

Research Paper

Cite this article: Gomez-Cabellos S, Toorop PE, Fernández-Pascual E, Iannetta PPM, Pritchard HW, Visscher AM (2022). (Epi)genetic control of secondary seed dormancy depth and germination in *Capsella bursa-pastoris*. *Seed Science Research* **32**, 200–221. <https://doi.org/10.1017/S0960258522000265>

Received: 30 May 2022

Accepted: 14 October 2022

First published online: 29 November 2022

Key words:


Capsella bursa-pastoris; ecotypes; epigenetics; histone deacetylase inhibitors; secondary seed dormancy depth; transcriptome

***Author for Correspondence:**

Anne M. Visscher,

E-mail: a.visscher@kew.org

(Epi)genetic control of secondary seed dormancy depth and germination in *Capsella bursa-pastoris*

Sara Gomez-Cabellos^{1,2}, Peter E. Toorop¹, Eduardo Fernández-Pascual^{2,3}, Pietro P. M. Iannetta⁴, Hugh W. Pritchard¹ and Anne M. Visscher^{1*} 

¹Royal Botanic Gardens, Kew, Wakehurst, Ardingly, Haywards Heath, West Sussex RH17 6TN, UK; ²Departamento de Biología de Organismos y Sistemas, Universidad de Oviedo, C/Catedrático Rodrigo Uría, 33006 Oviedo, Spain; ³IMIB – Biodiversity Research Institute, University of Oviedo, Mieres, Spain and ⁴The James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK

Abstract

Despite the importance of secondary dormancy for plant life cycle timing and survival, there is insufficient knowledge about the (epigenetic) regulation of this trait at the molecular level. Our aim was to determine the role of (epi)genetic processes in the regulation of secondary seed dormancy using natural genotypes of the widely distributed *Capsella bursa-pastoris*. Seeds of nine ecotypes were exposed to control conditions or histone deacetylase inhibitors [trichostatin A (TSA), valproic acid] during imbibition to study the effects of hyper-acetylation on secondary seed dormancy induction and germination. Valproic acid increased secondary dormancy and both compounds caused a delay of t50 for germination (radicle emergence) but not of t50 for testa rupture, demonstrating that they reduced speed of germination. Transcriptome analysis of one accession exposed to valproic acid *versus* water showed mixed regulation of ABA, negative regulation of GAs, BRs and auxins, as well as up-regulation of *SNL* genes, which might explain the observed delay in germination and increase in secondary dormancy. In addition, two accessions differing in secondary dormancy depth (deep *vs* non-deep) were studied using RNA-seq to reveal the potential regulatory processes underlying this trait. Phytohormone synthesis or signalling was generally up-regulated for ABA (e.g. *NCED6*, *NCED2*, *ABCG40*, *ABI3*) and down-regulated for GAs (*GA20ox1*, *GA20ox2*, *bHLH93*), ethylene (*ACO1*, *ERF4-LIKE*, *ERF105*, *ERF109-LIKE*), BRs (*BIA1*, *CYP708A2-LIKE*, probable *WRKY46*, *BAK1*, *BEN1*, *BES1*, *BRI1*) and auxin (*GH3.3*, *GH3.6*, *ABCB19*, *TGG4*, *AUX1*, *PIN6*, *WAT1*). Epigenetic candidates for variation in secondary dormancy depth include *SNL* genes, histone deacetylases and associated genes (*HDA14*, *HDA6-LIKE*, *HDA-LIKE*, *ING2*, *JMJ30*), as well as sequences linked to histone acetyltransferases (*bZIP11*, *ARID1A-LIKE*), or to gene silencing through histone methylation (*SUVH7*, *SUVH9*, *CLF*). Together, these results show that phytohormones and epigenetic regulation play an important role in controlling differences in secondary dormancy depth between accessions.

Introduction

The time between seed dispersal and completion of germination can be short or long, thus, seeds have evolved a series of strategies – for example, dormancy – to regulate this interval (Footitt and Finch-Savage, 2017). These strategies prevent responses to short-lived, out of season environmental changes, making it possible for plants to overcome periods that are unfavourable for seedling establishment (Bentsink and Koornneef, 2008). 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 exhibit a strong primary dormancy trait that is determined by the environment during seed development and physiological characteristics of 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). After the seed has left the mother plant and primary dormancy has been released in response to seasonal environmental changes, non-germinating seeds may enter secondary dormancy (Baskin and Baskin, 2014). This entrance into secondary dormancy can be induced in seeds with non-deep physiological dormancy, if the right set of external signals to germinate are absent (Hilhorst, 1998). 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 ecotypic differences (Finch-Savage and Footitt, 2017).

Wild plant species, especially annual ones which have seeds that persist in a soil seed bank, are better models for physiological studies of seed dormancy than domesticated crops (Hilhorst and Toorop, 1997). *Capsella bursa-pastoris* (L.) Medik. [shepherd's purse (Brassicaceae)] is an annual or biennial herb that can form a persistent seed bank in the soil (Hurka and Haase, 1982). The species displays primary seed dormancy (non-deep physiological) as well as an annual cycle of secondary dormancy/non-dormancy (Neuffer and Hurka, 1986; Baskin and Baskin, 1989; Toorop et al., 2012). It has a worldwide distribution, with the exception of extremely dry tropical environments (Neuffer and Eschner, 1995; Hurka and Neuffer, 1997), and 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). *C. bursa-pastoris* has a very small phylogenetic distance to the academic model species *Arabidopsis thaliana*. However, in comparison, the latter appears to be uncompetitive and is actually relatively rare in the wild (Hintz et al., 2006). Due to its wild nature, cosmopolitan distribution, and complex dormancy traits, we selected *C. bursa-pastoris* as a model species for our study on the possible (epi)genetic mechanisms involved in secondary seed dormancy induction, including differences in secondary seed dormancy depth between ecotypes.

The first aim of this study was to test the hypothesis that exposing *C. bursa-pastoris* seeds to the histone deacetylase (HDAC) inhibitors trichostatin A (TSA) and valproic acid during the induction of secondary dormancy prevents the seeds from entering this dormancy state. In addition, we reasoned that exposure to these compounds would lead to a higher germination speed compared with control conditions.

Acetylation of lysines 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 involved 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, such as embryo development, dormancy, germination and morphogenesis (Cadman et al., 2006; Wang et al., 2014). 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 (Koeller et al., 2003). HDACs can be pharmacologically inhibited by 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).

Valproic acid is known to be an inhibitor of histone deacetylases capable of reducing tumour growth and metastasis formation in animals (Göttlicher et al., 2001). 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 TSA (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 METHYLTRANSFERASE-1 and HETEROCHROMATIN PROTEIN-1 (Chateauvieux et al., 2010). It is also capable of inducing mono-, di- or tri- methylation of histone H3 at lysine 9 (H3K9) (Chateauvieux et al., 2010). However, no previous studies in plants have reported the effects of this HDAC inhibitor.

The second aim of this study was to test the hypothesis that genes implicated in epigenetic regulation processes would be differentially expressed between *C. bursa-pastoris* accessions previously shown to contrast in secondary dormancy depth (Gomez-Cabellos et al., 2021). In particular, histone acetylases were expected to be down-regulated in a relatively deep-dormant accession compared with a non-deep dormant one. In addition, differential gene expression induced by valproic acid exposure was studied in the non-deep accession to identify gene regulation by histone deacetylases.

While (genetic) research on the processes underlying primary dormancy is extensive, the molecular mechanisms controlling secondary seed dormancy are still poorly understood (Cadman et al., 2006; Finch-Savage and Leubner-Metzger, 2006; Holdsworth et al., 2008b; Footitt et al., 2013; Ibarra et al., 2016; Coughlan et al., 2017; Buijs, 2020; Laspina et al., 2020; Hourston et al., 2022). According to the hormonal balance model (Finkelstein et al., 2008), the ratio of abscisic acid (ABA) (promoting primary dormancy induction and dormancy maintenance) and gibberellic acid (GA) (promoting release of dormancy and germination) is the main determinant of the level of dormancy and seed germination (Finkelstein et al., 2008; Buijs et al., 2020). Environmental signals regulate the balance between ABA and GA levels, whose changes seem to be caused by alterations in the expression patterns of their metabolic genes and positive and negative regulators of both hormones (Finkelstein et al., 2008; Tuan et al., 2018). However, subsequent ABA and GA signalling and sensitivity are the more likely regulators of dormancy than the absolute level of these hormones (Ali-Rachedi et al., 2004; Ibarra et al., 2016; Laspina et al., 2020). In relation to secondary seed dormancy, Cadman et al. (2006) suggested that its control mechanisms differ from primary dormancy. Ibarra et al. (2016) demonstrated that the entrance into secondary seed dormancy reduced the content and sensitivity to GA, but not the content and sensitivity to ABA. However, Laspina et al. (2020) and Footitt et al. (2020) did find that ABA sensitivity plays an important role in secondary dormancy induction/dormancy cycling.

In addition to ABA and GA, there are other phytohormones with implications in the regulation of primary seed dormancy and germination. Brassinosteroids (BRs) are plant steroid hormones involved in stem elongation and leaf unfurling that promote germination. BR mutants are hypersensitive to inhibition of germination by ABA in comparison with wild-type seeds (Finkelstein et al., 2008). BRs were discovered to be implicated in the promotion of seed germination by modulating ABA signalling with a negative feedback loop (Xi et al., 2010). BR molecules may promote seed germination by enhancing embryo growth potential in a gibberellin-dependent manner (Leubner-Metzger, 2001). However, the detailed mechanisms underlying the BR and GA crosstalk are still not well understood (Shu et al., 2016).

Ethylene stimulates germination and breaks primary dormancy establishment in seeds by antagonizing the ABA pathway (Linkies and Leubner-Metzger, 2012; Corbineau et al., 2014). 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 final step in 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). On the other hand, whether ethylene affects GA biosynthesis and signalling in relation to seed dormancy and germination is still poorly understood (Shu et al., 2016).

Other hormones that are known for inhibiting seed germination and promoting primary seed dormancy are auxins (Holdsworth et al., 2008a; Liu et al., 2013). Auxins are required for the maintenance of *ABSCISIC ACID INSENSITIVE 3 (ABI3)* expression by recruiting *AUXIN RESPONSE FACTOR 10 (ARF10)* and *ARF16* (Liu et al., 2013). *ABI3* is recognized as a major regulator of primary seed dormancy and acts upstream of *ABI5* to execute ABA-responsive seed germination inhibition (Lopez-Molina et al., 2002). Another upstream regulator of *ABI5* is *RGL2*, which was found to be important for secondary dormancy induction in Arabidopsis (Ibarra et al., 2016). In addition, *ABI4* plays a role in regulating primary seed dormancy through the regulation of ABA and GA homeostasis (Shu et al., 2013). Together with *ABI3*, *FUSCA 3 (FUS3)*, *LEAFY COTYLEDON 1 (LEC1)* and *LEC2 (LAFL)* genes encode master transcriptional regulators that form a network that is key in regulating several important seed processes, including establishing primary dormancy (Carbonero et al., 2017; Lepiniec et al., 2018). For example, in *A. thaliana*, *lec1*, *lec2*, *fus3* and *abi3* mutants are severely affected in seed maturation and share some common phenotypes, such as decreased dormancy at maturation (Raz et al., 2001; Bentsink and Koornneef, 2008). *LEC1* and *LEC2* seem involved in maintaining or inducing a totipotent cell state during embryogenesis through the control of auxin biosynthetic genes (Stone et al., 2008; Wójcikowska and Gaj, 2015; Lepiniec et al., 2018). *FUS3* is known to modulate the ABA/GA balance by increasing ABA levels and repressing the synthesis of GA, while *ABI3* integrates ABA signalling (Curaba et al., 2004; Gazzarrini et al., 2004; Braybrook et al., 2006). In addition, *LAFL* activities are themselves modulated by hormone signalling feedbacks involving ABA, GA, BR or auxins (Braybrook and Harada, 2008; Carbonero et al., 2017; Lepiniec et al., 2018).

Another important gene known to regulate primary dormancy in Arabidopsis is *DELAY OF GERMINATION 1 (DOG1)* (Bentsink et al., 2006). In parallel to Arabidopsis, *DOG1* genes have been found in other species of Brassicaceae and some of *Latua*, with high similarity between amino acid sequences (Graeber et al., 2010, 2014; Huo et al., 2016; Carrillo-Barral et al., 2020). *DOG1* is linked to accumulation of thermal time (Footitt et al., 2015) and mutations in the gene can completely remove primary seed dormancy (Bentsink et al., 2006). The relationship between *DOG1* and phytohormones 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 (Née et al., 2017). 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). *DOG1* transduces environmental effects during seed maturation to alter the depth of primary dormancy (Kendall and Penfield, 2012), but its expression in Arabidopsis does not seem to determine the pattern of dormancy cycling in the soil seed bank (Footitt et al., 2013). Indeed, Footitt et al. (2020) demonstrated that seedling emergence timing is not directly controlled by the amount of *DOG1* or concentration of ABA, but through the ratio of *DOG1* to negative regulators of ABA sensitivity (e.g. *AHG1*, *PDF1*) (Footitt et al., 2011, 2020).

Evidence for 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. With respect to histone acetylation in seeds, different expression patterns of histone acetyltransferases and deacetylases were found between dormant and non-dormant seeds of Arabidopsis 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 showed that histone acetylation is involved in seed dormancy. For example, 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 with 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).

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. The *snl1 snl2-1* double mutant exhibited decreased dormancy and showed increased expression of genes involved in ethylene biosynthesis (like *ACO1*, *ACO4* or *ERF105*) and downstream ethylene-responsive genes (*B-1,3-GLUCANASE* and *EXPANSINS*) (Wang et al., 2013). Moreover, enhanced levels of *SNL1* and *SNL2* inhibited ABA hydrolysis and promoted its synthesis by histone deacetylation of certain target genes (Wang et al., 2013). In addition, Wang et al. (2016) discovered the regulation of radicle promotion and early growth in a manner dependent on *AUX1*, with *SNLs* involved in histone deacetylation of *AUX1* H3K9K18ac and repression of *AUX1* expression. After-ripened double *snl1 snl2-1* mutant seeds presented accelerated radicle protrusion and growth and increased transcript levels of a high number of auxin-related genes. Furthermore, *CYCLIN D-TYPE (CYCD) 1;1* and *CYCD 4;1*, which are involved in cell cycling and seed germination, showed an important role in radicle promotion and growth downstream of *AUX1*, *SNL1* and *SNL2*. With all these results, a final model was proposed in which the complexes associated with *SNL* proteins play an essential role in the establishment of primary seed dormancy and the regulation of germination in Arabidopsis seeds.

With respect to histone methylation, its implication 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). Sites of H3K9 methylation recruit DNA methyltransferases CMT3 and CMT2, forming a self-reinforcing loop of repressive epigenetic marks (Molitor et al., 2014). The seeds of *SUVH4* mutants had increased expression of *DOG1* and dormancy-associated genes (Liu et al., 2007), which indicates that H3K9me₂ caused by *KYP/SUVH4* induces their silencing through DNA methylation (Katsuya-Gaviria et al., 2020). In addition, PICKLE (PKL) is a chromatin-remodelling factor that affects the levels of H3K27me₃ and plays essential roles in regulating various developmental processes and environmental responses, including embryonic development, root meristem activity, photomorphogenesis, and thermomorphogenesis (Zhang et al., 2014; Zha et al., 2020). Mutants with a loss or reduced function of *PKL* showed increased seed dormancy as *PKL* inhibits *DOG1* transcription (Katsuya-Gaviria et al., 2020; Zha et al., 2020). Zhao et al. (2015) discovered that LYSINESPECIFIC DEMETHYLASE LIKE 1 and 2 (*LDL1* and *LDL2*) act redundantly in repressing seed primary dormancy. The *ldl1 ldl2* double mutant displayed increased seed dormancy, whereas overexpression of *LDL1* or *LDL2* caused reduced primary dormancy.

The POLYCOMB REPRESSIVE COMPLEX 2 (PRC2) is responsible for the deposition of H3K27me₃, which regulates major phase transitions in plant development such as the switch from embryonic to vegetative growth (Bouyer et al., 2011; Müller et al., 2012; Engelhorn et al., 2014). In Arabidopsis, there are 12 homologs of the Drosophila PRC2 subunits and, in particular, the histone methyltransferase EZ is encoded by the three homologs CURLY LEAF (CLF), MEDEA (MEA) and SWINGER (SWN). Different combinations of the four subunits result in three different PRC2-like complexes (Ruta et al., 2019). Mutants in *FERTILIZATION INDEPENDENT ENDOSPERM (FIE)*, which is an essential component of the PRC2, display a genome-wide abolition of H3K27me₃ and exhibit increased primary seed dormancy and germination defects (Bouyer et al., 2011). The *LAFL* genes are epigenetically repressed by the E3 H2A monoubiquitin ligase activity of PRC1 and by the H3K27me₃ activity of PRC2 (Carbonero et al., 2017). The canonical role of PRC1 is to recognize the H3K27me₃ marks and confer chromatin compaction (Lepiniec et al., 2018). *PRC1* mutants also exhibit delays in both germination and transcriptional repression, with a delayed switch in chromatin from the H3K4me₃-associated active to the H3K27me₃-associated repressive transcription state of *DOG1* and seed development genes (Molitor et al., 2014). Moreover, *ARABIDOPSIS TRITHORAX 4 (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).

The involvement of DNA methylation in dormancy has also been shown by different investigations. Research in cereals has implicated the RNA-directed DNA methylation pathway (RdDM) in silencing genes linked to seed dormancy (Katsuya-Gaviria et al., 2020). For example, the gene *AGO1003*, which is an *AGO4_9* class of ARGONAUTE, is differentially expressed in the embryos of primary dormant and non-dormant grains (Singh and Singh, 2012). In addition, DNA demethylation driven by the DNA glycosylase *REPRESSOR OF SILENCING 1 (ROS1)* regulates seed dormancy and the response to ABA by controlling the expression of *DOG1-LIKE 4 (DOGL4)* (Zhu et al.,

2018). However, the presence of specific DNA methylation markers associated with dormancy or germination transcriptomes remains to be elucidated (Matilla, 2020).

