The DELLA-LDL mediated control of germination via DELAY OF GERMINATION 1.

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1 Abstract

Germination is the process of a seed beginning to grow, and the proper timing of germination is vital to both plants and humans. Further understanding of the control of germination may contribute towards the improvement of human agriculture and the alleviation of major problems, such as pre-harvest sprouting in cereal crops. Seed dormancy ensures that germination occurs in favourable conditions and is regulated by the gibberellins and their repressors, the DELLA proteins. *DELAY OF GERMINATION 1 (DOG1)* has been labelled the master regulator of germination due to its considerable impact on seed dormancy. There is an emerging role for *LYSINE-SPECIFIC DEMETHYLASE LIKE 1* and *2* (*LDL1* and *LDL2*), chromatin remodelling enzymes (CREs) which regulate chromatin dynamics to repress *DOG1* expression and seed dormancy.

This thesis explored the possibility of interactions between the DELLAs and CREs through protein-protein interaction assays using a screening library designed to identify molecular interactions between hormone signalling genes and chromatin remodelling enzymes. The meaning of these interactions was explored through numerous experiments in the model plant species *Arabidopsis thaliana*, including analyses of germination timing combined with analyses of *DOG1* expression. These analyses were conducted in multiple conditions, including altering the balance of GAs, and the use of a *5PLE (quintuple DELLA)* mutant lacking all five DELLA proteins.

This thesis identifies multiple interactions between the DELLA and LDL proteins, amongst further interactions between the DELLAs and other chromatin remodelling enzymes. The combined germination and expression analyses report that the DELLA proteins are important in regulating *DOG1*, specifically through their role in cold-stratification mediated abolition of seed dormancy. Through integrating novel results reported with current research, this thesis proposes that the DELLA-LDL interactions are implicit in maintaining dormancy in unstratified seeds via *DOG1* and that the cold-stratification which breaks seed dormancy may also break the DELLA-LDL reaction, enabling germination.

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In dedication to my late grandfathers, John Burke and Kenneth West.

For getting me in the garden and supporting me always.

Thank you, for everything.

6 Introduction

6.1 Development Context

Though we possess many differences to plants, the plant kingdom excels in a particularly enviable characteristic. Plant development is plastic, allowing plants to respond to environmental changes, negating the disadvantages of their sessility and favouring survival (Casal *et al.*, 2004). Phenotypic plasticity can be defined as "*the ability of individual genotypes to produce different phenotypes when exposed to different environmental conditions"* (Pigliucci *et al.*, 2006).

Most of our understanding of phenotypic plasticity results from plant studies, due to their ease of cloning or inbreeding, and the dramatic effects environment has on plant development (Sultan, 2000). There also exists more rapidly reversible plasticity, of biochemical or physiological responses, which may feed into developmental alterations (Pigliucci *et al.*, 2006). Understanding the mechanisms of plasticity is key to our understanding of plant development.

6.2 Genetic Regulation and Plasticity

A plant must be able to perceive and respond to various external cues such as light quality, light quantity and temperature, to name but a few. For example, leaves grown in the light tend to be thicker, with a smaller surface area to both maximise photosynthesis and cool the leaf effectively (Rozendaal *et al.*, 2006) (***Figure 1***). Further, exogenous information must be integrated with endogenous, internal signals, and the genetic system to elicit a response (Davière *et al.*, 2016). It is the perception and processing of these cues that drives a change in phenotype, through epigenetic, transcriptional or post-transcriptional regulation (Nicotra *et al.*, 2010). The simplest and most effective method to modulate physiology is simply an alteration in gene transcription. This, however, requires a genome which can respond as the environment changes.

Figure 1: Phenotypic Plasticity

Two individuals of Polygonum cespitosum displaying phenotypic plasticity. ***Left:*** *Grown in dry soil and plentiful light, has developed multiple branches and extensive root tissue.* ***Right:*** *Grown in moist soil and simulated canopy conditions, developed extensive and large leaves in a more unbranched upright architecture. Image taken from Sultan, 2010.*

6.3 Chromatin

6.3.1 Chromatin Structure

Changes in chromatin dynamics are a prominent way in which transcriptional activity is regulated, and are emerging as an important factor in the flexibility of plant genomes.

The DNA inside one human cell would measure roughly one yard stretched out, therefore eukaryotic DNA is tightly wrapped around histones, being compressed into the nucleoprotein complex known as chromatin, which aids the storage of DNA. The nucleosome is the individual subunit of the chromatin complex and consists of 1.7 superhelical turns (147 base-pairs) of DNA wrapped around an octamer of four core histone proteins. An H3-H4 tetramer and two H2A-H2B dimers compose the nucleosome octamer ***(Figure 2)*** (Phillips and Shaw 2008*;* Jenuwein and Allis, 2001). These histone proteins are mostly globular in structure; however, they do also possess a protruding N-terminal “tail” (Kouzarides, 2007)**.**

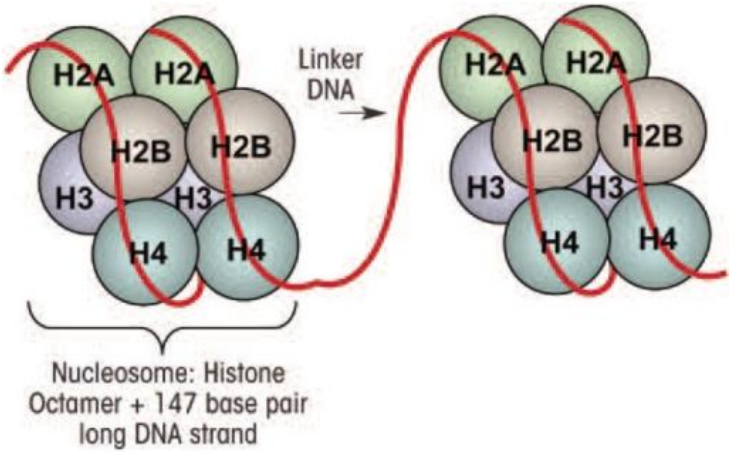

Figure 2: The Nucleosome

Visualisation of two linked nucleosome units in the chromatin structure and their individual histone subunits. Figure taken from Starkman et al., 2012.

6.3.2 Chromatin Modifications

Histones, in particular the N-terminal tails, are subject to a number of post-translational modifications, primarily targeting lysine residues. Such modifications may include, but are not limited to; methylation, acetylation, phosphorylation and ubiquitinylation. These modifications modify residue charges, altering chromatin architecture and, in turn, the accessibility of DNA for many genomic processes. Such genomic processes include, but are not limited to, DNA repair, replication, condensation, recombination, and transcription (Kouzarides, 2007).

Methylation, phosphorylation and acetylation are examples of epigenetic, N-tail histone modifications which provide dynamic and reversible changes to the histone, and further, to chromatin structure, without altering the DNA sequence (Goldberg *et al.*, 2007). These post-translational modifications affect transcriptional activity in both direct and indirect manners, modulating DNA accessibility to transcription factors or recruiting effector protein complexes to the nucleosome, to provide respective examples (Berger, 2007). Both direct and indirect effects may be key in how plant plasticity is directed by changes in both exogenous and endogenous signals.

Acetylation and deacetylation of N-terminal tails promote gene activation and repression, respectively. Addition and removal of acetyl groups to the histone proteins occurs at their lysine side chains, with histone acetyltransferases (HATs) depositing acetyl groups and

histone deacetylases (HDACs) removing the acetyl groups (Bannister and Kouzarides, 2011). The presence of acetyl groups neutralises the positively charged lysine side chains, reducing the strength of interaction between negatively charged DNA and histone proteins. This acetylation, therefore, allows the DNA to relax around the histone, permitting access to transcription factors and driving transcriptional activation of genes in close proximity to the modification (Berr, Shafiq and Shen, 2011). The relaxed and transcriptionally active state of chromatin is known as euchromatin, the compact inactive state is referred to as heterochromatin ***(Figure 3)***.

Histone methylation is a more nuanced example of modifications and, unlike acetylation, does not alter the charge of the histone. Methylation mainly occurs on the side chains of lysine and arginine residues and may yield more complex effects than acetylation (Bannister and Kouzarides, 2011). Methylations may be activating or deactivating, depending on the level of methylation. Lysine residues may be mono-, di- or tri-methylated; whereas arginines may be mono-, symmetrically or asymmetrically di-methylated (Ng *et al.*, 2009). The effects methylation has on the plant vary, depending on the specific residue that is methylated; for example, methylation at H3K27 (Histone 3, Lysine 27) and H3K9 is classically implied in silent chromatin, whereas H3K4 and H3K36 methylation is traditionally implicated in active chromatin (Kouzarides 2007) ***(Figure 3)***. Though histone methylation was considered a static modification for many years, like acetylation, methylations are reversible and are removed by Histone Demethylases (HDMs) and deposited by Histone Methyltransferases (HMTs) (Bannister *et al.*, 2002).

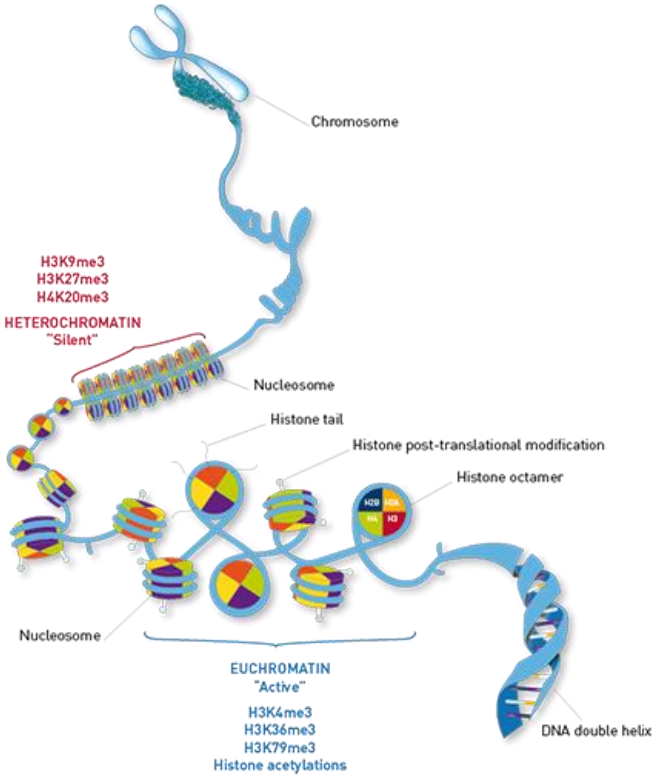

Figure 3: Chromatin State Modifications

Diagrammatic representation of the effects of chromatin modifications on chromatin state. Image taken from https://www.diagenode.com/en/categories/histone-antibodies

6.3.3 Writers, Erasers and Readers

Post-translational histone modifications such as those discussed above are regulated by "writers" and "erasers" which add and remove histone marks, respectively (Srivastava *et al.*, 2016). Writers are enzymes which add modifications to histones and include the HATs and HMTs mentioned earlier. Likewise, erasers remove modifications to histones, such as the HDACs and HDMs discussed (Gillette and Hill, 2015). The ways in which writers and erasers affect DNA transcription are controlled by "readers" which recognise and bind to specific active or repressive "marks" or modified sites. Readers may perceive methylated lysine residues differently depending on their level of methylation and also the neighbouring amino acid sequence (Hyun *et al.*, 2017).

Writers and erasers are expressed differentially in a spatio-temporal manner and display varying roles in directing plant development, for example in response to abiotic stresses (Asensi *et al.*, 2017). Histone methyltransferases play a role in many responses in plants, including stress responses. According to TAIR, there are currently 62 loci identified in *Arabidopsis*, representing 132 distinct genes which code for histone methyltransferases. There is evidence for partial redundancy in the function of these HMTs, such as redundancy between Su(var)3-9 homologs (SUVHs) in *Oryza sativa* (Qin *et al.*, 2010).

Named after the SuVar(3-9), E(z), and Trithorax domain proteins key to *Drosophila* development, the SET domain displays methyltransferase activity and lends its name to a group of HMTs (Yeates, 2002). For example, the histone methyltransferase SET DOMAIN GROUP8 (SDG8) is responsible for the conversion of H3K36me1 to H3K36me3, thereby being classed as a 'writer' which deposits histone marks. SDG8 is heavily involved in defence against fungal pathogens, with *sdg8* mutant plants being less resistant to both *Alternaria brassicicola and Botrytis cinerea*, which are necrotrophic fungal pathogens. *SDG8* is heavily expressed in the root tips of 8-day old Arabidopsis seedlings and also at the leaf margins and in the anther vasculature of the flower. However, *SDG8* expression is also induced in response to mechanical wounding (Berr *et al.*, 2010).

Similarly, the *Arabidopsis* histone deacetylase *HD2A (histone deacetylase 2 A)* is highly expressed in flowers and younger siliques, whilst *HD2B* is expressed in a more constitutive manner (Wu *et al.*, 2000). The *HD2* deacetylases are 'erasers', removing acetyl marks which impair seed development. This is evidenced by aborted seed development being observed in *Arabidopsis* plants where the *HD2A* gene was silenced.

The enzymes which drive histone modifications, chromatin configuration and therefore transcriptional activity, within *Arabidopsis* and further plant species, are evidently regulated differentially in time and space and exhibit a range of modification states. This level of complexity would suggest that changes in chromatin configuration can produce specific and detailed transcriptional changes, which play a role in allowing sessile plants to respond to the endogenous and exogenous signals relating to changes in the environment. The expression and action of specific enzymes must, therefore, have specific drivers behind their control.

6.4 Hormones and Control of Chromatin/Transcription

Plant meristems are groups of cells which retain the capacity to divide which is normally displayed by embryonic cells. They are a key component of plant plasticity, allowing the development of new organs, even in adult plants. Transcription factors play a key role in modulating meristematic activity, as do the writers and erasers which dictate chromatin structure (Lee *et al.*, 2019). Plant hormones (Phytohormones) are also implicit in regulating meristematic activity, linking with the genetic regulatory network. For example, cytokinins dictate cell division and specify stem cell fate in the meristems by inducing *WUSCHEL (WUS)* expression (Lee *et al.*, 2019*;* Wang *et al.*, 2017). WUS is a homeodomain transcription factor, which migrates from the Organizing Centre (the stem cell niche of shoot meristems) to the Central Zone, driving expression of *CLAVATA3 (CLV3).* CLV3, a peptide secreted from the *CLV3* gene, activates CLV1, a transmembrane receptor kinase in the Organizing Centre, which inhibits *WUS* expression, limiting the production of the WUS transcription factor. This feedback loop aids in maintaining the size of the stem cell pool in the Organizing Centre (Wang *et al.*, 2017). Abscisic acid (ABA) is a particularly important phytohormone in plant responses to environmental stresses, including osmotic stress. Limited water availability results in increased salinity, mirrored by a proportional increase in endogenous ABA concentration (Zhang *et al.*, 2006). This production of ABA regulates stomatal opening and leaf growth, integrating changes in environmental conditions into developmental changes (Dodd and Davies, 1994).

Phytohormones are, then, vital to how plants sense changes in their environment and help to modulate transcription of genes in order to respond to these changes, a valuable aid to plant plasticity. Phytohormones also modulate transcriptional activity through driving changes in chromatin state. MONOPTEROS (MP) is a transcription factor which specifies meristematic and primordium fate in the shoot apical meristem, many MP target sites are controlled via chromatin dynamics. When auxin is present, chromatin remodellers belonging to the SWITCH/SUCROSE NON-FERMENTABLE (SWI/SNF) subfamily of chromatin remodelling complexes interact with MP, revealing the promoter regions of key downstream genes for MP signalling (Lee *et al.*, 2019) ***(Figure 4)***. Phytohormones are key to the phenotypic plasticity which allows plants to modulate their developmental processes in response to exogenous stimuli.

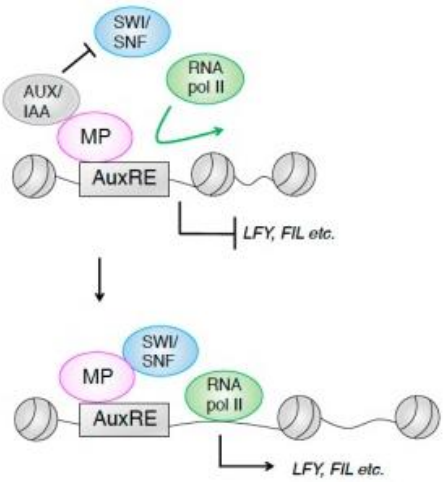

Figure 4: SWI/SNF and MONOPTEROS

The presence of auxin leads to the degradation of Aux/IAA. The interaction of SWI/SNF with MP, and resulting transcriptional activation, is dependent on phytohormonal changes. Image taken from Lee et al., 2019.

6.5 Gibberellins

Gibberellins (GAs) are an incredibly important class of phytohormones, their role as endogenous regulators is essential in regulating plant growth and development (Peng *et al.*, 1999). GAs are involved in many developmental processes including; seed germination, pollen maturation, and the induction of flowering, (Achard and Genschik, 2009). As a phytohormone with such a large role in plant development, gibberellins present a good focus for understanding the interplay between how plants respond to their environment, partially through chromatin remodelling.

The first gibberellin characterised, in the 1930s, was a metabolite produced by the pathogenic fungus *Gibberella fujikuroi,* which caused excessive elongation of rice stems (Silverstone and Sun, 2000). The discovery and application of mutants insensitive to GAs led to the 'Green Revolution', a period where cereal crop yields improved drastically, owing to less energy input into upwards growth and more into grain yield. Understanding

of GAs has significantly improved our agricultural processes and since the 1930s over 100 GAs have been identified in plants (Yamaguchi, 2008).

6.5.1 Biosynthesis of Gibberellins

Though there are over 100 GAs currently identified in plants, and three common bioactive GAs: GA_1, GA_3 and GA_4, which all stem from a GA_{12} precursor (Olszewski *et al.*, 2002; Yamaguichi, 2008). The biosynthesis of these bioactive GAs begins with geranylgeranyl diphosphate (GGDP), a common precursor for diterpene compounds such as those within GAs. GGDP is converted to GA_{12} through a series of enzymatic reactions involving terpene synthases (TSPs) and cytochrome P450 monooxygenases (P450s). This inactive precursor can be converted into bioactive GAs through a series of oxidation reactions, utilizing a range of 2-oxoglutarate-dependent dioxygenases (2ODDs) (***Figure 5***) (Sun, 2008).

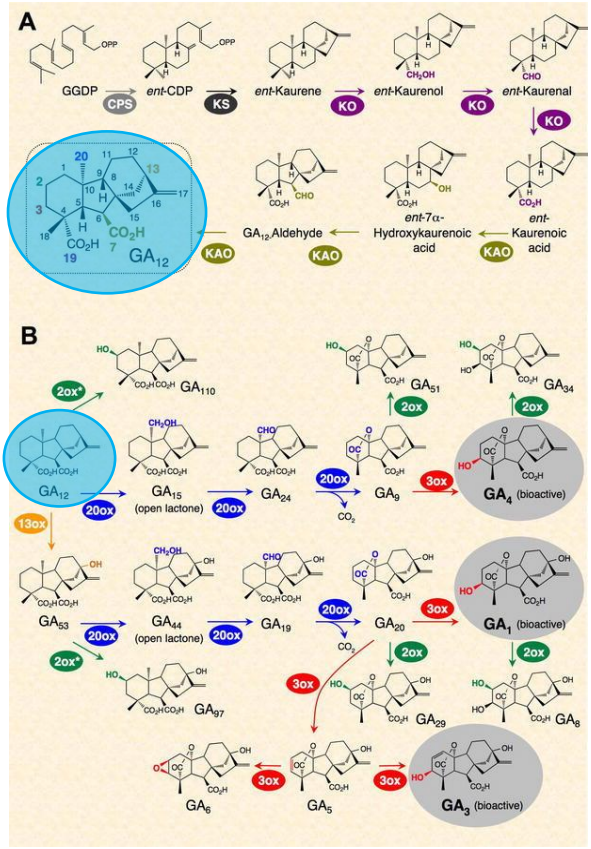

Figure 5: GA Metabolism and Catabolism

A: *Synthesis of GA_{12} precursor (blue circles) from GGDP.* ***B:*** *Biosynthesis of bioactive GAs (grey circles) and deactivation by GA2ox. Figure taken and adapted from Sun et al., 2008.*

6.5.2 Regulation of Gibberellin Biosynthesis

Changes in signalling activity, whether due to intrinsic genetic mutations or due to extrinsic treatments, have been observed to cause changes in some GA biosynthesis genes – namely the 2ODDs *GA20ox* and *GA3ox*, and the catabolic 2ODD gene *GA2ox* (Sun and Gubler, 2004). One such extrinsic treatment is the plant growth retardant paclobutrazol,

which can inhibit the biosynthesis of GAs by blocking one of the P450 enzymes, kaurene oxidase (Hedden and Graebe, 1985)

It, therefore, occurs that GA signalling might be an important component in how plants utilise their plastic development to respond to changes in their environmental conditions. The light-driven activation of Phytochromes PhyA and/or PhyB in *Arabidopsis thaliana* seeds leads to increased expression of the biosynthetic 2ODD genes *GA3ox1* and *GA3ox2* and decreased expression of catabolic gene *GA2ox2.* Thus, the perception of light consequently results in heightened GA signalling and elevated levels of the bioactive gibberellins such as GA_4, which promote germination (Seo *et al.*, 2006*;* Oh *et al.*, 2007).

6.6 Gibberellin Function and the DELLA Proteins

It would be remiss to discuss the importance of the gibberellins without also discussing the DELLA proteins and the integral part they play in GA signalling. The DELLA proteins are a subset of the GRAS family of putative transcription regulators, named after the first three family members: GIBBERELLIC-ACID INSENSITIVE (GAI), REPRESSOR OF GAI (RGA) and SCARECROW (SCR). The DELLAs are specific to plants, and act antagonistically to gibberellins, opposing almost all processes which GA signalling promotes, including seed germination and growth (Achard and Genschik, 2009)

6.6.1 DELLA Structure and Function

There are five DELLA proteins identified in Arabidopsis; GAI (GIBERELLIC-ACID INSENSITIVE), RGA (REPRESSOR OF ga1-3), RGL1 (RGA-like 1), RGL2, and RGL3, which all have individual functions within suppressing the downstream signalling response of GAs (Davière *et al.*, 2016). Belonging to the GRAS family of plant-specific proteins, the DELLAs all share a C-terminal GRAS domain which is key to how they interact with other proteins for DNA binding. The DELLA proteins are distinguished by their DELLA/TVHYNP N-terminal motif, which is involved in modulating the activity of DELLA proteins in response to gibberellins (Yoshida *et al.*, 2014) (Tyler *et al.*, 2004). The five DELLA proteins are believed to be functionally redundant with qPCR analyses supporting this. RGA and GAI are expressed at high levels across all tissues with *RGL1, 2*

and *3* genes showing peak expression in germinating seeds and/or developing flowers, whereas only low transcript levels were observed in vegetative tissues (Tyler *et al.*, 2004).

GAs are integral to many growth-regulating networks, which respond to environmental cues such as light, temperature or osmotic stress. In the absence of GAs, these responses are repressed by the DELLA proteins. The DELLA proteins are believed to function as transcriptional regulators but do not possess putative DNA-binding domains. There are multiple suggestions for how the DELLAs regulate transcription, though they can also function via non-transcriptional methods (Locascio *et al.*, 2013). DELLAs can form complexes with transcription factors, or inhibit other transcription factors, such as PHYTOCHROME INTERACTING FACTOR 4 (PIF4) which is a key component in how plant development responds to changes in light quality and also temperature (Claeys *et al.*, 2014).

Though the DELLA proteins inhibit GA driven responses, the presence and binding of GAs to their soluble receptor GA INSENSITIVE DWARF 1 (GID1), and subsequently the binding of GID1 to the DELLA proteins and the F-box protein SLEEPY (SLY1), targets the DELLAs for ubiquitination and subsequent degradation by the 26s-proteasome ***(Figure 6)*** (Davière *et al.*, 2016). Therefore, an important component of GA signalling is the degradation of the DELLA proteins which inhibit downstream GA signalling promoted development processes.

