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Sorbonne Université
Université Saint-Joseph de Beyrouth

ED 515 - Complexité du vivant
ED - Sciences Ingénierie et Technologie (EDSIT)

**Rôle de la signalisation oxydative et du métabolisme des ARNm
dans la réponse des semences d'*Arabidopsis thaliana* à l'éthylène**

Par **Rana JURDAK**

Thèse de doctorat de Biologie
Spécialité Physiologie Végétale

Dirigée par Pr. Christophe Bailly et Pr. Magda Bou Dagher Kharrat

Présentée et soutenue publiquement le 28/01/2021

Devant un jury composé de :

M. Christophe Bailly	Professeur, Sorbonne Université	Directeur de thèse
Mme Magda Kharrat	Professeur, Université Saint-Joseph de Beyrouth	Co-directrice de thèse
M. Martin Crespi	Docteur, Université Paris-Saclay	Rapporteur
Mme Gwyneth Ingram	Docteur, Ecole Normale Supérieure de Lyon	Rapporteur
Mme Mireille Kallasy Awad	Professeur, Université Saint-Joseph de Beyrouth	Examineur
Mme Isabelle Petropoulos	Professeur, Sorbonne Université	Examineur

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Résumé

Substantiel

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Ce manuscrit est constitué de 4 parties. La première partie est une synthèse bibliographique sur la dormance des semences et ses mécanismes régulateurs. Les 3 parties suivantes sont présentées sous la forme d'articles et concernent (1) le rôle de la signalisation rétrograde mitochondriale des semences dans leur réponse à l'éthylène, (2) l'étude de traitements de levée de dormance sur le métabolisme des espèces réactives de l'oxygène (ROS, reactive oxygen species) et (3) l'effet de l'éthylène sur le métabolisme des ARNm.

La graine, qui résulte de la fécondation, est l'organe de dissémination des Angiospermes et elle assure la pérennité et la dispersion des espèces. Après dispersion, si les conditions de l'environnement sont favorables, la graine va germer et donner un nouvel individu. La germination est dans une étape clé dans la dynamique de population de espèces végétales et sa 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). 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 celui-ci un blocage physiologique transitoire de la germination appelé dormance. La dormance est un mécanisme endogène qui empêche la graine de germer dans des conditions apparemment favorables (Bewley & Black, 1994). La dormance 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 (10-15°C), les graines sont toutes capables de germer. La levée de dormance se produit naturellement au cours de la conservation au sec (after-ripening) des graines après leur maturité ou par stratification, ce qui correspond

à un traitement à basse température des graines dormantes à l'état imbibé (Bewley, 1997; Probert, 2000).

La dormance est essentiellement sous le contrôle de deux 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). Toutefois les autres hormones végétales jouent également un rôle dans la régulation de ce phénomène (Shu *et al.*, 2016). L'éthylène, en particulier, a un rôle bénéfique sur la germination et la levée de dormance des semences de nombreuses espèces dont *Arabidopsis* (Corbineau *et al.*, 1990, 2014). Les mécanismes impliqués dans l'effet de l'éthylène sur la germination demeurent mal compris. Il a néanmoins été montré que celui-ci inhibait la biosynthèse et la signalisation de l'ABA alors qu'il agissait en synergie avec la signalisation des GAs (Bleecker *et al.*, 1988; Beaudoin *et al.*, 2000; Calvo *et al.*, 2004). Ces événements sont toutefois tardifs et l'on ne connaît pas la voie de transduction du signal éthylène qui permet d'agir sur la balance hormonale ABA/GA. Il a récemment été démontré que l'effet de l'éthylène sur la levée de dormance impliquait la voie N-end rule, spécifiquement impliquée dans l'adressage de protéines au protéasome (Wang *et al.*, 2018). Les hormones à elles seules n'expliquent pas le mécanisme de régulation de la dormance. Les ROS en particulier semblent également jouer un rôle clé dans ce phénomène. Il a en effet été démontré que leur homéostasie avait un impact direct sur le contrôle de la germination, en particulier par la dormance (Bailly, 2019). Lorsque la teneur des tissus séminaux en ROS est comprise dans ce qui a été défini comme la fenêtre oxydative alors divers processus cellulaires redox-sensibles, non encore formellement identifiés, sont activés ce qui conduit à orienter le fonctionnement cellulaire vers la germination. Par exemple il a été démontré que dans ce contexte les ROS pouvait oxyder spécifiquement des pools de protéines régulatrices et moduler les voies de signalisation de l'ABA, des GAs ou de l'éthylène. Ainsi il a été démontré que les ROS et l'éthylène agissaient en synergie pour lever la dormance des semences de tournesol (El-Maarouf-Bouteau *et al.*, 2014), notamment en inhibant l'action de l'ABA.

De nombreux travaux ont tenté de décrypter les mécanismes moléculaires de régulation de la germination. Ils se sont particulièrement développés avec l'essor des technologies à haut débit et ont amené à proposer des réseaux de co-expression de gènes qui intègrent les variations d'expression conduisant à la réalisation du processus de germination (Bassel *et al.*, 2011; Verdier *et al.*, 2013). En association avec des approches génétiques (recherche de QTLs notamment), ils ont permis d'identifier des acteurs clés dans

le contrôle de la dormance et de la germination. Il s'agit par exemple de *DOG1* (*Delay of Germination 1*, (Carrillo-Barral *et al.*, 2020, p. 1) qui code pour une protéine de fonction inconnue et qui maintient la dormance. Le même rôle a été attribué à divers acteurs de la signalisation de l'ABA comme *PYL*, *PP2C* ou *ABI5* (Kendall *et al.*, 2011, p. 1; Zhao *et al.*, 2016; Nishimura *et al.*, 2018, p. 1). Toutefois il s'avère que la régulation de la germination par la dormance n'est pas seulement transcriptionnelle et des travaux plus récents ont mis en avant des composantes épigénétiques et post-transcriptionnelles dans celle-ci. Ces études suggèrent notamment que le remodelage de la chromatine par ubiquitination, méthylation et acétylation des histones pourrait jouer un rôle important dans la régulation de la dormance des graines (Nonogaki, 2014, 2017). Les miRNA et les lncRNA pourraient également participer à cette régulation épigénétique de la germination. L'expression de *DOG1* est par exemple directement régulée par un ARN antisens (Fedak *et al.*, 2016). Des études comme celles de Bazin *et al.* (2011), Layat *et al.* (2014) Basbouss-Serhal *et al.* (2015) ou Galland *et al.* (2014) et Bai *et al.* (2017) ont montré que la régulation de la dormance n'était pas seulement associée à la transcription mais également à l'activité traductionnelle. Il a ainsi été montré que l'adressage d'un pool spécifique de transcrits aux polysomes était indispensable à la fois à la germination et au maintien de la dormance. Il semble également que la dégradation spécifique des transcrits au cours de l'imbibition des graines joue un rôle déterminant dans la germination (Basbouss-Serhal *et al.*, 2017).

Le premier chapitre de résultats ce manuscrit est consacré à l'étude de l'effet de l'éthylène sur le métabolisme des ROS lors de la levée de dormance. Comme attendu, une faible teneur en éthylène dans l'atmosphère (100 ppm) permet la germination de graines d'*Arabidopsis* dormantes à 25°C et à l'obscurité, alors que celles-ci sont pratiquement incapables de germer dans ces conditions en l'absence d'éthylène. Cet effet bénéfique de l'éthylène est associé à une surproduction de ROS dans les semences. Toutefois l'utilisation d'inhibiteurs de la chaîne respiratoire mitochondriale inhibe l'effet de l'éthylène et la production de ROS. Le rôle des mitochondries dans la production de ROS en réponse à l'éthylène a été montré en utilisant des graines ro-GFP mitochondriale. Cette construction permet de calculer l'état redox des mitochondries en se basant sur les propriétés redox des ro-GFP. Nous démontrons que l'application d'éthylène induit une transition vers un contexte plus oxydant au niveau des mitochondries. Des observations complémentaires réalisées en microscopie confocale confirment que les mitochondries sont bien le siège principal de la production de ROS lors de l'imbibition des semences, quelle soient dormantes ou en cours de germination. En présence d'éthylène toutefois la production de

ROS devient également nucléaire après 16 h et 24 h d'imbibition, ce qui n'est pas le cas pour les graines dormantes. De telles observations mettent en évidence l'existence d'une signalisation rétrograde entre la mitochondrie et le noyau en présence d'éthylène, ce qui est confirmé par l'étude de composant de cette voie de signalisation. Ainsi l'éthylène active l'expression des gènes *AOX1a* et *ANAC013*, et les graines des mutants *aox1a* sont moins sensibles à l'éthylène. De plus une analyse du transcriptome par RNA-seq montre que l'éthylène induit une signature transcriptionnelle spécifique des ROS et de la signalisation rétrograde mitochondriale. Ces données permettent pour la première fois de clairement identifier la dynamique temporelle et spatiale de la production de ROS lors de la germination. Elles montrent clairement que la mitochondrie est impliquée dans la perception du signal éthylène et que celle-ci est capable de moduler l'amplitude de la production de ROS sous l'effet d'un signal promoteur de germination. Nous mettons également en évidence l'existence d'une signalisation rétrograde mitochondrie-noyau dans le contexte de germination.

Le chapitre suivant porte sur l'étude de la production de ROS en réponse à divers traitements de levée de dormance. En effet, après avoir mis en évidence l'existence d'une signalisation rétrograde mitochondriale en réponse à l'éthylène, nous avons tenté de vérifier si ce mécanisme était ubiquitaire et commun à tous les systèmes de levée de dormance. Nous avons donc étudié la dynamique de production de ROS dans des graines dormantes traitées avec de l'acide gibbérellique, stratifiées à 4°C pendant 4 jours ou mises à germer en présence de lumière continue. Ces trois traitements stimulent fortement la germination des semences à 25°C qui atteint ainsi 100 % après 2 à 3 j, en inhibant la signalisation et le métabolisme de l'ABA et en ayant l'effet inverse vis à vis des GAs. Ces 3 traitements stimulent également fortement la production de ROS au cours de l'imbibition des semences, mais les inhibiteurs respiratoires ne diminuent pas cette production et ne réduisent pas leurs effets sur la germination. Il semble donc que les mitochondries soient moins impliquées dans la production de ROS en réponse à ces 3 traitements qu'en présence d'éthylène, ce qui est confirmé par l'utilisation des constructions ro-GFP mitochondriales qui ont permis de calculer les changements de l'état redox dans ces organites. En revanche, pour chacun des traitements, nous observons une accumulation de ROS nucléaires avant la sortie de la radicule, ce qui suggère que cette production de ROS dans le noyau est nécessaire à la germination. En utilisant des Grx1-GFP nous avons identifié qu'une des sources potentielles de ROS lors de l'imbibition était probablement les peroxysomes. L'étude de l'expression de gènes marqueurs de cet organite, notamment

celui codant pour la Glycolate Oxidase (*GOX2*) confirme cette hypothèse. Cette étude démontre donc l'unicité et la spécificité des mécanismes de levée de dormance par l'éthylène, qui diffèrent de ceux mis en œuvre lors de la levée de dormance par la lumière, la stratification ou l'application de GAs.

La dernière partie de ce travail a été consacrée à l'étude des mécanismes moléculaires de l'action de l'éthylène et les principaux résultats obtenus sont présentés dans le dernier chapitre. Grâce à des approches d'analyse transcriptomique par RNA-seq nous avons étudié l'effet de l'éthylène sur l'expression des gènes (le transcriptome), l'association des ARNm aux polysomes (le traductome) et la stabilité et la dégradation des ARNm. L'éthylène induit des changements majeurs d'expression de gènes et il a également un très fort impact sur la dynamique d'association des transcrits aux polysomes. Chez les graines traitées par l'éthylène il existe une certaine corrélation entre la surexpression d'un transcrit pendant l'imbibition et son adressage aux polysomes, ce qui n'est pas le cas chez les semences non-dormantes, où l'association aux polysomes se fait essentiellement à partir des ARNm stockés. Les bases moléculaires de cette sélectivité de l'association aux polysomes ont été étudiées, notamment en analysant la séquence en 5' UTR de ces transcrits. Nous avons identifié l'existence d'une séquence consensus riche en GA dans la région 5'UTR des transcrits associés aux polysomes en présence d'éthylène. De plus nous montrons que la dégradation spécifique de certains ARNm participe également à la réponse à l'éthylène. Cette dégradation ne semble pas être liée à un effet global de l'éthylène sur la stabilité des transcrits mais elle mobilise le système de dégradation de 5' en 3'. Ainsi des approches d'imagerie utilisant une construction DCP1-YFP montrent que l'éthylène induit la formation de foci attribuables à des P-bodies dans le cytoplasme des cellules de semences traitées avec l'éthylène. L'analyse de l'ensemble de ces données a permis d'identifier des acteurs clés de l'action de l'éthylène au niveau moléculaire. Comme attendu l'éthylène a un effet antagoniste marqué sur la biosynthèse et la signalisation de l'ABA, en combinant une action sur l'adressage des transcrits aux polysomes et leur dégradation. Divers autres régulateurs de la germination ont été identifiés et sont discutés dans ce chapitre.

L'ensemble des résultats obtenus dans le cadre de ce travail permet de mieux comprendre quels sont les mécanismes impliqués dans la transduction du signal éthylène chez les semences et comment celui-ci peut orienter le fonctionnement du génome vers la germination. Dans le dernier chapitre, ces données sont mises en perspective avec des

évolutions possibles de cette thématique de recherche afin d'ouvrir des pistes d'intérêt pour de futurs travaux.

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Introduction

Seeds represent vital components of life on earth for they are used as the foundation of agriculture to sustain the ever-growing demand for food. In fact, from the beginning of crop husbandry to our days, seeds have been the key elements in the establishment, diversification, development, and amelioration of yield production (Bewley, 1997). Seed quality is a determinant factor required to ensure the success of crop production. One of the main characteristics of seed quality resides in the ability of seeds to complete their germination (Sabry, 2018). Seeds represent a system for dispersion and distribution of genetic diversity in the plant kingdom (Finch-Savage & Leubner-Metzger, 2006). The embryo contains all the genetic information indispensable for growth; surrounded in some seeds by the endosperm whose function is to nourish the germinating embryo. The embryo and the cotyledons are protected against adverse environmental conditions by the seed coat (Boesewinkel & Bouman, 1984). The enormous diversity in seed structure is, to a certain extent, related to germination strategies. By definition, germination begins with water uptake by dry seeds and terminates with radical protrusion (Bewley, 1997). This pivotal process is essentially under genetic control without neglecting the substantial influence of the surrounding environment (Finch-Savage & Leubner-Metzger, 2006).

Since the main function of the seed is to ensure the perpetuation of a species by establishing a new plant, it seems peculiar that an opposing phenomenon to germination such as dormancy exists. Seed dormancy is a widely spread adaptive trait required to avoid germination in unsuitable environmental conditions. Nevertheless, seed dormancy becomes penalizing when an intact viable seed is unable to complete its germination under favorable conditions (Wareing, 1971; Bewley & Black, 1994; Bewley, 1997). Considerable effort has been put to decipher the mechanisms controlling the transition of a seed to a germinating state in the model plant *Arabidopsis thaliana*. The regulation of dormancy is mostly explained by the hormonal balance between abscisic acid (ABA) and gibberellins (GA). While ABA is known to enhance dormancy, GA, on the other hand, promotes seed germination (Finkelstein *et al.*, 2008; Shu *et al.*, 2016). The plant hormone "ethylene" can modify the crosstalk between ABA and GA and enable the completion of germination (Corbineau *et al.*, 2014). These hormonal mechanisms are also integrated into a more complex network of cell signaling, notably involving the homeostasis of reactive oxygen species (ROS) (Corbineau *et al.*, 2014; Bailly, 2019). ROS may act as signal molecules translating in an extremely precise way the environmental conditions (temperature, light, oxygen) present at the time of imbibition of the seeds and orienting the functioning of the

genome towards the completion of germination or the maintenance of dormancy (Bailly, 2019). ROS production in seeds is strongly stimulated by ethylene and also by other positive regulators of germination such as light, GA treatment, and cold stratification (Andley & Clark, 1989; Allan *et al.*, 2006; El-Maarouf-Bouteau *et al.*, 2014; Li *et al.*, 2018a). However, the mechanisms responsible for the burst of ROS following the onset of imbibition in these conditions are still very poorly understood.

Germination is also driven by the ability of the embryo to resume its metabolic activity following the onset of imbibition. Recent “omic” approaches have elucidated the pivotal role of messenger RNA in the success of seed germination (Bazin *et al.*, 2011; El-Maarouf-Bouteau *et al.*, 2013; Layat *et al.*, 2014; Basbouss-Serhal *et al.*, 2015, 2017). Understanding the molecular events that permit the progress towards germination refers to determining the multi-level molecular events involved in this process including transcriptional, post-transcriptional, and translational regulation (Basbouss-Serhal, 2015 ; Galland & Rajjou, 2015; Bai *et al.*, 2017, 2020). Changes in mRNA abundance and dynamics including transcription, translation, and decay heavily and excessively control seed germination (Kawaguchi & Bailey-Serres, 2005; Basbouss-Serhal, 2015; Basbouss-Serhal *et al.*, 2017; Bai *et al.*, 2017, 2020). Even if the positive effect of ethylene on germination has been well documented, how this hormone controls the fate of mRNA to enable the shift towards a germinating state remains a mystery.

The objectives of the present thesis were first centered around identifying the origin of ROS production following *Arabidopsis thaliana* seed treatment with ethylene. The work essentially focused on establishing the ROS signaling cascade involved in seed germination in response to this hormone. Secondly, this ROS signaling cascade was checked for similarities and differences following seed exposure to germination precursors such as light, GA, and stratification in an attempt to determine the specificity of the response to ethylene and identify the source of ROS production for the remaining treatments. Lastly, the multi-level molecular regulation of seed germination in response to ethylene was closely monitored to determine the influence of this hormone on mRNA metabolism following the onset of imbibition.

The first chapter summarizes the current knowledge on the subject in the form of a bibliographic review. The second chapter tackles the origin of ROS production in the presence of ethylene in *Arabidopsis thaliana* seeds. The source of ROS production in seeds exposed to light, treated with GA or stratified is detailed in the third chapter. The fourth

chapter elucidates the effect of ethylene on the mRNA metabolism through the study of the transcriptome, translome, and degradome of *Arabidopsis thaliana* seeds. The final chapter includes the general discussion, a conclusion on the work done and a suggestion of future research perspectives.

Chapter 1

Literature Review

Current knowledge on seed germination is summarized in this literature review. As a first step, we will start by defining seed germination and dormancy. We will concentrate next on elucidating the most prevalent environmental factors that control germination as well as the involvement of the key hormones in this process. Known dormancy alleviation treatments are then explained followed by the role of Reactive Oxygen Species in the progress towards germination. A short comprehensive summary about the involvement of mitochondria in germination is then given prior to explaining the molecular control of seed germination while tackling, by order, transcription, key transcriptional regulators, translation, decay and the N6-Methyladenosine epigenetic modification of mRNA before ending with a summary on the general context of research on seed germination.

1. Seed germination

Mature and dry seeds are often referred to as quiescent. These seeds have low moisture content (5-15% FW) and their metabolic activity is highly reduced. These features enable seeds to survive, often for many years, until the onset of imbibition. Germination begins when dry, viable seeds imbibe water and ends with the emergence of the embryonic axis through the structures surrounding it. Germination is known to be a triphasic process according to water uptake and the resulting metabolic changes (Bewley *et al.*, 2013). Phase I begins soon after imbibition and is often associated with osmotic changes leading to leakage of solutes such as sugars and ions into the surrounding medium. During the second phase, the water uptake slows and reaches a plateau and the water potential becomes less negative. This phase is accompanied by the restoration of cellular integrity, mitochondrial repair, initiation of respiration, and DNA repair. Phases I and II do not enable the differentiation between germinating and non-germinating seeds. Phase III is exclusive for seed that complete their germination and is associated with an exponential increase in water absorption to sustain the needs of the growing embryo (Bewley & Black, 1994; Bewley, 1997; Finch-Savage & Leubner-Metzger, 2006; Nonogaki *et al.*, 2010; Bewley *et al.*, 2013).

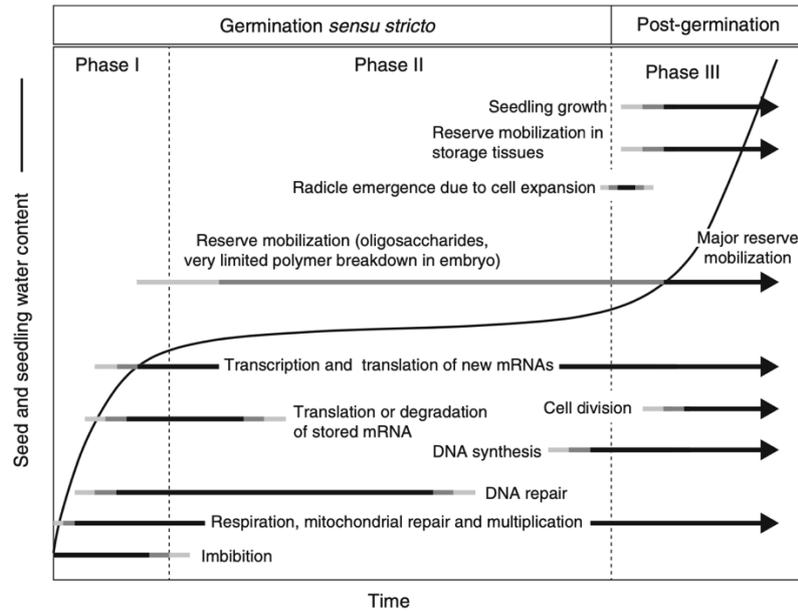


Figure 1-Time course summarizing water absorption and essential changes associated with germination and seedling development (Bewley *et al.* 2013).

2. Seed dormancy

Seed dormancy is defined by the inability of a viable and intact seed to complete germination under any combination of favorable environmental conditions (Wareing, 1971; Bewley, 1997; Baskin & Baskin, 2004; Finch-Savage & Leubner-Metzger, 2006; Nonogaki *et al.*, 2010). This inhibition of germination has differently evolved across species depending on the surrounding environment ensuring the occurrence of germination in optimal conditions (Hilhorst, 1995; Bewley, 1997; Baskin & Baskin, 2004; Finch-Savage & Leubner-Metzger, 2006). Thus, dormancy is highly dependent upon temporal and spatial environmental signals. Temporal environmental signals affect the sensitivity of the seeds while spatial environmental signals affect their germination completion. Several classification systems were proposed to group different dormant seeds based on different physiological, morphological, and anatomical mechanisms (Baskin & Baskin, 2004; Finch-Savage & Leubner-Metzger, 2006; Chahtane *et al.*, 2016; Footitt & Finch-Savage, 2017).

Primary dormancy is established at the end of seed development on the mother plant (Hilhorst, 1995; Bewley, 1997; Finch-Savage & Leubner-Metzger, 2006; Leprince *et al.*, 2016; Chahtane *et al.*, 2016; Buijs, 2020). The induction of primary dormancy is genetically determined through the expression of the *DELAY OF GERMINATION 1 (DOG1)* at the late stages of maturation (Nakabayashi *et al.*, 2012; Nonogaki, 2014) and its interaction with the

plant hormone ABA whose main function is to prevent precocious germination of embryos (Kermode, 1990, 2005).

Over time, primary dormant seeds may lose their dormancy status without being able to germinate due to their exposure to prolonged adverse conditions, resulting in their entrance into what is termed as secondary dormancy. This form of dormancy is not induced during seed maturation nor in dry seeds but rather in mature imbibed seeds. This dormancy cycling is essentially under the control of the immediate environmental conditions including temperature fluctuation, light and oxygen (when a seed becomes buried in the soil) rather than those experienced by the parent plant (Finch-Savage & Leubner-Metzger, 2006; Finch-Savage & Footitt, 2017; Buijs, 2020).

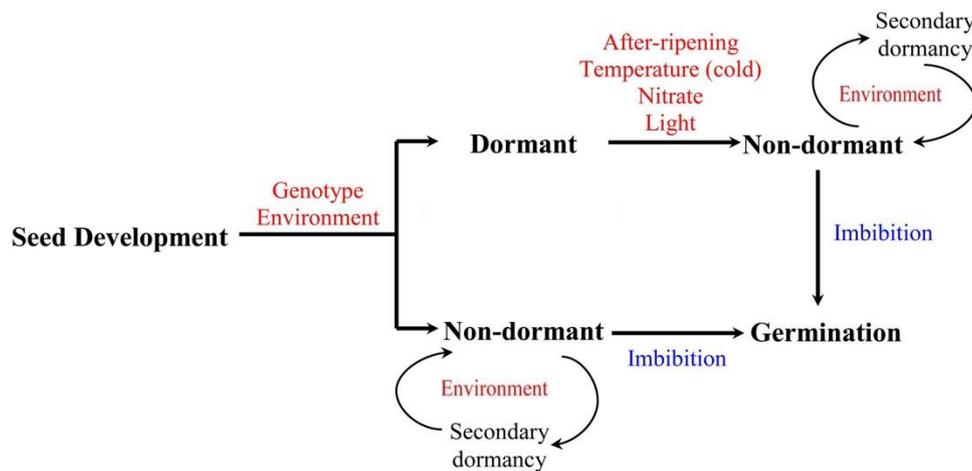


Figure 2-Scheme of seed dormancy and germination control and regulation in response to environmental conditions. Adapted from Gao & Ayele (2014).

3. Environmental control of germination

Germination is highly dependent on the ability of seeds to integrate various environmental signals (Bewley, 1997). The depth of seed dormancy at shedding is genetically dictated but is also greatly affected by the surroundings and the environment in which the mother plant is grown. Temperature, light, water supply, and oxygen represent the most important environmental factors that influence the germination potential of seeds. After being perceived by plants, these factors are often converted into internal cues that activate hormonal signaling cascades, which in turn regulate physiological processes in seeds (Footitt *et al.*, 2013; Footitt & Finch-Savage, 2017; Finch-Savage & Footitt, 2017; Yan & Chen, 2020a).

3.1 Temperature

Temperature plays a crucial role in determining seed behavior by influencing dormancy and germination timing (Finch-Savage & Leubner-Metzger, 2006; Chahtane *et al.*, 2016).

First of all, temperature has an important impact on the depth of dormancy at shedding. In some species, including *Arabidopsis thaliana*, exposure of the mother plant to low temperatures results in an increased dormancy rate whereas seeds that develop at warmer temperatures display reduced dormancy at harvest (Fenner, 1991; Footitt *et al.*, 2011; Burghardt *et al.*, 2016). This is partially mediated by ABA and GA metabolism. The exposure of developing seeds to low temperatures (15°C instead of 22°C) results in an increased ABA content and a decrease in GA levels (Kendall *et al.*, 2011; He *et al.*, 2014; Yan & Chen, 2020a). DOG1 also participates in enhancing seed dormancy by promoting GA catabolism during maturation at low temperatures (Nakabayashi *et al.*, 2012).

Secondly, temperature determines the germination potential within a population of seeds. Permissible germination temperatures are species-dependent and must range between the base temperature (T_b) (minimal temperature required for germination) and the maximal temperature (T_m) (temperature above which germination is inhibited). These cardinal temperatures define the thermal germination window which also includes the optimal temperature (temperature at which the germination rate is maximal) (Finch-Savage & Footitt, 2017). For *Arabidopsis thaliana*, base temperatures are set around 0 and 5°C while maximum temperatures are fixed around 30°C and the optimal germination temperature is around 15°C (Tamura *et al.*, 2006; Penfield & Springthorpe, 2012). Seed imbibition at supraoptimal temperatures often results in the suppression of germination, a phenomenon often referred to as thermoinhibition (Reynolds & Thompson, 1971). This phenomenon is important for the determination of appropriate seasonal timing for seed germination of winter annual plants (Baskin & Baskin, 2004).

