. ions and solutes) occur. Respiration increases dramatically within minutes of the start of imbibition along with a sharp increase in oxygen consumption (Leubner, 2006; Bewley et al, 2013).

Phase II of germination starts as water uptake decreases due to an increase in the seed Ψ. During this phase, two main processes occur in seeds: imbibitional damage repair (e.g. restoration of the cellular membranes and cytoskeleton, DNA repair, etc.) and control of germination (detailed in section 1.3). Finally, after germination, Phase III of imbibition starts due to the breakdown of the seed reserves and production of osmotically active substances, the Ψ of the seed starts to decrease leading to an increase in water uptake (Leubner, 2006; Bewley et al, 2013).

1.2 Seed Dormancy: Definition and General Features

The environmental conditions under which a seed germinates can be essential for seedling and plant survival and, consequently, controlled mechanisms have evolved to regulate germination (Linkies et al., 2010). One example is dormancy, which is defined as the failure of seeds to germinate even when the conditions are ideal. This is a strategy that some plant species use to spread germination of their seeds in time in order to guarantee the survival of the progeny under unfavorable environmental conditions. The inception of dormancy that occurs during seed development as a result of the interaction between the mother plant and the environment is called primary dormancy. Remarkably, dispersed non-dormant seeds (i.e. seeds that either lost or do not have primary dormancy) may enter into secondary dormancy if the conditions necessary for germination do not appear, which will lead to an inhibition of germination until secondary dormancy is broken (Bewley et al., 2013; Baskin and Baskin, 2014; Footitt et al., 2014). This dormancy cycling mechanism

underpins the bet-hedging strategy for the short- and long-term persistence of different species within the soil seed bank (Evans and Dennehy, 2005; Saatkamp et al., 2011). For over forty years, seed biologists have understood that the balance between the inhibitory actions of the phytohormone abscisic acid (ABA) and the stimulating actions of the gibberellins (GAs) phytohormones is the primary determinant of seed dormancy (primary and secondary) and germination (detailed in section 1.3) (Luckwill, 1952; Bewley and Black, 1982; Karssen and Laçka, 1985; Footitt et al., 2011; Rajjou et al., 2012; Bewley et al., 2013).

As briefly mentioned above, crop stand uniformity is a desired agronomic trait where seed germination plays an important role. Thus, dormancy is not a desirable trait when farmers need their seeds to germinate, and to do so as uniformly as possible. Hence, dormancy has been lost over centuries of plant domestication with the selection for seeds that would germinate fast and at the same time give rise to a growth-synchronized population. However, lack of dormancy can make cultivated varieties susceptible to preharvest sprouting, which reduces grain quality and results in significant economic losses (e.g. in cereals such as wheat and rice) (Bewley et al., 2013; Baskin and Baskin, 2014). Thus, there is still a real strategic requirement to carry out research aimed at understanding the mechanisms involved in the control of seed dormancy.

Textbooks describe two major categories of dormancy: coat- and embryo-imposed dormancy. In the first, the properties of the covering tissues (seed coat) are needed to prevent the expansion of the embryo that leads to the completion of germination; removal of these tissues is enough to induce embryo expansion and growth. However, when the embryo imposes dormancy, removal of the surrounding tissues does not promote embryo expansion and growth. Despite these two different classifications of dormancy, it is very common that both types exist concomitantly or sequentially (Bewley et al., 2013; Baskin and Baskin, 2014).

Germination has to be tightly regulated because it is a one-way process and seeds can do it only once. In order to break dormancy there are two major routes: one happens in dry seeds in the absence of metabolism; while the other occurs in a metabolic active seed. The route happening in dry seeds is called dry after-ripening, which breaks dormancy by an unknown mechanism. In nature, after-ripening is associated with dry/wet seasonal time, preventing seeds to germinate during the dry season (Bewley et al., 2013). For the metabolic active route, moist chilling is the best documented example, in which dormancy is broken by an increase in GA levels in seeds that experience low temperature for a certain period of time (Yamauchi et al., 2004). In nature, moist chilling is associated with winter, where seeds would break dormancy and germinate during spring. In addition to these seasonal time mechanisms, others such as light and chemicals can act more immediately and seem to be good indicators of the predominant environment (Bewley et al., 2013).

1.3 Regulation of Seed Germination and Dormancy in *Arabidopsis thaliana*

A. thaliana seeds have a single endosperm cell layer between the embryo and the coat (Penfield et al., 2004) (Figure 1.1 A) and, upon imbibition, germinate in a two-step process: first testa rupture followed by endosperm rupture (Figure 1.1 B and C) (Müller et al., 2006). Both events, the first and second, are consequence of embryo expansion, but testa rupture represents only the cracking of the testa while endosperm rupture represents only the protrusion of the radicle. Curiously, in non-dormant seeds, exogenously applied ABA has been shown to repress endosperm rupture but not testa rupture (Figure 1.1 D) (Müller, et al., 2006; Linkies et al., 2009). Nonetheless, endogenous ABA synthesis is required to repress testa rupture under low GA conditions (De Giorgi et al., 2015). Endosperm-imposed dormancy prevails in *A. thaliana* seeds and the phytohormones GA and ABA regulate germination in a tissue specific manner (Lee et al., 2010).

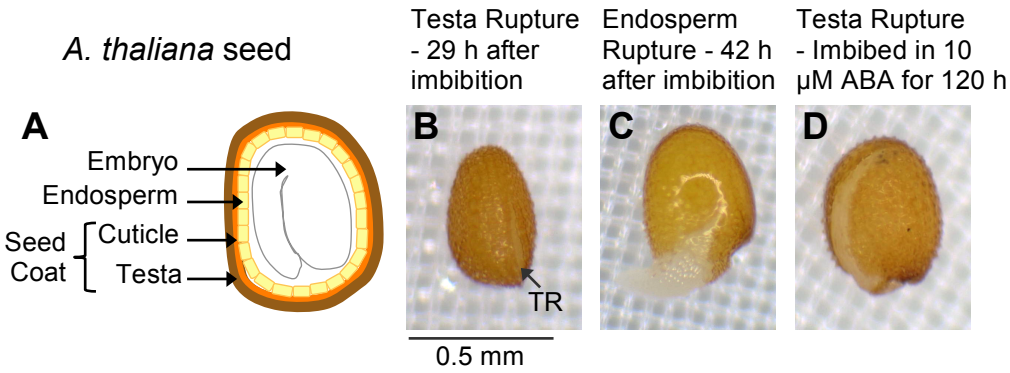


Figure 1.1 The two-step germination of *A. thaliana* seeds. (A) Scheme of a mature *A. thaliana* seed; the seed coat illustrated is composed of the testa (in brown) and the cuticle (in orange). (B-D) Upon seed imbibition, the first visual sign of germination is the rupture of the testa by expansion of the embryo (B), followed by the second step, which is marked by the rupture of the endosperm and protrusion of the radicle (C). In this process, ABA inhibits the second step but not the first (D). Seeds were imbibed in continuous light in the absence (control) or presence of 10 μ M ABA. Scale bar is 0.5 mm. This figure was generated as a repeat of previous published data (Müller, et al., 2006).

Seeds that are unable to synthesize GA, a hormone class that is represented by a large family of tetracyclic diterpenes, fail to germinate (Koornneef and van der Veen, 1980; Karssen et al., 1989; Nambara et al., 1991; Mitchum et al., 2006). GA biosynthesis is dependent on isopentenyl diphosphate (IPP) in the chloroplast, and the pathway also involves the endoplasmic reticulum and the cytosol. In the cytosol, three dioxygenases catalyze steps that are closely regulated: GA 20-oxidases (GA20ox) and GA 3-oxidases (GA3ox) catalyze the steps immediately prior to production of the bioactive GAs (GA_4 and GA_1); while GA 2-oxidases (GA2ox) are involved in GA deactivation. *GA3OX1* and *GA3OX2* encode enzymes that convert gibberellin A9 (GA_9) and GA_{20} into active forms GA_4 and GA_1 respectively, and their expression is required during germination (Mitchum et al., 2006; Yamaguchi, 2006; Taiz and Zeiger, 2010). Previous work performed with *A. thaliana* ecotype Cvi seeds indicate that the balance between biosynthesis and catabolism of GA and ABA will determine the dominance of either of the phytohormones in order to control germination (Ali-Rachedi et al., 2004; Cadman et al., 2006). For instance, dormancy breakage of Cvi seeds occurs effectively by some mechanisms (e.g. after-ripening and moist chilling), but exogenously applied GA induces a transient increase in

ABA levels in dormant Cvi seeds indicating that the net result of the dormant state involves an increase in ABA biosynthesis and GA deactivation (Ali-Rachedi et al., 2004).

GA biosynthesis occurs mainly in the embryo in Arabidopsis germinating seeds (Yamaguchi et al., 2001; Yamaguchi et al., 2007; Hu et al., 2008). GA induces germination via 26S proteasome degradation of the DELLA proteins, which are negative regulators of GA-inducible genes acting downstream of the GA receptor GA INSENSITIVE DWARF 1 (GID1) in the absence of GA (Figure 1.2) (Seo et al., 2009; Taiz and Zeiger, 2010; Davière and Achard, 2013). The *A. thaliana* genome has five DELLA genes with partly overlapping functions: *REPRESSOR OF GA1* (*RGA*); *GA INSENSITIVE* (*GAI*), *RGA-LIKE1* (*RGL1*), *RGL2* and *RGL3*. Among these DELLAs, RGL2 has a critical role not only in repressing the genes regulated by GA but also in promoting the biosynthesis of ABA (Piskurewicz et al., 2008; Lee et al., 2010). This removal of DELLA repression by GA is central to dormancy release and germination induction (Figure 1.2).

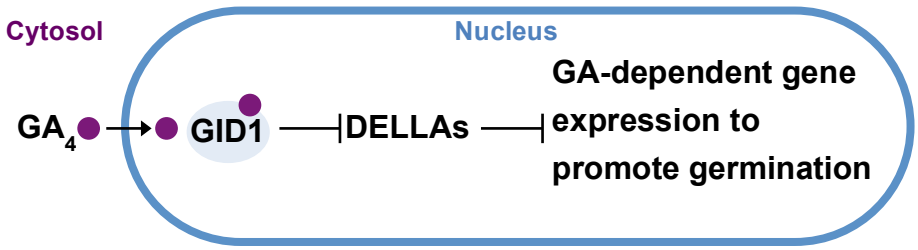


Figure 1.2 Simplified gibberellic acid (GA) signaling pathway in *A. thaliana*. GA_4 is thought to be the major active form in Arabidopsis (Yamaguchi 2006). GA_4 enters the nucleus and binds to its receptor GID1, stimulating the formation of a GA_4-GID1-DELLA complex. Formation of this complex induces ubiquitylation and subsequent destruction of DELLAs by the 26S proteasome, which allows genes to be expressed in response to GA_4. GA_4, Gibberellin A4; GID1, GA insensitive dwarf 1.

During the development of the seed in the mother plant, endogenous ABA that is synthetized by the seed itself induces and maintains seed dormancy, while maternal or exogenous ABA application during seed development fails to induce long lasting dormancy (Kucera et al., 2005). ABA is a sesquiterpene compound derived from the cleavage of carotenoids (Taiz and Zeiger, 2010). Its biosynthetic pathway begins in the chloroplast with IPP that leads to the synthesis of violaxanthin, catalyzed by the ABA

DEFICIENT 1 (ABA1) enzyme (Figure 1.3) (Taiz and Zeiger, 2010). The later steps of ABA biosynthesis occur in the cytosol, the penultimate step is catalyzed by an enzyme encoded by the *ABA DEFICIENT 2* (*ABA2*) locus that is involved in the synthesis of abscisic aldehyde (AB-aldehyde), which is the substrate of the ABSCISIC ALDEHYDE OXIDASE 3 (AAO3) to make ABA (Figure 1.3) (Taiz and Zeiger, 2010). Mutants in either the *ABA1* (Barrero et al., 2005) or *ABA2* (González-Guzmán et al., 2002) loci have reduced levels of ABA, leading to the production of non-dormant seeds. In imbibed dormant seeds, two isoforms, nine-*cis*-epoxycarotenoid dioxygenases (NCED) 6 and 9, are the rate-limiting regulatory step involved in the formation of xanthoxin, which is the first committed step for ABA biosynthesis (Figure 1.3) (Lefebvre et al., 2006; Taiz and Zeiger, 2010). In addition to this, Lefebvre and others (2006) also showed that NCED6 and NCED9 contribute to the induction of seed dormancy.

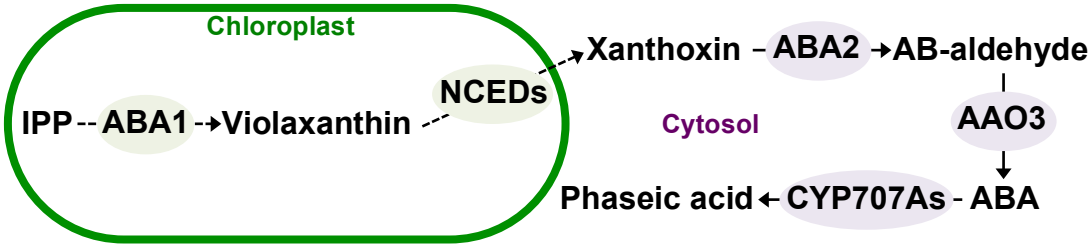


Figure 1.3 Simplified ABA biosynthetic and catabolic pathways in *A. thaliana*. Synthesis initiates in the plastid with IPP, which is catalyzed by several enzymes including zeaxanthin epoxidase (ABA1 in *A. thaliana*), to make violaxanthin. Cleavage of violaxanthin is catalysed by a family of NCEDs to make xanthoxin, which is then converted by a short-chain alcohol dehydrogenase (ABA2) into AB-aldehyde. Finally, AB-aldehyde is oxidized into ABA by AAO3. Then, ABA can be hydroxylated and inactivated by CYP707As, leading to the formation of phaseic acid. IPP, isopentenyl diphosphate; ABA1, ABA deficient 1; NCEDs, nine-*cis*-epoxycarotenoid dioxygenase 6 and 9; ABA2, ABA deficient 2; AB-aldehyde, abscisic aldehyde; AAO3, abscisic aldehyde oxidase 3; CYP707As, cytochrome P450 monooxygenases 1, 2 and 3. Dashed lines represent multiple enzymatic reactions that are not depicted.

Catabolism of ABA also plays a critical role in the control of seed dormancy and germination (Okamoto et al., 2006). Among the different ABA catabolic pathways in plants, the 8'-hydroxylation pathway that leads to the formation of phaseic acid is understood to be predominant and is catalyzed by cytochrome P450 monooxygenases (CYP707As) (Figure 1.3), as shown in *Echinocystis lobata* liquid endosperm (Gillard and

Walton, 1976) and in suspension cultures of maize (Krochko et al., 1998). Compared with wild type, *cyp707a1*, *cyp707a2* and *cyp707a3* accumulate more ABA levels in dry seeds and show low germination in *A. thaliana*. However, analysis of *CYP707A* (1, 2, and 3) transcript levels also suggest that they play distinct roles during other events in the life cycle of the plant (Okamoto et al., 2006).

ABA is biosynthesized mainly in the endosperm in dormant seeds and transported to the embryo in order to repress embryo expansion and therefore germination (Lee et al., 2010; Kang et al., 2015). When bound to its receptors, such as PYRABACTIN RESISTANCE 1 (PYR1) and PYR-LIKEs (PYLs), ABA triggers a signaling cascade that leads to inactivation of type 2C protein phosphatases (like ABA insensitive 1 [ABI1] and ABI2). Inactivation of ABI1 and ABI2 allows sucrose non-fermenting related kinase 2 (SnRK2) to phosphorylate downstream transcription factors such as ABA insensitive 5 (ABI5) (Figure 1.4) (Raghavendra et al., 2010; Taiz and Zeiger, 2010; Bewley et al., 2013). ABI3, ABI4 and ABI5 encode B3-, AP2-, and bZIP-type transcription factors, respectively, which are important for the ABA responses during seed maturation, germination and seedling establishment. For instance, ABI3 is a seed-specific regulator that plays a crucial role in all of these three different seed stages (Giraudat et al., 1992; Parcy et al., 1997; Nambara et al., 2000; Finkelstein et al., 2002; Lopez-Molina et al., 2002; Delmas et al., 2013). ABI5 has been shown to control germination downstream of ABI3 by directly binding and regulating genes involved in GA and ABA biosynthesis (Lopez-Molina et al., 2001; Lopez-Molina et al., 2002; Lee et al., 2012), while ABI4 has been shown to play a major role in controlling oil mobilization (detailed further in Chapter 4) in the embryo of germinating seeds (Penfield et al., 2006).

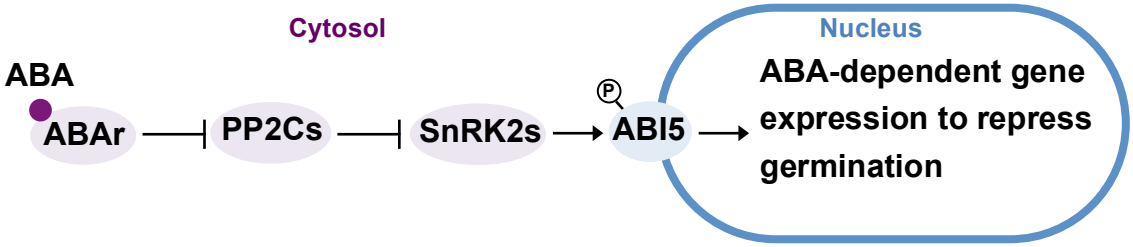


Figure 1.4 Simplified abscisic acid (ABA) signaling pathway in *A. thaliana*. In the cytosol, ABA binds to its receptors (ABAr) and form a complex with PP2Cs, freeing SnRK2s to perform auto-phosphorylation and phosphorylation of transcription factors such as ABI5. Finally, ABI5 regulates ABA-dependent gene expression in the nucleus. ABAr, ABA receptors (PYR1 and PYLs); PP2Cs, type 2C protein phosphatases (ABI1 and ABI2); SnRK2s, sucrose non-fermenting related kinases 2; ABI, ABA insensitive 5.

In 2011, Dave and others found that the forever-dormant phenotype of the *A. thaliana* peroxisomal ABC transporter (pxa) mutants comatose (*cts*) (Theodoulou et al., 2005; Footitt et al., 2002) and *pxa1* (Zolman et al., 2001) is caused by accumulation of the oxylipin *cis*-OPDA, a precursor of the wound-related phytohormone jasmonic acid (JA) (Wasternack and Hause, 2013). The oxylipin biosynthetic pathway starts with oxidation of polyunsaturated fatty acids (PUFAs) such as linoleic acid (18:2), octadecatrienoic acid (18:3n-3), and hexadecatrienoic acid in the chloroplast (Wasternack, 2007; Mosblech et al., 2009; Wasternack and Kombrink, 2010; Dave and Graham, 2012). However, the release of these fatty acids (FAs) from plastidial membrane lipids is still in debate (Wasternack and Hause, 2013). For instance, DEFECTIVE IN ANTHER DEHISCENSE 1 (DAD1) was shown to be involved in JA biosynthesis since *dad1* mutant plants showed reduced JA levels in flowers and male-sterility (Ishiguro et al., 2001). A homolog of DAD1, DONGLE (DGL) was also shown to be involved in basal JA biosynthesis (Yang et al., 2007; Hyun et al., 2008). However, Ellinger and others (2010) claimed that DAD1 and DGL are not necessary for wound- and pathogen-induction of JA synthesis, since *dad1* and DGL RNAi lines showed no differences in *cis*-OPDA and JA levels compared to wild type, indicating a redundancy within the lipase family.

The pathway that gives rise to JA starts with the oxidation of 18:3n-3 FA by 13-lipoxygenase to form 13-hydroperoxylinolenic acid, which is the substrate of ALLENE OXIDE SYNTHASE (AOS) and ALLENE OXIDE CYCLASE to make *cis*-OPDA. *cis*-

OPDA then moves from the plastid to the cytosol, where it is imported to the peroxisome, at least in part, by the PXA1/CTS transporter (Figure 1.5) (Zolman et al., 2001; Theodoulou et al., 2005). In the peroxisome, 12-oxophytodienoate reductase 3 (OPR3) reduces *cis*-OPDA (Sanders et al., 2000; Schaller et al., 2000; Stintzi and Browse, 2000), which is then activated to the CoA ester (Schneider et al., 2005; Koo et al., 2006; Kienow et al., 2008) prior to undergoing three rounds of β-oxidation (i.e. the catabolic process by which FAs are broken down 2 carbon units per round) to produce JA (Figure 1.5) (Cruz Castillo et al., 2004; Pinfield-Wells et al., 2005; Schilmiller et al., 2007). Finally, JA travels to the cytosol where it can be converted by jasmonic acid-amido synthetase (JAR1) into the active form jasmonoyl-l-isoleucine (JA-Ile) (Figure 1.5) (Staswick and Tiryaki, 2004), which is the effector of JA responses via the jasmonate ZIM domain (JAZ) repressor proteins (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007).

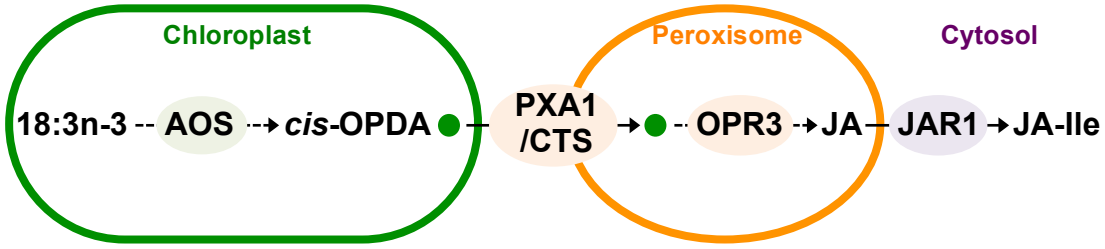


Figure 1.5 Simplified jasmonic acid related oxylipin biosynthetic pathway in *A. thaliana*. Biosynthesis initiates in the plastid with release of 18:3n-3 from membrane lipids by lipases, following sequential steps catalyzed by different enzymes including AOS. *cis*-OPDA is transported to the peroxisome via the PX/CT transporter, where after reduction by OPR3, is activated and undergoes three rounds of β-oxidation to give JA. JAR1 catalyzes formation of JA-Ile conjugate from JA in the cytosol. 18:3n-3, octadecatrienoic acid; AOS, allene oxide synthase; *cis*-OPDA, *cis*-12-oxo-phytodienoic acid; PXA1/CTS, PEROXISOMAL ABC TRANSPORTER 1/COMATOSE; OPR3, 12-oxophytodienoate reductase 3; JA, jasmonic acid; JAR1, jasmonic acid-amido synthetase; JA-Ile, jasmonoyl-L-isoleucine. Dashed lines represent multiple enzymatic reactions that are not depicted.

