Genetic and Environmental Regulation of Seed Longevity in *Arabidopsis thaliana*

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Natanael Viñegra de la Torre

aus Logroño, Spanien

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Berichterstatter: Prof. Dr. Maarten Koornneef

Prof. Dr. Ute Höcker

Prüfungsvorsitz: Prof. Dr. Wolfgang Werr

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Max Planck Institute for
Plant Breeding Research

EcoSeed
Seed performance in a changing climate

ABSTRACT

Seeds represent the link between one plant generation and the next and constitute the main system for dispersal of higher plants, both in space and time. One key trait for seed dispersal in time is seed longevity, which encompasses the period of time a mature seed remains viable and can germinate. The span of time that seeds remain quiescent and yet able to germinate when favourable conditions are perceived results from a combination of their genetic background and the environmental conditions they experienced both during development and once they shed from the mother plant. As such, seed longevity is an adaptive trait which can be under positive or negative selective pressure, affecting plant persistence and survivability. From a human perspective, seed longevity is fundamental for storage of seeds, be it as germplasm, as food sources or for planting in the coming seasons.

This PhD thesis describes a study of the regulatory mechanisms affecting seed longevity in *Arabidopsis thaliana* using diverse approaches. Temperature and drought stresses applied during seed maturation altered seed longevity and RNA-sequencing was used to evaluate how they modified the dry seed transcriptome. These analyses allowed the identification of several potential candidate genes that contribute to the regulation of seed longevity. Furthermore, the molecular regulation of seed longevity was investigated and two genes previously not associated with seed longevity were identified. Lastly, a novel procedure to evaluate seed quality was validated based on the use of biochemical probes. These probes helped to characterise two enzymatic activities which can be used to monitor seed longevity.

KEYWORDS

Seed longevity | Arabidopsis | DOG1 | Environmental stresses | ABPP | RNA-seq

ZUSAMMENFASSUNG

Samen stellen den vegetativen Übergang von einer Pflanzengeneration zur nächsten dar und bilden gleichzeitig die Grundlage für räumliche und zeitliche Ausbreitungsmechanismen von samentragenden Pflanzen. Die Langlebigkeit der Samen spielt bei der zeitlichen Ausbreitung von Pflanzengenerationen eine entscheidende Rolle und wird als die Zeitspanne definiert, in der ein reifer Samen die Fähigkeit zum Keimen besitzt. Die Zeitspanne, in der ein Samen in diesem Überdauerungszustand verbleiben kann, ohne seine Keimungsfähigkeit bei geeigneten Umweltbedingungen zu verlieren, wird durch eine Kombination aus dem jeweiligen genetischen Hintergrund der Pflanze und den Umwelteiflüssen, die während der Samenreifung und nach der Streuung des Samens von der Mutterpflanze vorherrschen, beeinflusst. Daher kann man die Langlebigkeit von Samen als ein adaptives Merkmal betrachten, welches durch positiven oder negativen Selektionsdruck die Persistenz und Überlebensfähigkeit von Pflanzenarten beeinflusst. Die Langlebigkeit von Samen stellt somit eine fundamentale Grundlage für die Lagerung von Samen als Saatgut, Lebensmittel oder in der Form von Keimplasma- als eine reichhaltige und vielfältige pflanzengenetische Ressource dar.

Diese Dissertation beschäftigt sich, unter Verwendung diverser Methoden, mit den regulatorischen Mechanismen, die die Langlebigkeit von Samen am Modellorganismus *Arabidopsis thaliana* beeinflussen. Um herauszufinden, wie diese Mechanismen das Transkriptiom von reifen Samen verändern, wurden die Samen während des Reifeprozesses Trocken- und Temperaturstress ausgesetzt. Die Auswirkungen dieser abiotischen Stressfaktoren in reifen Samen wurden zudem mittels RNA-Sequenzierung untersucht. Diese Analysen ermöglichten es, einige potenzielle Gene zu identifizieren, die an der Regulierung der Langlebigkeit beteiligt sein könnten. Zudem wurde die molekulare Regulation der Langlebigkeit von Saatgut untersucht. Dabei konnten zwei Gene identifiziert werden, die bisher noch nicht mit Saatgutlanglebigkeit in Zusammenhang gebracht werden konnten. Schließlich wurde eine biochemische Testmethode evaluiert, die zur Qualitätsüberprüfung von Saatgutlanglebigkeit eingesetzt werden kann. Dadurch konnten zwei Enzymaktivitäten im Zusammenhang mit Langlebigkeit von Saatgut näher charakterisiert werden.

SCHLÜSSELWÖRTER

Samen Langlebigkeit | Arabidopsis | DOG1 | Umwelteinflüssen | ABPP | RNA-seq

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ABBREVIATIONS

3-AT	3-Amino-1,2,4-triazole
ABA	Abscisic Acid
ABPP	Activity-Based Protein Profiling
ABRE	ABA-Response Element
AD	Activation Domain of GAL4
BD	Binding Domain of GAL4
BiFC	Bimolecular Fluorescence Complementation
bp	Base Pair
BRs	Brassinosteroids
CDS	Coding DNA Sequence
DAPI	4',6-Diamidino-2-phenylindole
DELLAs	DELLA domain proteins
DETs	Differentially Expressed Transcripts
dNTPs	Deoxynucleotides Triphosphate
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
FP	Fluorophosphonate
GAs	Gibberellins
GO	Gene Ontology
His	Histidine
HSPs	Heat Shock Proteins
LEAs	Late Embryogenesis Abundant proteins
Leu	Leucine
MES	2-(N-morpholino)ethanesulfonic Acid

MS	Mass Spectrometry
NPC	No Probe Control
PCD	Programmed Cell Death
PP2C	Protein Phosphatase type 2C
PQ	Plastoquinone
QTL	Quantitative Trait Locus
RH	Relative Humidity
ROS	Reactive Oxygen Species
RFOs	Raffinose Family Oligosaccharides
SDS	Sodium Dodecyl Sulfate
SOD	Superoxide Dismutase
SPSs	Seed Storage Proteins
T-DNA	Transfer DNA
TF	Transcription Factor
T_m	Melting Temperature
Trp	Tryptophan
UTR	Untranslated region
VPE	Vacuolar Processing Enzyme
v/v	Volume/volume
w/v	Weight/volume
YFP	Yellow Fluorescent Protein

1 INTRODUCTION

Seeds are the main structures for plant reproduction, allowing their perpetuation and distribution. Although highly diverse in shape, size and composition, seeds share the common feature of carrying the embryo, a miniaturised version of the plant that, upon germination, will develop and give rise to the new generation. Seeds enable plants to disseminate both in space and time, given they are equipped with different mechanisms that allow to stagger germination, increasing the chances that they will meet the optimal conditions for germination and subsequent seedling establishment (Finch-Savage and Bassel, 2016). Furthermore, they allow persistence in time, as some of them will remain in the soil as a part of the soil seed bank. This is especially relevant in those environments with changing or unstable conditions, in which it may occur that the next season does not bring favourable conditions for the seed to germinate.

From a human perspective, seeds constitute the foundation of man and animal feeding, with as much as 75 % of our food sources coming from seeds, specially cereals and legumes. In addition, *ex situ* conservation of plant germplasm is done mainly in the form of seeds (Li and Pritchard, 2009). This has promoted many studies to find the best conditions for storage as well as the processes taking place during this storage (Roberts, 1973; Ellis and Roberts, 1980b; Fu *et al.*, 2015). Besides, the seed industry has great interest in the study of seed storage, as they are expected to deliver seed batches which germinate close to 100 % and many of their economic loses originate from seed deterioration during storage.

A key contributor to seed storage and the main focus of this study is seed longevity. Specifically, seed longevity is the span of time seeds remain viable and able to germinate and it encompasses all those mechanisms present in the seed contributing to this end. The two most extreme cases of seed longevity reported were in seeds of sacred lotus (*Nelumbo nucifera*) and date palm (*Phoenix dactylifera*), which remained able to germinate and produce normal seedlings even after a thousand years (Shen-Miller J., 2002; Sallon *et al.*, 2008). Seed longevity is established during the last phases of seed maturation and the various factors contributing and modifying it will be discussed in the following sections.

1.1 SEED DEVELOPMENT

Seeds are composed of three differentiated tissues, including the embryo, the endosperm and the seed coat or testa, which are formed over the course of seed

development (Goldberg *et al.*, 1994). This process encompasses two major steps: embryogenesis and seed maturation. Seed embryogenesis includes all cell divisions following fertilisation that lead to the formation of the embryo and its surrounding tissues, *i.e.* endosperm and seed coat. Once the full size embryo has developed and stopped growing, seeds enter the maturation phase, during which seed reserves will accumulate, seed dormancy, longevity and desiccation tolerance will be established and the seed will lose most of its moisture content.

Seed embryogenesis starts with a double fertilization of the ovule by the pollen, which triggers a complex developmental process which encompasses several sub-phases. One of the nuclei within the pollen grain merges with the egg cell, creating a diploid (2n) zygote from which the embryo will form following several rounds of cell divisions (Baud *et al.*, 2002). Simultaneously, the second pollen nucleus merges together with the central cell producing a triploid (3n) cell from which the endosperm will develop, a nourishing tissue which supports the growth of the embryo and surrounds it (Olsen, 2001; Berger *et al.*, 2006). Cell divisions continue until the embryo reaches the heart stage (Mayer *et al.*, 1991). From this point, the embryo expands filling the embryo sac, after which cell divisions are arrested and the seed maturation phase begins (Goldberg *et al.*, 1994; Raz *et al.*, 2001). The fertilization process cues several other developmental switches, including the initiation of the seed coat development (Figueiredo *et al.*, 2015).

Contrary to the embryo and the endosperm, the seed coat is a tissue of maternal origin which does not involve the pollen. It develops from the outer and inner integument layers of the embryonic sac following fertilisation (Haughn and Chaudhury, 2005), regulated by signalling pathways involving auxins and gibberellins (GAs) (Kim *et al.*, 2005a; Figueiredo *et al.*, 2016). During seed coat development, several layers differentiate that accumulate different chemical compounds, which participate in seed protection (Debeaujon *et al.*, 2007). All these different layers contribute to the final degree of seed coat permeability, which has a great impact in the resulting seed longevity. In *Arabidopsis thaliana* (hereafter referred to as Arabidopsis), the innermost layer of the seed coat accumulates colourless proanthocyanidins (PAs) during early seed development, which are oxidised into brown pigments by *TRANSPARENT TESTA 10* (*TT10*) during seed desiccation (Pourcel *et al.*, 2005). The subepidermal cell layer thickens and accumulates yellow flavonoids termed flavonols (Pourcel *et al.*, 2007). The importance of these compounds in seed longevity was demonstrated analysing mutant Arabidopsis seeds with an altered flavonoid composition, which showed increased sensitivity to artificial and natural ageing (Debeaujon *et al.*, 2000; Clerkx *et al.*, 2004). The seed coat also accumulates

lignin, a polymer that reinforces it and reduces its water permeability and whose biosynthesis in Arabidopsis is also participated by *TT10* (Liang *et al.*, 2006). Two transcription factors affecting seed longevity in Arabidopsis have been described, which are speculated to do so by altering the seed coat composition or permeability. Increased expression of the transcription factor *ARABIDOPSIS THALIANA HOMEOBOX 25* (*ATHB25*) was reported to increase the expression of gibberellin biosynthetic genes and GA content of seeds, possibly related with alteration of the seed coat composition (Bueso *et al.*, 2014). Similarly, *COGWHEEL 1* (*COG1*)-overexpressing lines were reported to display decreased sensitivity to artificial ageing and greater levels of suberin accumulation in the seed coat, which showed reduced permeability compared to the wild type (Bueso *et al.*, 2016).

1.2 SEED MATURATION

After embryogenesis, the seed enters the phase of maturation, during which it will accumulate storage reserves, acquire germination capacity and lose most of its water content. Among storage reserves, that will support the embryo after germination, carbohydrates, lipids and storage proteins are the most common. Carbohydrates are mainly represented by starch, although other less abundant such as hemicellulose and raffinose family oligosaccharides (RFOs) are also present. The role of RFOs in seed longevity has been long speculated (Bentsink *et al.*, 2000; Buitink *et al.*, 2000). Raffinose biosynthesis requires galactinol, which is produced combining UDP-Galactose and myoinositol in a reaction catalysed by *GALACTINOL SYNTHASE* (*GOLS*) (Taji *et al.*, 2002). In Arabidopsis, only two *GOLS* are expressed in seeds. The galactinol content of seeds was demonstrated to correlate with seed longevity and the *gols2* mutant showed increased sensitivity to artificial ageing (de Souza Vidigal *et al.*, 2016). Transgenic Arabidopsis lines expressing the chickpea (*Cicer arietinum*) *CaGolS1* and *CaGolS2* transcripts under a seed specific promoter also showed an enhancement of seed longevity (Salvi *et al.*, 2016).

Lipids in seeds are mainly triacylglycerols (TAGs), which accumulate in oil bodies or oleosomes in the cytosol and become mobilised after seed germination. Seed storage proteins constitute the main source of N and S and are mainly composed of 12 S albumins and 2 S globulins, which are deposited in protein storage vacuoles. Apart from their role as storage compounds that will sustain the initial phases of seedling growth after germination, a recent study showed that cruciferins, a type of 12 S albumins, are also important for the establishment of seed longevity in Arabidopsis (Nguyen *et al.*, 2015). Organic phosphorus also accumulates during

development of the seed, mainly in the form of phytic acid and its derivatives, which were proposed to play a role in oxidative stress responses in seeds (Doria *et al.*, 2009).

Four main transcription factors govern the progression of seed maturation: *LEAFY COTYLEDON 1* (*LEC1*), *LEC2*, *ABA INSENSITIVE 3* (*ABI3*) and *FUSCA 3* (*FUS3*) (Nambara *et al.*, 1995; Parcy *et al.*, 1997; Kagaya *et al.*, 2005; To *et al.*, 2006). They all act coordinated, regulating each other's expression through feedback loops (Parcy *et al.*, 1997; To *et al.*, 2006). Mutations in any of these genes result in severe abnormalities during seed development and an array of pleiotropic effects, including intolerance to desiccation, failure to accumulate storage proteins and reduced seed dormancy and longevity (Meinke, 1992; Ooms *et al.*, 1993; Clerkx *et al.*, 2004; Tiedemann *et al.*, 2008). *HISTONE MONOUBIQUITINATION 1* (*HUB1*), a RING E3 ubiquitin ligase initially identified for its reduced dormancy phenotype (Peeters *et al.*, 2002; Liu *et al.*, 2007b), participates in transcriptional regulation through chromatin modifications, regulating the levels of gene expression (Cao *et al.*, 2008; Himanen *et al.*, 2012b; Ménard *et al.*, 2014). *hub1* mutants also exhibit pleiotropic phenotypes, including reduced seed dormancy and longevity alongside altered flowering time and chlorophyll content (Liu *et al.*, 2007b), and all of them are most likely a consequence of transcriptional misregulation (Himanen *et al.*, 2012a).

Late stages of seed maturation comprise the degradation of chlorophyll and other pigments present in seeds. This seems to be an important step in seed maturation, as seeds retaining chlorophyll were reported to exhibit low levels of quality and reduced longevity (Jalink *et al.*, 1998; Clerkx *et al.*, 2003; Nakajima *et al.*, 2012; Zinsmeister *et al.*, 2016). The program regulating chlorophyll degradation is under the control of *ABI3*, as *abi3* mutants retain most of their chlorophyll in mature seeds, and is intimately related with biosynthesis of tocopherols, antioxidant components that will play a role in preventing seed ageing (Clerkx *et al.*, 2003; Yang *et al.*, 2014; vom Dorp *et al.*, 2015). *ABI3* also controls the expression of seed specific vacuolar aquaporins (Mao and Sun, 2015). Two of these, named tonoplast intrinsic proteins (TIPs), were shown to be functionally redundant and expressed during late stages of seed maturation. These proteins are water and H_2O_2 transporters and it was shown that they contribute to the maintenance of seed longevity, as the *tip3* double mutant showed increased sensitivity to artificial ageing (Mao and Sun, 2015).

Late seed maturation encompasses the expression of heat shock proteins (HSPs), without the need of heat stress (Wehmeyer *et al.*, 1996). During heat responses, these proteins play a defensive role acting as molecular chaperones and preventing irreversible protein aggregation by promoting protein folding. Probably,

their function in maturation drying is similar, as indicated by recent studies (Kaur *et al.*, 2015). HSPs expression during maturation is promoted by heat shock factors. Specifically, it was shown how the seed-specific *HEAT SHOCK TRANSCRIPTION FACTOR A9* (*HSFA9*), directly regulated by *ABI3*, induces the expression of a whole network of HSPs during the later phases of seed maturation (Kotak *et al.*, 2007; Tejedor-Cano *et al.*, 2010). This is in agreement with the role of these proteins as protective chaperones and also with another report pointing towards enhanced seed longevity after overexpression of *HaHSF9* in sunflower (*Helianthus annuus*) seeds (Tejedor-Cano *et al.*, 2010).

1.3 DESICCATION TOLERANCE

Seed desiccation tolerance is intimately related with the acquisition of seed longevity, although their regulatory processes are separated (Leprince *et al.*, 2016). Based on their tolerance to desiccation, seeds are classified in two main types. On the one hand, recalcitrant seeds are defined as those which are unable to withstand desiccation and require an elevated moisture content to survive. They are usually present in fleshy fruits and their longevity is relatively low. On the other hand, orthodox seeds (including Arabidopsis) are defined as desiccation-tolerant, which lose most of their water content during the late phases of seed maturation (Roberts, 1973). Although highly variable between species, orthodox seeds are able to survive for relatively long periods of time in this dry state and germinate afterwards.

Desiccation tolerance is acquired through different mechanisms involving ABA and its regulatory networks, which trigger the accumulation of non-reducing sugars and late embryogenesis abundant proteins (LEAs). When seeds lose moisture content, the cytoplasm reduces its volume, which can induce several lesions and damages (Hoekstra *et al.*, 2001). To help to protect against these, the presence of previously accumulated RFOs and LEAs is crucial, since they confer stability to the membranes and contribute to the formation of a cytoplasmic glass, which allows metabolism to be stopped and prevents cell constituents from suffering irreparable damages (Ballesteros and Walters, 2011). During this process, water replacement occurs, a mechanism involving the substitution of water molecules by sugars, which helps to conserve the protein structure and lipid spacing, avoiding further membrane fusions (Buitink and Leprince, 2008).

LEA proteins accumulate during seed maturation and comprise a wide variety of protein families (Hundertmark and Hincha, 2008). Despite of their abundance, their molecular roles are not completely clear, although some evidence is available. In Arabidopsis, silencing of three specific dehydrins led to reduction of seed survival

after storage (Hundertmark *et al.*, 2011), whereas accumulation of four specific LEAs was correlated with an increase of seed longevity during seed maturation in *Medicago truncatula* (Chatelain *et al.*, 2012). It is speculated that LEAs can exert diverse roles in the cell based on the level of hydration. These functions include those expected from traditional chaperones, such as protecting the integrity of membranes and stabilizing proteins, but other studies speculate they can increase the density of the cellular glass after desiccation, therefore reducing molecular mobility and increasing seed longevity (Chakrabortee *et al.*, 2007; Tunnacliffe *et al.*, 2010).

1.4 SEED DORMANCY

It is also during the later stages of seed maturation when seed dormancy and longevity are established. These are two key traits that determine the life span of the seed. During the course of evolution, plants have developed strategies that allow them to time seed germination and make it coincide with the adequate environmental conditions (Finch-Savage and Leubner-Metzger, 2006). One such mechanism is seed dormancy, which is defined as the temporary inability of an intact, viable seed to germinate under favourable conditions (Bewley, 1997). Seed dormancy can be alleviated or completely released by dry storage of the seeds, a process referred to as after-ripening. From an ecological perspective, seed dormancy is key as it ensures that seeds will germinate at the correct environmental conditions, preventing them from germinating at a short period of favourable conditions amidst an unfavourable season. Besides, seed dormancy maximises the chances of seeds developing into an adult plant and producing offspring, given that even seeds from the same mother plant will exhibit different depths of seed dormancy. In crops, negative selection for seed dormancy was conducted during the domestication process, aiming for fast and uniform germination. However, a certain degree of seed dormancy is required to prevent pre-harvest sprouting.

The establishment and maintenance of seed dormancy is regulated by the balance between abscisic acid (ABA) and GAs. ABA is essential for the induction of dormancy, as indicated by the altered dormancy levels observed in different mutants with impaired ABA biosynthesis or signalling (Koornneef *et al.*, 1982; Karssen *et al.*, 1983; Koornneef *et al.*, 1984). Conversely, GAs promote seed germination, considering that Arabidopsis mutants impaired in GA biosynthesis fail to germinate, an effect mimicked after incubation with inhibitors of GA biosynthesis (Koornneef and van der Veen, 1980; Debeaujon and Koorneef, 2000). Both hormones are linked through a delicate balance, as they exert a negative influence on the other's biosynthesis and signalling pathways (Finkelstein *et al.*, 2008; Graeber *et al.*, 2012; Gazzarrini *et al.*,

2015). Aside from ABA and GAs, other hormones have been reported to affect seed dormancy and germination, including brassinosteroids, ethylene and nitric oxide (Steber and McCourt, 2001; Arc *et al.*, 2013a; Gazzarrini *et al.*, 2015).

DELAY OF GERMINATION 1 (*DOG1*) was initially described as a quantitative trait locus (QTL) for seed dormancy in Arabidopsis (Alonso-Blanco *et al.*, 2003). It has a major role in the regulation of seed dormancy considering that *dog1* mutants are completely non-dormant (Bentsink *et al.*, 2006). *DOG1* expression and accumulation are induced by low temperatures during seed maturation, causing enhanced levels of seed dormancy (Chiang *et al.*, 2011; Kendall *et al.*, 2011; Nakabayashi *et al.*, 2012). The DOG1 protein lacks domains with a known function, which complicated the elucidation of its molecular function. Several studies explored how *DOG1* is regulated, for example by alternative splicing and polyadenylation (Nakabayashi *et al.*, 2015; Cyrek *et al.*, 2016). It was also shown that *DOG1* can modify the expression of GA-related genes, thus altering cell wall properties (Graeber *et al.*, 2014). Other works showed it can affect the expression of genes involved in microRNA processing (Huo *et al.*, 2016) and its participation in seed development by affecting ABA signalling pathways (Dekkers *et al.*, 2016). However, only recently the molecular mechanism of action of *DOG1* was demonstrated (Née *et al.*, 2017). It was shown that DOG1 interacts with two PP2C phosphatases in seeds of Arabidopsis and negatively affects their function in dormancy release, although the exact mechanism of this regulation is not clear yet.

DOG1 also participates in the acquisition of seed longevity, as mutants of this gene exhibit reduced resistance to ageing (Bentsink *et al.*, 2006). However, if the mechanism underlying this process is similar as that described for seed dormancy remains to be determined. A recent publication showed that the *dog1-1* mutant enhances *abi3-1* phenotypes and phenocopies those from stronger *abi3* alleles, including a severe reduction in seed longevity and chlorophyll retention in seeds (Dekkers *et al.*, 2016). This suggests that *DOG1* actively participates in seed development and may explain its effect on seed longevity.

The connection between seed dormancy and seed longevity is not clear. Seed dormancy and longevity are studied as separate traits in plants and most of the mutants described showed a positive correlation between these traits, such as in *abi3*, *tt* mutants, *dog1* or *hub1* (Ooms *et al.*, 1993; Debeaujon *et al.*, 2000; Bentsink *et al.*, 2006; Liu *et al.*, 2007b). Contrary to this notion, the analysis of recombinant inbred lines (RILs) between different Arabidopsis accessions showed that several QTLs for seed longevity collocated with QTLs for seed dormancy and revealed a negative

correlation between these two traits (Nguyen *et al.*, 2012). These authors proposed that the apparent trade-off between seed dormancy and longevity may arise as a result of ecological variation and varying selective pressure on one or the other, but this is yet to be determined (Nguyen and Bentsink, 2015).

1.5 SEED DETERIORATION AND AGEING DURING STORAGE

Either buried in the soil seed bank or stored in the dry state, seeds deteriorate over time, a process referred to as seed ageing that involves a loss of vigour and viability and thereby directly related to seed longevity (Walters, 1998; Grappin *et al.*, 2008). As previously explained, orthodox seeds are both desiccation-tolerant and able to survive in this dry state for a prolonged period of time. The main factor contributing to this is the glassy conformation acquired by the cytoplasm of cells (Buitink and Leprince, 2008). In this state of extreme cytoplasmic viscosity, molecular diffusion is severely reduced and the low levels of available moisture deter most chemical reactions (Fernández-Marín *et al.*, 2013). However, and despite these protective mechanisms and quiescent metabolism, seeds age and deteriorate, eventually losing viability.

Environmental conditions are of key importance for seed storage, considering factors as temperature and relative humidity (RH) during this storage have profound effects on seed longevity and on the rate of seed ageing (Ellis and Roberts, 1980a; Dickie *et al.*, 1990; Ellis *et al.*, 1995). The combination of low temperatures and reduced seed moisture content ensures that seeds are in the glassy state, minimising the deleterious impact of ageing (Walters, 1998). Increasing temperature or moisture content of the seeds softens the cellular glass to a rubbery state or even back to the liquid state, reactivating metabolism and deteriorative processes. This is the theoretical basis for artificial seed ageing methods, which apply elevated temperatures and humidity levels to mimic the effects of the natural ageing process in a short period of time (Delouche and Baskin, 1973; Tesnier *et al.*, 2002).

Ageing damages during dry seed storage have been thoroughly investigated during the years. The main contributors to seed deterioration are free radical-mediated lipid peroxidation, loss of integrity of nucleic acids, disruption of cellular membranes, enzyme inactivation and protein degradation (Smith and Berjak, 1995; Walters, 1998; Murthy *et al.*, 2003; Rajjou *et al.*, 2008a). Most of these damages originate from oxidative processes, which in most cases lead to the production of reactive oxygen species (ROS) that subsequently can damage cellular components.

Evidence of these deteriorative processes during dry seed storage has been documented. For example, dry seeds were shown to release volatiles associated with lipid peroxidation or alcoholic fermentation (Buckley and Buckley, 2009; Colville *et al.*, 2012; Mira *et al.*, 2016). A particular case of chemical oxidation occurring in dry seeds are Maillard reactions (Murthy and Sun, 2000) in which, through a non-enzymatic process, reducing sugars or aldehydes react with the amino groups present in proteins. This results in glycosylated end-products whose accumulation negatively affects seed longevity (Wettlaufer and Leopold, 1991).

1.6 SEED LONGEVITY AND ITS REGULATION

The capacity of a seed to survive relatively long periods of time while retaining the ability to germinate is termed longevity. More specifically, seed longevity refers to the span of time mature seeds remain viable and it encompasses all those mechanisms present contributing to this end. Consequently, seed longevity is affected by those factors affecting seed deterioration and those which participate in seed ageing. Mechanisms regulating seed longevity can be divided into two main categories, according to their mode of action: protection and repair and detoxification (Rajjou and Debeaujon, 2008). Protection mechanisms are established during seed maturation and partially overlap with those involved in maturation drying, as demonstrated by mutants with impaired seed desiccation tolerance (Ooms *et al.*, 1993). On the other hand, repair and detoxification systems become active upon seed imbibition and contribute to minimise the effect of ageing-induced damages and compounds which are potentially harmful for seed viability.

1.6.1 Protective mechanisms

It was already discussed in the previous section that one of the main contributing factors to seed survival in the dry state is the glassy conformation of the cell cytoplasm established during seed maturation drying. The reduction of the water content of the cytoplasm together with the accumulation of non-reducing sugars and LEAs conforms a glassy matrix that severely impedes molecular mobility and chemical reactions. The accumulation of non-reducing sugars, particularly RFOs, is thought to be a key agent in the acquisition and maintenance of seed longevity. These compounds can be used as energy sources during germination and some reports linked them to the maintenance of membrane integrity and protection of labile proteins (Sano *et al.*, 2015).

Another fundamental contributor to seed longevity is the seed coat, as previously discussed. The seed coat is a tissue of maternal origin whose cells are dead

by the end of seed development. However, it acts as a chemical and mechanical barrier, limiting the interaction of the embryo with the environment. Flavonoids accumulated in the seed coat act as antioxidants and may help scavenge ROS produced during ageing, besides their contribution to the seed coat permeability (Debeaujon *et al.*, 2000).

Seed ageing is associated with oxidative damage to different cell constituents. Mild levels of oxidation during seed storage are linked with the release of seed dormancy (Bazin *et al.*, 2011; Arc *et al.*, 2013a), but a prolonged exposure may endanger seed survival. Consequently, seeds are equipped with an array of antioxidants to protect them from excessive oxidative damage. As dry seeds are devoid of ascorbic acid (vitamin C), its role in preventing oxidative damage is minor. However, mutants with a reduced redox buffering capacity were recently shown to display reduced seed longevity and germination speed (De Simone *et al.*, 2017). The glutathione system regulates the redox status of cells and the balance between the reduced and oxidised forms was used to monitor seed ageing (Kranner *et al.*, 2006). Tocopherols (vitamin E) and tocotrienols have drawn quite some interest, as they help preventing non-enzymatic lipid peroxidation and buffering oxidative stress at the photosynthetic apparatus (Sattler *et al.*, 2006a). Mutants involved in the biosynthesis of tocopherols, including *vitamin E deficient 1* (*vte1*), *vte2* and *vte6* displayed reduced seed longevity (Sattler *et al.*, 2004; vom Dorp *et al.*, 2015). Prevention of non-enzymatic lipid peroxidation is also contributed by lipocalins, a group of small proteins involved in the transport of lipophilic substrates. It was shown that *AtTIL*, a temperature-induced lipocalin and *AtCHL*, a chloroplastic lipocalin, prevent lipid oxidation and single and double mutants exhibited reduced seed longevity, whereas increased accumulation of AtCHL caused enhanced resistance to seed ageing (Boca *et al.*, 2014). Seed storage proteins (SSPs) were shown to be targets of oxidation in seeds, resulting in carbonylated proteins (Arc *et al.*, 2011). Besides, Arabidopsis mutants affected in 12 S globulin biosynthesis, a type of SSPs, were shown to have reduced seed longevity and that these proteins function as oxidation buffers in dry seeds, preventing carbonylation of other proteins (Nguyen *et al.*, 2015).

1.6.2 Repair and detoxification systems

The protective mechanisms of the dry seed are finite and consequently, prolonged storage can exhaust them. At this point, damages to different seed constituents will start accumulating, which could prevent seed germination. This is why seeds are equipped with several systems that, upon imbibition, allow them to deal with those damages incurred during storage. The extent of this damage is usually

accompanied by a reduction of seed vigour and a delay of seed germination (Matthews and Khajeh-Hosseini, 2007; Rajjou *et al.*, 2008a).

Imbibition of quiescent dry seeds allows for a rapid resumption of metabolic activity, alongside an increase in ROS production. In order to control this production and to process the hazardous free radicals accumulated during ageing, seeds have detoxification systems. These comprise antioxidant enzymes such as glutathione and dehydroascorbate reductases, catalases, superoxide dismutases (SOD) or glutathione and ascorbate peroxidases (Bailly *et al.*, 1996; Bailly, 2004). However, these enzymes can also be affected by the oxidative damages incurred during ageing, which would compromise seed vigour and viability. Contrary to this notion, a recent study in chickpea showed how galactinol synthases became active as a consequence of artificial ageing and helped to scavenge ROS species (Salvi *et al.*, 2016). A recent publication described how overexpression of a *Pseudomonas* aldo-keto reductase *PsAKR1*, a protein involved in detoxification of reactive groups resulting from lipid peroxidation, led to enhanced seed longevity in seeds of tobacco (*Nicotiana tabacum*) and rice (*Oryza sativa*) (Narayana *et al.*, 2017). Likewise, lipooxygenases were reported to affect the rate of lipid peroxidation in seeds during seed development and germination. Specifically, several studies conducted on rice reported that decreasing the expression level of *OsLOX2* and *OsLOX3* increased resistance of seeds to artificial ageing by reducing the levels of lipid peroxidation end-products (Huang *et al.*, 2014a; Ma *et al.*, 2015; Xu *et al.*, 2015). Along with ROS, accumulation of cyanide as a result of seed ageing was suggested as a possible contributor to the ageing process (Rajjou *et al.*, 2008a). In apple tree (*Malus domestica*) seeds, accumulation of hydrogen cyanide caused release of embryo dormancy by increasing the concentration of H_2O_2 (Krasuska *et al.*, 2014), although the long term effects on seed viability were not addressed.

Certain groups of cysteine-rich proteins are known to act as antioxidants, such as metallothioneins and peroxyredoxins, and were also reported to influence seed longevity. Specifically, two studies described how overexpression of sacred lotus metallothioneins *NnMT2a* and *NnMT3* and *1-CYSTEINE PEROXIREDOXIN 1* (*NnPER1*) in Arabidopsis seeds resulted in enhanced resistance to artificial ageing and seed vigour (Zhou *et al.*, 2012; Chen *et al.*, 2016). Overexpression of metallothioneins caused increased activity of SODs after artificial ageing compared to the wild type, whereas increased expression of *NnPER1* led to a reduction of ROS production and lipid peroxidation during the treatment. In both cases, expression of the transcripts was also detected during seed development, which suggest they may also contribute to buffer the oxidative stresses seeds undergo during maturation drying.

As already mentioned, proteins are also affected by oxidative damages and accumulate oxidations or covalent modifications, which can impose a loss of enzymatic activity or protein function (Rajjou *et al.*, 2008b). ROS oxidise methionine residues to methionine sulfoxide, a modification commonly reported in all ageing organisms (Stadtman, 2006). These modifications are repaired by methionine sulfoxide reductases (MSR) and accumulation of these proteins in *Medicago truncatula* and Arabidopsis seeds was positively correlated with seed longevity (Châtelain *et al.*, 2013). Covalent modification of proteins cause the alteration of L-aspartyl or asparaginyl residues to isoaspartyl (isoAsp) residues, causing a loss of protein function. These anomalous residues are repaired by L-isoaspartyl O-methyltransferases (PIMTs). Overexpression of *PIMT1* in Arabidopsis enhanced seed resistance to artificial ageing while reducing isoAsp accumulation (Oge *et al.*, 2008). In addition, Arabidopsis seeds overexpressing the chickpea *CaPIMT2* displayed increased resistance to artificial ageing (Verma *et al.*, 2013) and similar results were found for transgenic rice seeds (Wei *et al.*, 2015; Petla *et al.*, 2016).

Another group of macromolecules affected by oxidative damages during ageing are nucleic acids, whose integrity is crucial for seed germination and longevity (Waterworth *et al.*, 2015). ROS can induce breaks in DNA, either by desaturating the deoxyribose backbone or by covalent modification of the bases. One of the most abundant modifications is the hydroxylation of the C-8 position guanine into 7,8-dihydro-8-oxoguanine (8-oxoG), which is potentially mutagenic (Bray and West, 2005). In Arabidopsis, transgenic seeds overexpressing a bifunctional glycosylase/AP lyase, a protein participating in the repair of 8-oxoG modifications, showed increased resistance to artificial ageing and reduced levels of 8-oxoG (Chen *et al.*, 2012). DNA damage also includes the loss of integrity, represented by single and double strand breaks. In Arabidopsis, mutants impaired in DNA ligases *AtLIG4* and *AtLIG6*, which participate in strand-break repair, were shown to have reduced seed longevity and vigour (Waterworth *et al.*, 2010).

Translation but not transcription was demonstrated to be required for Arabidopsis seed germination, highlighting the importance of mRNAs stored during seed development that can partially compensate for those proteins damaged during ageing (Rajjou *et al.*, 2004; Kimura and Nambara, 2010). Similar to DNA, RNA is also affected by oxidative damages. Studies in pea (*Pisum sativum*) showed that artificial ageing induced RNA degradation, alongside DNA laddering (Chen *et al.*, 2013), an effect also observed in seeds of soybean (*Glycine max*) (Fleming *et al.*, 2017). Targeted degradation and decay of stored mRNAs was shown to contribute to dormancy alleviation in sunflower and Arabidopsis seeds during after ripening (Bazin *et al.*, 2011;

Basbouss-Serhal *et al.*, 2017). These studies reported how 5'UTR sequences carry specific motifs to which components of the cell machinery can bind, which opens the possibility for RNA-binding proteins as a protective mechanism. However, no such system has been described so far.

1.6.3 The influence of the environment

Apart from the specific mechanisms in place to protect the seed and assist it to cope with ageing, the maternal environment at which the seed develops plays a key role in the resulting seed longevity and quality. The impact of the environmental conditions experienced by the mother plant on traits of its offspring is referred to as maternal effects and they represent a major adaptive response in plants (Donohue, 2009). The contribution of maternal environments to the regulation of seed germination has been a topic of research for a long time now (Fenner, 1991).

Seed longevity is a plastic trait that exhibits natural variation and as such, it is likely that maternal effects can modify it (Miura *et al.*, 2002; Nguyen *et al.*, 2012). Some studies have explored the contribution of maternal effects to seed longevity. Temperatures experienced during seed maturation are known to affect the levels of seed dormancy. Likewise, some studies have reported that they can also alter seed longevity in a species-specific manner. Some investigations reported opposite effects of low temperatures experienced by the mother plant on the resulting seed longevity (Kochanek *et al.*, 2011; Mondoni *et al.*, 2014). As these studies were conducted in different species, it is possible that the underlying mechanisms to the altered seed longevity differ between them. In Arabidopsis, it has been shown that lower temperatures during seed maturation have a detrimental effect on seed longevity depending on the studied genotype (He *et al.*, 2014). These authors reported a similar genotype-dependent effect on seed longevity caused by the light intensity at which the seeds developed.

1.7 SEED GERMINATION

Germination starts with water uptake by the seed (imbibition) and continues until the radicle protrudes through the seed surrounding structures (Bewley, 1997). Seed germination represents a major phase transition in the life history of plants and as such, it is a tightly regulated process. Extensive literature is available on the regulation and mechanisms participating in germination, so this introduction will only highlight the key aspects of the process (Bewley, 1997; Holdsworth *et al.*, 2008; Nonogaki *et al.*, 2010; Rajjou *et al.*, 2012; Bewley *et al.*, 2013).

The water uptake process is divided in three distinct phases. During the first one, seeds rapidly take water as a result of the difference in water potential, which can cause solute leakage and imbibition damages in the seed. During this phase, metabolism will re-activate, alongside the repair mechanisms already described and protein translation from stored mRNAs will begin (Nonogaki *et al.*, 2010). Following this initial imbibition, water uptake is slowed down, representing the second or lag phase. Although the increase in water content during this phase is relatively minor, the seed cells expand as a result of diverse processes and seed volume increases, which can result in cracking of the seed coat. During this phase, mitochondrial respiration is already active as well as DNA repair systems. Therefore, transcription and translation of new mRNAs can occur. As the cells continue to grow and elongate, seeds enter the third phase of water uptake, which in fact corresponds to the final steps leading to radicle protrusion and the end of germination. Prior to radicle emergence, storage reserves remain mostly not mobilised, although a small fraction is used to feed the ongoing cellular respiration (Bewley *et al.*, 2013).

Plant phytohormones play fundamental roles during the process of seed germination (Holdsworth *et al.*, 2008). As described for the regulation of seed dormancy, ABA and GAs exert opposite roles in seed germination, with ABA associated with the inhibition of seed germination whereas GAs promote it. It is the balance between them what determines the resulting seed behaviour (Finkelstein *et al.*, 2008; Gazzarrini *et al.*, 2015; Topham *et al.*, 2017). Specifically, seed germination requires high levels of GAs and reduced amounts of ABA.

GAs are fundamental for seed germination. Early studies identified mutants impaired in GA biosynthesis which only germinated after addition of exogenous GAs (Koornneef and van der Veen, 1980). Alteration in GA biosynthetic genes can exhibit distinct phenotypes. For example, disruption of *GA REQUIRING 1* (*GA1*) completely prevents germination, considering it is a single-copy gene in Arabidopsis that catalyses one of the first committed steps in GA biosynthesis (Sun and Kamiya, 1994). Conversely, single mutants of *GIBBERELLIN 3-OXIDASE 1* (*GA3OX1*) and *GA3OX2*, which are expressed in the embryo following imbibition, exhibit germination levels comparable to that of the wild type as a result of functional redundancy, although the double *ga3ox1 ga3ox2* mutant is severely affected in its germination behaviour (Yamauchi *et al.*, 2004; Mitchum *et al.*, 2006). The activation of GA biosynthesis leads to accumulation of GAs, which in turn causes the degradation of DELLA-domain proteins, which are repressors of GA responses (Sun and Gubler, 2004). The DELLA protein *RGA-LIKE 2* (*RGL2*) was shown to be a repressor of seed germination in the absence of GA biosynthesis, but it is degraded when GAs are present (Tyler *et al.*,

2004). GA accumulation causes the activation of GA-responsive genes and the onset of germination. For example, *EXPANSIN* 2 (*EXP2*), a gene involved in cell wall loosening and active cell expansion is expressed in response to GAs but repressed in the presence of DELLAs (Yan *et al.*, 2014).