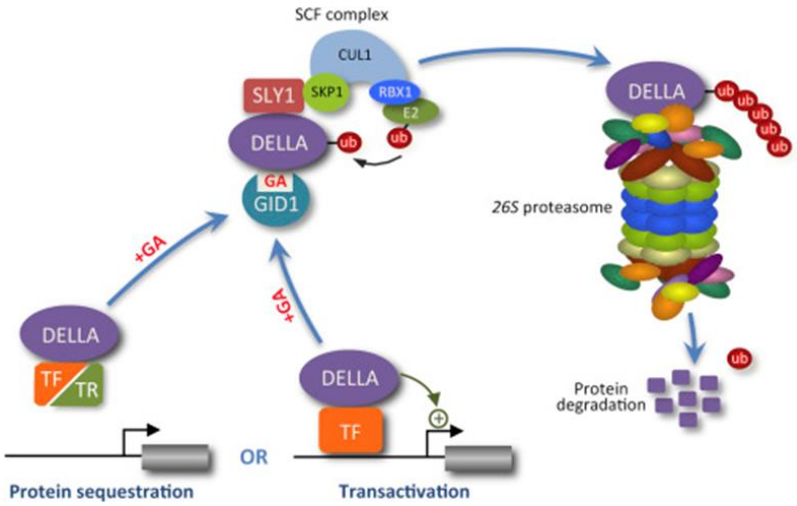

Figure 6: DELLA Degradation by the 26S Proteasome

The mechanism of DELLA degradation by the ubiquitin-proteasome pathway, triggered by the presence of GA binding to the GID1 receptor. Figure taken from Davière et al., 2016

Further, in a negative feedback loop, DELLAs also promote the activation of genes for the GA receptor GID1 and the 2ODD biosynthetic enzymes GA20ox and GA3ox ***(Figure 7)*** (Claeys *et al.*, 2014). Therefore, the DELLA proteins appear to play a role in their own downregulation by upregulating GA signalling, similar to how GAs self-regulate their homeostasis through regulating their own metabolism and catabolism.

6.6.2 Developmental Role of DELLA Proteins

Alongside their widespread pattern of expression, RGA and GAI regulate a large number of processes, including the expansion of shoot, root and hypocotyl cells (de Lucas *et al.*, 2008*;* Davière *et al.*, 2013). RGA, GAI and RGL1/2 function to regulate floral development, with experiments under GA-deficient conditions showing a further role for RGL2 in negatively regulating seed germination (Lee *et al.*, 2002*;* Cao *et al.*, 2005).

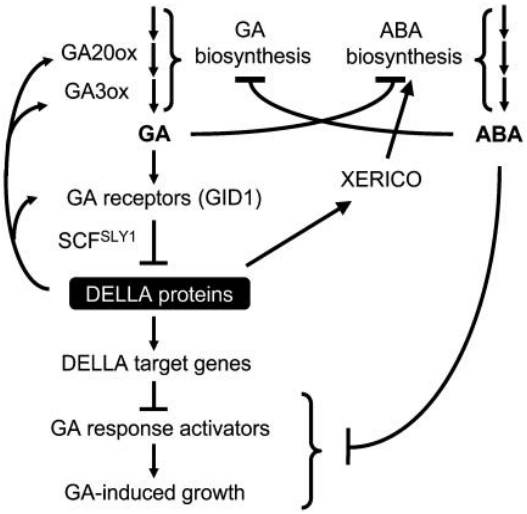

Figure 7: DELLA Feedback Mechanisms

A diagrammatic representation of the role of DELLAs in influencing their own expression, alongside their role in ABA and GA expression. Figure taken from Claeys et al., 2014

With the widespread expression of GAI and RGA and the pinpointed expression of the RGL DELLAs to germinating seeds, or developing flowers, these might be particularly pertinent areas of plant development to investigate within the GA-DELLA signalling framework.

Besides their roles in suppressing GA signalling, DELLA proteins are also thought to target the XERICO protein for upregulation, which in turn upregulates ABA metabolism (Eckardt *et al.*, 2002) ***(Figure 7)***. Therefore, DELLAs may be seen to play an important role in the balancing of GA and ABA signalling, key in the germination process and something this paper will discuss in further detail. qPCR, RT-PCR and T-DNA insertion mutant analyses all point towards RGL2 being the primary DELLA involved in the repression of germination (Tyler *et al.*, 2004). However, other studies have indicated that RGL1 may be implied in the regulation of seed germination, due to the germination of

RGL1 silenced seeds whilst in the presence of paclobutrazol, an inhibitor of GA biosynthesis (Wen and Chang, 2002). Whilst the role of RGL1 in the regulation of germination may need to be explored further, certain DELLA proteins appear to play a critical role in the germination process, perhaps illuminating a process in which the environment influences plant plasticity and variable expression within a flexible genome.

6.6.3 Gibberellins, DELLAs and Chromatin Remodelling

Thus, Gibberellins are incredibly important phytohormones, regulated both by themselves and the DELLA proteins. GAs play a critical role in how environmental changes may influence plant development through changes in endogenous signalling, driving alterations in gene expression. It would be nonsensical to discuss gene expression and not investigate links between gibberellins, DELLAs and chromatin remodelling.

Current understanding of chromatin remodelling is generally limited and even more so concerning the DELLAs. The SWI/SNF family of chromatin remodelling complexes are key to plant growth and development; without these subunits plant phenotypes are significantly impaired (Sarnowska *et al.*, 2013). The inactivation of SWI3C, the core component of SWI/SNF remodelers, inhibits DELLA dependent expression of *GID1*, the soluble receptor of GAs. This opposes both germination and growth, showing a way in which chromatin modifications may factor into the GA-DELLA signalling pathway (Claeys *et al.*, 2014) ***(Figure 6)***. Sarnowska's group, *via in vivo* bimolecular fluorescence complementation assays ***(Figure 8)***, showed that the SWI3C component can interact with RGL2 and RGL3 which affect the transcriptional activation of the GID1 (GA perception) and GA3ox (GA biosynthesis) genes.

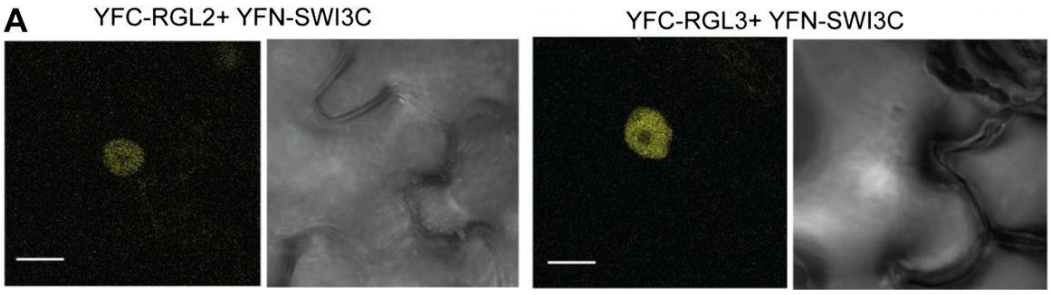

Figure 8: BiFC Assay of DELLA/SWI3C Interaction

Bimolecular fluorescence complementation assays showing how the SWI3C component of SWI/SNF CRCs interacts with the RGL2 and RGL3 proteins. Taken from Sarnowska et al., 2013

Furthermore, both SWI3C and DELLAs upregulate expression of SCARECROW LIKE 3 (SCL3), the complex containing SLY1, and GA biosynthetic enzymes (Zentella *et al.*, 2007*;* Archacki *et al.*, 2013). The physical interaction of SWI3C with DELLAs and SLY1 would indicate the SWI/SNF chromatin remodelling complexes may possess a necessary function to permit DELLA mediated activation of genes involved in GA signalling and vital developmental processes. However, the result of such interactions may be dependent upon the specific area of plant development investigated i.e. germination, root growth etc (Archaki *et al.*, 2013).

Recent literature has also proposed the involvement of the PICKLE (PKL) chromatin remodelling enzyme, also of the SWI/SNF family, in GA signalling within *Arabidopsis thaliana* (Zhang *et al.*, 2014). PKL inhibits the expression of genes specific to seeds during germination and drives the embryonic-to-vegetative transition.

The *pkl* mutant is characterised by a thick, green primary root displaying embryonic characteristics, a low penetrance of the 'pickle root' phenotype which is dramatically increased in GA deficient conditions. Adult *pkl* mutants are semi-dwarf, similar to mutants with reduced GA signalling. Together this evidence may suggest a positive role for PKL in regulating GA signalling (Park *et al.*, 2017). Through the use of a hextuple mutant, containing both the *pkl* mutant and the quintuple (*5PLE*) *della* mutant, Park *et al.* showed that PKL is required for most developmental processes promoted by GA signalling. Including hypocotyl and leaf elongation; and phase transitions from juvenile-to-vegetative and vegetative-to-reproductive phases. Further, the expression of 80% of GA-dependent genes in seedlings is PKL dependent.

PICKLE has also been shown to interact with PHYOTOCHROME-INTERACTING FACTOR 3 (PIF3) and BRASSINAZOLE-RESISTANT 1 (BZR1), critical players in light and brassinosteroid signalling, driving the interaction between the PICKLE remodelling enzyme and cell elongation-related genes (Zhang *et al.*, 2014) ***(Figure 9)***. Zhang *et al.* also reported PKL, PIF3 and BZR1 repressing H3K27me3 on target promoters, with GA_3 also involved in repression of H3K27me3 separately. Their findings suggest the PICKLE chromatin remodeler is critical for integrating the phytohormones brassinosteroids and gibberellins, and light signalling into the epigenetic regulation of plant development. Moreover, this provides a clear example of the interplay between phytohormonal

signalling, environmental cues such as light and regulation of gene expression through chromatin remodelling.

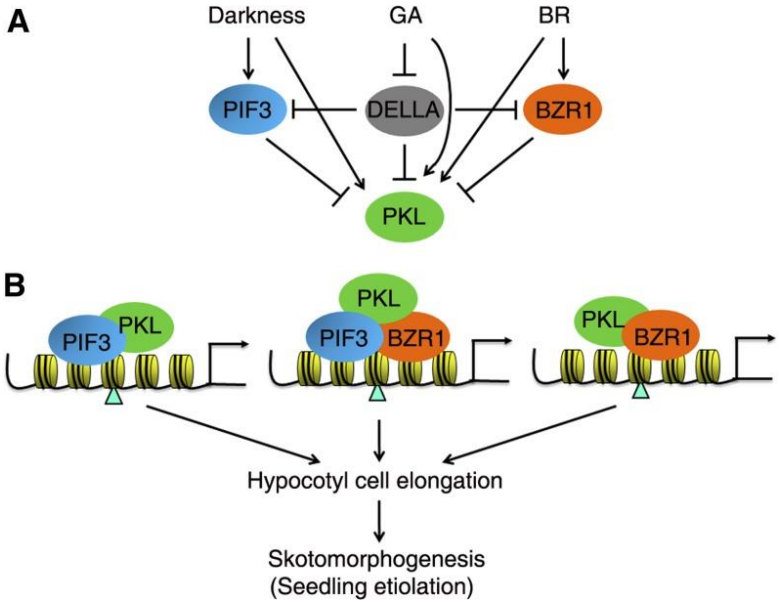

Figure 9: The Mechanism of DELLA-PICKLE Interaction

A: *Integration of light, gibberellins and brassinosteroids in the control of PKL.*

B: *Recruitment of PKL via transcription factors PIF3 and BZR1. Figure taken from Park et al., 2017*

6.7 Germination

Thus, the theme of our research is the critical role that chromatin state (and the modifications dictating chromatin state) hold in regulating gene expression within the flexible plant genome, thereby mediating responses to environmental changes and stimuli. Phytohormones such as the gibberellins are heavily involved in regulating gene expression, with both gibberellins and their DELLA repressors interacting with chromatin remodelling complexes.

Arguably the most important process for plant development is germination, the beginning of a plant's life. Phytohormones are intrinsically linked with the process of germination, DELLA expression is widespread throughout germinating seeds, and gibberellins accumulate in high concentrations during this stage. Germination is a critical moment for

both plants and human life, determining crop yield and crop quality. An in-depth understanding of germination is of great benefit not only to our academic understanding of plant development but also to the challenges which face agriculture.

6.7.1 What is Germination?

The process of germination begins with the uptake of water by the dry seed (imbibition), followed by the expansion of the internal embryo within the seed. This uptake is divided into three phases; the initial imbibition which is followed by a second phase where water uptake plateaus, and finally, water uptake recommencing in the third phase. In this third phase the embryonic axis elongates and protrudes through seed coat, thereby completing germination (Finch-Savage and Leubner-Metzger, 2006). Succinctly, germination encapsulates the transition from seed to seedling in the plant life cycle (Huo *et al.*, 2016). Germination is, therefore, a vital process in the development of both individual plants and also of plant populations.

6.7.2 Importance of High Specificity

In an evolutionary sense, the role of seeds is to produce viable, maintainable offspring. To promote successful germination, and therefore propagation of one's genetic material, plants employ a variety of strategies to ensure germination occurs in favourable conditions. Though the spatial distribution of germination may differ, most physiological attempts to promote successful germination come via temporal regulation (Nonogaki, H, 2010). Seeds may time germination in order to avoid competition with other germinating seeds, and also to ensure the seedling enters into favourable conditions.

Seed dormancy refers to the temporally-based suppression of germination and is one such evolved mechanism that plants utilise to promote a favourable germination. The triggering of such dormancy occurs as the seed begins its maturation stage; dormancy is released after a certain period of time known as "after-ripening". This is widely observed in a wide range of seed plants (Bewley *et al.*, 2012).

Proper timing of germination is not only vital for the survival of individual plants, but also holds great importance in our agricultural industry, providing scope for massive improvement not only in how we feed populations, but also the economic implications.

Dormancy is a somewhat undesirable trait in agriculture, where rapid germination and rapid growth are desirable, therefore modern crop species selected from wild relatives show reduced dormancy (Gao and Ayele, 2014). This does, however, run the risk of both crop quality and crop yield loss due to germination in unfavourable conditions.

In cereal crops, vivipary or pre-harvest sprouting (PHS) refers to germination of grains before harvesting ***(Figure 10)***, whilst still on the ear of the plant, which results in major losses to the cereal crop industry, totalling close to $1 billion annually (Bewley *et al.*, 2006). With wheat being a staple food source for over 40% of the world population, providing 19% of global calorie intake and 21% of global protein intake and heavy PHS occurring in anywhere from 6-20% of rice acreage in south China alone, understanding the timing of germination fully is a critical goal for agriculture (Singh and Upadhyaya, 2015*;* Guo *et al.*, 2004). Dormancy is therefore desirable for some cereals, however, malting of barley, a key process in the manufacturing of profitable products such as beer and whiskey, is reliant upon rapid and uniform germination of grains (Gubler *et al.*, 2005).

Figure 10: Pre-Harvest Sprouting in Triticum aestivum

Illustration of Pre-Harvest Sprouting in common wheat, Triticum aestivum. *Image taken from TradingFloor.com*

6.8 Seed Dormancy

6.8.1 Interactions Influencing Seed Dormancy

Seed dormancy is released at precise times, allowing germination at very specific points in time. This timing, therefore, requires precise sensing of the dynamic environment in which plants exist and the ability to respond to multiple varied inputs in order to time germination correctly to seasons (Huo *et al.*, 2016). Central players in the regulation of seed dormancy are the antagonistic effects of the phytohormones gibberellins (promotive) and abscisic acid (inhibitory), whose action and presence are varied as a response to environmental cues (Nonogaki, H, 2010) ***(Figure 11)***.

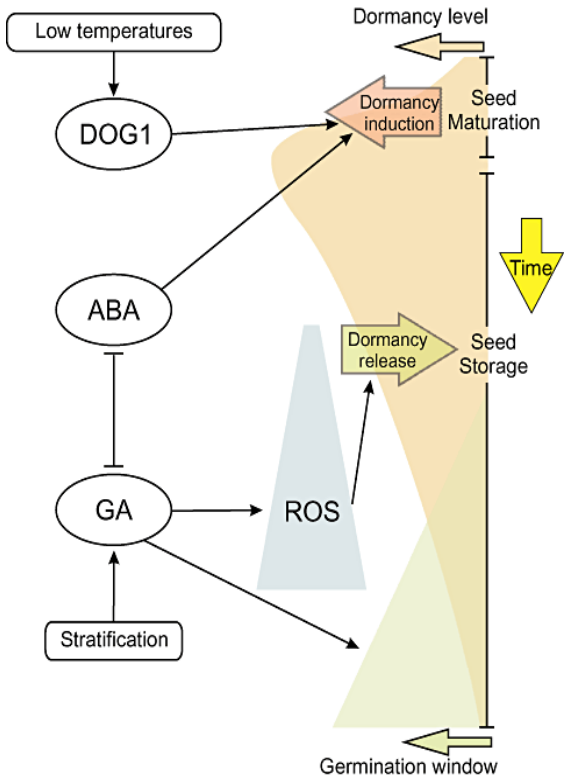

Figure 11: The Molecular Control of Seed Dormancy

The molecular mechanisms of seed dormancy, displaying the antagonistic action of GAs and ABA in impeding and promoting seed dormancy, respectively. Figure taken from Graeber et al., 2012.

The mechanism by which these antagonistic, phytohormonal effects are specified eluded us for quite some time, however, identification and analysis of two rate-limiting hormone metabolism genes, *NCED* (ABA biosynthesis) and *GA2ox* (GA deactivation), have provided a more detailed image of the roles of GAs and ABA in seed dormancy (Seo *et al.*, 2009).

6.8.2 Abscisic Acid and Seed Dormancy

Abscisic Acid (ABA) promotes the initiation and maintenance of seed dormancy. Qualification of this is observable in *abi1-1* and *abi2-1* mutants which results in ABI proteins being unable to bind to downstream ABA receptors, resulting in a phenotype displaying reduced dormancy (Graeber *et al.*, 2012).

The current proposed mode of action for ABA signalling in seed dormancy has its origins as recently as 2007. PYR1, a member of the PYR/PYL family of StAR-related Lipid Transfer (START) proteins, is proposed as a key regulator of the ABA signalling pathway. ABA binds to the PYR1 protein, facilitating binding of this complex to the Group-A Type-C Protein Phosphatases (PP2Cs), which negatively regulate ABA signalling (Allen *et al.*, 1999). This interaction relieves the repression of SNF1-Related Protein Kinase 2 (SnRK2) proteins, which are important positive regulators of ABA signalling and seed dormancy (Yoshida *et al.*, 2002*;* Fujii *et al.*, 2007). SnRK2 autophosphorylates, autoactivating itself to then phosphorylate downstream transcription factors (TFs) leading to transcriptional activation of ABA-responsive genes (Sheard and Zheng, 2009) ***(Figure 12)***.

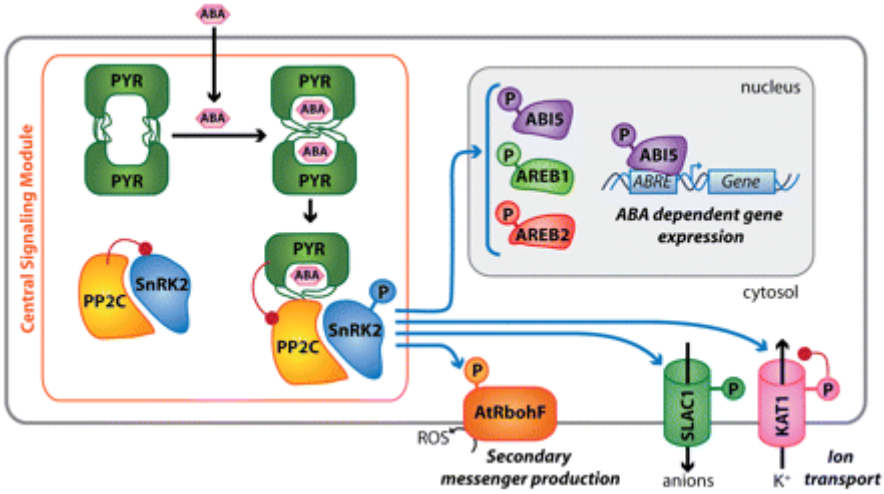

Figure 12: Model of ABA Signal Transduction

Figure taken from Hubbard et al., 2010.

ABA insensitive mutants named *aba insensitive 1-1 (abi1-1)* and *aba insensitive 2-1 (abi2-1)* do not bind PYR1 upon association with ABA. This allows the PP2C proteins to continue inhibiting the SnRK2s which promote the seed dormancy associated with ABA signalling. Further, mutations which abolish the catalytic activity of *abi1-1* also abolish the ABA insensitive phenotype (Park *et al.*, 2009). Without competent ABA signalling, seed dormancy is shortened, corroborating the idea that ABA signalling promotes seed dormancy.

Members of the SnRK2 family, SnRK2.2, 2.3, and 2.6 have been found to act redundantly in the transmission of ABA signalling during the maturation and dormancy stages of the

seed (Nambara *et al.*, 2010). Major targets of these three SnRK2 proteins include ABI5 and AREB3, with the triple mutant being almost completely insensitive to the presence of ABA. This abnormality in the seed's development serves to further corroborate the importance of ABA signalling for the processes during seed maturation (Nakashima *et al.*, 2009). However, this analysis does not provide quantitative evidence for the role of ABA in seed dormancy as the mutant does not show any large alterations to dormancy in their phenotypes, this is potentially due to the redundancy of the proteins.

The ABA/PYR1/PP2C/SnRK2 model is currently the most strongly supported model for a way in which ABA signalling promotes seeds dormancy. Other receptors including ABAR are also potentially involved in an ABA-mediated dormancy signalling pathway. The ABAR receptor crosses the chloroplast envelope, with the C-terminus interacting with a group of WRKY transcription factors which negatively regulate ABA signalling (Shang *et al.*, 2010). Some knockout mutant analyses have appeared to show no significant effect on ABA signalling, so the role of the ABAR receptor remains controversial (Sheard and Zheng, 2009).

The role of ABA in defence responses to abiotic stress may show crosstalk with the role of ABA in seed dormancy. ABA accumulates in osmotically stressful conditions, with several ABA biosynthesis genes upregulated by drought and salt stresses (Verma *et al.*, 2016). This may potentially link to the promotion of dormancy during unfavourable conditions for germination, such as osmotically stressful environments.

6.8.3 Gibberellins and Seed Dormancy

As opposed to the ABA-mediated initiation of seed dormancy, the release of such dormancy is less well understood, except that gibberellins (GAs) are thought to oppose seed dormancy, and promoting germination (Graeber *et al.*, 2012). There are fast and slow releases of seed dormancy, the fast release being in imbibed seeds (those which have taken up water) and the slow in dry seeds. Fast release of dormancy occurs at temperatures specific to each species in a process coined 'stratification'. Stratification is generally a short period of time in which seeds are subjected to cold, wet conditions, similar to those they would experience in nature (Bewley *et al.*, 2003).

The details of this temperature sensing mechanism and how it drives the release of seed dormancy is relatively unknown, except that transcription factors such as SPATULA (SPT) and PHYTOCHROME INTERACTING FACTOR 3-LIKE 5 (PIL5) which integrate light and temperature signals into GA biosynthesis have a role in stratification (Penfield *et al.*, 2005*;* Oh *et al.*, 2009). PIL5 represses germination in the dark after a cold treatment, while SPT is a negative regulator of germination which loses function after stratification ***(Figure 13)***. Both PIL5 and SPT inhibit GA biosynthesis genes such as *GA3ox1* and *GA3ox2*, indicating that without GA biosynthesis seed dormancy cannot be broken and germination is inhibited.

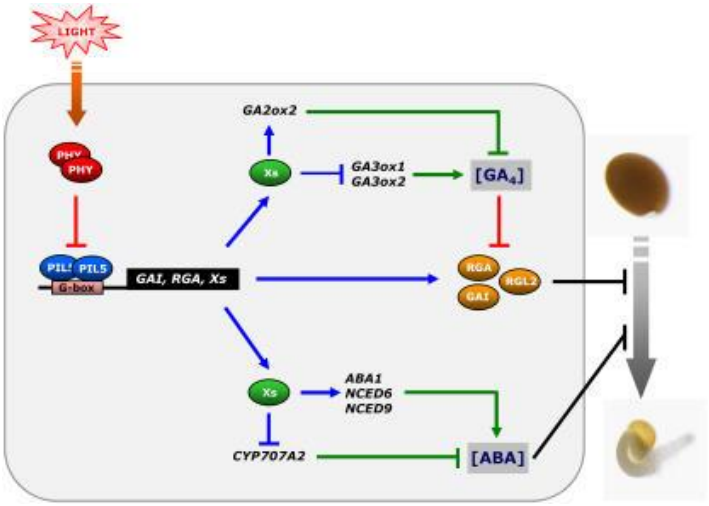

Figure 13: Mechanism for Germination upon Light Perception

Molecular events leading to germination upon light perception in plants. Figure taken from Oh et al., 2007.

