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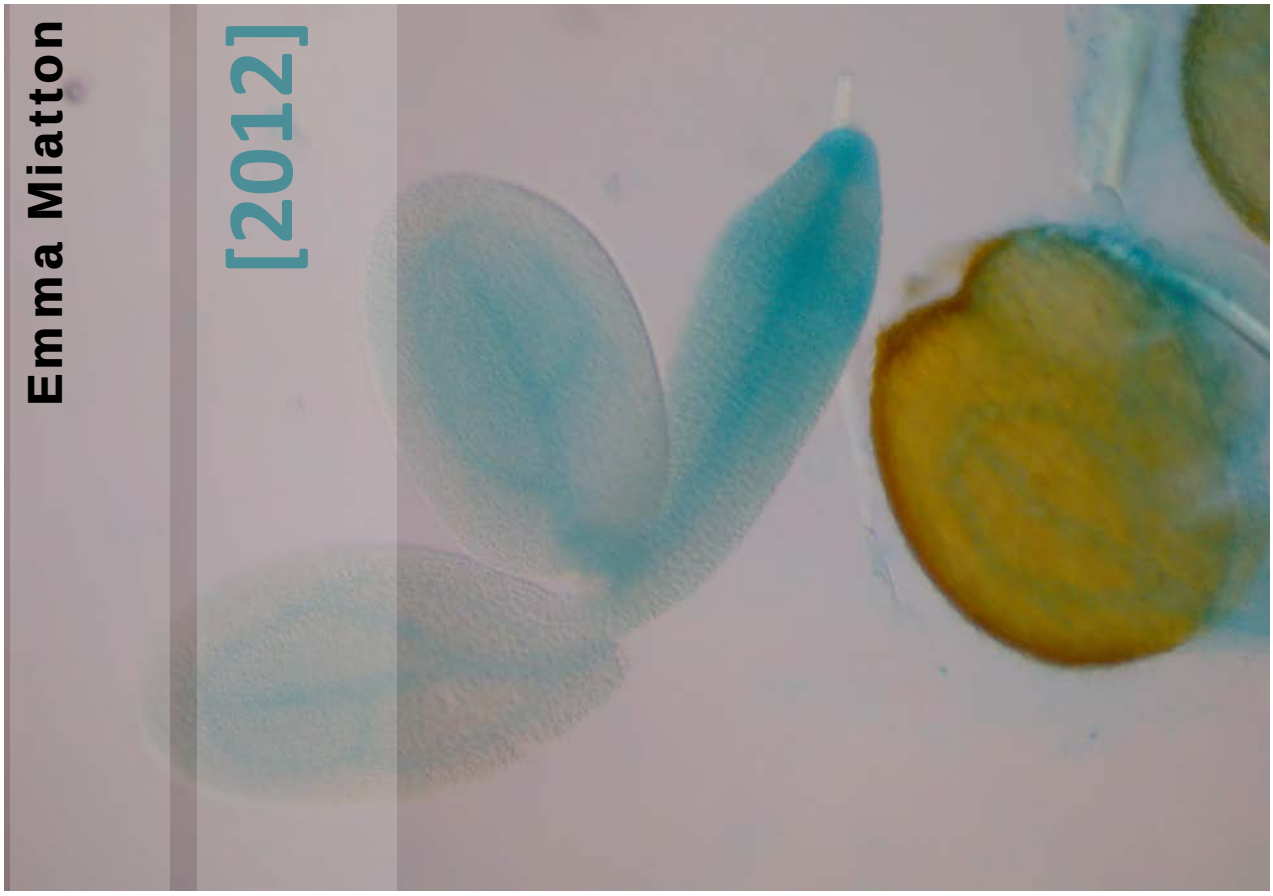

Characterization of PDF1 and its interaction with DELAY OF GERMINATION1 (DOG1) in the control of seed dormancy in *Arabidopsis thaliana*

Characterization of PDF1 and its interaction with DELAY OF GERMINATION1 (DOG1) in the control of seed dormancy in *Arabidopsis thaliana*

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Abstract

Seed dormancy is defined as the incapacity of a viable seed to germinate under favourable conditions. It is established during seed maturation and reaches high levels in mature dry seeds. Dormancy is a complex adaptive trait that assures germination at proper time of the year at the onset of the favourable growing season. This trait is regulated by hormonal and environmental cues such as temperature and light. In *Arabidopsis thaliana* dormancy can be released by imbibing seeds at cold temperatures (stratification) or by storing seeds in dry conditions (after- ripening). The molecular mechanisms that regulate the induction and the release of dormancy are still poorly understood.

Previous studies identified *DELAY OF GERMINATION1* (*DOG1*) as a key regulator of seed dormancy in Arabidopsis. The *dog1* mutant completely lacks seed dormancy and has no pleiotropic effects. *DOG1* shows a seed-specific expression pattern and the abundance of its protein correlates with the dormancy level in freshly harvested seeds. However, this correlation is lacking in after-ripened seeds, suggesting that the protein activity is lost during after ripening (Nakabayashi *et al*., 2012). DOG1 encodes a protein with unknown function and unknown regulation.

The phosphatase PDF1 was identified as an interactor of DOG1 in a yeast two hybrid assay. This thesis describes the relation between PDF1 and DOG1 which was investigated in order to gain further insights into the regulation of DOG1 and into the mechanisms controlling seed dormancy. A T-DNA insertion mutant named *pdf1-1* showed increased dormancy. *PDF1* and *DOG1* were co-expressed during seed maturation, interacted *in vivo* and were shown to function in the same pathway independent from ABA.

Two-dimensional gels analysis showed that DOG1 is targeted by two different post-translational modifications during after ripening and after imbibition. DOG1 shifted towards a lower pH during after-ripening, while imbibition caused a shift towards the basic side.

In the *pdf1-1* mutant DOG1 was detected at a lower pH in comparison to Columbia, indicating possible increased phosphorylation levels and implying a role of PDF1 in the dephosphorylation of DOG1. Moreover, the shift of DOG1 caused by the after-ripening was not observed in the *pdf1-1* mutant, suggesting that the post-translational modifications of DOG1 are interdependent.

Zusammenfassung

Das Ausbleiben der Keimung eines keimfähigen Samens unter vorteilhaften Bedingungen wird als Dormanz definiert. Diese bildet sich während der Reifung des Samens aus und erreicht ihren Höhepunkt in trockenen, ausgereiften Samen. Die Dormanz stellt ein komplexes adaptives Merkmal dar, welches die Keimung zur richtigen Jahreszeit und zu Beginn einer günstigen Wachstumsperiode sicherstellt. Ihre Steuerung erfolgt über Umgebungsreize, wie zum Beispiel Temperatur und Licht, sowie Pflanzenhormone. Bei *Arabidopsis thaliana* kann die Dormanz durch Imbibierung und Kühlung der Samen (Stratifizierung) oder längere Trockenlagerung (Nachreifung) durchbrochen werden. Die Mechanismen, welche die Induktion und das Durchbrechen der Dormanz steuern, sind auf molekularer Ebene immernoch wenig erforscht.

Vorrausgehende Studien haben *DELAY OF GERMINATION1* (*DOG1*) als einen Hauptregulator für Samendormanz in Arabidopsis identifiziert. Bei der *dog1*-Mutante bleibt die Dormanz vollständig und ohne pleiotrope Effekte aus. *DOG1*-Expression ist auf die Samen beschränkt und die Proteinmenge korreliert mit dem Ausmaß der Dormanz in frisch geernteten Samen. Jedoch fehlt diese Korrelation in nachgereiften Samen, was nahe legt, dass die Proteinaktivität während der Nachreife verloren geht (Nakabayashi *et al.,* 2012). DOG1 codiert für ein Protein dessen Steuerung und Funktion bislang unbekannt sind.

Die Phosphatase PDF1 wurde als DOG1-interagierendes Protein in einem Hefe-Zwei-Hybrid Versuch identifiziert. Diese Dissertation beschreibt das Verhältnis zwischen PDF1 und DOG1, welches untersucht wurde um tiefere Einsicht in die Steuerung von DOG1 und in Dormanz-regulierende Mechanismen zu erlangen. *pdf1-1*, eine T-DNA Insertionsmutante, wies erhöhte Dormanz auf. *DOG1* und *PDF1* wurden während der Reifung der Samen co-exprimiert, sie interagieren *in vivo* und es konnte eine Rolle im gleichen, ABA-unabhängigen Regulationsweg nachgewiesen werden.

Bei Untersuchungen auf zweidimensionalen Gelen konnte gezeigt werden, dass DOG1 Ziel von zwei unterschiedlichen posttranslationalen Modifizierungen während der Nachreife und nach der Imbibition ist. DOG1 verschiebt sich in Richtung eines sauren pH´s während der Nachreife, wohingegen Imbibition eine Verschiebung auf die basische Seite zur Folge hat.

In der *pdf1-1*-Mutante wurde DOG1 bei niedrigeren pH-Werten im Vergleich zu Columbia Wildtyp aufgefunden, was zum Einen die Möglichkeit einer stärkeren Phosphorylierung nahelegt und zum Anderen eine Rolle von PDF1 bei der Dephosphorylierung von DOG1 impliziert. Zudem wurde die pH-Verlagerung von DOG1 während der Nachreife in der *dog1-1*-Mutante nicht beobachtet. Das deutet darauf hin, dass die posttranslationalen Modifizierungen von DOG1 voneinander abhängig sind.

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Abbreviations

2D	Two-dimension gel
35S	35S promoter of the Cauliflower Mosaic Virus
aa	Amino acid
ABA	Abscisic Acid
bp	Base pair
Ca	Calcium
cDNA	Complementary Deoxyribonucleic Acid
Col	*Arabidopsis thaliana* Columbia accession
Cvi	*Arabidopsis thaliana* Cape Verde Island
d	days
DAP	Days After Pollination
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	*Escherichia coli*
e.g	Exempli gratia
EDTA	Ethylenediamine Tetraacetic Acid
et al.	Et alii/et aliae
F1, F2, F3	First, second, third generation after a cross
GA	Gibberellins
GUS	ß-Glucorinidase
h	Hours
Hv	Hordeum Vulgare
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
Kas	*Arabidopsis thaliana* Kashmir accession
kD	Kilo Dalton
LB medium	Luria Bertani medium
L*er*	*Arabidopsis thaliana* Landsberg *erecta* accession
-LW	Media without leucine and tryptophan
-LWH	Media without leucine, tryptophan and histidine
M	Molar

min	Minutes
mRNA	Messanger Ribounucleic Acid
MS	Murashige-Skoog
NIL	Near Isogenic Line
PCR	Polymerase Gel Electrophoresis
PD	Physiological Dormancy
PEG	Polyethylene Glycol
PTM	Post-translational modification
PVDF	Polyvinylidene Difluoride
qPCR	Quantitative Real time PCR
QTL	Quantitative Trait Locus/Loci
RH	Relative Humidity
RNA	Ribonucleic acid
ROS	Reactive Oxigen Species
rpm	Rotation per minute
SDS	Sodium Dodecyl Sulfate
Ser/Thr	Serine Threonine
TCA	Tricloroacetic acid
T-DNA	Transferred DNA
Tyr	Tyrosine
U	Units
v/v	Volume/volume
w/v	Weight/volume
wt	Wild type

Gene and names

ABA8'OH	*ABA 8' HYDROXYLASE*
ABI1	*ABA-INSENSITIVE 1*
ABI2	*ABA-INSENSITIVE 2*
ABI3	*ABA-INSENSITIVE 3*
ABI4	*ABA-INSENSITIVE 4*

ABI5	*ABA-INSENSITIVE 5*
AREB	*ABA-RESPONSIVE ELEMENT BINDING PROTEIN*
ATE	*ARGYNIL-tRNA:PROTEIN ARGYNILTRANSFERASE*
BZR1	*BRASSINAZOLE-RESISTANT 1*
FUS3	*FUSCA3*
GA3OX1	*GIBBERELLIN 3-OXIDASE 1*
GA3OX2	*GIBBERELLIN 3-OXIDASE 2*
HUB1	*HISTONE MONO-UBIQUITINATION 1*
LEA	*LATE EMBRYOGENESYS ABUNDANT*
LEC1	*LEAFY COTYLEDON 1*
LEC2	*LEAFY COTYLEDON 2*
NCED	*NINE-CIS-EPOXYCAROTENOID DIOXYGENASE*
CYP707A	*CYTOCHROME P450, FAMILY 707, SUBFAMILY A, POLYPEPTIDE*
PDF1	-
PDF2	-
PIL5	*PHYTOCHROME INTERACTING FACTOR 3-LIKE 5*
PIN	*PIN-FORMED*
PRT6	*PROTEOLYSIS 6*
RCAR	*REGULATORY COMPONENT OF ABA RECEPTOR 1*
RCN1	*ROOT CURL IN NAPHTHYLPHTHALAMIC ACID 1*
RDO2	*REDUCED DORMANCY 2*
RDO4	*REDUCED DORMANCY 4*
SDR4	*SEED DORMANCY 4*
SNRK	*SUCROSE NON FERMENTING1-RELATED PROTEIN KINASE*
SPT	*SPATULA*
TAP46	*2A PHOSPHATASE ASSOCIATED PROTEIN OF 46 KD*

1. INTRODUCTION

1.1 Dormancy: biological and economical relevance

Seeds act as dispersal units of the plant and provide energy for the establishment of a seedling.

Timing of germination determines the start of the life cycle of a plant: winter annuals, which include Arabidopsis and many weeds, germinate in autumn, flower in spring and produce new seeds in summer when the dormancy is released; summer annuals germinate, flower and produce seeds during the warmest period of the year. When the germination timing is artificially manipulated plants are significantly altered in post germination characters such as timing of reproduction and size at reproduction (Donohue, 2002).

If germination occurs under unfavorable environmental conditions, the resulting seedling might encounter mortality (Bewley, 1997). Seed dormancy is defined as the incapacity of a viable seed to germinate under favorable conditions (Finch-Savage & Leubner-Metzger, 2006) and is a fine-tuned mechanism that allows the seed to delay germination until the environmental conditions are optimal for the survival of the seedling and life cycle completion. The transition between dormancy and germination represents an important agricultural trait. Thus, strong dormancy limits the germination of the newly harvested seeds and during domestication crops have been selected to germinate uniform and fast in order to achieve rapid seedling establishment and good crop yield. As a negative consequence, selection for reduced dormancy resulted in undesirable traits as pre-harvest sprouting when grains germinate in humid conditions on the spike before harvest, causing major yield losses (Gubler *et al*., 2005). A better understanding of the molecular mechanisms regulating dormancy and germination is fundamental in order to obtain the desired level of dormancy in crops.

Dormancy can be classified by morphological or physiological properties of the seeds. The most common type of dormancy is physiological dormancy (PD): it is found in gymnosperm and all angiosperm clades. In particular the non-deep physiological dormancy (e.g. in *Arabidopsis thaliana*, later on Arabidopsis) is one of the most abundant form and it can be released by gibberellin application, after-ripening or stratification. When the coat is artificially removed the embryo produce normal seedlings (Finch-Savage & Leubner-Metzger, 2006).

Furthermore, dormancy can be classified in primary and secondary dormancy. Primary dormancy is acquired during seed maturation whereas secondary dormancy occurs in imbibed after-ripened seeds which are exposed to unfavorable environmental conditions. Up to nowadays, research has been focused almost exclusively on primary dormancy (Cadman *et al*., 2006).

Dormancy is a complex trait influenced by different environmental and endogenous factors. The model plant Arabidopsis shows great variability in dormancy level between different accessions. For this reasons QTL analysis is a powerful tool to identify gene underlying dormancy. QTLs for seed dormancy have been identified in Arabidopsis and crops; remarkably

the major dormancy QTL identified in Arabidopsis, *Delay of Germination1* (*DOG1*), has been cloned (Bentsink *et al.*, 2006).

1.1.1 Seed development in *Arabidopsis thaliana*

Arabidopsis thaliana is a small flowering plant belonging to the Brassicaceae family. It is widely considered a model organism in plant science, mostly because of its small size, short generation timing and the great availability of molecular and genetic tools. Moreover, it produces many seeds through self-pollination (reviewed in Koornneef and Meinke, 2010). Under optimal growth conditions (18-25 °C) Arabidopsis fruits, called siliques, contain 40 to 60 seeds.

Seed development in Arabidopsis takes 20 days under normal growth conditions and consists of two major phases: embryogenesis and seed maturation.

Embryogenesis begins with a double fertilization and is characterized by cell division and morphogenesis events which eventually lead to the development of a full size diploid embryo equipped with triploid endosperm (Mayer *et al*., 1991). At the end of the embryogenesis phase the cell division ceases and seed maturation begins. Dormancy is established during seed maturation and reaches high levels in mature seeds (Raz *et al*., 2001). Seed maturation is characterized also by the accumulation of storage compounds, acquisition of desiccation tolerance and decrease in water content (Goldberg *et al.,* 1994). ABA levels increase during this phase and its accumulation is required to impose dormancy, whereas maternal ABA or ABA application during seed development fail to induce dormancy (Karssen *et al*., 1983; Groot & Karssen, 1992; Koornneef & Karssen, 1994).

Four transcription factors play a fundamental role in the regulation of seed maturation. These transcription factors, named *ABA-INSENSITIVE3* (*ABI3*), *FUSCA3* (*FUS3*), *LEAFY COTYLEDON1* (*LEC1*) and *LEAFY COTYLEDON2* (*LEC2*), have partially redundant functions and are connected with ABA signaling (reviewed in Holdsworth *et al*., 2008). Mutants in these genes are severely affected in seed maturation and display decreased dormancy levels and reduced expression of seed dormancy proteins (Raz *et al*., 2001; Gutierrez *et al*., 2007).

1.1.2 Control of seed dormancy in *Arabidopsis thaliana*

Dormancy is regulated by a combination of different environmental and endogenous signals.

A major determinant of the dormancy status is the balance between abscisic acid (ABA) and GA. In general, ABA induces dormancy while GA promotes germination. Additionally, the importance of hormones-independent pathways started to unravel the complexity of this biological phenomenon.

Hormones pathways

ABA is an important positive regulator of dormancy induction and maintenance and it delays endosperm rupture (Leubner-Metzger G., 2003). Overexpression of ABA biosynthesis genes can increase ABA content, leading to enhanced dormancy or delayed germination (Finkelstein *et al*., 2002) while ABA deficiency during seed maturation results in the absence of primary dormancy in mature seed (Kucera *et al*., 2005). Moreover, as a consequence of ABA deficiency some mutants can display precocious germination on the mother plant (vivipary), e.g. *aba1* and *aba2* (Karssen *et al*., 1983; Koornneef & Karssen, 1994). Depending on the locus, also ABA-insensitive mutants (*abi1* to *abi8*) display a variable level of reduced dormancy and function in the ABA signal transduction pathway as phosphatases (e.g. *ABI1* and *ABI2*; see also section 1.2) or transcription factors (*ABI3*, *ABI4* and *ABI5*; reviewed in Kucera *et al*., 2005). Finally, high levels of ABA are present in the imbibed seed of the dormant accession Cvi and decrease when dormancy is released (Ali-Rachedi *et al*., 2004).

GA positively regulates seed germination by counteracting ABA and interacting with different environmental signals. GA deficient biosynthesis (e.g. *ga1*) mutants require GA addition in the media to be able to germinate. Kucera *et al*. (2005) proposed two different functions of GA during germination: first GA increase growth potential of the embryo by promoting cell elongation and secondly it is necessary to weaken the tissues surrounding the radicle in order to reduce the mechanical constraint by the embryo surrounding tissues. Finally, GA requirements for dormancy release and germination depend on the amount of ABA produced in the developing seeds and upon imbibition.

Ethylene also promotes seed germination by counteracting the effect of ABA effect. It has been proposed that it could act by promoting radial cell expansion or by decreasing seed base water potential. However, it appears to act after dormancy is released by GA (reviewed in Kucera *et al*., 2005; Linkies & Leubner-Metzger, 2012).

Hormones independent pathways

Chromatin remodeling has been shown to have an important role in the regulation of seed dormancy. *HISTONE MONO-UBIQUITINATION 1/ REDUCED DORMANCY 4 (HUB1/RDO4)* encodes a C3HC4 finger protein and its mutant shows several pleiotropic phenotype including reduced dormancy and reduced longevity. Liu *et al*. (2007) showed that *HUB1* and its homologue *HUB2* are required for monoubiquitination of *HISTONE MONO-UBIQUITINATION 2 (H2B)* which influences the expression of specific genes.

The gene *REDUCED DORMANCY 2* (*RDO2)* was isolated in the same mutagenesis screen for reduced dormancy (Peeters *et al.*, 2002) and encodes a transcription elongation factor SII (TFIIS). *RDO2* and *HUB1* are predicted to have a role in transcription elongation and to associate with the RNA polymerase II associated factor 1 complex (PAF1C). PAF1C associated factors are upregulated at the end of seed maturation, suggesting that they are required to

facilitate expression during this phase since is characterized by low metabolic activity, including gene transcription. This mechanism might counteract the negative effect of desiccation and chromatin condensation on gene expression (Liu *et al*., 2011).
Recently it has been shown that during seed maturation the size of embryonic cotyledon nuclei decreases in association with increased chromatin condensation. *ABI3* is required for the nuclear size reduction suggesting that this mechanism is part of the acquisition of desiccation tolerance (van Zanten *et al*., 2011).

The role of seed tissues in dormancy

Seed coverings represent a mechanical restraint for the embryo outgrowth. The growth potential of the embryo has to overcome this barrier to initiate the radicle protrusion upon imbibition.
In Arabidopsis the testa and the endosperms have a fundamental role in imposing dormancy. It has been shown that the aleurone is sufficient and necessary for dormancy of imbibed seeds (Bethke *et al*., 2007). A study conducted on several mutants affected in testa shape and pigmentation which displayed reduced dormancy confirmed the importance of the seed coat in the control of dormancy and germination (Debeaujon *et al*., 2000).
ABA catabolism occurs in tissues that surround the root (Okamoto *et al*., 2006) and it delays germination by inhibiting the seed envelopes rupture in Arabidopsis (reviewed in Finch-Savage & Leubner-Metzger, 2006; Lee *et al.,* 2010) and, interestingly, in very distantly related species (see 1.1.2.3).

