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Epigenetic control of seed dormancy using *Capsella bursa-pastoris* as a model species

Control epigenético de la dormición de semillas en *Capsella bursa-pastoris*

A thesis submitted by
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for the degree of Doctor of Philosophy
under the supervision of
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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
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RESUMEN (en español)

La dormición de semillas puede extender la germinación a través del tiempo para evitar condiciones ambientales desfavorables. Las transiciones de fase durante la vida de las semillas requieren de una reprogramación del genoma a gran escala, lo que se asocia con cambios en la estructura de la cromatina. Recientes investigaciones han desvelado el papel de la remodelación de la cromatina en la regulación de la dormición. A pesar de la gran importancia de esta, existe una enorme falta de conocimiento sobre su regulación a nivel molecular. La variación natural de las respuestas de los procesos de regulación epigenética al medio ambiente podría ser importante para la adaptación al clima. Es esencial comprender mejor la dormición de las semillas en un futuro en el que el impacto del cambio climático en las comunidades vegetales naturales es incierto.

Capsella bursa-pastoris tiene una distancia filogenética muy pequeña con *Arabidopsis thaliana*, especie relativamente rara en la naturaleza y que parece no ser competitiva. *C. bursa-pastoris* es una excelente elección como especie modelo para la investigación de la dormición de semillas. El objetivo principal de esta tesis doctoral fue determinar la posible implicación de procesos epigenéticos en la regulación de la inducción y el mantenimiento de la dormición secundaria en semillas y en las diferencias de profundidad entre los genotipos naturales de *C. bursa-pastoris*. La investigación se abordó utilizando un amplio conjunto de técnicas, incluida la cuantificación de la metilación global del ADN, inmunolocalizaciones y RNA-Seq.

Se encontró una variación sustancial en el potencial de inducción de dormición secundaria entre todas las accesiones estudiadas, siendo el genotipo el factor más importante. Se seleccionaron dos accesiones para un análisis RNA-Seq por sus respuestas extremas, la -367 (dormición profunda), y la -799 (dormición no-profunda).

Los patrones generales de H4Ac y 5-mC en las inmunolocalizaciones mostraron una amplia

variación dentro y entre los distintos tejidos de las semillas y entre los diferentes estados de dormición. Hubo un menor número de núcleos marcados con H4Ac en condiciones de dormición más profundas. Para las semillas con dormición primaria, hubo un mayor número de núcleos marcados con 5-mC y mayores niveles de metilación del ADN en estados de dormición más profundos. Cuando se indujo dormición secundaria, el nivel más alto de metilación del ADN se alcanzó después de 3 días en oscuridad, mostrando niveles decrecientes hacia una dormición más profunda. El mismo patrón se observó en las inmunolocalizaciones.

Los resultados del RNA-Seq mostraron una participación activa de la regulación epigenética en el establecimiento de las diferencias en la profundidad de dormición secundaria. La proteína *SNL1* y varias desacetilasas de histonas presentaron sobre-expresión y podrían estar afectando a la expresión de genes relacionados con la síntesis o señalización del ABA y de genes relacionados con el etileno, pero también podrían estar reprimiendo genes implicados en la síntesis, señalización y transporte de auxina. La comunicación cruzada de fitohormonas desempeña un papel importante en la regulación de la capacidad de germinación y en las diferencias en la profundidad de dormición secundaria entre accesiones. La hiper-acetilación causada por el ácido valproico provocó alteraciones en la expresión de genes implicados en la síntesis y señalización de diferentes fitohormonas. Las semillas embebidas en este inhibidor de desacetilasas de histonas tenían reprimida la biosíntesis de auxina.

Nuestros datos destacan el papel de la desacetilación de histonas en el establecimiento de las diferencias en la profundidad de dormición secundaria en semillas. Podría existir una vía común en la regulación de la inducción de dormición secundaria y en el retraso en la germinación a través de los complejos de desacetilización de histonas, con las proteínas SNL como componentes centrales.

RESUMEN (en Inglés)

The function of seed dormancy is to spread germination across time in synchrony with seasonal cycles to avoid unfavourable environmental conditions. Phase transitions during seeds' life require genome reprogramming at a large scale, which is often associated with major changes in chromatin structure. Recent advances have highlighted the role of chromatin remodelling in dormancy regulation. Despite the high importance of dormancy for plants competitiveness and life cycle timining, there is an enourmous lack of knowledge about its regulation at the molecular level. Natural variation in the responses of the epigenetic regulation processess to the environment could be informative regarding adapatation to climate. A greater understanding of seed dormancy is essential in a future where the impact of

climate change on natural plant communities is uncertain.

Capsella bursa-pastoris has a very small phylogenetic distance with *Arabidopsis thaliana*, but the latter appears to be uncompetitive and is actually relatively rare in the wild. We think that *C. bursa-pastoris* is an excellent choice as a model species for seed dormancy research. The principal aim of this doctoral thesis was to determine the possible implication of epigenetic processes in the regulation of secondary seed dormancy induction and maintenance and in the depth differences between natural genotypes of *C. bursa-pastoris*. Our research was approached using a broad set of techniques, including quantification of global DNA methylation, immunolocalizations and RNA-Seq.

Substantial variation in the potential of induction of secondary seed dormancy was found between the whole range of seed accessions studied, with genotype being the most important factor. Two accessions were selected for their extreme responses to the conditions tested, -367, (deep-dormant), and -799 (non-deep dormant), for an RNA-Seq analysis. The general patterns of H4Ac and 5-mC in the immunolocalizations showed wide varation within and between different tissues of the seeds and between different dormancy states. There was a lower number of H4Ac marked nuclei in deeper dormancy conditions. For primary dormant seeds, there was a higher number of 5-mC marked nuclei and higher DNA methylation levels in deeper dormant states. When secondary seed dormancy was induced, the highest DNA methylation level was reached after 3 d of imbibition in darkness, showing decreasing levels towards a deeper dormancy. The same pattern was observed in the immunolocalizations.

The RNA-Seq results showed an active involvement of epigenetic regulation in the establishment of different secondary seed dormancy depths. The up-regulated *SNL1*, and several other up-regulated histone deacetylases, could be affecting the expression of genes related to the synthesis or signalling of ABA and of genes related to ethylene, but also they could be implicated in the down-regulation of genes implicated in the synthesis, signalling and transport of auxin. The cross-talk between phytohormones plays an important role in the regulation of the germination capability and in the differences in secondary dormancy depth between accessions. The hyper-acetylation caused by valproic acid provoked alterations in the expression of genes implicated in the biosynthesis and signalling pathways of different phytohormones. Seeds imbibed in this histone deacetylase inhibitor had the biosynthesis of auxin repressed.

Our data clearly highlight the role of histone deacetylation in the establishment of secondary seed dormancy depth differences. These results could be indicating a common pathway in the regulation of secondary seed dormancy induction and in the delay of germination through

histone deacetylase complexes, with SNL proteins as central components.

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A mi familia

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List of Abbreviations

°C	Celsius Degrees
µL	Microliter
µM	Micromolar (µmol × L^{-1})
mM	Millimolar (mmol × L^{-1})
5-mC	5-methylcytosine
ABA	Abscisic Acid
ANOVA	Analysis of Variance
bp	Basepairs
BSA	Bovine Serum Albumin
BRs	Brassinosteroids
cm	Centimeters
CTAB	Cetyltrimethylammonium Bromide
d	Day
DAPI	4',6-diamino-2-phenylindole
DD	Days in Darkness
DEGs	Differently Expressed Genes
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
FDR	False Discovery Rate
g	Gram(s)
x g	Gravity, Unity of Relative Centrifugal Force (RCF)
GAs	Gibberellins
GLM	Generalised Linear Model
GO	Gene Ontology
GOs	Gene Ontology terms
h	Hour

H4Ac	Histone H4 Acetylated
HATs	Histone Acetylases
HDACs	Histone Deacetylases
IAA	Indole-3-Acetic Acid
m	Meter
mL	Milliliter
MPa	MegaPascal
ng	Nanogram
nm	Nanometer
PBS	Phosphate Buffer Saline
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PDS	Primary Dormant Seeds
RAM	Root Apical Meristem
RdDM	RNA-directed DNA Methylation
RH	Relative Humidity
ROS	Reactive Oxygen Species
RT	Room Temperature
rRNA	Ribosomal Ribonucleic Acid
mRNA	Messenger Ribonucleic Acid
siRNA	Small Interfering RNAs
miRNA	MicroRNAs
lncRNA	Long non-coding RNAs
RNA-Seq	RNA-sequencing
s	Second
SAHA	Suberoylanilide Hydroxamic Acid
SAM	Shoot Apical Meristem
SE	Standard Error
SEM	Scanning Electron Microscopy
TSA	Trichostatin A

TukeyHSD	Tukey Honest Significant Differences
V	Volt
WT	Wild Type

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CHAPTER 1

General Introduction

Plants are sessile organisms and seeds are the most common mobile phase in their life cycle. Seeds transport plant's genetic material through space and time and can remain dormant in the soil until the environmental conditions are suitable for germination and for the germinated plant to survive (Footitt et al., 2011). They act as highly effective sensors and interpreters of the spatial environment (Footitt et al., 2017). Most of them are well equipped for extended periods of unfavourable conditions, being potentially able to survive for years or decades (Cadman et al., 2006; Long et al., 2015; Footitt and Finch-Savage, 2017).

Seeds consist of three major regions, embryo, endosperm and testa (seed coat), which have different genetic origins, unique functions and distinct developmental pathways. The seed coat transfers nutrients from the maternal plant to the embryo during seed development and protects the seed in the soil and during dormancy. The endosperm provides nourishment to the embryo and in some dicots, such as *Arabidopsis* or *Capsella*, it degenerates and remains as a cell layer in the mature seed directly underneath the testa. Finally, the embryo (enclosed by the testa and endosperm), differentiates into axis and cotyledon regions, with the axis giving rise to the seedling after germination and the cotyledons accumulating storage reserves for the germinating seedling (*Figure 1.1.*) (Belmonte et al., 2013; Chen et al., 2018).

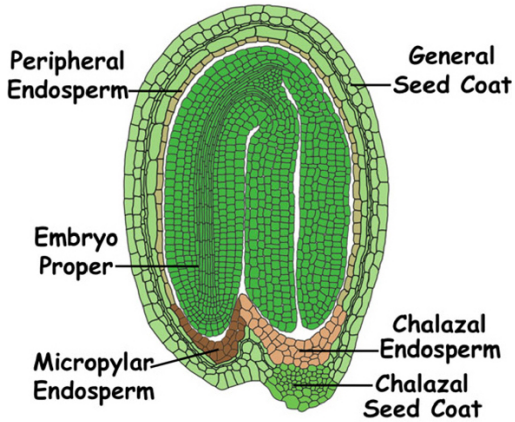

Figure 1.1. Section through an *Arabidopsis* seed depicting the different seed compartments (modified from The Seed Gene Network, www.seedgenenetwork.net/seeds).

Seeds of the model species *Arabidopsis* consist of a relatively large embryo surrounded by a single-cell layer of endosperm and a seed coat or testa and present non-deep physiological dormancy (Debeaujon and Koornneef, 2000).

Seed germination

Plants have evolved a series of strategies to regulate seed germination to ensure successful seedling establishment in the natural environment. Germination commences with the uptake of water by the dry seed and terminates with the elongation of the embryonic axis, which is determined by the balance of the growth potential of the embryo and the mechanical resistance of the covering tissues (Bewley and Black, 1994; Nonogaki, 2019).

The water uptake by a seed has three phases with an initial rapid uptake (phase I) followed by a plateau (phase II). Phase III consists of a further uptake of water as the embryonic axis elongates and breaks through the covering layers to complete germination (Finch-Savage and Leubner-Metzger, 2006). Phases I and II occur in dormant and germinating seeds, while phase III occurs only in the latter ones.

Germination occurs when a non-dormant, mature seed meets permissive environmental conditions regarding humidity, light and temperature and in *Arabidopsis* consists of two visible sequential events (Holdsworth et al., 2008). First, the testa splits (testa rupture) due to underlying expansion of the endosperm and embryo and thereafter, the radicle protrudes through the endosperm, completing germination *sensu stricto* (*Figure 1.2.*) (Finch-Savage and Leubner-Metzger, 2006; Holdsworth et al., 2008; Dekkers et al., 2013).

When seeds are hydrated and placed under conditions appropriate for germination, seed dormancy can be assessed (Finch-Savage and Footitt, 2017).

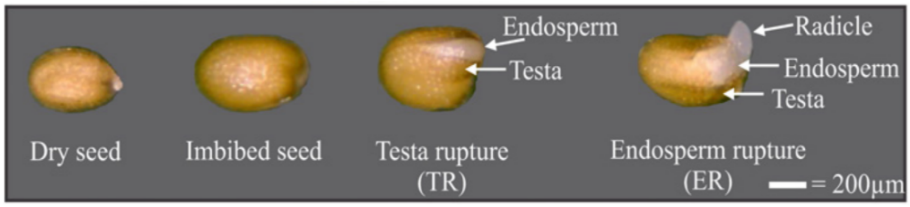

Figure 1.2. Different stages during seed germination including testa rupture (which exposes the underlying endosperm layer) and endosperm rupture (germination *sensu stricto*) [modified from Dekkers et al. (2013)].

Seed dormancy and seed dormancy cycling

Seed germination has to occur according to precise timing to ensure the survival of the seedling. The function of dormancy is to spread germination across time in synchrony with seasonal cycles to avoid unfavourable environmental conditions, in order to maximize competitive advantages and to ensure the establishment of the new generation of plants (Baskin and Baskin, 2014).

Seed dormancy is one of the most important adaptive traits in plants as it allows them to regulate when and where to grow (Ibarra et al., 2016). It has been defined as the failure of a viable seed to germinate in a specified period of time under any combination of normal physical environmental conditions that are otherwise favourable, with it being an innate property of the seed (Bewley, 1997; Baskin and Baskin, 2004). Any cue that widens the conditions that permit the completion for germination is therefore a dormancy release factor (Finch-Savage et al., 2007).

Different studies have demonstrated that dormancy is not a quiescent state and has characteristic transcriptional activity (Cadman et al., 2006; Finch-Savage et al., 2007; Dekkers et al., 2013, 2016). Seeds act as environmental sensors, adjusting their dormancy depth to a range of signals. Some of these signals (such as soil temperature or moisture) are related to slow seasonal changes and are integrated over time to alter the depth of dormancy and the sensitivity to a second set of signals (light, nitrate supply, alternating temperatures). This second set of signals indicates a more immediate situation where conditions are suitable to terminate dormancy and induce germination (Footitt et al., 2011).

Seed dormancy is considered to be a continuum with layers of dormancy that can be progressively removed by the correct order of environmental signals until only the final layer, light, is required (Finch-Savage and Leubner-Metzger, 2006). The precise response to the environmental signals necessary for dormancy release and germination induction is distinct between species and between genotypes of the same species. For example, in *A. thaliana*, the differences in responses to the environment can result in distinct seasonal patterns of germination, with different populations able to express a winter or a summer annual life history or sometimes both (Donohue et al., 2005). A winter annual is a genotype adapted to germination in autumn, while a summer annual is adapted to germination in spring (Finch-Savage and Leubner-Metzger, 2006).

Baskin and Baskin (2004) proposed a modified version of M.G. Nikolaeva's classification system for seed dormancy with five classes, including different levels and types (*Table 1.1.* and *Table 1.2.*).

Table 1.1. Classification system for seed dormancy [modified from (Nikolaeva, 1977; Baskin and Baskin, 2004)].

A. Class	Physiological dormancy (PD) Levels – deep, intermediate, non-deep Types – 1, 2, 3, 4 and 5 (of non-deep PD)
B. Class	Morphological dormancy (MD)
C. Class	Morphophysiological dormancy (MPD) Levels – non-deep simple, intermediate simple, deep simple, deep simple epicotyl, deep simple double, non-deep complex, intermediate complex and deep complex
D. Class	Physical dormancy (PY) (probably needs to be subdivided)
E. Class	Combinational dormancy (PY + PD) Level – non-deep PD (probably both Type 1 and Type 2 are represented)

Table 1.2. Characteristics of dormancy in seeds with deep, intermediate and non-deep physiological dormancy (Baskin and Baskin, 1998).

Deep	Excised embryo produces abnormal seedling Gibberellins do not promote germination Seeds require *c*. 3–4 months of cold stratification to germinate
Intermediate	Excised embryo produces normal seedling Gibberellins promote germination in some (but not all) species Seeds require 2–3 months of cold stratification for dormancy break Dry storage can shorten the cold stratification period
Non-deep	Excised embryo produces normal seedling Gibberellins promote germination Depending on species, cold (*c*. 0–10°C) or warm (≥ 15°C) stratification breaks dormancy Seeds may after-ripen in dry storage Scarification may promote germination

This classification system shows the great diversity of morphological and physiological features that have evolved to control dormancy in response to distinct environments (Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006).

Non-deep physiological dormancy is the most abundant, evolutionarily advanced and phylogenetically spread class of dormancy, occurring in gymnosperms and in all major clades of angiosperms and also being the only kind of dormancy found in the Poaceae and Asteraceae families (Baskin and Baskin, 2004). Moreover, it is the class and level of dormancy in seeds of wild populations

of *A. thaliana*, so its study at the biochemical, molecular and genetic levels can have broad applications in explaining the basic mechanisms of physiological dormancy in seeds (Baskin and Baskin, 2004).

The type of dormancy that freshly mature water-permeable seeds present at shedding from the mother plant is called primary dormancy. It is genetically determined and, as it develops during seed maturation, the environmental conditions experienced by the mother plant (temperature, nitrate, photoperiod) can influence its depth (Kendall and Penfield, 2012). A non-dormant seed is one with the capacity to germinate over the widest range of normal environmental factors possible for the genotype (Baskin and Baskin, 2004). After-ripening, dry storage of ripe seeds, leads to a reduction in dormancy and can take from few weeks up to several months depending on the accession (Bentsink et al., 2010). The precise mechanisms underlying after-ripening processes are not known, although they have been correlated with changes in gene expression, enzyme activity, and hormone accumulation, suggesting that transcription and translation can occur in dry seeds (Finkelstein et al., 2008). Dormancy can also be broken by environmental signals such as cold stratification in the presence of water (Graeber et al., 2012).

Secondary dormancy is referred to the dormancy state induced in non-dormant seeds by unfavourable conditions or re-induced in dormant seeds after a sufficiently low dormancy has been reached (Benech-Arnold et al., 2000). Only those seeds with the genetic capacity for primary dormancy can be induced into secondary dormancy (Baskin and Baskin, 2004).

Once primary dormancy is lost in response to environmental conditions, secondary dormancy can be induced if the conditions are not adequate for germination (Finch-Savage and Leubner-Metzger, 2006). The release from primary dormancy followed by the entrance into a deeper secondary dormant state leads to dormancy cycling in the soil seed bank (*Figure 1.3.*). Dormancy cycling involves repeated induction and release of secondary dormancy parallel to seasonal variation of the soil environment (Benech-Arnold et al., 2000). Only those seeds with non-deep physiological dormancy may cycle in the soil seed bank (Baskin and Baskin, 2004).

A portion of the seed population may contain seeds that are dormant, but at the same time, other portions may have non-dormant or conditionally dormant seeds. Those seeds that present an intermediate state of dormancy in this cycling in the soil seed bank are said to have a conditional or relative dormancy (Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006). All the dormant imbibed states are responsive at the molecular level to the soil environment signals by continually adjusting the dormancy depth (Footitt et al., 2011, 2013, 2014).

Thus, seeds in the soil seed bank are continually adjusting their dormancy depth in order to synchronize germination and seedling emergence to an appropriate climate space and time of the year, being able to compete within species-rich natural communities (Baskin et al., 2006; Footitt et al., 2015).

Despite the high importance of dormancy and dormancy cycling for plants competitiveness and life cycle timing, there is an enormous lack of knowledge about its regulation at the molecular level.

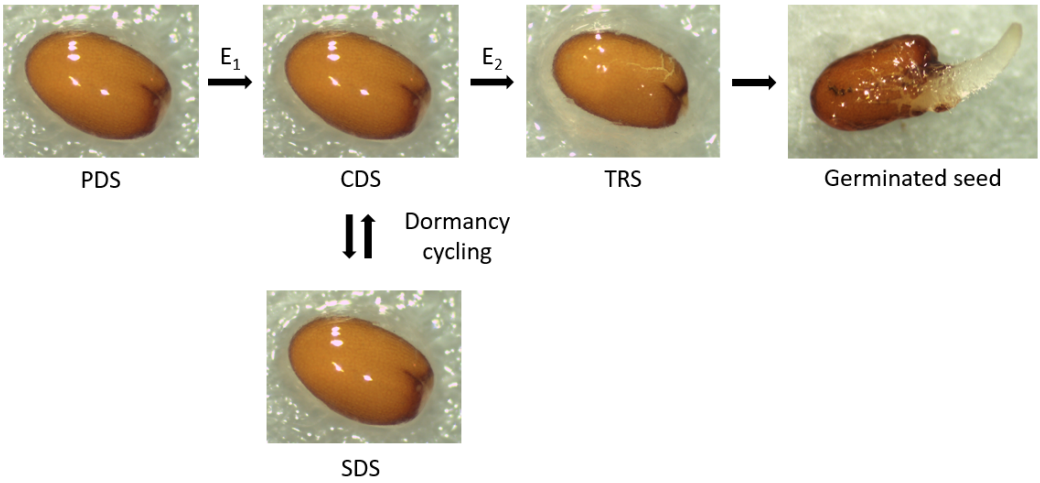

Figure 1.3. Model for transitions between dormant and after-ripened states in physiologically dormant *Capsella bursa-pastoris* seeds. Seeds present primary dormancy (primary dormant seeds, PDS) when shed from the mother plant. A set of environmental conditions (E1), e.g. dry after-ripening or moist chilling, is required to reduce dormancy. The conditionally dormant seeds (CDS) will germinate in the right set of environmental conditions (E2), e.g. light and optimal temperature. When E2 is not (fully) present, seeds will enter secondary dormancy (secondary dormant seeds, SDS). Secondary dormancy may be broken in the right set of conditions to enter conditionally dormancy again. This cycle may be repeated. TRS: testa ruptured seeds. [Adapted from Cadman et al. (2006)].

Molecular/genetic regulation of seed dormancy and germination

The central involvement of the abscisic acid (ABA)/gibberellins (GAs) balance in the ability of the seeds to interpret the environment and thereby regulate (primary) dormancy and germination completion has been accepted (Kucera et al., 2005). In this model, ABA and GAs simultaneously and antagonistically regulate the induction, maintenance and termination of (primary) dormancy (Baskin and Baskin, 2004). The dormancy status results from the response to this balance through hormone-signalling networks that influence sensitivity to ABA and GAs (Footitt et al., 2011).

Seed development is characterized by two peaks of ABA accumulation that occur during the mid- and late-phases of seed maturation, with the latter peak playing a key role in the induction of primary seed dormancy (Nambara et al., 2010). ABA is an important positive regulator of both the induction of dormancy and the maintenance of the dormant state in imbibed seeds following shedding (Finch-Savage and Leubner-Metzger, 2006). The application of exogenous ABA

inhibits seed germination and mutants defective in its biosynthesis or signalling have enhanced germination efficiency (Finkelstein et al., 2008).

The level of ABA in plant tissues is regulated by its biosynthesis and catabolism (Nambara and Marion-Poll, 2005). Key genes for ABA synthesis in the embryo and in the endosperm during seed development are the *9-CIS-EPOXYCAROTENOID DEOXYGENASE 6* (*NCED6*) and *NCED9*. Induction of *NCED6* during imbibition is sufficient to prevent seed germination (Martínez-Andújar et al., 2011). ABA levels decrease at the end of seed maturation and during imbibition due to the activity of ABA catabolism genes that belong to the Cytochrome P450, family 707, subfamily A (*P450 CYP707A*) (Bentsink and Koornneef, 2008). The *cyp707a2* and *cyp707a1* single mutants and the double mutant accumulate ABA in dry and imbibed seeds and exhibit enhanced seed dormancy (Okamoto et al., 2006). ABA perception by PYRABACTIN RESISTANCE 1 (PYR1)/PYR1-LIKE (PYL1) proteins plays a major role in the regulation of seed dormancy and germination. Plants lacking from three to six of these proteins present enhanced germination, indicating a quantitative regulation by this family of receptors (Gonzalez-Guzman et al., 2012).

GAs are other major phytohormones that promote germination by inducing hydrolytic enzymes that weaken the barrier tissues (endosperm or testa), inducing mobilization of seed storage reserves and stimulating expansion of the embryo (Kucera et al., 2005). The level of biologically active GAs in plant tissues is determined by the balance between their biosynthesis and inactivation (Yamaguchi, 2008). The biosynthesis of GAs is regulated mainly by reactions catalysed by GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox), while their inactivation is controlled primarily by GA 2-oxidase (GA2ox) (Tuan et al., 2018). However, GAs treatment alone does not stimulate germination in fully dormant seeds of *A. thaliana*. GAs signalling involves the DELLA (aspartic acid–glutamic acid–leucine–leucine–alanine) proteins REPRESSOR OF GA (RGA), GIBBERELLIC ACID INSENSITIVE (GAI) and RGA-LIKE 2 (RGL2) that play negative roles in seed germination (Finkelstein et al., 2008; Rajjou et al., 2012).

ABA and GAs signalling and sensitivity are the more likely regulators of dormancy than the absolute level of these hormones (Ali-Rachedi et al., 2004). Changes in the balance between ABA and GAs levels are associated with alterations in the expression patterns of their metabolic genes (Tuan et al., 2018). For example, although the maintenance of primary dormancy depends on high ABA/GAs ratios, dormancy release is characterized by an enhanced ABA degradation and increased GAs biosynthesis. This results in a low ABA/GAs ratio, which is followed by GAs promotion of germination (Ali-Rachedi et al. 2004; Cadman et al., 2006).

In addition to ABA and GAs, there are other phytohormones with implications in the regulation of seed dormancy and germination. Brassinosteroids (BRs) are plant steroid hormones involved in stem elongation and leaf unfurling

that promote germination. BRs mutants are hypersensitive to inhibition of germination by ABA in comparison to wild-type seeds (Finkelstein et al., 2008). BRs molecules may promote seed germination by enhancing embryo growth potential in a gibberellin-dependent manner (Leubner-Metzger, 2001).

Ethylene can stimulate germination and repress dormancy establishment in seeds by antagonizing the ABA pathway (Matilla and Matilla-Vázquez, 2008). Seeds of *ethylene resistant 1 receptor* (*etr1*) mutants display increased dormancy and their germination is ABA hypersensitive (Beaudoin et al., 2000). Mutations in *ENHANCED RESPONSE TO ABA 3* (*ERA3*)/*ETHYLENE INSENSITIVE 2* (*EIN2*) genes lead to an overaccumulation of ABA and increased seed dormancy, suggesting that *ERA3*/*EIN2* is a negative regulator of its synthesis (Ghassemian et al., 2000). The biosynthesis of ethylene during seed germination is regulated by 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) and is involved in counteracting the inhibitory effects of ABA (Linkies et al., 2009). However, the ethylene-ABA antagonism during germination and dormancy in seeds is still poorly understood.

Other hormones that are known for their implications in germination are auxins (Holdsworth et al., 2008). They have been shown to function both positively and negatively in seed germination depending on their dose. For example, exogenous application of high concentrations of indole-3-acetic acid (IAA) can inhibit seed germination in *Arabidopsis* (Brady et al., 2003; Liu et al., 2007a). On the contrary, low concentrations of IAA can promote seed germination and seedling establishment (He et al., 2011). Different studies indicate that distinct auxin signalling pathways are involved in seed germination by affecting ABA and/or GAs pathways, achieving these functions through the auxin transport carriers in the root tip (Ugartechea-Chirino et al., 2010).

In addition to the role of phytohormones, *ABSCISIC ACID INSENSITIVE 3* (*ABI3*), *FUSCA 3* (*FUS3*) and *LEAFY COTYLEDON 1* (*LEC1*) and *LEC2* are four key regulators that play prominent roles in controlling mid and late seed development. All four mutants, *abi3*, *fus3*, *lec1* and *lec2*, have altered seed maturation and decreased primary dormancy (Bentsink and Koornneef, 2008). These regulators interact as a network that controls various aspects of seed maturation, such as the expression of the late embryogenesis abundant (LEA) proteins and dehydrins, both related to desiccation tolerance (Gutierrez et al., 2007). Besides, *ABSCISIC ACID INSENSITIVE 4* (*ABI4*) and *ABI5* are also involved in many aspects of seed maturation through the interaction with *LEC1*, *ABI3* and *FUS3* (Reeves et al., 2011; Gao et al., 2012). LEC transcription factors also help to establish the high ABA/GAs ratio characteristic of the maturation phase by enhancing ABA levels (Braybrook and Harada, 2008). On the other hand, FUS3 and LEC2 were found to inhibit GAs biosynthesis through the repression of GAs biosynthetic genes (Gutierrez et al., 2007; Suzuki and McCarty, 2008).

DELAY OF GERMINATION 1 (*DOG1*) has been discovered to be essential for determining the depth of primary dormancy (Bentsink et al., 2006). It is a putative DNA-binding transcription factor, linked to accumulation of thermal time (Footitt et al., 2015) and its mutations can completely remove seed dormancy (Bentsink et al., 2006). The relationship between *DOG1* and the hormone balance previously described is beginning to be elucidated. DOG1 physically interacts with two phosphatases, ABA-HYPERSENSITIVE GERMINATION 1 (AHG1) and AHG3, to block their downstream roles in the release of seed dormancy. Besides, PROTEIN PHOSPHATASE 2A SUBUNIT A2 (PP2AA/PDF1) also physically interacts with DOG1, although acting upstream to have a negative role in seed dormancy (Née et al., 2017). The transcription factor DOG1 transduces environmental effects during seed maturation to alter the depth of dormancy (Kendall and Penfield, 2012), but its expression does not seem to determine the pattern of dormancy cycling in the soil seed bank (Footitt et al., 2013). Footitt et al. (2020) demonstrated that although the dormancy depth at the end of maturity (primary dormancy) is determined by DOG1, this is not the case for the post shedding annual dormancy cycle (including secondary seed dormancy) (*Figure 1.4.*).

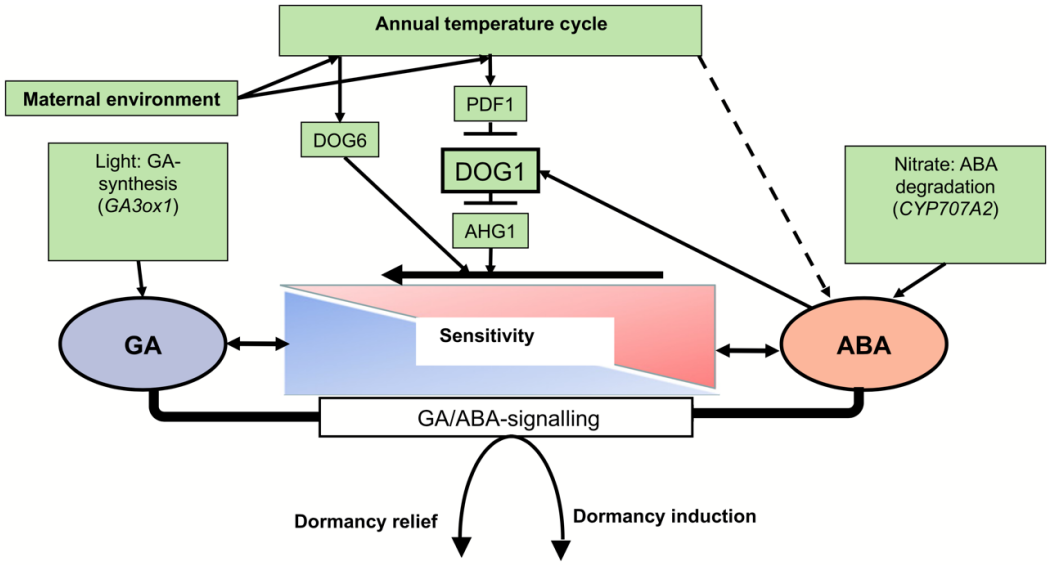

Figure 1.4. Schematic model for the regulation of dormancy cycling in *Arabidopsis thaliana*, proposed by Footitt et al. (2020). Maternal environment affects DOG1 and DOG6 (ANAC060) to determine initial depth of dormancy and their influence differs with accession. DOG1 and DOG6 expression patterns alter during the annual cycle, where DOG1 acts as a means of accumulating thermal time (Footitt et al., 2015; Finch-Savage & Footitt, 2017). PDF1 acts upstream of DOG1 to reduce depth of dormancy and may therefore facilitate the DOG1 environmental response (Née et al., 2017). In winter, DOG1 expression is high, AHG1 action is suppressed by DOG1, and seeds are not sensitive to spatial signals. During spring, DOG1 expression decreases to reduce suppression of AHG1, with a concurrent reduction in sensitivity to ABA. Presence of AHG1 therefore determines the timing of subsequent germination and seedling emergence. In response, there is increased sensitivity to spatial signals (light and nitrate) that further alter the hormone balance and remove the final layer of dormancy in favor of germination completion.

Epigenetic regulation in plants

Plants, as they have a sessile life, are often exposed to different environmental conditions. Not being able to move, and needing to cope with an unfavourable environment, has favoured the evolution of sophisticated developmental mechanisms to ensure a plastic response to these conditions (de la Paz Sanchez et al., 2015). Plasticity is responsible for allowing gene expression to adapt to biotic and abiotic stresses or variations in altitude, soil composition, day length, temperature, among others (Latzel et al., 2012). Various molecular pathways can be involved in plant plasticity, and some of them are mediated by epigenetic factors (Nicotra et al., 2010).

Epigenetics refers to the changes in gene expression that are stable and transmitted through cell divisions or across generations and that do not involve alterations in the DNA sequence (Berger et al., 2009). Different epigenetic modifications have been described, including DNA methylation, histone modifications, non-coding RNAs and chromatin remodelling (Gady et al., 2017). Multiple aspects of plant development, including flowering time, gametogenesis, stress response, light signalling, and morphological changes are modulated directly or indirectly by epigenetic marks (Feng and Jacobsen, 2011).