Another interesting link between GAs and germination is their role in managing reactive oxygen species (ROS) accumulation. Oxidation of particular mRNAs, resulting in the prevention of their translation and therefore altering the proteome, is associated with dormancy release, in particular, selective oxidation of stress response gene mRNAs (Bazin *et al.*, 2011). DELLA proteins, which negatively regulate GA signalling and are degraded in the presence of GAs, repress ROS accumulation, opposing the role of ROS in dormancy release (Achard *et al.*, 2008). Therefore, GAs might indirectly increase ROS accumulation, promoting seed release, via the inhibitive interaction towards DELLAs.

Reduction of GA levels and signalling has been shown to contribute to growth restrictions on exposure to cold, salt and osmotic stresses. Achard *et al.*, 2006 is key to our understanding of the interface between abiotic stress and GA signalling. *Arabidopsis thaliana* seedlings under high salinity conditions display reduced expression of bioactive GAs, corresponding with accumulation of DELLA proteins. Further to this, the growth reduction associated with wild-type seedlings under salt stress is not observed in a quadruple *della* mutant, however, these mutants showed reduced survival under such saline conditions. The *ga1-3* mutant, deficient in GA biosynthesis, showed increased survival under saline-stressful conditions (Achard *et al.*, 2006). GA signalling, therefore, is inhibited by salt stress, whilst ABA signalling is upregulated, presenting evidence that stress response pathways to unfavourable conditions correspond to the control of seed dormancy and germination. Similarly, prolonged cold stress yields accumulation of DELLAs, downregulation of bioactive GAs and restricted growth, in part due to upregulation of the GA inactivating, catabolic 2ODD *GA2ox* genes. Short periods of cold-stress (stratification) oppose DELLA accumulation (Achard *et al.*, 2008*;* Magome *et al.*, 2008).

The balance between ABA signalling and GA signalling is key to the optimally timed release of seed dormancy and the promotion of germination. There are various target pathways through which the two phytohormones may function in order to regulate the germination process, and also integrate their roles in the germination process and response to abiotic stresses. These stresses include osmotic stresses such as cold and salinity but also soil drying (Coelho Filho *et al.*, 2013*;* Wang *et al.*, 2008*;* Leach *et al.*, 2011).

6.8.4 The Importance of Chromatin Remodelling in Seed Dormancy and Germination

The importance of chromatin structure in developmental processes extends to include seed dormancy (Cooke *et al.*, 2012). Via genetic and biochemical analyses, a number of chromatin factors necessary for regulation of seed dormancy, and therefore germination, have been identified.

For example, *REDUCED DORMANCY-4* (*RDO4*), which was identified due to its reduced dormancy phenotype, encodes proteins necessary for histone ubiquitination, while *RDO2* encodes TFIIS, a factor in transcription elongation (Liu *et al.*, 2007). *RDO4/2* are thought

to interact with the PAF1 complex (PAF1C), regulating transcriptional elongation in order to influence seed dormancy. Mutants in other components of PAF1C have interestingly been shown to exhibit alterations in the regulation of ABA related genes, potentially contributing to the reduced dormancy observed, relating to the role of ABA in maintaining seed dormancy as mentioned earlier.

The roles of H3K27 and POLYCOMB REPRESSIVE COMPLEX 2 (PRC2) are also reflected in seed dormancy studies. FERTILISATION INDEPENDENT ENDOSPERM (FIE) is an essential component of PRC2, with the *fie* mutant being absent of H3K27 trimethylation and displaying a more dormant phenotype than the wild type, indicating that the H3K27me3 modification via PRC2 is important in terminating the period of dormancy shown by a seed during the maturation phase (Bouyer *et al.*, 2011). Many regulators of maturation including ABA/GA signalling proteins are repressed by PRC2, showing heavy involvement of the PRC2 chromatin remodelling complex in determining when seed dormancy is aborted and when germination is allowed to occur (Graeber *et al.*, 2012).

Further, the KRYPTONITE/Su(var)3-9 HOMOLOGUE-4 (KYP/SUVH4) and SUVH5 genes encode histone methyltransferases implicit in the demethylation of H3K9 (Jackson *et al.*, 2002). The mutants of each gene show longer dormancy and heightened expression of numerous dormancy genes, namely ABA INSENSITIVE-3 (ABI3) and DELAY OF GERMINATION-1 (DOG1) (Zheng *et al.*, 2012). KYP expression is also controlled by the antagonistic action of GAs and ABA, upregulated by GA and downregulated by ABA, therefore KYP is likely to be involved in the balancing of ABA/GA and their effects on seed dormancy.

6.9 DOG1 is a Master Regulator of Germination

6.9.1 Quantifying Seed Dormancy is Difficult

Understanding the control of germination has far-reaching implications; the desirable uniform germination in crops, the problem of pre-harvest sprouting and processes such as barley malting all stand to benefit from our improved understanding. The seed consists of three complex components, each with different genetic background: the endosperm and embryo are zygotic and the seed coat has its background in maternal tissues (Alonso-

Blanco *et al.*, 2003). This, combined with the environmental input in the control of seed dormancy, makes seed dormancy a complex and difficult trait to understand in a quantitative sense.

6.9.2 Emergence and Importance of DOG1

The *DELAY OF GERMINATION 1* (*DOG1*) gene emerged as a potential master regulator of seed dormancy, partly because out of the 7 *DOG* loci, the *DOG1* locus has been shown to collocate in three different experimental populations (Alonso-Blanco *et al.*, 2003*;* Clerkx *et al.*, 2004*;* Laserna *et al.*, 2008).

QTL analysis of the *Landsberg erecta* (*Ler*) lab strain of *Arabidopsis thaliana*, which shows extremely low dormancy, the highly dormant *CVI* accession from the Cape Verde Islands, and their crosses revealed that *DOG1* explained up to 12% of the variation in dormancy rates between the two accessions with opposite dormancy phenotypes (Bentsink *et al.*, 2006). Unlike many genes, *DOG1* is not named for the phenotypic effect of the mutant, but instead, for the wild-type effect, *DOG1* delays germination with *dog1* mutants lacking dormancy. The determination of *DOG1* in *Arabidopsis thaliana* is an important step in understanding a large part of dormancy control within plants, the large effect in *Arabidopsis* implicates a role within other plant species also with homologues being found in *Lepidium sativum, Brassica ripa* and also the rice *Oryza sativa* (Graeber *et al.*, 2010*;* Sugimoto *et al.*, 2010)

6.9.3 Expression of *DOG1*

Unlike many components of seed dormancy already discussed, which have various functions within plant development, *DOG1* appears to be specific to a role in seed dormancy. Analysis of various plant tissues including roots, flowers and seeds of various dormancy proved *DOG1* expression was seed-specific, beginning 9 days after pollination and peaking in the mid-stages of seed maturation before decreasing towards the end of seed maturation. *DOG1* transcripts are retained in after ripened, ready to germinate, non-dormant seeds, but both dormant and after-ripened seeds show a rapid decrease in these *DOG1* transcripts upon imbibition (Bentsink *et al.*, 2006). Imbibition does not affect ABA signalling however, indicating that *DOG1* may function solely in the initiation of dormancy within seed maturation, with ABA's function extending beyond imbibition

perhaps to maintain dormancy. Bentsink supports this niche for *DOG1* with evidence that *dog1* mutants still need the presence of GAs and light to initiate germination.

Little is currently known surrounding DOG1's mode of action. As a single master regulator implicit in large proportions of seed dormancy control, understanding of the mechanisms surrounding this regulation is incredibly important. As opposed to the decrease in *DOG1* transcript levels observed towards the end of the maturation, levels of the DOG1 protein remain high resulting in harvested seeds being high in the protein but low in the RNA transcript (Nakabayashi *et al.*, 2012). The levels of DOG1 protein are also unaffected by imbibition, in stark opposition to the rapid decline in *DOG1* transcripts. Nakabayashi's findings show that levels of DOG1 protein in freshly harvested seeds strongly correlate with the level or longevity of the dormancy period, however, this correlation is not supported in seeds allowed to after-ripen due to a change in the DOG1 protein, supported by a change observed in the isoelectric point. With the DOG1 protein primarily localised within the nucleus, elucidated in YFP imagery, it is possible that DOG1 may be a regulator of transcription with this study suggesting role in blocking or stimulating the translation of stored mRNAs, as germination does not require active transcription.

6.9.4 DOG1: Influencing ABA, GAs and Temperature Perception

More recently DOG1 has been implicated in controlling seed dormancy via the ABA signalling pathway, specifically by binding and inactivating the negative regulators of ABA signalling discussed earlier, the PP2C proteins AHG1 and AHG3 (Née *et al.*, 2017*;* Nishimura *et al.*, 2018). This frees protein kinases such as the SnRK2s from repression, promoting ABA signalling driven seed dormancy. Further, DOG1 has been reported to also bind to heme, independently of the binding to AHG1; this interaction is essential to the function of DOG1 in promoting dormancy (Nishimura *et al.*, 2018). This interaction scavenges heme, impairing the maturation of holocytochrome *c* required for redox changes in the mitochondria, resulting in reduced ABA sensitivity in the nucleus, reducing dormancy and favouring germination (Nonogaki, 2019) ***(Figure 14)***. Through independent interactions with PP2C proteins and heme, DOG1 may show duality in its functions as a positive master regulator of seed dormancy, however, the DOG1-heme pathway is still to be fully characterised.

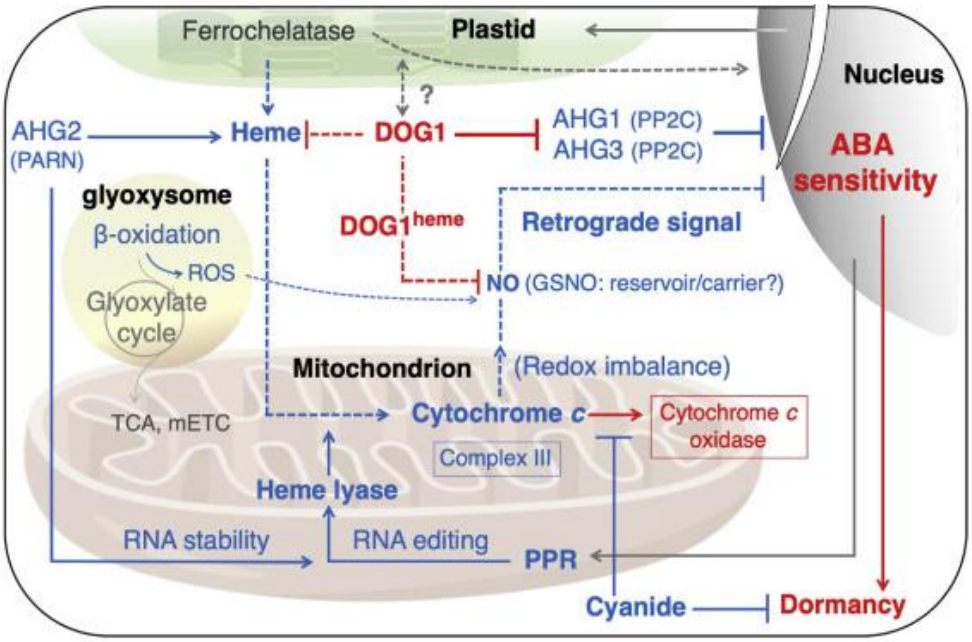

Figure 14: DOG1 in the Mitochrondria

Interactions between DOG1 and mitochondrial elements which may influence ABA signalling and seed dormancy. Figure taken from Nonogaki, 2019.

DOG1 expression is strongly correlated with a decrease in soil temperature, expression is higher in cold environments and decreases as environments become warmer (Footitt *et al.*, 2011). However, short periods of stratification stimulate a decline in *DOG1* expression (Footitt *et al.*, 2014). This, combined with the necessary presence of DOG1 in low temperature-induced dormancy, may suggest DOG1 functions in temperature-dependent promotion of ABA signalling and seed dormancy (Kendall *et al.*, 2011). CBF1 (C-REPEAT BINDING FACTOR 1), which plays a prominent role in cold acclimation, upregulates *GA2ox6*, an enzyme key for inactivating GA signalling and scavenging bioactive gibberellins. CBF1 also upregulates *DOG1* expression, with CBF1 loss-of-function mutants displaying decreased expression, the *DOG1* promoter region contains a CBF binding site which *GA2ox6* does not, indicating that *GA2ox6* is not a direct target for CBF1 and is instead regulated alongside *DOG1* (Achard *et al.*, 2008). This conclusion is supported by the *dog1* mutant which has a 10-fold decrease in *GA2ox6*, proposing a role for DOG1 in GA catabolism (Kendall *et al.*, 2011).

There is a host of support for DOG1 as a master regulator of seed dormancy, and therefore of germination. Influencing both GA and ABA signalling, a heavy involvement in the

difference in dormancy between dormant and non-dormant lines and the reported response to environmental conditions all make DOG1 an attractive candidate for furthering our understanding of the critical germination process.

6.10 LDL1, LDL2 and Understanding Control of Germination

The pervading subject within this thesis is that the environment in which a seed germinates is critical to plant success and has a significant impact on the agricultural industry. The ability of plants to modulate their development, and optimise their environmental response, is heavily dependent upon chromatin remodelling, the frontier of gene regulatory mechanisms.

Abscisic acid, gibberellins, DELLA proteins and the master regulator DOG1 all function to control the timing of germination, and each of these components are involved in chromatin remodelling. Ubiquitination of histone H2B through RDO4, promoting the expression of seed dormancy genes *DOG1* and NCED9, and repression of *DOG1* through the histone methyltransferases KYP/SUVH4 and SUVH5 are just two examples of the role of how histone modifications influence germination.

6.10.1 The Role of LDL1 and LDL2 in Plant Development

Arabidopsis thaliana expresses *LDL1* and *LDL2* are two homologues of the *LYSINE SPECIFIC DEMETHYLASE-1* (*LSD1)* expressed in humans. Alongside *FLD* (*FLOWERING LOCUS D*), another plant homologue of *LSD1*, *LDL1/2* are heavily influential in the control of flowering. The demethylase activity of LDL1 and LDL2 at H3K4 represses the expression *FLOWERING LOCUS C (FLC)* and *FWA*, key repressors of flowering (Jiang *et al.*, 2007). The roles of LDL1/2 have recently been further defined as essential for regulating seed dormancy, promoting stratification-based repression of *DOG1* expression, therefore opposing seed dormancy.

6.10.2 Structure and Function of LDL1 and LDL2

Very little has currently been reported about the function of the LDL proteins in seed dormancy and even less about their structure. *LDL1* and *LDL2* show 69% similarity,

whereas another observed homologue, *LDL3*, is less analogous (Jiang *et al.*, 2007). *LDL1* and *LDL2* expression is localised to the nucleus of the protoplast, this expression is prominent during seed development before gradually decreasing from 9 DPA (Days Post Anthesis) (Zhao *et al.*, 2015).

Mutational analyses revealed the LDL1 and LDL2 might act redundantly in how they repress seed dormancy, with single *ldl1* and *ldl2* mutant failing to show significant effects on germination, but with the *ldl1 ldl2* showing a significant decrease in seed germination (Zhao *et al.*, 2015). This redundant role is further supported by the observation that both *LDL1* and *LDL2* overexpressing plants individually display improved seed germination and strongly decreased dormancy versus wild-type samples.

There is precedent for the involvement of LDL1 and LDL2 in inhibiting the expression of *DOG1*, the transcription of *DOG1* being noticeably upregulated in the *ldl1 ldl2* double mutant when compared to wild-type *Arabidopsis thaliana* expression levels. With *DOG1* as a master regulator of seed dormancy, and therefore germination, these findings by Zhao *et al.* illustrate a critical role for the LDL histone demethylases in such a key developmental process. Though LDL1 and LDL2 may display some redundancy, the lack of analysis into the effect of both *ldl1* and *ldl2* single mutants on *DOG1* expression has failed to investigate whether this control of *DOG1* expression is dependent on just one LDL in particular.

6.10.3 LDL1 and LDL2 in Further Research

Whilst many chromatin remodelling enzymes function to actively repress seed maturation and dormancy genes, including *DOG1*, the LDL1/2 histone demethylases function to passively repress these genes by removing histone modifications which are activating (Xiao *et al.*, 2017). This, coupled with the large effect LDL1 and LDL2 have on seed dormancy and their effects on the master regulator *DOG1* presents these histone demethylases as valuable research targets.

6.11 Experimental Aims and Hypotheses

This study aims to examine the DELLA proteins which are crucial to plant development in mediating the balance between ABA and GA signalling. The DELLA proteins are therefore implicit in the control of seed dormancy and germination. This area of research then, may provide a novel understanding regarding control of germination which may contribute towards improving agricultural yields.

I present the hypothesis that the DELLA proteins affect the expression of master regulator *DOG1* through interactions with chromatin remodelling enzymes.

In order to explore and test this hypothesis this study will aim to answer the following questions:

1. ***"Do the DELLA proteins interact with chromatin remodelling enzymes in a meaningful way?"***
2. ***"Do the DELLA proteins regulate germination by modulating the expression of DOG1, the master regulator of germination?"***
3. ***"Are the newfound interactions between the DELLA proteins and chromatin remodelling enzymes implicit in the control of germination via DOG1?"***

Firstly, a yeast-two hybrid (Y2H) protein-protein interaction assay allowed the identification of interactions between the DELLA proteins and a selection of chromatin remodelling enzymes. Such positive interactions were quantified *in vivo* in *Arabidopsis thaliana* by biomolecular fluorescence complementation (BiFC) assays, allowing the localisation of such interactions.

To quantify the nature of such interactions, side by side phenotypic and genotypic analyses were carried out. Germination rates and timing of various mutants and various hormonal growth conditions was observed, next to the genotypic effect of such mutations on the expression of master regulator *DOG1* utilising quantitative PCR (qPCR) methods.

7 Materials and Methods

7.1 Chemical Suppliers

Details of the suppliers for all chemicals and reagents used in this study are recorded in Appendix 1. All restriction enzymes and their compatible buffers were purchased from Thermo Fisher Scientific unless otherwise stated.

7.2 Plant Materials and Growth Conditions

7.2.1 Plant Material

Wild-type *Arabidopsis thaliana* seeds were obtained from laboratory stocks of primarily *Landsberg erecta* ecotype (*Ler*), and also *Columbia* ecotype (*Col0*). All the results presented were attained using the *Ler* ecotype background. The only mutant used in this study *5PLE* DELLA was kindly provided by Prof. Miguel Angel Blázquez (IBMCP-CSIC, Valencia).

7.2.2 Plant Growth Medium

1x Murashige and Skoog (MS) medium was prepared as outlined in (***Table 1***) below and then autoclaved for 20mins at 121°C.

Table 1: Preparation of MS Medium

Reagent	**Amount (g/L)**
MS Basal Salt Mixture	4.3
2-(N-Morpholino)Ethanesulfonic Acid (MES)	0.5
Sucrose	10
Agar	8
KOH	Until pH 5.8

Hormones were filter sterilised through 0.22 µm nylon syringe filters and added to cooled, but still liquid, MS media after autoclaving and before pouring the plats. Stock solutions were prepared according to ***Table 2*** below, aliquoted and stored at -20°C.

Table 2: Hormone Stock Solution Preparation

Hormone	**Stock Solution**
GA3	10 mM in EtOH
Paclobutrazol (PAC)	10 mM in EtOH

7.3 Plant Growth Conditions

Seeds sown onto plates are addressed in the germination analyses section. For all seeds sown onto soil, the seeds were first stratified in dH_2O in the dark at 4°C for two days. Seeds were pipetted from these Eppendorf tubes into pots of soil plus sand. These pots were covered with a plastic lid and placed in a greenhouse with cycles of 16 hours of light at 22°C and 8 hours on dark at 18°C.

7.4 Plant Genotyping

7.4.1 DNA Extractions

A small secondary leaf from each plant was used for genotyping, and placed in a 1.5 ml Eppendorf tube. 2 steel bearings were added to the tube, alongside 100 µl of 1X DNA Extraction Buffer (***Table 3***). The tubes were then placed in a Qiagen TIssueLyser II and the tissues ground at 25 hertz for 2mins.

The tubes containing beads were then centrifuged at 15,000rpm for 10mins. After centrifugation, 50 µl of supernatant was removed to new tubes and mixed with 50 µl of DNAse-free dH_2O. The diluted supernatant was used to run a SALK Genotyping PCR in the Thermocycler.

Table 3: Preparation of 10x DNA Extraction Buffer

Reagent	**Stock Solutions**	**Volume for 10 ml**
200 mM Tris-HCL	1 M pH 7.5	200 µl
250 mM NaCl	5 M	500 µl
25 mM EDTA	0.5 M	500 µl
0.5% SDS	10%	500 µl
dH_2O		8.3 ml

7.4.2 Genotyping PCR

The genotyping PCR reaction mix was set up according to ***Table 4*** and run in the Eppendorf C1000 Touch Thermal Cycle according to the conditions outlined in ***Table 5*** All genotyping primers used are listed in the appendices.

Table 4: Salk Genotyping PCR Reaction Mix

Reagent	**Volume per Reaction**
TAQ Polymerase Master Mix	6 µl
Diluted extracted DNA	2 µl
Forward Primer (10 µM)	0.1 µl
Reverse Primer (10 µM)	0.1 ul
DNAse-free dH_2O	4 µl

Table 5: Salk Genotyping PCR Thermal Cycling Conditions

Temperature (°C)	**Time**	**Number of Cycles**
95	2mins	1
95	15s	41
50	30s/Kb	
72	1min	
72	5mins	1
12	Hold	

7.5 Agarose Gel Electrophoresis

All PCR product fragment sizes were tested using agarose gel electrophoresis, the agarose gels were prepared according to the particular fragments being separated. For a 1% standard gel, 1 gram of agarose was dissolved in 100 ml 1x TAE buffer (40 mM Tris pH 7.6, 20 mM acetic acid, 1 mM EDTA) via microwaving for approximately 2mins 10s. The liquid agarose was allowed to cool slightly before adding ethidium bromide (EtBr) to a final concentration of 05 µg/ml. The liquid gel was then poured into casting trays with the desired setting comb and allowed to set for approximately 20-30mins.

For genotyping PCR, 5 µl of sample mixed with 6x DNA loading buffer (60% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF, 150 mM Tris pH 7.6) was loaded into wells, for other PCR reactions the amount loaded was specific. Each gel also contained a well with 5 µl of hyperladder to determine the size of the separated fragments, this would be either 1kb or 100bp hyperladder depending on the fragment size expected to be detected. Gels were run from a BIO-RAD Power Pac Basic at 150V for approximately 20 minutes, or longer if sufficient fragment migration and separation had not occurred. Gels were then imaged using Syngen InGenius gel documentation, controlled by GeneSnap Software.

7.6 Yeast Two-Hybrid Assay

7.6.1 Yeast strains

The yeast (Saccharomyces cerevisiae) strains utilised for the yeast two-hybrid (Y2H) assay were:

AH109 (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, URA3::MEL1UASMEL1TATA-lacZ) (Holtz, unpublished)

Y187 (MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met–, gal80Δ, URA3::GAL1UAS-GAL1TATA-lacZ) (Harper *et al.*, 1993).

7.6.2 Reporter Genes

Confirmation of Y2H interactions relied on activation of the reporter genes *ADE2* and *HIS3*. The activation of such reporter genes facilitates the growth of yeast cells in media plates lacking adenine and histidine respectively. The reporter gene *MEL1*, which turns yeast colonies blue in response to *GAL4* activation, confirms protein interactions in an x-Gal assay. The X-Gal was used for a β-galactosidase assay which did not yield meaningful results.

7.6.3 Yeast Transformation and Mating

7.6.3.1 Media and Solutions

Media and solutions were prepared in advance of yeast transformation, these included; YPDA broth, YPDA solid, SD media solid, 40% D-Glucose and different drop out solutions (-Leucine (-L), -Tryptophan (-W), -Leucine and -Tryptophan (-LW) and -Leucine -Tryptophan -Alanine and -Histidine (-LWAH).

7.6.3.2 Preparation of Yeast Strains

Yeast strains were inoculated into YPDA broth overnight at 28°C whilst agitated. The OD600 of these cultures were checked in order to prepare further cultures in YPDA with a known starting OD of 0.1.

Further incubation at 28°C with agitation ended when an OD60 of 0.4-0.5 was reached (roughly 3-4 hours).

Yeast cells were harvested via centrifugation at 3000rpm, resuspended in Lithium Acetate (LiAc) Tris-EDTA (TE) buffer and then centrifuged again.

The remaining pellet was resuspended in more LiAc TE buffer, to which boiled salmon sperm was added.

The plasmids of interest were introduced to the resulting suspension and supplemented with PEG. This was then incubated whilst shaking for 30mins at 28°C, before being heat-shocked once more at 42°C for 15mins.

