

# Role of mRNA post-transcriptional metabolism in the regulation of Arabidopsis thalian dormancy

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## UNIVERSITE PIERRE ET MARIE CURIE - PARIS 6

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## **Thèse de Doctorat en Sciences**

Physiologie Végétale

Présentée par

## **ISABELLE BASBOUSS-SERHAL**

## ROLE OF mRNA POST-TRANSCRIPTIONAL METABOLISM IN THE REGULATION OF ARABIDOPSIS THALIANA DORMANCY

## ROLE DU METABOLISME POST-TRANSCRIPTIONNEL DES ARNM DANS LA REGULATION DE LA DORMANCE DES SEMENCES D'ARABIDOPSIS THALIANA

## Soutenue le 26 Juin 2015

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## Résumé

## Rôle du métabolisme post-transcriptionnel des ARNm dans la régulation de la dormance des semences d'*Arabidopsis thaliana*.

Une étude physiologique nous a permis d'identifier l'influence de la température et de l'humidité relative (HR) lors du stockage des graines dormantes d'Arabidopsis. Après une levée de dormance atteinte en 7 semaines avec des cinétiques variables selon les conditions, on observe une induction de la dormance secondaire à faible HR et une perte progressive de la viabilité à forte HR. La levée et l'induction de la dormance sont associées à la régulation de gènes liés aux voies de l'acide abscissique et des gibbérellines. Nous avons étudié la dynamique d'association des ARNm aux polysomes et comparé la transcription et la traduction des graines dormantes et non dormantes au cours de l'imbibition. Nous montrons qu'il n'y a pas de corrélation entre transcriptome et traductome et que la régulation de la germination est principalement liée à la traduction. Ceci suppose un recrutement sélectif et dynamique des ARNm liés aux polysomes dans les graines dormantes et non dormantes. Certaines caractéristiques de la région 5'UTR des transcrits semble impliquées dans la sélection des ARNm traduits pendant la germination. Les phénotypes de mutants d'éléments du catabolisme des ARN montrent que la dormance est également associée à une dégradation sélective des ARNm. Les P-bodies (localisés dans des lignées YFP-DCP1) sont d'ailleurs en quantité plus importante dans les graines non-dormantes. La comparaison des transcriptomes des mutants vcs-8 et xrn4-5 a permis l'identification de plusieurs transcrits dégradés via VCS ou XRN4, dont le rôle sur la dormance a été confirmé par génétique inverse. Certains motifs spécifiques semblent être impliqués dans la sélection de transcrits pour leur dégradation.

#### Arabidopsis, dégradation des ARNm, dormance, exosomes, germination, P-bodies, postmaturation, température, traduction, 5'UTR

## Abstract

#### Role of mRNA post-transcriptional metabolism in the regulation of *Arabidopsis thaliana* dormancy.

A physiological study allowed us to reveal the effect of temperature and relative humidity (RH) during Arabidopsis seed storage. Seven weeks of after ripening lead to alleviation of dormancy with different kinetics according to the conditions. Longer storage induced an induction of secondary dormancy at low RH and progressive loss of viability at high RH. Dormancy release and induction of secondary dormancy were associated with induction or repression of key genes related to abscissic acid and gibberellins pathways. We have studied the dynamics of mRNAs association with polysomes and compared transcriptome and translatome of dormant and non-dormant seeds. There was no correlation between transcriptome and translatome and germination regulation is largely translational, implying a selective and dynamic recruitment of mRNAs to polysomes in both dormant and non-dormant seeds. Some identified 5'UTR features could play a role in this selective. Dormancy is also associated with mRNA decay as demonstrated by phenotyping mutants altered in mRNA metabolism. Moreover we have shown that P-bodies were more abundant in non-dormant seeds that in dormant ones. Transcriptome analysis of xrn4-5 and vcs-8 mutants allowed us to identify several transcripts degraded via VCS ou XRN4 proteins, having a role in dormancy. This role was confirmed by reverse genetics for some of them. Some specific motifs were identified as involved in mRNA decay selection.

## After-ripening, Arabidopsis, dormancy, exosomes, germination, mRNA decay, P-bodies, temperature, translation, 5'UTR

#### Résumé substantiel

Ce travail effectué dans l'UMR 7622-UPMC-CNRS (Biologie de développement) au sein de l'équipe Biologie des semences est constitué de 4 parties. La première partie est une synthèse bibliographique sur la dormance des semences, d'une part, et sur le rôle du métabolisme posttranscriptionnel des ARN chez les plantes et en particulier au niveau des graines, d'autre part. Les 3 parties suivantes présentées sous la formes d'articles, concernent (1) l'évolution de la dormance des graines d'Arabidopsis selon leurs conditions de stockage (2) le rôle de la traduction dans la régulation de la dormance et (3) le rôle de la dégradation des ARNm dans ce processus.

La graine correspond à la phase du cycle de développement des végétaux supérieurs qui assure la pérennité et la dispersion des espèces. La régulation de la germination permet le développement d'une nouvelle génération dans les conditions optimales. La germination comprend trois phases successives: la phase d'imbibition, la phase de germination stricto sensu qui se termine par l'émergence de la radicule et la phase de croissance (Bewley, 1997). La germination des graines est influencée par des facteurs intrinsèques (la perméabilité des enveloppes à l'eau et à l'oxygène, la qualité des graines, par exemple) et des facteurs environnementaux (eau, oxygène, température, lumière). Ils participent à l'activation de voies de signalisation hormonales essentielles à la germination. Les graines dites « orthodoxes », qui sont capables de supporter la dessiccation et se caractérisent par une faible teneur en eau à la fin de leur développement, acquièrent au cours de leur développement un blocage physiologique transitoire de la germination appelé dormance. La dormance est un mécanisme qui empêche la graine de germer dans des conditions apparemment favorables (Bewley et Black, 1994). La dormance acquise au cours du développement sur la plante mère, sous l'influence de facteurs internes et externes, correspond à la dormance primaire qui est présente à la maturité de la semence. Elle s'exprime d'une façon relative et, dans le cas des graines d'Arabidopsis thaliana, elle se manifeste à température élevée (25°C), température à laquelle la germination est difficile, tandis qu'à des températures fraîches (10-20°C), les graines sont toutes capables de germer. Il existe également une dormance secondaire qui est induite, lorsque les semences matures sont placées dans des conditions environnementales défavorables à la germination (Finch-Savage and Leubner-Metzger, 2006). La dormance est généralement considérée comme la résultante d'une interaction antagoniste entre deux types d'hormones : l'acide abscissique (ABA) qui induit et maintient la dormance (Finkelstein et al., 2002; Kermode, 2005) et les gibbérellines (GAs) qui stimulent la germination (Daszkowska-Golec, 2011). La levée de dormance se produit naturellement au cours de la conservation au sec (after-ripening) qui suit généralement la récolte pour les plantes cultivées, ou par stratification, qui correspond à un traitement à basses températures à l'état imbibé ou au froid hivernal pour les graines maintenues dans le sol (Bewley, 1997; Probert, 2000). Les paramètres qui régulent la post-maturation sont nombreux et incluent l'humidité relative, la structure des semences et la température (Manz et al., 2005). Ils sont

spécifiques pour chaque espèce. Ce mécanisme de levée de dormance est associé à des réactions non-enzymatiques comme l'oxydation des protéines, des lipides ou des ARNm (Bazin *et al.*, 2011).

Comme de nombreux processus développementaux, la germination et de la dormance sont contrôlées par l'expression des gènes. Chez les eucaryotes, l'expression des gènes est principalement régulée au niveau de la transcription, mais également par des processus post-transcriptionnels, comme la séquestration des ARNm dans les complexes ribonucleoproteiques (mRNPs), la dégradation d'ARNm et la régulation de la traduction. Chez les plantes, et en particulier au niveau de la régulation de la germination, la régulation transcriptionnelle a été surtout étudiée. Il est largement admis que la dormance est régulée transcriptionellement et des réseaux d'interactions entre les transcrits impliqués dans la régulation de la germination des graines ont été définis (Bassel et al., 2011;. Verdier et al., 2013). Cependant des études récentes, comme celles de Bazin et al. (2011), Layat et al. (2014) ou Galland et al. (2014), ont montré que la régulation de la dormance n'est pas associée seulement à la transcription mais également à l'activité traductionnelle et l'on peut supposer que la dégradation des ARNm soit aussi impliquée. La régulation de la traduction a lieu principalement au niveau de son initiation. L'initiation de la traduction chez les eucaryotes débute par l'attachement d'un complexe comprenant la petite sous-unité ribosomale (40S) sur la coiffe, à l'extrémité 5' de l'ARNm lié aux différents facteurs d'initiation (eIF). Ce complexe progresse ensuite le long de la région 5' non traduite (5'UTR) jusqu'à atteindre le codon d'initiation (étape de «scanning») où le ribosome complet (80S) sera assemblé. Les ARNm recrutés liés au 80S forment le complexe polysomal et leur nombre par transcrit reflète l'efficacité de la traduction (Kawaguchi et al., 2004). En outre, les caractéristiques des régions 5'UTR et 3'UTR de l'ARNm jouent également un rôle clé dans la régulation de l'initiation (Wilkie et al., 2003). La régulation de la traduction chez les plantes est principalement décrite dans le contexte de la réponse au stress tels que l'hypoxie (Branco-Price et al., 2008), le stress thermique (Matsuura et al., 2010), les métaux lourds (Sormani et al., 2011) ou la lumière (Juntawong and Bailey-Serres, 2012).

La dégradation des ARNm joue un rôle important dans le développement des eucaryotes (Schier, 2007) et la réponse au stress (Hilgers *et al.*, 2006). Chez les plantes, le rôle de la dégradation des ARNm a été moins étudié mais a été mis en évidence dans certaines réponses environnementales ou développementales (Belostotsky et Sieburth, 2009; Chiba et Green, 2009). Chez les graines, la dégradation des ARNm a été décrite dans de la régulation de la qualité de transcrits comme ABH1 (Daszkowska-Golec *et al.*, 2013) ou AHG11 (Murayama *et al.*, 2012) impliqués dans la signalisation de l'ABA. La dégradation des ARNm se fait principalement par les deux extrémités des ARNm, en 5' ou en 3' (Mitchell and Tollervey, 2000; Parker et Song, 2004). La première étape de la dégradation de l'ARNm, est le processus de déadénylation qui réduit la queue poly (A) grâce au complexe CCR4-NOT (Chen *et al.*, 2002). Dans le cas de la dégradation de la 5' en 3', elle est suivie par le « decapping » qui est catalysé par un complexe, conservé parmi les

eucaryotes, constitué de deux protéines, DCP1 et DCP2, DCP2 étant la sous-unité catalytique (Dunckley et Parker, 1999; Xu et al., 2006). Plusieurs facteurs de régulation sont impliqués dans l'induction de l'activité de DCP2 comme Hedls/Ge1 chez l'homme qui est nécessaire pour l'interaction entre DCP1 et DCP2 (Fenger-Gron et al., 2005). VARICOSE (VCS) est l'orthologue chez Arabidopsis de Hedls/Ge1 et son interaction avec DCP1 et DCP2 est importante dans le développement post-embryonnaire (Xu et al., 2006). Les transcrits sans coiffe sont ensuite digérés par des 5'-3 exoribonucléases, comme XRN1 et XRN2 chez la levure et les animaux. Trois gènes d'Arabidopsis ont été identifiés comme homologues à XRN2 tandis qu'aucune homologie de séquence n'a étè trouvée pour XRN1. AtXRN2 et AtXRN3 fonctionneraient dans le noyau comme XRN2 chez la levure, alors que AtXRN4, localisé dans le cytoplasme, serait l'homologue fonctionnel de la protéine XRN1 de levure (Kastenmayer et Green, 2000). AtXRN4 est identique à EIN5 qui a été identifié comme un facteur impliqué dans la voie de signalisation de l'éthylène. AtXRN4 semble dégrader un ensemble limité de transcrits polyadénylés et miARN (Souret et al., 2004;. Rymarquis et al., 2011) et apparaît crucial pour l'adaptation au stress thermique (Merret et al, 2013). Les protéines de decapping et XRN4 s'accumulent avec d'autres protéines et des ARN pour former des complexes ribonucléoprotéiques appelés les Processing-bodies (P-bodies) (Parker et Sheth, 2007).

La voie de dégradation de l'extrémité 3' vers celle en 5' (3'-5') des ARNm est assurée par un complexe protéique localisé à la fois dans le cytoplasme et le noyau nommé exosome (Januszyk *et al.*, 2014. L'exosome est constitué de neuf sous-unités centrales et de co-facteurs (Lebreton et Séraphin, 2008). Un de ces co-facteurs, l'hélicase d'ARN MTR4 est nécessaire pour toutes les fonctions de l'exosome nucléaire chez l'homme et la levure (Lubas *et al.*, 2011;. Bernstein *et al.*, 2008). Arabidopsis possède deux homologues de MTR4, AtMTR4 et AtHEN2 (Lange *et al.*, 2011; Western *et al.*, 2002), chacun présentant des fonctions spécifiques. AtMTR4 est une protéine nucléolaire principalement impliquée dans la maturation des ARN ribosomaux en permettant la dégradation des précurseurs d'ARNr (Lange *et al.*, 2011). AtHEN2 est nucléoplasmique et est impliqué dans la dégradation des ARN non codants, d'ARNm aberrants et des précurseurs de miARN (Lange *et al.*, 2014). Le rôle des exosomes a été récemment montré dans la germination des graines d'Arabidopsis et la croissance des plantules avec RRP41L, une protéine de l'exosome, qui aurait un rôle dans la dégradation des ARNm cytoplasmiques spécifiques chez Arabidopsis (Yang *et al.*, 2013).

Le premier article présenté dans ce travail a consisté à déterminer comment les conditions de stockage des graines d'*Arabidopsis thaliana* à la récolte influencent la cinétique de la levée dormance et le vieillissement. Pour répondre à cette question, des graines d'Arabidopsis dormantes ont été stockées dans une large gamme de températures et d'humidités relatives et leur germination a été régulièrement évaluée pendant un an. Cette étude a été réalisée avec des graines d'Arabidopsis d'écotype sauvage Columbia et des mutants *mtr4-1* (ARN hélicase de l'exosome) et *cat2-1* (catalase 2). Le mutant *cat2-1* est moins dormant à la récolte tandis que les graines de mutant *mtr4-1* sont très

dormantes. Quels que soient le génotype et les conditions de stockage utilisées, la levée de dormance a eu lieu après 7 semaines mais sa cinétique dépend de l'humidité relative et de la température. En effet, la levée de dormance est plus rapide à 20 °C et à 56 % d'humidité relative. Nos résultats montrent que la levée de dormance dépend de la température et de la teneur en eau. Par exemple, la levée de dormance des graines peut être obtenue à 10 ° C ou 25 ° C si la teneur en eau est proche de 0,04 ou 0,08 g H<sub>2</sub>O g ms<sup>-1</sup>, respectivement. La levée de dormance d'Arabidopsis est obtenue par des mécanismes distincts dépendant de la teneur en eau. A basse teneur en eau, la levée de dormance serait liée à des réactions d'oxydation non enzymatiques alors qu'à de fortes teneurs en eau la levée dormance peut être associée à des réactions enzymatiques. Après 21 semaines de stockage, le taux de germination des graines diminue à différentes températures et humidité relative. A faible humidité relative, cela correspond à l'induction d'une dormance secondaire car les graines sont vivantes et peuvent germer dans d'autres conditions alors que les graines conservées à forte humidité relative (75-85%) présentent une perte de viabilité. Nous avons observé le même comportement pour les différents génotypes et l'intensité de dormance ne module pas la longévité des semences. Nous avons également suivi l'abondance des transcrits liés au métabolisme et à la signalisation de l'ABA et des GA dans les graines dormantes, non-dormantes et présentant une dormance secondaire, sèches ou imbibées durant 24 h à 25°C. Dans les graines sèches, il n'y a pas de différence d'abondance des transcrits selon les niveaux de dormance. Par contre dans les graines imbibées, la levée de dormance est associée à une accumulation des transcrits impliqués dans l'activation des GA (Ga3ox1 et Ga20ox4) et la dégradation d'ABA (CYP707A2). Les dormances primaire et secondaire sont associées à l'augmentation des transcrits impliqués dans la synthèse ou la signalisation de l'ABA (NCED3/6/9, ABI5) et la dégradation de GA (Ga2ox2). Les résultats présentés montrent clairement la régulation de la dormance par le métabolisme des GA et de l'ABA, et que l'expression des gènes impliqués dans ces métabolismes est similaire pour la dormance primaire et secondaire.

Dans le 2<sup>ème</sup> article, nous avons étudié la dynamique d'association des ARNm à la fraction polysomale et comparé le transcriptome et le traductome des graines dormantes et non dormantes d'*Arabidopsis thaliana* après 16 h et 24 h d'imbibition à 25 °C à l'obscurité. Les données de microarrays montrent que 4670 et 7028 transcrits sont différentiellement abondants entre les graines dormantes et non dormantes dans le transcriptome et dans le traductome, respectivement. Nous avons mis en évidence que la germination et la dormance sont associées à des changements qualitatifs d'adressage de transcrits spécifiques au cours de l'imbibition et qu'il n'y a pas de corrélation entre le transcriptome et le translatome dans la régulation de la dormance. La régulation de la germination dépendrait donc essentiellement du traductome en mettant en jeu un réseau de gènes différent de celui impliqué dans les régulations transcriptionnelles. Nos résultats montrent que l'activité traductionnelle globale est similaire chez les graines dormantes et non-dormantes, ce

qui implique une véritable régulation sélective et dynamique du recrutement des ARNm associés à la fraction polysomale. L'étude des régions 5'UTR des transcrits régulés par la dormance dans le traductome révèle que la teneur en GC et le nombre d' uORFs pourraient jouer un rôle dans cette traduction sélective. L'analyse des fonctions (Gene Ontology) des transcrits associés aux polysomes montre que ceux-ci sont différents entre les graines dormantes et non dormantes, ce qui a permis de révéler de nouveaux acteurs de la régulation de la dormance (Heat Shock Protein, Mitogen activated Proteins Kinases) et la germination (Expansins). En conclusion, nos données soulignent clairement un rôle fondamental de la traduction sélective dans la régulation de la dormance.

Dans le 3<sup>éme</sup> article, nous montrons que la régulation de la germination des graines est également liée à la dégradation des ARNm. Nous avons caractérisé le phénotype de germination de mutants affectés dans les différentes voies de dégradation des ARNm (5'-3' et 3'-5'). Les mutants d'exosomes (mtr4 et hen2) et d'exoribonuclease (xrn4) sont dormants par rapport au type sauvage Col-0 alors que le mutant de decapping varicose (vcs) est moins dormant. Certains composants des exosomes ou P-bodies semblent avoir un rôle dans la régulation de l'expression de gènes impliqués de la synthèse ou la signalisation d'ABA et de GA. En utilisant une construction transgénique (YFP-DCP1), nous avons pu observer les P-bodies dans les graines au cours de l'imbibition et nous avons montré que les P-bodies sont plus abondants dans les graines non-dormantes. Pour déterminer les cibles dégradées durant la dormance par les composantes de P-bodies (XRN4 et VCS), nous avons étudié le transcriptome après 24 h d'imbibition à 25°C des graines de mutants xrn4-5 et vcs-8. Les données de microarrays ont révélé que 973 et 2312 transcrits sont abondants plus dans xrn4-5 et vcs-8, respectivement. Ces transcrits sont potentiellement des ARNm dégradés par XRN4 et VCS respectivement impliqués dans la régulation de la dormance et la germination c'est-à-dire ceux qui sont spécifiquement dégradés chez un seul mutant, xrn4-5 ou vcs8. Cela représente 179 et 1518 transcrits chez xrn4-5 et vcs8 respectivement. L'analyse bioinformatique de séquence en 5'UTR montre que l'énergie secondaire et le contenu en GC pourraient jouer un rôle dans la sélection des transcrits dégradés par XRN4 et VCS. En conclusion ces résultats mettent en évidence un nouveau niveau de régulation post-transcriptionnel dans le contrôle de la dormance. L'ensemble de ces résultats permet d'établir un nouveau modèle de la régulation de la dormance chez Arabidopsis.

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## INTRODUCTION

Seed dormancy is defined as the inability to germinate in apparently favourable conditions (Bewley, 1997). It is a trait that allows germination and seedling establishment at the most suitable time. This process is a labile phenomenon since, depending in the species, it can be progressively released during dry storage (after-ripening), broken by a chilling treatment, or by various artificial treatments (Bewley and Black, 1994). Primary dormancy sets during the development of the seeds on the mother plant. Its intensity varies according to genotype and the environmental conditions experienced during seed development (Simpson, 1990; Bewley and Black, 1994). Moreover unfavourable conditions for germination induce a secondary dormancy on mature seeds leading to dormancy cycling in soil seed banks (Hilhorst et al., 2010). Both primary and secondary dormancies result mainly from a regulation through hormone-signalling networks that involve ABA and GA metabolism and sensitivity to these hormones (Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006). Under natural conditions, plants are exposed to a variety of environmental conditions and present various molecular mechanisms for a fine-tuned control of adaptive responses (Hirayama and Shinozaki, 2010). Thus, seeds are major organs in life cycle of higher plants because they optimize survival strategy. Control of gene expression in higher plants results from transcription but also from post-transcriptional processes including sequestration of mRNAs into cytosolic mRNA ribonucleoproteins (mRNPs), mRNA decay mechanisms and regulation of translation. Although less studied than transcription, mRNA degradation plays a critical role in eukaryote development (Schier, 2007) and response to stress (Hilgers et al., 2006). In plant, mRNA decay processes are involved in a wide variety of developmental and hormonal responses (Belostotsky and Sieburth, 2009; Chiba and Green, 2009). The main objectives of the thesis were to determine the role of post-transcriptional modifications in the regulation of Arabidopsis seed dormancy. After a general review on seed germination and post-transcriptional modifications, the results obtained are presented as three articles concerning (1) the effects temperature and relative humidity in dormancy release, (2) the comparison between transcriptome and translatome of dormant and non-dormant seeds and (3) role of mRNA decay in the regulation of dormancy. Finally, the results are discussed in the general conclusion, and some research perspectives are proposed. Additional results obtained are attached in annexes at the end of the manuscript.

## I. State of the art

## I.1 Seed dormancy and germination

#### I.1.1 Arabidopsis seed development and structure

Almost all plant cultivation in agriculture and horticulture is based on seeds. The vast majority of our domesticated plants are propagated via seeds and seeds provide most of our caloric intake either directly as food or indirectly as feed for our domestic animals. Higher-plant orthodox seed development is divided into 3 major phases: embryogenesis, seed maturation and desiccation (West and Harada, 1993; Gutierrez et al., 2007). Arabidopsis embryogenesis starts after fertilization with the formation of a single cell zygote and ends at the heart stage, when all embryo structures have been formed. It is followed by a growth phase until which the embryo fills the embryo sac. Then cell division in the embryo arrests and seed enters in maturation phase (Raz et al., 2001). Seed maturation includes the accumulation of reserves, the induction of dormancy, and the acquisition of desiccation tolerance. Desiccation phase is associated with a major loss of water, leading to a dry seed in preparation for a quiescent period and thereafter for germination (Kermode et al., 2002). The Arabidopsis mature dry seed consists of an embryo which is surrounded by a single cell layer of endosperm cells (Debeaujon et al., 2000) (Figure 1). A seed coat, which consists of five layers (derived from two ovular in teguments), surrounds the endosperm cells (Beeckman et al., 2000). Cells of the seed coat in the dry mature Arabidopsis seed are not alive, since these cells die during the late maturation.



Figure 1. Arabidopsis thaliana seed structure. (A) Arabidopsis seed. (B) Embryo anatomy of Arabidopsis seed. From Müller et al. (2006).

Mature dry seeds contain a large number of mRNAs that are translated during the seed maturation process (Sano *et al.*, 2012). These stored mRNAs were first identified in cotton seeds (Dure and Waters, 1965), and they have been found in the dry seeds of all angiosperms examined to date (Kimura and Nambara, 2010). Stored mRNAs are also called 'long-lived mRNAs' because they can survive severe desiccation and remain active for long periods in dry, quiescent seeds. However, little is known about the induction and regulation of translation of long-lived mRNAs after imbibition.

Gene transcripts stored in dry mature seeds represent residuals of mRNAs from seed developmental processes, and some of them only will serve as substrates for the synthesis of proteins during imbibition (Rajjou *et al.*, 2004; Kimura and Nambara, 2010). Sequence analysis suggests that the stored RNA comprises transcripts necessary for both late embryogenesis and seed germination (Comai *et al.*, 1990; Hughes and Galau, 1989, 1991).

#### I.1.2 Seed germination

The germination of a seed begins with the uptake of water by the dry seed and ends with embryo expansion (Bewley and Black, 1994). Under favorable conditions, rapid expansion growth of the embryo culminates in rupture of the covering layers and emergence of the radicle. Radicle emergence is considered as the completion of germination. Germination is divided to 3 phases: imbibition (phase I), germination sensu stricto (phase II) and growth (phase III) (Bewley, 1997; Figure 2). Water uptake occurs during the imbibition phase (Figure 2). The influx of water into the cells of dry seeds during phase I results in temporary structural perturbations, particularly to membranes, which leads to an immediate and rapid leakage of solutes and low molecular weight metabolites into the surrounding imbibition solution (Bewley, 1997). Glycolytic and oxidative pentose phosphate pathways both resume during phase I, and the Krebs's cycle enzymes become activated when oxygen is high enough in internal structures (Bewley, 1997). During germination sensu stricto, H<sub>2</sub>O and O<sub>2</sub> uptakes are not as rapid as during imbibition. Then translation of proteins begins with RNAs that were stored at the end of the seed development, and all cell components damaged by the dehydration and rehydration processes are repaired (mitochondria, DNA, etc) and new mRNA are transcribed (Figure 2). However it was shown that transcription is not strictly necessary for germination (Rajjou et al., 2004). Cell expansion during germination sensu stricto and radicle emergence is followed by a second water uptake. Cell division in the embryo and reserve mobilization begin during this third phase to obtain an autotrophic seedling with a short hypocotyl and development of green cotyledons under light conditions.



**Figure 2.** Time course of major events associated with germination and subsequent post-germinative growth. The time for events to be completed varies from several hours to many weeks, depending on the plant species and the germination conditions (from Bewley, 1997).

#### I.1.3 External factors regulating germination

#### Water content

Water is a basic requirement for germination. In their resting state orthodox seeds are characterized by low water content and are inactive metabolically, in a state of quiescence (Robert and Ellis, 1989). Water is the major factor needed for orthodox seeds but excess of water is often detrimental to germination because it deprives the embryo of oxygen. Water is essential for enzyme activation, breakdown, translocation, and use of reserve storage material. This is the reason why the seeds do not germinate when they are completely immersed except some seed of species like rice and aquatic plants (Corbineau and Côme, 1995). Once that critical seed moisture content is attained in the seed, sufficient water is present to initiate germination and the seed is committed to that event and cannot turn back. If the internal moisture content decreases below the critical moisture content, seeds will essentially decay in the soil (Bradford, 1995).

#### Oxygen

The vast majority of seeds of plant species requires oxygen to support germination, such as that found in air pockets between particles of lightly packed soil. Sensitivity of seed germination to oxygen partial pressure depends on the species (Corbineau and Côme, 1995). Al-Ani *et al.* (1985) identified two groups of seeds according to their responsiveness to low oxygen partial pressure: seeds with high lipid content (group I) are more sensitive to  $O_2$  deprivation than are seeds with high starch content (group II). The  $O_2$  requirement for seed germination is also strongly modulated by other environmental factors (e.g. temperature, water potential and light) (Corbineau and Côme, 1995). Generally, the sensitivity of seeds to O<sub>2</sub> deprivation decreases with decreasing temperature, because of reduced respiratory activity and the higher solubility of O<sub>2</sub> in water (Côme and Tissaoui, 1973; Corbineau and Côme, 1995). The sensitivity to oxygen of seed germination has been quantified using a population-based threshold model in 16 species and it was showed that the oxygen percentage required for 50% germination ranged from 21% to as low as 0.005%, depending upon the species, the temperature and the dormancy level (Bradford *et al.*, 2007).

#### Temperature

Seed germination is a complex process involving many individual reactions and phases, all affected by temperature. Each species is characterized by a temperature range over which germination is possible (Probert, 2000). The effect on germination can be expressed in terms of cardinal temperature: that is minimum, optimum, and maximum temperatures at which germination will occur. The minimum temperature is sometimes difficult to define since germination may actually be proceeding but at such a slow rate that determination of germination is often made before actual germination is completed. The optimum temperature may be defined as the temperature giving the greatest percentage of germination in the shortest time. The optimum temperature for most seeds is between 15 and 30°C. The maximum temperature is governed by the temperature at which denaturation of proteins occurs. The maximum temperature for most species is between 30 and 40°C. The response to temperature depends on a number of factors, including the species, variety, growing region, quality of the seed, and duration of time from harvest. As a general rule, temperateregion seeds require lower temperatures than do tropical region seeds, and wild species have lower temperature requirements than do domesticated plants. High-quality seeds are able to germinate under wider temperature ranges than low-quality seeds (Robert, 1988).

#### I.1.4 Seed dormancy

Seed dormancy is a common characteristic of wild plants which ensures their continued existence or survival under unfavorable conditions, decreases competition with other species and prevents damage to seedlings from out-of-season germination of the seed. Seed dormancy is defined as the inhibition of germination of an intact viable seed under favorable conditions (Hilhorst, 1995; Bewley, 1997; Li and Foley, 1997). The germination block has evolved in a different way from one species to another depending upon their habitat and conditions of growth. Both physiologists and ecologists have studied the factors controlling dormancy but the outcome is far from clear due to the fact that dormancy is affected by numerous environmental conditions (Walck *et al.*, 2005). Dormancy is an entire seed trait and, on this basis, can be classified into five classes, namely physiological,

morphological, morpho-physiological, physical and combinatorial dormancy (Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006). *Arabidopsis thaliana* seeds display a primary physiological dormancy, which is the most prevalent dormancy in temperate seed banks and the most abundant dormancy class. Two categories of dormancy are defined according to their establishment; *i.e.* primary dormancy and secondary dormancy (Hilhorst *et al.*, 2010) (Figure 3). Primary dormancy is acquired during the natural maturation when the seed is still attached to the mother plant and therefore the seed is dormant when shed by the mother plant. Primary dormant seeds are able to enter into a second stage of dormancy during imbibition if conditions for germination are unfavorable and this is known as secondary dormancy (Bewley and Black, 1994). Secondary dormancy is a safety mechanism that is implemented from the seed being exposed to adverse conditions once it falls from the plant and functions in imbibed seed. This dormancy can be promoted by high salt conditions, high or low water, hypoxia, high or low temperature, prolonged exposure to darkness, prolonged exposure to far red light, blue or white light (Khan and Karssen, 1980; Leymarie *et al.*, 2008; Hilhorst *et al.*, 2010; Hoang *et al.*, 2013).



**Figure 3.** Modulation of dormancy by environmental factors. Induction and maintenance of primary dormancy during seed development are influenced by both genetic and environmental factors. Transition of mature seeds from dormant to non-dormant state can be induced by after-ripening, a period of dry storage during which dormancy breaks down. Non-dormant seeds complete germination once imbibed or may enter secondary dormancy if the environmental conditions are unfavorable for germination (Kermode, 2005).

In Arabidopsis, dormancy is expressed in the dark when the temperature is above 20° (Figure 4A, Leymarie *et al.*, 2012). In contrast, non-dormant seed germinate fully in the dark at temperatures below 15°C or in light (Figure 4B).



**Figure 4.** Germination of dormant and non-dormant Col-0 seeds. (A) Freshly harvested seeds. (B) Seeds after-ripened for 5 weeks at 20°C. Germination was carried out at 10°C (diamonds), 15°C (squares), 20°C (open circles) and 25°C (crosses) in the dark and at 25°C under continuous light (rectangles). Means ± SD of triplicate experiments are shown. Adapted from Leymarie *et al.*, 2012.

#### Factors of dormancy

Dormancy is regulated by environmental factors such temperature, water content, light, oxygen and by phytohormones. Dormancy alters the sensitivity to the environmental factors leading to germination. Temperature signaling pathways in vegetative tissues are well understood, whereas in seeds, the knowledge is much more limited. In many summer annual plants, dormancy is usually lost during winter and, therefore, the exposure to cold during imbibition is required. Conversely, the germination of winter annuals often requires exposure to warm temperatures during imbibition, reflecting the loss of dormancy over summer and germination in autumn (Baskin and Baskin, 2004). In the seed bank, winter temperatures promote dormancy whilst spring temperatures alleviate it (Footitt et al., 2011). This suggests that if seeds have not germinated before the onset of winter, the low temperatures experienced during the winter induce secondary dormancy, which delays germination until the spring. In Arabidopsis, temperature is known to be an important factor in determining the levels of dormancy that are induced in the seed during maturation and this is extensively reviewed in Fenner (1991). This suggests that not only the environment which is directly experienced by the developing seed is important, but the parent plant is able to transmit signals that provide the seed with vegetative environmental information (Fenner, 1991). In Arabidopsis low temperature during seed maturation also promotes high dormancy levels (Schmuths et al., 2006; Donohue et al., 2008; Chiang et al., 2009), but the mechanism that regulates this process is currently unknown. Elwell et al. (2011) analyzed the effect of different parental environments on development throughout the plant life cycle, showing that the parental environment had the potential to affect germination, root growth, gravitropism and the time to produce the first floral bud.