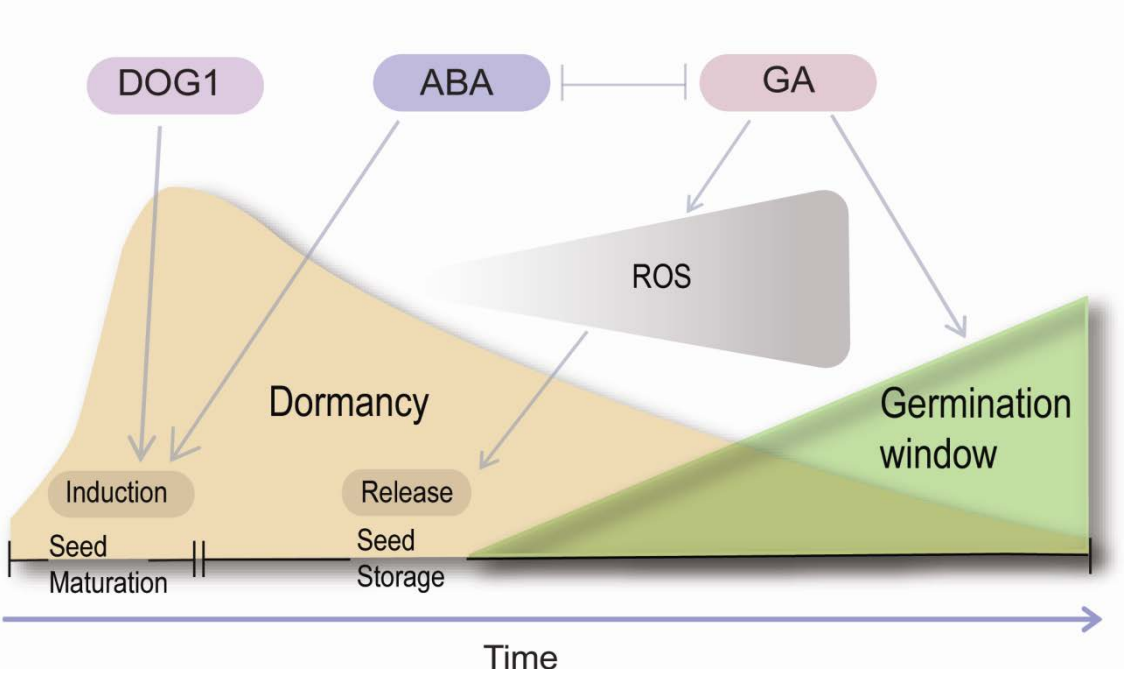


Figure 1. Scheme of the mechanism of dormancy induction and release.
Dormancy level (in beige) increases during seed maturation when dormancy is induced (rounded rectangle in brown). Dormancy is released (rounded rectangle in brown) when seeds are stored in the appropriate conditions, leading to a widening of the germination window (in green). The plant hormone ABA and the dormancy factor DOG1 induce dormancy, while GA releases it and promotes germination. GA and ABA have antagonistic roles. Increasing ROS level during seed storage reduces seed dormancy.
Time is indicated with a blue arrow. (Adapted from Graeber *et al.,* 2012)

1.1.3 Release of seed dormancy

Dormancy can either be quickly released in imbibed seeds (within a couple of days) or relatively slow in dry seeds (within weeks-months).

The fast release of dormancy requires imbibition at species-specific temperatures and is called stratification. It is largely unclear how stratification drives the release of seed dormancy and especially the temperature sensing mechanism is unknown, but a few genes with a role in this process have been identified. The basic helix-loop-helix transcription factors *SPT* and *PIL5* have a role in cold stratification (Penfield *et al.* 2005). *SPT* is a negative regulator of germination that loses its repressive activity after stratification, whereas *PIL5* is not responding to low temperatures, but represses germination in the dark after a cold treatment. Both transcription factors act by inhibiting the GA biosynthesis genes *GA3 OXIDASE 1* (*GA3OX1*) and *GA3OX2* expression, thereby preventing germination (Penfield *et al.* 2005).

Dormancy can be artificially released by removing constraints (i.e. embryo surrounding tissues) that prevent germination (scarification) or by storing seeds at room temperature under dry conditions (after-ripening). Increased time of after-ripening is associated with a widening of the conditions required for germination, resembling gradual dormancy loss (Finch-Savage & Leubner-Metzger 2006). The time required for a complete release of dormancy shows a high inter- and intra-species variation. For example, in Arabidopsis the accessions Landsberg *erecta* (L*er*) and Cape Verde Islands (Cvi) have very different after-ripening requirements. L*er* needs 12 to 17 days of dry storage to achieve 50% germination while Cvi needs 74 to 185 days (Alonso-Blanco *et al.* 2003). After-ripening is effective at low moisture contents (MC) of about 5-15%, but is prevented in very dry seeds (Probert, 2000). It is not well understood whether the changes that occur within the seed during after-ripening are predominantly happening at the transcript or protein level, but recent findings have started to shed some light on this issue.

Several transcriptome analyses showed that after-ripening affects transcript levels in dry seeds, resulting in the selective change of specific transcripts (Leymarie *et al.* 2007; Bove *et al.* 2005; Finch-Savage *et al.* 2007). An increase in transcript abundance during dry storage of seeds seems unlikely but could be explained by the occurrence of “humid pockets” whose existence has been proposed in dry seeds of tobacco. Such local areas with higher moisture levels within the seeds could allow transcriptional activities. Transient transcription and translation changes in dry tobacco seeds were shown for ß-1,3-glucanase (Leubner-Metzger, 2005). However, the presence of active transcription in dry seed has to be proved yet.

It is also possible that the quantity and quality of stored mRNAs is changed within the dry seed by mechanisms that do not require an active metabolism. A recent study showed that the selective oxidation of a subset of stored mRNAs is associated with dormancy release in sunflower seeds. Oxidation of mRNA can prevent their translation and lead to changes in the proteome after translation has been restarted during seed imbibition. Interestingly, there

seems to be a selective oxidation of mRNAs corresponding to genes involved in stress response (Bazin *et al.* 2011).

Oxidative processes within the dry seed also influence proteins. Proteomic approaches have been used as a tool to study the dynamics of posttranslational modifications (PTMs) during after-ripening. PTMs have a major role in the regulation of seed development and maturation (Arc *et al*, 2011). Carbonylation is an irreversible PTM that occurs in response to oxidative stress and leads to a change in the enzymatic and binding properties of the protein or to its degradation due to a higher sensitivity to proteolytic attacks. After-ripening results in an accumulation of reactive oxygen species (ROS) which is associated with the carbonylation of specific proteins in sunflower (Oracz *et al.* 2007) and in Arabidopsis (Job *et al.* 2005). It was suggested that the specific carbonylation of seed storage protein helps their mobilization during germination by promoting their proteolytic attack (Job *et al.* 2005). In mammals carbonylation is mainly associated with aging and diseases (Stadtman 1992; Agarwal & Sohal 1994) whereas Arabidopsis seeds still germinate and produce healthy plantlets when accumulating carbonylated proteins.

The importance of the ROS-dependent pathway in after-ripening was highlighted by the finding that the signal transduction of hydrogen cyanide (HCN), a compound used to break dormancy artificially, is ROS-dependent and results in an enhanced expression of genes involved in ethylene signalling (Oracz *et al.* 2009). Moreover, Müller *et al.* (2009) showed that the ROS-producing NADPH oxidase *AtrbohB* plays a role in Arabidopsis seed after-ripening. Interestingly, it has been shown that DELLA repressor proteins, which are negative regulators of GA signalling that are degraded by GA, repress ROS accumulation, leading to an enhanced tolerance to abiotic and biotic stress (Achard *et al.* 2008). Although this mechanism has not been demonstrated in seeds, it opens the possibility that GA can increase after-ripening by indirectly increasing ROS.

The ubiquitous signaling molecule nitric oxide (NO) releases seed dormancy in many species and has been proposed to be an endogenous dormancy regulator (Bethke *et al*. 2004) that decreases ABA sensitivity in the endosperm of imbibed seeds (Bethke *et al*. 2005). Holman *et al*. (2009) proposed that NO achieves this through the N-end rule pathway and two components of this pathway, *PROTEOLYSIS 6* (*PRT6*) and *ARGYNIL-tRNA:PROTEIN ARGYNILTRANSFERASE* (*ATE*), have been shown to regulate after-ripening and to reduce ABA sensitivity, implicating a role of targeted proteolysis in dormancy release.

1.1.4 Conservation of seed dormancy mechanisms

The ABA pathway is very conserved among species and genes involved in its biosynthesis *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE* (*NCEDs*) and degradation *CYTOCHROME P450, FAMILY 707, SUBFAMILY A, POLYPEPTIDE* (*CYP707As*) were found in all the plant studied so far (Nambara *et al.*, 2010). In barley the genes *HvNCED1* and *HvNCED2* are involved respectively in the control of primary and secondary dormancy (Leymarie *et al*., 2008) and in the grass model *Brachypodium distachyon BdNCED1* expression was higher in dormant compared to after-ripened imbibed grains (Barrero *et al*., 2012). Millar *et al*. (2006) showed that *HvABA8'OH-1*, the barley *CYP707A*, was differentially expressed in embryo of dormant and non-dormant seed and the expression was confined in the coleorhiza.

The mechanisms controlling seed dormancy are best understood in the model plant Arabidopsis (see 1.1.2.1). However, since PD is present among species over the entire phylogenetic tree, the knowledge about molecular mechanisms underlying dormancy in Arabidopsis has been used to understand how these mechanisms are conserved between species.

The *DOG1* gene was identified as the major dormancy QTL in Arabidopsis (see 1.1.3). Orthologous of *AtDOG1* were found in the Brassicaceae *Lepidium Sativum* and *Brassica rapa* and named respectively *LesaDOG1* and *BrDOG1.* Despite the shallow dormancy of these species, *DOG1* transcript is present in their seeds. Moreover, there is evidence that *LesaDOG1* is regulated by imbibition and ABA-inducible (Graeber *et al*., 2010). In rice *Seed dormancy 4* (*Sdr4*) was identified as a dormancy QTL and it was shown to be a key player in the regulatory network of seed maturation. *Sdr4* is a preharvest-sprouting-resistance gene which encode a protein with unknown function and its expression is dependent on *OsDOG1L-1*, a rice homologue of Arabidopsis *DOG1* (Sugimoto *et al.*, 2010). Ashikawa *et al*. (2010) showed that ectopic expression of wheat and barley *DOG1*-like genes induces dormancy in Arabidopsis, indicating that monocot *DOG1* is also functionally conserved.

In Arabidopsis, the seed structures have a fundamental role in imposing dormancy (see 1.1.2.1). The importance of seed tissues and how ABA affects them was highlighted in different species. In coffee seeds ABA regulates germination by inhibiting embryo growth potential and cap weakening (da Silva *et al*., 2004) and in *Lepidum sativum* it regulates the mechanical weakening of the endosperm (Graeber *et al*., 2010). ABA inhibits the endosperm weakening also in the wild tomato relative *Solanum lycocarpum* (Pinto *et al*., 2007). Barrero *et al*. (2009) suggested that the coleorhiza in barley might have the same function as the endosperm of Arabidopsis in regulating dormancy and germination, as it shows changes in ABA metabolism.

Substantially, the dormancy mechanisms are highly conserved between different species, however it is not clear yet if dormancy specific genes are as conserved as the hormonal pathways or the seed tissues.

1.1.5 The seed dormancy gene *DOG1*

DOG1 has been identified as a major QTL for seed dormancy in a natural variation study using the low dormant accession L*er* and the high dormant accession Cvi. *DOG1* and the underlying gene was isolated using a positional cloning strategy combined with mutant analysis. The mutant *dog1* is completely non dormant and does not show any obvious pleiotropic phenotype, apart from a reduced seed longevity (Bentsink *et al.,* 2006). The *DOG1* QTL influences the natural variation that exists in seed dormancy both in laboratory and field experiments (Bentsink *et al*., 2010: Huang *et al*., 2010), giving further evidences that DOG1 is key regulator and a true marker of seed dormancy.

DOG1 is encoded by the gene At5g45830 and is alternatively spliced into five different isoforms protein isoforms (Bentsink *et al*., 2006; Kazumi Nakabayashi, unpublished). It belongs to a small conserved family with unknown function; DOG1 homologues were found in *Lepidium sativum*, *Brassica rapa* and monocots (Sugimoto *et al*., 2010; Graeber *et al*, 2010; Ashikawa *et al*., 2010; see 1.1.2.3).

DOG1 encodes a protein of unknown function and unknown regulation. Recently, Nakabayashi *et al*., (2012) provided new insights into the regulation of DOG1.

The hypothesis that DOG1 has a crucial role in seed dormancy is supported also by its seed specific expression. The overall quantification of the *DOG1* transcripts showed that there is a peak in expression at 16 DAP with a subsequent reduction towards the end of seed maturation (Figure 2A). The transcripts quickly disappear upon imbibition.

DOG1 protein level gradually increases during seed maturation following the trend of the transcripts. However, in contrast to the transcript level, it did not exhibit a reduction at the end of seed maturation or upon imbibition of dormant and after ripened seeds (Figure 2A, 2B, 2C). DOG1 protein is still detectable in the strong dormant line NIL DOG1 seeds after 13 weeks of dry storage, when dormancy has been fully released (Figure 2B). Taken together, these data indicate that DOG1 accumulation correlates with dormancy in fresh but not in after ripened seeds. Further evidences to confirm this hypothesis came from the immunoblot analysis performed with the fresh seeds of the strong dormant NIL DOG1 and the less dormant L*er*, where it is shown that DOG1 is more abundant in the strong dormant line (Figure 2D). It is proposed that DOG1 protein becomes modified and loses its activity during after ripening. Moreover, in imbibed seeds of the loss-of-function *dog1-1* mutant, the induction of *DOG1* is not able to induce dormancy.

An *in situ* hybridization experiment showed that *DOG1* signal was present in the vascular tissue of the cotyledon, hypocotyl and radicle and, consistent with this, GUS activity was also observed in the vascular tissue of developing seeds (16-18 days after pollination). YFP transgenic lines showed that DOG1 is localized mainly in the nucleus (Figure 3).

DOG1 influences ABA and GA levels but does not regulate dormancy primarily via changes in hormone pathway. However, both ABA and DOG1 are required to induce seed dormancy, as shown from the evidence that the strong DOG1-Cvi allele does not induce dormancy in the

aba1 background and the high *DOG1* expression found in *aba1* mutant. Moreover, high ABA accumulation does not compensate for the absence of DOG1 function.

Finally, previous works performed in Wim Soppe laboratory identified PDF1 as an interacting protein of DOG1 in a yeast two hybrid assay (for detail about the yeast two hybrid see 3.1.1.2). PDF1 is a phosphatase belonging to the family 2A (see 1.2.1).

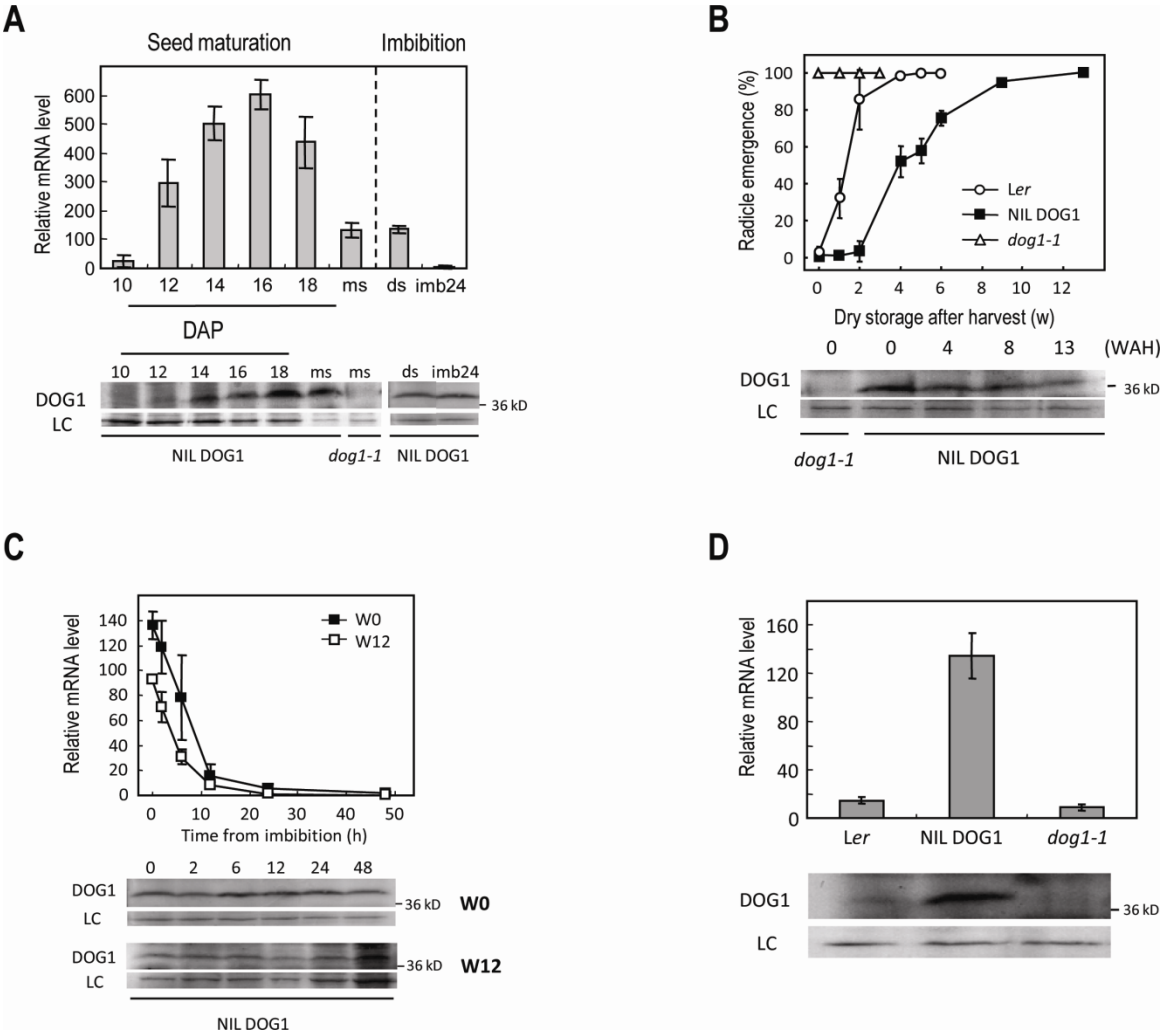


Figure 2. *DOG1* transcript and protein level during seed maturation and imbibitions.
A) Top panel: quantitative RT-PCR analysis of *DOG1* expression of freshly harvested seeds during seed maturation and imbibition. DAP, days after pollination; ms, mature seeds (20 DAP); ds, freshly harvested dry seeds, imb24, 24 hours imbibed seeds. Bottom panel: DOG1 accumulation in NIL DOG1 during seed maturation.
B) Top panel: germination percentage of the seeds used for the immunoblot analysis showed in the bottom panel. Bottom panel: DOG1 accumulation in NIL DOG1 during after ripening.
C) Top panel: quantitative RT-PCR analysis of imbibed after ripened seeds in NIL DOG1.
Bottom panel: DOG1 accumulation in imbibed after ripened seeds in NIL DOG1. W, week; LC, loading control.
D) DOG1 transcript (top panel) and protein level (bottom panel) in mature dry seeds of Ler, *dog1-1* and NIL DOG1. LC, loading control.
(Adapted from Nakabayashi *et al*., 2012)

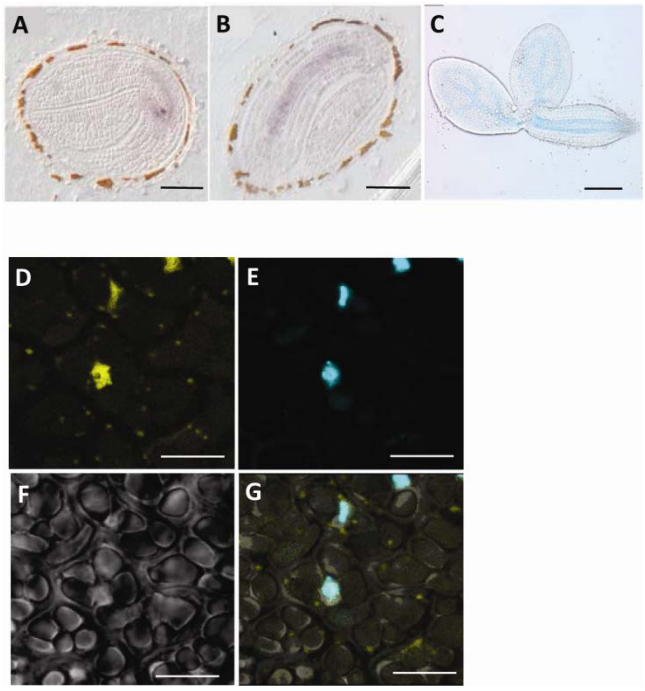


Figure 3. DOG1 localization.
A-B) Longitudinal sections of *in situ* hybridization of 14 DAP seeds. Bar= 100 micrometer.
C) GUS staining of embryo at 16-18 DAP. Bar= 200
D) Subcellular localization of DOG1 in 18 DAP seeds. D) YFP fluorescence, E) DAPI staining, F) transmission channel, G) merged channels. Bar= 10.
(Adapted from Nakabayashi *et al*., 2012)

1.2 Phosphorylation

Posttranslational modifications (PTMs) affect a broad range of biological processes, e.g. protein activity, structure, stability and localization. Among the PTMs, reversible phosphorylation is by far the most well studied in Arabidopsis and is involved in stress responses, hormonal signaling, stomatal closure, cell cycle and cytokineses (de la Fuente *et al*., 2007). Kinases catalyze the addition of a phosphate group while phosphatases remove it; phosphorylation occurs mainly on serine, threonine and tyrosine residues.

1.2.1 Protein phosphatases in *Arabidopsis*: nomenclature and functions

Phosphatases are divided in two major groups: Ser/Thr and Tyr phosphatases. The major types of Ser/Thr phosphatases are Type 1 (PP1) and Type 2 (PP2), depending on their substrate specificity and pharmacological properties.

PP2 group can be further classified in PP2A, 2B and 2C by their dependence on dicovalent cations: PP2B and PP2C needs Ca^{2+} and Mg^{2+} for activity while PP2A does not require any cations. Structural analysis showed that PP1, PP2A and PP2B are closely related and are defined as the PPP family while PP2C and other Mg^{2+} dependent Ser/Thr phosphatases are described as the PPM family (Luan, 2003).

PP2C is the representative group of the PPM family. Higher plants produce a large and diverse family of PP2C-like enzymes which share low homology to the PP2C in animals, where only few isoforms are present. Remarkably, each plant enzyme contains a unique N-terminal extension (Luan, 2003).

PP2A exist either in a heterodimer form consisting of a 36 kDa catalytic subunit and a 65 kDa scaffolding subunit (subunit A) or in a heterotrimer form with the catalytic subunit, the scaffolding subunit A and the regulatory subunit B. In general, PP2A are involved in a variety of different processes such as hormone-mediated growth regulation, control of cell shape and plant morphology, pathogen response and root cortical cell elongation (Rashotte *et al*., 2001; Kwak *et al*., 2002; Camilleri *et al*., 2002; Larsen & Cancel, 2003).

The A subunit acts as a scaffold for the formation of the heterotrimeric complex while the B subunit control the localization and substrate specificity of the different holoenzymes (Farkas *et al*., 2007; Shi, 2009).

In Arabidopsis, three genes encoding the A scaffolding subunit have been identified: *ROOT CURL IN NAPHTHYLPHTHALAMIC ACID1 (RCN1*, At1g25490), *PDF1* (At3g25800) and *PDF2* (At1g13320). Mutants lacking *RCN1* displays pleiotropic phenotypes including altered auxin transports, reduced elongation of seedling organs, increased ethylene production and sensitivity (Zhou *et al*., 2004). Also, *RCN1* functions as a positive transducer of ABA signalling and its mutant shows reduced sensitivity to ABA during seed germination (Kwak *et al*., 2002). Interestingly, although PDF1 and PDF2 share high amino acid sequence similarity with RCN1,

their loss of function mutants, referred in Zhou *et al*. (2004) as *pp2aa2-1* and *pp2aa3-1* respectively, exhibit normal phenotypes; even the double mutant does not show any evident phenotype. Expression analysis of the three A subunits indicated that the transcripts are present in every tissue with the highest abundance in roots and flowers (Zhou *et al*., 2004).

1.2.2 Role of phosphorylation in seed physiology

Although phosphorylation is very well studied, little is known about the phosphoproteome of dry and germinating seeds.

The predominant phosphorylated proteins in dry mature seeds of Arabidopsis are the 12S cruciferins which are phosphorylated during seed maturation; Wan *et al*., (2007) suggested that this may promote their disassembly and mobilization during germination. However, *de novo* specific protein phosphorylation occurs mainly during seed germination (reviewed in Arc *et al.*, 2011), because protein activities in the dry seed are reduced due to its quiescent state. For instance, Lu *et al*. (2008) conducted a phosphoproteomic analysis in germinating maize seeds and identified several phosphatases and kinases that showed upregulation during germination.