7.6.4 Yeast Selection

7.6.4.1 Selection for Vector Uptake

These cells were centrifuged a final time, before being resuspended in sterile water and plating on the appropriate dropout SD plates (SD-L for yeast with Lysine synthesis gene, SD-W for yeast with Tryptophan synthesis gene).

Plates were left to incubate at 28°C for two days for colonies to grow. Cells successfully transformed with the pGADT7 or pGBKT7 domain containing the Lysine or Tryptophan synthesis gene, respectively, would, therefore, survive on the dropout media lacking such amino acids, while unsuccessful transformants would not.

After two days, successfully grown colonies were mixed in with the liquid forms of their SD dropout media and left to incubate, agitated, at 28°C for four hours.

The resulting cultures were mixed in equal quantities and then plated on solid YPDA.

7.6.4.2 Selecting for Mated Yeast

The following day, the grown cells were cultured in liquid YPDA media for four hours at 28°C once more.

The resulting culture was diluted 1/10 in sterile water and subsequently plated on solid SD-LW media at 28°C for two days.

Therefore, only successfully mated cells contained both vector plasmids, and therefore the synthesis genes for both Lysine and Tryptophan, would survive and not those cells that simply contained one of the vector plasmids.

7.6.4.3 Selecting for Yeast Displaying Interactions between Genes of Interest

After two days, successful colonies were cultured once more in SD-LW liquid media at 28°C for four hours with agitation.

As before, the culture was diluted 1/10 and then further diluted 1/10, 1/100 and 1/1000 in different wells of a 96-well plate.

Using a 96-well plate replica plater, these samples were plated on SD-LWAH media therefore only the successfully mated yeast, which also showed an interaction between the genes of interest could survive and grow as the interaction caused reconstitution of the synthesis genes for Alanine (A) and Histidine (H).

7.6.5 Additional β-Galactosidase Assay

β-Galactosidase activity was quantified using a protocol resembling that of Möckli and Auerbach (2004). Colonies of mated transformants which were successful in establishing colonies on SD-LW plates were resuspended in 200 µl of liquid SD-LW and incubated overnight at 30°C. The suspension was then centrifuged at 4000rpm for 2mins and the supernatant removed from the pellet of cells. This pellet was resuspended in 10 µl of dH_2O and the cells lysed via three cycles of flash freezing in liquid nitrogen before being left to thaw at 30°C.

The lysed cells were mixed with 100 µl of Phosphate-Buffered Saline (PBS) containing 500 µg/ml of X-gal, 0.5% agarose, and 0.05% β-mercaptoethanol and incubated at 28°C. Images were taken when the blue activity of the β-galactosidase was strongest.

7.7 *E. coli* Hot Fusion Cloning

In order to see the *in vivo* effects of interactions suggested by the preliminary *in vitro* Yeast Two-Hybrid Assay, it is first important to have the genes of interest and any interacting genes of interest cloned in both the pGADT7 activation domain cloning vectors and pGBKT7 DNA-binding domain cloning vectors (Clontech). These vectors included either the C terminus (YFC) or N terminus (YFN) of the Yellow Fluorescence Protein (YFP) used to provide fluorescence *in vivo* upon interaction of genes of interest, resulting in the reconstitution of the two termini of the YFP.

7.7.1 Plasmid Preparation

Pre-prepared pYFC43 and YFN43 (Belda-Palazón *et al.*, 2012) plasmids were linearised by digestion with the AscI and SpeI restriction enzymes (***Table 6***) overnight at 37°C. Plasmid digestion was confirmed via gel electrophoresis and the confirmed digested plasmids were purified using AmpureBeads.

Table 6: Restriction Digestion Mix for Linearisation of pYFC43 and pYFN43

Reagent	**Volume per Reaction (µL)**
Plasmid DNA	10
dH_2O	6
10x Cut-Smart Buffer	2
AscI	1
SpeI	1

7.7.2 Phusion PCR

7.7.2.1 Phusion PCR Primers

The Phusion PCR used a universal BiYFC pGAD reverse primer, with the forward primer designed using a 22bp sequence homologous to the gene of interest tagged onto a universal 17bp pre-sequence, homologous to the target site of the plasmid vector. A list of all primers used can be found in Appendix 1.

7.7.2.2 PCR

Genes of interest were amplified via Phusion PCR quantities and timing conditions found in ***Table 7*** and ***Table 8*** below.

Table 7: Phusion PCR Reaction Mix

Reagent	**Volume (µl)**
dH_2O	35.5
5x Phusion Buffer	10
Designed Forward Primer (10µM)	1
Universal Reverse Primer (10µM) (pGADT7 pBiYFC)	1
dNTPs (10mM)	1
Phusion Polymerase	0.5
Template cDNA Gene of Interest	1

Table 8: Phusion PCR Thermal Cycling Conditions

Temperature (°C)	**Time**	**No. Cycles**
95	2mins	1
95	15s	10
55	30s	
72	(30s/kb)	
95	15s	30
65	30s	
72	(30s/kb)	

72	7min	1
4	Hold	

7.7.2.3 Purification of PCR Fragment

Once again, PCR fragments were confirmed via gel electrophoresis in a 1% agarose gel. Successful PCR fragments were classified as unique bands, the size of which corresponded to the length of the gene of interest's CDS.

Successful fragments were supplemented with 100 µl of AmpureBeads and left to incubate for 5 min at room temperature. The PCR tubes were placed onto a magnet until the beads congregated on the side and the remaining supernatant removed. The beads were washed and drained twice with 80% Ethanol and left to dry outside of the magnet.

Once dry the beads were resuspended in 10 µl dH_2O and placed on the magnet, the supernatant containing the clean PCR fragment was removed and the AmpureBeads left behind. The cleaned PCR fragments were nanodropped using a Labtech Nanodrop ND-1000 Spectrophotometer to confirm the concentration of each sample.

7.7.2.4 Vector Preparation

Table 9: Restriction Digestion Mix for pGBKT7

Reagent	**Volume per Reaction (µL)**
Plasmid DNA	8
dH_2O	9
10x Cut Smart Buffer	2
NdeI	0.5
PstI	0.5

Table 10: Restriction Digestion Mix for pGADT7

Reagent	**Volume per Reaction (µL)**
Plasmid DNA	8
dH_2O	9
10x Cut Smart Buffer	2
NdeI	0.5
XhoI	0.5

The restriction enzymes used for the linearization of pGADT7 and pGBKT7 vectors were supplied from New England BioLabs. The reaction mixes in ***Table 9*** and ***Table 10*** above were incubated at 37°C for 2 hours and the enzyme then inactivated via incubation at 70°C for 15mins. The linearised plasmids were purified using 2x volume of Ampure beads used for purification of Phusion PCR product.

7.7.3 Hot Fusion Reaction

This concentration is key to the follow-up Hot Fusion Reaction which requires ~50 ng of the cleaned PCR fragment alongside 1 µl of 100 ng linearised pYFN43/pYFC43 plasmid, as given in ***Table 11*** below, and placed in a thermal cycler with the conditions in ***Table 12***.

Table 11: Hot Fusion PCR Reaction Mix

Reagent	**Volume per Reaction (µl)**
Linearised BiYFC/N Plasmid (100 ng)	1
Cleaned PCR Fragment of Interest (~50 ng)	1
2x Hot Fusion Buffer	10
dH_2O	8 (To 20 µl final volume)

Table 12: Hot Fusion PCR Reaction Conditions

Temperature (°C)	**Time**	**No. Cycles**
50	1 hour	1
50 – 20	5mins (-0.1°C/s)	1
10	Hold	

7.7.4 Transformation of *E. Coli*

Table 13: Antibiotic Working Concentrations and Stock Solutions, all antibiotics purchased from Melford.

Antibiotic	**Working Concentration (µg/ml)**	**Stock Solution Preparation**
Carbenicillin	100	100 mg/ml in H_2O. Filter sterilised through 0.22µm nylon syringe filters and stored at -20°C.
Kanamycin	25	50 mg/ml in dH_2O. Filter sterilised as above and stored at -20°C.
Hygromycin	40	500 mg/ml in PBS. Filter sterilised as above and stored at 4°C.
Gentamycin	40	125 mg/ml in dH_2O. Filter sterilised as above and stored at -20°C.
Rifampicin	100	100 mg/ml in DMSO. Filter sterilised as above and stored at -20°C.

Hot Fusion Reaction products were introduced to aliquots of thawed Dh5α competent *Escherichia coli* (*E. Coli)* cells. The competent cells were incubated on ice, before receiving a heat shock at 42°C for 2mins. The cells were then left to incubate, shaking, for one hour at 37°C in Luria-Bertani (LB) liquid media (25g per litre of dH_2O of LB Broth High Salt Granulated, Table 13) with Kanamycin (Table 13), before being plated on solid LB agar plates (37g per litre of dH_2O of LB Agar High Salt Granulated, Table 14) supplemented with Kanamycin for selection of the plasmid and incubated overnight at 37°C to allow colonies to grow.

Table 14: Composition of LB Broth High Salt Granulated

Reagent	**Volume (g/L)**
Tryptone	10
Yeast Extract	5
NaCl	10

Table 15: Composition of LB Agar High Salt Granulated

Reagent	**Volume (g/L)**
Tryptone	10
Yeast Extract	5
NaCl	10
Agar	12
KOH	Until pH 7.2

7.7.4.1 Colony PCR

Successfully grown colonies were numbered and then used for Colony PCR, reagents and volumes given in ***Table 16*** below, thermal cycling conditions given in ***Table 17.***

Table 16: Colony PCR Reaction Mix

Reagent	**Volume per Reaction (µl)**
2x PCRBio Taq Mix	6
ddH20	4
Resuspended *E. Coli* Colony, Template DNA	2
Forward Primer	0.1
Reverse Primer	0.1

Table 17: Colony PCR Thermal Cycling Conditions

Temperature (°C)	**Time**	**Number of Cycles**
94	5min	1
94	15s	40
50	30s	
72	30s	
72	5min	1
12	Hold	

Once more the PCR product was confirmed via gel electrophoresis (observed bands roughly 300bp longer than the gene of interest CDS). Successful colonies were then grown up in liquid LB supplemented with Kanamycin overnight at 37°C in order to produce a culture of selected plasmid for miniprep.

7.7.4.2 Plasmid Extraction and Validation by Digestion

The plasmid DNA from two positive colonies was isolated from the host *E. coli* cells using Wizard® Plus SV Minipreps DNA Purification System following the instructions from Promega. The isolated plasmids were then nanodropped and confirmed via enzymatic digestion using the reaction mix below which was incubated at 37°C for 2 hours.

Table 18: Restriction Digestion Mix for Miniprep Confirmation

Reagent	**Volume per Reaction (µl)**
Isolated Plasmid of Interest	10
ddH20	7.5
Selected Enzyme	0.5
Buffer for Selected Enzyme	2

Enzymes were selected from ApE, A plasmid Editor, to cut at least twice, once inside the gene of interest insert and once outside, thus the enzyme used for digestion varied. Plasmids were digested using a reaction mix detailed in ***Table 18***, with incubation at 37°C for 2 hours.

Once the isolated plasmids were confirmed as successful by digestion and gel electrophoresis, they were sent for further sequencing to ascertain the presence of the genes of interest. All sequencing reactions were performed by the DNA Sequencing laboratory, School of Biological and Biomedical Sciences, Durham University. DNA sequence data were analysed using BLAST 2 sequencing tool (www.ncbi.nlm.nih.gov/blast/bl2seq) and ApE, A plasmid Editor, software (http://biologylabs.utah.edu/jorgensen/wayned/ape/).

7.8 Bimolecular Fluorescence Complementation (BiFC) Assay

7.8.1 Agrobacterium Transformation

100 µl aliquots of GV3101 *Agrobacteria* were allowed to thaw on ice.5 µl of BiFC binary plasmid, pBiYFPc and pBiYFPn, samples introduced to the 100 µl competent *Agrobacteria* aliquots, mixed, and left to incubate for 5mins on ice.

The incubated *Agrobacteria* aliquots were frozen in liquid nitrogen for 2mins, followed by heat shock at 37°C for 3mins. After heat shock, 150 µl of liquid LB media was added and incubated whilst shaking at 28°C for 2 hours.

The resulting culture was plated on selective solid LB media plates supplemented with Kanamycin and Rifampicin (see ***Table 13*** for working concentrations) and incubated at 28°C for two days until colonies formed.

7.8.2 Harvest of Agrobacteria

Once colonies had formed after roughly two days a single colony was selected and cultured overnight, with agitation, at 28°C in 10 ml of selective liquid LB media containing kanamycin and rifampicin antibiotics (see ***Table 13*** for working concentrations).

Cultures had their OD600 calculated and the result was used to ensure an OD600 of 0.2 once culture was added to 50 ml of liquid LB media containing kanamycin and rifampicin antibiotics, alongside 200µM of acetosyringone.

Cultures were placed back at 28°C to incubate whilst agitated until an OD600 of 0.4-0.6 reached, roughly 3-4 hours.

Cultures were centrifuged at 4000rpm for 10mins and the supernatant decanted. Remaining pellet of cells was resuspended in MMA buffer (***Table 19***) and supplemented with 200µM acetosyringone. The volume of buffer used for resuspension was dependent on culture volume and final OD600 value.

Table 19: MMA Buffer Composition

Reagent	**Amount (g/L)**
MS	5
MES	1.95
Sucrose	20
NaOH	Until pH 5.6

Resuspended cells were shaken at room temperature for one hour, inside a light excluding box to ensure the *Agrobacterium* cells were kept in the dark.

7.8.3 Infiltration of Agrobacteria into Nicotiana benthamiana

Agrobacterium resuspensions containing complementary BiFC constructs of the gene of interest (e.g. RGA-N vs LDL2-C) were mixed with cultures containing the p19 plasmid used to suppress gene silencing.

Agrobacteria were introduced into *Nicotiana benthamiana* plants grown for 3-4 weeks in standardised conditions at 21°C with light cycles of 16 hours in light and 8 hours in darkness. *Nicotiana benthamiana* plants kindly supplied by the Prof. Ari Sadanandom lab in the Durham University Biosciences Department.

Incision was made using a sterile razor blade in the epidermal cell layer of the lower leaf, taking care not to puncture through the leaf.

Mixed cultures of P19 and BiFC constructs of introduced were taken up into a syringe and then infiltrated into the incision made in the lower leaf, ensuring that the entire leaf was successfully saturated.

Only two leaves per plant were used for infiltration so that the stress of transformation would not harm the overall health of the plant. Plants were also infiltrated before floral development so that energy would be used to keep leaves healthy.

Plants were placed in a greenhouse in the dark overnight to ensure successful *Agrobacterium* infiltration, and then moved into the light and kept in the greenhouse for 3 days.

7.8.4 Confocal Microscopy to Observe Fluorescence

Nicotiana benthamiana leaves which had been infiltrated had a section cut out using a sterile razor blade. The leaf sample was mounted onto a glass microscope slide with water, gently so as not to damage the tissues.

A Zeiss 880 Confocal Laser Scanning Microscope was used to observe yellow fluorescence where interactions between genes of interest had taken place.

7.9 Germination Analyses

7.9.1 Seed Sterilisation and EtOH Washing

Seeds were sterilised before plating to ensure no contamination. 50 µl of Sterilisation Solution (70% EtOH + 0.1% Tween 20) was added to 1.5 ml Eppendorf tubes containing seed aliquots, the tubes were then shaken vigorously for 15mins.

In a sterile laminar flow hood the Sterilisation Solution was removed and 500 µl of 100% EtOH added. The tubes were vigorously shaken for a further 5 minutes, and the process repeated twice more.

On the third cycle of EtOH washing the seeds were decanted onto sterilised, dry filter papers to dry.

7.9.2 Seed Plating

All seeds were plated on Horizontal MS Agar using sterilised toothpicks as described in Salisbury faculty notes. All plates were sealed with Millipore tape and placed into a growth chamber with a 16h light/8h dark cycle at 21°C at midday. Roughly 20 seeds were plated per plate and roughly 6 plates were used per testing condition, ~120 seeds per condition.

Plates were observed every following day at midday under a Leica dissection scope. Germinated seeds were circled and labelled with the day on which germination was observed.

7.9.3 Treatment Conditions

Various analyses were carried out in order to ascertain the effect of various factors on the rate and percentage of germination.

Stratified seeds were plated and incubated in the dark at 4°C for two days before exposure to the growth chamber.

For hormonal analyses, MS plates were supplemented with 0.1 µl/ml of GA3 or 0.02 µl/ml of Paclobutrazol.

Quintuple (*5PLE*) DELLA mutants were acquired from Prof. Miguel Angel Blázquez (IBMCP-CSIC, Valencia) to be used in germination analyses.

After a pre-determined length of time, the number of seeds which had germinated on each particular day was calculated and recorded alongside which treatment or condition the seeds belonged to.

7.10 Quantitative PCR (qPCR) analysis

7.10.1 Seed Treatments.

8 individual 1.5 ml Eppendorf tubes containing ~25 mg of Landsberg wild type seeds each were used for this treatment. Half of the seed samples were stratified, with the other half left unstratified, providing 4 biological replicates for each condition. Stratification consisted of suspending the seeds in de-ionised water, with the tubes then wrapped in foil and left at 4°C for two days. The non-stratified tubes were left at room temperature, dry and in the dark. The same treatments of stratification applied also to mutant strains used in these germination analyses.

For hormone treatments seeds were suspended in H_2O once again containing either 0.1 µl/ml of GA3 or 0.02 µl/ml of Paclobutrazol, these aliquots were either stratified or unstratified.

7.10.2 RNA extraction

7.10.2.1 Buffer Preparation

Before the extraction could take place, several buffers and solutions were prepared as depicted below.

Table 20: Lysis/Binding Buffer (LBB) Composition

Solution Components	**Stock Solutions**	**Volumes for 50 ml**
100mM Tris-HCL	1M pH 8	5 ml
1M LiCl	8M	6.25 ml
1mM EDTA	500mM pH 8	1 ml
1% SDS	10%	5 ml
5mM DTT	1M	250 µl
Antifoam A		750 µl
dH_2O		31.75 ml

Table 21: Washing Buffer A (WBA) Composition

Solution Components	**Stock Solutions**	**Volumes for 50 ml**
100mM Tris-HCL	1M pH 8	5 ml
150mM LiCl	8M	940 µl
1mM EDTA	500mM pH 8	1 ml
0.1% SDS	10%	500 µl
dH_2O		42.56 ml

Table 22: Washing Buffer B (WBB) Composition

Solution Components	**Stock Solutions**	**Volumes for 50 ml**
100mM Tris-HCL	1M pH 8	5 ml
150mM LiCl	8M	940 µl
1mM EDTA	500mM pH 8	1 ml
dH_2O		43.06 ml

Table 23: Low Salt Buffer (LSB) Composition

Solution Components	Stock Solutions	Volumes for 50 ml
10mM Tris-HCL	1M pH 8	500 µl
150mM NaCl	5M	1.5 ml
1mM EDTA	500mM pH 8	100 µl
dH_2O		47.9 ml

Table 24: 10mM Tris-HCL Composition

Solution Components	Stock Solutions	Volumes for 50 ml
10mM Tric-HCL	1M pH 8	500 µl
dH_2O		49.5 ml

Table 25: 1M 2-Mercaptoethanol Composition

Solution Components	Stock Solutions	Volumes for 1 ml
1M 2-Mercaptoethanol	14.3M	7 µl
dH_2O		993 µl

Table 26: RNA Elution Buffer Composition

Solution Components	Stock Solutions	Volumes for 1 ml
10mM Tris-HCL	10mM (made previously)	999 µl
1M 2-Mercaptoethanol	1M (made previously)	1 µl
dH_2O		

Table 27: Reverse Transcriptase Master Mix

Solution Components	Volumes per Reaction (µL)
5x Reverse Transcription buffer	2
dNTPs	1
RevertAid Reverse Transcriptase	0.5
dH_2O	6.5

7.10.2.1 RNA Extraction

Before extraction, the number of samples was counted and multiplied by the approximate amount of Lysis/Binding Buffer required for the extraction (500 µl). 5 µl of 14.3M 2-Mercaptoethanol per ml of Lysis/Binding Buffer was added to prepare the buffer, which was then kept at room temperature to maintain the liquid state of the SDS.

RNA was extracted from seeds in these scenarios, to reflect the role of DOG1 in seed germination and so the standard RNA extraction described in Townsley *et al.* (2015), would not be sufficient as this took place on leaf tissue using Zirconia beads.

Seeds from the biological replicates were frozen with liquid nitrogen, if wet due to treatments seeds were dried before freezing. Mortars and pestles were cooled with liquid nitrogen before introducing the seeds and grinding to a fine powder, adding more liquid nitrogen when necessary. 200 µl of the prepared Lysis/Binding Buffer was added and grinding continued, adding another 200 µl of Lysis/Binding Buffer once homogeneous and grinding again.

The 400 µl of solution was pipetted from the mortar into a clean Eppendorf and centrifuged at 15,000 g for 10mins, the supernatant was collected ensuring not to collect too much of the lipid layer.

7.10.2.2 mRNA Capture

7.10.2.2.1 First Cycle

Biotin-linker-polyT oligo was prepared when needed, 12.5 µl from the 100 µM stock added to 87.5 µl RNAse-free dH_2O.

1 µl of 12.5 uM Biotin-linker-polyT oligo was added into each Eppendorf containing supernatant and incubated for 10mins at room temperature on the PTR-25 Mini Rotator.

Whilst the lysate incubates, new tubes were set up containing 20 µl of streptavidin beads per tube, resuspended in 100 µl of 2-Mercaptoethanol free Lysis/Binding Buffer to keep the beads from drying out. These tubes were then placed into a magnetic rack.

After 10mins of incubation, the Lysis/Binding Buffer was removed from the pellet of beads, and the beads then resuspended with the lysate that had been incubating. These tubes were then returned to incubate on the rotator for a further 10mins.

These tubes were placed on the magnetic rack and the supernatant removed. The beads were washed with 200 µl of Washing Buffer A, by mixing via pipette, and placing back in the magnetic rack to remove the supernatant. This process was repeated with 200 µl of Washing Buffer B and 200 µl of Low Salt Buffer.

At this point, the beads were resuspended in 16 µl of the RNA Extraction Buffer and heated at 80°C for 2mins. After heating the tubes were immediately placed on the magnetic rack with the supernatant transferred to clean tubes.

7.10.2.2.2 Second Cycle

For the second cycle, 200 µl of Lysis/Binding Buffer was added to each tube containing the RNA Extraction Buffer Supernatant and the process repeated, starting with adding 1 µl of 12.5 uM of Biotin-linker-polyT oligo and incubating for 10mins at room temperature. However, upon removing the Low Salt Buffer, the beads are resuspended in 10 µl of the Reverse Transcriptase Master Mix, instead of the RNA Extraction Buffer.

The streptavidin beads were re-used by being washed in 200 µl of 10 mM of Tris-HCL and then with 200 µl of Lysis/Binding Buffer.

7.10.2.3 Reverse Transcriptase Quantitative PCR (RT-qPCR)

At this point, the samples are placed into the ThermoCycler on a programme as detailed in ***Table 28*** below.

Table 28: RT-PCR Cycling Conditions

Temperature (°C)	**Time**	**No. Cycles**
42	60 mins	1
70	10 mins	1
4	Hold	

After the PCR reaction the samples were again heated at 80°C for 2mins, then placed immediately onto the magnetic rack, to remove the synthesised cDNA. cDNA was then stored at -20°C until needed for qPCR.

Table 29: qPCR Reaction Mix per Reaction

Reaction Component	**Volume per Reaction (µL)**
2X SYBR Green PCR Mix Lo-ROX	10
Forward Primer (100 µM)	0.1
Reverse Primer (100 µM)	0.1
cDNA (1:10 Dilution)	1.6
RNAse-free dH_2O	4.8

These amounts were multiplied based on the need for three technical replicates, the number of biological replicates and the number of different primers testes between, which would show expression of different genes (i.e. gene of interest vs control gene PP2A3). These multiplications left room for pipette error.

Reaction mixes were loaded into strips of 4 qPCR tubes, taking care not to leave a bubble which would affect the reading in the RotorgeneQ qPCR cycler. Once loaded into the RotorgeneQ, samples were labelled based upon the gene being tested and the conditions/biological replicate of the sample.

Table 30: qPCR Cycling Conditions

Temperature (°C)	**Time**	**No. Cycles**
94	3 mins	1
94	15s	40
50	30s	
72	30s	
72	5 mins	1
12	Hold	

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Gene expression levels were calculated from the average of the three technical replicates relative to the expression levels of a reference gene (*PP2A3*) and analysed using the comparative Ct method (ΔΔCt method) as described in Applied Biosystems User Bulletin No. 2. All primers mentioned are listed in Appendix 1.