#### After ripening

After-ripening occurs after seed maturation during a prolonged period of storage in dry condition and allows the dormancy release (Finch-savage and Leubner-Metzger, 2006). During this period, it is assumed that the seed undergoes certain changes, at the completion of which germination may take place. After-ripening is a widening or increased sensitivity of perception of seeds to environmental conditions (temperature, light, oxygen availability) promoting germination, at the same time as a narrowing or decrease in sensitivity of perception of conditions repressing germination (Finch-Savage and Leubner-Metzger, 2006). Seed water content and temperature an important factors to regulate this dormancy alleviation (Probert 2000, and references therein). Various studies have shown that seed dry after-ripening is effective at MCs from ca. 5–18% (on a fresh weight basis) (Steadman et al., 2004; Bair et al., 2006) corresponding to region 2 of water sorption isotherms which corresponds to weakly bound water (Probert, 2000). Temperature is the major environmental parameter influencing dormancy release in most species, with warm temperatures usually promoting dormancy loss in dry seeds (Baskin and Baskin, 1976). In most species, dormancy is alleviated faster with increasing afterripening temperature in agreement with the temperature dependence of most biological reaction rates (Gillooly et al., 2001; Brown et al., 2004). Although the seed MC during dry after-ripening is too low to detect any measurable metabolism, the temperature coefficient  $Q_{10}$ , which quantifies the temperature dependence, has been demonstrated to be constant within a species with positive values varying from c. 2–4 (Probert, 2000). Little is known about the molecular mechanism that control after-ripening. However, non-enzymatic reactions are likely candidates for seed dormancy release in these anhydrobiotic conditions. Oxygen can diffuse within glasses such as the vitreous cytoplasm at low seed moisiture levels, and ultimately lead to ROS has been shown in Oracz et al. (2007). For example during seed development and germination, non-ezymatic reactions can be induced at a lower water content at 0.25 g H<sub>2</sub>O.g MS<sup>-1</sup> (Bailly, 2004). This accumulation of ROS was associated with dormancy release in sunflower seeds. It was demonstrated that ROS caused lipid peroxidation and carbonylation of proteins that were associated with dormancy release. This type of modification can induce germination by the degradation of protein or the increase of sensitivity to proteolysis (Job et al., 2005). Lipids and proteins are not the only target of oxidative modification of ROS, mRNA is one the most sensitive molecule to ROS oxidation during after ripening (Bazin et al., 2011). mRNA bases are oxidized by hydroxyl radicals, and 8-OHG is the most abundant RNA lesion, as guanine has the lowest oxidation potential (Nunomura et al., 2006). The mRNA oxidation can produce an alteration of translation (Simms et al., 2014). Transcriptomic analysis have shown that after-ripening affects the abundance of specific transcripts (Finch savage et al., 2007; Carrera et al., 2008) occurring in dry seeds. A recent study shows that transcriptome of the mRNA of dry seed during after-ripeninng do not reflect the degree of dormancy but reflect the developmental context, such as seed maturation and desiccation (Meimoun *et al.*, 2014).

#### I.1.5 Hormonal regulation of dormancy and germination

Two phytohormones, ABA (abscisic acid) and GA (gibberellins), are the main hormones that are involved in regulating seed dormancy, although roles for additional hormones such as ethylene, brassinosteroid (BR), auxin and strigolactone have also been described (Kucera *et al.*, 2005). ABA acts as a positive regulator of dormancy and negatively regulates germination while GA and ethylene has the opposite effect.

#### Abscisic acid

The plant hormone ABA is ubiquitous among higher plants where it participates in the control of many crucial aspects of plant growth and development (Zeevaart and Creelman, 1988; Cutler et al., 2010). In seeds, ABA regulates several processes essential for seed viability and germination, in concert with other as yet unidentified developmental factors. These processes include the accumulation of protein and lipid reserves, the induction of dormancy, and the acquisition of tolerance to desiccation. During seed development, ABA levels peak during mid-maturation and this coincides with the induction of dormancy levels (Kermode, 2005). ABA which is synthesized during seed development can be of dual origin, the embryo and/or the maternal tissues (Kucera et al., 2005). The ABA biosynthesis pathway is well known and the germination phenotypes of a large number of mutants of its biosynthetic pathway have been characterized (Nambara and Marion-Poll, 2003) (Figure 5). Cellular ABA content is regulated by the balance between its biosynthesis and catabolism (Nambara and Marion-Poll, 2003). Nine-cis-epoxycarotenoid dioxygenases (NCEDs) catalyse the cleavage of C40 9-cis-epoxycarotenoids, 9-cis-neoxanthin and 9-cis-violaxanthin, which is a primary regulatory step in ABA biosynthesis (Schwartz et al., 1997). Zeaxanthin epoxidase (ZEP), which is a xanthophyll cycle enzyme, is also postulated to have a regulatory function in this process (Marin et al., 1996). Members of the CYP707A family of P450 mono-oxygenases encode ABA 8'hydroxylase, which is involved in a committed step in the ABA 8'-hydroxylation pathway, and they play a regulatory role in the control of ABA content (Kushiro et al., 2004; Saito et al., 2004; Millar et al., 2006). All plant species examined to date encode NCED and CYP707A as multigene families, and differential combinations of these members contribute to tissue- and environment specific regulation (Lefebvre et al., 2006; Seo et al., 2006). The ABA content in dry Arabidopsis seeds at harvest is not related to depth of dormancy (Ali-Rachedi et al., 2004) but expression of dormancy at high temperature is associated with maintenance of a high level of ABA content during the first 24-48 h of incubation at 25°C, while ABA decreases in seeds incubated at temperatures which allow

germination (Ali-Rachedi *et al.,* 2004). This ABA decrease depends largely on CYP707A2 activity (Ali-Rachedi *et al.,* 2004; Kushiro *et al.,* 2004). High ABA content and sensitivity are often associated in dormant seeds (Yoshioka *et al.,* 1998; Corbineau and Côme, 2003; Argyris *et al.,* 2011; Benech-Arnold *et al.,* 2006; Leymarie *et al.,* 2008).



**Figure 5.** ABA metabolic pathway (Adapted from Nambara *et al.,* 2010). Each box indicates an enzyme, grey boxes indicate enzymes with a regulatory function in seeds. ZEP, zeaxanthin epoxidase; NSY, neoxanthin synthase; NCED,9-cis-epoxycarotenoid dioxygenase, CYP707 A, ABA 8'-hydroxylase; PA, phaseic acid; DPA, dihydrophaseic acid.

#### Gibberellins

GA play an important role in diverse growth and developmental processes throughout the whole life cycle of plants, including seed germination, stem elongation, leaf expansion and flower development (Richards *et al.*, 2001). The biochemical pathway for GA biosynthesis is well characterized and the majority of genes encoding enzymes in this pathway has been identified (Sun, 2010). The activation and deactivation of GA have been extensively reviewed by Yamaguchi (2008). Briefly, terpene synthase and P450 enzymes are involved in early stages of GA biosynthesis, which leads to the production of GA12 (Figure 6). GA12 is then converted to a bioactive form, GA4 by GA20-oxidase (GA20ox) and GA3-oxidase (GA3ox). Key enzymes in the GA deactivation pathway are encoded by the *GA2ox* genes which use C-19 and C-20-GAs. The DELLA family of GRAS proteins, which are characterized by two leucine rich areas flanking a VHIID motif, are negative regulators of plant growth and have been shown to have a role in seed germination (Lee *et al.*, 2002; Tyler *et al.*, 2004; Cao *et al.*, 2006; Penfield *et al.*, 2006). In Arabidopsis there are five DELLA proteins, RGA, GAI, RGA

LIKE1 (RGL1), RGL2 and RGL3. DELLA proteins are nuclear transcriptional regulators, which repress GA signaling by causing transcriptional reprogramming (Sun, 2010). The GA signal targets DELLAs for degradation via ubiquitination and the 26S proteasome pathway, releasing the plant from the DELLA-mediated growth constraint (Silverstone *et al.*, 2001). This targeting of DELLAs for degradation occurs when GA binds to its soluble receptor, GA INSENSITIVE DWARF1 (GID1). The GID1-GA complex then causes the binding to DELLA and consequently degradation (Hirano *et al.*, 2007). Seed dormancy is alleviated through modulation of GA synthesis (Ogawa *et al.*, 2003). In Arabidopsis, a *de novo* biosynthesis of GAs is required during imbibition (Karssen *et al.*, 1989; Nambara *et al.*, 1991).



Figure 6. Principal pathway of GA metabolism in plants (Sakamoto et al., 2004).

#### ABA and GA balance

The integration of ABA and GA signaling can occur as shown previously through the regulation of one hormone content by the other (Seo *et al.*, 2006, 2009). In the *ga1-3* mutant, ABA biosynthetic genes are activated and ABA deactivation genes suppressed, suggesting that GA regulates ABA metabolism (Oh *et al.*, 2006). Additionally, RGL2 is responsible for promoting ABA synthesis when GA levels are reduced. The increase in ABA levels in turn forms a positive feedback loop by promoting *RGL2* expression (Piskurewicz *et al.*, 2008). Following transfer of imbibed seeds to 4°C and then 20°C, a decrease in ABA content occurs before germination (Ali-Rachedi *et al.*, 2004). This decrease in ABA levels is also apparent upon imbibition at warm temperatures (Chiwocha *et al.*, 2005), suggesting that the promotion of germination by cold stratification does not require a reduction in ABA levels. The balance between ABA and gibberellin levels and sensitivity is a major regulator of seed dormancy (Finch-Savage and Leubner-Metzger, 2006).

#### Ethylene

Ethylene is a gaseous plant hormone that regulates numerous developmental processes in higher plants, from seed germination to senescence and is important for plant responses to biotic and abiotic stresses (Mattoo and Suttle, 1991; Abeles *et al.*, 1992). Ethylene is implicated in the promotion of germination of non-dormant seeds of many species (Corbineau *et al.*, 1990; Esashi, 1991; Kepczynski and Kepczynska, 1997; Matilla, 2000). Ethylene biosynthesis pathway in germinating seeds is the same as the one described in other plant organs, 1-aminocyclopropane-1-carboxylic acid (ACC) is oxidized by ACC oxidase (ACO) to form ethylene. Responses to ethylene in Arabidopsis are mediated by a family of five receptors called Ethylene Response1 (ETR1), ETR2, Ethylene Insensitive 4 (EIN4), Ethylene Response Sensor 1 (ERS1), and ERS2 (Chang *et al.*, 1993; Hua and Meyerowitz, 1998; Hua *et al.*, 1998; Sakai *et al.*, 1998). CTR1 acts as a negative regulator of the pathway that, in turn, inhibits downstream components of the pathway, preventing ethylene responses (Kieber *et al.*, 1993).

#### I.2 Transcriptional regulation of gene expression

The growth, development, and function of an organism is a reflection of gene expression. Control of gene expression is exerted by multiple steps such as transcription, RNA splicing, mRNA export, mRNA degradation, translation, and posttranslational events (Figure 7). In the next chapters, the mechanisms of transcription, post-transcriptional and translation events are discussed in details.



Figure 7. An overview of the flow of information from DNA to protein in a eukaryote.

#### I.2.1 Transcriptional regulation in plants

Gene regulation is essential to life from the simplest virus to the most complex mammal. The transcription of DNA to make messenger RNA is highly controlled by the cell. In order for a higher organism to function, genes must be turned on and off in coordinated groups in response to a variety of situations. For a plant this may be "abiotic" stress such as drought, or heat, "biotic" stress such as insects, viral or bacterial infection. The job of coordinating the function of groups of genes falls to proteins called transcription factors.

Transcription factors are proteins that regulate gene expression. Each gene is preceded by a promoter region that includes a binding site for the RNA polymerase and a variety of other features, including the "TATA box" (a short segment of repeating thymidine and adenine residues) and one or more enhancer sites, which serve as a binding location for transcription factors (Roeder, 1996). For a messenger RNA to be created, an initiation complex must assemble in the promoter region. This complex consists of over 40 proteins, including the RNA polymerase, TATA binding protein, and one or more transcription factors. Transcription factors work by binding to DNA at the enhancer site and/or to other proteins in the initiation complex. Through these interactions, transcription factors are able to control whether RNA polymerase moves forward along the DNA to produce a message. Transcription factors play important and diverse roles in gene expression, including chromatin remodeling and recruitment/stabilization of the Pol II transcription-initiation complex (Karam *et al.*, 1998). Major classes of transcription factors are activators and repressors (Lefstin and Yamamoto, 1998).

Plant transcription factors contain a variety of structural motifs that allow for binding to specific DNA sequences. These two components of transcription are normally described as cis-acting and transacting factors. Cis-acting elements are defined like non-coding DNA sequence in the vicinity of the structural portion of a gene that are required for gene expression. Trans-acting factors were usually considered to be proteins that bind to the cis-acting sequences to control gene expression. The functional analysis of interactions between transcription factors and other proteins is very important for elucidating the role of these transcriptional regulators in different signaling cascades. In table 1, we present an overview of cis-element for the major families of transcription factors in plant.

Cis element	Sequence	Function	Reference
ABRE	ACGTGGC	ABA-responsive element	Choi <i>et al.,</i> 2000
ACE	ACACGT	UV/blue light-response	Hartman <i>et al.,</i> 1998
ATHB6	AATTAT	ABA-responsive element	Himmelbach et al., 2002
bZIP	ACGT	Light, stress and hormone response	Alves et al., 2014
CBF2	CCACGT	ABA signaling or stress response	Pla et al., 1993
DRE	TACCGACAT	ABA signaling or stress response	Kasuga <i>et al.,</i> 1999
ERE	TAAGAGCCGCC	ethylene-responsive	Shinshi et al., 1995
JASE2	CATACG	senescence and jasmonic acid response	He <i>et al.,</i> 2001
LTRE	CCGACA	Temperature response	Nordin <i>et al.,</i> 1993
MYC	CACATG	ABA signaling or stress response	Alves et al., 2014
MYB	AACNG	Response to dehydration, salt stress and ABA	Alves <i>et al.,</i> 2014
GARE	TAACAA(A/G)	Gibberellin responsive	Ogawa et al., 2003
GATA	(A/T)GATA(G/A)	Biotic and abiotic stress response	Terzaghi and Cashmore, 1995
WRKY	(T) (T) TGAC(C/T)	Signaling, transcription	Eulgem <i>et al.,</i> 2000

Table 1: Description of cis-element motifs from Arabidopsis gene regulatory information server (AGRIS)

The development of transcriptomics in seed science has led to considerable progress in the understanding of seed development and germination and its regulation by environmental factors. These studies revealed thousands of differentially expressed genes during these developmental phase transition and provided detailed maps of regulatory processes identified key transcription factors. They include seed maturation regulators such as ABSCISIC INSENSITIVE 3 (ABI3), genes involved in hormonal regulation or specific genes of dormancy such as DELAY OF GERMINATION 1 (DOG1) which encodes a protein of unknown function (Bentsink et al., 2006). Studies related to the expression of the Arabidopsis genome following imbibition of mature seeds indicate that there are very large changes in genome expression that are associated with the transition from dormancy to germination (Holdsworth et al., 2008). Using ABA and GA biosynthesis and signalling mutants, it has been demonstrated that these two hormones play essential but antagonistic roles in dormancy and germination. The balance between the levels of these two hormones and their respective signalling pathways regulate both induction and maintenance of dormancy, and promotion of germination in relation to the history of the maternal environment (Finkelstein et al., 2008). New developments in – omics approaches consist of generating gene regulatory networks that reveal developmental modules and key regulators related to seed germination (Bassel et al., 2011; Figure 8) and longevity (Verdier et al., 2013).



**Figure 8.** The SeedNet unweighted seed gene coexpression network. Regions outlined in yelow correspond to clusters associated with dormancy (region 1) or germination (regions 2 and 3). Distribution of the transcripts as more abundant in dormant seeds (red dots) or in non-dormant seeds (blue dots) in transcriptome. Adapted from Bassel *et al.*, 2011.

## I.3 Post-transcriptional regulation of gene expression

## I.3.1 Regulation of mRNA translation in plants

The control of mRNA translation plays a critical role in regulating protein production; indeed, its contribution is greater than those of the modulation of mRNA synthesis, degradation, or protein turnover (Schwanhausser *et al.*, 2011). In particular, controlling mRNA translation, rather than mRNA levels, allows protein synthesis to be regulated spatially at defined locations within the cell, and temporally, in response to particular stimuli or conditions. Translation of an mRNA into its cognate protein is accomplished in three successive steps (initiation, elongation and termination) controlled by a wide range of regulatory factors.

#### Regulation of translation initiation

Translation initiation is the process of assembly of elongation-competent 80S ribosomes, in which the initiation codon is base-paired with the anticodon loop of initiator tRNA (Met-tRNA<sup>Met</sup><sub>i</sub>). It requires at least nine eukaryotic initiation factors (eIFs; Figure 9) and comprises two steps: the formation of 48S initiation complexes with established codon–anticodon base-pairing in the P-site of the 40S ribosomal subunits, and the joining of 48S complexes with 60S subunits. On most mRNAs, 48S complexes form by a 'scanning' mechanism, whereby a 43S preinitiation complex (comprising a 40S subunit, the eIF2–GTP–Met-tRNA<sup>Met</sup><sub>i</sub> ternary complex (eIF2 TC), eIF3, eIF1, eIF1A and probably eIF5) attaches to the capped 5' proximal region of mRNAs in a step that involves the unwinding of the mRNA's 5' terminal secondary structure by eIF4A, eIF4B and eIF4F. The 43S complex then scans the 5'

untranslated region (5' UTR) in the 5' to 3' direction to the initiation codon (Figure 10). After initiation codon recognition and 48S complex formation, eIF5 and eIF5B promote the hydrolysis of eIF2-bound GTP, the displacement of eIFs and the joining of a 60S subunit. Although most mRNAs use the scanning mechanism, initiation of a few mRNAs is mediated by internal ribosome entry sites (IRES) which is a nucleotide sequence that allows for translation initiation in the middle of mRNA sequence as part of the greater process of protein synthesis (Hellen and Sarnow, 2001).



**Figure 9.** Schematic overview of the mRNA-dependent of cap-dependent translation initiation. eIFs are represented by their numbers, ribosomal subunits by 40S and 60S and methionyl-tRNA by Met (Parsyan *et al.,* 2011).

#### Regulation of eIF2 activity

The initiation factor eIF2 has a critical role in translational control. This is a heterotrimer of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits (Preiss and Hentze, 2003). The  $\alpha$ -subunit of eIF2 functions predominantly as a regulator of translation initiation via phosphorylation by eIF2 $\alpha$  kinases (Preiss and Hentze, 2003). eIF2 from plants does not differ significantly in composition or amino acid sequence from eIF2 from other eukaryotes, but there seem to be significant differences in the way eIF2 and protein synthesis are regulated in plants. The phosphorylation of eIF2 $\alpha$  in mammals and yeast is a significant regulatory pathway that prevents the interaction of eIF2-GDP with eIF2B to exchange GDP for GTP and shuts down protein synthesis (Goss *et al.*, 1984). Phosphorylation of  $\alpha$  subunit of eukaryotic translation initiation factor 2 (eIF2 $\alpha$ ) provides a key mechanism for down-regulating protein synthesis in response to nutrient starvation or stresses (Wek *et al.*, 2006). Vertebrates use four different eIF2 $\alpha$ -kinases (PKR, PERK, HRI and GCN2) to respond to various stresses, and typically one kinase has a predominant role in response to a specific cellular stress condition. Another eIF2 $\alpha$  kinase in yeast, GCN2 (positive general control of transcription 2), regulates translation during amino acid starvation (Gaba *et al.*, 2001). A GCN2 homologue has been reported in plants was also shown to be activated by amino acid deprivation condition (Lageix *et al.*, 2008).

#### Regulation of eIF4E and eIFisoE activity

The cap-binding translation factor, eIF4E, is a key component of the eIF4F cap-binding complex that binds directly to the m7G cap structure at the 5' end of a eukaryotic mRNA to facilitate 43S complex binding to the mRNA (Figure 10). The interaction of eIF4F with mRNA is supposed to be the first step in the initiation of translation. eIF4A, the original DEAD box helicase, is loosely associated with this complex and participates in ATP dependent unwinding of the mRNA (Rogers et al., 2004). Plant eIF4A was recently reported to be associated with a cyclin-dependent kinase during active cell proliferation (Hutchins et al., 2004). This finding opens a whole new arena for the regulation of translation during plant cell growth/development. Plants have a second form of eIF4F, termed eIFiso4F, which is not present in other eukaryotes. elFiso4F, similar to elF4F, consists of two subunits, a small cap-binding protein elFiso4E and a large subunit elFiso4G, and has in vitro activities similar to elF4F (Browning et al., 1996). The cap-binding subunits eIF4E and eIFiso4E show 50% similarity in their amino acid sequences. In mammals, eIF4E is phosphorylated in response to hormones (Morley and Traugh, 1989;) and growth factors (Bu and Hagedorn, 1991; Donaldson et al., 1991), but becomes dephosphorylated following heat-shock (Duncan et al., 1987) and viral infection (Huang and Schneider, 1991; Kleinj et al., 1996). Changes in the phosphorylation state of eIF4E mediate changes in overall rates of protein synthesis and recruitment of individual transcripts that differ in their dependence on eIF4E. The cap-binding subunits eIF4E and eIFiso4E have been shown to have a variety of roles during plant viral infection, primarily through interaction with the viral Vpg. EIF4E and eIFiso4E interact directly with RNA elements in the 3'untranslated region of satellite tobacco necrosis virus RNA (Gazo *et al.*, 2004). In Arabidopsis, the eIFiso4E transcript and protein are more abundant in roots, floral organs and tissues under development (Rodriguez *et al.*, 1998). In maize, eIFiso4E was particularly required for translation of the stored mRNAs from dry seeds, that eIF4E was unable to fully replace the eIFiso4E activity. The ratio of eIFiso4E to eIF4E in the corresponding eIF4F complex is critical for the mechanisms of translational control during germination (Dinkova *et al.*, 2011).

#### Control of mRNA translation by the 5' Untranslated Region

Structural features of the 5' Untranslated Region (5'UTR) of an mRNA can be important in controlling the level of translation initiation occurring on that transcript. They include:

(i) *The presence of a m7G cap structure*. The 5'cap structure is found on all known eukaryotic mRNAs and most viral mRNAs. This structure consists of a methylguanosine that is attached to the 5'end of the mRNA via a 5'-5' triphosphate linkage and is denoted m7G (5') ppp (5') N. The m7G cap structure protects mRNAs from 5'-3' exonucleolytic degradation (Furuichi *et al.*, 1977) and directs the entry of 40S ribosome subunits via interaction with the cap-binding complex eIF4F (Pestova *et al.*, 2002). In plants, the initiation of translation is independent of the 5'-terminal structure on the mRNA, since the mRNA recognition pathway would bypass the RNA 5' terminus. This suggests that the cap-binding function of the cap-binding complex is dispensable as the initial step in mRNA recognition and subsequent translation (Carrington *et al.*, 1990).

(ii) *The presence of upstream AUGs*. Upstream open reading frame (uORFs) are major regulatory elements in 5'UTRs. As their names suggest, uORFs are sequences defined by a start and stop codons upstream of the main coding region (Figure 10). uORFs are extremely diverses varying in position and in distribution. The preponderance of uORFs is clearly biased with respect to gene function. Current studies showed that there are around one third of mRNAs containing uORFs in different organisms. For example, 35 % of all annotated 5'UTRs contain uORFs in the human genome (lacono *et al.*, 2005) and 32 % in the yeast genome (Cvijovic *et al.*, 2007). uORFs correlate with significantly reduced protein expression levels because they reduce the efficiency of translation initiation of the main downstream ORF in unstressed conditions (Morris *et al.*, 2009), or trigger mRNA decay (Yepiskoposyan *et al.*, 2011). However, in response to cellular stress, the presence of uORFs can promote the increased expression of certain stress-related mRNAs (Spriggs *et al.*, 2010). About 35 % of Arabidopsis genes give rise to a uORF containing mRNA, and about half of these have multiple uORFs (von Armin *et al.*, 2014). Poorly expressed mRNAs such as mRNAs for transcription factors and kinases often have longer 5'UTRs and are rich in uORFs (Kim *et al.*, 2007). uORFs in

Arabidopsis are regulated by external signals and function as stop-go signals. They add an important regulatory ability in gene expression by the regulation of translation initiation (von Armin *et al.*, 2014).



**Figure 10.** Model of a uORF-containing transcript. Two uORFs (blue boxes) precede the main ORF of the CDS (white box). Ribosomes may initiate at the CDS initiation codon (white flag) after leaky scanning through both uORF initiation codons (blue flags), or may reinitiate after translating the first uORF and leaky scanning through the second uORF initiation site. Ribosomes translating the second overlapping uORF will not be available for translation of the CDS (Adapted from Wethmar *et al.*, 2014).

(iii) *The stability and position of secondary structure*. RNA secondary structure positioned between the cap structure and the AUG codon can be inhibit to translation initiation, with the extent of this inhibition depending on the thermodynamic stability and position of this structure. Secondary structure, positioned downstream from an AUG codon, can actually enhance recognition of the preceding initiation codon. Secondary structures have been determined in mammals to be particularly prevalent among mRNAs encoding transcription factors, protooncogenes, growth factors, and their receptors and proteins poorly translated under normal conditions. The scanning of the 5'UTR by the 43S pre-initiation complex is limited by the presence of a strong stem–loop structure, an effect that dependents on the location and stability of the structure (Kozak *et al.*, 2002). A stem-loop with a predicted free energy value of -20 kcal/mol near to the 5' end of the mRNA effectively inhibited ribosome entry *in vitro* and a stronger stem–loop structure (-30 kcal/mol) was necessary to abolish ribosome scanning (Kozak *et al.*, 1996), the effect of such structures has not been carefully evaluated.

(iv) *GC content*. In the prokaryotic genomes, the main source of GC content heterogeneity is the presence of highly expressed genes with strongly biased codon usage. Many studies have shown that the use of optimal codons is a decisive factor determining the level of gene expression in bacteria, yeast, flies, and worms and that genes requiring high translation levels are forced by selection to adopt a particular set of codons (Gouy and Gautier, 1982; Grosjean and Fiers, 1982). The situation is more complicated in mammals. The GC content of large genome fragments ranges from 30 % to 60 %, and the GC content at the third codon position of genes ranges from 25 % to more than 90 %

(Bernardi, 1995). In *Arabidopsis thaliana*, GC content can affect protein translation efficiency under stress condition (Kawaguchi *et al.*, 2005).

(v) The length of the 5' UTR. Progressive shortening of the 5'UTR of a eukaryotic mRNA usually promotes leaky scanning, unless there is a well-positioned downstream secondary structure. The average length of 5'UTRs is 100 to 220 nucleotides across species (Pesole *et al.*, 2001). In vertebrates, 5'UTRs tend to be longer in transcripts encoding transcription factors, protooncogenes, growth factors and their receptors, and proteins that are poorly translated under normal conditions. Such a structure suppresses leaky scanning, possibly by slowing the movement of the scanning 40S ribosomal subunit such that it is able to recognise the first AUG codon (Kozak, 1990). Seventy percent of higher eukaryotic mRNAs are 20-80 nucleotides long, with a mean length of 60 nucleotides.

#### Regulation by elements in the 3'Untranslated Region

It is becoming increasingly clear in eukaryotes that the 3'Utranslated Region (3'UTR) of an mRNA can have equally as important a role as has the 5'UTR in regulating gene expression post-transcriptionally (Munroe and Jacobson, 1990). Not only are specific regulatory elements present within the 3'UTR, but general regulatory elements, such as the poly (A) tail, are associated. The poly (A) tail is an important regulator of expression that is common to virtually all mRNAs. Postulated to regulate message stability and translational efficiency, its precise role as a cytoplasmic regulatory element remains controversial. Initially, poly A binding protein (PAB) was thought to play a passive role by functioning as a steric block to 3'-5' exoribonucleases. Approximately 20 members of PABP gene family genes were found in Arabidopsis (Belostotsky and Meagher 1996; Hilson et al., 1993). Four of the family members, including PAB1, PAB2, PAB3, and PAB5, have been identified and characterized so far and most of which show an organ-specific expression pattern (Belostotsky and Meagher, 1996). The role of the poly (A) tail as a regulator of translation is quantitatively greater than its role as a regulator of message stability (Gallie, 1991). The poly (A) tail is functionally dependent on the 5' cap structure (m7GpppN) in order to stimulate translational efficiency both in vivo (Gallie, 1991) and in vitro (Munroe and Jacobson, 1990), observations suggesting communication between the 5'UTR and 3'UTR an mRNA. Recent evidence has implicated cap-associated initiation factors as candidates for mediating the interaction (Gallie and Tanguay, 1994). The observation that exogenously added poly (A) repressed the translation of uncapped mRNAs to a greater extent than capped mRNAs in vitro cell lysates (Gallie and Tanguay, 1994; Munroe and Jacobson, 1990) suggests that exogenous poly (A) can sequester necessary translational components that also associate with the cap.

#### Regulation of translation elongation and termination

During the subsequent elongation phase in plant, the enzymatic peptidyltransferase activity of the ribosome catalyzes peptide bond formation between the two amino acids. The ribosome translocates by one codon, thereby shifting the spent initiator tRNA into its E(xit)-site, and opening up a new, empty, A-site. The elongation factors eEF1 and eEF2 mediate tRNA delivery and ribosome translocation, respectively. The elongation cycle repeats until the ribosome reaches a stop codon. At termination, peptide release requires ribosome release factors. Translation ends with the separation of the two subunits and their dissociation from the mRNA, a process known as recycling. The poly(A)binding protein (PABP) forms a bridge with the initiation factor eIF4G, thus bending the mRNA into a closed loop, which is thought to stimulate the recycling of the post-termination ribosomal subunits to the 5' end of the mRNA for the next round of translation (Cheng and Gallie, 2007, 2010).

#### I.3.2 mRNA stability

The rate of mRNA synthesis is not the only determinant of the steady-state levels of an mRNA, the mRNA decay rate is also a major determinant. Translation and degradation often show an inverse relationship (Coller and Parker, 2004; Jacobson and Peltz, 1996). Stress granules also can regulate the level of mRNA under stress condition which are aggregates of untranslating mRNAs in conjunction with a subset of translation initiation factors (eIF4E, eIF4G, eIF4A, eIF3, and eIF2), the 40S ribosomal subunit, and several RNA-binding proteins including the poly(A)-binding protein (PAB) (Anderson and Kedersha, 2006). The 5'7-methylguanosine cap structure and the 3'poly (A) tail (of up to 200 adenosine residues in length), protect the RNA chain from degradation by 5'-3' or 3'-5'exonucleases, or both (Figure 11).



**Figure 11.** Pathways for general cytoplasmic mRNA decay in plant. The mature RNAs poly (A) tail and 5'cap provide general stability determinants. The first step to initiate decay is deadenylation. Following deadenylation, decay can continue from

the 3'end through the activity of the exosome, or the 5'cap can be removed (by the decapping complex) and 5'-3' exoribonuclease activity of XRN4 can degrade the remaining transcript. Specialized decay pathways are also shown nonsense-mediated decay, silencing and internal cleavage (Belostostky *et al.*, 2009).

#### 5' to 3' Exoribonucleases

Efficient processing and degradation of RNA is a key process for the post-transcriptional control of gene expression. The cleavage of the 5' cap compromises mRNA to 5' to 3' exonucleolytic decay, apparently in an irreversible way, hence, decapping activity is tightly regulated (Li and Kiledjian, 2010). In yeast and animals, decapping complex consists on several subunits such as DCP1, DHH1p, EDC3, SCD6, PAT1, and LSM1-7 which mediate the activity of the catalytic subunit DCP2 (Kulkarni et al., 2010; Zheng et al., 2011). Several different regulatory factors are required for its activity such as Hedls/Ge1 which is required for the interaction of DCP1 and DCP2 (Fenger-Gron et al., 2005). The critical role of decapping in cell growth and development is suggested by the pleiotropic phenotype displayed by decapping component mutants in several species, such as Drosophila, Saccharomyces cerevisiae, and Schizosaccharomyces pombe (Beelman et al., 1996; Hatfield et al., 1996; Dunckley and Parker 1999; Sakuno et al., 2004; Lin et al., 2006). The decapping complex in Arabidopsis is formed by the catalytic subunit DCP2, and the subunits DCP1, DCP5, and varicose (VCS; Xu et al., 2006), although other proteins can potentially affect decapping activity. AtDCP2, AtDCP1, and VCS are isolated as proteins homologous to DCP2, DCP1, and Hedls/Ge1 in yeast and mammals, respectively (Table 2). AtDCP2 is an active subunit of a decapping complex, and the mutation analysis indicated that the Nudix domain is important for the activity. AtDCP1 and VCS can enhance the AtDCP2 activity in vitro. Analyses of interacting factors both in vivo and in vitro suggested that these three components interact with one another within a big complex. The important role of decapping process in postembryonic development has been indicated (Xu et al., 2006; Iwasaki et al., 2007). Global analysis of mRNA profiles in the mutant of AtDCP2 using microarrays indicated that putative targets of AtDCP2 include mRNAs of many different functional categories, such as signaling molecules, transcription factors, transporters, and metabolism-related proteins. Decapping component VCS is required for miRNA-mediated translational repression, as in animals (Eulalio et al., 2007; Brodersen et al., 2008), providing evidence of the link between mRNA decapping and action of at least some of the plant miRNAs. The decapping enzymes are concentrated in cytoplasmic foci called Processing bodies (P-bodies) which are cytoplasmic granules of 300-500 nm diameter, present from lower to higher eukaryotes, and composed of RNAs and proteins involved in mRNA degradation and storage. P-bodies are dynamic RNA protein aggregates found in yeast and mammalian cells with critical roles in mRNA decapping (Coller and Parker, 2004; Fenger-Gron et al., 2005), translation and translation suppression (Liu et al., 2005), RNA interference (Rehwinkel et al., 2005; Sen and Blau, 2005), RNA virus replication, and innate cellular immunity against retroviruses (Beliakova- Bethell et al., 2006). In Arabidopsis, P-body components consists of the mRNA decapping machinery, the activators of decapping and the 5' to 3' exonucleases (Xu et al., 2006). Yeast and mammals have two 5' to 3' exoribonucleases Xrn1 and Xrn2 (Nagarajan et al., 2013). Xrn1, localized in the cytoplasm, is a main enzyme catalyzing mRNA degradation following decapping, whereas Xrn2 localized primarily in the nucleus, is an essential enzyme involved in the processing of rRNA and small nucleolar RNAs (snoRNAs; Hsu and Stevens, 1993). Three members of the Arabidopsis XRN family were isolated as homologs of Xrn2, and no Xrn1 gene was identified. Among them, AtXRN4 is localized in the cytoplasm; therefore, it is the candidate for a functional homolog of the yeast Xrn1 (Kastenmayer and Green, 2000) (Figure 12). Although several substrates of AtXRN4 were identified using reverse genetics and microarray analysis, AtXRN4 is only required for the degradation of a few of the transcripts and not critical for the general degradation of unstable transcripts tested. Interestingly, the substrates of XRN4 include the 3' end products produced by miRNA mediated cleavage (Souret et al., 2004; German et al., 2008). During the search of Arabidopsis mutants that promote RNAdependent RNA polymerase (RdRp)-dependent cosuppression, a novel biological role of XRN4 in transgene-dependent gene silencing was revealed. XRN4 functions to negatively regulate gene silencing by degrading decapped transgene mRNA, which is required to initiate or maintain silencing by serving as a template for RdRp (Gazzani et al., 2004). Similar to XRN4, XRN2 and XRN3 also function as endogenous suppressors of transgene-induced silencing, probably by degrading aberrant transgene RNAs in the nucleus. In addition, XRN2 and 3 are involved in the maturation process of miRNA, such as degradation of looped end products derived from miRNA precursors (Gy et al., 2007).