Additional epigenetic regulators of primary dormancy have been identified through mutant screens, such as *HISTONE MONO UBIQUITINATION 1 (HUB1)* and *HUB2*, which encode two C3HC4 RING finger proteins with homology to the histone-modifying enzymes BRE1 in yeast and RNF20/RNF40 in humans (Liu et al., 2007). *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 lower primary dormancy through decreased expression of genes related to ABA metabolism and response, such as *NCED9* and *ABI4*, and also reduced ABA levels (Peeters et al., 2002).

Finally, *REDUCED DORMANCY 2 (RDO2)* is related to the POLYMERASE II-ASSOCIATED FACTOR 1 C (PAF1C) and encodes the TRANSCRIPTION ELONGATION FACTOR S-II (TFIIS). Mutations in *RDO2* and other PAF1C associated factors, such as *VERNALIZATION INDEPENDENT 4 (VIP4)*, *VIP5*, *EARLY FLOWERING 7 (ELF7)*, *ELF8* and *ARABIDOPSIS TRITHORAX-RELATED 7 (ATR7)*, 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 transcriptome analysis performed as part of our study aimed to elucidate whether (epigenetic regulatory) genes identified in primary and secondary dormancy research to date also play a role in secondary dormancy induction and depth of *C. bursa-pastoris*.

Materials and methods

Germination and secondary dormancy induction in the presence or absence of histone deacetylase inhibitors

For the first aim of our study, *C. bursa-pastoris* seeds from nine James Hutton Institute (then, 'Scottish Crop Research Institute') accessions (SCRI -156, -177, -367, -416, -469, -707, -773, -799 and -937: Champion et al., 2003; Iannetta et al., 2007; Gomez-Cabellos et al., 2021) were used to assess the effects of different HDAC inhibitors on the induction and maintenance of secondary dormancy. Seeds of all accessions were imbibed in either 1.5 ml of 10 mM valproic acid (control: water) or 33 µM TSA (control: 1:100 DMSO). The osmotic potential (MPa) of all solutions was tested with a Micro-Osmometer (Roebbling Autocal Type 13, Camlab, UK). Incubating conditions for secondary dormancy induction, subsequent germination testing and statistical analysis of the resulting data were previously described (Gomez-Cabellos et al., 2021). Briefly, seeds were incubated in darkness for 0 d (i.e. went directly into light conditions), 1, 2, 3 or 7 d at 30°C. Each treatment had three replicates (50 seeds each) in separate 50 mm Petri dishes on two layers of Whatman no. 1 filter paper soaked with 1.5 ml of the solution tested. Germination (radicle >1 mm) was scored at the end of each dark-incubation period and following transfer to a 12 h photoperiod (30°C). Non-germinated seeds were checked for

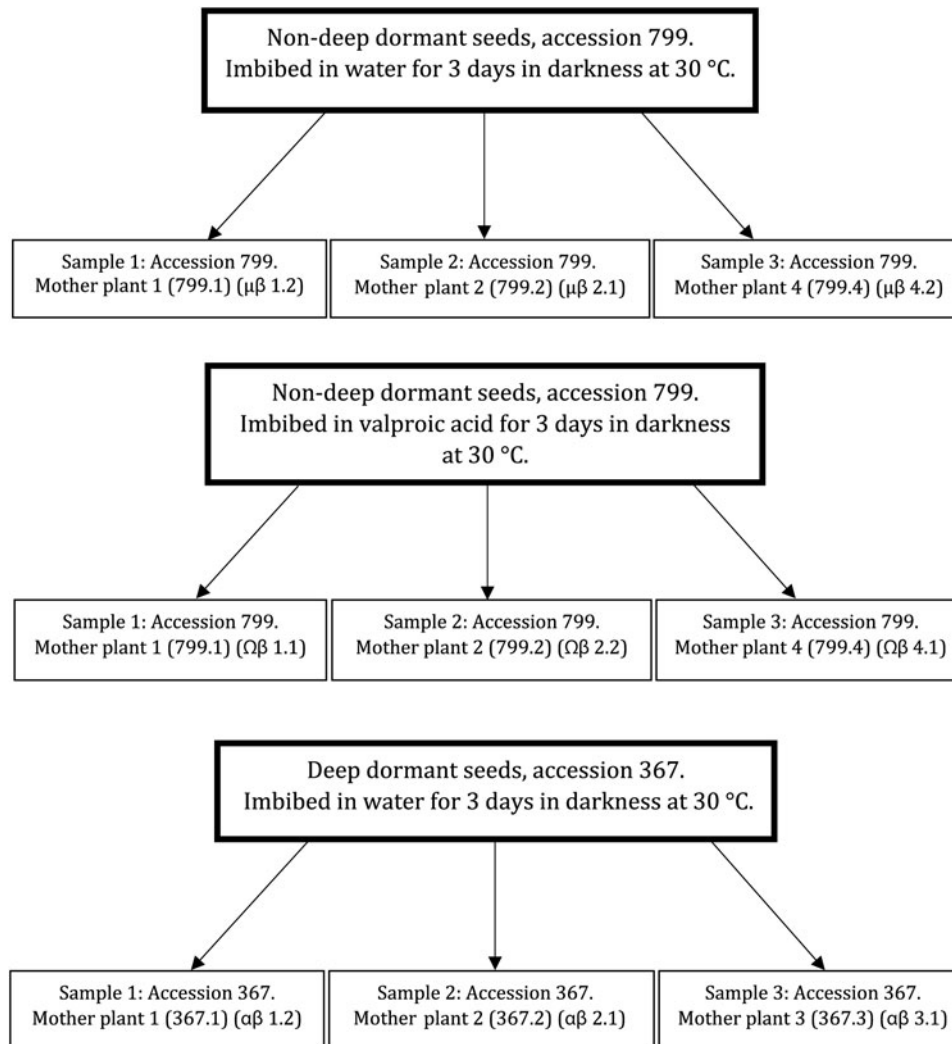


Fig. 1. Schematic representation of the nine samples used for transcriptome sequencing.

viability by after-ripening for 7 d, soaking in 1.5 ml of a 10 mM KNO_3 solution and germination scoring at 25/10°C (12/12 h light/dark). Seed germination was quantified as final germination (%), while times to 50% (of total number of seeds) testa rupture and germination (t_{50} s) were estimated by linear interpolation of the empirical cumulative germination curves. Results were analysed by fitting Generalized Linear Mixed Models (for germination %, with logit link and binomial error) or Linear Mixed Models (for t_{50} s); with compound, darkness (at 30°C), 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. We only used data points for which it was possible to calculate the t_{50} of both testa rupture and germination, that is, Petri dishes in which final germination had been at least 50% (=487 Petri dishes). The total number of Petri dishes was 540 (9 accessions \times 3 dishes/individuals \times 4 compounds tested \times 5 dark incubation times = 540).

Whole seed mRNA extraction and sequencing

For the second aim of our study, RNA was extracted from seeds of two accessions contrasting in secondary dormancy depth

(Gomez-Cabellos *et al.*, 2021) that were imbibed in either water or valproic acid during secondary dormancy induction (Fig. 1). Mother plants 367.1, 367.2, 367.3, 799.1, 799.2 and 799.4 were chosen for RNA extraction based on the individual germination tests (Supplementary Data S1). After 3 d of imbibition in darkness at 30°C in water or in valproic acid, seeds wrapped in aluminium foil were opened under a green safe light in a dark room. At this point, any germinated seeds were removed. For each extraction, 50–100 mg of seeds were weighed and immediately frozen and ground to a fine powder in liquid nitrogen with a mortar and pestle. The homogenized material was incubated with 1 ml of lysis extraction buffer AP1 (QIAGEN) at 56°C for 10 min. The sample was cooled down and mixed with 425 μl of potassium acetate (3 M $\text{C}_2\text{H}_3\text{KO}_2$) by vortex. After incubation on ice for 20 min, the solution was centrifuged at 22,000g for 5 min at 4°C. The supernatant was transferred to a new collection tube and the previous step repeated. To the solution, 450 μl of lysis extraction buffer RLT (QIAGEN) and 5% β -mercaptoethanol were added and the RNase Plant Mini Kit (QIAGEN) protocol was followed according to the manufacturer's instructions. Two samples of RNA from each mother plant were extracted, with each mother plant considered a biological replicate and the two samples per

mother plant technical replicates. The technical replicate from each mother plant that showed the best quality based on agarose gel assessment was used for sequencing (Fig. 1). The amount of RNA in each sample was quantified ($\geq 1 \mu\text{g}$ total RNA per sample) and its quality further analysed via RNA integrity number (RIN: ≥ 7 for each sample).

The isolation of mRNA and library construction of cDNA molecules was carried out by STAB VIDA (Caparica, Portugal) using a Kapa Stranded mRNA Library Preparation Kit (poly-A selected) and the generated DNA fragments were sequenced on an Illumina HiSeq 4000 platform, using 150 bp paired-end sequencing reads. The RNA-Seq raw reads were processed using CLC Genomics Workbench 11.0.1 and the bioinformatics analysis started with trimming of raw sequences to ensure the generation of high-quality data. 77.11–88.92% of the resulting paired high-quality reads were mapped against the reference genome of *C. bursa-pastoris* [GenBank assembly accession GCA_001974645.1 (Kasianov et al., 2017)] using the following parameters: length fraction = 8; similarity fraction = 8. The result of the mapping served to determine the gene expression levels based on the Transcripts per Million (TPM) method, which is a variation of the commonly used Reads per Kilobase of exon model per Million (RPKM).

mRNA sequencing data analysis: differential expression, annotation and GO-term abundance

A multi-factorial statistical tool based on a negative binomial model was used for the analysis of the differential expression of RNA-seq, using a Generalized Linear Model (GLM) approach influenced by the EdgeR method. Differential expression analysis was performed comparing (1) deep dormant accession -367 imbibed in darkness for 3 d at 30°C in water *versus* non-deep dormant accession -799 imbibed in darkness for 3 d at 30°C in water (D water *vs* ND water) and (2) non-deep dormant accession -799 imbibed in darkness for 3 d at 30°C in valproic acid *versus* non-deep dormant accession -799 imbibed in darkness for 3 d at 30°C in water (ND valproic acid *vs* ND water). Replicate sample 3 from the non-deep dormant accession -799 imbibed in water for 3 d was not included in this analysis as it clustered with the non-deep dormant samples imbibed in valproic acid and was considered an outlying sample by STAB VIDA (PCA: Supplementary Fig. S1). Those genes with a fold change $\geq |1|$ and a false discovery rate (FDR)-adjusted *P*-value < 0.05 were considered as statistically differentially expressed (differentially expressed genes, DEGs).

For functional annotation, the full set of 52,597 *C. bursa-pastoris* gene sequences was analysed with OmicsBox version 1.2.4 (BioBam Spain, <https://www.biobam.com/omicsbox/>) (Götz et al., 2008). *C. bursa-pastoris* sequences were used as queries in a BlastX search launched via CloudBlast. The Blast search was run against the non-redundant (nr) reference protein sequences database with normal speed BlastX, an expectation value (e-value) threshold of 1.0×10^{-3} , keeping the top 20 alignments for each sequence and a minimal alignment length (HSP length) cut-off of 33. In some cases, several *C. bursa-pastoris* sequences shared the same Blast result, possibly due to multiple gene copies (with sequence variation between the duplicated genes).

Following the first step, Gene Ontology (GO) mapping allowed the retrieval of the functional information for all of the Blast Hits, obtaining a set of GO candidate annotation terms for each shepherd's purse query sequence. Default weights of the evidence codes were used. The annotation algorithm in the Blast2GO

module selected GO-terms from the pool of candidate GOs obtained by the previous mapping step and assigned them to the query sequences (Conesa and Götz, 2008; Götz et al., 2008). The Blast2GO annotation module applies an annotation rule on the found ontology terms in order to find the most specific annotations with a certain level of reliability (Conesa and Götz, 2008; Götz et al., 2008). The default values of Blast2GO annotation parameters were chosen (e-value Hit filter of 1.0×10^{-6} ; annotation score of 55 as cut-off value; GO-weight of 5 to mapped children terms).

Afterwards, an InterPro domain and motif search were performed via CloudInterProScan (implemented in OmicsBox software version 1.2.4) with the default parameters. The identified domains and motifs were directly translated into GO-terms and this information was combined with the previous Blast searches. Finally, eggNOG mapper was run against the sequences followed by GO-enzyme code (both implemented in OmicsBox software version 1.2.4) and the results were merged with the previous data providing only one integrated functional annotation result. The possible parent-child relationships that had originated from the merging process were removed.

OmicsBox software version 1.2.4 was used for analysis of GO-term abundance. Only functionally annotated sequences from the DEGs and the whole genome were used to carry out the analysis. The GO-terms of the different up- and down-regulated subsets of annotated DEGs (water *vs* ND water and ND valproic acid *vs* ND water) were compared against those of the whole annotated genome using a Fisher's Exact Test with Multiple Test Correction of FDR at the significance threshold of < 0.05 . The setting of $FDR < 0.05$ is in general 1000 times more stringent than the $P\text{-value} \leq 0.05$ (Chang and Scharfenstein, 2014). FDR is used to control the expected proportion of incorrectly rejected null hypothesis, with a Benjamin-Hochberg correction. The results of the analysis show over- and under-represented GO biological process, cellular compartment, and molecular function categories for the different subsets of DEGs (Supplementary Data S5).

Results and discussion

The effect of histone deacetylase inhibitors on secondary seed dormancy induction and germination

A broad range in secondary seed dormancy potential was previously observed among the nine *C. bursa-pastoris* ecotypes examined, with accessions -367 and -799 showing the deepest and shallowest secondary dormancy, respectively (Gomez-Cabellos et al., 2021). For the current study, we tested the effect of two HDAC inhibitors (TSA and valproic acid) on secondary seed dormancy induction and germination of the nine genotypes. The final mean germination percentages in response to the different compounds and times of incubation in darkness at 30°C are shown in Fig. 2. Accessions -367 and -799 showed the lowest and highest final germination across treatments (Fig. 3A), while accessions -156 and -469 germinated the fastest (Fig. 3B).

In general terms, darkness and 30°C had a strongly significant and almost linear effect on final germination. The longer the period in darkness at 30°C, the lower the subsequent final germination percentages across all treatments of all the accessions studied (Fig. 3E). The period of time needed for 50% of the total number of seeds to germinate is denominated t50. If we compare the germination t50s, the longer the period of incubation

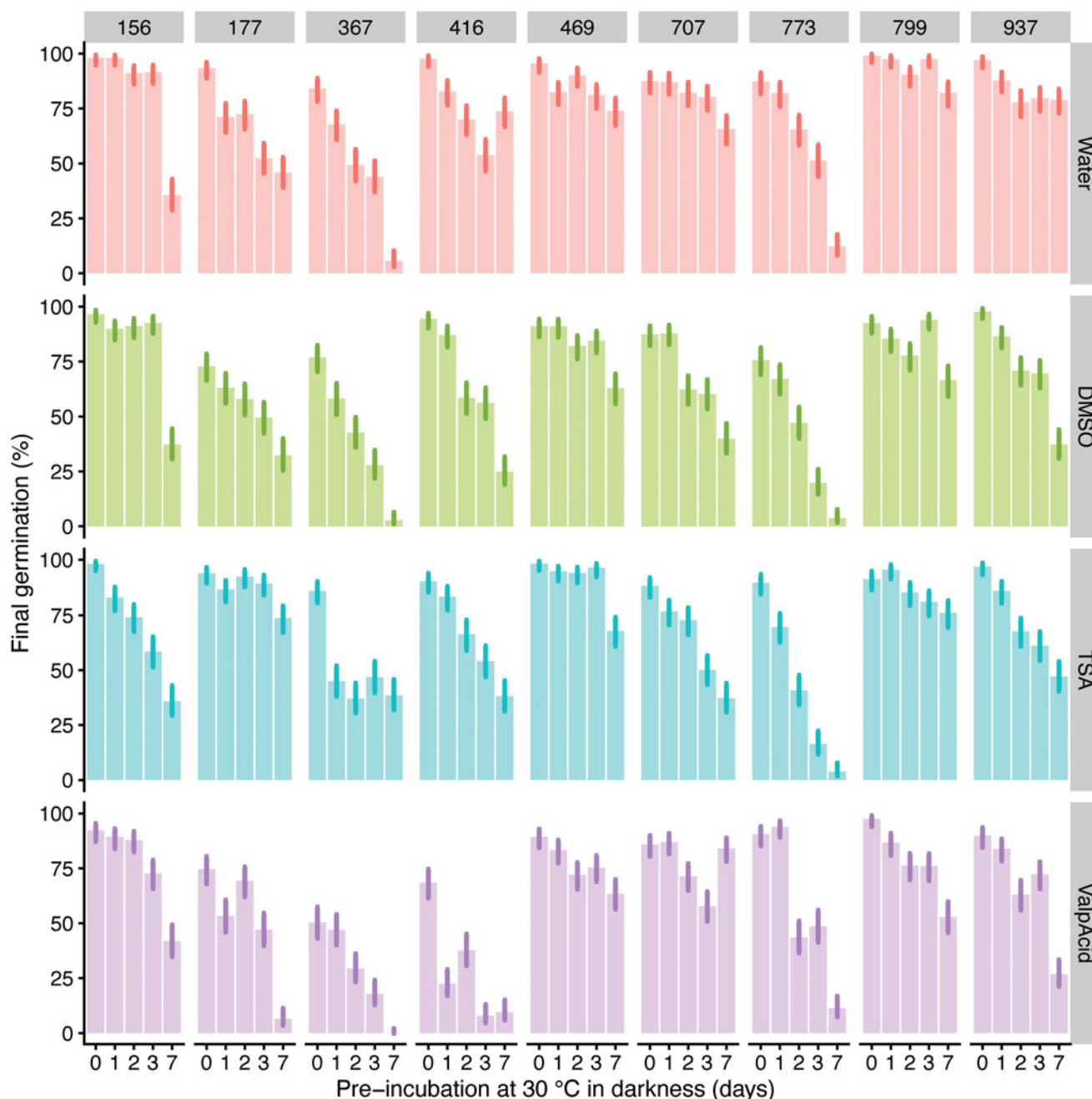


Fig. 2. Final mean germination percentages of seeds from all the accessions (SCRI -156, -177, -367, -416, -469, -707, -773, -799 and -937) of *C. bursa-pastoris* studied in the different compounds (water, DMSO, TSA and valproic acid) and times of incubation in darkness at 30°C tested (0, 1, 2, 3 and 7 d), after being exposed to germination-promoting conditions. Bars represent means and brackets the 95% binomial confidence interval. The most frequent number of viable seeds per condition tested was 200 ($4 \times 50 = 200$).

in darkness at 30°C, the longer the time for 50% of seeds to germinate, across all treatments and accessions (Fig. 3F). These results indicate that with longer incubation periods in darkness at 30°C, both final germination and germination speed are significantly reduced.