During wound and defense response against pathogens, JA and *cis*-OPDA appear to act together in order to fine tune the expression of genes, but show distinct roles when it comes to the male sterility phenotype of *opr3*, which can only be rescued by JA (Stintzi and Browse, 2000). Additionally, a growing body of evidence supports the idea that *cis*-OPDA has a distinct signaling role, despite the poor knowledge on the mechanisms that

mediate the downstream events (Weiler et al., 1993; Blechert et al., 1999; Taki et al., 2005; Mueller et al., 2008; Ribot et al., 2008; Böttcher and Pollmann, 2009; Dave et al., 2011; Schäfer et al., 2011; Dave and Graham, 2012; Park et al., 2013; Dave et al., 2016). It has been hypothesized that *cis*-OPDA acts through a redox-dependent signaling mechanism that activates TGA transcription factors (Böttcher and Pollmann, 2009). Indeed, Park and others (2013) found that *cis*-OPDA relays a retrograde signal from the chloroplast that changes the redox-state of the cell in order to control a subset of *cis*-OPDA responsive genes in the nucleus. Remarkably, they discovered that *cis*-OPDA binds to cyclophilin 20-3 (CYP20-3) in the chloroplast, which results in formation of a cysteine synthase complex (CSC), leading to an enhanced redox potential (thiols and GSH, for glutathione) in the cell under stress responses (Figure 1.6).

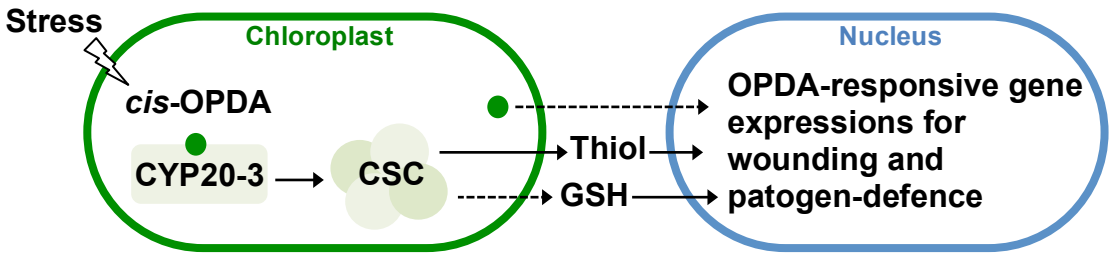


Figure 1.6 Simplified *cis*-12-oxo-phytodienoic acid (*cis*-OPDA) signaling pathway in *A. thaliana*. *cis*-OPDA binds to CYP20-3, which then promote CSC formation that shifts the redox potential, via an increase in thiol and GSH, that triggers expression of a subset of *cis*-OPDA responsive genes under wounding and pathogen stresses. CYP20-3, cyclophilin 20-3; CSC, cysteine synthase complex; GSH, glutathione. Dashed lines represent multiple steps that are not depicted.

Cellular redox status has often been proposed to regulate seed germination (El-Maarouf-Bouteau and Bailly, 2008), with thioredoxins and/or glutathione/glutaredoxin thought to be the two main cellular redox agents involved (Foyer and Noctor, 2005; Buchanan and Balmer, 2005). In more recent work, it has been shown that *cis*-OPDA acts through the germination inhibitory effects of ABA and RGL2, which promotes ABA accumulation during seed germination (Lee et al., 2010; Lee et al., 2012; Dave et al., 2016). For example, *aba1*-1 and *rgl2*-1 seeds showed insensitivity to exogenous

application of *cis*-OPDA suggesting that *cis*-OPDA requires ABA to repress germination in *A. thaliana* (Dave et al., 2016). However, under drought stress it has been reported that ABA and *cis*-OPDA may act independently in order to promote stomatal closure (Savchenko et al., 2014). Dave et al. (2016) have also demonstrated that *cis*-OPDA acts through the dormancy promoting factor MOTHER-OF-FT-AND-TFL1 (MFT) in germinating seeds. MFT belongs to the phosphatidyl ethanolamine-binding protein family and has been shown to control germination and dormancy in different species (Li et al., 2014; Nakamura et al., 2011; Vaistij et al., 2013; Xi and Yu, 2010; Xi et al., 2010). Furthermore, both ABA and MFT positively feed back into the *cis*-OPDA biosynthetic pathway promoting its accumulation (Dave et al., 2016).

Additionally, other important dormancy promoting factors, such as DELAY OF GERMINATION 1 (DOG1) and *cis*-12-oxo-phytodienoic acid (*cis*-OPDA), have also been shown to induce dormancy (Alonso-Blanco et al., 2003; Bentsink et al., 2006; Dave et al., 2011; Dave et al., 2016). DOG1 is a seed-specific protein with unknown function that was identified as a major Quantitative Trait Locus (QTL) for seed dormancy (Alonso-Blanco et al., 2003; Bentsink et al., 2006). Mutation of the *DOG1* gene leads to a loss of primary dormancy, and its transcript and protein abundance in freshly matured seeds correlate strongly with dormancy levels (Bentsink et al., 2006; Nakabayashi et al., 2012). In addition to this, DOG1 has been shown to act independently of ABA (Alonso-Blanco et al., 2003; Nakabayashi et al., 2012). Taken together, these results illustrate once more the complexity of interactions between all these phytohormones and dormancy-promoting factors creating a signaling network that ultimately controls seed germination.

Chapter 2 - Methods

2.1 Plant Material and Growth Conditions

All mutant lines used in this study were described previously: *aba2*-1 (González-Guzmán et al., 2002); *cyp707a1*-1 (Okamoto et al., 2006); *cyp707a2* (Kushiro et al., 2004); *cyp707a3* (Kushiro et al., 2004); *abi4*-1 (Penfield et al., 2006); *abi5*-7 (Nambara et al., 2002); *aos* (Park et al., 2002); *pxa1*-1 (Fulda et al., 2004); *opr3*-l (Chehab et al., 2011); *pif1*-l (Oh et al., 2004); *som*-3 (Kim et al., 2008); *cyp20-3* (Park et al., 2013). *aba2*-1, *cyp707a1*, *cyp707a2*, *cyp707a3*, *abi4*-1, *abi5*-7, *aos*, *pxa1*-1, *opr3*-l, *pif1*-l, *som*-3, *cyp20-3* was in the Columbia (Col-0) background. *aos aba2*-1 mutant seeds were obtained by crossing male *aba2*-1 with female *aos* plants. PIF1-myc (Oh *et al*., 2007; PIL5-myc) line was in Col-0 background and ABI4-ha transgenic line was in *abi4*-1 background.

Plants of *Arabidopsis thaliana* ecotypes Col-0, Ler, and Cvi, along with mutants and transgenic lines were grown side by side in a greenhouse supplemented with artificial light to give a photoperiod of 16 h light at a temperature of 20-22 °C. Seeds were harvested when plants had stopped flowering and siliques had started to dehisce. Freshly matured seeds indicate that experiments were performed with dormant seeds within 24h from harvest. After-ripened seeds indicate that experiments were performed with seeds that lost primary dormancy (two-to-eight weeks from harvesting time). Seeds were size sieved using a mesh size 250 μm. Male sterile *aos*, *aos aba2*-1 and *opr3*-1 plants were sprayed with 450 mM MeJA (Sigma-Aldrich) in 0.1% Tween 20 to obtain seeds.

For the after-ripening assay, freshly mature seeds were harvested and dry-stored in a box containing a solution of 15 M LiCl to give a relative humidity of 30 % at room temperature (around 20 °C) (Gold and Hay, 2008). Seed were dry-stored (after-ripened) for different times (according to experiment).

2.2 Germination Assays

Sterilized seeds were imbibed on water agar plates (0.9 % w/v) and incubated in a growth cabinet under continuous light (150 μmol.m^{-2} s^{-1}) at 20 °C. Plates were supplemented with ABA (Sigma-Aldrich), *cis*-OPDA (Larodan) and/or norflurazon (Sigma-Aldrich) according to the germination condition examined. For light treatment, after 4 h imbibed under white light, seeds were irradiated using an LED box (Enclosed box supplemented with far-red [LedEngin, Inc LZ4-40R200 740nm] and red [LedEngin, Inc LZ4-40R300 660nm] LEDs) with FR (4.5 μmol.m^{-2} s^{-1}) for 5 min and/or R (20 μmol.m^{-2} s^{-1}) for 5 min. Seeds were kept in darkness by wrapping plates in 5 layers of foil, and then placed in a 20°C incubator for different times. For the phyA-dependent germination assay, after 4 h imbibed under light, the seeds were irradiated with first FR (4.5 μmol.m^{-2} s^{-1}) pulse of 5 min and further irradiated with second FR (4.5 μmol.m^{-2} s^{-1}) pulse of 60 min after 44 h of dark incubation. Germination frequency was scored based on radical protrusion. Between 100 and 500 seeds were used to check radical emergence and repeated at least two times. Pictures of germinated seeds were taken using a GXMXTL3 stereo microscope (GT Vision) coupled with a GXCAM-HICHROME Camera (GT Vision).

2.3 Phytohormone and Oxylipin Analysis[1]

100-200 mg of seeds were ground and extracted overnight at 4°C with 2 mL 99:1 isopropanol:acetic acid together with internal standards (50 ng d_2-GA_4, 50 ng d_6-ABA, and 20 ng prostaglandin A1). The supernatant was transferred to a vial after centrifugation at 13,000 *g* for 5 min at 4°C. The remaining pellets were re-extracted with 1.1 mL extraction solvent for 2 h and the second supernatant pooled with that from the first extraction. The total supernatant was dried using a centrifugal solvent evaporator (GeneVac EZ-2 Series). The dried extracts were re-suspended in 50 μL methanol and 2 μL injected and analyzed on

[1] Method extracted from Dave et al. (2011).

an ultra performance liquid chromatography (UPLC)-MS system consisting of an Acquity UPLC system (Waters) coupled to a Finnigan LTQ ion trap mass spectrometer (Thermo Electron). Chromatographic separation of the phytohormones was performed on a Waters Acquity UPLC BEH C18 1.7 μm, 50 × 2.1-mm column using a gradient of mobile phases water + 0.1% acetic acid and acetonitrile + 0.1% acetic acid with a flow rate of 1 mL/minute. Eluted compounds were ionized on the mass spectrometer using a HESI source, and MS data were collected in full scan mode over the mass range m/z 100 to 500 in negative ionization mode. Ions at m/z 263.1 for ABA, 269.1 for d_6-ABA, 331.2 for GA_4, 333.2 for d_2-GA_4, 291.3 for *cis*-OPDA, 209.1 for Jasmonic Acid, 322.3 for JA-Ile, and 335.2 for Prostaglandin A1 were used for quantification. ABA, GA_4, *cis*-OPDA, JA and JA-Ile were quantified using response factors calibrated between internal standards (d_6-ABA, d_2-GA_4 and Prostaglandin A1) and phytohormone standards (ABA, GA_4, *cis*-OPDA, JA and JA-Ile). d_2-GA_4 was obtained from Lew Mander (ANU, Canberra, Australia), and d_6-ABA was purchased from ICON isotopes. ABA and GA_4 were purchased from Sigma-Aldrich. *cis*-OPDA was purchased from Larodan, and JA-Ile was a gift from Paul Staswick.

2.4 RNA Purification and cDNA Synthesis[2]

Using mortar and pestle and liquid nitrogen, 30 mg of seeds were ground to powder with 125 μL of extraction buffer (0.2 M sodium borate decahydrate, 30 mM EGTA, 1 % [w/v] SDS, 1 % [w/v] sodium deoxycholate, 10 mM DTT, 2 % (w/v) polyvinyl pyrrolidone, and 1 % (v/v) Igepal). The extract was thawed in a 1.5 mL Eppendorf tube and a further 125 μL of extraction buffer was added. To remove the proteins, the extract was incubated at 42 °C for 90 min with 10 μL of proteinase K (Roche Diagnostics). After protease incubation, 20 μL of 2 M KCl was added to samples, mixed and incubated on ice

[2] Method extracted from Dave et al. (2011).

for 60 min. Then, samples were centrifuged at 13,000 *g* for 20 min at 4 °C and the supernatant was transferred to a fresh tube with 90 µL of 8 M LiCl added. Samples were mixed and incubated at -20 °C for at least 3 h. After the incubation, samples were centrifuged at 13,000 *g* for 20 min at 4 °C, and the RNA pellet was dissolved in 100 µL RNase-free water. RNA was purified with the RNeasy plant mini kit (Qiagen).

The quality and quantity of RNA was assessed by the absorbance ratios (A260/280 and A260/030). Contaminating DNA was removed from RNA samples (1 µg) using the TURBO DNA-free kit (Ambion). First strand cDNA was synthesized from DNA-free total RNA using the SuperScript III first-strand synthesis system (Invitrogen) with oligo (dT)18 primer, following the manufacturer's instructions, and 180 µl water was added before the PCR step.

2.5 Quantitative RT-qPCR Analysis

2 µl of the diluted cDNA template was used, along with the primers described in Table 2.1. Quantitative RT-PCR (RT-qPCR) was performed using iQ SYBR Green Supermix (Bio-Rad) and the MyiQ Real-Time PCR detection system (Bio-Rad) according to the manufacturer's instructions. Expression of *UBQ11* was used for normalization.

Table 2.1 | Sequence of primers used in this thesis for RT-qPCR and ChIP-qPCR

Oligo name	Forward	Reverse
RT-qPCR		
AOS	5'AAGTCAAAGCCGGTGAAAT3'	5'CTTACCGGCGCATTGTTTAT3'
OPR3	5'TGGACGCAACTGATTCTGAC3'	5'CTCATCACTCCCTTGCCTTC3'
JAR1	5'TTGTGAGCCATGACTACGGT3'	5'CAACCGGTTTCTCCTCTCCT3'
CYP81D11	5'ATTGCCGAGGTAGTTGT3'	5'TTGCCTTTCGTAATACT3'
GST25	5'CTCGGTTGGGAAAAGTTTAG3'	5'AAACATTAAGTGACAGAAC3'
HSP17.6	5'CTTGCCTGGATTGAAGAAGG3'	5'CATCGCAGCCTTAACCTGAT3'
GRX480	5'TGATTGTGATTGGACGGAGA3'	5'TAAACCGCCGGTAACTTCAC3'
GAI	5'CCCGGTGACGCGATTCT3'	5'TCCGCCGCCTTGGTTAG3'
RGA	5'CTCTGAGCTTAATCCTCCTCCTCTT3'	5'CATAATCCGAAGCCGGAAAAC3'
RGL2	5'GTTGTGTGGATAGATCCGAATACG3'	5'TTCCTCGTCAAAGACTTCCTCTTC3'
NCED6	5'TTGAGTCCTCTATCGTTATTCCTATGG3'	5'GGAGCGAAGTTACCTGATAATTGAA3'
NCED9	5'CCACCATTTGTTCGACGGAG3'	5'TGTCCGTGAAGCTCTCCAAT3'
CYP707A1	5'TCATCTCACCACCAAGTA3'	5'AAGGCAATTCTGTCATTCTA3'
CYP707A2	5'ATCCATCACTCCTCCGAATTCTTCC3'	5'TCCATTTCCGAATGGCATGTACG3'

CYP707A3	5'CATGCCTTTTGGTAGTGGGATTCAT3'	5'CGGCCCATACTGAATTCCATCG3'
ABI3	5'CAGCAGAACCAAACCCAAAT3'	5'GCTCGGTCCATGGTAGGTAA3'
ABI4	5'TCCGCTCAACGCAAACG3'	5'TTGTCGAACGCCACGGTAA3'
EXPA1	5'GAACGATGGCTCTTGTCACC3'	5'TTCCGTATCCACAAGCACCT3'
EXPA2	5'CATAAACTCCGACGACAACG3'	5'TACCCACAAGCACCACCCAT3'
EXPA3	5'ATATTCCGGTGGCTTGTGGA3'	5'GCCCATTACACGGTCCATTC3'
EXPA8	5'ACACGCCGTCTCAATCAAAG3'	5'GCCAATTAGAAGGAGCCACG3'
EXPA9	5'TGGCGGTTATGGTGGTTACT3'	5'TTCACTCCGTAACCTTGGCT3'
EXPA10	5'CTTTGACCTTGCTCAGCCTG3'	5'GCTTGCCACACTGTTCTT3'
EXPA20	5'ACTATCTCATGCCGCCTTCA3'	5'CGAGGCCCACATTGCTTATC3'
UBQ11	5'TTCATTTGGTCTTGCGTCTG3'	5'GAAGATGAGACGCTGCTGGT3'
ChIP-qPCR		
For PIF1-myc		
AOS	5'CAAGCGTCGGACAAGAAGTT3'	5'ATCGCATGTGTCTTGTGCTT3'
OPR3	5'TGCCAATCCACTAAGAAACATCT3'	5'GTTGTTGTGTGGTCGTGGAA3'
RGA	5'CAGACTCGGTCCCTACCGTTT3'	5'GCCGTCATTAACGGCCTCTTTCT3'
For ABI4-ha		
AOS (A)	5'AGGCGTGACAAGTTATTTCTCG3'	5'GGTTGTGTATTCATGCTCATCGA3'
AOS (B)	5'GCAACAAGCTCGATAAAATATGA3'	5'CCTTTTTCAGACCACGCAGT3'
NCED6	5'TCACACACCGCATGAATTTT3'	5'AGATTATGTGGAACAGTGGAAAGA3'

2.6 Chromatin Immunoprecipitation (ChIP) Analysis

For the ChIP assay, 35S:PIF1-myc (PIL5-myc) or 35S:ABI4-ha seeds were imbibed in water agar plates for 4 h under white light, irradiated with FR (4.5 μmol.m^{-2} s^{-1}) for 5 min, wrapped in 5 layers of foil, placed in a 20°C incubator for 24 h. Samples (300 mg) of 24 h imbibed FR-treated seeds were crosslinked with 10 ml of 1% formaldehyde under vacuum infiltration conditions. The seeds were then ground to powder in liquid nitrogen and added to 1 ml of lysis buffer (50 mM HEPES pH 7.5; 150 mM NaCl; 1 mM EDTA; 0.04 % Triton X-100; 0.1 % Na deoxycholate; 0.1 % SDS; 1 mM PMSF; 200 μL of Sigma Protease Inhibitior Cocktail). Samples were centrifuged at 14,500 rpm for 2 min at 4°C and the pellets were resuspended with final 1.6 mL of lysis buffer. Sonication was performed (40 % duty cycle and 20 % power for 10 sec, 4 times) in order to generate DNA fragments around 500–1000 bp.

Protein A agarose beads (Upstate Biotechnology, http://www. upstate.com/) were equilibrated with ChIP dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl and 1 mM PMSF), and 40 μL of the equilibrated Protein A agarose beads were added to the sonicated samples in order to remove unspecific binding

fragments. Samples were then centrifuged, and 600 μL of the resulting supernatant was transferred to a new tube and immunoprecipitated with either anti-myc or anti-ha antibodies. After immunoprecipitation, samples were sequentially washed in four different buffers: Low wash buffer (150 mM NaCl, 0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tris-HCl pH 8), High Salt Wash buffer (500 mM NaCl; 0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tris-HCl pH 8), LiCl Wash Buffer (0.25 M LiCl; 1% v/v NP-40; 1% w/v sodium deoxycholate; 1 mM EDTA; 10 mM Tris-HCl pH 8), Tris/EDTA Wash Buffer (20 mM Tris-HCl pH 8; 1 mM EDTA). The immunoprecipitated DNA was recovered using an elution buffer (1 % SDS; 0.8 % Na_2CO_3). To reverse the crosslink, 20 uL of 5 M NaCl was added to the samples and incubated overnight at 65 C. In order to remove all the proteins, samples were incubated with 20 mg/ml of proteinase K at 45 C for 1 h. Finally, DNA was purified using a QIAquick PCR purification kit and DNA fragments were determined by RT-qPCR using specific primers (listed in Table 2.1).

2.7 Accession Numbers

Gene sequence information from this work can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: NCED6 (AT3G24220); NCED9 (AT1G78390); ABA2 (AT1G52340), CYP707A1 (AT4G19230); CYP707A2 (AT2G29090); CYP707A3 (AT5G45340); ABI4 (AT2G40220); ABI5 (AT2G36270); AOS (AT5G42650); PXA1 (AT4G39850); OPR3 (AT2G06050); JAR1 (AT2G46370); CYP20-3 (AT3G62030); GRX480 (AT1G28480); CYP81D11 (AT3G28740); GST25 (AT2G29420); HSP17.6 (AT5G12030); PIF1 (AT2G20180); SOM (AT1G03790); RGA (AT2G01570); RGL2 (AT3G03450); GAI (AT1G14920); EXPA1 (AT1G69530); EXPA2 (AT5G05290); EXPA3 (AT2G37640); EXPA8 (AT2G40610); EXPA9 (AT5G02260); EXPA10 (AT1G26770); EXPA20 (AT4G38210); UBQ11 (AT4G05050).

Chapter 3 - cis-12-oxo-phytodienoic acid is a light-dependent regulator of seed germination

3.1 Introduction

In plants, light is an essential environmental input affecting a diverse range of responses, including seed germination (Smith, 2000; Jiao et al., 2007; Linkies et al., 2010). The probability of seedling establishment is generally greater if germination occurs in full light rather than shade light (Lee and Lopez-Molina, 2012; Casal, 2013). Phytochrome photoreceptors distinguish between these types of light on the basis of their red/far-red (R/FR) intensities and ratios (Shinomura, 1997; Smith, 2000; Quail, 2002), with excess R triggering GA accumulation and germination, and excess FR, which is abundant in shade light, triggering ABA accumulation and a block in germination (Oh et al., 2006; Seo et al., 2006; Piskurewicz et al., 2009).