The *CYTOCHROME P450, FAMILY 707, SUBFAMILY A, POLYPEPTIDE 2* (*CYP707A2*) locus, involved in ABA degradation, was shown to be up-regulated following imbibition of the seed and mutant *cyp707a2* imbibed seeds accumulated six times more ABA and were hyperdormant (Kushiro *et al.*, 2004; Okamoto *et al.*, 2006). *ABI4* regulates both ABA and GA metabolic genes to repress seed germination and maintain seed dormancy and can directly bind to the promoter of *CYP707A2* (Shu *et al.*, 2013). This shows the importance of ABA degradation for seed germination. Moreover, it was shown that ABA produced by the endosperm can repress seed germination after embryo imbibition through the action of *RGL2* and *ABA DEFICIENT 1* (*ABA1*), whereas removal of the testa can alleviate this repression (Debeaujon and Koorneef, 2000; Lee *et al.*, 2010).

Extensive evidence is available that illustrates the cross-talk between ABA and GAs in seeds to control germination (Holdsworth *et al.*, 2008; Graeber *et al.*, 2012). For example, transcription factors *SPATULA* (*SPT*) and *PHYTOCHROME INTERACTING FACTOR 3-LIKE 5* (*PIL5*) were shown to repress the expression of *GA3OX1* and *GA3OX2*, thereby blocking seed germination (Penfield *et al.*, 2005). ABA also represses GA biosynthesis in imbibed seeds, as illustrated by the ABA-deficient mutant *aba2-2* (Seo *et al.*, 2006). *RGL2* enhances ABA accumulation and activity of *ABI5*, which results in the repression of germination (Piskurewicz *et al.*, 2008).

Apart from the balance between ABA and GAs, other plant hormones have been shown to contribute to seed germination. Ethylene and brassinosteroids (BRs) also participate in the regulation of seed germination, which they usually do through interactions with ABA signalling routes. Ethylene reduces ABA levels or sensitivity and in turn, ABA reduces ethylene biosynthesis and perception, resulting in an antagonistic mechanism in the regulation of seed germination (Ghassemian *et al.*, 2000; Cheng *et al.*, 2009; Linkies and Leubner-Metzger, 2012; Arc *et al.*, 2013b). BR-deficient or insensitive mutants were found to be ABA-sensitive, which suggest that BRs mode of action involves a modification of the ABA signalling pathway, which would agree with a proposed GA-parallel mechanism for BRs (Steber and McCourt, 2001; Leubner-Metzger, 2003). Interestingly, a recent publication showed that BR-deficient mutants are more resistant to artificial ageing before and after application of a priming treatment, which suggests that BRs have a negative impact on seed

longevity, apart from their role in seed germination (Sano *et al.*, 2017). Auxins were also reported to play a role in seed germination, again by interfering with ABA signalling. Specifically, altering auxin signalling by enhancing the expression of *AUXIN RESPONSE FACTOR 10* (*ARF10*) caused increased ABA sensitivity and exogenous application of auxin potentiated the inhibitory effect of ABA on seed germination (Liu *et al.*, 2007a).

Germination also represents one of the most vulnerable stages of the plant life cycle. If seeds are exposed to environmental stresses or adverse conditions, several repressive mechanisms can become active to arrest seed germination. One such example is the existence of a checkpoint immediately after seed germination. It was shown that at early stages of seed germination, if conditions of water stress are present, *ABI3* and *ABI5* can re-induce late seed maturation programmes and stop the germination process (Lopez-Molina *et al.*, 2001, 2002).

1.8 SEED QUALITY MARKERS

Seed quality is determined by several factors, such as genetic purity, germination capacity and uniformity, vigour, storability and performance under suboptimal conditions (McDonald, 1998). Seed vigour is defined as the sum of those properties of the seed which determine the potential level of activity and performance of seed lots during germination and seedling emergence in a wide range of environments (Finch-Savage and Bassel, 2016). From this definition, it is clear that seed vigour is not a single measurable trait but a concept associated with different aspects of seed performance.

Generally speaking, high quality seeds: a) germinate completely; b) germinate quickly and at the same time; c) generate normal and healthy seedlings; d) show little variation of germination in response to external factors; and e) have high storability (Corbineau, 2012). To obtain this is a major challenge for seed companies, which are always looking for novel ways to evaluate and discriminate the quality level of seed lots.

Ageing during seed storage was shown to negatively impact seed vigour, considering it involves a whole set of damages to cell constituents as well as disruption of the cell machinery. Based on this, the germination of seeds after artificial ageing is considered to be a vigour test (Delouche and Baskin, 1973). As mentioned above, seed storability is one of the factors contributing to the overall seed quality and it is the one on which this section will be focused. As artificial ageing combined

with germination tests are considered an estimator of seed vigour, the term seed quality will be used to refer to the seed germination percentage of a seed batch.

Classically, the approaches used to evaluate seed storability and viability have relied on the use of germination tests combined with accelerated ageing treatments (Delouche and Baskin, 1973) or the use of a tetrazolium test, which stains only living tissues and allows quantification of living seeds (Wharton, 1955). During the years, several methods have been proposed to investigate seed quality. In *Brassica*, the fluorescence of sinapine was used to distinguish dead seeds from those viable (Taylor *et al.*, 1990). Similarly, the chlorophyll fluorescence of *Brassica oleracea* seeds was also used as a marker of seed performance (Jalink *et al.*, 1998). Likewise, the abundance of HSP17.6I was positively correlated with seed performance in this species (Bettey and Finch-Savage, 1998) and changes in enzyme activities in four *Brassica* species were proposed as indicators of seed deterioration (Ramiro *et al.*, 1995).

The half-cell reduction potential of glutathione ($E_{GSSG/2GSH}$), a major cellular antioxidant and redox buffer, was also proposed as a valid marker to evaluate seed viability, as alterations of this indicator are usually associated with programmed cell death (PCD) events and loss of cell viability (Kranner *et al.*, 2006; Seal *et al.*, 2010). Thermal seed-profiling was demonstrated to be able to discriminate viable pea seeds upon imbibition in a non-destructive approach (Kranner *et al.*, 2010). Another non-invasive method showed a positive correlation between oxygen influx to the seed upon imbibition and seed viability (Xin *et al.*, 2013). Recently, the integrity of RNA in soybean seeds was proposed as a viability marker, although its resolution is limited (Fleming *et al.*, 2017).

The production of low molecular weight volatiles resulting from seed deterioration was also explored to monitor seed quality (Zhang and Roos, 1997). Methanol was suggested as a good marker candidate, since all studied species released it (Colville *et al.*, 2012). Similarly, seeds of *Brassica rapa*, *B. napus*, *B. oleracea* and Arabidopsis showed that ethanol release can be a good predictor of seed deterioration (Buckley and Buckley, 2009; Kodde *et al.*, 2012).

The role of sugars in seed longevity has been long speculated, although a satisfactory correlation could not be established (Bentsink *et al.*, 2000; Buitink *et al.*, 2000; Buitink and Leprince, 2008). However, recent studies have reported that accumulation of galactinol in mature, dry seeds of Arabidopsis, cabbage, tomato (*Solanum lycopersicum*) (de Souza Vidigal *et al.*, 2016) and chickpea (Salvi *et al.*, 2016) showed a direct correlation with seed longevity and can therefore be used to monitor it.

1.9 THE ECOSEED PROJECT AND SCOPE OF THIS THESIS

This PhD thesis is part of the European consortium EcoSeed, whose aim is to ensure the availability of future plant genetic resources across Europe and to provide the basis for improving seed quality traits and seed handling protocols. To this end, the impact of different environmental conditions during seed maturation and storage on seed quality was explored. This consortium is a joint venture bringing together 11 groups with expertise in different fields of seed biology. Using the plant model species *Arabidopsis thaliana* in combination with three major crops, *Brassica oleracea*, sunflower and barley (*Hordeum vulgare*), the project aims to determine how the changing climate impacts seed quality during production and storage, alongside the molecular mechanisms underlying it. Furthermore, the project aims to establish new tools to determine seed quality and to translate the generated knowledge to other species.

The aim of this thesis is to gain a better understanding on the genetic and environmental regulatory mechanisms that govern seed longevity in *Arabidopsis thaliana* and to evaluate novel tools to evaluate seed quality. For this, we conducted a multidisciplinary approach combining transcriptomics, molecular biology, cytology and biochemical labelling of proteins.

The first part of this thesis addresses the impact of the environmental stresses temperature and drought on seed resistance to artificial ageing and evaluates the accompanying modifications of the seed transcriptome by RNA-sequencing analyses. We show that temperatures experienced by the mother plant during seed development result in alterations of seed longevity and the transcriptome (**section 3.1.1**). Similarly, the application of drought during seed maturation severely reduces yield, but increases seed quality in two studied genotypes. We evaluated the modification of the seed transcriptome by drought and explored the possible role of cell wall modification in the observed responses to drought (**section 3.1.2**).

The genetic regulation of seed longevity in Arabidopsis is investigated in two separate approaches in this thesis. First, the role of *DOG1* in seed longevity is studied by analysing its interaction with other proteins *in vivo* and characterising T-DNA insertion mutants of these candidate genes (**section 3.2.1**). Second, the possible contribution to seed longevity of the flowering time regulator *FRIGIDA* (*FRI*) is addressed (**section 3.2.2**). *FRI* was initially identified together with 3 other genes contained within a natural modifier from the Shahdara accession that enhanced seed longevity in the longevity-deficient mutant *lec1-3*.

The last part of this thesis examines the feasibility of two distinct methods to monitor seed quality. The effect of maternal temperatures on seed quality was used as the starting point to determine whether nuclear size and chromatin compaction in Arabidopsis embryos correlated with the observed quality levels (**section 3.3.1**). Next, the labelling of Activity-Based Protein Profiling (ABPP) probes in protein extracts from seeds of different quality levels is evaluated as a possible predictor of seed quality (**section 3.3.2**).

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Plant material

The *Arabidopsis thaliana* accessions Columbia (Col-0), Landsberg *erecta* (L*er*) and Shahdara (Sha), together with NIL DOG1, originate from the laboratory of Prof. Dr Maarten Koornneef at the University of Wageningen. Accession C24 was kindly provided by Lei Zhang (Max Planck Institute for Plant Breeding Research; Cologne, Germany). All the *vpe* mutants were kindly provided by Dr Renier van der Hoorn (University of Oxford, United Kingdom). All T-DNA insertion lines and mutants used in this work are listed in **Supplemental Table 1** and were ordered from the Nottingham Arabidopsis Stock Centre (NASC). If not indicated otherwise, the accession used is Col-0.

2.1.2 Antibiotics

All the antibiotics used in this work were purchased from Duchefa (Haarlem, The Netherlands) and they were used for selection at the concentrations shown in **Table 1**. Stock solutions were prepared, aliquoted and stored at -20 °C.

Table 1. Antibiotics used in this study. The solvent and final working concentration are indicated.

Antibiotic	Solvent	mg/l
Ampicilin	H_2O	100
Gentamycin	H_2O	30
Hygromycin	H_2O	50
Kanamycin	H_2O	50
Rifampicin	DMSO	50
Spectinomycin	H_2O	100

2.1.3 Bacterial and yeast strains

For cloning purposes and plasmid multiplication, the chemically competent DH5α strain of *Escherichia coli* (Hanahan, 1983) was used. For plant transformation, the electrocompetent GV3101 strain of *Agrobacterium tumefaciens* carrying the

pMP90 helper plasmid (Koncz and Schell, 1986) was used. For yeast two-hybrid assays, *Saccharomyces cerevisiae* strain pJ69-4A was used (James *et al.*, 1996).

2.1.4 Buffers and culture media

Unless stated otherwise, all buffers and media mentioned were prepared as described by Sambrook *et al.* (1989). Yeast cells were grown on non-selective yeast extract, peptone and dextrose medium supplemented with adenine (YPDA) medium, whereas yeast two-hybrid assays were conducted using the selective synthetic define (SD) medium (**Table 2**).

Table 2. Media used for growth and selection of yeast cells.

YPDA	SD
20 g/l peptone	6.7 g/l yeast nitrogen base without amino acids
10 g/l yeast extract	0.64 **or** 0.62 g/l Drop out (DO) supplement (-Leu/-Trp **or** -Leu/-Trp/-His, respectively)
20 g/l glucose	20 g/l glucose
0.01 % adenine hemisulfate	18 g/l agar (for solid plates)
18 g/l agar (for solid plates)	

For some applications, additional solutions and buffers were required (**Table 3**).

Table 3. Additional solutions and buffers used.

Enzyme mix	High-salt solution for RNA precipitation
0.3 % (w/v) pectolyase	1.2 M sodium citrate
0.5 % (w/v) cytohelicase	0.8 M sodium chloride
0.5 % (w/v) cellulose	
Diluted in 10 mM citrate buffer at pH 4.5	

MES running buffer	0.5 X Murashige and Skoog (MS) media
50 mM MES	2.2 g/l MS basal salt mixture (Duchefa)
50 mM Tris-Base	0.5 g/l MES
1 mM EDTA	pH 5.7
0.1 % (w/v) SDS	10 g/l agar
pH 7.3	From Murashige and Skoog (1962)
4X SDS-PAGE loading buffer	**NuPAGE transfer buffer**
250 mM Tris-Hcl (pH 6.8)	25 mM Bicine
8 % (w/v) SDS	25 mM Bis-Tris
20 % β-mercaptoethanol	1 mM EDTA
40 % glycerol	pH 7.2
0.008 % bromophenol blue	10 % (v/v) ethanol

2.1.5 Chemicals

All the chemicals used in this work were purchased from the following suppliers: Becton Dickinson (Franklin Lakes, USA), Bio-Budget (Krefeld, Germany), Bio-Rad (Hercules, USA), Carl Roth (Karlsruhe, Germany), Invitrogen (Karlsruhe, Germany), Merck (Darmstadt, Germany) and Sigma-Aldrich (San Luis, USA).

2.1.6 Commercial kits and reagents

- ActivX™ Desthiobiotin-FP Serine Hydrolase Probe (ThermoFisher, Waltham, USA)
- Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Chicago, USA)
- Amino Acid -Leu/-Trp and -Leu/-Trp/-His Dropout Mixes (Clontech, Mountain View, USA)
- BioSprint 96 DNA Plant Kit (Qiagen, Hilden, Germany)
- Gateway® BP-Clonase® and LR-Clonase® (Invitrogen, Karlsruhe, Germany)
- iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, USA)
- NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany)
- NucleoSpin® Plasmid (Macherey-Nagel, Düren, Germany)

- O'GeneRuler 1 kb DNA Ladder (ThermoFisher, Waltham, USA)
- PageRuler™ Prestained Protein Ladder (ThermoFisher, Waltham, USA)
- Pierce™ BCA Protein Assay Kit (ThermoFisher, Waltham, USA)
- QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany)
- RNAqueous® Total RNA Isolation Kit together with Plant RNA isolation aid (Ambion™, Life Technologies, Austin, USA)
- RNeasy Plant Mini® Kit (Qiagen, Hilden, Germany)
- Streptavidin–Peroxidase Polymer, Ultrasensitive (Sigma-Aldrich, San Luis, USA)
- Streptavidin Sepharose High Performance beads (GE Healthcare, Chicago, USA)
- SYPRO® Ruby Protein Gel Stain (ThermoFisher, Waltham, USA)
- TURBO DNA-free™ Kit (Ambion™, Life Technologies, Austin, USA)
- Vectashield® mounting medium with DAPI (Vector Laboratories, Burlingame, USA)

2.1.7 Enzymes

All the restriction enzymes used were acquired from New England Biolabs® (Ipswich, USA) or ThermoFisher (Waltham, USA).

Taq DNA polymerase was purchased from Ampliqon (Odense, Denmark).

KOD Hot Start DNA polymersase was ordered from Merck Millipore (Billerica, USA).

Pectolyase and cellulase were acquired from Duchefa (Haarlem, The Netherlands).

Cytohelicase was ordered from Sigma-Aldrich (San Luis, USA).

Liquid RNase A (100 mg/ml) was purchased from Macherey-Nagel (Düren, Germany).

2.1.8 Primers and plasmids

All primers used in this work were ordered from Invitrogen (Karlsruhe, Germany) or Sigma-Aldrich (San Luis, USA) and are listed in **Supplemental Table 2** and **Supplemental Table 3**.

Entry vectors pDONR201 and 207 from Invitrogen (Karlsruhe, Germany) were used as donors for PCR-amplified fragments by Gateway® cloning (Invitrogen, Karlsruhe, Germany). Binary vector pGWB1 (Nakagawa *et al.*, 2007) was provided by

Dr Kazumi Nakabayashi (Royal Holloway, University of London, United Kingdom). Binary vectors R02 and R07 from the FAST collection (Shimada *et al.*, 2010) were also used. For yeast two-hybrid assays, pACT2-gateway (GAL4-AD fusion) and pAS2-gateway (GAL4-BD fusion) vectors (modified from Clontech) were used.

2.1.9 Software and websites

All DNA sequences were downloaded from the Arabidopsis 1001 genomes project (http://signal.salk.edu/atg1001/3.0/gebrowser.php) and The Arabidopsis Information Resource (www.arabidopsis.org).

Arabidopsis genome and transcript TAIR10 assemblies were downloaded from The Arabidopsis Information Resource (www.arabidopsis.org).

Alignment of sequences, design of primers and all *in-silico* cloning reactions were performed using different tools from the DNASTAR® Lasergene Core Suite. Primer design and characteristics were evaluated using Primer3 (Untergasser *et al.*, 2012).

Microarray expression data was obtained from the Arabidopsis eFP-browser (Winter *et al.*, 2007).

All software used for processing and analysing RNA-sequencing data is listed in the Methods section.

2.2 METHODS

2.2.1 Plant growth and seed storage conditions

Arabidopsis seeds were sown on 6-cm Petri dishes containing a water-saturated filter paper (Macherey-Nagel, Düren, Germany) and allowed to germinate in a MC785-VDB germination cabinet (Van den Berg Klimaattechniek, Reeuwijk, The Netherlands) under long day conditions (12 h light/12 h dark; 25/20 °C) for three to five days. Seedlings were then transferred to a soil mixture of substrate and vermiculite in a 3:1 proportion. Plants were grown in a growth chamber (Elbanton BV, Kerkdriel, The Netherlands) with long day cycles (16 h light/8 h darkness; 22/16 °C) with controlled humidity and allowed to set seeds and ripen. Seeds were harvested from plants which had at least two thirds of their siliques ripened, sieved and collected in paper bags. Plants used for seed propagation were grown in a greenhouse where the temperature was maintained close to 23 °C and 16 h of light were provided daily.

Plants used to evaluate dormancy phenotypes were allowed to bolt and immediately transferred to a low temperature growth chamber (Percival Scientific Inc., Perry, USA) with long day conditions at 16/14 °C, because this temperature regime allows an easier observation of differences in the depth of seed dormancy.

Freshly harvested seeds were immediately used in experiments or stored under constant conditions (21 °C, 50 % humidity, in the dark) in an Climacell 222 incubator (MMM Group, Planegg, Germany) for dormancy release. Unless indicated, seeds were not stratified in any of the experiments. Once dormancy was released, seeds were stored at room temperature on the bench.

Seeds used in sections **3.1.1** and **3.1.2** were produced and harvested in Warwick (United Kingdom), from Arabidopsis plants grown in a soil mixture of compost, sand and perlite (6:1:1) under long day conditions (16 h light/8 h dark; 22/18 °C). Before the first flower opened, plants were transferred to low (16/14 °C), control (22/18 °C) or elevated (28/25 °C) temperature regimes, maintaining long day conditions. For the drought treatment, a negative water potential of -1 MPa was applied by weighing pots and restraining water to a previously determined level.

Seeds from the wild species used in section **3.3.2.1** were produced and their viability constants determined by Dr Charlotte Seal at the Royal Botanical Gardens in Kew, United Kingdom.

2.2.2 Seed germination assays

To evaluate dormancy release or the effect of artificial ageing, the proportion of germinating seeds was determined. At least 50 seeds per replicate were distributed on 6-cm Petri dishes containing a round filter paper (Macherey-Nagel, Düren, Germany) soaked with 580 µl of water. Plates were then placed in transparent boxes with wet filter paper at the bottom to keep the moist atmosphere and prevent plates from drying. These moisture boxes were transferred to a MC785-VDB germination cabinet (Van den Berg Klimaattechniek, Reeuwijk, The Netherlands) with controlled conditions (16 h light /8 h darkness, at 25/20 °C). After seven days, the number of germinated seeds was counted with the help of a MZ6 dissecting microscope (Leica Microsystems, Wetzlar, Germany) and a KL1500LCD reflected light lamp (Schott, Mainz, Germany). Seeds were considered to have germinated when radicle emergence through the seed coat was observed (Bewley *et al.*, 2013).

For dormancy release assays, these experiments were conducted weekly until seed batches reached at least 95 % germination and were considered to be non-dormant. Seed stratification was conducted exactly as described for germination assays (either on moist filter paper or on MS media using previously sterilised seeds), but storing the imbibed seeds for three days at 4 °C in the dark.

For ABA germination assays, the procedure was as described but filter papers were soaked with a solution of the indicated ABA concentration instead of water.

Unless indicated otherwise, all germination experiments were conducted using independent biological replicates. A biological replicate is comprised by the whole batch of seeds harvested from a single mother plant.

2.2.3 Accelerated ageing of seeds

Seed longevity was studied by quantifying the number of germinated seeds after different periods of accelerated ageing (Delouche and Baskin, 1973). Accelerated ageing is a seed vigour test that evaluates the potential seed storability, as it provokes a rapid ageing of seeds. Specifically, aliquots of 100-200 seeds were placed on open PCR tube-stripes and incubated in hermetically sealed boxes containing a saturated solution of KCl at the bottom which, when placed at 37 °C, generates an atmosphere of 85 % RH within the container. Under these conditions, seeds lose their viability after a short period of time, which can then be monitored by periodic germination assays as described above.

Seed used in sections **3.1.1.3** and **3.3.1** were aged for 4 days at 40 °C and 75 % relative humidity (provided by a saturated NaCl solution) in Paris, France.

For the wild species assays described in section **3.3.2.1**, dry seeds were first equilibrated to 75 % relative humidity over a saturated NaCl solution in an air-tight container at 20 °C. Seeds were weighed every third day. The treatment was applied until the seed mass stopped increasing, meaning seeds had reached a 75 % relative humidity content. Once equilibrated, seeds were aged at 40 °C for the required period of time using the same container as for the equilibration, over the saturated NaCl solution.

2.2.4 Seed coat permeability assays

Seed coat permeability was determined as previously described (Debeaujon *et al.*, 2000).

2.2.5 Flowering time determination

The time required to flower was evaluated by counting the total number of rosette leaves (excluding cotyledons) present when the first flower opened (Koornneef *et al.*, 1991). All flowering time experiments were conducted under long day conditions (16 h light / 8 h dark).

2.2.6 Seed surface sterilization

Seed sterilisation was performed either with chlorine gas or liquid bleach, depending on the number of samples to be processed, always under sterile conditions. For chlorine gas sterilisation, aliquots of seeds were transferred to open 2-ml microcentrifuge tubes (Eppendorf, Hamburg, Germany) which were then placed into in a glass desiccator. 100 ml of bleach and 3 ml of concentrated HCl (37 %) were mixed in a beaker within the desiccator, which was then immediately sealed. Seeds were incubated for three to six hours in this atmosphere and then allowed to release the remaining fumes in the clean bench before sowing.

For bleach sterilisation, seeds in a microcentrifuge tube were incubated for 1 min with constant shaking in a 50 % commercial bleach solution including 0.05 % Tween® 20 (Sigma-Aldrich, San Luis, USA). After incubation and removal of the solution, the remaining bleach was removed by rinsing the seeds with sterile water five times. After discarding the last wash, seeds were suspended in either water or 40 % glycerol (Carl Roth, Karlsruhe, Germany) and sown on sterile MS medium.

2.2.7 Plant transformation and selection of transgenic lines

Arabidopsis plants were transformed following the floral dip method (Clough and Bent, 1998) with *A. tumefaciens* cells carrying the desired construct. For each transformation event, 6 to 10 pots (9 x 9 cm) with five plants each were used. These plants were clipped after the first shoot appeared in order to remove apical dominance and enhance the formation of additional, secondary shoots to increase the number of flowers available for transformation. Unless indicated otherwise, Arabidopsis wild type Col-0 was used for transformation.

For hygromycin-based selection of transgenic plants, seeds were first surface-sterilised (**section 2.2.6**), sown on half strength MS media (**section 2.1.4**) containing hygromycin and stratified for three days in the dark at 4 °C. Plates were then transferred to an incubator with long day conditions (12 h light/ 12 h dark; 25/20 °C) and incubated for 6 h with light and two days in the dark and then screened for elongated hypocotyls (Harrison *et al.*, 2006). T_2 segregation ratios were analysed to isolate T_3 homozygous plants with a single insertion using a χ^2 (chi-squared) test. For fluorescent-seed-coat markers (Shimada *et al.*, 2010), T_1 transformed seeds were manually selected using a MZ16 FA fluorescence stereomicroscope (Leica Microsystems, Wetzlar, Germany) combined with a LEJ EBQ 100 isolated mercury and xenon discharge lamp power supply (Hofstra Group, Santa Fe, USA) and further analysis of the fluorescent marker's segregation was conducted as described above.

2.2.8 Crossing of Arabidopsis

Young plants at the early stages of flowering were selected as parents. For the female parent, stems carrying three or four immature, closed flower buds were selected, further removing any open flowers, developing siliques, leaves and the apical meristem from these stems. Using a small pair of forceps and a MZ6 dissecting microscope (Leica Microsystems, Wetzlar, Germany), these flower buds were cleared from petals, sepals and stamens, leaving the pistil undamaged. A recently opened flower with dehiscent anthers was then selected from the male parent, gently removed with clean forceps and used for pollinating the female pistils by brushing its anthers against the stigma of the female flower. Each cross was labelled with a coloured thread for easy recognition. For each genetic cross, reciprocal crosses were conducted. These crosses were allowed to grow for two weeks and then bagged to avoid losing seeds.

2.2.9 Preparation of nuclear spreads of Arabidopsis embryos

Nuclei were isolated from embryos of Arabidopsis seeds, which were either dry or imbibed (72 h at 10 °C, continuous light). Seeds were artificially aged by storing them for 4 days at 40 °C and 75 % relative humidity in a sealed container. The area of the spread nuclei was measured as an indicator for nuclear size. To avoid radicle emergence, seeds were never allowed to be imbibed at room temperature longer than 2 hours.

For each sample, at least 75 embryos were isolated and processed as previously described (Liu *et al.*, 2011; Pavlova *et al.*, 2010). Slides were then mounted with 20 µl of 4',6-Diamidino-2-phenylindole (DAPI; 1.5 µg/ml) in Vectashield mounting medium (Vector Laboratories, Burlingame, USA) and covered with a coverslip. DAPI signals were detected using a Zeiss Axioplan 2 Imaging (Carl Zeiss, Oberkochen, Germany) fluorescence microscope and a 100X objective together with immersion oil. Pictures were acquired using a Leica DFC 490 digital camera (Leica Microsystems, Wetzlar, Germany).

The sizes of spread Arabidopsis nuclei from seeds of different treatments were measured by taking pictures of at least 50 nuclei per slide followed by measurement of their surface using the image analysis software *ImageJ* (Schneider *et al.*, 2012). After measurements, tests for statistical significance were conducted in order to determine whether the treatments under evaluation had a significant effect on nuclear size.

2.2.10 Bacterial transformation

Chemically competent DH5α *E. coli* cells were transformed by heat shock (Hanahan, 1983), whereas electrocompetent *A. tumefaciens* were transformed using electroporation (Weigel and Glazebrook, 2006). Transformed cells were plated on solid LB media including the corresponding antibiotics. Successful transformation was further confirmed by colony PCR and restriction analysis of the transformed construct.

2.2.11 Yeast culture and transformation

S. cerevisiae strain pJ69-4A cells were grown in YPDA at 30 °C in liquid culture. These cells were then co-transformed with the desired combination of vectors to test protein-protein interactions using the LiAc/SS carrier DNA/PEG method (Gietz *et al.*, 1992). Briefly, cells from a liquid culture were precipitated at 5,000 rpm for 10 min, rinsed with sterile water and pelleted again. The pellet was then re-suspended in 1 ml of 0.1 M LiAc, precipitated by centrifugation and suspended in 0.5 ml of 0.1 M LiAc, which rendered the cells competent for transformation. This suspension was

aliquoted in volumes of 50 µl. To each aliquot, the following mix was added: 240 µl of 50 % PEG 3350, 35 µl of 1 M LiAC and 25 µl of carrier salmon sperm DNA (2 mg/ml, previously boiled for 10 min) and a combination of both bait and prey DNA constructs (500 µg each in a final volume of 50 µl). Tubes containing these mixes were incubated for 30 min at 30 °C and immediately transferred to 42 °C during 20 min. Cells were then precipitated and re-suspended in 200 µl of 1 M sorbitol, from which 90 % of the volume was plated on SD -Leu/-Trp/-His plates and the remaining 10 % on SD -Leu/-Trp plates. These plates were incubated at 30 °C for five days.

2.2.12 Yeast two-hybrid assays

Yeast cells co-transformed with both bait and prey constructs (**section 2.2.11**) were plated in either SD -Leu/Trp or SD -Leu/-Trp/-His medium plates. The first medium allows to evaluate whether both constructs have been successfully delivered into the yeast cells whereas the second one evaluated if the protein-protein interaction is taking place and thus activating the system. Strength of the interactions was further tested by using increasing concentrations of 3-Amino-1,2,4-triazole (3-AT), which specifically inhibits histidine biosynthesis (Hilton *et al.*, 1965). The positive control used was previously described (Fields and Song, 1989).

2.2.13 Genomic DNA isolation

Plant genomic DNA from leaves was isolated using the BioSprint 96 DNA Plant Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. When the number of samples was not sufficient to fill up the entire Biosprint 96-well plate, genomic DNA was isolated as previously described (Edwards *et al.*, 1991).

2.2.14 Polymerase Chain Reaction (PCR)

For all genotyping and colony-PCR reactions, *Taq*-DNA polymerase from Ampliqon (Odense, Denmark) was used, whereas for high-accuracy cloning, KOD Hot Start DNA polymerase form Merck Millipore (Billerica, USA) was employed. In both cases, specifications from the manufacturers were followed, although genotyping reactions were usually down-scaled to a final volume of 10 µl, as follows: 1 µl of PCR buffer 10X (+15 mM $MgCl_2$), 0.2 µl of dNTPs mix (10 mM each), 0.2 µl of forward primer (10 µM), 0.2 µl of reverse primer (10 µM), 0.1 µl of *Taq* DNA polymerase (5 U/µl), 1 µl of template (ca. 100 ng/µl) and distilled water up to 10 µl.

All PCR reactions were performed in a Mastercycler® pro thermal cycler (Eppendorf, Hamburg, Germany). The general conditions used for PCR were: an initial denaturation phase of 2 minutes at 95 °C, followed by denaturation for 20 seconds

at 95 °C, primer annealing for 30 seconds at the specific T_m for the primer pair and an extension phase at 72 °C of variable duration, depending on the size of the amplicon and the processing speed of the DNA polymerase. These three steps were repeated for 30 cycles. A final extension phase of 3 minutes at 72 °C was included.

2.2.15 DNA fragment purification

After PCR amplification, DNA fragments were separated by electrophoresis in agarose gels. The band of interest was then excised from the gel and purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

2.2.16 Gateway® cloning and vector construction

Fragments of interest were first amplified by PCR and purified. In these PCR reactions, specific *att*B overhangs for Gateway® cloning (Invitrogen, Karlsruhe, Germany) were added to allow BP recombination into a pDONR201/207 entry vector (**Supplemental Figure 1**; Invitrogen, Karlsruhe, Germany). To clone promoter and coding sequences together, specific primers were designed to create an overlapping region between both sequences that allowed PCR amplification of the whole construct. When necessary, this approach was conducted in several sub-cloning steps.

Once the insert was cloned into the entry vector, this construct was further sequenced to ensure no DNA mismatches existed between the clone and the expected sequence. After confirmation by sequencing, constructs were further delivered into a destination vector containing a Gateway® cloning cassette by LR recombination (Invitrogen, Karlsruhe, Germany). Successful recombination was evaluated by PCR and band analysis after restriction.

A construct carrying the *DOG1* promoter from the Arabidopsis Cape Verde Islands (Cvi) accession was kindly provided by Dr Kazumi Nakabayashi. Construct pDOG1$_{Cvi}$::*FLC*$_{Col}$ was delivered into the binary vector pFAST-R07 (Shimada *et al.*, 2010). Construct pDOG1$_{Cvi}$::*FRI*$_{Sha}$ was cloned into the binary vector pGWB1 (Nakagawa *et al.*, 2007). The coding sequence of *VTE3* was cloned under control of the Cauliflower mosaic virus 35S promoter using the binary vector pFAST-R02 (Shimada *et al.*, 2010).

2.2.17 Plasmid purification

Plasmids were isolated from liquid cultures of DH5α *E. coli* cells using the NucleoSpin® Plasmid (Macherey-Nagel, Düren, Germany) kit and following the manufacturer's instructions.

2.2.18 DNA sequencing

All DNA sequencing reactions were performed by the Sanger Sequencing Group of the Max Planck Institute for Plant Breeding Research (Cologne, Germany) using an Applied Biosystems 3730XL Genetic Analyzer (ThermoFisher, Waltham, USA).

2.2.19 Total RNA isolation and purification

RNA from leaves was isolated using the RNeasy Plant Mini® Kit from Qiagen (Hilden, Germany), according to the manufacturer's instructions.

To extract RNA from Arabidopsis seeds, around 20 mg of seeds were used per extraction and the RNAqueous® Total RNA Isolation Kit together with Plant RNA isolation aid (Ambion™, Life Technologies, Austin, USA) was used as previously described (Kushiro *et al.*, 2004). Briefly, instructions from the manufacturer were followed that led to isolation of 100 µl of non-pure RNA, due to the high concentration of polysaccharides and lipids present in seeds of Arabidopsis. Isolated RNA was further purified by conducting two consecutive rounds of RNA precipitation, the first using 20 % isopropanol containing 0.24 M sodium citrate and 0.16 M NaCl (**Table 3**), and the second with 2 M LiCl. For each precipitation, isolated RNA was incubated at 4 °C overnight to allow for complete removal of contaminants. RNA was recovered by centrifugation at 20,000 g for 15 min at 4 °C. After removal of the supernatant, the pellet was rinsed with 70 % ice-cold ethanol, precipitated, allowed to air-dry and re-suspended in an adequate volume of water to reach a concentration of 200 ng/µl.

RNA purity was first determined using a Nanodrop ND-1000 spectrophotometer (PEQLAB Biotechnologie, Erlangen, Germany) and evaluated by its absorbance (A) ratios. The ratio A_{260}/A_{280} was used to estimate protein contamination and A_{260}/A_{230} for polysaccharides. RNA was considered to be clean when these two parameters were between $1.8 \leq (A_{260}/A_{280}) \geq 2.0$ and $2.0 \leq (A_{260}/A_{230}) \geq 3.0$. Extra rounds of precipitation were conducted when necessary. RNA quality was further evaluated by combining a small amount of pure RNA (ca. 400 ng) mixed with 0.5 volumes of formaldehyde loading buffer (Ambion™, Life Technologies, Austin, USA) and incubating it 5 min at 65 °C. This solution was then loaded on a 1 % agarose gel and

separated by gel electrophoresis. If RNA quality is good and it is not degraded, two major bands corresponding to the 28 S and 18 S ribosomal RNA bands should be visible, with no or very little smear (the rest of smaller bands are lost during LiCl precipitation and therefore do not appear in the gel).

2.2.20 DNAse treatment of RNA

A DNAse treatment was performed on all RNA samples prior to sequencing, using the TURBO DNA-free™ Kit (Ambion™, Life Technologies, Austin, USA) according to manufacturer's instructions.

2.2.21 cDNA synthesis

Synthesis of cDNa was done using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. For all reactions, 1 µg of total RNA template was used and primed with a combination of random hexamers and oligo-dT oligonucleotides. After synthesis, cDNA was diluted 10 times before using it for quantitative PCR analyses.

2.2.22 Quantitative PCR (qPCR)

All qPCR reactions were conducted using a CFX96 Touch™ Real-Time PCR Detection System (BioRad, Hercules, USA) and prepared using the iQ™ SYBR® Green Supermix according to the instructions provided by the manufacturer. All qPCR reactions included a melting curve analysis to ensure the primer pair used amplified a single product. All qPCR primers used were designed to cover exon-exon junctions to avoid genomic DNA contamination and their efficiency was tested before conducting any of the experiments. Efficiency values ranging from 85 to 110 % were considered valid. All primers used for relative quantification of gene expression are listed in **Supplemental Table 3**.

In all experiments, at least three biological replicates were used per data point and reactions of each biological replicate were conducted in sets of three technical replicates. For all reactions, 1 µl of cDNA was used as template. All reactions were prepared in Hard-Shell® 96-Well PCR Plates of low profile (BioRad, Hercules, USA) which were then sealed with Microseal® 'B' PCR Plate Sealing Film (BioRad, Hercules, USA).

Relative expression of target genes was quantified by the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001) and normalised with the expression of a reference gene. In each set of experiments, several reference genes were evaluated and the one with the most stable expression levels was used for normalisation.

2.2.23 Next Generation Sequencing of RNA (RNA-seq)

An aliquot of 1 µg total RNA was used for the RNA-seq analyses. RNA sequencing was performed using three biological replicates per treatment. Libraries were prepared from total RNA with a RNA integrity number > 7 (Bioanalyzer; Agilent, Santa Clara, USA) using the TruSeq RNA kit (Illumina, San Diego, USA) and including polyA-enrichment. Libraries were then sequenced as 100-bp single-end reads on a HiSeq 2500 (Illumina, San Diego, USA). Library preparation and sequencing were performed by the Max Planck Genome Centre (Cologne, Germany).

2.2.24 Trimming and mapping of the reads to a reference genome

Quality of the reads was evaluated using FASTQC (Andrews, 2010). Low quality reads were discarded and the remaining were trimmed for adapters and short reads (<25 bp) using the Python package CutAdapt (Martin, 2011). After trimming, each library produced at least 17 million good quality reads that were subsequently mapped to the TAIR10 Arabidopsis reference genome assembly using Tophat2 (Kim *et al.*, 2013) with default settings. Reads mapping to individual genes were calculated using the *HTseq-count* function from the HTseq Python package (Anders *et al.*, 2015).

2.2.25 Differential expression analyses

Differential expression of transcripts was determined using the *DeSeq2* package (Love *et al.*, 2014) for statistical analysis of transcriptomic data in R (R Development Core Team, 2008).

2.2.26 GO analyses

The identified subsets of differentially expressed transcripts were classified in Gene Ontology categories using the online analysis toolkit AgriGO (http://bioinfo.cau.edu.cn/agriGO) (Du *et al.*, 2010). GO analysis results were further summarised using ReviGO, which allows visual interpretation of large GO datasets and clusters GO terms based on semantic similarity (http://revigo.irb.hr) (Supek *et al.*, 2011).

2.2.27 Protein extraction

For protein extraction, 20 mg seeds of Arabidopsis were imbibed for 24 h, subsequently ground in liquid nitrogen to a fine powder and immediately suspended in 600 µl of the appropriate extraction buffer. For VPE labelling, this buffer consisted of 67 mM Tris-HCl at pH 7.5 including 10 mM dithiothreitol (DTT), whereas for serine hydrolases, the buffer contained 67 mM sodium acetate at pH 5.5 including 10 mM

DTT. Protein extracts were then centrifuged at least three times at 21,000 g for 20 minutes, recovering the supernatant in a new microcentrifuge tube every time, until no oil traces or debris were visible. For the wild species described in section **3.3.2.1**, 20-30 seeds were used per protein extraction, depending on the number of seeds available. The rest of the process was as described for Arabidopsis.

2.2.28 Protein labelling

Four different fluorescent probes were evaluated (**Table 13**). For the labelling, 60 µl of protein extract were incubated with the probe for 2 h in the dark. The final concentrations of probe used for labelling were: 1 µM AMS101, 1 µM MV151 and 0.2 µM FP-rhodamine (FP-Rh). After incubation, the labelling reactions were stopped by adding 20 µl of 4X SDS-PAGE loading buffer.

For negative-labelling controls, a pooled sample of protein extracts was first incubated for 30 minutes in the dark with a specific inhibitor of the enzymatic activity studied before adding the probe (**Table 4**). These inhibitors were used at a final concentration that allowed them to outcompete the tested probe and therefore inhibit the labelling.

An additional no probe control (NPC) was prepared for each set of samples by combining a small fraction from each proteome. In this control, neither probe nor inhibitor was added and they were substituted by dimethyl sulfoxide (DMSO), which was the solvent for both probes and inhibitors.

Table 4. Specific inhibitors used to supress ABPP-probe labelling.

Inhibitor	Final concentration (µM)	Inhibits
YVAD-cmk	50	AMS101
E64	20	MV151 (at pH 6.0)
Epoxomicin	50	MV151 (at pH 7.5)
DiFP	100	FP-Rh

2.2.29 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Labelled protein samples were boiled for 5 minutes at 95 °C prior to loading. These protein extracts were loaded in NuPAGE™ 4-12 % Bis-Tris Protein Gels (ThermoFisher, Waltham, USA) and separated by electrophoresis (Laemmli, 1970) applying 120-130 V for 65-75 minutes. To preserve the fluorescent probes from degradation, the electrophoretic chamber was fully covered so protein electrophoresis took place in the dark.