All the compounds, including the control for TSA (DMSO), reduced the final germination percentages in comparison with water significantly (Figs 3C and 4A). TSA dissolved in DMSO showed higher final germination than DMSO by itself (Figs 3C and 4A), while valproic acid seemed to cause a deeper secondary dormancy [i.e. lower final germination in comparison

with its control (water); Figs 3C and 4A], which was contrary to our hypothesis that blocking deacetylation would reduce secondary dormancy induction. Valproic acid and TSA had no significant interaction with darkness at 30°C (Fig. 4A), meaning that the negative response to darkness at 30°C seen in water was not affected by the presence of these compounds. However, DMSO (used as a control for TSA) was more negatively affected by darkness at 30°C than water ($P < 0.001$; Fig. 4A).

Analysing the germination t50s according to compound (vs water), significant effects were observed for valproic acid and

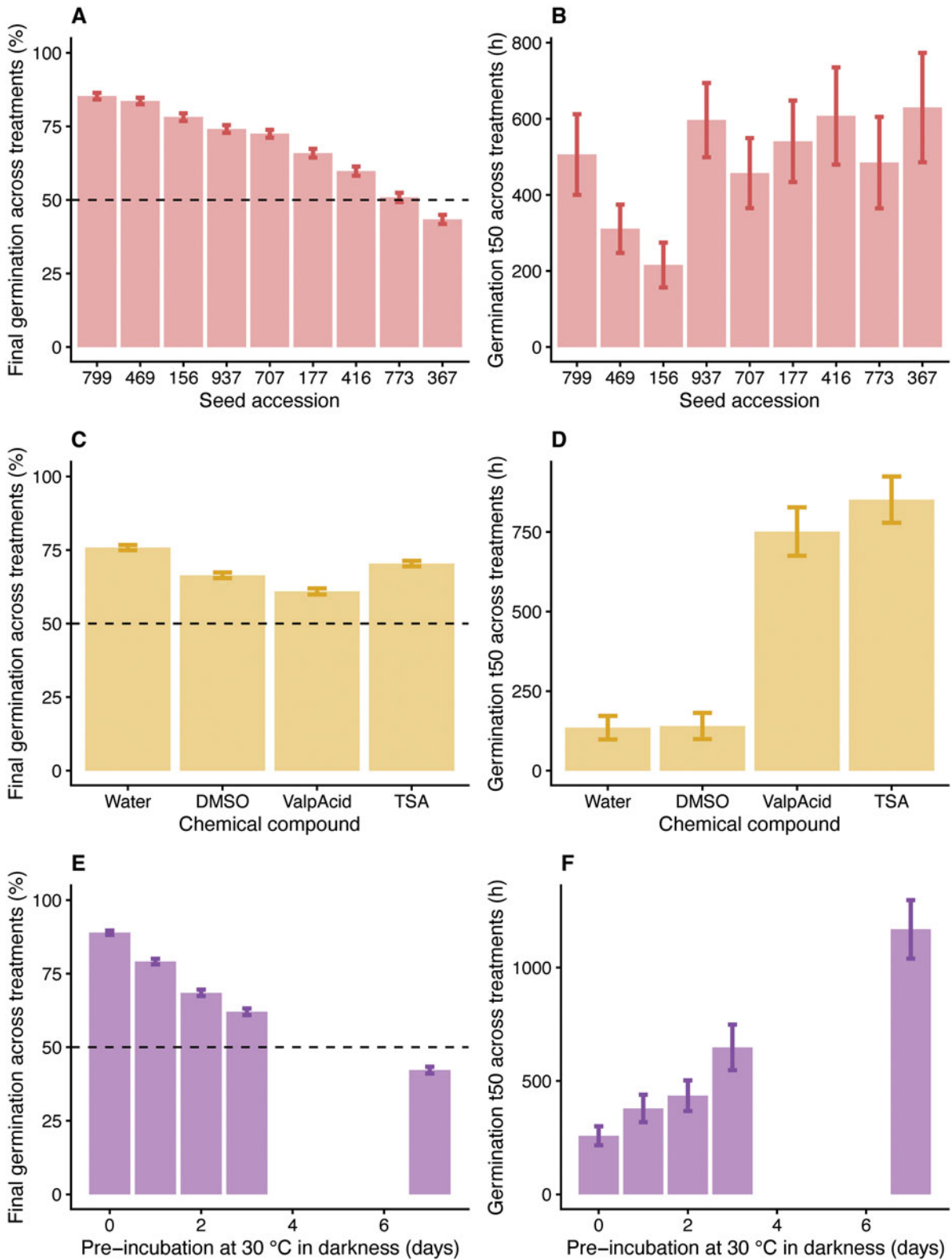


Fig. 3. Generalized Linear Model (GLM) fitted to the data, taking all the accessions analysed (SCRI -156, -177, -367, -416, -469, -707, -773, -799 and -937). (A) Final mean germination percentages (%) across all treatments studied in relation to the accession analysed. (B) t50s in hours to germination 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 hours 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 at 30°C. (F) t50 for germination in hours for all the accessions and compounds used in relation to the period of incubation in darkness at 30°C. For (A, C and E), brackets represent the 95% binomial confidence interval and for (B, D and F), brackets represent standard errors.

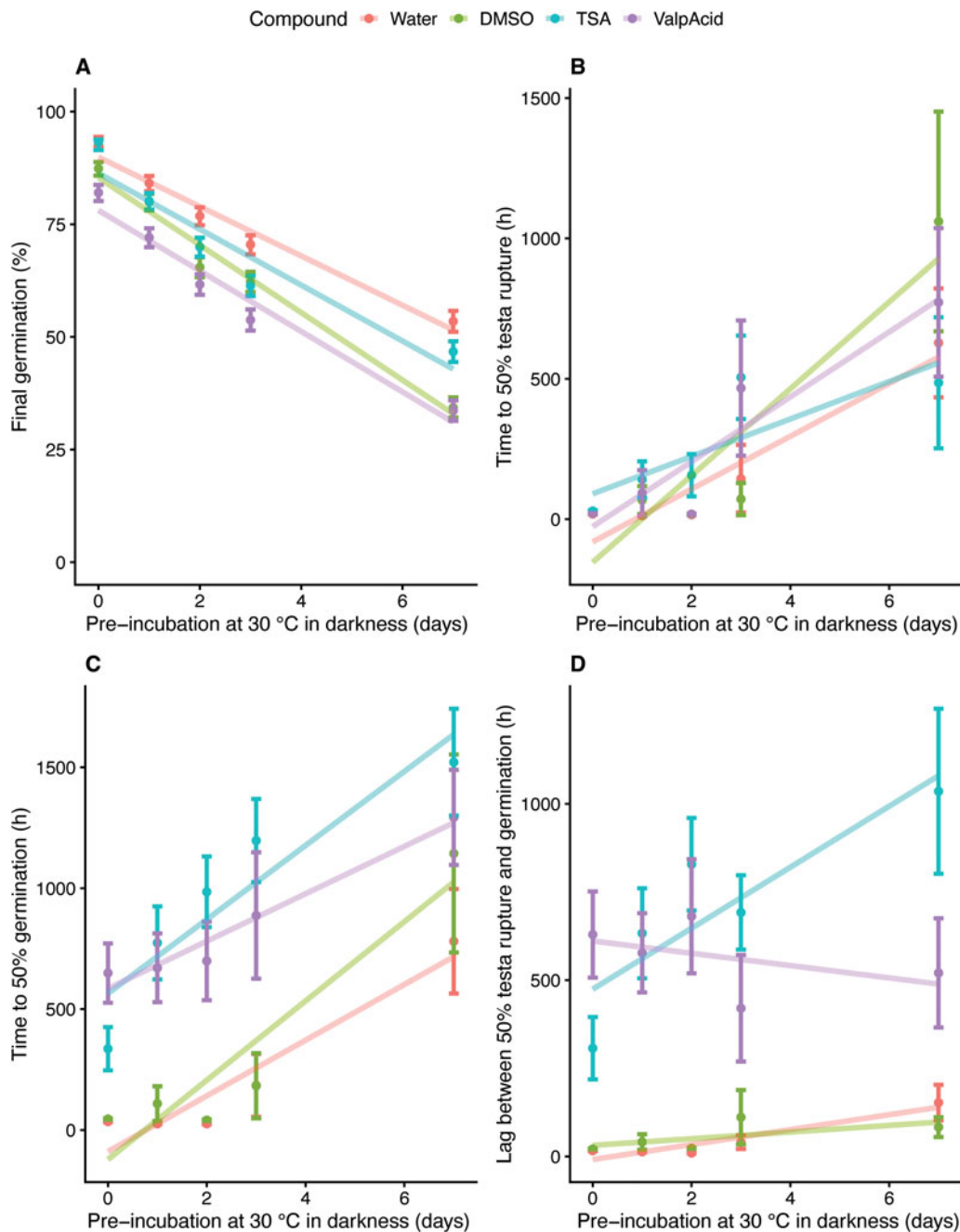


Fig. 4. Generalized Linear Model (GLM) fitted to the data, taking all the accessions analysed (SCRI -156, -177, -367, -416, -469, -707, -773, -799 and -937). (A) Final mean germination percentages (%) in relation to the period of incubation in darkness at 30°C and separated by the compound used. (B) t50s in hours to testa rupture in relation to the period of incubation in darkness at 30°C and separated by the compound used. (C) t50s in hours to germination in relation to the period of incubation in darkness at 30°C 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.

TSA ($P < 0.001$; $P < 0.001$), but not for DMSO (Figs 3D and 4C), revealing that both valproic acid and TSA reduced the speed of germination significantly compared with their controls. Testa rupture was also measured, with none of the compounds significantly affecting t50s for testa rupture in comparison with water (Fig. 4B). Therefore, valproic acid and TSA had a strongly significant retarding effect on the germination speed (germination t50s) but did not affect the speed of testa rupture (testa rupture t50s) (Figs 3D and 4B, C). The differences between germination t50s and testa rupture t50s in relation to the compounds are

represented in Fig. 4D. The osmotic potential of all solutions was 0 MPa, discarding an osmotic potential effect rather than the results being due to the compounds' inhibitory characteristics. The viability of the non-germinated seeds, after drying and treatment with KNO_3 , is represented in Supplementary Fig. S2, with 97% being the lowest viability. Application of exogenous nitrate alleviates seed dormancy and stimulates germination through transcriptional changes of several genes involved in ABA (e.g. *CYP707A2*) and GA metabolism and sensitivity (Matilla *et al.*, 2015; Sano and Marion-Poll, 2021).

In this study, TSA and valproic acid caused a higher t50 for germination but the speed of testa rupture (t50 for testa rupture) was not affected (Figs 3D and 4B, C). 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). Our results indicate that, contrary to our hypothesis, exposure to HDAC inhibitors slowed germination speed (higher t50 for radicle emergence) of the non-dormant seeds (Figs 3D and 4C), while valproic acid also increased secondary dormancy depth (lower final germination) (Figs 3C and 4A).

Several reports on germination of non-dormant seeds have shown inhibiting effects of TSA. For example, Tanaka et al. (2008) exposed non-dormant *Arabidopsis* seeds to 5–50 μ M TSA and found a delay in germination in comparison with the control after 3 d of sowing. Zhang et al. (2011) applied 1–50 μ M TSA to non-dormant *Zea mays* L. (maize) seeds, observing lower germination rates in seeds imbibed in TSA compared with seeds imbibed in the control (a DMSO control was not indicated). However, Nelson et al. (2017) rescued germination of dormant and gibberellic acid (GA) insensitive *sly1-2* mutant seeds with 2 μ M TSA, while 4 and 6 μ M led to decreasing germination capacity.

The probable involvement 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 increased 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*, *LEC2*, *FUS3* or *ABI3*, hence promoting a delay of germination (Carbonero et al., 2017; Lepiniec et al., 2018). In addition, we hypothesized that enhanced secondary dormancy and delay in germination observed in seeds treated with HDAC inhibitors could involve mis-regulation of genes related to hormone (ABA, GAs) biosynthesis or signalling pathways (Lepiniec et al., 2018).

At the same time, these results could indicate that acetylation/deacetylation of histones plays a role 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 was unveiled by Wang et al. (2016), who demonstrated the negative regulation of radicle promotion and early growth of *Arabidopsis* seeds by PAIRED AMPHIPATHIC HELIX SWI-INDEPENDENT3 (SIN3)-LIKE (SNL) 1 and SNL2 in a manner dependent on AUXIN RESISTANT 1 (AUX1). SNL1 and SNL2 can act as components of a HDAC-SNL complex capable of modulating the transcription of genes through histone deacetylation (Wang et al., 2013). Thus, we could hypothesize that HDAC inhibitors, such as TSA and valproic acid, act on histone deacetylases that are implicated in the regulation of auxin pathways and signals, which at the same time regulate specific steps in the seed germination process.

(Epigenetic) gene expression associated with exposure to histone deacetylase inhibitors during secondary seed dormancy induction

In order to test the hypotheses mentioned in the section ‘The effect of histone deacetylase inhibitors on secondary seed

dormancy induction and germination’, we compared gene expression of a non-deep dormant accession (-799) exposed to either an HDAC inhibitor or a control. Valproic acid instead of TSA was used to treat the seeds as it caused a deeper secondary dormancy and stronger delay of germination in all the accessions and does not need to be dissolved in DMSO. In the case of the ND valproic acid *versus* ND water comparison, the number of genes differentially expressed was 393, of which 122 were up-regulated and 271 down-regulated (Table 1). The DEGs highlighted below are listed in Supplementary Data S2 and S3. GO-term abundance analysis was also performed, with results presented in Supplementary Data S4 and S5.

Our first hypothesis regarding the delay in germination caused by valproic acid involved a positive regulation of embryo-specific transcription factors implicated in the maintenance of embryonic properties. However, the results showed no significant differential expression of genes such as *LEC1*, *LEC2*, *FUS3* or *ABI3*, despite the up-regulation of *CHROMATIN REMODELING 5 (CHR5)* (1.62) (Shen et al., 2015). We, therefore, assessed the possibility that mis-regulation of phytohormone genes could underlie increased secondary dormancy depth and germination delay. The phytohormone ABA has been found to play a key role in the regulation of seed dormancy and germination. It can inhibit germination and its accumulation correlates with the onset of primary dormancy (Tuan et al., 2018). In the up-regulated DEGs, only phytohormone GOs related to ABA transmembrane transport were enriched. Overall, several positive regulators of ABA were down-regulated, including *PYL4* (-2.02), which is a receptor of ABA required for ABA-mediated response that is crucial for seed germination (Wang et al., 2020). Another example is *ABI5* (-4.05), which is essential to execute an ABA-dependent growth arrest that sets in after breakage of seed dormancy but prior to autotrophic growth (Lopez-Molina et al., 2001, 2002). In accordance with this, a sequence implicated in the degradation of *ABI5* was up-regulated: E3 ubiquitin-protein ligase KEEP ON GOING (KEG) (1.33) (Liu and Stone, 2010). UDP-GLUCOSYLTRANSFERASE 75B1 (*UGT75B1*) (1.88), which is the main enzyme responsible for pABA-Glc formation in *Arabidopsis*, was also up-regulated. ABA can be transformed into inactive forms by glycosylation, which is a flexible way of maintaining ABA homeostasis, and this is catalyzed by the plant family of UDP-GLYCOSYLTRANSFERASES (UGTs). Overexpression of *UGT75B1* leads to overproduction of ABA-Glc in *Arabidopsis* and lowers the active ABA levels, allowing seed germination and seedling greening (Chen et al., 2020). However, we also observed up-regulation of genes implicated in positive signalling of ABA [*SNL1* (1.39) (Wang et al., 2013, 2016)] and down-regulation of genes implicated in negative signalling, such as *WRKY29* (Zhou et al., 2020) (-7.94), *WRKY18-LIKE* (-1.89) and *WRKY60* (-1.69).

The enriched GOs related to phytohormones in the down-regulated DEGs were linked to cytokinin and indoleacetic acid, such as the GO-term ‘indoleacetic acid biosynthesis’. Analysing auxin-related sequences within the up-regulated DEGs, there was one gene implicated in auxins efflux: the protein BIG (1.63), which is involved in cell elongation, lateral root promotion and general growth and development (Kanyuka et al., 2003). Nonetheless, *TRYPTOPHAN N-MONOOXYGENASE 2 (CYP79B3)*, which catalyzes the first step of IAA biosynthesis (Wang et al., 2016), presented a down-regulation of -2.29-fold. In addition, *SNL1* (1.39), which is implicated in the repression of sequences related to the biosynthesis of auxins (Wang et al.,

Table 1. Number of DEGs between conditions in *C. bursa-pastoris* seeds

Differentially Expressed Genes (DEGs)	DEGs FDR $P \leq 0.05$	DEGs Up	DEGs Down	DEGs Annotated Up	DEGs Annotated Down
D water vs ND water	6,337	2,727	3,610	2,414	3,317
ND valproic acid vs ND water	393	122	271	110	244

Total number of DEGs between comparison pairs, significantly up-regulated and down-regulated and final number of annotated genes.

2016), was up-regulated. We could hypothesize that, due to the up-regulation of *SNL1*, there may be no up-regulation of auxins biosynthesis genes. The reason behind the up-regulation of this histone deacetylase when seeds were treated with valproic acid could potentially be a compensation for the inhibition of deacetylation provoked by the exogenously applied compound.