Temperature is also a determinant factor in the induction of secondary dormancy. By perceiving and integrating temperature signals, seeds alter between deep and shallow dormancy until favorable conditions are found, a phenomenon described as dormancy cycling. When seeds encounter prolonged inadequate signals during dormancy cycling, they may enter secondary dormancy (Finch-Savage & Leubner-Metzger, 2006; Footitt *et al.*, 2013; Finch-Savage & Footitt, 2017; Yan & Chen, 2020a).

3.2 Light

Light is not only absorbed by plants to sustain the energy demanding process of producing organic compounds, but it also constitutes an important source of information for plants to reorient their response in order to adapt to the everchanging environment. For seeds, sensing the quality and intensity of light signals plays a fundamental role in determining the length of the day and position in soil. For instance, seeds buried deep in the soil are not able to sense the light and their germination is therefore prevented since their stored energetic reserves will run out before the seedling reaches the surface and initiate photosynthesis. Therefore, light represents an important environmental factor orienting seeds towards germination or maintenance of dormancy (Ballaré *et al.*, 1992; Mérai *et al.*, 2018; Yan & Chen, 2020a).

Light is perceived in plants by photoreceptors. *Arabidopsis thaliana* possesses different types of photoreceptors that include phytochromes, cryptochromes and phototropins (Møller *et al.*, 2002; Montgomery & Lagarias, 2002; Sullivan & Deng, 2003; Bae & Choi, 2008; Yan & Chen, 2020a). Germination in response to light occurs following the activation of phytochromes (Casal & Sánchez, 1998; Hennig *et al.*, 2002; Moller *et al.*, 2002).

Phytochromes have two photo-reversible conformations: the red light absorbing (Pr) form and the far-red light (Pfr) absorbing form which is the bioactive form (Figure3). Upon exposure to red light, the Pr form switches into the active Pfr form determining the ratio red light to far-red light. Five phytochromes (phyA to phyE) are encoded within the genome of *Arabidopsis*, of which phyB plays a predominant role in regulating the germination of seeds (Shinomura *et al.*, 1996; Tognacca *et al.*, 2019). Light irradiation causes an increase in the red to far-red ratio which triggers the proteasome-mediated degradation of PIL5 (PIF1), a basic helix-loop-helix (bHLH) transcription factor modulating GA and ABA levels, and the DELLA proteins RGA and GAI. This degradation of PIL5 increases GA concentration and decreases the transcription of the two DELLA genes, which represent nuclear repressors of plant gibberellin responses (Oh *et al.*, 2006, 2007; Seo *et al.*, 2006, 2009). Exposure of seeds to red light is also associated with the down-regulation ABA biosynthesis genes and up-regulation of ABA catabolic genes (Seo *et al.*, 2006).

The role of other regions of the spectrum remains unknown, however blue light might also stimulate the germination of *Arabidopsis thaliana* seeds by modulating the expression of genes related to light, GA, and ABA metabolism.

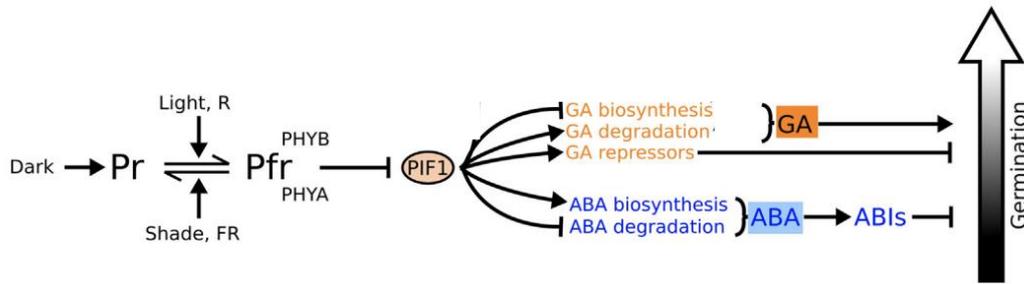


Figure 3- Schematic representation of the different events leading to light induced *Arabidopsis thaliana* seed germination. Adapted from Demotes-Mainard *et al.* (2015)

3.3 Water

Water is also an environmental factor that influences seed germination since by definition the progress towards germination begins with water uptake (Bewley & Black, 1994; Bewley, 1997; Finch-Savage & Leubner-Metzger, 2006; Nonogaki *et al.*, 2010; Bewley *et al.*, 2013). This initiating step in the germination of a seed is accompanied by the resumption of metabolism and the induction of cellular events leading to radicle protrusion. Therefore, the triphasic imbibition process already mentioned (section 1. Seed germination) will be detailed in the following paragraph. The duration of the different phases varies between seeds as it depends on certain properties such as size and seed coat permeability.

Moisture content of fully imbibed seeds can increase up to 75-100% (compared to 5-15% upon shedding) making up to 40-50% of their fresh weight. The movement of water from the soil to the seed depends on the water potential (Ψ), which represents the free energy status of the water, of both components (soil and the seed). Differences in Ψ between the soil and the seed determine the flow rate of water to the seed. Phase I begins when a dry seed comes in contact with water since Ψ is in favor for the movement of water into the seed. This process is not only accompanied by a leakage of solutes into the surrounding medium but also by the reactivation of cellular respiration (Bewley *et al.*, 2013). Indeed, the reactivation of cellular growth is energy dependent and requires the reactivation of primary metabolism (Stawska & Oracz, 2019). Promitochondria in dry seeds are structurally and functionally deficient, and lack internal structure. An extensive reactivation of mitochondrial dynamics occurs minutes after imbibition. These organelles rapidly regain membrane potential and resume their metabolic activity (Paszkiwicz *et al.*, 2017).

Phase II, also known as the lag phase, is characterized by the reduced rate of water uptake that reaches a plateau. This is mainly explained by the water potential that becomes less negative as cells become hydrated due to the exponential water uptake in phase I. The increase in seed volume can result in the rupture of the seed coat at this stage. Even if water uptake is minimal during phase II, important metabolic events occur. Several events that prepare for radicle emergence take place, such as the synthesis of mRNAs associated with germination, the repair of DNA damage that might happen between shedding and imbibition, and the incorporation of nucleotides into DNA prior to cell division. This phase is also characterized by the reformation of the cytoskeleton due to the reorganization of tubulins.

Phase III marks the initiation of embryo growth into a seedling indicating the completion of germination. Water uptake in this phase is a consequence to expanding plant cells.

3.4 Oxygen

As previously mentioned; germination highly relies on respiration, making oxygen availability a limiting factor in seed germination (Rosental *et al.*, 2014). Respiration in dry mature seeds is null compared to germinating seeds. Minutes after the onset of imbibition, respiration is resumed. This is associated with an increase in oxygen consumption which can be explained in part by the activation of mitochondrial enzymes involved in the cycle of Krebs or in the mitochondrial electron transport chain. Respiration tends to increase as more cells become hydrated after imbibition (Bewley *et al.*, 2013). Reduced oxygen levels may alter the proportion of seeds able to complete their germination and delay the mid germination time (time to reach 50% of germination). As the soil depth increases; O₂ availability gradually declines below the atmospheric concentration of 20.9% delaying germination speed and reducing the final germination rate (Yasin & Andreasen, 2016). Oxygen deprivation might also play an important role in the maintenance of physical dormancy alongside the inhibitory effects of the seed coat (Simon & Keith, 2008; McGill *et al.*, 2018).

4. Hormonal control of germination

Seed germination is under the control of several mechanisms required for the development of the embryo. In addition to the direct effect of the environment on the

establishment of a new seedling, there is a number of internal factors that control this process. Germination is apparently under the very strict regulation of plant hormones, mainly relying on the interplay between ABA, GA and ethylene (Finkelstein, 2010a; Davies, 2010; Corbineau *et al.*, 2014; Vishal & Kumar, 2018). Conclusion of studies that tackle the hormonal control of germination are based on the simultaneous occurrence of peak values in hormone levels and different physiological, morphological, and biochemical processes. The most important functions of plant hormones are centered around controlling and coordinating cell division, growth and differentiation (Hooley, 1994; Davies, 2010; Miransari & Smith, 2014).

4.1 Abscisic acid (ABA)

The involvement of abscisic acid in determining the fate of a seed starts during maturation and development, where a complex sequence of events occurs requiring multiple regulatory processes and control. Indeed, this hormone plays a crucial role in enhancing the synthesis of storage proteins and in the acquisition of desiccation tolerance during seed maturation. ABA also prevents precocious germination of seeds (vivipary) during seed development. In addition to its association with maturation, ABA also plays a key role in the induction and maintenance of seed dormancy. Like other hormones, ABA functions through a complex signaling network where cellular response is initiated by the perception of endogenous or exogenous ABA which triggers a signaling cascade to induce a final response. (Finkelstein *et al.*, 2002; Finch-Savage & Leubner-Metzger, 2006; Holdsworth *et al.*, 2008; Fang & Chu, 2008; Rodríguez-Gacio *et al.*, 2009; Finkelstein, 2010a; Bewley & Nonogaki, 2017).

ABA synthesis begins in the plastid and ends in the cytosol. The cleavage of C₄₀ carotenoids via the methylerythritol phosphate (MEP) pathway leads to the formation of zeaxanthin, the first oxygenated carotenoid precursor of ABA (Figure 4). A series of enzyme-catalyzed epoxidations mediated by Zeaxanthin epoxidase (ZEP/ABA1) and *nine-cis*-epoxycarotenoid dioxygenase (NCED) leads to the formation of xanthoxin, the proximal ABA precursor. The dehydrogenation of xanthoxin gives rise to abscisic aldehyde which is then selectively oxidized by the abscisic aldehyde oxidase (AAO) to ABA (Marion-Poll & Leung, 2007; Sah *et al.*, 2016).

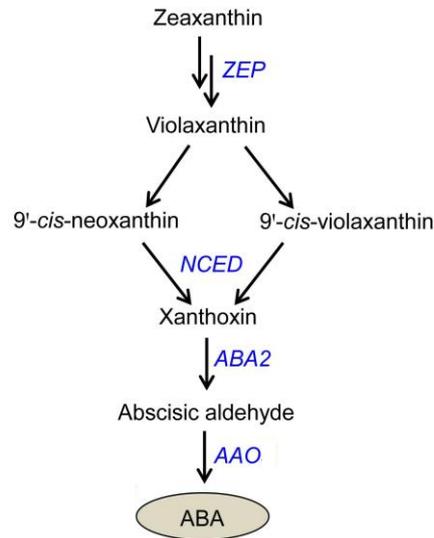


Figure 4- ABA synthesis pathway in plants (adapted from Tuan *et al.*, 2018).

ABA transport between cells plays a crucial role in the physiological response of plants. This weak acid can passively diffuse across membranes or through ABA transporters (ATP-binding cassette (ABC)-containing transporter proteins) (Kang *et al.*, 2010; Ng *et al.*, 2014). Three main components of ABA signaling have been identified in plants that include the pyrabactin resistance (PYR)/pyrabactin resistance-like (PYL) which are regulators of the ABA receptors (RCAR), the protein phosphatase 2C (PP2C) (negative regulator of ABA signaling) and the SNF1-related protein kinase 2 (SnRK2) (positive regulator of ABA signaling) (Figure 5). The activity of PP2C is inhibited in presence of ABA due to the formation of the PYR/PYL/RCAR-PP2C complex, allowing the activation of SnRK2 which phosphorylates downstream targets such as transcription factors leading to the transcription of ABA-responsive genes (Sah *et al.*, 2016).

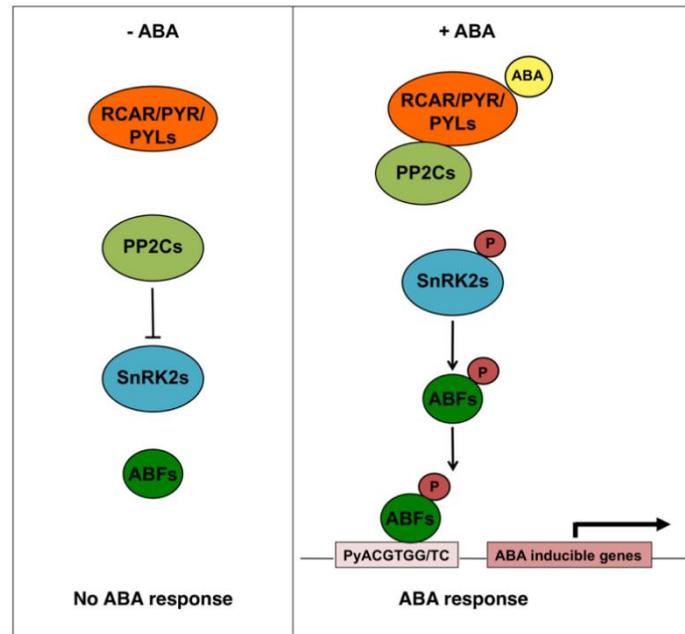


Figure 5- Schematic representation of the main components of the ABA signaling pathway. Normal arrows denote a positive, while T bars indicate a negative relationship (Fernando & Schroeder, 2016).

One APETALA2 (AP2)-type transcription factor, ABSCISIC ACID INSENSITIVE4 (ABI4), is widely described as a key player in the ABA-mediated inhibition of seed germination. In addition to favoring the ABA levels over GA during the crosstalk between these 2 hormones (elucidated in section 5. ABA and GA during seed germination), ABI4 suppresses the transcription of the *Arabidopsis* response regulators 6, 7, and 15 (negative regulators of the ABA-mediated inhibition of germination) (Finkelstein, 2010b; Shu *et al.*, 2016; Huang *et al.*, 2017).

ABA catabolism is accomplished by two pathways defined as hydroxylation and conjugation. In the first, ABA is hydroxylated via oxidation of three methyl groups (C-7', C-8' (dominant pathway), and C-9') by the Cytochrome P450 type enzyme (CYP707A) into phaseic acid (PA) and dihydro phaseic acid (DPA). In the latter, ABA and its hydroxylated catabolites are conjugated to glucose through the activity of glycosyltransferases (Nambara & Marion-Poll, 2005; Sah *et al.*, 2016).

4.2 Gibberellins (GAs)

Gibberellins are key regulators of different stages of plant development including germination, seedling growth, stem elongation, pollination and flower and fruit development. During germination, GA promotes embryo growth and facilitates the

(RGL1), RGL2, and RGL3. Following the binding of GA to the GA-INSENSITIVE DWARF1 (GID1) receptor, the degradation of DELLA proteins is induced through the SCF^{SLY1} or SCF^{SNE} (SCF complexes with the F-box protein subunit SLEEPY1 or SNEEZY) E3 ubiquitin ligases and the 26S proteasome (Sun, 2010; Schwechheimer, 2012; Hauvermale *et al.*, 2012; Colebrook *et al.*, 2014; Salazar-Cerezo *et al.*, 2018).

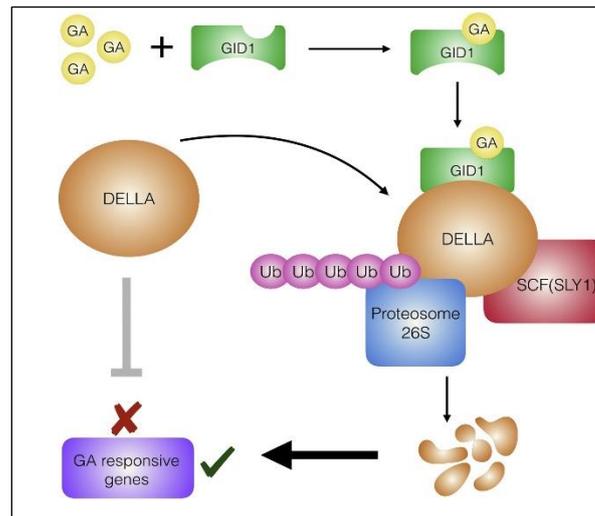


Figure 7- Model of GA signaling in plants. Normal arrows denote a positive, while T bars indicate a negative relationship (Salazar-Cerezo *et al.* 2018).

The 2 β -hydroxylation of bioactive GA, catalyzed by GA 2-oxidases (GA2ox) that belong to the 2-oxoglutarate-dependent dioxygenase family, results in the conversion of GA₄ and GA₁ to GA₃₄ and GA₈, respectively (Thomas *et al.*, 1999; Schomburg *et al.*, 2003; Sun, 2008). A novel inactivation mechanism, based on the methylation of GAs, has been recently identified in *Arabidopsis*. *GAMT1* and *GAMT2*, members of the SABATH methyltransferase gene family in the *Arabidopsis* genome, code for enzymes that utilize the S-adenosine-L-methionine (SAM) to methylate the carboxyl group of GAs leading to the formation of methyl esters of GAs (MeGAs) (Varbanova *et al.*, 2007; Sun, 2008).

5. ABA and GA crosstalk during germination

Plant hormones often coordinate to regulate growth and development rates during the life cycle of the plant by integrating internal and environmental signals. For instance, hormones can affect the levels or even the activity of other hormones by modulating their metabolism (activation or repression of key enzymes involved in pathways) or by altering their transport to specific tissues. In addition, hormones can also influence the sensitivity or

even the perception of other hormones through the modulation signaling components such as receptors or downstream targets, a process that defines nodes for crosstalk (Kuppusamy *et al.*, 2009; Gazzarrini & Tsai, 2015).

The progress towards germination is tightly controlled by multiple external and internal factors and is subject to hormonal crosstalk. This crosstalk essentially relies on the antagonistic interaction between ABA and GA (Figure 8). ABA maintains seed dormancy while repressing precocious germination by inhibiting GA metabolisms and *vice versa*. Nevertheless, the ratio of these two hormones, rather than the level of each hormone, plays a crucial role in regulating seed dormancy. This ABA/GA ratio is determined through the interaction of their signaling components, the involvement of other hormones and also by the environment (Yamaguchi, 2008; Holdsworth *et al.*, 2008; Nambara *et al.*, 2010).

The B3-domain transcription factor FUSCA3 (FUS3), an important regulator of seed maturation, represents a node of crosstalk between ABA and GA. This transcription factor promotes dormancy by increasing ABA levels and repressing GA biosynthesis. ABA positively regulates the activity of FUS3 through its phosphorylation by the SNF1-related protein kinase 1 (AKIN10) while the mechanisms of its deactivation by GA are yet to be understood (Tsai & Gazzarrini, 2012; Rodrigues *et al.*, 2013; Gazzarrini & Tsai, 2015). In addition, ABSCISIC ACID INSENSITIVE5 (ABI5), stabilized by ABA, is able to bind to the promoter of the GA 2-OXIDASE gene to reduce the accumulation of bioactive GA while negatively regulates the accumulation of ABI5 through repression of the DELLA protein Ral Guanine Nucleotide Dissociation Stimulator Like 2 (RGL2) (Piskurewicz *et al.*, 2008; Shu *et al.*, 2013; Cantoro *et al.*, 2013). Another hub between ABA and GA signaling is the RING-H2-type E3 ligase XERICO, a direct DELLA target and a positive regulator of ABA. In fact, XERICO promotes the accumulation of abscisic acid (ABA) by degrading a negative regulator of ABA biosynthesis or by affecting the activity of an ABA metabolic enzyme(s) (Zentella *et al.*, 2007).

Regarding the environmental control of this crosstalk, light-induced seed germination is also associated with the interplay between ABA and GA. Under far-red light, PIL5 maintains seed dormancy by repressing GA signaling through a direct transcriptional activation of DELLAs, which in turn induces ABA accumulation (Oh *et al.*, 2007; Piskurewicz *et al.*, 2009). High temperatures can also modulate the ABA/GA ratio in favor of ABA through the activity of FUS3. When seeds are exposed to high temperatures, FUS3 mRNA and protein synthesis is enhanced resulting in *de novo* ABA synthesis. Increased ABA/GA ratio at high

temperatures maintains an important FUS3 protein level in a positive feedback loop (Chiu *et al.*, 2012).

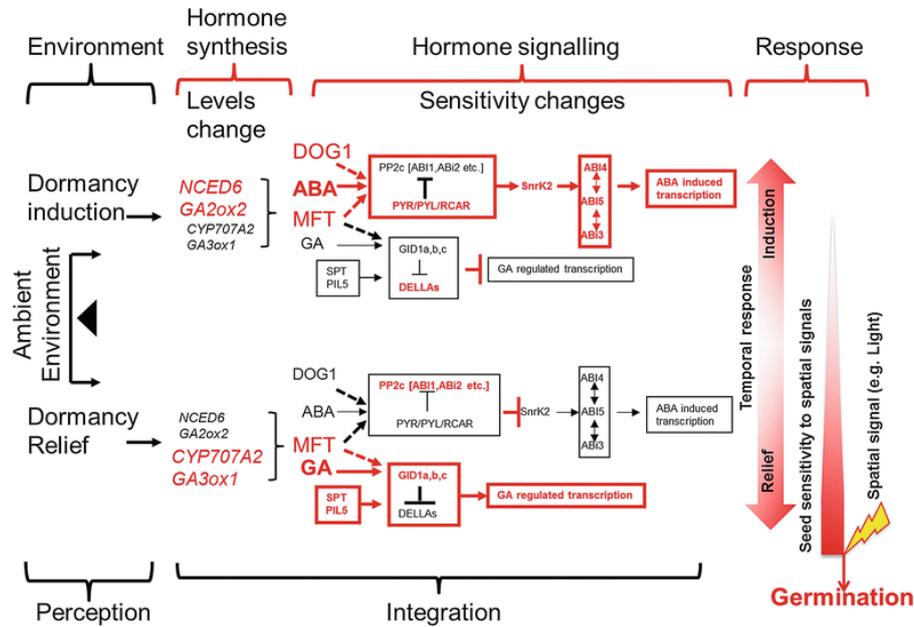


Figure 8- Model for the regulation of dormancy and germination by ABA and GA in response to the environment in *Arabidopsis*. The pathways indicated in red dominate when the induction or alleviation of dormancy are induced by environmental signals (Footit & Finch-Savage 2017).

6. Ethylene

Ethylene, an essential component of the gaseous environment, serves as a mediator to several environmental signal and plays a key role in the regulation of numerous developmental programs throughout the lifecycle of the plant. This hormone is actively involved in the regulation of seed germination, cell elongation, fertilization, fruit ripening, seed dispersal and defense against pathogens (Abeles *et al.*, 1992; Matilla, 2000; Wang *et al.*, 2002; Alonso & Stepanova, 2004; Corbineau *et al.*, 2014).

Ethylene, unlike other hormones, does not rely on the active transport in plant cells (due to its physicochemical properties which allow it to freely disuse through the cytoplasm and cellular membranes) making the tight regulation of its biosynthesis the only key point for plants to control ethylene levels (Bürstenbinder & Sauter, 2012). The pathway of ethylene biosynthesis starts with the production of S-adenosylmethionine (SAM) from methionine

(nearly 80% of cellular methionine), the precursor of endogenous ethylene formation, by the S-AdoMet synthetase (SAM synthetase) (Figure 9). SAM is then converted to 1-aminocyclopropane 1-carboxylic acid (ACC), which is the direct precursor of ethylene, by the ACC synthase. This conversion is considered to be the rate-limiting step of ethylene synthesis since active ACC synthase is present at low levels suggesting an important regulation of this process. 5'-methylthioadenosine (MTA), released as a byproduct during the conversion of SAM to ACC, is recycled into methionine by the intermediate of the Yang cycle due to the relatively low and stable abundance of methionine in plant cells. Nevertheless, the oxidation of ACC by the ACC oxidase results in ethylene production. (Yang & Hoffman, 1984; Wang *et al.*, 2002; Rzewuski & Sauter, 2008; Bürstenbinder & Sauter, 2012; Corbineau *et al.*, 2014; Wen, 2015; Booker & DeLong, 2015). The increase in ethylene production, documented in germinating seeds, is essentially explained by the increase in ACC oxidase activity (Iglesias-Fernández & Matilla, 2010; Linkies & Leubner-Metzger, 2012; Corbineau *et al.*, 2014).

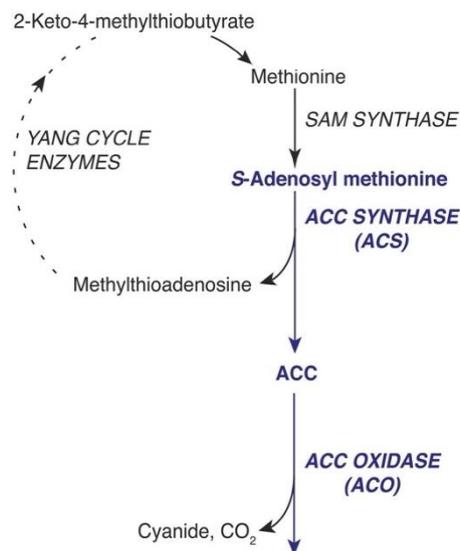


Figure 9- Schematic representation of ethylene biosynthesis in plants (adapted from Booker *et al.* 2015)

The mechanism that underlies ethylene perception and signal transduction to the nucleus, where the regulation of hundreds of genes occurs, has been elucidated (Figure 10) (Wang *et al.*, 2002; Stepanova & Alonso, 2005, 2009; Corbineau *et al.*, 2014; Wen, 2015). In *Arabidopsis*, ethylene is perceived by five receptors that could be divided into two groups depending on their structural characteristics (Sakai *et al.*, 1998; Hua *et al.*, 1998). The first group, consisting of ETR1 and ERS1, possesses a canonical histidine kinase in their N terminal end and three transmembrane domains that bind ethylene through coordination with copper (Rodriguez *et al.*, 1999; Hirayama *et al.*, 1999). The group II receptors, ETR2,

EIN4, and ERS2, have an additional hydrophobic domain in their N terminus and lack features of the canonical histidine kinases (Sakai *et al.*, 1998; Hua *et al.*, 1998). Group I receptors are considered to be more important for the response to ethylene rather than group II receptors (Qu *et al.*, 2007). These receptors localize in the endoplasmic reticulum where they interact with the copper transport protein CTR1, a negative regulator of the pathway. The binding of ethylene to its receptors results in the deactivation of CTR1, by inducing conformational changes in the receptors-CTR1 complex through histidine kinase activity in the receptors, which no longer exerts its inhibitory effect on EIN2 (Alonso, 1999; Alonso & Ecker, 2001; Hall *et al.*, 2012). ETHYLENE INSENSITIVE 2 protein is considered to be a central component in the ethylene signal transduction pathway since *ein2* mutants lose their sensitivity to ethylene (Alonso, 1999). The cleavage of the C-terminal end of EIN2 and its translocation to the nucleus activates downstream components of the pathway (Stepanova & Alonso, 2005). In the nucleus, components working downstream of EIN2 are the two transcription factors ETHYLENE INSENSITIVE 3 (EIN3) and its homolog EIN3-LIKE 1 (EIL1) which regulate the gene expression in response to ethylene (Chang *et al.*, 2013). EIN3-BINDING F-BOX 1 (EBF1) and EBF2 function in ethylene perception by regulating EIN3/EIL1 turnover. EIN2 acts to repress the proteasomal degradation of EIN3/EIL1 mediated by EBF1 and EBF2 (Binder *et al.*, 2007). In addition to its nuclear activity, EIN2 associates with the 3'UTRs of EBF1/2 mRNA in the cytoplasm and target them to processing-bodies (P-body) by interaction with multiple P-body factors such as the exoribonuclease EIN5 (Li *et al.*, 2015).