	Yeast	Human	Arabidopsis	Function
	DCP1	DCP1	DCP1	Decapping
5'-3' decay	DCP2	DCP2	DCP2	Decapping
		Hedls Ge1	VCS	Decapping
	XRN1	XRN1	XRN4	Exoribonuclease
	XRN2	XRN2	XRN2/3	Maturation of miRNA
	MTR4	MTR4	MTR4	Helicase
s-s uecay			HEN2	Helicase

 Table 2. Different proteins implicated in 5'-3' and 3'-5'decay in yeast, human and Arabidopsis.

#### 3'-5' Exoribonucleases

The main 3'-5' RNA degradation machine of eukaryotic cells is the exosome, a multi-subunit complex found in both cytoplasm and nuclear compartments (Figure 12). The exosome participates in a plethora of processing and degradation reactions, including the processing of ribosomal RNAs,
snoRNAs (Small nucleolar RNA) and snRNAs (Small nuclear ribonucleic acid), the turnover and quality control of mRNAs and the efficient elimination of RNA maturation by products and diverse RNA species generated from non genic regions (Schneider et al., 2012; Chekanova et al., 2002). In vivo, exosome activity requires the interaction of the exosome complex with associated RNA helicases. In yeast, cytoplasmic and nuclear exosomes are activated by the RNA helicases SKI2 and MTR4, respectively (Brown et al., 2000; De la Cruz et al., 1998). In both yeast and human MTR4 is an essential protein required for all functions of the nuclear exosome (Lubas et al., 2011; Bernstein et al., 2008). Interestingly, Arabidopsis thaliana has two MTR4 homologues, designated MTR4 and HEN2 (HUA enhancer 2). MTR4 is a predominantly nucleolar protein required for the efficient degradation of misprocessed 5.8S rRNA precursors and specific fragments of the 5' external transcribed spacer, a by product released during processing of three rRNAs from their common precursor transcript (Lange et al., 2014). The requirement for MTR4 in efficient rRNA production is reflected by the phenotype of *mtr4* mutants, which show a characteristic combination of developmental growth defects also observed in ribosomal protein mutants and in other Arabidopsis mutants lacking putative ribosome biogenesis factors such as nucleolin (Rosado and Raikhel, 2010). In contrast to AtMTR4, HEN2 is not required for processing or degradation of 5.8S rRNA precursors or the elimination of the 5' spacer (Lange et al., 2011; 2014). HEN2 is a nucleoplasmic protein associated with the Arabidopsis exosome core complex and it has a specific role in the exosomemediated degradation of noncoding RNAs, misprocessed mRNAs, introns and transcripts derived from retrotransposons and non-genic regions. Interestingly and as recently reported for human MTR4 (Lubas et al., 2011; Andersen et al., 2013), HEN2 associates with homologues of the NEXT (for Nuclear Exosome Targeting) complex components and also co-purifies with the cap binding complex. MTR4, by contrast, is associated with a distinct set of proteins, many of which appear to be involved in ribosome biogenesis.

#### Specialized decay pathways

Additional specialized cytoplasmic mRNA decay pathways also exist, for example, non sense mediated decay (NMD) is a cytoplasmic surveillance system that identifies old or aberrant mRNAs and targets them for decay. In plants, NMD is activated by either a long 3'UTR or by a premature termination codon in close proximity to an exon junction (Kertesz *et al.*, 2006). Plant NMD recognizes mRNAs having either an unusually long 3' UTR (Figure 12), or an intron at least ~50 nucleotides downstream of the stop codon as targets (Hori and Watanabe, 2007). RNA decay can also be elicited by an internal cleavage that is independent from RNA Induced Silencing Complex (RISC) and thus distinct from small RNA pathways. For example, the decay of CGS1 mRNA (encoding cystathionine synthase, an enzyme responsible for the key regulatory step of methionine biosynthetic pathway) is

regulated through feedback. This mRNA's decay initiates by internal cleavage, which is promoted by ribosomal stalling induced by the methionine metabolite AdoMet (Onouchi *et al.*, 2008). Internal cleavage is an especially effective method for the rapid loss of RNA as each fragment presents an unprotected end. In both bacteria and eukaryotes, large numbers of small regulatory RNAs have been characterized. Small RNAs (sRNAs) act to alter the translation of separate target RNAs, either positively or negatively (Huang *et al.*, 2008; Sittka *et al.*, 2008). Different sRNAs can functionally interact, and they can be regulated by poly (A) stimulated degradation (Urban and Vogel, 2008). sRNAs therefore show functional similarities to the endogenous, eukaryotic microRNAs (miRNAs) that also largely act by regulating mRNA translation.

#### **I.4.** Objectives of the thesis

Despite huge progress carried out this last decade, it becomes clear that the transcriptional level cannot provide a complete comprehensive understanding of the regulatory mechanisms governing seed development and germination. It is indeed well known that the abundance of a transcript does not necessarily reflect its translation (Bailey-Serres et al., 2009). The translation of individual messages is highly regulated in plants and represents a rapid and efficient way to adapt cell signalling in response to environmental stresses. This might be particularly relevant for seeds which store large amounts of mRNA during maturation. Indeed in developing seeds, during the preparation for the dry state, mRNA dynamics undergoes extensive modification resulting in the accumulation of so-called 'long-lived' or 'stored' mRNA (Bazin et al., 2011). More than 12,000 transcripts are being stored in mature dry seeds of Arabidopsis (Nakabayashi et al., 2005). De novo protein synthesis during the initial phase of germination relies on these stored mRNA templates since germination can occur in the presence of transcription inhibitors (Rajjou et al., 2004). Thus the set of mRNAs that will be mobilized during seed imbibition to ensure germination are specifically synthesized during maturation and must be correctly stored in an appropriate, yet unknown form (Bazin et al., 2011; Xu and Chua, 2009). A recent study demonstrated that germination results from a timely regulated and selective recruitment of mRNAs to polysomes (Layat et al., 2014) in sunflower. The main objective of this study is to better understand the role of post-transcriptional modifications in the regulation of Arabidopsis thaliana seed dormancy. Gene expression can be modulated at multiple levels from chromatin modification to mRNA translation. The first aim of this study was to determine the conditions of after-ripening of Arabidopsis seeds. We have determined the effect of various combinations of temperature and relative humidity on dormancy alleviation of Arabidopsis seeds during dry after-ripening. The second objective was to study the changes in the transcriptome and in the translatome of dormant and non-dormant Arabidopsis seeds during their imbibition at 25°C in the darkness. To determine the main process in the regulation of dormancy a comparison of transcriptome and translatome of dormant and non-dormant seeds was done. In the second time, we have compared translatome of dormant and non-dormant seeds to demonstrate the role of translatome in the germination of Arabidopsis seeds and its regulation by the dormancy status. To characterize the translatome of dormant and non-dormant seeds, we have used some bioinformatics approaches to study the main features of promoters, 5'UTR, 3'UTR and uORF of genes which are likely to play a key regulatory role in germination. Finally, we determined the role of mRNA decay in the regulation of Arabidopsis seeds dormancy. To that purpose the phenotypes of mutants affected in mRNA decay, particularly at exosome and P-bodies components, have been characterized. Then, we focused on P-bodies to determine their potential targets in Arabidopsis seeds and to identify the role of this complex in the regulation of seed dormancy. Transcriptomic analysis of *xrn4-5* and *vcs8* mutants were analyzed to reveal mRNA targets of 5'-3' mRNA decay components.

# II. Fluctuation of Arabidopsis seed dormancy with relative humidity and temperature during dry storage

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## **II.1 Introduction**

Orthodox seeds are anhydrobiotes, they withstand severe desiccation at the end of their development on the mother plant and they can survive under very limited amounts of water in a resting state for years or decades. The desiccated state allows the cytoplasm to enter the highly viscous glassy state in which active metabolism is virtually not possible since it is prevented by low molecular mobility (Buitink and Leprince, 2008). Molecular diffusion and probability of chemical reactions are so decreased in the glassy state that they prevent enzymatic reactions (Fernández-Marín *et al.*, 2013). However, despite their resting state, orthodox seeds undergo major physiological changes during their storage, such as dormancy alleviation and loss of viability, but the underlaying mechanisms of these processes remain largely unknown.

Seed dormancy is defined as an inability to germinate even though all the necessary environmental conditions for germination are apparently satisfied (Bewley, 1997). This is an adaptive trait that permits the novel generation of plants issued from seeds to develop in the favourable season. Dormancy determines the seasonal timing of germination and plays a major role in the dynamics of plant establishment in ecosystems (Baskin and Baskin, 1998). Primary dormancy is induced during seed maturation but for many species it decreases with time under dry storage through a process called after-ripening (Finch-Savage and Leubner-Metzger, 2006). Seed after-ripening depends on seed moisture content (MC) (*i.e.* relative humidity (RH) of storage) and temperature. It is generally favored when seed MC falls within the range 5-18 % fresh weight basis, which corresponds to the intermediate region of water sorption isotherms (Probert, 2000). Bazin et al. (2011) demonstrated that the rate of dormancy alleviation during dry after-ripening depended on the relationship between seed MC and temperature and they proposed that there existed an optimal MC for dormancy release at every different temperature. Seeds can also display secondary dormancy which is induced after seed dispersal when the conditions required for completing germination are absent (Baskin and Baskin, 1998). For example, prolonged imbibition at warm temperatures induces secondary dormancy in Arabidopsis thaliana (Donohue et al., 2008). Interestingly secondary dormancy can be lost and re-introduced with season changes until environmental conditions become favorable for allowing germination (Finch-Savage and Leubner-Metzger, 2006). Secondary dormancy participates to the dormancy cycling which is defined as the annual pattern of changing dormancy status in the natural environment, i.e. in the soil seed bank (Footitt *et al.*, 2014). In Arabidopsis it has been demonstrated that depth of dormancy of buried seeds was correlated with seasonal variation of soil temperature (Footitt *et al.*, 2011).

Mutants altered in dormancy induction or expression and studies related to the expression of the Arabidopsis genome following imbibition of mature seeds indicate that there are very large changes in genome expression that are associated with the transition from dormancy to germination (Holdsworth et al., 2008). It is widely admitted that abscisic acid (ABA) and gibberellins (GAs) are the hormones playing essential but antagonistic roles in dormancy and germination, and the relative prevalence of their metabolism and signaling pathways regulates the ability to germinate (Finch-Savage and Leubner-Metzger, 2006). Many genes have been involved in induction and maintenance of seed dormancy (see Graeber et al., 2012). They include seed maturation regulators (Graeber et al., 2012), genes regulating reactive oxygen species (ROS) homeostasis (Leymarie et al., 2012) or specific genes of dormancy such as DELAY OF GERMINATION 1 (DOG1) which encodes a protein of unknown function (Bentsink et al., 2006). The molecular mechanisms involved in seed dormancy release during dry after-ripening are far much less known. Even though some changes in transcript abundance have been evidenced during dry after-ripening (Finch-Savage et al., 2007), it has been demonstrated that active transcription was prevented in dry seeds (Meimoun et al., 2014). Oracz et al. (2007), Bailly et al. (2008) and Bazin et al. (2011) showed that seed dormancy release in the dry state was associated with ROS accumulation which in turn triggered specific oxidations of proteins and mRNA, thus modifying cell functioning during subsequent seed imbibition.

The other major modification occurring during prolonged seed dry storage is loss of viability, the ultimate consequence of seed ageing. Seed ageing is an irreversible process initiated at seed shed but its kinetics greatly depends upon RH and temperature during storage, initial seed vigor, genetic background, and growth environment of the seed (Ellis and Roberts, 1980; Walters, 1998). Interdependence of RH, *i.e.* seed MC, and temperature has been evidenced by Harrington (1973) who proposed 3 "rules of thumbs" regarding optimal seed storage. The first one, applicable for seeds with MC ranging from 5 to 14 % states that each 1 % reduction of seed MC doubles seed longevity; the second one states that for each 5-6 °C decrease in storage temperature seed storage life is doubled and the third one states that arithmetic sum of relative humidity and storage temperature should not exceed 100 for safe seed storage, or 120 as later reported (Bewley and Black, 1985; Copeland and McDonald, 1999). Similar biochemical and molecular changes are likely to occur during prolonged seed storage than during after-ripening which suggests that there is a continuum for the chemical reactions taking place at the onset of seed dispersal through dormancy release, ageing and

loss of viability. Since enzymatic reactions are not possible in dry seeds, ROS accumulation and oxidative modifications of biomolecules have often been considered as one of the most important factors influencing seed ageing (Bailly, 2004). Thus, Bailly *et al.* (2008) suggested that seed germination occurred when the seed ROS content was enclosed within an oxidative window allowing ROS signaling, *i.e.* dormancy alleviation, but not ROS damage, *i.e.* seed ageing.

The objective of the present study was to determine how the conditions of storage of Arabidopsis thaliana seeds at harvest influence the kinetics of seed dormancy release (after-ripening) and ageing. To address this question, dormant Arabidopsis seeds were stored within a large range of temperatures and RH and germination was regularly assessed for one year. This study was carried out with seeds of the Arabidopsis ecotype Columbia and with seeds from mutants mtr4-1 (exosome RNA helicase, Lange et al., 2011) and cat2-1 (catalase 2, Queval et al., 2007). Seeds of the mutant cat2-1 are low dormant at harvest (Leymarie et al., 2012) whereas seeds of mtr4-1 are deep dormant (Basbouss-Serhal et al., unpublished data). The use of seeds from mutants was undertaken to precise if the genetic background might influence the storage behaviour of the seeds and if initial dormancy level could modulate seed longevity. Water sorption isotherms and Arrhenius plots were built-up from the data to get insights on the mechanisms involved in release of primary dormancy. We also followed the changes in the abundance of transcripts related to ABA and GA metabolism and signalling during seed storage. They include NCED3 (Nine-Cis-Epoxycarotenoid Dioxygenase 3), NCED6 (Nine-Cis-Epoxycarotenoid Dioxygenase 6), and NCED9 (Nine-Cis-Epoxycarotenoid Dioxygenase 9), coding for 9-cis-epoxycarotenoid dioxygenases involved in ABA biosynthesis (Tan et al., 2003, Lefebvre et al., 2006), and ABI5 (ABA Insensitive 5) which codes for a member of the basic leucine zipper transcription factor family and is involved in ABA signaling during seed maturation and germination (Finkelstein and Lynch, 2000). CYP707A2 codes for an ABA 8-hydroxylase and is involved in ABA catabolism (Millar et al., 2006). GA3ox1 (GA3 oxidase 1) and Ga20ox4 (GA 20-oxidase 4) codes for dioxygenases which catalyzes the conversion of gibberellin precursors to their bioactive forms (Yamaguchi, 2008) while Ga2ox2 (GA2 oxidase 2) is involved in gibberellin inactivation (Yamaguchi, 2008). SLP2 (Subtilisin-Like serine Protease 2) is induced by gibberellins (Ogawa et al., 2003). Here we demonstrate that dormancy level of Arabidopsis seeds fluctuates during dry storage in a complex manner dependent upon seed MC and temperature of storage.

## **II.2 Material and methods**

#### Plant material and after-ripening conditions

*Arabidopsis thaliana* seeds were grown and harvested dormant as described by Leymarie *et al.* (2012). Col-0 (Columbia) was used as wild-type. The *mtr4-1* mutant (GABI-048G02, provided by Dr Hervé Vaucheret, Institut Jean-Pierre Bourgin, INRA, Versailles, France) is characterized by a T-DNA insertion in *MTR4* gene (AT1G59760) (Lange *et al.*, 2011). The *cat2-1* mutant (accession No. SALK\_057998) is characterized by a T-DNA insertion in the *CAT2* gene (AT4G35090) in the third exon from the 5'end (Queval *et al.*, 2007). After-ripening was performed by placing seeds at 10, 15, 20 and 25 °C over silicagel or satured solutions of ZnCl<sub>2</sub>, LiCl, KAc, MgCl<sub>2</sub>, CaCl<sub>2</sub>, CaNO<sub>3</sub>, NaCl and KCl, in tightly closed jars thus giving relative humidities (RH) of approximately 1, 5.5, 14, 23.3, 36, 45, 56, 75 and 85 %, respectively (Vertucci and Roos, 1993; Sun, 2004).

#### Germination assays

Germination assays were performed at 15 °C and 25 °C in darkness by placing 100 seeds of each genotype in 9 cm Petri dishes on a layer of cotton wool moistened with water or with a solution of gibberellic acid (10<sup>-4</sup>M). Ethylene treatment was carried out by placing open Petri dishes with seeds imbibed on water in a closed container containing 200 ppm ethylene. A seed was considered as germinated when the radicle protruded the envelopes. Germination was scored daily for 7 days and the results presented correspond to the mean of the germination percentages obtained for 3 replicates of 100 seeds ±standard deviation (SD).

#### Tetrazolium test

After 48 h of imbibition in water at room temperature seeds were transferred to 1 % (w/v) tetrazolium chloride (TZ) solution and incubated in darkness for a further 24 h at 25 °C before staining evaluation (Delouche *et al.,* 1962). Results presented correspond to the % of seeds developing red staining in presence of TZ solution and are the means of 3 replicates of 25 seeds ±standard deviation (SD).

#### Moisture content determination

Seeds (approx. 15 mg fresh weight, in 3 replicates) were dried at 105 °C for 24 h and dry weight was subsequently measured with a microbalance. Seed MC was determined as follow: fwt (fresh weight)-dwt (dry weight)/dwt. Results are expressed as g  $H_2O$  g<sup>-1</sup>on a dry weight basis.

#### RNA extraction and real-time quantitative RT-PCR

Thirty mg of seeds (3 biological replicates) were ground in liquid nitrogen and total RNA was extracted by a hot phenol procedure according to Verwoerd *et al.* (1989). Reverse transcription was performed with 1 µg total RNA as described by Leymarie *et al.* (2012). The relative expressions were calculated according to Hellemans *et al.* (2007) with 2 reference genes *Ubiquitin 5* and *AT4G12590* and expressed in arbitrary units with a value of 100 to the dry dormant seeds after harvest which was used as control sample for normalization (Pfaffl, 2001). Primer sequences are presented in Supplemental Table S1. The statistical analysis was performed with StatBox 6.40 software (Grimmer logiciel, France). Data were normally distributed and with equal variance Newman–Keuls tests ( $\alpha$ = 0.05) were performed after analysis of variance (ANOVA).

Supplemental Table S1. Sequences of primers used for	qRT-PCR exp	periments. The	AGI for e	each gene	were
determined by Tair and the sequence of primer by Primer	rs 3				

Genes	AGI	Primers sequenes
	471010400	F:TTGAAGTCAAGGGCACAACC
ΑΟΙΟ	A11016460	R:CGGGTTCCTCATCAATGTCC
CVD70742	AT2G29090	F:GGCACCAAAACCTTACACG
CIFTOTAL		R:TCTCCAATCACTTCCCATCTG
	AT1C72100	F:GCTGCGGTTTCTGGGAGAT
NCEDS	A11072100	R:GGCGGGAGAGTTTGATGATT
	AT3G51600	F: TTCAAGATACCGACACTTCCTG
NCEDO		R: GGCGATTCTGCTCCATAGG
NCED9	AT1G69530	F:TCCCCTGCTATGTTTCTTCC
		R:AGACGGTGGTTTGAATGTCG
SLP2	AT4G34980	F: AAAATCGGGAATGTCATCAGC
		R: CAACCAATCCTTTGGCTACG
Ca2ov1	AT1G18250	F:TTGGGGTCAGCGAAGAAGA
GUSOXI		R:CAGAATGGTTAGGAGGGTGGA
Ga20ox4	AT5G64100	F:GCGAGACGACAAGGAAGACA
		R:TCGGGATACGCTCTCTCACC
Calori	AT1G74670	F: TGTTGGAGATGGTTGCCGAA
Guzuxz		R: CCATCTTCTCCGCCTCTTCC

## **II.3 Results**

#### Effect of relative humidity and temperature on seed dormancy release

Figure 1 shows the germination of Col-0, *mtr4-1* and *cat2-1* seeds stored in various combinations of temperature and RHs for more than one year (63 weeks) after their harvest. Germination was evaluated after 7 days at 25 °C in darkness, conditions in which Arabidopsis seed dormancy is

expressed (Leymarie *et al.*, 2012). At harvest seeds from Col-0 were dormant since germination did not exceed 20 %. Seeds of *mtr4-1* were more dormant those from Col-0 since only 5 % germinate, while *cat2-1* seeds were less dormant (75 % of germination; Figure 1; Supplemental Figure 1). Storage of seeds first resulted in a progressive increase of germination at 25 °C for all genotypes. In all cases maximum germination percentage was reached after ca 7 weeks of storage but the efficiency of dormancy alleviation greatly depended on temperature and relative humidity. When temperature of storage increased from 10 to 25 °C, the optimal RH for dormancy alleviation increased from 33 to 75 %, whereas the lowest RH (1 %) prevented full dormancy release at all temperatures. Conversely high RH (85 %) also inhibited break of dormancy when temperature was lower than 25 °C.



**Supplemental Figure S1.** Germination after 7 days at 25 °C of Col-0, *mtr4-1* and *cat2-1* seeds after harvest (A), after storage for 2 weeks at 56 % of relative humidity and 20 °C (B), after storage for 3 weeks at 56 % of relative humidity and 20 °C (C) and after storage for 4 weeks at 56 % of relative humidity and 20 °C (D). Mean ± standard deviation of three biological repetitions for each measurement.



**Figure 1.** Seed germination percentages at 25 °C in darkness of wild type Col-0 (A, B, C, D), *mtr4-1* (E, F, G, H) and *cat2-1* (I, J, K, L) after different durations of storage at 10 °C (A, E, I), 15 °C (B, F, J), 20 °C (C, G, K) and 25 °C (D, H, L) under relative humidities of 1 % (black circle), 33 % (diamond), 45 % (black square), 56 % (triangle), 75 % (white circle) and 85 % (white square) Mean ± standard deviation of 3 biological replicates for each measurement.

Prolonged storage of seeds was associated with a dramatic decrease of germinability at 25°C, which occurred after 9 weeks of storage at 10, 15 and 25 °C and did not depend on RH. At 20 °C, however, germinability decreased more slowly when seeds were stored at 56 % RH and it became null only after ca 50 weeks. This RH also extended germinability after storage at 15 °C to ca 12 weeks (Figure 1).

The relationship between RH and seed MC at different temperatures is given by the water sorption isotherms shown in Figure 2. Whatever the genetic background, changes in seed moisture content as a function of relative humidity displayed the classical reverse sigmoidal curves of sorption isotherm allowing identifying 3 regions of water binding within seed tissues, as shown by arrows on graphs. They correspond to bound water, which includes structural and monolayer water, intermediate water, where water is less firmly bound than in the first region, and free water. Above 85 % RH, seed MC dramatically increased whereas it decreased at lower RHs (*i.e.* below ca 15 % RH) to reach MCs as low as 0.037 g H<sub>2</sub>O g dw<sup>-1</sup>. RHs delimitating regions of isotherms were similar at all temperatures and did not vary for the mutants. As expected, decreasing temperatures were associated with higher water binding capacities, particularly within the range 40-85 % RH. For example at 56 % RH, seed MC almost doubled when temperature decreased from 25 to 10 °C.

We determined the optimal moisture content for seed dormancy release at 10, 15, 20 and 25°C (Figure 3), which was evaluated by germinating seeds at 25 °C after 7 weeks of after-ripening, this duration being the most efficient for dormancy release (see Figure 1). Our results show a dependency of this value on temperature. The higher the seed MC, the higher the optimal temperature allowing breaking of dormancy was (Figure 3A, C, E), and reciprocally. For example at 25 °C, the optimal MC for dormancy release of Col-0 seeds was close to 0.07 H<sub>2</sub>O g dw<sup>-1</sup>, but it decreased to 0.06 at 20 °C, 0.05 at 15 °C and 0.04 at 10 °C (Figure 3A). Similar values were obtained for seeds of *mtr4-1* and *cat2-1* mutants (Figure 3C, D; respectively). One can assume that the optimal MC for seed dormancy alleviation increased by approximately 0.02 g H<sub>2</sub>O g dw<sup>-1</sup>when temperature increased by 5 °C (Figure 3A, C, E).

Arrhenius plots allow to determine the temperature dependence of dormancy alleviation rate on RH (Figure 3B, D, F). The relationship between seed MC, temperature and dormancy alleviation is shown by the straight lines obtained when plotting ln(germination) against reciprocal temperature (1/T) and by the high correlation coefficients (Figure 3B, D, F). The temperature dependence for dormancy release was similar for all genotypes (Figure 3B, D, F). Below ca 0.06 g H<sub>2</sub>O g dw<sup>-1</sup>, the activation energy, determined by the slope of the plots was negative (slope=-Ea/R), *i.e.* the rate of dormancy alleviation increased when temperature decreased (Figure 3B, D, F). Above this value, the activation energy became positive and increased when MC increased, *i.e.* the higher the seed MC, the higher the slope of the curves (Figure 3B, D, F).



**Figure 2.** Water sorption isotherms measured at 10, 15, 20 and 25 °C of seeds of Col-0 (A), *mtr4-1* (B) and *cat2-1* (C). Arrows within graphs indicate limits of regions of water binding. Mean ± standard deviation of 10 replicates of 10 seeds.



**Figure 3.** Germination after 7 days at 25 °C of Col-0 (A), *mtr4-1* (C) and *cat2-1* (E) seeds stored for 7 weeks at 10 °C (circle), 15 °C (diamond), 20 °C (triangle) and 25 °C (square) at various relative humidities (1, 33, 45, 56, 75 and 85 %) which produced the indicated moisture contents. Arrhenius plots of seed dormancy release, where ln(germination), determined after 7 days at 25 °C, is plotted against the inverse of the temperature [Temperature<sup>-1</sup> (1000/K)] for Col-0 (B), *mtr4-1* (D), *cat2-1* (F). Seed moisture content during after-ripening (which lasted for 7 weeks) and R<sup>2</sup> values of the linear regression lines are indicated within the graph.

The temperature coefficient (Q10) allows measuring the rate of change of a biological system when increasing the temperature by 10 °C. The coefficient Q10 has been calculated at different seed MC within the range 10-25 °C (Table 1). It increased regularly from ca 0.3 to more than 2 when seed MC increased from 0.04 to 0.15 g  $H_2O$  g dw<sup>-1</sup> (Table 1), which indicated that rate of seed dormancy alleviation decreased when temperature increased at MC lower than 0.06 g  $H_2O$  g dw<sup>-1</sup>, then did not vary at intermediate MCs, *i.e.* when Q10 value was close to 1 and increased for higher MC. Q10 values of 2 or more, obtained when seed MC was higher than 0.13, indicated that rate of dormancy alleviation doubled when temperature increased by 10 °C.

**Table 1.** Temperature coefficients  $(Q_{10})$  of Col-0, *mtr4-1* and *cat2-1* seeds, calculated between 10 and 25 °C at different moisture contents.

Seeds	Values of $Q_{10}$ at the seed moisture content (gH <sub>2</sub> O g dw <sup>-1</sup> )					
	0.04	0.05	0.06	0.1	0.13	0.15
Col-0	0.49	0.71	0.82	1.58	2.4	2.55
mtr4-1	0.31	0.61	0.75	1.2	1.96	2.14
cat2-1	0.36	0.69	0.9	1.44	2.16	2.29

#### Physiology of seed germination after prolonged storage

Since after 63 weeks of storage in all conditions germination percentages at 25 °C were always very low or null (Figure 1), we investigated whether seeds stored for this duration were died or deeply dormant. Germination at 15 °C, the optimal temperature for Arabidopsis seeds, revealed that seeds stored at 75 and 85 % RH were died after prolonged storage at all temperatures (Figure 4), which was also confirmed by the absence of staining in the presence of tetrazolium chloride (TZ) (Table 2). Lower RHs maintained seed viability when seed were stored at 10 or 15 °C, since seeds all germinated at 15 °C and almost all developed red staining in presence of TZ (Table 2). This revealed a process of secondary dormancy induction. In contrast, increasing temperature of storage to 20 and 25 °C lead to a decrease in seed viability, as shown Figure 4 and by lower staining of tissues by TZ (Table 2). At these temperatures only RHs close to 50 % allowed seeds survival (Figure 4 and Table 2). The use of several methods known to alleviate dormancy, such as cold stratification, GA and ethylene treatments permitted seeds stored for 63 weeks to germinate at 25 °C (Table 2) and confirmed the data shown in Figure 4.

To gain more insights about the changes in dormancy level we investigated the expression of genes known to be involved in the regulation of primary and secondary dormancy in freshly harvested seeds (D1), and seeds stored for 7 (ND) and 63 (D2) weeks at 56 % RH and 20 °C. Gene expression

was evaluated in dry seeds and in seeds imbibed for 24 h at 25 °C in the dark, using real-time reverse transcription-PCR (RT–PCR) (Supplementary Table S1). The abundance of all genes studied did vary significantly when assessed in dry seeds (Figure 5). The expression of *NCED3*, *NCED6*, *NCED9* and *ABI5* followed the same pattern. It decreased significantly in non-dormant imbibed seeds (*i.e.* seeds after-ripened for 7 weeks) but increased markedly in seeds imbibed after prolonged storage (*i.e.* 63 weeks). In contrast, abundance of *CYP707A2*, *Ga20ox4*, *Ga3ox1* and *SLP2* transcripts in imbibed seeds was higher in seed that had been for 7 weeks than in freshly harvested seeds. After 63 weeks of storage, expression of these genes in imbibed seeds decreased to the same level as the one found in freshly harvested seeds, excepted for *CYP707A2* whose expression did not decrease significantly. At last, expression of *Ga2ox2* was similar in all imbibed samples.



**Figure 4.** Germination after 7 days at 15 °C of Col-0, *mtr4-1* and *cat2-1* seeds stored for 63 weeks at 10 °C (A), 15 °C (B), 20 °C (C) and 25 °C °C (D) under different relative humidities. Mean ± standard deviation of 3 biological replicates for each measurement.

**Table 2.** Percentages of germination and viability of Col-0 seeds stored for 63 weeks at various relative humidities at 10 °C, 15 °C, 20 °C and 25 °C. Germination assays were carried out at 25 °C for 7 days on gibberellic acid solution (GA<sub>3</sub> 10<sup>-4</sup>M), on water in presence of ethylene (200 ppm) or after a stratification treatment of seeds at 4 °C for 2 days. The viability of seeds was assessed using tetrazolium chloride. Means  $\pm$  SD of triplicate experiments.

Storage temperature	Assay	Germination	Relative humidities (%)					
		conditions						
		-	1	33	45	56	75	85
		GA <sub>3</sub>	87.5±2.3	91±1.5	87.5±2.4	85±2.1	0	0
10 %	Germination (%)	Ethylene	85.7±1.7	92±1.4	86±1.2	86.5±1.4	0	0
10 C		Stratification	86.5±1.8	90.5±2.8	87±2.4	87.4±1.7	0	0
	Viability (%)	-	86.5±0.6	90±1.2	85.6±1.4	85.6±0.8	0	0
		$GA_3$	82.5±2.5	81	85	82.5±2.5	0	0
15 °C	Germination (%)	Ethylene	82.5±1.4	82	84	83.6±1.1	0	0
15 C		Stratification	81.5±1.7	82.5±2.4	83.5	82.4±1.2	0	0
	Viability (%)	-	83.2±1.4	83.2±1.5	84±0.7	83.5±0.5	0	0
20 °C		GA <sub>3</sub>	83.5±3.5	91	85	70	0	0
	Germination (%)	Ethylene	85±3.5	89	84±1.2	70±1.2	0	0
		Stratification	84.5±3.7	88.5±2.4	85	73	0	0
	Viability (%)	-	83.2±1.3	90±0.7	84.5±0.8	71±1.3	0	0
25 °C		GA <sub>3</sub>	69.5±3.2	88.5±3.5	81.5±4.1	82.5±3.1	0	0
	Germination (%)	Ethylene	70.0±2.5	87.2±2.1	82.5±3.2	80±1.2	0	0
		Stratification	71.5±1.7	89.5±3.4	83.5±3.4	82.5±2.4	0	0
	Viability (%)	-	72±0.7	90±0.7	82±1.8	81±1.2	0	0

To gain more insights about the changes in dormancy level we investigated the expression of genes known to be involved in the regulation of primary and secondary dormancy in freshly harvested seeds (D1), and seeds stored for 7 (ND) and 63 (D2) weeks at 56 % RH and 20 °C. Gene expression was evaluated in dry seeds and in seeds imbibed for 24 h at 25 °C in the dark, using real-time reverse transcription-PCR (RT–PCR) (Supplementary Table S1). The abundance of all genes studied did vary significantly when assessed in dry seeds (Figure 5). The expression of *NCED3*, *NCED6*, *NCED9* and *ABI5* followed the same pattern. It decreased significantly in non-dormant imbibed seeds (*i.e.* seeds after-ripened for 7 weeks) but increased markedly in seeds imbibed after prolonged storage (*i.e.* 63 weeks). In contrast, abundance of *CYP707A2*, *Ga20ox4*, *Ga3ox1* and *SLP2* transcripts in imbibed seeds of storage, expression of these genes in imbibed seeds decreased to the same level as the one found in freshly harvested seeds, excepted for *CYP707A2* whose expression did not decrease significantly. At last, expression of *Ga2ox2* was similar in all imbibed samples.



**Figure 5.** Transcript abundance of *NCED3*, *NCED6*, *NCED9*, *ABI5*, *DOG1*, *CYP707A2*, *Ga3ox1*, *Ga20ox4* and *Ga2ox2*in Col-0 seeds, dry (white bars) or imbibed for 24 h at 25 °C (black bars). Analysis was performed on dormant seeds after harvest (D1), non-dormant seeds after-ripened at 56 % relative humidity (RH) and 20 °C for 7 weeks (ND) and secondary dormant seeds stored for 63 weeks at 56 % RH and 20 °C (D2). The relative expression was calculated from RT-PCR data with 2 references genes Ubiquitin 5 and AT4G12590 and expressed in arbitrary units with a value of 100 attributed to the dry dormant seeds after harvest. Means ± SD of 3 biological replicates are shown.a, band c indicate homogeneous groups in a corresponding class (Anova test and Newman-Keuls tests, P=0.05).