A. thaliana has five phytochromes, A to E (Clack et al., 1994), with phytochrome B (phyB) the main promotor of seed germination under R light treatment (Shinomura et al., 1994). Successive pulses of FR and R reversibly deactivate and activate phyB respectively, inducing germination, while a single FR pulse inhibits (Shinomura et al., 1994; Jiao et al., 2007). In the last 12 years, a phytochrome signal transduction pathway that regulates GA and ABA turnover has been described (Oh, et al. 2004; Oh, et al. 2006; Oh, et al. 2007; Bae and Choi, 2008; Kim et al., 2008; Piskurewicz et al., 2009; Lee et al., 2012). Upon activation by R, phyB is converted to the biologically active P_{fr} form ($phyB_{fr}$) and translocated to the nucleus, inducing degradation of PHYTOCHROME INTERACTING FACTOR 1 (PIF1) via the 26S proteosome (Figure 3.1) (Shen, et al. 2005; Park et al., 2012). PIF1 is a basic helix-loop-helix transcription factor that binds to DNA with high affinity for the G-box (CACGTG) motif (Oh et al., 2007; Oh et al., 2009). Thus, PIF1

inhibits germination of *A. thaliana* seeds under FR light conditions through both direct and indirect regulation of gene expression (Oh et al., 2004; Kang et al., 2010).

Inactivation of phyB by a single FR pulse leads to accumulation of the PIF1 protein in the nucleus (Oh et al., 2004). PIF1 directly induces expression of *ABI3*, and both PIF1 and ABI3 proteins have been shown to directly bind to the promoter and induce the expression of *SOMNUS* (*SOM*) (Figure 3.1) (Kim et al., 2008; Park et al., 2011). *SOM* encodes a CCCH-type zinc finger protein with unknown function that is part of the phytochrome signal transduction pathway (Figure 3.1) (Bae and Choi, 2008; Kim et al., 2008). Remarkably, seeds of *pif1*, *abi3* and *som* single mutants have the ability to germinate under FR light conditions (Oh et al., 2004; Kim et al., 2008; Piskurewicz et al., 2009). Oh et al. (2007) showed that PIF1 also directly induces *RGA* and *GAI* expression in seeds under FR light, suggesting that these DELLAs play an important role in the GA responses during germination upon R conditions. Yet, only seeds of a triple DELLA loss-of-function mutant (*gai rga rgl2*; this mutant is in a *ga1* background) are able to germinate under FR conditions (Piskurewicz et al., 2009).

Piskurewicz and others (2009) demonstrated that germination of *ga1 gai rga rgl2* seeds under FR light is due to a decrease in both ABA levels and *ABI3* transcript abundance that ultimately leads to a decrease in ABI5 protein abundance (Figure 3.1). Under white light, ABI5 has been shown to act downstream of ABI3 in order to block germination in the presence of ABA (Park et al., 2011). Under FR conditions, ABI5 directly binds and induces the expression of genes involved in ABA (*NCED9*) synthesis and GA inactivation (*GA2OX2*), while it represses the expression of genes involved in GA (*GA3OX1*, *GA3OX2*) synthesis (Figure 3.1) (Lee et al., 2012). However, FR-treated seeds of *abi5* mutant are not able to germinate, showing that ABI3 still plays a major role in germination under FR conditions that is independent of ABI5 (Piskurewicz et al., 2009). Changes in the expression of genes involved in ABA and GA biosynthetic and catabolic

genes have been attributed to PIF1 and SOM light-dependent germination factors (Oh et al., 2007; Kim et al., 2008; Park et al., 2011). Based on this body of work GA and ABA phytohormones are currently understood to represent the core of the light-response mechanism that controls seed germination in *A. thaliana* (Lee et al., 2012; Lee and Lopez-Molina, 2012).

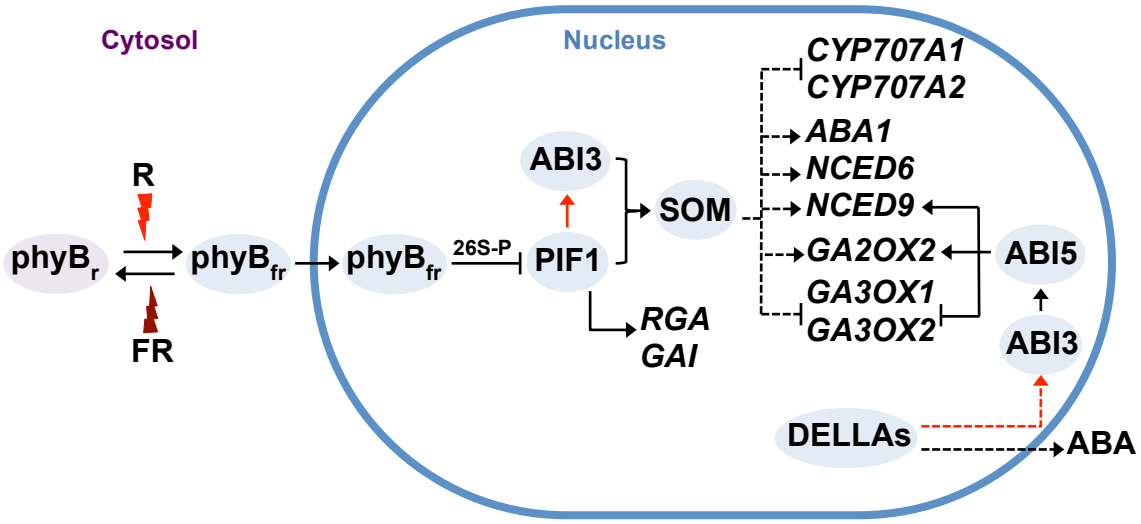


Figure 3.1 Model of the light-dependent control of germination in *A. thaliana*. R light activates phyB$_r$ leading to PIF1 degradation via 26S proteasome. Conversely, FR light inactivates phyB$_{fr}$ leading to PIF1 accumulation into the nucleus. In turn, PIF1 directly promotes expression of *ABI3*, which protein together with PIF1 directly promotes *SOM* expression. In addition to this, PIF1 promotes expression of *RGA* and *GAI* directly. These DELLA proteins then indirectly induce expression of *ABI3*. ABI5 acts downstream of ABI3 and directly regulates the expression of *NCED9*, *GA2OX2*, *GA3OX1* and *GA3OX2*. phyB$_r$, phyB inactive form P$_r$; 26S-P, 26S proteasome; DELLAs, for RGA, GAI proteins. Names that are circled represent proteins; names in italic represent genes; solid lines represent direct regulation; dashed lines represent indirect regulation; red lines represent regulation of transcription.

Intriguingly, seeds deficient in ABA levels still show low germination under FR conditions (Seo et al., 2006; Piskurewicz et al., 2009; Lee et al., 2012). For instance, ABA-biosynthesis deficient *aba1*-1 and *aba2*-1 mutant seeds germinate about 20 (Piskurewicz et al., 2009) and 40 % (Seo et al., 2006), respectively, under FR conditions, while wild-type seeds treated with the ABA-biosynthesis inhibitor norflurazon barely germinate (Lee et al., 2012). More recently, evidence from both genetic and pharmacological approaches have shown that *cis*-OPDA strongly represses seed germination (Dave et al. 2011; Dave et al., 2016) (see section 1.3 for more details on *cis*-OPDA). However, a mechanism that regulates *cis*-OPDA accumulation during seed imbibition has never been described.

CYP20-3 was found to strongly bind *cis*-OPDA in the chloroplast relaying a *cis*-OPDA-dependent retrograde signal cascade in response to wounding and pathogen stresses (Park et al., 2013). This signaling cascade was shown to trigger the expression of *cis*-OPDA responsive genes such as *GRX480*, *CYP81D11*, *GST25* and *HSP17.6* (Park et al., 2013; Stotz et al., 2003). Nonetheless, it is unknown if CYP20-3 plays a role during light-dependent seed germination. A series of experiments were performed to establish if *cis*-OPDA has a role in the control of light-dependent seed germination.

3.2 Results

3.2.1 *cis*-OPDA represses seed germination independently of ABA under shade (FR) light

The first objective was to determine whether or not *cis*-OPDA is also involved in the blockage of seed germination under FR conditions, taking into consideration that ABA deficient mutant seeds poorly germinate (below 40 %). Seeds of ABA- and oxylipin-deficient single and double mutants were imbibed in water agar plates, supplemented or not with norflurazon, and light treatment was performed according to Figure 3.2. Norflurazon was used as an ABA biosynthesis inhibitor in order to easily assess germination of FR-treated *aos* single mutant seed under low ABA conditions.

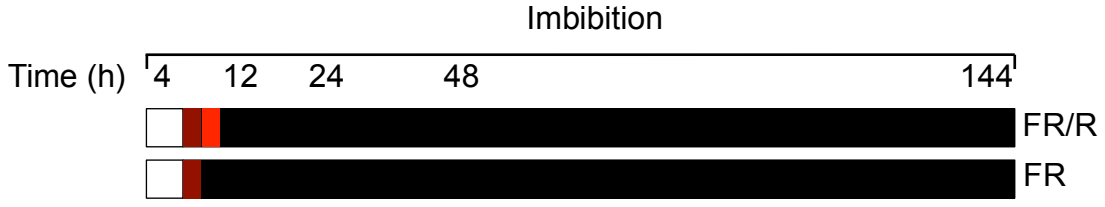


Figure 3.2 Light treatment scheme for experiments shown in chapter 3. Seeds were imbibed on water-agar plates for 4 h under white light, treated with either two successive 5 minutes FR and R pulses (FR/R), or only one FR pulse as indicated and then placed in dark until germination was counted or photographed. Samples were also collected at 12 and 24 h (after imbibition) for mRNA and oxylipin analysis.

In the absence of norflurazon, non-dormant seeds of both Col-0 and *aos* germinated at nearly 100 % after FR/R treatments, whereas FR-treated seeds of Col-0 failed to

germinate and *aos* germinated 2 % (Figure 3.3 A). However, in the presence of norflurazon, while FR-treated Col-0 seeds still failed to germinate as previously reported (Lee et al., 2012), germination rates of *aos* seeds increased greatly compared to the wild type in a dose dependent manner, reaching 76 % in the presence of 100 µM norflurazon (Figure 3.3 A). Furthermore, germination of FR-treated *aos* seeds in the presence 100 µM norflurazon was completely inhibited by direct application of either 10 µM *cis*-OPDA or 10 µM ABA (Figure 3.3 B).

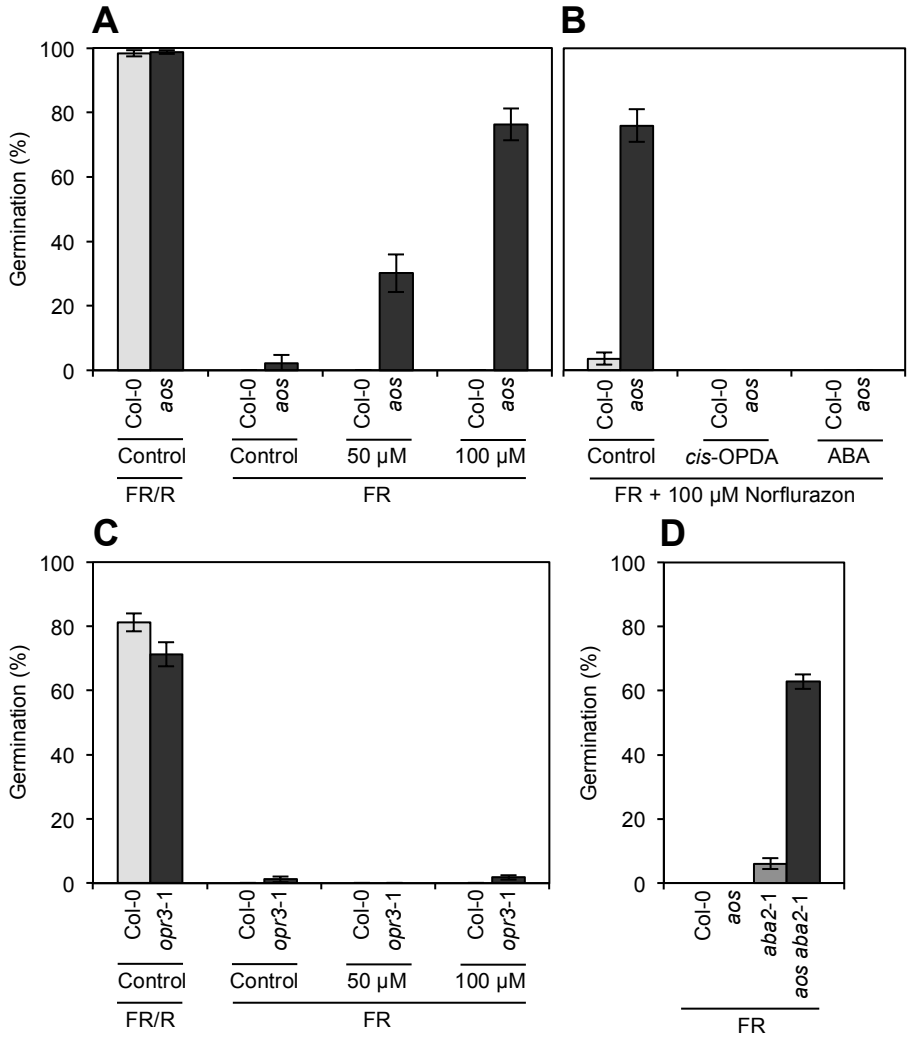


Figure 3.3 *cis*-OPDA represses seed germination independently of ABA under shade (FR) light. (A) Germination of FR/R- or FR-treated Col-0 and *aos* seeds in the presence or absence of 50 or 100 µM norflurazon. (B) Germination of Col-0 and *aos* FR-treated seeds in the presence of 100 µM Nor, supplemented with either 10 µM *cis*-OPDA or 10 µM ABA. (C) Germination of FR/R- or FR-treated Col-0 and *opr3*-1 seeds in the presence or absence of norflurazon. (D) Germination of FR-treated Col-0, *aos*, *aba2*-1 and *aos aba2*-1 seeds in water agar plates. Data are mean ± SD of three biological replicates.

Because in the *aos* mutant the whole JA/JA-Ile biosynthetic pathway is compromised, it could be argued that the lack of JA/JA-Ile might allow seeds to germinate under these conditions. In order to rule out this possibility, *opr3*-1 mutant seeds, which are deficient in JA and JA-Ile but not *cis*-OPDA (Dave et al., 2011; Chehab et al., 2011), had their germination assessed. In the absence of norflurazon, FR/R-treated Col-0 and *opr3*-1 non-dormant seeds germinated 81 and 71 % respectively (Figure 3.3 C). However, FR-treated Col-0 and *opr3*-1 seeds barely germinated, irrespective of the norflurazon treatment (Figure 3.3 C). In order to avoid any unspecific effect of norflurazon, a genetic approach was carried out using an *aos aba2*-1 double mutant. Thus, in the absence of norflurazon and under FR conditions, Col-0, *aos*, *aba2*-1, and *aos aba2*-1 seeds germinated 0, 0, 6, and 62 % respectively (Figure 3.3 D).

3.2.2 Differential expression of oxylipin pathway genes results in *cis*-OPDA accumulation under FR light

In order to investigate whether FR light is involved in the regulation of *cis*-OPDA levels in seeds, oxylipin accumulation, in FR/R- and FR-treated seeds at 12 and 24 h (as described in Figure 3.2), was measured. Compared to the FR/R treatment, *cis*-OPDA levels were more than 5- and 10-fold higher in FR-treated seeds at 12 and 24 h respectively (Figure 3.4 A), whereas JA and JA-Ile levels were unaffected (Figure 3.4 B and C).

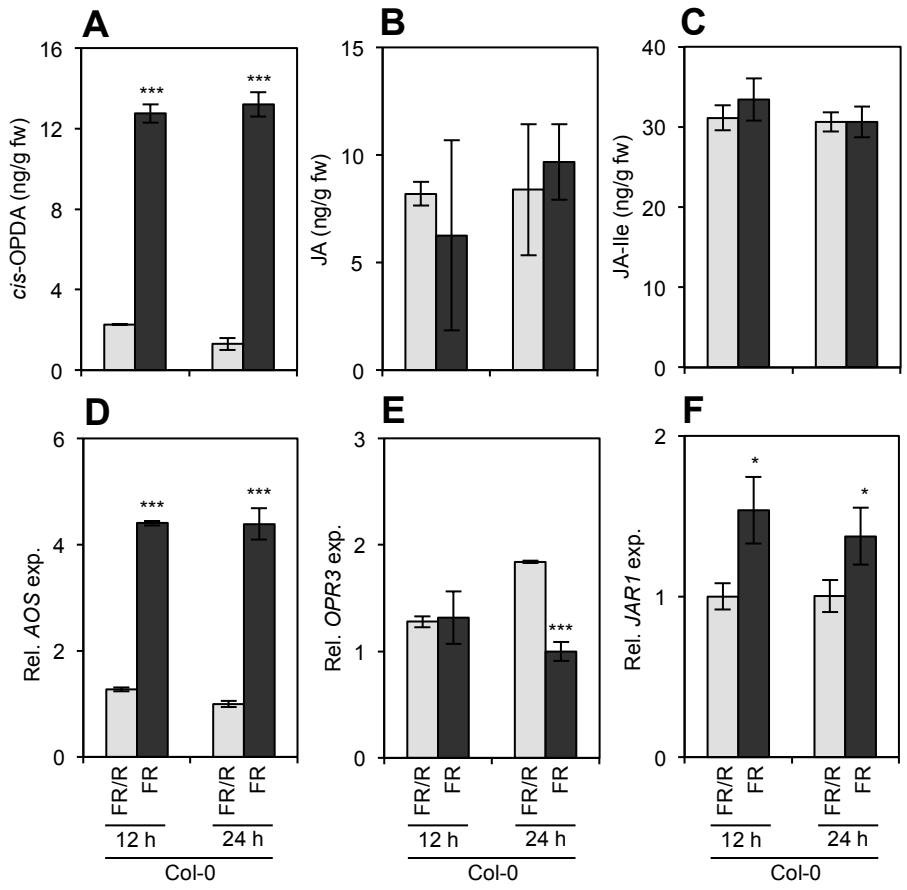


Figure 3.4 *cis*-OPDA accumulates under FR light via differential expression of oxylipin pathway genes. (A–C) Levels of oxylipins (A, *cis*-OPDA; B, JA; C, JA-Ile) in 12 and 24 h FR/R- or FR-treated WT seeds (as described in Figure 3.2). (D–F) Transcript levels of oxylipin pathway genes (D, *AOS*; E, *OPR3*; F, *JAR1*), normalized to *UBQ11* expression and expressed relative to the sample with the lowest expression, in 12 and 24 h FR/R- or FR-treated WT seeds (as described in Figure 3.2). Data are mean ± SD of three biological replicates. Asterisks indicate statistically significant difference according to two-tailed Student's t-test (* $P < 0.05$; *** $P < 0.001$).

Because of the increased *cis*-OPDA levels under FR-conditions, expression of oxylipin biosynthetic pathway genes was assessed in seeds under FR/R and FR conditions. By measuring the transcript levels of *AOS* and *OPR3*, it was determined that, compared to FR/R-treated seeds, *AOS* transcript levels were approximately 4-fold higher in FR-treated seeds at 12 and 24 h (Figure 3.4 D). Differences in *OPR3* transcript levels were only observed at 24 h, with them being 1.8-fold lower in FR-treated seeds compared to the FR/R treatment (Figure 3.4 E). Transcript levels of *JAR1* were 1.5- and 1.3-fold higher in FR-treated seed at 12 and 24 h, respectively, compared with FR/R (Figure 3.4 F).

3.2.3 Expression of oxylipin pathway genes is controlled by the light-dependent regulators of germination, PIF1 and SOM

Oxylipin levels and oxylipin pathway gene expression in *pif1*-1, *som*-3 and WT were investigated to establish what if any role these factors play in the FR response of *cis*-OPDA in 24 h imbibed seeds. It was found that *cis*-OPDA levels were 1.7- and 2.3-fold lower in *pif1*-1 and *som*-3, respectively, compared to WT (Figure 3.5 A), while JA and JA-Ile levels were unaffected (Figure 3.5 B and *C*).

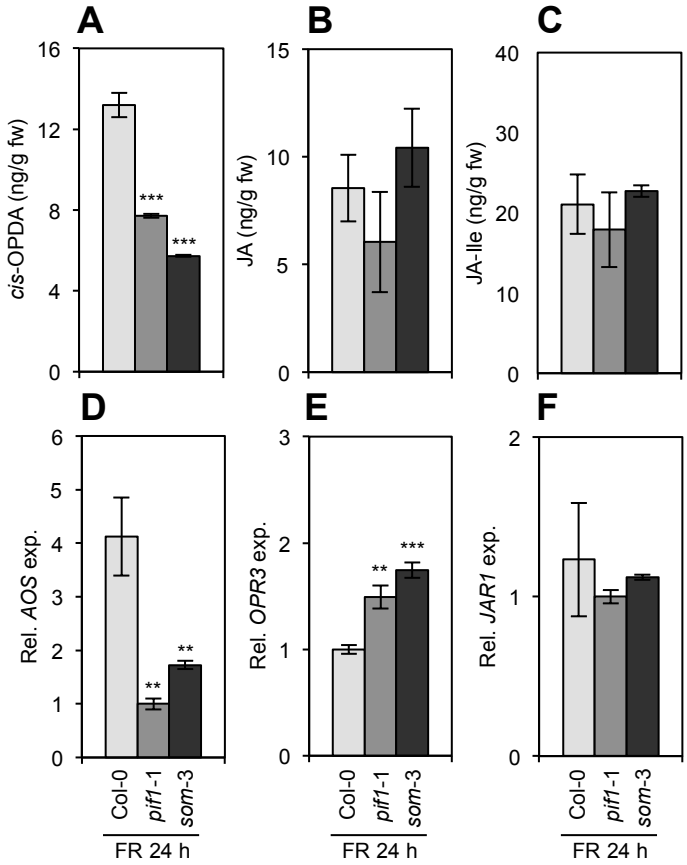


Figure 3.5 *cis*-OPDA levels are controlled by the light-dependent regulators of germination, PIF1 and SOM. (A–C) Levels of oxylipins (A, *cis*-OPDA; B, JA; C, JA-Ile) in 24 h FR-treated WT, *pif1*-1 and *som*-3 seeds (as described in Figure 3.2). (D–F) Transcript levels of oxylipin pathway genes (D, *AOS*; E, *OPR3*; F, *JAR1*), normalized to *UBQ11* expression and expressed relative to the sample with the lowest expression, in 24 h FR-treated WT, *pif1*-1 and *som*-3 seeds (as described in Figure 3.2). Data are mean ± SD of three biological replicates. Asterisks indicate statistically significant difference according to two-tailed Student's t-test (** $P < 0.01$; *** $P < 0.001$).