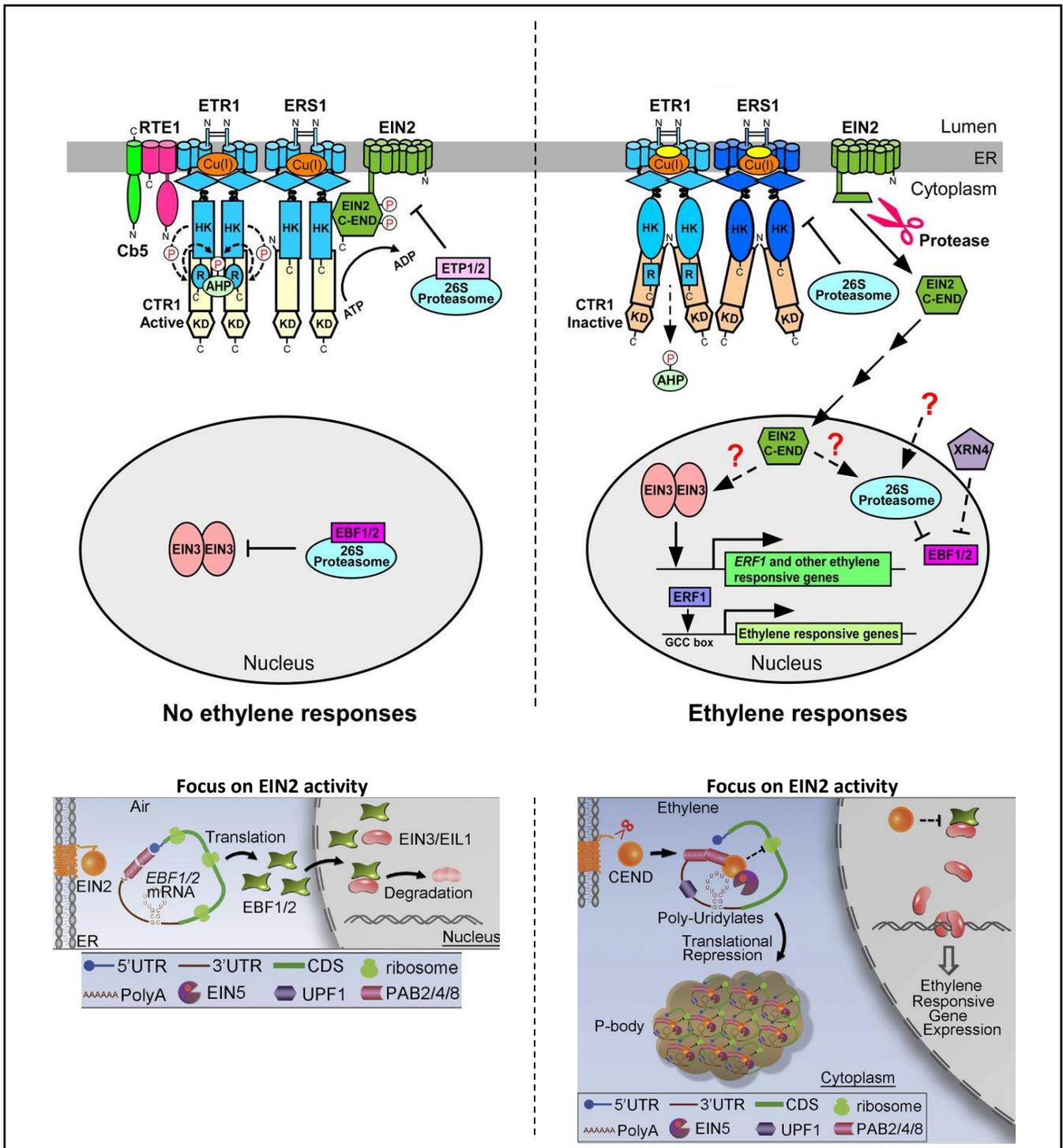


Figure 10- Schematic representation of the ethylene signaling pathway in *Arabidopsis* with a focus on the activity of the key component EIN2 in the signal transduction (adapted from Yang *et al.* 2015 and Li *et al.* 2015).

Exogenous ethylene can also stimulate the germination of several species including *Arabidopsis thaliana* as it can break its primary dormancy (Siriwitayawan *et al.*, 2003; Corbineau *et al.*, 2014). The beneficial effect of ethylene is dose-dependent, the hormone

exerts its positive effect when applied at concentrations ranging between 0.1 and 200 $\mu\text{L.L}^{-1}$ depending on the species, the surrounding environment and the depth of dormancy (Corbineau *et al.*, 2014).

The ability of a seed to germinate often correlates with ethylene production implying that this hormone is highly involved in the regulation of seed dormancy and germination (Matilla & Matilla-Vázquez, 2008; Arc *et al.*, 2013). For example, high temperature induced dormancy has been associated with reduced ethylene production in sunflower seeds (Corbineau *et al.*, 1988) while seed dormancy alleviation treatments such as chilling and nitric oxide are associated with ethylene production (Kepczynski & Kepczynska, 1997; Arc *et al.*, 2013). In addition, seed imbibition in presence of ACC, the direct precursor of ethylene, enhances germination of several species such as lettuce and sunflower. In contrast, seed treatment with inhibitors of ACS activity (aminoethoxyvinylglycine (AVG) and aminooxyacetic acid (AOA)) highly impacted the germination of seeds (Corbineau *et al.*, 2014).

7. Ethylene interplay with ABA and GA

Seed germination in response to ethylene is associated with the modulation of both ABA and GA metabolisms. Ethylene extensively reduces the inhibitory effect of ABA on the germination of several species including *Arabidopsis thaliana* (Leubner-Metzger *et al.*, 1998). The antagonistic actions between these two hormones converts stimulation of endosperm weakening and rupture prior to radicle protrusion in presence of ethylene (Linkies *et al.*, 2009). The relationship between both hormones is also supported by observations on various mutants affected in the signaling pathways of these hormones. For example, ethylene insensitive mutants such as *etr1*, *eni2* and *eni6* are hypersensitive to ABA while mutants with increased ethylene production such as *eto1*, *eto3*, or constitutive ethylene response *ctr1* show reduced dormancy and decreased ABA sensitivity (Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000; Subbiah & Reddy, 2010; Corbineau *et al.*, 2014; Gazzarrini & Tsai, 2015). Furthermore, ABA-deficient and ABA-insensitive mutants highly express ethylene biosynthetic genes and show an increased ethylene production since ABA inhibits the expression of *ACO1* (ethylene biosynthesis gene) (Linkies & Leubner-Metzger, 2012; Arc *et al.*, 2013). *FUS3* also modulated the crosstalk between ABA and ethylene by downregulating the expression of several ethylene signaling genes (*ERF*) that

contain B3-domain binding sites (Lumba *et al.*, 2012). These data demonstrate that ethylene and ABA inhibit each other's biosynthesis and signaling even if the exact mechanisms that regulate this crosstalk are not fully elucidated yet.

Regarding the interplay between GA and ethylene, gibberellins are able to stimulate the germination of seeds whose dormancy is broken by ethylene such as *Arabidopsis* (Ogawa *et al.*, 2003; Siriwitayawan *et al.*, 2003). The interaction between these two hormones is also evidenced by the studies conducted on mutants affected in the signaling pathways of these hormones. Ethylene stimulates the germination of the GA-deficient mutant *ga1-3* while GA enhances that of the *etr1* mutant (Karssen *et al.*, 1983; Bleecker *et al.*, 1988). The expression of *ETHYLENE RESPONSE SENSOR 1 (ERS1)* is also increased in presence of GA₄ (Ogawa *et al.*, 2003). On the other hand, GA₁, GA₄, and GA₇ are shown to accumulate in mature seeds of *etr1-2 Arabidopsis* mutant in comparison to wild-type seeds suggesting that the lack of ethylene alters the GA biosynthesis pathway and requires a higher concentration of GA to enable the progress towards germination (Chiwocha *et al.*, 2005).

All published data indicate that ethylene stimulates seed germination by negatively regulating ABA synthesis and signaling, and by promoting its catabolism while improving GA metabolism and signaling (Corbineau *et al.*, 2014).

8. Dormancy alleviation treatments

Following their shedding from the mother plant, seeds must lose their dormancy state before being able to germinate. In addition to environmental cues or exogenous hormonal treatments that help break dormancy, seeds of many species may require variable periods of dry after-ripening or moist chilling (cold stratification), varying from as little as a couple of days to as long as a couple of months, to release their dormancy (Bewley *et al.*, 2013).

8.1 After-ripening

After-ripening consists of a period of dry storage of freshly harvested seeds at room temperature during which physiological changes may occur leading to the gradual loss of dormancy and eventually seed germination (Bewley, 1997; Finch-Savage & Leubner-Metzger, 2006; Bewley *et al.*, 2013; Finch-Savage & Footitt, 2017). In nature, dry after-ripening may occur in winter annuals whose dormancy is broken by high summer temperatures (Bewley *et al.*, 2013). The efficiency of this process is conditioned by

environmental conditions such as moisture, temperature and oxygen (Manz *et al.*, 2005; Finch-Savage & Leubner-Metzger, 2006; Bewley *et al.*, 2013). Indeed, after-ripening requires a certain seed moisture content above a species-specific threshold since this process is prevented in very dry seeds or when water content is too high (Finch-Savage & Leubner-Metzger, 2006; Bazin *et al.*, 2011; Bewley *et al.*, 2013). In addition, this process is delayed when oxygen levels are low and it accelerates as the available oxygen increases (Bewley *et al.*, 2013). After-ripening is also conditioned by the surrounding air humidity since high RH % could lead to the induction of secondary dormancy (Basbous-Serhal *et al.*, 2016). The molecular mechanisms of after-ripening are not fully understood yet. Non-enzymatic reactions that enable germination and reactive oxygen species have been proposed to play a role in this process (Bailly, 2004). In fact, seed after-ripening is accompanied by ROS accumulation leading to a targeted mRNA oxidation and protein carbonylation of transcripts and proteins involved in cell signaling (Bazin *et al.*, 2011) and protein storage (Oracz *et al.*, 2007) (El-Maarouf-Bouteau *et al.*, 2013). Additional mechanisms controlling after-ripening have been proposed including membrane alterations (Hallett & Bewley, 2002), protein degradation via the proteasome (Borghetti *et al.*, 2002; Finch-Savage & Leubner-Metzger, 2006). Seeds may also exhibit a decrease in ABA content during after-ripening. For example, a 10 % and a 40 % decrease were observed in after-ripened *Arabidopsis* and tobacco seeds, respectively (Bewley *et al.*, 2013). Seed after-ripening results in a widening of the temperature range for germination and an increase in GA sensitivity along with the loss of exogenous GA requirement for the completion of germination. Light is no longer needed for germination of light-dependent seeds while the sensitivity to light increases in seeds that are unable to germinate even in presence of light. Lastly, an increase in germination velocity is observed in after-ripened seeds that no longer require nitrate to complete their germination (Finch-Savage & Leubner-Metzger, 2006).

8.2 Stratification (Cold chilling)

Seed imbibition at low temperatures, generally ranging between 1 to 10°C (15°C in some cases) is associated with dormancy alleviation for a wide range of species (majority of non-tropical seeds) (Bewley *et al.*, 2013). Imbibition of *Arabidopsis* seeds for 4 days at 4°C is sufficient to induce germination (Wang *et al.*, 2018). This process is significant for seeds dispersed in autumn that experience winter temperatures before being able to successfully germinate during warmer seasons (Footitt & Finch-Savage, 2017). While little information is

available on the mechanisms by which stratification leads to germination, the beneficial effects of this treatment are mostly explained by the GA/ABA balance (Bewley *et al.*, 2013; Footitt & Finch-Savage, 2017). Cold is able to induce genes that encode for enzymes involved in the GA biosynthetic pathway. *GA3ox1* is highly upregulated by cold treatment leading to the formation of bioactive GA₄ (Yamauchi *et al.*, 2004; Finch-Savage & Leubner-Metzger, 2006; Holdsworth *et al.*, 2008). In parallel, stratification also reduces the expression of genes involved in ABA signaling and synthesis and may even reduce endogenous ABA content through the induction of ABA 8'-hydroxylase activity involved in ABA catabolism (Umezawa *et al.*, 2006; Matakias *et al.*, 2009; Wang *et al.*, 2016). A recent study also demonstrated that chilling might also increase the sensitivity of seeds to ethylene prior to germination (Wang *et al.*, 2018).

9. Reactive oxygen species (ROS)

Life on earth evolved to an oxidizing environment with the proliferation of cyanobacteria, the first photosynthetic organisms. In other words cyanobacteria became the first organisms to release oxygen into the environment (Schopf *et al.*, 2007). While oxygen supports aerobic life, the outermost orbital of the dioxygen (O₂) molecule possesses two unpaired electrons with the same spin quantum number enabling it accept electrons and give rise to reactive oxygen species (ROS) (Bekker *et al.*, 2004; Bailly, 2019). Various contradictory roles have been attributed to ROS depending on their concentrations in organelles. High ROS concentrations can have damaging effects on cells while, at lower levels, ROS can act as signaling molecules in plants to help regulate developmental pathways, control redox homeostasis and enhance defense responses against pathogens or adverse environmental conditions (Wood *et al.*, 2003; Apel & Hirt, 2004). Increasing evidence suggests that the previously elucidated hormonal mechanisms are also integrated into a more complex network of cell signaling, notably involving the homeostasis of reactive oxygen species (ROS) (Bailly, 2019).

9.1 ROS metabolism in seeds

Under normal conditions, oxygen is involved in different biochemical reactions, notably the reduction of oxygen to water. However, incomplete oxygen reduction gives rise to extremely reactive ROS capable of oxidizing DNA, proteins, and lipids (Kurek *et al.*, 2019). Reactive oxygen species content in seeds is subject to constant and dramatic variations

depending on the physiological state of the seed. ROS production in seeds can be traced back to early stages of development. During this process, ROS content is at its highest at the beginning since the moisture content is still sufficient to allow metabolic activity before decreasing at the end of seed maturation (Bailly *et al.*, 2008a; Bailly, 2019). ROS generated during desiccation on the mother plant create an oxidative environment within tissues of mature seeds (Bailly, 2004). In addition, oxygen can be found stored in void spaces creating air networks within cells of mature dry seeds serving as means for further ROS production (Cloetens *et al.*, 2006). ROS productions in dry seeds is mainly non-enzymatic since desiccation inhibits most, if not all, enzymatic activities (El-Maarouf-Bouteau & Bailly, 2008a). Those non-enzymatic mechanisms are often explained by the Amadori and Maillard reactions (Priestley, 1986; Sun & Leopold, 1995) and lipid peroxidation (Bailly, 2004; El-Maarouf-Bouteau & Bailly, 2008a). Non-enzymatic ROS production, mostly targeting lipids that serve as sources of free radicals, can occur in dry seeds upon oxygen availability (Karel, 1980; Bailly, 2019). The shift from non-enzymatic to enzymatic ROS production is likely to take place shortly after water uptake (Basbouss-Serhal *et al.*, 2016). Seed imbibition is also accompanied by a burst in ROS production due to the resumption of cellular metabolism, thus the resumption of oxygen consumption (elucidated in section 3.4 Oxygen) (Bailly, 2019). Numerous ROS can be detected within imbibed seeds notably superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (*OH) (Apel & Hirt, 2004; Tiwari *et al.*, 2017; Bailly, 2019). Nevertheless, hydrogen peroxide is the most probable cellular messenger in plants mainly because of its stability, relatively long half-life (1ms compared to 1 μ s for superoxide) and its ability to passively diffuse across membranes (Apel & Hirt, 2004; Bienert *et al.*, 2006). It is possible for H_2O_2 to react with reduced Fe^{2+} to give rise to *OH through the Fenton reaction (Richards *et al.*, 2015).

It is admitted that almost 2 % of the total oxygen utilized by plants is dedicated for ROS production in various organelles (del Rio, 2002; Mittler, 2002), chiefly chloroplasts, mitochondria and peroxisomes. There is also a little contribution from the endoplasmic reticulum, apoplast and the cell wall (Figure 12) (Mittler, 2002; Tiwari *et al.*, 2017; Bailly, 2019).

9.1.1 Chloroplasts

Chloroplasts are not fully differentiated in embryos of imbibed seeds, thus do not actively participate in the production of ROS during germination (Liang *et al.*, 2018). It is worth mentioning that they represent major ROS production sites since electrons leaked through

photosynthesis are transferred to an ultimate acceptor where the production of ROS occurs (Noctor & Foyer, 1998).

9.1.2 Mitochondria

Mitochondria are believed to be one of the principle sites of ROS production, even in non-green plant tissues (Sweetlove et al., 2002; Tiwari et al., 2017). The mitochondrial electron transport chain consists of two partially overlapping pathways which consist of the cytochrome respiratory pathway with the cytochrome oxidase (COX) as the terminal oxidase and the alternative respiratory pathway, with the alternative oxidase (AOX) as the terminal oxidase (Figure 11). The Q pool represents the diverging point for both pathways where electron proceed through the cytochrome pathway if they are transferred through complex III or move through the alternative oxidase (Rhoads & Subbaiah, 2007). Electron leakage from complex I and III of the mitochondrial electron transport chain harbors enough energy to reduce oxygen and form superoxide radicals. Two pathways of oxygen reduction have been identified to date. The first pathway relies on O_2 consumption via the cytochrome oxidase to produce water and the second depends on the direct reduction of O_2 to $O_2^{\cdot-}$ in the NADPH dehydrogenase segment of the respiratory chain (Ježek & Hlavatá, 2005; Murphy, 2009a). The generated superoxide radicles are subject to dismutation via the Superoxide (SOD) dismutase to give rise to hydrogen peroxide (Murphy, 2009a). Recent data points to the possible involvement of the mitochondrial electron transport chain as a major source of ROS during germination (Ma et al., 2019). A more detailed description about the role of mitochondria in germination is elucidated in section 11. Involvement of mitochondria in seed germination.

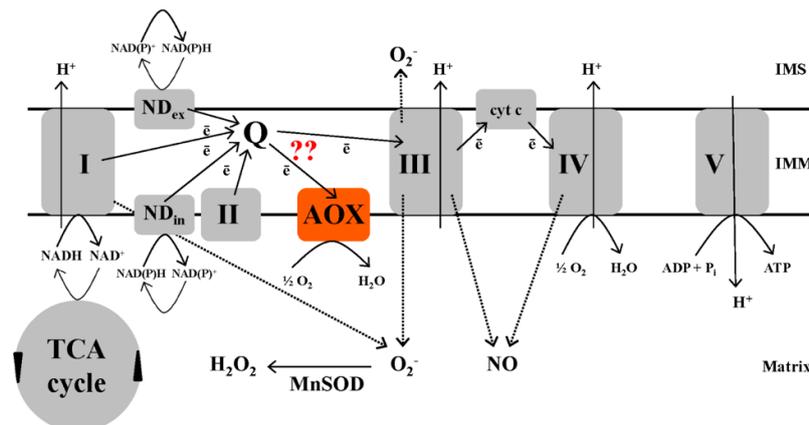


Figure 11- Schematic representation of the plant electron transport chain and its associated ROS production. I, II, III, IV, V: complexes I to V, IMS, inner membrane space; IMM, inner mitochondrial membrane; MnSOD, manganese superoxide dismutase; ND_{in}, internal-oriented alternate NAD(P)H dehydrogenases; ND_{ex}, external-oriented alternate NAD(P)H dehydrogenases; Q, ubiquinone pool (Vanlerberghe 2013).

9.1.3 Peroxisomes

Peroxisomes are ubiquitous subcellular organelles bounded by a single membrane and exempt of DNA, they have an oxidative metabolism and are considered as major sites of intracellular hydrogen peroxide production (del Rio, 2002; Corpas *et al.*, 2020). Plant peroxisomes have a significant number of enzymatic systems capable of producing hydrogen peroxide such as glycolate oxidase (GOX), acyl-CoA oxidase (AOX), urate oxidase (UO), polyamine oxidase, copper amine oxidase (CuAO), sulfite oxidase (SO), sarcosine oxidase (SOX), or superoxide dismutase (SOD) (Hauck *et al.*, 2014; Corpas *et al.*, 2017). This is mainly due to the involvement of peroxisomes in diverse key physiological functions such as fatty acid β -oxidation, glyoxylate cycle, purine catabolism and sulfur metabolism (del Río & López-Huertas, 2016).

Hydrogen peroxide is produced following different enzymatic reactions that include in a decreasing order the photorespiratory glycolate oxidase (GOX) reaction (usually found in green tissues), the main enzyme of fatty acid β -oxidation, acyl-CoA oxidase, the enzymatic reaction of flavin oxidases and the spontaneous or enzymatic dismutation of $O_2^{\cdot-}$ radicals (del Río, 2013). It has been demonstrated that peroxisomes produce superoxide radicals ($O_2^{\cdot-}$) in 2 different sites. The first site of $O_2^{\cdot-}$ generation is located in the organelle matrix, in which the generating system was identified as XOD, and the other site is the peroxisomal membrane where a small electron transport chain appears to be involved. A recent study evoked the production of singlet oxygen in the peroxisomes of non-photosynthetic tissues (Mor *et al.*, 2014). ROS levels within peroxisomes are regulated due to the presence of antioxidant systems. The main enzyme involved in this process is the catalase, which apart from its function in the control of hydrogen peroxide generated in peroxisomes is also involved in the maintenance of cellular redox homeostasis (Mhamdi *et al.*, 2012). In addition to the catalase, other antioxidative systems have been found in plant peroxisomes and they mainly include the superoxide dismutases (SODs) which catalyze the dismutation of superoxide radicals into oxygen and H_2O_2 (Fridovich, 1997).

9.1.4 Plasma membrane

Plant NADPH oxidases, known as respiratory burst oxidase homologues (RBOHs), belong to a family of transmembrane proteins able to transport electrons across a membrane originating from a cytosolic electron donor to oxygen (extracellular acceptor) and catalyzing the generation of superoxide (Jiménez-Quesada *et al.*, 2016; Qu *et al.*, 2017). The lifespan of superoxide radicals is relatively short since these molecules are rapidly

disrupted to hydrogen peroxide through the action of the apoplastic superoxide dismutases (SODs) (Bowler *et al.*, 1994). It has been shown that ROS deriving from NADPH oxidases accumulate and loosen the cell wall since they directly cleave cell wall polysaccharides (Li *et al.*, 2017a). A study conducted by Leymarie *et al.* (2012) showed that Arabidopsis seed treatment with the NADPH oxidase inhibitor, diphenyleneiodonium (DPI), significantly reduced the germination of non-dormant seeds imbibed at 25°C to almost 20%. It is worth saying that the *rbohD* gene has been shown to be involved in ethylene and ABA signaling and that *rbohD* is specifically needed for the initiation and diffusion of ROS signals (Miller *et al.*, 2009; Leymarie *et al.*, 2012).

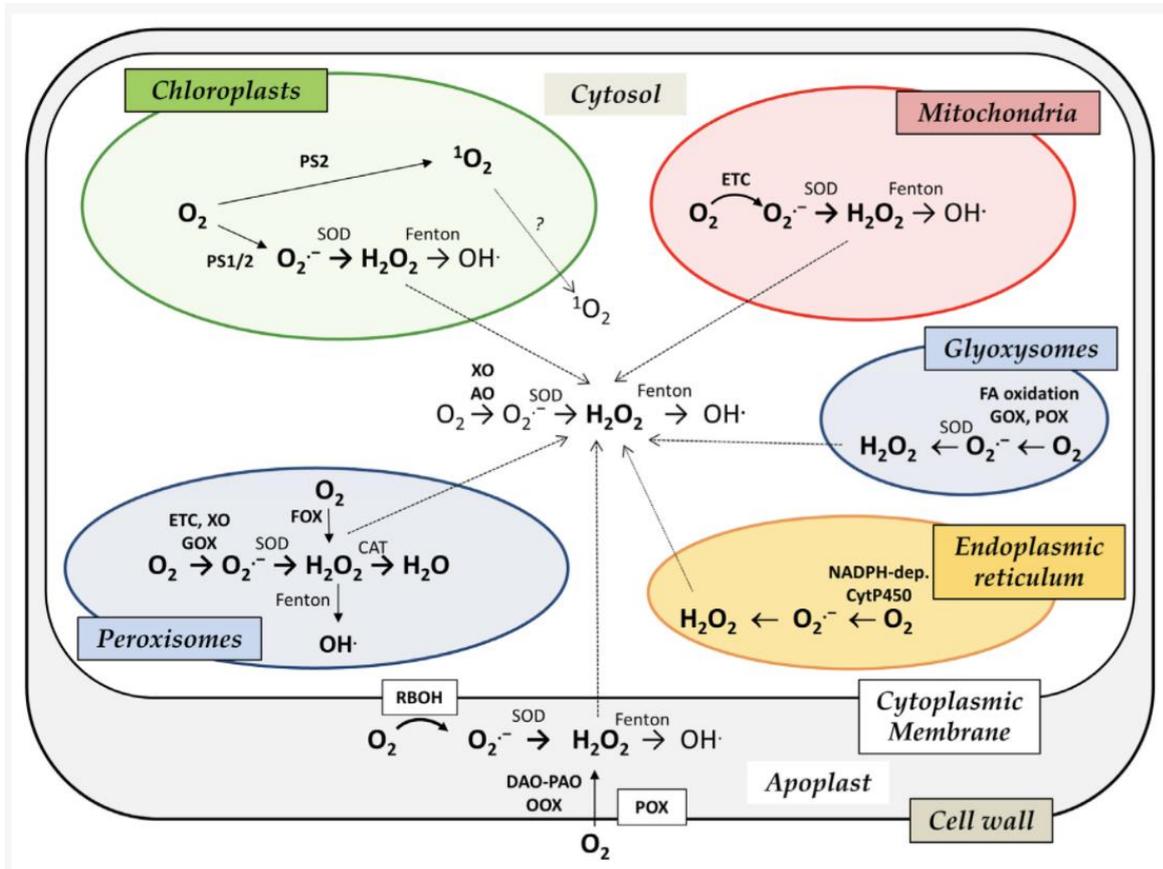


Figure 12- Schematic representation of different ROS-producing compartments in plant cells. AO, aldehyde oxidase; CAT, catalase; DAO, diamine oxidase; GOX, glycolate oxidase; ETC, electron transport chain; FOX, flavin oxidases; OOX, oxalate oxidase; PAO, polyamine oxidase; POX, peroxidases; PS, photosystem; RBOH, NADPH oxidase; SOD, superoxide dismutase; XO, xanthine oxidase/dehydrogenase (Janku *et al.* 2019).

ROS levels within cells do not only depend upon efficient production for they are subject to constant regulation through enzymatic and non-enzymatic antioxidants processes (Bailly, 2004, 2019; Tiwari *et al.*, 2017). For instance, superoxide dismutase is

responsible for the conversion of superoxide to hydrogen peroxide which is eliminated through the intermediate of catalases (CAT) located in glyoxysomes and peroxisomes (Willekens *et al.*, 1997; Bailly, 2004). The two-stage mechanism of catalase action begins with the reaction of the high spin ferric (Fe III) state with hydrogen peroxide to form an oxy-ferryl intermediate (compound I) and porphyrin π -cation radical containing Fe IV. This step is accompanied by a proton transfer from one end of the H₂O₂ molecule to the other which polarizes and breaks the O-O bond in hydrogen peroxide. In the next stage, a second hydrogen peroxide molecule is used as a reductant to regenerate the enzyme, producing water and oxygen (Mhamdi *et al.*, 2010). The ascorbate-glutathione (AsA-GSH) cycle (also called the Halliwell-Asada cycle) plays an important role in H₂O₂ breakdown. Hydrogen peroxide is reduced to water through the action of the ascorbate peroxidase (APX) which uses ascorbate (AsA) as the specific electron donor (Bailly, 2004; Tiwari *et al.*, 2017). APX can be found in different organelles such as the chloroplast (chlAPX), mitochondria (mitAPX), peroxisome (mAPX), and cytosol (cAPX) (Mittler, 2002). Enzymes involved in this cycle can be used to regenerate other antioxidants such as ascorbic acid (vitamin C), reduced glutathione and α -tocopherol (vitamin E) (Bailly, 2004). Peroxidases are also part of the enzymatic antioxidants since they function to reduce lipid hydroperoxides to alcohols and reduce free hydrogen peroxide to water (Noctor *et al.*, 2002). The plant glutathione peroxidase (GPX) family harbors many isozymes with different localizations named AtGPX1 to AtGPX8 in *Arabidopsis* (Milla *et al.*, 2003).