Although no enrichment of GOs related to GAs were found, genes such as *GA20ox1* (-8.24 and -4.17) and *GIBBERELLIN-REGULATED-LIKE PROTEIN* (-331.29, -91.56, -88.50 and -17.47) were found in the down-regulated list of DEGs. GAs are known to stimulate seed germination in a wide range of plant species (Tuan et al., 2018). The same situation was found for BRs, with genes implicated in their biosynthesis or response within the down-regulated DEGs, such as *CYTOCHROME P450 CYP708A2* (-32.09 and -7.62), *EXORDIUM-LIKE 1 (EXL1)* (-7.19), *EXORDIUM-LIKE 5 (EXL5)* (-2.44) or *EXORDIUM-LIKE (EXO)* (-2.68). BRs are known for promoting seed germination and both BRs and GAs induce the expression of cell elongation-associated genes such as distinct expansin family members (Finkelstein et al., 2008).

Altogether, it could be said that the hyper-acetylation caused by the use of valproic acid provoked alterations in the expression of genes implicated in the biosynthesis and signalling pathways of different phytohormones. As mentioned above, ABA signalling gene expression was both repressed and stimulated in seeds treated with valproic acid. However, genes related to synthesis of GAs, BRs and auxins were down-regulated (Table 2), which might explain the slowing of germination (higher t50) in response to valproic acid.

With respect to epigenetic regulation and chromatin remodelling, GOs related to histone acetylation and histone acetyltransferase complexes were within the enriched terms for the up-regulated DEGs, including 'chromatin remodelling', 'lysine-acetylated histone binding' and 'acetylation-dependent protein binding'. However, the histone deacetylases *SNL1* (1.39) and *SNL4* (1.34) were also up-regulated. These results seem to be contradictory but could be indicating that seeds are trying to stabilize histone acetylation levels. In addition, the up-regulation of *SNL1* might help explain the increase in secondary dormancy caused by valproic acid exposure.

Regarding methylation, we observed both up-regulation of genes involved in demethylation [*LYSINE-SPECIFIC DEMETHYLASE JMJ18* (5.87)] as well as methylation [*HISTONE-LYSINE N-METHYLTRANSFERASE ATXR3-LIKE* (1.36)]. Finally, the DNA helicase *INO80-LIKE* was also up-regulated (1.34). *INO80* is a chromatin remodelling complex that modulates, together with *SWRI*, the incorporation of H2A.Z in nucleosomes. The deposition of this histone variant at gene bodies is associated with lower transcription levels (Wang et al., 2019). Overall, the genes involved in epigenetic regulation of transcription show a dynamic pattern of expression in response to valproic acid.

Expression of (epigenetic regulatory) genes associated with secondary seed dormancy depth

Taking two ecotypes that presented extreme responses to the induction of secondary dormancy in water (-367 and -799), a transcriptome analysis was performed. The number of genes with statistically significant differences (fold change $\geq |1|$ and FDR P -value < 0.05) in expression between D water versus ND water was 6,337, of which 2,727 were up-regulated and 3,610 down-regulated (Table 1; Supplementary Data S3). The focus of the results and discussion is on DEGs with GO terms related to dormancy, phytohormones, as well epigenetic processes that could potentially explain differences in secondary dormancy depth between the accessions analysed. Given that the two ecotypes contrast in their response to secondary seed dormancy induction, the results may reflect differences in germination potential/status as well as secondary dormancy depth. The full results of the GO analysis are available in Supplementary Data S4 and S5. In addition, manual categorization of DEGs according to function was also performed (Supplementary Data S6).

Dormancy and phytohormones

DOG1 has been described as an essential and necessary protein in the establishment of primary seed dormancy over the last decade (Bentsink et al., 2006; Footitt et al., 2015; Carrillo-Barral et al., 2020). Although *DOG1* was not found to have direct control of secondary dormancy depth in Arabidopsis, Footitt et al. (2020) proposed a model for the regulation of dormancy cycling where a lower *AHG1/ANAC060/PDF1:DOG1* ratio is linked to deeper dormancy/lower germination potential. The results in this study on *C. bursa-pastoris* did not reveal differential expression of *DOG1* (there were no sequences annotated as *DOG1*), while *AHG1 (PROBABLE PROTEIN PHOSPHATASE 2C 75)* and *ANAC060 (NAC DOMAIN-CONTAINING PROTEIN 60 ISOFORM X1)* were more highly expressed (1.36 and 1.41) in the deeper dormant ecotype. Several *DOG1-LIKE* genes were also up-regulated in the deep versus non-deep dormant genotype: *DOG1-LIKE 3* (2.02, 1.74), *DOG1-LIKE 4* (1.54) and *DOG1-LIKE 1* (1.51). Ectopic expression of wheat and barley *DOG1-LIKE* genes promoted seed dormancy in Arabidopsis (Ashikawa et al., 2010). *DOG1-LIKE 3 (DOGL3)* is capable, like *DOG1*, of binding to *AHG1 PP2C*, thereby playing a similar role to *DOG1* in ABA sensitivity and dormancy enhancement (Nonogaki et al., 2020). For example, the *OsDOG1-LIKE 3* gene was found to up-regulate ABA biosynthesis and signalling-related genes, suggesting that its promotion of primary seed dormancy likely occurs by enhancing the ABA pathway (Wang et al., 2020). However, *DOGL4* is a negative regulator of primary seed dormancy and the ABA response, with mutations in *DOGL4* enhancing dormancy (Zhu et al., 2018; Katsuya-Gaviria et al., 2020).

Table 2. DEGs potentially explaining the observed delay in t50 for germination in *C. bursa-pastoris* seeds treated with HDAC inhibitors

DEGs	Max group mean TPM	Log ₂ fold change	Fold change	FDR P-value	Sequence description
Cbp51055	2.46	-2.06	-4.18	1.05 × 10 ⁻⁰²	Gibberellin 20 oxidase (GA20ox1)
Cbp5936	1.25	-3.04	-8.24	3.79 × 10 ⁻⁰⁴	Gibberellin 20 oxidase (GA20ox1)
Cbp4536	0.35	-5.00	-32.10	7.01 × 10 ⁻⁰³	Cytochrome P450, family 708, subfamily A, polypeptide 2 (CYP708A2)
Cbp4529	1.52	-2.93	-7.62	8.50 × 10 ⁻⁰⁵	Cytochrome P450, family 708, subfamily A, polypeptide 2 (CYP708A2)
Cbp48289	2.33	-1.19	-2.29	1.89 × 10 ⁻⁰²	Tryptophan N-monooxygenase 2 (CYP79B3)
Cbp23520	20.84	0.48	1.39	7.08 × 10 ⁻⁰³	Paired amphipathic helix protein Sin3-like 1 isoform X1 (SNL1)

The selected DEGs are from a comparison of a non-deep dormant accession (-799) exposed to an HDAC inhibitor (valproic acid) versus a control (water): the seeds were imbibed in darkness for 3 d at 30°C in water to induce secondary seed dormancy.

A high number of over-represented GO-terms in the down-regulated DEGs were related to water and water transport. Footitt et al. (2019) revealed a role for aquaporins in the induction and relief of secondary seed dormancy. *TIP3-2* was identified as a negative regulator of ABA in Arabidopsis, but a *PROBABLE AQUAPORIN TIP3-2* was expressed more highly in the deeper dormant accession of *C. bursa-pastoris* in this study (1.61 and 1.93). In general, the majority of differentially regulated aquaporins showed higher expression in the less deep dormant accession: aquaporin *TIP2-1* (-439.48, -286.70 and -223.97), aquaporin *TIP1-1* (-221.79 and -199.42), aquaporin *TIP1-2* (-90.19, -49.16 and -35.77), aquaporin *PIP1-3* (-10.90 and -3.81), probable aquaporin *PIP2-5* (-10.38) and probable aquaporin *NIP5-1* (-9.86 and -6.51).

With respect to phytohormones, we found that the number of over-represented GO-terms in the up-regulated DEGs was much lower than in the down-regulated list, with the former presenting only the two GO-terms: 'positive regulation of cytokine production' and 'response to ABA'. The GO-term 'response to ABA' was also found within the enriched GO-terms of the down-regulated genes. Therefore, there are genes implicated in the positive and negative regulation of this hormone within the up- and the down-regulated sequences.

Looking at specific genes within the up-regulated DEGs, some sequences that have been described as important in the positive regulation of ABA pathways and signalling were found: *SERINE/THREONINE-PROTEIN KINASE SRK2A-LIKE* (142.97), *ABC TRANSPORTER G FAMILY MEMBER 40 (ABCG40)* (15.16 and 10.65), *9-CIS-EPOXYCAROTENOID DIOXYGENASE NCED6* (8.51), *NCED2* (1.68 and 1.51), *B3 DOMAIN-CONTAINING TRANSCRIPTION FACTOR ABSCISIC ACID-INSENSITIVE 3 (ABI3)* (2.49) and *ABSCISIC ACID-INSENSITIVE 5-LIKE PROTEIN 7 (ABF4)* (2.28).

NCED6, together with *NCED9*, were shown to be up-regulated in secondary dormant versus non-dormant seeds (Cadman et al., 2006). The same pattern was found for *ABI3*, which presented higher expression in dormant (primary and secondary) compared with non-dormant states (Cadman et al., 2006). *SWI-INDEPENDENT 3 (SIN3)-LIKE (SNL) 1* and *SNL2* are histone deacetylases that have an important function in the regulation of primary seed dormancy (Wang et al., 2013). In research by Wang et al. (2013), enhanced levels of *SNL1* and *SNL2* inhibited ABA hydrolysis and promoted its synthesis by histone deacetylation of certain target genes. In this study, a potential *SNL1*

(Cbp42606: Data S7) was highly up-regulated in the deep dormant accession in comparison with the non-deep dormant one (1633.68). In addition, the protein *CRUCIFERIN CRU1-LIKE* was differentially expressed with a fold change of 2.98. *CRUCIFERIN A1* is a storage protein and a downstream target of ABA found to be related to dormant seeds by Gao et al. (2012).

On the other hand, within the down-regulated DEGs related to ABA, there were mostly negative regulators of its pathways, such as *NINJA-FAMILY PROTEIN AFP4* (-211.51), *RAC-LIKE GTP-BINDING PROTEIN ARAC7* and *ARAC10* (-26.70 and -3.79, respectively), *ZINC FINGER PROTEIN 8* and *3 (ZFP8* and *ZFP3)* (-17.17 and -3.48, respectively), *PROTEIN TERMINAL EAR1 HOMOLOG (ENHANCER OF ABA CO-RECEPTOR 1)* (-13.42), *ABSCISIC ACID 8'-HYDROXYLASE 3 (CYP707A3)*: -4.96) and *1 (CYP707A1)*: -2.21), *ENHANCED DISEASE RESISTANCE 2-LIKE (EDR2-LIKE)* (-6.60 and -2.46) and the key negative regulator *PROTEIN PHOSPHATASE 2C 56 (ABI1)* (-3.41 and -3.30). Nevertheless, there were also down-regulated sequences related to positive responses to ABA such as the previously mentioned *SERINE/THREONINE-PROTEIN KINASE SRK2A-LIKE* (-11.94), *HVA22E* (-10.37 and -4.23), *PHOSPHOINOSITIDE PHOSPHOLIPASE C1 (PLC1)* (-6.56), *ABSCISIC ACID-INSENSITIVE 5-LIKE PROTEIN 1* (-5.98 and -4.75), *ABSCISIC ACID-INSENSITIVE 5-LIKE PROTEIN 6* (-4.69), *ABI4-LIKE* (-4.01 and -2.19), *PYL4* (-2.00) and *F-BOX/KLECH-REPEAT PROTEIN AT3G16740-LIKE (FOA2)* (-505.45 and -471.55). However, the down-regulation of *FOA2* expression might be caused by ABA through a feedback regulation mechanism (He et al., 2016). Although a fivefold higher expression of *PYL4* was found in seeds induced into secondary dormancy compared with an array of primary dormant states (Laspina et al., 2020), our results show no evidence for enhanced expression of *PYL4* being associated with deeper secondary dormancy.

A large part of ABA accumulation in seeds relies on the regulation of the *NCED* gene family as the enzymes they encode carry out the first step in the synthesis of ABA (Nambara et al., 2010). The induction of *NCED6* during imbibition is sufficient to prevent seed germination (Martínez-Andújar et al., 2011). On the other hand, the major catabolic route is via the *ABA 8'-HYDROXYLASE* (Matilla et al., 2015). The up-regulation of *NCED6* and *NCED2* and the down-regulation of *ABSCISIC ACID 8'-HYDROXYLASE 3* might be indicating higher ABA levels in the deep-dormant accession than in the non-deep one. Taking into account all the ABA-related genes of the DEGs, in the up-regulated list, most of them are promoters of its synthesis

or signalling, while in the down-regulated set, the most abundant are repressors or sequences related to its catabolism. However, the number of ABA-related genes with differential expression is more limited than may have been expected, which was also observed in several earlier studies (Cadman et al., 2006; Matilla et al., 2015; Ibarra et al., 2016; Laspina et al., 2020). Footitt et al. (2020) proposed a model in which dormancy cycling is regulated via a negative response to ABA.

With respect to the over-represented GO-terms of the down-regulated DEGs that involve hormones, we observed a high number of GOs related to biosynthesis, signalling pathways or metabolism. For example, changes in the balance of catabolism and synthesis of GAs are necessary for the promotion of germination. Genes such as *GIBBERELLIN 20-OXIDASE 1 (GA20ox1)* (-12.24 and -5.22) and *GIBBERELLIN 20-OXIDASE 2 (GA20ox2)* (-6.22) were within the down-regulated DEGs. These are key players in the biosynthesis of gibberellins, act partially redundantly and are the most highly expressed of the genes implicated in the synthesis of GAs during vegetative and early reproductive development (Rieu et al., 2008; Tuan et al., 2018). The transcription factor *bHLH93*, which is implicated in regulation of flowering time in short days (SD), was also within the down-regulated genes with fold changes of -5.87 and -4.13. Mutants of this gene presented down-regulation of GA biosynthetic genes (*GA3ox1*, *GA3ox2*, *GA20ox1*) and up-regulation of the GA catabolic (*GA2ox2* and *GA2ox7*) and receptor genes in comparison with wild-type plants (Sharma et al., 2016). DEGs related to GA-mediated signalling presented contrasting results. For example, DELLA proteins (repressors of GA responses) showed both up- and down-regulation, involving relatively small fold changes [e.g. DELLA proteins *GAI* (1.48 and 1.39), *RGL2* (-1.43 and -1.91) and *RGL3* (-1.53 and -1.67)]. Although *RGL2* plays an important role in secondary dormancy induction in Arabidopsis as shown by mutant analysis (Ibarra et al., 2016), our results indicate that its expression is lower in the deeper dormant genotype during secondary dormancy induction. In addition, *F-BOX PROTEIN GID2*, a positive regulator of gibberellin signalling through degradation of DELLA proteins [including *RGL2* (Tyler et al., 2004; Yang et al., 2020)], was highly up-regulated (68.91). Loss of *GID2 (SLY1)* results in increased primary seed dormancy (Ariizumi et al., 2011), and therefore, its up-regulation in the deeper secondary dormant accession indicates that this gene may also not be a candidate for explaining differences in secondary dormancy depth.

Overall, the down-regulation of genes implicated in the biosynthesis of GAs in the deep dormant accession in comparison with the non-deep dormant one and the up-regulation of genes implicated in the synthesis or signalling of ABA suggest an active involvement of the ABA/GAs balance in the differences in capacity of induction of secondary seed dormancy found between the accessions. However, a larger number of differentially expressed genes between the accessions, especially those related to ABA biosynthesis and signalling pathways, was expected based on the literature related to primary seed dormancy of the last decades (Finkelstein et al., 2008; Dekkers and Bentsink, 2015; Matilla et al., 2015; Tuan et al., 2018; Sano and Marion-Poll, 2021).

There is abundant evidence that ABA and ethylene play important roles in the regulation of seed dormancy (Finkelstein et al., 2008). Ethylene can promote seed germination and repress seed dormancy establishment as it antagonizes the ABA pathway (Finkelstein et al., 2008; Linkies and Leubner-Metzger, 2012). However, the

ethylene-ABA antagonism during seed dormancy and germination is still poorly understood (Wang et al., 2013). Ethylene's biosynthesis is controlled by the 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID OXIDASE (ACO), which is involved in counteracting the inhibiting effects of ABA on endosperm cap weakening and endosperm rupture (Linkies et al., 2009). The main role of ethylene could be related to the promotion of radial cell expansion in the embryonic hypocotyl, decreasing the seed water potential and increasing the activity of cell wall hydrolases in the endosperm cap (Kucera et al., 2005). In research carried out by Wang et al. (2013), seeds of the *snl1 snl2-1* double mutant (with higher germination than non-mutant Arabidopsis Columbia seeds) presented affected transcription levels of genes regulating the ethylene pathways, including the up-regulation of the genes *ACO1*, *ACO4*, *ETHYLENE-RESPONSIVE TRANSCRIPTION FACTOR (ERF) 105*, *ERF9* and *ERF112*. This pointed to the involvement of *SNL1* and *SNL2* in seed dormancy by repressing ethylene synthesis and response. As mentioned above, great differences in expression of a potential *SNL1* [Cb42606 (1633.68)] were found within our study, a result that will be discussed later on.

Within the over-represented GO-terms of the down-regulated DEGs, some were related to responses and signalling pathways of ethylene. Specific genes related to its synthesis or responses could be found, such as *ETHYLENE RESPONSE FACTORS (ERFs)*. Some of the down-regulated sequences were 1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE 1 (*ACO1*) (-526.98 and -244.23), *ERF4-LIKE* (-1123.97 and -10.10), *ERF109-LIKE* (-14.21), *ERF105* (-12.27 and -4.61), *APETALA 2 (AP2)-LIKE ETHYLENE-RESPONSIVE TRANSCRIPTION FACTOR AIL1* (-4.38 and -1.58), *ETHYLENE-RESPONSIVE TRANSCRIPTION FACTOR CRF1* (-3.51), among others. These results are in accordance with the hypothesis of Paul et al. (2014) and Wang et al. (2013), in which ethylene signalling pathways are important in the regulation of dormancy, and also with previous works that demonstrate that ethylene expression is partially regulated by histone acetylation and deacetylation (Wang et al., 2020).