After separation, labelled proteins were visualized by in-gel fluorescence scanning using a Typhoon FLA 9000 scanner (GE Healthcare, Chicago, USA) with excitation and emission at 532 and 580 nm, respectively. After scanning, gels were stained with SYPRO® Ruby (ThermoFisher, Waltham, USA) for total protein assessment following the manufacturer's indications and scanned with excitation and emission at 450 and 610 nm, respectively.

2.2.30 Serine hydrolase pull-down experiments

100 µl of protein extract (in 67 mM Tris-HCl, pH 7.5 buffer including 10 mM DTT) from 24 h imbibed, non-aged Arabidopsis seeds were first incubated with 50 µl streptavidin sepharose beads (GE Healthcare, Chicago, USA) for 1 h with constant mixing on a rotator at room temperature in order to remove the excess of biotinylated background proteins. After incubation, samples were centrifuged for 3 minutes at 1,000 g and the supernatant collected in a clean tube, after which 1 volume of freshly-prepared 10 M urea dissolved in extraction buffer was added. This mixture was then labelled with 2 µM ActivX™ Desthiobiotin-FP Serine Hydrolase Probe (ThermoFisher, Waltham, USA) for 2 h. An identical no-probe control was prepared following these instructions, but replacing the volume of probe with DMSO. After incubation, 50 µl of streptavidin sepharose beads were added and samples were incubated for 1 h with constant mixing on a rotator. After incubation, samples were centrifuged for 3 minutes at 1,000 g and the supernatant collected in a clean tube. For elution of bound proteins, an appropriate volume of 2X SDS-PAGE loading buffer was added and samples incubated for 5 minutes at 95 °C. Proteins bound to the streptavidin sepharose beads were sent for mass spectrometry (MS) analysis to the Analytics Core Facility Essen (ACE), at the University of Duisburg-Essen, Germany.

Protein concentration was determined for all eluted fractions (except those eluted with 2X SDS-PAGE loading buffer) using the Pierce™ BCA Protein Assay Kit (ThermoFisher, Waltham, USA).

2.2.31 Western Blot

All eluted fractions were separated by SDS-PAGE and proteins were then blotted on an Immobilon-P PVDF membrane (Merck Millipore, Billerica, USA) using semi-dry electrotransfer for 70 minutes at constant 1.25 mA/cm^2 provided by a PowerPac 3000 power source (Bio-Rad, Hercules, USA). After transfer, membrane was blocked with 2 % BSA in PBS including 0.005 % Tween® 20 (Sigma-Aldrich, San Luis, USA; PBS-T) for 1 h with constant shaking. Membrane was then washed two times for 5 minutes each with PBS-T and afterwards incubated with Streptavidin–Peroxidase Polymer conjugate (Sigma-Aldrich, San Luis, USA) diluted 1:10,000 times in PBS-T for 1 hour. After incubation, six rounds of 5-minute washing with PBS-T were performed prior to gel imaging. Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Chicago, USA) was used following the manufacturer's instructions and the blot was visualised using a ChemiDoc XRS+ (Bio-Rad, Hercules, USA).

3 RESULTS

3.1 THE IMPACT OF ENVIRONMENTAL STRESSES DURING SEED DEVELOPMENT ON SEED LONGEVITY AND THE SEED TRANSCRIPTOME

The transcriptome of a cell comprises the whole set of transcripts present in that cell at a certain time. Contrary to the genome, which is fixed and relatively stable, the cellular transcriptome is a dynamic entity, which varies in response to different stimuli and stresses. Therefore, the transcriptome reflects the different transcriptional responses and its analysis allows a better understanding of how living organisms deal with perturbations.

3.1.1 The effect of temperature during seed development and of artificial ageing on the seed transcriptome of Arabidopsis

Maternal environments play a key role in determining how a plant's offspring will perform, as they can exert a determinant role in processes such as germination and dormancy behaviour (Donohue, 2009). Previous work characterised seed longevity as a plastic trait that is responsive to parental effects (Kochanek *et al.*, 2011). In addition, parental effects were shown to affect seed longevity of the following generation (Mondoni *et al.*, 2014). A previous study in Arabidopsis showed that different temperatures applied to the mother plant do modulate seed performance (He *et al.*, 2014).

Artificial ageing of seeds is a vigour test that allows to predict the storage life of seed batches in a comparatively short period of time (Delouche and Baskin, 1973). Similarly to the natural deterioration process, artificial ageing encompasses modifications of the seed transcriptome and proteome (Rajjou *et al.*, 2008b; Chen *et al.*, 2013).

The combined effects of temperature during seed maturation, artificial ageing and time of imbibition on the transcriptome of Arabidopsis Col-0 seeds were investigated. To this end, seeds that matured under different temperature regimes (low, 16/14 °C; control, 22/18 °C; and elevated, 28/25 °C), and that were artificially aged (4 days at 40 °C and 75 % RH) were used to assess the effect of these factors on the seed transcriptome. To evaluate the contribution of imbibition, three different time points were included: dry seeds, 6 h (from here on referred to as early imbibition) and 72 h after seed imbibition (from here on referred to as late imbibition) at 10 °C with constant light. These time points were experimentally determined and

correspond to the end of phase 1 of water uptake and to 80 % of the time required for the first radicles to emerge, respectively (**section 1.7**).

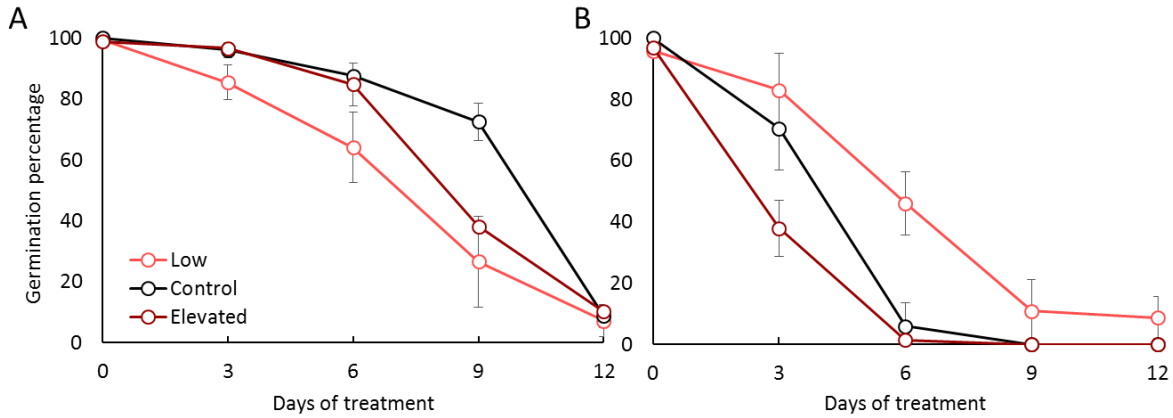

Figure 1. Different temperatures during seed development affect sensitivity to artificial ageing. Seed longevity phenotypes of Col-0 (**A**) and *dog1-2* (**B**) in response to three different temperature regimes during seed maturation: low (16/14 °C, light red), control (18/22 °C, black) and elevated (28/25 °C, dark red). Each data point represents the mean of three biological replicates and error bars correspond to standard deviation.

First, the effect of artificial ageing on seed lots grown at the conditions mentioned above was evaluated. Specifically, the wild type Col-0 and the *dog1-2* mutant (Nakabayashi *et al.*, 2012), which has a reduced seed longevity phenotype (Bentsink *et al.*, 2006) were compared and the effect of each maternal environment on sensitivity to artificial ageing evaluated (**Figure 1**). In the wild type, low and elevated temperatures during seed maturation led to reduced resistance to artificial ageing, in comparison to the control (**Figure 1A**). Conversely, *dog1-2* mutant seeds were able to resist the treatment better when grown at lower temperatures, while elevated temperatures at seed maturation caused a marked decline in resistance to ageing (**Figure 1B**). These observations confirm that the maternal environment during seed maturation does affect subsequent seed performance after accelerated ageing in a genotype-dependent manner, as previously reported (He *et al.*, 2014).

After confirming the phenotypes, RNA-sequencing and mapping of individual reads were conducted for Col-0 and transcriptional expression profiles were compared by principal components analysis (PCA, **Figure 2**), which facilitates visual interpretation of data (Ringnér, 2008). Component 1 separates the late imbibed seeds on the right side of the plot and dry, whereas early imbibed samples cluster on the left side. This component accounts for almost 90 % of the observed variability. Component 2 separates samples based on their imbibition status. Additionally, within each imbibition cluster, it allows to distinguish between aged and non-aged samples, although in the case of dry seeds a certain overlap is present. Samples from the early

imbibition point have more similar transcriptional profiles, i.e. are less variable than those from late imbibition samples, which are more scattered. This indicates that differences at the transcriptional level between seeds produced under different temperature regimes become more pronounced after longer imbibition times (**Figure 2**).

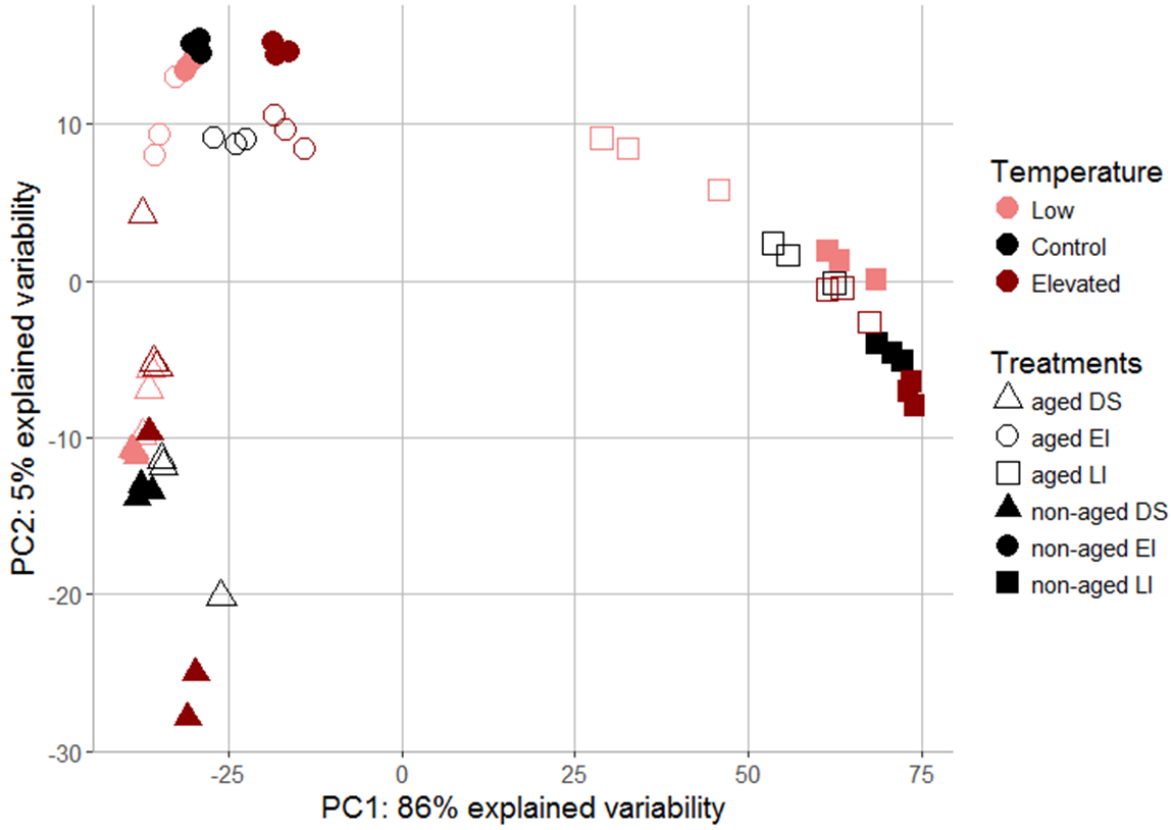

Figure 2. Principal Component Analysis plot of samples. Three different temperature regimes were applied during seed maturation and are represented with different colours. Three different imbibition time points were evaluated: dry seed (DS, triangles), early imbibition (EI, 6h imbibed; circles) and late imbibition (LI, 72 h imbibed; squares). Filled symbols correspond to non-aged seeds and open symbols to seeds aged for 4 days at 40 °C and 75 % RH.

To identify differentially expressed transcripts (DETs), an additional log_2-fold-change (log_2FC) threshold of 1.0 was established in addition to the calculated p-value. For all comparisons, dry, non-aged seeds grown at control temperature were used as the reference. **Table 5** shows the number of differentially expressed transcripts found for each studied condition. It was observed that imbibition causes a greater number of genes to be differentially expressed than any of the other treatments (**Table 5**). Nevertheless, considering that the main aim of this work was to determine how maternal environments alter seed performance, as reflected by the effect they had on

the germination phenotype after artificial ageing (**Figure 1**), special focus will be put onto these factors.

Table 5. Number of differentially expressed transcripts identified for each condition. For a transcript to be considered differentially expressed, a cut-off for $log_2FC \geq |1|$ and adjusted p-value ≤ 0.05 were applied.

Condition	Up-regulated transcripts	Down-regulated transcripts
Low temperature	74	264
Elevated temperature	111	175
Artificial ageing	68	132
Early imbibition	503	1790
Late imbibition	5,364	5,403

3.1.1.1 The effect of low temperature during seed maturation on the seed transcriptome

Low temperature during seed maturation caused increased sensitivity to artificial ageing in Col-0 (**Figure 1A**), despite the number of transcripts differentially expressed was not very high. For those transcripts upregulated by this temperature regime, only two gene ontology (GO) categories related to molecular function were enriched: transcription factor (TF) activity and DNA binding (**Table 6**). Closer examination of these transcripts showed several interesting genes. Within the enriched GO category transcription factor activity, the presence of *TEMPRANILLO 1* (*TEM1*) is surprising. This gene was previously linked to the regulation of flowering time under long days, as well as the repression of GA biosynthesis (Castillejo and Pelaz, 2008; Osnato *et al.*, 2012). Specifically, it was shown that *TEM1* represses the two main genes responsible for the biosynthesis of bioactive gibberellic acid (GA) *GA3OX1* and *GA3OX2*. Also part of this GO category and related to flowering time regulation, *DWARF AND DELAYED FLOWERING 1* (*DDF1*) was reported to affect flowering time and GA biosynthesis and its overexpression caused delayed flowering time and reduced GA content (Magome *et al.*, 2004, 2008). Furthermore, it was shown to be responsive to several abiotic stresses, including low temperatures (Kang *et al.*, 2011). Other upregulated TFs are related with auxin and ethylene signalling. For example, *INDOLE-3-ACETIC ACID INDUCIBLE 28* (*IAA28*) and *ETHYLENE RESPONSE FACTOR 10* (*ERF10*) were induced by this treatment. *ERF10* was reported to be a transcriptional repressor (Ohta *et al.*, 2001) and has been related to auxin and ethylene

responses (Chilley *et al.*, 2006). Similarly, *IAA28* belongs to the Aux/IAA family of auxin-response repressors (Pierre-Jerome *et al.*, 2013) and has been shown to regulate lateral root development in response to auxin (Rogg *et al.*, 2001; De Rybel *et al.*, 2010; Wang and Guo, 2015). Additionally, *iaa28-1* displayed delayed and reduced germination, together with a certain degree of ABA insensitivity (Rinaldi *et al.*, 2012). Apart from TFs, transcript abundance of the cold and ABA-inducible gene *KIN1* (Kurkela and Franck, 1990) was also increased in the transcriptome of seeds grown at lowered temperatures.

For transcripts downregulated by lowered temperatures, numerous GO categories were overrepresented (**Table 6**). As for molecular function, transcription factor activity, catalytic and hydrolase activities were enriched. The presence of transcripts involved in responses to auxin was remarkable and represented by those directly involved with auxin signalling, such as *IAA9*, *IAA17* (Ouellet *et al.*, 2001) and *SMALL AUXIN UPREGULATED RNA 3* (*SAUR3*) and *SAUR4* (Paponov *et al.*, 2008). In addition, several genes in this set were reported to be involved in embryo patterning together with auxins. For example, *WUSCHEL RELATED HOMEOBOX 2* (*WOX2*) and *DORNRÖSCHEN-LIKE* (*DRNL*). Both these genes are transcription factors that coordinate or affect the auxin signalling. *WOX2* was demonstrated to participate in embryo patterning, regulating the establishment of the auxin gradients required for embryo development and cotyledon primordia initiation (Haecker *et al.*, 2004; Palovaara and Hakman, 2009; Lie *et al.*, 2012). *DRNL* is also implicated in the patterning of the embryo of Arabidopsis and its expression is also required for cotyledon organogenesis (Chandler *et al.*, 2008, 2011). Related to embryo patterning, the expression of *MONOPOLE* (*MNP*) was also down-regulated. *MNP* is involved in the establishment of the boundary between two distinct developmental domains that precedes the development of the radicle, to which auxins also contribute (Nawy *et al.*, 2010). *MYB DOMAIN PROTEIN 34* (*MYB34*) is another transcription factor which is involved in the biosynthesis of tryptophan, which is a precursor in auxin biosynthesis (Smolen and Bender, 2002; Zhao *et al.*, 2002). *XYLOGLUCAN ENDO-TRANSGLUCOSYLASE / HYDROLASE 19* (*ATXTH19*) is found to be expressed in imbibed seeds, probably associated with germination. This gene was downregulated by low temperatures, which is possibly linked to the observed reduction in auxin responses, provided its expression has been connected to auxin (Pitaksaringkarn *et al.*, 2014). Interestingly, the transcription factor *AINTEGUMENTA* (*ANT*) was found downregulated. Transgenic lines overexpressing this gene produce bigger seeds with enhanced resistance to drought, as a result of ABA imbalances and it was proposed as a downstream target in the auxin signalling pathway (Meng *et al.*, 2015). A recent

publication further linked this gene to regulation of auxin accumulation and cell wall composition (Krizek *et al.*, 2016).

Aside from auxins, lipid metabolism appeared as an enriched GO category. Within this category, three genes were found to be relevant regarding the observed phenotypes. *FATTY ACID REDUCTASE 1* (*FAR1*) is involved in the biosynthesis of fatty alcohols which are components of suberin (Domergue *et al.*, 2010), a polymer found in cell walls of different organs, including the seed coat. Seeds with altered suberin composition were reported to display increased permeability and sensitivity to ABA (Vishwanath *et al.*, 2013). Also in this category, *DELTA 9 DESATURASE 1* (*ADS1*) was previously shown to be downregulated by low temperatures in leaves of Arabidopsis (Fukuchi-Mizutani *et al.*, 1998). *ADS1* is involved in lipid biosynthesis in diverse organs and mutants for this gene exhibited reduced seed lipid content (Smith *et al.*, 2013). *LONG-CHAIN ACYL-COA SYNTHETASE 2* (*LACS2*) was also downregulated by low temperature. *LACS2* participates in wax and cuticle synthesis in Arabidopsis (Schnurr *et al.*, 2004) and *lacs2* mutants were reported to show increased cuticle permeability (L'Haridon *et al.*, 2011). Interestingly, this gene is downregulated in the *hub1* mutant, which was previously linked to increased sensitivity to artificial seed ageing (Liu *et al.*, 2007b; Ménard *et al.*, 2014; De Giorgi *et al.*, 2015).

A GO category related to hormone responses was enriched within this set. Two genes involved in the biosynthesis of the active form of GAs were downregulated. Specifically, *GA3OX1* and *2* were downregulated by low temperatures during seed maturation, although it is possible that the upregulated *TEM1* is contributing to this reduced expression. These two genes are involved in the final step catalysing the formation of bioactive gibberellins and their expression is associated with a diverse range of developmental processes, especially seed germination (Mitchum *et al.*, 2006). Besides, the GA-responsive gene *EXP2*, involved in seed germination (Yan *et al.*, 2014), was also present in this set.

ARABIDOPSIS THALIANA HOMEOBOX 1 (*ATH1*), included in the category of light responses (Quaedvlieg *et al.*, 1995) was downregulated by cold. This gene was reported to be a transcriptional activator of the expression of the floral repressor *FLOWERING LOCUS C* (*FLC*) (Proveniers *et al.*, 2007).

3.1.1.2 The effect of elevated temperatures during seed maturation on the seed transcriptome

Elevated temperatures during seed maturation caused reduced levels of seed dormancy and altered resistance to artificial ageing of the seeds (**Figure 1**). Two GO

categories related to biological processes were significantly enriched by this treatment: regulation of biological process and multi-organism process. Interestingly, some GO categories related to molecular function were also enriched: namely, hydrolase activity affecting glycosyl bonds and compounds (**Table 6**). This could be indicative of cell wall modification mechanisms and, taken together with various genes related to seed coat development and composition, might be related to the observed phenotypes. Two genes involved in mucilage synthesis were present in this set. Mutants of *BETA-XYLOSIDASE 1* (*BXL1*) displayed delayed germination and patchy production of mucilage upon imbibition (Arsovski *et al.*, 2009). *MUCILAGE-MODIFIED 2* (*MUM2*) participates in biosynthesis of the seed mucilage and mutants of this gene fail to expand their mucilage upon hydration (Dean *et al.*, 2007). Expression of the orphan gene *QUA-QUINE STARCH* (*QQS*) also increased as a result of elevated temperature. *QQS* has been linked to starch biosynthesis (Li *et al.*, 2009), modulating seed composition by interacting with other transcriptional regulators (Li *et al.*, 2015).

Among the upregulated transcripts, some were linked to hormone signalling. *DHFS-FPGS HOMOLOG B* (*DFB*) participates in seed reserve accumulation (Meng *et al.*, 2014) and mutants for this gene were reported to display altered auxin signalling in the quiescent centre of the root tip and altered amino acid profiles (Srivastava *et al.*, 2011). *SLEEPY2* (*SLY2*) is involved in the degradation of DELLA proteins and therefore acts as a positive regulator of GA signalling (Ariizumi *et al.*, 2011). Also linked with GA responses, expression of *GA-STIMULATED ARABIDOPSIS 6* (*GASA6*) was increased. This gene is a positive regulator of seed germination, which promotes by enhancing the expression of *EXP1*, therefore contributing to cell wall loosening (Zhong *et al.*, 2015). *NITRATE REDUCTASE 2* (*NIA2*) is involved in nitrate metabolism and participates in the synthesis of the gaseous free radical nitric oxide, which is involved in the release of seed dormancy and promotes germination (Arc *et al.*, 2013a; Yu *et al.*, 2014; Albertos *et al.*, 2015).

Transcripts downregulated by elevated temperatures during seed maturation showed no overrepresentation of any GO categories. However, included in this group there were some interesting candidates. *DELAY OF GERMINATION 1* (*DOG1*), together with *ABSCISIC ALDEHYDE OXIDASE 3* (*AAO3*), appeared downregulated. *AAO3* is involved in the biosynthesis of ABA in seeds and leaves (Seo *et al.*, 2000, 2004) and mutants of this gene displayed reduced seed dormancy (González-Guzmán *et al.*, 2004). *DOG1* was first described as a major QTL regulating seed dormancy and the *dog1* mutant has reduced seed longevity (Bentsink *et al.*, 2006). In addition, its expression has been shown to be responsive to temperature (Chiang *et al.*, 2011). *BANYULS* (*BAN*), which is a negative regulator of anthocyanin and flavonoid

biosynthesis and accumulation in the seed coat was downregulated, and mutations in this gene were reported to cause reduced germination after storage (Albert *et al.*, 1997).

3.1.1.3 Modification of the seed transcriptome by artificial ageing

Contrary to the effects of maternal temperatures, artificial ageing is applied to the seeds once they are dry and mature. Therefore, its ability to modify the transcriptome could be assumed to be relatively low. However, artificial ageing of seeds is conducted at elevated levels of relative humidity, which allow a certain degree of moisture content within the seed, thus allowing activation of metabolism and signalling (Chen *et al.*, 2013).

No GO category was overrepresented for the set of upregulated transcripts. Artificial ageing of seeds and their subsequent deterioration is associated with oxidative damage caused by the treatment. Therefore, genes involved in these responses would be expected. Expression of *GLUTATHIONE S-TRANSFERASE 6* (*GSTF6*), which belongs to a family of redox-responsive genes (Sappl *et al.*, 2009), was found to be increased. A gene coding a 17.6 kDa class II heat shock protein (*HSP17.6II*) showed enhanced expression as well. These proteins were initially described for their role in heat responses, but have been shown to play fundamental roles during seed maturation and drying as a part of transcriptional networks regulated by *ABI3* (Kotak *et al.*, 2007). Interestingly, *AAO3* was also upregulated by ageing, which could relate to stress responses activated by ABA as a result of the ageing treatment. This enzyme also participates in the biosynthesis of H_2O_2, which could in turn contribute to the redox imbalance already present (Zarepour *et al.*, 2012). Transcripts of the ROS-responsive gene *HIGH AFFINITY K^+ TRANSPORTER 5* (*HAK5*) were increased as well. This gene is involved in potassium uptake and its expression has been shown to be responsive to low levels of potassium (Kim *et al.*, 2010). Another potassium transporter, *K^+ EFFLUX ANTIPORTER 2* (*KEA2*), was upregulated by artificial ageing. It is involved in the maintenance of plastid ionic homeostasis and correct plastid development (Zheng *et al.*, 2013; Aranda-Sicilia *et al.*, 2016), besides being activated in response to osmotic stresses in an ABA-dependent manner (Kunz *et al.*, 2014). The increased expression of these transporters may be reflecting an ionic imbalance as a result of the oxidative damage to cellular membranes.

In addition to oxidative stress responses, genes related to cell wall modification were also upregulated. *GALACTURONOSYLTRANSFERASE 6* (*GAUT6*) has been speculated to participate in pectin biosynthesis, given that the mutants exhibit altered cell wall composition (Caffall *et al.*, 2009). The expression of *3-BETA*

HYDROXYSTEROID-DEHYDROGENASE/DECARBOXYLASE ISOFORM 1 (*3BETAHSD/D1*) was also upregulated. Overexpression of this gene affects auxin signalling by altering the plasma membrane composition and thereby interfering with the mobility of auxin transporters (Kim *et al.*, 2012).

For transcripts downregulated by artificial ageing, GO categories related to hormone, water and abiotic stimuli responses were enriched (**Table 6**). Included in this group, *ARABIDOPSIS ZINC-FINGER PROTEIN 2* (*AZF2*) is a transcriptional repressor induced by abiotic stresses that acts as a negative regulator of ABA and auxin responses (Drechsel *et al.*, 2010; Kodaira *et al.*, 2011). Also related to ABA, *CBL-INTERACTING PROTEIN KINASE 3* (*CIPK3*) was shown to exert a negative regulation of ABA responses, including germination and seed dormancy (Pandey *et al.*, 2008). *SIN3-LIKE 1* (*SNL1*) has been previously reported to affect seed dormancy and longevity and shown to be involved in ABA and ethylene responses, but with opposed effects (Wang *et al.*, 2013). Its role as a hormone repressor was further demonstrated, given that it contributes as a negative regulator of auxin responses during seed germination (Wang *et al.*, 2016). In addition to this, the regulator of auxin transport and homeostasis *PIN-FORMED 6* (*PIN6*) was also downregulated by artificial ageing (Cazzonelli *et al.*, 2013), as was the auxin-regulated and senescence-responsive *XTH24* (Shi *et al.*, 2015). *ANT* also appeared downregulated by artificial ageing, as did the positive regulator of GA signalling *SLY2*. *1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE 7* (*ACS7*) participates in the biosynthesis of ethylene and its expression is responsive to GAs, ABA and ethylene (Mishra *et al.*, 2008; Dong *et al.*, 2011). Surprisingly, both abiotic stress-responsive genes *KIN1* and *KIN2* appeared downregulated by artificial ageing.

Table 6. Gene Ontology categories enriched for each set of differentially expressed transcripts. The last column shows the log_{10} of the p-value associated with each overrepresented category.

GO ID	Description	log_{10} p-value
	Upregulated low temperature	
GO:0003677	DNA binding	-0.6198
GO:0003700	transcription factor activity, sequence-specific DNA binding	-0.6198
	Downregulated low temperature	
GO:0007275	multicellular organism development	-0.3468
GO:0010468	regulation of gene expression	-0.3565
GO:0008610	lipid biosynthetic process	-0.3565
GO:0032501	multicellular organismal process	-0.3979
GO:0019222	regulation of metabolic process	-0.3979
GO:0032787	monocarboxylic acid metabolic process	-0.4559
GO:0031323	regulation of cellular metabolic process	-0.4685
GO:0006351	transcription, DNA-templated	-0.4685
GO:0006790	sulphur compound metabolic process	-0.5086
GO:0051171	regulation of nitrogen compound metabolic process	-0.5086
GO:0050789	regulation of biological process	-0.5376
GO:0032502	developmental process	-0.5528
GO:0050794	regulation of cellular process	-0.5528
GO:0009719	response to endogenous stimulus	-0.5528
GO:0042221	response to chemical	-0.5528
GO:0009628	response to abiotic stimulus	-0.5528
GO:0006355	regulation of transcription, DNA-templated	-0.5528
GO:0019219	regulation of nucleobase-containing compound metabolic process	-0.5528
GO:0080090	regulation of primary metabolic process	-0.5528
GO:0010556	regulation of macromolecule biosynthetic process	-0.5528
GO:0009605	response to external stimulus	-0.5686
GO:0009725	response to hormone	-0.585
GO:0046394	carboxylic acid biosynthetic process	-0.585
GO:0016053	organic acid biosynthetic process	-0.585
GO:0009889	regulation of biosynthetic process	-0.585
GO:0031326	regulation of cellular biosynthetic process	-0.585
GO:0006629	lipid metabolic process	-0.6383
GO:0065007	biological regulation	-0.6383
GO:0009791	post-embryonic development	-0.7696
GO:0009733	response to auxin	-0.7696
GO:0051704	multi-organism process	-0.7959
GO:0009639	response to red or far red light	-1.2218
GO:0051707	response to other organism	-1.2218

GO:0009620	response to fungus	-1.2218
GO:0009416	response to light stimulus	-1.2291
GO:0010114	response to red light	-1.2291
GO:0050896	response to stimulus	-1.2291
GO:0009607	response to biotic stimulus	-1.2291
GO:0009314	response to radiation	-1.2291
	Upregulated elevated temperature	
GO:0046914	transition metal ion binding	-0.4437
GO:0004553	hydrolase activity, hydrolysing O-glycosyl compounds	-0.4437
GO:0016798	hydrolase activity, acting on glycosyl bonds	-0.585
	Downregulated elevated temperature	
GO:0043167	ion binding	-0.3098
GO:0043169	cation binding	-0.3098
	Downregulated artificial ageing	
GO:0009628	response to abiotic stimulus	-0.5086
GO:0009719	response to endogenous stimulus	-0.5086
GO:0009725	response to hormone	-0.5528
GO:0009415	response to water	-0.5528

3.1.2 The influence of drought during seed maturation on seed quality and the seed transcriptome

The effect of drought stress or water scarcity has been extensively studied in plants, as this is one of the most common problems that plants are faced with worldwide. The lack of water imposes severe restrictions to plant growth and seed production, therefore being one of the main threats to agriculture and industry due to the severe economic loses it implicates. Many studies have been conducted to elucidate the molecular mechanisms and transcriptional networks underlying plant acclimation and adaption to this condition, as well as the signalling processes that it involves (Shinozaki and Yamaguchi-Shinozaki, 2007; Farooq *et al.*, 2009). However, the effects of drought on seed traits have been largely overlooked, considering its overall detrimental effect for the adult plant. Some studies already showed, nonetheless, that drought can exert a positive effect on seed quality traits, such as modifying seed composition and increasing responses to oxidative stresses (Britz and Kremer, 2002; Li *et al.*, 2013).

The impact of drought stress imposed during the course of seed development and maturation on seed dormancy and sensitivity to artificial ageing was evaluated in a mutant with reduced seed longevity. Specifically, seeds from the seed longevity-deficient mutant *dog1-1* and its background NIL DOG1 (in L*er* wild type) were used and their response to water deficit during seed maturation evaluated. NIL DOG1 carries a chromosomal region from the Cvi accession containing the *DOG1* gene introgressed into L*er* (Alonso-Blanco *et al.*, 2003). Whereas the *dog1-1* mutant has reduced seed longevity and is non-dormant, NIL DOG1 shows deep seed dormancy levels due to the strong *DOG1* allele from the Cvi accession. The *dog1-1* mutant is the result of a nucleotide substitution that introduces a premature stop codon, therefore rendering the plant devoid of DOG1 (Bentsink *et al.*, 2006).

Drought treatment caused an alleviation of seed dormancy in the NIL DOG1 control (**Figure 3A**), as determined in a germination test of freshly harvested seeds. Furthermore, when seed longevity was evaluated, it was observed that seeds from drought-treated plants showed reduced sensitivity to accelerated ageing compared to the non-treated controls and that this effect was comparable in both the mutant and the control genotypes (**Figure 3B**). Control NIL DOG1 seeds were partially dormant (**Figure 3A**), so when subjected to artificial ageing, a first phase of dormancy release was observed prior to the ageing deterioration (**Figure 3B**, 0-2 days of treatment).

RNA-sequencing of dry seeds was used to elucidate the transcriptional responses underlying this enhanced resistance to artificial ageing. The expression profiles of treated and control samples were first compared using a principal component analysis. **Figure 4** shows that the main component that separates samples is genotype, which accounts for 80 % of the observed variability and determines a clear clustering of samples. The drought treatment, represented by component 2, explains 10 % of the observed variability and separates control samples at the bottom of the chart from those grown under drought stress at the top.

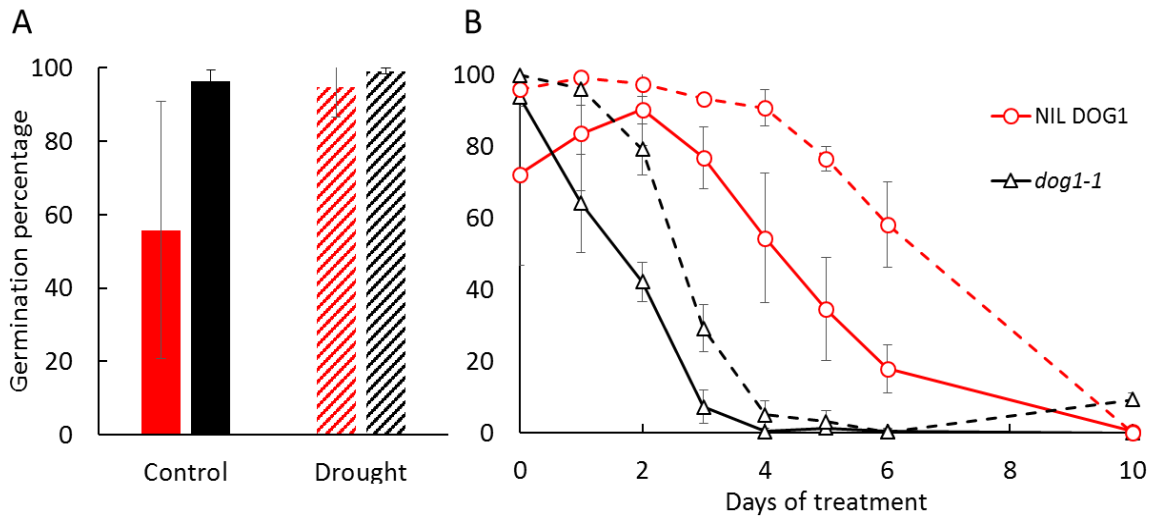

Figure 3. Drought treatment during seed maturation affects seed dormancy and longevity. Panel **A** shows the mean germination percentages of freshly harvested seeds used for this study. Panel **B** shows the germination proportion after different periods of artificial ageing of the same seed batches. Data points represent the mean value of three biological replicates. Error bars correspond to the associated standard deviation. Solid lines represent control samples, whereas drought-treated samples are represented with dashed lines.

All comparisons were made using the NIL DOG1 untreated control as a reference. **Table 7** shows the number of differentially expressed transcripts identified for each condition. It is apparent that the *dog1-1* mutant profoundly affects the dry seed transcriptome, causing over 2,000 genes to be differentially expressed, whereas the effect of drought led to the identification of 582 differentially expressed transcripts.

3.1.2.1 The effect of drought during seed maturation on the dry seed transcriptome

Water scarcity imposed during seed set and maturation led to reduced levels of seed dormancy and increased resistance to artificial ageing (**Figure 3**), as well as an increase in seed weight (**Supplemental Figure 2**). Transcriptomic analysis of drought treated seeds and their corresponding controls led to the identification of 501

differentially expressed transcripts (**Table 7**). **Figure 5** illustrates that determination of differentially expressed transcripts using either NIL DOG1 or *dog1-1* as the reference for the comparisons led to very similar results, which means that the effect of the drought treatment in both genotypes is comparable. The number of DETs might be slightly higher in the mutant as a result of its altered transcriptome (**Table 7**). GO enrichment analyses of upregulated transcripts found several overrepresented categories (**Table 8**).

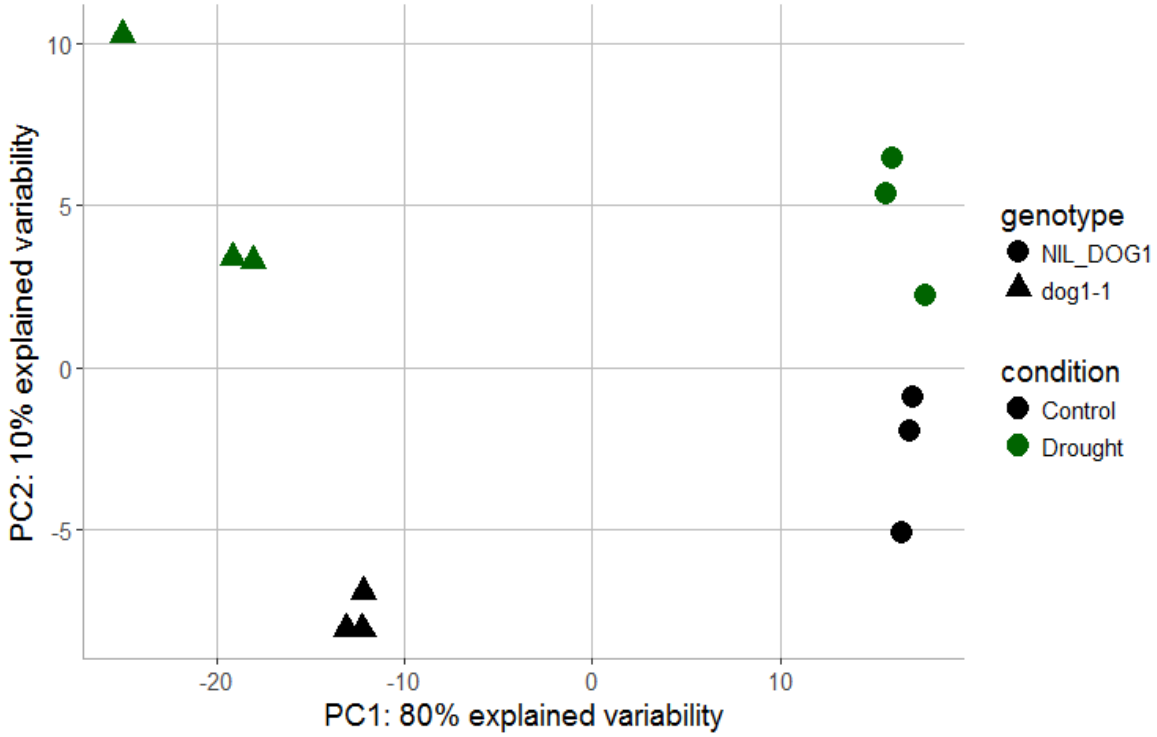

Figure 4. Principal Component Analysis plot of samples. Drought stress was applied during seed maturation to the *dog1-1* mutant and its wild type control NIL DOG1. Shapes represent genotypes and colours represent treatment, as indicated.

As mentioned above, drought imposes stresses to the plant that result in overall detrimental effects. Therefore, the presence of enriched GO categories related to abiotic stimulus responses was predictable. In this group, *HSP22* and *HSP17.6II* were previously linked to these stress responses and to play a part during seed development (Helm *et al.*, 1995; Kotak *et al.*, 2007). Also linked to abiotic stress responses, *RESPONSIVE TO ABA AND SALT 1* (*RAS1*) was upregulated by drought. This gene encodes a plant-specific protein responsive to ABA and which expression is indirectly enhanced by drought (Ren *et al.*, 2010; Yin *et al.*, 2017). *TOUCH 4* (*TCH4*) participates in cell wall modification and was shown to be responsive to environmental stresses (Xu *et al.*, 1995; Iliev *et al.*, 2002). The latter study showed that the promoter of *TCH4* is enriched in motifs associated with different responses, including touch and several phytohormones. Two transcripts encoding genes

participating in responses to oxidative stress were also present. *ASCORBATE PEROXIDASE 5* (*APX5*) and its close relative genes participate in detoxification of hydrogen peroxide (Panchuk *et al.*, 2002; Chen *et al.*, 2014a), whereas *VITAMIN E DEFECTIVE 4* (*VTE4*) participates in the degradation of chlorophyll linked to the biosynthesis of tocopherols, a set of compounds involved in protection of lipids from oxidative stress (Bergmüller *et al.*, 2003). In addition, this gene was reported to participate in oxidative stress responses by affecting the pool of tocopherols available in the cell (Semchuk *et al.*, 2009; Cela *et al.*, 2011). Also included in this category was *LIPID TRANSFER PROTEIN 4* (*LTP4*), which participates in lipid transport and was shown to be upregulated by salinity stress (Chae *et al.*, 2010). In close connection with stress responses, two transcripts related with ABA were upregulated. First, *MECHANOSENSITIVE CHANNEL OF SMALL CONDUCTANCE-LIKE 9* (*MSL9*), which is responsive to touch and a downstream target of ABA. Second, *DROUGHT-INDUCED 21*, reported to be responsive to ABA (Gosti *et al.*, 1995).