Non-enzymatic antioxidants often regroup cellular redox buffers such as ascorbate (AsA) and glutathione (γ -glutamyl-cysteinyl-glycine, GSH) as well as tocopherols and carotenoids. Ascorbic acid is the most abundant water-soluble antioxidant (Wang *et al.*, 2005) present in all the organelles of plant cell and in the apoplast (Smirnoff, 2000). Ascorbic acid is able to donate electrons to numerous enzymatic and non-enzymatic reactions which confer it the ability to directly scavenge O₂, \cdot OH, and reduce H₂O₂ to H₂O via the APX reaction (Smirnoff, 2000). Tocopherols are exclusive to green plant tissues and serve to protect lipids by reacting with oxygen and quenching its energy (Das & Roychoudhury, 2014). Carotenoids are part of the lipophilic antioxidants present in plastids of photosynthetic and non-photosynthetic plant tissues that scavenge ¹O₂ and generate heat as a by-product (Das & Roychoudhury, 2014).

10. ROS in the context of seed germination

ROS, at certain levels, appear to be central components of plant response to their surrounding environment including germination cues (Figure). ROS seem to play key signaling roles in this process for several reasons especially their high reactivity which confers ROS the ability to directly oxidize numerous cellular components and eventually modify cell functioning, and their capacity to reach targets farther from their production sites in imbibed seeds (water allows the transport of long-living ROS such as hydrogen peroxide) (Bailly *et al.*, 2008a; Bailly, 2019). Germination and ROS appear to be closely linked since germination success relies on internal ROS contents in many species including maize (Hite *et al.*, 1999), wheat (Caliskan & Cuming, 1998) or sunflower (Bailly *et al.*, 2002).

Many functions have been attributed to ROS in the context of seed germination such as the contribution to cell wall loosening during endosperm weakening. For instance, apoplastic OH radicals mediate the breakdown of chitosan, pullulan-like polysaccharides, and hyaluronate by activating calcium (Ca^{2+}) channels and mitogen-activated protein kinases (MAPKs) leading to the deterioration of the cell wall polysaccharides to allow radicle emergence (Stern *et al.*, 2007; Diaz-Vivancos *et al.*, 2013).

Seed germination and radicle emergence represent critical steps in the establishment of a seedling due to the presence of pathogens in the soil. ROS production might help protect the emerging radicle against pathogens (El-Maarouf-Bouteau & Bailly, 2008a).

Dormancy alleviation also relies on selective mRNA oxidation (El-Maarouf-Bouteau *et al.*, 2013). In fact, mRNAs are prone to oxidation (more than DNA) because of their single-stranded structures and their presence in the cytoplasm within the absence of effective repair mechanisms for oxidative damages (Kong & Lin, 2010). mRNA oxidation has been reported in sunflower seeds where the amount of 8-hydroxyguanosine (8-OHG) in poly(A)-RNA increases by 50% during seed after-ripening (in non-dormant seeds compared to dormant seeds) (Bazin *et al.*, 2011). Guanine is commonly oxidized in RNA through its reaction with $\cdot\text{OH}$ which results in the formation of 8-hydroxyguanosine (8-OHG). The oxidation of mRNAs was highly selective and most of the oxidized mRNAs corresponded to genes involved in signaling.

Oxidation is known to alter translation (premature termination) and to address truncated proteins to P-bodies (El-Maarouf-Bouteau *et al.*, 2013).

In addition, ROS may enhance the oxidation of several components to stimulate transcription factors for signaling (Laloi *et al.*, 2004). For example, hydrogen peroxide relies on the redox state of active proteins to accelerate the activation of redox sensitive transcription factors that will further activate downstream signaling cascades (Foyer & Noctor, 2013).

Proteins also display an increased sensitivity to oxidation by ROS (Shacter, 2000). Different oxidative modifications are known to target proteins including carbonylation (irreversible) and the oxidation of cysteine and methionine (Shacter, 2000; El-Maarouf-Bouteau *et al.*, 2013). Carbonyl group oxidation (aldehydes and ketones) results from oxidative action on the aminoacyl moiety (Lys, Arg, Pro, and Thr), while the oxidation of Cys and Met is mediated by their sulfur atoms. Selective protein oxidation was observed in germinating seeds including *Arabidopsis*. The carbonylation of seed storage protein is thought to enhance their mobilization by altering compact seed storage protein complexes and orienting them toward proteolytic cleavage (Job *et al.*, 2005; El-Maarouf-Bouteau *et al.*, 2013). In addition, sunflower seed imbibition in presence of dormancy alleviation treatments such as hydrogen cyanide or methylviologen resulted in the carbonylation of specific proteins (Oracz *et al.*, 2007).

ROS represent signal molecules that could possibly mediate the crosstalk between ABA, GA and ethylene during seed imbibition even if the detailed mechanisms have not been fully elucidated yet (El-Maarouf-Bouteau & Bailly, 2008a; Corbineau *et al.*, 2014). An increase in hydrogen peroxide levels was also accompanied by a decrease in ABA levels in germinating barley (Wang *et al.*, 1995) and apple seeds (Bogatek *et al.*, 2003). In fact, hydrogen peroxide accumulation in germinating seeds induced the activation of ABA-8-hydrolase, an ABA catalytic enzyme (Bailly, 2019). The interaction between ROS and GA has also been reported in elongating stem cells (Wigoda *et al.*, 2006). On the other hand, ethylene stimulates ROS accumulation in sunflower embryos (El-Maarouf-Bouteau *et al.*, 2014). In parallel, ROS produced in soybean embryos stimulated endogenous ethylene production (Ishibashi *et al.*, 2013).

Lastly, ROS have been shown to interact with other signaling pathways such as activating MAPKs or Ca²⁺ channels to relay ROS signals (Bailly, 2019).

Taken all together, these data suggest that ROS levels maintained within the oxidative window for germination can act as signaling messengers that enable the progress towards germination (Bailly *et al.*, 2008a).

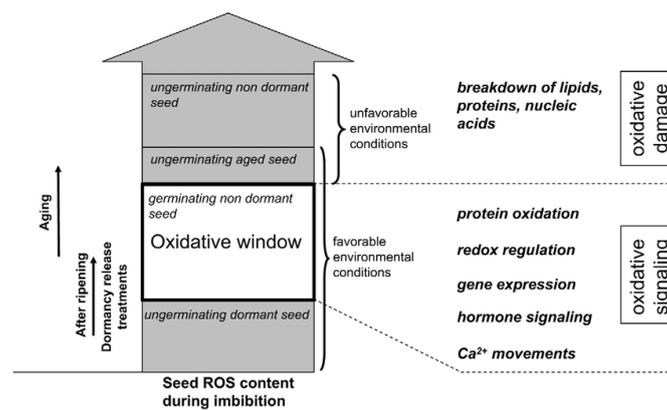


Figure 13- The dual role of ROS in seeds. ROS levels within the oxidative window enable the progress towards the germination while higher or lower levels diminish the beneficial effects of ROS on this process (Bailly *et al.* 2008).

11. Involvement of Mitochondria in seed germination

Seed germination is tightly associated with a rapid reestablishment of numerous biochemical activities localized in a number of distinct organelles (Bewley, 1997). Functional mitochondria play a crucial role in seed germination since it is an energy demanding process (Ma *et al.*, 2019). In dry seeds, mitochondria appear as circular organelles where both membranes are very close to each other (Howell *et al.*, 2006) to form the metabolically quiescent promitochondria (Logan *et al.*, 2001; Howell *et al.*, 2006). After imbibition, a progressive transition into metabolically and energetically active mature mitochondria occurs (Carrie *et al.*, 2013; Ma *et al.*, 2019). This structural change is accompanied by an early molecular switch characterized by a transient burst in the expression of genes that encode mitochondrial proteins. Factors involved in mitochondrial transcription and RNA processing were among the early expressed genes and they are followed by a burst in the transcript abundance of genes encoding proteins involved in mitochondrial DNA replication and translation (Law *et al.*, 2012, 2014). In addition to sustaining the energy demanding germination process, mitochondria are capable of sensing functional imbalances. For instance, the respiratory machinery of mitochondria, based on redox chemistry, can sensitively react to changing conditions (Schwarzländer & Finkemeier, 2013). A recent study published by Ma *et al.* (2019) showed that mitochondria are able to regulate ROS production during seed germination. They identified a mitochondrion-localized plant small heat shock protein (sHSP), GhHSP24.7, which is known to interact with GhCcmFc that

is a subunit of the cytochrome bc1 (Cytbc1) complex and is able to modulate ROS production within the mt-ETC. Interestingly, they also demonstrated that Cytbc1 plays a key role in mitochondrial biogenesis. Since Mitochondrial biogenesis is implicated in germination (Law et al., 2014), the activity of cytochrome bc1 might contribute to germination. A very interesting find was that at warm temperatures the increased level of GhHSP24.7 leads to the inhibition of the cytochrome oxidase (COX) pathway provoking an additional release of ROS to promote seed germination through a possible activation of the alternative oxidase (AOX) (Ma et al., 2019). It has been shown in recent years that altered mitochondrial status can even trigger signaling defining what is known as mitochondrial retrograde signaling (MRS). In mitochondrial retrograde signaling, mitochondria are the source of specific signaling molecules that relay information to the nucleus, thus modify the expression of nuclear genes (Ng et al., 2014; Law et al., 2014). Key markers of mitochondrial retrograde signaling in plants such as the alternative oxidase (AOX) have been recently reported. The alternative oxidase is an interfacial membrane protein, oriented towards the matrix of the inner mitochondrial membrane, bypasses proton-pumping complexes III and IV of the mtETC and is responsible for coupling the oxidation of ubiquinol to the four-electron reduction of O₂ to water. The plant AOX is encoded by a small gene family, consisting of two distinct subfamilies termed AOX1 and AOX2 (Dojcinovic et al., 2005; Vanlerberghe, 2013). Nevertheless, AOX1a is often used as a marker of MRS in *Arabidopsis thaliana* (Rhoads & Subbaiah, 2007; De Clercq et al., 2013). Mitochondrial retrograde signaling is associated with an upregulation of the alternative oxidase gene. The activity of the AOX is positively regulated by upstream transcription factors of the NAC (No apical meristem, ATAF1/2 and CUC2 cup-shaped cotyledon) family, mainly NAC013 and NAC017 located in the endoplasmic reticulum. The proteolytic cleavage of these transcription factors is followed by their translocation to the nucleus (Vanlerberghe, 2013; De Clercq et al., 2013; Ng et al., 2013a). Another positive regulator of the alternative oxidase 1a is the CYCLIN-DEPENDENT KINASE E1 and the WRKY63 transcription factor whereas negative regulators include the ABSCISIC ACID INSENSITIVE4 (ABI4) and WRKY15/40 (De Clercq et al., 2013; Ng et al., 2013a; Selinski et al., 2018b). It is known that ROS probably act as MRS-triggering signaling molecules (Vanlerberghe et al., 2002; Rhoads et al., 2006). The activation of mitochondrial retrograde signaling has been largely studied in the context of response to stress but information about its possible role in seed germination remain sparse (Maxwell et al., 2002; Rhoads et al., 2006; Giraud et al., 2009a).

12. Molecular control of seed germination

Huge efforts and progress were made in the last decade, notably using -omics approaches, to decipher the molecular mechanisms regulating seed germination and dormancy given their importance in this transition (Kawaguchi & Bailey-Serres, 2005; El-Maarouf-Bouteau *et al.*, 2013; Basbouss-Serhal, 2015; Basbouss-Serhal *et al.*, 2017; Bai *et al.*, 2017, 2020). For instance, protein synthesis relies on stored mRNAs and newly transcribed mRNAs to serve as matrices (Basbouss-Serhal *et al.*, 2015; Bai *et al.*, 2017, 2020) while the down-regulation of several genes is essentially dependent upon mRNA decay (Basbouss-Serhal *et al.*, 2015). Understanding the connection between these multi-level molecular events during the transition from metabolic quiescence to the resumption of metabolism during germination is therefore required to uncover the molecular aspects of this process.

12.1 Transcriptional regulation of germination

The reactivation of the cellular metabolism is associated with intensive cellular activity that requires *de novo* RNA synthesis prior to seed germination. The transcriptional activity in seeds resumes few hours after the onset of imbibition (1 to 3 hours) as evidenced by high throughput transcriptome analysis (Bewley *et al.*, 2013). The expression of genes associated with germination has been shown to increase during imbibition while comparing the transcriptome of dry and imbibed seeds. Indeed, phase II of seed germination is characterized by the expression of genes encoding transcription factors, hormone metabolism enzymes and signaling proteins, and cell wall modification enzymes (Bewley *et al.*, 2013). For instance, the expression of many ABA signaling components is regulated at the transcriptional level such as the protein phosphatase 2C (PP2C) (Wang *et al.*, 2019) and the PYL receptors (Li *et al.*, 2018b) whose expression is enhanced in presence of ABA. As for GA, an increase in the expression of genes coding for transcription factors involved in the regulation of the activity of expansins (EXPAs) has been documented (Xu *et al.*, 2020). This early resumption of transcriptional activity induces different signatures or profiles in the transcriptome of dormant and germinating seeds. The use of bioinformatic tools such as Pathifier enables the discrimination between dormant and germinating *Arabidopsis* seeds. This algorithm calculates Pathway Deregluation Scores (PDS) from gene expression values (extent to which the activity of a pathway, measured in a seed sample, differs from the activity of the pathway estimated in imbibed seeds undergoing germination) that enable the clustering of samples. Observed differences in the transcriptome of dormant and

germinating seeds involved different categories such as RNA metabolism, hormones, proteins, primary and secondary metabolism emphasizing on the importance of transcription during the transition to a germinating state (Ponnaiah *et al.*, 2019). The role of transcription during germination has been further investigated through the inhibition of novel mRNA synthesis in imbibed seeds. In fact, seed treatment with α -amanitin, an inhibitor of RNA synthesis in *Arabidopsis*, still enabled the progress towards germination but with consequences compared to normal seeds. The germination of the treated seeds occurred at a slower rate and was accompanied by a decreased sensitivity to GA. Nevertheless, seedling establishment was totally blocked and this could be explained by the repression of novel enzyme synthesis required for reserve mobilization (Rajjou *et al.*, 2004). A network model of global transcriptional interactions (SeedNet) has been generated in order to visualize and discriminate sets of interactions associated with dormancy and germination (Bassel *et al.*, 2011).

13. key molecular regulator of seed dormancy

Molecular analysis has identified many loci involved in seed dormancy and germination (Nishimura *et al.*, 2018). The induction of these genes, notably *DOG1* and *ABI4*, is always associated with the maintenance of seed dormancy (Bentsink *et al.*, 2006, p. 1; Carrillo-Barral *et al.*, 2020, p. 1; Chandrasekaran *et al.*, 2020). Moreover, the involvement of *DOG1* and *ABI4* in multiple pathways known for their ability to control germination such ABA signaling enables their detection in most studies that target dormancy and germination making of them key regulators and markers of this complex process (Wang *et al.*, 1995, p. 1; Nakabayashi *et al.*, 2015, p. 1; Huang *et al.*, 2017, p. 4; Carrillo-Barral *et al.*, 2020).

13.1 DELAY OF GERMINATION-1 (*DOG1*)

DELAY OF GERMINATION-1 (DOG1) is a heme-binding protein that belongs to a gene family (Carrillo-Barral *et al.*, 2020). In *Arabidopsis thaliana* the *DOG1* gene is composed of 3 exons and 2 introns and is alternatively spliced of the second intron thus producing five transcript variants (Nakabayashi *et al.*, 2015). It has been shown that *DOG1* is involved in many processes including seed maturation, seed longevity, primary dormancy (PD) release and germination timing (Graeber *et al.*, 2010; Nakabayashi *et al.*, 2012; Carrillo-Barral *et al.*, 2020). An important function of *DOG1* may be the induction of PD. For instance, *DOG1* is considered to be a major regulator of primary seed dormancy that interacts with

ABA to delay germination (Nishimura *et al.*, 2018). It is established that the expression of *DOG1* is required for the induction of PD, yet *DOG1* is reduced in fully mature dry seeds (Dekkers *et al.*, 2016) and its mRNA is practically undetectable in seedlings (Nonogaki, 2019a). The expression of *ABI5* is responsible for the decrease in the expression of the raffinose family oligosaccharides (RFO), an increase of SSPs, heat-shock proteins (HSPs), and late embryogenesis abundant (LEA) in the *dog1-1* mutant resulting in a reduced level of PD (Dekkers *et al.*, 2016; Li *et al.*, 2020). Additionally, *DOG1* and *ABI3* work in parallel to stimulate the maturation of seeds since the analysis of *dog1* and *dog1 abi3* mutants suggests that *DOG1* function is not limited to PD but is required for several aspects of seed maturation (Dekkers *et al.*, 2016). *DOG1* can also regulate dormancy by physically interacting with two phosphatases (ABA-HYPERSENSITIVE GERMINATION 1 and 3; AHG1 and AHG3) that act as negative regulators of ABA signaling to maintain primary dormancy (Nishimura *et al.*, 2018). Moreover, *DOG1* suppresses the actions of certain PP2C phosphatases which serve as a convergence point of ABA and *DOG1* pathways (Née *et al.*, 2017). A recent study evidenced the antagonistic interaction between ethylene and *DOG1* where they showed that the ethylene transcription factor ERF12 directly binds to the *DOG1* promoter, recruiting the co-repressor TOPLESS (TPL) in this nuclear process and inhibiting *DOG1* expression and by repressing its signaling pathway (Li *et al.*, 2019, p. 1).

13.2 ABSCISIC ACID INSENSITIVE 4 (*ABI4*)

ABSCISIC ACID INSENSITIVE 4 (*ABI4*) is a key positive regulator of the ABA signaling cascade. Recent studies show that *ABI4* is also involved in the regulation of other signaling pathways including several phytohormones biogenesis and signaling cascade, diverse developmental processes including seed dormancy and germination, seedling establishment and root development. It is also involved in disease resistance and lipid metabolism (Bulgakov *et al.*, 2019). *ABI4* is a member of the AP2/ERF family that is capable of binding to ABRE, CE1, S-box, G-box, CCAC motif and CACCG elements in the promoters of stress-responsive genes and regulate their expression (Chandrasekaran *et al.*, 2020). *ABI4* plays a key role in the regulation of primary seed dormancy by binding to the CCAC and/or CACCG *cis* elements causing a repression of the expression of ABA inactivating genes such as *CYP707A1* and *CYP707A2* (decline in ABA degradation) and enhancing the expression of *GA2ox7* to promote GA catabolism (Shu *et al.*, 2013). In addition, *ABI4* mediates the transcriptional repression of ethylene biosynthesis genes such as *ACS4*, *ACO2*, and *ACS8* by binding to the CCAC elements present in their promoters,

thus reducing ethylene levels (Dong *et al.*, 2016). Interestingly, ABI4 seems to be involved in mitochondrial retrograde signaling where it represses the transcript abundance of *AOX1a* by targeting the CGTGAT elements in its promoter (Giraud *et al.*, 2009a).

13.3 Translational regulation of germination

Translational regulation of seed germination plays a pivotal role in the multilayered molecular processes triggered in response to germination cues (Galland & Rajjou, 2015; Bai *et al.*, 2017, 2020; Sajeev *et al.*, 2019; Sano *et al.*, 2020). Translation represents a mean for cells to rapidly and reversibly regulate gene expression in response to environmental cues (Sajeev *et al.*, 2019). The importance of translation in germination was further put forward when seed treatment with cycloheximide, a cytosol translational inhibitor, completely blocked germination (Rajjou *et al.*, 2004; Sajeev *et al.*, 2019). Protein synthesis during early seed germination is dependent on pre-existing mRNA templates stored during maturation. These mRNAs are referred to as “long lived RNAs” because of their long half-life that enables them to remain translatable for long periods even after seed exposure to stressful conditions (Rajjou *et al.*, 2012; Sajeev *et al.*, 2019; Bai *et al.*, 2020). Thousands of stored mRNA have been detected in seeds, including 12,000 and 17,000 mRNAs in *Arabidopsis* and rice, respectively (Sano *et al.*, 2020). In addition to their involvement in germination, these stored mRNAs take into account the history of the mother plant to help seeds cope with their environment (Sano *et al.*, 2020). The time course of seed germination relies on selective and temporal mRNA translation (Galland *et al.*, 2014). For instance, the early steps of water uptake are accompanied by the translation of mRNAs associated with storage proteins and desiccation tolerance whereas in the lag phase of germination translated mRNAs are involved in energy and amino acid metabolism. At the end of this phase, mRNAs associated with nitrogen remobilization are translated in preparation for seedling growth (Galland & Rajjou, 2015). Selective translation is proven to be dependent on selective and timely regulated mRNA recruitment to polysomes (Layat *et al.*, 2014; Basbouss-Serhal *et al.*, 2015; Bai *et al.*, 2017). The polysome occupancy, defined as the ratio of mRNA in the polysome pool over total mRNA (Bailey-Serres, 1999), is determined by some key features of the mRNA. These features represent several characteristics of the 5'Untranslated Region (5'UTR), the presence and location of specific motifs, the length of the transcript, the secondary structure and stability of the mRNA (Basbouss-Serhal *et al.*, 2015; Bai *et al.*, 2017). Key regulatory elements in the 5'UTR that control polysome loading mainly consist of its length and GC% as well as the presence of upstream Open Reading

Frames (uORF) and their characteristics (number, length, GC%) (Browning & Bailey-Serres, 2015; Basbouss-Serhal *et al.*, 2015; Bai *et al.*, 2017). Polysome loading negatively correlates with increasing length and GC% of both 5'UTR and uORFs and eventually with the increase in the number of uORFs per 5'UTR (Basbouss-Serhal *et al.*, 2015; Bai *et al.*, 2017). It is admitted that short transcripts with low GC% are usually better translated than long transcripts (Liu *et al.*, 2012). mRNAs are also known to have a sharp decrease in structure complexity at the start and stop codons in order to facilitate the binding of the ribosomes for the initiation of translation (Li *et al.*, 2012). Upregulated mRNAs have been shown to be less structured in the 5'UTR suggesting that simpler structures are favored during translation (Bai *et al.*, 2017). Changes in the recruitment of mRNA to polysomes have been reported between in dormant and germinating *Arabidopsis* seeds (Basbouss-Serhal *et al.*, 2015; Bai *et al.*, 2017). For instance, polysome-associated transcripts in germinating *Arabidopsis* seeds were associated with transcription, hormone metabolism, cell wall and signaling. These categories were not significantly induced in dormant seeds (Basbouss-Serhal *et al.*, 2015). Thus, the findings of Basbouss-Serhal *et al.* (2015) and Bai *et al.* (2017) demonstrate that polysome loading changes to a large extent during the germination process.

13.4 mRNA decay during seed germination

mRNA dynamic turnover, a key process in the post-transcriptional gene regulation, plays an important role in controlling the depth of dormancy and eventually the transition of seeds to a germinating state (Sano *et al.*, 2020). The 5' m⁷G cap and the 3' poly(A) tail are primary determinants of mRNA stability. Both ends are subject to nuclease activity leading to mRNA decay (Zhang & Guo, 2017). While the 5' end is subject to the action of XRN4 (detailed later on in this section), the 3' end of mRNAs is hydrolyzed by a complex of 3'-5' exoribonucleases called the exosome. In addition to the activity of exoribonucleases at both ends of the mRNA, none-sense mediated decay (NMD) serves as a quality control coupling aberrant translation or premature termination to accelerated RNA decay (Shaul, 2015). The core of NMD machinery consists of UP FRAMESHIFT (UPF) proteins UPF1, UPF2, and UPF3 and may occasionally contain other factors such as the SUPPRESSOR OF MORPHOLOGICAL DEFECTS ON GENITALIA (SMG) proteins (Drechsel *et al.*, 2013).

Information regarding the mechanisms of mRNA decay in the context of germination is still missing. So far, recent findings evidenced the role of 5' to 3' decay in the germination of *Arabidopsis* seeds (Basbouss-Serhal *et al.*, 2017). Prior to 5'-3' decay, the 5' m⁷G cap is removed from capped transcripts through the catalytic activity of the decapping complex

VARICOSE (VCS) - Decapping protein 1 (DCP1) - Decapping protein 2 (DCP2) (Xu *et al.*, 2006). While DCP2 represents the catalytic subunit, DCP1 acts as its essential activator (Xu *et al.*, 2006) and VCS is required for the interaction of both DCP1-DCP2 subunits (Fenger-Grøn *et al.*, 2005). 5'- 3' mRNA decay is mediated by the XRN family of exoribonucleases. The Arabidopsis genome encodes for two nucleus-localized XRN (XRN2 and XRN3) and XRN4 located in the cytoplasm (Nagarajan *et al.*, 2013). The activity of XRN4 is essential for the transition of seeds to a germinating state. For instance, the *xrn4* mutant showed an enhanced dormancy compared to wild-type seeds (Basbouss-Serhal *et al.*, 2017). Even dormancy alleviation treatments such as 2 weeks of dry storage were only sufficient to reach a germination rate of 40 % at 25 °C for this mutant compared to 100 % for wild-type seeds and those of the *vcs* mutant (Basbouss-Serhal *et al.*, 2017). The phenotype of *xrn4* was associated with an increase in the abundance of transcripts related to dormancy (ABA synthesis and response, GA inactivation) and a decrease in the level of mRNAs involved in germination (ABA catabolism, GA activation and response). The opposite pattern was observed in seeds of the non-dormant *vcs* mutant. The slowed germination of both mutants in presence of exogenous ABA and the ability of seeds to reach the T_{50} more rapidly in presence of GA confirmed that the 5' to 3' mRNA decay is associated with the canonical hormonal pathway of dormancy regulation. Transcriptomic analysis on both mutants also showed that a strong secondary structure and a higher GC % in the 5'UTR made the transcripts prone to degradation (Basbouss-Serhal *et al.*, 2017). The components of the decapping complex as well as XRN4 can be found in processing bodies (P-bodies) (Xu & Chua, 2011a; Chantarachot & Bailey-Serres, 2018). Thus 5' to 3' decay may occur in but is not confined to P-bodies. Processing bodies consist of dynamic macromolecular assemblies of translationally inactive mRNAs and proteins involved in translation repression and RNA turnover processes (Chantarachot & Bailey-Serres, 2018).

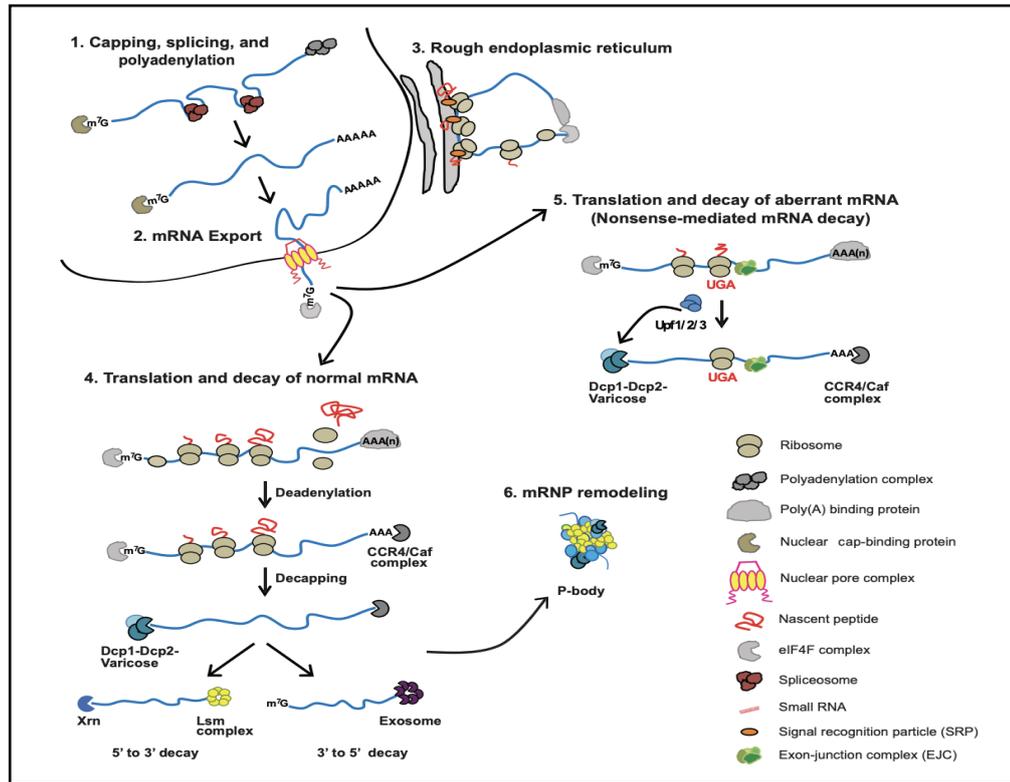


Figure 14-Overview of mRNA maturation, translation and decay in plant cells. Newly transcribed mRNA undergoes the maturation process in the nucleus (1) before being exported to the cytoplasm (2). mRNA is then translated via ribosomes associated to the endoplasmic reticulum (3) or free cytosolic ribosomes (4,5). Aberrant mRNA is subject to NMD (5). After translation termination, mRNA are degraded and addressed to P-bodies (6). Adapted from Roy & Arnim (2013).