Aside from GAs and ethylene, the roles of BRs in the promotion of germination by improving growth potential in a GA-independent manner have begun to be elucidated (Leubner-Metzger, 2001; Hao et al., 2017; Xu et al., 2020). They constitute another antagonist of ABA (Kucera et al., 2005). Although no GO-terms related to BRs were within the over-represented GOs, multiple key genes implicated in BRs synthesis, signalling pathways and homeostasis were markedly down-regulated. Some of them could be highlighted, for example those encoding BAHD ACYLTRANSFERASE *BIA1* (-226.23 and -11.17), CYTOCHROME P450 708A2-LIKE (-161.72 and -89.40), PROBABLE WRKY TRANSCRIPTION FACTOR 46 (-25.35 and -6.99), BRASSINOSTEROID INSENSITIVE 1 (*BRI1*)-ASSOCIATED RECEPTOR KINASE 1 (*BAK1*) (-4.47), PROTEIN *BRI1-5 ENHANCED 1 (BEN1)* (-3.97 and -3.78), BRASSINOSTEROID SIGNALLING POSITIVE REGULATOR (*BZR1*) FAMILY PROTEIN (*BES1*) (-3.80 and -3.16) and *BRI1* (-2.00 and -1.95), although a negative regulator was also down-regulated: *BRI1 KINASE INHIBITOR 1-LIKE (BK11)* (-3.22). Most of these genes were previously found to be up-regulated during the germination of peanut seeds (Xu et al., 2020). *BES1* forms a transcriptional repressor complex with *TPL-HDA19*, which directly facilitates the histone deacetylation of *ABI3* chromatin, leading to the transcriptional repression of *ABI3* and consequently *ABI5* (Ryu et al., 2014). *BRI1* has been proposed to play a role in the cold stratification pathway for releasing primary seed

dormancy and triggering germination (Kim et al., 2019). These differences of expression in several genes of the BR signalling pathways are consistent with a lower dormancy/higher germination capability of the non-deep accession in comparison with the deep dormant one.

Overall, auxins were the phytohormones with the greatest number of associated GO-terms. They are hormones that can regulate plant development by induction of cell elongation and division (Campanoni and Nick, 2005; Chapman and Estelle, 2009). Their biosynthesis has been found to be of great importance in seed germination as it is essential for hypocotyl elongation (Kucera et al., 2005; Bai et al., 2018). Auxins can also affect ABA and GAs signalling pathways through their transport in the root tip, where *AUX1* plays an essential role (Wang et al., 2016). Low auxins levels during the deep dormant stage of tea buds and other species have been observed (Li et al., 2003; Nagar and Sood, 2006), suggesting the need of changes in auxins content and auxin-associated genes for dormancy transitions in these systems (Hao et al., 2017). Moreover, Carrera et al. (2008) found auxins efflux and influx transporters up-regulated in after-ripened seeds compared with dormant seeds.

A high number of auxin-related genes was found in the list of down-regulated DEGs. Some examples of genes known to play an important role in auxin synthesis or their signalling pathways should be highlighted, such as those encoding *INDOLE-3-ACETIC ACID-AMINO SYNTHETASE GH3.6* (-133.53 and -2.38) and *GH3.3* (-24.06 and -8.72), *ABC TRANSPORTER B FAMILY MEMBER 19 (ABC19)* (-3266.98), *MYROSINASE 4 (TGG4)* (-1406.78 and -190.45), *AUXIN TRANSPORTER PROTEIN 1/AUXIN RESISTANT 1 (AUX1)* (-487.14), *AUXIN EFFLUX CARRIER COMPONENT 6 (PIN6)* (-421.66), *PROTEIN WALLS ARE THIN (WAT1)* (-172.76) and *ERF109-LIKE* (-14.21). However, two repressors of auxin response genes were also down-regulated: *AUXIN-RESPONSIVE PROTEIN IAA17* and *IAA27* (-23.74 and -23.53, respectively). The up-regulation of a potential *SNL1* [Cbp42606 (1633.68)] and down-regulation of *AUX1* in this study (i.e. lower expression of a potential *SNL1* and higher expression of *AUX1* in the non-deep accession) would be in accordance with Wang et al. (2016), where *AUX1* was proven to be positively implicated in seed germination and negatively regulated by the histone deacetylases *SNL1* and *SNL2*.

Overall, phytohormone synthesis or signalling was generally up-regulated for ABA (e.g. *NCED6*, *NCED2*, *ABCG40*, *ABI3*) and down-regulated for GAs (*GA20ox1*, *GA20ox2*, *bHLH93*), ethylene (*ACO1*, *ERF4-LIKE*, *ERF105*, *ERF109-LIKE*), BRs (*BIA1*, *CYP708A2-LIKE*, probable *WRKY46*, *BAK1*, *BEN1*, *BES1*, *BRI1*) and auxin (*GH3.3*, *GH3.6*, *ABC19*, *TGG4*, *AUX1*, *PIN6*, *WAT1*), while several ABA repressors or sequences related to its catabolism were down-regulated (e.g. *AFP4*, *ARAC7*, *ARAC10*, *ZFP8*, *ZFP3*, *ABII*, *CYP707A3* and *CYP707A1*) (Table 3). Together, these results suggest that phytohormones play an important role in controlling differences in secondary dormancy depth between accessions.

Epigenetic regulation and chromatin remodelling

While the up-regulated DEGs included terms related to epigenetic regulation in the over-represented GOs, the down-regulated DEGs included these terms in the under-represented GOs. This indicates an active expression and implication of these processes in differences in secondary seed dormancy depth between the accessions. The up-regulated DEGs were enriched for 'chromatin

remodelling', 'lysine-acetylated histone binding' and 'acetylation-dependent protein binding'. Chromatin remodelling factors *PROBABLE ATP-DEPENDENT DNA HELICASE CHR23* and *CHR12* were both up-regulated (2.82 and 2.29). Overexpression of *CHR12* or *CHR23* reduced the frequency of seed germination in *Arabidopsis* up to 30% relative to wild-type (Leeggangers et al., 2015).

Several sequences encoding histone deacetylases were within the up-regulated genes. As HDACs lack intrinsic DNA-binding activity, they are recruited to target genes through association with transcription factors or by incorporation into large multiprotein transcriptional complexes (Luo et al., 2017). Up-regulated HDACs included *HISTONE DEACETYLASE 14 (HDA14)* (35.98), *HISTONE DEACETYLASE 6-LIKE (HDA6-LIKE)* (29.62) and *HISTONE DEACETYLASE-LIKE PROTEIN (HDA-LIKE)* (3.50 and 2.58). *HISTONE DEACETYLASE 6 (HDA6)* and *HDA19* have partially redundant functions in regulating seed germination, embryo development and salt resistance (Tanaka et al., 2008) and both can interact with *HISTONE DEACETYLASE COMPLEX 1 (HDC1)*, *SNLs* and *MULTICOPY SUPPRESSOR OF IRA1 (MSI)* in order to repress gene expression and regulate plant development (Chen and Wu, 2010). The *AT-HOOK MOTIF NUCLEAR-LOCALIZED PROTEIN 22 (AHL22)* was also highly up-regulated (241.89 and 28.15). This protein acts as a chromatin remodelling factor, interacting with *HDA6*, and modulating both H3 acetylation (through deacetylation of acetylated histones) and methylation of *FLOWERING LOCUS T (FT)* (Yu et al., 2011). Another up-regulated gene, the *PHD FINGER PROTEIN ING2* (88.43), codes for a protein that is a native subunit of the repressive complex mSin3a-HDAC1 in mammalian cells (Shi et al., 2006).

The proteins *PAIRED AMPHIPATHIC HELIX SWI-INDEPENDENT3 (SIN3)-LIKE (SNL) 1* and *SNL2* can act as components of a HDAC-SNL complex capable of modulating the transcription of genes through histone deacetylation (Wang et al., 2013). Out of 45 annotated *PAIRED AMPHIPATHIC HELIX* proteins in the *C. bursa-pastoris* genome, two were up-regulated [Cbp42606 (1633.68) and Cbp47861 (1.41)]. The annotation results identified them as most closely related to *SNL4* and *SNL6* (Supplementary Data S3), although the most similar sequences in *Arabidopsis* were *SNL1* and *SNL5* (Supplementary Data S7). Further research is needed to establish their exact identity in *C. bursa-pastoris* and their potential role in secondary seed dormancy depth variation. The high up-regulation (1633.68) of a potential *SNL1* indicates possible similarities between epigenetic regulation of primary and secondary seed dormancy.

However, in the up-regulated genes, there were also sequences implicated in histone acetyltransferase activity, such as the *CHROMATIN MODIFICATION-RELATED PROTEIN EAF6* (3.48). *EAF6* is a small 13-kDa protein that in yeast forms part of the *PICCOLO NUCLEOSOME ACETYLTRANSFERASE OF HISTONE H4 (NuA4)* complex but its contribution to the transcriptional regulation mediated by NuA4 has not been fully addressed (Espinosa-Cores et al., 2020). Another example is the gene transcription regulator of RNA polymerase II, *SPT-ADA-GCN5-ACETYLTRANSFERASE (SAGA) SUBUNIT*, with a fold change of 2.55.

As mentioned above, the down-regulated DEGs presented under-representation of GO-terms related to epigenetic regulation, with terms such as: 'histone acetylation', 'peptidyl-lysine acetylation', 'internal peptidyl-lysine acetylation', 'histone

Table 3. DEGs potentially explaining the difference in secondary seed dormancy depth between two accessions of *C. bursa-pastoris*

DEGs	Max group mean TPM	Log ₂ fold change	Fold change	FDR P-value	Sequence Description
Cbp42600	16.30	3.09	8.51	$0.00 \times 10^{+00}$	9-Cis-epoxycarotenoid dioxygenase NCED6, chloroplastic
Cbp16271	9.53	0.75	1.68	1.59×10^{-03}	9-Cis-epoxycarotenoid dioxygenase NCED2, chloroplastic
Cbp22042	9.44	0.59	1.51	1.67×10^{-02}	9-Cis-epoxycarotenoid dioxygenase NCED2, chloroplastic
Cbp24455	0.82	3.92	15.16	9.96×10^{-04}	ABC transporter G family member 40 (ABCG40)
Cbp41780	0.82	3.41	10.65	8.11×10^{-03}	ABC transporter G family member 40 (ABCG40)
Cbp42531	63.27	1.31	2.49	2.65×10^{-14}	B3 domain-containing transcription factor ABI3
Cbp30923	22.12	-7.72	-211.51	$0.00 \times 10^{+00}$	Ninja-family protein AFP4
Cbp31262	0.44	-4.74	-26.70	1.60×10^{-02}	Rac-like GTP-binding protein ARAC7
Cbp39709	3.36	-1.92	-3.79	1.96×10^{-06}	Rac-like GTP-binding protein ARAC10
Cbp13725	0.39	-4.10	-17.17	2.03×10^{-02}	Zinc finger protein 8 (ZFP8)
Cbp3057	0.81	-1.80	-3.48	4.58×10^{-02}	Zinc finger protein 3 (ZFP3)
Cbp5869	8.51	-1.77	-3.41	6.18×10^{-06}	Protein phosphatase 2C 56 (ABI1)
Cbp37985	5.92	-1.72	-3.30	1.43×10^{-04}	Protein phosphatase 2C 56 (ABI1)
Cbp26900	8.78	-2.31	-4.96	6.05×10^{-06}	Abscisic acid 8'-hydroxylase 3 (CYP707A3)
Cbp22128	4.61	-1.15	-2.21	4.80×10^{-03}	Abscisic acid 8'-hydroxylase 1 (CYP707A1)
Cbp5936	0.89	-3.61	-12.24	6.22×10^{-04}	Gibberellin 20 oxidase (GA20ox1)
Cbp51055	1.74	-2.38	-5.22	2.95×10^{-03}	Gibberellin 20 oxidase (GA20ox1)
Cbp7789	0.76	-2.64	-6.22	2.91×10^{-03}	Gibberellin 20 oxidase 2 (GA20ox2)
Cbp19645	2.15	-2.55	-5.87	1.37×10^{-05}	Transcription factor bHLH93
Cbp28889	7.22	-2.05	-4.13	1.06×10^{-08}	Transcription factor bHLH93
Cbp39064	1.56	-9.04	-526.98	4.21×10^{-03}	1-Aminocyclopropane-1-carboxylate oxidase 1 (ACO1)
Cbp12844	0.95	-7.93	-244.23	1.93×10^{-02}	1-Aminocyclopropane-1-carboxylate oxidase 1 (ACO1)
Cbp19872	4.51	-10.13	-1123.97	1.38×10^{-03}	Ethylene-responsive transcription factor 4-like (ERF4-like)
Cbp32893	1.14	-3.34	-10.10	1.91×10^{-03}	Ethylene-responsive transcription factor 4-like (ERF4-like)
Cbp37700	17.85	-3.62	-12.27	$0.00 \times 10^{+00}$	Ethylene-responsive transcription factor ERF105
Cbp7837	20.10	-2.20	-4.61	2.16×10^{10}	Ethylene-responsive transcription factor ERF105
Cbp9675	0.74	-3.83	-14.21	2.28×10^{-03}	Ethylene-responsive transcription factor ERF109-like
Cbp45862	0.49	-7.82	-226.23	1.99×10^{-02}	BAHD acyltransferase BIA1
Cbp21867	0.52	-3.48	-11.17	1.37×10^{-03}	BAHD acyltransferase BIA1
Cbp4525	0.30	-7.34	-161.72	4.08×10^{-02}	Cytochrome P450 708A2-like (CYP708A2-like)
Cbp45864	1.10	-6.48	-89.40	7.99×10^{-07}	Cytochrome P450 708A2-like (CYP708A2-like)
Cbp42351	0.54	-4.66	-25.35	1.59×10^{-03}	Probable WRKY transcription factor 46
Cbp12534	0.45	-2.81	-6.99	2.56×10^{-02}	Probable WRKY transcription factor 46
Cbp37729	0.88	-2.16	-4.47	1.44×10^{-04}	BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 (BAK1)
Cbp12623	1.02	-1.99	-3.97	9.69×10^{-03}	Protein BRI1-5 ENHANCED 1 (BEN1)
Cbp42444	1.15	-1.92	-3.78	5.22×10^{-03}	Protein BRI1-5 ENHANCED 1 (BEN1)
Cbp2918	2.31	-1.93	-3.80	2.02×10^{-04}	Brassinosteroid signaling positive regulator (BZR1) family protein (BES1)
Cbp32638	1.39	-1.66	-3.16	1.15×10^{-02}	Brassinosteroid signaling positive regulator (BZR1) family protein (BES1)
Cbp20939	9.07	-1.00	-2.01	5.05×10^{-06}	Protein BRASSINOSTEROID INSENSITIVE 1 (BRI1)
Cbp15487	7.44	-0.97	-1.95	4.55×10^{-05}	Protein BRASSINOSTEROID INSENSITIVE 1 (BRI1)

(Continued)

Table 3. (Continued.)

DEGs	Max group mean TPM	Log ₂ fold change	Fold change	FDR P-value	Sequence Description
Cbp3699	0.20	-7.06	-133.53	4.98 × 10 ⁻⁰²	Indole-3-acetic acid-amido synthetase GH3.6
Cbp23761	1.82	-1.25	-2.38	3.56 × 10 ⁻⁰²	Indole-3-acetic acid-amido synthetase GH3.6
Cbp2631	0.45	-4.59	-24.06	8.38 × 10 ⁻⁰⁵	Indole-3-acetic acid-amido synthetase GH3.3
Cbp48208	1.13	-3.12	-8.72	3.27 × 10 ⁻⁰³	Indole-3-acetic acid-amido synthetase GH3.3
Cbp3272	2.33	-11.67	-3266.98	5.04 × 10 ⁻⁰⁵	ABC transporter B family member 19 (ABCB19)
Cbp4100	2.41	-10.46	-1406.78	4.59 × 10 ⁻⁰⁴	Myosinase 4 (TGG4)
Cbp52610	0.44	-7.57	-190.45	2.70 × 10 ⁻⁰²	Myosinase 4 (TGG4)
Cbp4566	0.97	-8.93	-487.14	6.95 × 10 ⁻⁰³	Auxin transporter protein 1/auxin resistant 1 (AUX1)
Cbp14764	0.67	-8.72	-421.66	6.01 × 10 ⁻⁰³	Auxin efflux carrier component 6 (PIN6)
Cbp14621	0.39	-7.43	-172.76	3.14 × 10 ⁻⁰²	Protein WALLS ARE THIN 1 (WAT1)
Cbp42606	0.99	10.67	1633.68	1.20 × 10 ⁻⁰⁴	Paired amphipathic helix Sin3-like protein, SIN3-like 1 (SNL1: Best BLAST result with Arabidopsis)
Cbp38639	3.22	5.17	35.98	0.00 × 10 ⁺⁰⁰	Histone deacetylase 14 isoform X1 (HDA14)
Cbp19750	8.99	4.89	29.62	0.00 × 10 ⁺⁰⁰	Histone deacetylase 6-like (HDA6-like)
Cbp8867	3.48	1.81	3.50	1.06 × 10 ⁻⁰³	Histone deacetylase-like protein (HDA-like)
Cbp32728	11.88	1.37	2.58	9.39 × 10 ⁻⁰⁴	Histone deacetylase-like protein (HDA-like)
Cbp3768	4.54	6.47	88.43	0.00 × 10 ⁺⁰⁰	PHD finger protein ING2
Cbp29763	8.45	-2.02	-4.05	0.00 × 10 ⁺⁰⁰	Lysine-specific demethylase JMJ30 isoform X1
Cbp43411	19.81	-1.22	-2.33	5.45 × 10 ⁻¹⁰	Lysine-specific demethylase JMJ30 isoform X1
Cbp9692	1.68	-3.11	-8.66	1.35 × 10 ⁻⁰³	bZIP transcription factor 11 (bZIP11)
Cbp38739	1.39	-3.03	-8.19	2.83 × 10 ⁻⁰³	bZIP transcription factor 11 (bZIP11)
Cbp23560	1.78	-2.89	-7.41	7.68 × 10 ⁻⁰⁵	AT-rich interactive domain-containing protein 1A-like (ARID1A-like)
Cbp43511	0.09	3.61	12.22	1.87 × 10 ⁻⁰²	Histone-lysine N-methyltransferase, H3 lysine-9 specific SUVH7
Cbp4106	4.19	2.57	5.93	0.00 × 10 ⁺⁰⁰	Histone-lysine N-methyltransferase family member SUVH9
Cbp2612	0.52	-1.38	-2.60	3.69 × 10 ⁻⁰²	Histone-lysine N-methyltransferase CLF isoform X1

The selected DEGs are from a comparison of a deep dormant (-367) versus a non-deep dormant accession (-799); both were imbibed in darkness for 3 d at 30°C in water to induce secondary seed dormancy.

modification', 'histone acetyltransferase activity' or 'peptide-lysine-N-acetyltransferase activity', as well as 'chromosome organization' and 'chromatin organization'. Despite the fact that the acetylation categories were under-represented, a few examples were still found, such as *BZIP TRANSCRIPTION FACTOR 11* (-8.66 and -8.19), which physically interacts with transcription factor ADAPTOR PROTEIN ADA2b to promote recruitment of SAGA-like histone acetyltransferase complexes to specific auxin-responsive genes (Weiste and Dröge-Laser, 2014). In this way, the bZIP11 transcription factor is able to recruit the histone acetylation machinery to open up packed chromatin. Another example of a down-regulated sequence implicated in histone acetylation is *AT-RICH INTERACTIVE DOMAIN-CONTAINING PROTEIN 1A-LIKE (ARID1A-LIKE)* (-7.41), which is responsible for maintaining the levels of histone acetylation between the vegetative nucleus and the sperm nuclei in pollen by associating with a histone deacetylation machinery (Zheng et al., 2014).