Table 7. Number of differentially expressed transcripts identified for each condition. For a transcript to be considered differentially expressed, a cut-off for $\log_2$FC ≥ |1| and adjusted p-value ≤ 0.05 were applied.

Condition	Up-regulated transcripts	Down-regulated transcripts
Drought	210	372
Drought (*dog1-1* reference)	324	475
dog-1-1 mutation	1944	460

Upregulated transcripts also showed several overrepresented categories related to cell wall organisation, modification and loosening. A detailed examination of genes present in these categories showed that they are also included in categories related to cellular and developmental growth (**Table 8**). Specifically, *EXPANSIN 2*, *3*, *9*, *10* and *20* were upregulated by the drought treatment. These enzymes are involved in cell wall loosening associated with growing organs and some of them have been linked to responses to abiotic stresses (Cho and Cosgrove, 2000; Kwon *et al.*, 2008; Zou *et al.*, 2013; Yan *et al.*, 2014). *TCH4* was also present in this group. Also present here was the transcription factor *WEREWOLF* (*WER*), which was described as a positive regulator of *TRANSPARENT TESTA GLABRA 2* (*TTG2*) and in turn may affect composition of the seed coat (Ishida *et al.*, 2007), as well as *XTH19*, which is upregulated by drought.

Within the upregulated transcripts, GO categories for gene expression, translation and translational elongation were also found overrepresented. Several chloroplast-encoded ribosome constituents (Supplemental Dataset 1), as well as some encoded in the nucleus (At1g22110, At5g12110) were present, together with two RNA polymerases from both the chloroplast (AtCg00740) and nucleus (*RNA-DEPENDENT RNA POLYMERASE 6*, *RDR6*). The latter was shown to mediate gene silencing and to help degrading incorrectly processed transcripts (Luo and Chen, 2007; Hoffer *et al.*, 2011; De Alba *et al.*, 2015). *DEFECTIVE IN MERISTEM SILENCING 3* (*DMS3*) was also present in this category. This gene was reported to contribute to *de novo* DNA methylation, acting as a transcriptional repressor (Ausin *et al.*, 2009; Lorković *et al.*, 2012).

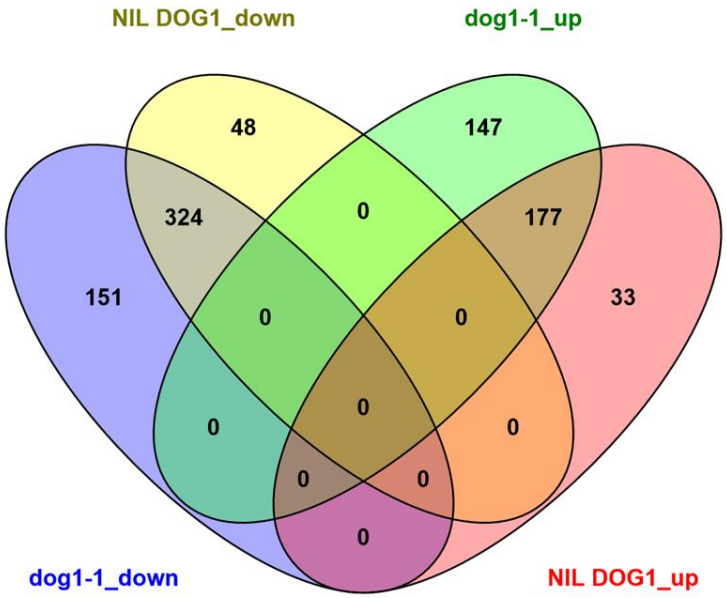

Figure 5. Number of differentially expressed transcripts caused by drought. Venn diagram illustrating the number of differentially expressed transcripts determined for each genotype. For each set of genes, the genotype from which it was determined is indicated. Down indicates downregulation and up refers to upregulation.

Intriguingly, another overrepresented GO category was photosynthesis, together with the closely related generation of precursor metabolites and energy, with which most genes are common. Included here, several transcripts encoding subunits of the photosystem II (PSII), electron transport chain and ATP biosynthetic pathway were upregulated (Supplemental Dataset 1). Furthermore, several subunits of the NAD(P)H dehydrogenase complex were present as well (Supplemental Dataset 1). Also related, *2-CYS PEROXIREDOXIN B* (*2CPB*), which participates in the responses to oxidative stresses within the chloroplast and which has been demonstrated to

participate in the protection of the photosynthetic apparatus (Rey *et al.*, 2007; Awad *et al.*, 2015), was upregulated. Furthermore, *DEGP PROTEASE 5* (*DEG5*) transcripts showed increased abundance. This protease was linked to repair and degradation mechanisms affecting photo-damaged components of the PSII (Sun *et al.*, 2007; Kato *et al.*, 2012).

Table 7 illustrates how the number of transcripts downregulated by drought during seed maturation is higher than that of those upregulated. When evaluating the overrepresented GO categories in this dataset, several related to pollen tube growth and development were present, along with other more general categories such as cell morphogenesis and developmental cell growth. All these categories included several transcripts involved in the development of the pollen tube and the pollination process. These transcripts comprise genes related to synthesis and expansion of the pollen tube, such as *KINKY POLLEN* (*KIN*), *VANGUARD 1* (*VGD1*) and *EXPA4* (Procissi *et al.*, 2003; Jiang *et al.*, 2005); or modification of the pollen cell wall, represented by an elevated numbers of transcripts, including several *ARABINOGALACTAN PROTEINS* (*AGPs*; Pereira et al., 2016) and *MICROTUBULE ASSOCIATED PROTEINS* (*MAPs*). In addition, *LORELEI* (*LRE*) transcripts, previously demonstrated to play a central role in fertilisation (Tsukamoto *et al.*, 2010), was also downregulated. One of the most well-known effects of drought is its detrimental effect on seed yield and gamete development (Barnabás *et al.*, 2008), which would fit with the enrichment of downregulated transcripts in GO categories related to pollen development.

Another group enriched within the set of downregulated transcripts is related with toxin metabolism. A closer examination of the genes present revealed that this category exclusively includes transcripts encoding different glutathione S-transferases. This is a striking result, considering most of these genes are known to be responsive to stresses (Sappl *et al.*, 2009). Connected with the previous, response to chitin, a polymer found in the cell wall of fungi, was also enriched. Transcripts within this group are mainly represented by *ETHYLENE RESPONSIVE ELEMENT BINDING FACTORS* (*ERFs*) *2*, *5*, *6* and *11*. *ERF5* was previously linked to chitin responses (Son *et al.*, 2012). It is a close relative of *ERF6* and together they were shown to participate in responses to water deprivation in growing leaves of Arabidopsis, where *ERF6* promotes the expression of *GA2OX6*, thus limiting growth by stabilising DELLA proteins (Dubois *et al.*, 2013, 2015). In addition, two genes involved in the biosynthesis of ethylene were also found downregulated: *ETHYLENE-FORMING ENZYME* (*EFE*) and AT1G12010 (Gómez-Lim *et al.*, 1993). *GA2OX6* is involved in active GA catabolism, a role partially activated by *DOG1* (Kendall *et al.*, 2011), which was also found

downregulated by drought treatment and fits with the observed reduction in dormancy depth (**Figure 3A**). Also related to the induction of seed dormancy, *9-cis-EPOXYCAROTENOID DIOXYGENASE 6* (*NCED6*), which participates in ABA biosynthesis during seed development, was downregulated (Martínez-Andújar *et al.*, 2011). Two close relatives of this gene appeared downregulated as well. On the one hand, *NCED4*, which plays a role in thermoinhibition of seed germination in lettuce (Huo *et al.*, 2013). Interestingly, this study reported that drought did not alter *NCED4* expression in leaves, contrary to the observed downregulation occurring in seeds. On the other hand, *CAROTENOID CLEAVEAGE DEOXYGENASE 7* (*CCD7*), which was linked to the biosynthesis of strigolactones, appeared downregulated as well (Booker *et al.*, 2004). This would be in agreement with the reduced ethylene responses, as these phytohormones were described to enhance strigolactone biosynthesis (Ueda and Kusaba, 2015). Nevertheless, it is possible that these genes are downregulated because of their role in carotenoid degradation i.e., to enhance carotenoid accumulation. Carotenoids act as antioxidants protecting the photosynthetic apparatus (Zakar *et al.*, 2016), which could also fit with the observed upregulation of photosynthesis-related GO categories in the group of upregulated transcripts.

Transcripts of two genes involved in seed coat composition were downregulated as well. Specifically, *TT5* and *TT10*, involved in flavonoid biosynthesis and in lignin and soluble proanthocyanidin (PA) oxidation, respectively (Pourcel *et al.*, 2013; Liang *et al.*, 2006), were found downregulated by drought. PAs were demonstrated to be able to enhance ABA accumulation by enhancing expression of *NCED6* (Jia *et al.*, 2012). Interestingly, a recent study reported that, similar to *tt10*, the mutant *nitrate transporter 2.7* (*nrt2.7*) also accumulates increased amounts of soluble PAs (David *et al.*, 2014). Within the dataset of downregulated transcripts, *NRT1.1*, *1.2* and *2.5* were present. The *nrt1.1* mutant was described to show reduced transpiration and therefore to be more resistant to drought stress (Guo *et al.*, 2003), whereas *NRT1.2* was reported to participate in ABA transport (Kanno *et al.*, 2012).

Although not represented by any enriched category, several transcripts involved in chromatin modification had reduced transcript abundance. Specifically, *VARIANT IN METHYLATION* (*VIM*) *2, 3* and *4* were downregulated by drought. These genes are involved in transcriptional silencing by contributing to the maintenance of DNA methylation levels (Woo *et al.*, 2008; Shook and Richards, 2014). This would agree with previous work showing that abiotic stresses modified epigenetic marks in the promoter of *TT5*, altering its expression (Bharti *et al.*, 2015) and with previous studies reporting the effect of abiotic stresses on chromatin marks (Kim *et al.*, 2015).

Among transcripts downregulated by drought, red light and far-red light responses were also enriched. *TYPE-A RESPONSE REGULATOR 4* (*ARR4*) is involved in the crosstalk between light and cytokinin responses (Sweere, 2001; Mira-Rodado *et al.*, 2007). Another study, however, showed that this gene also contributes to ethylene-mediated stomatal closure in leaves (Mira-Rodado *et al.*, 2012). Also included in the category of light responses, *SIGMA FACTOR E* (*SIGE*) is a nuclear-encoded regulator of plastid transcription (Noordally *et al.*, 2013) whose expression was shown to be responsive to abiotic stresses (Nagashima *et al.*, 2004). Specifically, *SIGE* expression is induced by ABA treatment (Yamburenko *et al.*, 2015) and its presence in this group of transcripts would fit the observed reduction in ABA biosynthetic genes.

Table 8. Gene Ontology categories enriched for each set of differentially expressed transcripts. The last column shows the $\log_{10}$ of the p-value associated with each overrepresented category.

GO ID	Description	$\log_{10}$ p-value
	Upregulated Drought	
GO:0051179	localization	-0.3279
GO:0032502	developmental process	-0.4437
GO:0009791	post-embryonic development	-0.4437
GO:0046483	heterocycle metabolic process	-0.4437
GO:0009409	response to cold	-0.4815
GO:0006811	ion transport	-0.5229
GO:0065008	regulation of biological quality	-0.5376
GO:0006796	phosphate-containing compound metabolic process	-0.5686
GO:0006793	phosphorus metabolic process	-0.5686
GO:0032989	cellular component morphogenesis	-0.6383
GO:0032535	regulation of cellular component size	-0.699
GO:0090066	regulation of anatomical structure size	-0.699
GO:0040007	growth	-0.8539
GO:0048869	cellular developmental process	-1
GO:0009266	response to temperature stimulus	-1.1487
GO:0006508	proteolysis	-1.1871
GO:0007166	cell surface receptor signalling pathway	-1.3665
GO:0055085	transmembrane transport	-1.4202
GO:0048589	developmental growth	-1.4318
GO:0007169	transmembrane receptor protein tyrosine kinase signalling pathway	-1.699
GO:0044260	cellular macromolecule metabolic process	-1.7959
GO:0009628	response to abiotic stimulus	-2.1367
GO:0009059	macromolecule biosynthetic process	-2.3098

GO:0010467	gene expression	-2.3188
GO:0034645	cellular macromolecule biosynthetic process	-2.3279
GO:0034220	ion transmembrane transport	-3.2291
GO:0043170	macromolecule metabolic process	-3.2441
GO:0044238	primary metabolic process	-3.3468
GO:0009825	multidimensional cell growth	-3.4815
GO:0009827	plant-type cell wall modification	-3.4815
GO:0009058	biosynthetic process	-3.5086
GO:0044249	cellular biosynthetic process	-3.585
GO:0044267	cellular protein metabolic process	-3.6198
GO:0009987	cellular process	-3.7212
GO:0006091	generation of precursor metabolites and energy	-3.7212
GO:0044237	cellular metabolic process	-3.7959
GO:0019684	photosynthesis, light reaction	-3.8239
GO:0042547	cell wall modification involved in multidimensional cell growth	-4.0862
GO:0008152	metabolic process	-4.3188
GO:0006412	translation	-4.9586
GO:0015979	photosynthesis	-5.1308
GO:0019538	protein metabolic process	-5.1308
	Downregulated Drought	
GO:0043933	macromolecular complex subunit organization	-0.3188
GO:0009653	anatomical structure morphogenesis	-0.3188
GO:0032535	regulation of cellular component size	-0.3665
GO:0090066	regulation of anatomical structure size	-0.3665
GO:0009639	response to red or far red light	-0.3665
GO:0050896	response to stimulus	-0.4949
GO:0051704	multi-organism process	-0.4949
GO:0005975	carbohydrate metabolic process	-0.4949
GO:0070271	protein complex biogenesis	-0.4949
GO:0048589	developmental growth	-0.4949
GO:0042545	cell wall modification	-0.5086
GO:0065003	macromolecular complex assembly	-0.6576
GO:0010200	response to chitin	-0.699
GO:0019748	secondary metabolic process	-0.7447
GO:0060560	developmental growth involved in morphogenesis	-0.8239
GO:0010114	response to red light	-1.1135
GO:0007267	cell-cell signalling	-2.0088
GO:0000904	cell morphogenesis involved in differentiation	-2.1938
GO:0009404	toxin metabolic process	-2.2757
GO:0048868	pollen tube development	-2.2757
GO:0035295	tube development	-2.2757
GO:0009407	toxin catabolic process	-2.2757

3.1.2.2 The seed transcriptome of the *dog1-1* mutant

A recent publication conducted microarray analysis comparing the *dog1-1* mutant to its background NIL DOG1 (Dekkers *et al.*, 2016). The authors proposed a role of *DOG1* as a repressor of genes associated with germination, considering many of these were upregulated in the mutant. In addition, they described how several genes whose expression is induced during the late stages of seed maturation fail to do so in this mutant. These observations prompted the hypothesis that *DOG1* could be a regulator of seed maturation, which was further supported by the altered metabolic profile of the mutant.

In this study, the dry seed transcriptome of *dog1-1* was evaluated by RNA-seq in both control and drought conditions. Comparisons between microarray and RNA-seq approaches showed that, although comparable, the latter allows for more detailed analyses, as this sequencing approach captures a larger fraction of the low-abundance transcripts (Izadi *et al.*, 2016). In agreement with this, the number of differentially expressed transcripts detected is three times higher than that described by Dekkers and colleagues (**Table 7**).

Among the transcripts upregulated in the mutant, several GO categories were overrepresented related to stress responses and hormones, such as gibberellins and ethylene and oxidative and osmotic stresses, which is in agreement with previous work (Dekkers *et al.*, 2016). Furthermore, this group also included categories linked to germination processes, such as growth, modification of the cell wall and ribosome biogenesis (Supplemental Dataset 2). Nevertheless, other overrepresented categories were present that were not previously reported. Among these, response to cold was found overrepresented. Within this category, the abiotic stress responsive genes *KIN1* and *KIN2* were present (Kurkela and Franck, 1990), together with *LEA5*. The latter was shown to be responsive to several abiotic stresses and its expression proposed to be linked to ROS signalling in roots (Salleh *et al.*, 2012). This category also included *SENSITIVE TO FREEZING 2* (*SFR2*), a gene that participates in the protection of chloroplast membranes by modification of their lipid composition (Moellering *et al.*, 2010). Likewise, *STARCH EXCESS 1* (*SEX1*) participates in degradation of starch and was shown to contribute to freezing tolerance (Yano *et al.*, 2005), besides of affecting the resulting seed composition and thus, post-germination growth (Andriotis *et al.*, 2012). Surprisingly, the transcription factor *SPT* was also upregulated and present in this category. *SPT* was described as a repressor of seed germination that contributes to the maintenance of seed dormancy (Penfield *et al.*, 2005). Further work characterised its regulatory role as affecting the expression of *ABI4* and *ABI5*, together

with DELLA proteins (Vaistij *et al.*, 2013). Intriguingly, this study reported the induction of *ABI5* and the repression of *ABI4* expression by *SPT*, whereas our results showed that *ABI5* is downregulated and *ABI4* is upregulated in *dog1-1*, in agreement with the previous study (Dekkers *et al.*, 2016). Furthermore, previous research reported that DELLA proteins can exert a negative regulation on *SPT* expression (Josse *et al.*, 2011), but the *dog1-1* dry seed transcriptome showed upregulation of most DELLA-encoding genes, including *REPRESSOR OF GA1-3 1* (*RGA1*), *RGL1*, *RGL2* and *GIBBERELLIC ACID INSENSITIVE* (*GAI*). Upregulation of these transcripts might seem unexpected, especially considering that several genes related to GA biosynthesis had increased expression in the mutant as well, such as *GA1*, *GIBBERELLIN 20-OXIDASE 1* (*GA20OX1*), *GA20OX2*, *GA20OX3* and *GA3OX2*. Besides, DELLA proteins were shown to repress the expression of *EXP2* (Yan *et al.*, 2014), but this gene and its close relatives *EXP3*, *8*, *9*, *10*, *15* and *20* were upregulated in the mutant as well. However, a previous study showed that DELLA proteins target genes involved in GA biosynthesis and signalling and suggested that DELLAs could contribute to GA homeostasis (Zentella *et al.*, 2007). Another study reported that exogenous application of GAs induced mRNA accumulation of *RGA1* in *ga1-3* mutants, an observation further confirmed in the wild type L*er* after treatment with an inhibitor of GA biosynthesis (Ariizumi *et al.*, 2008). This would agree with the observed increases in both GA biosynthetic and repressive transcripts. In addition, it is possible that other feedback loops are exerting their regulation simultaneously, including those affected by protein accumulation, which could in turn be affecting expression in the mutant. It is then difficult to draw conclusions from transcriptomic data alone.

Similarly, auxin responses appeared overrepresented in the group of upregulated transcripts. *PINOID* (*PID*) modulates auxin effluxes through phosphorylation of auxin carriers such as *PIN-FORMED 1* (*PIN1*) and *PIN2*, which in turn affect different processes, including the establishment of cell polarity (Zhang *et al.*, 2010; Zourelidou *et al.*, 2014; Weller *et al.*, 2017). *MONOPTEROS* (*MP*) is a transcription factor also involved in auxin signalling by modulating the expression of *PIN* genes (Schuetz *et al.*, 2008; Krogan *et al.*, 2016). This gene was recently linked to the regulation of cell fate by chromatin switches in response to auxins (Wu *et al.*, 2015). The transporter *AUXIN RESISTANT 1* (*AUX1*) was present in this category as well. This gene was shown to be involved in regulating germination speed and its expression to be controlled through chromatin modifications (Wang *et al.*, 2016). The transcript encoding for *POLARIS* (*PLS*), a small peptide linking the crosstalk between auxins, ethylene and cytokinins in roots was also upregulated (Chilley *et al.*, 2006; Liu *et al.*, 2013a). *TT4* is also present in this category and mutants for this gene were

reported to show altered auxin transport, as well as light and gravity responses (Buer and Muday, 2004), categories which also appeared overrepresented (Supplemental Dataset 2).

For downregulated transcripts, the results from RNA-seq data accommodate well with those previously described. As pointed out by Dekkers *et al.* (2016), one of the overrepresented categories within the set of downregulated transcripts is response to heat. This group comprises a broad array of heat shock proteins. Of special interest here is the presence of *HEAT SHOCK TRANSCRIPTION FACTOR A9* (*HSFA9*). This is a transcription factor whose expression is induced during the later stages of seed maturation under the control of *ABI3* (Kotak *et al.*, 2007). As described in this work, *HSFA9* regulates the expression of several downstream targets, such as *HSP17.4CI*, *HSP17.7CII*, *HSP70* and *HSP101*, all of which are also part of the category of response to heat. Furthermore, this transcriptional network was linked to the acquisition of seed longevity in sunflower, through a separate pathway from that involved in the acquisition of desiccation tolerance (Tejedor-Cano *et al.*, 2010).

3.1.2.3 The influence of drought during seed maturation on cell wall composition

Drought imposed during seed set in Arabidopsis causes a considerable reduction in the overall seed yield. The possible reasons for this reduction include ovule and pollen abortion, deficient seed filling and overall reduced plant growth, which reflects in a reduced number of seeds produced (Farooq *et al.*, 2009). In Arabidopsis plants, seed production occurs gradually over time. Imposing drought during this phase causes a reduction in plant growth, which implies that the subsequent formation of floral shoots and seeds becomes compromised. The results from the RNA-seq data analysis revealed that many of the differentially expressed transcripts caused by drought were related to cell wall composition and modification. This prompted the hypothesis that the observed enhanced resistance to artificial ageing might be caused by these modifications. To evaluate this, cell wall composition analyses were conducted. As drought imposed a strong limitation on the amount of available seeds for the experiments, these studies were conducted pooling all three biological samples together in order to reach the minimum amount of sample required for these analyses. All the cell wall composition analysis were conducted by the group of Professor Simon McQueen-Mason, at the University of York (United Kingdom).

Analysis of the monosaccharide profile of the non-crystalline fraction of the seed cell walls between control and drought-treated samples showed that differences in monosaccharide content were already present at control conditions (**Figure 6**). The *dog1-1* mutant exhibited higher amounts of monosaccharides than NIL DOG1. However, the application of drought during seed maturation alleviated these differences, resulting in similar profiles between the control and the mutant.

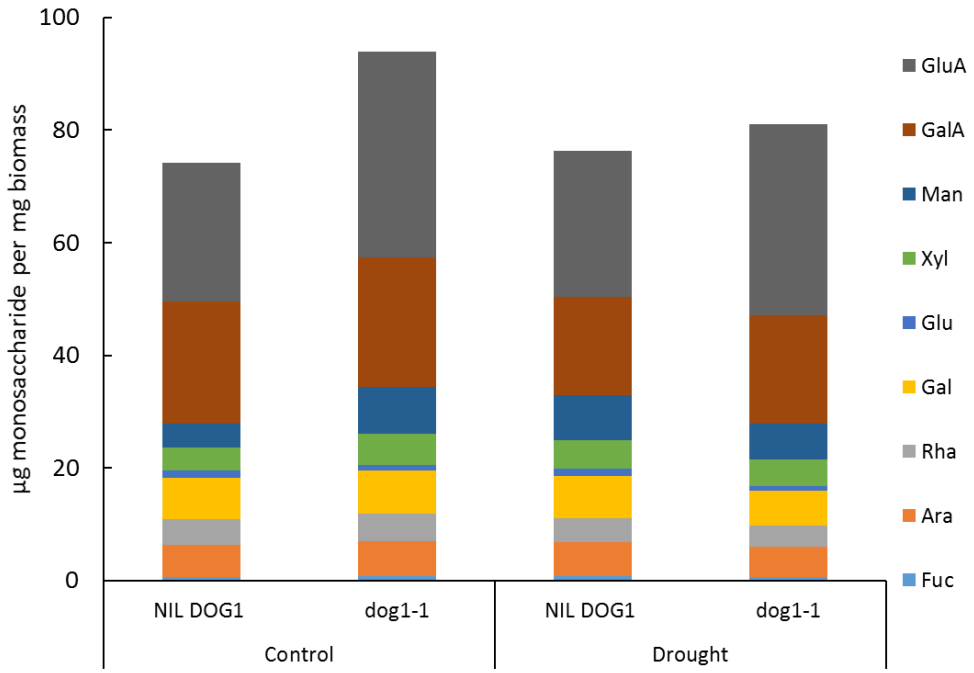

Figure 6. Monosaccharide profile of the non-crystalline fraction of seed cell walls. Total amount of monosaccharides in the xyloglucan fraction in control and drought treated seeds of NIL DOG1 and *dog1-1*. GluA: glucuronic acid; GalA: galacturonic acid; Man: mannose; Xyl: xylose; Glu: glucose; Gal: galactose; Rha: rhamnose; Ara: arabinose; Fuc: fucose.

Monosaccharides present in the pectin-enriched fraction were also evaluated and found to exhibit the same trend. Analysis of monosaccharides present in the esterified-pectin fraction showed a higher amount of these already in the non-treated mutant (**Figure 7A**). Similarly to the non-crystalline fraction, drought treatment led to a reduction of the monosaccharide content in *dog1-1*, whereas in this specific fraction, it led to the exact opposite pattern in NIL DOG1, as it showed increased monosaccharide content after drought treatment (**Figure 7A**). As for monosaccharides present in the non-esterified pectin fraction, the initial situation was comparable, with *dog1-1* having the highest content and the drought treatment leading to a considerable reduction in both genotypes (**Figure 7B**). It is interesting that in control seeds, NIL DOG1 had galactose (Gal), but *dog1-1* did not and,

conversely, whereas the control had reduced pool of glucose (Glu), this monosaccharide was more baundant in the mutant (**Figure 7B**).

Taken together, these results do not accommodate with the initially observed phenotypes, considering both genotypes showed enhanced resistance to artificial ageing when drought was applied during seed maturation (**Figure 3B**). From these observations, a similar behaviour of the seed cell wall composition could be expected if this was the underlying factor for this phenotype. However, cell wall composition analyses revealed that *dog1-1* shows an altered composition at control conditions, displaying a higher abundance of monosaccharides and an increased overall amount in comparison to NIL DOG1 (**Figure 6** and **Figure 7**).

Drought treatment applied during seed maturation led to a reduction of the monosaccharides present in cell walls of *dog1-1* seeds, reducing them to proportions similar to those of the control. *dog1-1* seeds were shown to have an altered composition (Dekkers *et al.*, 2016), which is in agreement with the observed cell wall composition. Altered seed coat composition was demonstrated to have a detrimental effect on seed longevity (Debeaujon *et al.*, 2000; Clerkx *et al.*, 2004), which could support the increased monosaccharide content in the mutant as a contributing factor to its reduced longevity due to a weaker seed coat. It is striking, however, that water deprivation applied either to the control or the mutant resulted in a similar phenotypic response. Moreover, the monosaccharide profiles behave with opposite trends in each genotype. It is possible that drought treatment leads to a certain cell wall composition involving a specific proportion of monosaccharides, thus allowing seeds to deal better with water scarcity. This specific composition would then explain the opposite observed behaviours, as each genotype starts from a different composition, although further experiments are required to substantiate this.

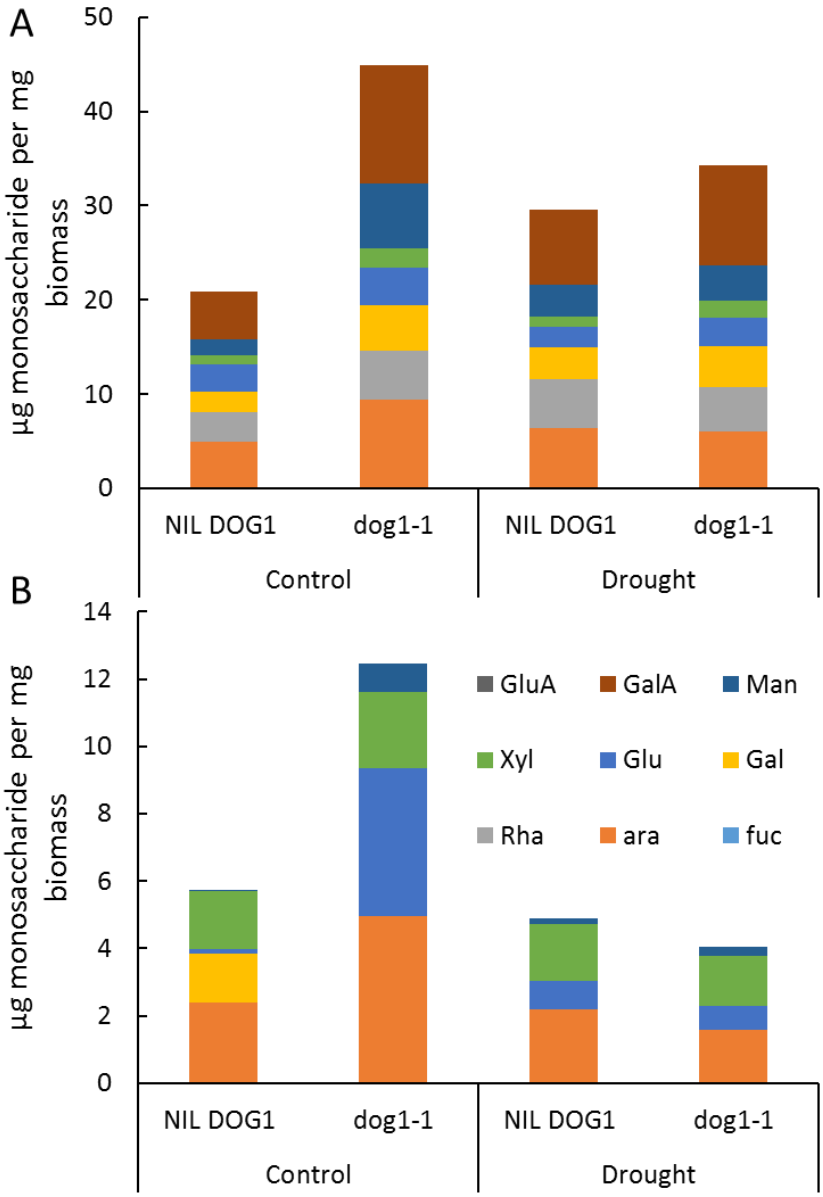

Figure 7. Monosaccharide profile of the pectin-enriched fraction of seed cell walls. Total amount of monosaccharides in the esterified (**A**) and non-esterified (**B**) pectin fraction in control and drought treated seeds of NIL DOG1 and *dog1-1*. GluA: glucuronic acid; GalA: galacturonic acid; Man: mannose; Xyl: xylose; Glu: glucose; Gal: galactose; Rha: rhamnose; Ara: arabinose; Fuc: fucose.

3.2 IDENTIFICATION OF GENES AFFECTING SEED LONGEVITY IN *ARABIDOPSIS THALIANA*

3.2.1 The role of DOG1-interacting proteins in seed longevity

DELAY OF GERMINATION 1 (*DOG1*) was initially described as a major quantitative trait locus regulating seed dormancy in recombinant inbred lines that originated from a cross between low and high dormant accessions of *Arabidopsis thaliana* (Alonso-Blanco *et al.*, 2003). *DOG1* was cloned over a decade ago and reported to be involved in the regulation of seed dormancy and longevity (Bentsink *et al.*, 2006), but its molecular role has only started being elucidated. A recent publication showed that *DOG1* regulates seed dormancy together with ABA through genetic and *in vivo* interactions with clade A PP2C protein phosphatases (Née *et al.*, 2017). This study showed that both ABA and *DOG1* negatively regulate the actions of these phosphatases in the release of dormancy. However, if this mechanism underlies the role of *DOG1* in the acquisition of seed longevity remains to be determined.

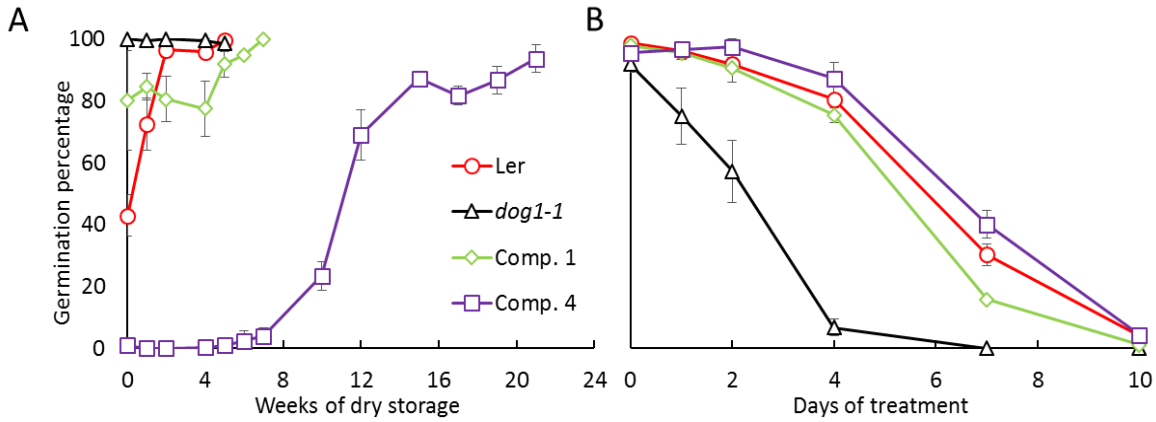

Figure 8. DOG1 protein accumulation affects the depth of seed dormancy but not sensitivity to artificial ageing. Germination proportion after different periods of dry storage (**A**) and after different lengths of accelerated seed ageing treatment (**B**) of wild type L*er* (circles, red line), *dog1-1* mutant (triangles, black line) and two transgenic lines of the *dog1-1* mutant complemented with pDOG1$_{Cvi}$:YFP:*DOG1*$_{Cvi}$, accumulating DOG1 at relatively low (Comp. 1, diamonds, green line) or high (Comp. 2, squares, purple line) levels (Nakabayashi *et al.*, 2012). Data points represent the mean of three biological replicates and error bars the associated standard deviation.

DOG1 protein accumulation directly correlates with the depth of seed dormancy. Nevertheless, this effect was never established for seed longevity. The seed longevity phenotype of previously generated complemented *dog1-1* mutants over-accumulating DOG1 (Nakabayashi *et al.*, 2012) was evaluated (**Figure 8**). Although DOG1 accumulation has a profound effect on the depth of seed dormancy (**Figure**

8A), its protein abundance levels do not correlate with sensitivity to artificial ageing. The presence of DOG1 complements seed longevity in *dog1-1*, confirming its role the establishment of this trait, but enhanced accumulation does not further improve seed longevity (**Figure 8B**). This raises the possibility that the role of *DOG1* in determining seed longevity is different from the one it plays in the establishment and maintenance of seed dormancy (Nguyen *et al.*, 2012).

Considering the participation of *DOG1* in the acquisition of seed longevity, proteins that interact *in vivo* with it could also be involved in this process. Hence, a recently produced DOG1 pull-down dataset, consisting of proteins able to interact with DOG1 in dry and imbibed seeds (Née *et al.*, 2017), was used to select possible candidates according to their expression profiles and available literature. **Table 9** shows the list of genes selected to evaluate their role in seed longevity.

Table 9. DOG1-interacting proteins selected to evaluate their role in seed longevity.

Locus	Symbol	Description
At4g26740	*CLO1*	*CALEOSIN 1*
At5g42980	*TRX3*	*THIOREDOXIN 3*
At2g24420	*ATPrel*	*DNA repair ATPase-related*
At4g39730	*PLAT1*	*Lipase/lipooxygenase, PLAT/LH2 family protein*
At3g63410	*VTE3*	*VITAMIN E DEFECTIVE 3*
At1g48130	*PER1*	*1-CYSTEINE PEROXIREDOXIN 1*
At1g56070	*LOS1*	*LOW EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1*

Candidate genes were first cloned and their ability to physically interact with DOG1 *in vivo* was confirmed in a yeast two-hybrid assay. Firstly, successful delivery of both constructs into the yeast cells was evaluated by plating them on SD -Leu/-Trp medium, in which all of them grew. Auto-activation tests for each construct were conducted and no construct showed ability to auto-activate the system. After this, the interactions were evaluated in SD -Leu/-Trp/-His and it was observed that all tested candidates could interact with DOG1 *in vivo* (**Figure 9**), as indicated by growth of yeast cells in the selective medium. All interactions were tested in both directions, fusing all genes to both the activation domain (pACT2 vector) and the DNA-binding domain (pAS2 vector). All interactions were further evaluated in plates including 2 and 5 mM of 3-AT, as an indicator for the strength of the interaction (**Figure 9**).

From these results, it can be concluded that all tested candidates can interact *in vivo* with DOG1, confirming the results from the pull-down assay. CLO1, PLAT1 and

VTE3 showed the strongest interactions, as they were able to grow up to 5 mM of 3-AT. Surprisingly, when LOS1 is fused to the binding domain instead, its interaction with DOG1 is also maintained up to 5 mM of 3-AT. Interestingly, TRX3 only could interact with DOG1 when it was fused to the binding domain (**Figure 9**).

After confirming the interactions, T-DNA insertion lines for these genes were ordered (**Figure 10**). Homozygous T-DNA insertion mutants were isolated for most of the candidates, with the exception of *CLO1* and *VTE3*. Only one T-DNA insertion line was available for *CLO1* (GK_823D08) and neither homozygous nor heterozygous lines could be identified. For *VTE3*, a previous report showed that the homozygous T-DNA insertion line used (SALK_105903) was soil lethal and does not reach the adult phase (Cheng *et al.*, 2003). A second allele was also described in the same study, but was no longer available.

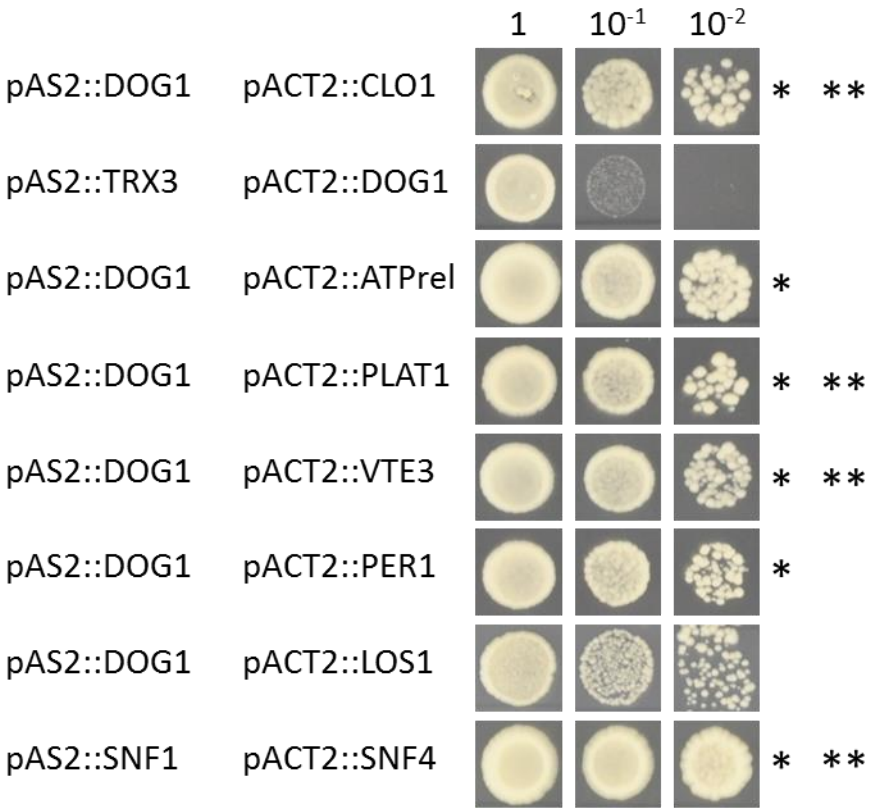

Figure 9. Interactions between DOG1 and the selected candidate proteins in a yeast two-hybrid assay. Yeast cultures co-expressing the indicated protein combinations were plated as 1:1, 1:10 and 1:100 dilutions from left to right on SD –LWH selective media. All pictures correspond to medium without 3-AT. Growth of yeast cells indicates protein-protein interaction. * and ** indicate that the interaction is maintained at 2 and 5 mM 3-AT, respectively. The interaction between SNF1 and SNF4 was used as a positive control.