Under the same conditions, *AOS* transcript levels were 4.1- and 2.4-fold lower in *pif1*-1 and *som*-3, respectively, compared to WT (Figure 3.5 D). In contrast, *OPR3*

transcript levels were 1.4- and 1.7-fold higher in *pif1*-1 and *som*-3, respectively, compared to WT (Figure 3.5 E), while *JAR1* transcript levels were unaffected (Figure 3.5 F).

3.2.4 *cis*-OPDA rely on CYP20-3 to control light-dependent germination

Because *cis*-OPDA accumulates in FR-treated seeds, it was hypothesized that expression of *cis*-OPDA responsive genes would be increased in dark-imbibed seeds. Thus, expression of *GRX480*, *CYP81D11*, *GST25* and *HSP17.6* were assessed in FR/R- and FR-treated Col-0 seeds after 24 hours of imbibition. Compared to FR/R-treated seeds, *CYP81D11*, *GST25* and *HSP17.6* were increased 7-, 2- and 13.7-fold respectively under FR conditions (Figure 3.6 A).

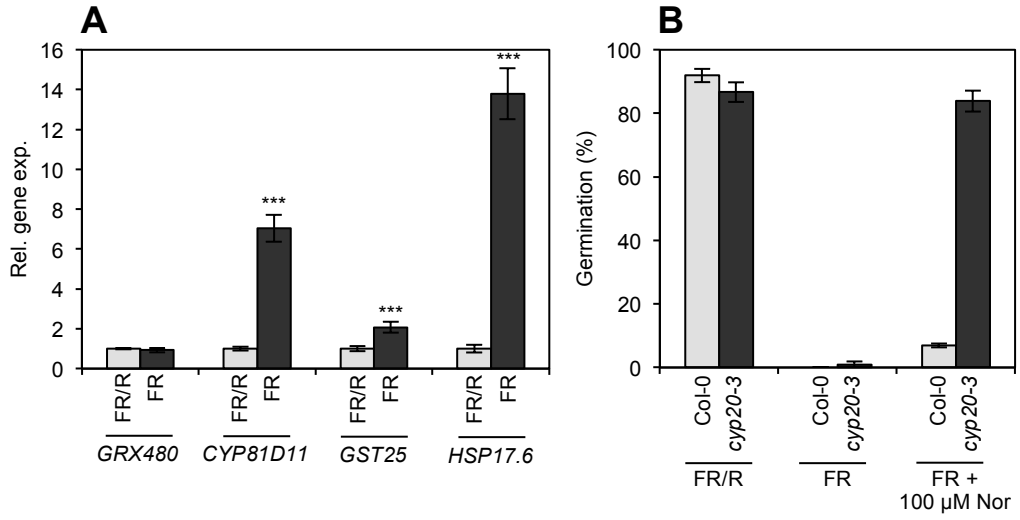


Figure 3.6 *cis*-OPDA responsive genes and receptor are involved in the control of light-dependent seed germination. (A) Transcript levels of *GRX480*, *CYP81D11*, *GST25* and *HSP17.6*, normalized to *UBQ11* expression and expressed relative to FR treatment, in 24 h FR/R- and FR-treated Col-0 seeds (as described in Figure 3.2). (B) Germination of FR/R- or FR-treated Col-0 and *cyp20-3* seeds in the presence of 100 µM norflurazon (Nor). Data are mean ± SD of three biological replicates. Asterisks indicate statistically significant difference according to two-tailed Student's t-test (** $P < 0.01$; *** $P < 0.001$).

Since expression of *CYP81D11*, *GST25* and *HSP17.6* were induced in FR-treated seeds, germination of *cyp20-3* seeds was investigated to establish if disruption of this known OPDA receptor affects light-dependent germination under low ABA levels. For this, *cyp20-3* seeds were imbibed in the absence or presence of 100 µM norflurazon under

FR conditions. In the absence of norflurazon, non-dormant seeds of Col-0 and *cyp20-3* germinated 91 and 86 % respectively after FR/R treatments, whereas FR-treated seeds of Col-0 failed to germinate and *cyp20-3* germinated 0.8 % (Figure 3.6 B). However, in the presence of 100 μM norflurazon, while FR-treated Col-0 seeds germinated 7 %, *cyp20-3* seeds germinated 83 % (Figure 3.6 B).

3.3 Discussion

Seo and others (2006) have suggested that ABA plays a critical role in the repression of seed germination under FR conditions. For instance, they have shown that *nced6*-1, *aba2*-1, and *aao3*-4 mutant seeds, which are deficient in ABA biosynthesis (detailed in section 1.3), germinated approximately 25, 32, and 25 % under FR conditions (Seo et al., 2006). The current study shows that *aba2*-1 seeds germinate poorly under FR conditions (Figure 3.3 D). Because ABA-deficient seeds exhibited low germination (below 40 %) under FR conditions, I hypothesized that *cis*-OPDA might be playing a role in the control of light-dependent germination.

Dave and others (2011 and 2016) showed that *cis*-OPDA promotes dormancy and represses germination in seeds. For example, mutation in the *AOS* locus, which leads to deficiency in both *cis*-OPDA and JA levels (detailed in section 1.3), rescues the deep dormancy phenotype of *pxa1*-1 seeds (Dave et al., 2011). Dave *et al*., (2016) also demonstrated that the *cis*-OPDA effect is dependent on ABA to promote seed dormancy and repress germination. As expected, FR-treated *aos* non-dormant seeds did not germinate (Figure 3.3 A). However, a substantial increase in germination was observed when *aos* seeds were imbibed in the presence of norflurazon under FR conditions, but not in the germination of the wild type (Figure 3.3 A). Supporting this, FR-treated *aos aba2*-1 double mutant seeds, which are deficient in both *cis*-OPDA and ABA levels, germinated significantly in comparison with individual single mutants and wild type (Figure 3.3 D).

Either *cis*-OPDA or ABA was sufficient to block germination of FR-treated *aos* seeds in the presence of norflurazon (Figure 3.3 B). This finding suggests that *cis*-OPDA does not rely on ABA to repress seed germination under FR conditions, in contrast to what was found during germination of after-ripened seeds under continuous white light (Dave et al., 2016). One possible explanation for this might be that the role of *cis*-OPDA in repressing seed germination increases under FR conditions. As Dave et al. (2011) demonstrated that the *cts*-2 *opr3*-1 double mutant did not rescue the deep-dormancy phenotype of *cts* single mutant seeds, *opr3*-1 seeds imbibed in the presence of norflurazon did not recover germination under FR conditions (Figure 3.3 C). Put together, these results indicate that both *cis*-OPDA and ABA, rather than just ABA, block seed germination under FR conditions and that *cis*-OPDA does so independently of ABA.

Since this data indicates that *cis*-OPDA represses seed germination under FR light, oxylipin levels and expression of key genes involved in the oxylipin biosynthetic pathway were measured. Importantly, these measurements were performed prior to when FR/R-treated seeds start to germinate (Appendix A; A1). Remarkably, *cis*-OPDA levels accumulated under FR light independently of JA and JA-Ile (Figure 3.4 A, B and C). These results suggest that there is a mechanism controlling the oxylipin biosynthetic pathway under FR light. Transcript levels of *AOS* were higher, and *OPR3* were lower, in wild type seeds imbibed for 24 h under FR in comparison with FR/R conditions (Figure 3.4 D and E).

AOS and *OPR3* encode enzymes leading to the synthesis and further transformation of *cis*-OPDA, respectively. Hence, it is likely that these single genes are important targets for the regulation of *cis*-OPDA levels under FR light. However, this does not rule out the possibility that the rapid increase in the pool of *cis*-OPDA under FR could also come from another source such as Arabidopsides, i.e. galactolipids containing esterified *cis*-OPDA (Dave and Graham, 2012). Despite the fact that JA-Ile levels were unaffected under FR/R-

and FR-treatments (Figure 3.4 C), transcript levels of *JAR1* were slightly higher in FR-treated seeds (Figure 3.4 F). Put together, these data indicate that differential expression of *AOS* and *OPR3* leads to specific accumulation of *cis*-OPDA in seeds under FR conditions.

As stated in the introduction to this chapter, PIF1 and SOM induce an increase in ABA and a decrease in GA levels in seeds by regulating the expression of their biosynthetic and catabolic genes under FR conditions (Oh et al., 2007; Kim et al., 2008; Park et al., 2011). Remarkably, *pif1*-1 and *som*-3 showed significant down-regulation of the *AOS* (Figure 3.5 D) and up-regulation of *OPR3* (Figure 3.5 E) genes accompanied by a decrease in the levels of *cis*-OPDA (Figure 3.5 A) at 24 h, at a time point prior to when FR-treated seeds start to germinate (Appendix A; A.2). These results demonstrate that, as is the case for ABA (Oh et al., 2007; Kim et al., 2008), PIF1 and SOM control *cis*-OPDA levels under FR light conditions. In the case of *cis*-OPDA differential regulation of *AOS* and *OPR3* genes is essential, reinforcing the critical role of these genes as the main targets for regulation of *cis*-OPDA levels in *A. thaliana* seeds.

The promoter regions of *AOS* and *OPR3* have binding sites known to be targets of PIF1 (Appendix A; A3), but based on chromatin immunoprecipitation (ChIP) assays, the regulation of these genes by PIF1 is indirect: transcript levels of *AOS* and *OPR3* promoter regions did not change in the presence, compared to the absence, of the anti-myc antibody (Appendix A; A.4); in these experiments *RGA* was used as a positive control (Appendix A; A.4). Therefore, it is tempting to suggest that SOM might be the direct regulator of *AOS* and *OPR3*. However, despite the fact that SOM is a nucleus-localized CCCH-type zinc finger protein, which is part of a large family of zinc finger containing C3H-type motifs with the potential to bind RNA and regulate its processing (Li et al., 2001; Delaney et al., 2006), its molecular function is unknown (Kim et al., 2008). Nonetheless, the possibility remains that other factors such as *GAI*, *RGA* and *RGL2* are involved in the direct regulation of *AOS* and *OPR3* expression.

Park and others (2013) found that *cis*-OPDA binds to CYP20-3 and relays a signal that ultimately controls a subset of *cis*-OPDA responsive genes under stress responses. Quite remarkably, norflurazon-treated *cyp20-3* mutant seeds germinated under FR conditions (Figure 3.6 B), suggesting that the *cis*-OPDA mediated repression of seed germination in response to FR light is dependent on CYP20-3. In addition to this, known *cis*-OPDA responsive genes such as *CYP81D11*, *GST25* and *HSP17.6* (Park et al., 2013; Stotz et al., 2003) were found to be up regulated in wild-type seeds imbibed under FR compared with FR/R conditions (Figure 3.6 A). Thus, these results strongly suggest that components of the receptor and transduction mechanism involved in *cis*-OPDA mediated responses to wounding and pathogen stresses are also involved in the *cis*-OPDA mediated response of seeds to FR light. This implies a redox based retrograde signaling pathway triggered by *cis*-OPDA (detailed in section 1.3) is involved in the repression of seed germination under FR conditions.

3.4 Conclusions

In *A. thaliana*, both *cis*-OPDA and ABA block germination under FR conditions. Similar to ABA (Kim et al., 2008), levels of *cis*-OPDA are regulated by light conditions through a mechanism that involves the phytochrome-associated factors PIF1 and SOM in the differential control of *AOS* and *OPR3*. Furthermore, the results shown support a role for CYP20-3 as a receptor that binds *cis*-OPDA in the plastid, triggering a redox based retrograde signaling response that ultimately blocks germination under FR treatment.

Chapter 4 - Control of seed germination and oil mobilization by ABI5 and ABI4

4.1 Introduction

Seed germination is tightly controlled by environmental cues such as light quality, temperature and water potential, and these cues eventually determine the relative levels of GA and ABA in order to control germination (Olszewski et al, 2002; Nambara and Marion-Poll, 2005). In *A. thaliana*, seed germination is chronologically marked by testa rupture followed by endosperm rupture, which can both be induced by exogenous GA (Piskurewicz et al., 2008). As detailed in section 1.3, Müller et al. (2006) showed that exogenous ABA blocks endosperm rupture but not testa rupture.

Similar to germination, GA and ABA are also known to promote and repress, respectively, seedling establishment by controlling the mobilization of the three major storage reserves in seeds: starch, oils and proteins (Graham, 2008; Tan-Wilson and Wilson, 2012; Eastmond et al., 2015; Barros-Galvão et al., 2016). Production of most of the crops grown worldwide starts with the sowing of seeds and seedling establishment represents a critical step for crop production, determining the success or failure of the future plant. For several species, including oil crops (e.g. sunflower, oilseed rape, soybean), triacylglycerol (TAG; i.e. oil) represents a major component of the seed storage reserves. To mobilize this reserve, a very specialized metabolism has evolved in plants, converting TAGs into substrates for respiration and gluconeogenesis. For a seedling to become photoautotrophic, the stored reserves have to be successfully mobilized. For example, *A. thaliana* seeds that fail to mobilize oil upon imbibition also fail to establish (Graham, 2008).

Oil mobilization starts with TAG breakdown catalyzed by lipases, specifically by SUGAR-DEPENDENT1 (SDP1) and SDP1-LIKE (Eastmond, 2006; Kelly et al., 2011), producing free fatty acids (FAs) and glycerol in the cytosol. Following this lipolysis, FAs

enter organelles called glyoxysomes (i.e. modified peroxisomes) where β-oxidation and part of the glyoxylate cycle occur (Cooper and Beevers 1969). β-oxidation converts FAs into acetyl-CoA, which is then converted into 4-carbon compounds via the glyoxylate cycle. These compounds are then transported to the mitochondria, where they can either be used for respiration or converted to malate and transported to the cytosol for gluconeogenesis (reviewed in Graham, 2008).

ABA responsive factors such as ABI5 and ABI4 have been shown to play important roles in the control of seed germination and oil mobilization respectively (Lopez-Molina et al., 2001; Penfield et al., 2006). The germination that is dependent on FR light is known as phyA-dependent germination (Reed et al., 1994; Shinomura et al., 1994; Shinomura et al., 1996). PhyA, in contrast to phyB, can be activated by FR light and, in a very specific manner, promote germination. Lee and others (2012) have demonstrated that an early FR pulse activates phyA in the embryo, but is not able to promote germination due to the still low GA/ABA ratio. After the first FR pulse, ABI5 directly regulates the expression of ABA metabolic genes that leads to a marked repression of seed germination (Lee et al., 2012) However, ABA levels decrease and the response become weaker after 48 hours of the first FR pulse, allowing a second FR pulse to promote and repress expression of embryonic GA and ABA synthesis genes, respectively, in a phyA-dependent manner. Hence, in *abi5*-7 mutant seeds, a second FR pulse applied after 12 hours of imbibition greatly stimulated germination and expression of GA biosynthetic genes in comparison with the wild type (Lee et al., 2012). While ABI5 has been shown to play a role in phyA-dependent germination, nothing is known about ABI4.

Curiously, ABI4, but not ABI5, has been shown to repress oil mobilization in the embryo in the presence of ABA (Penfield et al., 2006). This tissue-specific effect of ABA was observed in *A. thaliana* (Penfield et al., 2006) and tobacco seeds (Manz et al., 2005), suggesting a wide conservation among flowering plants (Graham, 2008). Penfield et al.

(2006) have also stated that the insensitivity of the endosperm to ABA can be attributed specifically to the lack of ABI4 in this tissue, therefore suggesting that ABI4 is a key regulator of lipid catabolism in response to ABA. This chapter is aimed at a further investigation of the roles of ABI5 and ABI4 in the control of seed germination and oil mobilization.

4.2 Results

4.2.1 ABA represses testa rupture in nicked seeds via the ABI5 transcription factor

Because nicking affects the integrity of the seed coat (Figure 4.1 A), it was questioned whether or not nicking would also affect the appearance of testa rupture. Nicking was performed in Col-0 seeds after 1 hour of imbibition using a needle to perforate the seed coat and the endosperm in order to generate a direct contact between the embryo and the external media (Figure 4.1 A). Intact Col-0 (Control) seeds showed testa and endosperm rupture after 29 and 42 hours of imbibition respectively, while Col-0 nicked seeds showed testa and endosperm rupture after 24 and 29 hours of imbibition respectively (Figure 4.1 B).

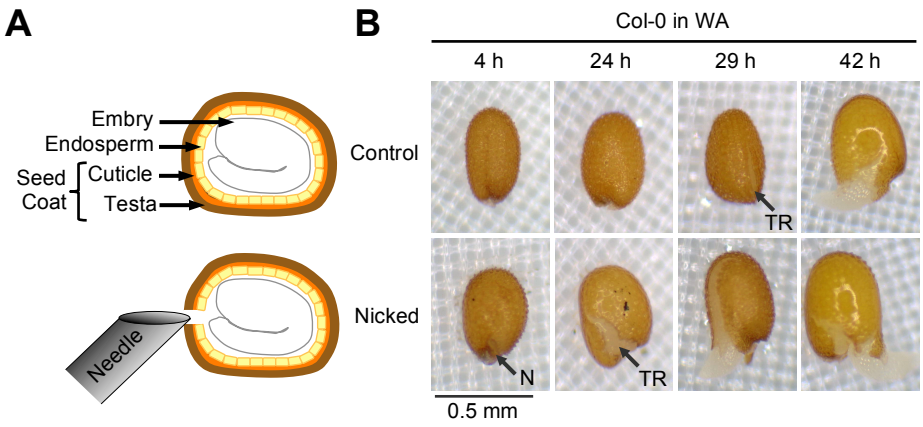


Figure 4.1 Effect of nicking on the two-step germination of *A. thaliana* Col-0 seeds. (A) Scheme of a mature *A. thaliana* seed showing the different tissues (on top) and the nicking technique applied to a seed using a 0.3 mm ø needle. Nicking (N) was performed after 1 hour of imbibition. (B) Pictures show intact (control) and nicked Col-0 seeds that were imbibed in water agar (WA) plates. TR, testa rupture. Scale bar is 0.5 mm.

Non-dormant Col-0 intact seeds imbibed in water-agar plates supplemented with 10 µM ABA for 120 hours showed testa rupture, but not endosperm rupture as previously reported (Figure 4.2 A, top panels). However, *abi4*-1 and *abi5*-7 intact seeds showed both testa and endosperm rupture in the presence of ABA (Figure 4.2 B and C, top panels).

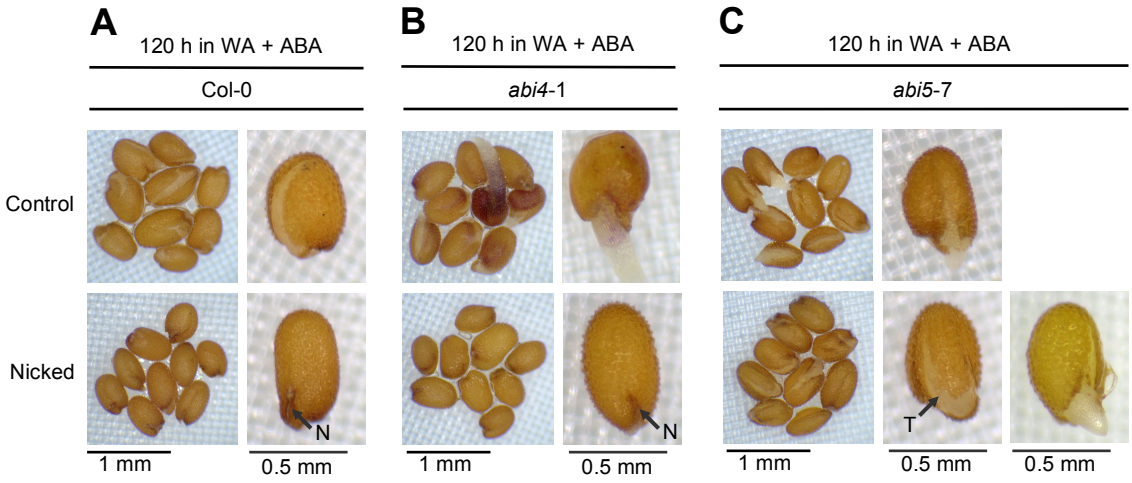


Figure 4.2 Effect of nicking on the two-step germination of *A. thaliana* mutant seeds in the presence of ABA. Pictures show intact (control) and nicked Col-0 (A), *abi4*-1 (B) and *abi5*-7 (C) seeds that were imbibed in water agar (WA) plates supplemented with 10 µM ABA for 120 h. Nicking (N) was performed according to Figure 4.1. TR, testa rupture. Scale bar is 1 mm for multiple seeds and 0.5 mm for single seeds.

Because ABA treated seeds showed testa rupture, it was hypothesized that intact seeds do not uptake enough ABA in order to repress testa rupture. Hence, nicking was performed to investigate whether or not an early embryo contact with ABA would block testa rupture. Surprisingly, Col-0 nicked seeds showed no testa rupture and no radicle protrusion in the presence of ABA (Figure 4.2 A, bottom panels), and *abi4*-1 and *abi5*-7 nicked seeds in the presence of ABA displayed opposite phenotypes (Figure 4.2 B and C, bottom panels). Behaving like Col-0 nicked seeds in the presence of ABA (Figure 4.2 A, bottom panels), *abi4*-1 nicked seeds no longer show testa rupture and radicle protrusion (Figure 4.2 B, bottom panels). On the other hand, ABA-treated *abi5*-7 nicked seeds still show testa rupture and radicle protrusion after 120 h (Figure 4.2 C, bottom panels).