In eukaryotic cell nuclei, genomic DNA is packed in a highly organized nucleoprotein complex named chromatin. For the compaction to happen, 147 bp of DNA are wrapped into an octamer of core histones building the basic unit, the nucleosome. A single nucleosome is composed of two H3-H4 histone dimers bridged together forming a stable tetramer that it is flanked by two separated H2A-H2B dimers and then linked by H1 at the entry/exit point (Liu et al., 2014; Gady et al., 2017).

Nucleosomes are not static structural units, as they can be moved, stabilized/destabilized or disassembled/reassembled at particular genome locations in response to specific signals (Andrews and Luger, 2011). Different mechanisms can alter the chromatin structure and the properties of nucleosomes in order to control gene expression, including DNA methylation, covalent posttranslational modifications of histones (PTMs), ATP-dependent chromatin remodelling and replacement of canonical histones with specialized variants.

DNA methylation

DNA methylation consists of the covalent addition of a methyl group at the C-5 position of cytosine (5-mC). It is also possible to find methylated adenine in small amounts in the DNA (Vanyushin, 2006). In the genome of higher plants it is widely found and is catalysed by DNA methyltransferases in CG, CHG and CHH (where H = A, T or C) sequence contexts (Law and Jacobsen, 2010). DNA methylation can be heritable through cell division. DNA replication adds unmethylated cytosines, resulting in hemimethylation at previously methylated

sites in all contexts. Methylation of the newly synthesized strand can occur through context-specific mechanisms and the *Arabidopsis* genome codifies DNA methyltransferases with different biological functions and target specificities:

1) DNA methyltransferase 1 (MET1): catalyses methylation at CG dinucleotides. Enzymes such as MET1 recognize the hemimethylated DNA and direct the maintenance methyltransferase to methylate the symmetrical unmethylated cytosine.

2) Chromomethylases (CMT3 and CMT2): catalyse the methylation of CHG sites. CHG methylation can be transmitted to newly replicated DNA in a similar fashion as with CG contexts.

3) Domains rearranged methyltransferases (DRM1 and DRM2): *de novo* methyltransferases that catalyse methylation in the non-symmetrical CHH contexts, with some contribution of CMT3. For these sites, inherited or newly generated small interfering RNAs (siRNAs) can direct DNA methylation via the RNA-directed DNA methylation (RdDM) pathway, where DRM2 plays a central role.

4) In addition to the enzymes that can directly transfer methyl groups, there are proteins with catalytic activities that can indirectly affect DNA methylation levels, such as decreased in DNA methylation 1 (DDM1) that encodes the chromatin remodelling factor SWI2/SNF2.

Euchromatin is associated with hypo-methylated DNA in actively transcribed regions, while heterochromatin contains silenced genes which are frequently hyper-methylated (Zhang et al., 2018). In the genic regions, CG and non-CG methylation at the promoter are associated with gene silencing whereas CG methylation is also detected within the transcribed region of moderately expressed genes (Zhang et al., 2006). However, DNA methylation marks are reversible, which confers plasticity by facilitating the modulation of RNA transcription from regions or specific loci.

Demethylation also influences DNA methylation patterns. Passive demethylation occurs through the lack of active maintenance of DNA methylation, while active DNA demethylation happens through catalytic removal of 5-mC (Zhang and Zhu, 2012). This latter process is catalysed by a family of glucosidases, which can act in a developmentally programmed way to induce locus-specific loss of DNA methylation (Eichten et al., 2014).

Therefore, the level and pattern of DNA methylation is not only the consequence of a homeostatic balance between DNA replication, *de novo* methylation and maintaining methylation and demethylation (Hsieh, 2000), but also the coordinated expression and function of distinct DNA methyltransferases,

glycosylases and other chromatin remodelling factors (*Figure 1.5.*) (Zhu, 2009; Zhang et al., 2018).

Methylation of the DNA plays a predominant role in the normal development of plant ontogenesis in response to internal cues but also as an intermediate in the transduction cascade of external stimuli leading to gene expression changes (Chinnusamy and Zhu, 2009).

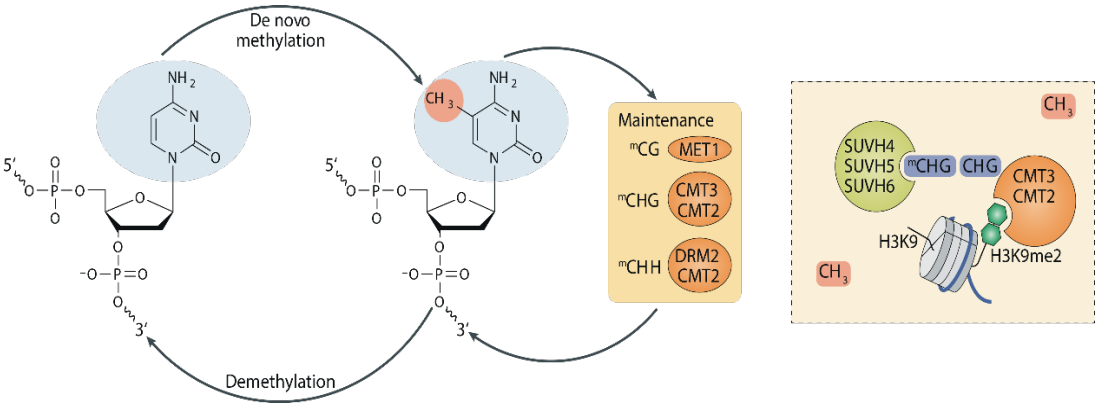

Figure 1.5. *De novo* DNA methylation can occur in all cytosine contexts. Following DNA replication, methylation in the symmetric CG context is maintained by METHYLTRANSFERASE 1 (MET1), whereas CHG (H represents A, T or C) methylation is maintained by CHROMOMETHYLASE 3 (CMT3) or CMT2. Methylated CHG recruits the histone H3 lysine 9 (H3K9)-specific methyltransferases SUPPRESSOR OF VARIEGATION 3-9 HOMOLOGUE PROTEIN 4 (SUVH4), SUVH5 and SUVH6, and, in turn, dimethylated H3K9 (H3K9me2) facilitates CMT3 and CMT2 function, thereby forming a reinforcing loop between CHG methylation and H3K9 methylation. Methylation in the asymmetric CHH context is maintained by DOMAINS REARRANGED METHYL ASE 2 (DRM2) through RNA-directed DNA methylation (RdDM) or by CMT2. DNA methylation can be removed by active demethylation that is initiated by DNA demethylases or by passive demethylation owing to failure in methylation maintenance following DNA replication [Adapted from Zhang et al. (2018)].

Post-translational modifications (PMTs) of histones

Histones comprise three domains that include a globular domain involved in the histone-histone interaction, an N-terminal tail for histones H3 and H4, as well as the C- and N-terminal tails for histones H2A and H2B. The terminal tails that extend from the core of the nucleosome provide sites for distinct posttranslational modifications, such as acetylation, methylation, phosphorylation, ubiquitination and ADP-ribosylation (Berger, 2007). Posttranslational modifications at a specific residue confer distinct physical properties to chromatin, altering the accessibility of the general transcription machinery including DNA or RNA polymerases to the DNA strand (Itabashi et al., 2018).

Different histone marks have different functions and even the same mark can have different functions in distinct organisms (Feng and Jacobsen, 2011). Histone modifications, alone or in combination, provide binding platforms for chromatin-associated proteins that initiate or block gene transcription (*Figure 1.6.*) (Fuks, 2005; Pfluger and Wagner, 2007). These PMTs are reversible and maintained by the action of different histone modifying enzymes.

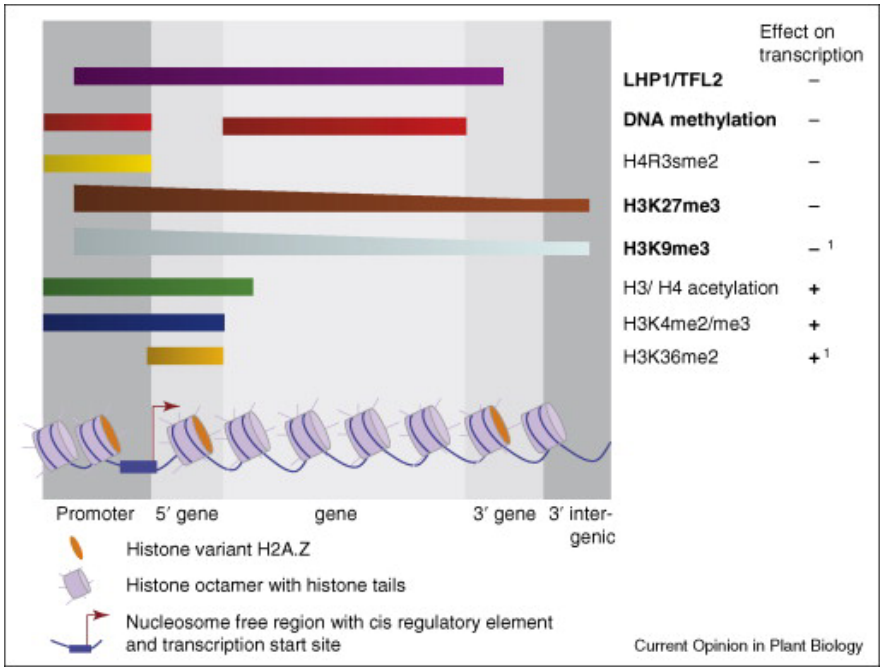

Figure 1.6. Spatial arrangement of chromatin modifications important for gene expression. Shown is a generic euchromatic *Arabidopsis thaliana* gene in the context of chromatin modifications known to affect gene expression. Histone octamers are shown as purple cylinders. The histone variant H2A.Z is shown as an orange ellipse. The promoter is partially depleted of nucleosomes, which is observed in many constitutively expressed genes and in inducible genes after the combined activity of chromatin remodeling ATPases and histone modifiers. Grey shading highlights the promoter region, the 50 end of the gene, the central gene region, the 30 end of the gene, and the 30 intergenic region. Spatial distribution of DNA methylation and of the Heterochromatin Protein 1 homolog LHP1/TFL2, two other chromatin regulators whose distribution was mapped genome-wide, is also shown [Adapted from Pfluger and Wagner (2007)].

Histone methylation

Histone methylation mainly occurs at lysine and arginine residues at the N-terminal tails of H3 and H4, being associated with both transcriptional activation and repression depending on the number of methyl groups and the location of the residues (Shen et al., 2016; Itabashi et al., 2018). For example, dimethylation of H3K9 (H3K9me2) is almost exclusively associated with repetitive genomic sequences and is involved in heterochromatin formation, H3K27me3 is found mainly in gene bodies and negatively correlates with gene expression, whereas

H3K4me3 and H3K36me3 are markers of active genes when they are in gene bodies (Liu et al., 2010).

Unlike acetylation, histone methylation does not change the charges of the histone tails, but it increases the affinity of the histones for negatively charged DNA (Yamamuro et al., 2017).

Methylation of histones is mediated by histone methyltransferases (HMTs) that contain a conserved SET domain (a conserved motif originally in *Drosophila*: SU(VAR)3-9, Enhancer of zeste and Trithorax). They catalyse these reactions using S-adenosyl methionine as a methyl group donor to produce N-methyl protein adducts (Shen et al., 2016).

There are also histone demethylases to remove methyl groups from histones, reactions catalysed by two groups of enzymes:

1) Lysine-specific demethylase 1 (LSD1), which is a flavin adenine dinucleotide (FAD)–dependent amino oxidase.

2) Jumonji C (jmjC) demethylases, which remove methyl groups from dimethylated and trimethylated lysines using the ion [Fe (II)] and 2-oxoglutarate as co-factors.

Histone acetylation

The acetylation state of the amino group of lysine residues within all four core histones is regulated by the opposing activities of histone acetyltransferases (HATs) and histone deacetyltransferases (HDACs) (Liu et al., 2014).

HATs can be classified in four categories:

1) HAG for HATs of the GNAT [GCN5 (general control nonderepressible 5)-related acetyltransferases] superfamily.

2) HAM for HATs of the MYST superfamily.

3) HAC for HATs of the CREB-binding protein (CBP) family.

4) HAF for HATs of the TATA-binding protein-associated factor ($TAF_{II}250$) family.

GCN5 is the best characterized HAT in yeast, mammals and plants. In *Arabidopsis*, this acetyltransferase and the transcription factor ADAptor proteins ADA2a and ADA2b interact with each other and are the subunits of the transcriptional adaptor complex SAGA (Spt-Ada-Gcn5-Acetyltransferase) (Servet et al., 2010). GCN5 is implicated in the maintenance of the root stem cell niche (Kornet and Scheres, 2009).

Histone deacetylation is mediated by the HDAC complexes. The *Arabidopsis* genome encodes for 18 HDACs and they are grouped in three families:

1) Histone deacetylase 1 (HDAC1), which is homologous to the yeast Reduced Potassium Deficiency 3 (RPD3). It is present throughout eukaryotes.

2) Histone deacetylase 2 (HD2)-related families [also called HD-tuins (HDT)], only present in plants.

3) Sirtuins, which are homologous to the yeast Silent Information Regulator 2 (SIR2).

Among the 18 HDACs of *Arabidopsis*, 12 belong to the RPD3/HDAC1 family, which is subdivided into three classes. Class I includes HDA19, HDA6, HDA7 and HDA9. Class II are HDA5, HDA15 and HDA17. HDA2 and its two additional isoforms compromise class III. HDA8, HDA14, HDA10 and HDA17 are not classified (Liu et al., 2014). *Arabidopsis* has four members of the HD2 family and two SIR2 members (SRT1 and SRT2).

Both HATs and HDACs can function in protein complexes as transcriptional co-repressors and co-activators or associated with chromatin remodelers as modulators of the accessibility of DNA to different machineries (Wang et al., 2014).

Histone variants

Besides the canonical histone proteins, there are multiple histone variants encoded in the plant genome. Their presence influences the physical properties of the nucleosome and its dynamics. They are deposited into chromatin by various histone chaperones and chromatin remodelling complexes (March-Díaz et al., 2008). Some variants of H2A and H3 are involved in various processes including transcription, DNA repair and chromatin remodelling (Talbert et al., 2012). For example, H3.1 is enriched in transcriptionally silent regions, whereas H2A.Z and H3.3 are predominantly enriched in actively transcribed genes (Stroud et al., 2012).

Non-coding RNAs

Non-coding RNAs are RNA molecules that do not code for proteins but have very important roles in the regulation of different developmental processes (Gady et al. 2017).

Small interfering RNAs (siRNAs) function as part of the RNA-directed DNA methylation (RdDM) pathway machinery, where they direct the RdDM complex to specific DNA locations that are going to be methylated. Micro RNAs (miRNAs) are small molecules of 21 nucleotides that induce transcriptional gene silencing by specifically binding to mRNA with a near-perfect sequence complementarity and provoking their degradation. Long non-coding RNAs

(lncRNAs) are transcripts over 200 bp that regulate gene expression in multiple ways as cis- or trans-regulatory elements (Liu et al., 2015).

The epigenetic pathway RdDM involves a large number of components, especially Pol IV and Pol V transcripts. Pol V transcribes lncRNAs that are later converted into double strand RNAs (dsRNAs) by RNA-dependent RNA polymerase 2 (RDR2) and then processed by DICER-like 3 (DCL3) into siRNAs (Haag et al., 2012). The siRNAs guide ARGONAUTE 4 (AGO4) to scaffold transcripts of Pol V. The complex formed by siRNA, AGO4 and Pol V-derived lncRNA scaffolds recruits HDACs and DNA methyltransferases that silence the genomic loci transcribed by Pol V (*Figure 1.7.*) (Law and Jacobsen, 2010; Gady et al., 2017).

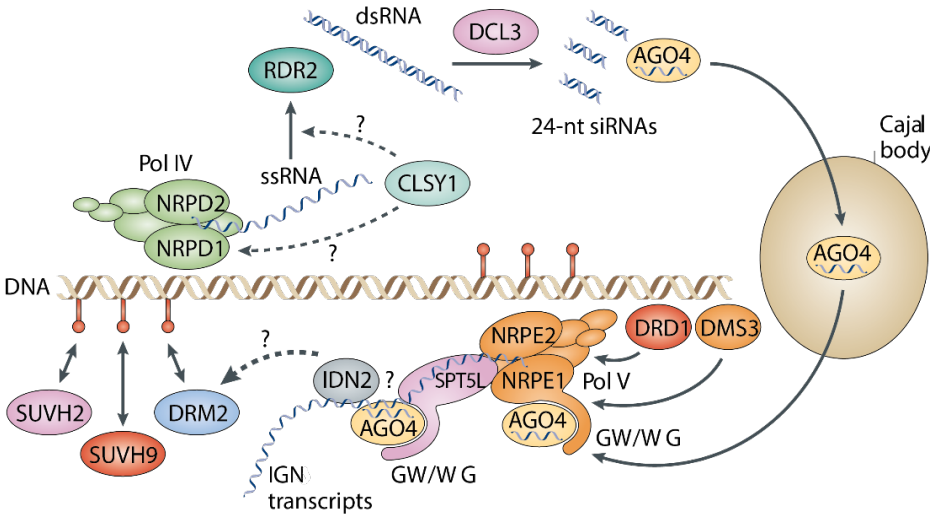

Figure 1.7. Model for RNA-directed DNA methylation. Single-stranded RNA transcripts corresponding to transposons and repeat elements are thought to be generated by RNA polymerase IV (Pol IV). CLASSY 1 (CLSY1) is likely to function early in RNA-directed DNA methylation (RdDM), possibly recruiting Pol IV to chromatin or aiding in ssRNA transcript processing. RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) is proposed to generate dsRNA from the ssRNA transcripts. DICER-LIKE 3 (DCL3) is thought to process the dsRNAs into 24-nucleotide (nt) small interfering RNAs (siRNAs), which are bound by an Argonaute protein, AGO4. AGO4 colocalizes with two Pol V subunits — NUCLEAR RNA POLYMERASE E1 (NRPE1) and NRPE2 — and DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) at a distinct nuclear focus, the AGO4–NRPE1 body, which may represent a site of active RdDM. Pol V is thought to transcribe intergenic non-coding (IGN) regions throughout the genome. NRPE1 association with chromatin requires another putative chromatin-remodelling factor, DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1), and a structural maintenance of chromosome (SMC) domain protein, DEFECTIVE IN MERISTEM SILENCING 3 (DMS3). IGN transcripts may serve as a scaffold for recruiting AGO4, which interacts with the GW/WG motifs of NRPE1 and SUPPRESSOR OF TY INSERTION 5-LIKE (SPT5), possibly through interactions between AGO4-bound siRNAs and the nascent transcript. An RNA-binding protein, INVOLVED IN DE NOVO 2 (IDN2), is proposed to recognize the siRNA–nascent transcript duplex. These associations may aid in targeting DRM2 to genomic loci that produce both 24-nt siRNAs and IGN transcripts. Recruitment or retention of DRM2 at such loci may be aided by SUPPRESSOR OF VARIEGATION 3-9 HOMOLOGUE 9 (SUVH9) and SUVH2, two proteins that bind methylated DNA and are likely to act late in RdDM. '?' indicates a putative function. The red circles represent DNA methylation [Adapted from Law and Jacobsen (2010)].

Epigenetic regulation of seed dormancy

Phase transitions during seeds' life require genome reprogramming at a large scale. These are often associated with major changes in chromatin structure and involve tight and dynamic transcriptional control imposed by epigenetic modifications (Van Zanten et al., 2013).

Evidence for an epigenetic regulation of gene expression in controlling dormancy has only emerged recently. Early studies were carried out by Law and Suttle (2002, 2004) elucidating the implications of 5-mC and histone H3 and H4 multi-acetylation in potato meristems during dormancy progression. A screen for low primary dormant mutant seeds led to the identification of *HISTONE MONO UBIQUITINATION 1* (*HUB1*) and *HUB2*, that encode for two C3HC4 RING finger proteins with homology to the histone-modifying enzymes BRE1 in yeast and RNF20/RNF40 in humans (Liu et al., 2007b). *HUB1* is required for monoubiquitination of histone H2B at Lys-143 (H2BK143), which is a prerequisite for histone H3 methylation at Lys4 (H3K4me3) and Lys79 (H3K79me3), both associated with gene activation (Du, 2012). Elimination of HUB1 in seeds causes decreased expression of genes related to ABA metabolism and response, such as *NCED9* and *ABI4*, and also reduced ABA levels (Peeters et al., 2002).

REDUCED DORMANCY 2 (*RDO2*) is related to the polymerase II-associated factor 1 complex (PAF1C) and encodes the transcription elongation factor S-II (TFIIS). Mutations in RDO2 and other PAFC1 associated factors, such as VERNALIZATION INDEPENDENT 4 (VIP4), VIP5, EARLY FLOWERING 7 (ELF7), ELF8 and ARABIDOPSIS TRITHORAX-RELATED 7 (ATRX7), cause a reduced seed dormancy phenotype and several dormancy-related genes, such as *DOG1*, are down-regulated in the *rdo* mutant (Liu et al., 2011).

TFIIS (*RDO2*) and *HUB1* are induced during the same stages of seed maturation, and a significant overlap of differentially expressed genes was observed in *tfIIs* and *hub1* mutants, which indicates that they share common targets, such as *DOG1* (Liu et al., 2011). The implication of other histone modifications in seed dormancy was demonstrated when mutations in *KRYPTONITE/SU(VAR)3-9 HOMOLOG4* (*KYP/SUVH4*), encoding a histone methyltransferase required for H3K9me2, resulted in increased primary seed dormancy (Jackson et al., 2002). These mutants had increased expression of *DOG1* and dormancy-associated genes (Liu et al., 2007). *A. thaliana* plants overexpressing *KYP/SUVH4* showed decreased dormancy (Zheng et al., 2012), which suggests that it is putatively involved in the ABA/GAs balance regulation.

In *Arabidopsis*, H3K4me2/3 marks are deposited by the *Arabidopsis* TRITHORAX (ATX) family while H3K27me3 marks are deposited by CLF and SWN members of the Polycomb Repressive Complex 2 (PRC2), which regulates major phase transitions in plant development (Calonje, 2014; Engelhorn et al., 2014). H3K27me3 deposition has been described as an important epigenetic mark

for the transition from seed dormancy to germination (Müller et al., 2012). Mutants in *FERTILIZATION INDEPENDENT ENDOSPERM* (*FIE*), which is an essential component of the PCR2, display a genome-wide abolition of H3K27me3 and exhibit increased primary seed dormancy and germination defects (Bouyer et al., 2011). *PRC1* mutants also exhibit delays in both germination and transcriptional repression, with a delayed switch in chromatin state from H3K4me3 to H3K27me3 enrichment of *DOG1* and seed development genes (Molitor et al., 2014). The dynamic role of histone methylation in the adjustment of the dormancy state during dormancy cycling was analysed by Footitt et al. (2015), who proposed the regulation of *DOG1* transcription by the proportions of H3K4me3 and H3K27m3 marks as part of a sensing mechanism of soil temperature. Moreover, *ATX4*, *CLF* and *SWN* are expressed in opposite phases to each other during the dormancy cycle (Footitt et al., 2015) and during dormancy breaking and germination (Müller et al., 2012).

Different expression patterns of histone acetyltransferases and deacetylases were found between dormant and non-dormant seeds of *A. thaliana* by Cadman et al. (2006). The HD2-like family are plant specific deacetylases with specific roles in seeds and seedling growth (Berr et al., 2011; Colville et al., 2011; Yano et al., 2013). Mutation analysis of genes encoding for this family of HDACs also showed that histone acetylation is involved in seed dormancy. Berr et al. (2011) demonstrated that HD2A deacetylates Histone 3 Lysine 9 (H3K9), a methylation target for KYP/SUVH4. Moreover, while seed germination is enhanced in *hd2a* mutants, *hd2c* mutants are restrained in germination in comparison to wild type seeds (Colville et al., 2011). Overexpression of *HD2C* confers an ABA-insensitive phenotype, as seeds present enhanced germination and expression of *LATE EMBRYOGENESIS ABUNDANT PROTEIN* (*LEA*) class genes (Sridha and Wu, 2006).

While activation of dormancy loci through transcription elongation seems to be critical for dormancy induction, continuous repression of seed germination-associated genes is possibly essential for dormancy maintenance. SWI-INDEPENDENT 3 (SIN3)-LIKE 1 (SNL1) and SNL2 belong to a protein family that contains a paired amphipathic helix repeat (Bowen et al., 2010). In *Arabidopsis*, Wang et al. (2013) demonstrated their redundant role in the regulation of seed dormancy as components of the HDAC-SNL complex, regulating the transcription of genes implicated in the antagonism between ethylene and ABA pathways (Linkies et al., 2009) by modifying their histone acetylation levels. HDAC-SNL complexes can have different combinations of SIN3 and HDACs (Wang et al., 2013), although only the histone deacetylase HDA19 was analysed in their investigation. Seeds from the *snl1 snl2* double mutant exhibit decreased dormancy, which was also observed in *hda19* mutant seeds. Global gene expression analysis for the double mutant and wild-type seeds identified targets of SNL-HDA19, such as genes involved in the ethylene pathway. This indicates a regulation of seed dormancy by suppression of the ethylene signalling pathway through histone deacetylation (*Figure 1.8.*).

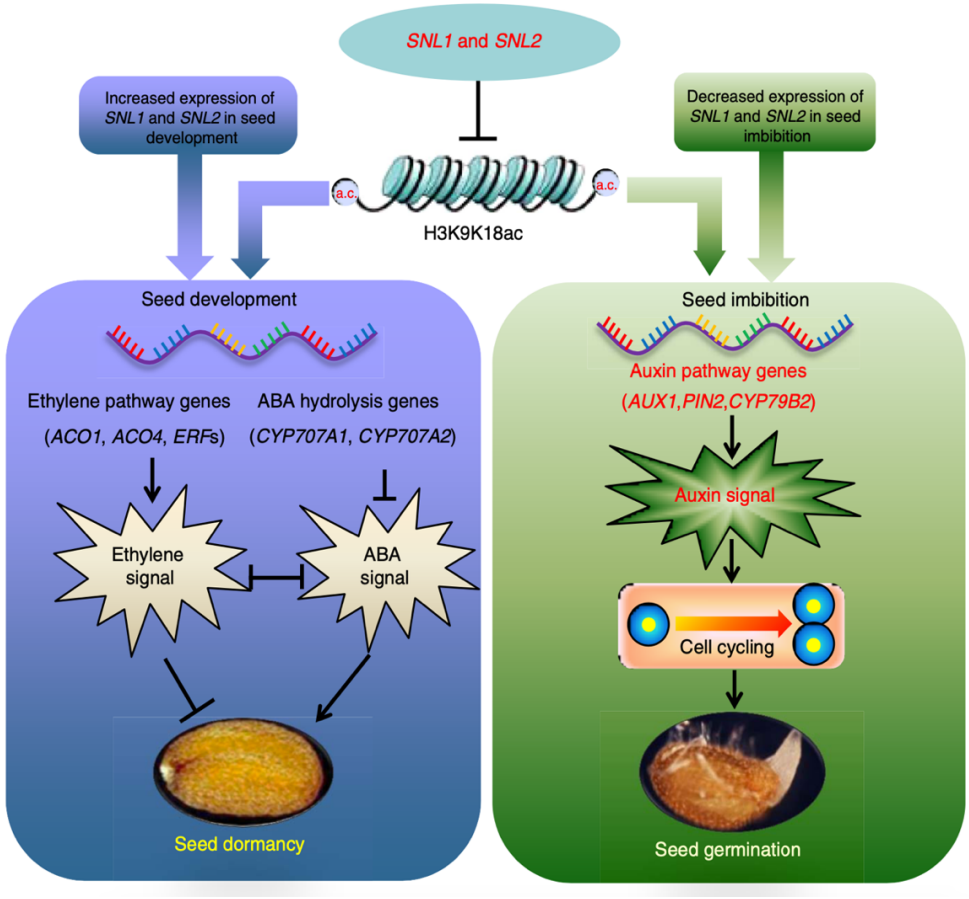

Figure 1.8. Hypothetical model for the regulation of seed dormancy and germination by *SNL1* and *SNL2* proposed by Wang et al. (2016). The expression of *SNL1* and *SNL2* increases gradually during embryo development and seed maturation causing a decrease in acetylation level of ABA hydrolysis genes (*CYP707A1* and *CYP707A2*) and some ethylene pathway genes (*ACO1* and *ACO4*). This promotes ABA signalling and represses ethylene signalling leading to the establishment of seed dormancy. During imbibition of after-ripened seeds, the expression of *SNL1* and *SNL2* declines, causing an increase in acetylation levels of auxin pathway genes [for example, *AUXIN RESISTANT 1* (*AUX1*)]. Subsequent activation of their transcription leads to increased auxin levels and signalling, followed by enhanced cell division promoting seed germination. *ERF*s: ethylene response factors; *PIN2*: auxin efflux carrier component 2.

In the model plant *A. thaliana*, seed development takes 20 days and can be divided in two stages. The first 10 days are dedicated to embryo development and the remaining days, defined as the seed maturation phase, are committed to accumulation of storage reserves, acquisition of desiccation tolerance and establishment of primary seed dormancy (Van Zanten et al., 2014). Hyper-methylation of dry seeds as they enter dormancy could be related to a halt in transcription (Bouyer et al., 2017; Kawakatsu et al., 2017). During seed maturation, nuclear size is reduced, and nuclei are highly condensed in seeds, while the opposite is observed during germination as nuclei regain their size and chromatin is decondensed (Van Zanten et al., 2011).

It is likely that DNA methylation is involved in these processes of chromatin condensation and decondensation. However, it has recently been shown that CG- and CHG-context methylation do not change significantly during seed development but, on the contrary, CHH-context methylation increases primarily within transposons during the period leading up to dormancy. This appears to be a mechanism to ensure that transposons remain silent and do not inactivate genes essential for seed development and germination (Lin et al., 2017; Chen et al., 2018).

Selection of a model species: *Capsella bursa-pastoris*

Capsella is a small genus within the Brassicaceae family. Its name translates from Latin as "little box" due to the characteristic fruits of its members (siliques) that are flattened and "heart shaped" with two seed chambers (Iannetta, 2011) (*Figure 1.9.*). The genus includes the self-fertilizing tetraploid *C. bursa-pastoris* (2n = 4x = 32) and three other diploid species: the self-incompatible *C. grandiflora*, and two self-compatible species, *C. rubella* and *C. orientalis* (2n = 2x = 16). These species differ in their geographical distribution (Douglas et al., 2015).

C. bursa-pastoris (shepherd's purse) has a worldwide distribution, with the exception of extremely dry tropical environments (Neuffer and Eschner, 1995; Hurka and Neuffer, 1997) (*Figure 1.10.*). It has become one of the five most widely distributed flowering plants on our planet, preferring disturbed, "man-made" habitats, like the margins of agricultural fields (Hintz et al., 2006). As a pioneer of disturbed ground, it is an opportunistic species and when established in open spaces it grows quickly. However, when perennial grass species enter the succession, its abundance declines and disappears (Aksoy et al., 1998). This tetraploid plant is an allopolyploid which resulted from a very recent hybridization event (in evolutionary terms) between the *C. orientalis* and the *C. grandiflora*/*C. rubella* lineages. The maternal parent came from the former lineage and the paternal contribution from the latter (Douglas et al., 2015).

Figure 1.9. Herbarium specimen collected at the Royal Botanic Gardens, Kew, UK, left (http://specimens.kew.org/herbarium/K000914151), and real picture, right (by Ignacio Fernández Villar, www.asturnatura.com), of *Capsella bursa-pastoris* (shepherd's purse) plants.

The vegetative and reproductive structures are very variable between habitats, including characteristics such as dry weight, plant height, number of siliques, number of seeds per silique and seed weights (Aksoy et al., 1998). Seeds of *C. bursa-pastoris* are on average 0.8 – 1.0 mm long and 0.6 – 0.8 mm wide. The number of seeds per silique and the number of siliques per plant vary depending on the genotype and habitat. They produce mucilage, which becomes sticky when moistened, therefore allowing the seeds to be transported by humans or birds (Aksoy et al., 1998). Seeds germinate at temperatures between 5 to 30 °C and alternating temperatures of 10/30 °C or 20/30 °C enhance germination and have a pronounced light-requirement.

Freshly matured seeds of *C. bursa-pastoris* present primary dormancy (Baskin and Baskin, 1989) and a period of stratification can be used for breaking it (Popay and Roberts, 1970). They have coat-enhanced, non-deep physiological dormancy, where the embryo is fully developed. Shepherd's purse seeds form a persistent seed bank in the soil (Hurka and Haase, 1982) and exhibit an annual dormancy cycle, losing their ability to germinate first at high and then at lower temperatures as they enter secondary dormancy (Baskin and Baskin, 1989). Many genotypes of *C. bursa-pastoris* are facultative winter annuals, being able to survive as leaf rosettes over the season (Linde et al., 2001) and germinating both in autumn and spring if seeds are exposed to the right conditions (Baskin and Baskin, 1989). The half-life of a seed in the ground is around 5 years (Roberts and Feast, 1973).