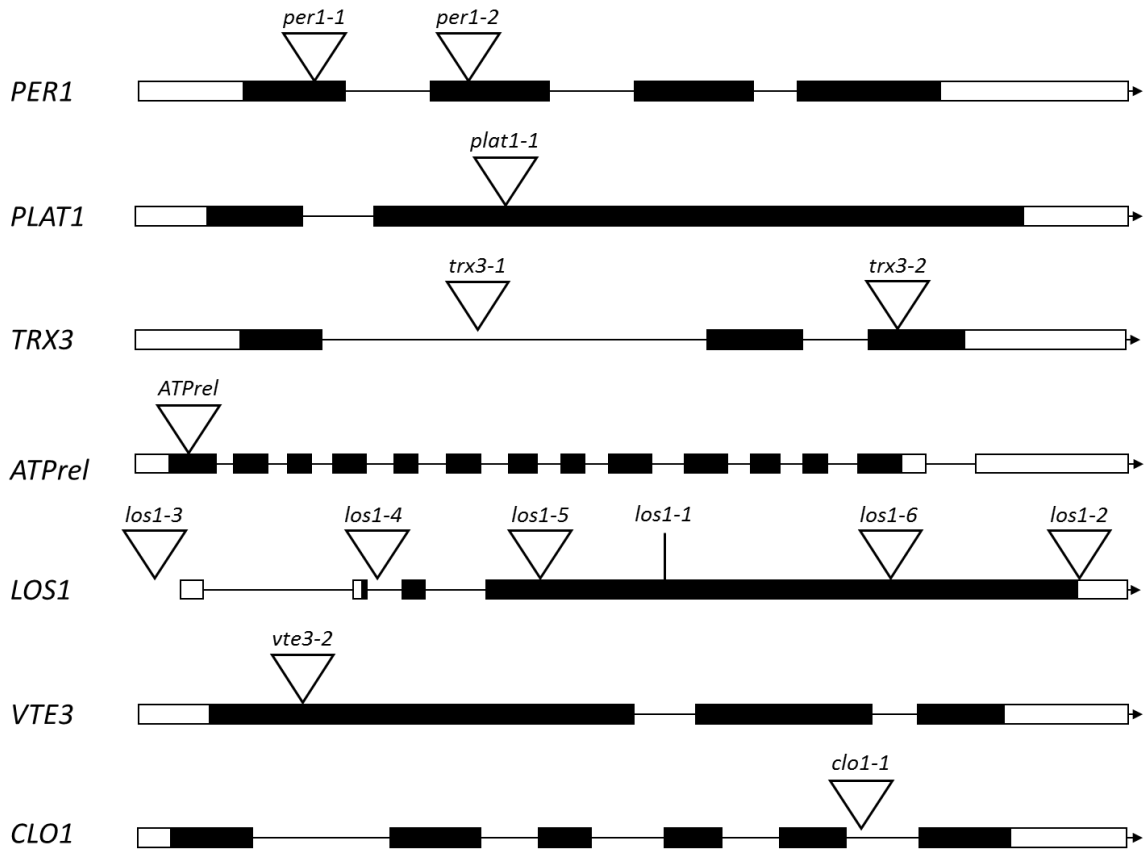

Figure 10. Schematic representation of the gene structure of selected candidates and the relative position of T-DNA insertion lines used. White boxes represent the untranslated regions (UTR). Black boxes represent exons and the lines connecting them correspond to introns. White triangles represent the T-DNA insertion line and the name given to the allele. *los1-1* carries a single amino acid mutation (Guo *et al.*, 2002). All genes are represented in 5'-3' orientation, with an arrowhead at the 3'-end.

All isolated homozygous T-DNA lines were evaluated for their seed longevity phenotype. **Figure 11A** shows that no significant differences were found between these lines and the wild type control. Several available alleles of *los1* were evaluated, but they did not exhibit significant differences in seed longevity (**Figure 11B**). *los1-2* carries an insertion at the 3'UTR region and *los1-3* carries the T-DNA insertion at the promoter region (**Figure 10**) but expression of the gene was unaffected when evaluated by qPCR (**Supplemental Figure 3**).

Evaluation of seed longevity on the progeny of heterozygous lines of *vte3-2* showed that this trait is affected, although this also included one third of wild type seeds as well as some lethal *vte3* homozygotes (**Figure 12A**). As homozygous lines for this insertion mutant were not viable, transgenic lines overexpressing the *VTE3* coding sequence under the control of a 35S promoter were constructed and their seed longevity phenotype evaluated. Interestingly, as depicted in **Figure 12B**,

overexpression of *VTE3* in the wild type causes a reduction of seed longevity. These transgenic lines were further analysed and qPCR confirmed that they were overexpressing *VTE3*, contrary to *vte3-2*, which showed reduced expression (**Figure 12C**).

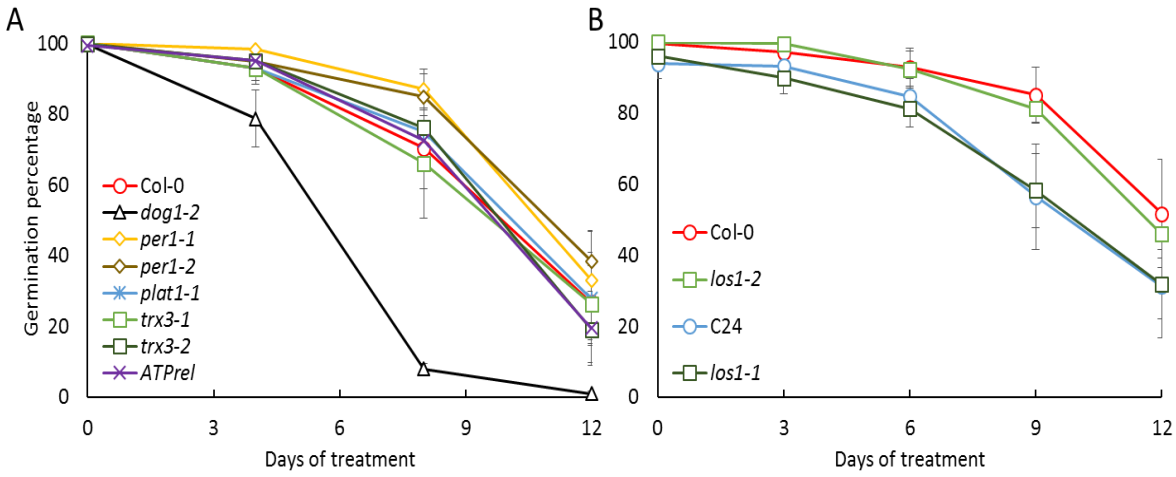

Figure 11. Seed longevity phenotypes of selected DOG1-interacting-protein insertion mutants. Germination after different periods of accelerated ageing (days of treatment) of T-DNA insertion lines of candidate genes. In panel B, *los1-1* is in C24 background and *los1-2* in Col-0. Data points represent the mean of three (A) or five (B) biological replicates. Error bars represent standard deviation.

To determine the possible cause of the reduced longevity in these overexpressing lines, germination in the presence of ABA was evaluated. ABA regulates many abiotic responses related to oxidative stress, and germination sensitivity to ABA can indicate altered responses or perception. Germination assays using increasing concentrations of ABA showed no differences in germination behaviour between transgenic lines and the wild type (**Figure 12D**). Permeability of the seed coat is another possible cause of reduced seed longevity, but these lines showed no differential staining with tetrazolium chloride when compared with the wild type (**Figure 13**).

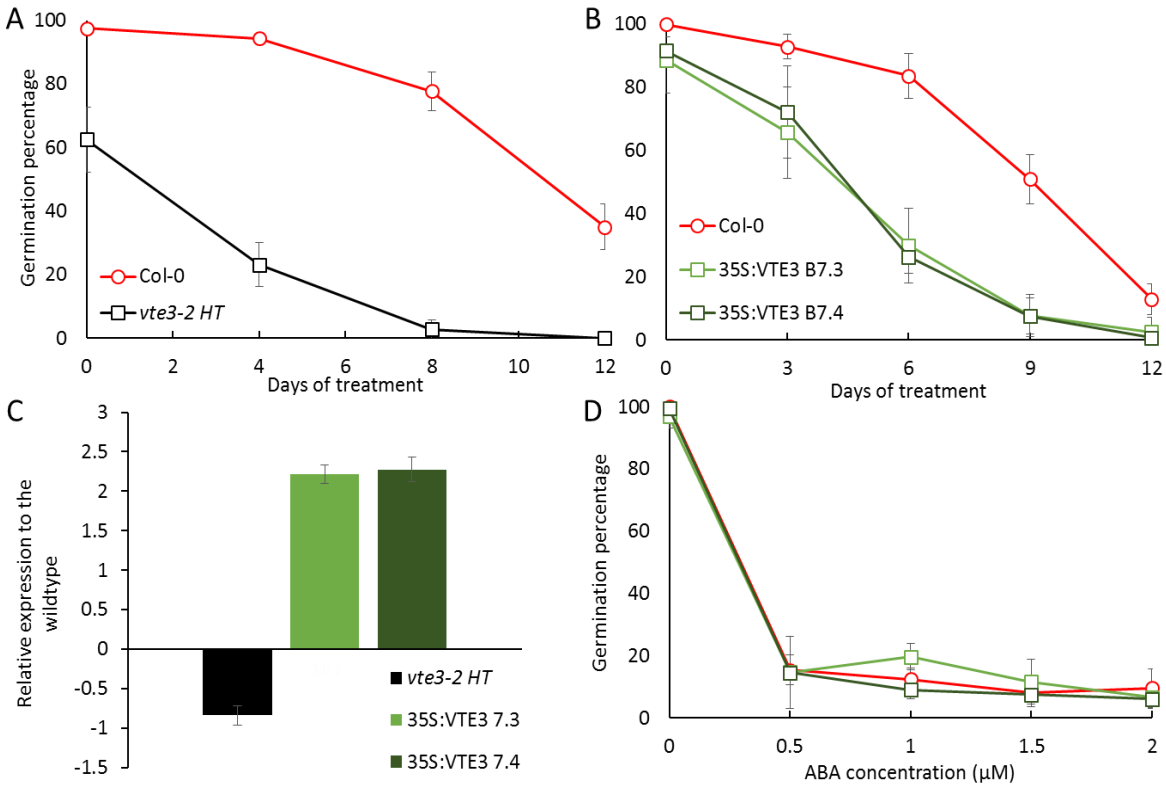

Figure 12. *VTE3* expression affects seed longevity. Panel **A** shows the germination after accelerated ageing of heterozygous *vte3-2* mutants. Panel **B** shows germination after accelerated ageing of two independent transgenic lines overexpressing *VTE3*. Panel **C** shows the relative expression in dry seeds of *VTE3* in the *vte3-2* mutant and two lines overexpressing it compared to the wild type Col-0 (represented by the baseline 0). Values plotted correspond to the $\log_2$-fold-change compared to Col-0. Panel **D** shows the germination of these transgenic lines in increasing concentrations of ABA. Each data point corresponds to the average of three (A, C) or four biological replicates (B and C). Error bars in panels correspond to standard deviation (A, B and D) or standard error of the mean (C).

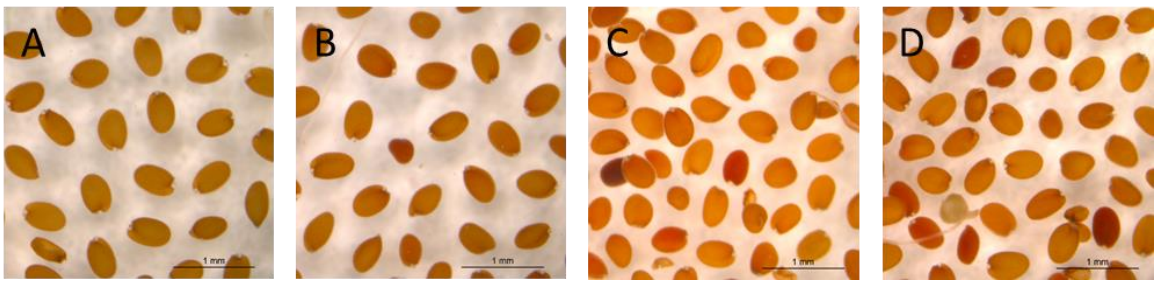

Figure 13. *VTE3* overexpression does not alter seed coat permeability. Representative pictures of seeds of Col-0 and two transgenic lines overexpressing *VTE3*. Panel **A** shows seeds of Col-0 imbibed in water for 48 h in the dark. Panels **B-D** show seeds from the control Col-0 (B) and two independent transgenic lines expressing the 35S:*VTE3* construct (C and D) after 48 h of tetrazolium staining. Scale bars correspond to 1 mm.

3.2.2 The role of the flowering repressors *FRI* and *FLC* in the regulation of seed longevity

Previous work exploiting the existing natural variation in Arabidopsis combined with the use of mutants with very poor seed longevity identified several seed longevity modifiers (Sugliani *et al.*, 2009). Specifically, three independent modifiers introgressed from the Shahdara accession contributed to enhance seed longevity in the *lec1-3* mutant. From these three modifiers, one of them named *lec*/Sha1 and located at the top of chromosome 4 conferred the strongest increase in seed longevity. Further fine-mapping of this region narrowed it down to a segment of 18 kb containing four full-length genes (At4g00620, At4g00630, At4g00650 and At4g00651). Three of these genes did not show complementation of the longevity phenotype when individually cloned and transformed. The fourth one, *FRIGIDA* (*FRI*), showed varying results when seed longevity in these complementation lines was evaluated, a variation that may originate from the influence of this gene in the regulation of flowering time.

Together with germination, the transition to flowering is the most important life-history decision that plants adopt during their life cycle. Similar to the former, it is regulated by a broad range of factors that fine-tune this decision and time it according to different environmental cues and genetic determinants (Amasino, 2010). Proper timing of flowering is of utmost importance, as it will determine in which way a plant will commit its resources for reproductive success. *FLOWERING LOCUS C* (*FLC*) is a MADS-box transcription factor that acts as a repressor of the floral transition in Arabidopsis (Michaels and Amasino, 1999) and whose action is repressed by exposing the vegetative plant to a long period of cold, a process termed vernalisation (Sheldon *et al.*, 2000). *FRIGIDA* (*FRI*) is an enhancer of *FLC* expression which does so by acting as a scaffolding protein in a transcriptional activator complex that mediates chromatin modification of the *FLC* locus (Johanson *et al.*, 2000; Choi *et al.*, 2011).

The role of the floral regulator *FRI* on seed traits was evaluated. To this end, a first approach was conducted making use of genetic material carrying functional and non-functional alleles of this gene. Specifically, lines of *flc-3*, *flc-3 FRI*$_{Sf2}$ and Col-0 were evaluated. The *flc-3* mutant, in Col-0 background, was produced using fast-neutron mutagenesis and carries a 104 bp deletion at the 5'UTR region of the *FLC* gene that removes the start codon (Michaels and Amasino, 1999). It was used because it lacks a functional *FRI* allele (*FRI* is non-functional in the Col-0 background) and to avoid any possible interference of flowering time regulation. The line *flc-3 FRI*$_{Sf2}$ is the same mutant but carrying an introgressed functional *FRI* allele from the San Feliu

accession (Michaels and Amasino, 1999). The wild type Col-0 was included as an additional control. It carries a non-functional *FRI* allele due to a premature stop codon (Johanson *et al.*, 2000), besides a strong, functional *FLC* allele. Evaluation of the phenotypes from this material showed no differences either for seed dormancy or longevity (**Figure 14**). These results prompted the idea that it might be necessary that both *FRI* and *FLC* have to be present in order to affect seed longevity. This raised the issue of the role of these two genes as repressors of flowering, as they strongly delay flowering in Arabidopsis (Michaels and Amasino, 2001). The problem is that even when conducting staggered seed sowing to achieve simultaneous seed set and harvesting, the age of the plant and physiological status would not be equivalent between lines with strong differences in flowering time, which might cause distortions when evaluating seed longevity.

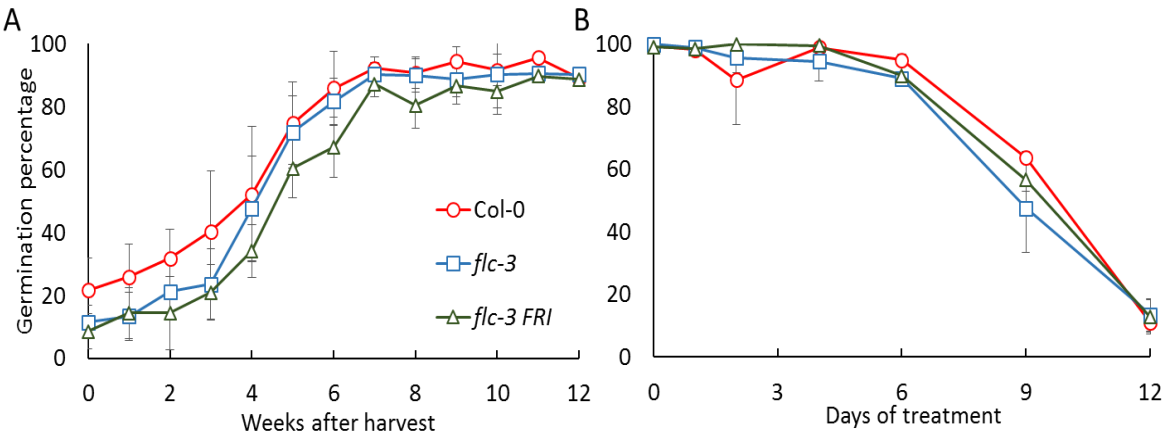

Figure 14. Different combinations of *FRI* alleles do not affect seed dormancy and sensitivity to artificial ageing. Germination proportion after different periods of dry storage (**A**) and after different lengths of accelerated seed ageing treatment (**B**) of Col-0 (circles, red line), *flc-3* (squares, blue line) and *flc-3 FRI*$_{Sf2}$ (triangles, green line). Data points represent the mean of six (A) or three (B) biological replicates. Error bars represent standard deviation.

An experimental approach using transgenic lines expressing either *FRI* or *FLC* under the control of a seed specific promoter was chosen, considering these constructs were not expected to influence flowering time. The promoter of *DOG1* was shown to be seed specific (Bentsink *et al.*, 2006; Nakabayashi *et al.*, 2012). As this gene displays a range of natural variation, causing different levels of seed dormancy between accessions, the promoter from the Cvi accession was used, considering *DOG1* expression in this specific background is strong, as reflected by its deep dormancy levels (Alonso-Blanco *et al.*, 2003). The *FRI* allele from Sha was used, as it was included in the *lec*/Sha1 modifier conferring enhanced seed longevity to the *lec1-3* mutant (Sugliani *et al.*, 2009). Moreover, Sha was reported to carry a functional *FRI* allele, contrary to the one in Col-0 (Johanson *et al.*, 2000). This study also reported

that Col-0 carries a strong *FLC* allele, which was the one used for this work. Only the coding sequence of these genes was used, as the first intron of *FLC* is known to be under a strong regulation (Crevillén and Dean, 2011; Berry and Dean, 2015). Both constructs were transformed in the *flc-3* background, which lacks both *FRI* and *FLC* functional alleles (Johanson *et al.*, 2000). After transformation, plants were selected based on marker segregation until homozygous T_3 transgenic plants for both constructs were isolated.

Four independent transgenic lines expressing the pDOG1$_{Cvi}$:*FLC*$_{Col}$ construct were isolated, named A5.9, C5.9, D2.6 and G1.1. These lines were first evaluated for their seed phenotypes. It was observed that all of them released dormancy faster than the *flc-3* control (**Figure 15A**). In addition, they all showed increased sensitivity to artificial ageing (**Figure 15B**), although line C5.9 had an intermediate phenotype.

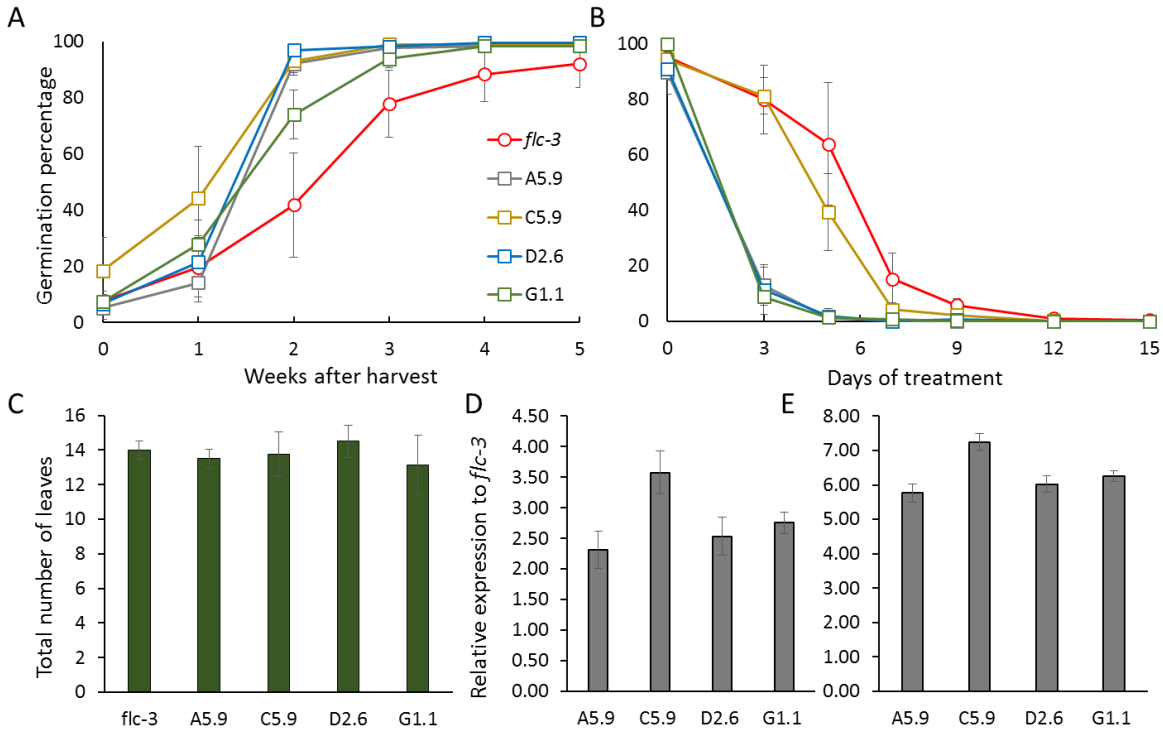

Figure 15. Phenotypic characterisation of transgenic lines expressing the pDOG1$_{Cvi}$:*FLC*$_{Col}$ construct. Germination proportion after different periods of dry storage (**A**) and after different lengths of accelerated seed ageing treatment (**B**) of four independent transgenic lines and their control *flc-3*. Panel **C** shows the flowering time of transgenic lines represented as the total number of leaves when the floral bud appeared. Panels **D** and **E** show relative expression of *FLC* in dry seeds and leaves, respectively, of these transgenic lines compared to the *flc-3* background (represented as the baseline 0). Values plotted correspond to the $\log_2$-fold-change compared to *flc-3*. Data points represent the mean value of six (A), eight (B, C) or three (D, E) biological replicates. Error bars represent standard deviation (A-C) or standard error of the mean (D, E).

To further confirm that the construct was not affecting the flowering time of these transgenic lines, this trait was evaluated (**Figure 15C**) and no differences between transgenic lines and the control were observed. Next, the expression of *FLC* in these lines was assessed. All four lines showed enhanced expression of this gene in dry seeds in comparison to the mutant background *flc-3* (**Figure 15D**). Furthermore, expression of *FLC* was evaluated in leaves of these lines. Expression of the construct was expected to be seed-specific but strikingly, levels of *FLC* expression in rosette leaves were even higher than those of dry seeds (**Figure 15E**). Based on the reduced seed dormancy levels and increased sensitivity to artificial ageing, the germination behaviour of the transgenic lines in the presence of increasing concentrations of ABA was evaluated, as a proxy of altered ABA perception or signalling. However, none of the lines differed significantly from the control (**Supplemental Figure 4**).

Confirmation that the pDOG$_{Cvi}$:*FLC*$_{Col}$ construct caused leaky expression of the gene in leaves complicated the evaluation of its specific role in seeds, as the construct did not drive *FLC* expression as anticipated. Nevertheless, all transgenic lines displayed similar trends regarding dormancy release and sensitivity to artificial ageing, besides showing no differences in flowering time (**Figure 15**) or sensitivity to ABA (**Supplemental Figure 4**). This reflects that, although not only restricted to seeds, ectopic expression of *FLC* was achieved and caused phenotypes not described before. Based on these observations, the expression of several targets of *FLC* was evaluated in the transgenic lines. These candidates were chosen based on literature (Chiang *et al.*, 2009; Deng *et al.*, 2011) and seed-specific co-expression networks (Bassel *et al.*, 2011) and are listed in **Table 10**.

Table 10. Candidate *FLC* targets evaluated for altered expression in transgenic lines expressing pDOG1$_{Cvi}$:*FLC*$_{Col}$. The effect of *FLC* regulation on the target gene expression is indicated, when known, with + for upregulation and – for downregulation.

Locus	Symbol	Description	Regulation
At3g15500	*ANAC055*	*NAC DOMAIN CONTAINING PROTEIN 55*	
At1g75390	*bZIP44*	*BASIC LEUCINE-ZIPPER 44*	
At3g56660	*bZIP49*	*BASIC LEUCINE-ZIPPER 49*	
At4g36910	*CDCP2*	*CYSTATHIONE [BETA]-SYNTHASE DOMAIN-CONTAINING PROTEIN 2*	
At4g11140	*CRF1*	*CYTOKININ RESPONSE FACTOR 1*	

At2g29090	*CYP707A2*	*CYTOCHROME P450, FAMILY 707, SUBFAMILY A, POLYPEPTIDE 2*	+
At4g25480	*DREB1A*	*DEHYDRATION RESPONSE ELEMENT B1A*	+
At4g25490	*DREB1B*	*DEHYDRATION RESPONSE ELEMENT B1B*	+
At1g65480	*FT*	*FLOWERING LOCUS T*	-
At4g25420	*GA20OX1*	*GIBBERELLIN 20-OXIDASE 1*	+
At4g22920	*NYE1*	*NON-YELLOWING 1*	
At3g54990	*SMZ*	*SCHLAFMÜTZE*	+
At2g45660	*SOC1*	*SUPPRESSOR OF OVEREXPRESSION OF CO 1*	-
At3g57920	*SPL15*	*SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 15*	-

Although the expression of some of these genes is expected to be very low or absent in dry seeds, all genes were evaluated as they are known targets of *FLC*. Out of these 14 genes, expression in dry seeds could not be detected for *SOC1* and *SPL15*. Among the rest, different expression patterns were observed. Firstly, several of these candidates displayed reduced expression in dry seeds of all transgenic lines compared to *flc-3* (**Figure 16**). Specifically, expression of the flowering promoter *FT* was found downregulated in all four transgenic lines. Similarly, expression of *CDCP2*, a regulator of the cellular redox balance appeared downregulated, as well as that of *NYE1*, a gene involved in the degradation of chlorophyll in senescing leaves. *CRF1*, which is involved in cytokinin signalling, showed reduced expression in three of the lines (**Figure 16D**). The rest of evaluated genes did not show a consistent expression between transgenic lines (**Supplemental Figure 5**).

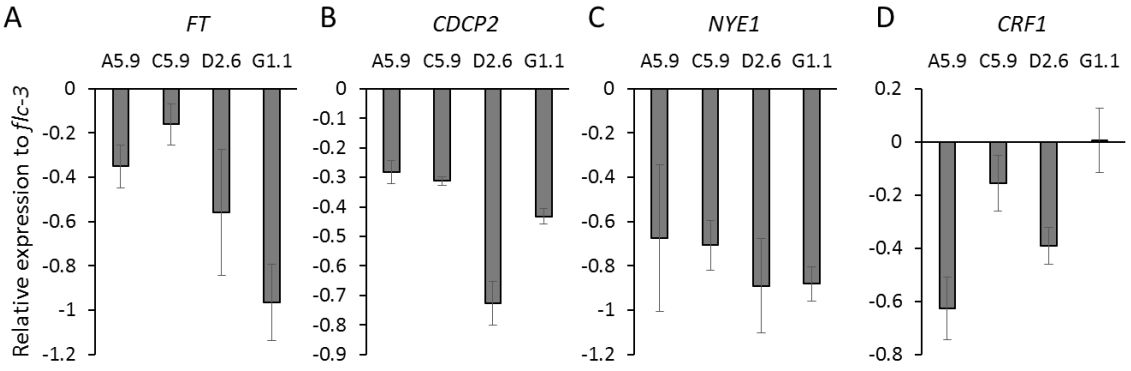

Figure 16. Genes with reduced expression in transgenic lines expressing the pDOG1$_{Cvi}$:*FLC*$_{Col}$ construct. Relative expression of *FT* (**A**), *CDCP2* (**B**), *NYE1* (**C**) and *CRF1* (**D**) in dry seeds of these transgenic lines compared to the *flc-3* background (represented as the

baseline 0). Values plotted correspond to the log_2-fold-change compared to *flc-3*. Data points represent the mean value of at least two biological replicates. Error bars represent standard error of the mean.

FT is a main regulator of flowering in Arabidopsis and its expression is partially repressed by *FLC* (Searle *et al.*, 2006). Besides, it is strongly expressed during the first stages of embryogenesis, where it has been shown to integrate temperature cues and alter seed coat composition, which is later reflected by seed dormancy depth (Chen *et al.*, 2014b). This work showed that *FT* contribution to flowering time and establishment of seed dormancy could be uncoupled and showed a maternal effect, in agreement with it affecting the seed coat. This scenario would also fit with the transgenic lines described, which have enhanced levels of *FLC*, which would in turn reduce *FT* expression. Modifications of the seed coat could then be linked to the observed reduction in seed dormancy and longevity.

CDCP2 was shown to participate in activation of thioredoxins (TRXs) and its overexpression caused deficient lignin deposition in pollen of Arabidopsis due to a lack of available H_2O_2 (Yoo *et al.*, 2011). It is then plausible that the opposite situation happened in transgenic lines expressing the $pDOG_{Cvi}$:FLC_{Col} construct. As the expression of *FLC* reduced expression of *CDCP2*, an increased abundance of ROS could be expected that led to the observed reduction in seed dormancy and longevity.

NYE1 has been linked to chlorophyll degradation in senescing leaves (Ren *et al.*, 2007). It was shown before that chlorophyll degradation is necessary for the acquisition of seed longevity, as seeds retaining chlorophyll display reduced longevity (Ooms *et al.*, 1993; Nakajima *et al.*, 2012). This gene was also linked to the production of tocopherols in maturing seeds of Arabidopsis, where the *nye1* mutant was shown to have reduced levels of these compounds, a phenotype fitting with the observed reduction in resistance to artificial ageing.

CRF1 is a member of a transcription factor family involved in cytokinin signalling and responses (Raines *et al.*, 2016). Single mutants of these genes showed no phenotypic differences, which points to a certain degree of functional overlap among them. In this work, the authors showed that some of them participate in the regulation of leaf senescence.

However, these results should be addressed with caution. Firstly, the construct did not confine *FLC* expression to seeds and therefore it might be exerting its regulation in different organs. Secondly, the qPCR results showed that the reduction of expression is small and might not relate to the observed phenotypes.

To determine if *FRI* is affecting seed longevity, five independent transgenic lines, named B2.2.2, B2.6.3, C3.8.1, D1.1.1 and F4.4.1, expressing the pDOG1$_{Cvi}$:*FRI*$_{Sha}$ construct in the *flc-3* mutant background were isolated and their phenotypes evaluated. Seed dormancy release of these transgenic lines showed an uncommon pattern (**Figure 17A**). Although a general trend of dormancy release was observed, two of them exhibited reduced dormancy levels right after harvest (lines D1.1.1 and F4.4.1), but their germination rates did not reach 100 % during the after-ripening process (**Figure 17A**). The control *flc-3* showed peaks in its germination rate during dormancy release, which might be caused by the conditions at the after-ripening chamber being not stable during the entire storage period or that conditions at the germination chamber were not constant during the experiments.

To ensure a complete release of seed dormancy, seeds were stratified for three days after the artificial ageing treatment. None of the five independent transgenic lines showed differences compared to *flc-3*, similar to the observed number of leaves at flowering (**Figure 17**). Evaluation of the expression of *FRI* in dry seeds of these transgenic lines showed no consistent expression of *FRI* between lines, with most of them showing decreased expression in contrast with the expected enhanced expression (**Figure 17D**). Inconsistent expression patterns were also observed in leaves of these lines (**Figure 17E**). No conclusion on the role of *FRI* in seed longevity could be drawn from the results presented in **Figure 17**, considering no phenotypic differences were observed after accelerated ageing (**Figure 17B**) and that irregular expression patterns of the gene were found between transgenic lines. Most certainly, this points to an unsuccessful assembly of the construct or the subsequent delivery, even though the selectable marker did segregate as expected and sequencing of the construct matched the cloned sequence.

Based on these results it cannot be concluded whether *FRI* is responsible for the enhanced longevity phenotype observed in the *lec*/Sha1 modifier lines (Sugliani *et al.*, 2009). A second revision of this work revealed an additional mistake. The *FRI*$_{Sha}$ sequence was retrieved from the Arabidopsis 1001 genomes project website (http://signal.salk.edu/atg1001/3.0/gebrowser.php) and primers were designed based on it. This database uses Col-0 as the background reference, which caused the mistake, considering the *FRI* allele from Col-0 carries a 16 bp deletion that causes an early stop codon (Johanson *et al.*, 2000; Schmalenbach *et al.*, 2014). This deletion is not included in the gene sequence in this database. For this reason, the primers designed covered the CDS corresponding to the Col-0 allelic version, and not the functional one from Sha. Therefore, this experiment was not properly designed and cannot conclude about the role of the *FRI* allele from Sha in seed longevity.

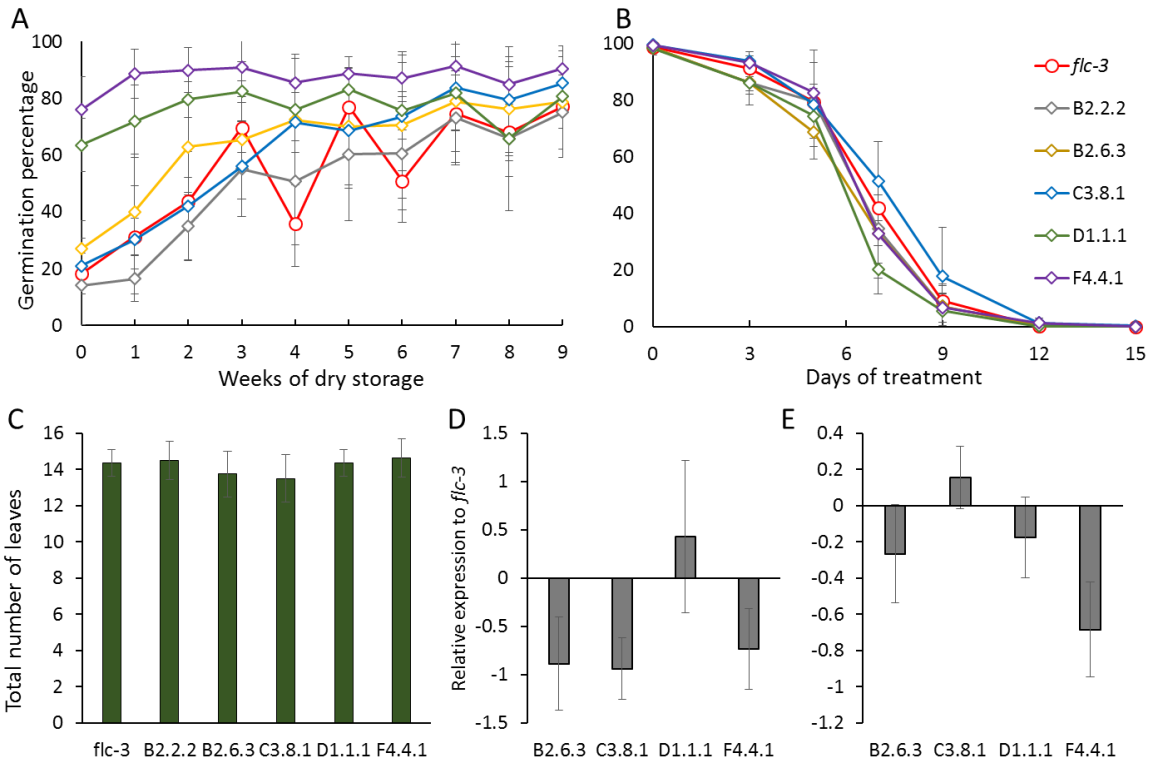

Figure 17. Phenotypic characterisation of transgenic lines expressing the pDOG1$_{Cvi}$:*FRI*$_{Sha}$ construct. Germination proportion after different periods of dry storage (**A**) and after different lengths of accelerated seed ageing treatment (**B**) of five independent transgenic lines and their control *flc-3*. Panel **C** shows the flowering time of transgenic lines represented as the total number of leaves when the floral bud appeared. Panels **D** and **E** show relative expression of *FRI* in dry seeds and leaves, respectively, of these transgenic lines compared to the *flc-3* background (represented as the baseline 0). Values plotted correspond to the $\log_2$-fold-change compared to *flc-3*. Data points represent the mean value of six (A), eight (B, C) or three (D, E) biological replicates. Error bars represent standard deviation (A-C) or standard error of the mean (D, E).

3.3 IDENTIFICATION OF NOVEL SEED QUALITY MARKERS IN ARABIDOPSIS

Overall seed quality results from the combination of several factors, such as genetic purity, germination capacity and uniformity, vigour, storability and performance under suboptimal conditions (McDonald, 1998). As seeds deteriorate during storage and lose vigour, different methods have been proposed to determine the quality level of a given seed batch (Corbineau, 2012). In this section, two distinct approaches to evaluate seed quality are presented.

3.3.1 The influence of temperature during seed development and of artificial ageing on nuclear size and chromatin compaction in embryos of Arabidopsis

Chromatin is a conglomerate of DNA, RNA and proteins found within the cell nucleus and named after its staining properties. Chromatin is classically divided into eu- and heterochromatin, mainly depending on the degree of staining. In Arabidopsis nuclei, heterochromatin is organised in visible chromocenters (Fransz *et al.*, 2002), which comprise highly condensed and repeat-rich heterochromatin. Previous studies have shown that Arabidopsis dry seeds exhibit reduced nuclear size and increased chromatin compaction (van Zanten *et al.*, 2011), probably due to their reduced moisture content (van Zanten *et al.*, 2012). Abiotic stresses, including heat, have been reported to cause decondensation of certain chromatic regions in rice (Santos *et al.*, 2011) and prolonged heat stresses also led to decondensation of chromocenters in leaves of Arabidopsis (Pecinka *et al.*, 2010).

The effect of different temperatures applied during seed maturation on nuclear size and degree of chromatin compaction was evaluated to assess the suitability of these phenotypes as predictors of seed quality. In addition, the influence of accelerated ageing and imbibition on these nuclear phenotypes was measured. Nuclei were isolated from Arabidopsis embryos, for which either dry (referred to as DS) or imbibed (72 h at 10 °C, referred to as imb) seeds were used that matured under low (16/14 °C), control (22/18 °C) or elevated (28/25 °C) temperatures. In addition, the effect of artificial ageing (4 days at 40 °C and 75 % RH) in these same seed batches was investigated. The area of the spread nuclei was measured as an indicator for nuclear size.

Evaluating the degree of chromatin compaction requires a clear distinction of brightly stained heterochromatic chromocenters from their darker euchromatic background, measured as the difference in DAPI-fluorescence intensity (Soppe *et al.*,

2002). However, the highly compacted nuclei of Arabidopsis dry seeds did not show distinct differences between heterochromatic chromocenters and euchromatin (**Figure 18**) and hence, a reliable measurement of the heterochromatin fraction was not feasible.

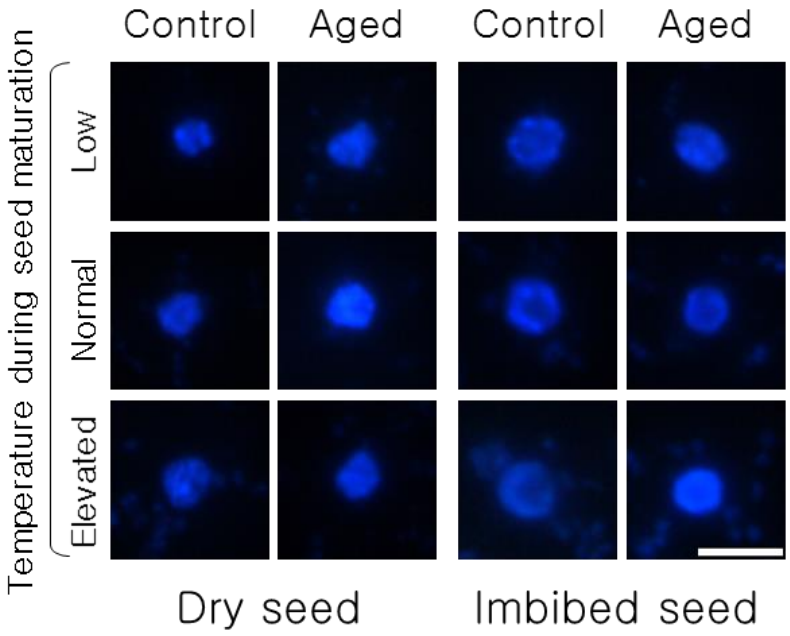

Figure 18. Representative nuclei from Arabidopsis embryos. Nuclei dissected from stress-treated Arabidopsis seeds. The lighter spots within the nuclei correspond to the chromocenters. The white scale bar at the bottom right panel corresponds to 1 μm.