With respect to histone methylation, several sequences were within the up-regulated genes, such as *HISTONE-LYSINE N-METHYLTRANSFERASE* (1.55), *SUVH7* (12.22) and *SUVH9*

(5.93), although others were down-regulated, including *CLF ISOFORM X1* (-2.60), *ASHH1* (-1.92) and *SUVH3-LIKE* (-1.56). However, *CLF* was suggested to be a repressor of a positive regulator of primary dormancy (*DOG1*), as it was found to be negatively correlated with seed dormancy in the soil seed bank by Footitt et al. (2015) in the Cape Verdi Islands (Cvi) Arabidopsis deep dormant genotype. Demethylases were also both up- and down-regulated, as shown by *LYSINE-SPECIFIC HISTONE DEMETHYLASE 1 HOMOLOG 1 (LDL1)* (4.06 and -4.43), *LYSINE-SPECIFIC DEMETHYLASE JMJ30* (-4.05, -2.33), *JMJ25 ISOFORM X2* (-2.80) and *JMJ14 ISOFORM X1* (1.53). *JMJ30* is a histone demethylase that demethylates Lys-36 of histone H3 with a specific activity for H3K36me3 and H3K36me2. H3K36me3 acts as a mark for HDACs to bind to and deacetylate the histone, which would prevent run-away transcription. Thus, the down-regulation of this demethylase is preventing the removal of methylation from H3 and hence allowing HDACs to bind to and deacetylate the histone (Yan et al., 2014).

Regarding DNA methylation, we observed differential regulation of DNA (*CYTOSINE-5)-METHYLTRANSFERASE CMT3* (-3.42), but not others (e.g. *DRM2*, *MET1*). Finally, *HEN1*

SUPPRESSOR 1 (HESO1) uridylylates miRNAs and siRNAs, thereby leading to their degradation. Its low fold change in our results (-158.33) could be indicating an over-accumulation of small RNAs being stabilized by methylation catalyzed by HEN1 (small RNA methyl transferase) (Ren et al., 2014).

Altogether, these results indicate the need of a general up-regulation of genes related to histone deacetylation and a down-regulation of specific sequences implicated in histone acetylation processes in the deep dormant accession in comparison with the non-deep dormant one in order to establish a deeper secondary seed dormancy state (Table 3). Nevertheless, as indicated before, some contradictions could be found, such as the up-regulation of sequences implicated in histone acetylation or the down-regulation of *HISTONE DEACETYLASE 14* (-16.92), which was also within the up-regulated DEGs (35.97). These contradictions might be explained either by the functions of the genes they control (positive or negative regulators of dormancy), or by variations in the acetylation patterns found between different areas or parts of the seeds when imbibed in water in darkness at 30°C, as described previously (Gomez-Cabellos et al., 2021). In that work, the immunolocalizations showed that after different periods of imbibition in darkness at 30°C, certain parts of the seed presented higher H4Ac signals than others, which would be in accordance with the apparent contradictions described here.

With respect to histone (de)methylation, we observed both up- and down-regulation of genes involved, although the expression patterns were generally in accordance with the difference in secondary dormancy depth between the accessions (Table 3). However, this was not always the case, such as for the *LYSINE-SPECIFIC HISTONE DEMETHYLASE 1 HOMOLOG 1* (*LDL1*), which presented both down- (-4.43) and up-regulation (4.06). *LDL1* acts redundantly with *LDL2* in repressing primary seed dormancy (Zhao et al., 2015). The *ldl1 ldl2* double mutant displays increased seed dormancy, whereas overexpression of *LDL1* or *LDL2* in *Arabidopsis* causes reduced dormancy. Our results show that *LDL1* may not play a significant role in secondary dormancy depth between accessions. Regarding DNA methylation, the fact that only one DNA methylase was differentially regulated seems to be in accordance with results from previous work that showed elevated global DNA methylation levels after 3 d in darkness at 30°C in seeds from both the deep and non-deep dormant accessions studied here (Gomez-Cabellos et al., 2021).

Thus, these results indicate an active involvement of epigenetic regulation in the establishment of different secondary seed dormancy depths. Moreover, it seems that histone (de)acetylation and (de)methylation play a larger role in establishing differences in secondary seed dormancy depth than DNA methylation. However, the effect of specific epigenetic processes on the level of secondary seed dormancy depth could potentially depend on the function of the genes they regulate (negative or positive regulators of dormancy), the area or part of the seed, or the time period since secondary dormancy induction. Future studies could focus on particular genes, sub-sections of seeds or different time points to further elucidate the epigenetic mechanisms underlying differences in secondary seed dormancy depth.

Conclusion

The HDAC inhibitors TSA and valproic acid caused a delay of t50 for germination but not of t50 for testa rupture in comparison with their respective controls, demonstrating they were lowering

the speed of germination. In addition, valproic acid exposure led to an increase in secondary dormancy depth. Transcriptome analysis of non-deep dormant seeds exposed to valproic acid or water revealed that even though sequences related to ABA showed complex regulation in seeds treated with valproic acid, the synthesis of GAs (*GA20ox1*), BRs (*CYP708A2*) and auxins (*CYP79B3*, *SNL1*) was negatively regulated (Table 2), thereby providing a potential mechanistic explanation for the observed delay in t50 for germination in seeds treated with HDAC inhibitors. Among those genes implicated in epigenetic regulation, *SNL1* could be involved in the enhanced secondary seed dormancy observed.

Transcriptome analysis of the deep dormant versus non-deep dormant seeds comparison showed that differential regulation of phytohormone-related genes may play an important role in secondary dormancy depth of *C. bursa-pastoris* (Table 3). Taking into account all the ABA-related DEGs, most of the up-regulated genes were promoters of its synthesis or signalling (e.g. *NCED6*, *NCED2*, *ABCG40* and *ABI3*), while in the down-regulated set, the most abundant were repressors or sequences related to its catabolism (e.g. *AFP4*, *ARAC7*, *ARAC10*, *ZFP8*, *ZFP3*, *ABI1*, *CYP707A3* and *CYP707A1*). In contrast, several key (regulators of) genes related to the biosynthesis of gibberellins were down-regulated, such as *GA20ox1*, *GA20ox2* and *bHLH93*. Down-regulation was also observed for important genes involved in the synthesis and signalling pathways of ethylene (*ACO1*, *ERF4-LIKE*, *ERF105*, *ERF109-LIKE*), BRs (*BIA1*, *CYP708A2-LIKE*, probable *WRKY46*, *BAK1*, *BEN1*, *BES1* and *BRI1*) and auxin (*GH3.3*, *GH3.6*, *ABCB19*, *TGG4*, *AUX1*, *PIN6* and *WAT1*). Future research could confirm whether the differential regulation of a potential *SNL1*, several histone deacetylases and associated genes (*HDA14*, *HDA6-LIKE*, *HDA-LIKE*, *ING2*, *JMJ30*), sequences linked to acetylases (*bZIP11*, *ARID1A-LIKE*), or to gene silencing through histone methylation (*SUVH7*, *SUVH9*, *CLF*), may be responsible for the observed differential expression of genes related to phytohormones between accessions of *C. bursa-pastoris* varying in secondary dormancy depth.

Author contributions. S.G.-C.: conceptualization, methodology, investigation, formal analysis, visualization, writing (original draft), writing (review and editing); P.E.T.: supervision, project administration, conceptualization, methodology, investigation, writing (review and editing); E.F.-P.: formal analysis, visualization, writing (original draft), writing (review and editing); P.P.M.I.: resources (ecotypes), writing (review and editing); H.W.P.: project administration, resources, supervision, writing (review and editing); A.M.V.: supervision, conceptualization, methodology, formal analysis, writing (original draft), writing (review and editing).

Supplementary material. To view supplementary material for this article, please visit: <https://doi.org/10.1017/S0960258522000265>.

Acknowledgements. We would like to thank STAB VIDA for the sequencing of our mRNA samples and the analysis of differentially expressed genes. In addition, we thank Mauro Maver for critical reading of the manuscript.

Financial support. This work was supported by grant-in-aid from the Department for Environment, Food and Rural Affairs to the Royal Botanic Gardens, Kew. E.F.P. was supported by the Jardín Botánico Atlántico (SV-20-GIJON-JBA). P.P.M.I. and The James Hutton Institute were supported by the Rural and Environmental Science and Analytical Services (RESAS), a division of the Scottish Government. The funders had no role in study design, report writing and article submission.

Conflicts of interest. The authors declare that they have no conflict of interest.

References

- Ali-Rachedi S, Bouinot D, Wagner MH, Bonnet M, Sotta B, Grappin P and Julien M (2004) Changes in endogenous abscisic acid levels during dormancy release and maintenance of mature seeds: studies with the Cape Verde Islands ecotype, the dormant model of *Arabidopsis thaliana*. *Planta* **219**, 479–488. doi:10.1007/s00425-004-1251-4
- Ariizumi T, Lawrence PK and Steber CM (2011) The role of two F-box proteins, SLEEPY1 and SNEEZY, in Arabidopsis gibberellin signaling. *Plant Physiology* **155**, 765–775. doi:10.1104/pp.110.166272
- Ashikawa I, Abe F and Nakamura S (2010) Ectopic expression of wheat and barley DOG1-like genes promotes seed dormancy in Arabidopsis. *Plant Science* **179**, 536–542. doi:10.1016/j.plantsci.2010.08.002
- Bai B, Novák O, Ljung K, Hanson J and Bentsink L (2018) Combined transcriptome and translome analyses reveal a role for tryptophan-dependent auxin biosynthesis in the control of DOG1-dependent seed dormancy. *New Phytologist* **217**, 1077–1085. doi:10.1111/nph.14885
- Baskin JM and Baskin CC (1989) Germination responses of buried seeds of *Capsella bursa-pastoris* exposed to seasonal temperature changes. *Weed Research* **29**, 205–212. doi:10.1111/j.1365-3180.1989.tb00860.x
- Baskin CC and Baskin JM (2014) *Seeds: ecology, biogeography, and, evolution of dormancy and germination*. Elsevier Inc. doi:10.1016/C2013-0-00597-X
- Beaudoin N, Serizet C, Gosti F and Giraudat J (2000) Interactions between abscisic acid and ethylene signaling cascades. *The Plant Cell* **12**, 1103–1115. doi:10.1105/tpc.12.7.1103
- Benech-Arnold RL, Sanchez RA, Forcella F, Betina KC and Ghersa CM (2000) Environmental control of dormancy in weed seed banks in soil. *Field Crops Research* **67**, 105–122. doi:10.1159/000142249
- Bentsink L and Koornneef M (2008) Seed dormancy and germination. *Arabidopsis Book* **6**, e0119. doi:10.1199/tab.0119
- Bentsink L, Jowett J, Hanhart CJ and Koornneef M (2006) Cloning of DOG1, a quantitative trait locus controlling seed dormancy in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 17042–17047. doi:10.1073/pnas.0607877103
- Berr A, Shafiq S and Shen WH (2011) Histone modifications in transcriptional activation during plant development. *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms* **1809**, 567–576. doi:10.1016/j.bbagr.2011.07.001
- Bewley JD and Black M (1994) *Seeds: physiology of development and germination* (2nd edn). New York, Springer Science+Business Media.
- Bouyer D, Roudier F, Heese M, Andersen ED, Gey D, Nowack MK, Goodrich J, Renou JP, Grini PE, Colot V and Schnittger A (2011) Polycomb Repressive Complex 2 controls the embryo-to-seedling phase transition. *PLoS Genetics* **7**, e1002014. doi:10.1371/journal.pgen.1002014
- Bowen AJ, Gonzalez D, Mullins JGL, Bhatt AM, Martinez A and Conlan RS (2010) PAH-domain-specific interactions of the Arabidopsis transcription coregulator SIN3-LIKE1 (SNL1) with Telomere-binding Protein 1 and ALWAYS EARLY2 Myb-DNA binding factors. *Journal of Molecular Biology* **395**, 937–949. doi:10.1016/j.jmb.2009.11.065
- Braybrook SA and Harada JJ (2008) LECs go crazy in embryo development. *Trends in Plant Science* **13**, 624–630. doi:10.1016/j.tplants.2008.09.008
- Braybrook SA, Stone SL, Park S, Bui AQ, Le BH, Fischer RL, Goldberg RB and Harada JJ (2006) Genes directly regulated by *LEAFY COTYLEDON2* provide insight into the control of embryo maturation and somatic embryogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 3468–3473. doi:10.1073/pnas.0511331103
- Buijs G (2020) A perspective on secondary seed dormancy in *Arabidopsis thaliana*. *Plants* **9**, 1–9. doi:10.3390/plants9060749
- Buijs G, Vogelzang A, Nijveen H and Bentsink L (2020) Dormancy cycling: translation-related transcripts are the main difference between dormant and non-dormant seeds in the field. *Plant Journal* **102**, 327–339. doi:10.1111/tj.14626
- Cadman CSC, Toorop PE, Hilhorst HWM and Finch-Savage WE (2006) Gene expression profiles of Arabidopsis Cvi seeds during dormancy cycling indicate a common underlying dormancy control mechanism. *Plant Journal* **46**, 805–822. doi:10.1111/j.1365-313X.2006.02738.x
- Campanoni P and Nick P (2005) Auxin-dependent cell division and cell elongation. 1-naphthaleneacetic acid and 2,4-dichlorophenoxyacetic acid activate different pathways. *Plant Physiology* **137**, 939–948. doi:10.1104/pp.104.053843
- Carbonero P, Iglesias-Fernández R and Vicente-Carbajosa J (2017) The AFL subfamily of B3 transcription factors: evolution and function in angiosperm seeds. *Journal of Experimental Botany* **68**, 871–880. doi:10.1093/jxb/erw458
- Carrera E, Holman T, Medhurst A, Dietrich D, Footitt S, Theodoulou FL and Holdsworth MJ (2008) Seed after-ripening is a discrete developmental pathway associated with specific gene networks in Arabidopsis. *Plant Journal* **53**, 214–224. doi:10.1111/j.1365-313X.2007.03331.x
- Carrillo-Barral N, Del Carmen Rodríguez-Gacio M and Matilla AJ (2020) Delay of germination-1 (DOG1): a key to understanding seed dormancy. *Plants* **9**, 480. doi:10.3390/plants9040480
- Champion LG, May MJ, Bennett S, Brooks DR, Clark SJ, Daniels RE, Firbank LT, Houghton AJ, Hawes C, Heard MS, Perry JN, Randle Z, Rossall MJ, Rothery P, Skellern MP, Scott RJ, Squire GR and Thomas MR (2003) Crop management and agronomic context of the Farm Scale Evaluations of genetically modified herbicide-tolerant crops. *Philosophical Transactions of the Royal Society B Biological Sciences* **358**, 1801–1818. doi:10.1098/rstb.2003.1405
- Chang P-K and Scharfenstein LL (2014) *Aspergillus flavus* Blast2GO gene ontology database: elevated growth temperature alters amino acid metabolism. *Journal of Genetics and Genome Research* **1**, 1–7. doi:10.23937/2378-3648/1410005
- Chapman EJ and Estelle M (2009) Mechanism of auxin-regulated gene expression in plants. *Annual Review of Genetics* **43**, 265–285. doi:10.1146/annurev-genet-102108-134148
- Chateauxvieux S, Morceau F, Dicato M and Diederich M (2010) Molecular and therapeutic potential and toxicity of valproic acid. *Journal of Biomedicine and Biotechnology* **2010**, 479364. doi:10.1155/2010/479364
- Chen LT and Wu K (2010) Role of histone deacetylases HDA6 and HDA19 in ABA and abiotic stress response. *Plant Signaling and Behavior* **5**, 1318–1320. doi:10.4161/psb.5.10.13168
- Chen TT, Liu FF, Xiao DW, Jiang XY, Li P, Zhao SM, Hou BK and Li YJ (2020) The Arabidopsis UDP-glycosyltransferase75B1, conjugates abscisic acid and affects plant response to abiotic stresses. *Plant Molecular Biology* **102**, 389–401. doi:10.1007/s11103-019-00953-4
- Cigliano RA, Cremona G, Paparo R, Termolino P, Perrella G, Gutzat R, Consiglio MF and Conicella C (2013) Histone deacetylase AtHDA7 is required for female gametophyte and embryo development in Arabidopsis. *Plant Physiology* **163**, 431–440. doi:10.1104/pp.113.221713
- Colville A, Alhattab R, Hu M, Labbé H, Xing T and Miki B (2011) Role of HD2 genes in seed germination and early seedling growth in Arabidopsis. *Plant Cell Reports* **30**, 1969–1979. doi:10.1007/s00299-011-1105-z
- Conesa A and Götz S (2008) Blast2GO: a comprehensive suite for functional analysis in plant genomics. *International Journal of Plant Genomics* **2008**, 619832. doi:10.1155/2008/619832
- Corbinau F, Xia Q, Bailly C and El-Maarouf-Bouteau H (2014) Ethylene, a key factor in the regulation of seed dormancy. *Frontiers in Plant Science* **5**, 1–13. doi:10.3389/fpls.2014.00539
- Coughlan JM, Saha A and Donohue K (2017) Effects of pre- and post-dispersal temperature on primary and secondary dormancy dynamics in contrasting genotypes of *Arabidopsis thaliana* (Brassicaceae). *Plant Species Biology* **32**, 210–222. doi:10.1111/1442-1984.12145
- Cui L, Miao J, Furuya T, Fan Q, Li X, Rathod PK, Su XZ and Cui L (2008) Histone acetyltransferase inhibitor anacardic acid causes changes in global gene expression during in vitro *Plasmodium falciparum* development. *Eukaryotic Cell* **7**, 1200–1210. doi:10.1128/EC.00063-08
- Curaba J, Moritz T, Blervaque R, Parcy F, Raz V, Herzog M and Vachon G (2004) *AtGA3ox2*, a key gene responsible for bioactive gibberellin biosynthesis, is regulated during embryogenesis by *LEAFY COTYLEDON2* and *FUSCA3* in Arabidopsis. *Plant Physiology* **136**, 3660–3669. doi:10.1104/pp.104.047266
- Dekkers BJW and Bentsink L (2015) Regulation of seed dormancy by abscisic acid and *DELAY OF GERMINATION 1*. *Seed Science Research* **25**, 82–98. doi:10.1017/S0960258514000415
- Du H-N (2012) Transcription, DNA damage and beyond: the roles of histone ubiquitination and deubiquitination. *Current Protein and Peptide Science* **13**, 447–466. doi:10.2174/138920312802430617
- Engelhorn J, Blanvillain R and Carles CC (2014) Gene activation and cell fate control in plants: a chromatin perspective. *Cellular and Molecular Life Sciences* **71**, 3119–3137. doi:10.1007/s00018-014-1609-0