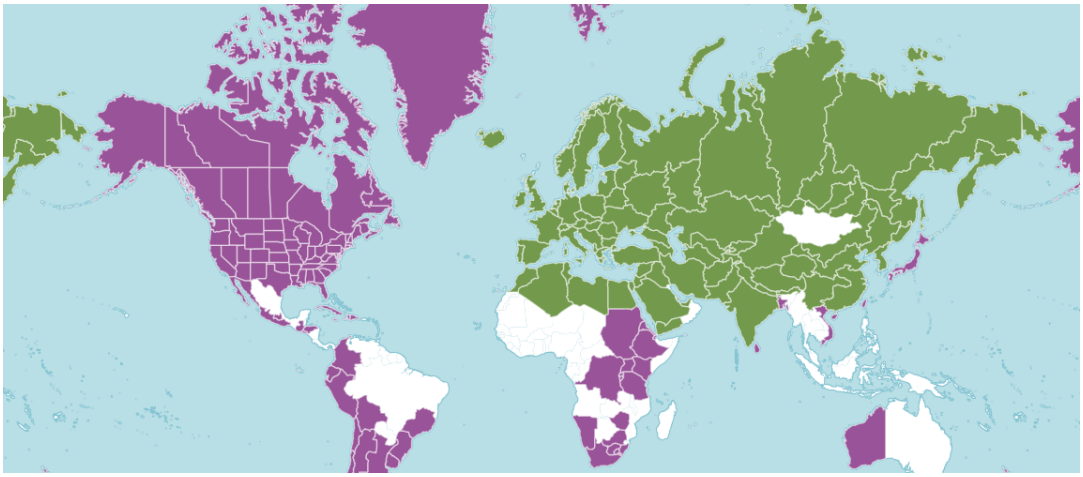

Figure 1.10. Worldwide distribution of *C. bursa-pastoris* (www.plantsoftheworldonline.org). Native (green) and introduced (purple).

C. bursa-pastoris has a very small phylogenetic distance with the academic model species *A. thaliana*. However, in comparison, the latter appears to be uncompetitive and is actually relatively rare in the wild (Hintz et al., 2006). Polyploid plants usually show more coping skills with a wider range of conditions compared to their progenitors and as it is self-fertile it produces a higher number of progenies without the negative effects of inbreeding depression (Hintz et al., 2006). Although the life cycle of *C. bursa-pastoris* is longer than that of *A. thaliana*, it is still sufficiently short to allow the production of three or four generations per year.

It has been argued that wild species, especially annual ones which have seeds that persist in the soil seed bank, are better models for physiological studies of seed dormancy than domesticated crops (Hilhorst and Toorop, 1997). Besides, a whole seed population of *C. bursa-pastoris* can be uniformly induced to different dormant states.

For all these reasons, we think that *C. bursa-pastoris* is an excellent choice as a model species for seed dormancy research which could be used as a companion to *A. thaliana*.

Approach and aims

The principal aim of this doctoral thesis is to gain insight into the epigenetic regulation processes involved in the induction and maintenance of secondary seed dormancy in *C. bursa-pastoris*. For achieving this goal, a great advantage has been taken from our research group at the University of Oviedo, Asturias, Spain, in studying epigenetic marks associated with different physiological processes, such as reproduction, flowering or aging, and the incredible resources available at Wakehurst Place, Royal Botanic Gardens, Kew, UK, to assist any investigation related to seed dormancy. This general goal was achieved through the following individual objectives:

1) Extensively investigate natural variation for seed dormancy among different *C. bursa-pastoris* accessions, determining the contribution of genotype to the variation in secondary seed dormancy by characterizing the differences in secondary dormancy induction and depth among them (Chapter II).

2) Test the hypothesis that acetylation of histones is involved in the induction and maintenance of secondary dormancy (Chapter II).

3) Optimization of a low-cost, reliable and fast large-scale DNA extracting protocol suitable for seed material of *C. bursa-pastoris* (Chapter III).

4) Quantification of global genomic DNA methylation of primary dormant seeds and seeds belonging to the two *C. bursa-pastoris* accessions previously selected with differences in their secondary dormancy depth (Chapter IV).

5) Analysis of the anatomy of *C. bursa-pastoris* embryos and of the spatio-temporal distribution of 5-mC and H4Ac epigenetic marks by immunolocalization in dry, imbibed and dormant seeds (Chapter V).

6) Overview of the transcriptomic dynamics that two selected accessions, with differences in secondary dormancy depth, present by performing an RNA-sequencing (RNA-Seq) analysis to detect differentially expressed genes (DEGs) associated with secondary seed dormancy depth and induction. Optimization of an RNA extracting protocol for whole seeds of *C. bursa-pastoris*. (Chapter VI)

The regulation of dormancy cycling upstream of the hormone signalling pathways is still poorly understood. Recent advances have highlighted the role of chromatin remodelling at the histone and DNA level in dormancy regulation. Natural variation in the responses of the epigenetic regulation processes to the environment could be informative regarding adaptation to climate.

A greater understanding of seed dormancy is essential in a future where the impact of climate change on natural plant communities is uncertain. Besides,

climate change could alter the behaviour of secondary dormant weed seeds, including *Capsella*, as it has a short life cycle and longer growing seasons could lead to more life cycles in a season (Ketner, 1990).

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2

CHAPTER 2

Contribution of genotype to secondary seed dormancy variation among accessions of *Capsella bursa-pastoris*

Introduction

Seeds are the most common mobile phase of a plant's life cycle, transporting its genetic material through space and time, with their function being the establishment of a new plant in a suitable environment. The time between seed dispersal and completion of germination can be short or long, thus, seeds have evolved a series of strategies - e.g. dormancy - to survive this time (Footitt and Finch-Savage, 2017). These strategies prevent responses to short-lived, out of season environmental changes. Different species have adapted to the climates and habitats they have colonized and their dormancy mechanisms have evolved at the same time, making it possible for plants to overcome periods that are unfavourable for seedling establishment (Bentsink and Koornneef, 2008).

In the last two decades there has been great progress in understanding the molecular and physiological mechanisms that control seed dormancy. In a very simple way it could be defined as a block to the completion of germination of an intact viable seed under favourable conditions (Hilhorst, 1995; Bewley, 1997), although it is a very complex trait. A seed is dormant when it does not have the capacity to germinate in a specific period of time under any combination of normal physical environmental factors that are otherwise favourable for its germination (Baskin and Baskin, 2004). On the other hand, a completely non-dormant seed has the capacity to germinate over the widest range of normal physical environmental factors possible for that genotype (Baskin and Baskin, 2004). An individual seed can be dormant or non-dormant and in a population of seeds in the soil seed bank, individual seeds can have different levels of dormancy (Footitt and Finch-Savage, 2017).

The germination of a non-dormant seed is an important stage, as it starts the regeneration of the mother plant (Finch-Savage and Leubner-Metzger, 2006). Germination *sensu stricto* commences with the uptake of water by the quiescent dry seed and terminates with the elongation of the embryonic axis (Baskin and Baskin, 2004). This early phase of imbibition occurs in dormant and non-dormant seeds (Carrillo-Barral et al., 2013). Germination has two visible stages in physical terms, testa rupture, as cells of the lower hypocotyl/radicle transition zone expand, followed by endosperm rupture, and it is finally completed when the micropylar endosperm is broken by the emerging radicle (Bentsink and Koornneef, 2008). *Capsella bursa-pastoris* (L.) Medik. (shepherd's purse) seeds are surrounded by a testa (a layer of cells of maternal origin) and a single-layer endosperm (a layer of living nutritive cells).

Primary dormancy is the innate dormancy possessed by seeds when they are dispersed from the mother plant (Benech-Arnold et al., 2000). Freshly matured water-permeable seeds can have strong primary dormancy that depends on the

environment during seed development and physiological characteristics imposed by the mother plant (Bewley and Black, 1994). These seeds do not germinate in light or darkness over a range of temperatures (Baskin and Baskin, 1989). Its release may involve after-ripening in the dry state or dormancy-release treatments (chilling, warm stratification, nitrate, light, smoke substances, gibberellins and other hormones) in the imbibed one (Finch-Savage and Leubner-Metzger, 2006).

Secondary dormancy occurs after the seed has left the mother plant and primary dormancy has been released. Subsequent entrance into secondary dormancy could be induced in seeds with non-deep physiological dormancy, if the right set of external signals to germinate are absent (Hilhorst, 1998). Secondary dormancy may be relieved and non-dormant seeds may germinate if the right conditions are encountered. Induction and relief of secondary dormancy can occur during successive seasons, leading to an annual dormancy cycle in the seed bank (Hilhorst, 1998). Subtle differences in this behaviour could result in local adaptation and genotypic differences (Finch-Savage and Footitt, 2017). The rate of loss of primary dormancy by after-ripening, seed-maturation temperature and inducing and reducing factors of secondary dormancy, all interact in a way that integrate environmental information and regulate very precisely seed germination time (Auge et al., 2015).

Time of germination in nature can vary within and among populations and can be attributed to variation in primary dormancy levels and sensitivity to secondary dormancy induction (Coughlan et al., 2017). The environmental conditions under which germination can occur are called the germination window (Footitt and Finch-Savage, 2017). Different genotypes within the same species may have different depths of dormancy, presenting a different response to environmental cues. These differences can result in distinct seasonal patterns of germination, so different populations can express either a winter or a summer annual life history or even both (Donohue et al., 2005). When a seed population is losing its dormancy, the germination permissive range of environments begins to open (Benech-Arnold et al., 2000). Conversely, if dormancy is induced to a deeper state due to a change in the environment, this germination window closes.

Seed dormancy is a common attribute of weed populations. *Capsella bursa-pastoris* is a weed that is a facultative winter annual, germinating in autumn and spring if seeds are exposed to light (Baskin and Baskin, 1989). In regions with a cool and moist summer, seeds can germinate throughout the growing season, if they are brought to the soil surface and exposed to light and adequate moisture (Popay and Roberts, 1970). Its buried seeds exhibit an annual conditional cycle of dormancy/non-dormancy (Baskin and Baskin, 1989).

Shepherd's purse seeds have non-deep physiological dormancy (Toorop et al., 2012). Non-deep physiological dormancy is the most abundant form of seed dormancy, especially in temperate seed banks. It has a profound impact on the structure and development of plant communities across the major climate regions

(Cadman et al., 2006). Most of the seed model species (e.g., *Arabidopsis thaliana*, *Avena fatua*, *Nicotiana spp*.) present this type of dormancy. It can be divided into deep, intermediate and non-deep categories (Baskin et al., 2004), with the non-deep category having five types, where most seeds belong to type 1, e.g. *A. thaliana*, or 2 (Finch-Savage and Leubner-Metzger, 2006). In seeds with non-deep physiological dormancy, embryos that are released from surrounding structures grow normally and dormancy is lost by moist chilling or after-ripening (Baskin and Baskin, 2004).

C. bursa-pastoris displays primary dormancy, secondary dormancy and relative dormancy (Neuffer and Hurka, 1986). Seeds in the intermediate states are said to have conditional or relative dormancy as the range of environmental factors that are permissive to germination is limited (Finch-Savage and Leubner-Metzger, 2006). Primary dormancy in shepherd's purse may be terminated by chilling imbibed seeds (Popay and Roberts, 1970) or by after-ripening of dry seeds. Secondary dormancy in this species develops in dispersed seeds in response to unfavourable germination conditions. Although secondary dormancy is of great importance in germination ecophysiology, little is known about this phenomenon for *Capsella* (Neuffer and Hurka, 1986).

In several species, exit from dormancy is completed by factors such as after-ripening, light and/or cold treatment (stratification). Besides, application of chemicals such as gibberellins, KNO_3 and NO also have releasing effects. This requirement for exogenous factors depends very much on the genotype (Bentsink and Koornneef, 2008). The seeds first become sensitive to nitrate, then to cold and finally to light (Finch-Savage et al., 2007), showing that dormancy is a continuum with layers that can be progressively removed by environmental signals until only light is required (Footitt et al., 2011, 2013). Release from dormancy by exposure of the seeds to alternating temperatures occurs in a lot of different species. Its ecological significance has been attributed to the possibility of detecting canopy gaps and the depth of burial (Thompson and Grime, 1983), i.e., as a spatial signal. Although termination of dormancy in seeds can be triggered by light in many species (Baskin and Baskin, 1985), depending on the dormancy state, this and other environmental cues can promote, inhibit or not have any effect on seeds (Bewley and Black, 1982). In species presenting a dormancy cycle, when secondary dormancy is induced, seeds become increasingly insensitive to light, but when seeds enter the shallow dormancy phase, they become increasingly sensitive to it, acquiring the maximum light sensitivity when the level of dormancy is at its minimum (Batlla and Benech-Arnold, 2014). In species with small seeds, such as *C. bursa-pastoris*, seeds are light sensitive as it informs them that they are returned to the surface by soil disturbance. Seeds at or near the soil surface can potentially sense and interpret a variety of light environments (Footitt and Finch-Savage, 2017).

In shepherd's purse, Popay and Roberts (1970) were the first to demonstrate that when seeds were exposed to alternating temperatures imbibed in nitrate at

10^{-1} and 10^{-2} mM, there was a dramatic increase in germination, demonstrating that *Capsella* was similar to other species.

The genetic regulation of secondary dormancy induction by specific environmental factors is poorly understood (Footitt et al., 2013). Although key environmental and genetic factors associated with natural variation in primary dormancy have been identified, there is little information about genetic variation for secondary dormancy dynamics and the relative importance of seed maturation conditions, post-dispersal environment and genotype in creating variation in secondary dormancy (Coughlan et al., 2017). As seed-maturation conditions and conditions experienced by seeds immediately after dispersal interact to influence dormancy and germination, the seasonal timing of flowering can influence the germination behaviour of seeds.

Arabidopsis, a species with a small phylogenetic distance with *C. bursa-pastoris* (Toorop et al., 2012), is a plant with large differences in dormancy among accessions collected from nature (Bentsink and Koornneef, 2008). In different accessions within the same species, several cues and physiological mechanisms can operate with differing importance (Cadman et al., 2006). Natural variation within dormancy reduces extinction risk within a new habitat (Footitt and Finch-Savage, 2017). The genetic variation for germination can be assessed by comparing genotypes in the same environmental conditions. This implies that besides the germination test conditions, the growing conditions during seed development and the storing conditions and time must be the same (Bentsink and Koornneef, 2008).

In different species, such as *A. thaliana* (Donohue et al., 2007) and *C. bursa-pastoris* (Toorop et al., 2012), imbibition of seeds at high temperatures under darkness followed by exposure to light has been shown to be effective for studying differences in secondary dormancy between genotypes. The environmental context of these responses is poorly understood, but they could be important for dormancy cycling under natural conditions (Footitt et al., 2011). Secondary dormancy induction by high temperatures stimulates the up-regulation of ABA-mediated dormancy pathways and of genes involved in GAs catabolism (Martel et al., 2018).

The implication of epigenetic processes in the regulation of dormancy has been demonstrated recently (Law and Suttle, 2002, 2004; Liu et al., 2007; Zheng et al., 2012). Among them, acetylation of lysines has been one of the most studied mechanisms. This modification at the N-terminal tails of histones removes their positive charge, altering the histone-histone and DNA-histone interaction and changing the accessibility of DNA to the chromatin-binding proteins (Turner, 2000). It is associated with an open chromatin state (euchromatin) and activation of gene transcription, while hypo-acetylation is related to chromatin condensation (heterochromatin) and consequently gene silencing (Wójcikowska et al., 2018).

Acetylation of lysine residues is a reversible process and there are two families of enzymes implicated in the acetylation state of histones: histone

acetyltransferases (HATs) and histone deacetylases (HDACs). The interplay between these enzymes is implicated in the control of many biological processes, like embryo development, germination and morphogenesis (Wang et al., 2014). Moreover, different expression patterns of histone acetyltransferases and deacetylases were detected by Cadman et al. (2006) between dormant and non-dormant seeds. The best studied HDACs belong to RPD3 Class 1, which includes HDA6, HDA19, HDA7, HDA9 and the pseudogenes HDA10 and HDA17 (Van Zanten et al., 2014).

HDAC inhibitors shift a reversible histone acetylation/deacetylation state towards a condition of histone hyper-acetylation. HDCAs can be pharmacologically inhibited by trichostatin A (TSA) (Yoshida et al., 1995) and other compounds, such as valproic acid (Göttlicher et al., 2001), suberoylanilide hydroxamic acid (SAHA) (Richon et al., 1998) and anacardic acid (Cui et al., 2008). They can, for example, be used for determining the role that histone acetylation plays in chromatin structure and remodelling and for finding genes regulated by histone acetylation (Yoshida et al., 1995; Wójcikowska et al., 2018).

The basic and principal aim of this study was to extensively investigate natural variation in seed dormancy among different accessions. In particular, determining the contribution of genotype to the variation in secondary seed dormancy by characterizing the differences in secondary dormancy induction and depth among accessions of *C. bursa-pastoris* (previously categorized by flowering time and divided into early, intermediate or late flowering) (Iannetta et al., 2007). In addition, studies by Toorop et al. (2012) demonstrated a co-adaptation between flowering time and secondary dormancy depth in different genotypes of shepherd's purse, so we checked whether we could corroborate this co-adaptation for our range of accessions.

The second aim was to test the hypothesis that acetylation of histones is involved in the induction and maintenance of secondary dormancy. We imbibed seeds of *C. bursa-pastoris* in HDAC inhibitors during the induction and maintenance of secondary dormancy, expecting to observe an obstacle to the entrance into secondary seed dormancy. In our initial hypothesis, seeds imbibed in conditions for induction of secondary dormancy with HDAC inhibitors would not be able to enter this dormancy state. Moreover, they would present higher germination capacity (after a short period of after-ripening and exposure to alternate temperatures, KNO_3 and light) than seeds that were only imbibed in water under the same secondary dormancy induction conditions.

The same collections of seeds as those used for the analyses of secondary seed dormancy depth and co-adaptation of flowering time (see above) were employed in order to elucidate whether these compounds would affect different accessions of *C. bursa-pastoris* differently. Seeds from all the accessions used for these experiments were produced under the same growing conditions and kept at

15 °C and 15 % RH for the same period of time. The germination tests were carried out under the same conditions for all the accessions.

Even though this work is of physiological nature, it is important to consider the ecological implications of seed dormancy, especially in relation to climate change. Climate change could be beneficial for weed species as it will alter the temperature regime. These changes could alter the behaviour of secondary dormant weed seeds such as *Capsella*, which is a facultative winter annual with capacity to survive as leaf rosettes over the season (Linde et al., 2001) and to germinate both in autumn and spring if seeds are exposed to the right conditions (Baskin and Baskin, 1989). Moreover, weed species have a short life cycle and longer growing seasons could lead to more life cycles in a season. Understanding dormancy molecular mechanisms is therefore essential for control of weeds.

Material and methods

Plant material

The capacity for induction of secondary seed dormancy in *C. bursa-pastoris* seeds was analysed in nine accessions (SCRI -156, -177, -367, -416, -469, -707, -773, -799 and -937 from the Scottish Crop Research Institute). These were derived originally from soil samples taken from 34 fields around the Farm Scale Evaluation sites in 2001 in the UK (Champion et al., 2003). Plants were then grown from these seeds over the summer/autumn of 2005 and 52 genotypes were classified based on previous knowledge of their flowering times (Iannetta et al., 2007). Seeds from the accessions studied were stored at 15 °C with 15 % of RH in the Millennium Seed Bank, Wakehurst Place, Royal Botanic Gardens, Kew, UK. The nine genotypes were originally classified by their time of flowering, with three classified as early flowering (-156, -707, -773), three as intermediate (-177, -367, -469) and three as late flowering (-416, -799, -937).

Seeds belonging to a commercial genotype of *C. bursa-pastoris* from Herbiseed (Twyford, Berkshire, UK) from 2013 (kindly provided by Dr. Pietro Iannetta, The James Hutton Institute, Scotland, UK), were also used to assess the effects of different compounds, known for affecting different epigenetic processes, on the induction and maintenance of secondary dormancy. Seeds were kept at 4 °C during the entire period following purchase.

Germination tests

The dormancy state of the commercial batch of *C. bursa-pastoris* seeds was corroborated by germination tests. Three conditions were used: 1) seeds imbibed in water at 30 °C with a 12 h photoperiod as an optimum test for non-dormant seeds; 2) seeds imbibed in water but exposed to alternate cycles of light/dark (12 h) and high/low temperatures (25/10 °C), as this treatment is known to partially

break primary dormancy; 3) seeds imbibed in 10 mM KNO_3 with alternate cycles of light/dark (12 h) and high/low temperatures (25/10 °C), as a way of completely breaking primary dormancy. This demonstrated that they had some residual primary dormancy.

Different compounds were tested for their effect on the induction and maintenance of secondary dormancy, using the commercial batch. The concentrations used for the different compounds were based on bibliography, a preliminary study with different concentrations for some of the compounds and availability in the laboratory at that moment. Seeds were imbibed in water; dimethyl sulfoxide (DMSO) (1:100 and 1:1000) dissolved in water; 10 mM (1:100) and 1 mM (1:1000) valproic acid; 33 μM (1:100) and 3.3 μM (1:1000) trichostatin A (TSA); 250 μM (1:100) and 25 μM (1:1000) genistein; 250 μM (1:100) and 25 μM (1:1000) SAHA or 25 μM (1:1000) anacardic acid and incubated in darkness at a constant temperature of 30 °C. TSA, genistein, SAHA and anacardic acid are dissolvable in DMSO, therefore a control of DMSO was necessary for all the experiments in the same concentrations used for the dilution of the compounds (1:100 or 1:1000). Based on the germination tests with the commercial batch, TSA and valproic acid at concentrations of 33 μM and 10 mM, respectively, were selected for further studies.

At the time of the study, seeds from the different accessions (SCRI -156, -177, 367, -416, -469, -707, -773, -799 and -937) did not present any type of dormancy. During their storage at 15 °C and 15 % RH they lost any primary dormancy left by after-ripening as corroborated by germination tests at 30 °C (described above). This allowed us to identify positive and negative effects of the inhibitors used.

To test seed lot capacity for secondary dormancy induction, seeds of all genotypes were imbibed in either 1.5 mL of water, 1:100 DMSO, 10 Mm valproic acid or 33 μM TSA, and incubated in darkness by wrapping Petri dishes with three layers of aluminium foil for different periods of time [0 (directly in light conditions), 1, 2, 3, 5, 7 or 14 d] at a constant temperature of 30 °C. For each experiment, three replicates of approximately 50 seeds per treatment (each replicate corresponding to a different mother plant of the same accession) were placed in separate 50 mm Petri dishes on two layers of Whatman no. 1 filter paper soaked with 1.5 mL of the relevant solution applied. Moisture was maintained throughout all the treatments by placing the petri dishes in clear polystyrene boxes and distilled water was added to the dishes whenever needed during the study.

Germination in darkness was scored at the end of each dark-incubation period (1 to 14 d), and the seeds transferred to a 12 h photoperiod at the same temperature (30 °C). Germination was scored twice daily under a binocular microscope. Seeds with a protruding radicle > 1 mm were considered to have germinated. To exclude loss of viability as a possible cause of any differences in the germination level, non-germinated seeds were left to dry for 7 d (4 d in the laboratory; and 3 d in a room at 15 °C and 15 % RH). Thereafter, seeds were soaked

in 1.5 mL of a 10 mM KNO_3 solution and placed at 25/10 °C with a 12 h photoperiod coinciding with the warm temperature phase. Seeds that had not previously germinated but did under these conditions were scored as viable and previously dormant; those that did not germinate, as inviable. All experiments were carried out in temperature- and light-controlled incubators. Light was provided by white fluorescent tubes (radiometric flux density of 50-100 $W.m^{-2}$).

Statistical analysis

Seed germination was quantified as final germination percentage. A fully factorial Generalized Linear Model (GLM) was fitted to the final germination percentages, with logit link and binomial distribution (accession or darkness as fixed factors, plus their interaction) using R (2019).

The reciprocal time a population needs to reach 50 % of germination of viable seeds, is the t50. Times to 50 % testa rupture and germination (t50s) were estimated by linear interpolation of the empirical cumulative germination curves. t50s were analysed by fitting Linear Mixed Models; with compound, darkness, and their interaction as fixed factors; accession and individual nested within accession as random factors; and 0 d of incubation in water as the contrast level.

Differences in the final seed germination percentages of accessions -367 and -799 imbibed in water for all the periods in darkness studied (0, 1, 2, 3 and 7 d) were assessed for significance ($P < 0.05$) by fitting a fully factorial Generalized Linear Model with binomial distribution and logit link (accession or darkness as fixed factors) using R.

Results

Germination tests

Screening of different compounds

A preliminary screening carried out with different concentrations of valproic acid, TSA, anacardic acid, genistein and SAHA, with a control of water and another of two different dilutions of DMSO, in order to test the capacity for induction into secondary dormancy of the commercial batch, allowed us to select the most suitable concentrations.

As can be observed in *Figure 2.1.*, the final germination percentages of seeds from the commercial batch at 0 d in darkness imbibed in water (meaning directly in a regime of a constant temperature of 30 °C with a 12 h photoperiod) was of 71.78 ± 8.00 %. This indicated, along with the germination tests for assessing their dormancy state described before, that seeds from the commercial batch presented some residual primary dormancy.

After 1 d in darkness, radicle emergence was delayed by genistein 250 μM, TSA 33 μM, SAHA 250 μM and valproic acid 10 mM in comparison with water and the dilution 1:100 of DMSO. In water the percentage of germinated seeds was 57.40 ± 9.21 %, whilst in DMSO 1:100 it was 60.36 ± 8.13 %, in genistein 56.45 ± 13.95 %, in TSA 42.67 ± 15.75 %, in SAHA 39.21 ± 15.03 %, and in valproic acid 2.05 ± 0.14 %. The same pattern was observed for 3, 7 and 14 d in darkness for all the compounds used, except for DMSO as the percentage of germinated seeds from 3 to 14 d in darkness was lower than in water. There was a small singularity at 5 d in darkness, since genistein 250 μM (11.81 ± 3.63 %) had a stronger effect than SAHA 250 μM (13.75 ± 2.22 %).

Based on the results obtained, the concentration of 33 μM for TSA and 10 mM for valproic acid were selected for further investigation.

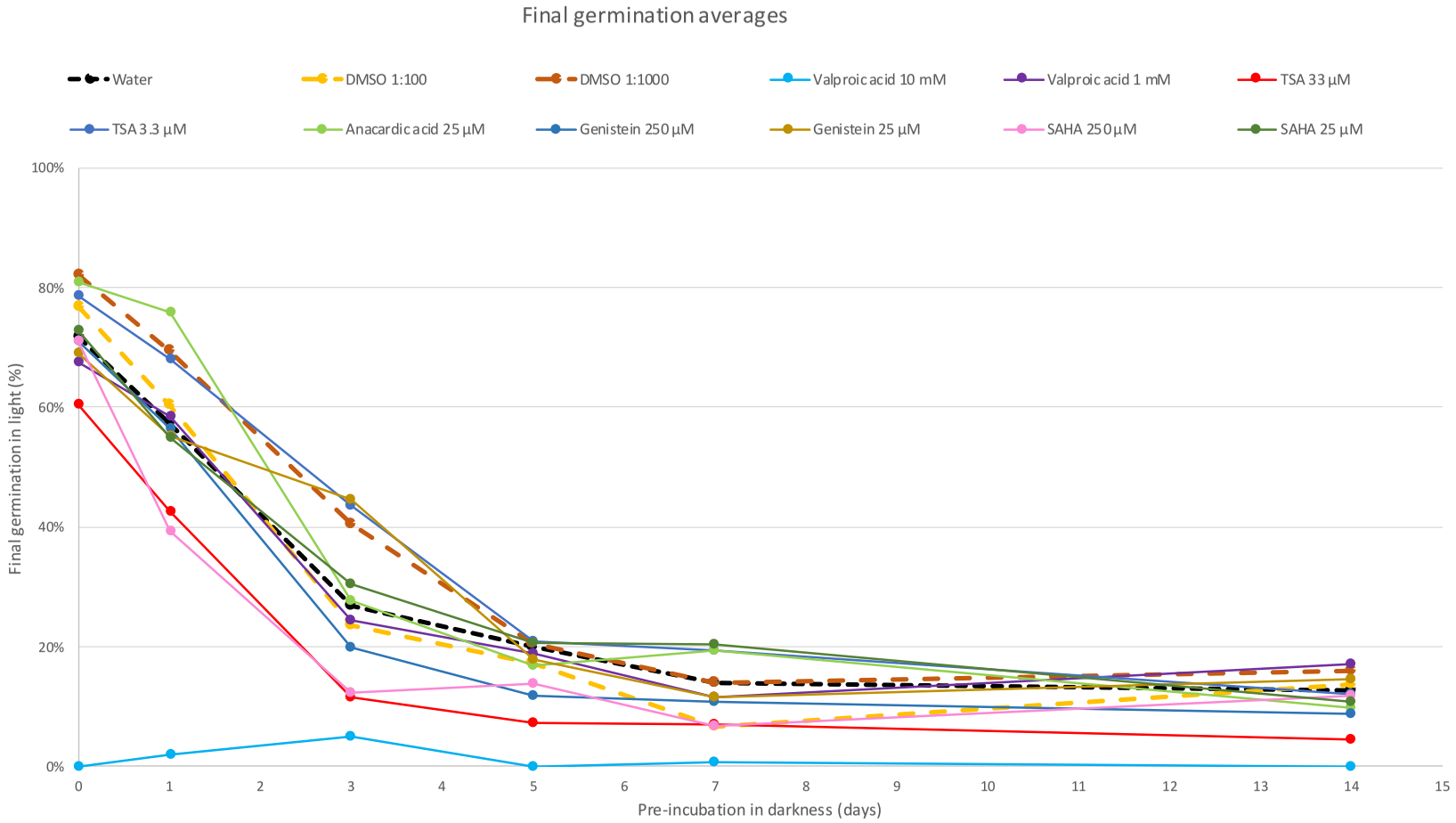

Figure 2.1. Final germination averages of the commercial batch of shepherd's purse seeds imbibed in distinct solutions for different periods of time (0, 1, 3, 5, 7 and 14 d) in darkness. Concentrations of the solutions were: DMSO 1:100 and 1:1000; valproic acid 10 mM and 1 mM; TSA 33 μM and 3.3 μM; anacardic acid 25 μM; genistein 250 μM and 25 μM; SAHA 250 μM and 25 μM.

Induction of secondary seed dormancy

With the selected compounds, TSA and valproic acid, and their respective controls at the concentrations determined in the preliminary screening, *C. bursa-pastoris* seeds from nine different accessions (testing 3 different mother plants per accession) were incubated in darkness at 30 °C for different periods of time. *Figure 2.2.* is a general representation of all the accessions and their final mean germination percentages in response to the different compounds and times of incubation in darkness tested.

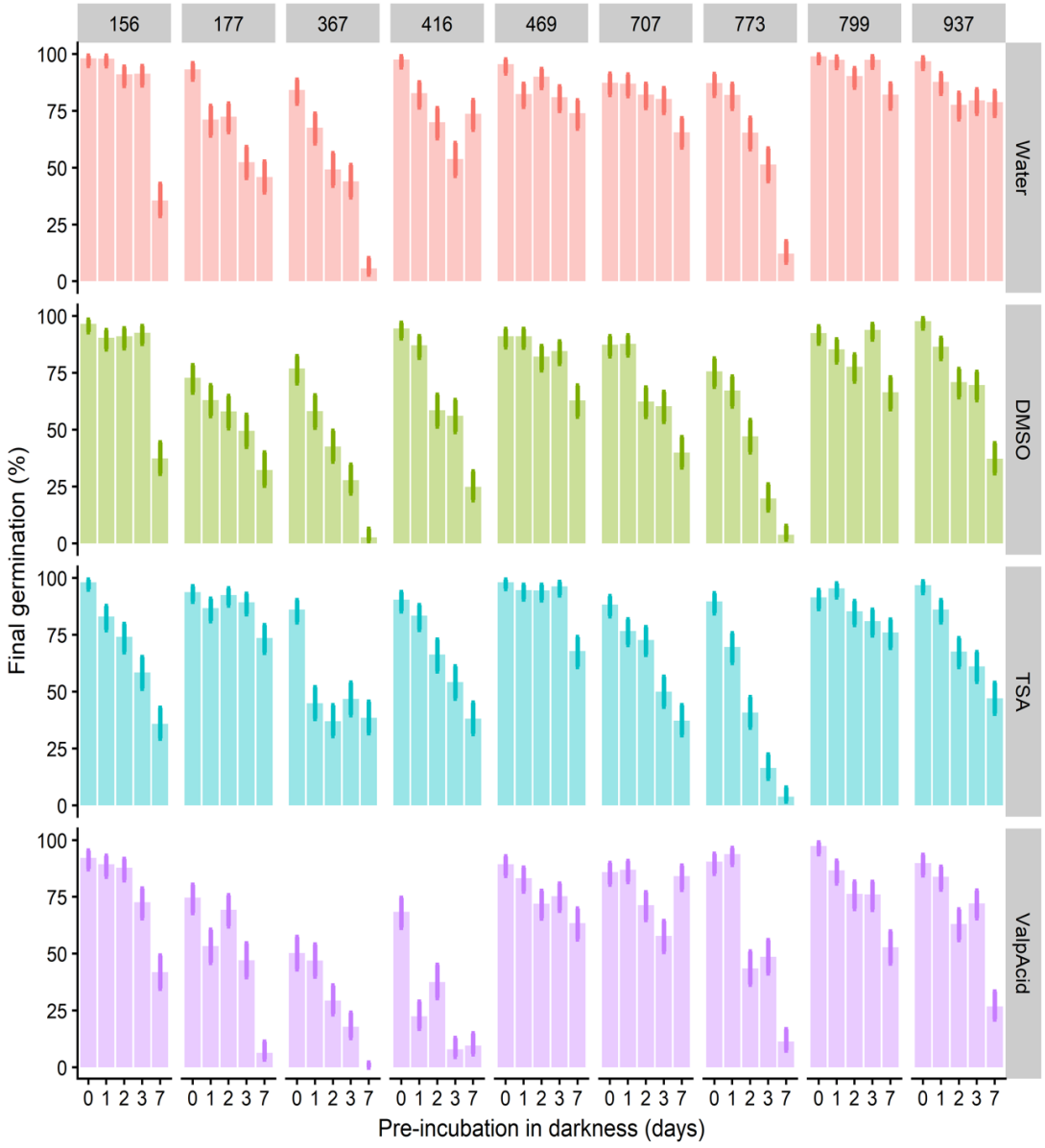

Figure 2.2. Final mean germination percentages of seeds from all the accessions (SCRI -156, -177, -367, -416, -469, -707, -773, -799 and -937) of *C. bursa-pastoris* studied in the different compounds (water, DMSO, TSA and valproic acid) and times of incubation in darkness tested (0, 1, 2, 3 and 7 d), after being exposed to germination-promoting conditions. Bars represent means and brackets the 95 % binomial confidence interval.