Several studies showed that the activity of many kinases and phosphatases is associated with ABA response. Members of the sucrose nonfermenting1-related protein kinases (SnRKs) are key regulators of ABA signaling in seeds and they can be divided into three subfamilies: SnRK1, SnRK2 and SnRK3.

In particular, SnRK2 is involved in the positive regulation of ABA by promoting the phosphorylation of the transcription factors family ABFs/AREB, including ABI5 whose phosphorylation affects dormancy through the regulation of dormancy related genes (Fujii *et al*., 2007; Nakashima *et al*., 2009). Three Arabidopsis SnRK2s (SnRK2.2, SnRK2.3, and SnRK2.6) are expressed mainly during seed development and germination. The triple mutant is highly ABA insensitive and viviparous when grown at high humidity. Strikingly the severity of the phenotype of the triple mutant is far stronger than the single mutants, indicating that these kinases are involved in different aspects in the control of dormancy (Nakashima *et al*., 2009).

ABA-INSENSITIVE1 (ABI1) and ABA-INSENSITIVE2 (ABI2) belong to the family of the protein phosphatases PP2Cs and they function as negative regulator of the SnRK2-mediated phosphorylation. The mutants *abi1-1* and *abi2-1* are dominant negative and display a reduced dormancy (Koornneef *et al*., 1984). The newly discovered family of ABA receptor PYR/PYL/RCAR inhibits the activity of PP2Cs, including ABI1 and ABI2, in an ABA-dependent manner and triggers the phosphorylation of downstream factors (SnRK2) together with ABA transcriptional responses (Ma *et al*., 2009; Park *et al*., 2009). Moreover, insertion mutants of these receptors are ABA-insensitive, indicating that they play a central role in seed dormancy.

1.3 Objectives of the thesis

Seed dormancy prevents the seed from germinating in an unfavorable season. So far, the molecular mechanisms controlling seed dormancy are not well understood. The purpose of this thesis was to improve our understanding of seed dormancy and the molecular mechanisms underlying it in the model plant *Arabidopsis thaliana*, in order to apply this knowledge to agronomical important species.

Previous studies identified *DOG1* as a key regulator of seed dormancy in *Arabidopsis thaliana*. DOG1 encodes a protein of unknown function, although significant progress in our understanding of its regulation has been made (Nakabayashi *et al*., 2012). DOG1 protein accumulates during seed maturation and remains stable during imbibition and after ripening. The protein level correlates with dormancy in fresh seeds. However, its accumulation does not correlate with dormancy level in after ripened seeds. Also, preliminary experiments performed in Wim Soppe laboratory identified PDF1, a phosphatase 2A, as DOG1 interacting protein.

As DOG1 accumulation does not correlate with dormancy level in after ripened seeds, one objective of this thesis was to understand how DOG1 is regulated during after ripening and test the hypothesis that DOG1 becomes modified and loses its activity in this stage.

Secondly, a phosphatase 2A was identified in a preliminary yeast two hybrid screening, therefore the role of phosphorylation in regulating DOG1 was investigated in order to better understand its protein function.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and antibiotics

The chemicals were purchased from the following companies: Biorad (Hercules, USA), Invitrogen (Karlsruhe, Germany), MBI Fermentas (St. LeonRoth, Germany), Merck (Darmstadt, Germany), Promega (Mannheim, Germany), Roche (Mannheim, Germany), Sigma (Deisenhofen, Germany) and Carl Roth (Karlsruhe, Germany).

The antibiotics were supplied by Duchefa (Haarlem, Netherlands) and used as indicated in the table 2.1.

Table 2.1

Name	Solvent	Final concentration in *E.coli* selection medium (mg/l)	Final concentration in *A. tumefaciens* selection medium (mg/l)
Ampicillin (Amp)	Water	100	-
Gentamycin (Gen)	Water	10	10
Hygromycin	Water	50	50
Kanamycin	Water	50	25
Rifampicin	DMSO	-	50
Spectinomycin	Water	100	-

2.1.2 Buffers and culture media

Buffers and culture media were prepared according to Sambrook and Russel (2001).
In addition, special solutions were prepared as following:

High salt solution for RNA precipitation

Sodium citrate	1.2 M
NaCl	0.8 M

DNA extraction buffer

Tris HCl	0.2 M, pH 7.5
NaCl	0.25 M
EDTA	25 mM
SDS	0.5%

GUS staining buffer

Triton X-100	0.2% (v/v)
$NaPO_4$	50 mM, pH 7.2
$K_4Fe(CN)_6*H_20$	2 mM
$K_3Fe(CN)_6$	2 mM
X-Gluc	2 mM

Infiltration medium

MS salt	1. 35 gr
Sucrose	25 gr
Silwet L-77	100 µl
Water	Up to 500 ml

Blocking solution (pH= 7.5)

Tris-Cl	50 mM
NaCl	150 mM
Tween 20	0.25 % (v/v)
Skim milk	5% (w/v)

IEF rehydration Buffer

Urea	9M
Thiourea	2 M
CHAPS	2%
Ampholyte	1% (v/v)
Bromophenol blue	0.002%
DTT	20 mM

Dense SDS solution

Tris-HCl (pH=8)	100 mM
Sucrose	30% (w/v)
SDS	2% (w/v)
Mercaptoethanol	5% (v/v)

Alkaline Phosphatase (AP) Visualization Solution

Tris	100 mM
NaCl	100 mM
Nitro Blue Tetrazolium (NBT)	0.1 mg/ml
5-Bromo-4-Chloro-3-Indoryl-Phosphate (BCIP)	0.05 mg/ml
$MgCl_2$	4 mM
N,N-Dimethylformamide	1%

Enzyme solution in mannitol solution

Cellulase	1%
Pectinase	0.3%

Mannitol solution

mannitol	0.4 M
KCl	20 mM
Mes pH 5.7	20 mM

PEG/Ca solution

PEG 4000	4 g
mannitol	0.36 g
1M Ca $(NO_3)_2$	1 ml
Water	Up to 10 ml

W5 solution	
NaCl	154 mM
$CaCl_2*2H_2O$	125 mM
KCl	5 mM
Mes (pH=5.7)	2 mM

MMg solution	
Mannitol	0.2 M
MgCl2*6H20	15 mM
Mes	4 mM

2.1.3 Enzymes

All restriction enzymes were ordered from New England Biolabs (Schwalbach/ Taunus, Germany) or Fermentas (Massachussets, USA), Platinum Pfx DNA-Polymerase (Invitrogen, Karlsruhe, Germany), Taq DNA Polymerase (Ampliqon).

2.1.4 Commercial Kits

Bio-Safe Coomassie G-250 stain (BIORAD, Hercules, USA)

BP-Clonase and LR Clonase (Invitrogen, Karlsruhe; Germany)

cDNA synthesis

Miniprep™ kit (Qiagen, Hilden, Germany)

RNAqueous RNA isolation aid (Ambion, Austin, USA)

pENTR™ Directional TOPO Cloning Kit

Protein assay (BIORAd, Hercules, USA)

2.1.5 Primers

The primers used in this thesis are listed in the tables below:

Primers for *DOG1*	Sequence 5´ to 3´
DOG1overall_F	GAGCTGATCTTGCTCACCGATGTAG
DOG1overall_R	CCGCCACCACCTGAAGATTCGTAG
Dog1-2 Mse F	TTCTTTAGGCTCGTTTATGCTTTGTGTGGTT
Dog 1-2 Mse R	CTGACTACCGAACCAAAAAATTGAATTTTAGTC

Primers for *PDF1*	
FW-570	TGAATCAATTTAATTTGTTAGTATACACACATATTGTCGT
FW-1200	GATGAAATGTATTAGTGAAAGTAAATCGTTCTGAAATTGT
FW-2400	CGC TGC CGT TTT AGC AAG TCT CTC TCA GA
Start_FW	ATGTCTATGATCGATGAGCCGTTGTACCCAATCGC
Stop_RW	TTAGCTAGACATCATCACATTGTCAATAGATTGTAGAGCTTG
BF_pdf1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGTCTATGA TCGATGAGCC
BR_1_pdf1	GGGGACCACTTTGTACAAGAAAGCTGGGTGGCTCTACAATCTATTGACAATGTGAT G
BR_2_pdf2	GGGGACCACTTTGTACAAGAAAGCTGGGTGGCTAGACATCATCACATTGTCAATA
3UTR1_r	CAAGACAATGGACAAAACCCGTACCGAGGCACTCTAGTAG
3UTR2_r	TTAGTAGCAAGACAATGGACAAAACCCGTACCGAGGCAC
1633_F	TTGATCAATCGGTTGTGGAGAAGACGATTCGTCCTGGGCTTGTGG
100_rv	CCC CAA GAG CAC GAG CGA TCG TAG
Salk_LP_pdf1	CGATGTTACGTGCCCTCTTAC
Salk_RP_pdf1	TCTACCGAATGACCATTTTGC
1518FW	GGGCTCAGAGATTACATGCTCTAAGCTCTTACC
600FW	GAATCAGCGCATTTGAAGACCGACG

Vectors	
Gal4F_AD	GATGATGAAGATACCCCACCAAACCCA
GAL4_BD	AAGGTCAAAGACAGTTGACTGTATCGC
pDONR_rv	ACGTTTCCCGTTGAATATGGCTCAT
35S-F	AAGACGTTCCAACCACGTCTTC
Yfp_156FW	GACAAGCAGAAGAACGGCATCA
Yfp_20R_rev	GTCCAGCTCGACCAGGATGG
Yfp_201R_rev	GTAGTGGTTGTCGGGCAGCA

Primers for *RCN1*	
Rcn1-start	ATGGCTATGGTAGATGAACCGTTGTATCCC
Rnc1-stop	TCAGGATTGTGCTGCTGTGGAACCAT
500F	GGGGAAATTTGCTACAACTGTCGAGTC
1000F	GGGGATGGCTCCTATCCTTGGGA
1400F	ATGCAGCACTTAGTTCCCCAGGTATTG
Salk_LP_rcn1	TATAGGATTTTTCGATGACAAGCTC
Salk_RP_rcn1	ATCTAGAGGGGTTGGATAAAGTTTG

Primers for *PDF2*	
Salk_LP_pdf2	TATTTCCAAACTTTGGGGGAC
Salk_RP_pdf2	ATGGACACAGCTTGAAGATGG
500F	GGGGAAGTTTGCTGCTACAATTGAATCAG
1000F	GGAATTATCATCAGACTCTTCTCAGCACGTCAG
1400F	AATGCAGCATATAGTTCCTCAGGTTCTAGAGA
Pdf2_start	ATGTCTATGGTTGATGAGCCTTTATACCCGAT
Pdf2_stop	TTAGCTAGACATCATCACATTGTCAATAGATTGGAG
Pdf2_nostop	GCTAGACATCATCACATTGTCAATAGATTGGAGAG

Primers for *ACTIN8*	
RT_actin8_fw	CTCAGGTATTGCAGACCGTATGAG
RT_actin8_rev	CTGGACCTGCTTCATCATACTCTG

2.1.6 Plasmids used in this thesis

Vector	Aim	Provided by
pDONR^{TM} 207	Cloning of PCR fragments with Gateway® technology	Invitrogen, Karlsruhe, Germany
pLeela GW	Overexpression in planta	Joachim Uhrig (MPIPZ)
pGWB3 GUS	Promoter activity analysis in planta with GUS assay	Kazumi Nakabayashi (MPIZ)
pGreen	DOG1 detection with HA antibody	Kazumi Nakabayashi (MPIZ)
pBatTL-B-sYFPn	Split YFP assay in planta	Joachim Uhrig (MPIPZ)
pBatTL-B-sYFPc	Split YFP assay in planta	Joachim Uhrig (MPIPZ)
pENSG-YFP	In vivo localization study	Vittoria Brambilla
pENSG-CFP	In vivo localization study	Vittoria Brambilla
pACT2-attR	Yeast two hybrid	Csaba Koncz (MPIPZ)
pAD2-attR	Yeast two hybrid	Csaba Koncz (MPIPZ)
pENTR	Cloning of PCR fragments with Gateway® technology	Invitrogen

2.1.7 Bacterial and yeast strains

Escherichia coli strain DH5α was used for standard cloning procedures (Hanahan, 1983). *Agrobacterium tumefaciens* strain GV3101 was used for plant transformation; in addition, when plants were transformed with the vectors pGreen and pLeela, strains carrying the helper plasmid pSoup or pMP90RK were used, respectively (Koncz & Shell., 1986; Koncz *et al*., 1990; Hellens & Mullineaux., 2000). For the two hybrid screen the yeast strain pj69-4a was used.

2.1.8 Antibodies

Anti-HA primary antibody: HA Clone 16B12 Monoclonal Antibody (Covance).
Secondary antibody: Alkaline Phosphatase conjugated goat affinity purified antibody to mouse (MP Biomedicals).

2.1.9 Plant material

Arabidopsis accession: Columbia (Col).
Mutants: *dog1-2* (isolated by V. Raz in Wageningen University and identified as a non-dormant mutant carrying two nucleotide changes from C to A at the positions 332 and 334 in the first exon, which causes a premature stop codon), *pdf1-1* (Salk_037095), *pdf2* (Salk_014113), *pdf1-2 pdf2* (Salk_042724 for *pdf1-2*) and *rcn1* (Salk_059903). All the mutants are in Columbia background.

2.1.10 Software and websites

Analysis of sequences, sequencing results, alignments, aminoacidic sequences and in silico cloning: Gene Runner 3.05, Clone Manager Suite 7.
Protein analysis in silico: Phosphat, NetPhos (prediction of phosphroylation sites), Expasy (prediction of isolelectric point).
Bioinformatic tools: www.ncbi.nlm.nih.gov

2.2 Methods

2.2.1 Plant methods

2.2.1.1 Plant growth conditions

Plant were grown in the greenhouse where the temperature was kept in the range 18-25 °C and light was provided for a minimum of 12 hours per day. When controlled growing conditions were required, plants were grown in special chambers (Elbanton BV, Kerkdriel, Netherlands) with 16h light/8h dark cycle (22°C/16°C).

2.2.1.2 Seed germination

For propagation, seeds were incubated for 3-4 days at 4°C in order to break dormancy and then directly sown on soil.
For the dormancy test (see 2.2.1.4), freshly harvested seeds were sown on filter paper soaked with water in 6cm Petri Dishes and incubated in a germination chamber in controlled conditions (16 hours light at 25°C/ 8 hours darkness at 20°) for 2 days.

2.1.3 Seed coat sterilization

Seeds were rinsed few times with a solution containing 40% Sodium Hypochloride and 0.02% Triton X-100. Afterwards the seeds were washed with sterile water and mixed with 0.1% Agar to be sown on MS agar plates (1x Murashige Skoog salts (MS), 1% sucrose, 0.8% Daishin Agar, ph 5.7 and appropriate antibiotics). All the operations were performed in sterile conditions.

2.2.1.4 Seed dormancy measurement

Freshly harvested seeds were stored in small bags in special incubators at 21°C, 50% relative humidity and periodically checked on their ability to germinate. For every time point, 30-40 seeds were sown on filter paper and incubated as described in 2.2.1.2. The total number of germinating and non-germinating seeds was then counted using a stereomicroscope (MZ6 from Leica, Germany) and the ratio plotted to a graph.

2.2.1.5 Crossings of plants

Open flowers and developing siliques were removed from the inflorescence of the mother plant which served as a pollen acceptor. On this inflorescence, the closed and immature buds were cleared from sepals, petals and stamens. The stigma was then pollinated by rubbing the anthers of the father plant using fine and clean forceps.

2.2.1.6 Plant transformation

Plants were transformed by floral dipping (Clough & Bent., 1998). These plants were grown in the greenhouse in short day conditions (8 hours light/ 16 hours dark) until the first bolts were developed and then transferred to long day conditions. In addition, to promote the growth of many secondary bolts, plants were clipped when the first shoots appeared.

Agrobacterium was preinoculated in 1 ml of YEB (5g/l Difco, 1g/L yeast extract, 5g/L peptone, 5g/L sucrose, 2 mM MgSO4) containing the appropriate selective antibiotics and shaked at 28 °C for one day. This was added to a 100 ml culture which was subsequently grown in the same conditions for few days. Cells of this culture were precipitated at 5000 rpm for 30 minutes and resuspended in 150 ml of infiltration media. The inflorescences were dipped in this mixture and wrapped in plastic bags for 2 days, in order to keep the optimal moisture condition to allow the infection. Finally the plants were transferred to the greenhouse.

2.2.1.7 Selection of transformants

Plants carrying the BASTA resistance gene were grown on soil for one week and then sprayed with a 200 mg/l solution of the herbicide Glufosinat.
In order to select plants resistant to antibiotics, the seeds of these plants were sterilized as described in 2.2.1.3 and sown on half strength MS plates containing the appropriate antibiotics.

2.2.2 Microscopy

2.2.2.1 GUS assay

Isolated embryos from plants carrying the constructs p*PDF1_2*:GUS and p*DOG1*_Cvi:GUS were tested for GUS activity as described in Sessions & Yanofski (1999).
Seeds were imbibed for 1 to 2 hours in order to be able to remove the seed coat. The resulting isolated embryos were submerged into vials containing the GUS staining buffer and vacuum was applied for 1 hour to allow the penetration of the buffer. Samples were then incubated at 37 °C overnight and washed several times with ethanol. Embryos were clarified with a few drops of chloral hydrate and analysed using a Axioplan 2 microscope from Zeiss, Germany.

2.2.2.2 *Arabidopsis* leaf protoplasts transient transformation

Leaves from 4-5 weeks old plants were cut in very small parts with a clean scalpel and placed in a Petri dish. Afterwards the leaves were incubated with 15 ml of Enzyme Solution for 2 hours at 100 rpm on a shaker platform. Before proceeding with the transformation, protoplasts were released by swirling the Petri dish by hand for 1 minute and filtrated with a nylon mesh (35-100 µm), collecting the flow-through in a round bottom tube. The mixture obtained after the filtration was centrifuged for 3 minutes at 500 rpm and washed once with 5 ml of W5 solution. A second centrifugation step followed and, after removing the supernatant, the protoplasts were resuspended in 1.2 ml of MMg solution. For every transformation 200 µl of protoplasts were pipetted in a separated round bottom tube where the plasmid DNA (15-30 µg) was added. An incubation of 5 minutes followed. The transfection was stopped by adding 500 µl of W5 solution. Afterwars, the protoplasts were washed 2 times with W5 solution and finally resuspended in 500 µl of W5 containing 1mM of glucose in 8-well plate ready to use for confocal laser scanning microscopy (CLSM 510 from Zeiss, Germany).

2.2.2.3 *Nicothiana benthamiana* leaves transient transformation

The Agrobacterium strain carrying the construct of interest was grown overnight in 5 ml of LB with the appropriate selective antibiotics. The bacterial cells were centrifuged at 5000 rpm for 10

minutes at 4 °C and resuspended in Induction Medium (10mM $MgCl_2$, 10mM MES pH 5.6, 0.15mM Acetosyringon). The culture was then left at room temperature in the dark for 2 hours. Young healthy leaves of *Nicotiana benthamiana* were infiltrated with a needled syringe from the lower side, opposing pressure with the finger on the other side. After three-five days the infiltrated leaves were harvested and analyzed using a confocal microscope.

2.2.3 Molecular biology methods

2.2.3.1 Genomic DNA extraction

Genomic DNA was extracted according to Edwards *et al*.(1991). 400 µl of extraction buffer were placed in a tube together with a piece of leaf. The leaf was disrupted quickly with the help of a small pestle. After 5 minutes centrifugation at 14000 rpm, the supernatant was transferred in a new tube and mixed with 300 µl of isopropanol. The mixture was left 2 minutes at room temperature and centrifuged as described before. The pellet was washed with 70% ethanol and dried. DNA was then resuspended in 70 µl of water. 1µl was used as a template for PCR reactions.

2.2.3.2 Plasmid DNA extraction

Plasmid isolation from *E. coli* was done using the Plasmid Isolation Mini kit (Qiagen) according to the company protocol.

2.2.3.4 Purification of PCR products and gel extraction of PCR fragments

PCR fragments and gel exctracted PCR fragments were purified using the Gel Extraction kit (Qiagen).

2.2.3.5 Total RNA extraction from dry seeds and siliques

In order to obtain RNA from seeds and siliques, a special protocol was required to purify the RNA from the polysaccharides, oils and protein storage present in these tissues. 10 to 20 mg of siliques or seeds were grinded in a mortar with liquid nitrogen and the initial step for the exctraction was performed using the RNAqueous™ total RNA isolation kit (Ambion, USA).
The resulting 100 µl of eluted RNA were quantified using the nanodrop ND-1000 Spectrophotometer and precipitation step followed. Here, 250 µl of isopropanol and 250 µl of high salt precipitation solution were added to the RNA solution in a final volume of 1ml. RNA was then recovered by centrifugation at 14000 rpm for 30 min at 4 °C and washed with 70% ethanol. The pellet was dried

and dissolved with water in order to have a concentration of 200 ng/µl. Afterwards a quarter volume of 10M lithium chloride was added to the RNA and the solution was kept on ice overnight. Pellet was recovered by centrifugation at 14000 rpm for 30 min at 4 °C and washed once with 2M lithium chloride. An additional washing with 70% ethanol followed and the pellet was finally dissolved in 10 µl of water.

2.2.3.6 cDNA synthesis

First strand cDNA synthesis was performed using the QuantiTect Reverse Transcription kit following the manifacturer's protocol and using 1 µg of total RNA.

2.2.3.7 Standard PCR conditions

In general, the PCR conditions were:

5 minutes	95 °C	
30 seconds	95 °C	35-40 cycles
30 seconds	55-65 °C	
30 second to 1 minute	68 or 72 °C	
5 minutes	68 or 72 °C	

All the reactions were performed using a Eppendorf Mastercycler (Hamburg, Germany) and the general conditions as well as the enzyme used were different depending on the purpose of the PCR reaction.
Standard PCRs (colony pcr and genotyping) were performed with a Taq polymerase (Ampliqon, Denmark) and for accurate amplification the *pfx50*™ (Invitrogen) or KOD (Toyobo, Japan) were used.

2.2.3.8 Yeast two hybrid

The yeast strain PJ69-4a was grown overnight in a 1 ml preculture of YPDA (20 g/L Difco peptone, 10 g/L yeast extract, 2% glucose, 0,003% adenine hemisulfate solution) shaken at 30°C . This preculture was used to obtain a bigger culture of 50 ml shaken at the same temperature for 3-5 hours. The yeast cells were then precipitated at 5000 rpm for 10 min and washed few times with sterile water in sterile conditions. Afterwards the cells were resuspended in 1 ml of 0.1M Lithium Acetate and, after a quick spin, dissolved in 0.5 ml of 0.1M Lithium Acetate in oder to obtain the competent cells. This amount would serve for 10 transformations of 50 µl each. Every aliquot was mixed with 240 µl of PEG 3350 (50%), 1M lithium acetate and salmon sperm DNA (2mg/ml) which

was boiled 10 minute before using. Finally the plasmid DNA, consisting of the bait and the prey (500 µg each, diluted to 50 µl final volume) was added to this mixture in order to have 400 µl as a total volume. The transformations were incubated at 30 °C for 30 min and 42 °C for 20 min. The cells were then pelleted and resupended in 200 µl of 1M sorbitol, of which 180 µl were plated on the –LWH plates and 20 µl were instead plated on the –LW plates. Plates were incubated at 30 °C for one week.