- Espinosa-Cores L, Bouza-Morcillo L, Barrero-Gil J, Jiménez-Suárez V, Lázaro A, Piqueras R, Jarillo JA and Piñero M (2020) Insights into the function of the NuA4 complex in plants. *Frontiers in Plant Science* **11**, 1–18. doi:10.3389/fpls.2020.00125
- Finch-Savage WE and Footitt S (2017) Seed dormancy cycling and the regulation of dormancy mechanisms to time germination in variable field environments. *Journal of Experimental Botany* **68**, 843–856. doi:10.1093/jxb/erw477
- Finch-Savage WE and Leubner-Metzger G (2006) Seed dormancy and the control of germination. *New Phytologist* **171**, 501–523. doi:10.1063/1.1661985
- Finkelstein R, Reeves W, Ariizumi T and Steber C (2008) Molecular aspects of seed dormancy. *Annual Review of Plant Biology* **59**, 387–415. doi:10.1146/annurev.arplant.59.032607.092740
- Footitt S and Finch-Savage WE (2017) Dormancy and control of seed germination, pp. 1–30 in Clemens S (Ed.), *Plant physiology and function. The plant sciences*, New York, NY, Springer. doi:10.1007/978-1-4614-7611-5_7-1
- Footitt S, Douterelo-Soler I, Clay H and Finch-Savage WE (2011) Dormancy cycling in Arabidopsis seeds is controlled by seasonally distinct hormone-signaling pathways. *Proceedings of the National Academy of Sciences* **108**, 20236–20241. doi:10.1073/pnas.1116325108
- Footitt S, Huang Z, Clay HA, Mead A and Finch-Savage WE (2013) Temperature, light and nitrate sensing coordinate Arabidopsis seed dormancy cycling, resulting in winter and summer annual phenotypes. *Plant Journal* **74**, 1003–1015. doi:10.1111/tjp.12186
- Footitt S, Müller K, Kermodé AR and Finch-Savage WE (2015) Seed dormancy cycling in Arabidopsis: chromatin remodelling and regulation of DOG1 in response to seasonal environmental signals. *Plant Journal* **81**, 413–425. doi:10.1111/tjp.12735
- Footitt S, Clewes R, Feeney M, Finch-Savage WE and Frigerio L (2019) Aquaporins influence seed dormancy and germination in response to stress. *Plant Cell and Environment* **42**, 2325–2339. doi: 10.1111/pce.13561.
- Footitt S, Walley PG, Lynn JR, Hambidge AJ, Penfield S and Finch-Savage WE (2020) Trait analysis reveals DOG1 determines initial depth of seed dormancy, but not changes during dormancy cycling that result in seedling emergence timing. *New Phytologist* **225**, 2035–2047. doi:10.1111/nph.16081
- Fu X and Harberd NP (2003) Auxin promotes Arabidopsis root growth by modulating gibberellin response. *Nature* **421**, 740–743. doi:10.1038/nature01387
- Gao F, Jordan MC and Ayele BT (2012) Transcriptional programs regulating seed dormancy and its release by after-ripening in common wheat (*Triticum aestivum* L.). *Plant Biotechnology Journal* **10**, 465–476. doi:10.1111/j.1467-7652.2012.00682.x
- Gazzarrini S, Tsuchiya Y, Lumba S, Okamoto M and McCourt P (2004) The transcription factor FUSCA3 controls developmental timing in Arabidopsis through the hormones gibberellin and abscisic acid. *Developmental Cell* **7**, 373–385. doi:10.1016/j.devcel.2004.06.017
- Ghassemian M, Nambara E, Cutler S, Kawaide H, Kamiya Y and McCourt P (2000) Regulation of abscisic acid signaling by the ethylene response pathway in Arabidopsis. *The Plant Cell* **12**, 1117–1126. doi:10.1105/tpc.12.7.1117
- Gomez-Cabellos S, Toorop PE, Cañal MJ, Iannetta PPM, Fernández-Pascual E, Pritchard HW and Visscher AM (2021) Global DNA methylation and cellular 5-methylcytosine and H4 acetylated patterns in primary and secondary dormant seeds of *Capsella bursa-pastoris* (L.) Medik. (shepherd's purse). *Protoplasma* **259**, 595–614.
- Göttlicher M, Minucci S, Zhu P, Krämer OH, Schimpf A, Giavara S, Sleeman JP, Lo Coco F, Nervi C, Pelicci PG and Heinzl T (2001) Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO Journal* **20**, 6969–6978. doi:10.1093/emboj/20.24.6969
- Götz S, García-Gómez JM, Terol J, Williams TD, Nagaraj SH, Nueda MJ, Robles M, Talón M, Dopazo J and Conesa A (2008) High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Research* **36**, 3420–3435. doi:10.1093/nar/gkn176
- Graeber K, Linkies A, Müller K, Wunchova A, Rott A and Leubner-Metzger G (2010) Cross-species approaches to seed dormancy and germination: conservation and biodiversity of ABA-regulated mechanisms and the Brassicaceae *DOG1* genes. *Plant Molecular Biology* **73**, 67–87. doi:10.1007/s11103-009-9583-x
- Graeber K, Linkies A, Steinbrecher T, Mummenhoff K, Tarkowská D, Turečková V, Ignatz M, Sperber K, Voegele A, De Jong H, Urbanová T, Strnad M and Leubner-Metzger G (2014) DELAY OF GERMINATION 1 mediates a conserved coat-dormancy mechanism for the temperature- and gibberellin-dependent control of seed germination. *Proceedings of the National Academy of Sciences of the United States of America* **111**, E3571–E3580. doi:10.1073/pnas.1403851111
- Hao X, Yang Y, Yue C, Wang L, Horvath DP and Wang X (2017) Comprehensive transcriptome analyses reveal differential gene expression profiles of *Camellia sinensis* axillary buds at para-, endo-, ecodormancy, and bud flush stages. *Frontiers in Plant Science* **8**, 1–19. doi:10.3389/fpls.2017.00553
- He R, Yu D, Li X, Duan G, Zhang Y, Tang D, Zhao X and Liu X (2016) F-box gene FOA2 regulates GA- and ABA- mediated seed germination in Arabidopsis. *Science China Life Sciences* **59**, 1192–1194. doi:10.1007/s11427-016-0098-3
- Hilhorst HWM (1998) The regulation of secondary dormancy. The membrane hypothesis revisited. *Seed Science Research* **8**, 77–90. doi:10.1017/S0960258500003974
- Hilhorst HWM and Toorop PE (1997) Review on dormancy, germinability and germination in crop and weed seeds. *Advances in Agronomy* **61**, 112–165.
- Hintz M, Bartholmes C, Nutt P, Ziermann J, Hameister S, Neuffer B and Theissen G (2006) Catching a 'hopeful monster': shepherd's purse (*Capsella bursa-pastoris*) as a model system to study the evolution of flower development. *Journal of Experimental Botany* **57**, 3531–3542. doi:10.1093/jxb/erl158
- Holdsworth MJ, Finch-Savage WE, Grappin P and Job D (2008a) Post-genomics dissection of seed dormancy and germination. *Trends in Plant Science* **13**, 7–13. doi:10.1016/j.tplants.2007.11.002
- Holdsworth MJ, Bentsink L and Soppe WJJ (2008b) Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination. *New Phytologist* **179**, 33–54. doi:10.1111/j.1469-8137.2008.02437.x
- Hourston JE, Steinbrecher T, Chandler JO, Pérez M, Dietrich K, Turečková V, Tarkowská D, Strnad M, Weltmeier F, Meinhard J, Fischer U, Fiedler-Wiechers K, Ignatz M and Leubner-Metzger G (2022) Cold-induced secondary dormancy and its regulatory mechanisms in *Beta vulgaris*. *Plant Cell and Environment* **45**, 1315–1332. doi:10.1111/pce.14264
- Huo H, Wei S and Bradford KJ (2016) DELAY OF GERMINATION1 (*DOG1*) regulates both seed dormancy and flowering time through microRNA pathways. *Proceedings of the National Academy of Sciences* **113**, E2199–E2206. doi:10.1073/pnas.1600558113
- Hurka H and Haase R (1982) Seed ecology of *Capsella bursa-pastoris* (Cruciferae): dispersal mechanism and the soil seed bank. *Flora* **172**, 35–46. doi:10.1016/S0367-2530(17)31309-9
- Hurka H and Neuffer B (1997) Evolutionary processes in the genus *Capsella* (Brassicaceae). *Plant Systematics and Evolution* **206**, 295–316. doi:10.1007/BF00987954
- Iannetta PPM, Begg G, Hawes C, Young M, Russell J and Squire GR (2007) Variation in *Capsella* (shepherd's purse): an example of intraspecific functional diversity. *Physiologia Plantarum* **129**, 542–554. doi:10.1111/j.1399-3054.2006.00833.x
- Ibarra SE, Tognacca RS, Dave A, Graham IA, Sánchez RA and Botto JF (2016) Molecular mechanisms underlying the entrance in secondary dormancy of Arabidopsis seeds. *Plant Cell and Environment* **39**, 213–221. doi:10.1111/pce.12607.
- Jackson JP, Lindroth AM, Cao X and Jacobsen SE (2002) Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. *Nature* **416**, 556–560. doi:10.1038/nature731
- Kanyuka K, Praekelt U, Franklin KA, Billingham OE, Hooley R, Whitelam GC and Halliday KJ (2003) Mutations in the huge Arabidopsis gene BIG affect a range of hormone and light responses. *Plant Journal* **35**, 57–70. doi:10.1046/j.1365-313X.2003.01779.x
- Kasianov AS, Klepikova AV, Kulakovskiy IV, Gerasimov ES, Fedotova AV, Besedina EG, Kondrashov AS, Logacheva MD and Penin AA (2017) High-quality genome assembly of *Capsella bursa-pastoris* reveals asymmetry of regulatory elements at early stages of polyploid genome evolution. *Plant Journal* **91**, 278–291. doi:10.1111/tjp.13563

- Katsuya-Gaviria K, Caro E, Carrillo-barral N and Iglesias-fernández R** (2020) Reactive oxygen species (ROS) and nucleic acid modifications during seed dormancy. *Plants* **9**, 1–14. doi:10.3390/plants9060679
- Kendall S and Penfield S** (2012) Maternal and zygotic temperature signalling in the control of seed dormancy and germination. *Seed Science Research* **22**, S23–S29. doi:10.1017/S0960258511000390
- Kim SY, Warpeha KM and Huber SC** (2019) The brassinosteroid receptor kinase, BRI1, plays a role in seed germination and the release of dormancy by cold stratification. *Journal of Plant Physiology* **241**, 153031. doi:10.1016/j.jplph.2019.153031
- Koeller KM, Haggarty SJ, Perkins BD, Leykin I, Wong JC, Kao MCJ and Schreiber SL** (2003) Chemical genetic modifier screens: small molecule trichostatin suppressors as probes of intracellular histone and tubulin acetylation. *Chemistry and Biology* **10**, 397–410. doi:10.1016/S1074-5521(03)00093-0
- Krämer OH, Zhu P, Ostendorff HP, Golebiewski M, Tiefenbach J, Peters MA, Brill B, Groner B, Bach I, Heinzl T and Göttlicher M** (2003) The histone deacetylase inhibitor valproic acid selectively induces proteasomal degradation of HDAC2. *Embo Journal* **22**, 3411–3420. doi:10.1093/emboj/cdg315
- Kucera B, Cohn MA and Leubner-Metzger G** (2005) Plant hormone interactions during seed dormancy release and germination. *Seed Science Research* **15**, 281–307. doi:10.1079/SSR2005218
- Laspina NV, Batlla D and Benech-Arnold RL** (2020) Dormancy cycling is accompanied by changes in ABA sensitivity in *Polygonum aviculare* seeds. *Journal of Experimental Botany* **71**, 5924–5934. doi:10.1093/jxb/eraa340
- Law RD and Suttle JC** (2002) Transient decreases in methylation at 5'-CCGG-3' sequences in potato (*Solanum tuberosum* L.) meristem DNA during progression of tubers through dormancy precede the resumption of sprout growth. *Plant Molecular Biology* **51**, 437–447. doi:10.1023/a:1022002304479
- Law RD and Suttle JC** (2004) Changes in histone H3 and H4 multi-acetylation during natural and force dormancy break in potato tubers. *Physiologia Plantarum* **120**, 642–649. doi:10.1111/j.0031-9317.2004.0273.x
- Leeggangers HACF, Folta A, Muras A, Nap JP and Mlynarova L** (2015) Reduced seed germination in Arabidopsis over-expressing SWI/SNF2 ATPase genes. *Physiologia Plantarum* **153**, 318–326. doi:10.1111/ppl.12231
- Lepiniec L, Devic M, Roscoe TJ, Bouyer D, Zhou DX, Boulard C, Baud S and Dubreucq B** (2018) Molecular and epigenetic regulations and functions of the LAFL transcriptional regulators that control seed development. *Plant Reproduction* **31**, 291–307. doi:10.1007/s00497-018-0337-2
- Leubner-Metzger G** (2001) Brassinosteroids and gibberellins promote tobacco seed germination by distinct pathways. *Planta* **213**, 758–763. doi:10.1007/s004250100542
- Li C, Junttila O, Ernstsen A, Heino P and Palva ET** (2003) Photoperiodic control of growth, cold acclimation and dormancy development in silver birch (*Betula pendula*) ecotypes. *Physiologia Plantarum* **117**, 206–212. doi:10.1034/j.1399-3054.2003.00002.x
- Linkies A and Leubner-Metzger G** (2012) Beyond gibberellins and abscisic acid: how ethylene and jasmonates control seed germination. *Plant Cell Reports* **31**, 253–270. doi:10.1007/s00299-011-1180-1
- Linkies A, Muller K, Morris K, Tureckova V, Wenk M, Cadman CSC, Corbineau F, Strnad M, Lynn JR, Finch-Savage WE and Leubner-Metzger G** (2009) Ethylene interacts with abscisic acid to regulate endosperm rupture during germination: a comparative approach using *Lepidium sativum* and *Arabidopsis thaliana*. *The Plant Cell* **21**, 3803–3822. doi:10.1105/tpc.109.070201
- Liu H and Stone SL** (2010) Abscisic acid increases Arabidopsis ABI5 transcription factor levels by promoting KEG e3 ligase self-ubiquitination and proteasomal degradation. *The Plant Cell* **22**, 2630–2641. doi:10.1105/tpc.110.076075
- Liu Y, Koornneef M and Soppe WJJ** (2007) The absence of histone H2B monoubiquitination in the Arabidopsis hub1 (rdo4) mutant reveals a role for chromatin remodeling in seed dormancy. *The Plant Cell* **19**, 433–444. doi:10.1105/tpc.106.049221
- Liu Y, Geyer R, van Zanten M, Carles A, Li Y, Hörold A, van Nocker S and Soppe WJJ** (2011) Identification of the Arabidopsis REDUCED DORMANCY 2 gene uncovers a role for the Polymerase Associated Factor 1 complex in seed dormancy. *PLoS ONE* **6**, 1–8. doi:10.1371/journal.pone.0022241
- Liu X, Zhang H, Zhao Y, Feng Z, Li Q, Yang HQ, Luan S, Li J and He ZH** (2013) Auxin controls seed dormancy through stimulation of abscisic acid signaling by inducing ARF-mediated ABI3 activation in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 15485–15490. doi:10.1073/pnas.1304651110
- Lopez-Molina L, Mongrand S and Chua NH** (2001) A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 4782–4787. doi:10.1073/pnas.081594298
- Lopez-Molina L, Mongrand S, McLachlin DT, Chait BT and Chua NH** (2002) ABI5 acts downstream of ABI3 to execute an ABA-dependent growth arrest during germination. *Plant Journal* **32**, 317–328. doi:10.1046/j.1365-313X.2002.01430.x
- Luo M, Cheng K, Xu Y, Yang S and Wu K** (2017) Plant responses to abiotic stress regulated by histone deacetylases. *Frontiers in Plant Science* **8**, 1–7. doi:10.3389/fpls.2017.02147
- Martínez-Andújar C, Ordiz MI, Huang Z, Nonogaki M, Beachy RN and Nonogaki H** (2011) Induction of 9-cis-epoxycarotenoid dioxygenase in *Arabidopsis thaliana* seeds enhances seed dormancy. *Proceedings of the National Academy of Sciences* **108**, 17225–17229. doi:10.1073/pnas.1112151108
- Matilla AJ** (2020) Seed dormancy: molecular control of its induction and alleviation. *Plants* **9**, 1–6. doi:10.3390/plants9101402
- Matilla AJ, Carrillo-Barral N and Rodríguez-Gacio MDC** (2015) An update on the role of NCED and CYP707A ABA metabolism genes in seed dormancy induction and the response to after-ripening and nitrate. *Journal of Plant Growth Regulation* **34**, 274–293. doi:10.1007/s00344-014-9464-7
- Molitor AM, Bu Z, Yu Y and Shen WH** (2014) Arabidopsis AL PHD-PRC1 complexes promote seed germination through H3K4me3-to-H3K27me3 chromatin state switch in repression of seed developmental genes. *PLoS Genetics* **10**, e1004091. doi:10.1371/journal.pgen.1004091
- Müller K, Bouyer D, Schnittger A and Kermode AR** (2012) Evolutionarily conserved histone methylation dynamics during seed life-cycle transitions. *PLoS ONE* **7**, e51532. doi:10.1371/journal.pone.0051532
- Nagar PK and Sood S** (2006) Changes in endogenous auxins during winter dormancy in tea (*Camellia sinensis* L.) O. Kuntze. *Acta Physiologiae Plantarum* **28**, 165–169. doi:10.1007/s11738-006-0043-9
- Nambara E, Okamoto M, Tatematsu K, Yano R, Seo M and Kamiya Y** (2010) Abscisic acid and the control of seed dormancy and germination. *Seed Science Research* **20**, 55–67. doi:10.1017/S0960258510000012
- Née G, Kramer K, Nakabayashi K, Yuan B, Xiang Y, Miatton E, Finkemeier I and Soppe WJJ** (2017) DELAY of GERMINATION1 requires PP2C phosphatases of the ABA signalling pathway to control seed dormancy. *Nature Communications* **8**, 72. doi:10.1038/s41467-017-00113-6
- Nelson SK, Ariizumi T and Steber CM** (2017) Biology in the dry seed: transcriptome changes associated with dry seed dormancy and dormancy loss in the Arabidopsis GA-insensitive sleepy1-2 mutant. *Frontiers in Plant Science* **8**, 1–21. doi:10.3389/fpls.2017.02158
- Neuffer B and Eschner S** (1995) Life-history traits and ploidy levels in the genus *Capsella* (Brassicaceae). *Canadian Journal of Botany* **73**, 1354–1365. doi:10.1139/b95-147
- Neuffer B and Hurka H** (1986) Germination behaviour in populations of *Capsella bursa-pastoris* (Cruciferae). *Plant Systematics and Evolution* **161**, 35–47. doi:10.1007/BF00936010
- Nonogaki H, Nishiyama E, Ohshima K and Nonogaki M** (2020) Ancient memories of seeds: ABA-dependent growth arrest and reserve accumulation. *Trends in Genetics* **36**, 464–473. doi:10.1016/j.tig.2020.04.009
- Paul A, Jha A, Bhardwaj S, Singh S, Shankar R and Kumar S** (2014) RNA-seq-mediated transcriptome analysis of actively growing and winter dormant shoots identifies non-deciduous habit of evergreen tree tea during winters. *Scientific Reports* **4**, 1–9. doi:10.1038/srep05932
- Peeters AJM, Vries HBD, Hanhart CJ, Léon-Kloosterziel KM, Zeevaert JAD and Koornneef M** (2002) Characterization of mutants with reduced seed dormancy at two novel rdo loci and a further characterization of rdo1 and rdo2 in Arabidopsis. *Physiologia Plantarum* **115**, 604–612. doi:10.1034/j.1399-3054.2002.1150415.x