14. Epigenetic control of seed germination: Example of N^6 -methyladenosine

Posttranscriptional regulation of gene expression is required for the completion of many developmental processes notably the response to external cues. Among the different cellular mechanisms that regulate mRNA fate, covalent nucleotide modification has recently emerged as a powerful modulator of the processing, localization, stability, and translatability of mRNAs (Yue *et al.*, 2019). Epigenetic modifications play crucial and dynamic roles in different processes including embryo development, stem cell fate determination, floral transition, stress responses, fruit ripening, and root development (Liang *et al.*, 2020). Massive epigenomic transformations have been recently shown to be associated with *Arabidopsis thaliana* seed germination (Narsai *et al.*, 2017; Bhat *et al.*, 2018). These reversible modifications allow a rapid response to external cues (Hewezi, 2018).

The methylation of adenosine in the N^6 position (m^6A) is the most understood and widespread internal modification present in rRNAs, mRNA, tRNAs, miRNA and long non-

coding RNA (Arribas-Hernández & Brodersen, 2020). For instance, m⁶A accounts for almost 80% of all RNA methylation out of which 50% occur in polyadenylated mRNA (Yue *et al.*, 2019). m⁶A is able to modify RNA metabolism at different levels including translation, translation efficiency, 3'-end processing and alternative splicing. In plants, little information is yet available on the regulation of m⁶A especially in the context of seed germination (Liang *et al.*, 2020).

This reversible modification is catalyzed by a conserved m⁶A methyltransferase complex. In *Arabidopsis*, the m⁶A writer complex consists of the mRNA adenosine methyltransferase catalytic subunit MTA (ortholog of the mammals METTL3), MTB (ortholog of the mammals METTL14) responsible for the orientation of the mRNA towards MTA and the FKBP12 INTERACTING PROTEIN 37 KD (FIP37) (ortholog of WTAP) (Zhong *et al.*, 2008; Yue *et al.*, 2019; Arribas-Hernández & Brodersen, 2020; Liang *et al.*, 2020). While MTA, MTB and FIP37 represent the core of the writer complex, a homolog of human VIRMA (KIAA1429) (*Drosophila*–Virilizer (VIR)) along with the homolog of human HAKAI (an E3 Ubiquitin ligase) have also been shown to be involved in m⁶A methylation (Růžička *et al.*, 2017). The m⁶A writer complex recognizes the consensus motif RRACH (Růžička *et al.*, 2017). It is worth noting that not all RRACH motifs are associated with m⁶A modifications, thus implying that the mechanisms behind this process are still unknown (Luo *et al.*, 2014). It is however admitted that m⁶A sites are enriched towards stop codons and the 3' UTR (Yue *et al.*, 2019). Methyl groups can be removed by m⁶A demethylases. The proteins ALKBH9A/B/C and ALKBH10A/B/C (orthologs of ALKBH5) function as m⁶A erasers. m⁶A exerts its function by recruiting reader proteins. 11 EVOLUTIONARY CONSERVED C-TERMINAL REGIONS (ECTs) have been identified in *Arabidopsis* to date and ECT2 has been shown to bind to m⁶A *in vivo* (Yue *et al.*, 2019; Arribas-Hernández & Brodersen, 2020; Liang *et al.*, 2020). Considering the evolutionary conservation and similarities in key elements of writers, erasers and readers, it is highly possible that this mRNA modification might control mRNA fate in a very tight manner (Liang *et al.*, 2020).

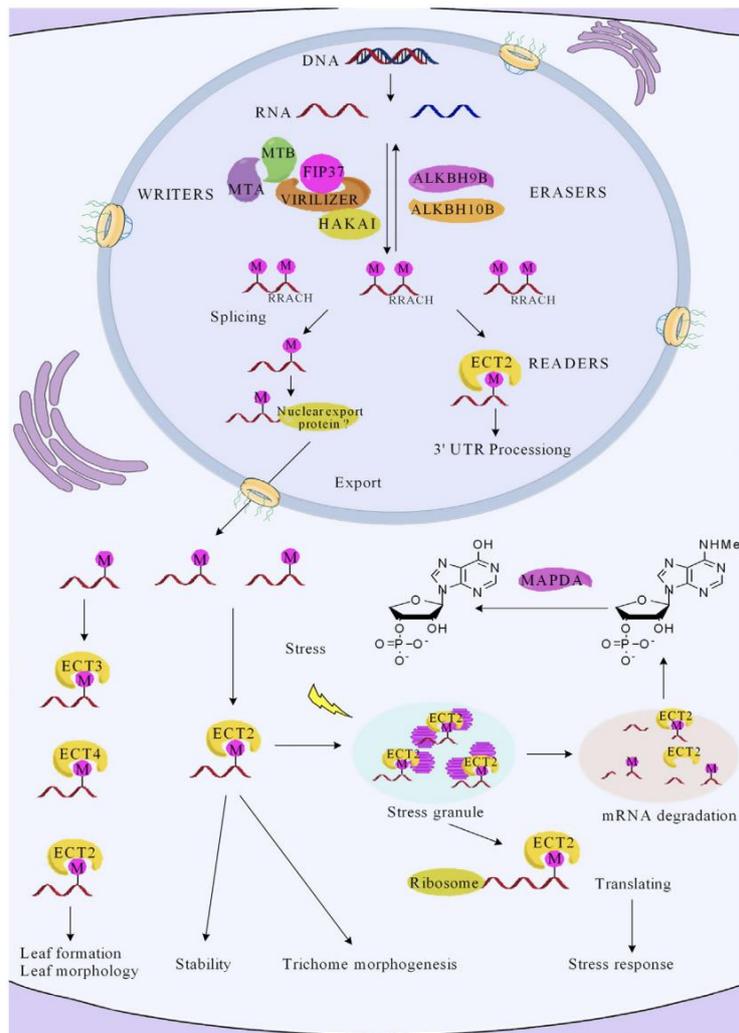


Figure 15- A representation view of the functioning of m6A in plant cells. (Yue et al 2019)

15. General context of the research on seed germination

In a context of global warming and changing climate, acquiring new knowledge in seed biology is a major challenge in the forthcoming years. Expected shifts in germination phenology resulting from global climatic changes will directly influence population dynamics in natural ecosystems and productivity of agrosystems (Walck *et al.*, 2011). In this context seed dormancy, which regulates seed germination, will be strongly modified, which makes research on its mechanisms critical since this trait plays a fundamental role in agriculture performance, food security and shaping of natural landscapes.

Dormancy has been studied for decades and its physiology is well documented and investigated (Baskin et Baskin). The theory of the hormonal balance, which postulates that the antagonist relationship of ABA and GA regulates germination, has also been proposed years ago and is still valid. Recent and significant progresses in the understanding of their

mechanisms of action have been made due to the development of high throughput technologies, from sequencing to phenotyping. Despite these advances, the influence of environmental or endogenous signals fine-tuning ABA and GA metabolisms is far from being known. These two hormones must also be considered in a large cellular signalling network where other hormones, nutritional factors, and ROS are also likely to play a key role. In addition, the dogma of transcriptional regulation of seed germination is less and less pertinent. It becomes clear that mRNA fate is also playing a key role in this germination but we are relatively ignorant of the molecular components involved in their metabolism during seed germination. Similarly, very little is known about the role of epigenetics in dormancy regulation, despite the increasing evidence claim for a strong involvement of these mechanisms in seed germination.

It is within the framework of this general research context that the present work has been designed and carried-out, with the main objective to bring new pieces of understanding of this fascinating regulatory event of plant life that is dormancy. Using a wide array of approaches and techniques, we provide here insights, from cell signalling to downstream molecular events, that should open numerous perspectives of research in seed biology.

Chapter 2

**Retrograde signaling from the
mitochondria to the nucleus
translates the positive effect of
ethylene on dormancy breaking
of *Arabidopsis thaliana* seeds**

Retrograde signaling from the mitochondria to the nucleus translates the positive effect of ethylene on dormancy breaking of *Arabidopsis thaliana* seeds

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NB: Supporting figures for this chapter are provided in the Annex Chapter 1 and the table are available on google drive using the following link: <https://drive.google.com/drive/folders/1wr387PSsQ2iaoT6dzSyZ1shltEAu2Cdn?usp=sharing>

NB: This chapter contains 6 Figures, 6 Supplemental figures and 2 Supplemental tables in Annex, and 3 Supplemental tables on Google Drive.

1. Summary

Ethylene and Reactive Oxygen Species (ROS) regulate seed dormancy alleviation, but the molecular basis of their action and crosstalk remains largely unknown.

Here we studied the mechanism of *Arabidopsis* seed dormancy release by ethylene using cell imaging, genetic and transcriptomics approaches, in order to tackle its possible interaction with ROS homeostasis.

We found that the effect of ethylene on seed germination required ROS production by the mitochondrial electron transport chain. Seed response to ethylene involved mitochondrial retrograde response (MRR) through nuclear ROS production and up-regulation of the MRR components *AOX1a* and *ANAC013*, but it also required the activation of the ethylene canonical pathway.

Together our data allow deciphering the mode of action of ethylene on seed germination and the associated dynamics of ROS production. Our findings highlight the occurrence of retrograde signaling in seed germination.

Keywords: *Arabidopsis*, dormancy; ethylene; mitochondrial retrograde signaling; reactive oxygen species; seed

2. Introduction

Reactive oxygen Species (ROS) are one of the major regulators of seed germination. Their homeostasis is likely to play a role in the translation of environmental factors into molecular mechanisms associated with germination (see Bailly, 2019, for review). It is postulated that controlled ROS production during seed imbibition is a prerequisite for radicle elongation during the germination process and that the prevention of seed germination under inappropriate environmental conditions would result from an excessive ROS accumulation causing oxidative damage (Bailly *et al.*, 2008; Bailly, 2019). Seed germination is also controlled by seed dormancy which is an endogenous blockage of seed germination and is defined as an inability of seeds to germinate under apparently favorable environmental conditions (Bewley, 1997). The prevention of germination of dormant seeds would be associated with insufficient ROS production during their imbibition, ROS levels being then too low for activating the redox-related molecular events involved in the completion of germination.

Despite increasing evidence suggesting that ROS are regulatory actors of seed germination, the mechanisms involved in the control of their homeostasis during seed

imbibition are far from being understood. Mitochondria, through the mitochondrial electron transport chain (mETC), and NADPH oxidases (also known as respiratory burst oxidase homologues, RBOHs) are suspected as being the major sources of ROS production in imbibed seeds before radicle protrusion (Bailly, 2019). In the late phases of seed germination, i.e. at the time of cell elongation, apoplastic ROS resulting from the activity of amine oxidases or of other apoplastic enzymes play a role in cell wall loosening (Bailly, 2019). However, the role of mitochondria as a central hub for ROS production during the germination has never been clearly addressed, despite increasing attention paid to the role of this organelle in the germination process (eg Paszkiewicz *et al.*, 2017; Ma *et al.*, 2019; Nonogaki, 2019; Nietzel *et al.*, 2020). Mitochondrial metabolism rapidly resumes during seed imbibition and is absolutely required to provide energy to the germinating embryo (Law *et al.*, 2014; Paszkiewicz *et al.*, 2017). Whether the ROS generated by the mETC during seed germination play a regulatory role in this process is nevertheless not known. In addition, mitochondrion, alongside the nucleus, is the core component of a process termed mitochondrial retrograde regulation (MRR), or mitochondrial retrograde signalling, and it is now widely admitted that mitochondria play a major role in cell signalling (Law *et al.*, 2014; Ng *et al.*, 2014). MRR describes signals originating from mitochondria which induce nuclear transcriptional reprogramming (Butow & Avadhani, 2004; Rhoads & Subbaiah, 2007; Ng *et al.*, 2014). MRR has mostly been described in response of plants to biotic (Colombatti *et al.*, 2014) and abiotic stresses (Rhoads & Subbaiah, 2007). Although the nature of the signal relaying information from mitochondria to nucleus is not known, it has been suggested that mitochondrial ROS (mtROS) might play this role (Huang *et al.*, 2016; Wagner *et al.*, 2018). Multiple upstream regulatory factors of MRR have also been identified these last years (see Giraud *et al.*, 2009; Ng *et al.*, 2013; De Clercq *et al.*, 2013 for review). For example they include transcription factors from the NAC (No apical meristem, ATAF1/2 and CUC2 cup-shaped cotyledon) family, notably ANAC017 and ANAC013 which localize to the ER and which proteolytic cleavage translocate them to the nucleus (De Clercq *et al.*, 2013; Ng *et al.*, 2013). At the downstream level, MRR trigger the up-regulation of AOX (*ALTERNATIVE OXIDASE*), which is commonly used as a marker to investigate MRR. AOX is an alternative mitochondrial terminal oxidase which couples the oxidation of ubiquinol with the reduction of oxygen to water in mitochondria (Vanlerberghe, 2013). In *Arabidopsis* AOX is encoded by the two gene family AOX1 and AOX2 and AOX1a is the most expressed (Vanlerberghe, 2013).

Interestingly the possible role of an interaction between ROS and plant hormones in the context of seed germination progressively emerged these former years. It has been demonstrated that ROS would interact with the metabolic and signalling pathways of the hormones involved in the regulation of seed germination and dormancy, in particular abscisic acid (ABA), a promotor of dormancy, and gibberrelins (GAs) and ethylene, promoters of germination (Finch-Savage & Leubner-Metzger, 2006; Finkelstein *et al.*, 2008; Nambara *et al.*, 2010; Corbineau *et al.*, 2014). Links between MRR, ROS and hormone signaling have been recently demonstrated (Berkowitz *et al.*, 2016). It has been proposed that mtROS could play a direct role in ABA signaling and sensitivity (He *et al.*, 2012; Nonogaki, 2019). It has also been shown that ethylene could promote signaling from mitochondria to nucleus in *Arabidopsis* (Berkowitz *et al.*, 2016). Ethylene is a gaseous plant hormone which regulates a number of developmental processes including vegetative growth, flowering time, fruit ripening, organ senescence and abscission, and seed germination (Yoo *et al.*, 2009; Muday *et al.*, 2012; Arc *et al.*, 2013; Corbineau *et al.*, 2014). Ethylene improves the germination of non-dormant seeds incubated in non-optimal environmental conditions (see Corbineau *et al.*, 2014, for review) including *Arabidopsis* (Wang *et al.*, 2018). The ethylene signaling cascade is well described in *Arabidopsis* (Alonso & Stepanova, 2004; Guo & Ecker, 2004). Ethylene is perceived by a group of receptors which include ETR1 (ethylene receptor 1), ETR2, ERS1 (ethylene response sensor 1), ERS2, and EIN4 (ethylene insensitive 4) (Chang & Stadler, 2001). In the presence of ethylene, the inactivation of the receptors and CTR1 (constitutive response 1) suppresses the phosphorylation state of a membrane protein located at the endoplasmic reticulum, ETHYLENE INSENSITIVE 2 (EIN2), resulting in its proteolysis and its transition to the nucleus (Qiao *et al.*, 2012; Wen *et al.*, 2012). In the nucleus, the components acting downstream from EIN2 are two major transcription factors, ETHYLENE INSENSITIVE 3 (EIN3) and its homolog EIN3-LIKE 1 (EIL1), which regulate the majority of gene expression mediated by ethylene, including ethylene response factors (ERFs). The positive effect of ethylene on seed germination can be explained by its antagonistic effect with ABA metabolic and signaling pathways (Arc *et al.*, 2013) and by a crosstalk with GAs signaling pathway (Corbineau *et al.*, 2014; Wang *et al.*, 2018). In addition it has also been proposed that ethylene and ROS could act in concert to control seed dormancy release (Corbineau *et al.*, 2014; El-Maarouf-Bouteau *et al.*, 2014).

Here we have taken advantage of the beneficial effect of ethylene on *Arabidopsis* seed dormancy alleviation to investigate the dynamics and localisation of ROS production in germinating seeds. Our findings highlight the mechanisms involved in the crosstalk

between ROS and ethylene and show that ethylene stimulates germination through the mitochondrial production of ROS and activation of MRR.

3. Material and methods

3.1 Plant Material

Arabidopsis (*Arabidopsis thaliana*) wild type seeds (ecotype Columbia, Col-0) were used in this study. Seeds of various mutants, were obtained from the same genetic background (see list of accessions in Table S1). The provided *rbohDB* double mutant seeds were obtained by crossing the *rbohB* and *rbohD* mutants together (Wang *et al.*, 2012). RoGFP-expressing lines were in Col-0 background and have been described by Jiang *et al.* (2006). All seeds were sown in soil and placed in growth chambers (21°C) under a photoperiod of 16 h per day with a light intensity of 150 and 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Seeds were harvested at maturity and stored at -20°C to maintain their dormancy until further use.

3.2 Germination assays

Seeds (75 seeds x 3 replicates) were sown in 9 cm Petri dishes on a filter paper moistened with 10 ml deionized water or with solutions indicated in the text and placed in the darkness at 15 or 25°C. Respiratory inhibitor solutions were prepared by first dissolving the powder in 2mL dimethylsulfoxide and the volume was completed to 100 mL with deionized water. Ethylene treatment was carried out by placing seeds into 360 mL gas-tight Petri dishes in which was injected 0.08 or 0.8 mL gaseous ethylene (5 %, Air Liquide, Paris, France) with a syringe, in order to obtain a final concentration of 10 or 100 ppm of ethylene, respectively. Germination was monitored on a daily basis for 10 days and a seed was considered as germinated when the radicle pierced the seed coat.

3.3 Hydrogen peroxide measurements

The quantification of hydrogen peroxide was carried out with ca. 30 mg FW of dry seeds using a peroxidase based assay in the presence of MBTH (3-methyl-2-benzothiazolinone hydrazone) and DMAB (3-dimethylaminobenzoic acid) as described by Leymarie *et al.* (2012). Results correspond to the mean \pm SD of 3 replicates and are expressed per mg of the initial FW of dry seeds.

3.4 Cell imaging

Fluorescence of mitochondrial-roGFP (mit-roGFP) seeds and of the various fluorochromes used in this study seeds was visualized by confocal laser using a Leica TCS SP5 confocal microscope. In all cases seeds were imbibed in the conditions indicated in the text and the seed coat was gently removed under a binocular prior confocal imaging.

Dormant mit-roGFP seeds were used for assessing mitochondrial redox status. Mit-roGFP seeds were issued from plants genetically transformed to express the reduction-oxidation-sensitive green fluorescent protein roGFP1 in mitochondria as described by Jiang *et al.* (2006). Decoated mit-roGFP seeds were excited at 405 nm (oxidized roGFP) and 488 nm (reduced roGFP) and the resulting fluorescence was detected in a 492-560 nm window. Total amount of reduced and oxidized roGFP was determined by measuring fluorescence at 405 and 488 nm after 10 min incubation of mit-roGFP seeds in 10 mM Dithiothreitol (DTT) or in 10 mM hydrogen peroxide, respectively. These values were used for calculation of the degree of oxidation and the redox potential. Fluorescence was quantified using ImageJ software. The degree of oxidation of the ro-GFP and redox potential were calculated according to Schwarzländer *et al.* (2008).

DCFH-DA (2',7'-Dichlorodihydrofluorescein diacetate, 25 μ M) was used for ROS localization as described by Leymarie *et al.* (2012). Mitochondria were detected using MITO-ID Red dye according to the protocol provided by the manufacturer (Enzo Life Sciences Inc, USA). Seeds were incubated for 20 min at 20 °C in the MITO-ID dye and washed twice for 20 min at 20 °C in the MITO-ID buffer. Seeds were excited at 561 nm and the fluorescence was detected in a 571-800 nm window. Nuclei were detected using 4',6-diamidino-2-phenylindole (DAPI, 1 μ g/ μ L) (Merck KGaA, Darmstadt, Germany). Seeds were placed on the slide for 10 min in darkness in presence of DAPI (1 μ g/ μ L). They were excited at 405 nm and the fluorescence was detected in a 415-470 nm window. The image treatment was performed using imageJ software.

3.5 RNA extraction and qRT-PCR

Fifty mg of seeds were ground in liquid nitrogen and total RNA was extracted according to Zhang *et al.* (2019). Reverse transcription was performed with 2 μ g total RNA as described by Leymarie *et al.* (2012). Relative expression was calculated according to Hellemans *et al.* (2007) with *CBE-5* (AT5G53560), *TIP41* (AT4G34270) and *RHIP1* (AT4G26410) as reference genes. Primer sequences are listed in Table S2.

3.6 Transcriptomic analysis

Total RNA was extracted from 50 mg of seeds, treated with DNase (ThermoFisher, Waltham, MA, USA) and purified using the NucleoSpin™ RNA clean-up XS (MACHEREY-NAGEL). The sequencing technology used was an Illumina NextSeq500 (IPS2 POPS Platform). RNA-seq libraries were performed by TruSeq_Stranded_mRNA_SamplePrep_Guide_15031047_D protocol (Illumina®, California, U.S.A.). The RNA-seq samples were sequenced in single-end (SE) with a read length of 75 base pairs. 21 samples by lane of NextSeq500 using individual bar-coded adapters and giving approximately 20 million of SE reads by sample were generated. All steps of the experiment, from growth conditions to bioinformatic analyses, were managed in CATdb database (Gagnot *et al.*, 2007), <http://tools.ips2.u-psud.fr/CATdb/> ProjectID NGS2018_10_Ethylene according to the international standard MINSEQE 'minimum information about a high-throughput sequencing experiment'. Transcriptomic data from this article were deposited at Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>, accession GSE151223).

The transcripts were classified according to the Gene ontology Tool within the Bioinformatics Group at the Lewis-Sigler Institute (Princeton University). GO results were visualized in 2D scatterplots using the software REVIGO (Supek *et al.*, 2011). REVIGO relies on the algorithmic clustering of the data according to semantic similarity measurements. The disc color gradient indicates the degree of GO enrichment according to the p-value while the disc size is proportional to the frequency of the GO in the database.

Differentially abundant transcripts (Fold change $> |2|$, Bonferroni < 0.05) after 24 h of imbibition were compared to the ROS clusters generated by Willems *et al.* (2016) in search of matches for each cluster. The same analysis was conducted for the clusters corresponding to the mitochondrial retrograde signalling (gene list in Table S3, established from De Clercq *et al.*, 2013), the nuclear genes encoding mitochondrial proteins (gene list in Table S4, established from Ng *et al.*, 2014) and the genes encoding the mitochondrial genome (gene list in Table S5, extracted from the RNA-Seq data).

Heatmaps corresponding to differentially expressed genes (DEG) (Fold change $> |2|$, Bonferroni < 0.05) belonging to ABA and GAs metabolism and signaling were obtained using R software from the gene lists provided as Tables S6 (Wang *et al.*, 2011) and S7 (Ogawa *et al.*, 2003).

4. Results

4.1 The effect of ethylene on germination of dormant seeds requires mitochondrial ROS production

Wild-type fully germinated within 2 days at 15°C whereas their germination was limited to 20 % at 25°C thus revealing seed dormancy (Leymarie *et al.*, 2012) (Fig. 1a). Ethylene treatment (100 ppm) broke *Arabidopsis* seeds (Col-0) dormancy since dormant seeds became able to germinate as well at 25°C as at 15°C (Fig. 1a). In order to determine whether the mitochondrial activity could participate in the response to ethylene, wild-type seeds (Col-0) were incubated in the presence of different respiratory inhibitors and treated with 10 ppm of ethylene (instead of 100 ppm to avoid an ethylene saturation which could mask the effect of the respiratory inhibitors).

Seeds were treated with either rotenone (ROT, $5 \cdot 10^{-5}$ M), a mitochondrial complex I inhibitor (Garmier *et al.*, 2008), salicylhydroxamic acid (SHAM, 10^{-4} M) an inhibitor of the mitochondrial alternative oxidase (Selinski *et al.*, 2018a), and antimycin A (AA, $5 \cdot 10^{-6}$ M) a mitochondrial complex III inhibitor (Sweetlove *et al.*, 2002). At all the low concentrations used here, we verified that the inhibitors did not have any inhibitory effect on seed germination at 15°C (Fig. 1b), but that they had a negative effect on ROS production (Fig. S1). AA, ROT and SHAM markedly decreased the beneficial effect of ethylene on germination of dormant seeds since in all cases germination in the presence of ethylene did not exceed 50 % (Fig. 1c).

Hydrogen peroxide (H_2O_2) content increased during imbibition of dormant seeds at 25°C and reached a maximum of $40 \mu\text{mol} \cdot \text{g}^{-1}$ FW after 24 h (Fig. 1d). It followed a similar trend in seeds treated with ethylene but it increased sharply at 24 h and reached a higher value than in dormant seeds, close to $60 \mu\text{mol} \cdot \text{g}^{-1}$ FW (Fig. 1d). It is also worth noting that at after 6 h of imbibition, ethylene treated seeds produced more than 2 times hydrogen peroxide than the untreated ones (Fig. 1d). When the ethylene treatment was performed in the presence of the respiratory inhibitors, ROT, SHAM and AA, H_2O_2 production was very low and did not exceed $20 \mu\text{mol} \cdot \text{g}^{-1}$ FW at 24 h (Fig. 1d).