7.11 Statistical Analysis

All statistical analyses in this study were performed in R, apart from statistical analyses of relative expression in qPCR which were analysed using the comparative Ct method (ΔΔCt method) as described in Applied Biosystems User Bulletin No. 2. Data collected in this study were tested for distribution normality both using Kolmogorov-Smirnov and Shapiro-Wilks tests. When appropriate as highlighted, non-normal distributions were analysed using the non-parametric Kruskal Wallis test followed by *ad hoc* testing with the Wilcoxon Pairwise Comparisons test. Probability of error was defined at 0.05 throughout i.e. $p<0.05$ in the Kruskal Wallis test indicates a significant difference occurs and in the Wilcoxon Pairwise Comparisons tests indicates that these differences are significant.

8 Results

8.1 Characterising the possible molecular interactions between the DELLA proteins and chromatin remodelling enzymes (CREs)

This study explored how the DELLA proteins which oppose GA signalling may interact with chromatin remodelling enzymes to do so. Aside from a few landmark studies (Sarnowska *et al.*, 2013; Zentella *et al.*, 2007; Archaki *et al.*, 2013), the current understanding of how DELLAs may interact with chromatin remodelling enzymes to oppose GA signalling is limited. Such limited understanding provides an attractive opportunity to explore this potentially important relationship further.

To aid our understanding of how the DELLA proteins may influence chromatin changes in suppressing the germination promoting activity of gibberellins, a Yeast Two-Hybrid (Y2H) assay was performed to identify protein-protein interactions between chromatin remodelling enzymes and the DELLAs which negate GA signalling. Y2H assays revolve around the function of GAL4, a transcriptional activator in *Saccharomyces cerevisiae*, yeast. One protein of interest, the bait, is fused to the N-terminal of GAL4, containing the Binding Domain (BD) and the other protein of interest, the prey, is fused to the C-terminal of GAL4, containing the Activation Domain (AD). An interaction between the bait and prey proteins results in the reconstitution of the GAL4, which drives the transcription of reporter genes (Brückner *et al.*, 2009). This activation of reporter genes is often characterised by auxotrophy, the ability to grow on selective mediums lacking certain amino acids, or by causing a change in colour of yeast colonies, such as the blue tint observed in X-Gal assays.

8.1.1 Identifying *In Vitro* interactions between the DELLAs and CREs

A Yeast Two-Hybrid library specifically designed to identify protein-protein interactions between chromatin remodelling enzymes and signalling genes, such as the DELLA proteins, had already been prepared (***Table 31*** and ***Table 32***), with these proteins cloned both into the BD domain-containing pGBKT7 plasmid and the AD domain-containing pGADT7 plasmid. The previous work of my supervisor Dr Miguel de Lucas, his PhD student Joey Nelson and his previous master's student Grace Brewer meant that all the

vectors I needed for exploring interactions between the DELLA proteins and CREs were already prepared. The library of chromatin remodelling enzymes was screened against the five DELLA proteins (RGA, GAI, RGL1, 2 and 3) to elucidate how the DELLA proteins and chromatin remodelling enzymes may interact to regulate the germination process.

Table 31: The DELLA Proteins Used in Y2H Screening

Accession Number	**Name**	**Function**
AT2G01570	**RGA**	DELLA Subfamily Member
AT1G14920	**GAI**	DELLA Subfamily Member
AT1G66350	**RGL1**	DELLA Subfamily Member
AT3G03450	**RGL2**	DELLA Subfamily Member
AT5G17490	**RGL3**	DELLA Subfamily Member

Table 32: The Chromatin Remodelling Enzymes Used in Y2H Screening

Accession Number	**Name**	**Function**
AT2G31650	**ATX1**	Histone-Lysine Methyltransferase
AT1G05830	**ATX2**	Histone-Lysine Methyltransferase
AT1G26760	**ATXR1**	Histone-Lysine Methyltransferase
AT5G09790	**ATXR5**	Histone-Lysine Methyltransferase
AT5G24330	**ATXR6**	Histone-Lysine Methyltransferase
AT1G76710	**ASHH1**	Histone-Lysine Methyltransferase
AT2G30580	**BMI1A**	Histone-Lysine E3 Ubiquitin Ligase
AT1G06770	**BMI1B**	Histone-Lysine E3 Ubiquitin Ligase
AT2G23380	**CLF**	Histone-Lysine Methyltransferase

AT5G51230	**EMF2**	Histone-Lysine Methyltransferase
AT3G20740	**FIE**	Histone-Lysine Methyltransferase
AT1G62310	**JMJ**	Histone-Lysine Demethylase
AT1G30810	**JMJ18**	Histone-Lysine Demethylase
AT1G78280	**JMJ21**	Histone-Lysine Demethylase
AT5G06550	**JMJ22**	Histone-Arginine Demethylase
AT4G00990	**JMJ27**	Histone-Lysine Demethylase
AT3G20810	**JMJ30**	Histone-Lysine Demethylase
AT5G17690	**LHP1**	Maintains State of Methylation
AT1G62830	**LDL1**	Histone-Lysine Demethylase
AT3G13682	**LDL2**	Histone-Lysine Demethylase
AT5G58230	**MSI1**	Histone-Lysine Methyltransferase
AT1G08620	**PKDM7D**	Histone-Lysine Demethylase
AT2G19670	**PRMT1A**	Histone-Arginine Methyltransferase
AT4G29510	**PRMT1B**	Histone-Arginine Methyltransferase
AT4G31120	**PRMT5**	Histone-Arginine Methyltransferase
AT4G16570	**PRMT7**	Histone-Arginine Methyltransferase
AT1G04870	**PRMT10**	Histone-Arginine Methyltransferase
AT5G44280	**RING1A**	Histone-Lysine E3 Ubiquitin Ligase
AT1G03770	**RING1B**	Histone-Lysine E3 Ubiquitin Ligase
AT2G33290	**SUVH2**	Histone-Lysine Methyltransferase
AT2G35160	**SUVH5**	Histone-Lysine Methyltransferase
AT3G04380	**SUVR4**	Histone-Lysine Methyltransferase
AT2G23740	**SUVR5**	Histone-Lysine Methyltransferase
AT4G02020	**SWN**	Histone-Lysine Methyltransferase
AT4G16845	**VRN2**	Histone-Lysine Methyltransferase

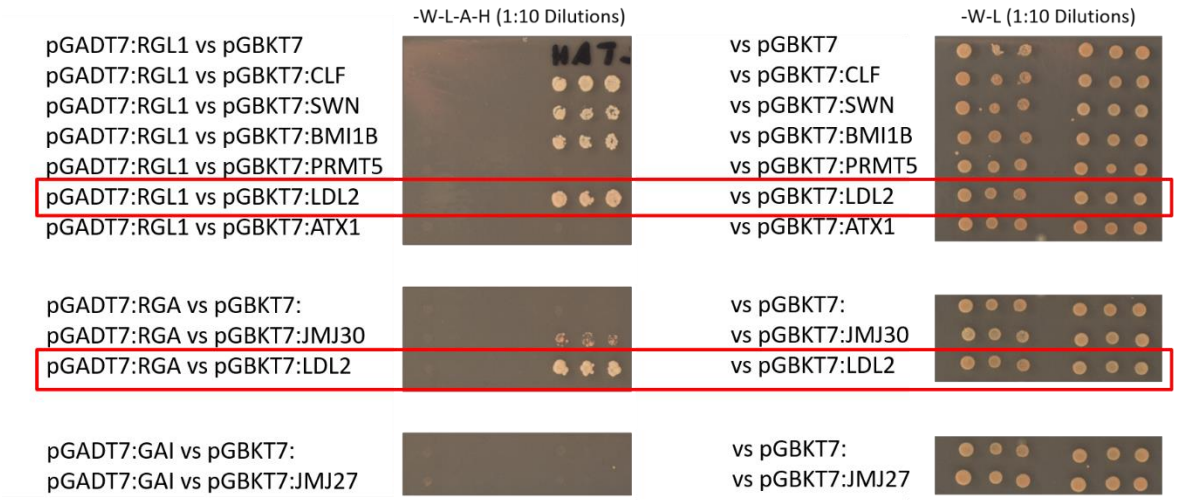

Figure 15: Y2H Assay Between DELLA Proteins and Chromatin Remodelling Enzymes

Yeast-Two Hybrid (Y2H) assay displaying successful Y2H transformations (-WL) and interactions (-WLAH) between the DELLA proteins (RGL1, RGA, GAI) and chromatin remodelling enzymes. Red box outlines interactions between LDL2 and the DELLA proteins RGL1 and RGA.

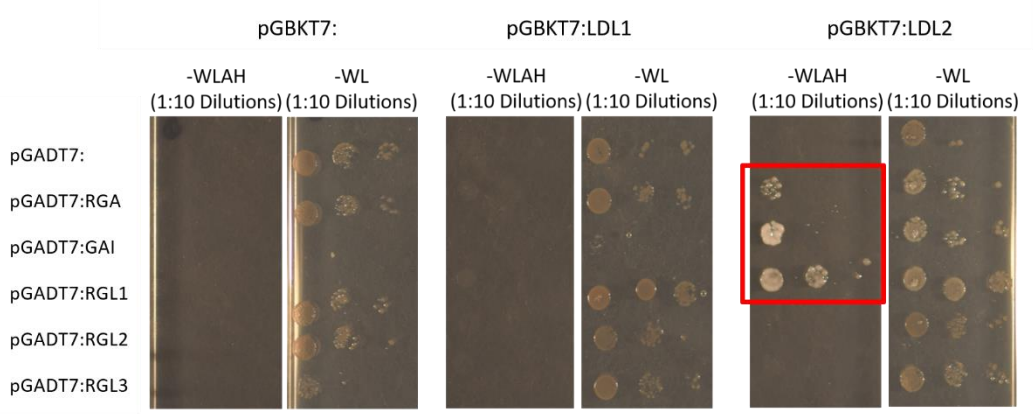

Figure 16: Y2H Assay Between DELLA Proteins and LDL1/2

Yeast-Two Hybrid (Y2H) assay displaying successful Y2H transformations (-WL) and interactions (-WLAH) between the DELLA proteins (RGA, GAI, RGL1/2/3) and LDL1/2. Red box outlines interactions between LDL2 and the DELLA proteins RGA, GAI and RGL1.

These Y2H were intended to answer the first question posed for this research: ***"Do the DELLA proteins interact with chromatin remodelling enzymes in a meaningful way?".*** To answer this a Yeast Two-Hybrid assay was performed in order to identify protein-protein interactions between the DELLAs and chromatin remodelling enzymes which, through their deposition of active or repressive histone marks, control the 'tightness' with which chromatin is wrapped around the histones. In doing this, the chromatin remodelling enzymes dictate whether gene expression is promoted, due to a looser conformation, or repressed, due to a tighter conformation.

pGADT7:DELLA vs pGBKT7 were used as negative controls. Both cultures on either non-selective media (SD-W-L) or selective media (SD-W-L-A-H) were incubated at 30^{o}C for 2 days. pGADT7:DELLA was used for screening images as pGBKT7 resulted in autoactivation, giving false-positive results.

In the right-hand column of ***Figure 15,*** *Saccharomyces cerevisiae* growth on non-selective SD-W-L plates confirms the presence of both pGADT7 and pGBKT7 plasmids of interest after successful transformation of strains. The pGADT7 plasmid contains the *LEU2* gene, required for the synthesis of leucine, and the pGBKT7 plasmid contains the *TRP1* gene, required for the synthesis of tryptophan.

In the left-hand column of ***Figure 15*** growth on the selective SD-W-L-A-H media, signals successful interaction of the proteins of interest within the pGADT7 and pGBKT7 plasmids, reconstituting and resulting in the expression of the reporter genes *ADE2* and *HIS3*, which drive the synthesis of adenine and histidine respectively. In the case of growth on the selective media, the Yeast Two-Hybrid interaction was therefore considered positive, identifying six different interactions between chromatin remodelling enzymes and the DELLA proteins. The interactions displayed are: RGL1 interacting with CLF, SWN, BMI1B and LDL2, and RGA interacting with JMJ30 and LDL2. Therefore RGA/RGL1 interact with histone lysine methyltransferases (SWN, CLF), histone lysine demethylases (LDL2, JMJ30) and histone lysine E3 ubiquitin ligases (BMI1B).

A further Y2H was carried out later after *in vivo* validation of the first Y2H had already occurred, screening specifically between the DELLAs and LDL1/2, growth on SD-W-L-A-H validated the interactions of RGA and RGL1 with LDL2 and identified an additional interaction between GAI and LDL2 (***Figure 16*****).**

8.1.2 Verification of interactions between the DELLAs RGA and RGL1 and chromatin remodelling enzymes *in vivo*

Having initially identified six different *in vitro* interactions between RGA, RGL1 and five different chromatin remodelling enzymes, it was decided to focus on the interaction of RGA and RGL1 with LDL2, a histone-lysine demethylase, because LDL2 was the chromatin remodelling enzyme showing more consistent interaction across DELLA proteins (***Figure 15)***.

To confirm that DELLA and LDL2 interaction occurs in vivo, we used bimolecular fluorescence complementation (BiFC). BiFC displays interactions through reconstitution of a fluorophore, in this study Yellow Fluorescent Protein (YFP). Each protein of interest in the interaction is fused to either the N-terminal fragment of YFP (YFP-N) or to the C-terminal fragment (YFP-C). A positive interaction between the two proteins of interest results in the reconstitution of the YFP protein, thus forming a bimolecular fluorescence complex which can be observed as yellow fluorescence.

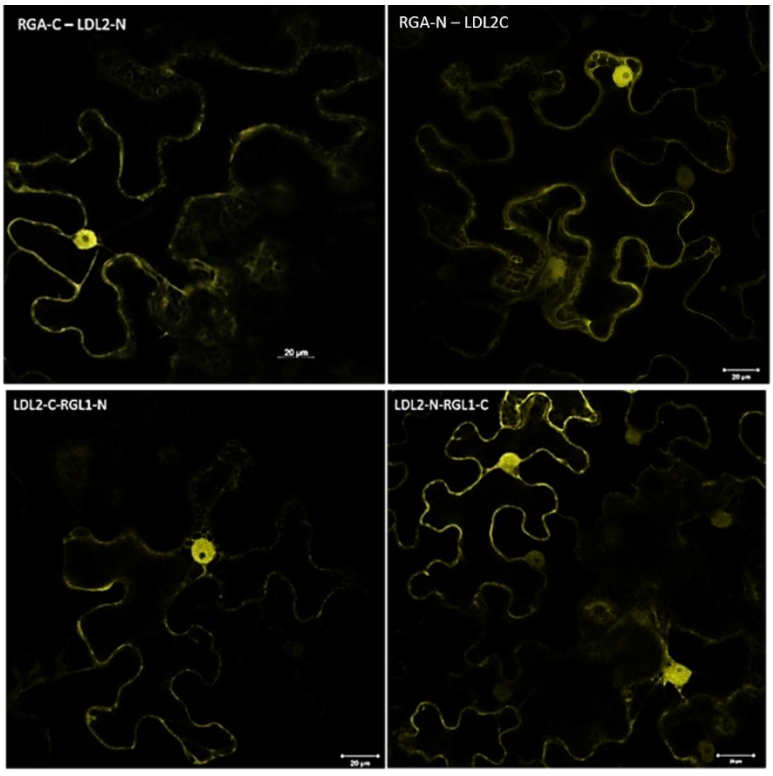

Figure 17: BiFC Between LDL2 and the DELLAs RGA and RGL1

Validation of interaction between the DELLAs RGA and RGL1 with the histone-lysine demethylase LDL2. BiFC interaction assays were performed through Agrobacterium transformation of Nicotiana benthamiana leaf epidermal cells. Confocal micrographs were imaged 3d post-infiltration. Scale bars = 20 µm.

Particularly strong fluorescence was observed in the nucleus of *Nicotiana benthamiana* leaf epidermal cells, which had been infiltrated with successfully cloned YFP plasmids containing the genes of interest, verifying the interaction of the histone-lysine demethylase LDL2 with the DELLA proteins RGA and RGL1 (***Figure 17***).

8.2 Understanding how the interaction between the DELLA proteins and LDL2 impacts germination

The substantial finding of LYSINE-SPECIFIC HISTONE DEMETHYLASE 2 (LDL2) as interacting with the DELLA proteins RGA and RGL1 both *in vitro* and *in vivo*, presents a stimulating frontier to further our understanding of how chromatin remodelling enzymes aid in the control of the germination process. LDL1/2, and their function as histone lysine demethylases, are highly implicated in the repression of seed dormancy, and the repression of the master regulator of germination *DOG1* and so this interaction makes an attractive research target (Zhao *et al.*, 2015).

Due to the shared roles of LDL1/2 and GAs in breaking seed dormancy and promoting germination this study sought to investigate if the DELLA-LDL interaction identified has a function in the timing of germination. This idea was explored both through analysis of the time in which it took seeds to germinate under specific conditions and through the analysis of how this DELLA-LDL interaction may impact the expression of *DOG1*, the master regulator of germination. Unfortunately, due to time constraints, there are aspects of this research that have not yet reached their conclusion. Namely, unsuccessful attempts to create mutants, through both CRISPR and surgical means, which do not express the five DELLA Proteins (RGA, GAI, RGL1, 2 and 3), nor LDL1/2. This will be discussed further in this thesis.

Both the visual observations of germination and the analyses of *DOG1* expression occurred simultaneously and the most valuable way to present these results is also side-by-side.

8.2.1 Stratified seeds germinate significantly earlier than unstratified seeds, *DOG1* expression is also significantly lower in stratified seeds

DOG1 is key to the repression of germination through maintaining seed dormancy, the importance of cold stratification (a brief exposure to cold, wet conditions) is well understood in promoting germination and breaking dormancy. There is evidence that LDL1/2s function in repressing *DOG1* and seed dormancy is vital to the stratification process, with their demethylase activity acting to demethylate the active H3K4me3 and H3K36me3 marks at the *DOG1* locus, thus functioning to repress *DOG1* expression in response to stratification (Footitt *et al.*, 2014). The DELLA-LDL interaction identified in this study may be implicated in this process.

These initial analyses set out to display the importance of cold stratification in promoting germination within wild-type seeds with normally functioning DELLAs, LDL2 and DOG1. Analyses of the day of germination between treatments served to present the phenotypic effects of stratification of germination time, while analyses of expression analyses indicated the genotypic effect of stratification on *DOG1* expression.

Figure 18 shows that the stratified Wild-Type *Landsberg erecta* (*Ler*) seeds tend to germinate earlier, seeds were plated on day zero and the bulk of germination occurred over days one and two, before becoming much less prominent from day three onwards. The unstratified seeds, however, experience most of their germination later on, between days two and four. The mean day of germination for stratified seeds vs unstratified seeds are day 1.89 and 2.52 respectively.

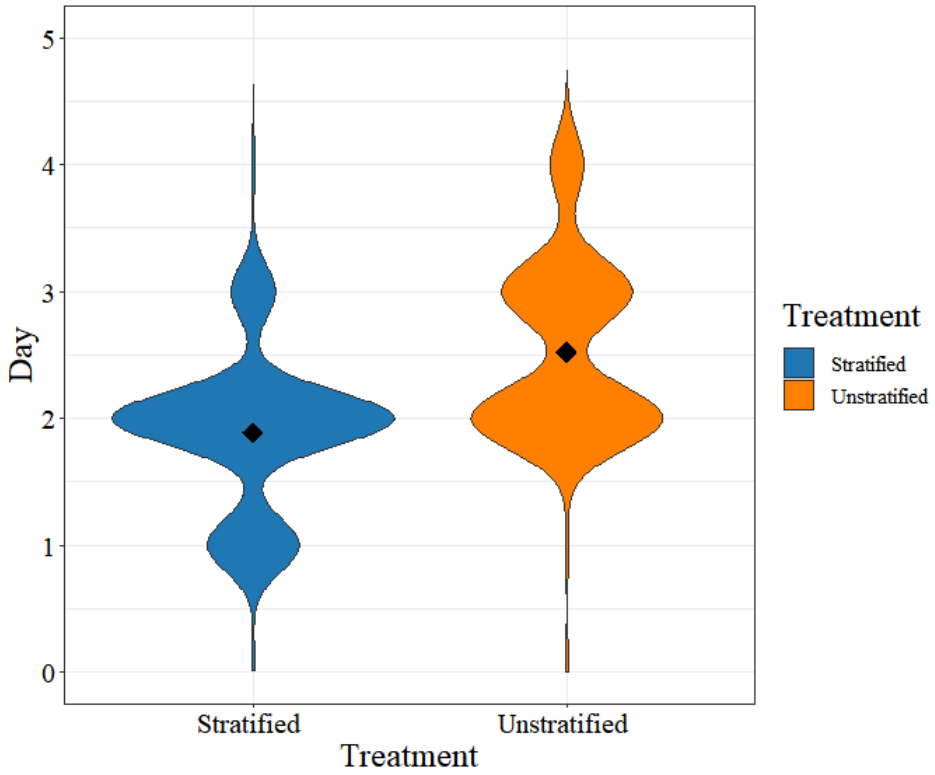

Figure 18: Germination Timing in Stratified and Unstratified Seeds

Violin Plot showing the distribution of the timing of germination for seeds of both stratified and unstratified WT Landsberg erecta (Ler) Arabidopsis thaliana. Black diamond shows the mean time of germination.

The two distributions of germination across both stratified and unstratified seeds were significantly non-normal according to both the Kolmogorov-Smirnov test and the Shapiro-Wilk test (***Table 33***). It may appear as though there are diel patterns with spikes of germination at certain times, however, analyses were only recorded once per day and so this effect is just due to the distribution of the violin plot.

Table 33: Tests of normality for data distributions of the day of germination across different treatment groups of Arabidopsis thaliana seeds.

	Kolmogorov-Smirnov			**Shapiro-Wilk**		
Treatment	Statistic	df	**Sig.**	Statistic	df	**Sig.**
WT *Ler* Stratified	0.361	123	**.000**	0.733	123	**.000**
WT *Ler* Unstratified	0.338	117	**.000**	0.733	117	**.000**

Thus, the Kruskal-Wallis test was conducted to examine the differences on day of germination according to the treatment to which seeds were subjected. Significant differences (Chi-square = 48.666, p = $3.035e^{-12}$, df = 1) were found between the stratified and unstratified wild-type *Ler* seeds. The Wilcoxon Pairwise Comparisons test reported that the mean day of germination in the Wild-Type *Ler* Stratified group was significantly lower than in the Wild-Type *Ler* Unstratified (p = $3.1e^{-12}$) group (***Table 34***).

Table 34: The mean day of germination across different treatment groups of Arabidopsis thaliana seeds.

Treatment	**Mean Day of Germination**
Wild-Type *Ler* Stratified	1.89
Wild-Type *Ler* Unstratified	2.52

The qPCR analyses of the expression of *DOG1* found significantly lower levels of *DOG1* expression in the stratified seeds than in the unstratified seeds when compared to the reference gene *PP2A-A3* (***Table 35***).

Table 35: Relative expression of DOG1 in stratified WT Landsberg erecta (Ler) seeds, compared to unstratified WT Ler seeds. PP2A-A3 used as reference.

Gene	**Relative Expression**	**Standard Error of Relative Expression**	**95% Confidence Interval**	**P-Value**
PP2A-A3	1			
DOG1	0.264	0.226-0.313	0.204-0.363	0.00

These experiments show that stratification has a significant effect on promoting early germination and decreasing *DOG1* expression, this corroborates the findings in studies by Footit *et al.*, (2014) and Nakabayashi *et al.*, (2012). This begins to suggest that the DELLA-LDL2 interaction identified in wild-type seeds contributes to regulating germination, further analyses were carried out to better understand the precise effect of the DELLA-LDL2 interaction on germination.

8.2.2 Treatment of Wild-Type *Ler* seeds with phytohormones has a significant effect on germination time

Endogenous treatment with bioactive GAs such as GA3 has been shown to promote early germination, while treatment with Paclobutrazol (PAC), which inhibits GA biosynthesis, has been proven to inhibit germination, maintaining seed dormancy (Piskurewicz *et al.*, 2008*;* de Mello *et al.*, 2009). This second set of germination and expression analyses sought to further understand the effects of combining cold stratification treatment with phytohormones which alter the levels of GAs in plants.