### **II.4 Discussion**

At harvest, seeds of *Arabidopsis thaliana* were dormant since they were unable to germinate at 25 °C in darkness (Supplementary Figure S1). Germination of seeds of the wild type Col-0 did not exceed 20 % under these conditions. Freshly harvested seeds of the mutant *cat2-1* where less dormant since they germinated to 70 % at 25 °C whereas seeds of the mutant *mtr4-1* displayed higher dormancy, their germination being lower than 10 % at harvest. After-ripening carried out by placing dormant

seeds at 56 % RH and 20 °C induced dormancy alleviation and seeds of the 3 genotypes became able to fully germinate at 25 °C after 4 weeks under these conditions (Supplementary Figure S1). Arabidopsis seed dormancy release by after-ripening is a classic mechanism but the consequences of modifying RH and temperature during this period are largely unknown. Here we demonstrate that there exist a strong dependency of the physiological changes occurring in the dry state on both RH and temperature. We also show that the genetic background, and the level of dormancy at harvest, do not influence the pattern of fluctuation of dormancy level during storage. Therefore the results obtained will be discussed below without making distinction between the wild type, Col-0, and the 2 mutants *cat2-1* and *mtr4-1*.

#### Primary dormancy alleviation dependency on temperature and RH

Studying the role of storage conditions of primary dormant Arabidopsis seeds revealed the complex relationship between seed MC and temperature for the control of dormancy release (Figure 1). Whatever the genotype and the conditions of storage used here, total or partial dormancy release occurred within 7 weeks, but its kinetics greatly depended on RH and temperature. As already demonstrated (see Finch Savage and Leubner Metzger, 2006) we show that dormancy alleviation is favoured at intermediates RHs, *i.e.* close to 50 %, but that it is slowed for extremes RHs, *i.e.* in very dried seeds or in high air humidity. We also show that dormancy release is faster at 20 °C, which allows proposing that the optimal combination for alleviation of dormancy in Arabidopsis seeds is 20 °C and 56 % RH. From the sorption isotherms shown Figure 2, one can determine the corresponding optimal MC which is close to 0.05 g H<sub>2</sub>O g dw<sup>-1</sup> at 20 °C (corresponding to 56 % RH). This MC fell within the water-binding region 2 of sorption isotherms, which corresponds to weakly bound water but nevertheless limits biochemical reactions. Water sorption isotherms shown here displayed the classical sigmoidal shapes and the RHs at which the transitions occurred from different water-binding regions were roughly similar to those found in other ecotypes of Arabidopsis (Hay, *et al.* 2003) or in oily seeds such as sunflower (Bazin *et al.*, 2011).

The results presented here reveal a tight and complex relationship between temperature and RH in the kinetics of after-ripening, and Figure 3 demonstrates that the higher the seed MC, the higher the optimal temperature for after-ripening. For example, similar kinetics of seed dormancy alleviation can be obtained at 10 °C or 25 °C if seed MC is close to 0.04 or 0.08 g H<sub>2</sub>O g dw<sup>-1</sup>, respectively. Thermodynamic component of seed dormancy release can be appreciated using Arrhenius plots (Figure 3B, D, F) and calculation of Q10 values (Table 1). The effect of temperature on enzyme activities is well described by the Arrhenius activation energy, which represents the effect of temperature on the catalytic rate constant, and it is widely admitted that rates of biological activity increase exponentially with temperature (Brown *et al.*, 2004). However dry seeds are a case species

in which absence of free water prevents active metabolism. Arrhenius plots shown Figure 3B, 3D and 3F allow proposing the seed MC value of 0.06 g  $H_2O$  g dw<sup>-1</sup> as being critical for governing the nature of the mechanisms leading to seed dormancy release. Indeed, below 0.06 g H<sub>2</sub>O g dw<sup>-1</sup> dormancy release in Arabidopsis was associated with negative activation energies, as shown by the negative slot of the plots (Figure 3B, D, F). The rate of dormancy release thus decreased with increasing temperature. In sunflower seeds it was also found that dormancy alleviation during after-ripening was associated with negative activation energies, when seed MC was below 0.1 g  $H_2O$  g dw<sup>-1</sup> (Bazin et al., 2011). For biological models, negative activation energies have been shown in mammalian cell lines exposed to hypothermia, which causes chilling injury and killing and they have been attributed to gel-to-liquid crystalline transition of lipids and protein denaturation (Muench et al., 1996). However, in the present study the major determinant was MC, but not temperature, since negative and positive activation energy occurred within the range 10/25 °C, as also shown by the Q10 values which are clearly below 1 at lowest MC (Table1). In plants, metabolic rates double with every 10 °C increase in temperature usually giving Q10 values close to 2 (Criddle et al., 1994). This was the case here but only when seed MC was higher than 0.12 g  $H_2O$  g dw<sup>-1</sup> (Table 1). Altogether these data therefore suggest that dormancy alleviation at low MC does not involve metabolic activities, contrary to what occur when seed MC increases and when Q10 values are close to 2 (Table 1). As suggested for dormancy alleviation of sunflower seeds, dormancy release of Arabidopsis may be achieved by distinct mechanisms as a function of seed MC. At low MC, we propose that seed dormancy release occurs through non-enzymatic oxidative reactions which are known to be associated with negative activation energies (Cheney, 1996; Cunningham et al., 1999; Olson et al., 2002; Bazin et al., 2011). Elementary reactions exhibiting negative activation energies are reactions for which increasing the temperature reduces probability of the colliding molecules capturing one another. This is in agreement with the concept of "oxidative window" which postulates that a certain oxidative load is needed to break dormancy (Bailly et al., 2008). When seed MC is above ca 0.12 g  $H_2O$  g dw<sup>-1</sup>, metabolic reaction are likely to occur and to participate to dormancy alleviation. Then, the activation energy (given by the slope of the plots) increases when seed MC increases thus showing that changes in temperature have a significant effect on the rate of dormancy alleviation. At last, between 0.06 and 0.12 g  $H_2O$  g dw<sup>-1</sup>, the activation energy is low within the studied temperature range which shows that changes in temperature have then little effect on the rate of dormancy alleviation.

#### Loss of seed germinability during prolonged storage

Following partial or full dormancy release during the first weeks of after-ripening, seed germination at 25 °C abruptly decreased and became very low after 12 weeks of storage (Figure 1). Nevertheless, storing seeds for longer periods induced again an increasing ability to germinate at this temperature,

and maximum germination percentages were reached after 21 to 28 weeks of storage, even though they never exceeded 35 %. Interestingly, the combination of RHs and temperatures which were the more efficient for triggering release of primary dormancy (see Figure 3) did not permit a marked increase of germination after 21-28 weeks of storage (Figure 1). Finally, the ability to germinate at 25 °C declined again and was fully prevented after one year of storage, whatever the conditions of storage. These fluctuating changes in the germination potential during dry storage were rather unexpected. Indeed, it is very well known that seed germinability decreases during storage as a result of ageing (Rajjou and Debeaujon, 2008) but what is shown here is a re-induction of the potential to germinate during storage after an initial loss of germinability. This suggests that loss of germinability after 12 weeks of storage did not result from a loss of viability but from an induction of secondary dormancy. This hypothesis was verified by germinating seeds stored for 63 weeks, i.e. whose germination was totally prevented at 25 °C, at lower temperature (Figure 4) or at 25 °C in the presence of ethylene or GA or after stratification at 5 °C (Table 2). The high germination rates observed under these conditions show that stored seeds were fully viable, as also observed with TZ staining, but that they were unable to germinate at 25 °C, provided that RH during storage did not exceed 56 % (Table 2). In this former case, seeds were no more viable as shown by the absence of TZ staining or by the inefficiency of dormancy release treatments (Table 2).

Our data therefore allow identifying two different mechanisms occurring during prolonged storage of Arabidopsis seed. First, the decline of seed viability at higher RH (> 56 %) (Table 2) was a consequence of seed ageing. Seed ageing was also favored at 20 and 25 °C at the lowest RH (Figure 4) which might result from increased lipid oxidations, which are known to be enhanced within region 1 of sorption isotherm and with increasing temperature (Labuza, 1980). Secondly, we demonstrate that secondary dormancy can be induced at low MC. While primary dormancy is set during seed development, secondary dormancy develops after harvest when environmental conditions do not allow germination (Hilhorst, 2007). In natural conditions, when seeds are buried in the soil, light, temperature and moisture content, through their natural seasonal variation are the key factors regulating dormancy cycling (Baskin and Baskin, 1998; Footitt et al., 2011). In our experiments these factors were maintained constant throughout storage and changes in dormancy level thus resulted from intrinsic properties. This was also rather surprising that all seed batches, *i.e.* stored in various conditions or issued for different genetic background, displayed synchronized and similar fluctuations in dormancy level. A relatively similar behavior has been evidenced for seeds of Mesembryanthemum nodiflorum L. stored in dried conditions (Gutterman and Gendler, 2005), who displayed an annual rhythm of germination. Seasonal or circadian rhythms are well described in plants but they generally concern metabolically active organisms (see McClung, 2006). The

mechanisms underlying fluctuating changes in dormancy level in the dry state during storage are totally unknown and difficult to explain with regards to the actual knowledge related to life rhythms. Gaining insights in the changes of dormancy level during storage was achieved by studying the expression of key regulator genes of dormancy. We show that seed dormancy cycling in the dry state relied on quantitative changes during seed imbibition of transcripts related to hormone synthesis and signaling. As expected, abundance of the transcripts studied did not vary during long term seed storage (1 year) at 20 °C and 56 % RH (Figure 5), a condition that retained seed viability but that induced secondary dormancy (Table 2 and Figure 4). Indeed at this RH, seed MC was close to 0.05 g H<sub>2</sub>O g dw<sup>-1</sup> thus preventing transcription (Meimoun *et al.*, 2014). However seed imbibition at 25 °C for 24 h revealed major changes in gene expression, as regulated by dormancy status. Release of primary dormancy, *i.e.* after 7 weeks of storage, was associated with the repression of genes related to ABA biosynthesis (AtNCED3, AtNCED6, AtNCED9) and signaling (AtABI5) and with the induction of AtCYP707A2, a gene involved in ABA catabolism (Figure 5). Simultaneously expression of AtGA200x4 and AtGA3ox1, both involved in GA activation (Yamaguchi, 2008), increased concomitantly to the one of the AtSLP2, a gene known to be induced by GA (Ogawa et al., 2003). This shows that alleviation of primary dormancy is regulated by the hormonal balance ABA/GA, as already well known for seeds of this species (Finch-Savage and Leubner-Metzger, 2006). Interestingly induction of secondary dormancy during subsequent storage globally restored the same pattern of gene expression, estimated after 24 h of imbibition at 25 °C, that the one found in primary dormant seeds. It can be conclude that this similarity in both the primary and secondary dormant states imposes maintenance of dormancy through ABA synthesis and signalling and repression of GA synthesis and signalling thus inducing a negative regulation of germination during seed imbibition (Cadman et al., 2006; Finch Savage and Leubner Metzger, 2006). Mostly it demonstrates that the period of storage experienced by the seeds is critical for regulating the expression of regulators during seed subsequent imbibition.

In conclusion, this study brings a comprehensive view of the combined effects of temperature and RH on the physiology of Arabidopsis seeds during prolonged storage. From a practical point of view our results should lead scientists interested by studies on dormancy using the model plant Arabidopsis to pay attention to the conditions of storage used in their experiments. This work also raises up fundamental questions about the molecular bases of the changes that can take place in the dry state, but they will request further investigations.

# III. Germination potential of dormant and non-dormant Arabidopsis seeds is driven by distinct recruitment of mRNAs to polysomes

## In revision in Plant Physiology

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## **III.1 Introduction**

Regulation of gene expression in higher plants results from transcription but also from posttranscriptional processes including sequestration of mRNAs into cytosolic mRNA ribonucleoproteins (mRNPs), mRNA decay mechanisms and regulation of translation. Initiation is the key regulatory step of translation which also includes elongation and termination. Initiation starts when a small (40S) ribosomal unit is loaded on the 5' cap of an mRNA and forms a 43S pre-initiation complex with an eIF2 $\alpha$ -GTP-tRNAmet ternary complex which scans the RNA template down in a 5' to 3' direction until an AUG codon (Browning, 1996). Then the coupling of 40S subunit with a 60S ribosomal subunit gives an 80S ribosome and the elongation phase starts (Bailey-Serres, 1999; von Arnim et al., 2014). It is worth noting that mRNAs recruit not one but multiple ribosomes to form polysomal complexes (polyribosomes) and their number per transcript therefore reflects the efficiency of translation and rate of elongation (Kawaguchi et al., 2004). Initiation of translation requires the assembly of a circular mRNA protein complex and is tightly regulated by various eukaryotic Initiation Factors 4 (eIF4) and by their phosphorylation, by RNA helicases and RNA binding proteins and by the availability in ATP and GTP, since translation is an energetically costly cellular process (Bailey-Serres, 1999). In addition the characteristics of the 5'- and 3'-untranslated regions (5'UTR and 3'UTR, respectively) of mRNA also play key regulatory roles in the initiation process (Wilkie et al., 2003). For example, the length and the nucleotide composition of the 5'UTR modulates the entry of the 43S pre-initiation complex, probably by modifying RNA secondary structure (Kawaguchi and Bailey-Serres, 2005). The presence of upstream open reading frames (uORF) in the 5'UTR also generally reduces translation in a length dependent manner (von Arnim et al., 2014).

The regulation of translation in plants is mainly documented in the context of response to stresses. Indeed, translation, which requires high energy biochemical processes, is often impaired in defavourable environmental conditions such as hypoxia (Branco-Price *et al.*, 2008), heavy metals (Sormani *et al.*, 2011) or excess heat (Matsuura *et al.*, 2010), but response to stress also involves differential translation of specific mRNAs (von Arnim *et al.*, 2014; Juntawong *et al.*, 2014). The role of translational control of plant development is documented in Arabidopsis in the context of pollen tube growth (Lin et al., 2014) and light signaling (Piques et al., 2009; Juntawong and Bailey-Serres, 2012; Liu et al., 2013) and in sunflower for seed germination (Layat et al., 2014). In this former study it was demonstrated that the translatome differs between germinating and non-germinating sunflower embryos, thus highlighting a timely regulated and selective recruitment of mRNAs to polysomes. This pioneering work opened fields of investigation for the understanding of germination since until now the molecular regulators of seed germination were identified using large scale data sets of total transcriptomes (see Holdsworth et al., 2008 for review). It is indeed widely admitted that seed germination and dormancy are regulated transcriptionally and are under the control of a balance between hormonal pathways associated with negative (abscisic acid) and positive (ethylene, gibberellins) regulators of germination. Recently, system approaches have led to design networks of transcriptional interactions that help to define seed germination regulators (Bassel et al., 2011; Verdier et al., 2013). However, the sequence of cellular events underlying germination, i.e. occurring before radicle emergence (Bewley, 1997), and their regulation by dormancy are far from being resolved, in particular when considering the putative roles of post-transcriptional mechanisms. Recent studies, such as the ones of Bazin et al. (2011), Layat et al. (2014) or Galland et al. (2014), had led to reconsider the role of mRNA metabolism in seed germination since these authors proposed that germination would not only rely on the transcription of specific subsets of mRNAs, but also on translational activity or on RNA decay.

We propose that the modulation of seed germination by dormancy would therefore be related to the association of specific mRNAs with polysomes, thus initiating their translation during seed imbibition, as already shown in sunflower by Layat *et al.* (2014). However this former work did not consider the relative parts of transcriptional versus translational regulation of germination and did not provide elements for explaining the molecular bases of selective translation. Here, we have studied the changes in the transcriptome and in the translatome of dormant and non-dormant Arabidopsis seeds during their imbibition at 25 °C in the darkness, conditions preventing germination of dormant seeds only (Leymarie *et al.*, 2012). We have isolated and identified the mRNAs in polysomal complexes, *i.e.* the translatome, and compared them to the transcripts identified at the same time points. Using bioinformatics approaches we have characterized the main features of 5'UTR, 3'UTR and uORF of genes which were likely to play a key regulatory role in germination. Results from this study provide insights in the understanding of seed germination regulation.

#### III.2 Results

#### Polysome profiling of dormant and non-dormant Arabidopsis seeds

At harvest, Arabidopsis seeds were dormant since they only germinated to ca 25 % at 25 °C in darkness (Figure 1A). After 3 weeks of storage at 56 % relative humidity (RH) and 20 °C, dormant seeds germinated to 75 % and after 4 weeks of after-ripening under these conditions seeds became non-dormant since they fully germinated at 25 °C (Figure 1A). In non-dormant seeds, germination at 25 °C in darkness did not begin before 2 days and was completed after 7 days (Figure 1A). At 15 °C both dormant and non-dormant seeds fully germinated after 4 days (data not shown, see Leymarie et al., 2012). In the present study, we choose data points at durations lower than 24 h at 25 °C because they corresponded to time points during germination sensu stricto, i.e. before radicle protrusion and allowed the comparison between dormant and non-dormant seeds in a similar physiological state. Sucrose gradient separation (Mustroph et al., 2009) was used to purify the polysomal fraction of RNA at regular time points during seed imbibition at 25 °C. The absorbance profiles of ribosome complexes showed that dry seeds contained a large amount of 80S monosomes and few or no polysomes (Figure 1B). In dormant seeds, early imbibition, from 3 to 6 h, was first associated with a decrease in the amount of 80S monosomes but was followed by an increase of this peak after 16 and 24h (Figure 1B). After 24 h of imbibition, the amount of 80S was significantly higher in non-dormant than in dormant seeds (Figure 1C). However, the amount of polysomal mRNAs, which increased during seed imbibition, was similar in dormant and non-dormant-seeds (Figure 1C). The amounts of polysomal components, estimated by measuring heights of peaks relative to the baseline, confirmed the visual evaluation of absorbance profiles (Supplemental Table S1). We determined whether the 80S monosomes that accumulated in dry seeds and after 24 h of imbibition were associated with mRNA. Eukaryotic ribosomes that are not associated with mRNA dissociate into subunits in solutions of high ionic strength (0.8 M KCl) (Martin, 1973) while free ribosomes are dissociated in these solutions. In dry dormant seeds, or imbibed for 24 h, the height of 80S peak was similar with KCl 0.2 M or 0.8 M (Supplemental Figure S1, A and B) which showed that monosomes were associated with RNA. In contrast, in non-dormant seeds imbibed for 24 h, treatment with 0.8 M KCl reduced the height of 80S peak (Supplemental Figure 1C) thus suggesting that a part of monosomes were RNA free. The increase of the 80S peak in non-dormant seeds therefore did not correspond to active ribosomes bound to mRNA.

The purified polysomal mRNAs and total mRNAs were characterized in dormant and non-dormant seeds after 16 h and 24 h of imbibition at 25 °C using CATMA microarrays. These time points were chosen because they allowed efficient isolation of polysomal fractions and because they respectively corresponded to 50 and 75 % of the amount of time required for the initiation of germination of

non-dormant seeds (Figure 1). MA plots representing the distribution of expression ratios of all transcripts at 16 h and 24 h of imbibition in either total or polysomal fractions showed that major variations were comprised between log2ratios values ranging from -2 and +2 (Supplemental Figure S2). Gene expression was considered to be different after Benjamini and Hochberg (BH) correction (P<0.05) and for a log2ratio above 0.5 or under -0.5, at least for one comparison. Among the 16616gene-specific sequence tags (GSTs) displaying signal levels above the limit of detection in at least one of the samples (representing 67 % of GSTs present on the array), 4670 and 7028 transcripts in total or polysomal mRNA, respectively, were significantly different between dormant and nondormant seeds after both 16 h and 24 h of imbibition (Figure 1, D and E, Supplemental Data S1). Analysis of the transcriptome showed that 1708 transcripts were more abundant in dormant seeds, and 2962in non-dormant ones, when considering both durations of imbibition (Figure 1D). Among these transcripts, 535 and 1065 displayed the same change in abundance at 16 h and 24 h of imbibition in dormant and non-dormant seeds, respectively (Figure 1D). In the translatome, 3872 transcripts were more abundant in dormant seeds and 3156 were more abundant in non-dormant seeds at 16 and 24 h of imbibition (Figure 1E). However, 1/3 of the polysome-associated transcripts (1333) were similar at 16 and 24 h of imbibition in dormant seeds when only 139 (among 3156, i.e. around 4 %) were common between 16 and 24h of imbibition in non-dormant seeds (Figure 1E).



**Figure 1.** Seed germination, polysome profiling and changes in abundance of mRNAs in transcriptome and translatome. A, Germination of Arabidopsis seeds on water at 25 °C in darkness during 8 days. (1) Freshly harvested seeds (dormant seeds). (2) Dormant seeds after-ripened for 3 weeks at 20 °C at 56 % relative humidity. (3) Dormant seeds after-ripened for 4 weeks at 20 °C at 56 % relative humidity (non-dormant seeds). Means  $\pm$  SD of triplicate experiments are shown. B and C, Sucrose density gradient profiles of RNA (absorbance at 254 nm) from Arabidopsis seeds. Peaks 1 and 2 correspond to 60S ribosomal subunits and to 80S monosomes, respectively. (B) Absorbance profiles of mRNA obtainedfrom dormant dry seeds and during their imbibition (3 h, 6 h, 16 h and 24 h) at 25 °C. (C) Comparison of absorbance profiles obtained with dormant (D, black solid line) and non-dormant (ND, red dotted line) seeds after 16 and 24 h of imbibition at 25 °C. D and E, Analysis of microarrays data: Venn diagrams showing the distribution of mRNAs presenting abundance variations (after BH correction with P<0.05 and log2 ratio above 0.5 or under -0.5) in (D) total RNA (T) and in (E) polysomal RNA (P) after 16 h and 24 h of imbibition at 25 °C. Numbers in brackets correspond to the sum of all transcripts in a category.



**Supplemental Figure S1.**Sucrose density gradient RNA profiles of Arabidopsis seeds in presence of KCl 0.2 M and 0.8 M. The 60S ribosomal submits and the 80S monosomes are indicated with (1) or (2), respectively. A, RNA profiles of dry dormant (D) seeds; B, dormant seeds after 24 h of imbibition at 25 °C and C, non-dormant (ND) seeds after 24 h of imbibition at 25 °C obtained with 0.2 M KCl (red curves, up) and 0.8 M KCl (black curves, down) in the sucrose gradient.

**Table S1.** Evolution of 80S and 60S abundance during time course of imbibition. Amounts of 80S and 60S components were evaluated with height (H) of 80S relative to baseline and the rapport of height of 80S relative to height to 60S. Amount of polysomal components were estimated with height relative to baseline.

Seeds	Duration of imbibition	Quantification	of 80S	Quantification of polysomal mRNA
		H80S (cm)	H80S/H60S	H polysomal (cm)
	0	7.57 ± 0.19	7.22 ± 0.54	nd
D	3h	$1.33 \pm 0.15$	$1.31 \pm 0.23$	nd
	6h	$2.49 \pm 0.14$	2.02 ± 0.19	nd
	16h	4.32 ± 0.27	3.36 ± 0.44	$1.56 \pm 0.05$
	24h	$5.3 \pm 0.08$	4.18 ± 0.25	1.533 ± 0.05
ND	16h	$4.68 \pm 0.05$	3.99 ± 0.2	1.5 ± 0.1
	24h	6.56 ± 0.18	6.39 ± 0.47	1.533 ± 0.05



**Supplemental Figure S2.** Microarray analysis. A to D, MA plot of total (T) and polysomal (P) mRNAs of dormant (D) and nondormant (ND) seeds after 16 and 24 h of imbibition at 25 °C. Data represented are Log2 (expression ratio of non-dormant seeds / expression ratio of dormant seeds). Positive values of log2(ND/D) correspond to transcripts more expressed in nondormant seeds and negative values to transcripts more expressed in dormant seeds.

#### Regulation of transcriptome and translatome by dormancy

This work was highly appropriate to determine whether the molecular regulation of seed germination is mainly transcriptional or translational. Therefore we compared the abundance of individual transcripts in the transcriptome and in the translatome by plotting the polysomal RNA ratios (ND/D) against total RNA ratios (ND/D) for all GSTs (Figure 2, A and B). In this representation, the diagonal line represents the behavior of transcripts that are regulated in the same way in the translatome and in the transcriptome. There was no correlation between the transcriptome versus the translatome after 16 h (R<sup>2</sup>=0.045, Figure 2A) or even 24 h (R<sup>2</sup>=0.019, Figure 2B) of imbibition. We constructed Venn diagrams for assessing dynamics of polysome loading with regards to dormancy release (Figure 2, C and D). At both points of imbibition we compared the differentially abundant

transcripts in transcriptome and translatome for dormant and non-dormant seeds. At 16 h of imbibition, 529 transcripts (ca 26 %) more abundant in the transcriptome of dormant seeds were found in the translatome of non-dormant seeds suggesting that after-ripening allowed this subset of mRNAs to become associated with polysomes (Figure 2C). Among the 2022 transcripts specifically more abundant in the transcriptome of non-dormant seeds, 699 (ca 34 %) were associated with polysomes in non-dormant seeds whereas 496 (ca 25 %) were found in the translatome of dormant seeds (Figure 2C). Conversely, only 317 transcripts (ca 28 %) among 1113 found in the transcriptome of dormant seeds were addressed to polysomes in these seeds (Figure 2C). At 24 h of imbibition, 450 (ca 39 %) transcripts that were more abundant in the transcriptome of dormant seeds became addressed to polysome when seeds became non-dormant (Figure 2D). Fifteen % (305) of the transcripts identified as being specific of the transcriptome of non-dormant seeds were in the translatome of dormant seeds and 40 % (415) in the one of non-dormant seeds (Figure 2D). At last, only 15 % (175) of the transcripts from the dormant transcriptome were addressed to polysomes in dormant seeds (Figure 2D). Transcriptome and translatome of dormant and non-dormant seeds were compared using SeedNet, a model designed from transcriptomic data of seed samples in relation with germination and dormancy. SeedNet topology allows to identify 3 regions: region 1, specific of dormancy, and regions 2 and 3, associated with germination (Bassel et al., 2011). Figure 2, E and F show localization within SeedNet model of transcripts identified in the transcriptome and translatome of dormant and non-dormant seeds, combining data of both durations of imbibition. For transcriptome, the transcripts more abundant in dormant seeds were mainly found in region 1, while those more abundant in non-dormant seeds were plotted in regions 2 and 3 (Figure 2E). Transcripts associated with polysomes in non-dormant seeds were mainly localized in region 3 but transcripts associated with polysomes in dormant seeds were localized randomly in region 1, 2 and 3 (Figure 2F).



**Figure 2.** Comparison of changes in mRNA abundance between transcriptome and translatome. A and B, Scatter plots show the change (abundance in non-dormant seeds / abundance in dormant seeds, ND/D) in total mRNA abundance (x-axis, transcriptome) *versus* change in polysomal mRNA (y-axis, translatome) seeds after 16 h (A) or 24 h (B) of imbibition at 25 °C. All transcripts present on the microarray are shown as dots on the graph which represents their ratio of log 2 (abundance ND) / log2 (abundance D). The transcripts being above or below the solid diagonal line are positively or negatively regulated at the level of translation, respectively. C and D, Venn diagrams show number of transcripts displaying significant change in abundance (after BH correction with P<0.05 and log2 ratio above 0.5 or under -0.5) in transcriptome (T) and translatome (P) at 16 h (C) or at 24 h (D) of imbibition at 25 °C. E and F, Localization of transcripts associated with transcriptome and translatome in the SeedNet transcripts co-expression network (www.vseed.nottingham.ac.uk). Regions outlined in orange correspond to clusters associated with dormancy (region 1) or germination (regions 2 and 3) (see Bassel *et al.*, 2011). The red dots represent the transcripts identified in the present study as more abundant in dormant seeds and the blue dots the transcripts more abundant in non-dormant seeds in transcriptome (E) or translatome (F) after 16 and 24 h of imbibition at 25 °C.

## Regulation of polysome loading by mRNA sequence features and by translational efficiency

The polysomal ratio (proportion of individual mRNA species in polysomes, P/T, Figure 3A) was determined for transcripts detected in polysomal fractions. While the distribution of polysomal ratios was similar for both durations of imbibition, it appeared significantly (p<0.0001) different between dormant and non-dormant seeds (Figure 3A). This distribution shows that the transcripts which were more abundant in dormant seeds presented a lower polysomal ratio (i.e. a higher number of transcripts with a polysomal ratio lower than 80 %) than the transcripts more abundant in nondormant seeds (a higher number of transcripts with a polysomal ratio of 92 %), whatever the duration of imbibition (Figure 3A). Major characteristics of 5'UTR and 3'UTR (length, GC content) of differentially abundant transcripts in translatome with a described 5'UTR described in TAIR database (Supplemental Table S2) have been analyzed in relation with their polysomal ratio. At both duration of imbibition, higher values of polysome ratios were observed when 5'UTR length was between 50-80 nucleotides (nt) and P/T was lower for mRNAs with short (<25 nt) or long (> 100 nt) 5'UTR lengths, whatever seeds were dormant or not (Figure 3B). However, the relationship between P/T and 5'UTR length was not affected by dormancy since the slopes of the curves shown in Figure 3B were similar. The relationship between polysome loading and GC content in 5'UTR revealed differences between dormant and non-dormant seeds (Figure 3C). Low GC contents (20-30 %) in 5'UTR were associated with high polysomal ratio and higher GC content significantly decreased ribosome loading (Figure 3C). The effect of GC content on P/T was considerably more pronounced in dormant than in nondormant seeds since P/T decreased to 25 % in dormant seeds but only to 55 % in non-dormant ones when GC content increased to 60 % (Figure 3C). The effect of 3'UTR features on polysomal ratio was also investigated but did not allow to evidence any relationship between length or GC content and the ability of seeds to germinate (Supplemental Figure S3). Over-represented motifs in 5'UTR of polysomal transcripts more abundant (analysis on 100 transcripts) in dormant and non-dormant seeds were determined with the unsupervised multiple expectation maximization for motif elicitation (MEME) algorithm (Bailey et al., 2009) (Figure 3D). We identified U-rich motifs in the 5'UTR of polysomal RNAs from dormant seeds and the same consensus motif "AGAGAGAAGA" at both durations of imbibition in polysomal transcripts from non-dormant seeds (Figure 3D). In all cases, the values of motif enrichment (ME, which corresponds to the factor of enrichment of this motif in comparison with its occurrence in the whole Arabidopsis genome) and of E-value (which refers to its statistical significance) demonstrated the high specificity of the motifs (Figure 3D). Putative uORFs were searched in 5'UTR of all the transcripts associated with polysomes (listed in Supplemental Dataset S4). More than 75 % of dormant seeds displayed few uORFs (1-3), whereas transcripts with more than 3 uORFs were more frequent in dormant seeds (Table I). Figure 3E shows that the polysomal ratio decreased with the increasing number of uORF per transcript but this effect was more pronounced in dormant seeds. The longer the length of uORF the lower the the transcripts did not present any uORF (Table I) but transcripts from non- polysomal ratio was (Figure 3F); however, the inhibition of polysome loading by the length of uORF appeared to be stronger for transcripts more abundant in non-dormant seeds (higher slope values on the graph).



**Figure 3.** Regulation of polysome loading by 5'UTR. For all graphs, filled symbols and black lines correspond to non-dormant (ND) seeds, while open symbols and gray lines correspond to dormant seeds (D). Triangles and circles correspond to 16 h and 24 h of imbibition respectively. A, Distribution of the transcripts according to their polysomal ratio (P/T) in D (n=2582 at 16 h and n=2581 at 24 h of imbibition) and ND (n=1629 at 16 h and n=1601 at 24 h of imbibition) seeds. Distributions were statistically different between D and ND seeds at both durations of imbibition as determined by the Student's-test (P<0.0001). B, Relationship between length of 5'UTR and polysomal ratio (P/T) calculated for transcripts that were found in in polysomal fractions of D and ND seeds. Transcripts were grouped based on 45 nucleotides classes. C, Effect of GC content in 5'UTR on polysomal ratio. mRNAs were grouped based on 5 % GC content windows and the polysomal ratio were calculated for each subset. D, Consensus sequences of motifs over-represented in the 5'UTR for the first 100 mRNA identified as differentially abundant in polysomal fraction of dormant (D) and non-dormant (ND) seeds at 16 h and 24 h of imbibition. The motif enrichment (ME) represents the frequency of the motifs over-represented in the 100 first differentially abundant transcripts list relative to their frequency in 5'UTR from whole genome of *Arabidopsis thaliana*. The

E-value calculated by MEME reveals the statistical significance of the motif.E, Relationship between the number of uORF per gene and the polysomal ratio in polysomal fraction of dormant (D) and non-dormant (ND) seeds at 16 h and 24 h of imbibition. F, Relationship between the length of uORF and the polysomal ratio in polysomal fraction of dormant (D) and non-dormant (ND) seeds at 16 h and 24 h of imbibition.



**Supplemental Figure S3.**Characterization of 3'UTR sequence features from RNA associated with polysomes in dormant (D; n=2582 at 16 h and 2581 at 24 h of imbibition) and non-dormant (ND; n=1629 at 16 h and 1601 at 24 h of imbibition) seeds. For all graphs, filled black symbols correspond to RNA from ND seeds, while open gray symbols correspond to RNA from D seeds. Triangles and circles correspond to changes after 16 h and 24 h, respectively. A, Effect of length of 3'UTR on polysomal ratio (P/T) in D and ND seeds after 16 h and 24 h of imbibition at 25 °C. B, Effect of GC content in the 3'UTR on polysomal ratio (P/T) in D and ND seeds after 16 h and 24 h of imbibition at 25 °C.

**Table I.** Number of uORF (Upstream Open Reading Frame) per transcript found in the translatome of dormant (D) and nondormant (ND) seeds at 16 h and 24 h of imbibition at 25 °C. <sup>a</sup> and <sup>b</sup> indicate homogeneous groups in a corresponding class (Fisher test, P=0.05).