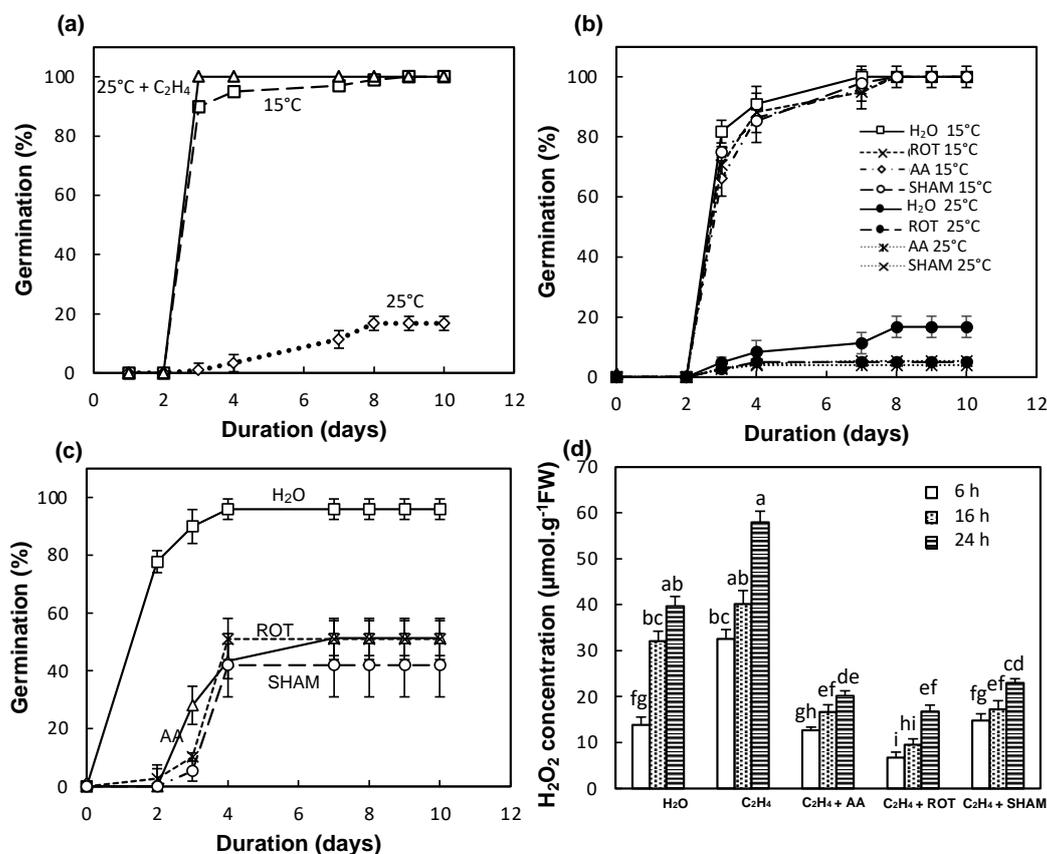


Fig. 1 Ethylene releases seed dormancy and induces ROS production. (a) Germination of dormant Col-0 seeds on water at 15°C and at 25°C in the dark with or without ethylene (100ppm). (b) Effect of Rotenone (ROT, 5.10⁻⁵ M), Antimycin A (AA, 5.10⁻⁶ M) and Salicylhydroxamic Acid (SHAM, 10⁻⁴ M) on the germination of dormant Col seeds incubated on water at 15°C and 25°C in the dark. (c) Effect of Rotenone (ROT, 5.10⁻⁵ M), Antimycin A (AA, 5.10⁻⁶ M) and Salicylhydroxamic Acid (SHAM, 10⁻⁴ M) on the germination of dormant Col seeds incubated on water at 25°C in the dark with ethylene. (d) Hydrogen peroxide production in Col-0 seeds incubated on water at 25°C in the dark in presence or in absence of ethylene and in presence of AA (5.10⁻⁶M) ROT (5.10⁻⁵M) and SHAM (10⁻⁴M) with ethylene. Data are means of 3 replicates ± SD. Different letters represent significantly different means with P < 0.05 according to Kruskal Wallis.

Lastly, we also investigated whether the response to ethylene could involve the activation of the ROS producing enzymes NADPH oxidase and monoamine oxidase. Dormant seeds of the double mutant *rbohDB* and the single mutant *mao* fully germinated at 25°C in the presence of ethylene (Fig. S2). We also determined whether EIN2, a canonical component of ethylene response in plants was involved in seed dormancy alleviation in presence of ethylene. Seeds of *ein2* mutants were insensitive to ethylene and unable to germinate at 25°C in the presence of ethylene (Fig. S2).

Since our results suggested that mitochondrial activity and ROS production might play a role in the response of dormant seeds to ethylene we next investigated ROS

production within the mitochondria using mit-roGFP constructs. Fluorescent foci on Fig. 2a to 2d revealed fluorescence production at the mitochondrial level in embryonic axes of mit-roGFP seeds placed for 24 h at 25°C, in the presence or absence of ethylene. Fluorescence emitted after excitation at 405 nm (Fig. 2a and 2c) corresponded to the oxidized state of ro-GFP whereas the one emitted in response to an excitation at 488 nm (Fig. 2b and 2d) revealed its reduced state. In the presence of ethylene ro-GFPs were predominantly in an oxidized state as shown by fluorescence emission after excitation at 405 nm (Fig. 2c), whereas in the absence of ethylene ro-GFPs were mostly in a reduced state as shown by fluorescence emission after excitation at 488 nm (Fig. 2b). This observation was confirmed by the increase of both oxidation degree and redox potential with time, which was magnified in the presence of ethylene (Fig. 2e). From 6 to 24 h of imbibition at 25°C, the mitochondrial redox potential increased from -325.8 mV to -311.32 mV in the absence of ethylene and from -298.88 mV to -276.49 mV in the presence of ethylene (Fig. 2e). Corresponding images are shown as Fig. S3.

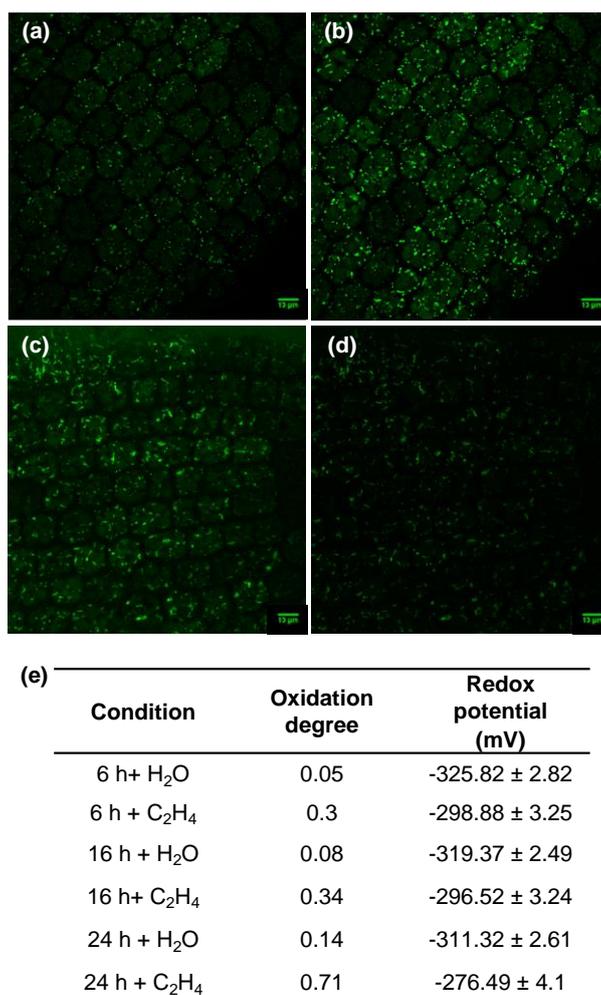


Fig. 2 Ethylene modifies seed mitochondrial redox status. Fluorescence images of embryonic axes of dormant mitochondrial ro-GFP seeds incubated on water for 24 h at 25°C in the dark and in absence (a and b) or in presence (c and d) of 100 ppm ethylene after excitation at 405 nm (a and c) or 488 nm (b and d) (e) Oxidation degree and redox potentials calculated from fluorescence data obtained with dormant mit ro-GFP seeds incubated for 6, 16 and 24 h on water at 25°C in the dark and in absence or presence of 100 ppm ethylene. Data are means of 12 replicates ± SD.

4.2 Involvement of retrograde signaling in the response to ethylene

To get further insights on the spatiotemporal ROS production in response to ethylene we investigated intracellular ROS localization within the hypocotyl region of the embryonic axes of seeds imbibed with and without ethylene (Fig. 3). After 6 h of imbibition at 25°C, with or without ethylene, ROS production sites, estimated by DCFH-DA fluorescence, were mostly localized within mitochondria which appeared as small green foci along the cell wall (Fig. 3 a,e). Mitochondria were formally identified using the mitochondrial marker Mito-ID Red (Fig. 3b,f,j,n,r,v). DAPI fluorescence identified nuclei but also revealed non-specific autofluorescence in the cell wall (Fig. 3 c,g,k,o,s,w). After 16 h of imbibition in

the presence of ethylene, DCFH-DA localized in both mitochondria and in the nucleus, as shown by the light blue colour (Fig. 3p), whereas ROS production of untreated dormant seeds remained exclusively mitochondrial (Fig. 3l). The same pattern of ROS localization was observed after 24 h of imbibition for treated and untreated seeds (Fig. 3t and 3x). Control images are shown in Fig. S4. Importantly we also examined whether nuclear ROS production could be induced by ethylene in the presence of respiratory inhibitors, since these compounds abolished the beneficial effect of ethylene on breaking of dormancy (Fig. 1). Figs S5 and S6 show that 16 h (Fig. S5) and 24 h (Fig. S6) of ethylene treatment in the presence of ROT, SHAM or AA prevented nuclear ROS production.

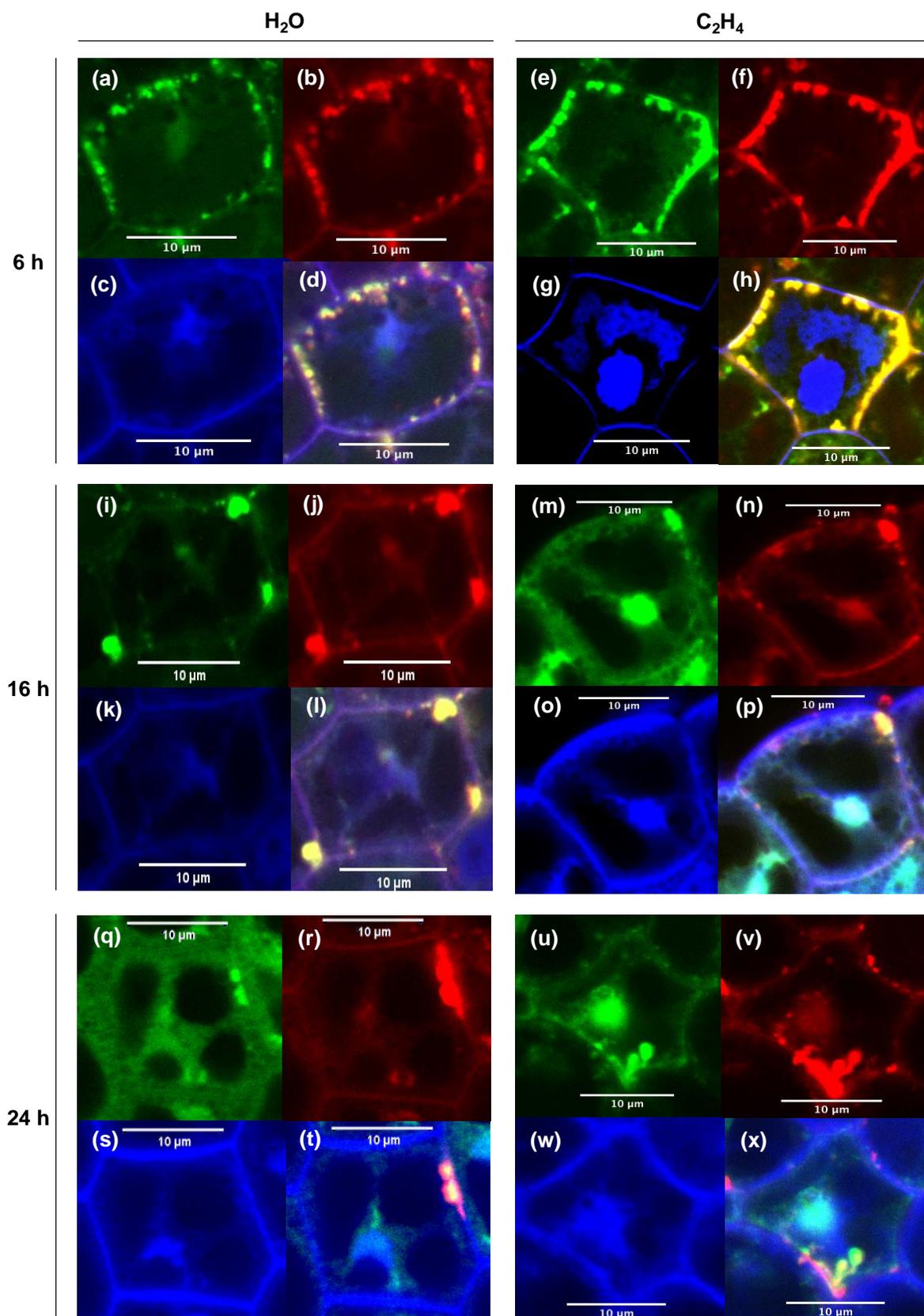


Fig. 3 Ethylene induces nuclear ROS production. Fluorescence images of cells of embryonic axis of dormant Col-0 seeds incubated for 6, 16 and 24 h on water at 25°C in the dark in absence (a-d, i-l, q-t) or presence (e-h, m-p, u-x) of 100 ppm ethylene and treated by DCFH-DA (a, e, i, m, q, u), Mito-ID (b, f, j, n, r, v), DAPI (c, g, k, o, s, w). (d, h, l, p, t, x) merged images.

At this stage the presence of ROS within mitochondria and subsequently into the nucleus of ethylene treated seeds suggested a possible involvement of mitochondrial retrograde signalling in the germination process. To test this hypothesis, we next investigated the response to ethylene of a set of dormant seeds of mutants altered in the components of the retrograde signaling pathway (Fig. 4). We also investigated the expression of genes known to be associated with MRR in plants (Fig. 5). They included *AOX1a* (which codes for the alternative oxidase in the mitochondrial electron transfer chain and is classically used as a retrograde signaling marker (Ng *et al.*, 2013b)), *ANAC017* (encodes the transcription factor of the NAC family, located in the endoplasmic reticulum, and is a positive regulator of the alternative oxidase (Ng *et al.*, 2013b)), *ANAC013* (encodes a transcription factor of the NAC family located in the endoplasmic reticulum and acts downstream of *ANAC017* to regulate the expression of the alternative oxidase (De Clercq *et al.*, 2013)), *NDUFS4* (codes for a fragment of the subunit 4 of NADH dehydrogenase of the complex I of the electron transfer chain (Meyer *et al.*, 2009)), *ABO6* (codes for a DEXH box RNA helicase involved in the splicing of several complex I genes of the electron transfer chain (He *et al.*, 2012)), *PrxIIIF* (encodes an enzyme that breaks down hydrogen peroxide in mitochondria (Finkemeier *et al.*, 2005)), *TIM23-2* (codes for the subunit 2 of the mitochondrial membrane translocase, is present in complex I and ensures the transport of proteins in the mitochondrial membrane (Wang *et al.*, 2012)), *MSD1* (codes for a superoxide dismutase involved in the conversion of ROS present in the mitochondrial matrix (Kliebenstein *et al.*, 1998)) and *ABI4* (codes for a member of the DREB subfamily A-3 of ERF/AP2 transcription factor family and is involved in ABA signal transduction (Wind *et al.*, 2013)).

At 15°C, seeds of *anac013*, *anac017*, *tim23-2*, *prxIIIF* and *aox1a* mutants completed their germination after 4 days of imbibition (Fig. 4a). At 25°C germination did not exceed 30 % for seeds of Col-0, *anac017*, *tim23-2* and 20% for seeds of *anac013* and *aox1a* (Fig. 4b), thus revealing dormancy. Following the addition of 100 ppm ethylene at 25°C, seeds of wild-type and mutants fully germinated after 4 days of imbibition except for seeds of

aox1a mutant whose germination did not exceed 75 % (Fig. 4c). Similar results were obtained in the presence of a lower concentration of ethylene, i.e. 10 ppm (Fig. 4d)

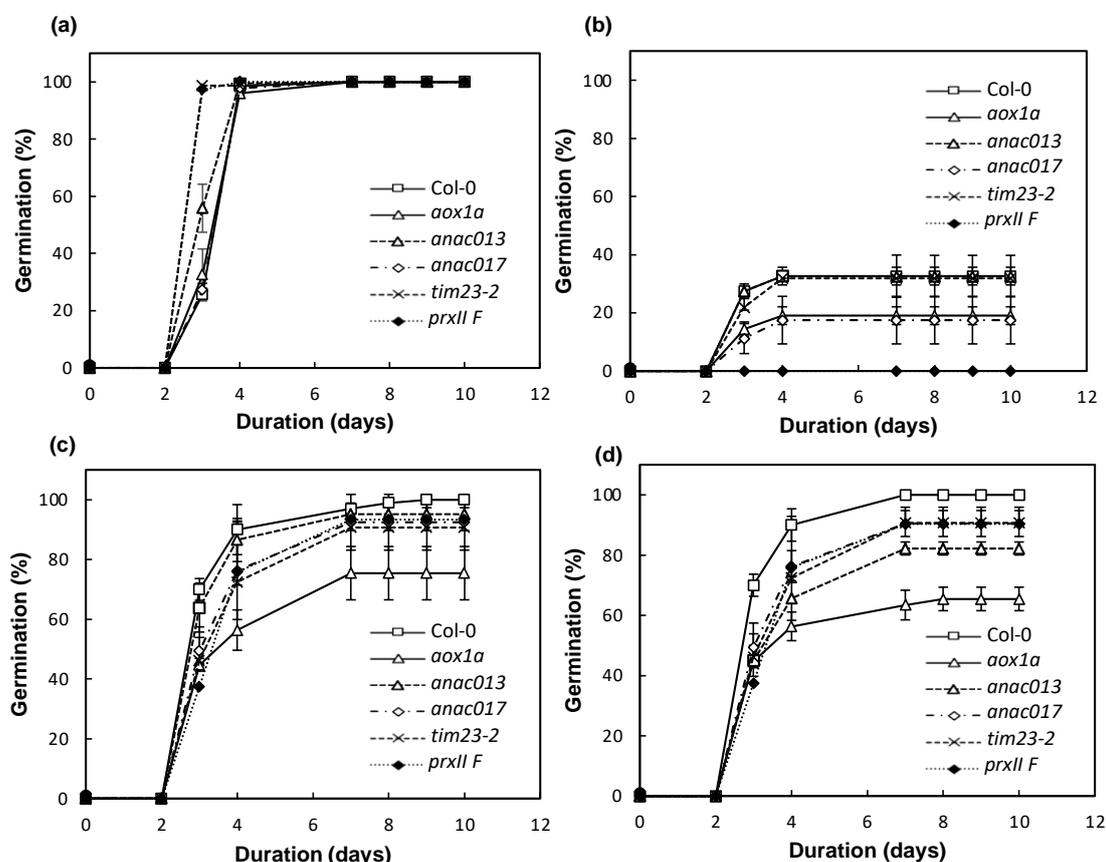


Fig 4 Seeds of *AOX1a* mutants do not fully respond to ethylene. Germination of freshly harvested seeds of Col-0, *aox1a*, *anac013*, *anac017*, *tim23-2* and *prxII F* on water and in the dark at 15°C (a) and 25°C (b) and at 25°C in presence of 100 ppm (c) and 10 ppm (d) ethylene. Data are means of 3 replicates \pm SD.

In the absence of ethylene few genes displayed significant changes in expression during seed imbibition at 25°C (Fig. 5b). The transcript level of *AOX1A* and *ANAC013* peaked after 6 h, the one of *MSD1* after 16 h and then their expression decreased (Fig. 5b). The abundance of *TIM23-2* transcripts increased regularly until 24 h of imbibition (Fig. 5b). Ethylene significantly stimulated the transcription levels of *AOX1A* and *ANAC013* after 6 h of imbibition (Fig. 5a). It also increased the transcription levels of *ABO6*, *TIM23-2* and *MSD1* (Fig. 5a). In contrast the abundance of *ABI4* transcripts was always lower in the presence of ethylene than in its absence (Fig. 5a and 5b). Fig. 5c and 5d shows the relative abundance of *AOX1a* (c) and *ANAC013* (d) transcripts in Col-0 seeds imbibed for 3 h and 6 h and in absence or in presence of ethylene, ROT and SHAM and AA. Ethylene clearly enhanced the transcript level of these 2 genes for both durations of imbibition, but, after 6 h, thi

stimulatory effect disappeared when seeds were imbibed in the presence of ROT, SHAM or AA.

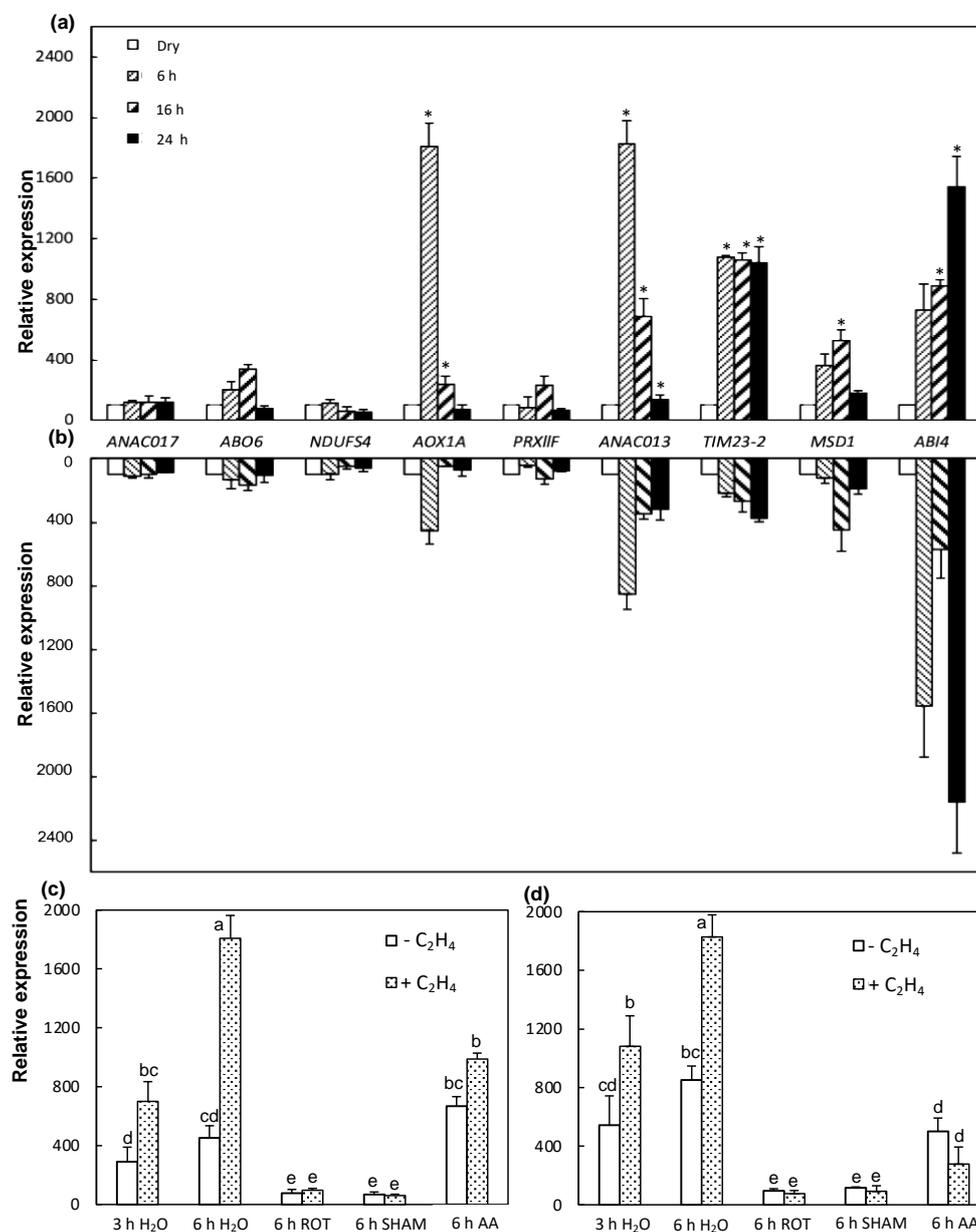


Fig 5 Ethylene positively regulates molecular markers of MRR. (a and b) Transcript abundance of *ANAC017*, *ABO6*, *NDUFS4*, *ABO6*, *PRXIIIF*, *ANAC013*, *TIM23-2*, *MSD1*, *ABI4* in dormant Col-0 seeds imbibed on water at 25°C in the dark for 6, 16 and 24 h in presence (a) or in absence (b) of 100 ppm ethylene. An arbitrary value of 100 was assigned to dry seeds. Data are means of 3 replicates \pm SD. Asterisks represent significantly different means when comparing water to ethylene with $P < 0.05$ according to Kruskal Wallis. (c and d) Transcript abundance of *AOX1a* (c) and *ANAC013* (d) in dormant Col-0 seeds imbibed on water at 25°C in the dark for 3 and 6 h and in absence or presence of ethylene, Rotenone (ROT, 5.10^{-5} M), Antimycin A (AA, 5.10^{-6} M) and Salicylhydroxamic Acid (SHAM, 10^{-4} M). Data are means of 3 replicates \pm SD. Different letters represent significantly different means with $P < 0.05$ according to Kruskal Wallis.

4.3 Transcriptome analysis confirmed the role of ROS and retrograde signaling in the response to ethylene

Lastly we performed a transcriptomic analysis in order to identify putative ROS and retrograde signaling transcriptional footprints among the differentially expressed genes in the presence of ethylene. The Venn diagram (Fig. 6a) compares the distribution of mRNA abundance after 24 h of seed imbibition in absence or in presence of ethylene (100 ppm). In the presence of ethylene, the abundance of 2092 transcripts specifically increased, whereas 1605 transcripts were less abundant after 24 h of imbibition (Fig. 6a). In contrast 591 genes were up-regulated and 646 were repressed after 24 h of imbibition on water (Fig. 6a). Putative functions were assigned to the ethylene up-regulated genes using gene ontology (GO) categorization and they were visualized as functional clusters following redundancy reduction using REVIGO (Fig. 6b). On this representation, colors represent the p-value and the size of the circles is relative to the frequency of GO terms. The ethylene up-regulated genes fell in various categories such "cellular process", "cellular metabolism", "cellular component organization" or "small molecule metabolism" (Fig. 6b).

We also used the so-called "ROS wheel" to investigate the specific transcriptional ROS signature in response to ethylene (Willems *et al.*, 2016a). The ROS wheel relies on transcriptional footprints and clusters together co-regulated genes in response to ROS. This approach clearly revealed a marked ROS transcriptional footprint associated with ethylene (Fig. 6c). In total, in presence of ethylene, 221 up-regulated genes and 45 down-regulated genes were identified within the ROS wheel, when less than 50 ROS related genes were identified among the transcripts found in untreated seeds. Among the ethylene over-expressed genes a large majority (163 genes) fell into the 3rd cluster (High Light (HL) early), 22 genes were specific to the 4th cluster and interestingly 12 genes were associated with retrograde signalling (Fig. 6c). Among the less abundant transcripts (C₂H₄ down, Fig. 6c), 11 were specific to retrograde signaling and 25 to HL signaling. In the absence of ethylene, no significant changes were observed for any of the clusters, excepted for the cluster III in which 27 less abundant transcripts were identified (Fig. 6c).

Fig. 6d shows the effect of ethylene on the regulation of genes affiliated to mitochondrial metabolism after 24 h of imbibition (Tables S3-S5). A total of 142 mitochondria-related genes were up-regulated in presence of ethylene compared to 25 in its absence and 62 genes were repressed in presence of ethylene and 31 in its absence. Ethylene dramatically affected expression of genes belonging to the 3 subcategories "mitochondrial retrograde signalling", "nuclear genes coding for mitochondrial proteins"

and “mitochondrial genome”. The progress towards seed germination induced by ethylene specifically modified the expression of genes from the mitochondrial genome, whereas seed imbibition on water had no effect. We also found that ethylene modified the expression of 3 times more nuclear genes coding for mitochondrial proteins than when seeds were imbibed on water.

At last, to complete this study, and because ABA and GAs are key players of the germination process, we also determined whether ethylene treatment could alter the expression of genes related to ABA and GA metabolism and signaling (Tables S6 -S7, Fig. 6e and 6f). The effect of ethylene on ABA related genes was mostly apparent after 24 h of imbibition, when the level of 17 transcripts among 57 in this cluster, became altered, either stimulated or repressed (Table S6; Fig. 6e). Among the 51 GAs-related genes, ethylene mainly lead to an over expression of 7 genes and repressed 2 genes (Table S7; Fig. 6f). The function and role of these differentially expressed genes are discussed below.