Figure 19 (overleaf) shows that the stratified seeds germinate earlier across all treatments than their respective unstratified counterparts. Within both stratified and unstratified groupings, the GA3 treated seeds germinate earliest and the Paclobutrazol treated seeds germinate last. The differences in mean germination day between treatments are more prominent in the stratified seeds than the unstratified seeds.

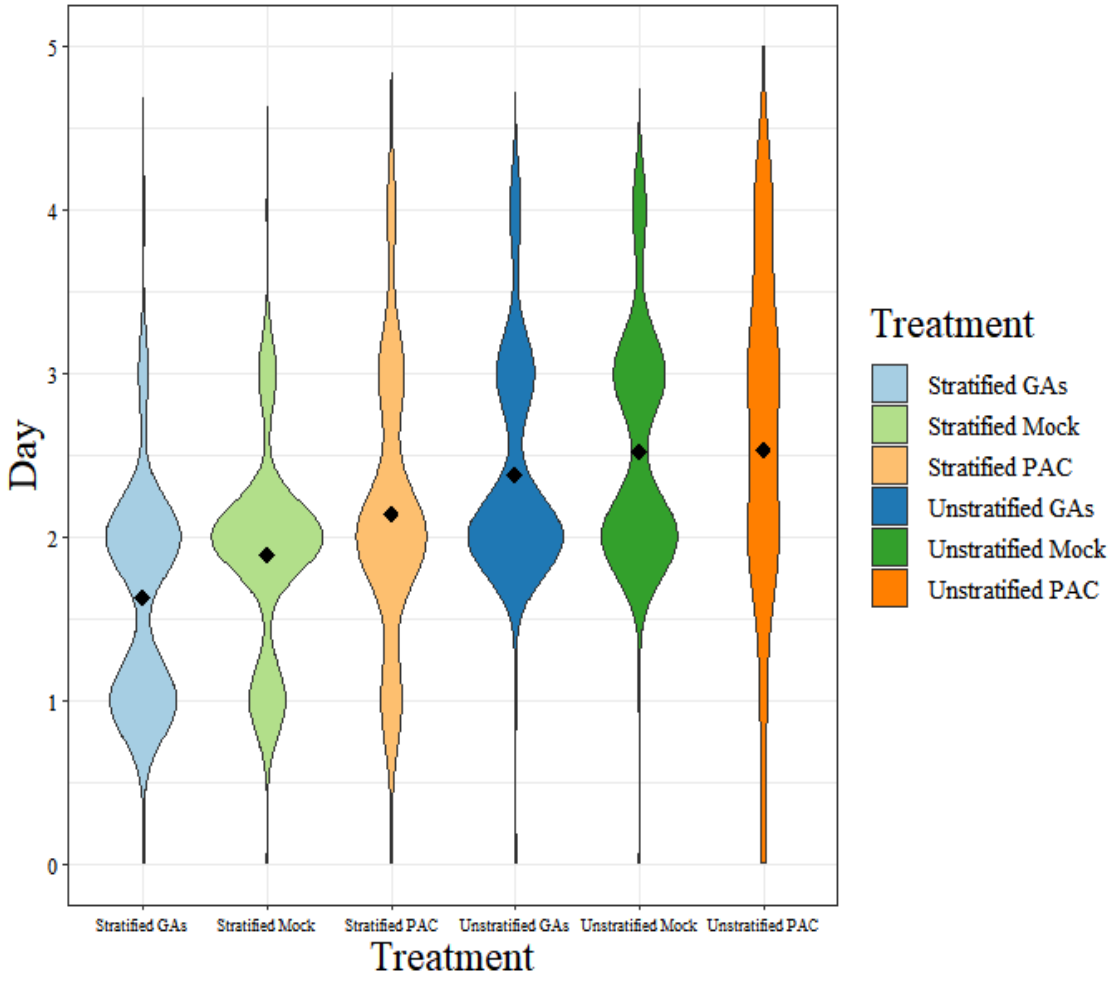

Figure 19: Germination Timing in Stratified and Unstratified Hormone Treated Seeds

Violin Plot showing the distribution of the timing of germination for seeds of WT Landsberg erecta (Ler) either treated with the hormones GA3 (GAs), Paclobutrazol (PAC) or left untreated (mock). Analyses includes both stratified and unstratified seeds. Black diamond shows the mean time of germination.

The distributions of germination in these treatments were all significantly non-normal according to both the Kolmogorov-Smirnov test and the Shapiro-Wilk test (***Table 36***).

Thus, the Kruskal-Wallis test was conducted to examine the differences on day of germination according to the treatment to which seeds were subjected. Significant differences (Chi-square = 116.12, $p = 2.2e^{-16}$, df = 5) were found among the six types of treatments (Mock) Wild-Type Unstratified, (Mock) Wild-Type Stratified, GA3 Treated Unstratified, GA3 Treated Stratified, Paclobutrazol Treated Unstratified, Paclobutrazol Treated Stratified).

Table 36: Tests of normality for data distributions of the day of germination across different treatment groups of Arabidopsis thaliana seeds.

	Kolmogorov-Smirnov			**Shapiro-Wilk**		
Treatment	Statistic	df	**Sig.**	Statistic	df	**Sig.**
(Mock) WT *Ler* Stratified	0.361	123	**.000**	0.733	123	**.000**
(Mock) WT *Ler* Unstratified	0.338	117	**.000**	0.733	117	**.000**
GA3 *Ler* Stratified	0.293	111	**.000**	0.744	111	**.000**
GA3 *Ler* Unstratified	0.405	106	**.000**	0.657	106	**.000**
PAC *Ler* Stratified	0.315	78	**.000**	0.829	78	**.000**
PAC *Ler* Unstratified	0.239	13	**.040**	0.812	13	**.010**

Table 37: The mean day of germination across different treatment groups of Arabidopsis thaliana seeds.

Treatment	**Mean Day of Germination**
GA3 *Ler* Stratified	1.63
(Mock) Wild-Type *Ler* Stratified	1.89
PAC *Ler* Stratified	2.14
GA3 *Ler* Unstratified	2.38
(Mock) Wild-Type *Ler* Unstratified	2.52
PAC *Ler* Unstratified	2.53

The Wilcoxon Pairwise Comparisons ad-hoc test was performed to further understand the meanings of these differences (***Table 38)***. The table reads left to right and shows the p-value for treatments which display a later mean day of germination than the principal (left-hand column).

Table 38: p-values from the Wilcoxon Pairwise Comparisons ad-hoc test for phytohormone treatments. U = unstratified, S = stratified. Orange highlight = non-significant.

	PAC U	WT U	GA3 U	PAC S	WT S	GA3 S
GA3 S	p = 0.0008	$p = 2e^{-16}$	$p = 9e^{-14}$	$p = 1.4e^{-5}$	p = 0.0013	
WT S	p = 0.0043	$p = 1.5e^{-11}$	$p = 8.6e^{-8}$	p = 0.032		
PAC S	p = 0.0968	p = 0.0009	p = 0.0321			
GA3 U	p = 0.3121	p = 0.0968				
WT U	p = 0.7000					
PAC U						

These germination analyses indicate that stratification significantly accelerates germination, whether the plant experiences high GA concentrations (GA3 treatment) or low GA concentrations (PAC treatment). The consistently earlier germination of stratified seeds indicates that even without the GA dependent repression of DELLA mediated seed dormancy, stratification removes seed dormancy, further implicating the idea that the DELLA-LDL2 interaction regulates the early germination promoted by stratification. Due to the early germination seen in the absence of GAs, it is possible that stratification allows LDL2 to shed its repressive DELLA interaction, allowing LDL2 to repress seed dormancy.

8.2.3 Treatment of Wild-Type *Ler* seeds with phytohormones does not significantly impact *DOG1* expression

Due to the effects observed in the germination analyses, that, even in the absence of GAs, stratification drives accelerated germination, these expression analyses were carried out in order to ascertain the phenotypic effects of how stratification regulates *DOG1* across hormonal treatments.

Though substantial differences were present in the germination of stratified seeds regardless of treatment, the qPCR analyses found no significant differences in the expression of *DOG1*

between treatments of stratified seeds. Whilst there were some observable differences in the mean day of germination that may be expected, these were not statistically significant as outlined above.

8.2.3.1 Expression of *DOG1* in WT *Ler* seeds is not significantly higher than in GA3 treated seeds

The qPCR analyses of *DOG1* expression found no significant difference between *DOG1* expression when untreated stratified seeds were compared to GA3 treated seeds. There were no statistically significant differences between the expression of *DOG1* in wild-type stratified seeds versus GA3 treated stratified seeds (p=0.654) (***Table 39***).

Table 39: Relative expression of DOG1 in untreated stratified WT Landsberg erecta (Ler) seeds, compared to GA3 treated stratified WT Ler seeds. PP2A-A3 used as reference.

Gene	Relative Expression	Standard Error of Relative Expression	95% Confidence Interval	P-Value
PP2A-A3	1			
DOG1	1.172	0.675-2.017	0.484-2.768	0.654

The first conclusion that can be drawn from these results is that the general pattern is as expected, however, this is confuscated by the level of error. The untreated seeds which germinate later than GA3 treated seeds do not show significantly higher *DOG1* expression, i.e. there are not significantly different abundances of DOG1 transcripts. This result could also suggest that the significant differences in germination are not mirrored by *DOG1* transcript abundances as *DOG1* is not the sole regulator of germination. Indeed, whilst being responsible for 12% of genetic difference between highly dormant and non-dormant seeds, this leaves 88% of the effect to be regulated by other factors (Bentsink *et al.*, 2006). Further, there is the possibility that although transcript abundances are not changed there may be post transcriptional regulation occurring which impacts the operation of the *DOG1* protein. Altered function of the DOG1 protein upon treatment could limit the suppression of germination typical of DOG1.

8.2.3.2 Expression of *DOG1* in WT *Ler* seeds is not significantly lower than in Paclobutrazol treated seeds

The qPCR analyses of *DOG1* expression found no significant difference between *DOG1* expression when untreated stratified seeds were compared to PAC treated seeds (p=0.92) (***Table 40***).

Table 40: Relative expression of DOG1 in untreated stratified WT Landsberg erecta (Ler) seeds, compared to Paclobutrazol treated stratified WT Ler seeds. PP2A-A3 used as reference.

Gene	Relative Expression	Standard Error of Relative Expression	95% Confidence Interval	P-Value
PP2A-A3	1			
DOG1	0.96	0.546-1.743	0.343-2.501	0.92

Again, the general pattern is as expected, but so is the high level of error. Those PAC treated stratified seeds which germinate later than untreated stratified seeds do not exhibit significantly higher *DOG1* expression which would result in later germination. Again, this could suggest that the significant differences in germination are not mirrored by *DOG1* suppression as *DOG1* does not suppress germination alone. Whilst accounting for 12% of genetic difference between highly dormant and non-dormant seeds, this leaves 88% of the effect to be regulated by other factors (Bentsink *et al.*, 2006). Likewise, this result mirrors the possibility that treatment may not necessarily alter the transcript abundance. But potentially the operation of DOG1.

8.2.3.3 Expression of *DOG1* in GA3 treated seeds is not significantly lower than in Paclobutrazol treated seeds

The qPCR analyses of *DOG1* expression found no significant difference between *DOG1* expression when GA3 stratified seeds were compared to PAC treated seeds. The relative expression value indicates that *DOG1* expression was approximately 20% lower in GA3

treated stratified seeds than PAC stratified seeds, but the level of error renders this result statistically insignificant (p=0.399) (***Table 41***).

Table 41: Relative expression of DOG1 in GA3 treated stratified WT Landsberg erecta (Ler) seeds, compared to Paclobutrazol treated stratified WT Ler seeds. PP2A-A3 used as reference.

Gene	**Relative Expression**	**Standard Error of Relative Expression**	**95% Confidence Interval**	**P-Value**
PP2A-A3	1			
DOG1	0.818	0.548-1.304	0.441-1.370	0.399

Once more the general pattern is as expected, however, this is again insignificant due to the level of error. Those GA3 treated stratified seeds which germinate earlier than PAC treated stratified seeds do show lower *DOG1* expression which results in earlier germination. Looking past the error this result could also suggest that the significant differences in germination are not correlated by *DOG1* suppression as *DOG1* is not the only regulator of germination. As aforementioned, the large effect of *DOG1* on seed dormancy still leaves 88% of the effect to be regulated by other factors (Bentsink *et al.*, 2006).

8.2.4 Quintuple DELLA Mutants (5PLE) germinate significantly earlier than Wild-Type *Ler* seeds

The final set of analyses was carried out to better answer the final two research questions posed at the start of this study: ***"Do the DELLA proteins regulate germination by modulating the expression of DOG1, the master regulator of germination?"*** and ***"Are the newfound interactions between the DELLA proteins and chromatin remodelling enzymes implicit in the control of germination via DOG1?"***.

The role of DELLA proteins in repressing germination is well documented as is the lifting of this repression upon cold stratification of seeds. Similarly, although the role of LDL1/2 in promoting germination through repressing *DOG1* and seed dormancy is also well

documented, the effect of stratification on promoting this repression of *DOG1* via chromatin remodelling utilising LDL1/2 is just beginning to emerge. By using a *5PLE* mutant which lacks all five DELLA proteins, we can better understand how the DELLA-LDL interactions identified in this study may integrate into the model of germination control via stratification.

Figure 20 (below) depicts that the germination time for stratified seeds within their treatments is again lower than their unstratified counterparts, with the stratified groups of seeds showing a lower mean time of germination than their unstratified counterparts. However, in this instance, the unstratified *5PLE* seeds germinate earlier than the stratified WT *Ler* seeds, with mean days of germination of 3.84 and 4.35 respectively. Both stratified and unstratified *5PLE* seeds germinate earlier than the WT *Ler* seeds, interestingly the unstratified seeds appear to lack, to an extent, the seed dormancy usually associated with seeds before stratification.

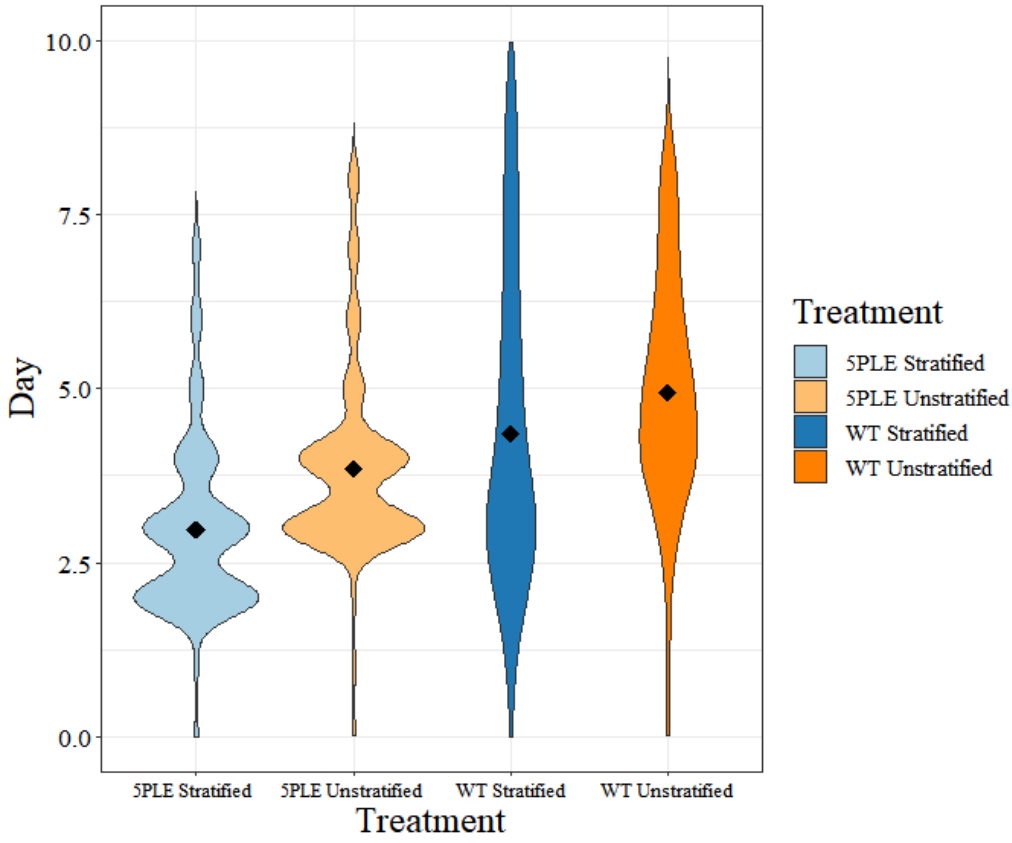

Figure 20: Germination Timing in Stratified and Unstratified 5PLE and WT Seeds

Violin Plot showing the distribution of the timing of germination for seeds of both WT Landsberg erecta (Ler) and quintuple DELLA mutant (5PLE) Arabidopsis thaliana. Analyses includes both stratified and unstratified seeds. Black diamond shows the mean time of germination.

The distributions of germination amongst these four treatment sets were all significantly non-normal according to both the Kolmogorov-Smirnov test and the Shapiro-Wilk test (***Table 42***)

Table 42: Tests of normality for data distributions of the day of germination across different treatment groups of Arabidopsis thaliana seeds.

	Kolmogorov-Smirnov			**Shapiro-Wilk**		
Treatment	Statistic	df	**Sig.**	Statistic	df	**Sig.**
5PLE Stratified	0.262	84	**.000**	0.788	84	**.000**
5PLE Unstratified	0.298	87	**.000**	0.724	87	**.000**
WT *Ler* Stratified	0.246	75	**.000**	0.863	75	**.000**
WT *Ler* Unstratified	0.197	62	**.000**	0.903	62	**.000**

Thus, a Kruskal-Wallis test was conducted to examine the differences in the mean day of germination (***Table 43***) according to the treatment to which seeds were subjected. Significant differences (Chi-square = 64.831, $p = 5.452e^{-14}$, df = 3) were found among the four types of treatments (Wild-Type Unstratified, Wild-Type Stratified, *5PLE* Unstratified, *5PLE* Stratified).

Table 43: The mean day of germination across different treatment groups of Arabidopsis thaliana seeds.

Treatment	**Mean Day of Germination**
5PLE Stratified	2.98
5PLE Unstratified	3.84
Wild-Type *Ler* Stratified	4.35
Wild-Type *Ler* Unstratified	4.94

The Wilcoxon Pairwise Comparisons ad-hoc test was again used to elaborate upon these differences (***Table 44***)**.**The table reads left to right and shows the p-value for treatments which display a later mean day of germination than the principal (left-hand column).

Table 44: p-values from the Wilcoxon Pairwise Comparisons ad-hoc test for mutant analyses. U = unstratified, S = stratified. Orange highlight = non-significant.

	WT U	WT S	5PLE U	5PLE S
5PLE S	$p = 1.6e^{-12}$	$p = 1.2e^{-5}$	$p = 5.4e^{-7}$	
5PLE U	$p = 9.8e^{-7}$	p = 0.60		
WT S	p = 0.01			
WT U				

Considering that the function of LDL1/2 in suppressing seed dormancy directly opposes the role of the DELLAs in promoting seed dormancy, the first implication in these results that *5PLE* mutants lack dormancy in unstratified seeds may indicate that the DELLA-LDL2 interaction identified in this study may contribute to how stratification breaks seed dormancy and promotes germination.

8.2.5 *5PLE* mutant seeds display significantly higher *DOG1* expression than Wild-Type *Ler* seeds

To further investigate such a difference, these observable differences were quantified via expressional analysis of *DOG1* expression. Somewhat contrary to what would be expected, though the *5PLE* seeds germinate much earlier, the expression of *DOG1* is 10.4 times higher (p=0.022) in the stratified *5PLE* seeds than in the stratified WT *Ler* seeds (***Table 45***).

Table 45: Relative expression of DOG1 in stratified quintuple knockout (5PLE) DELLA mutant seeds, compared to stratified WT Landsberg erecta (Ler seeds). PP2A-A3 used as reference.

Gene	**Relative Expression**	**Standard Error of Relative Expression**	**95% Confidence Interval**	**P-Value**
PP2A-A3	1			
DOG1	10.414	3.246-21.184	2.864-23.009	0.022

Conversely, there is no statistically significant difference between the expression of *DOG1* in unstratified *5PLE* seeds when compared to unstratified WT *Ler* seeds, owing to the large error values (p=0.14) (***Table 46***).

Table 46: Relative expression of DOG1 in unstratified quintuple knockout (5PLE) DELLA mutant seeds, compared to unstratified WT Landsberg erecta (Ler) seeds. PP2A-A3 used as reference.

Gene	**Relative Expression**	**Standard Error of Relative Expression**	**95% Confidence Interval**	**P-Value**
PP2A-A3	1			
DOG1	2.813	1.418-5.630	0.831-8.550	0.14

Likewise, there are no statistically significant difference in *DOG1* expression in stratified *5PLE* seeds versus unstratified *5PLE* seeds. The high p-value would indicate that any difference is extremely non-significant (p=0.863) (***Table 47***).

Table 47: Relative expression of DOG1 in stratified quintuple knockout (5PLE) DELLA mutant seeds, compared to unstratified 5PLE DELLA mutant seeds PP2A-A3 used as reference.

Gene	Relative Expression	Standard Error of Relative Expression	95% Confidence Interval	P-Value
PP2A-A3	1			
DOG1	1.096	0.612-1.760	0.530-2.260	0.863

As reported earlier, the expression of *DOG1* in stratified WT *Ler* seeds was lower than in unstratified *Ler* seeds, however, due to large standard error in this qPCR run this was seen as non-significant (***Table 48***). However, it does provide comfort that this qPCR matches the lower expression of *DOG1* in the stratified versus unstratified treatments as earlier observed, a consistent finding.

Table 48: Relative expression of DOG1 in stratified WT Landsberg erecta (Ler) seeds, compared to unstratified WT Ler seeds. PP2A-A3 used as reference.

Gene	Relative Expression	Standard Error of Relative Expression	95% Confidence Interval	P-Value
PP2A-A3	1			
DOG1	0.289	0.104-0.927	0.069-1.634	0.263

Though the unexpected upregulation of *DOG1* in *5PLE* seeds is intriguing, the real interest lies in the lack of difference stratification makes in *5PLE* seeds (***Table 47***). In wild-type plants stratification is key to repressing *DOG1* (***Table 35*** and ***Table 48***), this result would suggest that the lack of DELLAs in unstratified seeds results in *DOG1* levels much closer to that of stratified seeds. This would suggest that stratification may allow LDL2 to free itself from interaction with the DELLAs in order to repress *DOG1* and seed dormancy. This could suggest an involvement of the DELLA proteins in the chromatin remodelling enzyme-mediated control of seed dormancy, via *DOG1*.

9 Discussion

This study aimed to elaborate on our understanding of the germination process, and how this critical stage of plant development is so stringently regulated to avoid improper timing of germination. A greater understanding of the germination process would help to alleviate the issue of pre-harvest sprouting (PHS) in cereal crops, responsible for losses of approximately $1 billion per year, affecting key crops such as wheat, a staple food for 40% of the global population (Bewley *et al.*, 2006*;* Singh and Upadhyaya, 2015). These insights could further aid in the production of beer and whiskey, important products that rely upon rapid and uniform germination during barley malting (Gubler *et al.*, 2005).

The gibberellins (GAs) are well-characterised phytohormones, implicit in many aspects of plant development, including the germination process (Achard and Genschik, 2009). The relationship between the GAs and the DELLA proteins, which repress GA signalling, has been extensively studied, and the importance of this relationship in the control of germination is not to be understated. Research into the importance of chromatin dynamics in plant development, and the chromatin remodelling enzymes (CREs) which drive changes in chromatin dynamics, is becoming ever more prevalent, yet, there is limited research into how CREs may regulate the germination process. This brings us to the first of the three questions this study proposes, ***"do the DELLA proteins interact with chromatin remodelling enzymes in a meaningful way?"***.

9.1 The DELLA proteins RGA and RGL1 interact with LDL2, amongst multiple chromatin remodelling enzymes

To explore how the GA signalling process incorporates chromatin remodelling to regulate the timing of germination, a Yeast Two-Hybrid (Y2H) assay for protein-protein interactions was performed. This Y2H was designed to screen for interactions between the five DELLA proteins (RGA, GAI, RGL1/2/3), which suppress GA signalling and germination, and a library of chromatin remodelling enzymes which account for approximately 30% of the estimated number of histones modifying enzymes (***Table 32***). The results of the Y2H were validated by bimolecular fluorescence complementation (BiFC) assays.