2.2.4 Biochemical methods

2.2.4.1 Protein extraction

10 to 15 mg of seeds were placed in a mortar and grinded with liquid nitrogen and 10% sand (w/w). The fine powder obtained was transferred in a tube and vortexed after adding 1 ml of ice-cold acetone; the mixture was then centrifuged at 10000 rpm for 10 min at 4°C and the supernatant discarded. This step was repeated and a chilled solution of 10% TCA/acetone was added to the pellet; a brief sonication followed. The mixture was then washed with chilled 10% TCA/acetone, 10% TCA/water and two times with 80% acetone. The pellet was centrifuged one last time in order to remove the residual acetone and suspended in 0.8 ml of dense SDS solution and 0.8 ml of phenol. This phenol phase was collected after 45 minutes centrifugation at room temperature and transferred in a new tube. Proteins were precipitated overnight at -20 °C by adding 5 times the sample volume 0.1 M ammonium acetate/methanol. The following day the pellet was centrifuged at 4°C and washed two times with 0.1 M ammonium acetate/methanol and two times with 80% acetone. Finally the pellet was resuspended at room temperature in 50 µl of rehydration buffer and the total protein concentration was quantified with Bradford assay.

2.2.4.3 Rehydration of the immobilized pH gradient (IPG) strips and isoelectrofocusing (IEF)

The rehydration step was performed overnight on the bench at RT by applying 135 µl of rehydration buffer to each IPG strips (Biorad, pH 4.7-5.9) and covering them with 1 ml of mineral oil to avoid evaporation of the sample. The following day 40 µg of protein were resuspended in 50 µl of rehydration buffer to achieve the suitable volume to be loaded in the sample cups. The rehydrated strips were placed in a cup loading tray and the IEF was performed according to the following conditions:

	Start Voltage	End Voltage	Set time	Ramp	Temperature
Step1	0 V	250 V	30 min	Rapid	20°C
Step2	250 V	4000 V	180-300 min	Slow	20°C
	Start Voltage	End Voltage	Volt-Hours	Ramp	Temperature
Step3	4000 V	4000 V	24000 V-hr	Rapid	20°C

2.2.4.5 Two-dimensional gels and Western Blot

The separation on the second dimension was obtained by positioning the strip on the top of a small precast polyacrylamide gel (Nupage, Invitrogen) and applying 170 V tension for 75 min. After separation the gel was blotted on PVDF membrane through wet electrotransfer for 2 hours at 30 V. Before proceeding with the immunological reactions, the membranes were washed for 1 hour with Blocking Solution. Primary antibody reaction was performed by incubating the membrane overnight with anti-HA monoclonal antibody while for the secondary antibody reaction the membrane was incubated overnight with a mouse anti-IgG antibody conjugated with alkaline phosphatase.

3. RESULTS

3.1 Molecular characterization of the DOG1 interactor PDF1

3.1.1 Identification of DOG1 interacting proteins

3.1.1.1 Preliminary work

In search of putative interacting proteins of DOG1, a yeast two hybrid library screening was performed. Two cDNA libraries, one from cell culture and one from whole plant, were used in the screening, leading to the identification of several candidate interactors (Table 3.1).

Among the clones showing an interaction with DOG1, many independent ones were found to contain the same cDNA encoding *PDF1* (At3g25800), a Ser/Thr phosphatase 2A.

Therefore, a T-DNA insertion line for *PDF1* was obtained from the Arabidopsis Stock Centre. As shown in Figure 3.1 this line, named *pdf1-1*, displayed enhanced dormancy indicating that the phosphatase could play a role opposite to DOG1 in controlling seed dormancy.

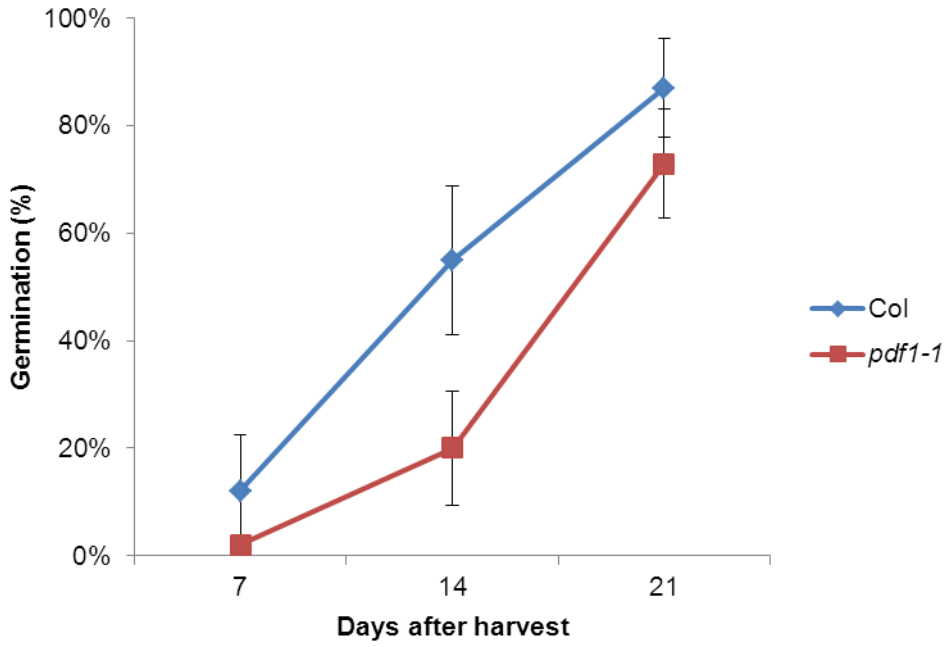


Figure 3.1. Germination assay showing that *pdf1-1* displayed enhanced dormancy in comparison to the wild type. Data points show the average of 10 plants. Standard errors are indicated by the bars.

ID	Symbol	Description
AT1G43170.3	*ARP1*	Similar to 60S ribosomal protein L3 (RPL3B)
AT1G55360.1	none	Expressed protein, unknown function
AT1G56045.1	none	Ribosomal protein L41 family protein, contains Pfam domain PF05162: Ribosomal protein L41
AT3G03960.1	none	Chaperonin, putative
AT3G16640.1	*TCTP*	Translationally controlled tumor family protein
AT3G25800.1	***PDF1***	**Serine/threonine protein phosphatase 2A (PP2A) 65 KDa regulatory subunit A, identi to protein phosphatase 2A 65 kDa regulato subunit (pDF1) GI:683502 from (Arabidops thaliana)**
AT4G03190.1	*GRH1*	F-box family protein (FBL18)
AT4G18400.1	none	Expressed protein
AT5G11790.1	none	Ndr family protein
AT5G38420.1	none	Ribulose bisphosphate carboxylase small chain 2 RuBisCO small subunit 2B (RBCS-2B)

Table 3.1 An initial yeast two-hybrid screening led to the identification of several putative interacting proteins of DOG1. Column ID, ATG number of the putative interactors. Column Symbol, abbreviation of the gene name. In bold is PDF1.

These preliminary data, showing that PDF1 interacts with DOG1 in yeast and influences dormancy, have been used as a starting point for the work described in this chapter.

3.1.1.2 Yeast two hybrid screen

To confirm the interaction between PDF1 and DOG1 a yeast two hybrid co-transformation was performed. The study of interaction between two proteins using yeast two hybrid takes advantage of the properties of the GAL4 protein in *Saccharomyces cerevisiae* (Fields & Song, 1989). GAL4 is a transcription factor required for the expression of genes encoding enzymes for galactose utilization. This transcription factor is split in two domains, the binding domain (BD) and the activating domain (AD) which can be fused to putative interacting partners. When the fusion proteins are interacting, the reporter gene is transcribed and yeast growth occurs on selective medium.

According to these principles, *PDF1* cDNA was fused to the binding domain and co-transformed in yeast with a vector containing *DOG1* fused to the activation domain. Yeast growth was restored on selective

medium (Figure 3.2), indicating that DOG1 and PDF1 were interacting in yeast. The result was confirmed also by exchanging the binding and the activation domain fusions.

As mentioned in 1.2.1, *PDF1* has two homologues in Arabidopsis, *PDF2* and *RCN1*. Their amino acid sequences show a high homology with PDF1, of respectively 94% and 86% (Zhou *et al.*, 2004). Therefore, also PDF2 and RCN1 have been tested for the interaction with DOG1. Figure 3.3 shows that PDF2 but not RCN1 is able to restore yeast growth. As for PDF1, the binding and the activation domain fusions were exchanged in order to confirm the interaction.

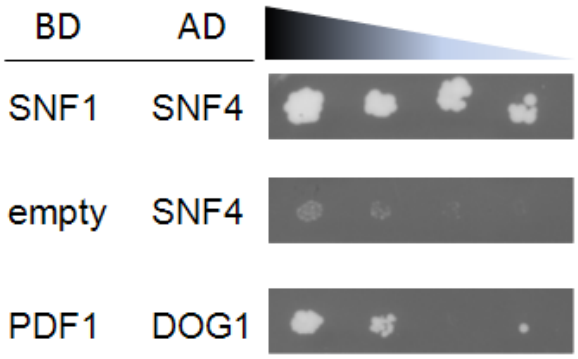


Figure 3.2. Dilution series (indicated by the triangle) of the yeast two hybrid assay showing the interaction of DOG1 with PDF1. The interaction SNF1-SNF4 is used as positive control while the empty vector functions as negative control. BD: binding domain; AD: activating domain. Yeast was grown in presence of 5 mM AT.

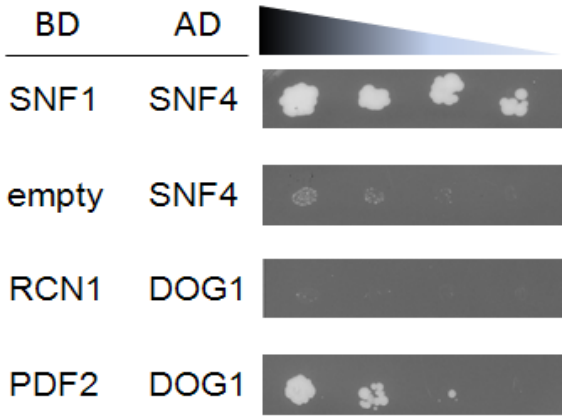


Figure 3.3. Yeast two hybrid assay to test the interaction of DOG1 with the *Arabidopsis* homologues of PDF1, PDF2 and RCN1. Dilution series are indicated by the triangle. Yeast was grown in presence of 5 mM AT.

3.1.2 Phenotypic studies of *pdf1-1, pdf2*, *rcn1*, *pdf1-1 dog1* and *pdf1-2 pdf2*

The 2A phosphatases PDF1 and PDF2 were identified as interacting protein of DOG1. Therefore, a germination assay with *pdf1-1* and several related mutants was performed in order to understand whether *PDF1* and its homologues have a role in the control of dormancy (Figure 3.4).

The mutant *pdf1-1* was tested in this experiment to confirm the enhanced dormancy shown in the preliminary study. In addition, *pdf1-1* was crossed with *dog1-2* to generate the double mutant *pdf1-1dog1* with the purpose of understanding the relationship between the two genes. The loss of function mutant *dog1-2* was chosen because it shares the same Columbia background of *pdf1-1*.

A T-DNA insertion line was obtained for *RCN1* from the Nottingham Arabidopsis Stock Center.

Homozygous mutant lines were selected by PCR using specific primers annealing to the T-DNA border and to the gene. This line carries an insertion in the last intron (Figure 3.5D).

Finally, the double mutant *pdf1-2pdf2* was included in the assay to understand whether PDF1 and PDF2 have redundant function. This mutant was generated in Alison DeLong's lab (Brown University) by crossing the SALK lines *pdf2* and *pdf1-2* (referred in Zhou *et al.* as *pp2a3-1* and *pp2a2-1* respectively) and provided as a line heterozygous for *PDF1* and *PDF2*. Plants carrying the double T-DNA insertion for *PDF1* and *PDF2* were selected by PCR following the same procedure as for *RCN1*. As some of these plants were heterozygous, it was possible to obtain homozygous mutants carrying a single *pdf2* mutation and include them in the germination assay. The mutant *pdf1-2* carries an insertion in the 9th exon and it is different from the one used in the preliminary germination assay (Figure 3.5A and 3.5B).

All the above mentioned mutants were grown together with the wild type in the greenhouse under the conditions described in 2.2.1.1. The adult plants did not show any evident growth phenotype (Figure 3.6), confirming the observations of Zhou *et al*. (2004) for *pdf1-1*, *pdf2*, *rcn1* and *pdf1-2 pdf2*.

The germination assay showed that *pdf1-1* displayed enhanced dormancy in comparison to the wild type Columbia. The mutant *pdf2* didn't display any evident phenotype, suggesting that *PDF2* is not involved in the regulation of dormancy. In support to this, the double mutant *pdf1-2 pdf2* showed the same phenotype as *pdf1-1*, indicating that *PDF1* and *PDF2* are not redundant and the enhanced dormancy displayed by *pdf1-1* is not an allele specific phenomenon, as the double mutant was obtained by crossing *pdf2* with *pdf1-2.*

The mutant *rcn1* showed an intermediate phenotype between *pdf1-1* and *pdf2.* A dormant phenotype for *RCN1* was expected, as it is known to function as a positive transducer of early ABA signaling (Kwak *et al.,* 2002).

The double mutant *pdf1-1 dog1* displayed the same phenotype as *dog1-2*, indicating that the *dog1* mutant is epistatic to *PDF1*. This finding, together with the absence of a dormancy phenotype in *pdf2*, suggests that PDF1 but not PDF2 could play a role in seed dormancy by interacting with DOG1.

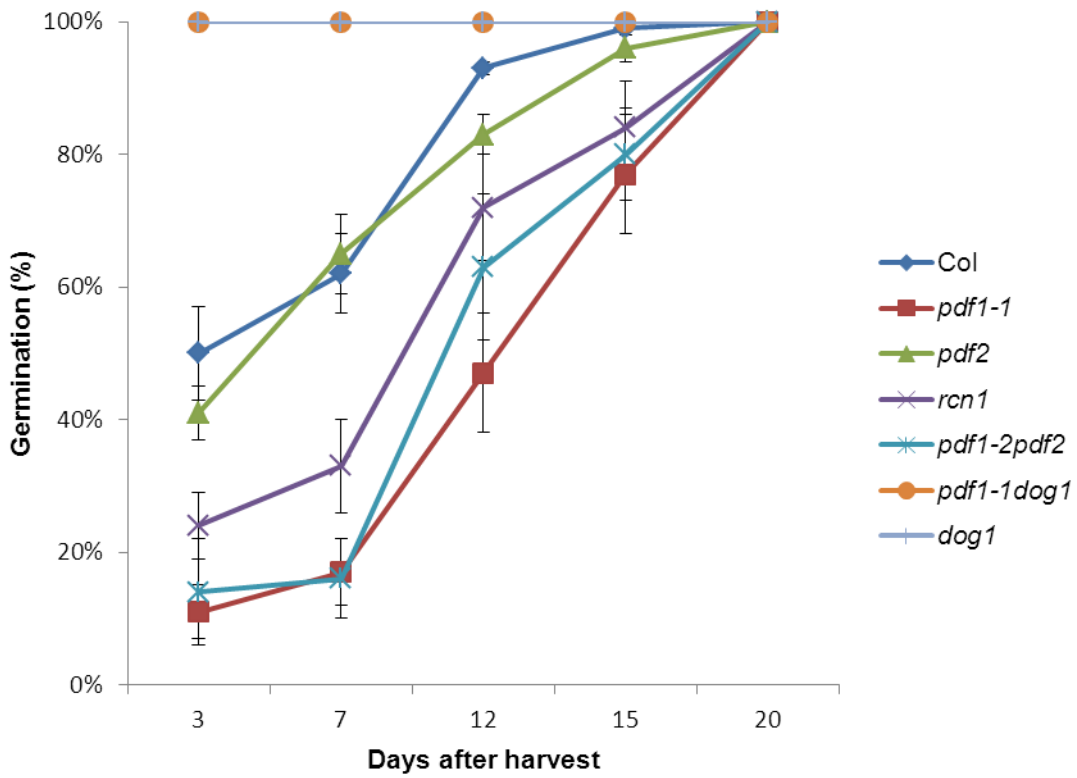


Figure 3.4. Germination test comparing the wild type Columbia with *pdf1-1* and its related mutants. Data points show the average of three biological replicates; ten plants were grown for each genotype. Standard errors are indicated.

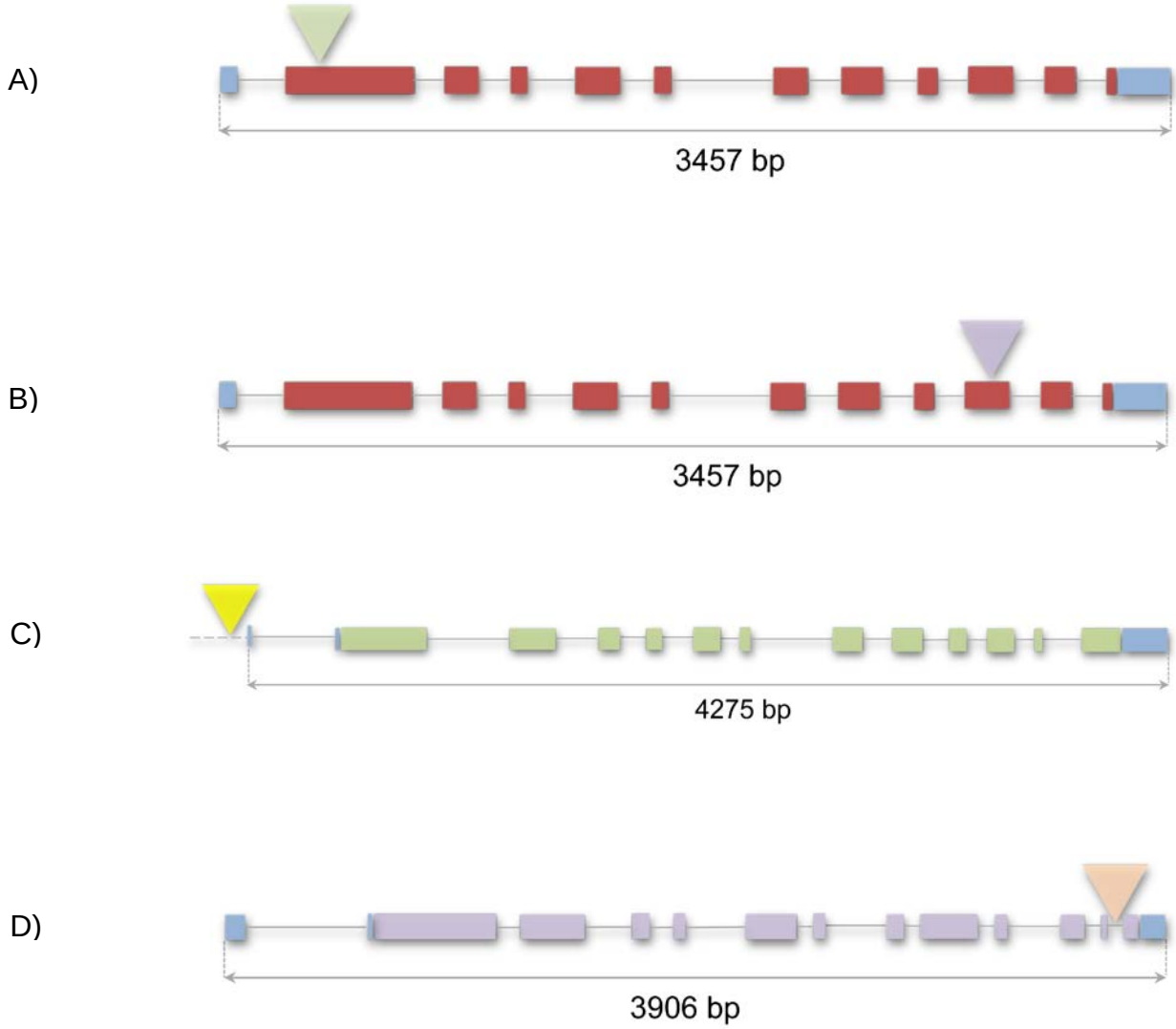


Figure 3.5. Graphic representation of *PDF1*, *PDF2*, *RCN1* and the position of their T-DNA insertions. For every gene, the untranslated regions are depicted in blue and the introns are represented by a line.
A) Representation of the *PDF1* gene and the position of the T-DNA insertion of the mutant named *pdf1-1* (Salk_037095). The green triangle indicates the T-DNA insertion in the first exon.
B) Representation of the *PDF1* gene and the position of the T-DNA in the mutant named *pdf1-2* (Salk_042724). The purple triangle indicates the insertion in the 9th exon.
C) Graphic representation of the *PDF2* gene and the position of the T-DNA insertion in the promoter (dashed line) (Salk_014113).
D) Graphic representation of the *RCN1* gene and the position of the T-DNA insertion in the last intron (Salk_059903).

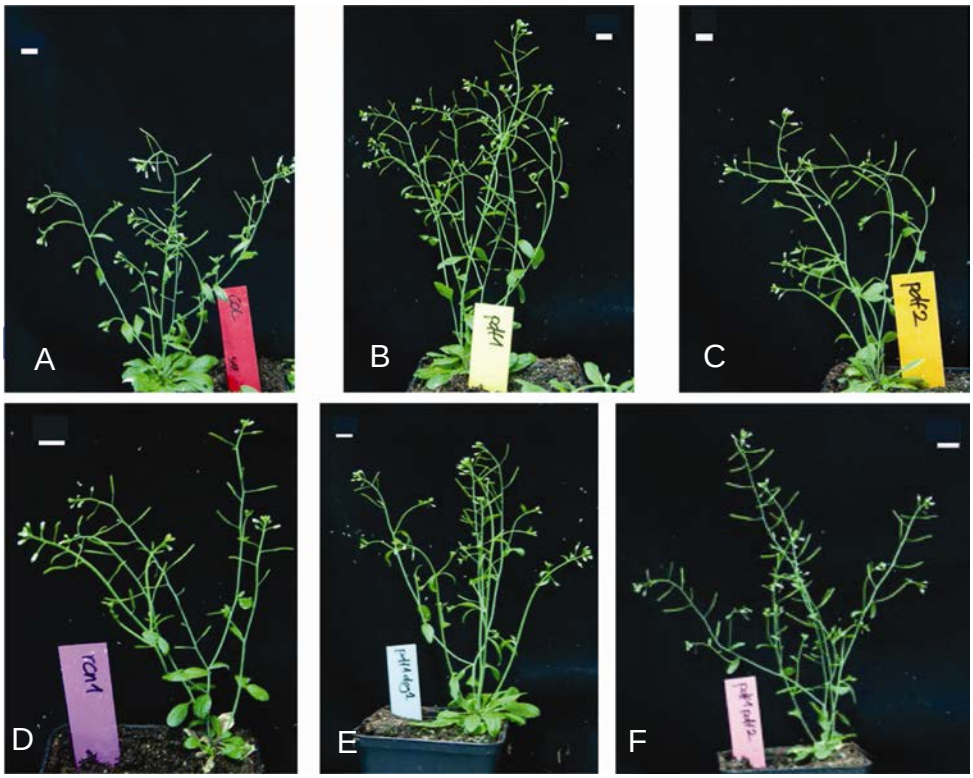


Figure 3.6. Adult plant phenotype of wild type Columbia (A), *pdf1-1* (B), *pdf2* (C), *rcn1* (D), *pdf1-1 dog1* (E), *pdf1-2 pdf2* (F). All plants were grown in the greenhouse under controlled conditions. Bars= 5cm.