The results after applying a General Linear Model (GLM) are represented in *Figure 2.3*. and *Figure 2.4*.

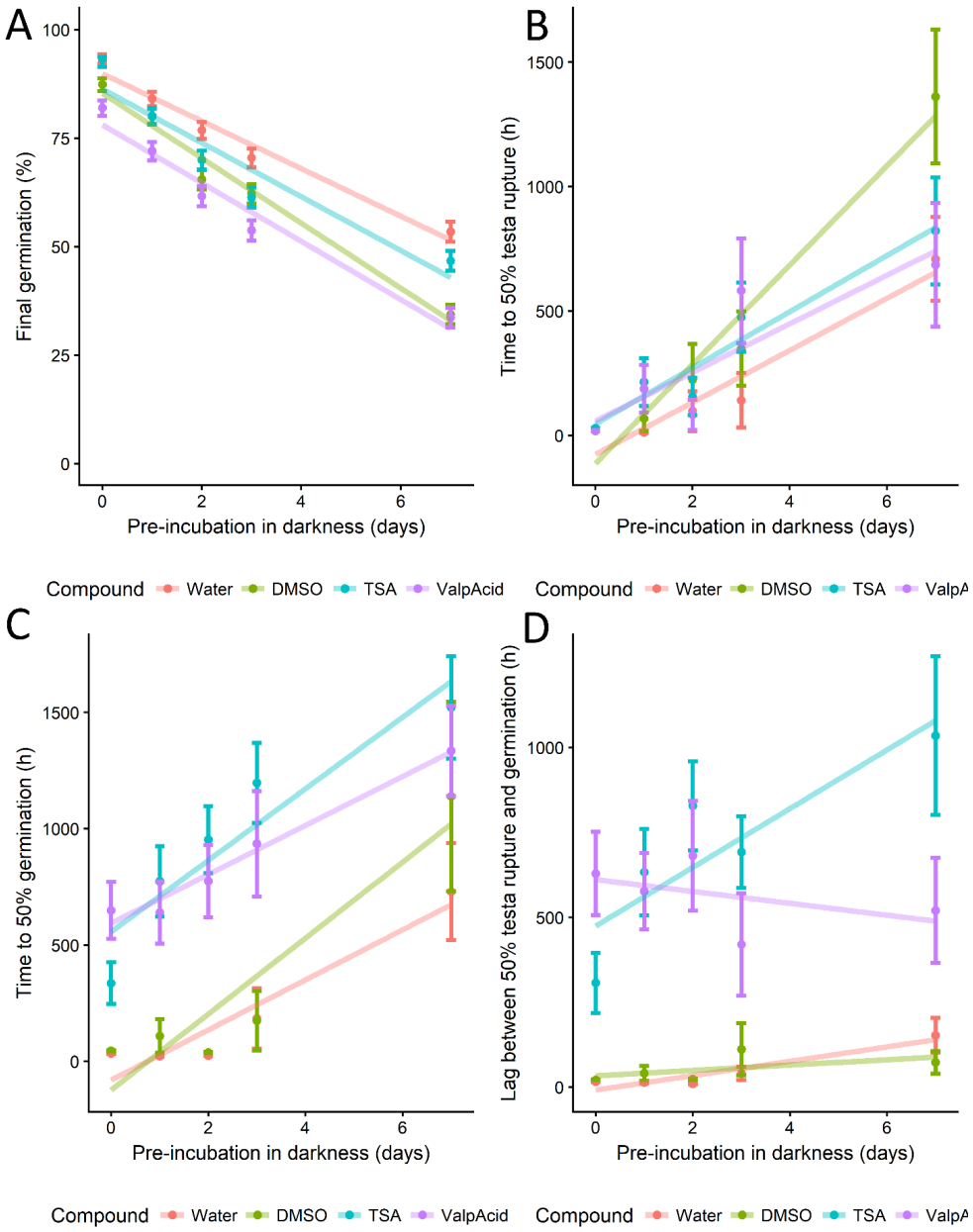

Figure 2.3. Generalised Linear Model (GLM) fitted to the data, taking all the accessions analysed (SCRI -156, -177, -367, -416, -469, -707, -773, -799 and -937). (**A**) Final mean germination percentages (%) in relation to the period of incubation in darkness and separated by the compound used. (**B**) t50s in hours to testa rupture in relation to the period of incubation in darkness and separated by the compound used. (**C**) t50s in hours to germination in relation to the period of incubation in darkness and separated by the compound used. (**D**) Differences between t50s of testa rupture and germination, with the mean of all the accessions together. For (**A**), brackets represent the 95 % binomial confidence interval and for (**B**, **C** and **D**), brackets represent standard errors

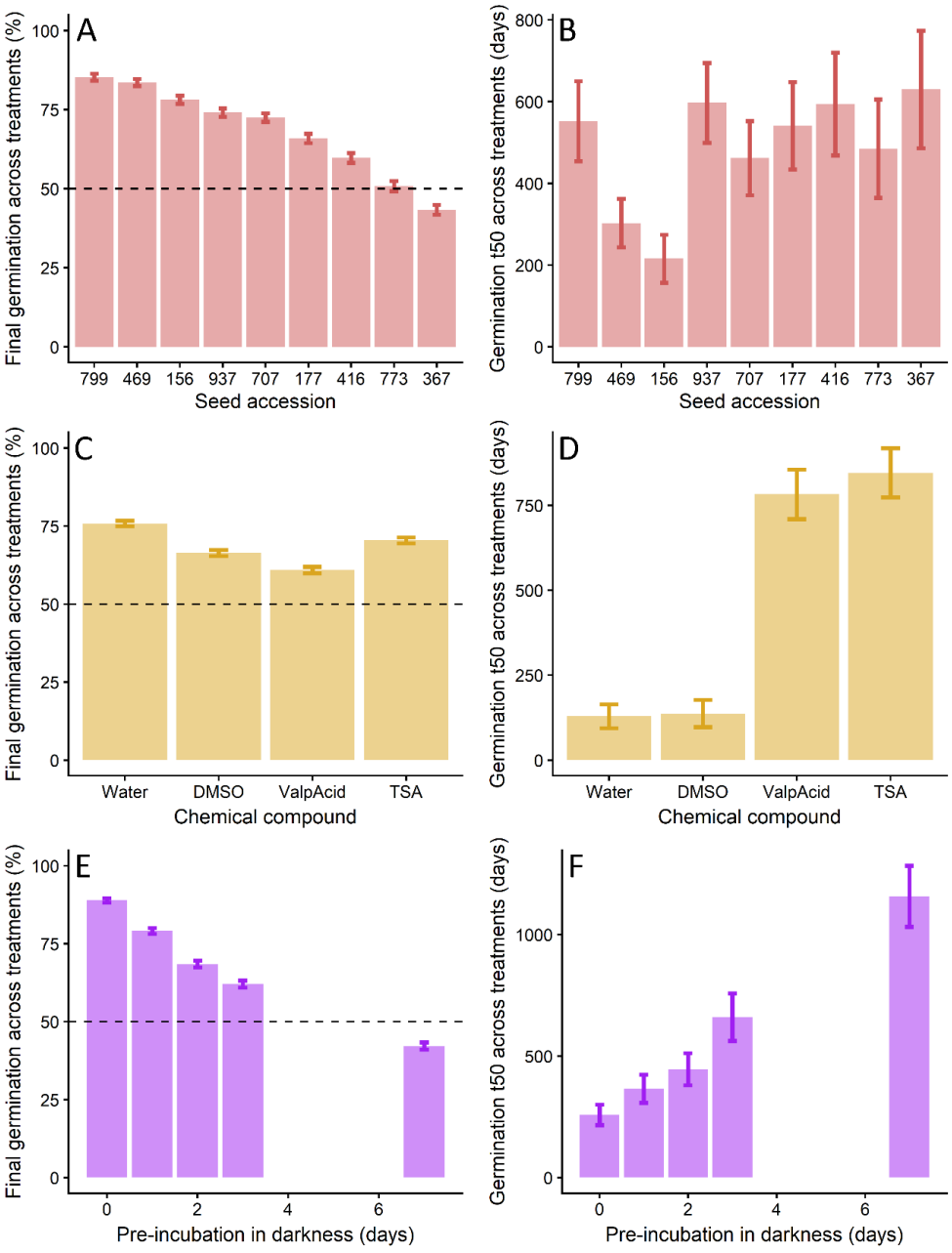

Figure 2.4. Generalised Linear Model (GLM) fitted to the data, taking all the accessions analysed (SCRI -156, -177, -367, -416, -469, -707, -773, -799 and -937). (**A**) Final mean germination percentages (%) across all treatments studied in relation to the accession analysed. (**B**) t50s in days to germination across all treatments studied in relation to the accession analysed. (**C**) Final mean germination percentages (%) across all treatments studied in relation to the compound used. (**D**) t50 for germination in days for all the accessions and treatments analysed in relation to the compound used. (**E**) Final mean germination percentages (%) for all the accessions and compounds used in relation to the period of incubation in darkness. (**F**) t50 for germination in days for all the accessions and compounds used in relation to the period of incubation in darkness. For (**A**, **C** and **E**), brackets represent the 95 % binomial confidence interval and for (**B**, **D** and **F**), brackets represent standard errors

Effect of incubation in darkness and selected compounds

In general terms, darkness had a strong and almost linear effect on final germinations. The longer the period in darkness, the lower the final germination percentages of all the accessions studied (*Figure 2.4.*, E).

The period of time needed for 50 % of the seeds to germinate is denominated t50. If we compare the germination t50s, the longer the period of incubation in darkness, the longer the time for 50% of seeds to germinate, across all treatments and accessions (*Figure 2.4.*, F). The same result was obtained with final germination percentages (*Figure 2.4.*, E), indicating that with longer incubation periods in darkness the germination speed is reduced.

Comparing the germination t50s among compounds, significant differences between valproic acid and TSA with their controls (water and DMSO, respectively), were observed ($P < 0.00001$; $P < 0.00001$) (*Figure 2.3.*, C; *Figure 2.4.*, D). These results indicate that valproic acid and TSA reduce the speed of germination significantly.

Testa rupture was also measured. Comparing t50s for testa rupture between the compounds for all the treatments and accessions, no great differences were observed among them (*Figure 2.3.*, B). All the compounds, including the control DMSO, reduced the final germination percentages in comparison with water (*Figure 2.3.*, A; *Figure 2.4.*, C). Valproic acid had a significant interaction with darkness ($P = 0.02$), meaning that imbibition in darkness caused a stronger delay of germination in the presence of valproic acid than water. Even though DMSO is the compound used to dissolve TSA, it had a strong interaction with darkness with respect to delay of germination ($P = 5.58$ x 10-7). In addition, TSA had less effect on the delay of germination than DMSO (*Figure 2.3.*, A; *Figure 2.4.*, C).

The differences between germination t50s and testa rupture t50s in relation to the compounds are represented in *Figure 2.3.*, D. While valproic acid and TSA had a strongly significant retarding effect on the germination speed (germination t50s), they did not affect the speed of testa rupture (testa rupture t50s) in comparison with water and DMSO. Conversely, DMSO caused no significant difference between the speed of testa rupture and the speed of germination in comparison with water.

The osmotic potential of all solutions was 0 MPa, discarding an osmotic potential effect rather than the effects being due to the compounds' inhibitory characteristics. The viability of the non-germinated seeds, after drying and treatment with KNO_3, is represented in *Figure 2.5.*, with 97 % being the lowest viability.

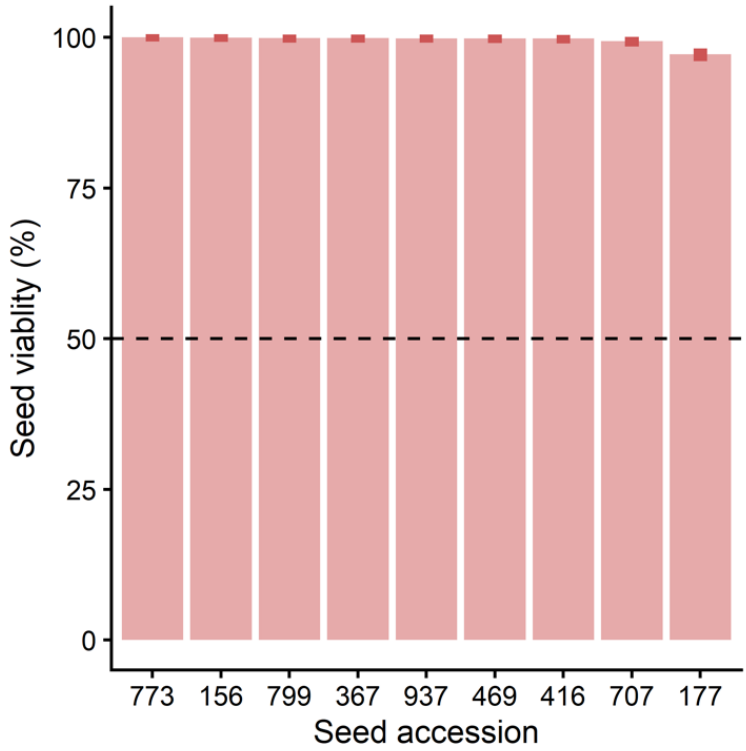

Figure 2.5. Mean seed viability of the different accessions among all the treatments studied. Brackets represent the 95 % binomial confidence interval.

Differences between accessions and mother plants

A broad range in secondary seed dormancy potential was observed among the genotypes examined. Average germination was significantly variable among the nine accessions studied and also among the different mother plants analysed from each accession.

Out of nine accessions tested, -367 and -799 had the most extreme responses to secondary seed dormancy induction (*Figure 2.6.*). Comparing these two, the differences in the final seed germination after dark-induction were significant ($P < 0.05$; main effect's $X^2 = 291.2$, $P < 0.001$) (*Table 2.1.*). Accession -367 showed less germination than accession -799 at all time intervals (main effect's $X^2 = 89.8$, $P < 0.001$) and it also responded more to dormancy induction (interaction's $X^2 = 19.5$, $P < 0.001$), with the rate of secondary dormancy induction approximating to a fall of 11 % in germination per day in -367, compared with 2% per day for -799. This indicates that accession -367 has about a five-time higher propensity to enter secondary dormancy (deep dormant accession) than accession -799 (non-deep dormant accession). The other seven seed accessions had intermediate sensitivities to secondary dormancy induction. Thus, these two accessions were selected for further analyses described in Chapters IV and VI.

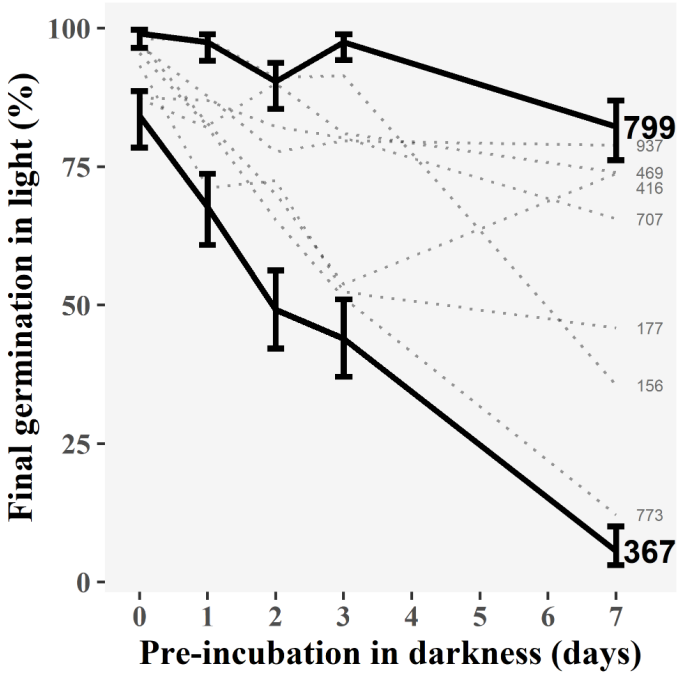

Figure 2.6. Differential rate of dark-induced dormancy amongst accessions (SCRI -156, -177, -367, -416, -469, -707, -773, -799 and -937) of *C. bursa-pastoris* in water and times of incubation in darkness tested (0, 1, 2, 3 and 7 d). Brackets represent the 95 % binomial confidence interval.

Table 2.1. Differences in the final seed germination proportions of accessions -367 and -799 imbibed in water for all the periods in darkness studied (0, 1, 2, 3 and 7 d). Asterisks represent significant differences in the interaction of each accession and darkness in the induction of secondary seed dormancy.

	X^2	p-value	
Accession	89.792	$< 2.2E^{-16}$	***
Darkness	291.161	$< 2.2E^{-16}$	***
Accession:Darkness	19.454	$1.03E^{-05}$	***

However, the co-adaptation described by Toorop et al. (2012) between the flowering times of the different genotypes and their secondary dormancy depth, was no longer applicable. The results obtained indicated that, for example, an intermediate flowering accession, -367, was the genotype with the strongest secondary dormancy depth and a late flowering accession, -799, was the genotype with the weakest one.

Discussion

Seed dormancy is a complex evolutionary ancient trait innate to the seed. It is a function of genotype, location of progeny on the mother plant, as well as environmental conditions during seed maturation (Gulden et al., 2004; Baskin and Baskin, 1998). It is common within wild plants and a major determinant of the ability of a species to survive from year to year. It could be defined as a block to germination that has evolved with the adaptation of plants to different climates and habitats (Footitt and Finch-Savage, 2017).

Seeds that remain in the soil seed bank could be cycling through different depths of dormancy or could persist in a state that only requires light to break dormancy. Disturbance of the soil terminates dormancy as it exposes seeds to light. Seed populations present variable degrees of dormancy as the permissive window for germination opens or closes constantly with each seed somewhere in the continuum from deeply dormant (secondary dormant) to non-dormant (Finkelstein et al., 2008). The sensitivity of seeds to the environmental factors that influence germination is a species or genotype-specific adaptation to the habitat (Finch-Savage and Leubner-Metzger, 2006).

In the present work experiments were carried out to characterize the response of *C. bursa-pastoris* seeds from a set of accessions to different treatments that affect the induction and maintenance of secondary dormancy. These treatments consisted on imbibing seeds at high temperatures (30 °C), in darkness and with compounds known to have an influence on different epigenetic modifications. The temperature of 30 °C was established by Dr. Peter Toorop (personal communication) as the optimum for germination of after-ripened (no residual primary dormancy) *C. bursa-pastoris* seeds.

Genotypic differences in secondary dormancy in a species like *C. bursa-pastoris*, with seeds with a long-lived character in the soil, seem to be more relevant for its life-history characteristics than primary dormancy (Toorop et al., 2012). In this investigation, we found substantial variation in the potential for the induction of secondary dormancy when all accessions were considered, which is influenced by several factors amongst which we found genotype to be the principal and most important. The broad range of secondary dormancy potential was not unexpected as seed dormancy is a very complex phenomenon (Gulden et al., 2004) and considerable variation exists in the degree of seed dormancy among genotypes or cultivars in closely related species such as *Arabidopsis* (Alonso-Blanco et al., 2003; Bentsink et al., 2010).

On both extremes for secondary dormancy depth we found accessions -367 and -799 (deep and non-deep dormant, respectively). Accession -367 presented the lowest final germination percentage across treatments and the longest t50 for germination compared to the rest of accessions. On the opposite side, accession -799 presented the highest germination percentage across treatments.

Sheldon et al. (2002) demonstrated the epigenetic regulation of the *FLOWERING LOCUS C* (*FLC*) gene, with its level of expression being a major determinant of time of flowering. Later, Cao et al. (2008) proved the regulation of flowering time in *Arabidopsis* by the histone epigenetic modification H2B mono-ubiquitination, which controls the binding of histone H3 lysine methylases. The observation of a concurrent existence of co-adaptation in the flowering time and the depth of secondary dormancy found in different genotypes of *C. bursa-pastoris* seeds (Toorop et al., 2012), suggested a possible epigenetic role as its cause. However, this relationship was not confirmed for our seed material after analysing the results obtained in the germination tests using the same seeds as Toorop et al. (2012), but which had remained at 15 % RH and 15 °C for another seven years.

One possible explanation for obtaining different results than this previous investigation, could be due to the fact that Toorop et al. (2012) assessed primary seed dormancy levels of the accessions studied using alternating temperatures of 25/10 °C with light. These conditions are known to break primary dormancy in *C. bursa-pastoris* seeds and are therefore not appropriate to assess primary dormancy levels. However, in their research, primary dormancy of those same seeds was considered marginal and not differing between times to flowering categories. This means that we do not know the actual primary dormancy levels of the distinct accessions when they were analysed by Toorop et al. (2012), as they did not test non-dormant after-ripened seeds entering into secondary dormancy, but seeds with some residual primary dormancy. This implicates that we cannot completely accept that the co-adaptation found between secondary seed dormancy depth and time to flowering was true for the seeds used. Consequently, we cannot compare their results to our study.

The use of compounds with a known effect on epigenetic modifications is considered to be an appropriate tool for studying the biological role of this type of genetic regulation. We tested compounds that are known to alter cytosine methylation [genistein, Arase et al. (2012)] or histone acetylation levels [SAHA, Richon et al. (1998); TSA, Yoshida et al. (1995); valproic acid, Göttlicher et al. (2001); anacardic acid, Cui et al. (2008)] by carrying out germination tests with seeds belonging to a commercial batch. The results obtained indicated that the strongest responses were with incubation of the histone deacetylases inhibitors TSA and valproic acid.

The effective hyper-acetylation caused by TSA was proven with Western blotting by Murphy et al. (2000). The inhibition activity of HDACs by valproic acid has also been demonstrated (Phiel et al., 2001). However, no studies of its effects on germinating seeds have been done before. It has been described as a stronger and more specific inhibitor of histone deacetylases than other compounds such as TSA or SAHA in animal models (Kostrouchová and Kostrouch, 2007). Nonetheless, the specificity of these HDAC inhibitors has not been fully tested.

The implication of histone deacetylases in seed dormancy and germination in *Arabidopsis thaliana* has been demonstrated in different studies, with some of them using TSA or other HDACs inhibitors with seeds (Tanaka et al., 2008; Zhang et al., 2011; Wang et al., 2013; Van Zanten et al., 2014; Nelson and Steber, 2017; Pagano et al., 2017) or with other systems such as pollen (Cui et al., 2015) or plant explants (Wójcikowska et al., 2018) to obtain a first glimpse of their possible implication.

Tanaka et al. (2008) demonstrated that the repression of both histone deacetylases HDA6 and HAD19 resulted in arrested growth after germination through repression of embryonic phase transition genes such as *FUSCA 3* (*FUS3*) and *ABSCISIC ACID INSENSITIVE 3* (*ABI3*). In these studies, they used concentrations of TSA on germinating *Arabidopsis* seeds from 5 to 50 μM. In all of the tested conditions, seeds showed a delay of germination in comparison with the control after 3 d of sowing, however the final germination after 7 and 14 d was higher than the control. On the other hand, post-germination growth was always inhibited in a TSA concentration dependent manner.

Zhang et al. (2011) applied different concentrations of TSA (10 μM) to *Zea mays* L. (maize) germinating seeds, observing lower germination rates in seeds imbibed in TSA compared to seeds imbibed in the control. The seeds used did not present primary dormancy as 90 % of them germinated at a constant temperature of 25 °C in all the conditions. Nevertheless, TSA needs to be dissolved in DMSO but a DMSO control was not indicated. Although in our results DMSO had a stronger effect on the inhibition of testa rupture t50 than TSA (dissolved in DMSO) (*Figure 2.3.*, B), only TSA had an increased germination t50 (*Figure 2.4.*, C). However, both of them reduced the final germination percentage compared to water (*Figure 2.4.*, C). Pagano et al. (2017) observed the same response in *Medicago truncatula* seeds, where TSA caused a 24 h delay in radicle protrusion of treated seeds. However, this effect was only seen when TSA concentration was higher than 10 μM.

Wang et al. (2013) showed that histone deacetylation mediated by SWI-INDEPENDENT3 (SIN3)-LIKE (SNL) 1 and SNL2 is involved in the regulation of primary seed dormancy in *Arabidopsis* through affecting the balance between abscisic acid (ABA) and ethylene. In their work, post germination growth was inhibited with 30 μM of TSA and *snl1 snl2-1* double mutant seeds presented the highest sensitivity to this compound.

Following treatment with TSA, pollen germination and tube growth were inhibited in *Picea wilsonii* (Cui et al., 2015). Although the germination rates after TSA treatment were significantly lower than under the control, DMSO, more pollen grains germinated in response to TSA over time than to DMSO.

In the research carried out by Van Zanten et al. (2014), freshly harvested seeds of *A. thaliana* were used and TSA enhanced germination in a dose-dependent manner compared to the control, DMSO. Although the primary dormancy level of

these seeds was considered low by the authors, they only germinated to 50 % in water (in conditions of 25 °C for 16 h and of 20 °C for the remaining 8 h), and this percentage increased drastically after stratification for 3 d at 4 °C, both results indicating that the seeds used presented high levels of primary dormancy. This is an essential difference between our study and the research by Van Zanten et al. (2014) as the physiological conditions of the seeds used in the studies differ. Nonetheless, our results present a relation with their results as TSA increased the final germination percentage of seeds, as discussed before. However, another crucial difference with Van Zanten et al. (2014) is that to treat *Arabidopsis* seeds with TSA, their testa was ruptured manually with a dissection needle to aid the penetration of the chemical. In our research this process was not applied as it could have affected the dormancy status of the seeds. This small difference could have had an enormous effect on the results.

Nelson and Steber (2017) studied the treatment of dormant and gibberellic acid (GA) insensitive *sly1-2* mutant seeds with TSA, a treatment that rescued their germination at an optimum concentration of 2 μM, showing decreasing germination capacity at 4 and 6 μM. The use of TSA with *Arabidopsis* explants was carried out by Wójcikowska et al. (2018) with concentrations from 0.1 to 1.0 μM. Their results suggest an involvement of histone acetylation in the release of the embryonic development program mediated by auxins.

Although differences in the results of all these investigations can be observed, what seems to be the main reason behind the differences is the concentration of TSA used. Our study tested higher concentrations of TSA than those used in the previously cited experiments, which would explain the high delay of t50 for germination observed in all the accessions when treated with TSA in comparison with the control, DMSO, or water (general control and control of valproic acid). However, the final germination percentag of seeds treated with TSA was superior to the one of DMSO, although lower than with water, which could also be explained by the high concentration of TSA.

TSA, as indicated before, is dissolved in DMSO and in our results DMSO had a clear interaction with darkness. Moreover, the final germination average of all the treatments and accessions was lower in DMSO than in water, a result in accordance with Van Zanten et al. (2014), although in their study the difference was found to be not significant. The reasons behind this strong effect of DMSO, supposedly being just a solvent, are not very clear. It has however the ability to induce cell fusion and cell differentiation and enhance the permeability of lipid membranes. It also causes the membrane to become floppier which would reduce the barrier to molecular transport, and at high concentrations induces water pores formation in the membrane (Notman et al., 2006).

As mentioned before, the specificity of these compounds has not been completely characterized. For instance, TSA presents genotoxic effects provoking DNA damage (Pagano et al., 2017), blocks the cell cycle and induces reactive

oxygen species (ROS) accumulation (Hou et al., 2015). All of these events affect different molecular processes, making the general picture extremely complicated.

In our results, TSA and valproic acid caused a delay of t50 for germination but the speed of testa rupture, t50 for testa rupture, did not present significant differences with their respective controls. As previously mentioned, germination has two visible physical stages: testa and endosperm rupture, the latter being completed with the micropylar endosperm rupture by the radicle (Bentsink and Koornneef, 2008). These results indicate that the compounds do not affect secondary dormancy as it was originally hypothesised (because of the testa rupture) and therefore the results will be discussed in relation to the observed delay in the speed of germination.

The probable implication of a deacetylation event in seed germination has been described in previous studies (Perrella et al., 2010; Cigliano et al., 2013; Wang et al., 2013). HDACs contribute to the repression of embryogenesis related gene expression during germination (Tai et al., 2005; Tanaka et al., 2008). This indicates that the action of TSA and valproic acid in the delay of t50 for germination could possibly be related to the positive regulation of embryo-specific transcription factors implicated in the maintenance of embryonic properties, such as LEC1, FUS3 or ABI3, hence promoting a delay of germination.

At the same time, these results are indicating that acetylation/deacetylation of histones is implicated in the elongation of the transition zone and lower hypocotyl cells necessary for radicle protrusion (Sliwinska et al., 2009). The main hormones implicated in these processes are auxins (Fu and Harberd, 2003). Their relation with histone deacetylation has been unveiled by Wang et al. (2016), who demonstrated the regulation of radicle promotion and early growth of *Arabidopsis* seeds by SNL1 and SNL2 in a manner dependent on AUXIN RESISTANT 1 (AUX1). Thus, we could hypothesize that HDACs inhibitors, such as TSA and valproic acid, act on histone deacetylases that are implicated in the regulation of auxins pathways and signals, which at the same time regulate specific steps in the seed germination process. In accordance with this theory, differences in the expression of histone deacetylases between accessions with differences in their germination response to HDAC inhibitors may be observed when seeds are imbibed in water and induced into secondary dormancy.

The focus in these germination tests has been on elucidating the role of epigenetic regulators in natural dormancy depth. Taking two accessions which presented extreme responses to the induction of secondary dormancy in water (-367 and -799), a transcriptome analysis that is described in Chapter VI was done. In addition, it was tested whether exposure of a particular accession to a HDAC inhibitor (versus a control) leads to differential expression of epigenetic regulator genes similar to those potentially involved in differences in natural dormancy depth. Valproic acid instead of TSA was used to treat the seeds as it caused a

stronger and faster delay of germination in all the accessions and does not need to be dissolved in DMSO.

Valproic acid was discovered as an inhibitor of histone deacetylases by Göttlicher et al. (2001), who proved its effect as a reducer of tumour growth and metastasis formation in animals. It is used as an anti-epileptic drug and to treat bipolar disorder (Phiel et al., 2001). It inhibits the catalytic activity of class I HDACs in mammals but also induces the proteasomal degradation of HDAC2, in contrast to trichostatin A (Krämer et al., 2003). It has been shown to down-regulate the expression of proteins essential for chromatin maintenance in animal cells such as the Structural Maintenance of Chromatin 1 to 6, DNA methyl transferase-1 and Heterochromatin Protein-1. It is also capable of inducing mono-, di- or tri-methylation of histone H3 at lysine 9 (H3K4) (Chateauvieux et al., 2010). However, no previous studies in plants on the effects of this HDAC inhibitor have been reported before, indicating that this research could be used as a preliminary study of its possible future applications in plant physiology research.

This works provides novel insights into the effects caused by the histone deacetylase inhibitors TSA and valproic acid during the induction of secondary seed dormancy in seeds of *C. bursa-pastoris*, potentially mimicking the effects of chromatin remodelling genes.

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3

CHAPTER 3

Optimization of a genomic DNA extraction protocol for *Capsella bursa-pastoris* seeds

Introduction

Even though isolating high-quality DNA from different plant tissues is often a routine procedure, it can also present great problems, therefore being still an important and critical issue in plant molecular studies. Plants may have rigid polysaccharide walls, pigments or secondary metabolites that can interfere with DNA extraction and purification (Varma et al., 2007). Obtaining high-quality non-contaminated genomic DNA was essential for reliable results in this doctoral thesis.

Numerous protocols have been developed in the preceding decades to isolate DNA from different plant species. These methods dilute, selectively precipitate or inactivate the contaminating substances. Some very common procedures are Doyle and Doyle (1987, 1990), Murray and Thompson (1980) or Dellaporta et al. (1983). These methods avoid the co-precipitation of polysaccharides or proteins and nucleic acids with the use of cetyltrimethylammonium bromide (CTAB) or sodium dodecyl sulphate (SDS). Although they have been successful in different experiments, there is not an universal protocol that works for all plant tissues and species.

Even closely related species may require different isolating protocols. In most cases, researchers need to adapt a protocol, or even use a combination of protocols, to obtain DNA from a particular material source. Commercially available kits do not always deliver good results.

Homogenization of the material is a crucial step in the extraction process, especially with hard tissues like seeds. Fine grinding the tissue in liquid nitrogen using a mortar and pestle is rapid and applicable to a wide range of sources. Besides, it is a good choice for an improved separation of DNA from the contaminating compounds if the ground material is afterwards incubated with the extraction buffer (Jobes et al., 1995).

The choice of the source tissue can be critical, with fresh, young and expanded leaves being the most convenient organ to sample (Murray and Thompson, 1980). Problems encountered in the extraction and purification of DNA from seeds include co-isolation of highly viscous polysaccharides and other substances that bind tightly to nucleic acids during the isolation. The contaminated DNA stays in the wells during gel electrophoresis. Complex polysaccharides can make up a high percentage of tissue weight. As far as possible, it is necessary to avoid the co-precipitation of polysaccharides with DNA, which prevents its redissolution.

Here the evaluation of several DNA extraction protocols that were developed to overcome the problems of polysaccharide contamination is reported and a solution for a new, inexpensive and fast large-scale DNA protocol suitable for seed material of *Capsella bursa-pastoris* is presented.

The newly developed protocol combines traditional lysis with anion exchange chromatography along with modifications to minimize polysaccharide co-isolation. This method is a low-cost, reliable and a rapid technique for extracting high-quality and -quantity DNA from seeds.

Material and methods

Plant material

Non-dormant, primary or secondary dormant *C. bursa-pastoris* seeds, either dry or imbibed in water, were used to optimize a total genomic DNA extraction protocol. The non-dormant seeds used had some residual primary dormancy, as was detected with germination tests (data not shown). They belonged to a commercial batch from Herbiseed (Twyford, Berkshire) from 2013 (kindly provided by Dr. Pietro Iannetta, The James Hutton Institute, Scotland).