Gibberellins are integral to many growth-regulating processes, particularly in response to environmental cues, germination is one such process regulated by GA signalling. In the absence of GAs, however, these growth-producing responses are inhibited by the DELLA proteins. This is achieved through the DELLAs activity as transcriptional regulators which via modulation of transcription factors (TFs) drive gene expression opposing GA responses, though Locascio *et al.*, 2013 also suggests the DELLAs may also operate via non-transcriptional means. However, the presence of GAs and their binding to the soluble receptor GID1 results in the DELLAs being targeted for ubiquitination by the F-box protein SLEEPY (SLY1), and subsequent degradation by the 26S-proteasome (Davière *et al.*, 2016). The DELLA proteins also promote the transcription of GA biosynthetic genes and genes for the soluble receptor GID1, in this way upregulating GA production and perception, and downregulating their own expression (Claeys *et al.*, 2014) (***Figure 7***).

There have been few studies indicating a role for CREs in the GA signalling pathway, and these have mainly focussed on the SWI/SNF (SWITCH/SUCROSE-NON-FERMENTABLE) family of chromatin remodellers, which promote the DELLA dependent expression of the GA receptor *GID1* (***Figure 7***). The Y2H assay in this study has indicated the interaction of five different CREs with the DELLAs. Specifically, there are interactions between RGA-LIKE 1 (RGL1) and; LYSINE-SPECIFIC DEMETHYLASE LIKE 2 (LDL2), BMI1B (a component of PRC1, the Polycomb repressive complex 1), CURLY LEAF (CLF) and SWINGER (SWN), and further, there are interactions between REPRESSOR OF GA (RGA) and; LDL2 and JMJ30 (a Jumonji C domain-containing protein). LDL2 is implicit in the stratification-mediated degradation of *DOG1*, using its demethylase activity to remove the active H3K4me3 and H3K36me3 histone marks. CLF and SWN are also implicated in this same model, activating as histone methyltransferases, methylating H3K27, a repressive mark. The interaction identified between LDL2 and RGA/RGL1 was of particular interest, not solely due to interacting with both RGA and RGL1, but also in light of the recent research surrounding the role of LDL2 in repressing seed dormancy (Zhao *et al.*, 2015).

The BiFC assay validated these interactions *in vivo*, an important result as *in vitro* analyses such as the Y2H cannot replicate the physiological and biochemical conditions experienced within a cell. Such cellular context cannot be ignored as this may result in reporting a false positive interaction from the Y2H (Waadt *et al.*, 2014). The strong yellow fluorescence observed in the nuclei of *Nicotiana benthamiana* cells validates the

interactions between LDL2 and RGA/RGL1. The characterisation of these interaction widens our scope of understanding regarding how many CREs and which CREs interact with the DELLA proteins, and how this may impact the upon GA signalling critical in plant development.

9.1.1 RGA and RGL1 may facilitate LDL2 suppressing seed dormancy

Though Jiang *et al.*, 2007 explained the role of both LDL1 and LDL2 in promoting flowering, via the repression of *FLOWERING LOCUS C (FLC)* and *FLOWERING WAGENINGEN (FWA)* which repress flowering, a more recent study by Zhao *et al.*, (2015) made a case for LDL2 in the regulation of seed dormancy. The expression of both *LDL1/2* is highest during the early stages of seed development in the silique, decreasing from nine days post-anthesis onwards. LDL1/2 act as histone demethylases, removing the active marks H3K4me3 and H3K36me3 from above the *FLC* locus, thereby repressing *FLC* expression (Jiang et al., 2007). There is evidence for a role of the demethylating LDL proteins in repressing seed dormancy, and redundantly, with the *ldl1 ldl2* double mutants showing significant decreases in germination, whilst *ldl1* and *ldl2* single mutants displayed no significant effect. The observation that plants singularly overexpressing *LDL1* or *LDL2* displayed strongly reduced dormancy, alongside the 69% similarity in amino acid sequence between *LDL1* and *LDL2* does provide strong evidence that LDL2, even in isolation, plays a key role in the regulation of seed dormancy. Zhao *et al.* go on to propose a role for LDL1 and LDL2 in inhibiting the expression of *DELAY OF GERMINATION1* (*DOG1*), the master regulator of germination which accounts for up to 12% of the variation in dormancy between the highly dormant *CVI* accession and *Landsberg erecta* (*Ler*) which shows incredibly low dormancy (Bentsink *et al.*, 2006).

Therefore, the identification of the interactions between both RGA and RGL1 with the histone lysine demethylase LDL2 provides a connection between the DELLA proteins and an emerging role for chromatin remodelling enzymes in the repression of seed dormancy via the master regulator *DOG1*, (Footitt *et al.*, 2014*;* Zhao *et al.*, 2015).

The initial Yeast Two-Hybrid assay in ***Figure 15*** was performed early into the period of research, the results were somewhat skewed by researcher skill with an absence of successful transformations for both RGL2 and RGL3. Therefore, it is possible that there may be interactions between RGL2/3 and LDL1/2 that were missed initially.

In light of this, a further Y2H assay (***Figure 16***) was performed further along in the research period, specifically investigating interactions between the DELLA proteins and LDL1/2. This assay yielded consistently successful transformations for all DELLA proteins and provided the insight of a further interaction between LDL2 and GIBBERELLIC ACID INSENSITIVE (GAI) which was not validated in *vivo*. However, though interactions were observed between LDL2 and RGA/GAI/RGL1, these three DELLAs did not interact with LDL1 (***Figure 16***) RGL2 and RGL3, even once successfully transformed, did not show interactions with either LDL1 or LDL2. LDL1 and LDL2 show significant sequence similarity of ~74% and have been shown to act redundantly in their functions within the germination and flowering (Zhao *et al*., 2015). As such both LDL1 and LDL2 could be expected to display similar interactions, including with the DELLAs. The fact that DELLA interactions were seen with LDL2 but not LDL1, may be due to their 26% sequence dissimilarity. This may indicate that the functional redundancy of LDL1/2 in the germination process is not through the regulation of the DELLAs in particular, but other regulatory factors.

9.1.2 Interaction of RGA, GAI and RGL1 may support a theory of germination control established in seed development

An interesting question that this research raises is the specificity of LDL2 interacting with RGA, GAI and RGL1 but not with RGL2 or RGL3, it is, therefore, prudent to consider the functions of each of the DELLA proteins. The DELLAs are incredibly complex and can act as transcriptional regulators, despite lacking a direct DNA binding domain. The DELLAs, therefore, dictate gene transcription and plant development through interactions with an ever-growing list of transcription factors (Davière *et al.*, 2008). DELLA-protein interactions broadly exist in two categories, where a DELLA may 'kidnap' transcription factors which meddle with its function (De Lucas *et al.*, 2008; Feng *et al.*, 2008), or DELLAs may collaborate with transcription factors to regulate their binding to target gene promoters (Vera-Sirera *et al.*, 2016). To add further complexity, DELLAs also operate non-transcriptionally, interacting with PREFOLDIN 3 and 5, which lack transcriptional activity (Locascio *et al.*, 2013), and DELLAs may also act positively in response to disadvantageous conditions, such as de-etiolation, rather than solely as repressors (Vera-Sirera *et al.*, 2016).

Considering the role of LDL2 in suppressing seed dormancy and promoting germination through demethylating H3K4me3 and H3K36me at the *DOG1* locus, it is surprising that LDL2 showed no interaction with RGL2. Numerous studies propose RGL2 as the major DELLA involved in the control of germination via promoting secondary seed dormancy. Lee *et al.*, (2002) report that while the GA biosynthesis inhibitor paclobutrazol (PAC) suppresses germination, the loss-of-function *rgl2* mutation confers resistance to this effect. Although the loss of *RGL2* abolishes seed dormancy, equivalent loss-of-function mutants lacking *RGA, GAI* and *RGL1* fail to have the same effect. Similar observations are noted in Tyler *et al.*, (2004) which notes that although all five DELLAs are expressed during germination only the expression of *RGL2* increases in response to PAC treatment, with RGA and GAI diminishing. More recent research states that although in low GA conditions only RGL2 is necessary for repressing germination in the light, in the dark GAI and RGA are also required for repressing germination (Piskurewicz *et al.*, 2008). It would, therefore, be interesting to investigate whether the interaction of RGA, GAI and RGL1 with LDL2, might modulate the effect of LDL2 on suppressing dormancy and promoting germination and whether light or dark conditions impact this *in vivo*. With the DELLAs promoting dormancy, could it be possible that RGA, GAI and RGL1 are 'kidnapping' LDL2 in order to interfere with its function, similar to how DELLAs are reported to 'kidnap' transcription factors?

Another avenue to explore would be the origins of primary dormancy in seed development in the silique, as *LDL2* is reported to be highly expressed in the early stages of seed development and was first identified as a regulator of floral development (Jiang *et al.*, 2007). The late-flowering mutants *constant* (*co*) and *flowering locus* (*ft*) are characterised by highly dormant seeds, *FT* represses dormancy via suppressing the production of proanthocyanidins, condensed tannins which are secondary metabolites responsible for the pigmentation of fruits and defence against biotic and abiotic stresses (Rauf *et al.*, 2019). The *ft* mutant, therefore, produces seed coats with a greater tannin content, decreasing the permeability of the seed, perhaps desensitizing the seeds to stratification which is integral to breaking seed dormancy (Chen *et al.*, 2014). *FT* acts downstream of *FLC* which is repressed by LDL1 and LDL2, proposing a redundant role for the LDLs in the regulation of seed dormancy from the beginnings of seed development (Zhao *et al.*, 2015). RGA, GAI and RGL1 also regulate seed development, mutants lacking these DELLAs develop fruits (siliques) without seeds, this is one of the few developmental processes which implicates

RGA, GAI and RGL1 together (Fuentes *et al.*, 2012). Compiling research regarding the function of LDL1/2 in flowering and primary seed dormancy, with the roles of RGA/GAI/RGL1 may suggest that the interaction identified here functions to regulate primary seed dormancy established in the silique, though further analysis is required to understand the precise implications of these interactions in development.

9.1.3 The interactions of DELLA proteins with CLF, SWN, BMI1B and JMJ30 provide backing to the theory of seed development established control of germination

This study only focussed on the interactions observed with LDL2, however, the Yeast Two-Hybrid assays did characterise interactions with other CREs (***Figure 15***) which may provide interesting targets for research. An interaction between RGA and JMJ30 was observed, alongside RGL1 interacting with CLF, SWN and BMI1B. Interestingly, each of these CREs have been reported to function in some capacity, within seed development and/or seed dormancy. Transcriptomic profiling reports that CLF represses ~11.6% of genes in the *Arabidopsis thaliana* genome, 54% of which were repressed specifically in the silique. These gene aggregate into sets which regulate not only the composition and accumulation of lipids which act as energy stores in seeds but also seed size (Liu *et al.*, 2016). SWN, alongside CLF and other components of the Polycomb Repressive Complex-2 (PRC2). represses expression of *FLC*, which ensures floral development does not begin before winter, upon prolonged cold exposure. Both SWN and CLF are critical in the initiation of reproductive development resulting in seed production and germination and are implicit in the downregulation of *DOG1* in response to cold stratification (Berry and Dean, 2015*;* Footitt *et al.*, 2014). JMJ30 also regulates seed development, inhibiting the switch to reproductive development by repressing *FT* (Lu *et al.*, 2011), BMI1B, however, is a component of the Polycomb Repressive Complex-1 (PRC1), vital to the proper timing of germination and the developmental switch to seed development (Molitor *et al.*, 2014).

Taken together, the identification of interactions between LDL2 (amongst other chromatin remodelling enzymes) and RGA/GAI/RGL1 provides exciting additions to the emerging list of CREs implicit in the GA signalling which drives plant developmental processes including seed development and germination. The background research surrounding the function of the interacting CREs, alongside the expression patterns and functions of the

DELLA proteins, may serve to reinforce an emerging theory that seed dormancy is influenced by the conditions experienced during seed development. This would propose a cyclical nature of how DELLA mediated control of flowering and seed production, influences DELLA mediated control of seed dormancy and germination, however, further research would be necessary to confirm this.

9.2 Stratification causes earlier germination, accompanied by a decrease in *DOG1* expression

Identifying multiple novel interactions between the DELLA proteins and chromatin remodelling enzymes, presented an opportunity to further understand how chromatin dynamics influence the regulation of seed dormancy and germination. The interactions of RGA, GAI and RGL1, which are most involved in the reproductive development of *Arabidopsis*, with LDL2, which is reported to regulate flowering and seed dormancy via master regulator *DOG1*, provided an interesting target for research. The first question this study posed ***"Do the DELLA proteins interact with chromatin remodelling enzymes in a meaningful way?"*** was answered with a resounding yes; this answer opened the door for new questions to be asked such as ***"Do the DELLA proteins regulate germination by modulating the expression of DOG1, the master regulator of germination?"*** and ***"Are the newfound interactions between the DELLA proteins and chromatin remodelling enzymes implicit in the control of germination via DOG1?"***.To answer these questions this study analysed both the germination timing and expression profile of *DOG1* when seeds were subjected to various treatments. These observations were recorded simultaneously in order to understand the link between the two observations.

The first set of observations showed that cold stratified wild-type *Landsberg erecta* (*Ler*) germinated significantly earlier than their unstratified counterparts, and the coordination of germination was also different. ***Figure 18*** displays that the cold stratified seeds tended to be more condensed in when they germinated, whereas the unstratified seeds showed more variation. The level of *DOG1* expression was significantly altered also, *DOG1* expression was approximately 3.8 times lower in the cold stratified seeds than in the unstratified seeds (***Table 35***).

The importance of a short period of cold-stratification is well understood, the short period of cold, wet conditions simulates the conditions experienced in nature by a high proportion of species (particularly non-tropical species). Cold-stratification increases a seed's sensitivity to exogenous factors such as light and endogenous factors such as gibberellins (GAs) and has been observed to prompt a larger accumulation of bioactive GAs, resulting in rapid germination in the spring (Bewley *et al.*, 2013). Considering the basis of this study in furthering our understanding of the GA-signalling mechanism in seed dormancy, this made sense as a starting point. The observed effect is consistent both with current literature and with the decrease in *DOG1* expression. As DOG1 significantly promotes seed dormancy (Murphey *et al.*, 2015) it stands to reason that a treatment such as cold stratification which breaks dormancy would do so at least partially through reducing *DOG1* expression. Indeed, there is evidence that imbibition and cold stratification initiate a decline in *DOG1* transcript levels (Footitt *et al.*, 2014*;* Nakabayashi *et al.*, 2012).

Not only do these observations provide a useful reference for the effects of stratification in treatments observed later in the study, but they demonstrate demonstrates the importance of stratification in breaking seed dormancy, in part through chromatin remodelling. Certainly, in Figure 3 of Zhao *et al.*, (2015) it is observable that the germination of stratified seeds is normal even in the *ldl1 ldl2* loss-of-function double mutant, whilst the same *ldl1 ldl2* mutant displays heavily arrested germination when left unstratified. However, this effect is confuscated somewhat as the unstratified seeds were not freshly harvested, whilst the stratified seeds were, extensive study is therefore required to precisely understand the effect of LDL1/2 in breaking dormancy upon stratification.

9.3 Treatment with GA3 and PAC results in earlier and later germination, respectively, particularly when partnered with stratification treatment

This second set of observations demonstrated that, relative to untreated *Ler* seeds, treatment with endogenous GA3, a bioactive gibberellin results in an earlier mean day of germination, whilst treatment with PAC, which inhibits GA biosynthesis, results in a later mean day of germination. This pattern of GA3-treated/Untreated/PAC treated, from earliest to latest germination is repeated in two sets; seeds which were also stratified alongside the treatment and seeds that were not (***Table 37***). The stratified seeds all germinated significantly earlier than their unstratified counterpart, apart from the PAC

treated seeds. Although the stratified seeds treated with PAC had an earlier mean day of germination than the unstratified, this was deemed not statistically significant by the Wilcox Pairwise Comparisons test. Likewise, although the differences between treatments were deemed statistically significant in the stratified set of seeds, the differences between treatments in the unstratified set of seeds were not statistically significant.

The findings that PAC treated seeds germinate later and GA treated seeds earlier is not in and of itself a novel observation. There is extensive literature reporting that PAC prevents the germination of wild-type seeds through its inhibition of an enzyme key to GA biosynthesis, *ent*-kaurene oxidase (Piskurewicz *et al.*, 2008*;* Tyler *et al.*, 2004). Similarly, the germination expediting effects of exogenous treatment with GA3 is well documented, as would be expected due to the role of endogenous GAs in promoting germination (de Mello *et al.*, 2009).

The effects of combining GA3 treatment with cold stratification were reviewed by Pipnis *et al.*, (2016) who surmised that those seeds which were stratified germinated earlier than their unstratified counterparts across all variations of GA3 treatment. Likewise, there is some limited evidence that stratified seeds treated with PAC, germinate earlier than those seeds not subjected to stratification (Jin *et al.*, 2018). This is in keeping with the results confirmed in this study, that stratification begets earlier germination regardless of phytohormonal treatments.

9.3.1 Treatment with GA3 and PAC does not, however, affect *DOG1* expression

Due to the lack of difference observed in the mean germination day between unstratified treatments, qPCR analyses were carried out between the stratified seeds of each treatment, which were statistically different from one another. Unlike the qPCR analyses between stratified and unstratified seeds, the qPCR analyses here did not show a significant difference in *DOG1* expression as a result of treatment with either GA3 or PAC. Due to the differences in mean germination day observed, it might be expected that this would be accompanied by discrepancies in *DOG1* expression which is so important in the control of germination.

There were differences in the relative expression of *DOG1* between treatments, the expression is; ~15% lower in GA3 treated seeds than in untreated seeds, ~4% higher in PAC treated seeds than in untreated seeds, and ~20% lower in GA3 treated seeds than in

PAC treated seeds (***Table 39, Table 40, Table 41***). These follow the patterns we would expect to see based upon the germination analyses above, however, these differences were not statistically significant.

There are several possible explanations for the discrepancy between the germination analyses and the lack of significant difference in *DOG1* expression. Simply, the statistical error in these qPCR runs were quite high and so this would obscure whether the differences observed in *DOG1* expression could be classed as significant. Further, even upon ignoring the large statistical error, the variation in the expression levels were not so high as one might expect given the results of the germination analyses. In the first qPCR performed (***Table 35***) the expression of *DOG1* in stratified seeds was almost four times higher than in unstratified. The differences observed in these treated analyses are not of the same magnitude.

Though *DOG1* accounts for up to 12% of the difference in dormancy between highly dormant (*CVI*) and highly non-dormant (*Ler*) accessions and this is a substantial amount of variation to attribute to one gene, this still leaves 88% of variation to be dictated by other mechanisms (Bentsink *et al.*, 2006). It is unlikely that the effects GA3 and PAC treatments have on seed dormancy and germination are solely mediated by DOG1, in fact, Murphey *et al.*, (2015) also muse that whilst *DOG1* is highly involved in the induction of secondary dormancy, via the downregulation of GA metabolism and upregulation of GA catabolism (Kendall *et al.*, 2011*;* Hedden and Thomas, 2016), it is improbable to be the sole dictator.

It is also important to address the possibility that, as qPCR analyses measure transcript abundance, post transcriptional regulation could explain the unexpected results. Although the differences in germination analyses are not accompanied by the expected changes in *DOG1* expression, the action of the DOG1 protein may be changed by post transcriptional regulation spurred by the hormone treatments. Therefore, DOG1 may still be dictating the differences in germination timing, not through sheer transcript abundance, but via an altered protein function.

A review of the literature suggests no other studies have used qPCR analysis to compare the effects of treatment with GA3 and PAC on the expression of the master regulator of germination, *DOG1*.

9.4 5PLE mutant seeds germinate earlier than wild-type seeds

The final set of observations sought to answer the remaining research questions: ***"Do the DELLA proteins regulate germination by modulating the expression of DOG1, the master regulator of germination?"*** and ***"Are the newfound interactions between the DELLA proteins and chromatin remodelling enzymes implicit in the control of germination via DOG1?"***. The design of these experiments encompasses how the action of DELLA proteins in repressing germination may be linked to the same repression of germination as a result of *DOG1* expression, with a focus on how the interaction between LDL2 and the DELLAs may bridge this gap.

Consistent with current consensus, the seeds of mutant *Ler* plants lacking all five DELLA proteins (termed *5PLE*, quintuple or pentuple) germinated significantly earlier than those seeds from wild-type *Ler* (Tyler *et al.*, 2004). This effect was consistent whether the seeds were stratified or not with the order of the mean day of germination, from earliest to latest, being: *5PLE* stratified, *5PLE* unstratified, WT *Ler* stratified, WT *Ler* unstratified (***Table 43***). The Wilcox Pairwise Test confirmed that all the differences between each combination of these treatments were significant, displaying that unstratified seeds did not struggle to germinate. This is a stark contrast to earlier observations in which the lack of stratification significantly inhibited the ability of seeds to germinate, even combined with the germination promoting effects of endogenous GA3 application (Pipnis *et al.*, 2016).

The early germination of *5PLE* seeds is not a novel observation, the most interesting result from these germination assays is the lack of large difference in the germination timing of stratified and unstratified seeds. This indicates that the lack of DELLAs may suppress the disparity between stratified seeds germinating earlier than unstratified seeds, potentially due to the lack of interaction between the DELLAs and the many interactions that mediate the DELLAs repressive activity (Claeys *et al.*, 2014). Amongst these interactions is the interaction with LDL2 identified in this study, encouragingly, *ldl1 ldl2* double mutants experience the opposite effect to our DELLA mutants displaying a much larger disparity in germination success between seeds that were stratified or unstratified (Zhao *et al.*, 2015). Considering that LDL1 and LDL2s roles in suppressing seed dormancy directly oppose the role of the DELLAs in promoting seed dormancy, this first indication that stratification does not impact germination in *5PLE* mutants may begin to suggest a role for the DELLA-LDL2 interaction in mediating control of germination through stratification. To further

investigate such a difference, these observable differences were quantified via expression analysis of *DOG1* expression.

9.4.1 Unexpectedly, *5PLE* seeds display significantly higher *DOG1* expression than wild-type seeds

At first glance, it appears as though the expression analyses are contrary to what we would have expected to see, our germination analyses show unequivocally that, stratified *5PLE* mutant seeds germinate far earlier than their stratified wild-type counterparts, and so one would expect that the expression of *DOG1* a master repressor of germination had also dropped. However, the qPCR results show the opposite, the expression of *DOG1* in *5PLE* seeds is 10.4 times higher than in the wild-type seeds (***Table 45***). It is appropriate to acknowledge that the standard error in this test is large, the confidence intervals show that the upregulated *DOG1* expression in *5PLE* seeds could fall from anywhere within ~3 times as high, to ~23 times as high as the expression in wild-type seeds. Error of this proportion would suggest that repeating the qPCR would be prudent, however, regardless of the high error observed this difference in expression is statistically valid (***Table 45***).

DOG1 was also upregulated when unstratified *5PLE* seeds compared to wild type (***Table 46),*** the increased expression of *DOG1* in unstratified seeds matches the changes in expression when comparing stratified seeds. As already seen in Section 2 *DOG1* was downregulated in stratified wild-type seeds compared to unstratified wild-type seeds (***Table 48***), the relative expression was very similar to that observed in the first qPCR performed in Section 2 (***Table 35***). Both of these results support that this unexpected increase in *DOG1* expression seen in *5PLE* seeds is legitimate.

However, the most interesting result occurs when comparing stratified *5PLE* seeds to unstratified *5PLE* seeds, there is barely any difference in their expression of *DOG1* (***Table 47***). This is in stark contrast to the effect seen in wild-type seeds, where stratification drastically reduces the expression of *DOG1*, this aligns with the germination analyses which display that the delay of germination associated with unstratified seeds wasn't so prevalent in *5PLE* seeds. It is interesting to see the unexpected rise of *DOG1* in seeds which germinate earlier, as these effects seem somewhat antithetical to one another. However, this could be due to a whole host of factors, though *DOG1* is vital for regulating germination, 88% of the difference in seed dormancy is explained by factors other than

DOG1 regulation, this upregulation then isn't very informative (Bentsink *et al.*, 2006). Further, as aforementioned, post transcriptional regulation may alter the function of the DOG1 protein, and drive germination differences, rather than transcript abundance.