3.1.3 Expression analyses

DOG1 is a key regulator of seed dormancy with a seed specific expression pattern (Bentsink *et al*., 2006; Nakabayashi *et al*., 2012). The 2A phosphatase PDF1 was identified as an interacting protein of DOG1 in a yeast two hybrid screen and its T-DNA insertion line showed enhanced dormancy. These data indicated that PDF1 is likely to play a role in seed dormancy through its interaction with DOG1. However, to confirm that *DOG1* and *PDF1* are co-expressed, the transcript level of *PDF1* during seed maturation was analyzed with qPCR assay.

For the expression analysis, plants were grown under controlled conditions in special growth chambers (Elbanton) and their siliques harvested in a time course from 10 to 20 days after pollination, covering the complete seed maturation phase during which seed dormancy is induced. In addition, fresh and after ripened mature seeds of these plants were imbibed for 12 hours and included in the experiments. Total RNA was extracted from each sample following the procedure described in 2.2.3.5.

Publicly available microarray data on the eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) indicated that *PDF1* is ubiquitously expressed in every tissue and its transcript abundance is constant throughout Arabidopsis life cycle. These data also indicate that *PDF1* is expressed in dry mature seeds (Figure 3.7).

Specific primers were designed to avoid cross amplification with *PDF2* and *RCN1*, since they share high sequence similarity. Additionally, the qPCR conditions were optimized to have high stringency with SYBR green detection.

Quantitative PCR confirmed that *PDF1* was expressed in dry seeds. Moreover, it revealed that its expression level is constant throughout seed maturation with a decrease in imbibed seeds (Figure 3.8). This result confirmed that *PDF1* is expressed during seed maturation and imbibition and suggests that *PDF1* plays a role in the control of dormancy.

In addition, the expression levels of *DOG1* were checked in the wild type Columbia and in *pdf1* mutant. For the amplification of *DOG1* the primers published in Nakabayashi *et al*., (2012) were used; these primers amplified all the splicing variants of the gene.

In the wild type Columbia *DOG1* showed a peak in expression levels at mid stage of seed maturation while in imbibed seeds it quickly disappeared (Figure 3.9). These results are in agreement with Nakabayashi et al (2012) where it is shown that *DOG1* has a similar pattern in the highly dormant genotype NIL DOG1 (see 1.1.6).

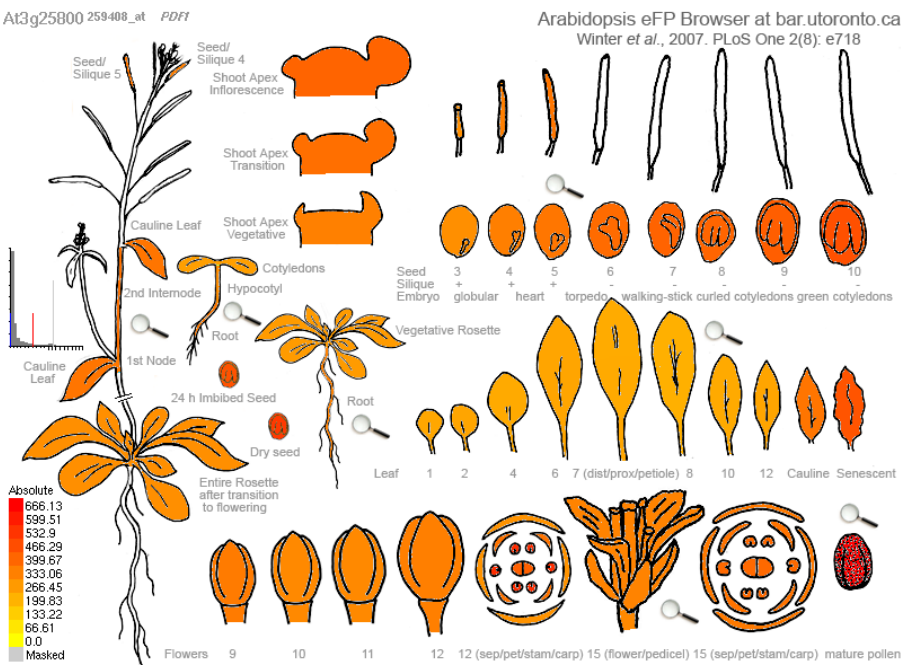


Figure 3.7. *PDF1* expression in different Arabidopsis tissues. Picture from the website http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi.

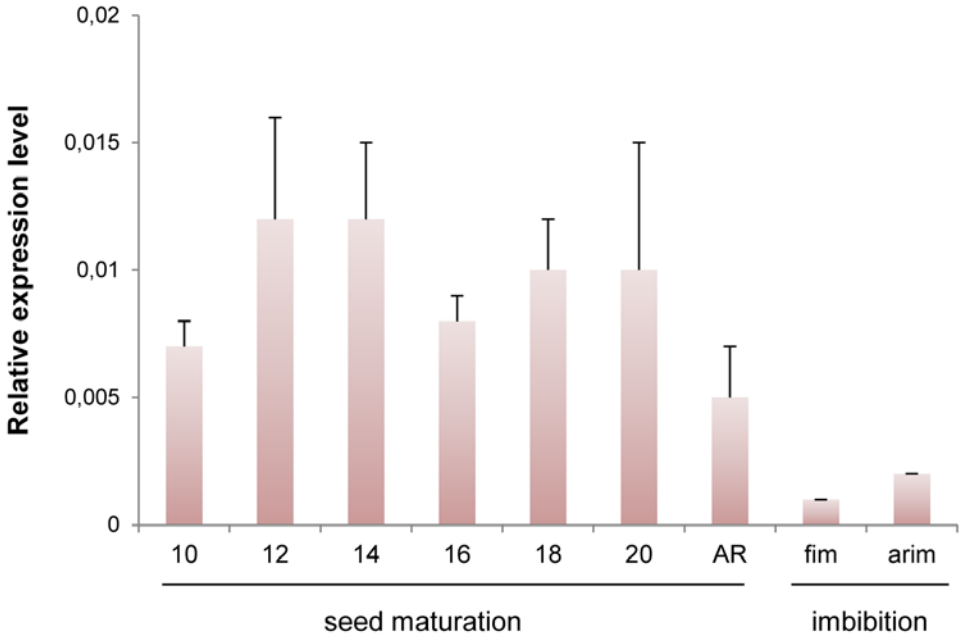


Figure 3.8. Expression levels of *PDF1* in Columbia. *PDF1* expression was checked during seed maturation, in after ripened seeds and in imbibed seeds. Values were normalized by the housekeeping gene *ACTIN8* and represent the average and the standard deviation of a qPCR assay of two representative biological replicates. AR, after-ripened seeds; fim, fresh mature imbibed seeds; arim, after-ripened imbibed seeds.

In *pdf1-1* mutant *DOG1* transcript levels is reduced in comparison to the wild type (Figure 3.10). However its expression shows a similar tendency by having a peak around 16-18 days after pollination and a fast decrease upon imbibition.

Taken together, these data indicate that *DOG1* and *PDF1* are co-expressed during seed maturation and show a decrease in transcript levels upon imbibition. *DOG1* expression in *pdf1-1* mutant is not altered in comparison to the wild type, suggesting that PDF1 is affecting DOG1 mostly at the protein level, possibly by influencing its activity or stability.

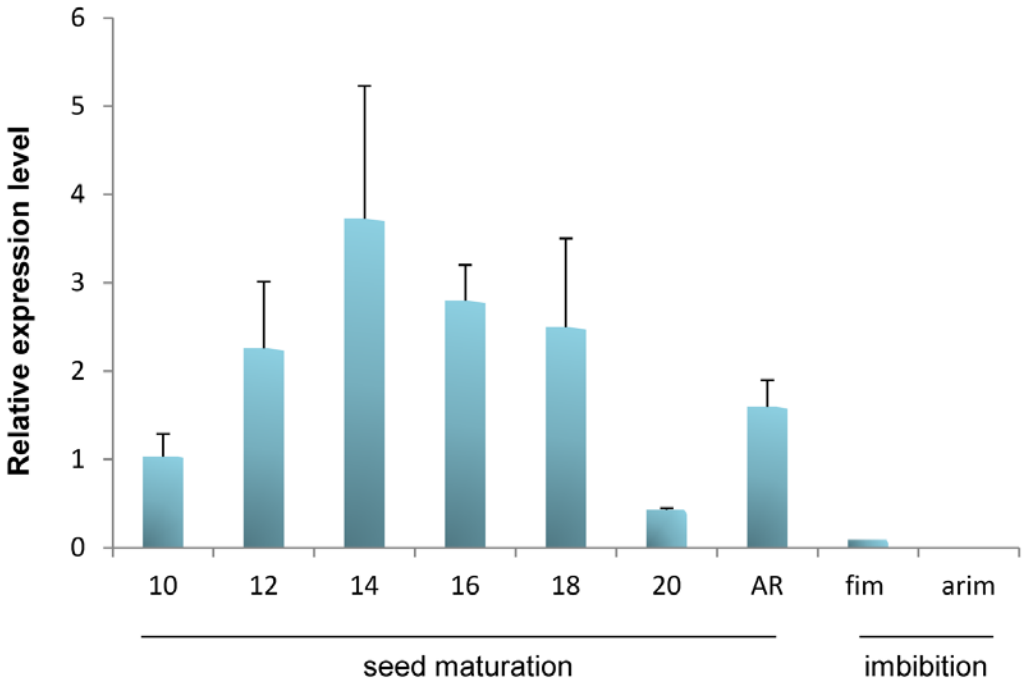


Figure 3.9. Expression levels of *DOG1* in Columbia. DOG1 expression was checked during seed maturation, in after ripened seeds and in imbibed seeds. Values were normalized by the housekeeping gene *ACTIN8* and represent the average and the standard deviation of a qPCR assay of two biological replicates. AR, after-ripened seeds; fim, fresh mature imbibed seeds; arim, after ripened imbibed seeds.

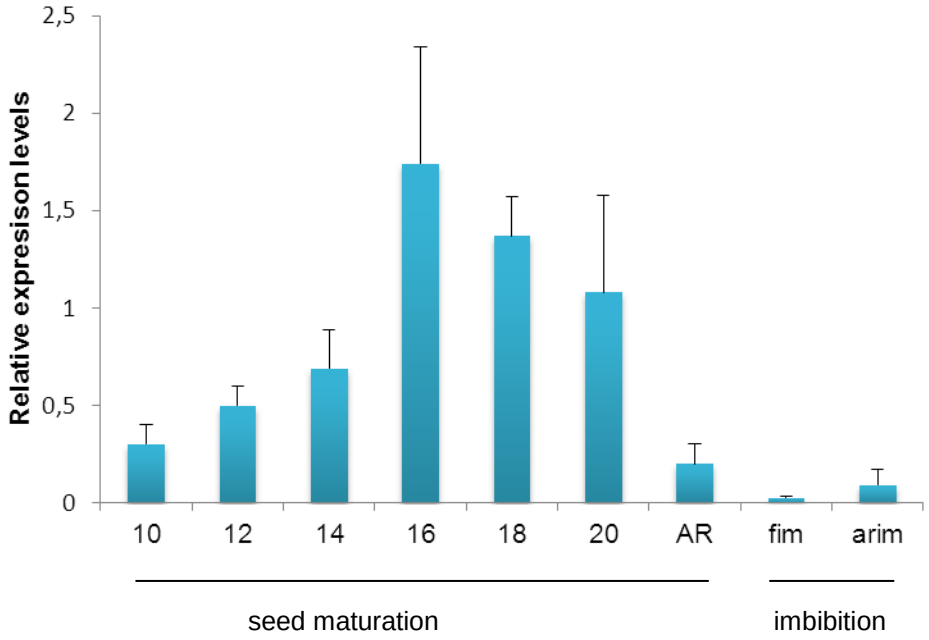


Figure 3.10. Expression levels of *DOG1* in the *pdf1* mutant. DOG1 expression was checked during seed maturation, in after ripened seeds and in imbibed seeds were used. Values were normalized by the housekeeping gene *ACTIN8* and represent the average and the standard deviation of a qPCR assay of two biological replicate. AR, after ripened seeds; fim, fresh mature imbibed seeds; arim, after ripened imbibed seeds.

3.1.4 Overexpression of *PDF1*

To analyze the effect of constitutive increased expression of *PDF1*, its coding sequence was cloned in a vector containing the double 35S promoter.

The construct was transformed in Columbia and several transformants were obtained. Of these transgenic plants, two homozygous independent lines with single insertions were selected and tested in a germination assay with Columbia to see how the overexpression of *PDF1* could influence dormancy, since its loss of function leads to enhanced dormancy.

The assay (Figure 3.11) revealed that one of the transformant lines (line 3.1) showed a significant reduction in the dormancy level in comparison to the wild type and *pdf1-1*. However the other line tested was very similar to Columbia and did not show any significant difference.

One possible explanation for this could be that *PDF1* was silenced in these lines and a qPCR experiment will be useful in a further investigation of these lines.

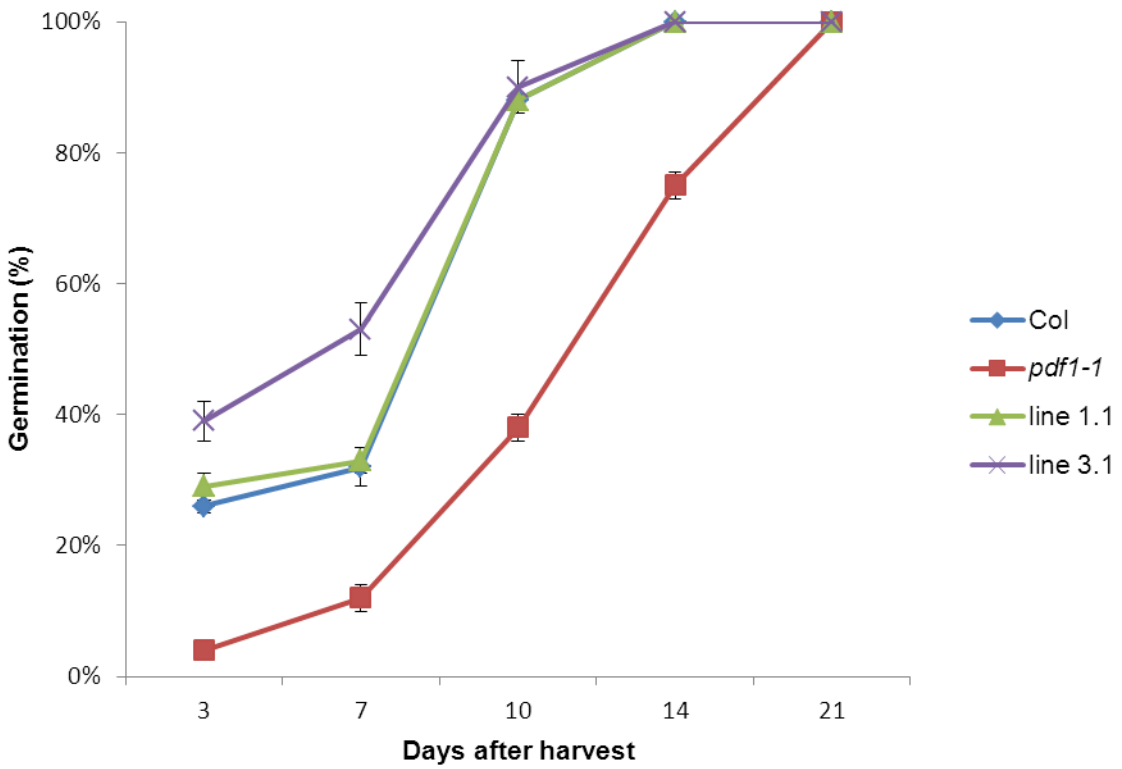


Figure 3.11. Germination assay comparing the wild type Columbia with two independent homozygous *PDF1* overexpressor lines. Values are means of ten plants, bars represent standard errors.

3.1.5 The influence of hormones on germination of *pdf1-1* and *pdf2* mutants

3.1.5.1 ABA sensitivity of *pdf1-1* and related mutants at germination

Plant hormones have an essential role in the control of dormancy. In particular, ABA and GA are the main regulators.

ABA is a positive regulator of seed dormancy; it accumulates during seed maturation when desiccation tolerance and primary dormancy are established.

Germination assay in presence of increasing amounts of ABA is a common method used in many screenings to identify ABA-insensitive mutants (Koornneef *et al.,* 1984) and, more in general, genes involved in the ABA signal transduction.

Also, Kwak *et al*. (2002) showed that a T-DNA insertion line for *RCN1* has a reduced ABA sensitivity at germination. This finding, together with the evidence that the *rcn1* mutation impairs ABA-related induced stomatal closing, ABA activation of slow anion channels and ABA-induced gene expression, suggested that *RCN1* is a general component of the ABA signaling pathway.

The sensitivity of *pdf1-1*, *pdf2* and *pdf1-2 pdf2* was checked by performing a germination test in presence of ABA, in order to determine whether *PDF1* and *PDF2* are also involved in the ABA signaling

pathway. After ripened seeds of these mutants were grown with increased concentrations of ABA up to 5 µM and their germination rate was counted after 7 days (Figure 3.12).
The test revealed that the ABA sensitivity detected in *pdf1-1* and *pdf1-1pdf2* is similar to the one of the wild type, indicating that *PDF1* and *PDF2* are unlikely to be involved in ABA signaling.

3.1.5.2 Paclobutrazol sensitivity of *pdf1-1* and related mutants at germination

GA plays a key role in dormancy release by promoting germination and counteracting ABA effects. In the seed it is required to overcome seed coat restriction and endosperm weakening.
When germinated on ABA solution, *pdf1-1* and *pdf2* do not show difference in comparison to the wild type, indicating that the enhanced dormancy observed is not caused by an altered ABA signaling.
Paclobutrazol (PAC) is a gibberellins biosynthesis inhibitor. Germination assay in presence of PAC has been used in many screening to identify ABA-deficient mutant, which show resistance to PAC due to their low ABA content (Nambara *et al*., 1992; Leòn-Kloosterziel *et al*, 1996). As a consequence, this method has been extensively used to indirectly measure the ABA content in seeds.
After ripened seeds of *pdf1-1*, *pdf2* and *pdf1 pdf2* were germinated in presence of PAC up to 100 µM to understand whether the dormant phenotype observed was induced by a change in the ABA content.
Figure 3.13 shows that no significant differences were detected in the sensitivity to PAC of the mutants in comparison to the wild type. Taken together, these results indicate that *PDF1* and *PDF2* are not involved in the ABA signaling pathway and do not have an altered ABA content.

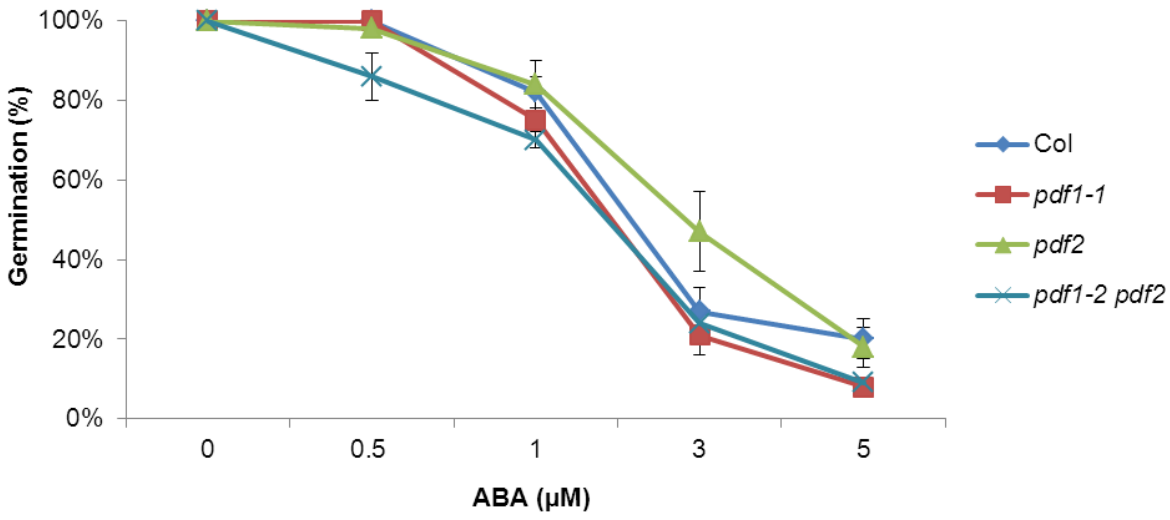


Figure 3.12. ABA sensitivity at germination in the mutants *pdf1-1*, *pdf2*, *pdf1 pdf2* compared to the wild type. Data points are the average of 10 plants from each line, standard errors are indicated by the bars.

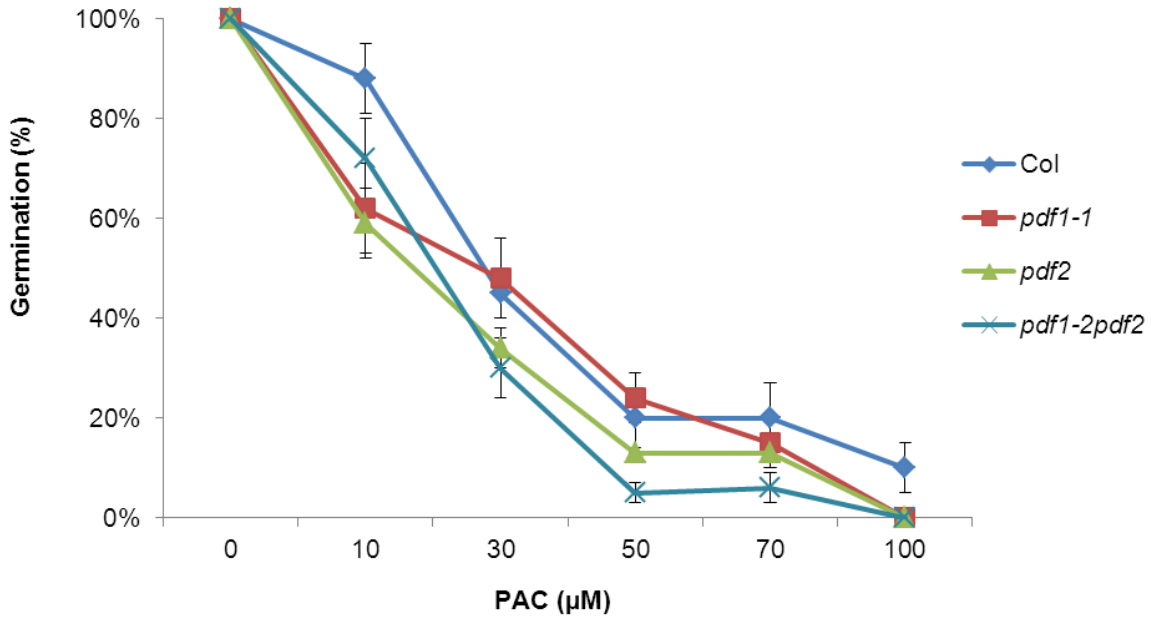


Figure 3.13. PAC sensitivity at germination of *pdf1-1*, *pdf2*, *pdf1 pdf2* compared to the wild type. Data points are the average of 10 plants from each line, standard errors are indicated by the bars.

3.1.6 Localization studies

Previous studies detected *DOG1* promoter activity in the vascular tissue of freshly harvested seeds; additionally, DOG1 protein was located in the nucleus based on the fluorescence of transgenic plants containing YFP::DOG1 fusion (Nakabayashi *et al*., 2012; see 1.1.6).

The promoter of *PDF1* and its coding sequence were fused to different reporter genes in order to compare the spatial expression pattern of *PDF1* with *DOG1* and test their *in vivo* interaction. These constructs were transformed in the wild type Columbia and for each of them several transgenic plants were obtained. Independent homozygous lines carrying a single copy insertion were selected for further analysis.