Number of uORF	% of transcripts having the corresponding number of uORF in				
per transcript	the translatome of				
	D seeds imbibed for		ND seeds imbibed for		
	16 h	24 h	16 h	24 h	
0	77.90 <sup>a</sup>	77.63 <sup>a</sup>	76.23ª	76.10 <sup>a</sup>	
1	1.8 <sup>a</sup>	1.96 ª	7.55 <sup>b</sup>	7.41 <sup>b</sup>	
2	2.66 <sup>a</sup>	2.75 °	6.14 <sup>b</sup>	6.16 <sup>b</sup>	
3	3.42 <sup>a</sup>	3.33 <sup>a</sup>	4.63 <sup>b</sup>	4.70 <sup>b</sup>	
4	3.98 <sup>a</sup>	3.83 <sup>a</sup>	2.42 <sup>b</sup>	2.50 <sup>b</sup>	
5	4.59 <sup>a</sup>	4.41 <sup>a</sup>	1.82 <sup>b</sup>	1.88 <sup>b</sup>	
6	6.07 <sup>a</sup>	6.09 <sup>a</sup>	1.21 <sup>b</sup>	1.25 <sup>b</sup>	

In addition, we compared the translation efficiency in dormant and non-dormant seeds. We adapted the method from David *et al.* (2012) where the amount of puromycin-labeled proteins detected in gel is directly related to the translational activity. Puromycylation is based on incorporation of puromycin into the C terminus of elongation nascent chains and the ribosome-bound nascent chains are detected with a puromycin specific antibody, as shown in Figure 4. Incubation of seeds with sodium arsenite (SA, an irreversible inhibitor of translation initiation (Kedersha *et al.*, 2002) and puromycylation (David *et al.*, 2012)) and absence of puromycin treatment (C) suppressed the signal thus demonstrating the efficiency of this technique for assessing translation in seeds (Figure 4). Densitometric quantification of the labeled-bands showed that translation remained unchanged between 16 and 24 h of imbibition was similar in dormant and in non-dormant seeds (Figure 4). Nevertheless longer duration of imbibition, *i.e.* 72 h, resulted in a higher translational activity in non-dormant seeds, but at this time point germination had occurred in non-dormant seeds (Figure 4).



**Figure 4**. Quantification of translational activity by incorporation of puromycin and western blot detection (anti-puromycin antibodies) after 16 h, 24 h and 72 h of imbibition at 25 °C (with 4 h with puromycin) with dormant (D) and non-dormant (ND) seeds. Dormant seeds were incubated for 4 h with 500  $\mu$ M sodium arsenite and puromycin (SA) or without puromycin (control, C). Means ± SD of 3 biological replicates.

#### Identification of transcripts in the transcriptome and the translatome

The microarrays data were validated by performing qRT-PCR with 10 selected transcripts. This showed a high correlation between qRT-PCR and microarrays transcripts abundance, with R<sup>2</sup> coefficients ranging from 0.71 to 0.88 (Supplemental Figure S4).



Microarray value (ratio ND/D) arbitrary unit)

**Supplemental Figure S4.** Validation of microarrays results. Scatterplot showing correlations between microarray results (x-axis) and qRT-PCR experiments (y-axis) on *SLP2* (At4g34980), *ABA1* (At5g67030), *CYP707A2* (At2g29090), *LEA* (At2g21490), *LTP5* (At3g51600), *EXPA1* (At1g69530), *ACO1* (At2g19590), *EXPA4* (At2g39700), *GASA6* (At1g74670), *peroxidase* (At1g05240), *RGL2*, (At3g03450), *PIF6*, (At3g62090), *MPK4* (At4g01370), *ABI2* (At5g57050), and *HSP70* (At5g02500) genes. The UBQ5 (At3g62250) and At4g12590 genes were used as reference genes. Regression lines are drawn for each gene and the R<sup>2</sup> values corresponding are indicated within the graph. For each point of qRT-PCR, ratios of log2 (abundance) ND/D were calculated to compare with microarray data. For both methods 3 independent biological samples were used.

Full list of differentially abundant transcripts in transcriptome and translatome is given in Supplemental Dataset S2/S3; however we focused the functional analysis of gene identity on the transcripts that were identified in the translatome only, since we previously demonstrated the discrepancy between transcriptome and translatome (see Figure 2). Putative functions were assigned to transcripts from Supplemental Dataset S3 using the Gene Ontology (GO) TAIR categorization and were visualized as functional clusters after redundancy reduction via REVIGO (Figure 5). On this representation, colors indicate the user-provided p-value (see legend) and the size of the circlesis relative to the frequency of GOterms in underlying GO database. Polysomal RNAs associated with germination fell essentially in the categories "cell wall", "hormone metabolism" and "redox pathway" whatever the duration of imbibition (Figure 5, B and D). Furthermore the categories



"response to stress", and "hormone metabolism pathways "were associated to polysomal RNAs related with dormancy (Figure 5, A and C).

**Figure 5.** Gene ontology (GO) classification of mRNAs specifically found in the translatome of dormant (D) and non-dormant (ND) seeds after 16 h (A-B) and 24 h (C-D) of imbibition at 25 °C. TAIR accession numbers were used to perform the classification according to Gene Ontology (GO) categories with the Classification SuperViewer Tool in Bio-Analytic Resource for Plant Biology (BAR) and plots were obtained using REVIGO (<u>http://revigo.irb.hr</u>; Supek *et al.*, 2011) that allows a 2D-space representation according to semantic similarity of GO terms (semantically similar GO terms remain close together in the plot). Size of circles represents indicates the frequency of the GO term in the GO database (more general terms are represented by larger size circles) and color of circles indicates the p-value (scale on the right, blue and green circles are GO terms with more significant p-values than the orange and red circles).

The more relevant GO categories are discussed in the discussion section, as well as the sequential modifications of the transcripts belonging to the different GO categories in translatome after 16 h and 24 h of imbibition (Table S4). At last, the abundance of some transcripts being involved in the translational regulation of dormancy and germination were analyzed by qRT-PCR in dormant and in non-dormant seeds after 16 h and 24 h of imbibition (Figure 6). Figure 6 shows that abundance of
these transcripts was generally much higher in the translatome than in the transcriptome either in dormant (Figure 6A) and non-dormant seeds (Figure 6B).



**Figure 6.** Pattern of expression in transcriptome and translatome of key transcripts more abundant in dormant seeds (log2ratio ND/D <0; A) and more abundant in non-dormant seeds (log2ratio ND/D>0; B) after 16 h and 24 h of imbibition. Data of expression were obtained by qRT-PCR on three biological replicates. *RGL2*, At3g03450; *ABI2*, At5g57050; *PIF6*, At3g62090; *MPK4*, At4g01370; *HSP70*, At5g02500; *ABA1*, At5g67030; *LEA*, At2g21490; *SLP2*, At4g34980; *LTP5*, At3g51600; *EXPA4*, At2g39700 ; *EXPA1*, At1g69530; *ACO1*, At2g19590 and *GASA6*, At1g74670. Green color corresponds to changes measured in total RNA (T, transcriptome) and blue color correspond to changes in polysomal RNA (P, translatome) at 16 h (full bars) and 24 h of imbibition (striped bars).

**Table S4.** Changes in polysomal transcripts, clustered by GO categories, during imbibition at 25 °C of dormant (D, black bars) and non-dormant (ND, grey bars) seeds. GO functions and trends of evolution of transcript abundance were determined using REVIGO which provided P-values and frequency of the GO terms (Figure 5). Red and blue bars are indicated the GO functions whose P value and frequency evolved similarly in dormant and non-dormant seeds, respectively.

GO function	Seeds	Trends of chang transcripts during se 16 to 24 h e	ge in polysomal eed imbibition from valuated by
		P-value	Frequency
Hormono motoboliam	D		
Hormone metadolism	ND		
Datas	D		
Redox	ND		
C' 1'	D		
Signaling	ND		
	D		
Cell wall	ND		
	D		
Transport	ND		
	D		
Lipid metabolism	ND		
	D		
Photosystem	ND		
Transmission DNA his dias	D		
Transcription, KINA binding	ND		
Cell organisation and	D		
biogenesis	ND		
Other highering process	D		
Other biological process	ND		
Aming gold motchelism	D		
Annio acid metabolishi	ND		
Pageona to strass	D		
Response to suess	ND		
Secondary metabolism	D		
	ND		
Transcription, DNA	D		
dependent	ND		
Protein metabolism	D		
	ND		
Developpement process	D		
	ND		

## **III.3** Discussion

The control of translational activity in plants has emerged these last years consecutively to increasing experimental evidence demonstrating low coupling between mRNAs and proteins (Bailey Serres *et al.*, 2009; von Arnim *et al.*, 2014). Translation is mainly regulated at its initiation step when 60S and 40S ribosomal subunits join together at the initiating AUG codon of an mRNA before formation of a polysome on the translated mRNA due the recruitment of additional ribosomes (Bailey-Serres *et al.*, 2009). The role of this process in seed germination has been documented recently by Layat *et al.* (2014) who were the first showing that translatomes of dormant and non-dormant sunflower seeds differed markedly. Here we demonstrate the role of translatome in the germination of Arabidopsis seeds and its regulation by the dormancy status.

In Arabidopsis, the Columbia ecotype is dormant at harvest since it germinates poorly at 25 °C in darkness (Figure 1A, Leymarie et al., 2012). Dormancy is alleviated by germinating seeds under continuous light, stratification at 5 °C or by 4-5 weeks of after-ripening (Leymarie et al., 2012). Polysomal profiles of dry dormant and non-dormant seeds showed that monosomes were abundant at the end of seed maturation but that RNA associated-polysomes were scarce or absent (Figure 1B), in agreement with results obtained with endosperm of castor bean seeds (Marrè et al., 1965). Increase of salt concentration in sucrose gradients revealed that monosomes in dry seeds were already associated with mRNA (Supplemental Figure S1A). They probably contained the so called "long-lived mRNA" that are stored at the end of seed development and thought to be translated after the onset of imbibition (Kimura and Nambara, 2010). This would mean that translation in the early steps of seed imbibition is determined during late seed development and it would be worth identifying the mRNA associated to these monosomes. Seed imbibition was associated with a transient decrease of monosomes followed by their progressive recovery at 24 h of imbibition (Figure 1B) and with an increase of polysomal mRNAs thus revealing translational activity. This is in accordance with polysome profiles obtained from seeds of other plant species such as castor bean (Marrè et al., 1965), wheat (Spiegel et al., 1975) and sunflower (Layat et al., 2014). The similarity of polysome profiles in dormant and non-dormant seeds suggests that the translational activity was similar in both kind of seeds even if non-dormant seeds contained more free monosomes at 24 h of imbibition (Figure 1C). These former were not all bound to mRNA (inactive ribosome) (Supplemental Figure S1C), but they may accumulate to sustain the increased protein synthesis which is associated with the subsequent radicle protrusion (Beltran-Pena et al., 1995). In yeast, it has been proposed that inactive ribosomes that are accumulated during stress, dissociate upon stress release promoting recovery (van den Elzen et al., 2014). Moreover, this mechanism was also identified in non-stressed cells and would be a new level of translation regulation (van den Elzen et al., 2014). We suggest that

the storage of ribosomes in an unproductive state in non-dormant seeds might serve as a reservoir of active ribosomes for sustaining the active translation which occurs at the onset of radicle protrusion. Microarrays analysis allowed identifying transcripts in transcriptome and translatome of dormant and non-dormant seeds at the 2 time points of imbibition. After BH correction, 4670 and 7028 transcripts were found as differentially abundant in total and polysomal RNA, respectively, for dormant and non-dormant seeds after 16 h and 24 h of imbibition (Figure 1, D and E). By comparison, a time course analysis of transcriptomic changes during Arabidopsis seed germination also revealed that ca 8000 transcripts were similarly up- or down-regulated at each time point studied (i.e. 1, 6, 12 and 24 h imbibition) (Narsai et al., 2011). In sunflower, ca 7300 transcripts were continuously associated with polysomes from 3 to 24 h in both dormant and non-dormant embryos (Layat et al., 2014), a number which is very similar to the one determined here. This is also worth noting that germination was associated with a marked change in the nature of the transcripts associated with polysomes since they differed noticeably between 16 and 24 h (Figure 1E). This clearly demonstrates that completion of the germination program relies on dynamic and qualitative changes in translation. Recent papers (Galland et al., 2014; Layat et al., 2014) also revealed the sequential regulation of protein synthesis during seed germination. In addition, the microarray data demonstrate that dormancy was associated with an active association of transcripts with polysomes, but contrary to non-dormant seeds, many polysome-associated transcripts were similar at 16 and 24 h of imbibition (Figure 1E). This is interesting because it demonstrates that dormancy cannot be explained by the absence of translation of some transcripts, but by an active association of selected transcripts with polysomes, as already pointed out by Layat et al. (2014).

The divergence between the number of transcripts associated with dormancy and germination in the transcriptome (4670) and in the translatome (7028) clearly raises the question of transcriptional vs translational regulation of germination. Figure 2, A and B shows that there was little or no correlation between translatome and transcriptome at both durations of seed imbibition. The discrepancy between transcript abundance and translation of specific mRNAs was also shown for example in yeast (Lackner*et al.*, 2007), mouse fibroblasts (Schwanhaüsser *et al.*, 2011), transformed cancerous cells (Spence *et al.*, 2006), primary T cells (Grolleau *et al.*, 2002) and in Arabidopsis (Sormani *et al.*, 2011; Juntawong and Bailey Serres, 2012). This suggests that RNA in the transcriptome were not necessarily recruited to polysomes and that dormancy can regulate this process. The Venn diagrams (Figure 2, C and D) are of highest interest since they bring unexpected conclusions about the dynamics of transcripts association with polysomes. First, they demonstrate that the regulation of germination is both transcriptional and translational and that the relative part of each process evolves during germination. At 16 h of imbibition, 25 % (418 among 1646) of the polysomal transcripts more abundant in non-dormant seeds were not found as up-regulated in the

transcriptome of either dormant or non-dormant seeds, suggesting a translational regulation. This percentage increased to 47 % (784 among 1649) at 24 h which indicates that the regulation at the translation level is more important in the latter phases of germination. Secondly, this analysis demonstrates that numerous transcripts found in the transcriptome of either dormant or nondormant seeds are not addressed to polysomes or even, more surprisingly, can be found in the polysomes of their counterparts. For example, many transcripts (529 and 450 at 16 h and 24 h, respectively) of dormant seeds were also found in the polysomes of non-dormant seeds (Figure 2C). Their association with polysomes occurred after dormancy alleviation which suggests that afterripening would modify the ability of preexisting transcripts to become polysomal. Similarly, 496 and 305 transcripts found as being specific of non-dormant seeds at 16 h and 24 h, respectively, were in fact associated with polysomes when seeds were dormant, *i.e.* before after-ripening (Figure 2, C and D). This suggests that dormancy alleviation may also trigger the dissociation of polysomal complexes, leading the released transcripts to be present in the non-dormant transcriptome. These are major findings since the current knowledge of molecular regulation of dormancy is based on transcriptomic studies and we show here that they are not fully relevant for studying this process. The use of SeedNet provided additional insights into the relationship between transcriptome and translatome in germinating seeds. While our transcriptome data clearly fitted with the model, the translatome did not match with the regions designed for transcriptomic data (Figure 2, E and F). Particularly the transcripts belonging to the translatome of dormant seeds were not preferentially found in region 1 of the model, which is defined as the dormancy region. Using sunflower seeds, Layat et al. (2014) also pointed out that SeedNet failed predicting functional areas of translated transcripts.

These data clearly demonstrate that germination, as modulated by dormancy, relies on the specific and timely regulated association of transcripts with polysomes. This is confirmed by the distribution of transcripts in relation to their polysomal ratio, which was affected by dormancy (Figure 3A), and which indicates that there exists differences in selective translation between dormant and non-dormant seeds, even though they displayed similar polysomal profiles and translational activities (Figs. 1C and 4). The use of the puromycin method (Figure 4) nicely demonstrated that the efficiency of the translation machinery was similar during imbibition of either dormant or non-dormant seeds. This is in accordance with measurements of translational activity obtained by radiolabeled methionine incorporation by dormant and non-dormant seeds after 1 day of imbibition (Chibani *et al.,* 2006). Since global translation was not repressed by dormancy, but polysomal ratio was (Figs. 4 and 3A), we propose that dormancy regulates the selective translation of individual transcripts. In other words, dormancy-associated translational control (which can be seen in 16h and 24h) is selective to particular sets of genes, and is not a general translational repression. This means that transcripts in non-dormant seeds must be translated with a higher efficiency, as shown by their

higher polysome loading values, and this can be explained by the regulatory role of 5'UTR. Selective translation in eukaryotes is known to be driven by 5'UTR or 3'UTR (Kawaguchi and Bailey-Serres, 2005; Wilkie et al., 2003) but here the 5'UTR sequences only appeared as playing a role in seed dormancy regulation. The relationship between 5'UTR length and polysomal ratio (Figure 3B) corresponds to classical observations in eukaryotes (Jackson et al., 2010; Pestova et al., 2002; Kawaguchi and Bailey Serres, 2005). Short 5'UTR (20 nt) are known to inhibit the entry of 40S preinitiation complex or recognition of AUG initiation codon whereas moderately long 5'UTR (60-100 nt) promoted initiation (Kozak, 1991) but longer lengths decreased polysomal ratio, as it is shown in Figure 3B. However the effect of 5'UTR on translation cannot be directly related to germination since dormant and non-dormant seeds displayed the same response to this parameter (Figure 3B). Besides, the GC content of 5'UTR appeared as being more important for regulating polysomal ratio of transcripts in relation with dormancy. Indeed, the increase of GC content dramatically altered polysomal loading in dormant seeds whereas it had only a slight effect in non-dormant seeds (Figure 3C). Polysomal ratio has been shown to be negatively correlated with 5'UTR GC content in Arabidopsis plants submitted to dehydration stress which can be related to a higher requirement for ATP during the initiation step (Kawaguchi and Bailey-Serres, 2005). Our results also show that the 5'UTR of transcripts more abundant in translatome of dormant and non-dormant seeds contain specific over-represented motifs (Figure 3D), thus suggesting that translated mRNA can be selected according to these motifs. The UUUC motif appeared as being specific for transcripts addressed to polysomes in dormant seeds (Figure 3D). To our knowledge no similar motif was found in eukaryotes but in viruses pyrimidine-rich motifs are involved in translation initiation enhancement (Carneiro et al., 1995). The AG-rich motif found in 5'UTR of non-dormant seeds transcripts has already been identified as a regulator of exon recognition in splicing process in plants (McCullough and Schuler, 1997). In our data set, around 75 % of the polysomal transcripts had no uORF (Table I), which is quite similar to the proportion found in the whole Arabidopsis genome (Kim et al., 2007). However, there were more transcripts containing a high number of uORFs in the translatome of dormant seeds than in non-dormant seeds, which might explain the lower polysome loading evidenced in these seeds (Figure 3E). In addition, our results clearly show the major influence of length of uORF on polysome loading, particularly in non-dormant seeds (Figure 3F). Although uORFs are known to repress in vitro translation in a length dependent manner (Kozak, 2002), only a moderate correlation between inhibition of gene expression and uORFs length had been shown in plants up to now (von Arnim et al., 2014). We therefore suggest that the presence and the length of uORFs also contribute to the selective translation of mRNA in dormant and non-dormant seeds. Our data therefore allow proposing a key regulatory role of 5'UTR for the regulation of seed germination through selective translation.

The clusters representative of transcripts found in dormant and non-dormant polysomal fractions have been visualized as scatterplots (Figure 5). Some GO categories appear as being largely represented in both dormant and non-dormant seeds (Figure 5). Similarly to what was shown by Layat et al. (2014), translatomes of dormant and non-dormant seeds contain many up-regulated transcripts (196 and 153, respectively) belonging to the GO category "transport" but dormant seeds were markedly enriched into transporters from the subcategories "metabolite transporters at the mitochondrial membrane" or "sugars". The role of transporters in seed germination is poorly described, but among the transcripts identified here, one can point out the ABC transporters, which are a large family transporting different molecules including hormones. For example, ABCG25 (At1g71960), found in the translatome of dormant seeds at 16 and 24 h is an efflux transporter that exports ABA out of the vessels thus mediating ABA import into cells (Kuromori et al., 2010). Interestingly, Comatose (CTS, At4g39850) is also found in the translatome of dormant seeds whereas it has been proposed that its expression was associated with the late phases of germination (Footit et al., 2002; Carrera et al., 2007). The GO categories "transcription, DNA dependent" and "transcription RNA binding" were also largely represented in the translatome of dormant and non-dormant seeds. They include many transcription factors which demonstrates that germination, but also dormancy maintenance, both require an active transcription at some time points of the whole process, as suggested by Layat et al. (2014), but the nature of the transcripts drives the cellular functioning towards germination or not. For example, 13 WRKY transcription factors were identified in this work (4 in non-dormant seeds and 9 in dormant seeds) and they are known to act as negative or positive regulators of ABA signaling (Tripathi et al., 2014). WRKY33 (At2g38470), which is found in the translatome of dormant seeds, has already been shown to be involved in salt stress response and ABA signaling (Jiang and Deyholos, 2009). Among the transcription factors, MYC2 (At1g01260), specifically present in the translatome of dormant seeds, is known as an activator of jasmonic acid responses (Fernández-Calvo et al., 2011), a phytohormone related to germination inhibition (Oh et al., 2004). Our set of data also underlines that many chromatin remodeling factors are present in the translatome and are likely to play a role in both germination and dormancy (Supplemental Dataset S2 and S3). Chromatin structure regulates gene transcription but is still poorly characterized in the context of seed germination since only few studies have addressed this point (e.g. Molitor et al., 2014). The prominence of this GO category suggests that this mechanism should deserve more attention, as proposed by Nonogaki (2014).

The microarray analysis of translatomes also allowed to identify functional GO categories which were specific of either dormant or non-dormant states (Figure 5 and Supplemental Data S3). Clustering analysis shows that polysome-associated transcripts specific of non-dormant embryos fell mainly into the categories "transcription, RNA binding", "hormone metabolism", "cell wall" or "redox"

(Supplemental Data S3). In the GO category "hormone metabolism", we found regulators of the hormonal balance which drives the germination process (Finch-savage and Leubner-Metzger, 2006). This category contains for example the inhibitors of acid abscissic (ABA) signaling HAI1 (At5g59220; Bhaskara et al., 2012) and AHG1 (At5G51760; Nishimura et al., 2007), positive regulators of ethylene synthesis ACO1(At2g19590, Figure 6) and signaling ERF4 (At5g61600; Yang et al., 2005) or of gibberellin synthesis Ga3ox3 (At5g07200; Yamaguchi, 2008) and gibberellin signaling SLP2 (At4g34980; Ogawa et al., 2003; Figure 6) and GASA6 (At1g74670; Roxrud et al., 2007; Figure 6). Reactive oxygen species have been proposed as playing a signaling role in Arabidopsis seed germination (Leymarie et al., 2012). This assumption is confirmed here by the over-representation of the GO category "redox" in the polysomal fraction of non-dormant seeds which contained transcripts related to the enzymatic reactive oxygen species (ROS) scavenging, such as CAT2 (At4g35090), MDAR4 (At3g27820) or GPX8 (At1g63460) (Bailly et al., 2004) but also GRXS13 (At1g03850) and GRXC1 (At5g63030) which regulate the cellular redox state through the formation of reversible disulfide bonds from thiol groups (Buchanan and Balmer, 2005) thus playing a role in seed germination (Montrichard et al., 2003; El-Maarouf-Bouteau et al., 2013). As proposed by Bailly et al. (2008) and Leymarie et al. (2012), regulation of enzymatic and non-enzymatic detoxifying systems would result from an increased ROS production in germinating seeds. Transcripts involved in cell wall modifications were found as more abundant in the translatome of non-dormant seeds than dormant seeds. Over-representation of cell wall-related transcripts in the transcriptome was shown in Lepidium sativum (Morris et al., 2011) and Arabidopsis (Endo et al., 2012) germinating seeds and in the translatome of sunflower seeds (Layat et al., 2014). Transcripts for expansins such as EXPA1, EXPA10, EXPA4 and EXPA2 (At1g69530, At1g26770, At2g39700 and At5g05290; Figure 6), mannanase such as MAN7 (At5g66460) and pectin lyases (At4g33440 and At3g15720) were highly abundant in the translatome of non-dormant seeds. Enzymes coded by these transcripts participate in cell wall extensibility modification which subsequently allows cell elongation, a process often mentioned as being critical for allowing radicle elongation in Arabidopsis seeds (Bewley, 1997; Nonogaki et al., 2007). For example, EXPA2 (At5g05290), was recently shown to be involved in GAinduced seed germination in Arabidopsis (Yan et al., 2014). The GO category "lipid metabolism" also appeared as being more important in the translatome of non-dormant seeds at 24 h of imbibition (Figure 5, Table S4). For example, the transcripts for LTP6 and LTP5 (At3g51600 and At3g08770, respectively; Figure 6), which ranged within this category, correspond to lipid transfer proteins and they have been shown to participate in the intracellular transfer of lipids during seed germination (Pagnussat et al., 2012).

Attention has also to be paid to the transcripts which were specifically present in the translatome of dormant seeds since their active translation was associated with the inhibition of germination. They

mainly ranged in the categories "response to stress" and "signaling". For example, RGL2 (At3g03450; Figure 6), which encodes a DELLA protein known to repress germination (Lee et al., 2002), was highly abundant in the translatome of dormant seeds at 24 h of imbibition, suggesting that GA signaling might be impaired in those seeds. In addition, signaling components of ABA are over-represented in the translatome of these seeds, such as for example ABI3 (At3g24650), ABI2 (At5g57050, Figure 6), and ABA1 (At5g67030), major negative regulators of germination (Finkelstein et al., 2002). Interestingly, PIF6 (At3g62090, Figure 6), a Phytochrome Interacting Factor (PIF), which was suggested to play a role in Arabidopsis primary dormancy (Penfield et al., 2009) was more abundant in the translatome of dormant seeds. Translatome profiling of dormant seeds also revealed that many mitogen-activated protein kinases (MPKs) were involved in the inhibition of germination, such as MPK1 (At1g10210) and MPK4 (At4g01370, Figure 6) or upstream kinases such as MKK2 (At4g29810) and MKK9 (At1g73500). MPKs are involved in numerous signaling pathways that control plant development via the phosphorylation of target molecules and are involved in hormone signaling pathways (Colcombet and Hirt, 2008). MPK1 has been shown to be activated by ABA (Umezawa et al., 2013) and MPK4 is activated by jasmonic acid (Gomi et al., 2005) but their role in the regulation of germination is poorly described. Inhibition of germination was also related to the association of many transcripts of Heat Shock Proteins (HSPs) with polysomes such as Hsp70 (At3g09440, At5g02500 and At1g11660, Figure 6) as shown in sunflower (Layat et al., 2014), thus suggesting that chaperon activity can play a role in maintenance of dormancy. Interestingly it has been shown recently in mammals that chaperones, and particularly HSP70, would regulate translation elongation by affecting ribosome dynamics (Shalgi et al., 2013). LEA (At2g21490, Figure 6), also involved in the response of stress and activated by ABA (Chen et al., 2007) was also more abundant in dormant seeds (Table S3).

Further insights about the dynamic changes of GO categories between 16 h and 24 h of seed imbibition are given Table S4. This table permits to see the dynamics of association of the various categories of transcripts with polysomes taking into account changes in both p-values and frequency within a kind of seed. Even if it is difficult to precise what is the more relevant parameter at the biological level (*i.e.* frequency or p-value), this table allows identifying GO categories whose both abundance and number of individual transcripts increased or decreased during seed imbibition. For example, in non-dormant seeds "photosystem" and "cell organization and biogenesis" increased while dormancy maintenance was associated with a decrease of the GO categories "Redox" or "Lipid metabolism" (see Table S4). This demonstrates that the selective association of transcripts with polysomes led to the regulation of major cellular events preparing subsequent seedling growth after radicle protrusion or dormancy maintenance. For example, the GO category "photosystem" includes

cellular components preparing autotrophy acquisition (Supplemental Data S3) which will take place in the growing seedling.

At last, the relevance of our findings was confirmed when studying by PCR the abundance of key transcripts in the transcriptome and translatome (Figure 6). For some key genes identified as being important for regulating germination and dormancy (see above) we confirmed by qRT-PCR that they well all highly abundant in the polysomal fraction of either dormant or non-dormant seeds. As demonstrated previously in some cases there was a correlation between the amount of transcripts and their abundance in the translatome (*ACO1, GASA6, HSP70*) but in most cases they were not related demonstrating again the translational component of the regulation of germination and dormancy.

In conclusion, this comparison between transcriptome and translatome of dormant and nondormant Arabidopsis seeds clearly emphasizes the key fundamental role of the polysomal fraction in dormancy which should lead to consider under a new light the dogma of a transcriptional regulation of seed germination. We demonstrate that germination results for a timely and selective recruitment of mRNA to polysomes and bring comprehensive insights related to the regulation of this process. GO analyses of translatome data allowed the identification of key players in the active regulation of either germination or dormancy. Figure 7 summarizes the major findings of this study and provides a comprehensive model showing how the dynamics of association of transcripts with polysomes regulates Arabidopsis seed germination. The robustness of "omics" analysis using the plant model Arabidopsis permitted to demonstrate that the 5'UTR sequence regulates the translation efficiency at multiple levels and underlined in particular the major contribution of its GC content and uORF. We are convinced that the future studies dealing with the molecular regulation of dormancy will have to focus on this novel scale of post-transcriptional mechanisms in order to provide cutting edges theories for explaining this complex phenomenon.



**Figure 7.** A simplified model showing the dynamics of transcripts association with polysomes and their related GO categories during germination or dormancy maintenance at 25 °C. In the dry state, dormancy release occurs during afterripening, but the stored mRNA are roughly similar in both categories of seeds (Meimoun *et al.*, 2014). Imbibition results in transcription of genes specifically associated with dormant (D) and non-dormant (ND) seeds, while a pool of mRNA is similar in D and ND seeds (non differentially expressed mRNA and stored mRNA). Horizontal colored bars indicate the % of transcripts recruited by polysomes among the different RNA pools in D and ND seeds at 16 and 24 h of imbibition (calculated from transcriptome and translatome analysis presented in Figure 2). This demonstrates that up-regulated transcripts in D seeds participate only in a very minor way in dormancy maintenance (12 % and 7 % addressed to polysomes at 16 and 24 h, respectively) and that up-regulated transcripts in ND are more likely to be become polysome associated (45 % and 25 % addressed to polysomes at 16 and 24 h, respectively). This suggests that regulation of germination and dormancy is mainly translational. Three major GO categories specifically translated at each time points in D and ND seeds are indicated. Vertical bars on the right show that the transcripts translated at 16 and 24 h have 36 % similarity in dormant seeds but only 4 % in non-dormant ones, demonstrating the dynamics of translational activity in germinating seeds.

# **III.4 Materiels and methodes**

### Plant material and germination assays

*Arabidopsis thaliana* (ecotype Col-0) were grown in a greenhouse at 20-22 °C under long days (16 h light/8 h dark). Seeds were first sown in a mixture of soil and vermiculite (9:1) and plantlets were transferred after 12–15 days on soil/perlite/vermiculite (2:1:1). Floral stems were cut at silique maturity (about 3 months after sowing), dried at room temperature for 4 days in a paper envelope and seeds were collected. Freshly harvested seeds were either stored at -20 °C to preserve their dormancy or after-ripened at 20 °C and 56 % relative humidity in the dark for 4 weeks to alleviate dormancy. Non-dormant seeds were then stored also at -20 °C. Germination assays were performed by placing seeds in darkness in 9 cm Petri dishes (100 seeds per dish, 3 replicates) on a filter paper on the top of a layer of cotton wool moistened with deionized water. Germination was evaluated daily, a seed being considered as germinated when the radicle protruded through testa. Dormant and non-dormant seeds used for other experiments were imbibed at 25 °C in darkness in Petri dishes on a filter paper placed on a layer cotton wool imbibed with water for durations mentioned in each experiment description. After imbibition, seeds were collected, frozen in liquid nitrogen and stored at -80 °C. For each experiment a germination test was always performed to check the maintenance of the germination rate for dormant and non-dormant seeds during the storage in freezer.

#### Polysome preparation and RNA extraction

All chemicals used in this study without specific annotation were furnished by Sigma. Polysomes were prepared according to Mustroph *et al.* (2009). Frozen imbibed dormant or non-dormant seeds were ground in a mortar into a fine powder in liquid nitrogen and then solubilized (1 ml/100 mg of seeds) in polysome extraction buffer (PEB) containing 200 mM Tris-HCl pH 9.0, 200 mM KCl, 25 mM EGTA, 36 mM MgCl<sub>2</sub>, 1 % (v/v) octylphenyl-polyethylene glycol (Igepal CA-630), 1 % (v/v) polyoxyethylene(23) laurylether (Brij 35), 1 % (v/v) Triton X-100, 1 % (v/v) Tween-20, 1 % (v/v) polyoxyethylene 10 tridecyl ether, 1 % (v/v) sodium deoxycholate, 1 mM dithiothreitol (DTT), 0.5 mM Phenylmethylsulfonyl fluoride,50  $\mu$ g.ml<sup>-1</sup> cycloheximide, 50  $\mu$ g.ml<sup>-1</sup> chloramphenicol, 10  $\mu$ l. ml<sup>-1</sup> protease inhibitor cocktail. Cells debris were eliminated by centrifugation at 14000 *g* for 15 min at 4 °C and the supernatant was filtrated with Miracloth. An aliquot of 800  $\mu$ l of the supernatant was reserved for isolation of total RNA. Four ml of extracts were loaded on an 11 ml 15-60 % sucrose gradient (2 M sucrose, 0.4 M Tris-HCl pH 8.4, 0.2 M KCl, 0.1 M MgCl<sub>2</sub>, 100 mg. ml<sup>-1</sup> chloramphenicol and 100 mg. ml<sup>-1</sup> cycloheximide). After ultracentrifugation at 16 000 *g* for 2.5 h in a Beckman (SW41 rotor), the sucrose gradient was fractionated in 10 fractions with a Density Gradient Fractionator system (Teledyne ISCO) with a 254 nm detector and 2 M sucrose as displacement fluid. The

concentration of KCl used in sucrose salt solution of sucrose gradient was 0.2 M for the polysomes preparation and extraction of mRNA and 0.8 M of KCl was used to separate free monosomes from monosomes that are associated with mRNA (Martin, 1973; Fennoy and Bailey-Serres, 1995). The four fractions corresponding to the polysomes (7 to 10) were pooled. The total and polysomal RNA were extracted by addition of 500  $\mu$ l of 5 % SDS-0.2 M EDTA and one volume of phenol/chloroform/isoamyl alcohol (Barkan, 1993). After 5 min centrifugation at 2200*g* (room temperature), 1 ml of ethanol 100 % was added to the aqueous phase and the mix was centrifuged at 2200 *g* for 15 min. After resuspension of the pellet in water, the RNA was purified with Macherey-Nagel nucleospin XS columns according to manufacturer's instruction. The RNA quantity was measured with Nanovue (GE Healthcare) and RNA quality was checked with Experion electrophoresis system (BioRad).