Comparison of nuclear surfaces between different treatments revealed that the main factor affecting nuclear size is imbibition (**Figure 19**), in agreement with previous reports (van Zanten *et al.*, 2011). The combined effect of imbibition with either low or elevated maturation temperatures caused significant differences in size, in contrast with the mild effects observed for control samples. This suggests that the nuclei of seeds matured at low and high temperatures increase faster in size during imbibition compared to seeds matured at control temperatures. Conversely, artificial ageing treatment exerted a buffering effect on nuclear size increase following imbibition.

To evaluate the statistical significance of each treatment's contribution to nuclear size, a factorial ANOVA was conducted (**Table 11**). Interestingly, each stress treatment analysed showed a significant contribution, including several of the interactions between factors. However, it is more informative to evaluate the overall contribution of each factor to explain the observed variability. One way to do this is

by evaluating the eta-squared (η^2) value, which provides an indication of the proportion of variance explained by each factor (Richardson, 2011). **Table 11** shows that, as observed in **Figure 19**, imbibition explains most of the observed changes in nuclear sizes. However, its contribution is still small, accounting for only 7.8 % of the variance. Interestingly, temperature and artificial ageing also appear as significant factors, but their total contribution is below 2 %.

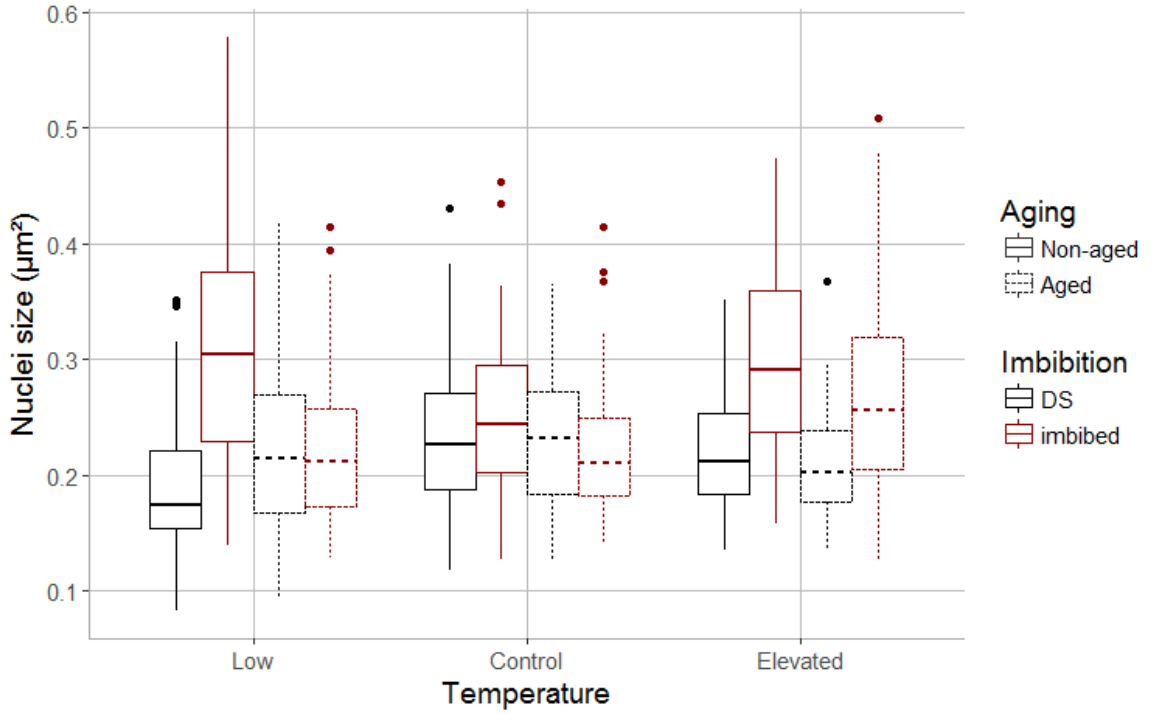

Figure 19. Temperature differences during seed maturation do not influence nuclear size. Nuclear sizes of measured Arabidopsis spread nuclei. The three temperatures applied are shown (low, 16/14 °C; control, 22/18 °C; or elevated, 28/25 °C) and the imbibition status is displayed (dry seed, DS, black boxes; imbibed seed, red boxes), as well as the ageing treatment (non-aged, continuous lines; aged, dotted lines). The horizontal line in the boxes represents the median. Whiskers represent 1.5 times the interquartile range. Values outside this range are depicted as dots.

The only non-significant factor is the interaction between temperature and ageing, with the rest of interactions being significant and in some cases having a higher η^2 score than temperature or ageing alone. Taken together, these results indicated that, although the studied stresses contribute to the overall observed variance, there are still other factors which account for 81 % of the observed variance.

Table 12 shows pairwise comparisons of all treatments after a post-hoc Tukey's test and those comparisons that were found to be statistically significant. An analysis of the effects of the three different temperature regimes during seed maturation on nuclear size revealed several significant effects (**Table 12**), but did not result in a clear,

consistent pattern related to temperature. Dry, non-aged seeds that had matured at low temperatures had smaller nuclei compared to seeds matured at standard temperatures. In contrast, imbibed seeds matured at low temperatures had larger nuclei compared to the control temperature regime (**Figure 19**).

Table 11. Summary statistics of factorial ANOVA test on nuclear size differences. Underlined values correspond to those found to be statistically significant. df: degrees of freedom.

Factor	Sum Sq.	df	Mean Sq.	F	Signif.	η^2
Temperature	0.141317	2	0.070659	4.950195	7.27E-03	0.008656
Imbibition	1.281746	1	1.281746	89.79652	0.00E+00	0.078513
Ageing	0.254806	1	0.254806	17.8512	2.62E-05	0.015608
Temperature x Imbibition	0.461546	2	0.230773	16.16749	1.25E-07	0.028272
Temperature x Ageing	0.001657	2	0.000829	0.058051	9.44E-01	0.000102
Imbibition x Ageing	0.429048	1	0.429048	30.05823	5.40E-08	0.026281
Temperature x Imbibition x Ageing	0.358114	2	0.179057	12.54437	4.21E-06	0.021936
Residuals	13.33182	934	0.014274	NA	NA	0.816636

Overall, we observed that our model does not explain most of the observed variability, which means there are more factors contributing to the observed nuclear size phenotypes than those addressed in this study. Considering that the unexplained variability accounts for 81 % of the total, it cannot only be attributed to biological variation between samples, but it necessarily implies that extra sources of variation are present which were not covered by the experimental approach. These results, together with the concerns just mentioned, lead us to conclude that nuclear size phenotypes are not good predictors of seed quality. A more in-depth study will be required to characterise these extra factors affecting nuclear size and establish a more comprehensive model which could then be linked with seed quality.

Table 12. Pairwise comparisons of all treatments after a post-hoc Tukey's test. Underlined values correspond to statistically significant comparisons.

			Low				**Control**				**Elevated**			
			Non-aged		Aged		Non-aged		Aged		Non-aged		Aged	
			DS	imbibed	DS	imbibed	DS	imbibed	DS	imbibed	DS	imbibed	DS	imbibed
Elevated	Aged	imb	0	0.11642	0.00035	0.00465	0.23207	0.98735	0.11670	0.00314	0.00826	0.34815	1.69E-05	-
		DS	0.38727	0	0.99858	0.98993	0.42676	0.00767	0.84091	0.96127	0.99289	0	-	-
	Non-aged	imb	0	1	0	0	2.83E-05	0.01607	1.55E-05	0	0	-	-	-
		DS	0.01891	0	1	1	0.97815	0.18327	0.99985	1	-	-	-	-
Control	Aged	imb	0.00574	0	1	1	0.9942	0.25615	0.99999	-	-	-	-	-
		DS	0.00466	1.7E-06	0.99805	0.99996	1	0.82090	-	-	-	-	-	-
	Non-aged	imb	1E-07	0.00285	0.07764	0.25924	0.95785	-	-	-	-	-	-	-
		DS	0.00021	2.8E-06	0.91515	0.98948	-	-	-	-	-	-	-	-
Low	Aged	imb	0.01979	0	1	-	-	-	-	-	-	-	-	-
		DS	0.02681	0	-	-	-	-	-	-	-	-	-	-
	Non-aged	imb	0	-	-	-	-	-	-	-	-	-	-	-
		DS	-	-	-	-	-	-	-	-	-	-	-	-

3.3.2 Assessment of seed quality in Arabidopsis by Activity-Profiling of Proteases

Activity-Based Protein Profiling (ABPP) is a powerful biochemical approach that allows to monitor the functional status of proteins in a proteome extract or in living cells (Cravatt *et al.*, 2008). This technique relies on the use of small, reporter-tagged probes that specifically react with the active site of an enzyme in a mechanism-dependent manner (Cravatt *et al.*, 2008; Edgington *et al.*, 2011). Probes are designed to bind only certain enzyme subfamilies in their active state. During the ageing process, seeds endure oxidative stresses, which damage cell constituents and progressively reduce the seed capacity to germinate (**section 1.5**). Among those cellular components, proteins are also affected by these oxidative damage and rendered inactive by processes such as carbonylation (Rajjou *et al.*, 2008b). Considering the specificity of ABPP probes for active targets and the above-mentioned effects of ageing, it is plausible that these probes and their target proteins could be used to monitor seed quality.

Plant proteases are a wide group of enzymes involved in the degradation of non-functional proteins by cleaving specific peptide bonds. This processing also occurs as a response to environmental and developmental cues, which makes proteases interesting candidates in different studies (van der Hoorn and Kaiser, 2012). To determine the feasibility of ABPP as a novel approach for evaluating seed quality, several probes targeting different subsets of proteases in Arabidopsis were evaluated (**Table 13**).

Table 13. ABPP probes tested in this study. The probe target, pH value used for labelling, inhibitor used and reference are indicated.

Probe	Target	pH	Inhibitor	Reference
AMS101	Vacuolar Processing Enzymes	5.5	YVAD-CMK	Misas-Villamil *et al.* (2013)
MV151	26 S proteasome	7.5	Epoxomicin	Gu *et al.* (2010)
MV151	Papain-like cysteine proteases	6.0	E64	Gu *et al.* (2010)
FP-Rh	Serine proteases	7.5	DiFP	Kaschani *et al.* (2012)

ABPP assays were conducted using artificially aged seeds from the Arabidopsis accessions Col-0 and L*er* representing different levels of seed quality, as determined

in a germination assay (**Figure 20**). Evaluation of these four probes in dry seeds showed no changes in labelling between non-aged and aged seeds. Consequently, these seeds were imbibed for 24 hours and proteins were extracted in the corresponding buffer depending on the targeted enzyme (**Table 13**). Testing of the four probes showed that only two of them (AMS101 and FP-Rh) exhibited a differential band pattern in the differently aged seed batches after 24 h of imbibition.

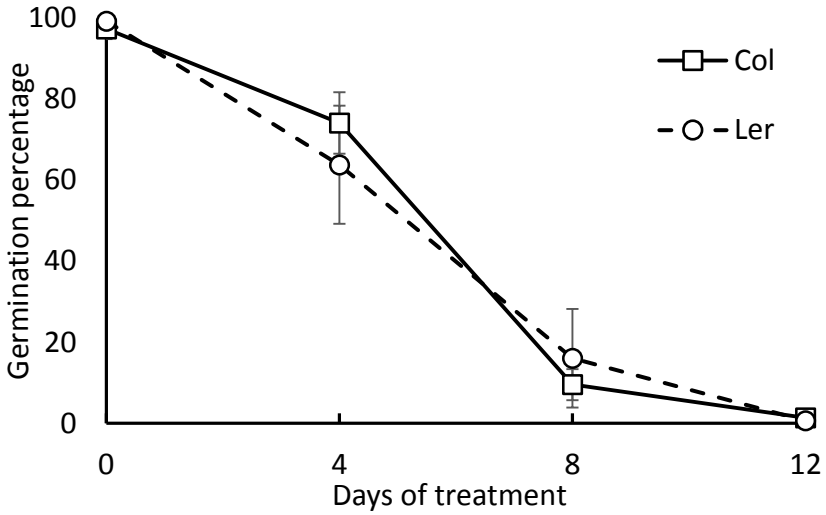

Figure 20. Germination proportion after different periods of accelerated seed ageing treatment of seed lots from L*er* and Col-0 accessions. Each data point represents the mean of three biological replicates. Error bars represent the standard deviation.

Fluorophosphanate (FP)-based probes bind the active site of serine hydrolases, which are hydrolytic enzymes involved in a wide range of physiological processes, including development, defence and homeostasis (Kaschani *et al.*, 2009). This group of enzymes comprises more than 200 members and their activity is tightly regulated by several mechanisms, such as post-translational modifications and the presence of cofactors or inhibitors. **Figure 21** illustrates that serine protease activities are gradually lost the longer the artificial ageing treatment is applied, in both Col and L*er*. Two high-molecular weight bands (> 70 kDa, **Figure 21**) virtually disappear after 8 days of treatment whereas lower bands that appear strong in non-aged samples become fainter after 8 and 12 days. This profile of decreased activity can be caused by the ageing treatment damaging these enzymes, thus rendering them inactive and unable to bind the probe.

Considering that FP probes have a wide range of action and can therefore bind several targets, a pull-down assay was conducted in order to identify the proteins underlying the observed band patterns. A combination of biotin-tagged FP probe and streptavidine beads (**section 2.2.30**) was used in protein extracts of non-aged

seeds to purify several bands (**Supplemental Figure 6**) which were submitted for MS analyses. The reason to use non-aged seeds was to maximise the number of bands isolated, as they become fainter during ageing (**Figure 21**). These analyses identified twenty candidate hydrolases enriched bound to the beads (**Table 14**).

Probe AMS101 specifically targets Arabidopsis Vacuolar Processing Enzymes (VPEs, Misas-Villamil et al., 2013), which are cysteine proteases responsible for the processing and maturation of seed storage proteins (Hara-Nishimura *et al.*, 1998; Gruis *et al.*, 2002). Four VPE-encoding genes (α, β, γ and δ) have been described in Arabidopsis, which are involved in plant immune responses (Misas-Villamil *et al.*, 2013), stress responses (Albertini *et al.*, 2014) and development (Kinoshita *et al.*, 1999; Nakaune *et al.*, 2005). Interestingly, although initially classified into seed and vegetative types, it was observed that all of them are expressed during seed maturation (Gruis *et al.*, 2004).

Table 14. List of candidate serine proteases identified by MS after pull-down assays. Analysis of the pull-down MS data was conducted by Dr Farnusch Kaschani at the Analytics Core Facility Essen (ACE), Germany.

Locus	Alias	Description
AT4G18970	-	GDSL esterase/lipase
AT4G20850	*TPP2*	Tripeptidyl-peptidase 2
AT2G23590	*MES8*	Methylesterase 8
AT2G23610	*MES3*	Methylesterase 3
AT1G09390	-	GDSL esterase/lipase
AT1G77440	*PBC2*	Proteasome subunit beta type-3-B
AT2G27920	*SCPL51*	Serine carboxypeptidase-like 51
AT5G45920	-	GDSL esterase/lipase
AT4G22300	-	Probable carboxylesterase SOBER1-like
AT4G12910	*SCPL20*	Serine carboxypeptidase-like 20
AT5G20060	-	Phospholipase/carboxylesterase
AT2G23620	*MES1*	Methylesterase 1
AT3G50440	*MES10*	Methylesterase 10
AT5G45670	-	GDSL esterase/lipase
AT1G49660	*CXE5*	Probable carboxylesterase 5
AT3G05180	-	GDSL esterase/lipase
AT3G48690	*CXE12*	Probable carboxylesterase 12
AT3G09405	*PAE4*	Pectin acetylesterase 4

AT3G09410	*PAE5*	Pectin acetylesterase 5
AT2G27360	-	GDSL esterase/lipase

Contrary to the observed band pattern found for serine hydrolases, VPEs increase their activity as a result of artificial ageing (**Figure 22**) in both L*er* and Col-0. The observed band pattern includes several bands at different sizes, which probably correspond to different maturation stages of the enzymes (Kuroyanagi *et al.*, 2002). In addition, the four VPEs in Arabidopsis have very similar molecular sizes, making it complicated to discriminate whether the increased activity is caused by one or more of them (Misas-Villamil *et al.*, 2013).

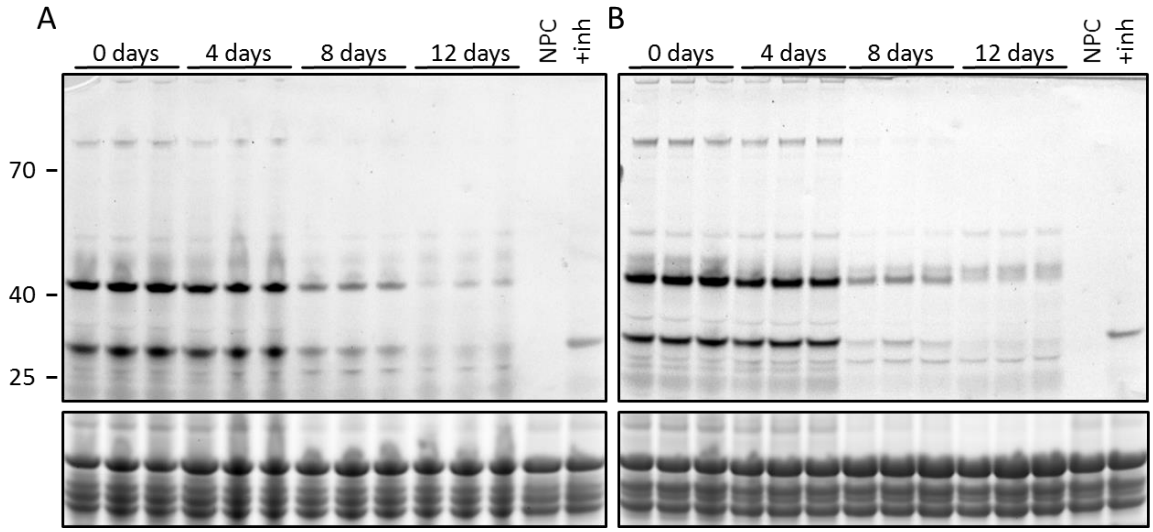

Figure 21. Serine hydrolases turn inactive during artificial ageing. Protein extracts from Arabidopsis Col (**A**) and L*er* (**B**) accessions labelled with FP-Rh. For each time point, three biological replicates are shown. The NPC column corresponds to a non-labelled sample (no-probe control) prepared combining aliquots of all proteomes. The inhibitor used was DiFP, corresponding to the last column (+inh). The bottom section in each panel corresponds to total protein loaded stained with SyproRuby Protein Gel stain.

To determine which VPE activity is enhanced during the treatment, seeds from different *vpe* mutant lines were evaluated (Gruis *et al.*, 2002). These mutants were first checked for any phenotype related to seed longevity, but their behaviour was not significantly different from that of the control (**Figure 23A**). Seeds from these mutant lines were then artificially aged for 8 days to reach a strong activation of VPEs (**Figure 22**), subsequently protein extracts were isolated and labelled. It was expected to discriminate which of the four Arabidopsis VPEs is behind the observed activation pattern. Nevertheless, a clear distinction based on the observed labelling could not be achieved (**Figure 23B**).

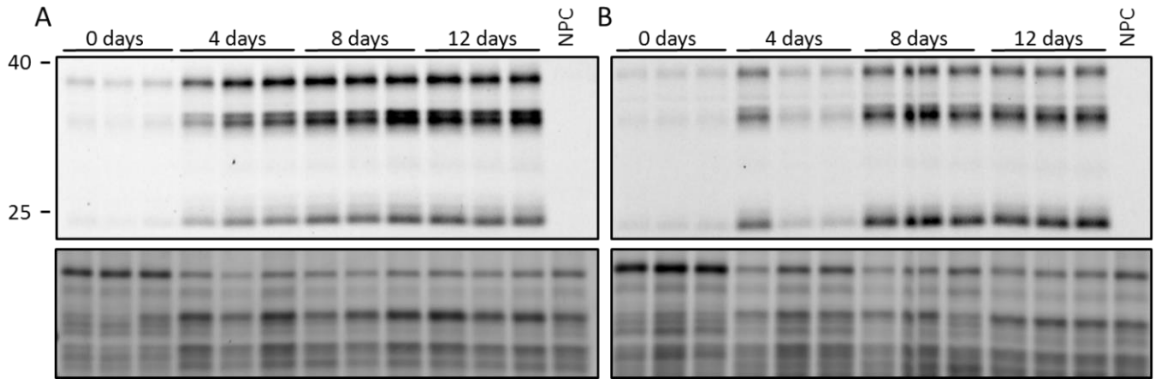

Figure 22. VPEs become active during artificial ageing. Protein extracts from Arabidopsis L*er* (**A**) and Col-0 (**B**) accessions labelled with AMS101. For each time point, three biological replicates are shown. The NPC column corresponds to a non-labelled sample (no-probe control) prepared combining aliquots of all proteomes. The bottom section in each panel corresponds to total protein loaded stained with SyproRuby Protein Gel stain.

A publication described that γ-VPE becomes active after heat stress (Li *et al.*, 2012) as part of the signalling pathway leading to programmed cell death. Specifically, this study described how heat treatments of 1 hour at 40 °C caused significant increases in γ-VPE activity. These conditions are quite similar to those used in this study for the artificial ageing of seeds (several days at 37 °C).

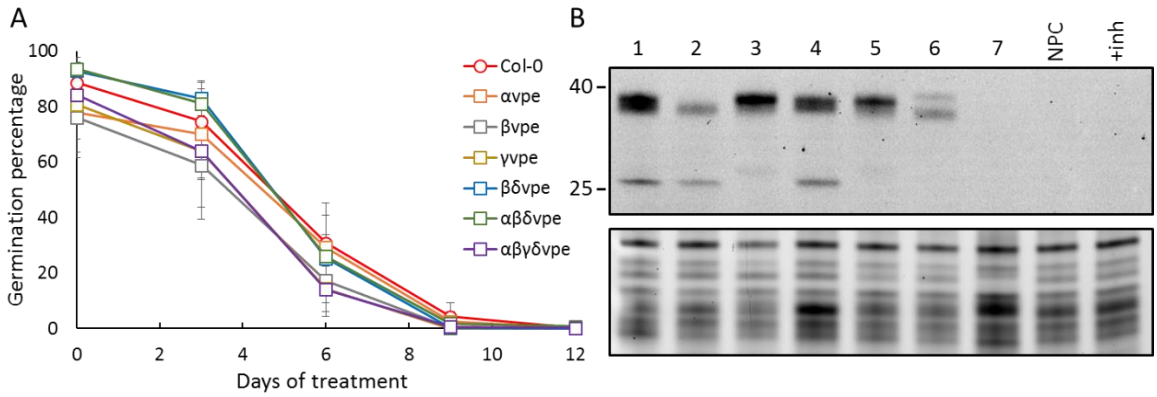

Figure 23. Seed longevity phenotype and labelling profile of several *vpe* mutants. Panel **A** shows the germination proportion of several *vpe* mutants after different periods of artificial seed ageing. Each data point represents the mean of five biological replicates. Error bars represent the standard deviation. Section **B** shows protein extracts from eight-day-aged seeds of these mutants labelled with AMS101 (top panel) and the total protein loaded stained with SyproRuby Protein Gel stain (bottom panel). **1**: Col-0; **2**: α-*vpe*; **3**: β-*vpe*; **4**: γ-*vpe*; **5**: βδ-*vpe*; **6**: αβδ-*vpe*; **7**: αβγδ-*vpe*. The NPC column corresponds to a non-labelled sample (no probe control) prepared combining aliquots of all proteomes. The inhibitor used was YVAD-cmk and corresponds to the last column (+inh).

To verify that the observed activation pattern reflects the effect of accelerated ageing and is not caused by a response to high temperature, the labelling pattern of naturally aged seeds was evaluated. Specifically, seeds naturally aged for eight years

from L*er* and *dog1-1* were used to confirm the previous results. First, a germination kinetics assay was conducted to determine the quality level of these seed batches (**Figure 24A**). It was observed that L*er* seeds germinated up to 40 %, whereas the longevity-deficient mutant *dog1-1* was unable to germinate. The comparison with proteins isolated from fresh, viable seeds and these naturally aged seeds confirmed that the activation of VPEs observed after artificial ageing also holds true in naturally aged samples (**Figure 24B**, **C**).

Taken together, these results confirm ABPP as a valid approach to evaluate seed quality. Using this experimental approach, two different markers with opposite behaviours were identified. Specifically, the activity of VPEs was demonstrated to be induced by both artificial and natural seed ageing.

Serine hydrolases exhibited the exact opposite trend, decreasing their activity as the ageing treatment progressed. Furthermore, several interesting candidates were identified which can now be further analysed and their role in seed quality and longevity studied. However, some concerns regarding the reliability of these data were raised, as the MS analysis found that the non-labelled control was also enriched for serine hydrolases (**Supplemental Figure 7**).

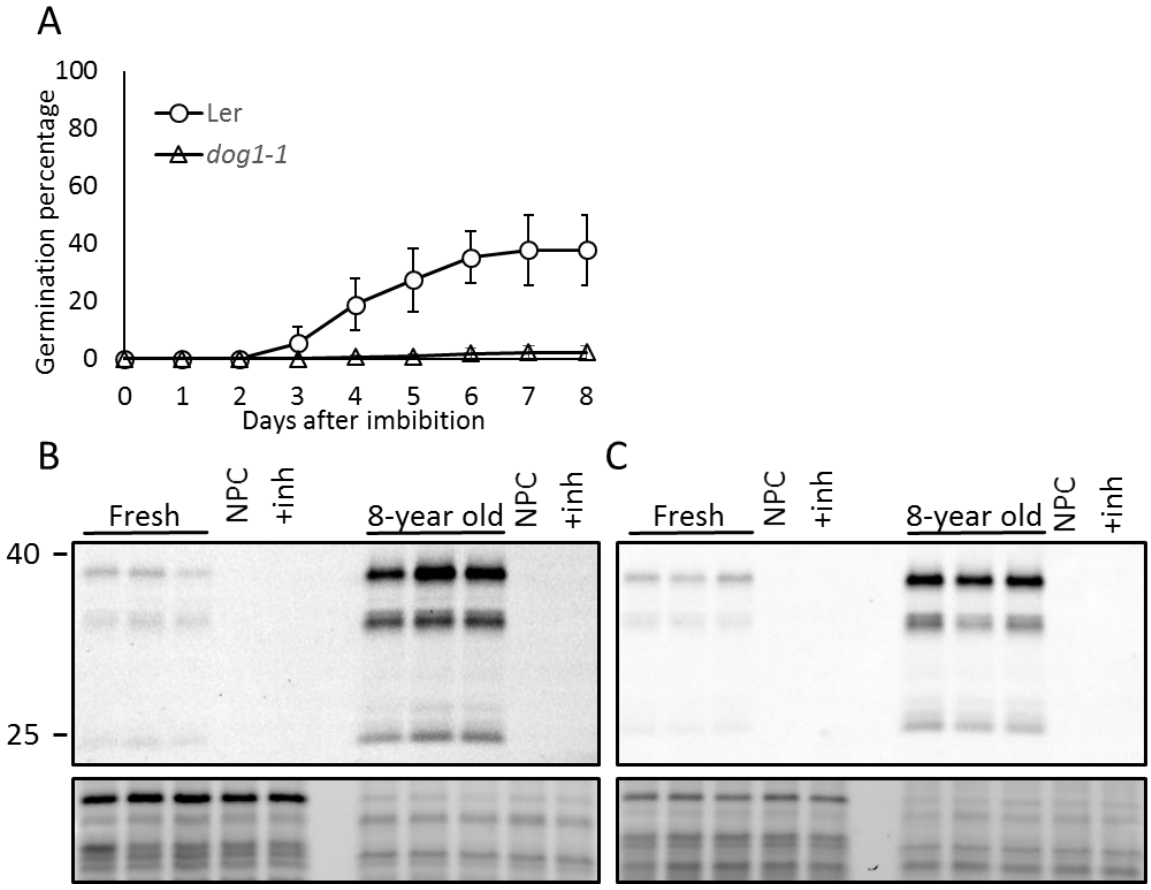

Figure 24. VPEs show increased activity in naturally aged seeds. Panel **A** shows the germination kinetics of 8-year-old naturally aged seed batches of L*er* and *dog1-1*. Each data point represents the mean of three biological replicates. Error bars correspond to standard deviation. Panels B and C show AMS101-labelled protein extracts from eight-year old seeds of L*er* (**B**) and *dog1-1* (**C**). In each panel, three biological replicates are shown from either fresh (left side) or naturally aged (right side) seeds. The inhibitor used was YVAD-cmk and corresponds to the last column (+inh). The NPC column corresponds to a non-labelled sample (no probe control) prepared combining aliquots of all proteomes. The bottom section of panels B and C corresponds to the total protein load stained with SyproRuby Protein Gel stain.

3.3.2.1 Validation of the identified seed quality markers on wild species

Transitioning the results found in model species as *Arabidopsis thaliana* to a wider range of species is among the aims of the EcoSeed consortium. To provide further substantiation for the identified markers as robust predictors of seed quality, eight wild species, closely related to well-established crops, were selected and their viability constants determined as a part of the project (**Table 15**).

The two probes identified to exhibit a differential labelling pattern in aged seeds of Arabidopsis were further tested in seed protein extracts of these eight wild species. It was decided to compare non-aged seeds and seeds aged to reach a proportion of 25 % germination, as this point showed a strong differential labelling in Arabidopsis (**Figure 20** and **Figure 22**). Due to the reduced amount of seeds available, the germination proportion of seeds following the artificial ageing treatment could not be determined except for *Brassica rapa* spp. *campestris* (**Supplemental Figure 8**). Similarly, the labelling conditions used were those described to be optimal for Arabidopsis, because there were not enough seeds available to conduct extra trials. Due to a mistake during sample preparation, only the seeds of *Brassica rapa* spp. *campestris* were imbibed for 24 h prior to protein extraction. The rest of the extracts were directly isolated from dry seeds.

Table 15. List of the eight wild species used to evaluate the markers identified in Arabidopsis. Initial viability is presented as the mean germination percentage ± standard deviation. Days of ageing refers to the estimated number of days of incubation required for seeds to reach a germination proportion of 25 %. All data presented in this table were experimentally determined by Dr Charlotte Seal at the Royal Botanic Gardens in Kew, United Kingdom.

Species	Initial viability (%)	Days of ageing
Brassica rapa spp. *campestris*	100 ± 0	18
Brassica rapa spp. *sylvestris*	100 ± 0	8
Brassica tournefortii	90 ± 1	16
Hordeum marinum	77 ± 25	2
Hordeum brachyantherum	97 ± 6	5
Helianthus argophylus	93 ± 12	2
Helianthus angustifolius	100 ± 0	2
Helianthus gracilentus	37 ± 21	2

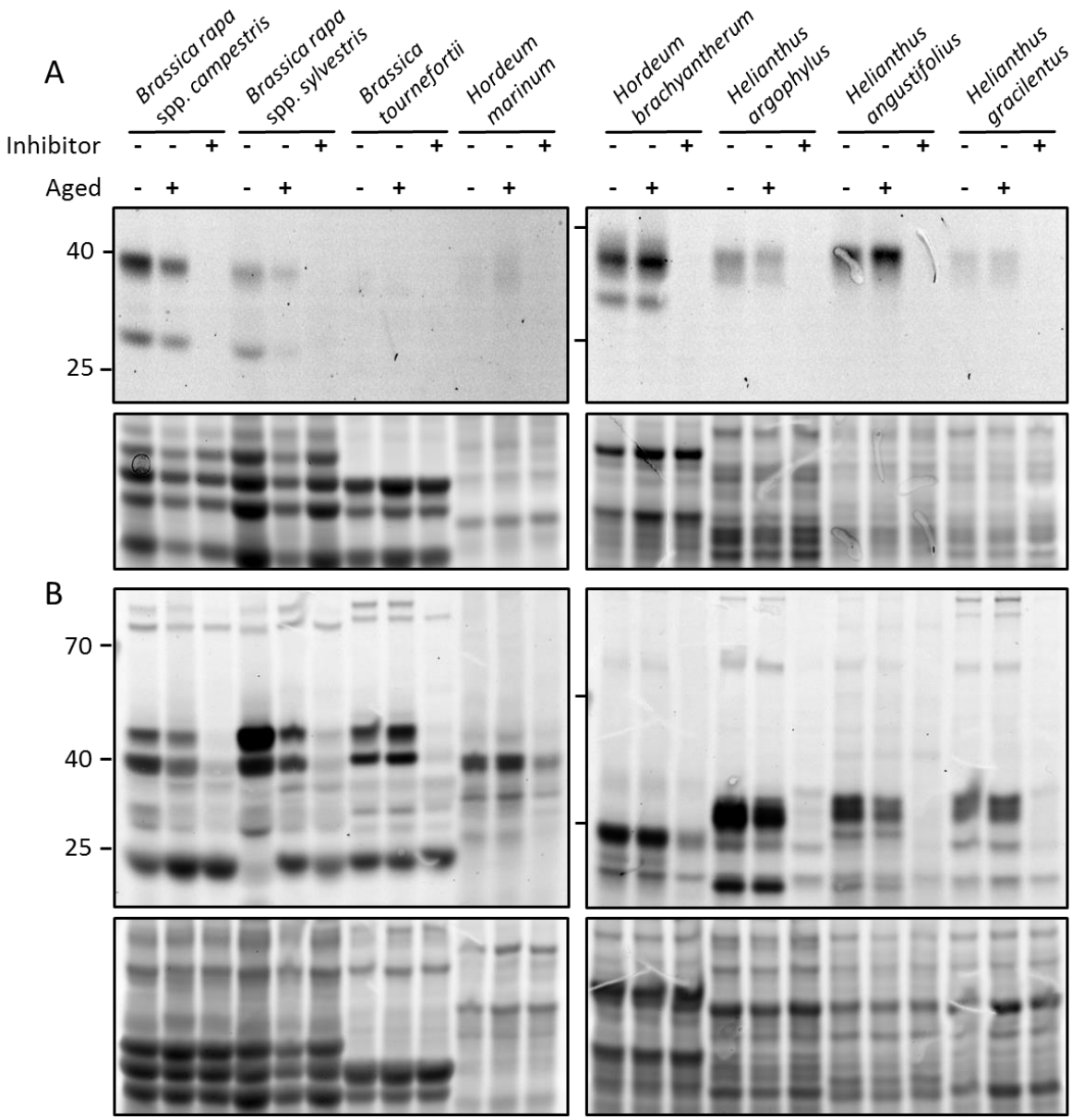

Figure 25. Characterisation of the identified seed quality markers on seeds of eight wild species. Section **A** shows protein extracts from the indicated eight wild species labelled with AMS101 (top panels) and the total protein loaded stained with SyproRuby Protein Gel stain (bottom panels). Section **B** shows protein extracts from the indicated eight wild species labelled with FP-Rh (top panels) and the total protein loaded stained with SyproRuby Protein Gel stain (bottom panels). For each species, a non-aged and an aged sample were used, together with an inhibitor control prepared combining aliquots from both samples. The inhibitor used in section A was YVAD-cmk. The inhibitor used in section B was DiFP. The molecular weight markers at the top right panel in section B represent 70 (upper band) and 40 kDa (lower band).

Probe AMS101 was able to label protein extracts from most of the wild species, although the labelling in *Brassica tournefortii* and *Hordeum marinum* was very faint (**Figure 25A**). For the rest of species, a specific labelling could be observed, which hypothetically corresponds to VPE-like proteins in these species. This is also

supported by the fact that these enzymatic activities are supressed in the presence of the specific inhibitor YVAD-cmk. Nevertheless, comparison between control and aged samples from these species did not show major differences in the labelling. For the two *Brassica rapa* subspecies, the fluorescence gel shows a slightly stronger signal in the control compared to the aged sample, although the total protein loaded in the *Brassica rapa* ssp. *sylvestris* non-aged sample was higher and therefore the differential labelling could be caused by that (**Figure 25A**).

Based on the results from Arabidopsis, it was expected that extracts from aged seeds would display stronger signals than those from non-aged seeds, but none of the tested species exhibited this pattern. On the contrary, no differences were observed for most of the species (**Figure 25A**). This absence of differential labelling probably originated from the mistake of using dry instead of 24 h-imbibed seeds.

Protein extracts labelled using the FP-Rh probe showed an abundance of bands, as expected for a probe with a less stringent target specificity (**Figure 25B**). Contrary to the observations for the AMS101 probe, it is clear that the addition of the specific FP-Rh inhibitor could not supress all signals, indicating a certain degree of unspecific labelling in all eight species. In all *Brassica* species, strong signals were detected around 40 kDa, but no evident reduction of the labelling caused by the ageing treatment could be observed. None of the *Hordeum* species exhibited differential labelling between control and aged samples. Similarly, no strong differences could be appreciated between treatments in samples from *Helianthus* species. It looks that the signals around 25 kDa decrease their intensity in the aged sample in *Helianthus argophylus* and *Helianthus angustifolius*, but this reduction is relatively minor (**Figure 25B**).

From these observations, it can be concluded that both probes identified in Arabidopsis are functional in the evaluated wild species. However, further research will be required in order to establish the most adequate conditions to conduct the labelling in these species. It is clear that probe AMS101 conserves a very specific pattern of labelling in most species, meaning the identified activities most certainly correspond to VPE-like proteins in those species. However, no increased signals were detected in aged samples, which could stem from a variety of factors, such as the lack of the 24 h imbibition period or inappropriate labelling conditions. Nonetheless, seeds from *Brassica rapa* spp. *campestris* were imbibed and did not show a differential labelling (**Figure 25A**). It is then plausible that seeds of these wild species have different mechanisms to cope with ageing damages from those of Arabidopsis, which again will require further investigation.

The same reasoning is valid for the results observed in samples labelled with FP-Rh. Identically, the lack of a consistent reduction in the signals of aged samples, as was expected based on Arabidopsis results, can originate from the above-mentioned concerns. It is conceivable that seeds from these species are equipped with different mechanisms to deal with damages incurred during the ageing process that result in serine hydrolases being more protected than in Arabidopsis thus explaining the absence of differential labelling. However, more studies are required to substantiate these speculations.

Lastly, it is worth mentioning that the seed biology and physiology from these species is not as well-known and established as in *Arabidopsis thaliana*. Therefore, growing these plants at optimal conditions and obtaining high quality seeds from them is challenging, as reflected in **Table 15**.

4 DISCUSSION

Seed longevity is a complex trait governed by a combination of genetic and environmental factors. Furthermore, its role in seed dispersal and persistence in natural environments as well as during seed storage stresses the importance of improving our understanding of the factors and mechanisms regulating it. In this thesis, we have conducted a study on different molecular and environmental factors that affect seed longevity using multiple approaches. Moreover, we have explored novel strategies to evaluate the quality levels of seed batches with different levels of quality.

4.1 THE EFFECT OF MATERNAL TEMPERATURE DURING SEED DEVELOPMENT ON THE SEED TRANSCRIPTOME OF ARABIDOPSIS

Modifications of a particular trait of the offspring exerted by conditions experienced by the mother plant are referred to as maternal effects. These effects determine how a plant's offspring will perform in the next growing season and represent a major adaptive response in plants (Donohue, 2009). Maternal temperatures were previously shown to be able to affect different traits in seeds. In Arabidopsis, it is now well established that seeds that matured at lower temperatures exhibit increased levels of seed dormancy, contrary to those matured at higher temperatures, which are usually non-dormant or have very shallow levels of dormancy.

Although not many studies have explored the contribution of maternal temperatures to seed longevity, some examples are available. In *Plantago cunninghamii*, low-temperature maternal environments led to longer-lived seeds as a result of improved seed quality (Kochanek *et al.*, 2011). Further work in *Silene vulgaris* reported that seed longevity, although genetically determined, exhibits great plasticity in response to the maternal environment (Mondoni *et al.*, 2014). In this work the authors explored how seeds of alpine plants, which exhibit reduced seed longevity as this trait is under no selective pressure in this natural environment, exhibited improved longevity when grown at warmer temperatures. They suggest that this effect could be caused, at least partially, by an altered provisioning of mRNAs in the developing seed. In Arabidopsis, the contribution of maternal temperatures to resistance to artificial ageing was previously reported, with low temperatures increasing seed sensitivity to artificial ageing and elevated temperatures having the opposite effect, in a genotype-dependent manner (He *et al.*, 2014).

The experimental approach conducted in this thesis led to the same observed phenotypes, as depicted in **Figure 1A**. Lower seed maturation temperatures caused increased sensitivity to artificial ageing. It was expected that elevated temperatures would result in comparable resistance levels to those of the control, but increased sensitivity was also observed for this treatment. This could be caused by experimental variability, as experiments conducted by project collaborators using the same biological material revealed a similar pattern of resistance to that of the control (**Supplemental Figure 9**). RNA-sequencing was conducted to elucidate the transcriptomic responses underlying the observed phenotypes and it was found that maternal effects altered the seed transcriptome (**Table 5**).