3.1.6.1 Analysis of the *DOG1* and *PDF1* promoter activities

PDF1 and its neighboring gene (At3g25805) are separated only by a very small region of 231 bp and the exact size of the *PDF1* promoter was not clear. Therefore, three different constructs where the genomic sequence of the neighboring gene was included partially or entirely were used to analyze *PDF1* promoter activity (Figure 3.14).

In the first construct, named pPDF1-1, a small insert containing the 5′ UTR of PDF1 and the 3′ UTR of the neighbor gene (At3g25805) was cloned. In the second and the third constructs, named pPDF1-2 and pPDF1-3, the genomic sequence of the neighbor gene was included partially or entirely.

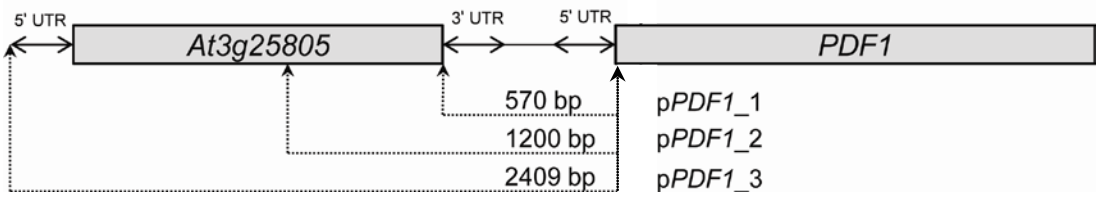


Figure 3.14. *PDF1* promoter regions used for the analysis of promoter-reporter gene fusions.
The three different constructs used for the promoter analysis of *PDF1* are indicated as p*PDF1*_1, p*PDF1*_2, p*PDF1*_3.

T3 after ripened seeds of stable transformants with all three promoter constructs were analyzed for their histochemical GUS activity and two independent lines were used for every construct. This showed that *PDF1* promoter activity was present in the vascular tissue and showed some additional activity throughout the whole embryo, with a peak of intensity in the radicle tip (Figure 3.15A) and no difference in the expression pattern of the different constructs was detectable.

PDF1 promoter activity was compared to a GUS fusion construct containing *DOG1* promoter of the strong dormant accession Kashmir (provided by K. Nakabayashi). *DOG1* signal in after ripened seeds was clearly detectable in the vascular tissue (Figure 3.15B), indicating that *DOG1* and *PDF1* promoter activities are overlapping.

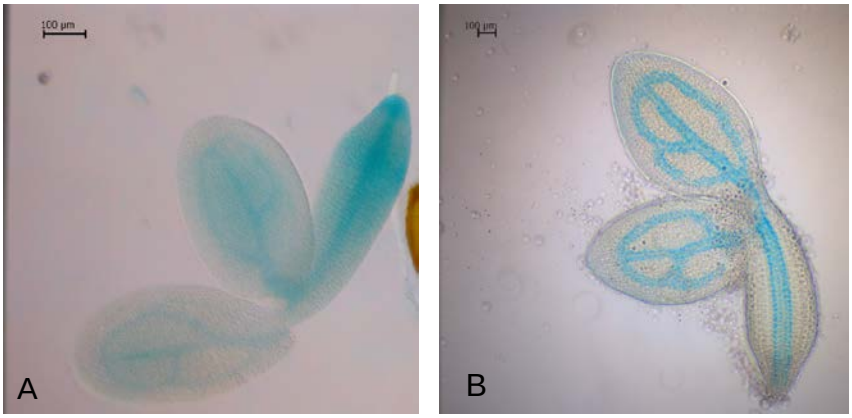


Figure 3.15. Localization of GUS activity in embryos of dry after ripened seeds. Plants were transformed with the constructs p*PDF1_2*:GUS (A) and p*DOG1*_Cvi:GUS (B)

3.1.6.2 Cellular localization of DOG1 and PDF1

To analyze the subcellular localization of PDF1, a binary vector with the coding sequence of PDF1 under the control of a 35S promoter and fused to the 3' end with the *CYAN FLUORESCENT PROTEIN* gene (*CFP*) was produced and transformed in wild type protoplasts with a PEG-mediated transient assay as described in 2.2.2.2. After the transfection, protoplasts were incubated at room temperature overnight and analyzed by confocal laser scanner microscopy (CLSM). CFP signal could be detected in the nucleus and in the cytoplasm of leaf protoplasts (Figure 3.16B).

The construct YFP::DOG1δ was also transformed in leaf protoplasts and confirmed that DOG1 is localized mainly in the nucleus (Figure 3.16A) as previously shown by Nakabayashi *et al*., 2012. Moreover, this result showed that DOG1 and PDF1 co-localize in the nucleus and cytoplasm.

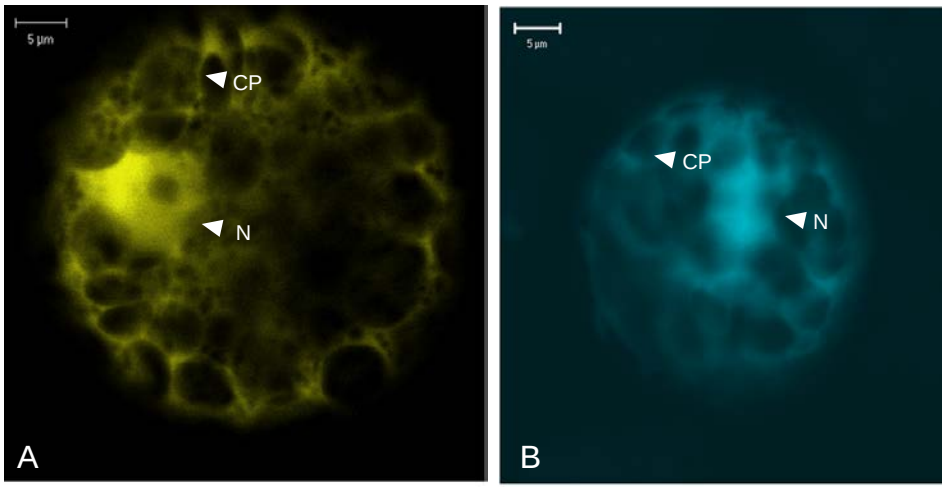


Figure 3.16. Sub-cellular localization of DOG1 (A) and PDF1 (B). The constructs YFP::DOG1δ and CFP::PDF1 were transformed into leaf protoplasts and the fluorescence was detected after overnight incubation. White triangles indicate the nucleus (N) and the cytoplasm (CP)

3.1.6.3 Interaction studies in *Arabidopsis* protoplast

DOG1 and PDF1 co-localize in Arabidopsis leaf protoplasts, indicating that they could interact *in vivo*. To confirm this hypothesis, a split YFP assay was performed.

The split YFP assay is based on the principle that the N- and the C- terminus of YFP do not spontaneously reconstitute a functional fluorophore. However, when fused to interacting proteins, the two non-functional halves are able to reconstitute the fluorophore and generate *de novo* fluorescence (reviewed in Bhat R.A. et al., 2006). This assay offers the possibility to detect interactions between proteins *in vivo* and allows the visualization of the sub-cellular location of the interaction.

The cDNA of *PDF1* was cloned in two different binary vectors containing the N-terminus and the C-terminus of the *YELLOW FLUORESCENT PROTEIN* (*YFP*) under the control of the 35S promoter. These constructs were transformed in leaf protoplast either with a construct carrying the N-terminus or the C-terminus of the YFP fused to DOG1 using the same transient assay as in 3.1.3.2.

Confocal microscopy confirmed that the fluorescence was restored in the nucleus and in the cytoplasm, indicating that the interaction occurred *in vivo* (Figure 3.17).

All three DOG1 protein variants (α, β, δ) were tested in the assay in order to see whether they could differentially interact with PDF1 but no significant difference could be detected.

Following the same procedure, the interaction between DOG1 and PDF2 was checked. As for PDF1, YFP fluorescence was detected in the nucleus and in the cytoplasm but the signal appeared less intense (Figure 3.18).

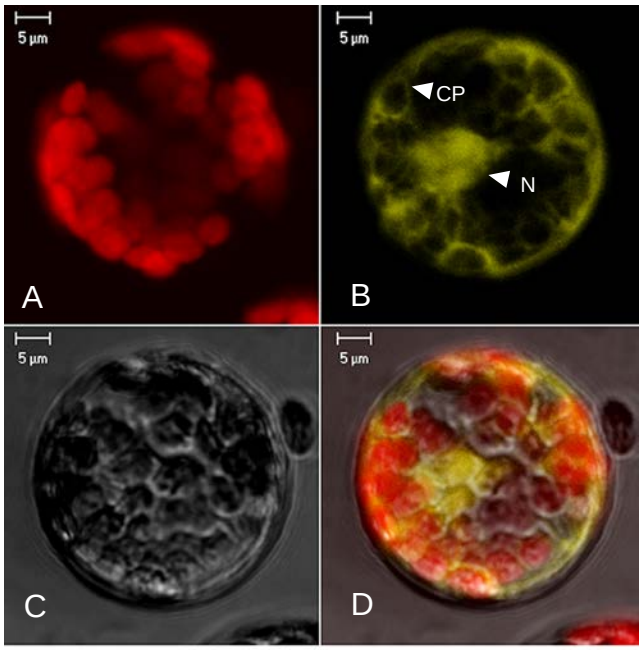


Figure 3.17. Confocal image of the split YFP assay showing the interaction between DOG1-YFP^{C} and PDF1-YFP^{N} in leaf protoplasts. A) Chlorophyll channel; B) YFP channel; C) Transmission channel; D) Merged channels. White triangles indicate the nucleus (N) and the cytoplasm (CP). Pdf1n-dog1c

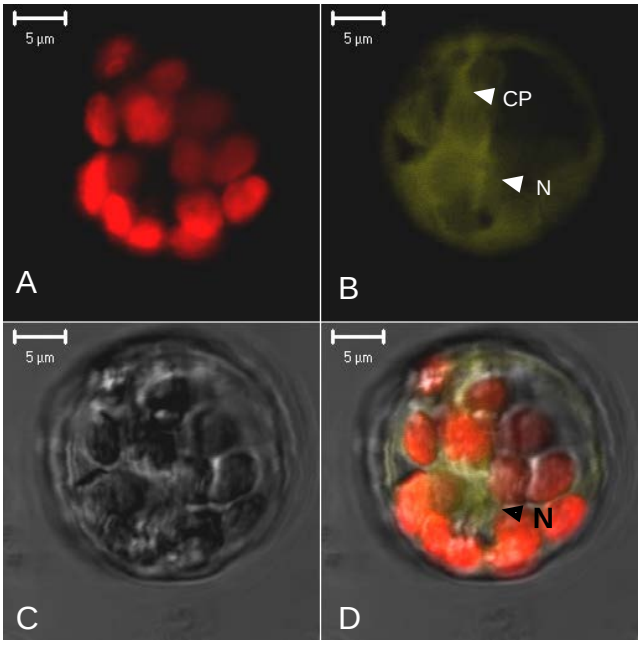


Figure 3.18. Confocal image showing the split YFP assay of the interaction between DOG1-YFP^{N} and PDF2-YFP^{C}. A) Chlorophyll channel B) YFP channel C) Transmission channel D) Merged channels. White triangles indicate the nucleus (N) and the cytoplasm (CP).

3.1.6.4 Interaction studies in *N. benthamiana* leaves

The interaction between DOG1 and PDF1 and the localization of the single proteins were not determined in seeds of stable transformants.

However, the split YFP assay performed in protoplasts was carried out also in N. *benthamiana* leaves using the same constructs, in order to confirm the protoplast result.

Figure 3.19 shows interaction of DOG1-PDF1 in the nucleus and in the cytoplasmatic strands, confirming what was already observed in Arabidopsis protoplasts.

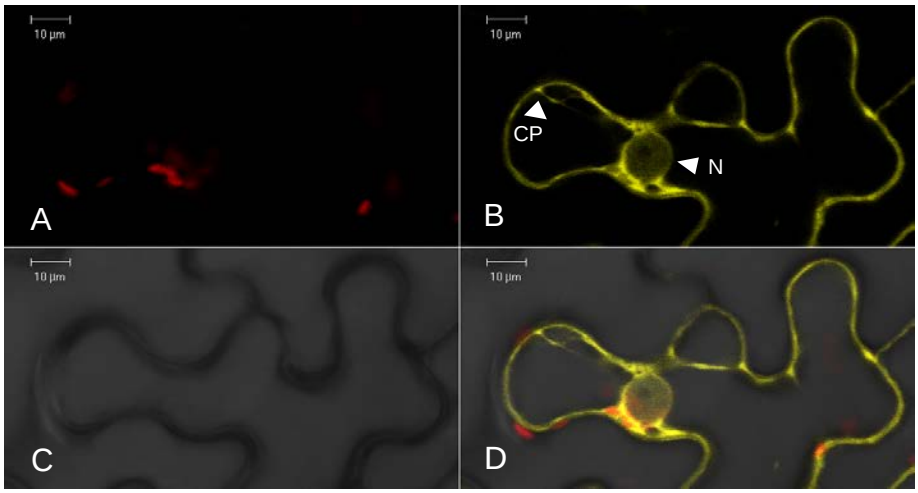


Figure 3.19. Confocal image of *Agrobacterium*-mediated transient expression assay in N. *benthamiana* leaves. As for *Arabidopsis*, DOG1-YFP^N and PDF1-YFP^C interaction occurs in nucleus (N) and cytoplasm (CP). A) Chlorophyll channel B) YFP channel C) Transmission channel D) Channels merged.

3.2 DOG1 protein studies

3.2.1 Analysis of DOG1 protein in Columbia with two-dimensional gels

Seed dormancy in Arabidopsis can be released by a period of cold treatment (stratification) or by storing seeds in dry conditions (after ripening). *DELAY OF GERMINATION1* (*DOG1*) plays a central role in the control of seed dormancy. Remarkably, the DOG1 protein levels correlate with dormancy in freshly harvested seeds; however, this correlation is lacking in after ripened seeds, suggesting that the protein activity is lost in this stage (Nakabayashi *et al*., 2012). In addition, PDF1, one the three genes coding for the scaffolding subunit A of a PP2A phosphatase, was identified as interacting protein of DOG1 and therefore a possible role of phosphorylation in affecting DOG1 function was investigated.

Proteins are subjected to a large number of enzymatic and non-enzymatic post translational modifications that affect different properties such as localization, stability and activity. These protein modifications can be investigated using two-dimensional gels by looking at quantitative or qualitative changes in the isoelectrofocusing of specific proteins detected as spots. Therefore, DOG1 protein was studied qualitatively with two-dimensional gels by comparing fresh and after ripened seeds in order to detect alterations related to its loss of activity during after ripening.

As demonstrated by previous experiments performed in the Soppe lab, DOG1 is a low abundant protein in seeds and for this reason it has to be detected with a specific antibody. A polyclonal antibody was raised against DOG1; however, the antibody was not fully specific and therefore a construct with a tagged version of the protein was generated in the lab. This construct contains 3xHA-tagged DOG1 under the control of the DOG1 Cvi promoter and used for transformation of Columbia and *pdf1-1*.

Homozygous transgenic plants carrying the construct ProDOG1:3xHA:DOG1 in Columbia background were grown under controlled conditions and total proteins were extracted from fresh seeds, 16 hours fresh imbibed seeds and 12 weeks after-ripened dry and imbibed seeds. In order to be able to detect the protein effectively, two-dimensional gel electrophoresis was followed by electroblotting on PVDF membrane and immunostaining; DOG1 fusion protein was detected with a monoclonal anti-HA antibody at the expected size of 40 kDa. HA:DOG1 in fresh dry seeds of Columbia was focused at the isoelectric point (pI) of ~5.0 while in 16 hours fresh imbibed seeds the protein had shifted towards the basic side at the isoelectric point of ~5.15. In after ripened imbibed seeds it was focused at two major spots at pI ~4.75 and 5.0 (Figure 3.20).

These results indicate that DOG1 is targeted by two different post-translational modifications. The first modification occurs during after ripening and causes a shift towards the acidic side in dry and imbibed after ripened seeds (see A.1 and A.2 for the shift in dry seeds), indicating that the modification occurs primarily in dry seeds and can still be observed upon imbibition. This shift is likely to be responsible for DOG1 loss of activity during after-ripening.

The second modification occurs upon imbibition and induces a shift toward the basic side that is consistent with a dephosphorylation event. In order to investigate the role of PDF1 in the possible dephosphorylation event, DOG1 protein was analyzed in the *pdf1-1* mutant.

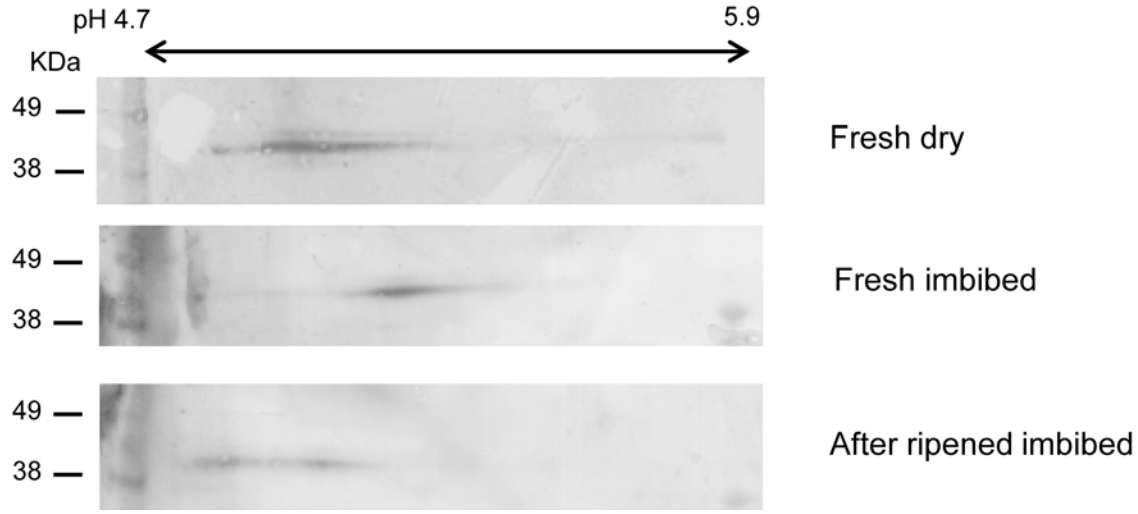


Figure 3.20. Two-dimensional immunodetection showing DOG1 altered isoelectrofocusing during imbibition and after ripening of Columbia. Total proteins were extracted from fresh and after ripened seeds of plants containing the ProDOG1:3xHA:DOG1 construct and DOG1 was detected with an anti-HA antibody. Here, 40 µg of total proteins were separated using a narrow range IPG strip and on a bis-tris 4-12% acrylamide gradient gel.
Freshly harvested seeds or 12 weeks after-ripened seeds were imbibed for 16 hours. The shift has been observed in three different biological replicates.
pH and molecular mass marker are indicated respectively on the top and on the left of the blots.

3.2.2. Analysis of DOG1 protein in *pdf1-1* with two-dimensional gels

PDF1 was identified as an interacting protein of DOG1 and encodes a scaffolding subunit A of 2A phosphatase. A homozygous T-DNA insertion line named *pdf1-1* showed increased dormancy, supporting the hypothesis that PDF1 plays a role in controlling seed dormancy.

Localization studies confirmed that PDF1 and DOG1 co-localize and interact *in vivo*; RT-PCR analysis showed that *DOG1* mRNA accumulation pattern was similar to the one observed in the wild-type, although reduced. This suggested that *PDF1* was influencing *DOG1* at the protein level.

During imbibition of Columbia seeds, DOG1 had altered isoelectrofocusing and shifted towards the basic side. This shift is consistent with a dephosphorylation event because upon removal of negative phosphates the protein is likely to accumulate positive charges and migrates through the negative side.

Total proteins were extracted from homozygous transgenic plants carrying the construct ProDOG1:3xHA:DOG1 in *pdf1-1* background to be able to detect DOG1 with a specific antibody. Figure 3.21 shows that in *pdf1-1* HA:DOG1 was detected at the expected size of 40 kDa and was focused at pI ~4.9 in fresh and after ripened imbibed seeds.

In fresh imbibed seeds of *pdf1-1*, HA:DOG1 was focused at a lower pH in comparison to Columbia, indicating possible higher phosphorylation levels, consistent with a role of PDF1 in DOG1 desphosphorylation. Also, the shift towards lower pH during after-ripening did not occur anymore,

suggesting that this shift depends on DOG1 phosphorylation levels and raises the possibility that these two post-translational modifications observed are interdependent.

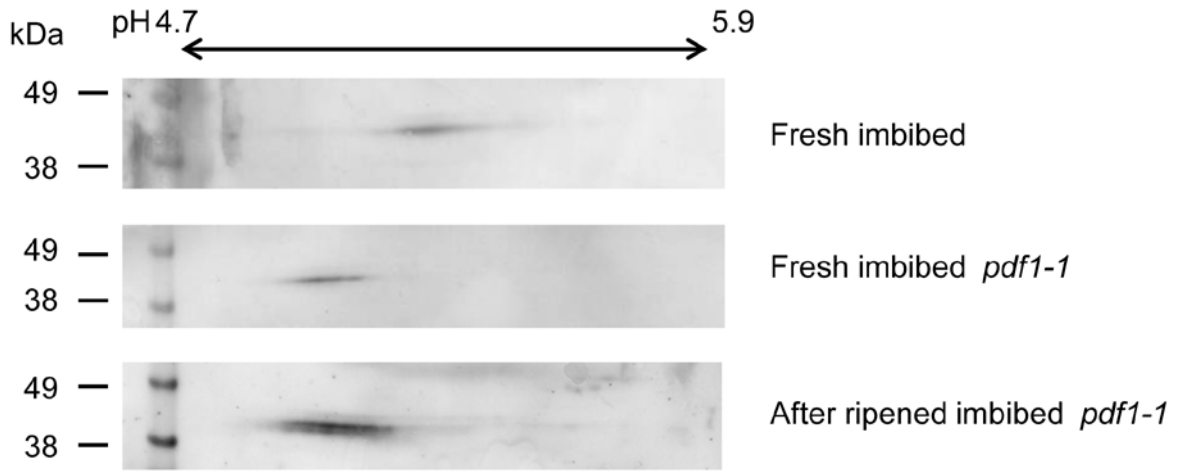


Figure 3.21 Two-dimensional immunodetection comparing DOG1 isoelectrofocusing in *pdf1-1* and Columbia. Total proteins were extracted from fresh and after ripened seeds of *pdf1-1* transgenic plants containing the ProDOG1:3xHA:DOG1 construct to detect DOG1 with an anti-HA antibody. Here, 40 µg of total proteins were separated using a narrow range IPG strip and on a bis-tris 4-12% acrylamide gradient gel.
Seeds either freshly harvested or 12 weeks after-ripened were imbibed for 16 hours. The shift has been observed in two different biological replicates.
pH and molecular mass marker are indicated respectively on the top and on the left of the blots.

4. DISCUSSION

Seed dormancy is a complex adaptive trait regulated by several hormonal and environmental factors and prevents germination during temporary advantageous environmental conditions in an unfavorable season. The balance between the two plant hormones abscisic acid (ABA) and gibberellins (GA) plays an important role: ABA is involved in the induction and maintenance of the dormant state, while GA promotes germination. In Arabidopsis dormancy can be released by a period of cold treatment (stratification) or by storing seeds in dry conditions (after ripening).

Up to now, our understanding of the molecular mechanisms controlling seed dormancy is still limited. Recently, *DELAY OF GERMINATION1 (DOG1)* was identified as a major QTL for seed dormancy and it was shown to be a key regulator and a true marker for dormancy in fresh mature seeds of Arabidopsis (Bentsink *et al*., 2006; Nakabayashi *et al*., 2012). Its protein accumulation correlates with dormancy in fresh seeds but this correlation is lacking in after-ripened seeds and upon imbibition. DOG1 encodes a protein of unknown function and unknown regulation.