Because Piskurewicz and others (2008) have shown that DELLA proteins repress testa rupture and Lee et al. (2012) have shown that ABI5 directly control DELLA genes

under FR light condition, expression of *GAI*, *RGA* and *RGL2* genes were assessed in Col-0 and *abi5*-7 nicked seeds imbibed in 10 µM ABA for 24 h. Transcript levels of these DELLA genes did not show any differences between Col-0 and *abi5*-7 nicked seeds in the presence of ABA (Figure 4.3).

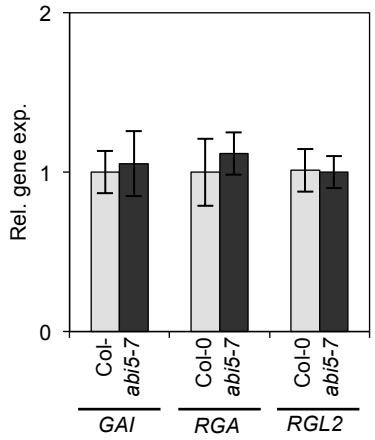


Figure 4.3 Expression of DELLA genes in nicked Col-0 and *abi5*-7 seeds in the presence of ABA. Transcript levels of *GAI*, *RGA* and *RGL2* genes, normalized to *UBQ11* expression and expressed relative to Col-0, in 24 h ABA-treated Col-0 and *abi5*-7 nicked seeds. Data are mean ± SD of three biological replicates.

4.2.2 ABI4 represses ABA and *cis*-OPDA biosynthesis under FR light to promote phyA-dependent germination

Previous work reported that *abi5*-7 seeds do not have any phyB-dependent phenotype in comparison with the wild type (Piskurewicz et al., 2008; Lee et al., 2012), but germinate more than wild type in a phyA-dependent manner (Lee et al., 2012). Hence, the question here is whether or not *abi4*-1 seeds also germinate more than the wild type in a phyA-dependent manner. PhyB-dependent germination was promoted by FR/R consecutive pulses and repressed by a single FR pulse (Figure 4.4 A), while a second FR pulse promoted phyA-dependent germination after 48 h of the first FR pulse performed after 4 h of imbibition (Figure 4.4 A). Similar to the wild type, in a phyB dependent germination, *abi4*-1 and *ABI4oe* seeds germination was 94 and 97 % under FR/R condition while germination was completely abolished under FR (Figure 4.4 B). Interestingly, in a

phyA-dependent germination triggered by a second FR pulse, seeds of Col-0, *abi4*-1 and *ABI4oe* germination was 57, 10 and 74 % respectively (Figure 4.4 B).

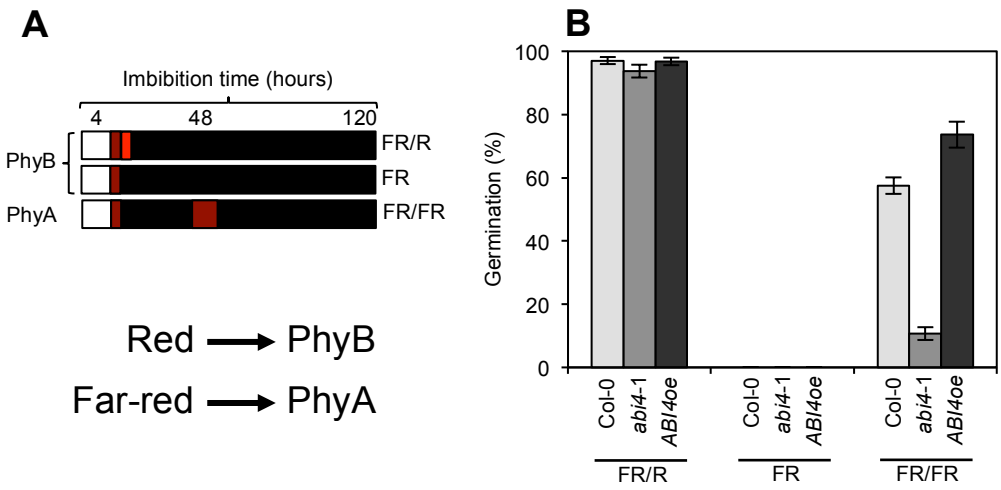


Figure 4.4 *abi4*-1 seeds show a deep dormancy phenotype under phyA-dependent germination. (A) Light treatment scheme: Col-0, *abi4*-1 and 35S:ABI4-ha over expressor (*ABI4oe*) seeds were imbibed on water-agar plates for 4 h under white light, treated with two successive 5 minutes FR and R pulses (FR/R), or either a single 5 minutes FR pulse or two FR pulses, with the second being for 30 min after 48 h of imbibition, and then placed in dark until germination was counted after 120 h of imbibition. Samples were also collected at 24 and 48 h (after imbibition) after a single FR pulse for mRNA and phytohormone analysis. (B) Germination of FR/R-, FR-, and FR/FR-treated Col-0, *abi4*-1 and *ABI4oe* seeds in water agar plates. Data are mean ± SD of three biological replicates.

Because *abi4*-1 seeds showed insensitivity to phyA-dependent germination, levels of ABA and *cis*-OPDA were measured in dry, and FR-treated seeds (24 and 48 hours imbibed) of Col-0, *abi4*-1 and *ABI4oe*. Dry seeds of Col-0 and *abi4*-1 showed no significant differences in ABA levels, but *ABI4oe* dry seeds showed 16 % lower ABA levels in comparison with Col-0 (Figure 4.5 A). Under FR conditions, prior to a second FR pulse, ABA levels in *abi4*-1 seeds imbibed for 24 h and 48 h were approximately 1.6-fold higher compared with Col-0 (Figure 4.5 A). ABA levels in FR-treated *ABI4oe* imbibed seeds showed no differences in comparison with the wild type, regardless of the time after imbibition (Figure 4.5 A).

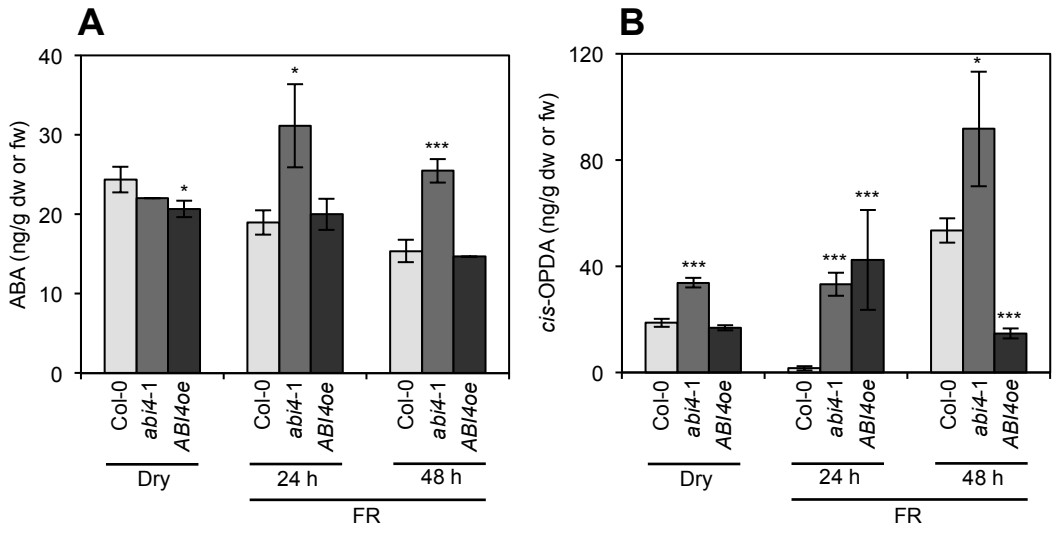


Figure 4.5 ABA and *cis*-OPDA levels accumulate in *abi4*-1 seeds under FR conditions. ABA (A) and *cis*-OPDA (B) levels in dry and FR-treated imbibed (24 and 48 h) Col-0 and *abi4*-1 seeds (as described in Figure 4.4 A). Data are mean ± SD of three biological replicates. Asterisks indicate statistically significant difference according to two-tailed Student's t-test (* $P < 0.05$; *** $P < 0.001$).

In addition to this, *cis*-OPDA levels were 1.8-, 20- and 1.7-fold increased in *abi4*-1 dry, 24- and 48-hours imbibed seeds, respectively, in comparison with the wild type (Figure 4.5 B). Compared with Col-0, *cis*-OPDA levels in *ABI4oe* dry seeds did not show any difference, but increased 20-fold after 24 hours and suddenly dropped 3.6-fold after 48 hours after imbibition under FR conditions (Figure 4.5 B).

Because ABA levels were increased in *abi4*-1 seeds under FR conditions, transcript levels of ABA biosynthetic and catabolic genes were assessed in FR-treated Col-0 and *abi4*-1 seeds after 24 hours of imbibition. Expression of *NCED6* was 2.1-fold increased in *abi4*-1 compared to Col-0 seeds (Figure 4.6 A). No significant differences were observed in the expression of *NCED9* between Col-0 and *abi4*-1 seeds (Figure 4.6 A). FR-treated *abi4*-1 seeds showed an increase of 1.9- and 1.8-fold in the expression of *CYP707A2* and *CYP707A3*, respectively, in comparison with Col-0 (Figure 4.6 A). However, no difference in the expression of *CYP707A1* was observed between FR-treated Col-0 and *abi4*-1 seeds (Figure 4.6 A).

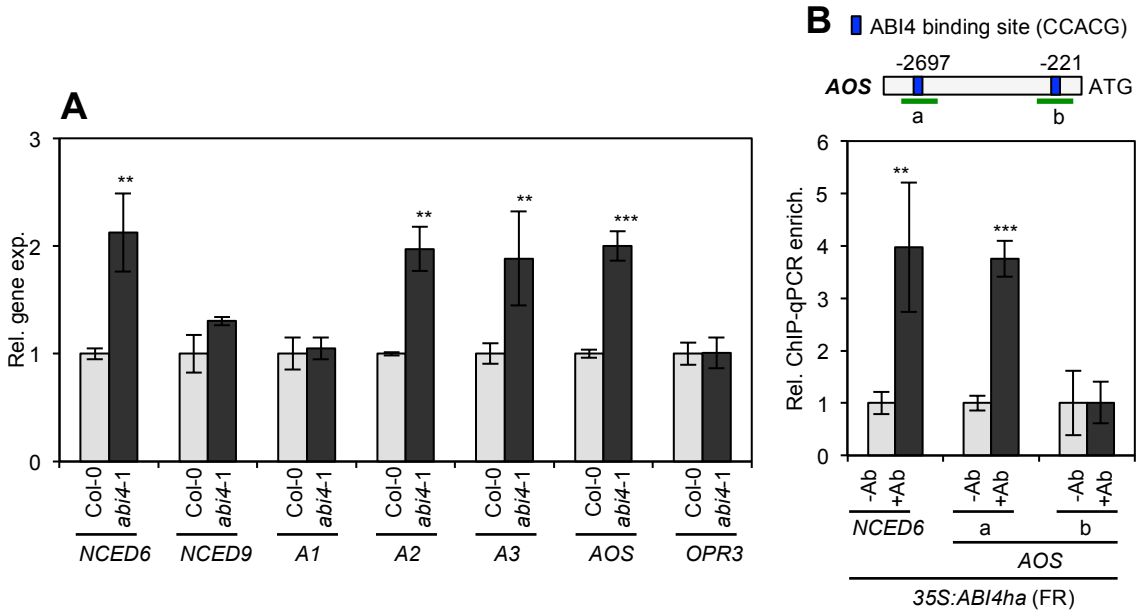


Figure 4.6 ABI4 represses ABA and *cis*-OPDA biosynthetic genes under FR conditions. (A) Transcript levels of *NCED6*, *NCED9*, *CYP707A1* (*A1*), *CYP707A2* (*A2*), *CYP707A3* (*A3*), *AOS* and *OPR3* genes, normalized to *UBQ11* expression and expressed relative to Col-0, in FR-treated imbibed (24 h) Col-0 and *abi4*-1 seeds (as described in Figure 4.4 A). (B) Relative ChIP-qPCR enrichment (Rel. ChIP enrich.) of the indicated promoter regions compared with the positive NCED6 control. Assays were performed on FR-treated seeds samples from a 35S:ABI4-ha epitope-tagged transgenic line with (+Ab) or without (−Ab) anti- HA antibodies. Data are mean ± SD of three biological replicates. Asterisks indicate statistically significant difference according to two-tailed Student's t-test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Because *cis*-OPDA levels were increased in *abi4*-1 seeds under FR conditions, expression of *AOS* and *OPR3* genes were assessed in FR-treated Col-0 and *abi4*-1 seeds after 24 hours of imbibition. Compared to Col-0, transcript levels of *AOS* in FR-treated *abi4*-1 seeds were approximately 2-fold higher while *OPR3* transcript levels were unaffected (Figure 4.6 A). The promoter region of *AOS* has binding sites known to be targets of ABI4, and using a 35S:ABI4-ha epitope-tagged transgenic line ChIP assays targeted to the *AOS* promotor region were performed showing a 3.7-fold increase in the presence compared to the absence of the anti-ha antibody (Figure 4.6 B). *NCED6* is shown as a positive control (Figure 4.6 B).

4.2.3 ABI4 plays a minor role in regulating oil mobilization in seeds

Oil mobilization in the embryo and endosperm of ABA-treated Col-0 and *abi4*-1 nicked seeds and light-treated Col-0 and *abi4*-1 seeds were assessed after 5 days of

imbibition to investigate whether or not oil mobilization is blocked by these two treatments (Figure 4.7 A). Col-0 and *abi4*-1 seeds were nicked after 1 hour of imbibition in the presence or absence of 10 µM ABA. Then, after 4 hours and 5 days of imbibition, seeds were dissected to separate embryo and endosperm for quantification of 20:1 fatty acid. Results are presented in percentage of 20:1 fatty acid in 5 days after imbibition in comparison with day 0 (after 4 hours after imbibition). Thus, ABA-treated Col-0 and *abi4*-1 nicked seeds had approximately 95 and 96 %, respectively, remaining 20:1 fatty acid levels in the embryo, while control (imbibed in water agar) Col-0 and *abi4*-1 embryos had 26 and 40 % respectively (Figure 4.7 B). In the endosperm, ABA-treated Col-0 and *abi4*-1 nicked seeds had approximately 58 and 79 %, respectively, remaining 20:1 fatty acid levels, while control Col-0 endosperm had only 5 % and control *abi4*-1 had nothing (Figure 4.7 C).

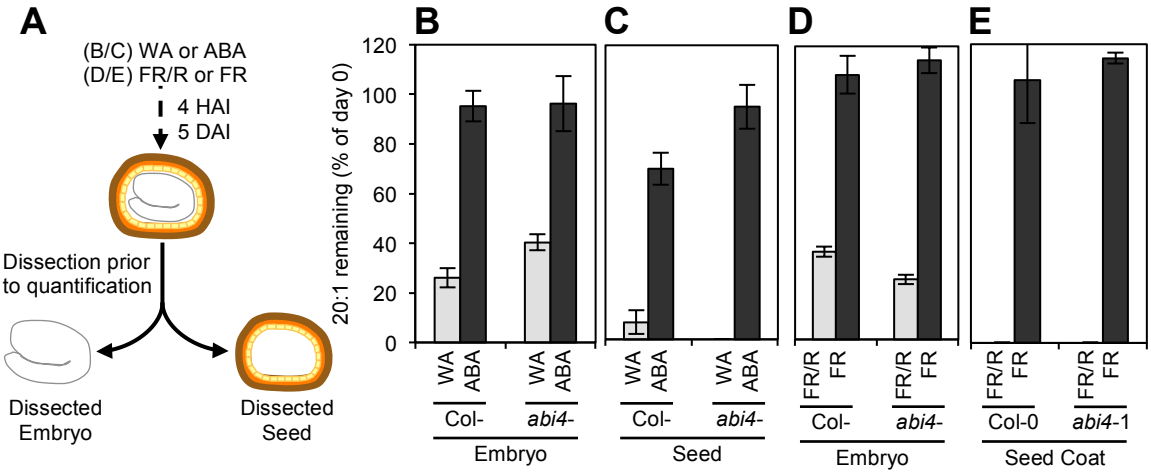


Figure 4.7 ABA-treated *abi4*-1 nicked seeds and FR-treated *abi4*-1 seeds do not mobilize oil. (A) Scheme of a mature *A. thaliana* seed showing the dissection into embryo and endosperm after nicking (nicked seeds imbibed in either water agar [WA] or WA supplemented with 10 µM ABA plates according to Figure 4.1 A) and light (FR/R- or FR-treated according to Figure 4.4 A) treatments, and prior to quantification. Dissection was performed using a syringe coupled with a 0.3 mm ø needle. (B-E) Eicosenoic Acid (20:1) levels in either nicked- and +- ABA-treated (B and C) or light-treated (D and E) Col-0 and *abi4*-1 embryos and endosperms 5 d after imbibition. Data are mean ± SD of six biological replicates.

Col-0 and *abi4*-1 seeds were treated with FR/R or FR pulses after 4 hour of imbibition under white light and maintained in the dark for 5 days after imbibition to

investigate if oil can be mobilized under these light regimes. Seeds were dissected to separate embryo and endosperm for quantification of 20:1 fatty acid prior to FR/R or FR treatments and after 5 days of dark imbibition (Figure 4.7 A). FR-treated Col-0 and *abi4*-1 nicked seeds had approximately 108 and 114 %, respectively, remaining 20:1 fatty acid levels in the embryo, while FR/R-treated Col-0 and *abi4*-1 embryos had 35 and 24 % respectively (Figure 4.7 D). In the endosperm, FR-treated Col-0 and *abi4*-1 nicked seeds had approximately 105 and 114 %, respectively, remaining 20:1 fatty acid levels, while FR/R-treated Col-0 and *abi4*-1 endosperms had nothing (Figure 4.7 E).

4.3 Discussion

In *A. thaliana*, seed coat nicking is a procedure used to stimulate germination of dormant seeds. The nicking treatment did not affect the appearance of testa rupture, but accelerated both testa rupture and radicle protrusion in comparison with intact seeds (Figure 4.1 B). Remarkably, Col-0 nicked seeds imbibed in the presence of ABA did not show any signs of both testa rupture and radicle protrusion (Figure 4.2 A, bottom panels), suggesting that exogenous ABA also blocks testa rupture. This result supports a role for exogenous ABA in the repression of testa rupture in A. thaliana seeds during imbibition as opposed to what was described by Müller and others (2006).

In a more recent study, a thick cutin-containing cuticular layer was found between the testa and the endosperm surrounding the whole endosperm of *A. thaliana* seeds (De Giorgi et al., 2015. These authors showed that a defect in the cuticle is associated with changes in seed permeability. They also showed that endogenous ABA is required to repress testa rupture, which is in agreement to what has been found in the present study with ABA-treated nicked seeds (Figure 4.2 A). Thus, it could be argued that exogenous ABA does not block testa rupture in Col-0 intact seeds due to the inability of ABA to

penetrate the cuticular layer, while it blocks testa rupture of Col-0 nicked seeds due to its early contact with the embryo and endosperm upon imbibition.

abi4-1 and *abi5*-7 mutant seeds were investigated to establish what if any role these factors played when seeds were nicked in the presence of ABA. As previously known (Finkelstein, 1994), *abi4*-1 and *abi5*-7 intact seeds showed insensitivity to exogenous application of ABA in comparison with the wild type Col-0 (Figure 4.2). One unanticipated finding was that *abi5*-7, but not *abi4*-1, nicked seeds were able to show testa rupture and radicle protrusion in the presence of exogenous ABA (Figure 4.2 B and C, bottom panels). Incompatible with this finding, Piskurewicz and others (2008) have shown that regulation of testa rupture by ABA during seed germination does not involve the ABI5 protein. They reported that RGL2, which is required for stimulation of ABA synthesis in imbibed seeds, RGA and GAI repress testa rupture under low GA conditions. Under FR light, ABI5 has been shown to directly regulate the expression of *RGL2* and two other DELLA proteins, *RGA* and *GAI* (Lee et al., 2012). In contrast to this result, however, no evidence of changes in transcript levels of *RGL2*, *RGA* and *GAI* between Col-0 and *abi5*-7 nicked seeds in the presence of ABA were observed (Figure 4.3). Understanding of these findings is somewhat limited by the lack of information on RGL2, RGA and GAI protein abundance in *abi5*-7 nicked seeds treated with ABA. Put together, regulation of testa rupture by ABI5 in nicked seeds is independent of the transcription regulation of DELLA genes during imbibition in the presence of ABA.

Expansins are proteins that constitute one of the four molecular mechanisms of cell wall loosening in plants (Cosgrove, 2005). They are encoded by a multigene family and are very conserved among higher plants (Shcherban et al., 1995; Hutchison et al., 1999; Cosgrove, 2005). Accumulated evidence throughout the past several years suggest that expansins play multiple roles during the life cycle of higher plants (McQueen-Mason et al., 1992; Brummell et al., 1999; Pien et al., 2001; Sabirzhanova et al., 2005; Gal et al., 2006;

Budzinski et al., 2011; Wei et al., 2011; Tabuchi et al., 2011; Zhao et al., 2012; Ma et al., 2013; Lü et al., 2013), including seed germination (Chen and Bradford, 2000; Chen et al., 2001; Yan et al., 2014).

A recent study showed that seeds of an endosperm-specific expansin mutant, *expa2* (i.e *exp2*), are delayed in germination in comparison with the wild type (Yan et al., 2014). In addition to this, using the β-glucuronidase (GUS) assay with a *pAtEXP2:GUS* construct these authors observed GUS staining in the micropylar endosperm prior to germination. In a different study, a tomato expansin, LeEXPA4, was found to be involved in the control of germination by contributing to cell wall loosening in the micropylar endosperm (Chen and Bradford, 2000). Interestingly, ABI5 has been shown to have a peculiar pattern of expression in the endosperm of *A. thaliana* in the presence of ABA, which is confined to the micropylar region (Penfield et al., 2006). This finding led to speculation about whether or not ABI5 plays an essential role in repressing cell wall loosening genes in the micropylar region.