Primary dormant seeds were generated and collected in Oviedo, Asturias from plants grown in a greenhouse from November 2013 to March 2014, when the plants were ready to be dried. Its production has been described in Chapter II. Seeds were harvested from individual plants and then mixed to form 8 different pools that were kept at 4 °C for the rest of the project. Pool 1, 3 and 5 were selected as they presented higher total amount of seeds. Secondary dormant seeds were non-dormant after-ripened seeds, stored at 15 °C and 15 % RH, which were then imbibed at 30 °C in darkness for different periods of time.

See *Table 3.1* for the source of plant material used for the subsequent DNA methylation studies using the described optimized protocol.

Table 3.1. Yields and absorbance ratios of extracted DNA

Sample	DNA Concentration (ng/μL)	A260/280	A260/230
Dry seeds Pool 1	42.15	1.86	1.45
Dry seeds Pool 3	30.01	1.90	1.29
Dry seeds Pool 5	17.93	2.05	1.08
1 Day 30 °C 12h light Pool 1	6.96	1.89	1.10
1 Day 30 °C 12h light Pool 3	8.91	1.75	0.62
1 Day 30 °C 12h light Pool 5	8.86	1.60	0.63
14 Days 30 °C 12h light Pool 1	53.73	1.75	1.07
14 Days 30 °C 12h light Pool 3	31.31	1.83	1.71
14 Days 30°C 12h light Pool 5	26.65	1.78	1.09
Dry seeds 367.1	39.48	1.88	1.63
Dry seeds 367.2	39.98	1.93	1.64
Dry seeds 367.3	35.63	1.94	1.54
Dry seeds 799.1	37.65	1.93	1.70
Dry seeds 799.2	27.59	2.00	1.64
Dry seeds 799.4	41.99	1.91	1.72
1 Day 30 °C 12h light 367.1	13.06	1.67	1.08
1 Day 30 °C 12h light 367.2	33.49	1.78	1.36
1 Day 30 °C 12h light 367.3	31.49	1.75	1.34
1 Day 30 °C 12h light 799.1	31.48	1.83	1.46
1 Day 30 °C 12h light 799.2	31.10	1.84	1.50
1 Day 30 °C 12h light 799.4	22.56	1.87	1.26
1 Day Dark 367.1	16.90	1.79	0.85
1 Day Dark 367.2	49.04	1.81	1.55
1 Day Dark 367.3	22.44	1.82	1.21
1 Day Dark 799.1	25.83	1.91	1.54
1 Day Dark 799.2	27.27	1.88	1.50
1 Day Dark 799.4	27.50	1.83	1.32
3 Days Dark 367.1	33.79	1.83	1.79
3 Days Dark 367.2	43.61	1.74	0.91
3 Days Dark 367.3	46.25	1.88	1.73
3 Days Dark 799.1	35.11	1.84	1.75
3 Days Dark 799.2	32.11	1.83	1.37
3 Days Dark 799.4	27.45	1.85	1.57
7 Days Dark 367.1	31.20	1.77	1.29
7 Days Dark 367.2	47.68	1.79	1.86
7 Days Dark 367.3	21.57	1.86	1.63
14 Days Dark 367.1	27.98	1.84	1.61
14 Days Dark 367.2	34.47	1.84	1.63
14 Days Dark 367.3	58.59	1.83	1.84

Testing DNA extraction protocols

Total genomic DNA from seeds was extracted using six different methods, with certain modifications, which have been described as efficient for isolating good-quality DNA from samples with large amounts of secondary compounds or polysaccharides. For DNA extraction, 50 to 100 mg of dry or imbibed seeds were used in all the protocols, except for Valledor et al. (2009), where up to one g of material was used in a modified version of the protocol. Four extraction buffers from different protocols [DNeasy® Plant Mini Kit (Qiagen); Dellaporta et al., 1983; Edwards et al., 1991; Jobes et al., 1995] were used to optimize a final protocol. Samples were frozen and ground in liquid nitrogen with a mortar and pestle immediately before procedures.

Method 1: DNeasy®Plant Mini Kit (Qiagen)

Extraction from frozen tissue was performed according to the manufacturer's instructions with some modifications. Increased amounts of buffers AP1 (up to 1 mL) and AP2 (up to 425 μL) were used as suggested in the troubleshooting guidelines, as well as a longer period of incubation (60 min) at 65 °C.

Method 2: Modified (Doyle and Doyle, 1987, 1990)

This protocol was modified after Doyle and Doyle (1987, 1990), which is a "classical" DNA purification protocol based on lysis and purification with CTAB, which selectively precipitates the DNA while maintaining carbohydrates, proteins and other contaminants in solution.

- **MODIFIED EXTRACTION BUFFER**: 2 % (w/v) CTAB, 100 mM Tris-HCl, 20 mM ethylenediaminetetraacetic acid (EDTA), 2.0 M NaCl, pH 8.0. 2 % (w/v) polyvinylpyrrolidone-40 (PVP-40), 5 % (v/v) β-mercaptoethanol and 1 % (v/v) RNase A were added just before use.
- **MODIFIED PROTOCOL**: homogenized material was incubated with 1 mL of the extraction buffer at 65 °C for 60 min. An equal volume of chloroform:octanol (24:1) was added, mixed and centrifuged at 18000 g for 10 min. The upper phase was removed and rewashed with chloroform:octanol. Sodium acetate and isopropanol were added, and the solution was left at -20 °C for 30 min to precipitate the nucleic acids. The solution was centrifuged at 18000 g for 10 min at 4 °C, the supernatant discarded, and the pellet washed with 70 % (v/v) ethanol. After centrifugation, ethanol was discarded and the pellet was dried in a Speed Vacuum concentrator, resuspended in $T_{10}E_1$ (Tris-HCl 10mM and EDTA 1mM) with RNase and incubated at 37 °C for 1 h. DNA was precipitated by adding NaCl and 95 % (v/v) ethanol and centrifuging at 18000 g at 4 °C for 10 min. Afterwards, the supernatant was discarded. The pellet containing the DNA was washed three times with 70 % Ethanol (v/v), dried with a Speed Vacuum and diluted in sterilized milliQ water.

Method 3: Valledor et al. (2009)

Method based on the protocol of Doyle and Doyle (1987, 1990), with lysis and purification by CTAB. Different modifications of the protocol were used, an increase in EDTA concentration in the extraction buffer from 50 mM to 500 mM or in the starting plant material from 100 mg to 1 g.

- **EXTRACTION BUFFER**: 2 % (w/v) CTAB, 100 mM Tris-HCl, 50 mM EDTA, 2.0 M NaCl, 8 mM ascorbic acid, 5 mM diethyldithiocarbamate (DIECA), pH 8.0. 2 % (w/v) PVP-40, 5 % (v/v) β-mercaptoethanol and 1 % (v/v) RNase A were added just before use.
- **PROTOCOL:** 50 to 100 mg of homogenized material were incubated with 800 μL of the extraction buffer at 65 °C for 30 min. After a centrifugation step at 5000 g for 5 min, the supernatant was mixed with an equal volume of chloroform:isoamylalcohol (24:1) and centrifuged at 7500 g for 5 min. The aqueous phase was transferred to a new collection tube, RNase A was added, and the mixture was incubated at 37°C for 20 min. The solution was washed twice with chloroform:isoamylalcohol (24:1) as described before. The upper phase was transferred to a new collection tube and the DNA was precipitated by addition of 1 volume of isopropanol and incubation at RT. Centrifugation at 12000 g was performed in order to pellet the DNA, which was cleaned 3 times with 70 % cold (-20 °C) ethanol. After centrifugation, the DNA was recovered forming a pellet, which was dried and dissolved in sterilized milliQ water. With 1 g of homogenized material, the procedure was performed in the same way except for the centrifugation step after the addition of isopropanol, where the DNA was "fished" with a Pasteur pipette.

Method 4: Piskurewicz and Lopez-Molina (2011)

Protocol based on lysis and purification with CTAB and CsCl.

- **EXTRACTION BUFFER:**
  - CTAB B Solution: 2 % (w/v) CTAB, 1.4 M NaCl, 0.1 M Tris-HCl (pH 8.0).
  - CTAB C Solution: 1 % (w/v) CTAB, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0).
  - CTAB D Solution: 1 % (w/v) CTAB, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 1 M CsCl.
- **PROTOCOL:** 100 mg of homogenized material were incubated with the extraction buffer CTAB B at 65 °C for 10 min. A mixture of 1:1 CTAB B and CTAB C was added and the sample was incubated at 65 °C with vortexing. The mixture was centrifuged at 18000 g for 15 min and the supernatant was transferred to another collection tube and mixed with chloroform. The solution was centrifuged at 18000 g and the upper phase collected. Washing with chloroform was repeated twice. The upper phase was transferred then to a new collection tube and incubated with one volume of CTAB C at RT. The solution was centrifuged at 18000 g for 15 min and the pellet was resuspended in CTAB D plus RNase A, incubated at 37 °C for 30 min and mixed with two

volumes of absolute ethanol. DNA was precipitated at -20 °C by centrifugation at 18000 g. The pellet was washed with 75 % ethanol and resuspended in milliQ water.

Method 5: Modified protocol of Russell et al. (2010) and Souza et al. (2012)

Modification of the protocol by Souza et al. (2012), that is a variation of Russell et al. (2010). Mucilaginous polysaccharides are removed with a sorbitol buffer and extraction and purification of DNA is performed with a CTAB buffer with high salt concentration to avoid co-precipitation of polysaccharides.

- **EXTRACTION BUFFERS**:
  - Sorbitol buffer: 100 mM Tris-HCl, 0.35 M sorbitol, 5 mM EDTA (pH 8.0). 1 % (w/v) PVP-40, 1 % (v/v) RNase A and 5 % (v/v) β-mercaptoethanol added just prior use.
  - CTAB buffer: 2 % (w/v) CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl. 2 % (w/v) PVP-40, 5 % (v/v) β-mercaptoethanol and 1 % (v/v) RNase A added just prior use.
- **PROTOCOL:** 100 mg of homogenized material were mixed by vortex with 1 mL of sorbitol buffer. The solution was centrifuged at 3500 g for 10 min at 4 °C. The pellet and the supernatant were separated and transferred to new collection tubes. One mL of sorbitol was added to each tube, the solutions were mixed, and the centrifugation step repeated. The darkest pellet with the testa residues was discarded, the lighter pellet was transferred to a new collection tube and kept on ice. The supernatants were transferred to new collections tubes, sorbitol was added and mixed and the centrifugation step repeated. The pellets were separated and kept on ice. Addition of sorbitol and centrifugation were repeated once more, and the pellets kept separately. The supernatants were finally discarded. All the pellets were transferred together to a collection tube whilst avoiding pipetting. A centrifugation step at 21000 g for 20 min at 4 °C was carried out. The supernatant was discarded, and the pellet washed with 70 % ethanol. The solution was centrifuged at 21000 g for 10 min at 4°C. The pellet was incubated in CTAB buffer at 65 °C for 1 hour. The solution was washed with chloroform:isoamylalcohol (24:1) and centrifuged at 21000 g for 10 min. The aqueous phase was recovered and transferred to a new collection tube where the DNA was precipitated with 1:10 volumes of sodium acetate 3 M and 2:3 volumes of isopropanol and centrifuged at 21000 g for 20 min. The pellet was washed three times with 70 % ethanol, dried and resolubilized in sterilized milliQ water.

Method 6: Either lysis buffer of Dellaporta et al. (1983), Edwards et al. (1991), Jobes et al. (1995) or AP1 (Qiagen) and modified protocol of DNase® Plant Mini Kit, Qiagen.

Dellaporta et al. (1983), Edwards et al. (1991), Jobes et al. (1995) or AP1 (Qiagen) extraction buffers along with the use of disposable chromatographic mini spin columns with an anion exchange matrix were used to perform this method. After lysis and a pre-purification step, the lysate is mixed with a chaotropic salt

that allows DNA to bind to the anion exchange matrix. Following this, DNA can be purified using washing steps and is eluted from the anion exchange column by adding sterilized milliQ water. Only the buffers described in these protocols were conserved, although modifications were made. Times and procedures were optimized for *C. bursa-pastoris* seed material.

Extraction buffer by Dellaporta et al. (1983)

- **EXTRACTION BUFFER**: 100 mM Tris-HCl (pH 8.0), 500 mM NaCl, 50 mM EDTA (pH 8.0). 10 mM β-mercaptoethanol and 1 % (v/v) RNase A were added just prior use.

Extraction buffer by Edwards et al. (1991)

- **EXTRACTION BUFFER:** 200 mM Tris-HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA, 0.5 % (w/v) SDS. 2 % (v/v) β-mercaptoethanol and 1 % (v/v) RNase A were added just prior use.

Extraction buffer by Jobes et al. (1995)

- **EXTRACTION BUFFER:** 100 mM sodium acetate (pH 4.8), 500 mM NaCl, 100 mM EDTA (pH 8.0), pH 5.5. 10 mM dithiothreitol (DTT), 2 % (w/v) PVP-40, 100 μg/mL proteinase K and 2 % (v/v) β-mercaptoethanol were added just prior use.

Extraction buffer AP1 (Qiagen)

- **EXTRACTION BUFFER:** Commercial buffer from the DNeasy® Plant Mini Kit of Qiagen, based on SDS. 5 % (v/v) β-mercaptoethanol and 1 % (v/v) RNase A were added just prior use.

Other solutions

- Potassium acetate: 3 M $C_2H_3KO_2$.
- Guanidine-HCl: 0.66 M CH_5N_3-HCl in 63% (v/v) ethanol.
- Washing solution: 0.025 M NaCl, 0.005 M Tris-HCl (pH 7.5) and 0.05 mM EDTA in 75 % (v/v) ethanol absolute.

- **PROTOCOL**: 50 mg of homogenized material were incubated with the extraction buffer by Edwards et al. (1991) (1 mL), by Dellaporta et al. (1983) (735 μL) or using the AP1 buffer (Qiagen) (1 mL) with 20 % (v/v) SDS solution (490 μL) at 65 °C for 1 h. In the case of using the extraction buffer by Jobes et al. (1995), the sample was incubated with 1 mL of the buffer for 1 h at 55 °C. Afterwards, 81 μL of 20 % (v/v) SDS solution were added, the sample was incubated for another hour at 55 °C and centrifuged at 10000 g for 10 min. The pellet was discarded, and the supernatant was transferred to a new collection tube. After the described extraction step, all the solutions from the different

treatments were cooled down and 1:3 of the volume of potassium acetate was added and mixed by vortex. The collection tube was placed on ice for 20 min. Centrifugation was carried out at 21000 g for 5 min at 4 °C and the pellet was discarded. The lysate was transferred to a new collection tube and another precipitation step with potassium acetate was performed. The pellet was discarded, and the lysate was transferred to a silica mini spin column for DNA purification (Econospin® mini spin column) and centrifuged at 18000 g for 2 min at RT. 1.5 volumes of Guanidine-HCl were then added to the flow through and mixed in by pipetting. The solution was transferred to a silica spin mini column and centrifuged at 11000 g for 1 min. The flow through was discarded and the column was transferred to a new collection tube. 500 μL of washing solution were added to the column and this was then centrifuged at 11000 g for 1 min. The flow through was discarded and another 500 μL of washing solution were added, centrifuged at 18000 g for 3 min before the flow through was again discarded. The column was transferred to a new collection tube where 30 μL of sterilized milliQ water at 70 °C were added and incubated for 5 min. The column on the collection tube was centrifuged at 11000 g for 1 min at RT and the eluted DNA was kept on ice. Addition of water, centrifugation and collection of the eluted DNA were repeated twice.

DNA analysis

Genomic DNA quantity and quality were evaluated by agarose gel electrophoresis and by measuring the 260/280 and the 260/230 absorbance ratios (A260/280 and A260/230, respectively) using a NanoDrop™ Spectrophotometer (ThermoScientific™). DNA was subjected to gel electrophoresis in 1 % agarose gels, stained with ethidium bromide and visualized using a UV transilluminator.

Results

DNA extracted with the modified methods described before [Doyle and Doyle (1987, 1990), Valledor et al. (2009), modified Russell et al. (2010) and Souza et al. (2012)] remained in the wells when subjected to electrophoresis in agarose gels, or only a part of the total DNA migrated. The DNeasy® Plant Mini Kit (Qiagen) and the Piskurewicz and Lopez-Molina (2011) methods did not yield DNA. Therefore, all these methods were not suitable for DNA extraction from *C. bursa-pastoris* seeds.

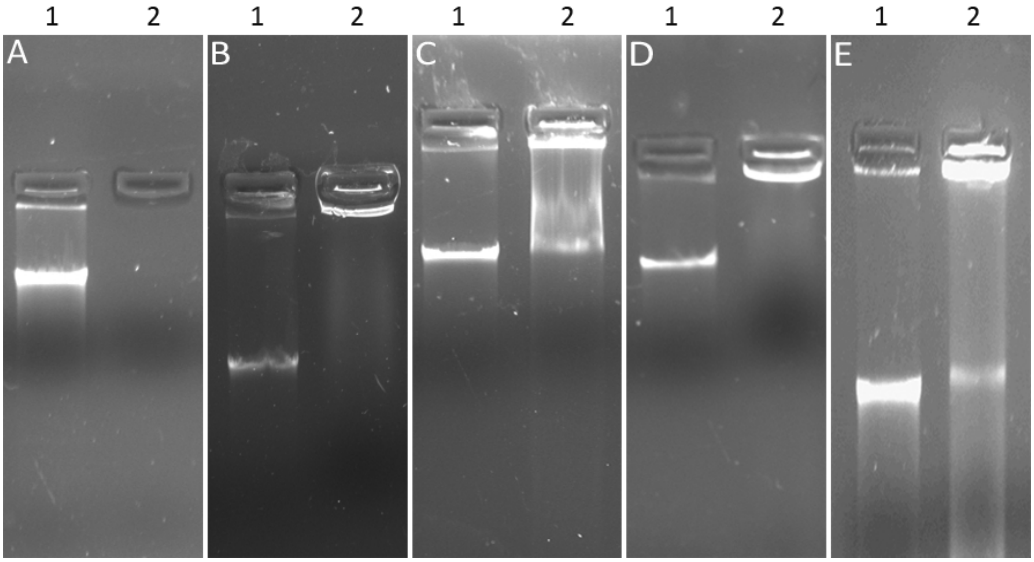

Figure 3.1. Agarose electrophoresis gels (1 %) of total genomic DNA extracted using different methods not suitable for *C. bursa-pastoris* seeds. (**A**) DNA extracted with DNeasy® Plant Mini Kit (Qiagen); lane 1: 75 ng of undigested *Lambda* DNA, lane 2: extracted DNA. (**B**) DNA extracted with modified Doyle and Doyle (1987,1990); lane 1: 50 ng of undigested *Lambda* DNA, lane 2: extracted DNA. (**C**) DNA extracted using Valledor et al. (2009); lane 1: 75 ng of undigested *Lambda* DNA, lane 2: extracted DNA. (**D**) DNA extracted using Valledor et al. (2009), with 1 g of starting material; lane 1: 75 ng of undigested *Lambda* DNA, lane 2: extracted DNA. (**E**) DNA extracted using modified Russell et al. (2010) and Souza et al. (2012); lane 1: 75 ng of undigested *Lambda* DNA, lane 2: extracted DNA.

DNA extracted with the optimized protocol described as Method 6, using any of the lysis buffers indicated [Dellaporta et al. (1983); Edwards et al. (1991); Jobes et al. (1995) or AP1 (Qiagen)] and the modified protocol of DNeasy® Plant Mini Kit (Qiagen), migrated adequately when subjected to electrophoresis in agarose gels. No contamination of RNA was detected. DNA preparations co-migrated with intact Lambda DNA, indicating a molecular weight on the order of 50 kbp or larger.

For the following studies in this thesis, total genomic DNA from 40 samples was extracted using the optimized protocol in Method 6 with the commercial buffer AP1. DNA quality was assessed by measuring absorbance ratios, A260/280 and A260/230. Yields and ratios of DNA samples are indicated in *Table 3.1*. DNA yields achieved by this method range from 58 to 8 ng/μL, derived from 50 to 100 mg of dry or imbibed seed material.

Normally, 260/280 absorbance ratios of 1.8 are expected for DNA, with values higher than 2.0 indicating the presence of RNA. Expected values of A260/230 ratios are usually in the range of 2.0 to 2.2. Lower ratios may indicate the presence of contaminants that absorb at 230 nm, such as EDTA, carbohydrates or phenol.

The lowest A260/280 ratio within the samples was 1.60 and the highest 2.05, indicating that none or little RNA contamination was observed in any of them. However, the minimum A260/230 ratio was 0.62 and the maximum 1.86, indicating the possible presence of some contaminants absorbing at 230 nm.

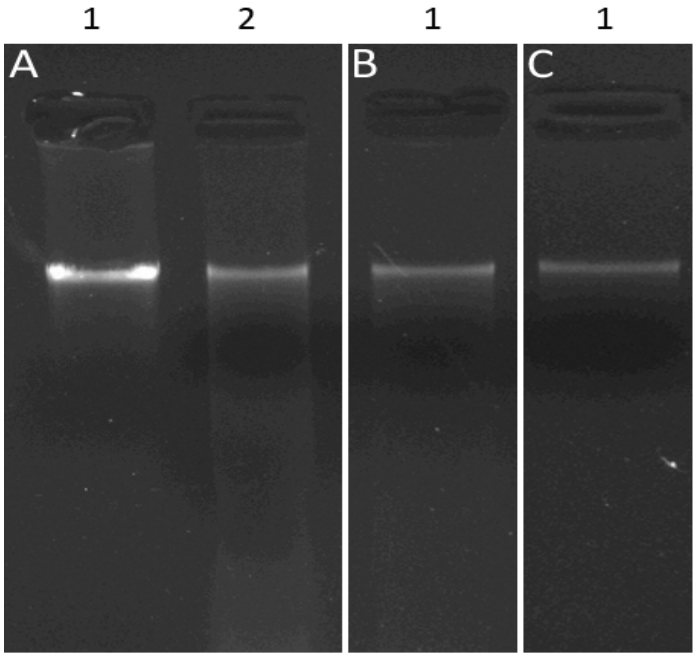

Figure 3.2. Agarose electrophoresis gels (1 %) of total genomic DNA extracted using the different lysis buffers indicated in Method 6 along with the use of chromatographic mini spin columns. (**A**) DNA extracted with buffer by Jobes et al. (1995); lane 1: 75 ng of undigested *Lambda* DNA, lane 2: extracted DNA. (**B**) DNA extracted with buffer by Edwards et al. (1991); lane 1: extracted DNA. (**C**) DNA extracted with Dellaporta et al. (1983); lane 1: extracted DNA.

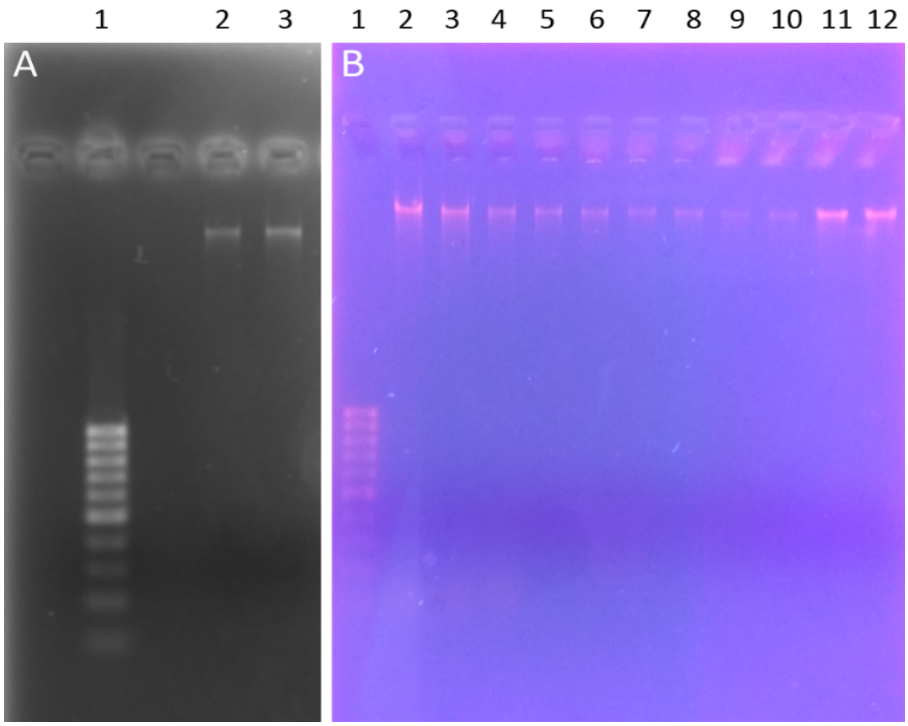

Figure 3.3. Agarose electrophoresis gels (1 %) of total genomic DNA extracted with the final optimized protocol described in Method 6 using the commercial lysis buffer AP1 (Qiagen). (**A**) Extracted DNA from seeds from the commercial genotype; lane 1: degraded 1 kbp size ladder, lanes 2 and 3: extracted DNA. (**B**) Extracted DNA from seeds in different physiological conditions; lane 1: degraded 1 kbp size ladder, lanes 2 to 4: DNA extracted from dry primary dormant seeds, lanes 5 to 7: DNA extracted from primary dormant seeds imbibed in water for 1 day with 12 h of light; lanes 8 to 10: DNA extracted from primary dormant seeds imbibed in water for 14 days with a 12 h photoperiod; lanes 11 and 12: DNA extracted from secondary dormant seeds imbibed in water for 3 days in darkness.

Discussion

Any genomic study using seeds as initial plant material can be complicated due to the large quantity of secondary compounds and polysaccharides that seeds accumulate. These compounds usually co-precipitate with DNA during extraction, affecting the quality and quantity of the isolated DNA.

The protocols by Doyle and Doyle (1987, 1990), Valledor et al. (2009), Piskurewicz and Lopez-Molina (2011), Russell et al. (2010) and Souza et al. (2012) produced DNA that was highly contaminated with impurities. CTAB or sorbitol in the extraction buffers did not prevent co-precipitation of polysaccharides. Their presence was evident from the viscous and sticky consistency of the precipitated DNA (Tel-Zur et al. 1999). The original protocol from the DNeasy® Plant Mini Kit (Qiagen) was also inefficient at handling plant material with high polysaccharide contents, such as seeds. Even though *C. bursa-pastoris* is a species closely related to

A. thaliana, protocols that were developed for extracting DNA from seeds of this species were not suitable for extracting pure DNA from shepherd's purse seeds.

Unlike the protocols mentioned above, the protocol described in Method 6, using any of the extraction buffers followed by the use of chromatographic mini spin columns, was consistent and produced very acceptable DNA yields of sufficient quality for further applications. SDS detergent in the lysis buffer and potassium acetate form a complex of proteins and polysaccharides prior to the precipitation of DNA. The removal of this complex in a precipitation step, followed by anion exchange chromatography, enabled a fast and reliable DNA extraction procedure from *C. bursa-pastoris* seeds under all physiological conditions covered in this study. Moreover, the resulting DNA was of high quality and yield in most of the cases, whilst only needing small seed samples ranging from 50 to 100 mg. Although AP1 was selected as extraction lysis buffer for further applications due to the high yields obtained, any other buffer from the ones described in Method 6 could have been used, making the procedure very cheap as well.

There were a few critical steps in the optimization of the protocol for extracting total genomic DNA from *C. bursa-pastoris* seeds. A proper and fine grinding of the samples in liquid nitrogen is essential for any type of material used in any kind of genetic study. An essential discovery was the use of SDS as a detergent in the lysis buffer instead of CTAB, the latter not being useful for the material analysed in this study. Even though there are many protocols available that describe how to extract total genomic DNA, some of them remove the polysaccharides after the first precipitation step has been done. This means that the DNA is precipitated along with the polysaccharides. For extracting DNA from seeds from *Capsella*, this procedure was not applicable, and polysaccharides needed to be separated from the DNA before any kind of precipitation step was done. Finally, another important step in obtaining DNA of high quality was the repeated precipitation with potassium acetate at the beginning of the extraction protocol to eliminate most of the contaminants.

Further analyses that address the suitability of this optimized protocol for other species and materials could involve a complete digestion with restriction endonucleases and specific polymerase chain reaction (PCR) amplification with selected primers. In addition, DNA from different plant species could be extracted to test the procedure, even though the heterogeneity of plants makes it unlikely that a single method is found to be valid for all of them.

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4

CHAPTER 4

Quantification of global DNA methylation and H4 acetylation for primary and secondary dormant *Capsella bursa-pastoris* seeds

Introduction

Reprogramming in response to environmental stresses through a complex network of response mechanisms is key for phenological and developmental plasticity in plants, making them capable of surviving unfavourable conditions (Chinnusamy and Zhu, 2009). These mechanisms help plants to adjust the duration of their developmental phases to avoid damage at critical moments (e.g. seed development, germination or seedling establishment) to stress (Chinnusamy and Zhu, 2009).

Seeds have functions of reproduction and dispersal, so they are complex structures with stress-resistant properties (Dekkers et al., 2016). They can remain dormant in the soil for many years until the conditions are suitable for the resulting plant to survive (Footitt et al., 2013). Seed dormancy is defined as a temporary failure of a viable seed to complete germination under normally favourable conditions (Bewley, 1997) and is a major determinant of the ecological adaptability, distribution and reproductive success of plants. In this way, seeds determine the time and place of plant establishment, synchronizing their life cycle with favourable environments (Footitt et al., 2017). *C. bursa-pastoris* seeds have non-deep physiological dormancy, which is defined as a physiological inhibiting mechanism that prevents radicle emergence (Baskin and Baskin, 2014).

When mature seeds are shed from the mother plant and get into contact with the soil, they may not immediately germinate, indicating that they present primary dormancy. It is only after seeds have been hydrated and placed in conditions favourable for germination that any dormancy status can be assessed (Finch-Savage and Footitt, 2017). Mature *C. bursa-pastoris* seeds present primary dormancy at shedding. Imbibed mature primary dormant seeds are sensitive to signals that inform them of the spatial environment (light, nitrate and temperature) from the moment of entering the soil (Footitt et al., 2017). Primary dormancy is established as a continuum with layers that need to be removed before the seed is able to germinate. If the seeds receive the signals in the right order, these layers will be completely removed and the seeds will germinate (Finch-Savage and Footitt, 2017). However, if the signals received are not adequate, the seeds can regain dormancy by induction of secondary dormancy and may alternate between dormant and non-dormant states, establishing a dormancy cycle (Cadman et al., 2006; Finch-Savage and Footitt, 2017). Shepherd's purse seeds are able to enter secondary dormancy and undergo seasonal dormancy cycles in the soil (Toorop et al., 2012).

DNA methylation is an epigenetic modification that consists of the addition of a methyl group onto the C5 position of cytosine to form 5-methylcytosine (5-mC). It is required for maintaining genomic structure and stability, regulating gene

expression, imprinting, growth, development (An et al., 2017), in addition to silencing transposable elements (TEs), repetitive sequences and transgenes (Bartels et al., 2018). It is implicated in the regulation of genes involved in leaf morphology, flowering time, floral organ identity, fertility, embryogenesis and even in seed development (An et al., 2017). The effects of this epigenetic mark on gene transcription relate to their differential distribution in the genes, being associated with repression in gene promoters and with activation in genes bodies, depending on the level of methylation (Zilberman et al., 2007).

Global DNA methylation has been studied in different plant species, showing high diversity of levels and sequence contexts, variation in different tissues and organs and changes during plant development. It is the most studied mechanism in the epigenetic control of gene expression in plants (Finnegan, 2010). Its implications in seed development and germination have been elucidated by different investigations. Hypo-methylation of the endosperm of rice seeds was studied by Zemach et al. (2010) and of *A. thaliana* by Hsieh et al. (2009). Bouyer et al. (2017) demonstrated the highly dynamic role of DNA methylation during *A. thaliana* embryogenesis and early growth and An et al. (2017) during the maturation of soybean seeds. High rates of demethylation events have been reported in seed germination of rapeseed, wheat, pepper, soybean and *Arabidopsis* (Portis et al., 2004; Guangyuan et al., 2006; Meng et al., 2012; Lin et al., 2017; Narsai et al., 2017).

The DNA is wrapped around nucleosomes which are the basic structure of chromatin (Finnegan, 2010). A nucleosome is formed by eight histone proteins, containing two copies each of H2A, H2B, H3 and H4 proteins, with variants of each histone encoded by different genes. Nucleosomes are dynamic structural units that can be moved, stabilized/destabilized, disassembled/reassembled in response to specific signals (Berr et al., 2011). Some histone post-translational modifications enhance transcription, such as acetylation, ubiquitination, phosphorylation and certain methylations, whilst others repress gene expression (Berger, 2007).

Post-translational histone modifications confer different physical properties to chromatin, altering the accessibility of transcription machinery to the DNA (Itabashi et al., 2018). Acetylation of a histone lysine residue neutralizes the positive charge of lysine, thereby reducing the interaction between histones and DNA and activating gene expression (Lee and Workman, 2007). Methylation of histones is more complex, as it is associated with activation and repression of transcription. For example, H3K4me1 (histone H3 Lysine 4 monomethylation), H3K4me2, H3K4me3, H3K36me2 and H3K36me3 up-regulate specific gene expression, whilst on the contrary, H3K9me2, H3K9me3, H3K27me2, H3K27me1 and H3K27me3 down-regulate it (Mathieu et al., 2005; Chen et al., 2010).

Gene expression is influenced by chromatin structure, which is controlled by processes often associated with epigenetic regulation (Chinnusamy and Zhu,

2009). While global acetylation of histones is linked to the "open" configuration of chromatin named euchromatin, global DNA methylation is linked to the "closed" configuration named heterochromatin. Interaction between DNA methylation and histone modification to jointly control gene expression has been demonstrated (Fuks, 2005; Finnegan, 2010; Wójcikowska et al., 2018). For example, *CHROMOMETHYLASE 3* (*CMT3*) and *CMT2* can be targeted by H3K9me2, leading to DNA methylation near the modified histones (Itabashi et al., 2018).