PDF1, one of the three genes coding for the scaffolding subunit of a PP2A phosphatase, was identified as interacting protein of DOG1. The relation between PDF1 and DOG1 was therefore investigated in order to gain further insights into the regulation of DOG1 and into the mechanisms controlling seed dormancy.

4.1 The structure and function of PP2A phosphatases

Plants contain large and diverse families of phosphatases which are classified according to their substrate specificity (serine, threonine or tyrosine) and to their structure: serine/threonine-specific phosphoprotein phosphatase (PPP), metal ion-dependent protein phosphatase (PPM) and phosphotyrosine phosphatase (PTP). PP2Cs are the representative group of the PPM family and are known to be involved in many signaling pathways, including seed dormancy. PP2As belong to the PPP family and were found as either a heterodimer consisting of the catalytic subunit (PP2Ac) and the scaffolding subunit A or as heterotrimer with the addition of the regulatory subunit B. This composition is very conserved and highly similar to the ones in mammalian enzymes (reviewed by Luan, 2003). Arabidopsis contains respectively 5, 3 and 17 genes coding for the catalytic subunit, the scaffolding subunit A and the regulatory subunit B, leading to a great variety of combinations with very different substrate specificity and functions.

In mammals PP2As are the major Ser/Thr phosphatases together with PP1; also, they are highly conserved from yeast to humans. As in Arabidopsis, they have a very complex structure and take part in many signaling pathways. Recent reports have focused primarily on their role as tumor suppressors. Remarkably, mutations in the gene coding for the subunit A are very common in human tumors and have been associated with primary lung tumors, breast tumors and primary colon tumors.

In Arabidopsis, the role of PP2As has up to now not been deeply investigated and they were mostly linked to developmental processes and hormonal signal transduction pathways (Kwak *et al*, 2002; Camilleri *et al*., 2002). Over the last few years, several papers have elucidated more precisely their roles in these pathways. Tang *et al*. (2012) showed that members of the B' regulatory subunit are key components of the brassinosteroids (BR) signaling pathway and they activate BR response by dephosphorylating *BRASSINAZOLE-RESISTANT* (*BZR*) transcription factors. The catalytic subunit PP2Ac-2 was identified as a negative regulator of ABA signaling and, interestingly, a T-DNA insertion line in this locus showed delayed seed germination and increased dormancy (Pernas *et al*., 2007). Another regulatory subunit, TAP46, is involved in the TARGET OF RAPAMYCIN (TOR) pathway that regulates cell growth and metabolism in response to growth factors, nutrients, energy and environmental conditions (Ahn *et al.,* 2011).

Zhou *et al*. (2004) carried out an extensive mutant analysis of *PDF1*, *PDF2* and *RCN1*, the three genes coding for the scaffolding subunit A of the Arabidopsis PP2A. The authors came to the conclusion that *RCN1* performs a crucial role in different pathways, as its insertion line shows several defects especially in hormone signaling, despite the fact that adult plants do not show any evident phenotype. Mutants lacking *PDF1*, *PDF2* or both exhibit a largely normal phenotype. A putative role of these genes in controlling seed dormancy or germination is not discussed in the paper. The work described in this thesis showed the function of *PDF1* in seed dormancy.

4.2 Analysis of the DOG1 interacting protein PDF1 and its homologous genes

To investigate the regulation and function of the key dormancy regulator DOG1, a yeast two hybrid library screening was performed. This assay has been developed over a decade ago and since then broadly used in protein-protein interaction analysis because it is a relatively fast and easy technique to perform. It provides a straight-forward approach to study protein interaction in vivo with a fair resemblance to higher eukaryotic systems and it requires only the cDNA of the genes of interest. However, the outcome of yeast two-hybrid experiment has to be carefully evaluated as it is common to get false positives. In the preliminary yeast two hybrid library screening many independent clones that showed complementation contained the cDNA coding for PDF1 and for this reason the phosphatase was picked as one of the most interesting candidates. Thereafter, the result was confirmed several times by checking its direct interaction with DOG1. Such interaction was consistent but relatively weak in yeast, possibly due to the fact that phosphorylation and dephosphorylation are known to be transient and reversible events. Moreover, the interaction could depend on certain post-translational modifications that might not occur in yeast.

Once identified as a putative DOG1 interactor, PDF1 was tested for a possible dormancy phenotype. The homozygous T-DNA insertion line *pdf1-1* showed enhanced dormancy in comparison to the wild type, indicating that PDF1 could play a role opposite to DOG1 in the control of seed dormancy. Also,

this result suggested that the phosphatase could function as negative regulator of DOG1 and the phosphorylated form of DOG1 could have increased activity. However, the enhanced dormancy phenotype of *pdf1-1* was not very strong. The lack of a strong dormancy phenotype of this mutant could be due to the presence of other phosphatases that influence DOG1 redundantly or it could imply that phosphorylation has a minor role for DOG1 function.

The double mutant *pdf1-1 dog1* was generated to understand the relation between *DOG1* and *PDF1* and showed the same phenotype as *dog1-2*, consistent with DOG1 and PDF1 functioning in the same pathway in which PDF1 requires DOG1 to exert its function.

PDF1 has two homologues in Arabidopsis, PDF2 and RCN1 of which only PDF2 also showed interaction with DOG1. Despite this interaction, the insertion mutant *pdf2* had a similar dormancy level as the wild type Columbia, suggesting that it does not have a role in seed dormancy. The absence of a dormancy phenotype could be explained by the fact that *PDF2* shows low expression in seeds (data from http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). This hypothesis has still to be confirmed by RT-PCR analysis. In contrast, RCN1 did not show any interaction with DOG1 but a shallow dormancy was present in its T-DNA insertion mutant. RCN1 is known to be part of the ABA signaling pathway (Kwak *et al*., 2002) and this result suggests that it plays a role in seed dormancy by decreasing ABA signaling.

To understand whether PDF1 and PDF2 could have redundant functions, the double mutant *pdf1-2 pdf2* was tested in a germination assay but it did not show any additive dormant phenotype. Altogether, these data suggest that PDF1 and RCN1 play a role in seed dormancy but function in different pathways, while PDF2 does not seem to have a role.

In addition, the mutants *pdf1-1*, *pdf2* and *pdf1-2 pdf2* were germinated in presence of ABA or PAC to understand if their enhanced dormancy was caused by an alteration in ABA signaling or content. No difference in sensitivity was detected, indicating that the pathway where DOG1 and PDF1 function is independent from ABA. This result is in agreement with Nakabayashi *et al*. (2012) where the authors conclude that *DOG1* and *ABA* function in largely independent pathways.

As previously mentioned, the yeast two hybrid system is a fast and easy technique to test protein-protein interaction. However, the chance to get false positives is high when, for example, the protein of interest is able to activate transcription by its own (auto-activation). Therefore, the ability of PDF1 to bind DOG1 was investigated *in vivo* with a split YFP transient assay in Arabidopsis protoplast. Here, the interaction was detected both in the cytoplasm and in the nucleus, where PDF1 and DOG1 were co-localizing.

In Nakabayashi *et al*., (2012) localization studies of plants containing the YFP:DOG1 fusion showed that the protein is mainly detected in the nucleus, while in the work described here DOG1 is present also in the cytoplasm, where the binding with PDF1 is occurring as well. Although reporter-gene fusions are widely used to analyze the spatial regulation of a gene, there is a chance to encounter overexpression artifacts (e.g. protein aggregations and saturation of protein targeting machinery) because all the fusion proteins were driven by 35S promoter. In order to clarify this issue, future experiments are required using constructs where YFP is fused to the coding region of *PDF1*

expressed under its own promoter; also, stable transformants will help to perform the localizations and the binding studies in embryos.
Promoter activity studies indicated that the *PDF1* promoter is able to drive expression in the vascular system of the embryo where it overlapped with *DOG1* promoter activity. This experiment was performed using three fusion constructs containing promoter regions with different sizes. The constructs gave the same localization pattern, suggesting that the cis-regulatory elements were located in the shortest region cloned.

The expression of *PDF1* was investigated during seed maturation. Publicly available microarray data showed that the phosphatase is ubiquitously expressed, consistent with the fact that PDF1 has different functions and does not have only DOG1 as target. Recent studies have highlighted how PDF1 and its gene family are involved in the regulation of PIN-FORMED (PIN) phosphorylation state and auxin transport (Dai *et al*., 2012; Michniewicz *et al*., 2007). Because of its ubiquitous expression and diverse function, it will be of interest to investigate PDF1 expression pattern during seed development more in detail.
The RT-PCR analysis performed indicated that *PDF1* expression level is constant throughout seed maturation and decrease upon imbibition. Interestingly, a phosphoproteomic study conducted in germinating maize seeds demonstrated that, among the phosphatases identified, the corresponding gene coding for the PP2A regulatory subunit A was downregulated (Lu *et al*., 2008).
In addition, the expression of *DOG1* was quantified in *pdf1-1* mutant and, surprisingly, its expression was reduced. The mutant *pdf1-1* showed enhanced dormancy, therefore *DOG1* would be more likely upregulated in this mutant. These data demonstrate that a negative feedback regulation exists between PDF1 and DOG1, as it was also observed between *aba1* and *dog1* mutant analyzed in Nakabayashi *et al*. (2012).

4.3 DOG1 undergoes different post-translational modifications

In Arabidopsis, seed development is completed in 20 days under standard growing conditions and ends with seed maturation when the desiccation tolerance is acquired. This stage is very important because it allows the seed to survive adverse environmental conditions. During seed maturation, dormancy is established and reaches high levels in dry seeds. The mature dry seed requires a period of dry storage (after-ripening) to break dormancy completely. Upon imbibition, when dormancy is fully released, the seed is able to restart all the metabolic activities.
Analysis of seed proteomes revealed the importance of protein post-translational modifications (PTMs). PTMs represent a major form of metabolic control in higher eukaryotes and recently "-omics" investigations have illustrated how they contribute to the high complexity of the seed proteome and generate a large diversity of protein functions. For example, in Arabidopsis only three genes encode for 12S cruciferins. However, more than 100 spots corresponding to these proteins have been identified on two-dimensional gels (Arc *et al*., 2011). In addition, a very poor correlation

between the proteins theoretical and the observed isoelectric point was found, demonstrating the major impact of PTMs (Galland et al., 2012; www.seed-proteome.com).
In the present study, the DOG1 protein was analyzed by two-dimensional gels in order to detect possible post-translational modifications responsible for its loss of activity during after-ripening.

4.3.1 DOG1 is modified during after ripening

DOG1 fused to the HA epitope was analyzed in fresh and after-ripened seeds on two-dimensional gels in order to investigate potential modifications occurring during after-ripening that could be responsible for its loss of activity, as hypothesized by Nakabayashi *et al*. (2012). The experiments conducted so far showed that DOG1 shifted towards a lower pH during after-ripening, indicating that the alteration occurred primarily in dry seeds and could still be detected upon imbibition. Moreover, these data indicated that DOG1 is targeted by a PTM that is likely to cause its loss of activity during seed storage.
Since a long time, non-enzymatic reactions like lipid peroxidation are known to occur in dry seeds where the absence of water does not allow metabolic activities (Wilson & McDonald, 1986). Recent studies have highlighted the importance of non-enzymatic oxidation during the after-ripening of Arabidopsis and sunflower seeds. Here, after-ripening was found to be associated with an accumulation of ROS and resulted in the carbonylation of specific proteins (Oracz *et al*., 2007). Irreversible oxidative carbonylation plays an important role in protein turnover and promotes the degradation or inhibition of aberrant, damaged or unnecessary proteins (Arc *et al*., 2011). In Arabidopsis dry seeds, specific subunits of the 12S cruciferins are the major target of carbonylation and this facilitates their mobilization during germination.
For the above mentioned reasons, oxidation is considered a likely candidate in DOG1 modification during after-ripening and the shift observed is consistent with this hypothesis.

4.3.2 DOG1 is modified upon imbibition

The comparison between dry and imbibed seeds revealed that DOG1 is targeted by a second PTM causing a shift towards the basic side. This shift was detected during imbibition of both fresh and after-ripened seeds. This work described the identification of PDF1, a 2A phosphatase interacting with DOG1 *in vitro* and *in vivo*. A homozygous T-DNA insertion line at this locus, named *pdf1-1*, showed increased dormancy, supporting the hypothesis that PDF1 plays a role in seed dormancy. The shift observed upon imbibition in wild type seeds is consistent with a dephosphorylation event, because the protein accumulates positive charges upon removal of negatively charged phosphates. In order to confirm that PDF1 is responsible for the shift observed during imbibition, the DOG1 protein was analyzed by two-dimensional gels in imbibed seed of *pdf1-1*. In fresh imbibed seeds of this mutant DOG1 was focused at a lower pH in comparison to Col fresh imbibed seeds, suggesting

that the absence of the PP2A phosphatase prevents dephosphorylation upon imbibition and leads to enhanced dormancy. According to this hypothesis, in dry seeds DOG1 would be phosphorylated and active, while it would become dephosphorylated and inactive after imbibition.

Reversible phosphorylation is involved in the regulation of different cellular functions, including protein activity, stability and localization. The auxin transporter PIN-FORMED3 (PIN3) requires phosphorylation for its biological function; mutations in the phosphorylatable residues disrupt its subcellular trafficking and caused severe defects in the PIN3-mediated processes (Ganguly *et al*., 2012). In addition, *RCN1*, *PDF1* and *PDF2* are part of a novel PP6-type phosphatase complex that directly regulates PIN phosphorylation (Dai *et al*., 2012).

In general, the phosphorylation proteome of dry and imbibed seeds is poorly documented. However, a recent comparative study of seed maturation in Arabidopsis, rapeseed and soybean showed that most of the phosphoproteins identified were observed at the first stages of seed maturation, while a large portion of the late-maturation phosphopeptides were corresponding to late-embryogenesis-abundant (LEA) proteins, indicating that phosphorylation might play a role in the transition from seed maturation to a quiescent state (Meyer *et al*., 2012). De novo phosphorylation occurs mainly during germination (Arc *et al*., 2011). However DOG1 could still be phosphorylated during seed maturation, similar to 12S cruciferins that are known to be phosphorylated during this stage (Wan *et al*., 2007). Lu *et al*., (2008) in a phosphoproteomic investigation of germinating maize seeds, showed that the regulatory subunit A of a PP2A phosphatase was identified during germination. Interestingly, this phosphatase is highly expressed right after imbibition and gradually downregulated thereafter. The same tendency was observed for *PDF1* and could suggest that the phosphatase has a peak of activity immediately at the start of imbibition when it dephosphorylates DOG1. To confirm this hypothesis, the isoelectric focusing of DOG1 will be analysed after different imbibition timing (e.g. 2, 4, 8, 12 and 24 hours after imbibition), as in the experiments performed up to now its isoelectrofocusing was investigated only after 16 hours of imbibition.

The data presented here provide indirect evidence that DOG1 is subjected to dephosphorylation upon imbibition. However, the mechanism by which PDF1 specifically affects DOG1 function is still an open question. According to the *pdf1-1* and *pdf1-1 dog1* phenotypes, PDF1 functions as a negative regulator of DOG1 but, on the other hand, the dormancy phenotype displayed by *pdf1-1* is not very strong and, as mentioned before, there might be other phosphatases that function redundantly with PDF1 in the regulation of DOG1. Another possibility could be that phosphorylation enhances DOG1 activity but it is not strictly required for its regulation. Moreover, the experiments performed in this thesis illustrated how DOG1 is affected after 16 hours imbibition. The conditions used in these experiments might not be the ones where PDF1 and phosphorylation are most important; therefore, in the future, the isoelectrofocusing of DOG1 will be analysed under different conditions. For example, the effect of PDF1 could be temperature-dependent and therefore stronger during stratification.

Nakabayashi *et al*. (2012) suggested that DOG1 acts during imbibition by inhibiting germination; therefore, removal of phosphates upon imbibition could result in a loss of function leading to a promotion of germination. Nevertheless, the possibility that DOG1 acts during seed maturation is still open, because the authors also show that it affects ABA and transcript levels in dry seeds.
Furthermore, the hypothesized dephosphorylation was observed also in fresh seeds where germination does not occur but DOG1 seems to be dephosphorylated as well, suggesting that this modification is not related to dormancy but prevent DOG1 activity during downstream processes triggered by germination. In the *pdf1-1* mutant, the shift during after-ripening was not observed, supporting the hypothesis that phosphorylation might reduce or prevent the oxidation effect. These data raise the possibility that the modifications that target DOG1 are interdependent, however more investigations are needed to confirm this hypothesis.

4.3.3. Future directions to confirm DOG1 post-translational modifications

The two-dimensional gels performed so far illustrated DOG1 changes only in a qualitative way. To identify precisely which kind of PTMs affects DOG1, additional experiments are required.
The HA antibody used to detect HA:DOG1 revealed to be very specific, therefore DOG1 will be immunoprecipitated and subsequently analyzed by mass spectrometry. This will identify also the amino acids which are specifically targeted by dephosphorylation and by oxidation. In addition, protein carbonylation is a widely used marker to detect oxidative damages and sensitive methods for its detection have been developed (Levine *et al*., 1994). It occurs by direct oxidative attack on lysine, arginine, proline and threonine or by secondary reactions via reactive carbohydrates or lipids on cysteine, histidine and lysine that lead to the formation of protein carbonyl derivatives (Johansson *et al*., 2004; Job *et al*., 2005).
DOG1 is known to be regulated at many different levels: transcriptionally, post-transcriptionally and at the protein level (Nakabayashi *et al*., 2012). The present study contributed to improve our understanding of the regulation of DOG1 on a post-transcriptional level during after-ripening and upon imbibition, where oxidative processes and phosphorylation are likely to be involved.

References

Agarwal S. & Sohal R.S. (1994) DNA oxidative damage and the expectancy in houseflies. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 12332-12335.

Ahn C.S., Han J.A., Lee H.S., Lee S., Pai H.S. (2011) The PP2A regulatory subunit Tap46, a component of the TOR signaling pathway, modulates growth and metabolism in plants. *The Plant Cell* **23**, 185-209.

Ali-Rachedi S., Bouinot D., Wagner M.H., Bonnet M., Sotta B., Grappin P., Jullien M. (2004) Changes in endogenous abscisic acid levels during dormancy release and maintenance of mature seeds: studies with the Cape Verde Islands ecotype, the dormant model of Arabidopsis thaliana. *Planta* **219**, 479-488.

Alonso-Blanco C., Bentsink L., Hanhart C.J., Blankestijn-de Vries H., Koornneef M. (2003) Analysis of natural allelic variation at seed dormancy loci of Arabidopsis thaliana. *Genetics* **164**, 711-29.

Arc E., Galland M., Cueff G., Godin B., Lounifi I., Job D., Rajjou L. (2011) Reboot the system thanks to protein post-translational modifications and proteome diversity: how quiescent seeds restart their metabolism to prepare seedling establishment. *Proteomics* **11**, 1606-1618.

Ashikawa I, Abe F, Nakamura S. (2010) Ectopic expression of wheat and barley DOG1-like genes promotes seed dormancy in Arabidopsis. *Plant Science* **179**, 536-42.

Barrero J.M., Jacobsen J.V., Talbot M.J., White R.G., Swain S.M., Garvin D.F., Gubler F. (2012) Grain dormancy and light quality effect on germination in the model grass *Brachypodium distachyon. New Phytologist* **193**, 376-386.

Barrero J.M., Talbot M.J., White R.G., Jacobsen J.V., Gubler F. (2009) Anatomical and transcriptomic studies of the coleorhiza reveal the importance of this tissue in regulating dormancy in barley. *Plant Physiology* **150**, 1006-1021.

Bazin J., Langlade N., Vincourt P., Arribat S., Balzergue S., El-Maarouf-Bouteau H., Bailly C. (2011) Targeted mRNA oxidation regulates sunflower seed dormancy alleviation during dry after-ripening. *The Plant Cell* **23**, 2196-208.

Bhat R.A. Lahaye T., Panstruga R. (2006) The visible touch: in planta visualization of protein-protein interactions by fluorophore-based methods. *Plant Methods* **2**, 12

Bentsink L., Hanson J., Hanhart C.J., Blankestijn-de Vries H., Coltrane C., Keizer P., El-Lithy M., Alonso-Blanco C., de Andrés M.T., Reymond M., van Eeuwijk F., Smeekens S., Koornneef M. (2010) Natural variation for seed dormancy in Arabidopsis is regulated by additive genetic and molecular pathways. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 4264-9.

Bentsink L., Jowett J., Hanhart C.J., Koornneef M. (2006) Cloning of *DOG1*, a quantitative trait locus controlling seed dormancy in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America* **103,** 17042-17047.

Bethke P.C., Gubler F., Jacobsen J.V., Jones R.L. (2004) Dormancy of Arabidopsis seeds and barley grains can be broken by nitric oxide. *Planta* **219**, 847-855.

Bethke P.C., Libourel I.G.L., Aoyama N., Chung Y.Y., Still D.W., Jones R.L. (2007) The Arabidopsis aleurone layer responds to nitric oxide, gibberellin and abscisic acid and is sufficient and necessary for seed dormancy. *Plant Physiology* **143**, 1173-1188.

Bethke P.C., Libourel I.G.L., Jones R.L. (2005) Nitric oxide reduces seed dormancy in Arabidopsis. *Journal of Experimental Botany* **57**, 517-526.

Bewley J.D. (1997) Seed germination and dormancy. *The Plant Cell* **9**, 1055-1066.

Bove J., Lucas P., Godin B., Oge L., Jullien M., Grappin P. (2005) Gene expression analysis by cDNA-AFLP highlights a set of new signaling networks and translational control during seed dormancy breaking in Nicotiana Plumbaginifolia. *Plant Molecular Biology* **57**, 593-612.

Cadman C.S.C., Toorop P.E., Hilhorst H.W.M. & Finch-Savage, W.E. (2006). Gene expression profiles of Arabidopsis Cvi seed during cycling through dormant and non-dormant states indicate a common underlying dormancy control mechanism. *The Plant Journal* **46**, 805-822.

Camilleri C, Azimzadeh J, Pastuglia M, Bellini C, Grandjean O, Bouchez D. (2002) The Arabidopsis TONNEAU2 gene encodes a putative novel protein phosphatase 2A regulatory subunit essential for the control of the cortical cytoskeleton. *The Plant Cell* **14**, 833–45.

Clough S.J., Bent A.F. (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant Journal* **16**, 735-743.

da Silva E.A.A., Toorop P.E., Nijsse J., Bewley J.D., Hilhorst H.W.M. (2004) Abscisic acid controls embryo growth and endosperm cap weakening during coffee (*Coffea arabica* vs Rubi) seed germination. *Planta* **220**, 251-261.

de la Fuente van Bentem S., Hirt H. (2007) Using phosphoproteomics to reveal signaling dynamics in plants. *Trends in Plant Science* **12**, 404-411.

Dai M., Zhang C., Kania U., Chen F., Xue Q., McCray T., Li G., Qin G., Wakeley M., Terzaghi W., Wan J., Zhao Y., Xu J., Friml J., Deng X.W., Wang H. (2012) A PP6-type phosphatase holoenzyme directly regulates PIN phosphorylation and auxin efflux in Arabidopsis. *The Plant Cell* **24**, 2497-514

Debeaujon I., Léon-Kloosterziel K.M., Koornneef M. (2000) Influence on the testa on seed dormancy, germination and longevity in Arabidopsis. *Plant Physiology* **122**, 403-413.

Donohue K. (2002) Germination timing influences natural selection on life-history characters in Arabidopsis thaliana. *Ecology* **83(4)**, 1006-1016.