The suggestion that the *5PLE* mutant shows reduced effects of stratification is greatly intriguing, more-so considering the identification of interactions between the DELLAs RGA, GAI and RGL1 with the histone lysine demethylase LDL2 (***Figure 16***).In wild-type plants brief cold stratification leads to the downregulation of *DOG1* (Née *et al.*, 2017) and the balancing of histone marks is key to this effect. The integration of CREs and DOG1 in the control of seed dormancy is reviewed by Footitt *et al.*, (2014), which suggests that this integration may function as a thermal sensing mechanism. The temperature mediated control of dormancy is achieved by influencing the expression of *DOG1*, the expression of *DOG1* is dictated by chromatin state which is modified by the addition of active and repressive marks. A decrease in *DOG1* and therefore dormancy is mirrored by the removal of the activating histone marks H3K4me3 and H3K36me3, from the *DOG1* locus. The demethylation of H3K4me3 and H3K36me3 is largely controlled by the histone lysine demethylase activity of LDL1/2, which therefore inhibits expression of *DOG1* by modifying chromatin state (Zhao *et al.*, 2015). Simultaneously, CLF and SWN, two further CREs that this study reported to interact with RGL1 (***Figure 15***), deposit repressive methylation marks at H3K27. Their histone lysine methyltransferase activity increases the proportion of repressive H3K27me3 at the *DOG1* locus at the same time the active marks are removed by LDL1/2 (Footitt *et al.*, 2014). Taken together, these studies indicate that the chromatin remodelling enzymes shown to interact with DELLAs in this research, are integral in dormancy cycling to dictate how temperature directs changes in *DOG1* expression, and therefore seed dormancy.

9.4.2 The lack of difference in *DOG1* expression between stratified and unstratified *5PLE* seeds suggests a role for the DELLAs in regulating *DOG1*

The crux of this result is that stratification normally causes an inhibition of *DOG1* and therefore seed dormancy, with LDL2 helping to drive this change. However, in the absence of the DELLA proteins (*5PLE*) stratification no longer results in the inhibition of *DOG1*. This is exemplified by the lack of difference between stratified and unstratified *5PLE* seeds

observed in both the germination analyses and expression analyses (***Table 43, Table 47***). Therefore, the second research question ***"Do the DELLA proteins regulate germination by modulating the expression of DOG1, the master regulator of germination?"*** is answered; yes, the DELLA proteins indirectly modulate the expression of *DOG1* as exhibited by both the upregulation of *DOG1* in *5PLE* seeds, but more importantly the lack of difference due to stratification of *5PLE* seeds.

These findings suggest that interaction of the DELLAs with LDL2 (and further interacting CREs identified in this work) functions to maintain the active histone marks H3K4me3 and H3K36me3 at the *DOG1* locus in unstratified seeds, opposing LDL2s function in repressing both *DOG1* expression in the seed and the resulting dormancy. This repression would usually be broken by cold stratification, however, in *5PLE* mutants, the DELLAs would not be present to maintain dormancy in the seed through inhibiting LDL2 (amongst other interacting CREs identified). This would explain the lack of difference observed upon stratification, as these processes are also occurring in unstratified due to the lack of DELLA mediated repression. Therefore, we can begin to hypothesise an answer to the final research question proposed: ***"Are the newfound interactions between the DELLA proteins and chromatin remodelling enzymes implicit in the control of germination via DOG1?"***. That via the control of chromatin remodelling enzymes, particularly LDL2 the DELLAs promote *DOG1* expression and therefore promote seed dormancy. This research does not provide conclusive evidence to this to the final research question and further research would be necessary to evaluate the hypothetical scenario proposed above, nevertheless, this study opens exciting leads to further our understanding of how interactions between DELLAs and CREs regulates *DOG1* and seed dormancy.

10 Concluding remarks and a view of the Future

Three research questions were proposed for investigation throughout this research:

1. ***"Do the DELLA proteins interact with chromatin remodelling enzymes in a meaningful way?"***
2. ***"Do the DELLA proteins regulate germination by modulating the expression of DOG1, the master regulator of germination?"***
3. ***"Are the newfound interactions between the DELLA proteins and chromatin remodelling enzymes implicit in the control of germination via DOG1?"***

This study has answered the first research question, identifying novel interactions between the DELLA proteins RGA, GAI and RGL1 with the histone lysine demethylase LDL2, amongst other chromatin remodelling enzymes including CLF and SWN. Integrating the expression and function of *LDL2* in regulating flowering and seed dormancy, with the roles of RGA, GAI and RGL1 in flowering and seed development, suggests that these novel interactions may regulate seed dormancy established during the early stages of seed development.

Analysis of germination timing and *DOG1* expression supported the consensus that cold stratification of seeds accelerates the timing of germination, at least in part through downregulation of *DOG1*. These analyses continued, with both promotive and repressive treatments of GA3 and PAC, respectively. These results further displayed the importance of cold stratification in abolishing seed dormancy and repressing *DOG1* expression. The culmination of these analyses concluded that the effects of cold stratification in the suppression of *DOG1* are abolished in *5PLE* mutants lacking all five DELLA proteins, answering the second research question that DELLA proteins likely regulate seed dormancy via *DOG1*. The suppression of *DOG1* by cold stratification is largely mediated by chromatin remodelling enzymes LDL2, CLF and SWN which target the chromatin at the *DOG1* locus, providing a hypothetical answer to the third research question, that the interactions of DELLA proteins and CREs (specifically LDL2) may be implicit in the control of seed dormancy, and therefore germination, via *DOG1*, the master regulator of germination. A prospective model for this pathway of interaction is provided overleaf (***Figure 21***). As discussed in regards to the unexpected qPCR results, it is worth noting that the DELLA-LDL2 interactions may not just impact the transcript abundance of DOG1, but also the function of DOG1, through post transcriptional regulation.

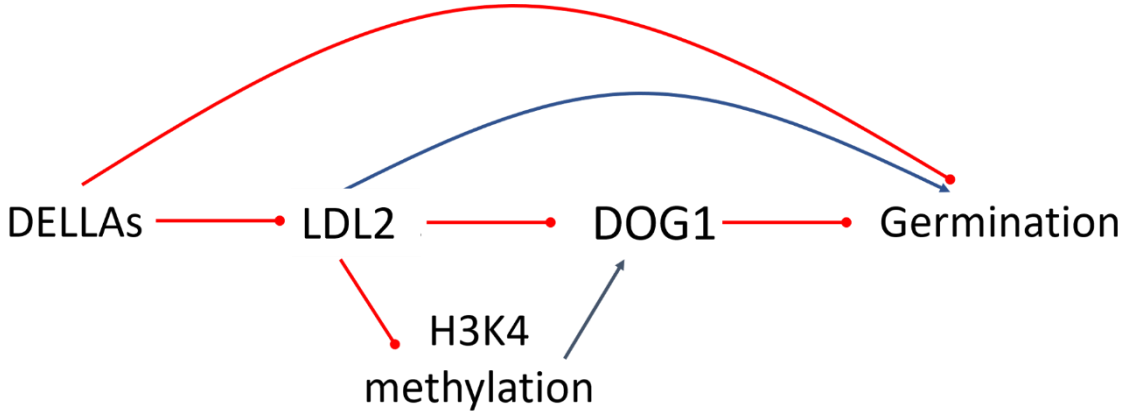

Figure 21: Proposed model of DELLA-LDL2 mediated control of germination.

Proposed pathway of how DELLA-LDL2 interactions mediate germination via the master regulator DOG1. Red, round ended arrows depict inhibitory interactions. Blue arrows indicate promotive interactions. Upper arrows display overall effects.

This research by no means singlehandedly provides all the answers to questions of seed dormancy or human agriculture sustainability, these issues require lots of future research to provide answers. This research does, however, open some interesting avenues by which the role of the DELLA proteins and chromatin remodelling enzymes in modulating seed dormancy can be better understood. Further enlightenment of the processes surrounding seed dormancy is not only important for academic knowledge of one of the most important processes in plant development, but it is also vital to tackling issues facing humans in regards to the sustainability of incredibly important food sources such as wheat and rice.

The timescale of this masters left further scope to elaborate upon this work, including a wider variety of mutant analyses. The creation of a *7PLE* mutant lacking all five DELLAs, LDL1 and LDL2 was attempted using both surgical and CRISPR based methods but was unsuccessful, this would have allowed for a more detailed understanding of how the interaction between LDL2 and the DELLAs impacts seed dormancy and *DOG1* expression. It is possible that the lack of LDL1/2 results in a significant upregulation of dormancy, making it difficult for such mutants to grow. Besides the *7PLE* mutant, specific mutants using individual DELLAs, such as *rga ldl1 ldl2* or *rgl1 ldl1 ldl2* may allow for a more precise understanding of the interactions reported here. In addition, Chip-Seq experiments would have allowed a more detailed image of where the DELLA-LDL interactions bind to and modify chromatin and how this influences the role of these interactions.

11 Bibliography

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12 Appendices

Appendix 1: List of all chemicals and reagents used

Reagent	**Suppliers**
2-(N-morpholino)ethanesulfonic acid (MES)	Melford
2-Mercaptoethanol	VWR
2X SYBR Green PCR Mix Lo-ROX	PCR Biosystems
3-Indolacetic Acid (IAA)	Duchefa Biochemie
5-Bromo-4-chloro-3-indolyl-ß-D-galactoside (X-GAL)	Melford
5X First Reaction Buffer	Thermo Fisher Scientific
5X Phusion HF Buffer	PCR Biosystems
Acetic Acid	Thermo Fisher Scientific
Acetone	Scientific Laboratory Supplies
Acetosyringone	Sigma Aldrich
Advance seed and modular compost + sand	Levington
Agar	Melford
Agarose	Bioline
Ammonium Sulphate	Melford
Bikinin	Selleckchem
Brassinazole (BRZ)	Sigma Aldrich
Bromophenol Blue	Sigma Aldrich
Calcofluor White/ Fluorescent Brightener 28	Sigma Aldrich
Carbenicillin Disodium	Melford
Carboxyl-modified Sera-Mag Magnetic Speed-beads	Thermo Fisher Scientific
Chloral Hydrate	Acros Organics
D-Glucose	Thermo Fisher Scientific
Dimethyl Sulphoxide (DMSO)	Melford

DNA Gel Loading Dye (6X)	Thermo Fisher Scientific
dNTPs	Thermo Fisher Scientific
DTT	Melford
Epibrassinolide (BL)	Sigma Aldrich
Ethanol (EtOH)	Thermo Fisher Scientific
Ethidium Bromide	Thermo Fisher Scientific
Ethylenediamine Tetraacetic Acid (EDTA)	Thermo Fisher Scientific
Gentamycin Sulphate	Melford
Glycerol	Melford
Hydrochloric acid (HCl)	Thermo Fisher Scientific
Hygromycin	Melford
Hyperladder 1kb	Bioline
Kanamycin Monosulphate	Melford
LB Agar High Salt Granulated	Melford
LB Broth High Salt Granulated	Melford
Lithium Acetate (LiAc)	Sigma Aldrich
Murashige & Skoog Medium	Duchefa Biochemie
NTI binding buffer	Macherey-Nagel
Paraformaldehyde	Agar Scientific
PEG 8000	Melford
Periodic acid	Honeywell
Phusion DNA polymerase	Thermo Fisher Scientific
Potassium Ferricyanide	Sigma Aldrich
Potassium Ferrocyanide	Sigma Aldrich
Potassium hydroxide (KOH)	Melford
Propidium Iodide	Sigma Aldrich
Random primers	Thermo Fisher Scientific
RevertAid Reverse Transcriptase	Thermo Fisher Scientific

RiboLock RNase Inhibitor	Thermo Fisher Scientific
Rifampicin	Melford
Sc Dropout minus Leu	Formedium
Sc Dropout minus Trp	Formedium
Sheared Salmon Sperm DNA	Invitrogen
Sodium Chloride (NaCl)	Scientific Laboratory Supplies
Sodium Deoxycholate	Sigma
Sodium Dodecil Sulfate (SDS)	Melford
Sodium Hydroxide (NaOH)	Melford
Sodium Metabisulphite ($Na_2S_2O_5$)	Thermo Fisher Scientific
ß-estradiol	Sigma Aldrich
Streptavidin Magnetic Beads	New England Biolabs
Sucrose	Melford
Synthetic Complete (Sc) Dropout minus ADE, HIS, LEU, TRP	Formedium
Taq Mix Red	PCR Biosystems
Trans-Zeatin	Sigma Aldrich
Tris pH 7.6	Melford
Tris-HCl pH8	Scientific Laboratory Supplies
Triton TX-100	Sigma Aldrich
Tween 20	Melford
Urea	Melford
X-alpha-gal	Apollo Scientific
Xylene Cyanol FF	Thermo Fisher Scientific
Xylitol	Sigma
Yeast Extract Peptone Dextrose (YEPD)	Formedium
Yeast Nitrogen Base (w/o amino acids)	Formedium
Zirconium silica beads	Thistle Scientific

Appendix 2.1: Hot Fusion Cloning Primers

Gene	**Vector**	**Primer Sequences**
ATXR1	pGADT7	GTACCAGATTACGCTCATATGAGAGGAGAGCAATTCGAGC ATTCATCTGCAGCTCGATTACTCTATGCCAAGAAGAGTC
ATXR5	pGADT7	GTACCAGATTACGCTCATATGGCCACATGGAACGCATCCT ATTCATCTGCAGCTCGATCAGAGGAAGTGATGAGTAGGA
ATXR6	pGADT7	GTACCAGATTACGCTCATATGGTGGCTGTGAGGCGAAGGA ATTCATCTGCAGCTCGATTATACAAAATGTTCAGTTGGA
ASHH1	pGBKT7	CAGAGGAGGACCTGCATATGCAATTTTCTTGTGATCCTG GCTAGTTATGCGGCCGCTCATTTGGCTTCCAAGAGTTTA
BMI1A	*pGADT7*	GTACCAGATTACGCTCATATGGAAGGAGACATGGTGGCTA ATTCATCTGCAGCTCGATTAGTTGTTGCATTCAGGGAGC
	pGBKT7	CAGAGGAGGACCTGCATATGGAAGGAGACATGGTGGCTA GCTAGTTATGCGGCCGCTTAGTTGTTGCATTCAGGGAGC
BMI1B	*pGADT7*	GTACCAGATTACGCTCATATGATGATTAAGGTGAAGAAGG ATTCATCTGCAGCTCGATTACATGTTGCACTCTGGTAGC
	pGBKT7	CAGAGGAGGACCTGCATATGATGATTAAGGTGAAGAAGG GCTAGTTATGCGGCCGCTTACATGTTGCACTCTGGTAGC
CLF	*pGADT7*	GTACCAGATTACGCTCATATGGCGTCAGAAGCTTCGCCTT ATTCATCTGCAGCTCGACTAAGCAAGCTTCTTGGGTCTA
	pGBKT7	CAGAGGAGGACCTGCATATGGCGTCAGAAGCTTCGCCTT GCTAGTTATGCGGCCGCCTAAGCAAGCTTCTTGGGTCTA
EMF2	*pGADT7*	GTACCAGATTACGCTCATATGCCAGGCATTCCTCTTGTTA ATTCATCTGCAGCTCGATCAAATTTGGAGCTGTTCGAGA
	pGBKT7	CAGAGGAGGACCTGCATATGCCAGGCATTCCTCTTGTTA GCTAGTTATGCGGCCGCTCAAATTTGGAGCTGTTCGAGA
FIE	*pGADT7*	GTACCAGATTACGCTCATATGTCGAAGATAACCTTAGGGA ATTCATCTGCAGCTCGACTACTTGGTAATCACGTCCCAG
	pGBKT7	CAGAGGAGGACCTGCATATGTCGAAGATAACCTTAGGGA GCTAGTTATGCGGCCGCCTACTTGGTAATCACGTCCCAG
ATX1	pGADT7	GTACCAGATTACGCTCATATGGCGTGTTTTTCTAACGAAA ATTCATCTGCAGCTCGATTATTCTGCGGTCCAGTCTATT
ATX2	pGADT7	GTACCAGATTACGCTCATATGATTTCAATGTCGTGTGTCC ATTCATCTGCAGCTCGATCAGGACTCTGTCCACTCTTTT
JMJ	pGADT7	GTACCAGATTACGCTCATATGGATTCTGGAGTTAAATTGG ATTCATCTGCAGCTCGATCAAAGAGATAAAAGACTTGCC
	pGBKT7	CAGAGGAGGACCTGCATATGGATTCTGGAGTTAAATTGG

		GCTAGTTATGCGGCCGCTCAAAGAGATAAAAGACTTGCC
	pGADT7	GTACCAGATTACGCTCATATGGAAAATCCTCCATTAGAAT ATTCATCTGCAGCTCGATTACATCAAATCTACTCCGAAA
JMJ18	pGBKT7	CAGAGGAGGACCTGCATATGGAAAATCCTCCATTAGAAT GCTAGTTATGCGGCCGCTTACATCAAATCTACTCCGAAA
	pGADT7	GTACCAGATTACGCTCATATGGATTCTGGAGTTAAATTGG ATTCATCTGCAGCTCGATCAAAGAGATAAAAGACTTGCC
JMJ21	pGBKT7	CAGAGGAGGACCTGCATATGGATTCTGGAGTTAAATTGG GCTAGTTATGCGGCCGCTCAAAGAGATAAAAGACTTGCC
	pGADT7	GTACCAGATTACGCTCATATGCCAAAGTGCAAGAATCTGT ATTCATCTGCAGCTCGATTAGAAAGAAAACTTGAAAGTA
JMJ22	pGBKT7	CAGAGGAGGACCTGCATATGCCAAAGTGCAAGAATCTGT GCTAGTTATGCGGCCGCTTAGAAAGAAAACTTGAAAGTA
JMJ27	pGADT7	GTACCAGATTACGCTCATATGGAGAAAATGAGAGGGAAGC ATTCATCTGCAGCTCGATTAGGTATCACTGCGTCGGGAG
	pGADT7	GTACCAGATTACGCTCATATGTCAGGAGCTACCACCGCTT ATTCATCTGCAGCTCGACTACGAGCTAGAAGATTCTGCT
JMJ30	pGBKT7	CAGAGGAGGACCTGCATATGTCAGGAGCTACCACCGCTT GCTAGTTATGCGGCCGCCTACGAGCTAGAAGATTCTGCT
LHP1	*pGADT7*	GTACCAGATTACGCTCATATGAAAGGGGCAAGTGGTGCTG ATTCATCTGCAGCTCGATTAAGGCGTTCGATTGTACTTG
	pGBKT7	CAGAGGAGGACCTGCATATGAAAGGGGCAAGTGGTGCTG GCTAGTTATGCGGCCGCTTAAGGCGTTCGATTGTACTTG
	pGADT7	GTACCAGATTACGCTCATATGTCAACAGAGACTAAAGAAA ATTCATCTGCAGCTCGACTAATCAAAGATCTGTCGATTC
LDL1	pGBKT7	CAGAGGAGGACCTGCATATGTCAACAGAGACTAAAGAAA GCTAGTTATGCGGCCGCCTAATCAAAGATCTGTCGATTC
LDL2	pGADT7	GTACCAGATTACGCTCATATGAATTCTCCGGCGTCGGATG ATTCATCTGCAGCTCGATCAATTAAAATGCAGGGGGTTT
	pGADT7	GTACCAGATTACGCTCATATGGGGAAAGACGAAGAGGAAA ATTCATCTGCAGCTCGACTAAGAAGCTTTTGATGGTTCT
MSI1	*pGBKT7*	CAGAGGAGGACCTGCATATGGGGAAAGACGAAGAGGAAA GCTAGTTATGCGGCCGCCTAAGAAGCTTTTGATGGTTCT
PKDM7D	pGADT7	GTACCAGATTACGCTCATATGGGGACAGAGCTAATGAGAA ATTCATCTGCAGCTCGATCAGCGACGGTTCTGGATCTCT
PRMT10	pGADT7	GTACCAGATTACGCTCATATGAGGAGCTCCCAAAACGGCG ATTCATCTGCAGCTCGATCACTCTATGAAGTAAGTCTTC

PRMT1A	pGADT7	GTACCAGATTACGCTCATATGACTAGTACGGAGAACAACA ATTCATCTGCAGCTCGATTAGCGCATCTTATAGAAGTGG
PRMT1b	pGADT7	GTACCAGATTACGCTCATATGACTAAGAACAGTAACCACG ATTCATCTGCAGCTCGATTAACGCATTTTGTAGTGTTGG
PRMT5	pGADT7	GTACCAGATTACGCTCATATGCCGCTCGGAGAGAGAGGAG ATTCATCTGCAGCTCGACTAAAGGCCAACCCAGTACGAA
	pGBKT7	CAGAGGAGGACCTGCATATGCCGCTCGGAGAGAGAGGAG GCTAGTTATGCGGCCGCCTAAAGGCCAACCCAGTACGAA
PRMT7	pGADT7	GTACCAGATTACGCTCATATGTCGCCTCTGTCTTCTCTTC ATTCATCTGCAGCTCGATCAAGAAATAGTATGAGTGACG
	pGBKT7	CAGAGGAGGACCTGCATATGTCGCCTCTGTCTTCTCTTC GCTAGTTATGCGGCCGCTCAAGAAATAGTATGAGTGACG
RING1A	*pGADT7*	GTACCAGATTACGCTCATATGTCTGTCAAGAATAATAGCT ATTCATCTGCAGCTCGATCACTCAGTTTGCTTCTTCCGG
	pGBKT7	CAGAGGAGGACCTGCATATGTCTGTCAAGAATAATAGCT GCTAGTTATGCGGCCGCTCACTCAGTTTGCTTCTTCCGG
RING1B	*pGADT7*	GTACCAGATTACGCTCATATGCCTTCCTTGAAGAGCTTCT ATTCATCTGCAGCTCGACTACGCGATTTGCTTTCTCCGG
	pGBKT7	CAGAGGAGGACCTGCATATGCCTTCCTTGAAGAGCTTCT GCTAGTTATGCGGCCGCCTACGCGATTTGCTTTCTCCGG
SUVH2	pGADT7	GTACCAGATTACGCTCATATGAGTACATTGTTACCATTTC ATTCATCTGCAGCTCGACTAGTTGCAGATGGCGAGCTTG
	pGBKT7	CAGAGGAGGACCTGCATATGAGTACATTGTTACCATTTC GCTAGTTATGCGGCCGCCTAGTTGCAGATGGCGAGCTTG
SUVH5	pGADT7	GTACCAGATTACGCTCATATGGTACATTCAGAGTCATCAA ATTCATCTGCAGCTCGATTAGTAGAGCCTACCACTACAC
SUVR4	pGADT7	GTACCAGATTACGCTCATATGATCAGTCTCTCCGGACTAA ATTCATCTGCAGCTCGATCATTTGCGCTTTTTAGACA
SUVR5	pGADT7	GTACCAGATTACGCTCATATGGAAGTTAAAATGGATGAGT ATTCATCTGCAGCTCGACTAACTTAAGAGACCTCTGCAA
SWN	*pGADT7*	GTACCAGATTACGCTCATATGGTGACGGACGATAGCAACT ATTCATCTGCAGCTCGATCAATGAGATTGGTGCTTTCTG
	pGBKT7	CAGAGGAGGACCTGCATATGGTGACGGACGATAGCAACT GCTAGTTATGCGGCCGCTCAATGAGATTGGTGCTTTCTG
VRN2	*pGADT7*	GTACCAGATTACGCTCATATGTGTAGGCAGAATTGTCGCG ATTCATCTGCAGCTCGATTACTTGTCTCTGCTGTTATTG
	pGBKT7	CAGAGGAGGACCTGCATATGTGTAGGCAGAATTGTCGCG GCTAGTTATGCGGCCGCTTACTTGTCTCTGCTGTTATTG

Appendix 2.2: Colony PCR Primers

Primer name	**Sequence**
T7 Promoter Forward	TAATACGACTCACTATAGGG
3'AD Reverse	AGATGGTGCACGATGCACAG
3'BD Reverse	TAAGAGTCACTTTAAAATTTGTATC

Appendix 2.3: BiFC Primers

Gene	**Primer sequences**
LDL2	ggaggtggatctcttggCATGAATTCTCCGGCGTCGGATGAAACGGC GGCCGCTCTAGAACTAGTACGATTCATCTGCAGCTCGAG
RGA	ggaggtggatctcttggCATGAAGAGAGATCATCACCAATTCCAAGG GGCCGCTCTAGAACTAGTACGATTCATCTGCAGCTCGAG
RGL1	ggaggtggatctcttggCATGAAGAGAGAGCACAACCACCGTGAATC GGCCGCTCTAGAACTAGTACGATTCATCTGCAGCTCGAG

Appendix 2.4: qPCR Primer

Gene	**Primer Sequences**
DOG1_Forward *DOG1_Reverse*	GACTGGAGCACGAGGACACT ACGTTAGGCTCTCCGACATT
PP2A-A3_Forward *PP2A-A3_Reverse*	TAACGTGGCCAAAATGATGC GTTCTCCACAACCGCTTGGT