To investigate if transcript levels of expansins were changed in *abi5*-7 compared to Col-0 nicked seeds in the presence of ABA, expression of 7 expansins were assessed. These expansins were selected according to their high levels of expression in imbibed seeds of *A. thaliana* using public transcriptomic data (Winter et al., 2007; Bassel et al., 2008). Remarkably, *EXPANSIN 1* (*EXPA1*), *EXPA2*, *EXPA3*, *EXPA9*, and *EXPA20* expression were 1.8-, 2.1-, 2.1-, 2.6-, and 1.6-fold increased in *abi5*-7 nicked seeds compared with Col-0 in the presence of ABA, while no significant changes were observed in the expression of *EXPA8* and *EXPA10* (Appendix B; B.1). This molecular evidence further supports the role of ABI5 in repressing the expression of expansins in order to block testa rupture in nicked seed in the presence of ABA. However, there is still a debate as to whether or not the expansins are essential for germination (Bewley et al., 2013).

One interesting finding is that similar to control seeds, *abi4*-1 nicked seeds treated with ABA did not germinate (Figure 4.2 B, bottom panels), suggesting that ABI4 might play a minor role in the control of seed germination. In 2012, Lee and others have demonstrated that *abi5*-7 seeds have a phenotype in a phyA-dependent assay showing more germination than the wild type. Because of this, the present study was also designed to determine if *abi4*-1 seeds, like *abi5*-7, germinate more than the wild type in a phyA dependent manner. Unexpectedly, *abi4*-1 seeds demonstrated a remarkable insensitivity to phyA-dependent germination in comparison with the wild type, while *ABI4oe* seeds not only recovered the wild type germination phenotype, but also showed a slightly higher germination (Figure 4.4 B). This may be explained by the fact that the first FR light pulse induces biosynthesis of ABA, which is known to modulate phyA-dependent response in seeds (Lee et al., 2012).

Prior studies have noted the role of ABI4 in inducing ABA biosynthesis during seed development and seedling establishment: in comparison to the respective wild types, dry seeds of *abi4*-1 have been reported to have less ABA levels (Shu et al., 2013), while 2-weeks old seedlings of an ABI4 overexpressor transgenic line have been shown to accumulate more ABA (Shu et al., 2016). Another important finding was that single FR pulse induces accumulation of *cis*-OPDA in the dark (Figure 3.4 A). Because of these reports, levels of ABA and *cis*-OPDA were determined in *abi4*-1 and *ABI4oe* dry and imbibed seeds treated with a single FR pulse (Figure 4.5). In contrast to what Shu et al. (2013) found, *abi4*-1 dry seeds did not show significant differences in ABA levels compared with wild type (Figure 4.5 A). Remarkably, FR-treated *abi4*-1 mutant seeds accumulated more ABA and *cis*-OPDA levels prior to a second FR pulse compared with Col-0 (Figure 4.5 A and B), consistent with the low germination phenotype (Figure 4.4 B). *ABI4oe* seeds did not show any differences in ABA levels and had less *cis*-OPDA amounts prior to a second FR pulse compared with Col-0 (Figure 4.5), consistent with the high

germination phenotype (Figure 4.4 B). It is difficult to explain these results, but it could be related to the fact that FR light induces *cis*-OPDA accumulation in seeds (Figure 3.4 A), with a possible role for ABI4 in this induction, and that *cis*-OPDA induces ABA synthesis (Dave et al., 2016).

To further investigate this increase in ABA and *cis*-OPDA levels in FR-treated *abi4*-1 seeds, expression of ABA biosynthetic and catabolic genes and *cis*-OPDA biosynthetic genes were assessed. ABI4 has been reported to directly induce the expression of *NCED6* (Shu et al., 2016) and repress the expression of the genes involved in ABA catabolism (*CYP707A1*, *CYP707A2* and *CYP707A3*) (Shu et al., 2013). In agreement with Shu et al. (2013), expression of *CYP707A2* and *CYP707A3* were up regulated in FR-treated *abi4*-1 seeds compared with the wild type (Figure 4.6 A). However, opposite to what was described in Shu et al. (2016), *abi4*-1 seeds showed high transcript levels of *NCED6* in comparison with Col-0 (Figure 4.6 A). It is possible that ABI4 represses *NCED6* specifically under FR conditions. Another possible explanation for this might be that the increase in *NCED6* occurred to balance out the increase in *CYP707A2* and *CYP707A3* in response to the increased *cis*-OPDA levels in FR-treated *abi4*-1 seeds. Remarkably, the current study suggests that ABI4 directly down regulates *AOS* expression in FR-treated seeds (Figure 4.6), consistent with the increase in both *AOS* transcripts and *cis*-OPDA levels in FR-treated *abi4*-1 seeds. In addition to this, *ABI4* expression was repressed by FR conditions in comparison with FR/R (Appendix B; B.2). Put together, these results strongly suggest that ABI4 represses ABA (directly or indirectly) and *cis*-OPDA synthesis under FR conditions, rendering seeds more susceptible to phyA-dependent responses.

ABA is known to greatly inhibit oil mobilization in *A. thaliana* germinated seeds (Graham, 2008). The current study found that ABA insensitivity is lost in nicked *abi4*-1 seeds during germination and establishment (Figure 4.2 B, bottom panels) and that FR-treated *abi4*-1 seeds were unable to germinate and establish (Figure 4.4 B). Because of

these, oil mobilization in ABA-treated *abi4*-1 nicked seeds and FR-treated *abi4*-1 seeds were examined. Penfield et al. (2006) have established that ABI4 blocks oil mobilization in the embryo in the presence of ABA. For this, they have demonstrated that 3 days stratified Col-0 and *abi4*-1 seeds imbibed in 20 μM ABA had 58 and 8 %, respectively, remaining 20:1 FA after 5 days of stratification. Unpublished results from others in the Graham lab indicated that blocking of oil mobilization can still occur in *abi4*-1 seeds with an increase in the concentration of ABA: non-stratified Col-0 seeds imbibed in 0, 2, 5, and 10 μM ABA had 1.4, 64, 66, 75 %, respectively, remaining 20:1 FA after 5 days of imbibition, while *abi4*-1 seeds had 0.3, 12, 28, 61 %, respectively (Appendix B; B.3). The differences in remaining 20:1 FA between Col-0 and *abi4*-1 under 2 μM (5-fold) and 5 μM (2-fold) ABA treatments are obvious, but under 10 μM ABA the differences dissapear.

It could be argued that the differences observed in published (Penfield et al., 2006) compared to unpublished (Appendix B; B.3) data are due to the presence or absence of stratification respectively. For instance, stratification induces biosynthesis of GA (Yamauchi et al., 2004), which in turn acts antagonistically to the ABA-responses in seeds (Bewley et al., 2013). In agreement with the unpublished data (Appendix B; B.3), *abi4*-1 mutant embryos treated either with nicking and ABA or with FR light did not mobilize oil compared with respective controls (Figure 4.7 B and D). In addition to this, endosperms of either Col-0 or *abi4*-1, which do not have ABI4, also failed to mobilize oil under either ABA plus nicking treatments or FR conditions (Figure 4.7 C and E). Put together, these results indicate that ABI4 plays a rather secondary role in blocking oil mobilization in either ABA-treated (in nicked or intact seeds) or FR-treated seeds.

Contrary to expectations, FR treatment showed a 1.6-fold induction of *SDP1* expression compared with FR/R treatment in Col-0 seeds imbibed for 24 hours (Appendix B; B.4). In addition to this, expression of *SDP1* does not increase upon seed imbibition in Col-0 non-dormant seeds (Appendix B; B.5). A possible explanation for these results may

be that the regulation of oil mobilization is at the post-translational level, as proposed by Eastmond (2007). In his paper, Eastmond (2007) showed that the *sugar-dependent2* mutant of *A. thaliana*, which fails to successfully mobilize storage oil, is deficient in the membrane-bound MONODEHYROASCORBATE REDUCTASE 4 (MDAR4) component of the ascorbate-dependent scavenging system. He suggested that MDAR4 function is critical to detoxify H_2O_2 generated during FA β-oxidation, which otherwise could escape from the peroxisome and inactivate the triacylglycerol lipase SDP1 blocking oil mobilization. In agreement with this, *MDAR4* expression was strongly repressed by FR treatment compared with FR/R (Appendix B; B.4), but was dramatically increased upon imbibition of Col-0 non-dormant seeds (Appendix B; B.5).

One interesting finding is that *ABI3* expression was not affected in FR-treated *abi4*-1 seeds compared with Col-0, while ABA-treated *abi4*-1 intact seeds showed a decrease in *ABI3* transcript levels compared with the wild type control (Appendix B; B.6). This result was previously reported by Söderman et al. (2000). Based on these observations, a model was designed in order to predict how oil mobilization is regulated in seeds: under FR conditions, expression of *ABI3* is up-regulated in an ABI4 independent-manner, accumulated ABI3 then represses expression of *MDAR4*, which would ultimately lead to blocking of SDP1 activity due to accumulation of non-scavenged H_2O_2; however, under ABA conditions, expression of *ABI3* is up-regulated in an ABI4 dependent-manner (Figure 4.8).

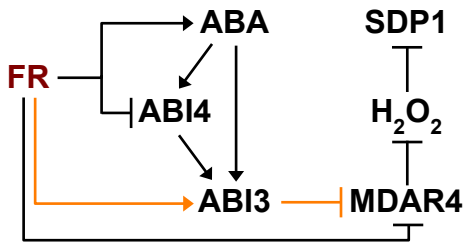


Figure 4.8 Tentative model for the regulation of oil mobilization in seeds.

The model predicts that MDAR4 is an important component for regulation of oil mobilization by ABA-responsive transcription factors such as ABI4 and ABI3 in seeds. Furthermore, the model raises the possibility that ABI3 is the main regulator of oil mobilization, which could be supported by the facts that *abi3* mutant seeds are able to germinate and establish under both FR (Piskurewicz et al., 2009) or ABA conditions (Finkelstein and Somerville, 1990; Lopez-Molina et al., 2002). These results however need to be interpreted with caution, especially because Lopez-Molina and others (2002) also reported that ABI5 is able to recover the growth arrest in *abi3*-1 seeds; a note of caution is due here since *abi3*-1 is considered a week allele (Clerkx et al., 2003).

4.4 Conclusions

By using the nicking technique, the presented data demonstrate that exogenous ABA is indeed able to block testa rupture in *A. thaliana* seeds, while analyses of the ABA insensitive mutants, *abi4*-1 and *abi5*-7, indicate that regulation of testa rupture by ABA involves only the ABI5 transcription factor. In contrast to ABI5, ABI4 showed a distinct function in blocking ABA and *cis*-OPDA biosynthesis in order to promote phyA-dependent germination in *A. thaliana* seeds. Furthermore, analyses of *abi4*-1 seeds under FR light and using the nicking technique combined with ABA suggest that ABI4 has a secondary role in the regulation of oil mobilization. Further work is however required to establish the role of ABA insensitive transcription factors in the control of oil mobilization in seeds.

Chapter 5 - After-ripening breaks dormancy by altering phytohormones levels in dry seeds

5.1 Introduction and Preliminary Results[3]

In nature, dormancy is very often linked with seasonal time, preventing seed germination during unfavorable conditions (Footitt et al. 2014; Burghardt et al. 2016). In a dry/wet climate, after-ripening plays an important role in breaking dormancy of seeds that are exposed to a specific set of environmental conditions and timing germination to the wet season (Bewley et al. 2013). In addition to its effects on breaking dormancy, De Casas and others (2012) have shown that shifts in germination time caused by after-ripening accelerates *A. thaliana* flowering.

After-ripening requires oxygen, specific ranges of temperature and seed moisture content (Roberts, 1962; Iglesias-Fernández et al., 2011; Bewley et al. 2013). For instance, low oxygen availability slows after-ripening and high oxygen conditions speeds it up (Simmonds and Simpson, 1971; Bewley et al. 2013). Besides oxygen, the rate of after-ripening also varies with temperature: a decrease in temperature positively correlates with a delay in after-ripening. After-ripening fails to occur when relative humidity is either too high (above 70 %), which could lead to decreased seed viability or even induction of secondary dormancy if above 90 % (Basbouss-Serhal et al., 2016), or too low (below 20%), where breaking of dormancy is significantly delayed (Bewley et al., 2013). Thus, seeds are considered dry for after-ripening if their water contents are in equilibrium with 70% relative humidity or less (Bewley et al., 2013).

Seeds with different depths of dormancy, such as Col-0 and Cvi, also after-ripen at different rates (Preston, 2009). In *A. thaliana*, after-ripening is generally performed at ~ 30

[3] Unpublished data generated by Anuja Dave, Fabián Vaistij and Alison Gilday that form the basis of this PhD project.

% of relative humidity, in which seeds have less than 10 % water content (Basbouss-Serhal et al., 2016). Under these conditions, they contain both strongly (< 4 %) and weakly bound (4–20 %) water but no free water available for metabolism. Because of this, there is general consensus that after-ripening occurs without changes in transcription and translation. Although enzymatic processes are unlikely to occur when seeds are in the dry state, the formation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$), may occur (Bewley et al. 2013).

There was a long time debate about whether or not changes in transcription and translation occur during after-ripening of dry seeds (Carrera et al., 2008; Holdsworth et al., 2008; Meimoun et al., 2014). Recently, comparisons between both dormant and non-dormant (after-ripened) dry seeds of sunflower and *A. thaliana* have demonstrated that dormancy alleviation does not correlate with changes in gene expression (Meimoun et al., 2014; Basbouss-Serhal et al., 2016). Additionally, in the last ten years, a robust set of data have shown that breakage of dormancy during after-ripening is associated with reactive oxygen species (ROS) and oxidized product accumulation in dry seeds (El-Maarouf-Bouteau and Bailly, 2008; Bazin et al., 2011a; Bazin et al., 2011b; Leymarie et al., 2012; El-Maarouf-Bouteau et al., 2013; Meimoun et al., 2014). In agreement with the accumulation of ROS, it has been shown that targeted protein and mRNA oxidation occur in dry seeds and that these processes could play a role in seed dormancy alleviation (Oracz et al., 2007; Bazin et al., 2011b). Despite this, after-ripening is still a poorly understood mechanism, especially because there are limitations when it comes to performing treatments on dry seeds.

Among the different factors involved in the regulation of seed dormancy, the phytohormones ABA and GA are widely accepted to play a significant role (Bewley et al. 2013; Baskin and Baskin 2014). In *A. thaliana*, as in several other species, dormant imbibed seeds are usually associated with low levels of GA and high levels of ABA

(Holdsworth et al. 2008). In agreement with this, based on genetic and physiological studies, it has been demonstrated that after-ripening changes GA and ABA metabolism and sensitivity upon seed imbibition (Bewley et al. 2013). Grappin and others (2000) reported that ABA levels in dry dormant seeds of *Nicotiana plumbaginifolia* are higher than in dry after-ripened seeds. Recently, it was found that the oxylipin *cis*-OPDA also plays an important role in repressing germination of primary dormant seeds (Dave et al. 2011; Dave et al., 2016). Additionally, ABA has been shown to positively feed back into the *cis*-OPDA pathway by promoting its accumulation in developing seeds in order to set dormancy (Dave et al. 2016).

To get new insights into how ABA and *cis*-OPDA levels influence the dormancy alleviation by after-ripening, seeds that over-accumulate either ABA or *cis*-OPDA were previously investigated by others in the Graham lab. A six-week after-ripening experiment using Col-0, *cyp707a1*-1 (*a1*), *cyp707a1*-1 *cyp707a2*-1 (*a1 a2*) and *cyp707a1*-1 *cyp707a3*-1 (*a1 a3*) dry seeds was conducted. Freshly matured seeds of Col-0 germinated about 1.7 %, while seeds of *a1*, *a1 a2* and *a1 a3* failed to germinate (Figure 5.1 A). In agreement to what was previously known (Okamoto et al., 2006), compared to wild type ABA levels in freshly matured dry seeds of *a1*, *a1 a2* and *a1 a3* were about 37-, 52- and 64-fold higher, respectively (Figure 5.1 C and D). After-ripened seeds of Col-0, *a1*, *a1 a2* and *a1 a3* germinated at about 77, 13, 0 and 11 % respectively (Figure 5.1 A). After-ripened dry seeds of Col-0, *a1*, *a1 a2* and *a1 a3* showed a decrease in ABA levels of 2.6-, 4.7-, 4.3- and 4.1-fold, respectively, compared with freshly matured seed (Figure 5.1 C and D). What is interesting about these data is that as well as the expected finding that high ABA levels correlated with low germination, after-ripening results in a decrease in ABA levels in dry seeds even in the absence of the CYP707As which are known to be involved in ABA catabolism.

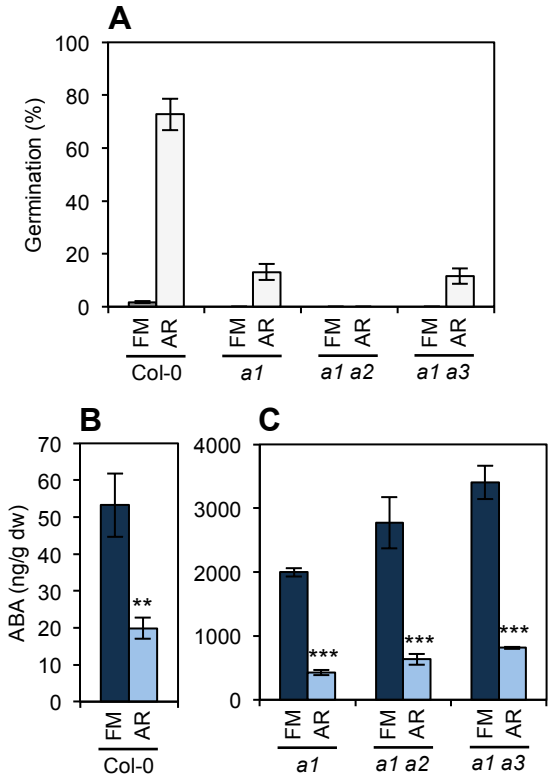


Figure 5.1 CYP707As are not involved in the decrease of ABA levels in dry seeds during after-ripening. (A) Germination of freshly matured (FM) and 6 weeks after-ripened (AR) Col-0 (WT), *cyp707a1*-1 (*a1*), *cyp707a1*-1 *cyp707a2*-1 (*a1 a2*) and *cyp707a1*-1 *cyp707a3*-1 (*a1 a3*) seeds. (B and C) ABA levels in FM and AR WT, *a1*, *a1 a2* and *a1 a3* dry seeds. Data are mean ± SD of three biological replicates. Asterisks indicate statistically significant difference according to two-tailed Student's t-test (** $P < 0.01$; *** $P < 0.001$) compared with FM. (Unpublished data obtained by Dr Anuja Dave and co-workers in the Graham lab and used to introduce this chapter of the current study).

To investigate the involvement of *cis*-OPDA Dave and co-workers in the Graham lab designed an experiment using *pxa1*-1 which accumulates *cis*-OPDA (Dave *et al*., 2011). Col-0 and *pxa1*-1 mutants seeds were after-ripened for six weeks after which they germinated at 78 and 18 % respectively (Figure 5.2 B). Levels of *cis*-OPDA in freshly matured *pxa1*-1 dry seeds were 2.2-fold higher compared to the wild type (Figure 5.2 E), in agreement to what was previously known (Dave et al., 2011). Col-0 and *pxa1*-1 after-ripened dry seeds showed a decreased in *cis*-OPDA levels of 3.9- and 1.6-fold respectively, compared with freshly matured dry seeds (Figure 5.2 E). For the data presented in Figure 5.2, it is clear that high *cis*-OPDA levels correlated with low germination of the seeds, and that after-ripening treatment decreases *cis*-OPDA levels in dry seeds.

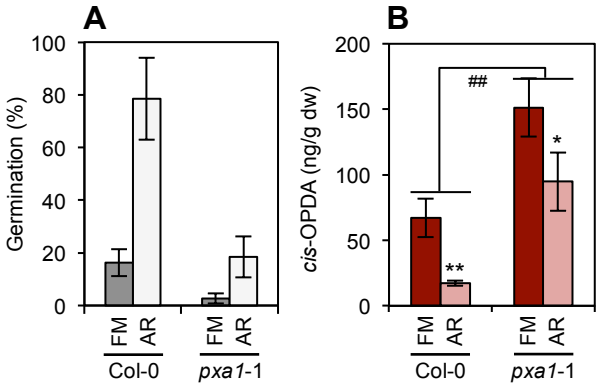


Figure 5.2 *cis*-OPDA levels decrease in dry seeds during after-ripening. (A) Germination of FM and AR Col-0 (WT) and *pxa1*-1 seeds. (B) *cis*-OPDA levels in FM and AR WT and *pxa1*-1 dry seeds. Data are mean ± SD of three biological replicates. Asterisks indicate statistically significant difference according to two-tailed Student's t-test (* $P < 0.05$; ** $P < 0.01$) compared with FM. Crosshatches indicate statistically significant difference according to two-tailed Student's t-test (## $P < 0.01$) compared with WT. (Unpublished data obtained by Dr Anuja Dave and co-workers in the Graham lab and used to introduce this chapter of the current study).

To further investigate how the levels of phytohormones change in dry seeds during after-ripening, GA_4, ABA and *cis*-OPDA levels in dry seeds of freshly matured (FM) and after-ripened (AR) Col-0 seeds were assessed in a heat treatment experiment by Dave and co-workers. Freshly-matured Col-0 dry seeds were treated with either 65 or 100 °C for either 2 or 5 minutes prior to 40-weeks of after-ripening. The aim of this experiment was to create two physiologically different groups: one composed of viable seeds (control and 65 °C treated); and the other composed of non-viable seeds (100 °C treated), in which protein denaturation is expected. Freshly matured control seeds, and seeds pre-treated with 65 °C for 2 min and 5 min germinated 49, 66 and 69 % respectively (Figure 5.3 A). After-ripened control seeds, and seeds pre-treated with 65 °C for 2 min and 5 min germinated 98, 98 and 87 % respectively (Figure 5.3 A). Freshly matured and after-ripened seeds pre-treated with 100 °C failed to germinate, despite the time (Figure 5.3 A).