Lowered temperatures during seed maturation were demonstrated to cause increased levels of seed dormancy in Arabidopsis (Schmuths *et al.*, 2006). We observed that lowered temperatures during seed maturation caused reduced resistance to artificial ageing (**Figure 1A**), in agreement with previous reports (He *et al.*, 2014). Our transcriptomic analysis of seeds that matured at lowered temperatures revealed an overall reduction in expression of GA biosynthesis transcripts, which may be expected to lead to reduced GA accumulation. This is consistent with the enhanced expression of *TEM1* and *DDF1*, flowering time regulators which were reported to repress the expression of *GA3OX* genes and reduce the final GA content (Magome *et al.*, 2004, 2008; Osnato *et al.*, 2012). In addition, this is further supported by the finding that *GA3OX1* and *2* were downregulated in this temperature regime. This reduced expression of GA biosynthesis transcripts in seeds could be responsible for an altered seed development and coat formation, as these phytohormones are required for completion of these processes (Singh *et al.*, 2002; Kim *et al.*, 2005b). Besides, considering that GAs are required for seed germination, it is likely that the observed effects of low temperature do alter the GA/ABA balance towards a more dormant status, in agreement with a recent report (Topham *et al.*, 2017).

Further support for an altered seed coat composition is provided by the downregulation of three genes known to participate in this regulation: *FAR1*, *ADS1* and *LACS2*. The first two were reported to affect suberin formation and seed lipid content, respectively (Domergue *et al.*, 2010; Smith *et al.*, 2013). *LACS2* participates in cuticle biosynthesis in Arabidopsis and mutant lines of this gene showed increased sensitivity to artificial ageing (De Giorgi *et al.*, 2015). Previous reports showed that low temperatures during seed maturation do contribute to an altered suberin and seed coat composition, as reflected by their degree of permeability to tetrazolium salts (MacGregor *et al.*, 2015; Fedi *et al.*, 2017). The first study showed that the altered seed coat permeability was, at least partially, resulting from enhanced expression of several

genes involved in the biosynthesis of phenylpropanoids. Intriguingly, our RNA-seq results showed no differential expression for any of these genes, even though both studies were conducted using the Col-0 accession. Whereas our study was conducted using mature, dry seeds, MacGregor *et al.* (2015) analysed green cotyledon stage seeds, i.e., an earlier stage of seed development, which can at least partially explain this discrepancy and the transcription of genes more abundant during seed maturation. In addition, the software used to analyse their RNA-seq data was also different, which can further substantiate the different genes found differentially expressed. The same transcriptomic dataset was used by Fedi *et al.* (2017) and, strikingly, they found that low temperatures during seed set enhanced the expression of *FAR1* and *LACS2*, contrary to the results exposed in this thesis. Public available microarray data illustrates that expression of these genes is considerably reduced from green cotyledon stage seeds to dry seeds (Winter *et al.*, 2007), so we hypothesise this is the causal reason for this disagreement.

The upregulation of *ERF10* by low temperatures during seed development could also contribute to the observed reduction of GA responses, as this TF was previously shown to be able to interact *in vivo* with DELLA proteins, repressors of GA signalling (Zhou *et al.*, 2016). In this study, Zhou and colleagues showed that *ERF11* and *4*, closely related genes to *ERF10* (Nakano *et al.*, 2006), are positive regulators of GA responses in Arabidopsis hypocotyls, by both enhancing expression of GA biosynthetic genes and interacting with DELLA proteins (Zhou *et al.*, 2016). Nevertheless, it is plausible that *ERF10* acts in an opposite manner in seeds, repressing GA responses, although further experiments will be required to evaluate this.

The role of auxins in seed development was initially related with embryo patterning during seed embryogenesis (Möller and Weijers, 2009; Locascio *et al.*, 2014). Moreover, this plant hormone was recently identified as the triggering signal inducing seed coat development in Arabidopsis (Figueiredo *et al.*, 2016). As for their regulation by temperature, several studies have explored these responses. Arabidopsis plants grown at elevated temperatures showed increased auxin content and responses, which led to elongated hypocotyls in the presence of light (Gray *et al.*, 1998; Miyazaki *et al.*, 2016). Consistent with these observations, another study found reduced expression of the auxin-responsive reporter DR5:GUS in response to low temperature in roots, which would correspond with reduced amounts of free auxins (Lee *et al.*, 2005) and could relate with the observed downregulation of transcripts involved in auxin responses which we observed.

Auxin responses are complexly regulated by an intricate network of positive and negative regulators. These include Aux/IAA proteins, which usually act as repressors of the second type, the auxin response transcription factors (ARFs). Both types of regulators have been extensively studied and shown to form homo- or heterodimers to maximise their regulatory specificity (Dreher *et al.*, 2006; Vernoux *et al.*, 2011; Piya *et al.*, 2014). This interlinked regulation could explain the presence in the dataset of *IAA9*, *IAA17* and *IAA28* among the upregulated transcripts. However, it still does not provide insights on how temperature is affecting these regulatory loops. The observed downregulation of *ANT* could be linked with the previous hypothesis that low temperatures during seed maturation cause an alteration of the seed coat composition, as this gene was shown to affect cell wall composition by altering the expression of pectin-modifying enzymes and auxin accumulation (Krizek *et al.*, 2016). In sunflower, *HaIAA27* was shown to participate in the regulation of seed longevity by repressing expression of *HaHSFA9* (Carranco *et al.*, 2010). Our results showed that lowered temperatures caused increased expression of *IAA28*, but further evidence is required to determine a similar role of this gene in the regulation of seed longevity, especially considering that no HSF transcript was found to be downregulated. It is as well possible that *IAA28* is not involved in this regulation.

Elevated temperatures during seed maturation cause shallow levels of seed dormancy (Kendall *et al.*, 2011). The results presented here showed that they led to increased sensitivity to artificial ageing compared to the control (**Figure 1A**). This phenotype was unexpected, considering previous reports showing how elevated temperatures during seed maturation caused similar levels of sensitivity to artificial ageing to those of the control (He *et al.*, 2014). This could be the result of experimental variability, provided that even though seeds are treated in the same manner, sometimes not all batches exhibit the exact same behaviour. Besides, seeds are highly sensitive to the environment, so many factors experienced by the seed could have caused the observed variation of the phenotype. Despite the lack of a differential phenotype between control and elevated temperatures with regard to sensitivity to artificial ageing, several differentially expressed transcripts were found, although no overrepresented GO categories were identified. However, a number of genes was found differentially expressed which can be linked, at least partially, with responses to the maternal environment. The presence of upregulated transcripts involved in mucilage biosynthesis might point towards an adaptive response to high temperatures. It seems fitting that increasing the amount of mucilage in the seeds could help them cope with the predictable lack of water usually encompassed by high

temperatures (Penfield *et al.*, 2001). This could be accompanied by altered seed composition, as hinted by upregulation of several genes affecting this trait.

The presence of genes related to the enhancement of germination and GA signalling is not surprising, considering the available literature pointing to the shallow dormancy caused by this temperature regime (Huang *et al.*, 2014b). Following this reasoning, the presence of *DOG1* among the downregulated transcripts by elevated temperature was expected, as *DOG1* partially contributes to the active degradation of GAs (Kendall *et al.*, 2011) and maintenance of seed dormancy (Née *et al.*, 2017). Also downregulated, *AAO3* is responsible for the final step in ABA biosynthesis. When seeds are non-dormant and prone to germinate, the GA/ABA balance is skewed towards GAs, so it is reasonable that ABA biosynthesis is not promoted (Holdsworth *et al.*, 2008; Topham *et al.*, 2017).

Based on these results and although elevated temperatures seem to participate in the establishment of seed dormancy and do cause differential expression of genes, their contribution to sensitivity to artificial ageing seems minor under the studied experimental conditions. Taken together, these observations and the cited literature illustrate an active effect of lowered temperatures during seed maturation on the seed cell wall composition that in turn results in altered seed phenotypes (MacGregor *et al.*, 2015), increasing seed dormancy while reducing seed longevity. The relationship between seed dormancy and seed longevity has been seldom studied. Recent work in Arabidopsis reported that both traits are negatively correlated (Nguyen *et al.*, 2012), an observation further supported by work with *Eruca sativa*, which showed opposite trends for seed dormancy and longevity (Hanin *et al.*, 2013). Some authors speculated that low temperatures exert selective pressure on seed dormancy rather than longevity. These environments, usually more humid and with sufficient water available, allow dormancy cycling and re-hydration phases, that would thus activate repair mechanisms. On the other hand, more extreme environments would impose harsher conditions, with less water availability and therefore an increased seed longevity would be favoured (He *et al.*, 2014). However, our data does not accommodate the second part of this hypothetical trade-off, at least in terms of seed longevity, considering that even though elevated temperatures reduced seed dormancy, no enhancement was observed for seed longevity.

4.2 MODIFICATION OF THE SEED TRANSCRIPTOME BY ARTIFICIAL AGEING

The effect of ageing damages on seed constituents is mainly associated with oxidative stress and the subsequent detrimental effects incurred on the cells (Kranner *et al.*, 2006; Hu *et al.*, 2012). Although not directly related with maternal effects, studies

in barley showed that abiotic stresses experienced during seed maturation can be aggravated by subsequent application of artificial ageing (Nagel *et al.*, 2015).

As a result of the oxidative stress imposed during artificial ageing, the presence of upregulated transcripts associated with responses to oxidative stresses was expected, such as *GSTF6* or *HDP17.6II*. Upregulation of the ABA biosynthetic gene *AAO3* may also fit with the role of ABA in abiotic stress responses. Additionally, its role in the production of H_2O_2 could also enhance the ageing process (Zarepour *et al.*, 2012). Considering that among downregulated transcripts there were several genes implicated in the negative regulation of ABA responses, such as *AZF2* and *CIPK3*, both options seem plausible. Moreover, previous studies reported that H_2O_2 is required for the initiation of programmed cell death processes, which would fit with the reduced viability (Kranner *et al.*, 2006; Hu *et al.*, 2012). However, more experiments will be required to verify these claims.

Considering that oxidative damage incurred during seed ageing causes leakage of solutes due to loss of membrane integrity (Buitink and Leprince, 2008), it makes sense that ionic transporters become upregulated, as a response to the resulting ionic imbalance. Conversely, the presence of genes related to cell wall modification is more controversial. Although artificial ageing is conducted at elevated levels of relative humidity, it seems rather unlikely that the seed's resources would be invested in cell wall remodelling rather than in repair mechanisms. A possible explanation could be that these transcripts are responsive to oxidative stress, in a similar manner to that occurring during cell wall loosening following apoplast acidification (Cosgrove, 2005).

The presence of *SNL1*, a member of a histone deacetylase complex (Wang *et al.*, 2013), among the downregulated transcripts hints the possibility that post-translational modifications of proteins are taking place during artificial seed ageing, which would agree with previous reports (Chen *et al.*, 2013). Although conducted in recalcitrant seeds, a recent study also reported that artificial ageing does induce profound modifications of the epigenetic landscape in seeds of *Quercus robur* (Michalak *et al.*, 2015).

A transcriptomic analysis of aged pea seeds found that many of the differentially expressed genes were related to translation (Chen *et al.*, 2013), a similar result to that found in proteomic analysis of artificially aged Arabidopsis seeds (Rajjou *et al.*, 2008b). In the transcriptomic study of pea seeds, the authors described how the number of identified differentially expressed genes directly correlated with the length of the ageing treatment. In our case, only one time point was used corresponding to four days, which might miss on some of the effects of this treatment, especially considering

that some of the maternal effects could be amplified (Nagel *et al.*, 2015). In addition, they reported that progressive artificial ageing of the seeds encompasses an increasing degradation of RNA, therefore threatening the robustness of the analyses. In our case, the short period of ageing applied ensured that the isolated RNA remained unharmed (the RNA integrity was evaluated prior to sequencing), but maybe it was at the cost of a more comprehensive picture of the transcriptome. The lack of GO categories related to translation could be the result of the ageing treatment not being long enough or that more time points were necessary to establish a more complete landscape of the aged seed transcriptome.

4.3 THE INFLUENCE OF DROUGHT DURING SEED MATURATION ON SEED QUALITY AND THE SEED TRANSCRIPTOME

Drought stress imposes strong limitations to plant growth and fertility, therefore threatening crop production (Gall *et al.*, 2015). Although many studies have been conducted to evaluate plant responses to drought, not so many have dealt with its effect on seed traits. Previous reports on the effect of water deprivation during seed maturation described that drought led to reduced levels of seed dormancy in wild oat (*Avena fatua*) seeds, but did not alter seed vigour after artificial ageing (Sawhney and Naylor, 1982; Gallagher *et al.*, 2013). Seeds of soybean that matured under drought conditions exhibited altered composition of seed storage compounds, enhanced accumulation of α-tocopherols and an earlier accumulation of seed-specific dehydrins (Dornbos and Mullen, 1992; Britz and Kremer, 2002; Samarah *et al.*, 2006). Similar results were also found for yellow lupine (*Lupinus luteus*) and triticale (*x Triticosecale*) seeds, in which drought modified the carbohydrate composition of seeds (Zalewski *et al.*, 2001). These responses could have positive effects on the resulting seed longevity, but none of these studies evaluated this trait. In Arabidopsis, only one study explored the relationship between artificial ageing and seeds matured under drought conditions (Bueso *et al.*, 2014), showing a detrimental effect of drought in resistance to artificial ageing.

In this thesis, the effects of drought imposed during seed maturation on the seed longevity-deficient mutant *dog1-1* and its control were evaluated. It was observed that for both the mutant and the control, drought treatment led to enhanced resistance to artificial ageing, besides of partially alleviating the levels of seed dormancy in the NIL DOG1 control (**Figure 3**). The reduction of seed dormancy depth does fit with the previously described effects in other species. However, our results on seed sensitivity to artificial ageing disagree with those previously reported for drought-grown Arabidopsis seeds (Bueso *et al.*, 2014). These differences could originate from different plant growing conditions, as this publication does not provide details on how the plants were taken care of after the interruption of watering. Besides, the protocol used for artificial ageing of the seeds was not the same and these authors only aged seeds for one day. In addition, they used a different genotype (Col-0). All these differences may explain the different phenotypes reported.

RNA-sequencing of dry seeds was conducted to elucidate the mechanisms underlying the enhanced resistance to artificial ageing caused by drought. The effect of drought during seed maturation led to the identification of around 500 differentially expressed transcripts (**Table 7**). We observed a big overlap in the

number of differentially expressed transcripts (at least 49 %) when using either NIL DOG1 or *dog1-1* as the reference for determining differential expression, which indicates that the transcriptomic response of both genotypes is similar (**Figure 5**), as expected from the similar phenotypes observed (**Figure 3**).

Early responses to drought stress are linked to the biosynthesis and accumulation of ABA, which subsequently triggers an array of transcriptional responses which will lead to adaption or resistance to water deprivation (Benech Arnold *et al.*, 1991; Huang *et al.*, 2008; Wang *et al.*, 2008; Farooq *et al.*, 2009). In our analysis, two genes involved in the biosynthesis of ABA (*NCED4* and *NCED6*) and an ABA transporter (*NRT1.2*) were found downregulated, which seems to contradict these reports. Nevertheless, several genes which expression is reported to be ABA-responsive were upregulated as well. It is plausible that the imposition of drought stress during seed maturation triggered ABA accumulation. However, considering that our transcriptomic analysis was conducted on mature, dry seeds, this initial upregulation of ABA synthesis may have been reduced during the process of seed maturation, which would explain the observed reduction in expression for these transcripts. *NCED6* participates in ABA biosynthesis during seed maturation and contributes to the establishment of seed dormancy (Lefebvre *et al.*, 2006; Martínez-Andújar *et al.*, 2011). Therefore, its downregulation accomodates with the observed reduction of seed dormancy caused by drought. Moreover, the presence of *DOG1* among the downregulated transcripts is in line with the reduced dormancy phenotype observed **Figure 3A**.

Increased levels of ABA signalling would fit with the observed reduction in ethylene-related genes. These two phytohormones are known to establish a complex crosstalk at different stages of plant development, such as seed germination, in which ABA and ethylene play antagonistic roles (Ghassemian *et al.*, 2000; Tanaka *et al.*, 2005). The presence of *ERF5* and *ERF6* among downregulated transcripts seems to agree with the enhanced growth responses represented by the upregulation of expansins, as these two *ERFs* were reported to reduce the GA levels present in water-deprived growing Arabidopsis leaves (Dubois *et al.*, 2013).

Our study also showed that *VTE4*, a gene implicated in chlorophyll degradation, was upregulated, and this process was demonstrated to be induced by ABA (Yang *et al.*, 2014). The presence of *VTE4* is of particular interest considering it is also involved in the biosynthesis of tocopherols, antioxidant compounds that protect lipids from oxidative damage and partial contributors to seed longevity (Sattler *et al.*, 2004). Specifically, this enzyme catalyses the conversion of ɣ-tocopherol into α-tocopherol

(Bergmüller *et al.*, 2003). *vte4* mutants displayed altered proportions of seed tocopherols, which in turn can modulate certain transcriptional responses (Semchuk *et al.*, 2009; Cela *et al.*, 2011). However, no strong claims can be extracted from this observation, as neither the ABA nor the tocopherol content of these drought-matured seeds were determined.

Several GO categories related to cell wall organisation and modification were found (**Table 8**). Specifically, five different expansins were found to have enhanced expression in seeds matured under drought stress. This could be related with the fact that seeds are sink organs, actively growing and as such, drought does not induce a cease of growth as it does in other vegetative tissues (Shao *et al.*, 2008; Harb *et al.*, 2010). The latter study also showed how expression of expansins is enhanced in the first stages of drought treatment, but became downregulated when water deprivation was prolonged in time. Similarly, previous studies described that root tips of maize plants (*Zea mays*) deprived of water exhibited increased expansin activity and expression (Wu *et al.*, 1996, 2001). Further work corroborated the induction of expansin activities upon dehydration of the resurrection plant *Craterostigma plantagineum* (Jones and McQueen-Mason, 2004). The enhanced expression of expansins also fits the observed increase in seed weight caused by drought, which could result from increased cell expansion and growth and which was observed in both genotypes (**Supplemental Figure 2**). This contrasts with a previous study in wheat (*Triticum aestivum*) that reported that drought applied during seed maturation caused a strong reduction in seed size (Begcy and Walia, 2015). This study also reported that drought treatment strongly enhanced expression of *NCED4*, contrary to the results we found for Arabidopsis seeds. These discrepancies possibly originate from the fact that the seed developmental program is different between cereals and dicotyledonous plants.

Our results also showed the presence of upregulated transcripts involved in epigenetic modifications and gene silencing as a result of restraining water availability during seed development. Drought imposed during seed maturation led to the upregulation of *RDR6* and *DMS3*. These two genes contribute to gene silencing through small interference RNA (siRNA)-mediated maintenance of methylation levels (Lorković *et al.*, 2012). On the other hand, three VIM genes involved in DNA methylation (Kim *et al.*, 2014) were found downregulated. Previous studies showed that drought stress leads to chromatin modifications that can contribute to adaption responses (Ding *et al.*, 2012; Kinoshita and Seki, 2014; Kim *et al.*, 2015). A previous study on mature seeds of pear (*Pyrus communis*) found that desiccation of these seeds altered their DNA methylation levels (Michalak *et al.*, 2013). It is then possible

that drought experienced during seed maturation helps seeds adapting to future exposure to this type of stress. Anyway, further research will be necessary to evaluate and confirm the impact of drought during seed maturation on the levels of DNA methylation, as *DMS3* and *RDR6* were also upregulated in the *dog1-1* mutant.

We have described several mechanisms that may underlie the observed enhancement of seed resistance to artificial ageing after drought treatment. Considering that seed longevity is a plastic trait with great capacity to respond to environmental cues (Nguyen and Bentsink, 2015), it is difficult to draw a single claim in the light of these results. We cannot rule out that several of the mentioned genes may be partially contributing to the observed final seed longevity, but additional experimental evidence is necessary prior to any final conclusion.

4.4 THE SEED TRANSCRIPTOME OF THE *DOG1-1* MUTANT

Although the aim of this study was to determine the effects of drought applied during seed maturation on seed quality and the dry seed transcriptome, RNA-seq analysis of the dry seed transcriptome of *dog1-1* was conducted as a part of the experimental design. This analysis revealed a set of differentially expressed transcripts which are in agreement with the ones previously described in microarray studies (Dekkers *et al.*, 2016). However, as RNA-seq approaches allow for more in-depth studies, some extra categories were identified which were not previously reported.

Dekkers and colleagues already described the downregulation of heat-responsive transcripts in *dog1-1* and linked it with the acquisition of seed longevity orchestrated by *HSFA9* (Tejedor-Cano *et al.*, 2010; Dekkers *et al.*, 2016), provided that this gene and several of its HSP targets are downregulated in the mutant, a result we also found in our analyses. In addition to this, we found that upregulated transcripts in *dog1-1* dry seeds showed overrepresentation of responses to cold. **Figure 1** shows data regarding the effect of maternal temperatures on seed sensitivity to artificial ageing treatments. Specifically, **Figure 1B** shows how these conditions affected seed performance of the *dog1-2* mutant. The phenotypes observed clearly match the responses identified with our RNA-seq analysis, as the mutant performed better than the control when grown at lowered temperatures and worse when elevated temperatures were applied.

Our results also showed that several GA biosynthesis genes, as well as multiple GA-response repressors were upregulated. We speculate that there might be positive and negative feedback regulatory loops, including at the protein level, which alter the expression of these genes and could underlie this apparent inconsistence in GA

responses. We applied the same reasoning for the observed *SPT* expression pattern. Initially, *SPT* was described as a repressor of seed germination and a contributor to the maintenance of seed dormancy, in cooperation with *ABI4*, *ABI5* and DELLA proteins (Josse *et al.*, 2011; Vaistij *et al.*, 2013). However, our transcriptomic approach showed opposite expression patterns of these transcripts compared to those previously described. Therefore we think that other regulatory loops are acting, giving as a result the observed expression patterns. In addition, a recent publication linked *SPT* to the regulation of gynoecium development, alongside cytokinins and auxins (Reyes-Olalde *et al.*, 2017). This could fit with the proposed role of *DOG1* as a developmental regulator (see the following paragraph), so when it is absent, the expression of other genes involved in developmental processes becomes altered.

The authors of the previous transcriptomic study on *dog1-1* seeds speculated on the role of *DOG1* as a regulator of seed maturation, based on the observation that the *dog1-1* mutation aggravates the phenotypes of *abi3-1* in *dog1-1 abi3-1* double mutants. We found that auxin-related transcripts were overrepresented among upregulated transcripts, especially auxin transporters. Two studies in *Medicago truncatula* have reported an abundance of auxin-related transcripts in co-expression networks associated with the acquisition of seed longevity (Righetti *et al.*, 2015; Pereira Lima *et al.*, 2017). Moreover, a previous study reported that auxins act as positive regulators of *ABI3* expression (Liu *et al.*, 2013b). The observed genetic interaction between *DOG1* and *ABI3* might indicate that in the absence of the first, auxin responses are enhanced to take over the role of *DOG1* and maintain the levels of *ABI3* expression. However, more research will be required to shed light on this regulatory mechanism.

4.5 THE INFLUENCE OF DROUGHT DURING SEED MATURATION ON CELL WALL COMPOSITION

The observation that many of the overrepresented GO categories were related to cell wall composition and modification made us consider the possibility that the enhanced resistance to artificial ageing we observed might be caused by modifications of this structure. To validate this, we conducted cell wall composition analysis of the drought-grown seeds and their controls.

Cell wall composition analyses revealed that at control conditions, the cell wall composition of the *dog1-1* mutant was different from that of the NIL DOG1 control (**Figure 6** and **Figure 7**). Specifically, it was observed that in both the non-crystalline and the pectin-enriched fractions, *dog1-1* exhibited a higher abundance of

monosaccharides than the control. Conversely, application of drought during the course of seed maturation led to a reduction of monosaccharide levels in *dog1-1*, bringing them closer to those observed for the NIL DOG1 control, which in turn remained relatively stable, apart from a slight increase in monosaccharide content in the esterified pectin fraction as a result of drought (**Figure 7A**).

This increased abundance of monosaccharides in control samples of *dog1-1* seeds could be one contributing factor explaining the reduced longevity phenotype of this mutant, as seed coat composition exerts a key role in the resulting seed longevity (Debeaujon *et al.*, 2000). Upon drought treatment, Arabidopsis seeds accumulate raffinose, galactinol and stachyose (Taji *et al.*, 2002). *dog1-1* mutant seeds were shown to have an altered seed sugar composition, with increased levels of glucose, fructose and xylose, whereas the relative amounts of galactinol, raffinose and stachyose were reduced in comparison to NIL DOG1 (Dekkers *et al.*, 2016). Raffinose and stachyose are oligosaccharides, whereas galactinol is a sugar alcohol derived from galactose. Stachyose is synthesised from galactinol and raffinose, and the latter requires galactinol for its biosynthesis as well (Taji *et al.*, 2002). Since the initial levels of these compounds in the mutant are low in comparison to the control, it is probable that upon drought treatment these levels increase at the expense of other monosaccharides, which would explain the relative reduction in monosaccharide content observed.

However, based on the initially observed phenotypes, it is difficult to associate the altered seed cell wall composition with increased resistance to artificial ageing in drought-matured seeds. This is especially stressed by the fact that most of the differentially expressed genes caused by drought were shared between genotypes, which seems to suggest a common regulation. From this point, we can only hypothesise that drought does induce changes in seed cell wall composition and that considering that each genotype has a different initial composition, they converge at a specific composition which would hypothetically increase the resistance of seeds to artificial ageing. Nevertheless, further experimental support will be required to substantiate this claim.

4.6 THE ROLE OF DOG1-INTERACTING PROTEINS IN SEED LONGEVITY

DOG1 plays a key role in the establishment and acquisition of seed dormancy. Besides, the presence of a functional allele is required for the establishment of seed longevity, as *dog1* mutants exhibit reduced seed longevity (Bentsink *et al.*, 2006). However, levels of DOG1 protein accumulation had not previously been studied in relation with seed longevity. We first evaluated if, like for seed dormancy (**Figure 8A**), accumulation of DOG1 correlated with the levels of seed longevity. We observed that, contrary to seed dormancy, increased DOG1 protein accumulation does not enhance seed longevity (**Figure 8B**). From this observation, we can conclude that DOG1 protein is necessary for the establishment of seed longevity (as seed longevity is reduced in *dog1* mutants), but further increasing its accumulation does not improve this trait beyond the levels of the L*er* wild type (**Figure 8B**), which was used as a control instead of NIL DOG1 (Bentsink *et al.*, 2006). In this section, we evaluated the involvement of proteins that interact with DOG1 *in vivo* to gain insight on the mechanisms through which *DOG1* regulates seed longevity.

Seven candidate genes were chosen (**Table 9**) which were identified in a previous pull-down assay conducted in dry and imbibed seeds of the *dog1-1* mutant complemented with pDOG1$_{Cvi}$:YFP:*DOG1* (Née *et al.*, 2017). We found that all the proteins evaluated were able to interact *in vivo* with DOG1 in a yeast two-hybrid assay (**Figure 9**). DOG1 localisation was shown to be restricted to the nucleus and the cytosol (Nakabayashi *et al.*, 2012; Née *et al.*, 2017). Similarly, PER1 was shown to be located in the nucleus and cytoplasm (Haslekås *et al.*, 2003), whereas TRX3 is restricted to the cytoplasm (Park *et al.*, 2009). However, CLO1 was described to be associated with the membrane of oil bodies (Næsted *et al.*, 2000), whereas PLAT1 was detected at the endoplasmic reticulum (Hyun *et al.*, 2014) and VTE3 is a nuclear-encoded gene targeted to the inner membrane of the chloroplast (Motohashi *et al.*, 2003). Web-based online tools for subcellular localisation prediction showed that ATPrel is expected to be located at the endoplasmic reticulum and LOS1 in the cytosol. Based on these data, some discrepancies exist regarding the reported localisation of DOG1. For CLO1, PLAT1 and ATPrel, their subcellular localisation could still match with that described for DOG1 as cytoplasmic, in case they are exposed in the outer membrane of the organelles. However, further research will be required to elucidate this. For *VTE3*, the protein carries a signal peptide that targets it to the inner chloroplast membrane facing the stroma, not the cytoplasm (Motohashi *et al.*, 2003). Considering that this interaction was detected *in vivo* by pull-down assays and further confirmed in yeast two-hybrid assays, it seems that it is a real interaction and not a

false positive. Nonetheless, further studies using bimolecular fluorescence complementation (BiFC) should determine the precise localisation of this interaction *in planta*.

To study the possible role of the interactors in seed longevity, we used T-DNA insertion mutants, although homozygous lines could not be isolated for all candidates. The reasons for unsuccessful isolation of T-DNA insertion mutants can be diverse. For *CLO1*, no insertion could be detected in the T-DNA insertion line used (**Figure 10**). This line was ordered in two separate occasions, so it is possible that the stock contains mislabelled wild type seeds. Disruptions in this gene might cause lethality and therefore, only wild type plants survive. However, a previous study described two mutant alleles of *clo1* with undistinguishable phenotypes from the wild type (Poxleitner *et al.*, 2006), so this reasoning is unlikely. For *VTE3*, the *vte3-2* mutant was shown to be soil lethal, so no homozygous lines could be isolated. The *albino or pale green mutant 1* (*apg1*) mutant is allelic to *vte3* and also soil lethal. This lethality is caused by a lack of plastoquinone (PQ), which prevents these plants from performing photosynthesis and autotrophic growth (Cheng *et al.*, 2003; Motohashi *et al.*, 2003). For *LOS1*, T-DNA insertion lines could only be isolated either at the promoter or the 3'UTR regions of the coding sequence (**Figure 10**). All the other lines carrying an insertion located within the gene coding sequence (including introns) could not be isolated, which suggests that this gene is essential for the correct growth of the plant or for a developmental process during seed formation. For the isolated *los1* alleles, qPCR evaluation of gene expressions showed no strong differences in expression levels (**Supplemental Figure 3**). This is why the longevity phenotype of *los1-3* was not assessed.

Homozygous T-DNA insertion lines for *ATPrel*, *LOS1*, *PLAT1*, *PER1* and *TRX3* were evaluated and showed no differences in sensitivity to artificial ageing (**Figure 11**). From all tested candidates, only *PER1* was previously linked to seed longevity. Specifically, it was shown that ectopic expression of *Nelumbo nucifera NnPER1* in Arabidopsis Col-0 enhanced seed resistance to artificial ageing (Chen *et al.*, 2016). However, our results showed no significant differences between *per1* mutants and Col-0 (**Figure 11A**).

One drawback in our experimental approach is functional redundancy. All genes chosen to evaluate their possible role in seed longevity belong to gene families. Therefore, when evaluating the phenotype of a single mutant, it is always possible that their function is being taken over by another member from the same gene family.

However, it is also possible that the chosen candidates do not contribute to the establishment of seed longevity, despite their ability to interact *in vivo* with DOG1.

Characterisation of seed longevity in heterozygous lines of *vte3-2* showed that this trait is impaired (**Figure 12A**). *VTE3* is a methyltransferase that catalyses the methylation of 2-methyl-6-phytylbenzoquinone (MPBQ) to yield 2,3-dimethyl-5-phytylbenzoquinone (DMPBQ). In addition, it participates in the biosynthesis of plastoquinone (Cheng *et al.*, 2003). We demonstrated that transgenic seeds overexpressing *VTE3* had reduced longevity (**Figure 12B**). Our results illustrate that either reduced or enhanced expression of *VTE3* led to reduced resistance to artificial ageing and that this reduction could not be linked with altered ABA perception or seed coat permeability (**Figure 12** and **Figure 13**). The *vte3-2* mutant only accumulates β- and δ-tocopherols, whereas transgenic lines overexpressing *VTE3* in seeds were reported to have increased levels of α- and ɣ-tocopherols (Van Eenennaam *et al.*, 2003). An altered composition of tocopherols was shown to influence the expression of transcriptional networks related to defence responses and ethylene and jasmonic acid (Sattler *et al.*, 2006b; Cela *et al.*, 2011). It is possible that our *VTE3*-overexpressing lines caused a similar effect resulting from enhanced accumulation of tocopherols that, in turn, had a detrimental effect on seed longevity. Anyhow, further analyses of the tocopherol levels and transcriptional responses in these lines will be required to confirm this. Likewise, *VTE3* is involved in the biosynthesis of plastoquinone, and the available pools of this compound were shown to be responsive to oxidative stresses, provided that they act as antioxidants (Szymańska and Kruk, 2010). In addition, the redox state of these pools was demonstrated to regulate gene expression under stress conditions (Adamiec *et al.*, 2008). Taken together, these observations may indicate that altering the expression of *VTE3* causes a modification of gene expression, as it affects both pools of tocopherols and plastoquinone.

From our observations, the role of the interaction between DOG1 and VTE3 in the regulation of seed longevity is yet unclear, apart from both *dog1* and *vte3* mutants having reduced seed longevity (**Figure 11** and **Figure 12**). A search through the transcriptome of *dog1-1* described in section **3.1.2.2** revealed that *VTE3*, *VTE4* and *VTE5* are differentially upregulated in the *dog1-1* mutant, which suggest that *DOG1* could affect transcription of these genes. *VTE3*, *VTE4* and *VTE5* participate in the biosynthesis of tocopherols in seeds, which requires compounds originating from chlorophyll degradation (Valentin *et al.*, 2006; vom Dorp *et al.*, 2015). Chlorophyll degradation during seed maturation is regulated by *ABI3*, as *abi3* mutant seeds retain chlorophyll (Ooms *et al.*, 1993). *ABI3* was shown to genetically interact with *DOG1*

(Dekkers *et al.*, 2016). However, transcriptome analyses of *abi3* mutant seeds did not find altered expression of these *VTE* genes (González-Morales *et al.*, 2016). Further research will be necessary to determine the exact role of the interaction between DOG1 and VTE3 and the possible cause of the transcriptional misregulation we observed.

We have shown that DOG1 is able to interact *in vivo* with several proteins involved in different processes. However, from all evaluated candidate genes, only *VTE3* showed an effect on seed longevity. Previous work proposed a role for *DOG1*, beyond its involvement in seed dormancy, as a regulator of seed development, considering that it interacts genetically with *ABI3* and affects the expression of other regulators such as *ABI5* (Dekkers *et al.*, 2016). A recent study showed that *DOG1* interacts *in vivo* with PP2C phosphatases, regulating seed dormancy release in Arabidopsis (Née *et al.*, 2017). Our data confirmed the ability of *DOG1* to physically interact with several proteins *in vivo*, which could be an additional function to this regulatory role. Further research will be necessary, nonetheless, to determine the biological meaning and importance of these interactions.

4.7 THE ROLE OF THE FLOWERING REPRESSORS *FLC* AND *FRI* IN THE REGULATION OF SEED LONGEVITY IN ARABIDOPSIS

We have explored the possible contribution of *FRI* to seed longevity, as it was previously shown that an introgressed fragment from Sha that contained this gene enhanced seed longevity in the longevity-deficient mutant *lec1-3* (Sugliani *et al.*, 2009). Complementation assays using the other genes contained within this chromosomal fragment showed no enhancement of seed longevity, whereas *FRI* showed fluctuating results between experiments. However, evaluation of genetic material carrying different allele combinations of this gene showed no differences in terms of seed dormancy or longevity (**Figure 14**), which prompted the idea that both *FRI* and *FLC* have to be present to affect these traits. To avoid interferences in terms of flowering time, transgenic lines expressing each gene's coding sequence under the *DOG1* seed-specific promoter were constructed, but neither of the constructs caused an improvement of seed longevity (**Figure 15** and **Figure 16**).

Our results showed that the *DOG1* promoter could not confine the expression of *FLC* nor *FRI* to the seeds, as their expression was also detected in rosette leaves (**Figure 14**). However, previous work reported successful seed-specific expression of constructs under the control of this promoter (Nakabayashi *et al.*, 2012; Née *et al.*, 2017). Besides, expression of *FLC* under control of an heterologous promoter was previously reported to be functional, as illustrated by the observed delay in flowering time (Searle *et al.*, 2006; Sheldon *et al.*, 2008). The reason for this absence of seed-specific expression in the transgenic lines described in this thesis is not clear.

We have shown that ectopic expression of *FLC* leads to reduced levels of seed dormancy and seed longevity, whereas flowering time remained unaltered (**Figure 15**). We evaluated if these seed phenotypes were related to an altered sensitivity to exogenous ABA, but we observed no differences compared to the *flc-3* control (**Supplemental Figure 4**). A previous study showed that *FLC* participates in the regulation of seed germination at low temperatures (Chiang *et al.*, 2009), a role that a second report linked to the levels of seed dormancy and the germination-promoting conditions present (Blair *et al.*, 2017). In both reports, the authors showed that enhanced expression of *FLC* causes increased germination. Our results are in agreement with this, as we observed reduced levels of seed dormancy and high *FLC* expression in our transgenic lines. However, none of these reports explored the contribution of *FLC* to seed longevity. We have shown that increased levels of *FLC* expression led to a dramatic reduction in seed resistance to artificial ageing (**Figure 15B**), whereas ABA sensitivity remained unaffected (**Supplemental Figure 4**).

Therefore, we examined the expression of several *FLC* targets, in case they could explain the increased sensitivity to artificial ageing of the transgenic lines. Despite the high levels of expression of *FLC*, we observed a relatively minor modification of expression of its targets. Among all evaluated targets, only four of them showed the same trend in terms of regulation in all transgenic lines evaluated (**Figure 16**), although their possible contribution to seed longevity has yet to be examined. One possible candidate from this group is *NYE1*, considering it participates in the degradation of chlorophyll (Ren *et al.*, 2007), a process known to affect the resulting seed longevity. However, further research should evaluate this possible role of *NYE1*.

Previous work showed that overexpression of *FLC* in L*er* caused a delay of flowering time (Michaels and Amasino, 1999). However, other studies reported that Col-0 and C24 accessions overexpressing *FLC* controlled by a 35S promoter could flower earlier or at the same time as the wild type (Sheldon *et al.*, 1999; Ratcliffe *et al.*, 2001). This is a possible explanation for the absence of a flowering delay despite of the elevated expression levels of *FLC* we observed (**Figure 15**). The unaltered flowering time observed could also be caused by the expression of the construct at a slightly different stage of development during seed maturation. *FLC* is expressed throughout embryo development, but it is also expressed later during plant development (Sheldon *et al.*, 2008). On the other hand, *DOG1* expression is seed specific and peaks during seed maturation (Bentsink *et al.*, 2006; Nakabayashi *et al.*, 2012). Nevertheless, since the expression of the construct was not restricted to the seeds, this explanation seems unlikely.

The high levels of *FLC* expression detected in leaves could also be associated with the absence of *cis*-regulatory elements. As we wanted to remove as many flowering time regulatory elements as possible, we only cloned the CDS of the gene. Several reports have explored the complex regulatory mechanisms fine-tuning the expression of *FLC* and found that many of the sequences responsible for stably repressing *FLC* expression are located within the first intron of the gene (Bastow *et al.*, 2004; Li *et al.*, 2014). This first intron also encodes the non-coding RNA COLDAIR, which recruits chromatin-modifying complexes that further repress *FLC* expression (Heo and Sung, 2011). Furthermore, the promoter of this gene also carries sequences which are necessary for vernalisation-independent expression of *FLC* (Sheldon *et al.*, 2002). For *DOG1*, it is know that its promoter carries RY repeats, involved in the seed-specific expression and *ABI3* regulation and ABA-response elements (ABRE) motifs, which are linked to ABA responses through *ABI5* (Bentsink *et al.*, 2006; Graeber *et al.*, 2010). The strong increase in *FLC* expression in rosette leaves could originate from

the altered regulation of gene expression introduced with the promoter switch, which drove *FLC* expression in a manner that we did not anticipate.

The aim of this section was to evaluate the possible role of the *FRI* allele from the Sha accession in the regulation of seed longevity. However, a mistake was made during the construction of these lines that led to the cloning of an allelic version identical to that of Col-0. This accession was reported to carry a 16 bp deletion that introduces an early stop codon and generates a truncated version of the protein (Johanson *et al.*, 2000). This truncated version is the one we cloned and constructed our transgenic lines with. Therefore, no conclusions on the role of the Sha allele of *FRI* on seed longevity can be drawn. A first evaluation using genetic material carrying the Sf-2 allele showed no effect on seed longevity (**Figure 14**), despite it is also a functional *FRIGIDA* (Johanson *et al.*, 2000). However, the effect of the Sha allele should be determined experimentally prior to any further conclusion, as these alleles differ in several SNPs. Previous work found different effects of *FRI* on the germination behaviour depending on the background accession. Chiang *et al.* described that *FLC*-mediated germination did not require the action of *FRI* in a L*er* background (Chiang *et al.*, 2009). Conversely, Blair *et al.* (2017) argued that this apparent lack of effect of *FRI* on germination resulted from the Cvi allele of *FLC* used, which does not require *FRI* to enhance its expression (Gazzani *et al.*, 2003). Moreover, this work reported that, in a Col background, disruption of *FLC* only affected seed germination in the presence of a functional *FRI* and that without a functional *FLC*, disruption of *FRI* slightly improved the germination behaviour (Blair *et al.*, 2017), an effect also confirmed on a second report (Auge *et al.*, 2017)

It is also possible that the observed enhancement of seed longevity was not caused by any of the genes present in the introgressed region alone but from a combination of them. Further studies should evaluate how different sub-fragments of this introgressed fragment affect seed longevity. Lastly, the presence of several long non-coding RNA (lnRNA) within this region should not be overlooked. These transcripts, such as At4g03675 or At4g03685, could underlie the initial observations. Anyhow, further investigation will be required to determine the basis of the observed enhancement of seed longevity initially reported (Sugliani *et al.*, 2009).