## DNA microarray hybridization studies and analysis of polysome loading

Microarray analysis was carried out at the Unité de Recherche en Génomique Végétale (Evry, France), using the CATMAv6.2 array based on Roche-NimbleGen technology. A single high density CATMAv6.2 microarray slide contains twelve chambers, each containing 219 684 primers representing all the Arabidopsis thaliana genes: 30834 probes corresponding to CDS TAIRv8 annotation (including 476 probes of mitochondrial and chloroplast genes) + 1289 probes corresponding to EUGENE software predictions. Moreover, it included 5352 probes corresponding to repeat elements, 658 probes for miRNA/MIR, 342 probes for other RNAs (rRNA, tRNA, snRNA, soRNA) and finally 36 controls. Each long primer is triplicate in each chamber for robust analysis and in both strand. For each comparison, one technical replicate with fluorochrome reversal (dye-swap) was performed for each of the 3 biological replicates (*i.e.*4 hybridizations per comparison). Starting from 500ng of total RNA, labeling of cRNAs with Cy3-dUTP or Cy5-dUTP (Perkin-Elmer-NEN Life Science Products) was performed as described in Lurin et al. (2004). The hybridization and washing were performed according to NimbleGen Arrays User's Guide v5.1 instructions. Two micron scanning was performed with InnoScan900 scanner (Innopsys, Carbonne, France) and raw data were extracted using Mapix software (Innopsys, Carbonne, France). For each array, the raw data comprised the logarithm of median feature pixel intensity at wavelengths 635 nm (red) and 532 nm (green). For each array, a global intensity-dependent normalization using the loess procedure (Yang et al., 2002) was performed to correct the dye bias. The differential analysis is based on the log-ratios averaging over the duplicate probes and over the technical replicates. Hence the numbers of available data for each gene equals the number of biological replicates and are used to calculate the moderated t-test (Smyth, 2004). Under, the null hypothesis, no evidence that the specific variances vary between probes is highlighted by Limma and consequently the moderated t-statistic is assumed to follow a

standard normal distribution. To control the false discovery rate, adjusted p-values found using the optimized BH approach of (Storey and Tibshirani, 2003) were calculated. We considered as being differentially abundant the probes with an adjusted p-value  $\leq$  0.05. Analysis was done with the R software. The function SqueezeVar of the library Limma has been used to smooth the specific variances by computing empirical Bayes posterior means. The library kerfdr has been used to calculate the adjusted p-values.

Microarray data from this article were deposited at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/, accessionGSE61809) and at CATdb (http://urgv.evry.inra.fr/CATdb/; Project: AU13-12\_Polysome) according to the "Minimum Information about a Microarray Experiment" standards.

"*In silico*" comparison allowed to compare transcriptome and translatome for each point and type of seeds and to compare the time points (16 h vs 24 h) for each type of sample. Relative RNA abundance in polysomal (P) and total (T) fractions was quantified with the polysomal ratio (P/T) which was calculated as follow:

P/T (%) = (Log abundance in polysomal RNA / Log abundance in total RNA) x100

Student's t- tests were used to determine whether polysomal ratios of dormant and non-dormant seeds for both duration of imbibition were significantly different from each other with P-value=0.0001. Fisher test was used to compare the distribution of transcripts in each category (according to uORF occurrences) with P-value=0.05.

#### Distribution of differentially abundant mRNAs in SeedNet network

Network was constructed using SeedNet online tool (www.vseed.nottingham.ac.uk; Bassel *et al.*, 2011) with the list of transcripts identified as up-regulated in dormant and non-dormant seeds for both duration of imbibition, in transcriptome or translatome.

# Quantitative real time PCR (qRT-PCR)

RNA (100 ng) was reverse transcribed, amplified and the relative expression was calculated as described in Leymarie *et al.* (2012). Primer sequences are presented in Supplemental Table S3.

Genes	AGI	Primers sequenes
5102	A+4a24080	F:AAAATCGGGAATGTCATCAGC
JLP2	A14824980	R:CAACCAATCCTTTGGCTACG
A D A 1	A+E ~67020	F:GGCTGATGCTCCAAATGGTA
ADAI	AL5807050	R:CGCATGCAACAAGTCGAGTA
CVDZ0ZAZ	A+2a20000	F:GGCACCAAAACCTTACACG
CIFTOTAZ	Alzgz9090	R:TCTCCAATCACTTCCCATCTG
ΙEΔ	Δ+1σ72100	F:TCCGACTAAGGTAGCGAGCA
LLA	A(1g/2100	R:CGTGACCAAACAAACCCACA
	At3g51600	F:CCGAGGCGGTTTCATTCCT
LIFS	A(3g)1000	R:ATCCTAACACGGCAAGCACC
EVDA1	A+1a60520	F:AACGCAGGAGGTTGGTGTAA
LAPAI	A(1803220	R:GCACGGCACTCTTCGGTAA
ACO1	A+2g10500	F:CTCAGCAAGACGATGGATGAA
ACOI	A(2g19590	R:CGTGGGCATTCTGGGTATTTAG
Derovidase	A+5a6/100	F:TTGAGAAGGCTTGTCCTCGT
FEIOXIGUSE	A(3804100	R:GCGAAGTCTTGCTTCTGCTT
GASAG	At1g7/670	F:TGCCTTTCCCTTTCTATTTATTGCT
0,0,0,0	Alig/40/0	R:CCTTGTGCATTGTCCTCCAC
RGL2	At3g03450	F:CGACCCGAATCTGAAACCT
		R:GTTGTGGTTCGCTTCTTGCT
ABI2	At5g57050	F:GGGCTCGTGTATTTGGTGTT
		R:GAGCCAAATCGCACACTTCT
MPK4	At4g01370	F:AAACCACCGCAGAGAGAGAA
		R:CCCACGCAACAACTGATACA
HSP70	At5g02500	F:CAACCATCCCAACCAAGAAG
		R:TTGTCCTTGGTTCTGGCTCT
PIF6	At3g62090	F:TCTCACAAGGACGACAACGA
		R:CACCCATTGCTAGACCCATC
EXPA4	At2g39700	F:TCGCTATTCCTCCTCCTTTT
		R:CCCTCATTTCCCCCCTTTATC

**Table S3**. Sequences of primers used for q RT-PCR experiments. The AGI foreach gene was determined by 1 of primer by Primer3.

## **Translation quantification**

Quantification of translation was adapted from David *et al.* (2012). Seeds (30 mg) were first imbibed for 12, 20 or 68 h in water at 25 °C, before being dried 1 h at 20 °C, and then incubated for 4 hours with 200  $\mu$ g.ml<sup>-1</sup> of puromycin at 25 °C, in order to increase penetration of the product, thus giving total durations of imbibition of 16, 24 and 72 h, respectively. Seeds were then frozen and ground in liquid nitrogen in a mortar and solubilized in 300  $\mu$ l of extraction buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl<sub>2</sub>, 25 mM KCl, 1 % x protease inhibitor cocktail, 0.2U/IRibolock (Thermo), 10 % Nonidet P-40). Cells debris were eliminated by centrifugation at 14 000 *g* for 15 min at 4 °C and the supernatant was filtrated with Miracloth. Proteins were precipitated by adding two volume of ethanol and incubated at 4 °C for 6 h (Merret *et al.*, 2013). After 15 min centrifugation at 14 000 *g* at 4 °C, the pellet was solubilized with 2X Laemli buffer. The protein concentration was determined using 2-D Quant Kit and 30 µg were separated by SDS-PAGE 10-20 % and electroblotted onto PVDF membrane, which was blocked with 1 % (w/v) casein in PBS-Tween for 1 h and incubated with the primary antibody (rabbit antibodies anti-puromycin from Merck Millipore used at 1/25 000 dilution) for 1 h at room temperature. The secondary antiserum anti-rabbit IgG-Alkaline Phosphatase (Sigma) was used at 1/30 000 dilution and incubated for 1 h at room temperature. Blots were revealed with NBT-BCIP and all the bands were quantified with Image J software.

#### Gene ontology analysis and clustering

Subsets of transcripts were classified according to Gene Ontology (GO) categories with the Classification SuperViewer Tool in Bio-Analytic Resource for Plant Biology (BAR, http://bar.utoronto.ca). GO analysis results were presented using the software REVIGO (http://revigo.irb.hr; Supek et al., 2011). REVIGO summarizes long, unintelligible lists of GO terms by finding a representative subset of the terms using a simple clustering algorithm that relies on semantic similarity measures. REVIGO allows to visualize the clusters in scatterplots in 2D-space, assigning x and y coordinates to each term so that more semantically similar GO terms are also closer in the plot. The disc color gradient (blue to red colors) indicates the degree of GO enrichment corresponding to P-values, while the disc size is proportional to the frequency of the GO term in the underlying database. Spatial arrangement of discs approximately reflects a grouping of GO categories by semantic similarity. The allowed similarity used was small (0.5), and the semantic similarity measure was 'SimRel'.

#### Characterization of mRNA sequence features

Information about 5' and 3'UTR was searched in TAIR (http://www.arabidopsis.org) for transcripts being differentially abundant in translatome of dormant and non-dormant seeds and analyses on the UTR sequences were performed on these genes (listed in Supplemental Table S2). Nucleotide composition of UTRs was determined using MFOLD (http://mfold.rna.albany.edu/).Over represented motifs in 5'UTR and 3'UTR were identified on UTRs of the 100 first differentially abundant transcripts (according to BH test) by use of the MEME suite (Multiple EM for Motif Elicitation, http://meme.nbcr.net/meme/; Bailey *et al.*, 2009) using the two component mixture (TCM) with set minimum and maximum widths 6 and 50 bases, respectively. The motif consensus obtained by MEME was represented using Weblogo application (http://weblogo.berkeley.edu/; Crooks *et al.*, 2004). The motif enrichment (ME) represents the ratio of the frequency of the motif over-

represented in the co-expressed transcripts (calculated with MEME)to the frequency of this motif in 5'UTR (or 3'UTR) in whole genome of Arabidopsis thaliana (calculated with TAIR). The uORFs were determined only for transcripts having a described 5'UTR (Supplemental Table S2). In the 5'UTR sequence, we considered having one uORF when an initiation codon (ATG) was followed by a stop codon (TAA, TGA and TAG). We determined the number of uORFs using Microsoft Excel and the length of uORFs was determined using MFOLD. Transcripts presenting one or several uORFs are listed in Supplemental Dataset S4.

## **Supplemental Data**

**Supplemental Dataset S1**. mRNA abundance in dormant and non-dormant seeds in total and polysomal fractions.

**Supplemental Dataset S2**. GO function of transcripts displaying changes in abundance in dormant and non-dormant seeds transcriptome.

**Supplemental Dataset S3**. GO clustering of transcripts differentially abundant in dormant and nondormant seeds in translatome.

**Supplemental Dataset S4.** List of transcripts differentially abundant in translatome of dormant and non-dormant seed having one or several uORFs.

# IV. 5' to 3' mRNA decay contributes to the regulation of Arabidopsis seed germination by dormancy

## In preparation

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# **IV.1 Introduction**

Under natural conditions, plants are exposed to a variety of environmental conditions and thus display various molecular mechanisms for a fine-tuned control of their adaptive responses (Hirayama and Shinozaki, 2010). Seeds are major organs in life cycle of higher plants. They serve to optimize plant survival strategy and seed dormancy, which is the inability to germinate in apparently favorable conditions (Bewley, 1997), allows germination to proceed at the right moment, according to seasons and environmental factors, thus permitting the best seedling establishment. Seed dormancy is mainly regulated by environmental and hormonal factors, mainly abscisic acid (ABA) and gibberellins (GA). Molecular regulators of seed germination were identified using transcriptome data (Holdsworth et al., 2008) which ultimately led to build integrative gene regulatory networks (Bassel et al., 2011). However, recent data have shown that post-transcriptional events are likely to be more appropriate to explain the germination process, since one can now admit that there is no correlation between transcritome and translatome in germinating seeds. Indeed, only a part of the mRNA stored during seed maturation or neo-synthesiszed during seed imbibition are effectively addressed to translation, as recently shown in sunflower (Layat et al., 2014) or in Arabidopsis (Basbouss-Serhal et al., 2015). This is rising-up the question of the molecular basis of seed germination and clearly questions the actual dogma of its transcriptional regulation.

Control of gene expression in eukaryotes results indeed from transcription, but also from posttranscriptional processes including regulation of translation, but also sequestration of mRNAs into cytosolic mRNA ribonucleoproteins (mRNPs) and mRNA decay. Although less studied than transcription or translation, mRNA degradation plays a critical role in eukaryote development (Schier, 2007) and response to stress (Hilgers *et al.*, 2006). In plants, mRNA decay processes are involved in a wide variety of developmental and hormonal responses (Belostotsky and Sieburth, 2009; Chiba and Green, 2009). The role of mRNA decay in seed germination is not very documented (Xu and Chua, 2011) but it was evidenced in the modulation of abscisic acid (ABA) response, a major regulator of seed germination and dormancy (Nambara *et al.*, 2010). Indeed, some mRNA-processing proteins such as ABH1 (Daszkowska-Golec *et al.*, 2013) or AHG11 (Murayama *et al.*, 2012), which are modulators of ABA signaling, are implicated in the regulation of seed germination.

Among eukaryotes, mRNA decay mechanisms have been first identified in yeast in which the majority of transcripts are degraded via two major pathways, 5'-3' and 3'-5' decays (Mitchell and Tollervey, 2000; Parker and Song, 2004). In multicellular eukaryotes, most components of RNA machinery are conserved thus suggesting similar basic mechanisms (Chiba and Green, 2009). The first step of mRNA decay, is the deadenylation process which shortens the poly(A) tail through the activity of the CCR4-Not complex (Chen et al., 2002). In the case of the 5'-3' degradation, it is followed by decapping which is catalyzed by a conserved complex among eukaryotes and which consists in two subunits, DCP1 and DCP2, DCP2 being the catalytic subunit (Dunckley and Parker, 1999; She et al., 2006, Xu et al., 2006). Several regulatory factors are involved in the induction of DCP2 activity, such as Hedls/Ge1 in human which is required for the interaction of DCP1 with DCP2 (Fenger-Gron et al., 2005). VARICOSE (VCS) is the Arabidopsis orthologue of human Heldls/Ge1 and its interaction with DCP1 and DCP2 is involved in postembryonic development (Xu et al., 2006). DCP2 is the component complex possessing catalytic activity which can be modulated by DCP1 and VCS (Xu et al., 2006). The decapped transcripts are then digested by 5'-3' exoribonucleases, named XRN1 and XRN2 in yeast and animals. Three genes of Arabidopsis have been identified as homologous to XRN2 while no sequence homology could be found for XRN1. AtXRN2 and AtXRN3 function in nucleus, similarly to yeast XRN2, while AtXRN4 is localized in the cytoplasm and would be the functional homolog of yeast XRN1 (Kastenmayer and Green, 2000). AtXRN4 is identical to EIN5, a factor involved in ethylene signaling (Olmedo et al., 2006; Potuschak et al., 2006). AtXRN4 was shown to degrade a limited set of polyadenylated transcripts and miRNAs (Souret et al., 2004; Rymarquis et al., 2011) and is involved in adaptation to heat stress (Merret et al., 2013). Decapping components DCP1, DCP2 and VCS and the 5'-3' exoribonuclease XRN4 localize in cytoplasmic foci called Processing Bodies (P-Bodies) (Xu and Chua, 2011). Arabidopsis DCP5 is also considered as an additional P-bodies constituent which associates with DCP1 and DCP2 and is required for mRNA decapping (Xu and Chua, 2009). Although it remains uncertain whether 5'-3' mRNA decay is exclusive to P-Bodies, they constitute specialized cellular compartments of mRNA turnover (Fillman and Lykke-Andersen, 2005). These dynamic complexes were first identified in yeast and human (Sheth and Parker, 2003; Cougot et al., 2004) and later in plants (Weber et al., 2008). They cannot be purified but localization of decapping proteins allows their visualization (Buchan et al., 2010). The phenotype of Arabidopsis mutants suggests that P-Bodies function is developmental or involved in response to environment rather than housekeeping (Xu and Chua, 2006; Merret et al., 2013).

The main 3'-5' RNA degradation machinery of eukaryotic cells is the exosome, a complex found in both cytoplasm and nuclear compartments (Januszyk *et al.*, 2014). The yeast exosome contains nine subunits and co-factors (Lebreton and Seraphin, 2008). A co-factor of the nuclear exosome is the RNA helicase MTR4, which is required for all functions of the nuclear exosome in human and yeast (Lubas *et al.*, 2011; Bernstein *et al.*, 2008). Arabidopsis has two MTR4 homologues, AtMTR4 and AtHEN2 (Lange *et al.*, 2011; Western *et al.*, 2002), having each specific functions. AtMTR4 is a predominantly nucleolar protein involved in rRNA processing, allowing degradation of rRNA precursors and rRNA maturation by-products (Lange *et al.*, 2011). AtHEN2 is nucleoplasmic and is involved in the degradation of non-coding RNA, misprocessed mRNA, miRNA precursors, incompletely spliced mRNAs and transcripts produced from pseudogenes and intergenic region (Lange *et al.*, 2014). The role of exosome has been recently shown in Arabidopsis seed germination and early seedling growth with RRP41L, a protein of the exosome core complex that would mediate specific cytoplasmic mRNA decay in Arabidopsis (Yang *et al.*, 2013).

The role of mRNA decay in seed germination and dormancy is largely unknown. Germination is a developmental step that requires major changes in gene expression program that suggests the involvement of mRNA decay (Xu and Chua, 2011). The objective of this work was therefore to determine whether Arabidopsis seed germination, as modulated by dormancy, might rely on a selective decay of transcripts. Using several mutants of 5'-3' mRNA degradation *dcp1*, *vcs*, *xrn4* and exosome mutants, *mtr4* and *hen2*, we reveal a possible role of mRNA decay in seed germination and dormancy. Then we focused this study on the involvement of the 5'-3' decay and we analyzed the transcriptome of *xrn4-5* and *vcs-8* mutants during seed imbibition at 25 °C in darkness, a condition preventing germination of dormant seeds only (Leymarie *et al.*, 2012). This leads us to identify the targets of XRN4 and VCS having a role in the regulation of dormancy and to characterize their main features. We conclude that decapping complex and 5'-3' decay play a critical role in the regulation of Arabidopsis seed dormancy.

## **IV.2** Results

#### RNA decay mutants have a dormancy phenotype

We have characterized the development of mutant plants affected in the metabolism of mRNA. Five weeks after sowing, *dcp1-3, xrn4-5* and *mtr4-1* present a faster development while *vcs-8* were smaller (Figure 1A). To confirm the phenotype observed, we quantified some development traits for each genotype (Supplemental Figure S1). The length of the floral stem of *dcp1-3, xrn4-5* and *hen2-4* was similar to Col-0 while floral stem was higher in *mtr4-1* and smaller in *vcs-8* (Supplemental Figure S1A). *Varicose* mutant plants produced less seeds per silique and per plant and smaller seeds

(Supplemental Figures S1B and S1D). *Dcp1-3, hen2-4,* and *mtr4-1* mutant plants produced a similar number of seeds per silique but less silique per plant than Col-0 (Supplemental Figures S1B and S1C). Seeds of *xrn4-5* were more abundant per silique and per plant than in Col-0 (Supplemental Figures S1B and S1C). Seeds of *hen2-4* were smaller than Col-0 while seed size of *dcp1-3, xrn4-5* and *mtr4-1* was similar to the one of Col-0 (Supplemental Figure S1). Whatever the mutant, the plants nevertheless produced morphologically normal seeds and in sufficient amounts for the rest of the study.

At harvest, Col-0 seeds were dormant since 100 % germinated at 15 °C but only 25 % germinated at 25 °C in darkness (Figures 1B and C). After-ripening treatment (at 20 °C and 56 % relative humidity) increased the germination rate of Col-0 seeds to 70 % after 2 weeks and 100 % after 3 weeks (Figures 1D and 1E). At 15 °C all mutant lines germinated after 5 days but *mtr4-1*, *hen2-4* and *xrn4-5* germinated more slowly (Figure 1B, Table 1). Germination at 25 °C revealed that *mtr4-1*, *hen2-4* and *xrn4-5* seeds were more dormant than Col-0 ones since only 5 % germinated after 8 days, while *vcs-8* was less dormant since seeds from this mutant germinated to 49 % (Figure 1C). Two weeks of after-ripening were enough to totally alleviate seed dormancy of *vcs-8* mutant while only 25 % of *mtr4-1*, *hen2-4* and *xrn4-5* seeds germinated after this duration of dry storage (Figure 1D). After 3 weeks of after-ripening, all genotypes became non-dormant (100 % germination after 7 days at 25 °C) but *mtr4-1*, *hen2-4* and *xrn4-5* seeds germinated more slowly (Figure 1E). In all conditions tested seeds of *dcp1-3* mutant germinated like the ones of Col-0 (Figures 1B and 1E). To confirm that the dormancy phenotypes observed were due to the mentioned mutations, a second allele of each mutant was characterized (*mtr4-2*, *xrn4-3*, *vcs-9*, *hen2-2* referenced in Table 2). Results are presented in Supplemental Figure 2.

The potential to germinate of freshly harvested seeds of Col-0 and mutants was studied in the presence of the major hormonal regulators of dormancy. ABA treatment ( $10^{-6}$  M) at 15 °C slowed down germination for all genotypes since it increased by 50-70 % the time to reach 50 % of germination (T50) for Col-0, *vcs-8* and *dcp1-3* mutants and had a stronger effect on *xrn4-5*, *hen2-4* and *mtr4-1* (Table 1). At 25 °C, GA<sub>3</sub> ( $10^{-3}$  M) and ethylene (50 ppm) promoted germination of all genotypes in a similar way. At 25 °C in control conditions, less than 50 % of seeds of Col-0 and *dcp1-3* germinated, while in presence of GA<sub>3</sub>, T50 value was 50 h. The *vcs-8* seeds mutant seeds germinated more than two times faster with GA<sub>3</sub> than in control conditions (Table 1). Seeds of *mtr4-1*, *hen2-4* and *xrn4-5* displayed a T50 of ca 66h. Similar results were obtained when using the other mutant alleles *mtr4-2*, *xrn4-3*, *vcs-9*, *hen2-2*.



**Figure 1.** Phenotypic Characterization of Arabidopsis Mutants Affected in mRNA Decay Compared to Their Background Columbia. All experiments were carried out on Col-0 (filled circles), *vcs-8* (open squares), *xrn4-5* (filled triangles), *hen2-4* (filled diamonds) *mtr4-1* (filled squares) and *dcp1-3* (+). Means  $\pm$  SD of triplicate experiments are shown. (A) Plant development 5 weeks after sowing. (B-E) Germination tests of Col-0 and mutants performed with freshly harvest seeds in darkness at 15 °C (B), at 25 °C (C), at 25 °C after 2 weeks of storage at 20 °C at 56 % of relative humidity (D) and at 25 °C after ripening (stored for 3 weeks at 20 °C at 56 % of relative humidity).



**Figure S1.** Phenotypic Characterization of Col and mutants. a, b, c and d indicate homogeneous groups in a corresponding class (Anova test and Newman-Keuls tests, P=0.05). (A) Floral stem measurements for Col and mutants (plants grown at 22°C, long days). Values shown means  $\pm$  SE of 10 spikes. (B) Number of seeds per silique. Values shown means  $\pm$  SE of 10 spikes. (C) Number of seeds per plant = number of siliques per plant number of seeds by silique. Values shown means  $\pm$  SE of 10 siliques. (D) Weight of 50 seeds. Values shown means  $\pm$  SE of three biological replicates.

**Table 1.** Effect of Abscisic acid (ABA), Gibberellic acid (GA<sub>3</sub>) and Ethylene on the T50 (Time to Reach 50 % of Germination) of Mutants of mRNA Decay vcs-8, xrn4-5, hen2-4, mtr4-1 and dcp1-3 Mutants Compared to Col-0 Wild-type at 15 °C and 25 °C. \*50 % of germination was not reached. Means  $\pm$  SD of triplicate experiments are shown.

		T5	0 (hours)		
	A	at 15 °C		At 25	5 °C
	Water	ABA (10 <sup>-6</sup> ) M	Water	$GA_3(10^{-3} M)$	Ethylene (50 ppm)
Col-0	$60\pm1.4$	91 ± 1.3	*	$55 \pm 1.2$	$55 \pm 1.4$
vcs-8	$50\pm0.7$	$84\pm1.5$	108	$48 \pm 1.3$	$43\pm1.2$
xrn4-5	$72\pm0.7$	$192\pm1.4$	*	$64\pm1.9$	$67\pm2.2$
hen2-4	$72\pm2.5$	*	*	$67\pm1.5$	$67 \pm 2.1$
mtr4-1	$72\pm1.8$	*	*	$67\pm1.4$	$66 \pm 1.4$
dcp1-3	$58\pm1.5$	$96\pm2.8$	*	$53\pm1.6$	$54\pm1.5$



**Supplemental Figures S2.** Seed Germination of seeds of Col and of seeds of a second allele of mutants presented in Figure 1 performed with freshly harvest seeds in darkness at 15 °C (A), at 25 °C (B), at 25 °C after 2 weeks of storage at 20 °C and 56 % of relative humidity (C) and at 25 °C 3 weeks at 20 °C and 56 % relative humidity (D). Col (filled circles), *vcs-9* (open squares), *xrn4-3* (open circles), *hen2-2* (filled diamonds) and *mtr4-2* (filled squares) seeds . Means ± SD of triplicate experiments are shown.

#### The abundance of transcripts involved in ABA/GA balance is altered in RNA decay mutants

We investigated the abundance of transcripts known to be involved in the hormonal metabolism or dormancy using quantitative (real-time) reverse transcription-PCR (qRT–PCR) in seeds of Col-0 and of the mutants previously described, after 24 h of imbibition in water in the dark at 25 °C, *i.e.* before radicle protrusion (Figure 2). In *vcs-8* mutant, the abundance of transcripts related to ABA synthesis (*NCED3, NCED6,* and *NCED9*), ABA signaling (*ABI5*) and GA catabolism (*Ga2ox2*) was reduced compared to Col-0, while the abundance of transcripts related to ABA catabolism (*CYP707A2*), GA activation (*Ga3ox1, Ga20ox4*) and GA signaling (*SLP2*) was more important (Figure 2). For *dcp1-3* mutant, the abundance of the transcripts *NCED3, CYP707A2, Ga3ox1, Ga20x2* and *SLP2* was similar to Col-0. *NCED6, NCED9* and *ABI5* transcript abundance was reduced while *Ga20ax4* abundance increased (Figure 2). For *hen2-4* and *xrn4-5*mutants, the abundance of transcripts related to ABA synthesis (*NCED3, NCED6,* and *NCED9*), and signaling (*ABI5*), and GA catabolism (*Ga20x2*) was more important than in Col-0 whereas the abundance of transcripts related to ABA catabolism (*CYP707A2*), GA activation (*Ga30x1, Ga200x4*) and GA signaling (*ABI5*), and GA catabolism (*Ga20x2*) was more important than in Col-0. The transcript abundance of transcripts related to ABA catabolism (*CYP707A2*), GA activation (*Ga30x1, Ga200x4*) and GA signaling (*SLP2*) was less important than in Col-0. The transcript abundance was similar in *mtr4-1* mutant and Col-0 for all genes (Figure 2).

#### P-bodies are more abundant in non-dormant seeds

DCP1 being a P-body marker in yeast and plants (Buchan et al, 2010; Maldonado-Bonilla, 2014), Pbodies were localized in the radicle tip of the embryos using a transgenic line YFP-DCP1 under the control of its endogen promoter. Fluorescent granules containing DCP1 (size up to 2  $\mu$ m) were visualized in dormant and non-dormant YFP-DCP1 seeds after 16 and 24 h of imbibition at 25 °C in water, while they were absent in Col-0 embryos and in a negative control (YFP-DCP1 seeds treated with cycloheximide which suppresses P-bodies (Motomura et al., 2014)) (Figure 3A). P-bodies were more abundant in non-dormant than in dormant embryos for both duration of imbibition (Figure 3A). This difference corresponds to a significant increase of 50 % and 30 % of intensity of fluorescence in non-dormant seeds after 16 and 24 h of imbibition, respectively (Supplemental Figure S3). The abundance of DCP1 protein was determined by western blot using anti-YFP. A protein with an apparent molecular mass of 75 kDa corresponding to the mass of the fusion-protein YFP-DCP1 was quantified (Figure 3B). DCP1 protein was more abundant in non-dormant than dormant seeds for both durations of imbibition. Another marker of P-bodies, XRN4, was also quantified by western blot. XRN4 protein was more abundant in non-dormant seeds than in dormant ones for both durations of imbibition tested (Figure 3C).



**Figure 2.** Transcript Abundance of ABA and GA Pathways Are Affected in mRNA Decay Mutants.Quantification of expression of *NCED3*, *NCED6*, *NCED9*, *CYP707A2*, *ABI5*, *Ga3ox1*, *Ga20ox4*, *Ga2ox2* and *SLP2* analyzed in mRNA decay mutants seeds after 24 h of imbibition at 25 °C. The analyses of the expression of ABA and GA genes was performed in on Col-0 (black), *vcs-8* mutant (orange), *dcp1-3* mutant (dark blue), *xrn4-5* mutant (green), *hen2-4* mutant (red) and *mtr4-1* mutant (pink). Means ± SD of triplicate experiments are shown. a, b, c, d and e indicate homogeneous groups in a corresponding class (Anova test and Newman-Keuls tests, P=0.05).



в С 40 🗆 D 🔳 ND ٥D ∎ND 120 Quantification of YFP-DCP1 proteins 100 Quantification of XRN4 proteins 30 (arbitrary unit) 80 (arbitrary unit) 20 60 40 10 20 0 0 xrn4-5 Col 16h 24h 24h 16h

**Figure 3.** Changes in P-bodies DCP1 and XRN4 Abundance in Dormant and Non-Dormant Seeds. (A) Localization of P-bodies in Embryonic axes of dormant (D) and non-dormant (ND) seeds after 16 and 24 h of imbibition on water at 25 °C. P-bodies were visualized with a transgenic line YFP-DCP1 which is under the control of its endogen promoter. Col-0 (Columbia) seeds (first column) and transgenic seeds treated with cycloheximide (CHX; 100 mg/ml) were used as a control. (B) Quantification of western blot analysis of DCP1 protein in dormant (D) and non-dormant (ND) seeds of YFP-DCP1 line after 16 and 24 h of imbibition. Col-0 (Columbia) and Cycloheximide (CHX; 100 mg/ml) was used as a control. Data represent the mean and SE of three biological replicates per treatment.

(C) Quantification of XRN4 protein levels by western blot in dormant (D) and non-dormant (ND) seeds after 3, 6, 16 and 24 h of imbibition. Data represent the mean and SE of three biological replicates per treatment.



**Figure S3.** Quantification of P-bodies intensity in dormant (D) and non-Dormant (ND) seeds of YFP-DCP1 line after 16 and 24 h of imbibition using ImageJ software. Col (Columbia) and cycloheximide (CHX; 100 mg/ml) were used as a controls. Data represent the mean and SE of 10 biological replicates per treatment.

#### Identification and characterization of transcripts putatively degraded by XRN4 and VCS

Since *xrn4-5* and *vcs-8* mutants presented contrasted seed dormancy phenotypes (see Figure 1), transcriptomics analysis was performed to compare these mutants to Col-0 in order to identify templates of XRN4 and VCS, both involved in 5' to 3' mRNA decay. RNA was analyzed in seeds after 24 h of imbibition at 25 °C in the dark using CATMAv7 microarrays. MA plots representing the distribution of expression ratios of all transcripts in total fraction showed that major variations were comprised between log2 ratios values ranging from -1.5 and +1.5 (Supplemental Figure S4). Gene expression was considered to be different after Benjamini and Hochberg (BH) correction (P=0.05) and for a log2 ratio above 0.5 or under -0.5, at least for one comparison.



**Figure S4.** MA Plot of microarray data of ratios of expression between *xrn4-5* mutant and Col (A) or *vcs-8* mutant and Col (B) in seeds after 24 h of imbibition at 25 °C. Data represented are Log2(expression ratio of *xrn4-5* or *vcs-8* seeds / expression ratio of Col seeds). Positive values of log2(*xrn4-5*/Col) and log2(*vcs-8*/Col) correspond to transcripts more expressed in *xrn4-5* and *vcs-8* mutant respectively, negative values correspond to transcripts more expressed in Col seeds.

The microarrays data were validated by performing qRT-PCR with 17 genes. They showed a high correlation between qRT-PCR and microarrays transcripts abundance, with R<sup>2</sup> coefficients ranging from 0.71 to 0.95 (Supplemental Figure S5). Full list of differentially abundant transcripts in *xrn4-5* and *vcs-8* mutants is given in Supplemental Data Set 1; however we focused the functional analysis of gene specifically more abundant in the mutants. Indeed, transcripts that are putative targets of mRNA decay mediated either by XRN4 or by VCS in the wild type seeds should be more abundant in the corresponding mutants than in Col-0. The microarrays data were validated by performing qRT-PCR with 17 genes. They showed a high correlation between qRT-PCR and microarrays transcripts abundance, with R<sup>2</sup> coefficients ranging from 0.71 to 0.95 (Supplemental Figure S5). Full list of differentially abundant transcripts in *xrn4-5* and *vcs-8* mutants is given in Supplemental Data Set 1; however we focused the functional analysis of gene specifically abundant transcripts in *xrn4-5* and *vcs-8* mutants is given in Supplemental Data Set 1; however we focused the functional analysis of gene specifically more abundant in the mutants. Indeed, transcripts that are putative targets of mRNA decay mediated either by XRN4 or by VCS in the wild type seeds should be more abundant in the corresponding mutants than in Col-0.