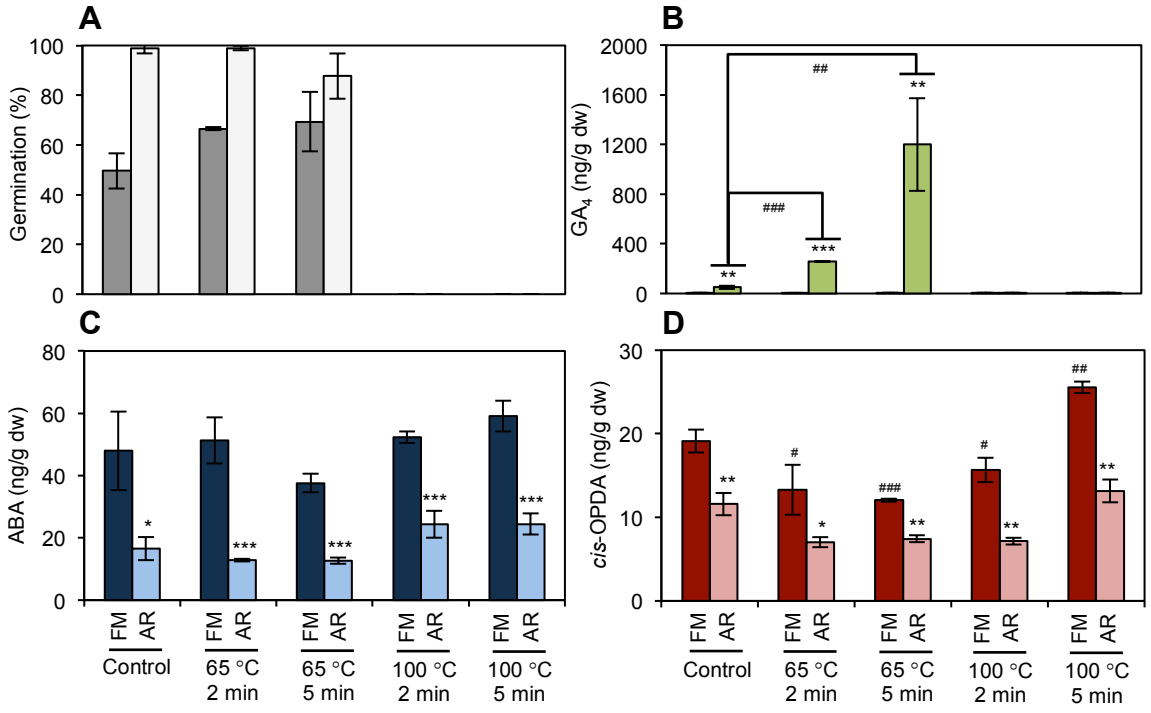


Figure 5.3 Pre-heating treatment in dry seeds affects phytohormones levels during after-ripening. (A) Germination of freshly matured (FM) and 40-weeks after-ripened (AR) Col-0 seeds that were pre-treated with 65 or 100 °C, or not (control), for 2 or 5 min. (B-D) Phytohormones levels (B, GA_4; C, ABA; D, *cis*-OPDA) in freshly matured (FM) and 10 months after-ripened (AR) Col-0 dry seeds that were pre-treated with 65 or 100 °C, for 2 or 5 min. Data are mean ± SD of three biological replicates. Asterisks indicate statistically significant difference according to two-tailed Student's t-test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) compared with FM. Crosshatches indicate statistically significant difference according to two-tailed Student's t-test (# $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$) compared with control. (Unpublished data obtained by Dr Anuja Dave and co-workers in the Graham lab and used to introduce this chapter of the current study).

Compared to freshly matured seeds, after-ripened control seeds showed a 8.9-fold increase in GA_4 content (Figure 5.3 B). After-ripened seeds that were pre-treated with 65 °C for 2 min and 5 min showed a 56- and 203-fold increase in GA_4 content compared to freshly matured seed (Figure 5.3 B). Surprisingly, after-ripened seeds that were pre-treated with 65 °C for 2 min and 5 min showed a 4.3- and 20-fold increase in GA_4 content compared to the control (Figure 5.3 B). However, no changes in GA_4 levels were observed between freshly matured and after-ripened seeds that were pre-treated with 100 °C. In freshly matured dry seeds, temperature treatment did not affect the initial levels of GA_4 compared with control (Figure 5.3 B).

Initial levels of ABA in freshly matured dry seeds were not affected by temperature treatment, but dry after-ripened seeds showed a decrease of at least 2-fold in the ABA

levels compared with freshly matured dry seeds, regardless of the temperature pre-treatment (Figure 5.3 C). Compared with the control, *cis*-OPDA levels in freshly matured dry seeds were 30, 37 and 18 % lower in 65 °C for 2 min, 65 °C for 5 min, and 100 °C for 2 min treatments respectively, while 100 °C for 5 min showed an increase of 33 % in *cis*-OPDA levels (Figure 5.3 D). Despite the fact that *cis*-OPDA levels were affected by temperature treatment in freshly matured dry seeds, *cis*-OPDA levels observed in after-ripened dry seeds were at least 1.6-fold lower than freshly matured dry seeds, regardless of the temperature pre-treatment (Figure 5.3 D).

In the current study the aim was to extend this work to gain a more complete understanding of the role and regulation of phytohormones levels during after-ripening of *A. thaliana* seeds.

5.2 Extended Results

5.2.1 Germination of *A. thaliana* Cvi ecotype correlates with an increase in GA_4 levels in dry seeds during after-ripening

Two *A. thaliana* ecotypes that after-ripen at different rates were used in order to investigate how changes in phytohormone levels in dry seeds contribute to germination. Germination was scored and GA_4, ABA and *cis*-OPDA levels were quantified in freshly matured and 12 weeks after-ripened dry seeds. During this time, dormancy was completely broken in Col-0 but not Cvi seeds (Figure 5.4 A). Freshly matured, dormant seeds of Col-0 and Cvi germinated at about 7 and 0.2 % respectively (Figure 5.4 A). After 12 weeks of after-ripening, Col-0 seeds germinated about 97 %, while Cvi seeds failed to germinate (Figure 5.4 A).

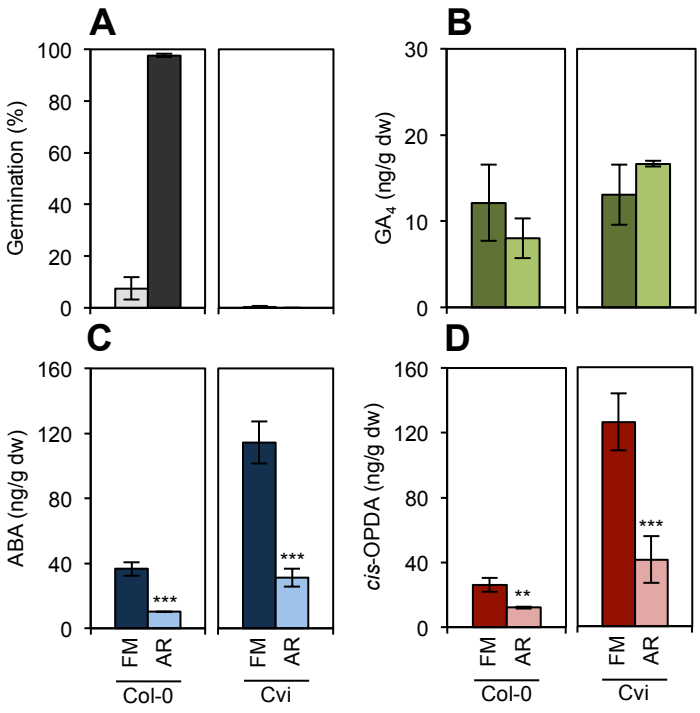


Figure 5.4 After-ripening changes phytohormone levels in dry seeds. Germination of freshly matured (FM) and 12 weeks after-ripened (AR) Col-0 and Cvi (A). GA_4 (B), ABA (C) and *cis*-OPDA (D) levels in FM and AR Col-0 and Cvi dry seeds. Data are mean ± SD of three biological replicates. Asterisks indicate statistically significant difference according to two-tailed Student's t-test (** $P < 0.01$; *** $P < 0.001$) compared with FM.

Freshly matured dry seeds of Col-0 and Cvi showed no significant differences in GA_4 levels compared with after-ripened seeds (Figure 5.4 B). ABA levels decreased 3.6-fold in both Col-0 and Cvi after-ripened compared with freshly matured dry seeds (Figure 5.4 C). Compared to freshly matured, *cis*-OPDA levels decreased 2.1- and 3-fold in Col-0 and Cvi after-ripened dry seeds respectively (Figure 5.4 D).

To further explore the correlation between changes in the phytohormones and germination of Cvi, dry seeds were analyzed after a period of 40 weeks of after-ripening. During this time, dormancy of Cvi seeds was broken (Figure 5.5 A). At time 0, i.e. freshly matured, and after 24 weeks of after ripening, seeds of Cvi failed to germinate, but germinated about 83 % after 40 weeks of after-ripening (Figure 5.5 A). Remarkably, GA_4 levels increased 18-fold in 40 weeks of after-ripened dry seeds compared with freshly matured (Figure 5.5 B). ABA (Figure 5.5 C) and *cis*-OPDA (Figure 5.5 D) levels

decreased 5.8- and 1.8-fold, respectively, in 40 weeks of after-ripened dry seeds compared with freshly matured.

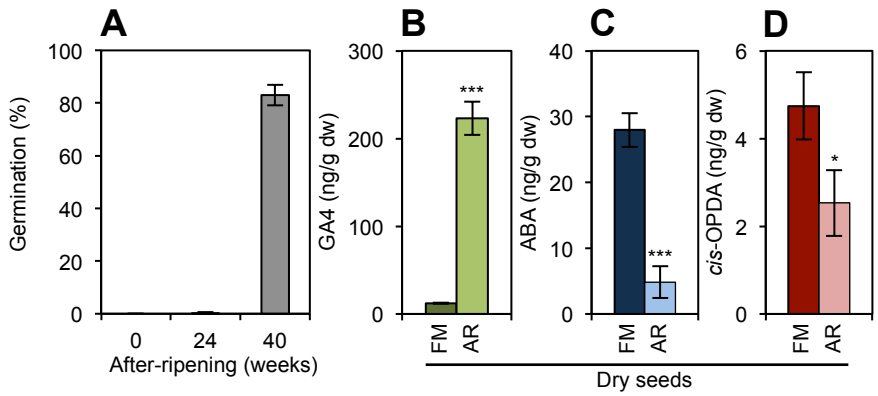


Figure 5.5 Germination correlates with increase in GA_4 levels in Cvi dry seeds during prolonged after-ripening. Germination of freshly matured (time 0), and 24- and 40-weeks after-ripened Cvi seeds (A). GA_4 (B), ABA (C) and *cis*-OPDA (D) levels in freshly matured (FM) and 40-weeks after-ripened (AR) Cvi dry seeds. Data are mean ± SD of three biological replicates. Asterisks indicate statistically significant difference according to two-tailed Student's t-test (* $P < 0.05$; *** $P < 0.001$) compared with FM.

5.2.2 Changes in phytohormone levels that occur in dry seeds during after-ripening are maintained upon imbibition

To investigate if the changes in phytohormone levels occurred in dry seeds during after-ripening are maintained when seeds are imbibed, levels of GA_4, ABA and *cis*-OPDA were measured in freshly matured and 40-weeks after-ripened Cvi seeds during four days of imbibition. Freshly matured and 40-weeks after-ripened Cvi seeds germinated 0 and 84 % respectively (Figure 5.5 A). 40-weeks after-ripened seeds showed 48-, 58-, 43- and 17-fold increase in levels of GA_4 after 1, 2, 3 and 4 days of imbibition, respectively, compared with freshly matured imbibed seeds (Figure 5.6 A). Compared to freshly matured imbibed seeds, ABA levels were 3.1- and 5.1-fold lower in 40-weeks after-ripened seeds after 1 and 2 days of imbibition respectively, being undetectable after three days of imbibition (Figure 5.6 B). Similar to ABA levels, *cis*-OPDA levels were maintained 2- and 3.8-fold lower after 1 and 2 days of imbibition of after-ripened seeds, respectively, compared to freshly matured imbibed seeds, being undetectable after 3 days of imbibition (Figure 5.6 C).

However, while GA_4 and ABA levels progressively decrease with time of imbibition, regardless of the dormancy state (Figure 5.6 A and B), *cis*-OPDA levels in freshly matured imbibed seeds increased 3.7-fold after 4 days compared with 3 days (Figure 5.6 C).

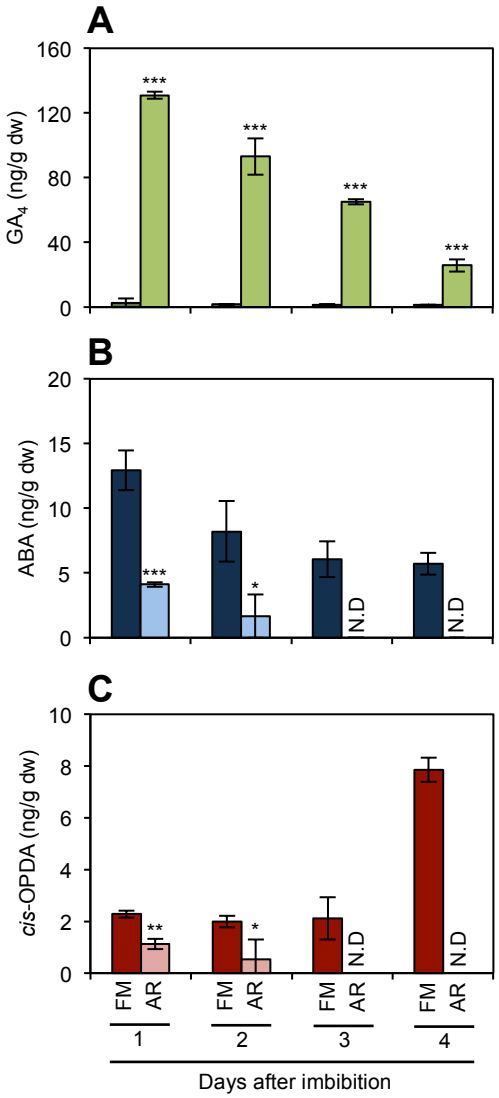


Figure 5.6 Changes in phytohormone levels during dry after-ripening are maintained upon imbibition in Cvi seeds. GA_4 (A), ABA (B) and *cis*-OPDA (C) levels in 1, 2, 3 and 4 days imbibed Cvi freshly matured (FM) and 40-weeks after-ripened (AR) seeds. Data are mean ± SD of three biological replicates. Asterisks indicate statistically significant difference according to two-tailed Student's t-test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) compared with FM.

5.2.3 Time to break dormancy of Cvi seeds during after-ripening correlates with the interval between wet seasons in Cape Verde

It was investigated whether the extended time for Cvi seeds to after-ripen correlates or not to the environmental conditions that Cvi seeds may encounter in their natural

habitats. *A. thaliana* Cvi ecotype is originally from Cape Verde, which has a dry/wet climate where temperature does not vary much throughout the year. Precipitation and temperature records presented in Figure 5.7 are an average of 5 years data (from 2010 to 2015) of Praia in Cape Verde (National Centers for Environmental Information, n.d.). In the last 5 years, the temperature in Praia varied from a minimum average of 23 °C to a maximum average of 27 °C throughout the year (Figure 5.7).

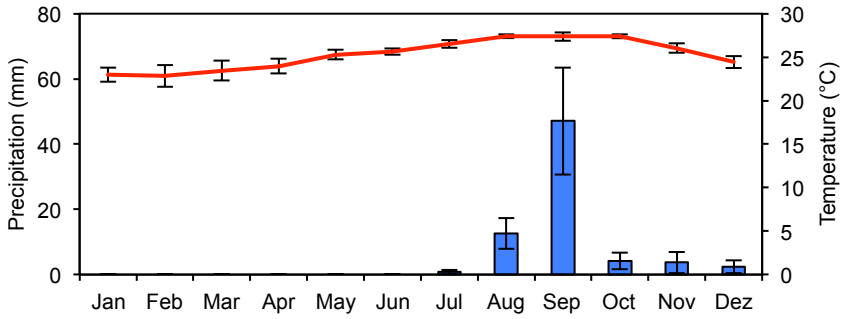


Figure 5.7 Climate in Praia, Cape Verde. Data was obtained from the National Centers for Environmental Information from year 2010 to year 2015 in Praia Cape Verde (W23.6/N14.9 70m). Variation in precipitation (blue bars; in mm) and temperature (red line; in °C) throughout the year.

During the same period, precipitation in Cape Verde occurred mainly between August and December, with six months of dry season, between January and June, throughout the year (Figure 5.7). The 1 mm of precipitation observed in the month of July (Figure 5.7) corresponded to an anticipation of the wet season observed only in the years 2011 and 2012 (data not shown).

5.3 Discussion

Col-0 ecotype (grown at temperatures above 20 °C) is well known to have a low dormancy phenotype seeds (Preston et al., 2009), which can be completely broken within a couple of weeks of dry storage to allow germination to take place (Appendix C; C.1 A). In Col-0, an increase in germination correlated with the decrease in ABA and *cis*-OPDA levels in dry seeds (Appendix C; C.1), suggesting a role for these phytohormones in the

alleviation of seed dormancy which would be in in addition to dormancy alleviation by protein and mRNA oxidation previously proposed by Oracz et al. (2007) and Bazin et al. (2011b) respectively. In agreement with this, previous work has already shown that catabolism of ABA plays an important role in promoting germination of seeds during imbibition (Rodríguez-Gacio et al., 2009). However, these changes in phytohormones levels in dry seeds do not seem, *a priori*, to play a major role in alleviating dormancy of Cvi ecotype during after-ripening (Figure 5.4). For instance, the decreased ABA and *cis*-OPDA levels during after-ripening of Cvi dry seeds did not correlate with the increase in germination (Figure 5.4 A, C and D). Seeds of Cvi ecotype are known for their deep dormancy phenotype (Preston et al., 2009), and DOG1 has been shown to play an important role in it (Alonso-Blanco et al., 2003).

It has been proposed that DOG1 dictates the time required for dormancy release in *A. thaliana*, and that would occur independently of GA_4 and ABA levels (Nakabayashi et al., 2012). However, in a study using *A. thaliana* and *L. sativum*, Graeber and others (2014) have demonstrated that regulation of germination by DOG1 indeed involves changes in GA levels. Remarkably, the data presented showed that germination of Cvi seeds correlate with an increase in GA_4 levels in dry seeds during prolonged after-ripening (Figure 5.5 A and B). In addition to this, unpublished data from the Graham laboratory also showed that GA_4 levels were increased in Col-0 and Ler dry seeds that were after-ripened for 40 weeks in comparison with freshly matured seed (Appendix C; C.2). These data suggest that, differently from Col-0 and Ler, a decrease in ABA and *cis*-OPDA levels in addition to an increase in GA levels greatly contribute to the release of dormancy in Cvi ecotype. In agreement with this, Bentsink et al. (2006) have demonstrated that after-ripened *ga1-3 dog1* double mutant seeds, unable to synthesize GA, require exogenous application of GA in order to germinate.

The current study found that the changes in phytohormone levels observed in Cvi dry seeds during after-ripening (Figure 5.5 B-D) were maintained when seed imbibition took place (Figure 5.6), supporting a role for the hormonal equilibria acquired in dry after-ripened seeds on the dormancy status upon seed imbibition. In addition to this, several reports have shown that after-ripening entails significant shifts in the sensitivity to phytohormone, decreasing and increasing the sensitivity to ABA and GA, respectively, upon seed imbibition (Leubner-Metzger, 2002; Liu et al., 2013; Hauvermale et al., 2015; Kaur and Zhawar, 2016). Thus, combination of the GA_4, ABA and *cis*-OPDA measurements in dry and imbibed seeds further supports the critical role for these phytohormones during after-ripening in order to control dormancy.

In a second set of experiments, germination of Col-0 and Ler freshly matured seeds were about 17 and 3 % respectively and most of the primary dormancy was broken after 6-weeks of after-ripening (Appendix C; C.2 A and B). These *A. thaliana* ecotypes are originally from cold/hot climates and precipitation is not a limiting factor. Thus, differences in temperature have been shown to play a critical role in controlling the life cycle, particularly in seed dormancy (Kendall et al., 2011; Auge et al., 2015). However, Cvi is originally from Cape Verde Island, where precipitation is a limiting factor (Figure 5.7). Hence, it is possible that temporally programmed after-ripening is the major route for breaking seed dormancy in Cape Verde. In agreement with this, the data here presented showed that the time required to break dormancy of freshly matured Cvi seeds by after-ripening (Figure 5.5 A) coincides with the interval between the wet seasons in Cape Verde Islands (Figure 5.7).

The temperature treatment experiment (pre-heated) performed in Col-0 dry seeds by Dave and co-workers in the Graham lab provides important information on how the changes in GA_4, ABA and *cis*-OPDA levels occur during after-ripening. Results from this experiment suggest that the observed decrease in ABA and *cis*-OPDA levels during after-

ripening is not due to enzymatic activity. This is supported by the fact that 100 °C pre-treated seeds, which are unviable dead seeds, still show a decrease in the levels of these two phytohormones (Figure 5.3 C and D). In addition to this, analyses performed on *cyp707a* (*a1*, *a1 a2* and *a1 a3*) and *pxa1*-1 mutants strongly indicate that ABA and *cis*-OPDA levels decrease in dry seeds during after-ripening in a non-enzymatic manner.

After-ripened seeds of *cyp707a*s, including single and double mutants (Figure 5.1 A), and *pxa1*-1 mutants (Figure 5.2 A) poorly germinate compared to the respective wild types, and ABA and *cis*-OPDA levels in after-ripened dry seeds of *cyp707a*s (Figure 5.1 B and C) and *pxa1*-1 (Figure 5.2 B) mutants were at least 5.5-fold higher compared to after-ripened respective wild types. All together, these reinforce the influence that the levels of these phytohormones in dry seeds have during germination upon seed imbibition.