4.8 THE INFLUENCE OF TEMPERATURE DURING SEED DEVELOPMENT AND OF ARTIFICIAL AGEING ON NUCLEAR SIZE AND CHROMATIN COMPACTION IN EMBRYOS OF ARABIDOPSIS

In this section, we evaluated whether changes in nuclear traits can be used as predictors of seed quality. Specifically, we evaluated how different temperature regimes applied during seed maturation, in combination with artificial ageing, affected seed nuclear size and the degree of chromatin compaction. Additionally, we established the effect of seed imbibition on these traits, in comparison with dry seeds. Previous studies showed that dry seeds of Arabidopsis are characterised by reduced nuclear size and increased chromatin condensation (van Zanten *et al.*, 2011). Furthermore, they identified that the shrinkage of the nucleus is developmentally controlled, in part by *ABI3*, as a part of the maturation drying process that seeds undergo. Upon germination, nuclear size increases again, a process partially contributed by *LITTLE NUCLEI 1* (*LINC1*) and *LINC2* (van Zanten *et al.*, 2011).

A clear distinction between the bright, stained heterochromatic chromocenters and the darker euchromatic background was not achieved (**Figure 18**), which did not allow further determination of the degree of chromatin compaction imposed by the evaluated stresses. The absence of clearly distinguishable heterochromatin could have been caused by the specific growing conditions of the plants during seed maturation or by the fact that we analysed nuclei isolated from the entire embryo, whereas previous work restricted these analyses to embryonic cotyledons (van Zanten *et al.*, 2011).

Nuclear sizes of embryos that matured under standard and high temperatures were similar in dry seeds, but imbibed seeds matured at high temperatures had bigger nuclei than those matured at control temperatures (**Figure 19**), similar to what we observed in imbibed seed that matured at lowered temperatures. It is possible that the nuclei of seeds matured either at low or high temperatures enlarge faster during imbibition compared to seeds matured at control temperatures. As the increase in nuclear size was previously linked with germination (van Zanten *et al.*, 2011), it could be connected with faster germination of seeds grown at these temperature regimes. However, this faster increase in size cannot be associated with improved seed quality because seeds matured at lower temperatures had lower quality and high dormancy whereas seeds matured at high temperatures showed a similar performance compared to seeds matured at control temperatures (**section 3.1.1**). Artificial ageing of seeds caused a decrease in nuclear size expansion upon

imbibition (**Figure 19**). This could be explained by the decreased germination capacity of the aged seeds, which after imbibition, instead of commencing the process or germination, are committed to cellular damage repair and is in agreement with previous reports which showed that aged seeds exhibit reduced germination speed and rate (Rajjou *et al.*, 2008b).

Imbibition of seeds for 72 h had the strongest effect on nuclear size, whereas maternal temperatures and artificial ageing exerted a relatively small impact on this trait. Strikingly, 81 % of the observed variability could not be explained by the variables we studied (**Table 11**). Since we could not evaluate the levels of chromatin compaction, it is possible we missed substantial information. High levels of chromatin compaction are associated with reduced levels of transcription (Fransz and De Jong, 2011). Environmental cues, such as light, were reported to be able to affect chromatin organisation and transcriptional activity (Bourbousse *et al.*, 2015). Therefore, it is possible that the maternal environments at which plants were grown exerted a modification on the chromatin landscape that we did not detect. Therefore, new studies will be required to establish a more comprehensive model that can better explain the observed variability in nuclear size and that includes an evaluation of chromatin compaction levels.

4.9 Assessment of seed quality in Arabidopsis by Activity-Profiling of Proteases

We have evaluated the feasibility of activity-profiling of proteases as a tool to monitor levels of seed quality. To this end, we used artificially aged seeds from the Arabidopsis accessions Col and L*er* and looked for differential labelling in protein extracts from these seeds. Seed germination after different periods of artificial ageing was used as a proxy for seed quality (**Figure 20**), considering artificial ageing reduces seed vigour and overall seed quality (Delouche and Baskin, 1973; McDonald, 1998).

ABPP probes are designed to covalently bind the active site of target enzymes in an activity-dependent manner, usually by substrate-mimicking or the use of tagged inhibitors (Morimoto and Van Der Hoorn, 2016). This means that if, for any reason, the protein loses its conformation or the active site becomes blocked, the labelling will not occur. Apart from the fragment binding the active site, these probes usually carry reporter tags that allow downstream analyses of the labelled proteome. In this work, we evaluated the activity-dynamics of several protease families during seed ageing, as they are involved in a wide array of processes within the cell (van der Hoorn and Kaiser, 2012).

We evaluated four biochemical probes targeting different subsets of plant proteases (**Table 13**) and found that two of them exhibited a differential pattern of labelling. However, this labelling was only observed when seeds had been imbibed for 24 h prior to protein isolation and labelling, whereas labelled protein extracts from dry seeds showed no differences. This could mean that imbibition and restoration of the metabolic activity of the cell are required to observe the changes induced by artificial ageing. In line with this, some proteases were shown to be synthesised in an inactive form, resulting from a propeptide masking the active site (Kuroyanagi *et al.*, 2002). This propeptide is removed during translation, a process requiring enzymatic activities and therefore a hydrated cytoplasm.

We observed that the activity of serine hydrolases is reduced during the ageing treatment in both Col and L*er* accessions (**Figure 21**). This is in agreement with previous reports that described the different damages incurred on the seed proteome during ageing (Rajjou *et al.*, 2008b). As a result of the oxidative damage caused by seed ageing, protein denaturalisation and carbonylation occur, so the observed loss of activity is possibly caused by these processes, although this should be experimentally confirmed.

The FP probe used labelled several enzymes. To determine the identity of the characterised bands, we conducted a pull-down assay using a biotin-tagged version of the FP probe. Our initial attempt identified 20 different serine hydrolases in protein isolates from non-aged seeds compared to an identical non-labelled sample (**Table 14**). However, the MS analysis showed that several of these candidates were also enriched in the non-labelled control (**Supplemental Figure 7**), which suggests that some mistake happened during sample preparation. For this reason, we will repeat this experiment using a differential-enrichment approach. We will employ non-aged and 12 days aged seeds, in order to determine those bands whose abundance is heavily reduced or disappear after the ageing treatment. This will allow to confirm the identity of these proteases.

Contrary to the pattern observed for serine hydrolases, VPEs showed a pattern of increasing activity during the ageing treatment (**Figure 22**). From these observations, we tried to elucidate which VPE was responsible for the increased activity. However, artificial ageing of seeds of different *vpe* mutants and subsequent labelling did not provide a clear answer (**Figure 23**). Previous studies showed that VPEs have redundant functions in protein processing and they can take over the role of others (Gruis *et al.*, 2002; Shimada *et al.*, 2003; Gruis *et al.*, 2004) and a similar compensation effect was reported in programmed cell death (PCD) responses (Rojo *et al.*, 2004). It is likely that a similar process happened in the responses induced upon artificial ageing and more than one VPE is involved in the observed activity. Furthermore, we confirmed that the increase in VPE activity also occurred in naturally aged seeds and was not an artifact caused by the artificial ageing treatment (**Figure 24**). The lack of a seed longevity phenotype in the *vpe* mutants is probably observed because they play no part in the acquisition of this trait. Even mutants in ɣ-VPE, required for seed coat development, or the quadruple *vpe* mutant are indistinguishable from the wild type (Nakaune *et al.*, 2005), which suggests VPEs do not contribute to seed longevity.

The observed increase in VPE activity can arise from the fact that ageing damages produce hazardous compounds within the cell, which would act as a trigger to enhance VPE activity in order to process or isolate them into the vacuole (Kinoshita *et al.*, 1999; Hatsugai *et al.*, 2015). Several studies have described that, apart from their role during seed maturation, VPEs play a role during PCD, both developmentally-regulated (Nakaune *et al.*, 2005) or as a result of pathogen attacks, including hypersensitive responses (Hatsugai, 2004; Kuroyanagi *et al.*, 2005; Hatsugai *et al.*, 2015). PCD processes can be elicited by vacuolar collapse (Hatsugai *et al.*, 2006) and VPE-silenced lines were shown to not commence tonoplast disintegration and

subsequent cell death (Hatsugai, 2004). The ageing process in seeds was also connected to PCD responses, considering DNA laddering, RNA degradation and membrane leakage were observed (Kranner *et al.*, 2006; El-Maarouf-Bouteau *et al.*, 2011; Hu *et al.*, 2012; Chen *et al.*, 2013). Interestingly, a recent study found decreasing VPE activities in senescing leaves of Arabidopsis (Pružinská *et al.*, 2017), although leaf senescence and PCD are not regulated in the same manner (Schippers *et al.*, 2015). Given the role of VPEs as proteases, it is conceivable they are contributing to process damaged proteins or that they are becoming active as a part of PCD responses, but this is not clear yet.

The two ABPP probes identified in Arabidopsis seeds were further evaluated in seeds of eight wild species (**Table 15**). We confirmed VPE-like activities in seeds of most of these species, in agreement with previous reports that labelled them in leaf extracts (Misas-Villamil *et al.*, 2013; Lu *et al.*, 2015). However, we did not observe the same pattern of increased activity in aged seeds (**Figure 25**) that was identified in Arabidopsis. This was possibly caused because we mistakenly used dry instead of imbibed seeds. Similarly, we could not identify a reduction of activity of serine hydrolases. From these observations, we confirmed that both probes are functional in protein extracts of other species. However, since no differential labelling was observed, we cannot conclude if they are valid to monitor seed quality in these species. Further research should characterise the labelling of 24 h-imbibed seeds to determine if the patterns observed in Arabidopsis are common to the studied wild species. We speculate with the possibility that the ageing process in these species could differ from that of Arabidopsis. Further investigation will be required to determine the optimal conditions of labelling in these species to establish robust markers of seed quality.

From these results, we conclude that two markers of seed quality in seeds of *Arabidopsis thaliana* were identified. The activities labelled by these two markers exhibited opposite trends, which could allow for a combination of these probes in order to achieve a more refined approach to determine levels of seed quality. In addition, several serine hydrolase activities were identified which could be linked to the initial levels of seed quality, although these connections remain to be determined.

4.10 Final remarks

The research presented in this PhD thesis has explored how seed longevity in *Arabidopsis thaliana* is controlled by both genetic and environmental factors. The temperatures applied during seed maturation altered the seed transcriptome. Specifically, lower temperatures, which resemble those faced by plants when growing in their natural environments, were able to induce differential expression of transcripts which can alter the seed coat composition. Even though some reports addressed some of these effects (MacGregor *et al.*, 2015; Fedi *et al.*, 2017), our study identified auxin and GA responses in response to cold, which may suggest additional mechanisms through which the environment can shape seed longevity.

We have described how drought applied during seed maturation, despite its overall detrimental effect on seed yield, increased seed quality in two studied genotypes. However, we could not determine the specific mechanisms that underlie this enhancement of seed resistance to artificial ageing. It seems that modifications of the seed cell walls originate from the treatment, but each genotype behaves differently, whereas their transcriptomic response was similar. Nevertheless, this opens a new field of opportunity to explore how water scarcity experienced by the plant can improve seed longevity, protecting the plant's offspring.

This thesis has documented the interaction between DOG1 and several proteins in Arabidopsis, including VTE3. Although additional experiments should address the exact subcellular localisation of these interactions, our observations support the proposition of DOG1 as a master regulator of seed development (Dekkers *et al.*, 2016), beyond its role in seed dormancy.

Our results could not determine the role of the Sha allele of *FRIGIDA* in the regulation of seed longevity in Arabidopsis. Nonetheless, as previous reports showed it can effect on seed germination independently from *FLC*, this possibility remains open (Blair *et al.*, 2017). However, we showed that overexpression of *FLC*, a repressor of the flowering transition in Arabidopsis, does alter seed longevity. This effect was not previously reported and adds an additional layer to the already complicated regulation that governs this locus (Rouse *et al.*, 2002; Deng *et al.*, 2011; Wu *et al.*, 2016).

We identified two probes which label two different enzymatic activies with opossed patterns of activity in aged seeds. This qualifies them as markers of seed quality in Arabidopsis. Adittionally, we confirmed that the activities identified were

also present in seeds of other eight wild species, which opens the possibility of using the marker probes in a wider range of species to evaluate seed quality.

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6 APPENDIX

Supplemental Table 1. Arabidopsis mutants and T-DNA insertion lines used in this thesis.

Locus	Allele	NASC code	ID	Reference
At5g45830	*dog1-1*	-	-	Bentsink *et al.* (2006)
	dog1-2	-	-	Nakabayashi *et al.* (2012)
At4g26740	*clo1*	N355586	GABI_823D08	
At5g42980	*trx3-1*	N682779	SALK_111160	Yamamoto and Nasrallah (2013)
	trx3-2	N877494	SAIL_864_G11	
At2g24420	*ATPrel*	N682426	SALK_061840	
At4g39730	*plat1-1*	N656873	SALK_112728	
At3g63410	*vte3-2*	N605903	SALK_105903	Cheng *et al.* (2003)
At1g48130	*per1-1*	N653878	SALK_133714	
	per1-2	N653600	SALK_036808	
At1g56070	*los1-1*	N24936	-	Guo *et al.* (2002)
	los1-2	N671080	SALK_004229	
	los1-3	N504041	SALK_004041	
	los1-4	N804803	SAIL_100_B12	
	los1-5	N834645	SAIL_775_C02	
	los1-6	N831700	SAIL_715_B12	
At2g25940	α-*vpe*	-	-	Gruis *et al.* (2002)
At1g62710	β-*vpe*	-	-	
At4g32940	ɣ-*vpe*	-	-	
At3g20210	δ-*vpe*	-	-	

Supplemental Table 2. Primers used in this work. For each primer, the purpose (P) is indicated: cloning (C), sequencing (S) or genotyping.

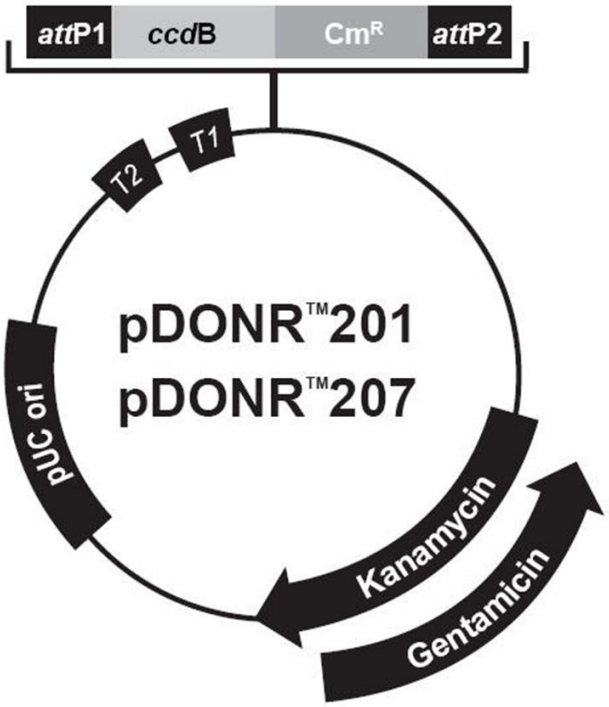
Primer name	Target	P	Sequence 5'-3'
pDOG1-F	At5g45830	C	AGGCTACCAAATTGTTTGTGCATGCTTCAG
pDOG1-Adapt-R	At5g45830	C	AAGGGACTGACCACCGATCTCTTTTGGTTTGCGTGTTTGTG
pDOG1_2F	At5g45830	S	GATCACCACCACTACTATAC
pDOG1_3F	At5g45830	S	GTGTCGAACTATCCTCATAC
pDOG1_4F	At5g45830	S	GTACAATCCGCTGTCTCAGGACATC
pDOG1_5F	At5g45830	S	GGAACAACAACTCGCACTCTC
pDOG1_6F	At5g45830	S	GACATTTGTCATTGTTTCCC
Adapt-FLC-F	At5g10140	C	GGTGGTCAGTCCCTTATGGGAAGAAAAAAACTAGAAATC
FLC-R	At5g10140	C	CTAATTAAGTAGTGGGAGAGTCACC
Adapt-FRI-F	At4g00650	C	GGTGGTCAGTCCCTTATGTCCAATTATCCACCGACGGTG
FRI-2R	At4g00650	C	CTATACCTGAGACCATAGGGAC
At5g42980 LP1	At5g42980	G	AGGTTTGAATTGTCCCCAATC
At5g42980 RP1	At5g42980	G	ATTGAAATTCCTCATGGCCTC
At5g42980 LP2	At5g42980	G	GCTGCGAGTAATCAAGTTTGC
At5g42980 RP2	At5g42980	G	ACCGACACAGAGACGAAGAAG
At4g26740 LP	At4g26740	G	GACAAAACCATCAAAAATTTCG
At4g26740 RP	At4g26740	G	ACGACCCAAGAAAGCTTTTTC
At4g39730 LP	At4g39730	G	ATGGATGGGTCCTAATCGATC
At4g39730 RP	At4g39730	G	GCTCGTGCTTGAGCTTTACTC
At2g24420 LP	At2g24420	G	ACACCAGTGACAGAACATCCC
At2g24420 RP	At2g24420	G	TTTGCAGAAAAATGCCAAAAC
At3g63410 LP	At3g63410	G	CAAACACACACTGTGGGAATG
At3g63410 RP	At3g63410	G	AACACTGTTGCTATGGTTGGG
At1g56070 LP	At1g56070	G	GACAAAACCATCAAAAATTTCG
At1g56070 RP	At1g56070	G	ACGACCCAAGAAAGCTTTTTC

LOS1_RP2	At1g56070	G	TCCCTTGTACAACATCAAGGC
LOS1_LP2	At1g56070	G	AACGAGTCAATGGACGTGTTC
VTE3_1Fw	At3g63410	C	ATGGCCTCTTTGATGCTC
VTE3_1Rv	At3g63410	C	TTAGATGGGTTGGTCTTTGG
LOS1_1Fw	At1g56070	C	ATGGTGAAGTTTACAGCTGATG
LOS1_1Rv	At1g56070	C	TTAAAGCTTGTCTTCGAACTC
CLO1_1Fw	At4g26740	C	ATGGGGTCAAAGACGGAGATG
CLO1_1Rv	At4g26740	C	TTAGTAGTATGCTGTCTTGTCTTCAC
ATPrel_1Fw	At2g24420	C	ATGGCGGCCGCGAAACTC
ATPrel_1Rv	At2g24420	C	TCATTTGTCAGAATGACCCC
PLAT1_1Fw	At4g39730	C	ATGGCTCGTCGCGATGTTC
PLAT1_1Rv	At4g39730	C	TTAAACGACCCAAGAAAGC
PER1_1Fw	AT1G48130	C	ATGCCAGGGATCACACTAGG
PER1_1Rv	AT1G48130	C	TCAAGAGACCTCTGTGTGACG
TRX3_1Fw	At5g42980	C	ATGGCCGCAGAAGGAGAAG
TRX3_1Rv	At5g42980	C	TCAAGCAGCAGCAACAACTG
SALK_LBb1.3	-	G	ATTTTGCCGATTTCGGAAC
SAIL LB2	-	G	GCTTCCTATTATATCTTCCCAAATTACCAATACA
GABI T-DNA	-	G	CCCATTTGGACGTGAATGTAGACAC
SelA	pDONR201	S	TCGCGTTAACGCTAGCATGGATCTC
SelB	pDONR201	S	GTAACATCAGAGATTTTGAGACAC
attB1-F	-	C	GGGGACAAGTTTGTACAAAAAAGCAGGCT
attB2-R	-	C	GGGGACCACTTTGTACAAGAAAGCTGGGT

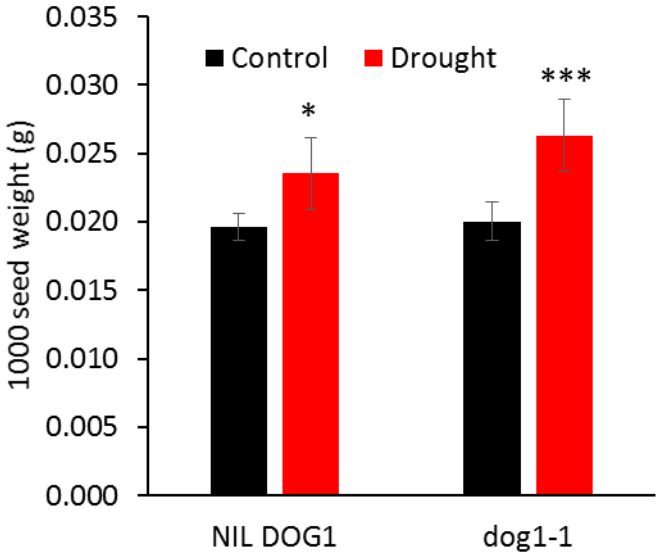
Supplemental Table 3. Primers used to evaluate relative expression in qPCR assays.

Primer name	Target	Sequence 5’-3’
ACT8_qPCR_2Fw	At1g49240	GCAGACCGTATGAGCAAAGAG
ACT8_qPCR_2Rv	At1g49240	ACATCTGCTGGAAAGTGCTG
At4g12590_qPCR_Fw	At4g12590	CTTCAGCAACGAGGAGAATGG

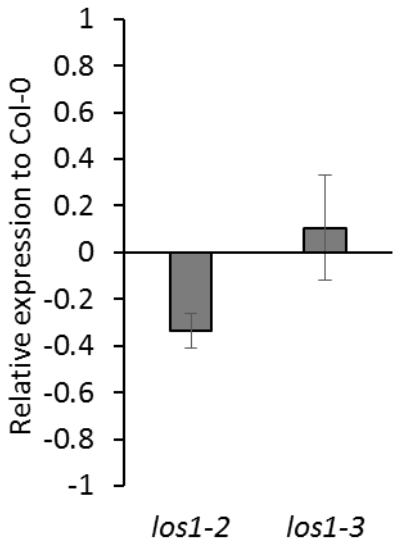
At4g12590_qPCR_Rv	At4g12590	AGAAGAAGAAGTTGACCCATGC
At1g16970_qPCR_Fw	At1g16970	AGAGCTAAGGATGCACAAGACC
At1g16970_qPCR_Rv	At1g16970	CTTCAGTTGGTCCTTCATGTCC
VTE3_qPCR_1Fw	At3g63410	AGTCGCCACATCAGCTGG
VTE3_qPCR_1Rv	At3g63410	GGCCAGTACTCAATGCTTCC
LOS1_qPCR_1Fw	At1g56070	GACCACGGGAAATCCACTC
LOS1_qPCR_1Rv	At1g56070	CACGTTCAGCCTCATCAGC
FLC_qPCR_2Fw	At5g10140	AGCCAAGAAGACCGAACTCA
FLC_qPCR_2Rv	At5g10140	TTTGTCCAGCAGGTGACATC
FRI_qPCR_2Fw	At4g00650	ATCTGAACAGCGACGAAGAG
FRI_qPCR_2Rv	At4g00650	CGCAGCTAATTCGTCTATGG
FT_qPCR_Fw	At1g65480	ATCATCACCGTTCGTTACTCG
FT_qPCR_Rv	At1g65480	ACAACTGGAACAACCTTTGGC
SOC1_qPCR_Fw	At2g45660	ATTCGCCAGCTCCAATATGC
SOC1_qPCR_Rv	At2g45660	TGAGCTGCTCAATTTGTTCC
DIN10_qPCR_Fw	At5g20250	CTTCTTCGCTTTCTGGCATTG
DIN10_qPCR_Rv	At5g20250	CGAACCGCCGGTTTAATCGT
DREB1B_qPCR_Fw	At4g25490	GGTTTCTGAAGTGAGAGAGCC
DREB1B_qPCR_Rv	At4g25490	GCGAAGTTGAGACATGCTGA
DREB1A_qPCR_Fw	At4g25480	TCGTCACCCAATATACAGAG
DREB1A_qPCR_Rv	At4g25480	GGTTTGAAATGTTCCGAGCC
SPL15_qPCR_Fw	At3g57920	CGCTCCATCTCTTTACGGAAAC
SPL15_qPCR_Rv	At3g57920	GAGATCCTACTGCGTGGTCAA
SMZ_qPCR_Fw	At3g54990	GGGAATCTCATATTTGGGATTG
SMZ_qPCR_Rv	At3g54990	CATGAGTTTTGAATTGGGTGCA
bZIP44_qPCR_Fw	At1g75390	CTTAACCACCGTCTCCAATCTC
bZIP44_qPCR_Rv	At1g75390	ACCGTCGAATAATCCCTGAC
bZIP49_qPCR_1Fw	At3g56660	TGCATTCCTCCTGACGCTAC
bZIP49_qPCR_1Rv	At3g56660	CACAATCGCCTGAAATTCCC
CRF1_qPCR_Fw	At4g11140	CGGCGGCGATTTATTTGGAG
CRF1_qPCR_Rv	At4g11140	GTTAAGACAGGATCCGACCCG
ANAC055_qPCR_Fw	At3g15500	ACGCGCTGCCTCATAGTC
ANAC055_qPCR_Rv	At3g15500	GAGTTCTGTCCAATCAAATTCGC
CDCP2_qPCR_Fw	At4g36910	AGCTCCACTAGTTGTTGAGGAA
CDCP2_qPCR_Rv	At4g36910	GGCTCTAACCACGTTTCCTCT
GA20ox1_qPCR_Fw	At4g25420	TACTTTCATGGCTCTATCGAACG
GA20ox1_qPCR_Rv	At4g25420	TGGAGAGTGTTCATGTCTGCT
CYP707A2_qPCR_Fw	At2g29090	ACTCGCCAAAACAAGTACGG
CYP707A2_qPCR_Rv	At2g29090	GCCTCTGGTCCAATCATACGC



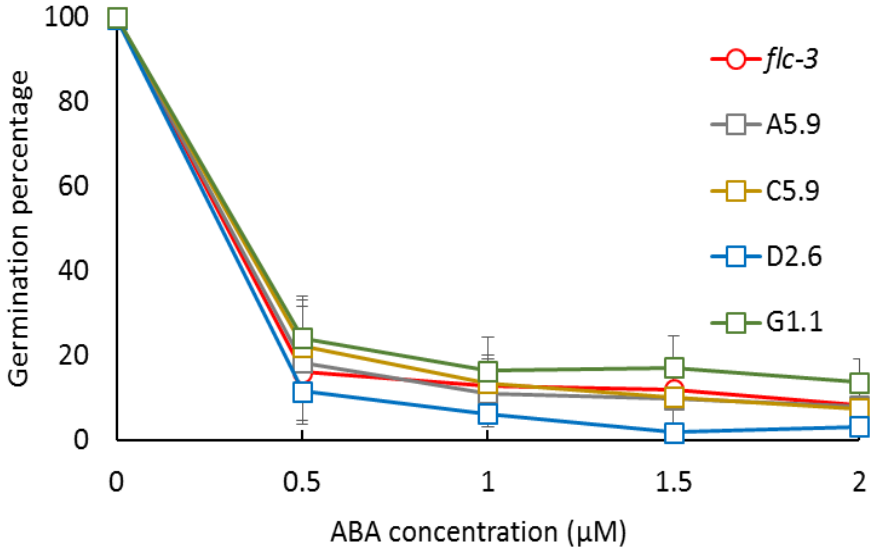
Supplemental Figure 1. Schematic map of the entry vectors pDONR201 and pDONR207. pDONR201 carries a cassette for kanamycin resistance whereas the selectable marker in pDONR207 is gentamicin. Location of the Gateway® recombination cassette is highlighted. Taken form the Invitrogen Gateway® pDONR™ Vector manual.



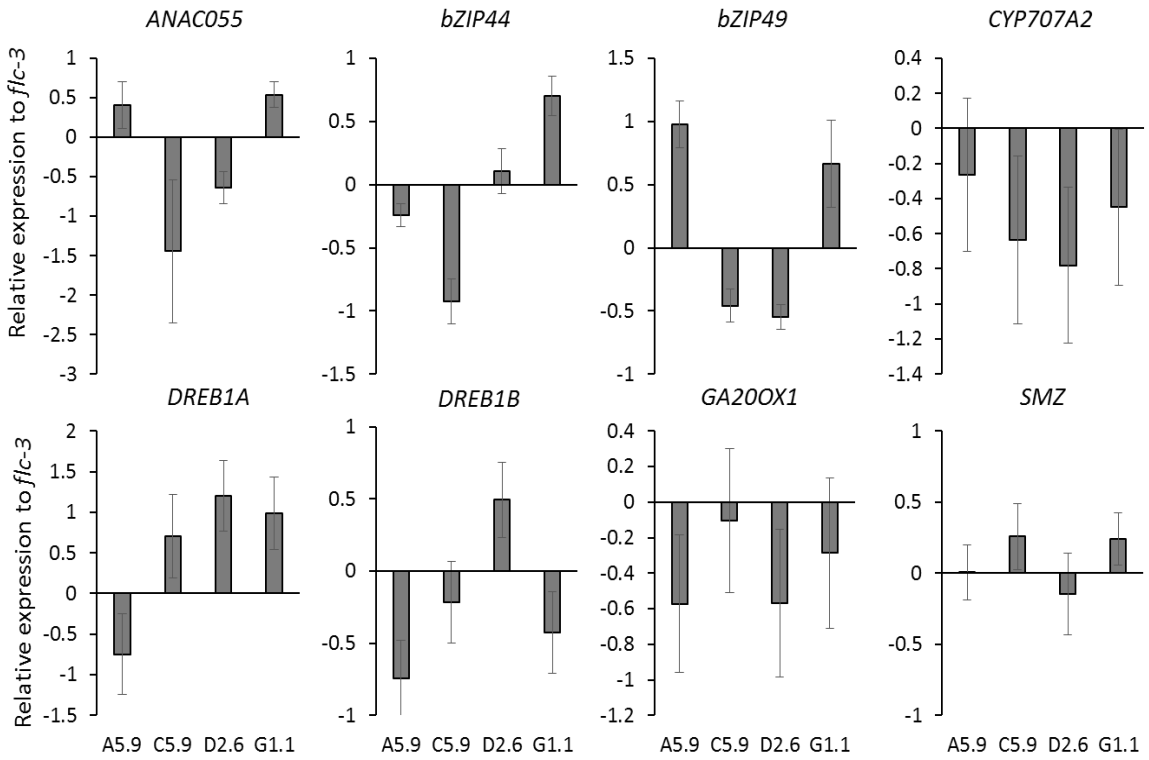
Supplemental Figure 2. Drought applied during seed maturation caused an increase in dry seed weight. Comparison of the 1000 seed weight between control and drought-treated dry seeds of NIL DOG1 and *dog1-1*. Statistical significance after a two-tailed T-test is represented by * ($p<0.05$) and *** ($p<0.001$). Each data point represents the mean of three biological replicates and error bars represent the standard deviation. All data presented in this figure was produced by Dr Sajjad Awan at the University of Warwick, United Kingdom.



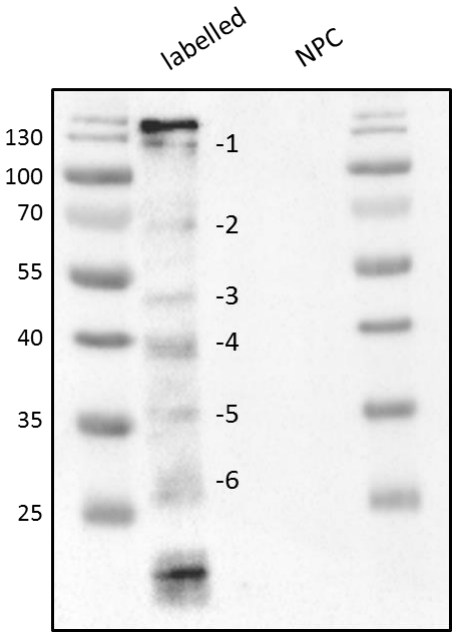
Supplemental Figure 3. Relative expression of *LOS1* in the T-DNA insertion lines *los1-2* and *los1-3*. Relative expression of *LOS1* in dry seeds of the *los1-2* and *los1-3* lines compared to the wild type Col-0 (represented by the baseline 0). Values plotted correspond to the log_2-fold-change compared to Col-0. Each data point corresponds to the average of three biological replicates. Error bars represent the standard error of the mean.



Supplemental Figure 4. Germination of transgenic lines expressing the pDOG1$_{Cvi}$:*FLC*$_{Col}$ construct in increasing concentrations of ABA. Each data point corresponds to the average of five biological replicates and error bars represent standard deviation.

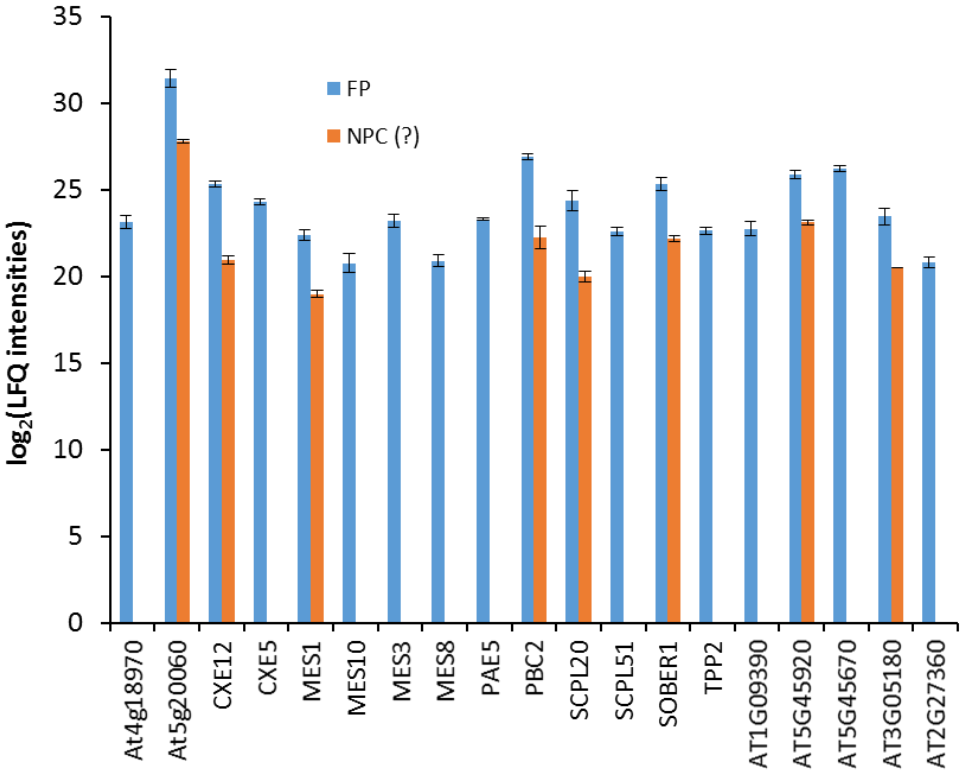


Supplemental Figure 5. Relative expression of *FLC* targets in four independent transgenic lines expressing the pDOG1$_{Cvi}$:*FLC*$_{Col}$ construct. Relative expression of the selected *FLC* targets in dry seeds of these transgenic lines compared to the *flc-3* background (represented as the baseline 0). Values plotted correspond to the log_2-fold-change compared to *flc-3*. Data points represent the mean value of at least two biological replicates. Error bars represent standard error of the mean.

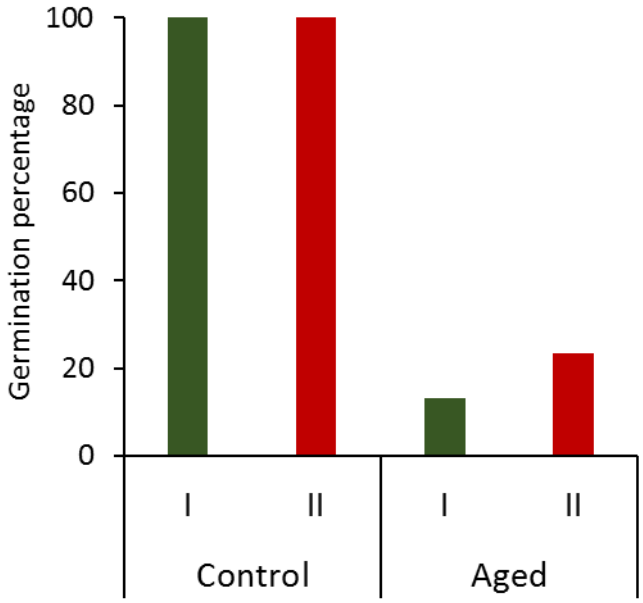


Supplemental Figure 6. Samples used for MS analyses of pulled-down proteins after FP-desthiobiotin labelling. Numbers 1-6 represent the bands that were excised from the gel

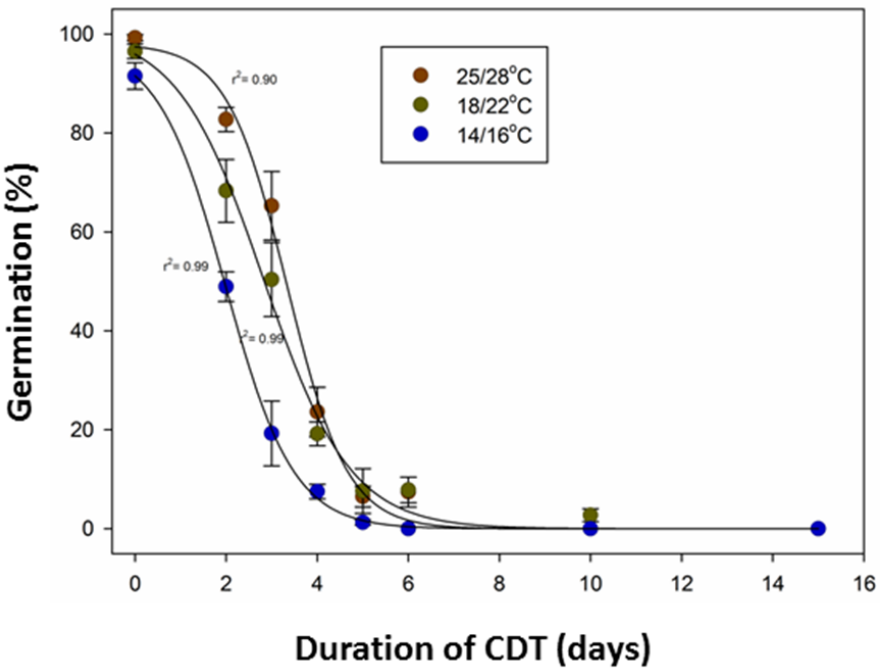
and sent for MS analyses. These bands were cut from both the labelled and the NPC samples. Molecular weights (in kDa) are indicated on the left side.



Supplemental Figure 7. Serine hydrolases identified after MS analyses of labelled (FP) and NPC samples. Some of the identified hydrolases were also enriched in the non-labelled control (NPC). All data presented in this figure was analysed by Dr Farnusch Kaschani at the University of Duisburg-Essen, Germany.



Supplemental Figure 8. Germination of *Brassica rapa* spp. *campestris* seeds. Germination of seeds from two technical replicates used for evaluation of ABPP probes before and after artificial ageing.



Supplemental Figure 9. Different temperatures during seed development affect sensitivity to artificial ageing. Seed longevity phenotypes of Col-0 in response to three different temperature regimes during seed maturation: low (16/14 °C, dark blue), control (18/22 °C, khaki) and elevated (28/25 °C, brown). Each data point represents the mean of three biological replicates and error bars correspond to standard deviation. All data presented in this figure was produced by Dr Sajjad Awan at the University of Warwick, United Kingdom.

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ERKLÄRUNG

"Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen – , die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist, sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Maarten Koornneef und Dr. Wim Soppe betreut worden".

Köln, September 2017

Natanael Viñegra de la Torre

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LEBENSLAUF

Persönliche Informationen

Name: Natanael Viñegra de la Torre

Geburtstdatum: 11.04.1988

Geburtsort: Logroño (La Rioja), Spanien

Ausbildung

2006: Abitur an der Sekundarschule Práxedes Mateo Sagasta in Logroño, Spanien.

2006 – 2011: Bachelorabschluss in Biologie an der Universidad de Salamanca in Salamanca, Spanien.

2011 – 2012: Masterabschluss in Agrobiotechnologie an der Universidad de Salamanca in Salamanca, Spanien. Titel der experimentellen Arbeit: "Characterisation of Plant Protein Interactions by Yeast Two-Hybrid and Bimolecular Fluorescence Complementation Assays".

2013: Forscher am Projekt: "Regulation of Leaf Abscission in London Plane Tree (*Platanus* x *acerifolia*)" in der Centro Hispano-Luso de Investigaciones Agrarias in Salamanca, Spanien. Leitung: Dr Óscar Lorenzo Sánchez.

2014: Doktorand am Max Planck Institut für Pflanzenzüchtungsforschung in Köln, in der Arbeitsgruppe von Dr Wim Soppe. Thema: "Genetic and environmental regulation of seed longevity in *Arabidopsis thaliana*".

Köln, September 2017