Microarrays value (ratio arbitrary unit)

**Figure S5**. Validation of Microarrays Results. Scatterplot showing correlations between microarray results (x-axis) and qRT-PCR experiments (y-axis) on a set of 17 genes: *LTP1* (At1g62500), *FDS1* (At4g25100), *PXL1* (At1g08590), *YSL5* (At3g17650), *Glycoside hydrolase* (At3g62110), *Asapartyl protease* (At1g66180), *DWA3* (At1g61210), *LP1* (At1g18250), *CYP707A2* (At2g29090), *ABI5* (At2g36270), *peroxidase* (At1g05240), *PAB4* (At2g23350), *RAP2* (At1g22190), *Gibberellin-regulated protein* (At2g14900), *LEA* (At3g0248), *EXP4* (At2g39700) and *ACO1* (At2g19590) genes (A). The UBQ5 (At3g62250) and At4g12590 genes were used as reference genes. Regression lines are drawn for each gene and the R<sup>2</sup> values corresponding are indicated within the graph. For each point of qRT-PCR, ratios of log2 (abundance) ND/D were calculated to compare with microarray data. For both methods 3 independent biological samples were used.

Venn diagram shows that 973 transcripts were more abundant in *xrn4-5* and that 2312 transcripts were more abundant in *vcs-8*, when compared to Col-0 (Figure 4A). Among these differentially abundant transcripts, 794 transcripts were common between the two mutants but they must not be related to dormancy since the phenotypes of the two mutants were opposite (Figure 4A). Attention was therefore paid to the transcripts specifically more abundant in each mutant, thus representing 179 transcripts in *xrn4-5* and 1518 in *vcs-8*. Putative functions were assigned to these specific transcripts using the Gene Ontology (GO) TAIR categorization and they were visualized as functional clusters after redundancy reduction via REVIGO (Figures 4B and 4C). On this representation, colors indicate the user-provided p-value (see legend) and the size of the circles is relative to the frequency of GO terms in underlying GO database. Interestingly, GO analysis of transcripts specifically abundant in *xrn4-5* mutant revealed that the biological functions "response to stress", "hormone metabolism", "transcription, RNA binding" and "signaling" were the most represented (Figure 4B). The transcripts more abundant in *vcs-8* mutant belonged to "lipid metabolism", "protein metabolism", "cell wall",

"transport" "transcription, RNA binding" and "redox" categories (Figure 4C). The more relevant GO categories will be detailed in the discussion section.

Α



**Figure 4.** Comparison of Changes in mRNA Abundance between *xrn4-5* and *vcs-8* Mutants. (A) Venn diagram show number of transcripts displaying significant increase in abundance (after BH correction, ratio > 0.5) in mutants as compared to Columbia (Col) after 24 h of imbibition at 25 °C. 973 and 2312 transcripts more abundant in *xrn4-5* and *vcs-8* mutants were identified. (B-C) Gene ontology (GO) classification of transcripts specifically more abundant in *xrn4-5* (B) and *vcs-8* (C) mutants after 24 h of imbibition at 25 °C. Size of circles represents the frequency of the GO terms and color of circles indicates the p-value (scale on the right).

We explored the various structural properties of mRNAs more abundant in *xrn4-5* and *vcs-8* mutants. The total length of mRNAs and of 5'UTR of all transcripts was not correlated with their abundance in the mutants (Figures 5A and 5D). Free secondary energy ( $\Delta$ G) and GC content of 5'UTR were calculated for the transcripts specifically more abundant in the 2 mutants and also for the transcripts the abundance of which was constant between Col-0 and mutants. To allow a valid comparison with a similar number of genes in the 2 categories (constant and up-regulated) we had to establish a list of constant genes for each mutant. It corresponded to a range of ratios between -0.2 and 0.2 for *xrn4-5* or between -0.3 and 0.3 for *vcs-8*. The major part of the transcripts more abundant in *xrn4-5* was characterized by a low  $\Delta$ G and a high GC content (Figures 5E and 5G) but this was not the case for

constant transcripts. Similar behaviour was observed for *vcs-8* (Figures 5F and 5K). We searched motifs over-represented in 5'UTR (up to 500 nucleotides) in transcripts specifically more abundant in *xrn4-5* and *vcs-8* mutants using the Arabidopsis Information Resource (TAIR) and the "Motif Analysis" tool (Table 2). The motif enrichment (ME) represents the factor of enrichment of the motif in 5'UTR of the transcript analyzed in comparison with its occurrence in the Arabidopsis genome. E represents the E (expect)-value corresponding to the significance of the motif. We identified 17 over-represented motifs in *xrn4-5* mutants characterized by ME values higher than 9.1 and E values lower than 2.22e-10. For *vcs-8* mutant, 10 over-represented motifs were found with ME values between 6.1 and 11.12 and E values lower than 7.3e-3 (Table 2). Three over-represented common motifs were found in motifs of transcripts more abundant in *xrn4-5* and *vcs-8* mutants (Table 2).



**Figure 5.** Correlation of Gene Transcript Features and Relative mRNA More Abundant in *xrn4-5* and *vcs8* Mutants. (A) and (B)Scatter plots represent the mRNA total length (x-axis) *versus* the ratio of change in mRNA in *xrn4-5* and *vcs-8* mutants (y-axis).Each point on the graphs represents against log10 (mRNA total length) of transcripts more abundant in mutants versus the ratio of change in mRNA log2 expression mutant/Col. (C) and (D)Scatter plots represent the length of mRNA 5'UTR (x-axis, (C)) *versus* the ratio of change in mRNA expression in *xrn4-5* and *vcs-8* in mutants (y-axis, (D)).Each point on the graphs represents plotted against log2 (5'UTR length) of transcripts more abundant in mutants versus the ratio of change in mRNA expression of specifically transcripts more abundant in *xrn4-5* (E) and *vcs-8* (F)mutants(stripped bars)and transcripts displayingno significantly change between mutant and Col-0 (black bars) based on minimal free energy of a secondary structure. mRNAs were grouped based on free energy of a secondary structure with 10 Kcal.mol<sup>-1</sup> windows. Distributions were statistically different between transcripts up and constant regulated in *xrn4-5/vcs-8* seeds at both durations of imbibition as determined by khi<sup>2</sup> test (P<0.05).

(G) and (H) Distribution of specifically transcripts up-regulated in *xrn4-5* and *vcs-8* mutants (stripped bars) and transcripts displaysno significantly change between mutant and Col-0 mutants (black bars) based on GC content. mRNAs were grouped based on 5 % GC content windows.

**Table 2.** Common Cis-Elements Identified in the 5'UTR Region of Transcripts Up-Regulated in *xrn4-5* and *vcs8*Mutants After 24 h of Imbibition. Analysis was performed on a subset of 988 and 2346 transcripts more abundant in *xrn4-5* and *vcs8* mutants respectively. The motif enrichment (ME) represents the factor of enrichment of the motif in the 5'UTR region (-500 bp) of the transcript analyzed in comparison with its occurrence in the Arabidopsis genome. E represents the E (expect)-value corresponding to the significance of the motif. The motifs in bold correspond to common motifs to both mutants.

	xrn4-5	
Motifs	ME	Е
GCTCAG	13.15	7.10e-21
TTGACT	22.14	8.84e-73
TGGTTA	21.19	1.41e-82
CTCAGT	18.61	2.74e-12
CGTCAA	17.14	5.11e-22
AGCTGG	15.16	2e-40
ITAGAG	15.15	5.82e-51
TCTCCG	15.41	2.22e-10
GGAGC	13.03	4.50e-20
CAGACG	9.55	4.36e-99
GCGCTG	9.55	4.36e-99
AGCGCT	9.40	4.44e-63
CCGCAG	9.34	3.20e-10
CTGCGG	9.35	1.45e-20
CAGCTG	9.22	3.20e-19
CCCCAG	9.10	2.70e-20

4.10e-15

ATGTAT 9.10

#### Functional analysis of putative targets of XRN4 and VCS

Given that XRN4 and VCS were implicated in 5' to 3'decay process, we postulate that the observed transcript up-regulated in mutants were linked to this degradation. To assess this point we then followed the stability or the degradation of some of these transcripts in the wild type and the mutants. This was achieved by determining the amount of these transcripts during seed imbibition in the presence of cordycepin, a transcriptional inhibitor (Gutierrez *et al.*, 2002), which allows studying their stability. We have analyzed the change in abundance of transcripts known to be targeted by XRN4 (*MYB33, MYB65and AP2-like*) or by XRN4 and VCS (*AGO1*), used as controls, and we also have chosen 6 other genes from our transcriptomic analysis: *aspartyl protease* (*ASP*, At1g66180), *glycoside hydrolase* (*GH*, At3g62110), Phloem Intercalated with Xylem-Like 1 (*PXL1*, At1g08590), *DWD*-*Hypersensitive-to-ABA3* (*DWA3*, At1g61210) and *Yellow-stripe-like-5* (*YSL5*, At3g17650). These former genes were chosen based on their high ratio changes (see dataset S1). In Col-0 background, the level of all the transcripts tested decreased after 6 h of imbibition in the presence of cordycepin (Figure 6A). As expected, decay of all control genes (*MYB33, MYB65, AP2-like* and *AGO1*) depended on XRN4 or VCS (Figures 6A and 6 D). *GH, PXL1, DWA3, YSL5* mRNA decay was VCS-dependent (Figures 6F and 6I), while the one of *ASP* was XRN4-dependent (Figure 6E).

In order to gain functional insights on the role of mRNA decay in seed dormancy, we characterized the phenotype of dormancy of two alleles of each of the mutants corresponding to the previously studied genes *asp*, *gh*, *pxl1*, *dwa3* and *ysl5* (Supplemental Table S1). The germination percentages, measured after 7 d at 25 °C in the dark, of seeds from these mutants was evaluated after different durations of after-ripening (Figures 6J and Supplemental Figure 6). The number of days of after-ripening required to reach 50 % germination (DSDS50) is a criteria which is related to the dormancy intensity. It corresponded to 25.5 d in Col-0, 16 d for *asp-1*, 28 d for *dwa3-1*, 29 d for *pxl1-1* and *ysl5-1*, and 30 d for *gh-1* (Figure 6J). This shows that seeds from *asp1* mutant were less dormant while seeds from *gh*, *pxl1*, and *ysl5* mutants were slightly more dormant than Col-0 seeds (Figures 6 and Supplemental Figure 6).



**Figure 6.** Analysis of Stability of Specific mRNA Regulated by XRN4 and VCS in Col-0, *xrn4-5* and *vcs-8* Mutants and Kinetics of seed dormancy alleviation in mutants affected in these genes. (A) Transcripts abundance of gene more abundant in *xrn4-5* or *vcs-8* mutants (*MYB33*, *MYB65*, *AP2-like*, *AGO1*, *ASP* (*aspartyl protease*), *GH* (*glycoside hydrolase*), *PXL1*, *DWA3* and *YSL5*) analyzed by real-time RT–PCR. Black bars, Col-0; orange bars, *vcs-8* mutant; greenbars, *xrn4-5* mutant. Means ± SD of triplicate experiments are shown. \* indicates difference between mutant and Col-0 (Anova test and Newman-Keuls tests, P=0.05). (B) Effect of duration of after-ripening treatment at 20 °C at 56 % of relative humidity on subsequent germination percentage after 7 d at 25 °C of Arabidopsis seeds. Col-0, *asp1* (salk\_025595), *gh1* (salk\_017839), *pxl1-1* (salk\_070153), *dwa3-1*
(salk\_092993) and ysl5-1 (salk\_105596) mutants during dry storage. The after-ripening requirement was estimated for Col-0 and mutant in experiment as the  $DSDS_{50}$  value given in the legends. Values are means of three biological replicates  $\pm$  SD.



**Supplemental Figure S6.** Kinetics of dormancy alleviation of seeds of Col and of second alleles of mutants presented in Figure 6 at 25 °C in darkness. *aspartyl protease-2 (asp-2;* salk\_045167), *glycoside hydrolase-2 (gh-2;* salk\_03912), *pxl1-2* (salk\_001782), *dwa3-2* (salk\_058391) and ysl5-2 (salk\_05856) seeds were stored for 5 weeks (35 days; d) at 56 % of relative humidity at 20 °C to release dormancy. The after-ripening requirement was estimated for Col and mutant in experiment as the DSDS<sub>50</sub> value given in the legends. Means  $\pm$  SD of triplicate experiments are shown.

## **IV.3** Discussion

Dormancy is a relative phenomenon controlled by environmental factors that prevail during seed imbibition, and its expression can therefore vary greatly with hydration level, temperature, oxygen availability or light (Bewley and Black, 1994). It requires a fine molecular regulation to allow germination at the right time, according to environmental factors. The mRNA decay was historically less studied than transcription to regulate gene expression in developmental and in environmental responses, but more and more evidence highlight its major role in these processes (Belestotsky and Sieburth, 2009; Maldonado-Bonilla, 2014). This is because degradation of RNA could be more efficient and rapid for fine-tuning gene expression than mobilizing the transcription machinery. It could play a major role in the case of germination since many mRNAs accumulated during seed development must not be translated for efficient germination (Xu and Chua, 2009).

To understand the role of mRNA decay in the regulation of germination of Arabidopsis seeds by dormancy, we have studied mutants affected in the 2 main degradation pathways in eukaryotes, *i.e.* deadenylation-dependent decapping pathway following by 5' to 3' decay by an exoribonuclease (Chiba and Green, 2009) and 3' to 5' decay by the exosome (Lange *et al.*, 2011). The effect on plant

development of the chosen mutations was assessed in order to exclude any strong phenotype that might have an indirect effect on seed germination. The effects were the most important for vcs mutants (Deyholos et al., 2003) while xrn4 mutations effects, already described elsewhere (Olmedo et al., 2006; Potuschak et al., 2006; Gregory et al., 2008), were less drastic in our growth conditions. For example, we did not observe late flowering as mentioned by Olmedo et al. (2006). The absence of strong pleiotropic effect of mutations on plant development and seed production therefore allowed comparisons of germination phenotypes. As shown previously, freshly harvested Arabidopsis Col-0 seeds germinated at 15 °C in dark, when dormancy is not expressed. In these conditions, 80 % of seeds of all mutants germinated also, but seeds of mtr4-1, hen2-4 and xrn4-5 mutants displayed a slight decrease of germination at the first day. At 25 °C, only 25 % of freshly harvested Col-0 seeds germinated confirming that they were dormant (Figure 1C). Classically, after-ripening for 4 weeks at 20 °C and 56 % RH allowed dormancy alleviation (Figure 1E). Seeds of vcs mutant were less dormant than Col-0 ones, whereas seeds of mtr4-1, xrn4-5 and hen2-4 were more dormant, as shown with freshly harvested seeds and during after-ripening (Figures 1C and 1E). These results indicate that VCS, XRN4, MTR4 and HEN2 proteins are likely to play a role in the regulation of dormancy while DCP1 did not appear as being important in this process. This was confirmed by studying the effect of hormone application (ABA, GA<sub>3</sub> or ethylene) during germination of freshly harvested seeds since this allowed to similarly rank the mutants according to their level of dormancy (Table 1). XRN4 has been identified as EIN5 (Ethylene-insensitive 5) that regulates the ethylene signaling factor EIN3 through mRNA decay of F-Box proteins (Olmedo et al., 2006) and insensitivity of some alleles of xrn4 mutants has been observed at the plant level in presence of 10 ppm of ethylene (Potuschak et al., 2006; Olmedo et al., 2006). However, at the seed level, the insensitivity to 50 ppm of ethylene of xrn4-5 and xrn4-3 was only partial (Table 1).

We analyzed the transcript abundance of genes related to Arabidopsis seed dormancy in the same mutants. We focused on ABA and GA metabolisms and responses as ABA/GA balance is the major regulator of Arabidopsis seed dormancy (Finch-Savage and Leubner-Metzger, 2006; Finkelstein *et al.*, 2008; Nambara *et al.*, 2010). Dormant mutants *xrn4-5* and *hen2-4* presented higher abundance of transcripts related to dormancy (ABA anabolism and GA degradation) and lower level of genes related to germination (ABA catabolism and GA anabolism) while non-dormant *vcs* mutants were characterized by the pattern of gene expression (Figure 2). However, the dormant mutant *mtr4-1* showed a similar amount of transcripts related to hormones metabolism than Col-0 (Figure 2). MTR4 and HEN2 are related RNA helicases of the Arabidopsis core exosome, the major role of MTR4 is the degradation of rRNA precursors and rRNA maturation by-products (Lange *et al.*, 2011), while HEN2 is involved in the degradation of a large number of polyadenylated nuclear exosome substrates such as snoRNA and miRNA precursors, incompletely spliced mRNAs, and spurious transcripts produced from

pseudogenes and intergenic regions (Lange *et al.*, 2014). This suggests that the dormancy phenotype of *mtr4-1* mutant was probably more related to ribosome biogenesis than to the hormonal regulation. In contrast, HEN2, which is involved in mRNA degradation, could be more important for dormancy regulation. Surprisingly, the expression of the genes related to ABA and GA metabolism was altered in seeds of *dcp1-3* mutant while they germinated like Col-0 seeds. However, changes in transcript abundance in seeds of *dcp1-3* were less important than in seeds of other mutants for most of genes analyzed, thus suggesting this was not enough to induce a phenotype. It is noteworthy that *dcp1-3* is a partial-loss-of function mutant (Martinez de Alba *et al.*, 2015) since *dcp1* null mutations are lethal at the seedling cotyledon stage (Xu *et al.*, 2006). These phenotypic results suggest that hormonal regulation of dormancy involves mRNA decay pathways particularly through VCS, XRN4 or HEN2.

Since mRNA decay localizes in P-bodies, we observed these granules in seeds using YFP-DCP1 lines, DCP1 being a good marker of P-bodies (Buchan *et al.*, 2010; Merret *et al.*, 2013). We show that P-bodies were more abundant in non-dormant than in dormant seeds after 16 h and 24 h of imbibition and that DCP1 and XRN4 protein abundance evolved similarly (Figures 3A and B). Our results suggest that P-bodies assembly could be associated with germination and that decapping and exoribonuclease activities would be involved in decay of specific transcripts that might prevent germination. This is in accordance with the high level of dormancy found in seeds of *xrn4-5* mutant but, in contrast, seeds of *vcs* mutant are less dormant, while VCS is known as being a regulatory component of the decapping machinery in P-bodies and Goeres *et al.* (2007) have shown that vcs mutant lacks P-bodies in seedlings. However the dynamics of association and composition of P-bodies is still unclear. For example it has been shown that DCP1 can form DCP1 bodies independently of DCP2 (Motomura *et al.*, 2014) and this rises up the specificity of the mechanisms of assembly of P-bodies as a function of the organs.

Nevertheless, since we identified distinct dormancy phenotypes clearly associated with changes in transcript abundance involved in hormonal regulation of dormancy, as well as a differential assembly of P-bodies in dormant and non-dormant seeds, we focused our study on 5'-3' RNA decay in P-bodies using the 2 mutants presenting a strong phenotype, *i.e. xrn4-5* and *vcs-8*. Moreover these mutants had opposite phenotypes of dormancy thus allowing the role of mRNA decay in either dormancy maintenance (VCS activity) or alleviation (XRN4 activity). We have studied the transcripts which were more abundant in the mutants since they are likely to be direct targets of decay (Souret *et al.*, 2004; Gregory *et al.*, 2008), while transcripts less abundant in mutants would correspond to secondary effects of the mutations (Rymarquis *et al.*, 2011). We have identified 973 transcripts more abundant in *xrn4-5* mutant, *i.e.* putative targets of XRN4 in wild type seeds, and 2312 transcripts more abundant in *vcs-8* mutant, *i.e.* putative targets of VCS. This discrepancy in the number of transcripts

has to be considered with regards to the stronger phenotype of *vcs-8* mutant for other development aspects (Figure1 and supplemental Figure S1; Deyholos *et al.*, 2003). The 794 common transcripts between the two mutants represent the major part of XRN4 targets (Figure 4A). This must be related to the fact that exoribonuclease activity of XRN4 is directed against mRNAs decapped by the decapping complex involving VCS. This overlap was not observed with the comparison of mRNA decay in *dcp2* and *xrn4* mutants, leading to the hypothesis that others exoribonucleases than XRN4 might exist (Goeres *et al.*, 2007) and could also explain why the two thirds of the transcripts affected in *vcs* were not more abundant in *xrn4*. The 179 transcripts found specifically as more abundant in *xrn4-5* could have been either not decapped but exposed to hydrolysis after endonucleotide cleavage or decapped in a VCS-independent manner. With regards to the opposite dormancy phenotypes of *vcs-8* and *xrn4-5* seeds (Figures 4 and 1C), this suggests that VCS is involved in decapping transcripts that can positively regulate germination (1518) or are not related to this process (794).

To gain insights in dormancy regulation, we focused this study on the transcripts that were specific for each mutation (179 in xrn4-5 and 1518 in vcs-8). The clusters of transcripts found specifically more abundant in xrn4-5 or in vcs-8 mutants were classified according to GO functions and visualized as "REVIGO" scatterplots (Figure 4B). The GO category "transcription, RNA binding" was common to both mutants, and contained many transcription factors which demonstrates that both germination and dormancy maintenance require specific transcription, although this process is not strictly necessary for sensu stricto germination. For example, several WRKY transcription factors (Tripathi et al., 2014) were identified only in the non-dormant vcs-8 mutants (see Supplemental Data Set 3). WRKY60 (At2g25000) and WRKY63 (At1g66600) have already been shown as negative regulators of ABA signaling (Liu et al., 2012; Ren et al., 2010) and WRKY22 (At4g01250) was significantly induced by  $H_2O_2$  treatment (Zhou *et al.*, 2011), which could be related to the role reactive oxygen species (ROS) in germination or dormancy alleviation (Bailly et al., 2008 and Leymarie et al., 2012). WRKY33 (At2g38470) and WRKY52 (At5g45260) identified in vcs-8 were also identified in biotic stress response that could be related with ROS (Merz et al., 2015). Auxin inducible transcription factors were also more represented in vcs-8 (ARF5; At1g19850, IAA4; At5g43700, IAA2; At3g23030, IAA11; At4g28640, IAA18; At1g51950, AXR5; At4g14560). They could be related to the growth process occuring after germination sensu stricto. Among the transcription factors regulated by XRN4, several transcripts related to ABA signaling were identified, such as ABA insensitive 5 (ABI5; At2g36270; Lopez-Molina *et al.*, 2002) or *MYB33* (Kim *et al.*, 2008).

Clustering analysis showed that the transcripts which were specifically more abundant in *xrn4-5* mutant fell mainly into the 3 categories "hormone metabolism", "response to stress" and "signaling". Here we identified transcripts that should be degraded in wild-type plants for permitting dormancy alleviation. In the GO category "hormone metabolism", we found regulators of hormonal pathways

related to dormancy (see Supplemental Data Set 2). For example, the ABRE binding factors *ABF3* and *ABF4* (At4g34000, At3g19290) are involved in ABA signaling (Muñiz García *et al.*, 2014) or At3g02480 encodes a protein of the LEA (Late Embryogenesis Abundant) family which is known to be often ABA induced (Parcy *et al.*, 1994). *PIF6* (Phytochrome Interacting Factor 6; At3g62090), more abundant in *xrn4-5* mutant, has already been suggested to play a role in Arabidopsis primary dormancy (Penfield *et al.*, 2009). Inhibition of germination was also related to the presence of many transcripts of Heat Shock Proteins (HSPs) such as *HSP17* (At5g12030) and *HSP20* (At1g52560, At1g53540, At1g59860 and At1g07400), *HSP70* (At3g12580) or *HSP90* (At5g52640). The involvement of HSP proteins in inhibition of germination has already shown in sunflower (Layat *et al.*, 2014) or Arabidopsis (Basbouss-Serhal *et al.*, 2015), suggesting that their chaperon activity can play a role in maintenance of dormancy. The XRN4-mediated decay was already shown to be involved in Arabidopsis heat tolerance (Merret *et al.*, 2013).

Transcripts that accumulated only in vcs-8 mutant are enriched for those encoding "hormone", "redox", "cell wall" and "transport" (Figure 4C, see supplemental dataset S3). In contrast to what was shown for xrn4-5 mutant, the degradation of these transcripts in the wild-type thus prevents germination. In the GO category "hormone metabolism" we found regulators of the hormonal balance which drives the germination process (Finch-Savage and Leubner-Metzger, 2006). The ABA hydroxylase CYP707A1 (At4g19230), involved in ABA degradation, has already been shown to be transcriptionally regulated and is considered as a key point of control of germination (Okamoto et al., 2006; Millar et al., 2006). The ACC oxidase ACO1 (At2g19590) represents a major step of ethylene synthesis (Corbineau et al., 2014) and At2g14900 is involved in gibberellin response. Involvement of ROS signaling in dormancy alleviation was confirmed here by the over-representation of the GO category "redox" in the vcs-8 mutant. Increase in ROS content could be related to an increase in transcripts related to the enzymatic ROS scavenging, such as monodehydroascorbate reductases MDAR1 (At3g52880) and MDAR4 (At3g27820) (Bailly et al., 2004) and several thioredoxins (At3g51030, At5g39950, At2g32920, At1g77510), a family of proteins known to regulate seed germination (Montrichard et al., 2003; Marx et al., 2003; El-Maarouf-Bouteau et al., 2013). Glutathione involvement was also suggested with the presence of the glutathione-disulfide reductase (GR1, At3g24170), the glutathione synthetase 2 (GSH2, At5g27380) and a glutaredoxin (At3g57070). Cell wall modifications (extensibility, degradation and synthesis) are critical to allow cell expansion allowing radicle elongation (Bewley, 1997; Nonogaki et al., 2007; Wang et al, 2015). Here we found transcripts for expansins such as EXPA20 (At4g38210), extensins (At1g76930, At2g43150), At5g48900, several pectin lyases (At3g06770, At3g16850, At1g67750, At3g62110) galacturonosyltransferase GAUT11 (At3g61130), cellulose synthase (At5g22740, At4g07960, At3g07330) and xylose transferase XGD1 (At5g33290) (see Supplemental Data Set 3). The

cytoskeleton elements were also overrepresented in this category with tubulin (At4g20890, At1g75780) and actin (At5g07740, At5g55400, At1g52080). Myosins (At3g19960, At3g56480, At2g34730, At5g07890, At2g20290) and dynein (At1g23220) accumulation could be related to stimulation of intracellular trafficking (Diaz-Camino *et al.*, 2005; Wang *et al.*, 2015). The GO category "transport" was also markedly represented in *vcs-8* mutant including for example sugar or oligopeptides transporters that may be associated with the germination process (Aluri & Buttner, 2007). For example the peptide transporter PTR1 (At3g54140) was already identified in germinating seeds (Dietrich *et al.*, 2004; Stacey *et al.*, 2006; Layat *et al.*, 2014) as the metal transporter YSL5 (At3g17650) (Waters *et al.*, 2006).

Besides the mRNAs putatively degraded via XRN4 and VCS, we also identified targets of miRNA using transcriptomic analysis of *xrn4-5* and *vcs-8 mutants*. This is particularly relevant because XRN4 and VCS have also been shown to be involved in the degradation of 3' products of miRNA-mediated cleavage (Souret *et al.*, 2004; Motomura *et al.*, 2012). Thus we have found 62 transcripts targets of miRNA in transcripts more abundant in *xrn4-5*. In particular, the accumulation of scarecrow-like (At2g45160) RNA proceeds through the miRNA-directed cleavage pathway mediated by miR171 (Llave *et al.*, 2002), *ARF8* (At5g37020) is a target of miR167, *AP2-like* (At4g36920) a target of miR172, *AGO1* (At1g48410) a target of miR168 and *MYB33/65* (At5g06100; At3g11440) targets of miR159 (Kasschau *et al.*, 2003; Rhoades *et al.*; 2002). Souret *et al.* (2004) and Rymarquis *et al.* (2011) identified also all these targets of miRNA as regulated by XRN4 in Arabidopsis. For *vcs-8* mutants, we identified 171 transcripts up-regulated related to miRNA targets such *AGO1* (At1g48410) that is a target of miR168 and *CUC1/2* (At3g15170, At5g53950), a target of miR164 (Laufs *et al.*, 2004). This targets of miRNA regulated by VCS were identified by Motumora *et al.* (2012). These results suggest that miRNA-dependent degradation process may play a role in seed dormancy as suggested by Nonogaki (2014).

Our attempt to identify mRNA properties associated with more abundant transcripts in *xrn4-5* and *vcs-8* mutants highlighted the 5'UTR role. Our analysis indicates that total mRNA length and 5'UTR length were not implicated in the selectivity of XRN4 and VCS (Figures A to D). Although a relationship between transcript length and mRNA decay has not been reported, the analysis of Arabidopsis mRNA half-life data in cultured cells has revealed that shorter mRNA have a longer half-life (Narsai *et al.*, 2007). However, we show that strong secondary structure and high GC content made the transcripts more sensitive to degradation (Figure 5E to H). In agreement with our results, it has been previously reported that strong secondary structures in the 5'UTR accelerated decay of yeast transcripts (Muhlrad *et al.*, 1995; Doma and Parker, 2006). In mammalian, Zhang *et al.* (2011) have shown also a negative correlation between secondary structure and RNA decay. To precise the role of 5'UTR on mRNA decay, we identified 17 hexamer motifs over-represented in the 5'UTR of

transcripts more abundant in *xrn4-5* mutants (Table 1). Nine motifs (GCTCAG, TTGACT, CTCAGT, CGTCAA, AGCTGG, TTAGAG, TCTCCG, TGGAGC and GCGCTG) were previously identified as overrepresented in targets of XRN4 in Arabidopsis plants (Rymarquis *et al.*, 2011). For the *vcs-8* mutant, we have identified 10 over-represented motifs (Table 1). Some of them (TTTTTC, ATTGCA and TGCTTG) were identified as specific for uncapped transcripts in Arabidopsis flower system (Jiao *et al.*, 2008). Three motifs were common between transcripts up-regulated in *xrn4-5* and *vcs-8* mutants (CAGACG, GCGCTG and AGCGCTT) and could directly interact with the decapping complex and XRN4.

At last, we confirmed that the transcripts more abundant in xrn4-5 and vcs8 mutants were really targets of XRN4 and VCS. We therefore assessed the stability of some mRNAs using the transcriptional inhibitor cordycepin. We show that MYB33, MYB65, AP2-like and AGO1 were targets of both XRN4 and VCS. The decay of ASP was XRN4-dependent while the degradation of GH, PXL1, DWA3 and YSL5 was VCS-dependent (Figure 6E-I). Mutants corresponding to these transcripts were used to demonstrate if they could play a role in the regulation of dormancy. Thus we grew plants of the corresponding mutants and characterized the physiology of the seed produced. While asp-1 seeds were less dormant than the ones of Col-0, seeds of *qh-1*, *ysl5-1*, *dwa3-1* and *pxl1-1* were more dormant (Figure 6J). This suggests that ASP protein might be involved in dormancy maintenance. ASP is a predicted A1 family aspartyl protease which responsive to ascorbic acid and light but its role in plants is not elucidated yet (Gao et al., 2011). The degradation of the related mRNA is made by XRN4. This is in accordance with the phenotype of xrn4-5 seeds which were more dormant. GH, YSL, PXL1 and DWA3, whose seeds of the mutants were dormant, might be involved in the germination process. Their transcript is degraded by VCS which is also related to the low dormancy phenotype of vcs8 seeds. Previous results show that GH play important roles in many diverse processes including hydrolysis of cell wall-derived oligosaccharides during germination (Xu et al., 2004). YSL1 and YSL3 were involved in Fe transport at seed level are Waters et al., 2006), but no data are available about YSL5. For the LRR kinase PXL1/PXL1, the role of this LRR kinase in temperature perception during germination was recently shown (Jung et al, 2015).DWA3 was involved in the negative regulation of ABA responses (Lee et al. 2010, 2011). Comparison of translatome between dormant and nondormant seeds shows that GH, YSL5, PXL1 and DWA3 transcripts were more associated with polysomes in germinating non-dormant seeds after 24 h of imbibition at 25 °C (Basbouss-Serhal et al., 2015). These transcripts must therefore be degraded to maintain dormancy.

## **IV.4 Methods**

#### Plant material and germination assays

All mutant lines from *Arabidopsis thaliana* were in Columbia (Col-0) background and are listed in Supplemental Table 1. T-DNA insertions in homozygous mutant plants were checked by PCR using two sets of primers designed on Salk database (http://signal.salk.edu/ tdnaprimers). Seeds were stratified at 4 °C for 2-4 d before sowing and plants were grown at 20-22 °C, under long days (16 h light/8 h dark). Seeds were harvested dormant and were either stored at harvest at -20 °C to maintain dormancy or dormancy was alleviated by storage in 56 % of relative humidity at 20 °C for 3 weeks. Germination assays were performed at 15 °C or 25 °C in darkness in 3 biological replicates of 50 seeds for each genotype in 9 cm Petri dishes on a layer of cotton wool covered by a filter paper sheet soaked with water or with abscisic acid (ABA, 10<sup>-6</sup> M) or gibberellic acid (GA<sub>3</sub>, 10<sup>-3</sup>M). Ethylene (50 ppm) was applied in gaseous form in sealed Petri dishes. A seed was considered as germinated when the radicle has protruded through the testa. Germination was scored daily for 8 days and the results presented correspond to the mean of the germination percentages obtained for 3 biological replicates.

Table S1. T-DNA Mutant Lines Used. The AGI for each gene was determined by TAIR. T-DNA insertion lines in Col background were kindly given by (a) Dr Hervé Vaucheret (Institut Jean-Pierre Bourgin, INRA, Versailles), (b) Dr Heike Lange (Institut deBiologie Moléculaire des Plantes du Centre National de la Recherche Scientifique, Université de Strasbourg), (c) obtained from the Nottingham Arabidopsis Stock Centre and (d) Dr Pascal Genschik (Biochemistry and Plant Molecular Physiology, IBIP, Montpellier).