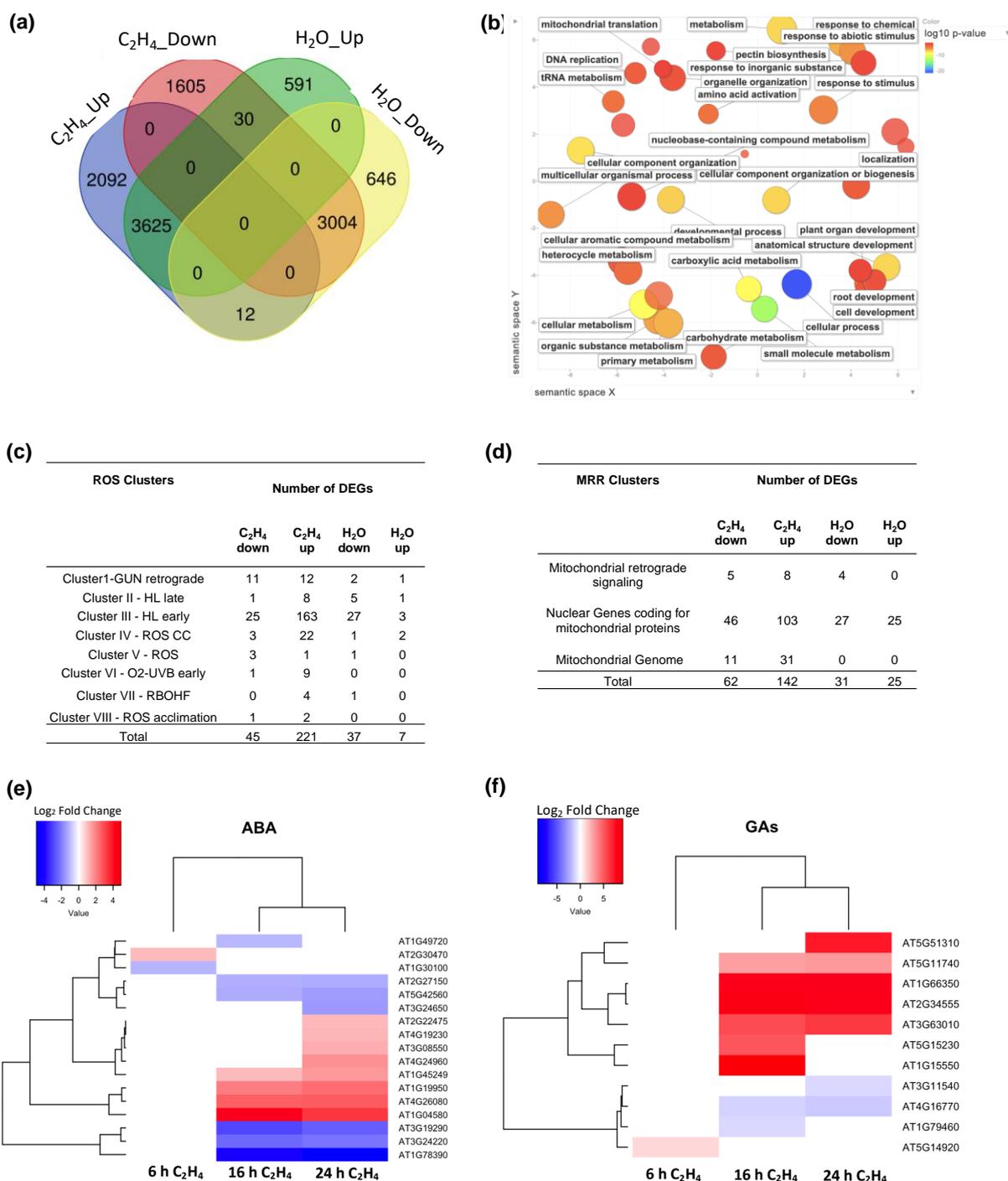


Fig. 6 Transcriptional footprints reveal involvement of MRR, ROS and hormone pathways in response to ethylene. (a) Venn Diagram showing the number of transcripts displaying significant increase in abundance (fold change absolute value >2, Bonferroni <0,05) in dormant Col-0 seeds after 24 h of imbibition at 25°C on water in the dark in presence or in absence of 100 ppm ethylene. (b) Gene ontology (GO) classification obtained using REVIGO of mRNAs specifically more abundant (fold change absolute value >2, Bonferroni <0,05) in the transcriptome of dormant Col-0 seeds imbibed on water in the dark at 25°C in presence of 100 ppm ethylene. (c) Number of up- or down- differentially expressed genes (DEGs) (fold change absolute value >2, Bonferroni <0,05) belonging to the transcriptional footprints of clusters I to VIII (columns) as defined by Willems et al. (2016) in dormant Col-0 seeds imbibed for 24 h on water in the dark at 25°C in absence or presence of 100 ppm ethylene. (d) Number of up- or down- differentially expressed genes (DEGs) (fold change absolute value >2, Bonferroni <0,05) belonging to the transcriptional footprints of MRR clusters (as defined in methods) in dormant Col-0 seeds imbibed for 24 h on water in the dark at 25°C in absence or presence of 100 ppm ethylene. (e, f) Heat maps showing DEGs (fold change absolute value >2, Bonferroni <0,05) related to ABA (E) or GAs (F) metabolism and signaling pathways in dormant Col-0 seeds imbibed for 24 h on water in the dark at 25°C in presence of 100 ppm ethylene.

5. Discussion

5.1 Relationship between ethylene signaling, ROS metabolism and mitochondria

Arabidopsis seeds are dormant at harvest, as shown by their inability to germinate at 25°C in darkness (Leymarie *et al.*, 2012). In the presence of ethylene, dormant seeds fully germinated in these conditions (Fig. 1) showing that ethylene released seed dormancy, as already demonstrated for several species (Mattoo, 2018; Kigel, 2017; Corbineau *et al.*, 2014), including *Arabidopsis* (Wang *et al.*, 2018). This effect has been proposed to rely, at least partially, on its antagonistic relationship with ABA metabolism and signaling (Arc *et al.*, 2013; Corbineau *et al.*, 2014). Ethylene has also been proposed to interact with ROS for regulating seed germination (Corbineau *et al.*, 2014; Oracz *et al.*, 2009; El-Maarouf-Bouteau *et al.*, 2014). In agreement with these results, we show here that the beneficial effect of ethylene on germination was associated with a stimulation of H₂O₂ production after only 6 h of imbibition (Fig. 1d). The sources of ROS production during seed germination are not clearly identified yet (Bailly, 2019). NADPH oxidases and apoplastic amine oxidases have been frequently proposed to be involved in ROS production during seed imbibition, even though apoplastic ROS production is likely to occur later in the germination process (Bailly, 2019). Importantly we showed that seeds of *rbohBD* double mutant or *mao* mutant fully responded to ethylene (Fig. S2) which suggested that mitochondria could be the main source of ROS production in this context, since they are often considered as a major source of ROS production during seed germination (Bailly, 2019). In this organelle, ROS are mainly produced in complexes I and III of the mitochondrial electron transport chain (mETC) (Murphy, 2009; Raha & Robinson, 2000; Moller, 2001). The superoxide anion is the main ROS produced in the mitochondria by the single-electron reduction of oxygen before being rapidly converted to hydrogen peroxide by superoxide dismutase (Fridovich, 1997; Murphy, 2009; Huang *et al.*, 2016). It is difficult to tackle the role of mETC in ROS production because mETC mutants often display strong pleiotropic phenotypes. For example seeds of mutants of complex I do not germinate (Fromm *et al.*, 2016). Thus, our strategy was to disrupt the electron transport chain using the respiratory inhibitors AA, SHAM and ROT. As expected, the respiratory inhibitors dramatically decreased ROS production in the presence of ethylene (Fig. 1d) and halved the beneficial effect of ethylene on germination (Fig. 1c). This shows that the mETC and the associated ROS production were crucial for the response to ethylene. This was confirmed by using seeds which constitutively expressed mit-roGFP.

We show that the redox status of the mitochondria shifted towards an oxidative state after 6 h (Fig. 2, Fig. S3) of seed treatment with ethylene, in agreement with changes in H₂O₂ content (Fig. 1d), thus suggesting a localized ROS production (Fig. 2). At the whole seed level, redox shifts towards more oxidizing states have already been demonstrated during germination and they were associated with H₂O₂ production (Gerna *et al.*, 2017; Gerna *et al.*, 2018). The values of mitochondrial redox potentials calculated from our experiments are in accordance with the ones found in the literature, reaching -276.49 mV after 24 h of ethylene treatment thus indicating a significant oxidized context (de Simone *et al.* 2017).

5.2 Mitochondrial retrograde signaling and response to ethylene

Here the enhanced ROS production in the mitochondria and the dynamics of ROS production strongly suggests that MRR might be involved in seed response to ethylene during germination (Petrov & Van Breusegem, 2012; Ng *et al.*, 2014; Dietz *et al.*, 2016). Whether seeds were treated or not with ethylene, mitochondria appeared to be the major intracellular source of ROS production during seed imbibition, thereby confirming the results obtained with mit-roGFP seeds. In seeds undergoing germination only, i.e. treated with ethylene, ROS were detected in the nuclei at the mid time to radicle protrusion, as already observed by Leymarie *et al.* (2012). Interestingly suppressing the effect of ethylene using respiratory inhibitors also suppressed nuclear ROS production (Figs S5 and S6), which suggests that the oxidative status of the nucleus might play a role in the completion of seed germination. Information about ROS production within the nucleus is still sparse (Mazars *et al.*, 2010). Although some data suggest that ROS could be produced within the nucleus, they are mostly thought as being transferred from other cell compartments to nucleus where they can activate gene expression (Exposito-Rodriguez *et al.* 2017; Martins *et al.*, 2018). H₂O₂ can leave mitochondria through permeability transition pores (Maxwell *et al.*, 2002) and can diffuse into the nucleus via nuclear pores (Rodrigues *et al.*, 2017). Foyer & Noctor (2016) also proposed that extensions from organelles (matrixules in that case) could associate the mitochondria to the nucleus thereby facilitating ROS exchange between organelles. Nevertheless, in our experiments, we did not observe that mitochondria were physically associated with nuclei upon ethylene treatment. Interestingly Kacprzak *et al.* (2020) very recently demonstrated that the involvement of ethylene in mitochondrial signalling in another plant system. This would suggest that ethylene might be a common factor controlling MRR.

Additional evidence of MRR were provided by studying the regulation of known molecular actors of this pathway and by following the germination of dormant seeds of the corresponding mutants in the presence of ethylene (Figs 4, 5). The transcript level of *AOX1a* dramatically increased in response to ethylene (Fig. 5a). Alternative oxidase is a well-known marker of the MRR response in plants (Clifton *et al.*, 2006; Rhoads & Subbaiah, 2007; Vanlerberghe, 2013; Ng *et al.*, 2014) and *AOX1a* is specifically used in *Arabidopsis* (De Clercq *et al.*, 2013). Recent studies have shown a strong correlation between the level of expression of *AOX1a* and mitochondrial ROS production (Maxwell *et al.*, 1999; Vanlerberghe, 2013). Here we show a dramatic and synchronous stimulation of mitochondrial ROS production (Figs 1d, 2e, 3h) and abundance of *AOX1a* transcripts (Fig. 5a) after 6 h of imbibition with ethylene. In contrast, *AOX1a* expression and nuclear ROS production were not synchronous (Fig. 3) which suggests that *AOX1a* induction relies on other mechanisms than a modulation of the nucleus redox status. *AOX1a* bypasses the complex III of the mETC and is responsible for the direct conversion of oxygen to water (Vanlerberghe, 2013) but one other key feature of this enzyme is its ability to control the levels of mitochondrial ROS (Maxwell *et al.*, 1999; Vanlerberghe, 2013). Interestingly it has been shown that AOX and ROS played an essential role in ethylene-induced drought tolerance in tomato (Zhu *et al.*, 2018). Accordingly, the germination of dormant seeds of *aox1a* mutant only partially responded to ethylene (Fig. 4c,d). In contrast, although ethylene strongly stimulated the transcription level of *ANAC013* (Fig. 5a), dormant seeds of *anac013* mutant fully responded to ethylene, which indicated that the effect of ethylene on seed germination was not strictly related to *ANAC013* signaling (Fig. 4c,d). It is worth noting that ethylene effect on seed germination is also likely to induce its canonical signaling pathway. Using the same experimental model Wang *et al.* (2018) demonstrated that the positive effect of ethylene on dormant seed germination was fully inhibited for dormant seeds of *etr1*, *ein2* and *ein4* mutants. Here we assessed the effect of exogenous ethylene on seeds of *ein2* mutants and observed the same behaviour (Fig. S2). This indicates that MRR involvement in ethylene responses requires a functional ethylene transduction pathway, at least from its early components, including *ein2*. Nevertheless, *ANAC013* is an MRR marker that is often detected in co-expression networks alongside *AOX1a* (Ng *et al.*, 2013; De Clercq *et al.*, 2013; Kleine & Leister, 2016). *ANAC013* acts downstream the alternative oxidase to positively regulate the expression of *AOX1a* (De Clercq *et al.*, 2013; Selinski *et al.*, 2018b) and it directly binds to the *AOX1a* promoter (Hofmann, 2013; Selinski *et al.*, 2018b). The straight correlation between ethylene response, ROS production and *AOX1a* and *ANAC013* expression was shown using respiratory inhibitors (Fig. 5c,d). Interestingly

the transcript level of *ABI4*, a repressor of *AOX1a* activated by ABA (Giraud *et al.*, 2009), decreased in presence of ethylene (Fig. 5), thus highlighting another regulatory module of the antagonist relationship between ethylene and ABA

5.3 Transcriptomic response to ethylene

The effect of ethylene on the transcriptome also confirmed the involvement of MRR in seed response to ethylene. After Bonferroni correction, 3697 and 1237 transcripts were found to be differentially abundant in presence and in absence of ethylene, respectively (Fig. 6a), which is comparable to changes in gene expression following the transition from dormancy to germination states published elsewhere (Holdsworth *et al.*, 2008; Dekkers *et al.*, 2013; Basbouss-Serhal *et al.*, 2015). The more abundant transcripts in presence of ethylene belonged to GO categories such as "cellular process" and "developmental process" and "cellular metabolism" (Fig. 6b). Nevertheless our objective was to gain further insight on the involvement of MRR in the specific seed response to ethylene using known transcriptomic footprints. The ROS wheel indicates how ROS are able to specifically reshape the transcriptome (Willems *et al.*, 2016a) and it clearly demonstrated the occurrence of ROS metabolism in response to ethylene (Fig. 6c). Most of the up-regulated genes by ethylene belonged to the cluster "Early HL", but this cluster has been described as containing a very large amount of transcripts (≤ 800 , Willems *et al.*, 2016) with a strong enrichment in genes responding to oxidative stress, thus this was not surprising to see it over-represented here. Interestingly, in the presence of ethylene, a set of transcripts fell into the cluster "GUN retrograde signalling", in accordance with our previous findings. We also investigated the involvement of ethylene in the regulation of genes related to mitochondrial metabolism. Ethylene clearly modified the transcript level of MRR related genes, of nuclear genes coding for mitochondrial protein and of mitochondrial genes (Fig. 6d, Tables S3-S5). This is to our knowledge one of the first evidence showing, through mitochondrial transcriptional footprint, the direct impact of ethylene on mitochondrial functions. In a meta-analysis of transcriptomic data, Berkowitz *et al.* (2016) identified very few mitochondrial genes responding to ethylene, but this was in another system than seeds (seedlings) and ethylene was not provided directly but through its immediate precursor 1-aminocyclopropane 1-carboxylic acid (ACC).

Finally, with regards to the pivotal roles of ABA and GA in the regulation of seed germination and their interaction with ethylene, we also investigated the possible effect of ethylene on the transcript level of a subset of genes involved in either metabolism and

signalling pathways of both hormones (Tables S6-S7, Fig. 6e,f). Moreover Nonogaki (2019) recently suggested that mitochondria could play a role in ABA sensitivity in the context of seed germination. Among the 57 ABA related genes which were studied here (Table S4), the abundance of 17 transcripts was significantly modified by ethylene (Fig. 6e). From these data one can postulate that the ethylene effect was associated with a decrease in ABA seed content, as suggested by the down regulation of genes coding for its biosynthesis, i.e. *NCED3*, *NCED9* and *AAO3* (Nambara & Marion-Poll, 2003). Ethylene also decreased the transcript level of *ABI3*, a key component of ABA signalling (Parcy *et al.*, 1994) and increased the one of *ABI1*, a protein phosphatase 2C which is a negative regulator of ABA signalling (Gosti *et al.*, 1999). Although these results clearly claimed for a down regulation of ABA synthesis and signaling in the presence of ethylene, as already proposed by Corbineau *et al.* (2014), ethylene also stimulated the transcription levels of *HVA22* and *ABF2* which are known to be positively regulated by ABA, but also by other effectors (Cheng *et al.*, 2002; Kim *et al.*, 2004). Ethylene modified the transcript level of 11 GAs-related genes among 51 (Table S7, Fig. 6f). Most of them were induced by ethylene, including genes involved in GAs biosynthesis (*GA3ox*, *GA20ox* (Binenbaum *et al.*, 2018)) or perception (*GID1B*; (Griffiths *et al.*, 2006)). However ethylene also increased the abundance of transcripts of *GA2ox*, which is involved in GAs catabolism, and of *RGL1*, a repressor of GAs signalling pathway, so that the relationship between ethylene and GAs is not so clear. It nevertheless remains difficult to precise at this stage whether the effects of ethylene on ABA metabolism and signaling are related to MRR or not.

To summarize, our findings confirm the pivotal role of ROS in the regulation of seed germination and they shed a new light on their dynamics and mechanisms of action during germination. They place the mitochondria as a central regulator of seed germination, at least in the context of ethylene response, and open novel perspectives of research. In particular, it would be of paramount importance to determine whether mitochondrial ROS homeostasis is also regulated by environmental factors involved in seed germination or by other endogenous signals. Similarly, it would be also interesting to decipher the possible effects of feedback messages (anterograde) from nucleus to mitochondria, and their role in the germination process, notably through the identification of all the components involved in the pathway, in order to fully understand its contribution to germination.

Chapter 3

Spatiotemporal production of Reactive Oxygen Species in response to seed dormancy breaking treatments in Arabidopsis

Spatiotemporal production of Reactive Oxygen Species in response to seed dormancy breaking treatments in *Arabidopsis*

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NB: this chapter contains 10 figures and 2 supplemental figures and tables in Annex Chapter 3.

1. Summary

Gibberellic acid (GA), light and cold stratification alongside Reactive Oxygen Species are positive regulators of seed germination, yet the dynamics of ROS production in response to these treatments remains largely unknown.

We investigated the germination of *Arabidopsis thaliana* seeds in response to GA, stratification and light using cell imaging and genetic approaches with the aim of deciphering their possible influence on the cellular ROS content.

We found that all 3 treatments were associated with a ROS burst within seed embryonic axes and that the mitochondria were responsible for ROS production during the early hours of imbibition. At half-way to germination, ROS presumably localized within the peroxisomes within an important induction of glycolate oxidase *GOX2* before localising in the nucleus prior to radicle protrusion.

Taken all-together, our data highlights the importance of mitochondrial ROS production during the early stages of germination and suggest a possible involvement of peroxisomal ROS in this process.

Keywords: *Arabidopsis*, seed germination, Gibberellic acid, stratification, light, Reactive Oxygen Species, mitochondria, peroxisomes.

2. Introduction

Seed dormancy is among the most important adaptive mechanisms in plants, leading to a delayed germination to avoid unfavorable seasons and conditions (Wareing, 1971; Bewley, 1997). It corresponds to an inability to germinate regardless of the stimulating environmental conditions (Bewley, 1997; Baskin & Baskin, 2004). Seed dormancy is a transient phenomenon and has therefore the intrinsic property to be alleviated, thus allowing germination to proceed in a wider range of environmental conditions (Bewley, 1997). In many seeds, such as *Arabidopsis*, physiological dormancy can be overcome by a period of dry storage known as after-ripening but it can also be broken by applying various treatments on imbibed dormant seeds (Finch-Savage & Leubner-Metzger, 2006). *Arabidopsis* seed dormancy can also be released by exposing imbibed seeds to low temperatures (i.e. cold stratification) (Baskin & Baskin, 2004). Effective cold stratification temperatures range between 1 and 10°C with the highest dormancy alleviation speed being between 2 and 5°C (Stokes, 1965). In *Arabidopsis* seeds, dormancy release due to cold stratification is known to occur after 2 to 4 days of imbibition at 4°C (Wang *et al.*, 2018). Dormancy release by stratification essentially relies on the promotion of gibberellins (GA) biosynthesis by enhancing the expression of *GA3ox* genes (Yamauchi *et al.*, 2004; Finch-Savage & Leubner-Metzger, 2006; Holdsworth *et al.*, 2008) and abscisic acid (ABA) catabolism through the induction of ABA 8'-hydroxylase activity involved in ABA catabolism (Umezawa *et al.*, 2006; Matakias *et al.*, 2009). Light is also one of the most relevant environmental factors which release seed dormancy and promote seed germination (Baskin & Baskin, 2004). *Arabidopsis thaliana* is among the plants whose seed germination is light dependent (Hennig *et al.*, 2002) and this beneficial effect occurs through the activation of five phytochromes which perceive red/far-red light (600 -750 nm) and are encoded by five genes named *phyA* to *phyE* (Casal & Sánchez, 1998; Hennig *et al.*, 2002; Møller *et al.*, 2002). In *Arabidopsis*, *phyB* plays a predominant role in regulating the seed germination since it mediates the red/far-red photo-reversible response (Shinomura *et al.*, 1996; Tognacca *et al.*, 2019; Yan & Chen, 2020b). Red light stimulates germination through the proteasome-mediated degradation of PIL5, a basic helix-loop-helix (bHLH) transcription factor modulating GA and ABA levels, and of the repressors of GA signaling DELLA RGA and GAI (Oh *et al.*, 2006, 2007; Seo *et al.*, 2006, 2009). As for cold stratification, light mostly controls seed dormancy and germination through its effect on ABA and GA metabolism and signaling. Exogenous GA can also be directly used to break seed dormancy and promote seed germination (Debeaujon & Koornneef, 2000; Yamauchi *et al.*, 2004; Hu

et al., 2008). Two mechanisms of action are thought to govern the role of GA in the control of seed germination. The first one relies on the expression of genes coding for enzymes involved in endosperm and embryo axis cell wall weakening (Groot *et al.*, 1988; Leubner-Metzger *et al.*, 1996; Finch-Savage & Leubner-Metzger, 2006). The second mechanism consists of stimulating the growth potential of the embryo through antagonizing the activity of ABA (Debeaujon & Koornneef, 2000; Finch-Savage & Leubner-Metzger, 2006; Finkelstein *et al.*, 2008). Finally, ethylene is a gaseous plant hormone which can break dormancy of seeds of many species, including *Arabidopsis* (Corbineau *et al.*, 2014; Wang *et al.*, 2018; Jurdak *et al.*, 2020). It has been demonstrated that this effect could be explained by its antagonistic effect with ABA synthesis and signaling and by stimulating GA response (Arc *et al.*, 2013; Corbineau *et al.*, 2014; Wang *et al.*, 2018) and its mechanism of action as dormancy breaking treatment has been recently described (Jurdak *et al.*, 2020).

The crosstalk between GA and ABA is considered as being a key actor mediating the environmental response to seeds following the onset of imbibition and is a common feature of the dormancy breaking treatment listed previously (Yamauchi *et al.*, 2004; Finch-Savage & Leubner-Metzger, 2006; Yamaguchi, 2008). However, the shift in the hormonal balance ABA/GA cannot be considered as the sole signaling event regulating seed dormancy and germination. Reactive oxygen species (ROS) have also progressively emerged as being highly involved in the regulation of seed germination and dormancy of several species including *Arabidopsis*, leading to the concept of the "oxidative window for germination" (Bailly, 2004, 2019; El-Maarouf-Bouteau & Bailly, 2008; Bailly *et al.*, 2008; Oracz *et al.*, 2009; Leymarie *et al.*, 2012; Cremers & Jakob, 2013, Bailly, 2019). Several sources of ROS production have been proposed to be associated with the germination process and they include the mitochondria and NADPH oxidases as major contributors, while peroxisomes and cell wall ROS producing enzymes seem to be involved to a lesser extent (Bailly, 2019). Recently Jurdak *et al.* (2020) demonstrated that the beneficial effect of ethylene on seed germination required ROS production by the mitochondrial electron transport chain and that it involved a mitochondrial retrograde response (MRR) leading to nuclear ROS production. In this system the mitochondria therefore appeared as a central regulator of seed germination, through the regulation of ROS homeostasis during seed dormancy breaking by ethylene. This study was one of the first to clearly identify where and when ROS are produced during the germination process and dormancy release, thus underpinning novel mechanisms of seed dormancy regulation.

The objective of the present work was to determine whether the spacio-temporal regulation of ROS homeostasis during breaking of seed dormancy would follow a common pattern, whatever the dormancy release treatment. To address this question, dormant *Arabidopsis* seeds were treated with light, GA or were cold stratified, and the dynamics of ROS production, as well as the regulation of known actors involved in cell signaling, was determined. Our data allow proposing a novel sketch describing the role of ROS in the regulation of seed germination by dormancy.

3. Materials and methods

3.1 Plant Material

Arabidopsis thaliana wild-type seeds used in this study were from the genetic background Columbia (Col-0). Mutants and roGFP-expressing lines used in this study were in the same genetic background (Table S1). Seeds were stratified for 4 days at 4 °C before being sown in soil and placed in growth chambers under controlled conditions with a temperature of 21°C and a photoperiod of 16 h per day with the light intensity of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Mature seeds were harvested and stored at -20 °C to preserve their dormancy until further use.

3.2 Germination assays

Approximately 75 seeds per replicate were sown in 9 cm Petri dishes on filter papers moistened with deionized water or with solutions indicated in the text and placed in the darkness at 15 or 25°C. Stratification was performed by placing seeds on a filter paper on the top of a cotton wool moistened with deionised water at 4°C for 4 days in the darkness, before their transfer to 25°C. Light treatment was performed under continuous light with an intensity of approximately 8 000 lux. Respiratory inhibitor solutions were prepared by first dissolving the powder in 2mL dimethylsulfoxide and the volume was completed to 100 mL with deionized water. Germination was daily monitored and seeds were considered as germinated when the radicle protruded through the envelopes. All assays were carried out in triplicates.

3.3 Quantitative RealTime -PCR

Total RNA was extracted from 50 mg of Arabidopsis seeds using the protocol developed by (Hellemans *et al.*, 2007), adapted by (Zhang *et al.*, 2019), as described by Jurdak *et al.* (2020). Relative gene expression was calculated according to (Hellemans *et al.*, 2007) with CBE-5 (AT5G53560), TIP41 (AT4G34270) and RHIP1 (AT4G26410) as reference genes. The relative expression of the triplicates was determined compared to dry seeds considered as reference in this study. Primer sequences are listed in Table S2.

3.4 Hydrogen peroxide quantification

Hydrogen peroxide quantification was performed using 30 mg of seeds as described by Jurdak *et al.* (2020) using a peroxidase based assay in the presence of DMAB (3-dimethylaminobenzoic acid) and MBTH (3-methyl-2-benzothiazolinone hydrazone). Assays were carried out in triplicates and the results are expressed in $\mu\text{mol.g}^{-1}\text{FW}$. 3

3.5 Confocal microscopy

Fluorescence of Mit-roGFP, Grx1-roGFP (provided by Professor Andread Meyer, University of Bonn) seeds and various fluorochromes of Col-0 seeds used in this study was detected by confocal microscopy using a Leica TCS SP5 confocal microscope. Seeds were imbibed according to conditions detailed in the text and the seed coat was gently removed under a binocular prior cell imaging. Assessment of mitochondrial redox status was performed using mit-roGFP seeds, as described by Jurdak *et al.* (2020). ROS were localized using DCFH-DA (2',7'-Dichlorodihydrofluorescein diacetate, 25 μM) (Leymarie *et al.*, 2012), mitochondria were visualized using MITO-ID (Enzo Life SciencesInc, USA) dye and nuclei were stained using 4',6-diamidino-2-phenylindole DAPI (Merck KGaA, Darmstadt, Germany). All procedures are described in Jurdak *et al.* (2020). Observations were carried out with at least 30 embryos per condition.