- Perrella G, Consiglio MF, Aiese-Cigliano R, Cremona G, Sanchez-Moran E, Barra L, Errico A, Bressan RA, Franklin FCH and Conicella C (2010) Histone hyperacetylation affects meiotic recombination and chromosome segregation in Arabidopsis. *Plant Journal* **62**, 796–806. doi:10.1111/j.1365-313X.2010.04191.x
- Phiel CJ, Zhang F, Huang EY, Guenther MG, Lazar MA and Klein PS (2001) Histone deacetylase is a direct target of valproic acid, a potent anti-convulsant, mood stabilizer, and teratogen. *The Journal of Biological Chemistry* **276**, 36734–36741. doi:10.1074/jbc.M101287200
- Raz V, Bergervoet JHW and Koornneef M (2001) Sequential steps for developmental arrest in Arabidopsis seeds. *Development* **128**, 243–252. doi:10.1242/dev.128.2.243
- Ren G, Xie M, Zhang S, Vinovskis C, Chen X and Yu B (2014) Methylation protects microRNAs from an AGO1-associated activity that uridylyates 5' RNA fragments generated by AGO1 cleavage. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 6365–6370. doi:10.1073/pnas.1405083111
- Richon VM, Emiliani S, Verdini E, Webb Y, Breslow R, Rifkin RA and Marks PA (1998) A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 3003–3007. doi:10.1073/pnas.95.6.3003
- Rieu I, Ruiz-Rivero O, Fernandez-Garcia N, Griffiths J, Powers SJ, Gong F, Linhartova T, Eriksson S, Nilsson O, Thomas SG, Phillips AL and Hedden P (2008) The gibberellin biosynthetic genes AtGA20ox1 and AtGA20ox2 act, partially redundantly, to promote growth and development throughout the Arabidopsis life cycle. *Plant Journal* **53**, 488–504. doi:10.1111/j.1365-313X.2007.03356.x
- Ruta V, Longo C, Boccaccini A, Madia VN, Saccoliti F, Tudino V, Di Santo R, Lorrai R, Dello Ioio R, Sabatini S, Costi R, Costantino P and Vittorioso P (2019) Inhibition of Polycomb Repressive Complex 2 activity reduces trimethylation of H3K27 and affects development in Arabidopsis seedlings. *BMC Plant Biology* **19**, 1–13. doi:10.1186/s12870-019-2057-7
- Ryu H, Cho H, Bae W and Hwang I (2014) Control of early seedling development by BES1/TPL/HDA19-mediated epigenetic regulation of ABI3. *Nature Communications* **5**, 4138. doi:10.1038/ncomms5138
- Sano N and Marion-Poll A (2021) ABA metabolism and homeostasis in seed dormancy and germination. *International Journal of Molecular Sciences* **22**, 5069. doi:10.3390/ijms22105069
- Sharma N, Xin R, Kim DH, Sung S, Lange T and Huq E (2016) NO FLOWERING IN SHORT DAY (NFL) is a bHLH transcription factor that promotes flowering specifically under short-day conditions in Arabidopsis. *Development (Cambridge)* **143**, 682–690. doi:10.1242/dev.128595
- Shen Y, Devic M, Lepiniec L and Zhou DX (2015) Chromodomain, Helicase and DNA-binding CHD1 protein, CHR5, are involved in establishing active chromatin state of seed maturation genes. *Plant Biotechnology Journal* **13**, 811–820. doi:10.1111/pbi.12315
- Shi X, Hong T, Walter KL, Ewalt M, Michishita E, Hung T, Carney D, Peña P, Lan F, Kaadige MR, Lacoste N, Cayrou C, Davrazou F, Saha A, Cairns BR, Ayer DE, Kutateladze TG, Shi Y, Côté J, Chua KF and Gozani O (2006) ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. *Nature* **442**, 96–99. doi:10.1038/nature04835
- Shu K, Zhang H, Wang S, Chen M, Wu Y, Tang S, Liu C, Feng Y, Cao X and Xie Q (2013) ABI4 regulates primary seed dormancy by regulating the biogenesis of abscisic acid and gibberellins in Arabidopsis. *PLoS Genetics* **9**, e1003577. doi:10.1371/journal.pgen.1003577
- Shu K, Liu XD, Xie Q and He ZH (2016) Two faces of one seed: hormonal regulation of dormancy and germination. *Molecular Plant* **9**, 34–45. doi:10.1016/j.molp.2015.08.010
- Singh M and Singh J (2012) Seed development-related expression of ARGONAUTE4_9 class of genes in barley: possible role in seed dormancy. *Euphytica* **188**, 123–129. doi:10.1007/s10681-012-0624-1
- Sliwinska E, Bassel GW and Bewley JD (2009) Germination of *Arabidopsis thaliana* seeds is not completed as a result of elongation of the radicle but of the adjacent transition zone and lower hypocotyl. *Journal of Experimental Botany* **60**, 3587–3594. doi:10.1093/jxb/erp203
- Sridha S and Wu K (2006) Identification of AtHD2C as a novel regulator of abscisic acid responses in Arabidopsis. *Plant Journal* **46**, 124–133. doi:10.1111/j.1365-313X.2006.02678.x
- Stone SL, Braybrook SA, Paula SL, Kwong LW, Meuser J, Pelletier J, Hsieh TF, Fischer RL, Goldberg RB and Harada JJ (2008) Arabidopsis LEAFY COTYLEDON2 induces maturation traits and auxin activity: implications for somatic embryogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 3151–3156. doi:10.1073/pnas.0712364105
- Tai HH, Tai GCC and Beardmore T (2005) Dynamic histone acetylation of late embryonic genes during seed germination. *Plant Molecular Biology* **59**, 909–925. doi:10.1007/s11103-005-2081-x
- Tanaka M, Kikuchi A and Kamada H (2008) The Arabidopsis histone deacetylases HDA6 and HDA19 contribute to the repression of embryonic properties after germination. *Plant Physiology* **146**, 149–161. doi:10.1104/pp.107.111674
- Toorop PE, Campos Cuerva R, Begg GS, Locardi B, Squire GR and Iannetta PPM (2012) Co-adaptation of seed dormancy and flowering time in the arable weed *Capsella bursa-pastoris* (shepherd's purse). *Annals of Botany* **109**, 481–489. doi:10.1093/aob/mcr301
- Tuan PA, Kumar R, Rehal PK, Toora PK and Ayele BT (2018) Molecular mechanisms underlying abscisic acid/gibberellin balance in the control of seed dormancy and germination in cereals. *Frontiers in Plant Science* **9**, 1–14. doi:10.3389/fpls.2018.00668
- Turner BM (2000) Histone acetylation and an epigenetic code. *BioEssays* **22**, 836–845. doi:10.1002/1521-1878(200009)22:9<836::AID-BIES9>3.0.CO;2-X
- Tyler L, Thomas SG, Hu J, Dill A, Alonso JM, Ecker JR and Sun TP (2004) DELLA proteins and gibberellin-regulated seed germination and floral development in Arabidopsis. *Plant Physiology* **135**, 1008–1019. doi:10.1104/pp.104.039578
- Van Zanten M, Zöll C, Wang Z, Philipp C, Carles A, Li Y, Kornet NG, Liu Y and Soppe WJJ (2014) HISTONE DEACETYLASE 9 represses seedling traits in *Arabidopsis thaliana* dry seeds. *Plant Journal* **80**, 475–488. doi:10.1111/tpj.12646
- Wang Z, Cao H, Sun Y, Li X, Chen F, Carles A, Li Y, Ding M, Zhang C, Deng X, Soppe WJJ and Liu Y-X (2013) Arabidopsis paired amphipathic helix proteins SNL1 and SNL2 redundantly regulate primary seed dormancy via abscisic acid–ethylene antagonism mediated by histone deacetylation. *The Plant Cell* **25**, 149–166. doi:10.1105/tpc.112.108191
- Wang Z, Cao H, Chen F and Liu Y (2014) The roles of histone acetylation in seed performance and plant development. *Plant Physiology and Biochemistry* **84**, 125–133. doi:10.1016/j.plaphy.2014.09.010
- Wang Z, Chen F, Li X, Cao H, Ding M, Zhang C, Zuo J, Xu C, Xu J, Deng X, Xiang Y, Soppe WJJ and Liu Y (2016) Arabidopsis seed germination speed is controlled by SNL histone deacetylase-binding factor-mediated regulation of AUX1. *Nature Communications* **7**, 1–14. doi:10.1038/ncomms13412
- Wang J, Gao S, Peng X, Wu K and Yang S (2019) Roles of the INO80 and SWR1 chromatin remodeling complexes in plants. *International Journal of Molecular Sciences* **20**, 1–17. doi:10.3390/ijms20184591
- Wang Z, Ren Z, Cheng C, Wang T, Ji H, Zhao Y, Deng Z, Zhi L, Lu J, Wu X, Xu S, Cao M, Zhao H, Liu L, Zhu J and Li X (2020) Counteraction of ABA-mediated inhibition of seed germination and seedling establishment by ABA signaling terminator in Arabidopsis. *Molecular Plant* **13**, 1284–1297.
- Wang Q, Lin Q, Wu T, Duan E, Huang Y, Yang C, Mou C, Lan J, Zhou C, Xie K, Liu X, Zhang X, Guo X, Wang J, Jiang L and Wan J (2020) OsDOGIL-3 regulates seed dormancy through the abscisic acid pathway in rice. *Plant Science* **298**, 110570. doi:10.1016/j.plantsci.2020.110570
- Wang L, Zhang F and Qiao H (2020) Chromatin regulation in the response of ethylene: nuclear events in ethylene signaling. *Small Methods* **4**, 1–7. doi:10.1002/smtd.201900288
- Weiste C and Dröge-Laser W (2014) The Arabidopsis transcription factor bZIP11 activates auxin-mediated transcription by recruiting the histone acetylation machinery. *Nature Communications* **5**, 3883. doi:10.1038/ncomms4883
- Wójcikowska B and Gaj MD (2015) LEAFY COTYLEDON2-mediated control of the endogenous hormone content: implications for the induction of somatic embryogenesis in Arabidopsis. *Plant Cell, Tissue and Organ Culture* **121**, 255–258. doi:10.1007/s11240-014-0689-8

- Wójcikowska B, Botor M, Morończyk J, Wójcik AM, Nodzyński T, Karcz J and Gaj MD (2018) Trichostatin A triggers an embryogenic transition in Arabidopsis explants via an auxin-related pathway. *Frontiers in Plant Science* **9**, 1–19. doi:10.3389/fpls.2018.01353
- Xi W, Liu C, Hou X and Yu H (2010) MOTHER OF FT and TFL1 regulates seed germination through a negative feedback loop modulating ABA signaling in Arabidopsis. *The Plant Cell* **22**, 1733–1748. doi:10.1105/tpc.109.073072
- Xu P, Tang G, Cui W, Chen G, Ma CL, Zhu J, Li P, Shan L, Liu Z and Wan S (2020) Transcriptional differences in peanut (*Arachis hypogaea* L.) seeds at the freshly harvested, after-ripening and newly germinated seed stages: insights into the regulatory networks of seed dormancy release and germination. *PLoS ONE* **15**, 1–21. doi:10.1371/journal.pone.0219413
- Yan Y, Shen L, Chen Y, Bao S, Thong Z and Yu H (2014) A MYB-domain protein EFM mediates flowering responses to environmental cues in Arabidopsis. *Developmental Cell* **30**, 437–448. doi:10.1016/j.devcel.2014.07.004
- Yang L, Jiang Z, Liu S and Lin R (2020) Interplay between REVEILLE1 and RGA-LIKE2 regulates seed dormancy and germination in Arabidopsis. *New Phytologist* **225**, 1593–1605. doi:10.1111/nph.16236
- Yano R, Takebayashi Y, Nambara E, Kamiya Y and Seo M (2013) Combining association mapping and transcriptomics identify HD2B histone deacetylase as a genetic factor associated with seed dormancy in Arabidopsis thaliana. *Plant Journal* **74**, 815–828. doi:10.1111/tpj.12167
- Yoshida M, Horinouchi S and Beppu T (1995) Trichostatin A and trapoxin: novel chemical probes for the role of histone acetylation. *BioEssays* **17**, 423–430. doi:10.1002/bies.950170510
- Yu CW, Liu X, Luo M, Chen C, Lin X, Tian G, Lu Q, Cui Y and Wu K (2011) HISTONE DEACETYLASE6 interacts with FLOWERING LOCUS D and regulates flowering in Arabidopsis. *Plant Physiology* **156**, 173–184. doi:10.1104/pp.111.174417
- Zha P, Liu S, Li Y, Ma T, Yang L, Jing Y and Lin R (2020) The evening complex and the chromatin-remodeling factor PICKLE coordinately control seed dormancy by directly repressing DOG1 in Arabidopsis. *Plant Communications* **1**, 100011. doi:10.1016/j.xplc.2019.100011
- Zhang L, Qiu Z, Hu Y, Yang F, Yan S, Zhao L, Li B, He S, Huang M, Li J and Li L (2011) ABA treatment of germinating maize seeds induces VP1 gene expression and selective promoter-associated histone acetylation. *Physiologia Plantarum* **143**, 287–296. doi:10.1111/j.1399-3054.2011.01496.x
- Zhang D, Jing Y, Jiang Z and Lin R (2014) The chromatin-remodeling factor PICKLE integrates brassinosteroid and gibberellin signaling during skotomorphogenic growth in Arabidopsis. *The Plant Cell* **26**, 2472–2485. doi:10.1105/tpc.113.121848
- Zhao M, Yang S, Liu X and Wu K (2015) Arabidopsis histone demethylases LDL1 and LDL2 control primary seed dormancy by regulating DELAY OF GERMINATION 1 and ABA signaling-related genes. *Frontiers in Plant Science* **6**, 1–9. doi:10.3389/fpls.2015.00159
- Zheng B, He H, Zheng Y, Wu W and McCormick S (2014) An ARID domain-containing protein within nuclear bodies is required for sperm cell formation in Arabidopsis thaliana. *PLoS Genetics* **10**, e1004421. doi:10.1371/journal.pgen.1004421
- Zhou C, Lin Q, Lan J, Zhang T, Liu X, Miao R, Mou C, Nguyen T, Wang J, Zhang X, Zhou L, Zhu X, Wang Q, Zhang X, Guo X, Liu S, Jiang L and Wan J (2020) WRKY transcription factor OsWRKY29 represses seed dormancy in rice by weakening abscisic acid response. *Frontiers in Plant Science* **11**, 1–15. doi:10.3389/fpls.2020.00691
- Zhu H, Xie W, Xu D, Miki D, Tang K, Huang C-F and Zhu J-K (2018) DNA demethylase ROS1 negatively regulates the imprinting of DOG1 and seed dormancy in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences* **115**, 201812847. doi:10.1073/pnas.1812847115