The implication of epigenetic processes in the regulation of seed dormancy has been demonstrated in different investigations. Early studies were carried out by Law and Suttle (2002, 2004), elucidating the implications of 5-mC and histone H3 and H4 multi-acetylation in potato meristems during dormancy progression. Different expression patterns of histone acetyltransferases and deacetylases were found between dormant and non-dormant seeds of *A. thaliana* by Cadman et al. (2006). A screening for low dormancy mutants by Liu et al. (2007) permitted the identification of *HISTONE UBIQUITANTION1* (*HUB1*) required for the monoubiquitination of H2B, which in itself is necessary for H3K4me3 and H3K79me3 histone modifications. The dynamic role of histone methylation in the adjustment of the dormancy status during dormancy cycling was analysed by Footitt et al. (2015), who proposed the regulation of *DOG1* transcription by the proportions of H3K4me3 and H3K27m3 marks as part of a sensing mechanism of soil temperature.

Global cytosine methylation levels can be analysed by several methods. However, these techniques require a high-quality annotated reference sequence. *C. bursa-pastoris* genome has been sequenced and annotated, but only its sequence is publicly available (www.capsella.org). An alternative analysis can be obtained by quantifying global DNA methylation with the use of an ELISA-based kit, providing an estimation of the percentage of total genomic cytosines that are methylated without requiring previous knowledge of the species' genomic features. This method does not reveal differential patterns of methylation at specific DNA sequences but is recommended for genome-wide quantification.

In spite of all the knowledge regarding the implications of epigenetics in the control of gene expression, a comprehensive characterization of the levels of global DNA methylation and histone H4 acetylation of seeds in different states of dormancy was still remaining. The aim of this thesis was to provide insights in the levels of epigenetic marks during the entrance and maintenance of seed dormancy. It was hypothesized that global DNA methylation and global H4 acetylation had a dynamic implication in the entrance and maintenance of seed dormancy, adjusting the seeds dormancy state through regulation of dormancy controlling genes in response to environmental signals. In this hypothesis, 5-mC levels increased in relation to the depth of dormancy and H4 acetylation levels decreased during this process. In other words, the deeper the dormancy, the higher the DNA methylation levels and the lower the H4 acetylation levels. Unfortunately, quantification of global H4 acetylation was not achieved due to technical problems

with the histone extraction methodology. In this doctoral thesis, quantification of global genomic DNA methylation of primary dormant seeds and seeds belonging to *C. bursa-pastoris* accessions with differences in their secondary dormancy depth is presented.

Material and methods

Quantification of global DNA methylation

Plant material

Seeds from shepherd's purse in different dormancy states were used as plant material. Seeds from accession SCRI -773 of shepherd's purse were sown and grown in Oviedo, Asturias, Spain, from November 2013 to March 2014, to produce primary dormant seeds. Seeds were surface sterilized in 1 mL of sodium hypochlorite (NaOCl) with 0.01 % (v/v) Tween 20 for 5 min with shaking. After washing three times with sterile distilled water, seeds were germinated in Petri dishes on two layers of filter paper Whatman no. 1 that were moistened with sterilized distilled water. Dishes were placed at 30 °C and the seeds exposed to cycles of light/dark of 12 h each, with light provided by white fluorescence tubes (radiometric flux density of 50-100 W m^{-2}). Once germinated, seeds were transferred to 2.5 cm^2 plastic modules in a greenhouse in Oviedo, Asturias, Spain. When seedlings were well established, they were transferred to medium size peat pots for 6 weeks and then to large size peat pots for the rest of their life cycle. These plants were considered replicate plants of the genotype. Plants were bagged with paper bags individually when siliques showed the first signs of dehiscence and left to dry without watering in a warm and dry location.

Seeds were harvested from individual plants and then mixed to form 8 different pools that were stored at 4 °C in hermetic glass containers with dried silica gel and a RH and temperature measuring dispositive for the rest of the project. Germination tests were carried out throughout that period to corroborate changes in dormancy state (data not shown). Three conditions were used: 1) seeds imbibed in water at 30 °C with a 12 h photoperiod as an optimum test for non-dormant seeds; 2) seeds imbibed in water but exposed to alternate cycles of light/dark (12 h) and high/low temperatures (25/10 °C), as this treatment is known to partially break primary dormancy; 3) seeds imbibed in 10 mM KNO_3 with alternate cycles of light/dark (12 h) and high/low temperatures (25/10 °C), as a way of completely breaking primary dormancy. For the subsequent experiments, seeds from the largest three pools (1, 3 and 5) were used as biological replicates.

The selection of accessions with contrasting secondary dormancy depth was based on germination tests under dormancy-inducing conditions (see Chapter II).

In brief, the experiments comprised nine seed accessions (SCRI -156, -177, 367, -416, -469, -707, -773, -799 and -937). All seed lots had been stored at 15 °C and 15 % RH since 2006, losing any residual primary dormancy by after-ripening as corroborated by germination tests at 30 °C. Accession -367 was identified as a deep dormant accession and -799 as a non-deep dormant accession. Seeds from three different mother plants per accession (-367.1, -367.2, -367.3, as well as -799.1, -799.2 and -799.4) were selected as biological replicates for the experiments described below.

Treatment conditions

DNA was extracted for the conditions indicated in *Table 4.1*. Primary dormant seeds (SCRI -773) were imbibed in water for 14 d with a 12 h photoperiod, conditions that do not promote the release from primary dormancy but induce a deeper primary dormant state than seeds imbibed for 1 d under the same conditions. At the same time, these conditions were compared to primary dormant dry seeds. Non-dormant seeds from the selected accessions (SCRI -367 and -799) were imbibed for 1 d in water at 30 °C with a 12 h photoperiod, conditions that are optimal and germination-promoting for non-dormant seeds. For the induction of secondary seed dormancy, non-dormant seeds were imbibed in darkness at high temperature (30 °C), conditions known for inducing secondary dormancy. Different times were tested, with longer periods in darkness inducing a deeper secondary dormancy state. The results obtained were compared to non-dormant dry seeds.

Table 4.1. DNA samples

Primary Dormant Conditions	Secondary Dormant Conditions -367	Secondary Dormant Conditions -799
Dry seeds Pool 1	Dry seeds 367.1	Dry seeds 799.1
Dry seeds Pool 3	Dry seeds 367.2	Dry seeds 799.2
Dry seeds Pool 5	Dry seeds 367.3	Dry seeds 799.4
1 Day 30 °C 12h light Pool 1	1 Day 30 °C 12h light 367.1	1 Day 30 °C 12h light 799.1
1 Day 30 °C 12h light Pool 3	1 Day 30 °C 12h light 367.2	1 Day 30 °C 12h light 799.2
1 Day 30 °C 12h light Pool 5	1 Day 30 °C 12h light 367.3	1 Day 30 °C 12h light 799.4
14 Days 30 °C 12h light Pool 1	1 Day Dark 367.1	1 Day Dark 799.1
14 Days 30 °C 12h light Pool 3	1 Day Dark 367.2	1 Day Dark 799.2
14 Days 30°C 12h light Pool 5	1 Day Dark 367.3	1 Day Dark 799.4
	3 Days Dark 367.1	3 Days Dark 799.1
	3 Days Dark 367.2	3 Days Dark 799.2
	3 Days Dark 367.3	3 Days Dark 799.4
	7 Days Dark 367.1	
	7 Days Dark 367.2	
	7 Days Dark 367.3	
	14 Days Dark 367.1	
	14 Days Dark 367.2	
	14 Days Dark 367.3	

DNA extraction

DNA extraction was performed as described in the optimized protocol in Chapter III.

Quantification of global DNA methylation

MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric) (Epigentek, NY) was used according to the manufacturer's instructions. One hundred ng of purified DNA from each sample were added to an ELISA plate where the methylated fraction of DNA was detected using capturing and detecting antibodies. Optical density (OD) intensity at 450 nm was read with a microplate spectrophotometer and this was proportional to the amount of methylated DNA. For quantification of global DNA methylation levels,

a standard curve was generated using methylated DNA standards provided in the kit. The value for each sample was calculated as a ratio of the OD of the sample relative to the OD of the standard, after subtracting the negative control readings (*Figure 4.1*.). The assay was performed in duplicate per biological replicate, meaning that per accession and condition, six samples were analysed.

$$5 - mC\% = \frac{Sample\ OD - NC\ OD}{Slope\ x\ DNA\ ng}\ x\ 100\%$$

Figure 4.1. Formula to calculate the percentage of methylated DNA. The slope is from the standard curve generated with the methylated DNA standards provided in the kit.

Global DNA methylation statistical analysis

Data were assessed for significance ($P < 0.05$) by one-way ANOVA (dormancy type as factor) and two-way ANOVA analyses (dormancy type and duration as main factors) using R software v3.3.0. The ANOVA analyses were combined with Tukey's HSD (Honest Significance Difference) test for post-hoc comparisons of means using "Agricolae" v.1.3-1 (Mendiburu, 2019) package in R.

Quantification of global H4 acetylation

Plant material

The same seed material used for the global DNA methylation analysis was used for the extraction of nuclear proteins.

Histone extraction

A general protocol for histone extraction was applied to seeds in all the different secondary seed dormancy conditions. However, no adequate results were obtained as characteristic patterns of histones were not observed in the protein gels. Subsequent Westerns with antibodies against histone H4 and histone H4 acetylated were performed, without any results, confirming the absence of histones in the protein extracts obtained from secondary dormant seeds.

However, an optimized protocol for primary dormant seeds was developed, although we did not use it for further studies.

General protocol

- **EXTRACTION BUFFERS**:
  - Buffer A: 0.44 M sucrose, 10 mM Tris-HCl (pH 8.0). 5 mM β-mercaptoethanol and 1 mM phenylmethanesulfonyl fluoride (PMSF) added just prior use.
  - Buffer B: 0.25 M sucrose, 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 % Triton X-100. 5 mM β-mercaptoethanol and 0.15 mM PMSF added just prior use.
  - Buffer C: 0.25 M sucrose, 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$. 5 mM β-mercaptoethanol and 0.15 mM PMSF just added prior use.
- **PROTOCOL:** 300 to 500 mg of seed material for each physiological condition studied were ground with a mortar and pestle in liquid nitrogen and transferred to a 50 mL polypropylene tube. 16 mL of Buffer A were gently added by letting them flow along the tube walls. The solution was gently mixed for 30 min at 4 °C in an overhead shaker, filtered twice through 8 layers of miracloth and centrifuged at 3000 g for 15 min at 4 °C. The supernatant was discarded, and the pellet dissolved in 10 mL of ice-cold Buffer B. The solution was incubated for 10 min on ice, being mixed gently by inversion. Using a pipette, the mixture was placed carefully on top of a sucrose gradient (2 M and 0.32 M) and centrifuged at 2000 g for 30 min at 4 °C. The layer on top of the 2 M sucrose layer was collected by pipetting and transferred to a new collection tube. The suspension was dissolved in 10 mL of Buffer B and incubated on ice for 10 min. The mixture was centrifuged at 3000 g for 15 min. This step was repeated another two times. The final pellet was resuspended in 8 mL of Buffer C. The final solution was centrifuged at 3000 g for 10 min at 4 °C. The supernatant was discarded and the pellet containing the nuclei stored at -80 °C overnight. Acid extraction of nuclear proteins following Shechter et al. (2007) with modifications was performed. The pellet stored at -80 °C was warmed up to RT, dissolved in 800 μL of H_2SO_4 0.4 N and transferred to a 2 mL collection tube. The solution was vortexed and mixed in an overhead shaker for 20 min at 4 °C. Afterwards, the solution was sonicated with 3 pulses of 15 s in a water sonicator and centrifuged at 16000 g for 10 min at 4 °C. The supernatant was collected and mixed with 280 μL of a saturated solution of trichloroacetic acid. The tube was incubated on ice for 30 min and centrifuged at 16000 g for 10 min at 4 °C. The supernatant was removed, and the pellet washed with cold acetone and centrifuged at 16000 g for 10 min at 4 °C. The supernatant was removed, and the pellet washed with acetone for a second time. The pellet was air-dried and resuspended in 100 μL of sterilized water. The extracted proteins were kept at -20 °C until further use.

Protocol optimized for primary dormant seeds

- **NUCLEAR ISOLATION BUFFER (NIB):** 0.46 M sucrose, 5 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 % (v/v) Ficoll, 1 % (v/v) Triton X-100. 1 mM DTT and 1 mM PMSF added just prior use.
- **PROTOCOL:** 300 to 500 mg of seed material were ground with a mortar and pestle in liquid nitrogen and transferred to a 50 mL polypropylene tube. 16 mL

of NIB were gently added by letting them flow along the tube walls. The solution was gently mixed for 30 min at 4 °C in an overhead shaker, filtered twice through 8 layers of miracloth and centrifuged at 3000 g for 15 min at 4 °C. The pellet was discarded, and the supernatant transferred to a new collection tube where it was centrifuged again at 3000 g for 15 min at 4 °C. The supernatant was removed, and the pellet resuspended very carefully in 10 mL of NIB by pipetting to avoid breaking the nuclei. The solution was incubated on ice for 10 min, mixed by gentle inversion every 2 min and centrifuged at 11000 g for 15 min at 4 °C. The supernatant was removed by inverting the tube and the pellet containing the nuclei was stored at -80 °C overnight.
The acid extraction of nuclear proteins was carried out as in the general protocol above.

Protein gels

Size fractionation of the extracted proteins from non-dormant and secondary dormant seeds was done by SDS-PAGE analysis using NuPage Bis-Tris precast protein gels (Invitrogen), with a 4-12 % (w/v) gradient system. Histones extracted from primary dormant seeds were separated by SDS-PAGE gels with a 15 % acrylamide concentration. 4-Morpholinepropanesulfonic acid (MOPS) was used as a running buffer (Invitrogen) following manufacturer's instructions. 6.5 μL of each sample were loaded per lane. Electrophoresis was carried out for 45 min at 200 V. The gels were stained with Coomassie Blue R250 for 1 h with orbital agitation. De-staining was carried out with a solution composed of 30 % (v/v) methanol and 10 % (v/v) acetic acid, by changing the solution each 15 min, with agitation in an orbital shaker. Afterwards, the gels were left overnight with 1:3 of the detaining solution and 2:3 of distilled water at 4 °C and visualized.

Western blots

Proteins extracted from non-dormant and secondary dormant seeds were transferred to nitrocellulose membranes for 1 h at 35 V. Membranes were blocked with a 4 % (w/v) non-fat milk solution in distilled water overnight under orbital agitation at 4 °C. Membranes were washed twice with PBS containing 0.05 % (v/v) Tween 20 and incubated with a 1:2000 dilution in a non-fat milk solution of the rabbit polyclonal anti-H4 antibody (Merk KGaA, Cat. N. 07-108, Darmstadt, Germany) or the rabbit polyclonal anti-acetylated H4 antibody (Merk KGaA, Cat. N. 06-866, Darmstadt, Germany) for 2 h at RT. The membranes were then washed three times in PBS containing 0.05 % (v/v) Tween 20 for 5 min. Blots were incubated with a 1:10000 dilution in a non-fat milk solution of the goat anti-rabbit secondary antibody peroxidase-conjugated (Abcam, Cat. N. ab97051) for 1 h in agitation. Afterwards, the membranes were washed three times for 5 min in PBS with 0.05 % (v/v) Tween 20. Western blots were developed with a chemiluminescent substrate and visualized.

Results

Quantification of global DNA methylation

Global DNA methylation was analysed with the MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric), which includes a standard curve with a range for quantification of methylation from 0.1 to 5.0 %. (*Figure 4.2.*). The lowest DNA methylation level measured in the seed lots was 1.63 %, and the highest 12.05 %. All the measurements higher than 5.0 % can therefore only be compared within this assay.

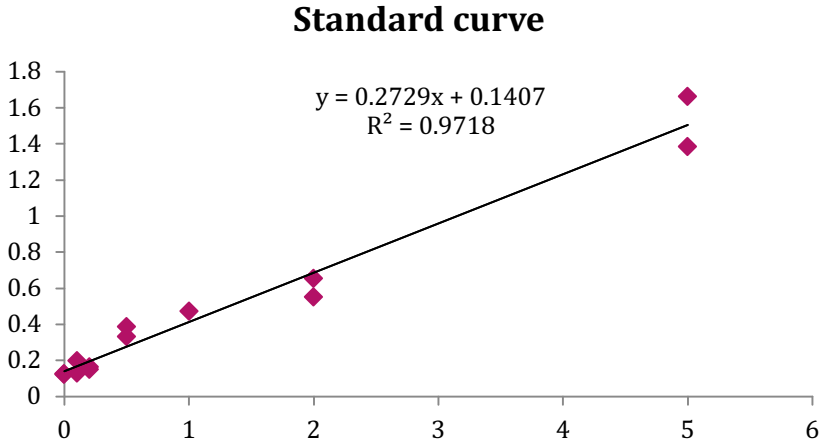

Figure 4.2. Standard curve obtained in this assay.

The average of global DNA methylation levels of the three biological replicates per dormancy condition with the two technical replicates per sample are summarized in *Figure 4.3*. DNA methylation levels in seeds from the deep and the non-deep dormant accessions (-367 and -799, respectively) revealed similar patterns across time (*Figure 4.3.*). The ANOVA statistical analysis showed that there was a significant interaction between the duration of the treatment to which seeds were exposed and the levels of global DNA methylation ($P < 0.001$), in the three types of dormancy studied (primary dormancy, deep and non-deep secondary dormancy).

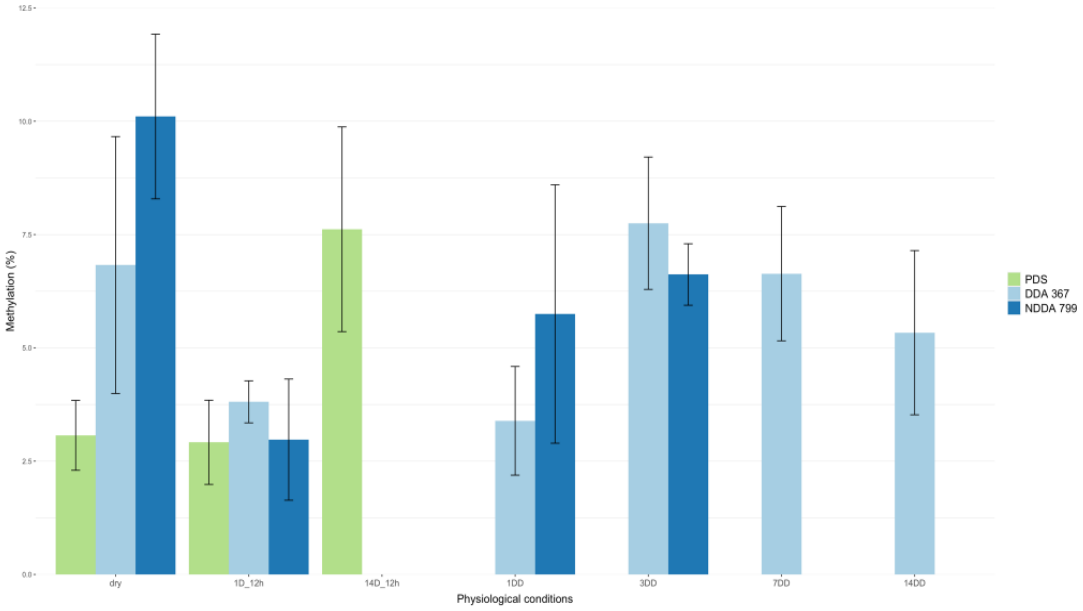

Figure 4.3. Global genomic DNA methylation percentage means for every three biological replicates per physiological condition with two technical replicates. Histogram representing the values of 5-mC percentage of total cytosines. Bars represent SD. PDS: primary dormant seeds; DDA 367: deep dormant accession -367; NDDA 799: non-deep dormant accession -799; DD: days in darkness.

Global DNA methylation levels were significantly different ($P < 0.01$) between primary dormant dry seeds (3.07 ± 0.82 %) and non-dormant dry seeds from the non-deep dormant accession -799 (10.11 ± 1.91 %). At the same time, non-dormant dry seeds from the deep dormant accession -367 had lower global DNA methylation levels (6.83 ± 3.15 %) than the non-dormant dry seeds from the non-deep dormant accession -799, although the difference was not significant (*Figure 4.4.*, A).

Global DNA methylation levels of seeds imbibed in water for 1 d with a 12 h photoperiod showed the lowest levels of DNA methylation throughout all the treatments studied. These levels did not differ significantly between the three types of seed dormancy states studied (*Figure 4.4.*, B).

With respect to primary dormant seeds, global DNA methylation levels of seeds imbibed in water for 1 d with a 12 h photoperiod (2.91 ± 1.02 %) did not differ significantly from those of primary dormant dry seeds (3.07 ± 0.83 %). However, when these seeds were imbibed for a longer period of 14 d under the same conditions, DNA methylation levels increased significantly compared to only 1 d (7.61 ± 2.26 %) ($P < 0.05$; *Figure 4.4.*, C).

Global DNA methylation levels of seeds from the deep dormant accession -367 did not differ significantly throughout all the treatments. However, seeds

imbibed in water for 1 d with or without a 12 h photoperiod presented lower levels than deep dormant dry seeds and very similar levels between them. In the induction of secondary dormancy of the deep dormant accession, seeds imbibed in water for 3 d in darkness had the highest global DNA methylation, with increasingly lower levels of DNA methylation after 7 and 14 d in darkness, although higher levels than seeds for 1 d in darkness.

Dry seeds from the non-deep dormant accession -799 had significant differences from non-deep dormant seeds imbibed for 1 d in water (12 h photoperiod) ($P < 0.001$). Non-deep dormant seeds imbibed in darkness for 1 or 3 d did not differ significantly from dry seeds or seeds imbibed in water for 1 d (12 h photoperiod), although their methylation levels were higher than the latter ones.

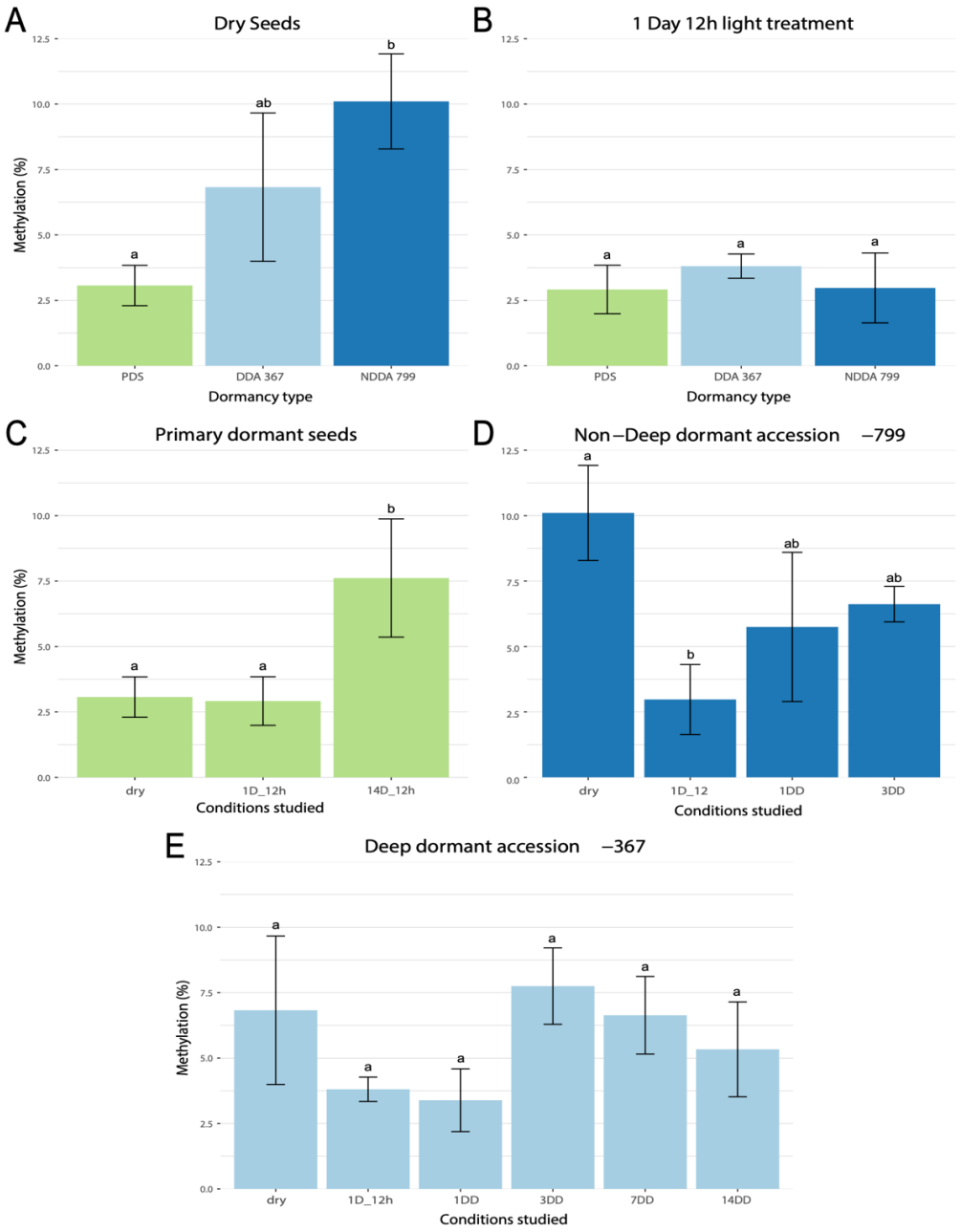

Figure 4.4. Histogram representing the values of 5-mC percentages of total cytosines. (**A**) Primary dormant dry seeds, dry seeds from the deep dormant accession -367 and dry seeds from the non-deep dormant accession -799. (**B**) Primary dormant seeds, non-dormant seeds from the deep dormant accession -367 and non-dormant seeds from the non-deep dormant accession -799 imbibed in water for 1 d with 12 h of light. (**C**) All the conditions studied for primary dormant seeds. (**D**) All the conditions studied for the non-deep dormant accession -799. (**E**) All the conditions studied for the deep dormant accession -367. Statistical analysis of global DNA methylation percentage means for every three biological replicates per physiological condition with two technical replicates. Different letters indicate significant differences at $P < 0.05$ (Tukey's HSD test). Bars represent standard deviation. PDS: primary dormant seeds; DDA 367: deep dormant accession -367; NDDA 799: non-deep dormant accession -799; DD: days in darkness.

Quantification of global H4 acetylation

Protein gels

SDS-PAGE gels with extracted proteins from primary dormant seeds, using the optimized method, showed a characteristic pattern for histones. However, this protocol did not successfully extract histones from non-dormant or secondary dormant seeds (*Figure 4.5.*, A).

Histones from non-dormant and secondary dormant seeds were extracted using the general protocol described previously. However, the proteins extracted were not histones as observed in the SDS-PAGE gels where the proteins had a very small size and did not present the characteristic pattern for histones (*Figure 4.5.*, B). Western blots with the extracted proteins and antibodies against histone H4 and acetylated histone H4 did not present any signal.

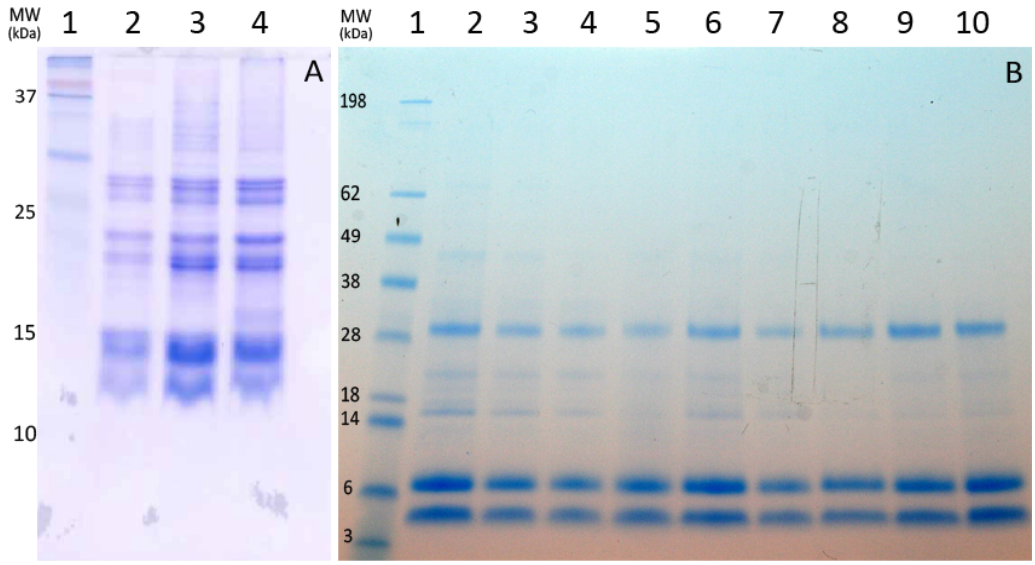

Figure 4.5. SDS-PAGE gels with the extracted proteins. (**A**) Proteins extracted from primary dormant seeds with the protocol optimized for their physiological condition; lane 1: pre-stained protein standard, lanes 2 to 4: extracted histones. (**B**) Proteins extracted from secondary dormant and non-dormant seeds; lane 1: SeeBlue™ pre-stained protein standard (Life Technologies), lanes 2 to 10: proteins extracted with the general protocol.

Discussion

The implications of an epigenetic regulation in the control of seed dormancy have only begun to be revealed. No other investigation has previously quantified the levels of global DNA methylation and histone H4 acetylation of whole seeds in different physiological states of their dormancy cycle. In the initial hypothesis, global DNA methylation levels increased in relation to the depth of dormancy and H4 acetylation levels decreased during this process. In other words, seeds imbibed for 14 d in darkness would have the highest 5-mC levels and the lowest H4 acetylation levels, in comparison with the rest of the conditions studied here.

The main goal of this study was to test the initial hypothesis by analysing the dynamics of global DNA methylation and global H4 acetylation during primary seed dormancy maintenance and secondary seed dormancy entrance and maintenance.

The use of seeds as starting material in molecular studies may present several technical difficulties. Here, these were encountered particularly in the extraction of acid nuclear proteins: histones. Even though strong efforts were made to quantify the acetylation levels during the physiological states studied, they were not successful. However, global DNA methylation levels were analysed and provided valuable information regarding the implications of epigenetic marks in the regulation of seed dormancy.

In prolonged imbibed primary dormant seeds (after shedding from the mother plant and in the soil seed bank), DNA methylation levels are higher than in primary dormant seeds imbibed for a short period of time (1 d). Primary dormant seeds which are maintained in a prolonged imbibed state in conditions that do not promote germination had a deeper primary dormancy than primary dormant seeds imbibed for a short period (1 or 2 d) (Cadman et al., 2006; Finch-Savage et al., 2007). Cadman et al. (2006) imbibed PDS for periods of 1, 2 or 30 d in darkness. These PDS did not germinate in the presence of light or nitrate separately but would germinate slowly when both conditions were present. In this study, PDS were imbibed for periods of 1 or 14 d with a 12 h photoperiod and very low germination was observed after 14 d without nitrate (data not shown). These conditions were chosen to avoid changing the light factor as the results were going to be compared with those seeds imbibed for 1 d under the same conditions. When these seeds were imbibed in nitrate with the presence of light, germination occurred. Because of this, we could consider that light treatments did not prevent the induction of a deeper primary seed dormancy state. Therefore, this result may be indicating that in primary dormant imbibed seeds, the deeper the dormancy state, the higher the DNA methylation levels.

On the other hand, in secondary dormant seeds this was not the case, as seeds in a deeper secondary dormancy state after 14 d imbibed in darkness had lower global DNA methylation levels than seeds imbibed for a shorter period of time, such as 3 d in darkness. These results are consistent with a dynamic role of

chromatin remodelling in the control of the entrance and maintenance of primary and secondary dormancy. Cadman et al. (2006) described that different intensities and cycles of dormancy are accompanied by changes in the expression of genes, indicating that transcripts in dormant seeds are not only those accumulated from the mother plant and that dormant seeds have a characteristic transcriptional activity. This is in accordance with the dynamic global DNA methylation patterns observed in this study.

In primary dormant seeds (PDS), the lowest 5-mC levels were found in seeds imbibed for 1 d at 30 °C with 12 h of light. Nonetheless, almost no differences were detected between these seeds and primary dormant dry ones (*Figure 4.4*., A and E). A high constant temperature is not adequate for germination of PDS (primary dormancy is not released) in shepherd's purse, which could explain the phenomena (Popay and Roberts, 1970). However, the reason behind the low methylation levels of primary dormant dry seeds in comparison with the non-dormant dry seeds of the deep and the non-deep dormant accessions is not clear. One possible explanation could be the water content. It has been demonstrated that seed water content impacts DNA methylation and a previous study correlated an increase in the global DNA methylation levels with the period of storage (Michalak et al., 2013). They analysed strongly dried seeds, after 1 year in storage with 2.2 % of moisture content, and seeds dried at 20 °C on a laboratory bench with a moisture content of 8.4 %. The first ones presented higher global methylation levels. PDS in this study presented morphological characteristics different to the non-dormant dry seeds, potentially indicating a higher seed moisture content (more voluminous), although no measurements were taken. Therefore, differences in seed moisture content might explain the differences found here between primary dormant dry seeds that did not pass through a period of after-ripening and were stored to conserve their dormancy state, and non-dormant dry seeds which were stored in a dry room, where they slowly lost their primary dormancy over time.

Different transcription patterns between newly imbibed PDS (24 or 48 h imbibed in water at 20 °C) and PDS imbibed for a longer period (30 d at 20 °C) were detected by Cadman et al. (2006). Long-term imbibition of PDS in water at a constant high temperature (such as 30 °C) results in a deeper dormancy than PDS imbibed in water for a short period (after they have been shed from the mother plant) (Cadman et al., 2006). The results here reported indicate significantly higher global DNA methylation levels in PDS imbibed in water for 14 d with a 12 h photoperiod than in PDS imbibed for only 1 d under the same conditions. It could be speculated that this large increase in global DNA methylation levels is directly correlated with a deeper dormancy in primary dormant imbibed seeds, after they have shed from the mother plant and formed part of the soil seed bank. However, further investigations need to test if these 5-mC levels are maintained, increased or decreased (as in secondary dormancy induction mentioned below) along with the time of imbibition at a constant temperature. In addition, imbibition in water at a constant temperature does not mimic natural conditions that occur in the soil

seed bank and a more realistic experiment including the different cycles that occur in nature would be able to elucidate more details of this hypothesis.