The results from the temperature treatment also indicate that GA_4 levels increase in dry seeds during prolonged after-ripening in an enzyme activity dependent manner, since 100 °C pre-treated after-ripened seeds show no increase in GA_4 (Figure 5.3 B). However, it is possible that this increase is due to a non-enzymatic conversion of a thermo-labile precursor that could have been accumulated during seed development. Thus, the evidence so far is not sufficient to explain the increase in GA_4 during prolonged after-ripening of dry seeds. Perhaps a future analysis using *ga3ox* mutant seeds, which are involved in the biosynthesis of active GAs (detailed in section 1.3) (Mitchum, 2006), will provide more information. Curiously, 65 °C pre-treated after-ripened seeds show a substantial increase in GA_4, in a time-dependent manner, compared with the control (Figure 5.3 B). For this, it could be suggested that the glassy state of the dry seed, with its extreme viscosity (Bewley et al., 2013), restricts the molecular motion and diffusion of substrates and reactants required for this increase in GA_4, and therefore the 65 °C treatment would accelerate rates of the movements for the reaction to occur.

5.4 Conclusions

Alleviation of seed dormancy during dry after-ripening involves a non-enzymatic decrease in ABA and *cis*-OPDA levels in *A. thaliana* seeds. Furthermore, biosynthetic increase in GA_4 levels in dry seeds may play an important role in breaking dormancy of the Cvi ecotype. The coincidence of after-ripening period with the length of the dry season in Cape Verde Islands suggests that the increase in GA may be temporally programmed to match the optimal environmental conditions for germination to occur.

Chapter 6 - Final remarks

This study set out to determine how *cis*-OPDA contributes to regulate light-dependent germination, how ABA responsive factors contribute to the regulation of germination and oil mobilization, and how ABA, *cis*-OPDA and GA_4 contribute to the release of seed dormancy during dry after-ripening. One of the more significant findings to emerge from this study is that *cis*-OPDA is essential for the control of light-dependent germination. The study has identified a mechanism, which involves the phytochrome-associated factors PIF1 and SOM, that regulates *AOS* and *OPR3* genes differentially in order to accumulate *cis*-OPDA and repress seed germination independently of ABA under FR conditions. At this point it is not obvious why plants have evolved two independent, phytohormone based repression signals to control the same process but it could be that because of the critical importance of the germination process it has been advantageous to adopt a 'belt and braces' approach to its control. Furthermore, this study connects the work performed in the field of plant-pathogen interaction with the work done in seed biology, suggesting that diverse *cis*-OPDA mediated responses rely on retrograde signaling. Taken together, this research provides a framework for the further exploration of the *cis*-OPDA signaling pathway in seed biology.

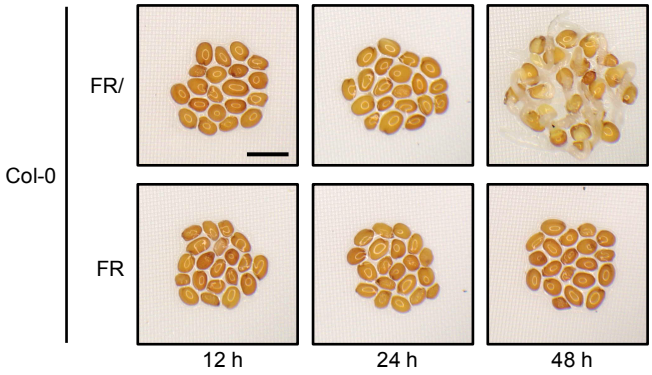
The second major finding of this thesis work was that exogenous ABA indeed is able to repress testa rupture in *A. thaliana* seeds. For this, investigations of *abi4*-1 and *abi5*-7 mutants have shown that ABI5, but not ABI4, plays a role in repressing testa rupture in nicked seeds treated with ABA. Whilst this study did not confirm how ABI5 ultimately blocks testa rupture in seeds, it did partially substantiate the role of expansins in this repression. The observed differences between germination of *abi4*-1 and *abi5*-7 in nicked seeds treated with ABA led to a reexamination of the role of ABI4 in controlling seed germination and establishment. Results obtained from FR-treated *abi4*-1 and ABI4

overexpressor seeds confirm that ABI4 directly down-regulates *AOS* and *NCED6* in order to block *cis*-OPDA and ABA accumulation, respectively, in dark imbibed seeds. This finding could be used to help understand the communication between *cis*-OPDA and ABA noted by Dave and others (2016). The research has also suggested that ABI4 acts as a second player in the control of oil mobilization, although it must be noted that these results are preliminary. It would be interesting to assess the effects of other ABA-responsive factors, such as ABI3, in the control of oil mobilization necessary for seedling establishment.

Last but not least, this thesis work has developed an understanding of the alleviation of seed dormancy by after-ripening that involve changes in ABA, *cis*-OPDA and GA_4 levels in dry seeds. The data from the thesis work highlight the importance of these changes in order to promote germination, especially regarding *A. thaliana* Cvi ecotype seeds of which require a prolonged after-ripening period the end of which is marked by an increase in GA_4 levels in dry seeds. Based on the literature, after-ripening remains one of the least understood aspects of seed germination and the current study provides some new insights into this important process.

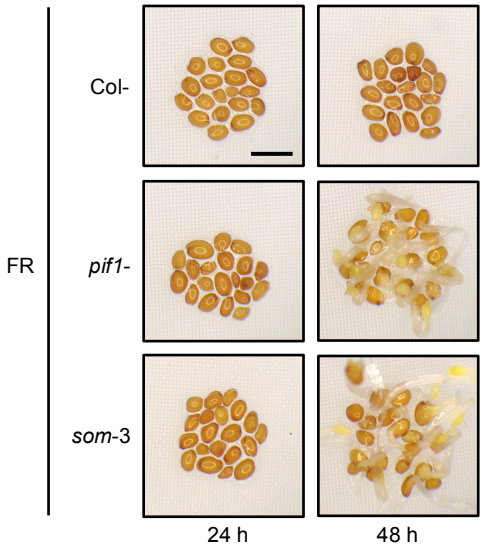
Appendix A - supplementary results for chapter 3

A.1 Germination of Col-0 seeds under light treatments



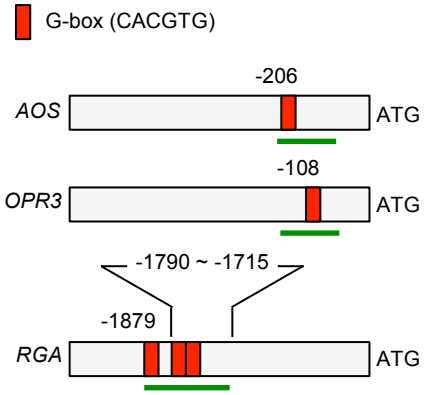
Pictures show WT (Col-0) FR/R- and FR-treated seeds at 12, 24 and 48 h after imbibition (as described in Figure 3.2). Scale bar is 1 mm.

A.2 Germination of Col-0, *pif1*-1 and *som*-3 seeds under FR light treatment.



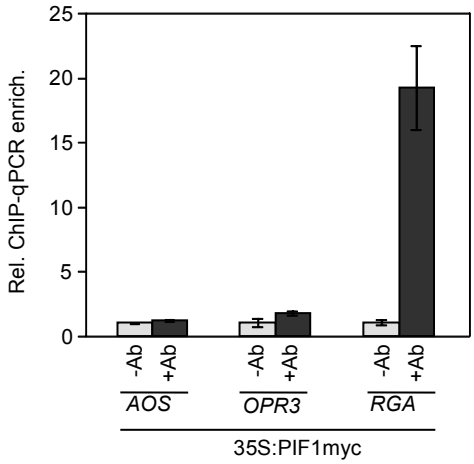
Pictures show Col-0, *pif1*-1 and *som*-3 FR-treated seeds at 24 and 48 h after imbibition (as described in Figure 3.2). Scale bar is 1 mm.

A.3 Diagram of *AOS*, *OPR3* and *RGA* promoters.



Red boxes represent a G-box element (CACGTG). Horizontal green lines indicate amplicons used to assess protein-promoter interactions by ChIP-qPCR.

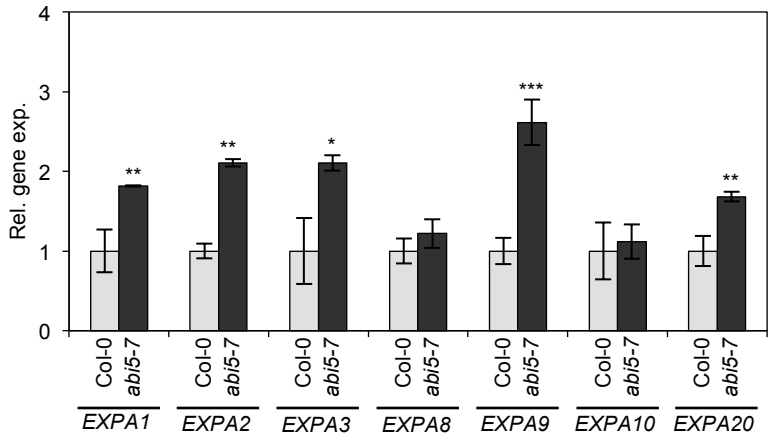
A.4 PIF1 regulation of *AOS* and *OPR3* is indirect.



Relative ChIP-qPCR enrichment of the indicated promoter regions (Appendix A; A3), normalized to an internal control (*UBQ11*) and relative to the negative control (-Ab). Assays were performed on FR-treated (24 h) seeds of a 35S:PIF1myc epitope-tagged transgenic line (Oh et al., 2007). The *RGA* amplicon was used as ChIP positive control (Oh et al., 2007). Data are mean ± SEM of three biological replicates.

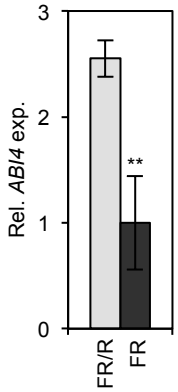
Appendix B - supplementary results for chapter 4

B.1 Expansin genes are up-regulated in ABA-treated *abi5*-7 nicked seeds.



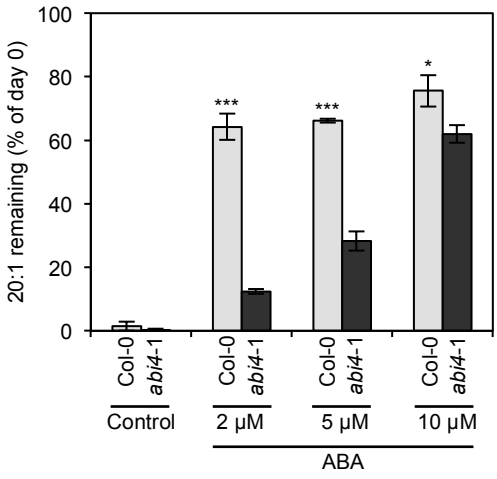
Transcript levels of *EXPA1*, *EXPA2*, *EXPA3*, *EXPA8*, *EXPA9*, *EXPA10* and *EXPA20* genes, normalized to *UBQ11* expression and expressed relative to Col-0, in 24 h ABA-treated Col-0 and *abi5*-7 nicked seeds. Data are mean ± SD of three biological replicates. Asterisks indicate statistically significant difference according to two-tailed Student's t-test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

B.2 *ABI4* expression is repressed by FR and induced by FR/R treatments in Col-0 seeds.



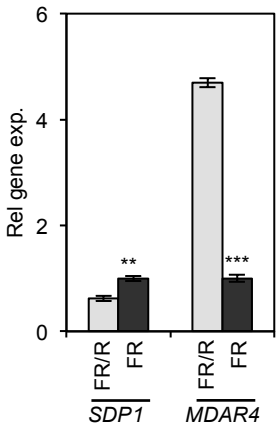
Transcript levels of the *ABI4* gene, normalized to *UBQ11* expression and expressed relative to FR/R, in 24 h FR/R- and FR-treated Col-0 seeds. Data are mean ± SD of three biological replicates. Asterisks indicate statistically significant difference according to two-tailed Student's t-test (** $P < 0.01$).

B.3 Oil mobilization in *abi4*-1 seeds becomes less efficient under high ABA levels.



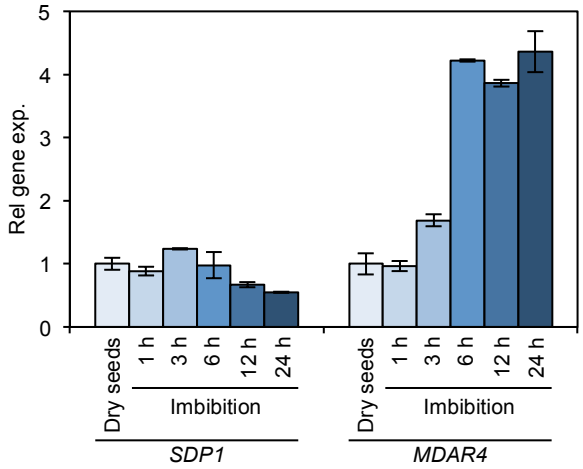
Eicosenoic Acid (20:1) levels in Col-0 and *abi4*-1 whole seeds 5 days after imbibition in the presence of 0, 2, 5 and 10 µM ABA. Data are mean ± SD of six biological replicates.

B.4 *SDP1* and *MDAR4* transcript levels are increased and reduced, respectively, by FR- compared to FR/R-treated Col-0 seeds.



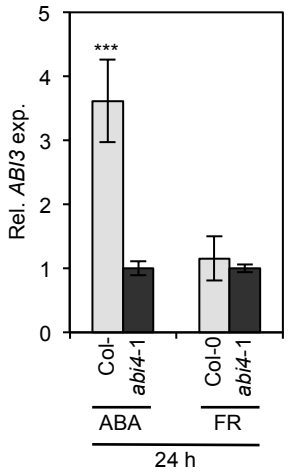
Transcript levels of *SDP1* and *MDAR4* genes, which were extracted from a transcriptomic data set comparing 24 h FR/R- and FR-treated Col-0 seeds and expressed relative to FR/R. Data are mean ± SD of three biological replicates. Asterisks indicate statistically significant difference according to two-tailed Student's t-test (*** $P < 0.001$).

B.5 *SDP1* and *MDAR4* transcript levels are reduced and increased, respectively, upon imbibition of Col-0 non-dormant seeds.



Transcript levels of *SDP1* and *MDAR4* genes, which were extracted from a public avaiable transcriptomic data set (Winter et al., 2007; Bassel et al., 2008) and expressed relative to dry seeds, in dry and imbibed (for 1, 3, 6, 12, and 24 hours after imbibition) Col-0 non-dormant seeds. Data are mean ± SD of three biological replicates. Asterisks indicate statistically significant difference according to two-tailed Student's t-test (*** $P < 0.001$).

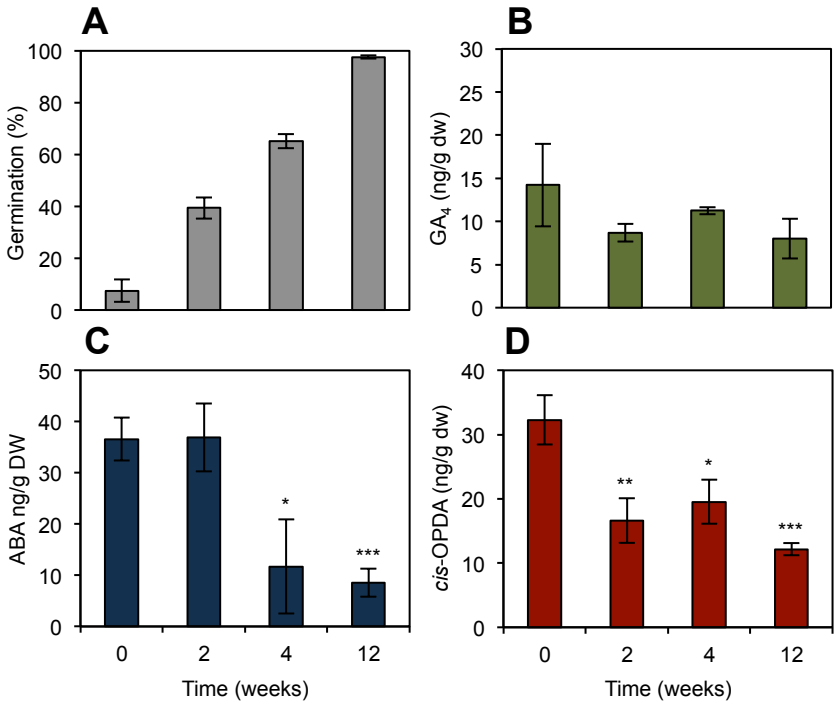
B.6 *ABI3* expression partially depends on ABI4 under ABA treatment but not under FR conditions.



Transcript levels of the *ABI3* gene, normalized to *UBQ11* expression and expressed relative to Col-0, in 24 hours imbibed Col-0 and *abi4*-1seeds under either 10 µM ABA or FR conditions. Data are mean ± SD of three biological replicates. Asterisks indicate statistically significant difference according to two-tailed Student's t-test (*** $P < 0.001$).

Appendix C - supplementary results for chapter 5

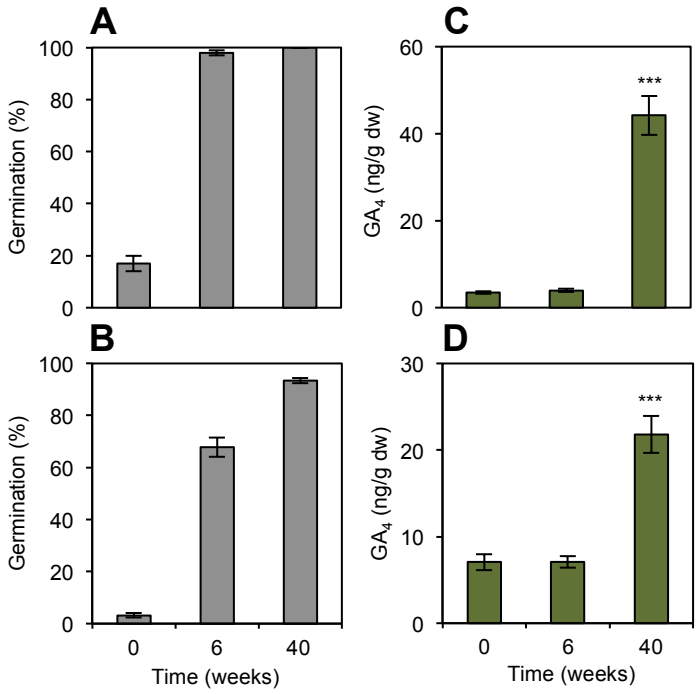
C.1 Decrase in ABA and *cis*-OPDA levels in dry seeds correlate with dormancy alleviation of Col-0.



(A) Germination of freshly matured (time 0), and 2-, 4- and 12-weeks after-ripened Col-0 seeds. Levels of GA_4 (B), ABA (C) and *cis*-OPDA (D) in 0, 2-, 4- and 12-weeks AR Col-0 dry seeds. Data are mean ± SD of three biological replicates. Asterisks indicate statistically significant difference according to two-tailed Student's t-test (*** $P < 0.001$) compared with time 0.

Appendix C

C.2 GA_4 levels increase in 40-weeks after-ripened Col-0 and Ler dry seeds.



Germination of freshly matured (time 0), and 6- and 40-weeks after-ripened Col-0 (A) and Ler (B) seeds. GA4 levels in 0, 6- and 40-weeks AR Col-0 (C) and Ler (D) dry seeds. Data are mean ± SD of three biological replicates. Asterisks indicate statistically significant difference according to two-tailed Student's t-test (*** $P < 0.001$) compared with time 0.

List of Abbreviations

AAO3	Abscisic aldehyde oxidase 3 (AAO3)
ABA	Abscisic acid
ABI (1/2/3/4/5)	ABA insensitive (1/2/3/4/5)
ABA1 (2)	ABA deficient 1 (2)
AOS	Allene oxide synthase
ChIP	Chromatin Immunoprecipitation
cis-OPDA	*cis*-12-oxo-phytodienoic acid
Col-0	Columbia
CTS	Comatose
Cvi	Cape Verde Islands
CSC	Cysteine synthase complex
CYP20-3	Cyclophilin 20-3
CYP707A1 (A2/A3)	Cytochrome P450 monooxygenase 1 (A2/A3)
CYP81D11	Cytochrome P450
DAD1	Defective in anther dehiscense 1
DNA / cDNA	Deoxyribonucleic acid / complementary DNA
DGL	Dongle
DOG1	Delay of germination 1
DW	Dry weight
EXPA (1/2/3/8/9/10/20)	Expansin (1/2/3/8/9/10/20)
FA	Fatty acid
FR	Far-red
FW	Fresh weight
GA / GA1 (A4/A9/A20)	Gibberellic acid / Gibberellins A1 (A4/A9/A20)
GAI	GA insensitive
GA3ox1 (2) / GA20ox	GA 3-oxidase 1 (2) / GA 20-oxidase
GID1	GA insensitive dwarf 1
GSH	Glutathione
GST25	Glutathione s-transferase 25
HSP17.6	Heat shock protein 17.6
IPP	Isopentenyl diphosphate
JA / JA-Ile	Jasmonic acid / Jasmonoyl-l-isoleucine
JAR1	Jasmonic acid-amido synthetase
JAZ	Jasmonate ZIM domain
LED	Light-emitting diode
Ler	Landsberg
MDAR4	Monodehydroascorbate reductase 4
MeJA	Methyl JA
MFT	Mother-of-FT-and-TFL1
NCED (6/9)	Nine-*cis*-epoxycarotenoid dioxygenase (6/9)
OPR3	12-oxophytodienoate reductase 3
PCR	Polymerase Chain Reaction
PhyA (B)	Phytochrome A (B)
PIF1	Phytochrome interacting factor 1
PUFA	Polyunsaturated fatty acid
PYR1	Pyrabactin resistance 1
PYL	PYR-like

List of Abbreviations

PXA1	Peroxisomal ABC transporter 1
QTL	Quantitative Trait Locus
R	Red
RCAR	Regulatory component of ABA receptor
RGA	Repressor of GA1
RGL1 (2/3)	RGA-like1 (2/3)
RNA / RNAi	Ribonucleic acid / RNA interference
RT-qPCR	Reverse Transcription quantitative PCR
SDP1	Sugar-dependent 1
SnRK2	Sucrose non-fermenting related kinase 2
SOM	Somnus
UBQ11	Ubiquitin 11
UPLC	Ultra Performance Liquid Chromatography

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