Gene	Mutant	AGI	T-DNA insertion	Source
putative ASPARTYL PROTEASE	asp-1	At1g66180	Salk_025595	с
	asp-2		Salk_041567	
PHLOEM INTERCALATED WITH XYLEM-LIKE 1	PXL1-1	At1g08590	Salk_070153	с
	PXL1-2		Salk_001782	
DECCAPPING 1	dcp1-3	At1g08370	Sail_377 B10	a
GLYCOSIDE HYDROLASE	gh1	At3g62110	Salk_017839	с
	gh2		Salk_031921	
HU ENCHANCER2	hen2-4	At2g06990	Salk_091606	b
	hen2-2		Gabi_774H07	
putative RNA HELICASE	mtr4-1	At1g59760	Gabi_048G02	а
	mr4-2		Sail_50_C11	
VARICOSE	vcs-8	A+2~12200	Sail_1257_H12	а
	vcs-9	Alog1000	Sail_218_E01	
DWD HYPERSENSTIVE TO ABA3	dwa3-1	441-(1210	Salk_092993	с
	dwa3-2	At1g01210	Salk_058391	
EXORIBONUCLEASE 4	xrn4-5	At1g54490	Sail_681_E01	а
	xrn4-3		Salk-014209	d
YELLOW STIPE LIKE 5	ysl5-1	At3g17650	Salk_105596	с
	ysl5-2		Salk_058656	

#### **RNA** extraction

RNA was extracted from dormant seeds after 24 h of imbibition at 25 °C in 3 biological replicates. For each extraction, 30 mg of seeds were ground in liquid nitrogen, and total RNA was extracted by a hot phenol and polyvinylpyrrolidone (PVP) procedure according to Verwoerd *et al.* (1989) and purified with Macherey-Nagel nucleospin XS columns according to manufacturer's instructions. RNA extract was analyzed by a spectrophotometer (Nanovue, GE Healthcare) to determine the RNA concentration at 260 nm and RNA quality (230-400 nm) that was also checked on agarose gel.

## Real-time quantitative reverse transcription-RT

Total RNA (1µg) was treated with DNase I (Sigma) and then was reverse transcribed with Revertaid<sup>TM</sup> Reverse transcriptase (Fermentas). The cDNAs obtained were checked by agarose gel electrophoresis and with PCR amplification of actin gene (26 cycles). The amplification was then performed with realtime PCR in Mastercycler epgradients Realplex<sup>2</sup> (Eppendorf) using 5 µL of 30X diluted cDNA solution. Real-time PCRs were performed with the Maxima SYBR Green/qPCR Master Mix (Fermentas) in a 15 µl reaction volume with 0.23 µM of each primer (Eurogentec). The program for running qPCR was initiated at 95 °C for 10 min followed by 30 cycles at 95 °C for 30 s, 56 °C for 30 s and at 72 °C for 30 s. As reference genes, fragments of *ubiquitin 5* and *At4g12590* genes were amplified. The sequences of the primers used are presented in Supplemental Table 2. Critical thresholds (Ct) were calculated using the Realplex 2.software (Eppendorf) and the relative expressions were calculated according to Hellemans *et al.* (2007). An arbitrary value of 100 was assigned to the Col-0 seeds, which were used as control sample for normalization (Pfaffl, 2001). Results presented are the means ± SD of three biological replicates. Statistical analyses were performed with StatBox 6.40 software (Grimmer logiciel, France) software. Data were normally distributed and with equal variance Newman-Keuls tests ( $\alpha$ = 0.05) were performed after analysis of variance (ANOVA). Table S2. Sequences of Primers Used for qRT-PCR Experiments. The AGI for each gene was determined by TAIR and the sequence of primer by Primer3. DOG1 universal primers for splice variants in Bentsink et al. (2006). Ga2ox2 and NCED6 primers were tacked from Footit et al. (2011) and Seo et al. (2004).

Genes	AGI	Primers sequences
ABI5	At2g36270	F: TTGAAGTCAAGGGCACAACC
		R: CGGGTTCCTCATCAATGTCC
ACCOX1	At2g19590	F: CTCAGCAAGACGATGGATGAA
		R: CGTGGGCATTCTGGGTATTTAG
AGO1	At1g48410	F: CAATCCAAGCATCACATTCG
		R: TGCCCTTCACTCCTAAATGG
AP2-like	At1g22190	F: GCGTTTGCGTTCTCTCTACC
		R: AATCTCAGCCACCCATTTTC
Aspartyl protease	At1g66180	F: CACTACTCTTACTTCTACGCTGATGG
		R:CCTATCATCAGAAGACTCAGTAGCAC
PXL1	At1g08590	F:GGAGTTAATAGCTGGGAAGAAACCTG
		R: ACCCACCTAACTATATCCACTCCTTC
CYP707A2	At2g29090	F: GGCACCAAAACCTTACACG
		R: TCTCCAATCACTTCCCATCTG
DOG1	At5g45830	F: GACGGCTACGAATCTTCAGG
	0	R: AGCTAGCTGCTCCGCACTTA
DWA3	At1g61210	F: TAATACTTCCTCCACCATCTCTCCTC
	<b>U</b> -	R: GATCTCTTCTCATATCCCTCCCTTTG
FXP4	At2g39700	F: TCGCTATTCCTCCTCCTTTT
	0	R: CCCTCATTTCCCCCCTTTATC
FDS1	At4g25100	F: GAAAACATCACAGAGCTTACGTGG
		R: GTAAGTGCTGTGGATAATGTGCTC
Ga20ox4	At5g64100	F: GCGAGACGACAAGGAAGACA
	110801200	R: TCGGGATACGCTCTCTCACC
Galov2	Δ+1σ30040	
002072	ALIESOOAO	
Ga3ox1	Δ+1σ15550	
603021	Aligi5550	
Gibberellin-regulated protein	A+2g1/000	
	A(2g14900	
Glucoside hydrolase	A+2g62110	
Giycoside nyurolase	A(3802110	
IEA	A+2a0248	
	AL320248	
1.01	A+1 a192E0	
LPI	ALIG10250	
	4+1~62500	
LIP	AL1802500	
A 4//D 2 2	445-00100	
МҮВЗЗ	At5g06100	
ANDCE	442-11440	
МҮВ65	At3g11440	
110500		R: TTACAGCGACCAAACAGGAG
NCED3	At3g14440	F: GCIGCGGIIICIGGGAGAI
		R: GGCGGGAGAGIIIGAIGAII
NCED6	At3g24220	F: CGTTATTCCTATGGAGCAGAAATCG
		R: GGAGCGAAGTTACCTGATAATTGAA
NCED9	At1g78390	F: TCCCCTGCTATGTTTCTTCC
		R: AGACGGTGGTTTGAATGTCG
PAB4	At2g23350	
		R: CCTCCTTTCTTCTTCCTCTG
Peroxidase	At5g64100	F: TTGAGAAGGCTTGTCCTCGT
		R: GCGAAGTCTTGCTTCTGCTT
RAP2	At1g22190	F: GTTCTCTCTACCCGCTCCAA
		R: CCACCCATTTTCCCCAGT
YSL5	At3g17650	F: TAATACTTCCTCCACCATCTCTCCTC
		R: CTCATGTTCCACTATCTGACGATCTC

### In situ localization of DCP1 protein

The localization of DCP1 proteins was visualized by using YFP lines. Transgenic line was made in Laboratoire Génome et Développement des Plantes, Univ. Perpignan. Dormant and non-dormant seeds were imbibed for 16 and 24 h in water, or in water (for 23 h or 15 h) and for 1 h in cycloheximide (100 mg/ml). Testa of seeds were removed before observation. Images were acquired (excitation, 515 nm; emission, 535-600 nm) with an apotome microscope (Zeiss Imager Z1) using a 60 numerical aperture objective. Z-series were performed with a Z-step of 1µm, and maximum projections of Z planes are displayed. For each embryo, a maximal projection of 10 planes on radicle apex was performed.

#### Protein extraction and western blot analysis

Proteins were extracted from dormant and non-dormant seeds after 3, 6, 16 and 24 h of imbibition. Seeds (30 mg) were frozen and ground in liquid nitrogen in a mortar and solubilized in 300 µl of extraction buffer (250 mM Tris-HCl, pH 7.5, 1 % protease inhibitor cocktail, 0.07 %  $\beta$ mercaptoethanol). The samples were centrifuged at 14 000 q for 15 min at 4 °C and clear supernatants were collected. The proteins were precipitated with 10 % (V/V) of TCA at 4 °C for 5 min. After 5 min centrifugation at 10 000 g, the supernatant was removed, the pellet was washed by acetone 80  $\,$  % and  $\beta$ -mercaptoethanol 0.07  $\,$  % to 15 min at -20 °C, twice. After the pellet dried, 50  $\mu$ l of Laemmli buffer 2X were added to solubilize proteins for 30 min. The samples were incubated for 2 min at 65 °C. The protein concentration was determined using 2-D Quant Kit (GE Healthcare). Three biological replicates were used. Total proteins (30 µg) were separated by SDS-PAGE 10 % (Miniprotean<sup>®</sup> TGX Gel, Biorad) and electroblotted onto PVDF membrane, which was blocked with 1 % (w/v) casein in PBS-Tween for 1 h and incubated with the primary antibody (rabbit antibodies anti-XRN4 1/500e and YFP 1/25 000e dilution) for 1 h at room temperature. XRN4 antibody was given by Cécile Antonelli, Laboratoire Génome et Développement des Plantes, Univ. Perpignan (Merret el al., 2013) and YFP antibody was obtained from Life Science. The secondary anti-rabbit IgG HRP conjugate (Sigma) was used at 1/10 000 dilution and incubated for 1 h at room temperature. HRP detection was performed with ECL Plus Chemiluminescence system (Amersham). Blots were revealed with Storm 860 and all the bands were quantified with ImageJ software.

#### DNA microarray hybridization studies and analysis

Microarray analysis was carried out at the Institut of Plant Sciences Paris-Saclay (Evry, France), using the CATMAv7 array based on AGILENT technology. The CATMAv7 design of *Arabidopsis thaliana* genome has been made with gene annotations include in FLAGdb++, an integrative database around

plant genome (http://urgv.evry.inra.fr/FLAGdb, Dérozier et al., 2011). The single high density CATMAv7 microarray slide contains four chambers, each containing 149 916 primers. Each 60 bp primer is triplicate in each chamber for robust analysis and in both strand. As part of all probes, 35 754 in triplicate correspond to gene TAIRv8 (among which 476 probes corresponding to mitochondrial and chloroplast genes) + 1289 probes corresponding to EUGENE software predictions + 658 probes for miRNA/MIR, and finally 240 controls. Three independent biological replicates were produced. For each comparison, one technical replicate with fluorochrome reversal was performed for each biological replicate (i.e. four hybridizations per comparison). The labeling of cRNAs with Cy3dUTP or Cy5-dUTP was performed as described in Two-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labeling manual (© Agilent Technologies, Inc.). The hybridization and washing were performed according to Agilent Microarray Hybridization Chamber User Guide instructions (© Agilent Technologies, Inc.). Two micron scanning was performed with InnoScan900 scanner (Innopsys<sup>R</sup>, Carbonne, FRANCE) and raw data were extracted using Mapix<sup>R</sup> software (Innopsys<sup>R</sup>, Carbonne, FRANCE). For each array, the raw data comprised the logarithm of median feature pixel intensity at wavelengths 635 nm (red) and 532 nm (green). For each array, a global intensity-dependent normalization using the loess procedure (Yang et al., 2002) was performed to correct the dye bias. The differential analysis is based on the log-ratios averaging over the duplicate probes and over the technical replicates. Hence the numbers of available data for each gene equals the number of biological replicates and are used to calculate the moderated t-test (Smyth, 2004). Under, the null hypothesis, no evidence that the specific variances vary between probes is highlighted by Limma and consequently the moderated t-statistic is assumed to follow a standard normal distribution. To control the false discovery rate, adjusted p-values found using the optimized FDR approach of Storey et al. (2003) were calculated. We considered as being differentially expressed the probes with an adjusted p-value  $\leq$  0.05. Analysis was done with the R software. The function SqueezeVar of the library limma has been used to smooth the specific variances by computing empirical Bayes posterior means. The library kerfdr has been used to calculate the adjusted p-values.

Microarray data from this article were deposited at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), accession no. GSE68268) and at CATdb (http://urgv.evry.inra.fr/CATdb/; Project: AU14-08\_xrn\_vcs) according to the "Minimum Information About a Microarray Experiment" standards.

### Gene ontology analysis

Subsets of genes were classified according to Gene Ontology (GO) categories with the Classification SuperViewer Tool in Bio-Analytic Resource for Plant Biology (BAR, <u>http://bar.utoronto.ca</u>). GO

analysis results were presented using the software REVIGO (<u>http://revigo.irb.hr</u>; Supek *et al.*, 2011) that allows to plot the GO data in 2D-space. The allowed similarity used was small (0.5), and the semantic similarity measure was 'SimRel'.

## Characterization of 5'UTR

A database of 5'UTR of transcripts being differentially abundant in *xrn4-5* and *vcs8* mutants was generated from TAIR (http://www.arabidopsis.org). The minimal energy for secondary structures was calculated using RNAfold. Nucleotide composition of UTRs was determined using MFOLD (<u>http://mfold.rna.albany.edu/</u>). Over represented motifs in 5'UTR were identified on UTRs of transcripts more abundant in mutants with TAIR. The motif enrichment (ME) represents the ratio of the frequency of the motif over-represented in the co-expressed genes to the frequency of this motif in 5'UTR in whole genome of *Arabidopsis thaliana* (calculated with TAIR).

#### mRNA stability

To assess mRNA stability, we have analyzed the level of several transcripts in Col-0, *xrn4-5* and *vcs-8* dry seeds and seeds incubated for 6, 16 and 24 h at 25 °C in darknesss, with 50 µM of cordycepin that inhibits transcription. Total RNA was extracted and mRNA levels were assessed by qRT-PCR analyses as described previously. The percentages of remaining mRNAs calculated relative to dry seeds for each genotype were plotted against time and a regression curve obtained.

## **Supplemental Data files**

Supplemental Data 1. mRNA Abundance in *xrn4-5* and *vcs-8*Mutants after 24 h of Imbibition at 25 °C.
Supplemental Data 2. GO Clustering of Transcripts more Abundant in *xrn4-5*Mutant.
Supplemental Data 3. GO Clustering of Transcripts more Abundant in *vcs-8*Mutant.
Supplemental Table 1. Characterization of T-DNA Mutant Lines Studied.
Supplemental Table 2. Sequences of Primers used for qRT-PCR Experiments

## **GENERAL CONCUSION**

This work brings novel findings in the field of seed biology and allows a better understanding of the mechanisms regulating dormancy. I will summarize here the major points that have been evidenced during my PhD.

In Arabidopsis thaliana seed, the loss of dormancy during after-ripening depends on the environmental conditions prevailing during seed maturation, seed storage and germination conditions. The effect of various combinations of temperature and relative humidity on dormancy alleviation of Arabidopsis seeds during dry after-ripening was investigated. Our data indicate that at each temperature corresponds a specific relative humidity to induce after-ripening and we propose a simple model predicting primary dormancy alleviation rate. As in sunflower seeds we demonstrate that after-ripening in the dry state depends on non-enzymatic reactions. Since ROS play an important role in the regulation of sunflower seed dormancy and germination (Oracz et al., 2007; 2009), particularly during after-ripening, it would be therefore important to determine if oxidative modifications may play a role in alleviation of dormancy of Arabidopsis seeds during storage. In particular, we have shown that the storage period could induce changes in the expression of key regulatory genes of germination. This would be interesting to determine if ROS could play a role in this process, as suggested by Bazin et al. (2011). Our results also demonstrate that induction of secondary dormancy can occur during long-term storage, which was completely unexpected and has never been reported. Explaining fluctuations in dormancy level is highly challenging because following intrinsic changes or rhythms in the dry state is technically unrealistic, but this finding rises up fundamental questions about the life in anhydrobiosis. At last, this study was an indispensable prerequisite for controlling the physiological parameters regulating seed dormancy in Arabidopsis.

At the molecular level, we provide a better understanding of the role of post-transcriptional modifications in the regulation of germination and dormancy in *Arabidopsis thaliana* seeds. We show that there is no correlation between transcriptome and translatome and that germination regulation is largely translational, implying a selective and dynamic recruitment of mRNAs to polysomes in both dormant and non-dormant seeds. This is a fundamental finding which clearly challenges the actual dogma of a transcriptional regulation of germination. We also demonstrate that dormancy maintenance, but not only germination, requires an active translation of specific genes. Gene Ontology (GO) clustering allowed identifying the functions of polysome-associated transcripts in dormant and non-dormant seeds and permitted to reveal novel actors of seed dormancy and germination. For example we have shown that Mitogen-activated Protein Kinases (MPKs) and Heat Shock Proteins (HSPs) are involved in the inhibition of germination whereas expansins are implicated in the induction of germination. Such genes should deserve more attention in future studies. The

study of 5'UTR features revealed that GC content and number of uORF could play a role in selective translation occurring during germination. Over-represented motifs in 5'UTR of polysomal transcripts in dormant and non-dormant seeds were also characterized. It might be interesting to prove the role of these 5'UTR motifs with transgenic reporter lines with altered motifs. In addition, since the most regulated step of translation initiation is the recruitment of mRNA by the translation machinery, we have used translational factors mutants to characterize this step of translation in seeds (results provided in annex 1). Our preliminary results show that seeds of translation factors mutants have strong dormancy phenotypes (annex 1), thus suggesting a role of these factors in the regulation of dormant and non-dormant seeds after different points of imbibition. We also aim to determine if the absence of different initiation factors has a discriminatory activity in mRNA translation during germination by comparing translatome profiles between mutants and Col.

Using different mutants of mRNA fate, we have clearly shown the role of mRNA decay in Arabidopsis seed germination. Our results demonstrate that xrn4-5 seeds are more dormant than Col seeds whereas vcs-8 seeds are less dormant. By analyzing the abundance of transcripts related to ABA and GA metabolism, we have found a relationship between hormonal metabolism and mRNA decay. Using a transgenic line and western blot analysis, we have shown that P-bodies or some components of P-bodies are more represented during imbibition of non-dormant seeds. To characterize the role of P-bodies in the regulation of dormancy, transcriptomics analysis was performed in xrn4-5 and vcs-8 mutants. In this study, attention was paid to the transcripts specifically more abundant in each mutant, representing 179 transcripts in xrn4-5 and 1518 in vcs-8. Gene Ontology (GO) clustering showed that the functions of transcripts differed between xrn4-5 and vcs8 mutants and allowed to reveal actors of seed dormancy or germination. The study of 5'UTR of degraded transcripts revealed that specific hexamer motifs,  $\Box G$  and GC content could play a role in their selective degradation by XRN4 and VCS. We also demonstrated that the stability of some transcripts related to dormancy depended on XRN4 and VCS activities. Then, it might be important to study the presence of the corresponding proteins by western blot in dormant and non-dormant seeds. Study of XRN4 and VCS mutants nevertheless only brings a partial view of the role of mRNA degradation in seed germination. Indeed this process is very complex and involves the recruitment of multiple actors. For example, this work did not assessed the role of the 3' to 5' degradation by the exosome in seed germination. Similar transcriptomics approaches could therefore be interesting to determine the targets of HEN2, a component of the exosome, since hen2 mutants produced deeply dormant seeds.

In conclusion, this study clearly emphasizes the key fundamental role of post-transcriptional modifications that includes mRNA decay pathway and translation in the regulation of dormancy. Figure 12 summarizes the major finding of this study and provides a model showing that germination regulation results from both a timely and selective recruitment mRNA by polysomes and a selective mRNA decay. Synchronized activation of the 2 pathways (mRNA translation and mRNA decay) leads to guide cell functioning towards radicle protrusion or dormancy maintenance. This work thus allows identifying molecular actors regulated by these mechanisms and brings novel insights in the understanding of the complex process of germination.



Figure 12. Model showing the role of post-transcriptional modification in the regulation of dormancy.

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### ANNEXES

## Annex 1. Addition analyses on transcripts identified in transcriptome and translatome (complement to chapter III)

I. Analysis of secondary structure of transcripts identified as differentially abundant in translatome between dormant an non-dormant seeds

Thermodynamically stable RNA secondary structures (evaluated by  $\Delta G$ ) in 5'UTR have an effect of initiation of translation, when  $\Delta G$  decreased the translation is inhibited (Kozak, 1989). The minimal energy for secondary structures was calculated using RNA fold (http://mfold.rna.albany.edu/). Folding free energies were computed for transcripts more abundant in translatome of dormant or non-dormant seeds at 16 h and 24 h of imbibition (Figure 1). This comparison revealed that mRNA with weak potential secondary structure in the 5'UTR ( $\Delta G$  >-10 kcal.mol<sup>-1</sup>) had an advantage to be translated while mRNA with higher secondary structure ( $\Delta G$  <-50 Kcal.mol<sup>-1</sup>) were less addressed to polysomes for all pools (Figure 1). The polysomal ratio was higher in non-dormant seeds than in dormant seeds ones as shown in the main document (see chapter III) but we cannot reveal another differential effect according to dormancy.



**Figure 1.** Relationship between  $\Delta G$  of 5'UTR and polysomal ratio (P/T) of transcripts more abundant in translatome of dormant (D; n=2582 at 16 h and 2581 at 24 h of imbibition) and non-dormant seeds (ND; n=1629 at 16h and 1601 at 24h of imbibition). mRNA were grouped based on the predicted  $\Delta G$  with 10 Kcal/mol.

### II. Identification of sequence motifs in promoters of genes identified in transcriptome and translatome

We searched specific promoters among the transcripts either up-regulated in transcriptome and translatome in dormant seeds or in non-dormant seeds. Among the transcripts being more abundant in translatome and in transcriptome of dormant seeds after 16 and 24 h of imbibition (317 and 175 transcripts, respectively), we identified several motifs related to ABA signaling or stress response, such as MYC Related Elements (MREs), MYB family, ABA-responsive element (ABRE), Crepeat-Binding Factor (CBF2), Homeobox-Leucine Zipper (ATHB6), Dehydration Responsive Element-Binding factor 1 (DREB1) and Low Temperature Responsive Element (LTRE) (Table 1). Several genes known to be regulated by these motifs and by ABA, such as GEAS6 (At2g40170; Gaubier et al., 1993), rab28 (AT1G03120; Pla et al., 1993) and RD29b (AT5G52300; Yamaguchi-Shinozaki and Shinozaki, 1994), were identified in our data. In addition, motifs such as CATACG, previously identified in promoters regions of JA/Senescence Element 2, involved in JA signaling (He and Gan, 2001), were also over-represented in dormant seeds and could be associated with the repression of germination (Table 1). The genes which were more abundant in both the translatome and transcriptome of nondormant seeds after 16 and 24 h of imbibition (699 and 415 transcripts, respectively) also displayed specific cis-elements in their promoter region (Table 2). The TGA binding site suggests a role for bZIP proteins in germination signaling (Schindler et al., 1992). Hormonal regulation of transcription was demonstrated by the presence of ethylene-(EIL1 and ERE) and gibberellin-responsive elements (GARE) (Table 2). The ACTTTT hexamer motif corresponds to several glutathione S-transferase genes (Chen et al., 1996), transcripts of which were over-represented. Interestingly we also identified the "evening element promoter" and a motif related to the circadian clock (Penfield and Hall, 2009) has been suggested to play a role in seed dormancy from our GO analysis.

**Table 1.** Common cis-elements identified in the promoters of up-regulated genes in transcriptome of dormant seeds after 16 and 24 h of imbibition at 25°C. Analysis was performed on a subset of 317 and 175 genes common in total and polysomal fractions in dormant seeds after 16 and 24 h of imbibition, respectively. The motif enrichment (ME) represents the factor of enrichment of the motif in the promoter (-1000 bp) of the genes analyzed in comparison with its occurrence in the Arabidopsis genome. E represents the E (expect)-value corresponding to the significance of the motif. nd, not determined.

Motif	Motif description	16 h of ir	16 h of imbibition		24 h of imbibition	
		ME	E	ME	E	
ACACGT	ACE	11.29	1.77E-17	10.93	1.03E-17	
CACATG	MYC	10.90	3.00E-04	8.02	2.01E-06	
AACAAT	MYB	9.54	1.12E-04	10.34	4.27E-07	
AACAGG	nd	8.20	5.39E-17	8.11	2.81E-06	
AGATGG	nd	7.47	6.38E-06	6.99	1.42E-07	
AACACG	nd	5.65	2.18E-06	5.61	1.07E-07	
CCACGT	CBF2	5.18	2.11E-05	4.83	1.51E-19	
AGAAGA	nd	4.87	2.41E-04	4.53	2.14E-06	
ACCGAC	DRE	3.81	4.64E-06	4.13	1.80E-06	
ACGTGG	ABRE	3.15	2.98E-05	4.09	2.37E-23	
AATTAT	ATHB6	3.08	1.60E-04	3.82	2.50E-06	
CCGACA	LTRE	3.01	1.50E-04	3.10	2.82E-07	
CATACG	JASE2	3.24	8.56E-06	2.92	5.37E-07	

**Table 2.** Common cis-elements identified in the promoters of up-regulated genes in transcriptome of non-dormant seeds after 16 and 24 h of imbibition. Analysis was performed on a subset of 699 and 415 genes common in total and polysomal fractions in non-dormant seeds after 16 and 24 h of imbibition, respectively. The motif enrichment (ME) represents the factor of enrichment of the motif in the promoter (-1000 bp) of the genes analyzed in comparison with its occurrence in the Arabidopsis genome. E represents the E (expect)-value corresponding to the significance of the motif. nd, not determined.

Motif	Motif description	16 h of imbibition		24 h of imbibition	
		ME	E	ME	E
TGACGT	TGA1	17.44	6.26E-07	10.26	4.50E-10
AGAGCC	ERE	11.26	2.25E-06	8.25	1.31E-04
ATCTCTC	nd	10.32	2.21E-04	8.18	1.14E-04
AAATAT	Evening Element promoter	9.36	8.50E-04	8.58	5.14E-06
ACTTTT	Glutathione S-transferase	9.12	5.31E-07	7.49	5.67E-06
	promoter				
AGAAAA	nd	8.85	1.38E-04	7.26	7.17E-08
AAAAC	nd	7.14	1.47E-08	6.11	1.17E-11
AAGGGG	EIL1 BS in ERF1	6.41	1.26E-05	5.10	2.21E-14
TAACAA	Gibberellin-responsive	5.16	2.15E-16	4.38	7.17E-20
	element (GARE)				
AGATAG	GATA	3.21	2.60E-24	3.04	4.23E-13

### Annex 2: Differential expression and regulation of translation initiation factors during germination

The recruitment of a ribosome to the initiating AUG codon of an mRNA is typically the rate-limiting step in polypeptide synthesis in eukaryotes, although polypeptide elongation can also be regulated (Dever *et al.*, 2002). Most mRNAs are translated via a mechanism that depends on the interaction of the 5'-7mGpppN-cap with the 3'-poly (A) tail. The initial step in translation is the assembly of a circular mRNA–protein complex. In plants, the 5'-7mGpppN-cap of the transcript is recognized by eukaryotic initiation factor 4E (eIF4E) or eIFiso4E, which are bound to their respective partners, eIF4G and eIFiso4G (Browning, 1996). A recent study have shown an important role of eIFiso4E and eIF4F in the regulation of maize (Dinkova *et al.*, 2014).

### I. Dormancy phenotyping of eiF mutants

To determine if eIF4E1 and eIFiso4E were implicated in the regulation of germination in *Arabidopsis thaliana* seeds, we have characterized the phenotype of *eif4e1* and *eifiso4e1* mutants with germination test 25°C in darkness (Figure 1). At harvest, in this condition 25% of Col seeds germinated (Figure 1A), while 45% of *eifiso4e1* seeds and only 7% of *eif4e1* seeds germinated (Figure 1A). To release dormancy, seeds were stored at 56% of relative humidity at 20°C as established in chapter II. After 2 weeks of storage, 51% of Col seeds, 85% of *eifiso4e1* seeds and 12% of *eif4e1* seeds germinated totally (Figure 1C). This kinetics of dormancy alleviation shows that *eifiso4e1* mutant was less dormant whereas *eif4e1* was more dormant than Col. These results suggest a role of eIF4E1 and eIFiso4E in the regulation of dormancy.

### II. Analysis of abundance of eIF4E1, eIFiso4e and eIF2α proteins

The abundance of eIF4E1, eIFiso4e and eIF2 $\alpha$  proteins was quantified in dormant and non-dormant seeds after 3, 6, 16 and 24 h of imbibition at 25°C by western blot. The quantity of eIFiso4E stayed constant during imbibition of dormant and non-dormant seeds (Figure 2A), although *eifiso4e* mutant was less dormant than Col. eIF4E1 was more abundant in non-dormant seeds than in dormant seeds (Figure 2B). This result is in agreement with the phenotype of *eif4e1* mutant which is more dormant than Col (Figure 2C). eIF4E1 could play a role in the release of dormancy. In contrast, eIF2 $\alpha$  protein was more abundant in dormant seeds (8 times more after 3h and 6h, and 50% more after 24h) than in non-dormant seeds (Figure 2C) suggesting an important role in the regulation of dormancy.



**Figure 1.** Germination of dormant Col, *eifiso4e* and *eif4e1* seeds during 8 days at 25°C. Germination was carried out after harvest (A), after 2 weeks of storage at 56% of relative humidity at 20°C (B) and after 5 weeks of storage at 56% of relative humidity at 20°C (C). Means ± SD of triplicate experiments are shown.



**Figure 2.** Quantification of elFiso4E (A), elF4E1 (B), and elF2 $\alpha$  (C) proteins by western blot. Proteins were extracted from dormant (D) and non dormant (ND) seeds after 3, 6, 16 and 24 h of imbibition on water at 25°C. The bands were quantified with Image J software. Antibodies anti elF4E1 and elFiso4 were given by Dr. Jean Luc Gallois (Institut National de la Recherche UR1052 Génétique et Amélioration des Fruits et des Légumes) and anti elF2 $\alpha$  by Dr. Karen Browning (Department of Chemistry and Biochemistry, University of Texas at Austin, TX).

# Annex 3. Additional results on role of mRNA decay in regulation of Arabidopsis thaliana seeds dormancy (complement to chapter IV)

### I- Germination of other mutants affected in mRNA metabolism

We have characterized other mutants than the ones presented in the chapter IV affected in the mRNA metabolism (Table 1). All these mutants were kindly given by Dr Hervé Vaucheret (Institut Jean-Pierre Bourgin, INRA, Versaille). At harvest, Col and mutant seeds can totally germinate after 4 days of imbibition at 15°C (Figure 1A) while at 25°C, only 25% of Col, *xrn3-3* and *rrp6l3* seeds and 60% of *pab2pab4* seeds were able to germinate within 8 days (Figure 1B). This result shows that *pab2pab4* double mutant was less dormant than Col and suggest that poly A binding proteins could be implicated in the regulation of dormancy. **Table 1.** Characterization of mutants affected in the metabolism of mRNA in Arabidopsis

Function	Genes	Cellular compartment	Mutants	Reference	
Exosome	RRP6	cytoplasmic	rrp6-l3	lange et al., 2011	
P-bodies	XRN3	nuclear	xrn3-3	Gy et al., 2007	
Stress Granules	PAB2	cytoplasmic	pab2pab4	Dufresne et al., 2008	
	PAB4		<u> </u>		



**Figure 1.** Germination of dormant Col, *xrn3-3, rrp6l3* and *pab2pab4* seeds after 8 days. Germination was carried out at 15°C (A) and 25°C (B). Means ± SD of triplicate experiments are shown.

### II- ROS effect on germination of RNA metabolism mutants

The possible involvement of ROS in germination of Arabidopsis seeds mutants affected in the metabolism of mRNA was assessed by using compounds that alter seed ROS content. Dormant seeds were treated with menadione (MN, 1 mM during 16 h) or H<sub>2</sub>O<sub>2</sub> (5 mM), two ROS-generating compounds known to be effective in releasing Arabidopsis seed dormancy (Leymarie *et al.*, 2012). MN and H<sub>2</sub>O<sub>2</sub> improved germination of Col at 25°C, which reached 85% (Figure 2A-B). For mutants, *pab2pab4*, *vcs-8*, *vcs-9*, *dcp1-3*, *xrn4-3*, *xrn3-3* and *mtr4-2* seeds germinated almost fully after 8 d at 25°C, whereas germination of *rrp6l3*, *mtr4-1* and *hen2-4* did not exceed 40% (Figure 2A-B). Germination of exosome mutants displayed marked phenotype of dormancy in response to ROS suggesting a role of MTR4, RRP6 and HEN2 in the response of ROS.



Figure 2. Germination of dormant seeds in presence of menadione for 16 h (A) and  $H_2O_2$  (B) at 25°C after 8 days. Means ± SD of triplicate experiments are shown.

#### III- Hypoxia effect on germination of RNA metabolism mutants

Dormancy is regulated by environmental factors such as oxygen. To test the effect of the oxygen in the germination of mutants affected in the metabolism of mRNA, seeds were incubated seeds at 15°C under different concentrations of oxygen with a system described by Côme and Tissaoui (1968) where gas mixtures containing 5, 10 and 21% oxygen were obtained through capillary tubes connected to sources of compressed air and nitrogen. With 5% of oxygen, 40% of Col can germinate as all mutants except *pab2pab4* seeds that germinated with a 80% rate (Figure 3). At 10 and 21% oxygen, all the mutants like Col totally germinate (Figure 3). This suggests that the difference in sensitivity to oxygen is related to dormancy but not to the perception of oxygen (Bradford *et al.*, 2008).



**Figure 3.** Effect of oxygen tension on the germination percentages obtained after 5 days at 15°C in dark of dormant seeds. Means ± SD of triplicate experiments are shown.

#### Annex 4. Expression of genes involved in mRNA metabolism by RT-qPCR

We have studied the relative expression of transcripts involved in the metabolism of mRNA such as VCS, DCP1, XRN4, XRN3, MTR4, RRP6 and PAB2. mRNA was extracted form seeds of mutants and Col imbibed 3, 6, 16 and 24 h at 25°C. Gene expression was evaluated in seeds, using real-time quantitative reverse transcription-PCR (RT–PCR). An arbitrary value of 100 was assigned to the seeds imbibed for 3h for each genotype, which were used as control sample for normalization. The level of expression the transcripts was not different between dormant and non-dormant seeds after 3, 6, 16 and 24 h of imbibition (Figure 4). These result indicates that transcript abundance of mRNP components do not show major changes during germination or in relation to dormancy level. This suggests a post-transcriptional regulation of mRNA metabolism in seeds.



**Figure 4.** Relative expression of *VCS*, *DCP1*, *XRN4*, *XRN3*, *MTR4*, *RRP6* and *PAB2* transcripts. RNA was extracted from the dormant (D) and non-dormant (ND) seeds of mutants after 3, 6, 16 and 24 h of imbibition on water at 25°C and relative expression was evaluated by qRT-PCR. Means ± SD of triplicate experiments are shown. \* indicates difference between dormant and non-dormant seeds (Anova test and Newman- keuls tests, P=0.05).