Dry seeds of the deep dormant accession -367 had global DNA methylation levels between those of the primary dormant dry seeds and those of the dry seeds of the non-deep dormant accession -799 (*Figure 4.4*., A). Dry seeds of the non-deep dormant accession had the highest DNA methylation levels of all the different plant lines and conditions analysed. It could be speculated that the higher values presented by the non-deep dormant accession dry seeds in comparison with the deep dormant accession dry seeds might be linked to a relationship between the 5-mC levels and the potential of germination. Whilst a drop in global 5-mC levels was associated with a decrease in the viability of *Quercus robur* L. recalcitrant seeds (Michalak et al., 2015), the two *Capsella* accessions analysed here (-367 and -799), both retained viabilities near 100 % (Chapter II, *Figure 2.5*.). Thus, any changes in methylation more likely reflect underlying differences in dormancy rather than seed quality.

A pronounced decrease in 5-mC levels was detected when dry seeds from the deep and the non-deep dormant accessions (-367 and -799, respectively) were imbibed in water for 1 d with 12 h of light (*Figure 4.4*., D and E), conditions that promote germination in non-dormant seeds of *C. bursa-pastoris*. This decrease in global DNA methylation levels, in comparison with the dry seed ones, corroborates previous studies in which a demethylation event accompanied the germination of seeds from different species (Portis et al., 2004; Guangyuan et al., 2006; Meng et al., 2012; Lin et al., 2017; Narsai et al., 2017). Besides, Lin et al. (2017) described that global CHH methylation increased through the entire seed of *Arabidopsis* from differentiation to dormancy and decreased in post-germination and seedling stages. These changes represent a dynamic epigenetic regulation and differences between a quiescent and a germinating state.

Induced secondary dormant seeds (seeds imbibed in darkness at high temperature) had the highest global DNA methylation after 3 d in both accessions (-367 and -799), showing decreasing levels towards a deeper dormancy in the deep dormant accession -367. This might indicate that there is a critical point in the induction of secondary dormancy in after-ripened seeds where the highest levels of DNA methylation are acquired. From that point, levels of methylation might decrease until they are maintained at a lower, stable level whilst environmental conditions do not change. This is not in concordance with the original hypothesis in which it was speculated that global DNA methylation levels were higher in a deeper secondary dormancy state. Hence, studies with seeds imbibed in darkness for a longer period with temperatures and moisture levels that mimic more realistic field conditions would need to be done to test this new hypothesis.

Accumulating evidence designates the control of dormancy and germination to the result of a balance between gibberellins (GAs) and abscisic acid (ABA), that act by promoting or inhibiting germination, respectively (Debeaujon

and Koornneef, 2000). Environmental signals affect the ABA/GAs balance and the sensitivity to these hormones (Finch-Savage and Footitt, 2017). The results from Cadman et al. (2006) linked differentially expressed genes in the dormant state, in comparison to after-ripened seeds with no residual primary dormancy, to high-ABA and low-GAs endogenous concentrations. They provided physiological evidence of the role of ABA synthesis in the maintenance of dormancy and of the synthesis and catabolism of GAs in the promotion of germination. Footitt et al. (2011) found a link between ABA signalling and endogenous ABA with deep dormancy, and GAs signalling and synthesis with shallow dormancy. However, they showed that there is not a direct link between the absolute amount of ABA and the depth of dormancy.

Although many other factors influence the dormancy state, it could be speculated that there might be a relationship between the global DNA methylation levels presented in this research and the regulation of the expression of genes implicated in ABA and GAs synthesis, catabolism or in genes that respond to these hormones. So far, there is no evidence for a direct link between ABA/GAs signalling and epigenetic components implicated in DNA methylation processes. Nevertheless, more indirect evidence indicates that histone modification and DNA methylation have substantial effects on stress and ABA sensitivity (Wong et al., 2017). Another question is whether these DNA methylation changes correspond to key regulatory genes whose expression may in turn coordinate the ABA/GAs balance.

In general terms, this study shows that DNA methylation is highly dynamic and probably necessary in regulating gene expression during the entrance and maintenance of seed dormancy. However, due to the high variations between samples belonging to different mother plants, further investigations using more replicates and studying more physiological dormancy states (more closely related to field conditions) would reveal a more detailed picture of the dynamics observed.

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5

CHAPTER 5

Cellular 5-methylcytosine and H4 acetylated patterns in primary and secondary dormant seeds of *Capsella bursa-pastoris*

Introduction

Seeds are the transition state between two phases in plants life cycle: embryo and seedling (Baud et al., 2002). Dry seeds are a quiescent stage with very low levels of water and metabolic activities and absence of growth and development (Van Zanten et al., 2013). These characteristics are associated with a high degree of chromatin condensation, which plays a critical role in gene regulation.

Dehydration, accumulation of storage reserves and induction of dormancy begins during the seed maturation phase (Vicente-Carbajosa and Carbonero, 2005). Different studies have demonstrated that developmental phase transitions in seeds are usually associated with major changes in chromatin structure and involve dynamic transcriptional control by epigenetic modifications (Cadman et al., 2006; Wolny et al., 2014; Bouyer et al., 2017). During the seed maturation phase, chromatin becomes more compact, reaching its maximum in dry seeds. However, after 24 h of imbibition, there is a significant decrease in chromatin compactness (Van Zanten et al., 2011).

Most of angiosperms present seeds that are dormant at maturity (Bewley, 1997). When seeds are shed from the mother plant, they usually experience imbibition for long periods of time in or on the soil. During this period, they are tracing environmental signals for the best conditions for germination and seedling emergence by adjusting their depth of dormancy (Footitt et al., 2015). Seed dormancy is an adaptive trait that optimizes the distribution of germination over time (Bewley, 1997), being essential for plants to establish a new generation. A seed is dormant when it fails to complete germination within a specific period of time under environmental factors that are normally favourable for its germination (Holdsworth et al., 2008).

In spite of the immense importance of seed dormancy, its control by epigenetic marks across the seed in space and time remains uncharacterized. This is the first study in plants of the epigenetic dynamics during the entrance and maintenance of seed dormancy. Early immunohistochemical studies with whole seeds were carried out by Zluvova et al. (2001), analysing DNA methylation dynamics in dry and germinating seeds of *Silene latifolia*. Wolny et al. (2017) recently studied the spatial distribution of histone H3 and H4 acetylation and DNA methylation in *Brachypodium distachyon* embryos during the four stages of seed development: maturation, desiccation, imbibition and germination. However, they did not study embryos in a dormant state.

Seeds of dicotyledonous plants possess an embryo with a root apex as well as a shoot apex surrounded by two lateral cotyledons (*Figure 5.1*, A). The root

apical meristem (RAM) is one of the most studied tissues in higher plants. Both the shoot apical meristem (SAM) and RAM (*Figure 5.1*., B and C, respectively) contain stem cells, and investigations have revealed the importance of chromatin remodelling in the regulation of their activity (Shen and Xu, 2009).

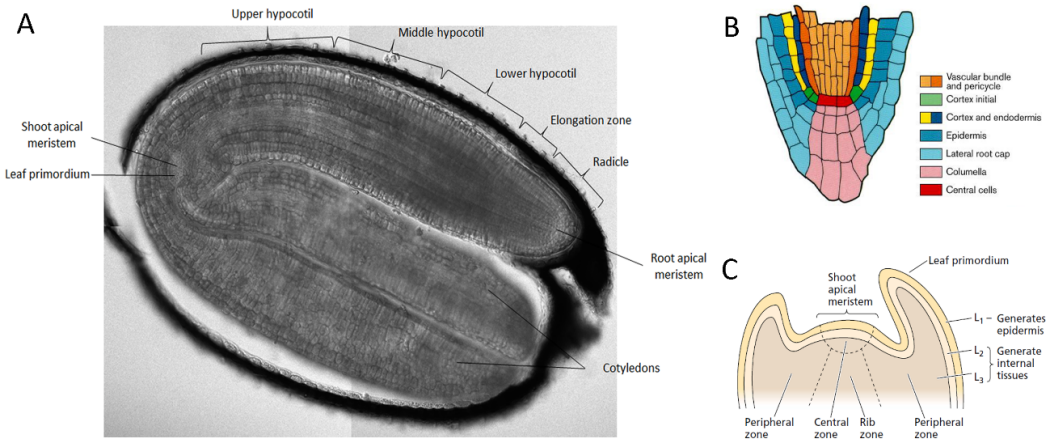

Figure 5.1. (**A**) Differential interference contrast (DIC) of a *Capsella bursa-pastoris* seed section. (**B**) Schematic representation of the root apex in *Arabidopsis thaliana*. Modified from [Weigel and Doerner (1996)]. (**C**) Schematic representation of *Arabidopsis thaliana* shoot apex. Modified from [Taiz and Zeiger (2002)].

In *Capsella bursa-pastoris* seeds, as in *Arabidopsis*, the endosperm consists of a single cell layer. Whole seeds of *Capsella bursa-pastoris*, including the testa and the endosperm, were used in this study due to the demonstrated importance of the combined effect of the aleurone layer and testa in the determination of dormancy (Bethke et al., 2007).

To analyse if global DNA methylation and histone H4 acetylation had a differential distribution pattern, immunolocalization of 5-mC and H4Ac was performed for the conditions defined in *Table 5.1*. Dry seeds were used as a control. In the initial hypothesis, DNA methylation and H4 acetylation had a dynamic role in the regulation of the entrance and maintenance of seed dormancy where the 5-mC signal increased in the whole seed in relation to the depth of dormancy and the H4 acetylation signal decreased.

In this study, the anatomy of *Capsella bursa-pastoris* seeds and the patterns of epigenetic modifications in dry, imbibed and dormant seeds were analysed. This is the first study with whole dormant seeds that links physiological aspects with their epigenetic status in a topographical context. One marker typical of euchromatin, H4 acetylation, and another typical of heterochromatin, 5-mC, have been chosen.

The global epigenetic patterns studied here can give us information about the role of specific organs and tissues and their regulation of gene expression during the entrance and maintenance of seed dormancy.

Material and methods

Scanning electron microscopy (SEM)

Fixation and processing for SEM

After 1 d of imbibition, *C. bursa-pastoris* seed testae were removed and from each whole mature embryo one cotyledon was dissected to expose the shoot apex (epicotyl). The material was fixed overnight at 4 °C under vacuum in 2.5 % (v/v) glutaraldehyde and 4 % (w/v) paraformaldehyde in 0.2 M cacodylate buffer (pH 7.2). Fixed embryos were washed three times in phosphate buffer saline (PBS) and stored in the fixative at 4 °C until use. Just prior to use, samples were washed in cacodylate buffer and post-fixated in 1 % (w/v) osmium tetroxide (OsO_4) cacodylate buffer for 1 h. The material was then dehydrated in an alcohol series and washed twice with acetone. The samples were critical-point dried in liquid CO_2 and sputter-coated with gold/palladium. Scanning electron microscopy was performed with a JSM 7400F field emission scanning electron microscope at an accelerating voltage of 5 kV.

Immunohistochemical detection of 5-mC and H4Ac

Plant material

C. bursa-pastoris seeds used were described in Chapter II. Seeds from accession SCRI -773, mother plant 6, were used as non-dormant seed material. Primary dormant seeds were harvested in Oviedo, Asturias, Spain, from plants of accession SCRI -773 (mother plant 6) grown in a greenhouse.

Fixation and processing for immunohistochemical detection

At least three replicate samples per treatment were studied. After each of the treatments indicated in *Table 5.1.*, whole seeds were fixed in 4 % paraformaldehyde in PBS (w/v) overnight at 4 °C under vacuum. To facilitate penetration of the fixative, seed testae were punctured with a needle under safe green light conditions before fixation.

Table 5.1. Immunolocalization conditions

Primary Dormancy Conditions	**Secondary Dormancy Conditions**
Dry seeds	Dry seeds
1 Day 30 °C 12h light	1 Day 30 °C 12h light
14 Days 30 °C 12h light	1 Day 30 °C Dark
	3 Days 30 °C Dark
	7 Days 30 °C Dark
	14 Days 30 °C Dark

Fixed seeds were washed three times in PBS and stored in 0.1 % paraformaldehyde in PBS at 4 °C until use. Just prior to use, samples were washed in PBS, embedded in Tissue-Tek compound and frozen for sectioning. Once frozen, seeds were sectioned at 40-50 μm thickness using a Leica CM1510-S cryomicrotome (Leica Instruments), mounted on coated microscope slides, air-dried and stored at -20 °C until use for immunohistochemistry.

Immunohistochemical detection of 5-mC and H4Ac

Permeabilization was required prior to immunohistochemical detection. After equilibration to RT, sections were washed three times in PBS, dehydrated in an ascending ethanol series (25 %, 50 %, 75 %, and 100 %; 5 min each) and subsequently rehydrated in a descending ethanol series (100 %, 75 %, 50 %, and 25 %; 5 min each). After a washing step with PBS, samples were subjected to an enzymatic digestion of cell walls by incubation in 2 % (w/v) Cellulase Onozuka R-10 and 2 % (w/v) Macerozyime R-10 (©Duchefa Biochemie) in PBS for 1.5 h at 30 °C followed by 45 min in 0.1 % Tween 20 in PBS (v/v) at RT. DNA was denatured for 5-mC detection with 2N HCl for 30 min and incubated with 5 % of bovine serum albumin (BSA) in PBS (w/v) and 0.1 % Triton (v/v) for 30 min to prevent unspecific binding.

Sections were incubated for 2 h with the mouse monoclonal anti-5-mC antibody (Diagenode, Cat. N. C15200081, Liege, Belgium) or with the rabbit polyclonal anti-H4Ac antibody (Merk KGaA, Cat. N. 06-866, Darmstadt, Germany) diluted 1:50 in 1 % BSA in PBS (w/v) with 0.1 % Triton X-100 (v/v). After two rinsing steps in 0.1 % Tween 20 in PBS (v/v), sections were incubated for 1 h in darkness with either the Alexa Fluor® 488-labeled anti-mouse polyclonal secondary antibody (Thermo Fisher Scientific Inc., Cat. N. A11001) for 5-mC detection or the Alexa Fluor® 488-labeled anti-rabbit polyclonal secondary antibody (Thermo Fisher Scientific Inc, Cat. N. A11008) for H4Ac detection. Secondary antibodies were diluted 1:25 in 1 % BSA in PBS (w/v) with 0.1 % Triton

X-100 (v/v). Nuclei were counterstained with a 4',6-diamidino-2-phenylindole (DAPI) solution [1 μg mL^{-1}, 0.1 % Triton in PBS (v/v)] for 30 min in darkness, washed in distilled water and mounted in Mowiol® 4-88 (Sigma-Aldrich).

A control was included for all section types and materials used by performing the whole immunohistochemical protocol with substitution of primary antibodies by PBS. Controls showed no fluorescence signal in the nuclei.

Image analysis

Fluorescence was examined under a confocal microscope (Leica Microsystems TCS-SP8-AOBS). Confocal optical sections were collected at 0.5 μm z-intervals. Images of maximum projections from each z-series were obtained with the open source image processing software ImageJ (version 1.52i) (Rasband, W.S., National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij). For confocal series capture, the same settings (pinhole size, gain, offset and laser intensity) were used in all the experiments performed using the same primary antibody (anti-5- mC or anti-acetylated histone H4). Visual assessment was used to compare differences in fluorescence intensity between areas of the seeds and across treatments, a procedure that has been applied by other authors such as Meijón et al. (2009) or Santamaría et al. (2009).

Results

Scanning electron microscopy (SEM)

The first immunolocalization images with confocal microscopy of *C. bursa-pastoris* seeds showed the presence of two true leaf primordia in dry seeds or seeds imbibed in water for just 1 d at 30 °C. To corroborate this, scanning electron microscopy of the shoot apex was carried out (*Figure 5.1.*). SEM images showed that the shoot apical meristem of shepherd's purse mature embryos is flanked by two leaf primordia (*Figure 5.1.*), that are between 35-40 μm wide and 20 μm long.

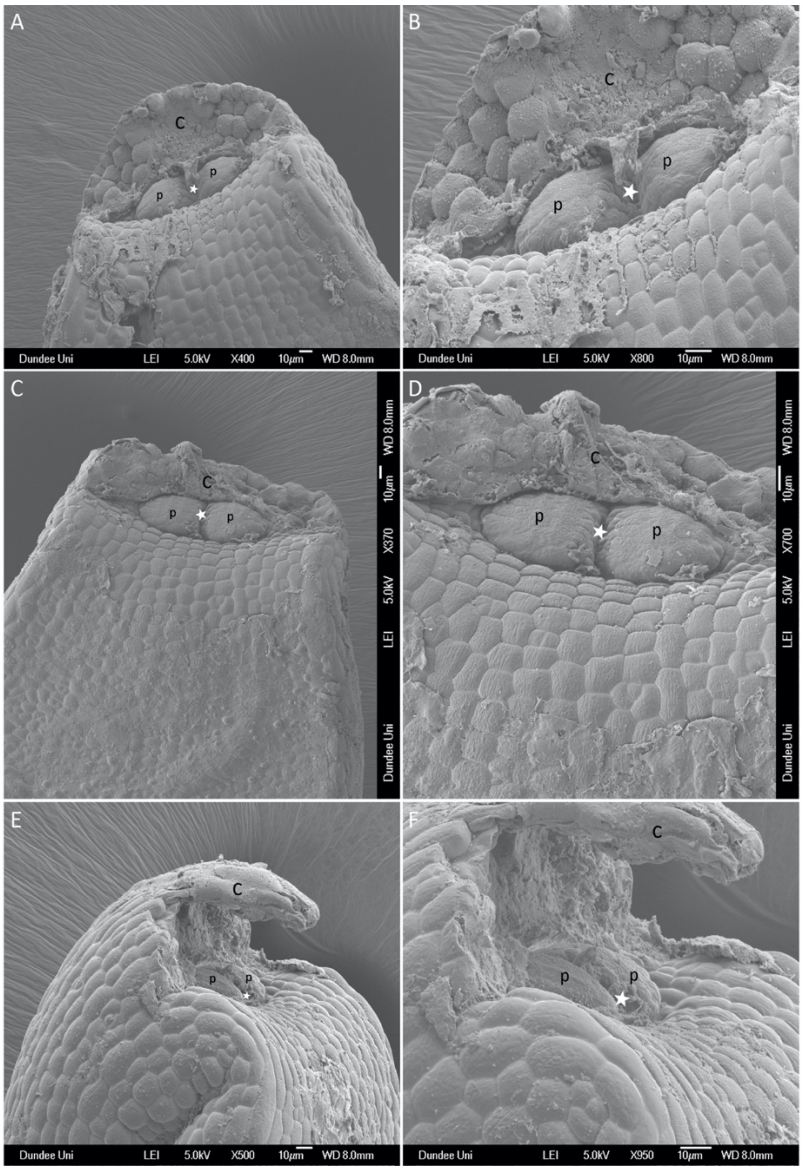

Figure 5.1. (**A**-**F**) SEMs of mature embryo apices of *C. bursa-pastoris* imbibed in water for 1 d where one cotyledon has been removed (**C**). First true leaf primordia (**p**) and SAM (white star) are indicated. Scale bars of 10 μM

Immunolocalizations

Primary dormant seeds

DNA methylation

The intensity of the 5-mC signal in primary dormant dry seeds was detected in different areas, although the intensity of the signal was low (*Figure 5.2.*, A). In the radicle, especially in the RAM, the immunosignal was the most intense (*Figure 5.2.*, C). On the other hand, in the shoot apex, even though the leaf primordia had 5-mC, immunofluorescence was not detected in the SAM (*Figure 5.2.*, B). After 1 d of imbibition with 12 h of light there was an increase in the signal intensity (*Figure 5.2.*, D) and the seed had an increase in the number of marked nuclei in comparison to dry seeds. Standing out was the shoot apex, where the SAM had immunosignal (*Figure 5.2.*, E).

With an imbibition time of 14 d with light, the immunofluorescence signal in the hypocotyl, elongation zone and radicle disappeared, compared to seeds imbibed for 1 d (*Figure 5.2.*, G and I). On the other hand, there was an increase in the number of marked nuclei in the SAM, vascular tissue of the shoot apex and in the cotyledons (*Figure 5.2.*, H).

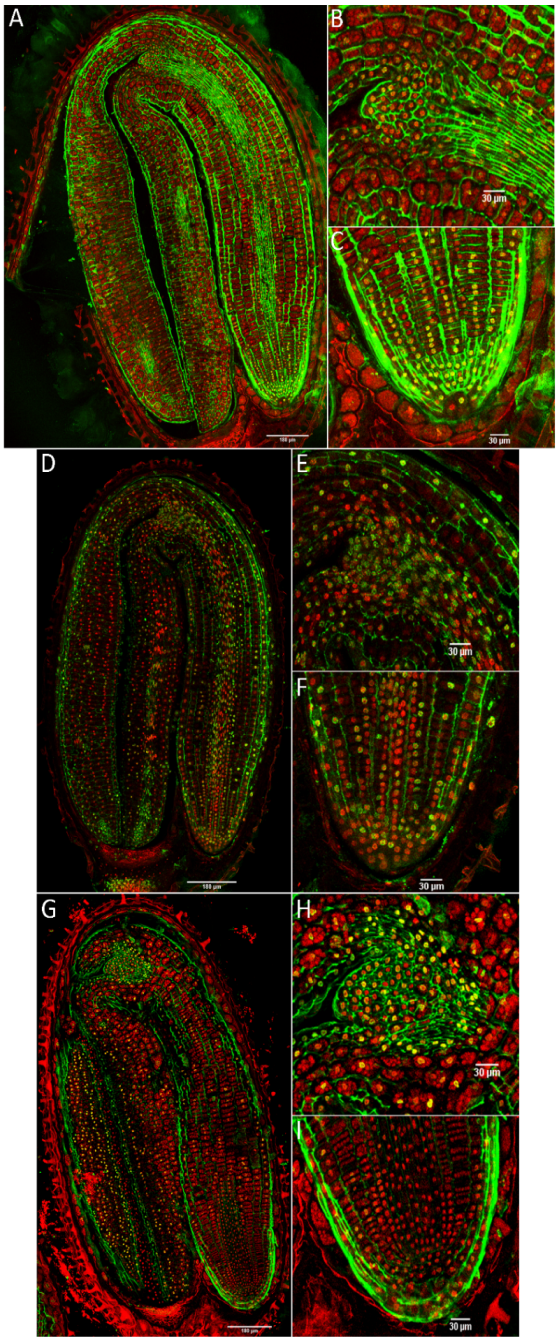

Figure 5.2. Merged images of immunodetection of 5-mC using confocal microscopy in primary dormant seeds. Longitudinal sections. (**A**) Primary dormant dry seeds with, (**B**) detail of the shoot apex and (**C**) detail of the root apex. (**D**) Primary dormant seeds imbibed in water for 1 d with 12 h of light with, (**E**) detail of the shoot apex and (**F**) detail of the root apex. (**G** and **H**) Primary dormant seeds imbibed in water for 14 d (12 h photoperiod) with, (**I**) detail of the shoot apex and (**J**) detail of the root apex. DAPI (in red, false colour) and Alexa 488, immunostaining 5-mC (in green). Scale bars of 180 and 30 μm.

Histone H4 acetylation

In mature dry seeds (*Figure 5.3.*, A), H4 acetylation was detected in moderate levels in the vascular tissue nuclei of the radicle, excluding the RAM (*Figure 5.3.*, C), and in the vascular tissue nuclei of the shoot apex, but not in the SAM or true leaf primordia (*Figure 5.3.*, B). After 1 d imbibed in water with 12 h of light, a large increase in the number of nuclei marked with H4Ac signal was detected in the whole seed (*Figure 5.3.*, D). The intensity of the immunosignal was the highest in the shoot apex, including the SAM and the leaf primordia (*Figure 5.3.*, E), the first part of the cotyledons and in the lower-middle hypocotyl. Moderate levels were detected in the radicle, including the RAM (*Figure 5.3.*, F).

In imbibed PDS, the number of nuclei with H4Ac signal decreased with the depth of dormancy. Seeds imbibed for 14 d (12 h photoperiod) had fewer marked nuclei within the whole seed in comparison with seeds imbibed for 1 d, with marks disappearing especially in the SAM and leaf primordia (*Figure 5.3.*, G, H and I). Nonetheless, the RAM was still acetylated (*Figure 5.3.*, J).

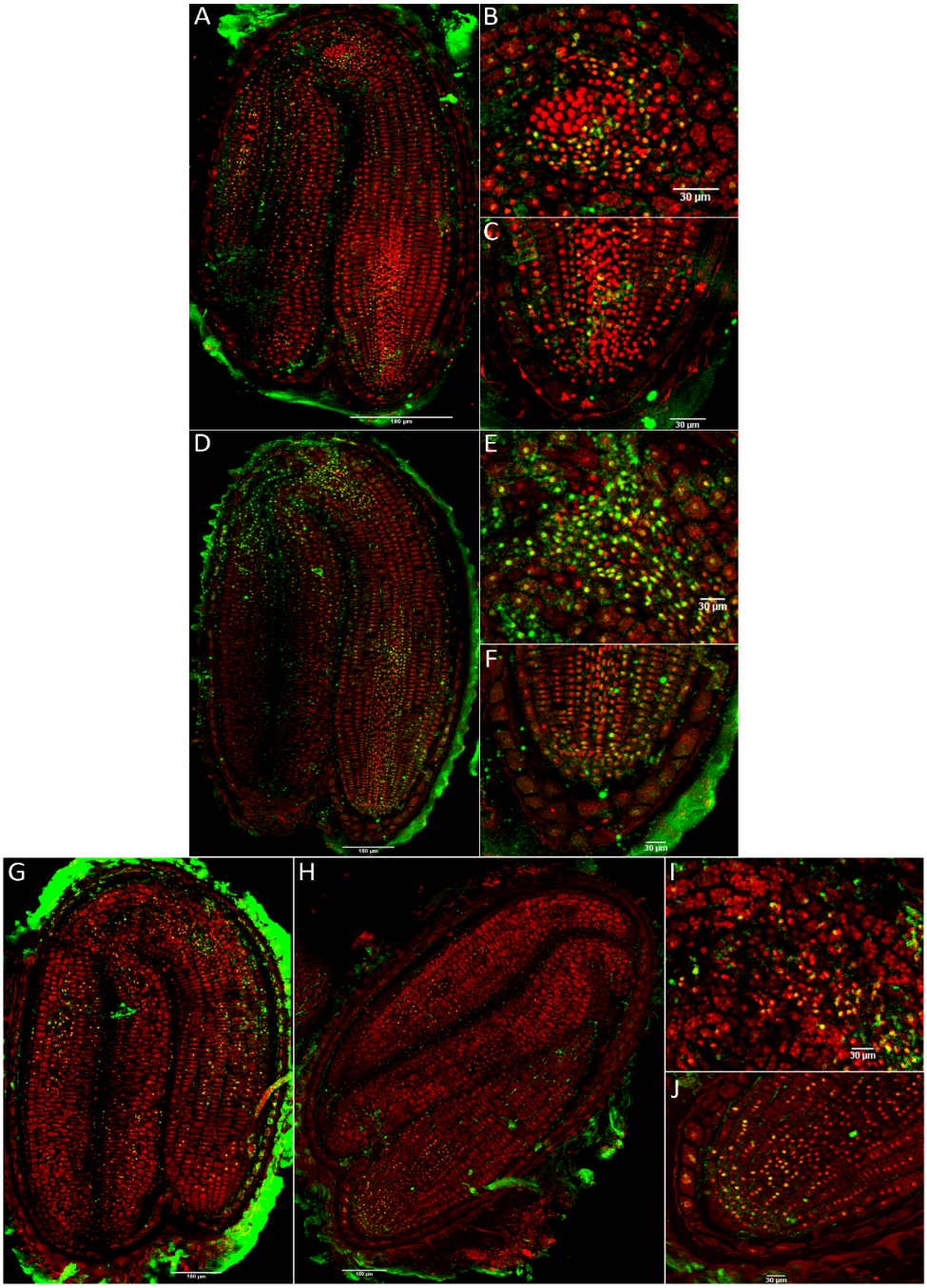

Figure 5.3. Merged images of immunodetection of H4Ac using confocal microscopy in primary dormant seeds. Longitudinal sections. (**A**) Primary dormant dry seeds with, (**B**) detail of the shoot apex and (**C**) detail of the root apex. (**D**) Primary dormant seeds imbibed in water for 1 d with 12 h of light with, (**E**) detail of the shoot apex and (**F**) detail of the root apex. (**G** and **H**) Primary dormant seeds imbibed in water for 14 d (12 h photoperiod) with, (**I**) detail of the shoot apex and (**J**) detail of the root apex. DAPI (in red, false colour) and Alexa 488, immunostaining of H4Ac (in green). Scale bars of 180 and 30 μm.

Secondary dormant seeds

DNA methylation

Whilst the DNA methylation signal in non-dormant dry seeds was highest in the leaf primordia (*Figure 5.4.*, A and B), and became more obvious in the shoot apex, leaf primordia and vascular tissue nuclei of the meristem after 1 d of imbibition in the light (*Figure 5.4.*, D and E), the signal in the whole seed was low (*Figure 5.4.*, C). By comparison, 1 d in darkness triggered an increase of the 5-mC immunosignal in the SAM, the cotyledons and in the radicle, with a particularly high immunosignal observed in the nuclei of the RAM in the dark (*Figure 5.4.*, G, H, I and J). By 3 d in the dark (*Figure 5.5.*, A and B), nuclei in the leaf primordia, the SAM and vascular tissue nuclei of the meristem were marked with 5-mC signal (*Figure 5.5.*, C), with these areas having the most intense immunofluorescence within the whole seed. A higher signal after 7 d in darkness (*Figure 5.5.*, E) had been hypothesized. However, leaf primordia revealed comparably high levels of this modification relative to seeds imbibed for 3 d, whilst the signal in the SAM, vascular tissues of the meristem, radicle and RAM was reduced (*Figure 5.5.*, F). Seeds imbibed in darkness for 14 d showed lower immunofluorescence levels in the whole seed in comparison with 3 or 7 d. There were fewer marked nuclei, especially in the leaf primordia, the SAM and in the cotyledons (*Figure 5.5.*, H and I), but also in the radicle and RAM. Nevertheless, the SAM presented higher methylation levels than the RAM and radicle.

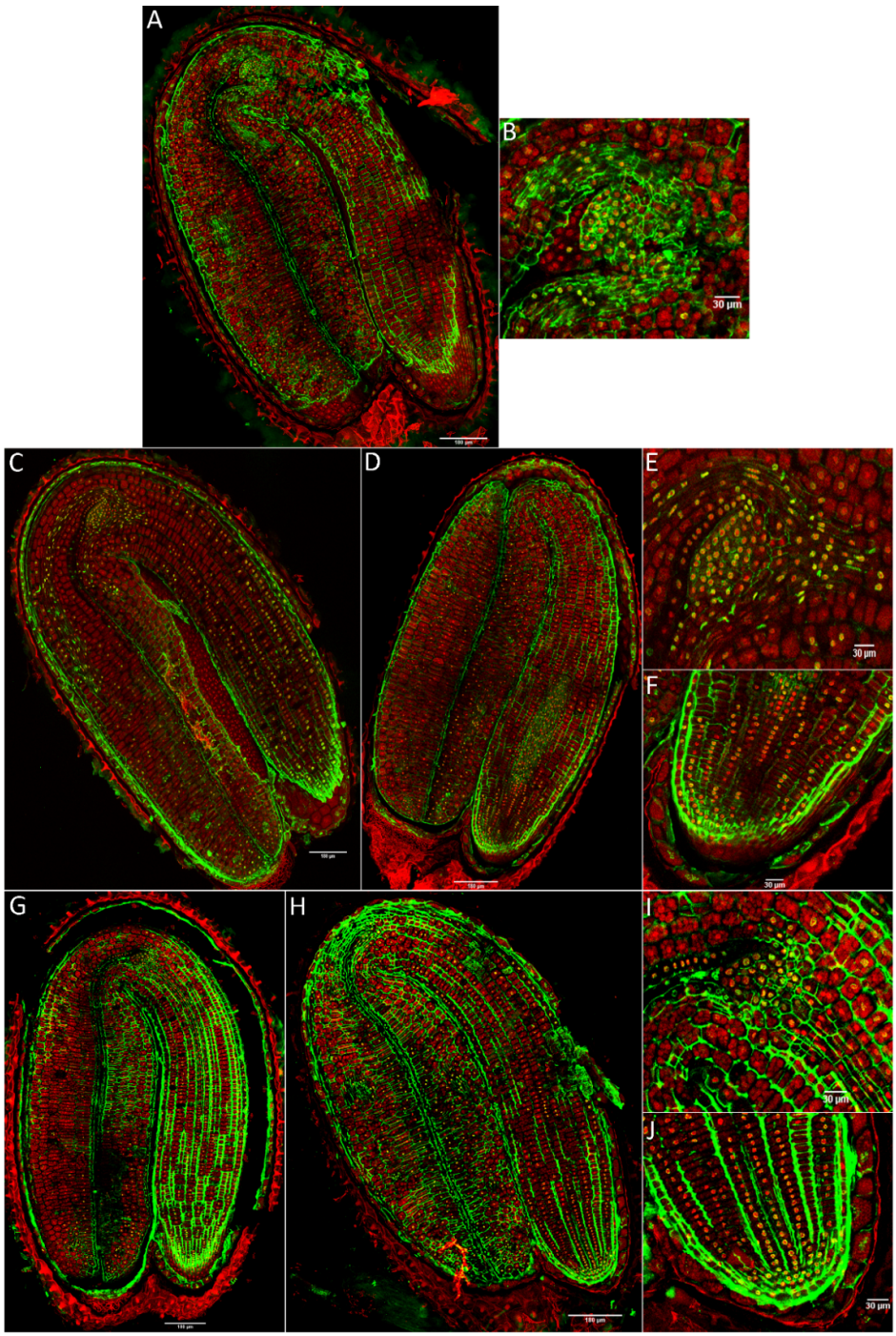

Figure 5.4. Merged images of immunodetection of 5-mC using confocal microscopy in non-dormant seeds. Longitudinal sections. (**A**) Non-dormant dry seeds with, (**B**) detail of the shoot apex. (**C**) and (**D**) Non-dormant seeds imbibed in water for 1 d with 12 h of light with, (**E**) detail of the shoot apex and (**F**) detail of the root apex. (**G**) and (**H**) Non-dormant seeds imbibed in water for 1 d in darkness with, (**I**) detail of the shoot apex and (**J**) detail of the root apex. DAPI (in red, false colour) and Alexa 488, immunostaining of 5-mC (in green). Scale bars of 180 and 30 μm.