Edwards K., Johnstone C., Thompson C. (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Research* **19**, 1349.

Farkas I., V Dombradi, M Miskei, L Szabados., Koncz C. (2007) Arabidopsis PPP family of serine/threonine phosphatases. *Trends in Plant Science* **12**, 169-176.

Fiels S., Song O. (1989) A novel genetic system to detect protein–protein interactions. *Nature* **340**, 245 – 246.

Finch-Savage W.E. & Leubner-Metzger G. (2006) Seed dormancy and the control of germination. *New Phytologist* **171**, 501–523.

Finkelstein R.R., Gampala S.S.L., Rock C.D. (2002) Abscisic acid signaling in seed and seedlings. *The Plant Cell* **14**, S15-S45.

Fujii H., Verslues P.E., Zhu J.K. (2007) Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in Arabidopsis. *The Plant Cell* **19**, 485-94.

Galland M., Job D., Rajjou L. (2012) The seed proteome web portal. *Frontiers in Plant Science* **3**, 98.

Ganguly A., Cho H.T. (2012) The phosphorylation code is implicated in cell type-specific trafficking of PIN-FORMEDs. *Plant, Signal & Behavior* **7**, 1-4.

Goldberg R.B., de Paiva G., Yadegari R. (1994) Plant embryogenesis: zygote to seed. *Science* **266**, 605-614.

Graeber K., Linkies A., Müller K., Wunchova A., Rott A., Leubner-Metzger G. (2010) Cross-species approaches to seed dormancy and germination: conservation and biodiversity of ABA-regulated mechanisms and the Brassicaceae DOG1 genes. *Plant Molecular Biology* **73**, 67-87.

Graeber K., Nakabayashi K., Miatton E., Leubner-Metzger G., Soppe W.J. (2012) Molecular mechanisms of seed dormancy. *Plant, Cell & Environment* **35**, 1769-86.

Groot S.P. & Karssen C.M. (1992) Dormancy and germination of abscisic acid-deficient tomato seeds: studies with the sitiens mutants. *Plant Physiology* **99**, 952-958.

Gubler F., Millar A.A. & Jacobsen J.V. (2005) Dormancy release, ABA and pre-harvest sprouting. *Current Opinion in Plant Biology* **8,** 183–187.

Gutierrez L., van Wuytswinkel O., Castelain M., Bellini C. (2007) Combined networks regulating seed maturation. *Trends in Plant Science* **12**, 294-300.

Hanahan D. (1983) Studies on transformation of Escherichia coli with plasmids. *Journal of Molecular Biology* **166**, 557-580.

Hellens R., Mullineaux P. (2000) A guide to Agrobacterium binary Ti vectors. *Trends in Plant Science* **5**, 446-451.

Holdsworth M.J., Bentsink L. & Soppe W.J.J. (2008) Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination. *New Phytologist* **179**, 33-54.

Holman T.J., Jones P.D., Russel L. (2009) The N-end rule pathway promotes seed germination and establishment through removal of ABA sensitivity in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 4549-4554.

Huang X., Schmitt J., Dorn L., Griffith C., Effgen S., Takao S., Koornneef M., Donohue K. (2010) The earliest stages of adaptation in an experimental plant population: strong selection on QTLS for seed dormancy. *Molecular Ecology* **19**, 1335-51.

Job C., Rajjou L., Lovigny Y., Belghazi M., Job D. (2005) Patterns of protein oxidation in Arabidopsis seeds and during germination. *Plant Physiology* **138**, 790-802.

Johansson E., Olsson O., Nyström T. (2004) Progression and specificity of protein oxidation in the life cycle of Arabidopsis thaliana. *The Journal of Biological Chemistry* **279**, 22204-8.

Karssen C.M., Brinkhorst-van der Swan D.L.C., Breekland A., Koornneef M. (1983). Induction of dormancy during seed development by endogenous abscisic acid: studies on abscisic deficient genotypes of Arabidopsis thaliana (L.) Heynh. *Planta* **157**, 158-165.

Koncz C., Mayerhofer R., Koncz-Kalman Z., Nawrath C., Reiss B., Redei G.P., and Schell J. (1990) Isolation of a gene encoding a novel chloroplast protein by T-DNA tagging in Arabidopsis thaliana. *EMBO Journal* **9**, 1337–1346.

Koncz C., Schell J. (1986) The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimeric genes carried by a novel type of Agrobacterium binary vector. *Molecular and General Genetics* **204**, 383–396.

Koornneef M. & Karssen, C. M. (1994). Seed dormancy and germination. In *Arabidopsis* (ed. E. M. Meyerowitz and C. Somerville) 313-334.

Koornneef M., Meinke D. (2010) The development of Arabidopsis as a model plant. *The Plant Journal* **61**, 909-921.

Koornneef M., Reuling G., Karssen C.M. (1984) The isolation and characterization of absicic acid insensitive mutants of Arabidopsis thaliana (L) Heynh. *Theoretical and Applied Genetics* **61**, 385-393.

Kucera B., Cohn M.A. & Leubner-Metzger G. (2005) Plant hormone interactions during seed dormancy release and germination. *Seed Science Research* **15**, 281–307.

Kwak J.M., Moon J.H., Murata Y., Kuchitsu K., Leonhardt N., DeLong A., Schroeder J.I. (2002) Disruption of a guard cell-expressed protein phosphatase 2A regulatory subunit, *RCN1*, confers ABA insensitivity in Arabidopsis. *The Plant Cell* **14**, 2849-2861.
Laemmli U. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Larsen P.B., Cancel J.D. (2003) Enhanced ethylene responsiveness in the Arabidopsis eer1 mutant results from a loss-of-function mutation in the protein phosphatase 2A A regulatory subunit, RCN1. Plant Journal **34**, 709-18.

Lee K.P., Piskurewicz U., Turecková V., Strnad M., Lopez-Molina L. (2010) A seed coat bedding assay shows that RGL2-dependent release of abscisic acid by the endosperm controls embryo growth in Arabidopsis dormant seeds. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 19108-13.

Léon-Kloosterziel K.M., Gil M.A., Ruijs G.J., Jacobsen S.E., Olszewski N.E., Schwartz S.H., Zeevaart J.A., Koornneef M. (1996) Isolation and characterization of abscisic acid-deficient Arabidopsis mutants at two new loci. *The Plant Journal* **10**, 655-61

Leubner-Metzger G. (2003) Functions and regulation of ß-1,3-glucanase during seed germination, dormancy and after ripening. *Seed Science Research* **13**, 17-34.

Leubner-Metzger G. (2005) ß-1,3-Glucanase gene expression in low hydrated seeds as a mechanism for dormancy release during tobacco after-ripening. *The Plant Journal* **41**, 133-145.

Levine R.L., Williams J.A., Stadtman E.R., Shacter E. (1994) Carbonyl assays for determination of oxidatively modified proteins. *Methods in Enzymology* **233**, 346–357.

Leymarie J., Bruneaux E., Gibot-Leclerc S., Courbineau F. (2007) Identification of transcripts potentially involved in barley seed germination and dormancy using cDNA-AFLP. *Journal of experimental Botany* **58**, 425-437.

Leymarie J., Robayo-Romero M.E., Gendreau E., Benech-Arnold R.L., Courbineau F. (2008) Involvement of ABA in induction of secondary dormancy release during tobacco after-ripening. *Plant and Cell Physiology* **49**, 1830-1838.

Linkies A., Leubner-Metzger G. (2012) Beyond gibberellins and ascisic acid: how ethylene and jasmonates control seed dormancy. *Plan cell reports* **31**, 253-270.

Liu Y., Geyer R., van Zanten M., Carles A., Li Y., Hörold A., van Nocker S., Soppe WJJ. (2011) Identification of Arabidopsis REDUCED DORMANCY 2 gene uncovers a role for the polymerase associated factor I complex in seed dormancy. *PLoS One* **6**, e22241.

Liu Y., Koornneef M., Soppe WJJ. (2007) The abscence of histone H2B monoubiquitination in Arabidopsis hub1 (rdo4) mutant reveals a role for chromatin remodeling in seed dormancy. *Plant Cell* **19**, 433-444.

Lu T.C., Meng L.B., Yang C.P., Liu G.F., Liu G.J., Ma W., Wang B.C. (2008) A shotgun phosphoproteomics analysis of embryos in germinated maize seeds. *Planta* **228**, 1029-4.

Luan S. (2003) Protein phosphatases in plants. *Annual Review of Plant Biology* **54**, 63-92.

Ma Y., Szostkiewicz I., Korte A., Moes D., Yang Y., Christmann A., Grill E. (2009) Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* **324**, 1064-1068.

Mayer U., Torrez Ruiz R.A., Berleth T., Miséra S., Jürgens G. (1991) Mutations affecting body organization in the *Arabidopsis* embryo. *Nature* **353**, 402-407.

Meyer L.J., Gao J., Xu D., Thelen JJ. (2012) Phosphoproteomic analysis of seed maturation in Arabidopsis, rapeseed, and soybean. *Plant Physiology* **159**, 517-28.

Michniewicz M., Zago M.K., Abas L., Weijers D., Schweighofer A., Meskiene I., Heisler M.G., Ohno C., Zhang J., Huang F., Schwab R., Weigel D., Meyerowitz E.M., Luschnig C., Offringa R., Friml J. (2007) Antagonistic regulation of PIN phosphorylation by PP2A and PINOID directs auxin flux. *Cell* **130**, 1044-56.

Millar A.A., Jacobsen J.V., Ross J.J., Helliwell C.A., Poole A.T., Scofield G., Reid J.B., Gubler F. (2006) Seed dormancy and ABA metabolism in Arabidopsis and barley: the role of ABA 8'-hydroxylase. *The Plant Journal* **45**, 942-954.

Müller K., Carstens A.C., Linkies A., Torres M.A., Leubner-Metzger G. (2009) The NADPH-oxidase AtrbohB plays a role in Arabidopsis seed after-ripening. New Phytologist **184**, 885-9.

Nakabayashi K., Bartsch M., Xiang Y., Miatton E., Pellengahr S., Yano R., Seo M., Soppe W.J. (2012) The Time Required for Dormancy Release in Arabidopsis Is Determined by DELAY OF GERMINATION1 Protein Levels in Freshly Harvested Seeds. *The Plant Cell* **24**, 2826-38.

Nakashima K., Fujiita Y., kanamori N., Katagiri T., Umezawa T., Kidokoro S., Maruyama K., Yoshida T., Ishiyama K., Kobayashi M., Shinozaki K., Yamaguchi-Shinozaki K. (2009) Three Arabidopsis SnRK2 proteins kinases, SRK2D/SnRK2.2, SRK2E/SnRK2.6/OST1 and SRK2I/SnRK2.3, involved in ABA signaling are essential for the control of seed development and dormancy. *Plant & Cell Physiology* **50**, 1345-1363.

Nambara E., Okamoto M., Tatematsu K., Yano R., Seo M., Kamiya Y. (2010) Abscisic acid and the control of seed dormancy and germination. *Seed Science Research* **20**, 55-67.

Nambara E., Naito S., McCourt P. (1992) A mutant of Arabidopsis which is defective in seed development and storage protein accumulation is a new abi3 allele. *The Plant Journal* **2**, 435-441.

Oracz K., El-Maarouf-Bouteau H., Farrant J., Cooper k., Belghazi M., Job C., Job D., Courbineau F., Bailly C. (2007) ROS production and protein oxidation as a novel mechanism for dormancy alleviation. *The Plant Journal* **50**, 452-465.
Oracz K., El-Maarouf-Bouteau H., Kranner I., Bogatek R., Courbineau F., Bailly C. (2009) The mechanism involved in seed dormancy alleviation by hydrogen cyanide unravel the role of reactive oxygen species as key factor of cellular signaling during germination. *Plant Physiology* **150**, 494-505.

Park S.Y., Fung P., Nishimura N., Jensen D.R., Fujii h., Lumba S., Santiago J., Rodrigues A., Chow T.F., Alfred S.E., Bonneta D., Finkelstein R., Provart N.J., Desveaux D., Rodrigues P.L., McCourt P., Zhu J.K., Schroeder J.I., Volkman B.F., Cutler S. (2009) Abscisic acid inhibits PP2C protein phosphatases via PYR/PYL family of START proteins. *Science* **324**, 1068-1071.

Peeters AJ, Blankestijn-De Vries H, Hanhart CJ, Léon-Kloosterziel KM, Zeevaart JA, Koornneef M. (2002) Characterization of mutants with reduced seed dormancy at two novel rdo loci and a further characterization of rdo1 and rdo2 in Arabidopsis. *Physiologia Plantarum* **115**, 604-612.

Penfield S., Josse E.M., Kannangara R., Gilday A.D., Halliday K.J., Graham I.A. (2005) Cold and light control seed germination through the bHLH transcption factor *SPATULA*. *Current Biology* **15**, 1998-2006.

Pernas M., García-Casado G., Rojo E., Solano R., Sánchez-Serrano J.J. (2007) A protein phosphatase 2A catalytic subunit is a negative regulator of abscisic acid signalling. *The Plant Journal* **51**, 763-78.

Pinto L.V., da Silva E.A., Davide A.C., De Jesus V.A., Toorop P.E., Hilhorst H.W. (2007) Mechanism and control of Solanum lycocarpum seed germination. *Annals of Botany* **100**, 1175-1187.

Probert R.J. (2000) The role of temperature in the regulation of seed dormancy and germination. In *Seeds: the Ecology of Regeneration in Plant Communities* (ed. Fenner M.) pp. 261-292 CAB International, Wallington, Oxon, UK.

Rashotte AM, DeLong A, Muday GK. (2001) Genetic and chemical reductions in protein phosphatase activity alter auxin transport, gravity response, and lateral root growth. *The Plant Cell* **13**, 1683–1697.

Raz V., Bergervoet J.H.W., Koornneef M. (2001) Sequential steps for developmental arrest in *Arabidopsis* seeds. *Development* **128**, 243-252.

Shi Y. (2009) Serine/Threonine phosphatases: mechanism through structure. *Cell* **139**, 468-484.

Stadtman E.R. (1992) Protein oxidation and aging. *Science* **257**, 1220-1224.

Sessions A., Yanofsky M.F. (1999) Dorsovenal patterning in plants. *Genes and Development* **13**, 1051-1054.

Sugimoto K., Takeuchi Y., Ebana K., Miyao A., Hirochika H., Hara N., Ishiyama K., Kobayashi M., Ban Y., Hattori T., Yano M. (2010) Molecular cloning of Sdr4, a regulator involved in seed dormancy and domestication of rice. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 5792-5797.

Tang W., Yuan M., Wang R., Yang Y., Wang C., Oses-Prieto J.A., Kim T.W., Zhou H.W., Deng Z., Gampala S.S., Gendron J.M., Jonassen E.M., Lillo C., DeLong A., Burlingame A.L., Sun Y., Wang Z.Y. (2012) PP2A activates brassinosteroid-responsive gene expression and plant growth by dephosphorylating BZR1. *Nature* **13**, 124-31

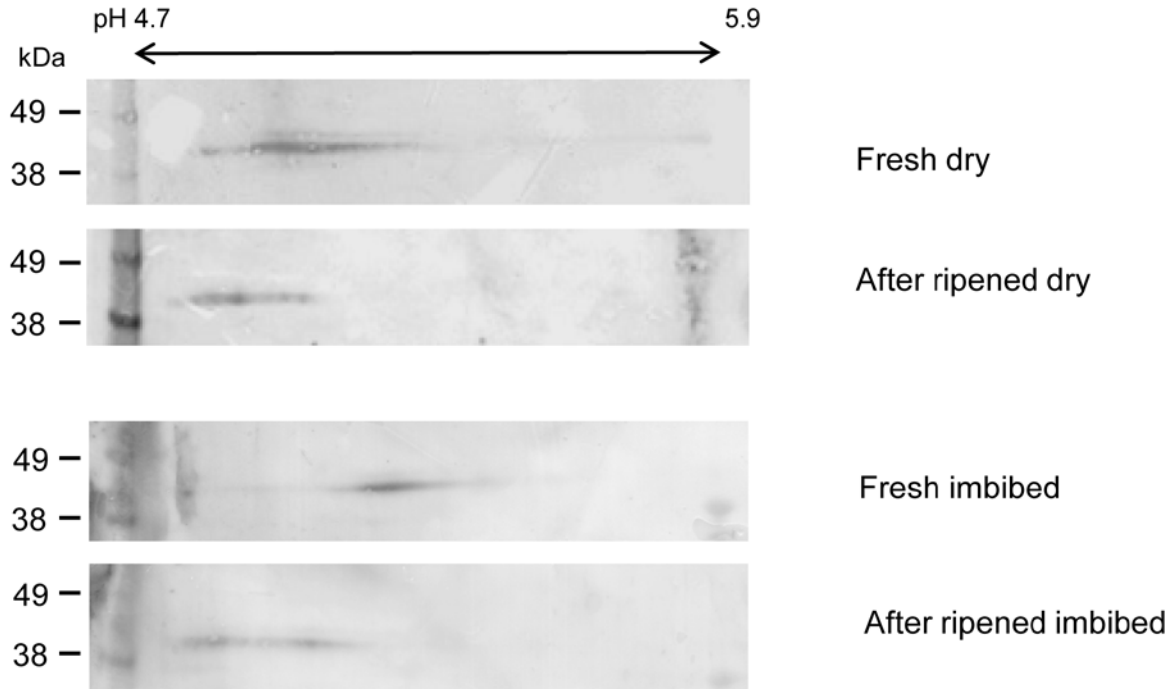
van Zanten M., Koini M.A., Geyer R., Liu Y., Brambilla V., Bartels D., Koornneef M., Fransz P. & Soppe W.J.J. (2011) Seed maturation in Arabidopsis is characterised by nuclear size reduction and increased chromatin condensation. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 20219-20224.

Wan, L., Ross A. R., Yang J., Hegedus D. D., Kermode A.R. (2007) Phosphorylation of the 12S globulin cruciferin in wildtype and *abi1-1* mutant *Arabidopsis thaliana* (thale cress) seeds. Biochemical journal **404**, 247–256.

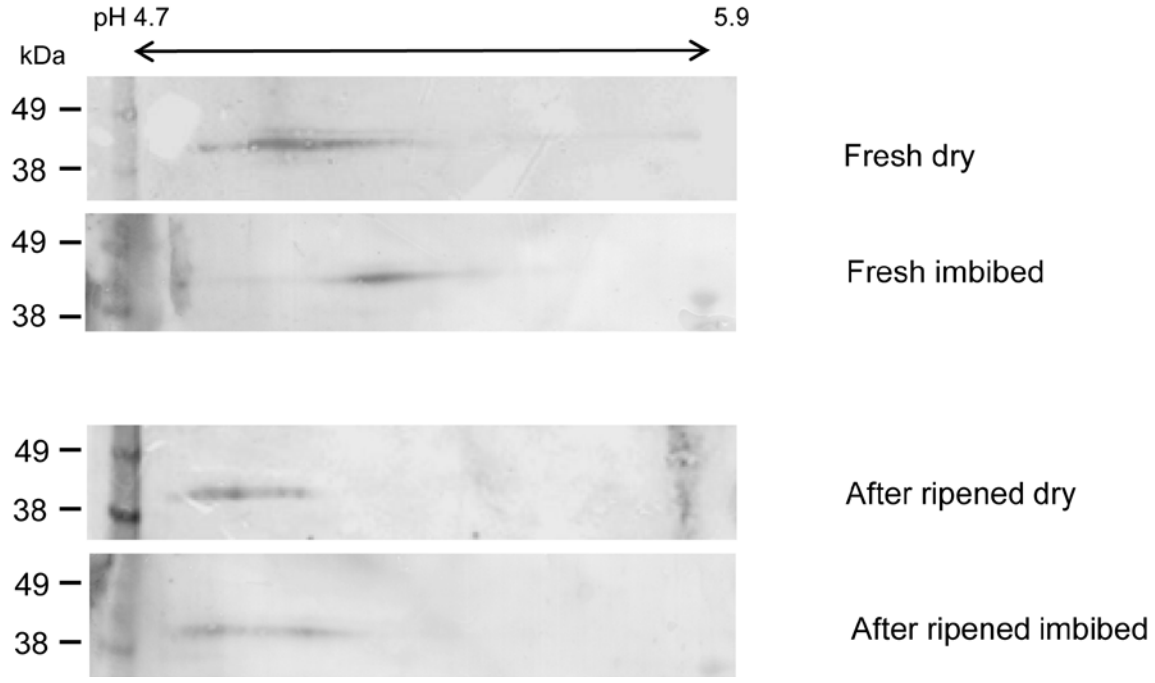
Wilson D.O. & McDonald M.B. (1986) The lipid peroxidation model of seed aging. *Seed Science & Technology* **14**, 269-300.

Zhou H.W., Nussbaumer C., Chao Y., DeLong A. (2004) Disparate roles for the regulatory A subunit isoforms in Arabidopsis protein phosphatase 2A. *The Plant Cell* **16**, 709-722.

Appendix



Appendix A.1. Two-dimensional immunodetection showing DOG1 altered isoelectrofocusing during after ripening in Columbia.
In after ripened dry seeds, DOG1 is detected as a major spot at pI ~4.85 plus several minor spots and it is shifted towards the acidic side as observed in after ripened imbibed seeds.
Total proteins were extracted from fresh and after ripened seeds of plants containing the ProDOG1:3xHA:DOG1 construct and DOG1 was detected with an anti-HA antibody. Here, 40 µg of total proteins were separated on a bis-tris 4-12% acrylamide gradient gel using a narrow range IPG strips. Seeds were imbibed for 16 hours and ripened for 12 weeks. The shift has been observed in three different biological replicates. pH and molecular mass marker are indicated respectively on the top and on the left of the blots.



Appendix A.2. Two-dimensional immunodetection showing DOG1 altered isoelectrofocusing upon imbibition in Columbia. When after ripened seeds are imbibed, DOG1 shifts towards the basic side as already oberserved with fresh seeds.
Total proteins were extracted from fresh and after ripened seeds of plants containing the ProDOG1:3xHA:DOG1 construct and DOG1 was detected with an anti-HA antibody. Here, 40 µg of total proteins were separated on a bis-tris 4-12% acrylamide gradient gel using a narrow range IPG strips. Seeds were imbibed for 16 hours and ripened for 12 weeks. The shift has been observed in three different biological replicates. pH and molecular mass marker are indicated respectively on the top and on the left of the blots.

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Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständing angefertig, die benutzen Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit- einschließlich Tabellen, Karten un Abbildungen-, die anderen Werken im Wortlaut oder dem Sinn nach entonommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfungs vorgelegen hat; dass sie – abgesehen von unten angegeben Teilpublikationen – noch nicht veröffentlich worden ist sowie, dass ich solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Maarten Koornneef betreut worden.

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Oktober 2005- Oktober 2007: Master Abschluss in Pflanzenbiotechnologie an der Universitá degli Studi di Milano, Italien. Praktische Arbeit am Parco Tecnologico Padano (Lodi, Italien). Titel der experimentellen Arbeit: "Identification and validation of candidate genes involved in development control of the spikelet in barley"

November 2007- September 2008: Stipendium für Absolventen der Biotechnologie an der Universitá degli Studi di Milano. Projekttitel: "Functional and morphological characterization of barley mutants affected in plant development". Praktische Arbeit am Parco Tecnologico Padano (Lodi, Italien)

Seit Oktober 2008: Promotion am Max-Planck-Institut für Pflanzenzüchtungsforschung. Köln, AG Wim Soppe, Leitung Prof. Dr. Maarten Koornneef. Thema: "Characterization of PDF1 and its interaction with DELAY OF GERMINATION1 (DOG1) in the control of seed dormancy in *Arabidopsis thaliana*".

Köln, September 2012