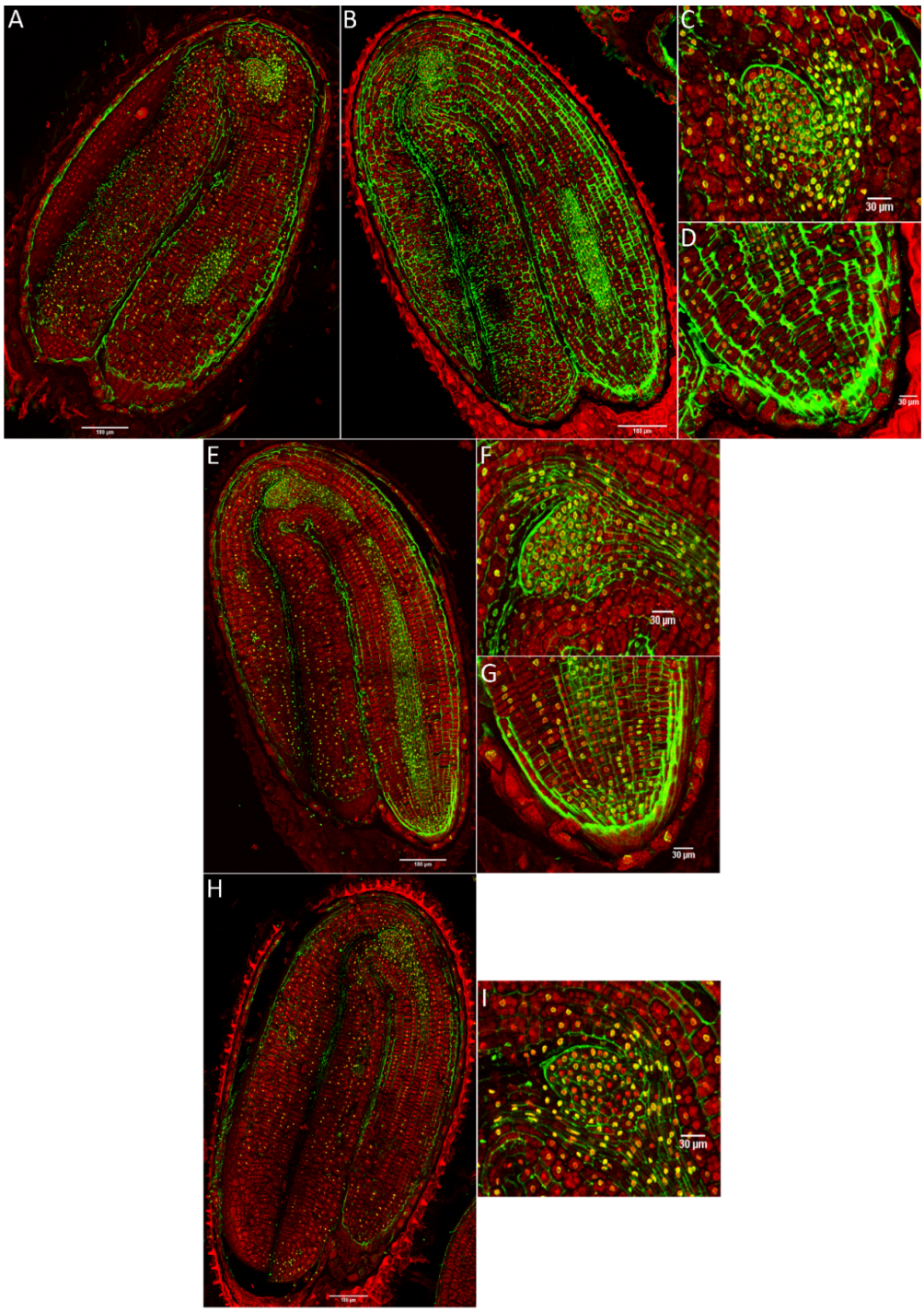

Figure 5.5. Merged images of immunodetection of 5-mC using confocal microscopy in induced secondary dormant seeds. Longitudinal sections. (**A**) and (**B**) Seeds imbibed in water for 3 d in darkness with, (**C**) detail of the shoot apex and (**D**) detail of the root apex. (**E**) Seeds imbibed in water for 7 d in darkness, (**F**) detail of the shoot apex and (**G**) detail of the root apex. (**H**) Seeds imbibed in water for 14 d in darkness with, (**I**) detail of the shoot apex. DAPI (in red, false colour) and Alexa 488, immunostaining of 5-mC (in green). Scale bars of 180 and 30 μm.

Histone H4 acetylation

H4Ac immunosignal in dry seeds without dormancy (*Figure 5.6*., A) was detected in the root apex, specifically in the nuclei of the RAM, the cortex and the pericycle of the radicle (*Figure 5.6*., C). When seeds were imbibed for 1 d with 12 h of light, conditions appropriate for germination for non-dormant seeds, there was a general increase in H4 acetylated nuclei (*Figure 5.6*., D). The shoot apex presented H4 acetylation in the leaf primordia but not in nuclei of the SAM (*Figure 5.6*., E) and the most intense immunofluorescence was localized in the vascular tissue nuclei of the hypocotyl and the elongation zone.

For the induction of secondary dormancy, seeds were imbibed in darkness at the same temperature. After 1 d in darkness, seeds had low levels of H4 acetylation in the vascular tissue nuclei of the hypocotyl and of the elongation zone (*Figure 5.6*., G), compared to seeds imbibed in the light. A comparatively high signal was found in the nuclei of the leaf primordia and the RAM (*Figure 5.6*., H and I).

When seeds were imbibed in darkness for 3 d, H4Ac modification was reduced in the SAM, leaf primordia, the hypocotyl and elongation zone, whilst H4 acetylation levels were maintained in the cotyledons (*Figure 5.7*., A and B), compared to seeds imbibed in darkness for 1 d. After 7 d (*Figure 5.7*., C and D), very low or no signal was observed in the leaf primordia and the SAM (*Figure 5.7*., E), whereas high intensity immunosignal was present in the upper and middle hypocotyl and in the RAM (*Figure 5.7*., F). Cotyledons had a weak signal.

Very weak or no immunosignal was observed in the leaf primordia, the SAM or cotyledons of seeds imbibed in darkness for 14 d (*Figure 5.7*., G, H and I). Nonetheless, immunofluorescence was visualized in the RAM, although not in the epidermis tissue nuclei, as it was in seeds imbibed for 7 d.

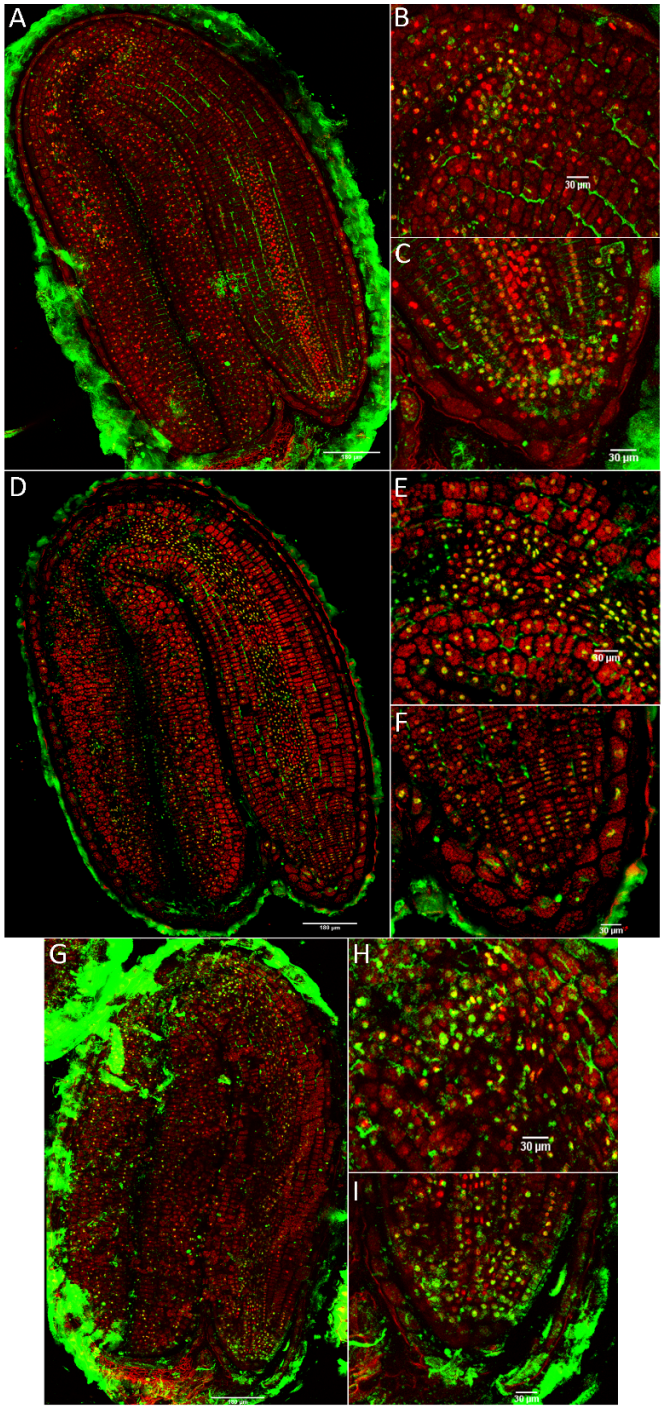

Figure 5.6. Merged images of immunodetection of H4Ac using confocal microscopy in non-dormant seeds. Longitudinal sections. (**A**) Non-dormant dry seeds with, (**B**) detail of the shoot apex and (**C**) detail of the root apex. (**D**) Non-dormant seeds imbibed in water for 1 d with 12 h of light with, (**E**) detail of the shoot apex and (**F**) detail of the root apex. (**G**) Non-dormant seeds imbibed in water for 1 d in darkness with, (**H**) detail of the shoot apex and (**I**) detail of the root apex. DAPI (in red, false colour) and Alexa 488, immunostaining of H4Ac (in green). Scale bars of 180 and 30 μm.

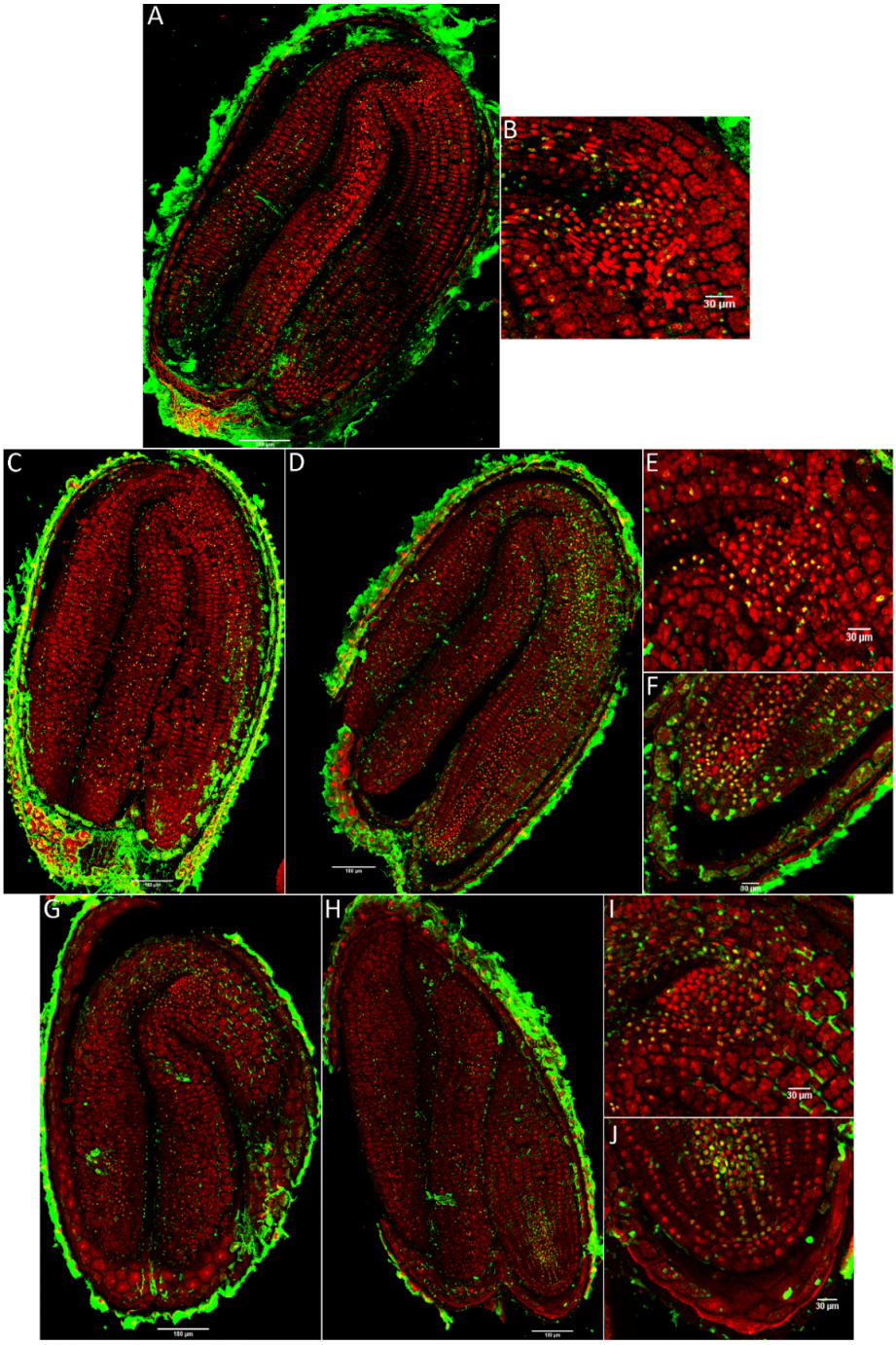

Figure 5.7. Merged images of immunodetection of H4Ac using confocal microscopy in induced secondary dormant seeds. Longitudinal sections. (**A**) Seeds imbibed in water for 3 d in darkness with, (**B**) detail of the shoot apex. (**C**) and (**D**) Seeds imbibed in water for 7 d in darkness with, (**E**) detail of the shoot apex and (**F**) detail of the root apex. (**G** and **H**) Seeds imbibed in water for 14 days in darkness with, (**I**) detail of the shoot apex and (**J**) detail of the root apex. DAPI (in red, false colour) and Alexa 488, immunostaining of H4Ac (in green). Scale bars of 180 and 30 μm.

Discussion

As indicated throughout this doctoral thesis, the role of epigenetic regulation in the control of seed dormancy has remained elusive and has only begun to be elucidated in recent years. Previous immunohistochemical investigations have used non-dormant dry seeds, germinating seeds or embryos (Zluvova et al., 2001; Wolny et al., 2014, 2017), but never dormant.

The main aim of this part of the thesis was to verify the dynamic roles of 5-mC and acetylation of H4 in the regulation of primary and secondary seed dormancy and to detect the levels of these epigenetic marks in specific organs and tissues throughout the process of dormancy induction and maintenance. The use of whole dormant seeds without removal of the testae was an approach methodologically demanding. Dormant seeds showed more obstacles to the passing of antibodies through cell walls than non-dormant or germinating seeds. Washing steps were increased with respect to other protocols, however that did not lead to an improvement in the elimination of artefacts. Longer incubation times in solutions with enzymes to digest the cell walls than in previous investigations were done. As a result, secondary antibodies, especially in 5-mC immunolocalizations, attached to the cell walls. In future studies, the analysis of only embryos instead of whole seeds would probably generate images with fewer artefacts. At the same time, using a monoclonal H4 acetylation antibody could possibly prevent these undesired non-specific signals.

Capsella bursa-pastoris embryo anatomy

The SEM images of shepherd's purse mature embryos showed that the shoot apical meristem is flanked by two leaf primordia (*Figure 5.1.*). This feature has been described for the close species *A. thaliana* (Irish and Sussex, 1992), but it had not been described for the *Capsella* genus before. However, in *Arabidopsis* seeds, the first two primordia appeared after 48 h of imbibition, while in shepherd's purse they were already observed in the dry state. These leaf primordia can also be clearly observed in the immunolocalization images (e.g., *Figure 5.5.*, A and C).

Overall dynamics of epigenetic marks in (secondary) seed dormancy

In the initial hypothesis for methylation and acetylation dynamics, an increase of global DNA methylation levels in relation to the depth of dormancy over time was proposed, as well as a decrease of H4 acetylation levels during this process. In this way, seeds imbibed for a long period in darkness, such as 14 d, would present higher 5-mC levels and lower H4 acetylation levels than seeds imbibed for a short period, such as 1 d.

The immunolocalizations showed that the general patterns of H4Ac and 5-mC in whole seeds were spatially complex. A wide variation of H4Ac and 5-mC was found within and between different tissues of the seeds and between different

dormancy states. The dynamics of H4 acetylation agreed with the initial hypothesis, in that a lower number of H4Ac marked nuclei were detected in deeper dormancy conditions. In the case of global DNA methylation, primary dormant seeds in a deeper dormancy state had an increased DNA methylation signal but only in specific parts of the seed. On the other hand, during the induction of secondary dormancy, seeds did not have a higher number of nuclei marked or higher intensity of the 5-mC signal as dormancy deepened. However, secondary dormant seeds showed temporal patterns of methylation similar to those found in the global methylation quantification analysis (Chapter IV).

5-mC and H4Ac dynamics in primary dormant seeds (PDS)

The genomes in dry seeds tend to be under-acetylated (Hodurková and Vyskot, 2003). The immunolocalization images for primary dormant dry seeds show H4 acetylation dynamics that concur with that perspective, and similar to that found in the nuclei of pollen with high chromatin compaction and H4 hypo-acetylation (Janousek et al., 2000).

In contrast, hyper-methylation is known in dry seeds (Hodurková and Vyskot, 2003) and global DNA methylation can be high in mature pollen (Janousek et al., 2000). This is not the case for *C. bursa-pastoris* seeds, based on 5-mC immunolocalization. Primary dormant dry seeds had fewer marked nuclei and lower intensity of the signal in general terms (*Figure 5.2.*, A) in comparison with PDS imbibed for 1 d with 12 h of light (*Figure 5.2.*, D). These results seem to indicate that DNA methylation levels increase after imbibition of seeds under conditions that do not promote germination. As the DNA methylation signal increased only in specific parts of the seed after 14 d of imbibition, whilst a significant increase in DNA methylation levels was observed in the global quantification, it seems that spatially significant changes in the DNA methylation signal can be both responsible for and potentially masked by the overall differences in the global DNA methylation quantification.

If we focus on the shoot and the root apex, the SAM and the leaf primordia, but not the RAM, lost their acetylation signals in the deep primary dormant state (*Figure 5.3.*, I and J). On the other hand, 5-mC disappeared from the radicle and the RAM, but increased in the SAM under the same conditions (*Figure 5.2.*, H and I). The shoot apex represents a permanent pluripotent cell line, with self-maintenance of meristematic cells and morphogenesis of the plant body (Zluvova et al., 2001). This could indicate that for the maintenance of a deeper primary dormancy state in seeds, the shoot apex needs to be maintained in a "silenced state" by hyper-methylation, whilst the radicle can present certain levels of histone acetylation. This acetylation of the radicle, including the RAM, may suggest a central role for the radicle/RAM in the response to any ambient changes perceived by the seed and in adjusting the seed dormancy state to them.

5-mC and H4Ac dynamics in non-dormant and secondary dormant seeds

With respect to the global DNA methylation signal, in non-dormant dry seeds it can only be said that the highest immunosignal was in the leaf primordia (*Figure 5.4.*, A and B), as the images obtained were not of enough quality. When *C. bursa-pastoris* seeds were sown under conditions that promote germination in non-dormant seeds (i.e. 1 d, 30°C, light), seeds had high numbers of marked nuclei in the shoot apex, leaf primordia and vascular tissue nuclei of the meristem but no signal was detected in the nuclei of the SAM (*Figure 5.4.*, D). However, in general terms, the number of nuclei presenting signal in the whole seed was low (*Figure 5.4.*, C).

After 1 d in complete darkness to start inducing secondary dormancy, there was an increase of the immunosignal in the SAM, the cotyledons and in the radicle, especially the RAM, in comparison with seeds imbibed in the same conditions but with 12 h of light (*Figure 5.4.*, G, H, I and J). When seeds were imbibed for another 2 d, a large increase in the nuclei marked with 5-mC signal in the leaf primordia, the SAM and vascular tissue nuclei of the meristem was detected (*Figure 5.5.*, C). Although a higher signal was expected after 7 d in darkness following the initial hypothesis, a lower number of marked nuclei in the SAM and in the vascular tissues of the meristem were detected (*Figure 5.5.*, F). Maintaining the seeds for another week in darkness resulted in even fewer marked nuclei, especially in the leaf primordia, the SAM and in the cotyledons (*Figure 5.5.*, H and I). Therefore, the DNA methylation signal after 3 d of dark imbibition was highest in some of the seed tissues, but reduced thereafter. This suggests a critical point in the induction of secondary dormancy. The significance of this pattern requires assessment at a range of ecologically meaningful temperatures and conditions of intermittent hydration to mimic realistic field conditions. The cellular 5-mC immunolocalization and global DNA methylation quantification patterns (Chapter IV) are in concordance during the induction and maintenance of secondary dormancy (from 3 to 14 d in darkness). After 3 d in darkness, deep and non-deep dormant seeds showed the highest levels of global DNA methylation, showing decreasing levels with longer periods of imbibition time. The immunolocalizations showed the same pattern, where the highest number of marked nuclei was detected after 3 d in darkness, descending after 7 or 14 d.

Comparing the H4Ac signals between the shoot and the root apex in non-dormant dry seeds, the shoot apical meristem did not present any signal opposite to the root apex, where the RAM was marked (*Figure 5.6.*, A and C). A high increase of H4 acetylated marked nuclei was detected in the whole seed in germinating conditions, particularly in the hypocotyl and the elongation zone (*Figure 5.6.*, D and F). This change in the spatial pattern for acetylation may indicate that the chromatin in these areas needs reorganisation to enable transcriptional reprogramming, and that these embryo regions are implicated in the induction of germination.

In contrast, after 1 d of imbibition in darkness, which induces secondary dormancy, seeds had lower levels of H4 acetylation in the hypocotyl and the elongation zone (*Figure 5.6*., G and I). By 3 d in the dark, H4 acetylation decreased further, except for the cotyledons (*Figure 5.7*., A). By 14 d in the dark, the signal had completely disappeared in the SAM, leaf primordia and the cotyledons (*Figure 5.7*., C, D, E, G, H and I), whilst the RAM and/or the epidermis tissue nuclei maintained the H4Ac mark (*Figure 5.7*., F and J). This is similar to the deeper primary dormant state, where the SAM did not show H4 acetylation signal and the RAM still maintained certain acetylation levels after 14 d of imbibition. This suggests, like for PDS described above, that the radicle could be a centre of control that integrates the environmental external signals received by the seed (such as light, temperature or moisture) during dormancy, thereby indicating the right time for germination.

Conclusions

The first investigation that interrelated the two epigenetic marks studied here was carried out by Law and Suttle (2004) studying dormancy in potato tubers. In Santamaría et al. (2009), where the epigenetic control of bud dormancy was analysed, a loss of H4 acetylation during bud set was accompanied by an increase in the nuclei marked with 5-mC and vice versa during bud burst. Other studies focused, for example, on the epigenetic control of winter dormancy in poplar stems (Conde et al., 2013) or on the epigenetic implications of floral differentiation gene regulation in azalea (Meijón et al., 2009).

With this study, DNA methylation and histone H4 acetylation patterns have been linked to the physiological changes that accompany *C. bursa-pastoris* seed germination and induction and maintenance of primary and secondary dormancy. The results obtained show that epigenetic marks are dependent on environmental conditions which impact temporal and spatial patterns. The immunolocalization results showed that deeply primary dormant seeds had a higher number of 5-mC marked nuclei only in specific parts of the seed, whilst secondary dormant seeds showed temporal patterns of 5-mC similar to those found in the global methylation analysis (Chapter IV). In addition, fewer H4Ac marked nuclei in deeper dormant states were observed, for both types of dormancy.

For primary dormant seeds, the 5-mC signal after 14 d of imbibition disappeared in the hypocotyl, elongation zone and radicle, but it increased in the SAM. The SAM lost its acetylation signal after the same period of imbibition, whilst the RAM still had marked nuclei. In the induction of secondary dormancy, the SAM and RAM showed fewer 5-mC marked nuclei after 7 and 14 d in darkness in comparison with 3 d. However, the SAM showed higher levels of DNA methylation than the RAM. Analysing the acetylation signal, the SAM had fewer H4 acetylated marked nuclei after 7 and 14 d in darkness in comparison with 3 d, whilst the RAM still had H4Ac signal. These results seem to indicate that for a deeper primary or secondary dormancy state, the shoot apex needs to be more

methylated than the RAM, whilst the latter can maintain certain histone acetylation levels. Keeping the radicle in a (partially) active epigenetic state may be necessary for a proper control of maintenance and release of primary and secondary dormancy.

There are several potential procedures that could be used for an automatic quantification of global DNA methylation or histone acetylation levels through quantification of the fluorescence signal based on single cell inflorescence values for each nucleus. The feasibility of the approach has been dramatically increased but the series of procedures required to lead to successful whole-tissue quantification is far from developed (Hirashima and Adachi, 2015). In previous studies in plants, the contour of each nucleus was manually outlined and the average fluorescence intensity per area obtained (Conde et al., 2013). In this way, single measures of total fluorescence on maximum projections in a representative sample size were taken, with those being the sum of the fluorescence on each pixel within the outlined area (a number from 0 to 255 per pixel) divided by the total number of pixels of the area. This procedure results in a representation of the total fluorescence intensity as histograms.

However, in this thesis, visual assessment was carried out to compare differences in fluorescence intensity between areas of the seeds and across treatments; a procedure that has been applied by other authors such as Meijón et al. (2009) or Santamaría et al. (2009). This approach was used instead of quantification methods because the quality of the images was not high enough, partly due to working with complete seeds; especially those that were in dormant states. In future studies, the analysis of only embryos instead of whole seeds would probably generate images with fewer artefacts. At the same time, using a monoclonal H4 acetylation antibody would be of immense help in avoiding these undesired non-specific signals.

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6

CHAPTER 6

RNA transcriptomic sequencing analysis

Introduction

The chemical composition of seeds is dominated by reserves (carbohydrates, lipids and proteins), containing high levels of starch, polysaccharides and secondary metabolites such as phenolics and mucilages (Birtić and Kranner, 2006; Meng and Feldman, 2010). Isolation of high-quality RNA from seeds requires complex and time-consuming methods and has been shown to be difficult, as these reserve compounds form complexes with the RNA during tissue extraction and co-precipitate. However, it is a critical step for gene expression experiments (Birtić and Kranner, 2006). This means that protocols for RNA isolation from tissues other than seeds need to be adapted.

Many procedures have been developed to cope with these problems. The use of chaotropic agents such as guanidinium isothiocyanate or proteinase K during homogenization often solves problems with RNases and RNA degradation (Wan and Wilkings, 1994). However, the extraction of RNA from starch-rich seed samples using guanidine-based buffers, such as TRIzol® reagent or the RNeasy® Plant Mini kit (Qiagen), can result in a solidification of the extract (Li and Trick, 2005). A rapid and simple method has been developed here by modification and combination of established techniques in order to overcome these difficulties.

Seed dormancy is one of the most important adaptive traits of seed plants. Therefore, an understanding of its genetic basis and the mechanisms controlling it is of great significance (Yano et al., 2013). Dormancy allows plants to avoid environmental factors not adequate for germination and can be divided into two categories: primary and secondary (Liu et al., 2019).

Primary dormancy refers to the level of dormancy that a seed presents when it is shed from the mother plant. It is induced during seed development on the parent plant and its maintenance is regulated by complex interactions between many endogenous and environmental factors (Fei et al., 2007). Natural accessions of *Arabidopsis thaliana* belonging to different habitats present high variation of primary dormancy phenotypes (Buijs et al., 2020).

Secondary seed dormancy is imposed on mature, non-dormant seeds by stresses such as light, temperature or moisture. After primary dormancy is progressively lost, secondary dormancy can be induced if the right conditions are not encountered and a dormancy cycle could be established and repeated until the proper conditions for germination are met (Finch-Savage and Leubner-Metzger, 2006; Footitt et al., 2011). This is a very important mechanism of persistence of seeds in the soil seed bank (Baskin and Baskin, 2014).

Most of the research about seed dormancy has been focused on primary dormancy and the mechanisms controlling secondary seed dormancy are still poorly understood (Cadman et al., 2006). Whether the mechanisms regulating primary dormancy are also behind the control of secondary dormancy is unclear, although recent studies have found genes that overlap (Footitt et al., 2011).

The balance between the hormones abscisic acid (ABA) and gibberellins (GAs) has been recognized as a major regulator of seed dormancy and germination, with ABA being a key hormone in the establishment of dormancy and GAs in dormancy loss and induction of germination (Finkelstein et al., 2008). However, the implication of other phytohormones, such as jasmonic acid, auxins or ethylene in these processes is beginning to be elucidated (Barrero et al., 2009). For example, the hormones auxins have been shown to be implicated in germination and early seedling growth (Wang et al., 2016) and ethylene revealed a role in endosperm weakening (Linkies et al., 2009).

The water uptake in seeds is influenced by the balance between ABA and GAs (Footitt et al., 2019). The expansion of cells from the hypocotyl to complete germination is blocked during dormancy and the water potential thresholds necessary for radicle emergence change and follow the dormancy cycle (Footitt et al., 2011).

The implication of epigenetic mechanisms and chromatin remodelling factors in controlling seed dormancy and germination has been demonstrated in previous studies (Liu et al., 2007; Tanaka et al., 2008; Wang et al., 2013, 2016). Histone acetylation and deacetylation, catalysed by histone acetyltransferases (HATs) and deacetylases (HDACs) respectively, are two of the most important modifying epigenetic mechanisms regulating gene activity (Liu et al., 2016). The use of HDAC inhibitors, such as trichostatin A (TSA), has been employed for studying the possible implication of these activities in the regulation of seed dormancy (Nelson et and Steber, 2017; Pagano et al., 2017).

An RNA-sequencing (RNA-Seq) analysis using two accessions with differences in their secondary seed dormancy depth (see Chapter II) to detect differentially expressed genes (DEGs) associated with secondary seed dormancy was performed. In an initial hypothesis, genes implicated in epigenetic regulation processes would be differentially expressed between the accessions studied. Moreover, whilst histone acetylases would be present within the down-regulated genes of the deep-dormant accession in comparison with the non-deep dormant one, DNA methyltransferases would be amongst the up-regulated DEGs.

At the same time, another comparision where the non-deep secondary dormant accession was imbibed in water or in valproic acid to study the possible genes regulated by this chemical was performed. The main goal was to determine the possible role that histone acetylation plays in the delay of germination and to identify specific genes which expression is partially regulated by histone deacetylases.

With RNA-Seq, it is possible to quantify whole-genome expression at high detection limits (Magalhães et al., 2016) and it can be very useful for having an overview of the transcriptomic dynamics that the different accessions present (Liu et al., 2019).

Material and methods

Optimization of an RNA extraction protocol

Plant material

Non-dormant, primary or secondary dormant *Capsella bursa-pastoris* seeds, dry or imbibed in water, were used to optimize an RNA extraction protocol.

Testing RNA extraction protocols

RNA from seeds was extracted following different methods, with certain modifications, which have been described as efficient for isolating RNA from seeds of different species. 30 to 50 mg of dry or imbibed seeds were used in all protocols. A final protocol was developed as a combination of different procedures. Samples were frozen and ground in liquid nitrogen with mortar and pestle under liquid nitrogen immediately before procedures.

Method 1: RNeasy®Plant Mini Kit (Qiagen)

Extraction from frozen tissue was performed according to the manufacturer's instructions without modifications.

Method 2: Chomczynski and Sacchi (2006)

Half of the samples were extracted following the original protocol by Chomczynski and Sacchi (2006), while the other half were extracted using a modified version including a polysaccharide elimination step based on the addition of 0.1 volumes of sodium acetate and 0.6 volumes of ethanol.

- **EXTRACTION BUFFER (DENATURING SOLUTION, D):** 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0) and 0.5 % (w/v) N-lauroslysarcosine (Sarkosyl). 5 % (v/v) β-mercaptoethanol was added just prior use.
- **PROTOCOL:** 50 mg of homogenized material were incubated with 1 mL of denaturing solution and mixed by vortex for 15 s; 0.1 mL of 2 M sodium acetate (pH 4.0) were added and mixed. Afterwards, 1 mL of water-saturated phenol was added and mixed by inversion and 0.2 mL of cloroform:isoamylalcohol (49:1) were added as a final step and mixed vigorously for 10 s. The sample was incubated on ice for 15 min and centrifuged at 10000 g for 20 min at 4 °C. The upper phase was transferred to a new collection tube and 1 mL of