4. Results

4.1 Hormonal regulation of seed germination in response to GA, light and stratification

Freshly harvested *Arabidopsis thaliana* wild type seeds poorly germinated at 25 °C in darkness and their germination did not exceed 20 % after 10 d of imbibition. However, seeds fully germinated at 25 °C in darkness in the presence of GA (1mM), after cold stratification and under constant light (Figure 1).

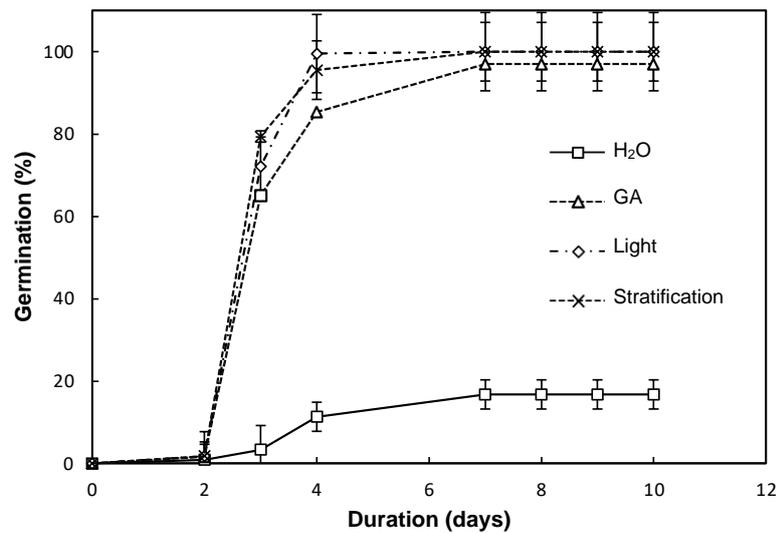


Figure 1. Germination of freshly harvested Col-0 seeds at 25°C in darkness on water, on a solution of gibberellic acid (GA) 1 mM at 25°C, after cold-stratification for 4 d at 4°C or at 25°C under constant light. Means of 3 replicates \pm SD.

With regards to the paramount role of ABA and GA in the regulation of seed germination we investigated the response of a set of genes involved in ABA and GA production, signaling and catabolism to the 3 dormancy breaking treatments by qRT-PCR (Figure 2). The chosen genes were *GA3OX1* and *GA3OX2* (code for the Gibberellin 3-oxidase responsible for catalyzing the final step in the synthesis of bioactive gibberellins, (Hu *et al.*, 2008), *EXPA3* (codes for the expansin 3 involved in the response to GA, (Marowa *et al.*, 2016), *GASA4* and *GASA14* (code for GA responsive transcripts part of the gibberellic acid-stimulated in *Arabidopsis* gene family, (Chen *et al.*, 2007; Sun *et al.*, 2013), *GA2OX2* (codes for the GA2 oxidase (GA2ox) whose function is to decrease the levels of active Gas, (Huang *et al.*, 2015), *AAO1* (codes for the aldehyde oxidase responsible of the oxidation of abscisic aldehyde, the last step of ABA biosynthesis, (Seo *et al.*, 2004), *ABA1* (codes for the zeaxanthin epoxidase that functions in first step of the biosynthesis of ABA,

(Barrero *et al.*, 2005), *ABI4* (codes codes for a member of the DREB subfamily A-3 of ERF/AP2 transcription factor family and is involved in ABA signal transduction (Wind *et al.*, 2013), *ABI5* (codes for ABA-insensitive 5 gene involved the response to ABA, (Brocard *et al.*, 2002), *CYP707A2* and *CYP707A3* (code for a protein with ABA 8'-hydroxylase activity involved in ABA catabolism, (Umezawa *et al.*, 2006; Matakias *et al.*, 2009). An induction of genes involved in GA biosynthesis was evidenced after 6 h of imbibition in germinating seeds, *i.e.* treated with GA, light or stratified, as shown by the dramatic increase in abundance of *GA3OX1* and *GA3OX2* transcripts (Figure 2a). Further seed imbibition was associated with a progressive decrease in the abundance of these transcripts (Figures 2c,e). GA responsive genes were also induced after 6 h of imbibition in response to the treatments since the expression *GASA4* and *GASA14* increased during the imbibition of germinating seeds (Figures 2a,c,e). Abundance of *EXPA3* transcripts also increased in response to the 3 dormancy breaking treatments but only after 16 h of imbibition (Figures 2a,c,e). No significant induction of *GA2OX2* was observed for any of the treatments and it is worth noting that the expression of all genes involved in GA metabolism and signaling remained low in dormant seeds imbibed on water (Figures 2a,c,e). Expression of *AAO1* and *ABA1* genes, involved in ABA biosynthesis, was evidenced in dormant seeds during their imbibition on water only. After 6 and 16 h in this condition the transcript abundance of *CYP707A2* and *CYP707A3* was relatively high but it decreased markedly after 24 h of imbibition (Figures 2b,d,f). In contrast the level of transcripts of *AAO1* and *ABA1* remained very low in treated germinating seeds (Figures 2b,d,f) while the one of *CYP707A2* and *CYP707A3* was globally higher in treated seeds than in non-treated ones (Figures 2b,d,f). At the first time point of imbibition, there was a marked accumulation of *ABI4* transcript in treated seeds when compared to dormant seeds imbibed on water, which was less clear at the other time points, except for GA treated seeds (Figures 2 b,d,f) which showed a 20 fold increase in the expression of this transcript. A similar pattern was observed after 16 and 24 h of imbibition when the expression levels of *AAO1* and *ABA1* were higher in dormant seeds and the transcripts abundance of *CYP707A2* and *CYP707A3* were more important in treated seeds.

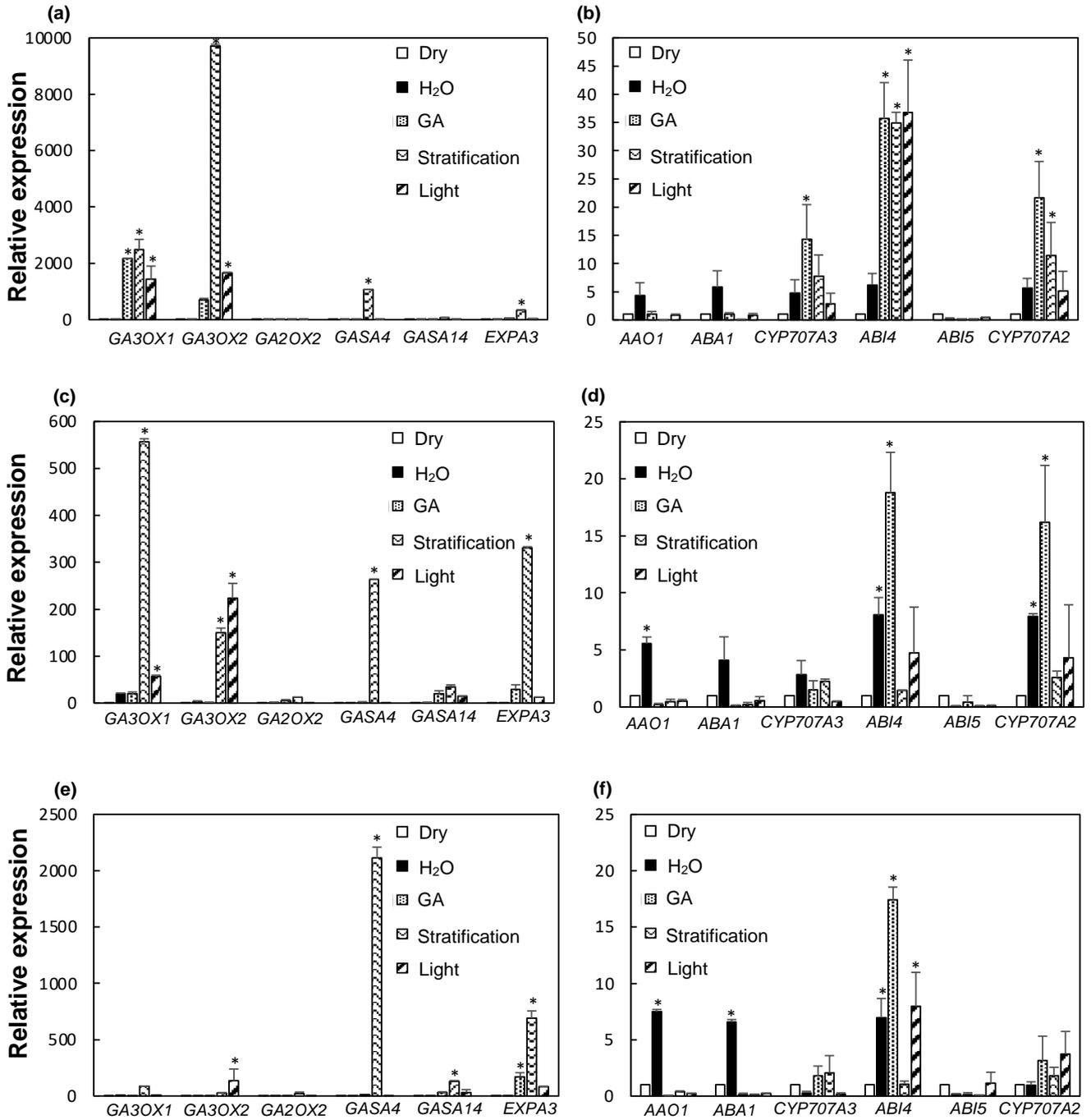


Figure 2. Transcript abundance of *GA3OX1*, *GA3OX2*, *GA2OX2*, *GASA4*, *GASA14*, *EXPA3*, *AAO1*, *ABA1*, *CYP707A3*, *ABI4*, *ABI5*, *CYP707A2* in freshly harvested Col-0 dry seeds or in seeds imbibed for 6 (a, b), 16 (c, d) and 24 h (e, f) on water at 25°C in darkness, in the presence of gibberellic acid (GA) (1 mM), after stratification for 4 days at 4°C or after exposure to constant light. An arbitrary value of 1 was assigned to dry seeds. Data are means of 3 replicates \pm SD. Asterisks represent significantly different means when comparing water to GA, stratification and light with $P < 0.05$ according to Kruskal Wallis.

4.2 Reactive oxygen species are produced in germinating seeds in response to GA, light and stratification and partially localize to the mitochondria

The production of hydrogen peroxide was evaluated in dormant seeds incubated for 6, 16 and 24 h at 25°C on water, with or without cold stratification, in the presence of GA (1 mM) or after exposure to light (Figure 3a). A dramatic increase in H₂O₂ production was observed in seeds after 16 and 24 h of imbibition, independently of any specific treatment (Figure 3a). H₂O₂ production was nevertheless higher in germinating seeds, *i.e.* following the 3 treatments. Indeed, after 16 h of imbibition, seed H₂O₂ content was 2 times higher in the presence of GA, light and stratification and reached ca 30 µmol.g⁻¹ FW, when compared to 15 µmol.g⁻¹ FW found in dormant seeds imbibed on water (Figure 3a). H₂O₂ production by germinating seeds ranged between 45 µmol.g⁻¹ FW and 50 µmol.g⁻¹ FW after 24 h of imbibition, whatever the treatment.

We investigated whether H₂O₂ production could result from the activity of the mitochondrial electron transport chain (mtETC) by treating seeds with the inhibitors rotenone (ROT, 5.10⁻⁵ M), a mitochondrial complex I inhibitor (Garmier *et al.*, 2008), salicylhydroxamic acid (SHAM, 10⁻⁴ M) an inhibitor of the mitochondrial alternative oxidase (Selinski *et al.*, 2018a), and antimycin A (AA, 5.10⁻⁶ M) a mitochondrial complex III inhibitor (Sweetlove *et al.*, 2002; Jurdak *et al.*, 2020). Despite the presence of inhibitors, H₂O₂ concentration increased during seed imbibition and reached values between 16 and 22 µmol.g⁻¹ FW after 16 h of imbibition and close to 35 and 40 µmol.g⁻¹ FW after 24 h of imbibition for the different treatments (Figure 3b). Nevertheless, a slight decrease in hydrogen peroxide production was recorded for seeds imbibed in presence of mitochondrial respiratory inhibitors compared to control untreated seeds only imbibed in presence of GA, light and stratification (Figure 3a).

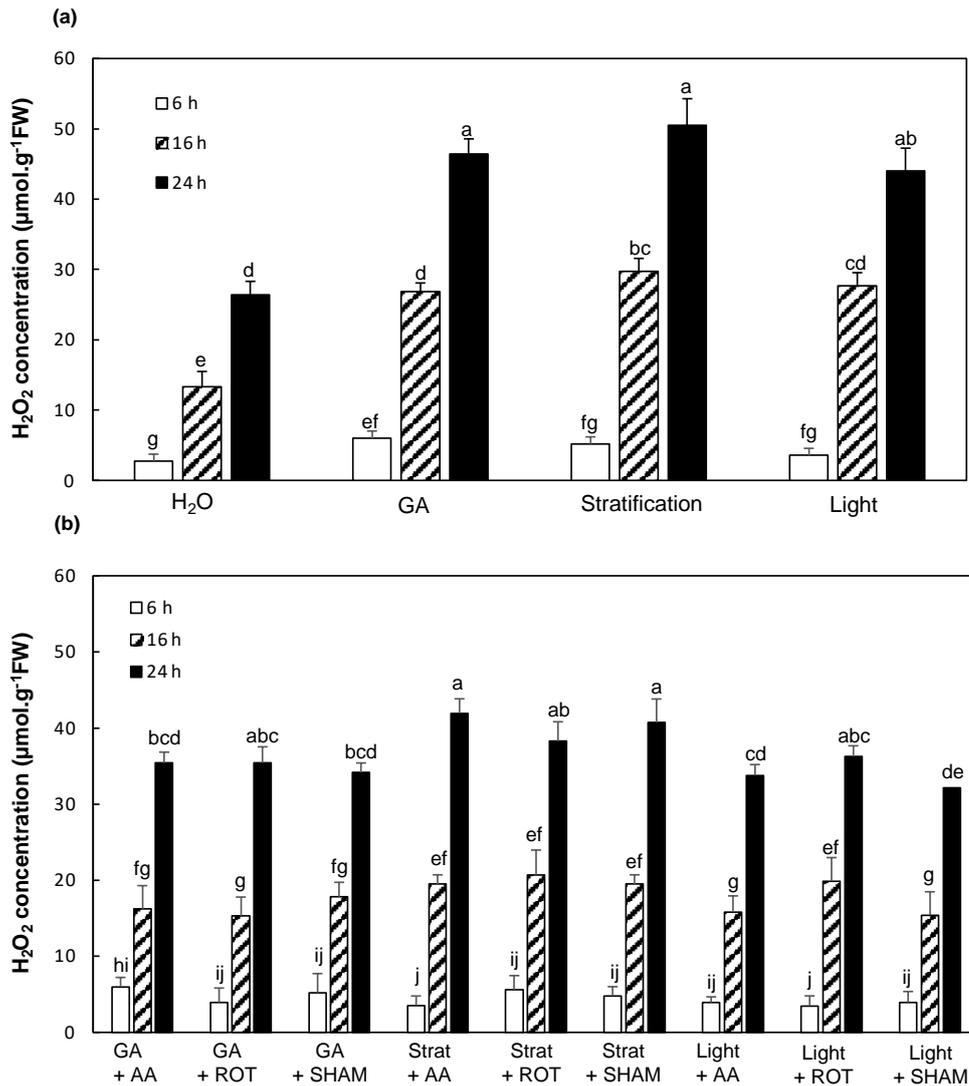


Figure 3. Hydrogen peroxide production in freshly harvested Col-0 seeds incubated for 6, 16 and 24 h (a) in darkness at 25°C on water, in the presence of gibberellic acid (GA) (1 mM), in light at 25°C, after stratification for 4 days at 4°C (Strat) and (b) in the presence of antimycin A (AA) (5.10^{-6} M), Rotenone (ROT) (5.10^{-5} M) or salicyhydroxamic acid (SHAM) (10^{-4} M). Means of 3 replicates \pm SD. Different letters represent significantly different means with $P < 0.05$ according to Kruskal Wallis.

Germination of wild-type seeds incubated in presence of the different respiratory inhibitors was also followed (Figure 4). Germination of dormant seeds at 25°C in the darkness was not modified by AA, ROT and SHAM and remained close to 20 % (Figure 4a). AA, ROT and SHAM slightly decreased the beneficial effect of the 3 treatments on the germination of dormant seeds since in all cases germination remained below 80 % (Figure 4b,c,d), but it remained higher than on water only.

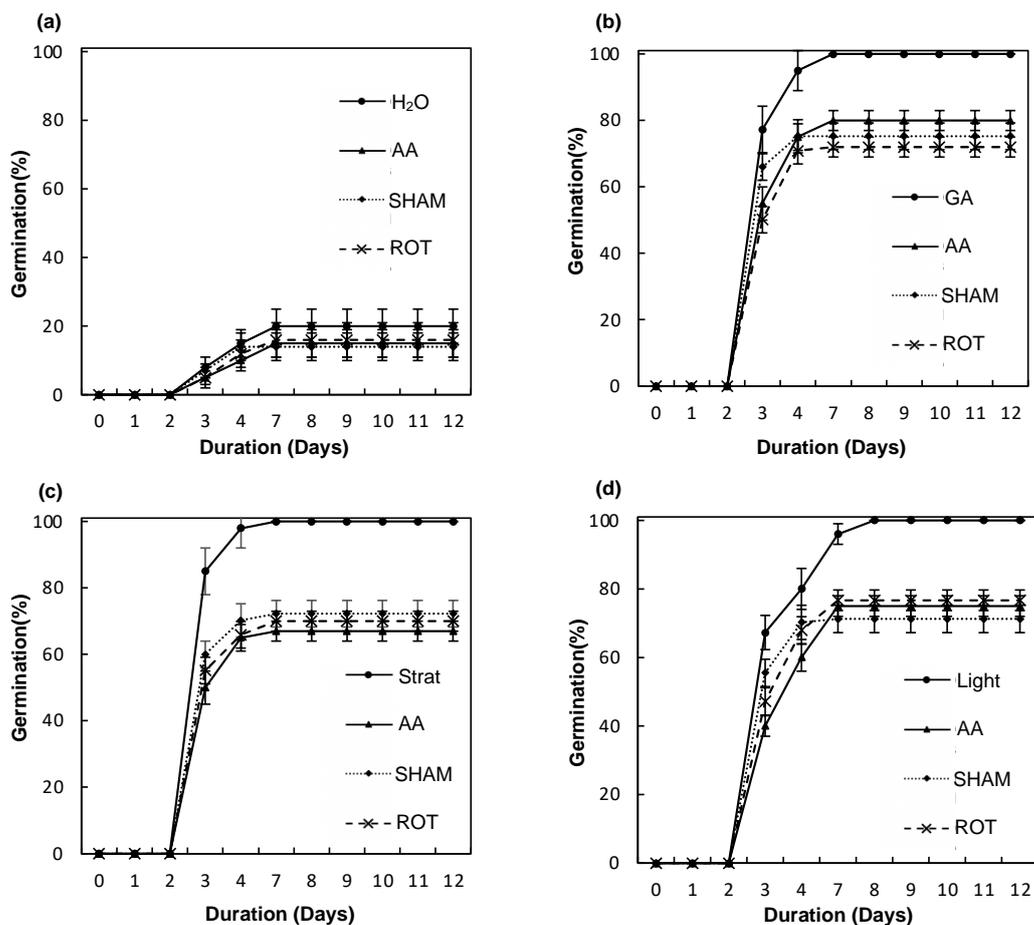


Figure 4- Germination of freshly harvested Col-0 seeds at 25°C in darkness on water (a), on a solution of gibberellic acid (GA) 1 mM at 25°C (b), after cold-stratification for 4 d at 4°C (Strat) (c) or at 25°C under constant light with or without antimycin A (AA) ($5 \cdot 10^{-6}$ M), Rotenone (ROT) ($5 \cdot 10^{-5}$ M), or salicylhydroxamic acid (SHAM) (10^{-4} M). Means of 3 replicates \pm SD.

We used mit-roGFP seeds to determine to what extent mitochondria could be involved in ROS production in response to dormancy breaking treatments (Figure 5). Fluorescent foci on Figure 5 a-h were used to evaluate the mitochondrial localization of ROS in cells of embryonic axes of dormant and germinating mit-roGFP seeds after 24 h at 25°C. Fluorescence emitted after excitation at 405 nm (Figure 5a,c,e,g) corresponded to the oxidized state of ro-GFP whereas the one emitted in response to an excitation at 488 nm (Figure 5b,d,f,h) revealed its reduced state. The fluorescence emission at 488 nm (Figure 4 b,d,f,h) showed that ro-GFPs were predominantly in a reduced state in dormant seeds imbibed on water and in seeds treated with GA, exposed to light or even stratified. Nevertheless, a slight increase in the fluorescence emitted at 405 nm was detected in treated seeds (mostly in GA treated seeds and light exposed seeds) compared to dormant

seeds uniquely imbibed on water. This was confirmed by the increase of both oxidation degree and redox potential with time, following exposure to light, GA treatment and stratification (Figure 5i).

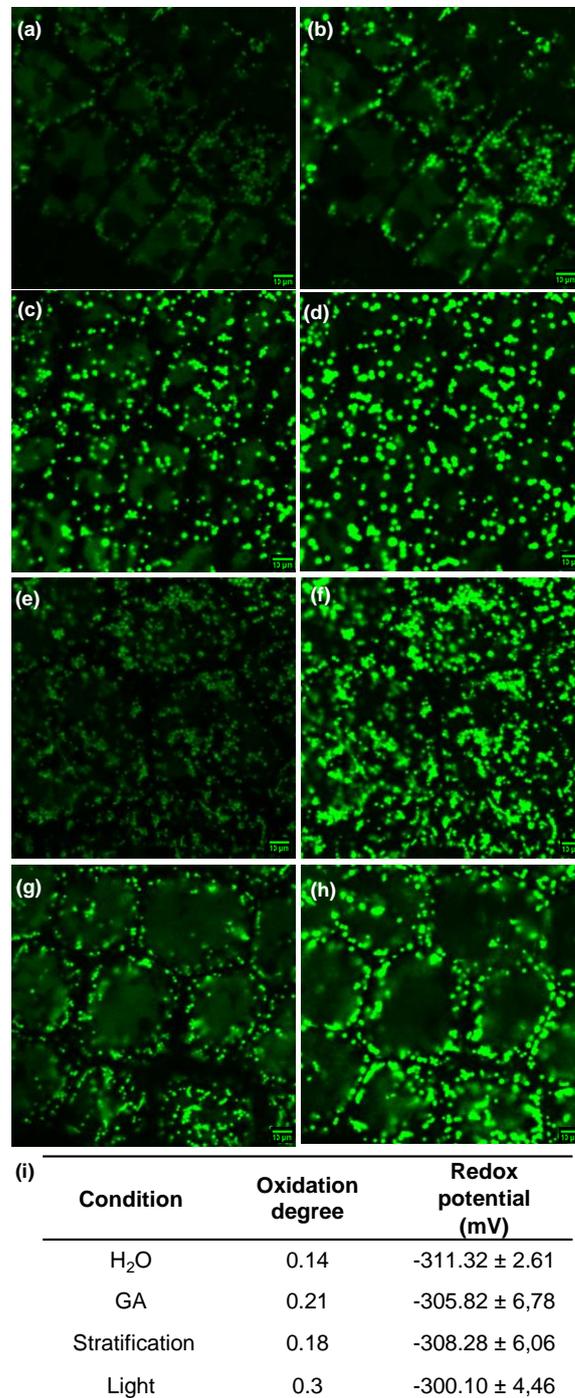


Figure 5. Fluorescence images of embryonic axes of dormant mitro-GFP seeds incubated on water for 24 h at 25°C in the dark (a-b) and in presence of gibberellic acid (GA) (1 mM) (c-d), after stratification for 4 days at 4°C (e-f) or at 25°C under constant light (g-h) after excitation at 405 nm (a- c- e- g) or 488 nm (b- d- f- h). (i) Oxidation degree and redox potentials calculated from fluorescence data obtained with dormant mitro-GFP seeds incubated for 24 h in the same conditions. Data are means of 14 replicates ± SD.

Since we previously demonstrated that breaking of dormancy by ethylene relied on mit-ROS production and MRR, we investigated the effect of the dormancy breaking treatments used here on the expression of MRR markers such alternative oxidase 1a (*AOX1a*) and the NAC transcription factor 13 (*ANAC013*) (Jurdak *et al.*, 2020). The expression of *AOX1a* and *ANAC013* was dramatically stimulated after 6 h of imbibition on water at 25 °C in the darkness (Figure 6). In contrast, the transcript abundance of both genes increased in a much lower extent in treated seeds. *AOX1a* decreased after GA treatment while a slight induction was noticed following stratification and exposure to light (2 fold increase compared to dry seeds). The opposite scenario was observed regarding the expression of *ANAC013* since it peaked in the presence of GA with a 3 fold increase compared to dry seeds, slightly decreased following the exposure to light and was diminished in stratified seeds. In addition, seeds of *aox1a* and *anac013* mutants did not show any specific phenotype, when germinated on water or after dormancy release treatments (Figure S1).

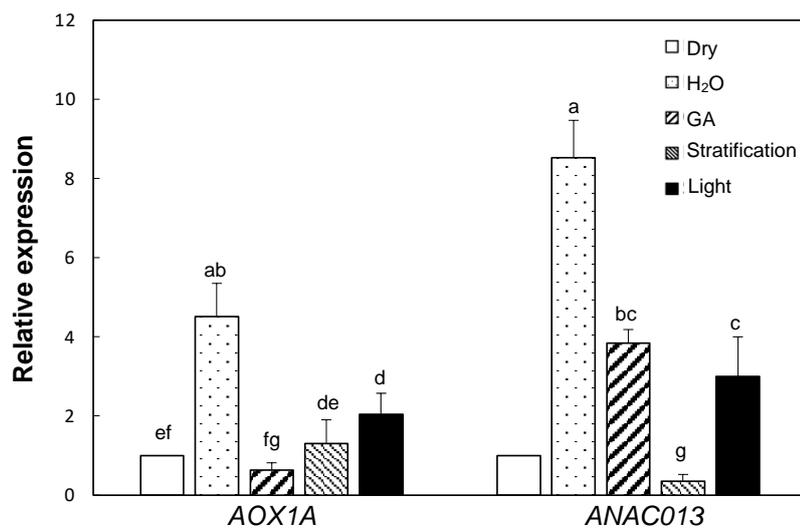


Figure 6. Transcript abundance of *AOX1A* and *ANAC013* in freshly harvested Col-0 dry seeds and after 6 h of imbibition at 25°C in darkness on water, on a solution of gibberellic acid (GA) 1 mM at 25°C, after cold-stratification for 4 d at 4°C or at 25°C under constant light. An arbitrary value of 1 was assigned to dry seeds. Different letters represent significantly different means with $P < 0.05$ according to Kruskal Wallis. Means of 3 replicates \pm SD.

4.3 Investigating the origin of ROS production following exposure to light, GA or stratification

Since mitochondria seemed to be only partially involved in ROS production in response to the different treatments, we next assessed the possible role of NADPH oxidase in seed dormancy breaking treatments. This was performed by following the germination phenotype of seeds of *rboh D* mutant, *rboh B* mutant, and of the double mutant *rboh DB* (Figure 7). At harvest, seeds of all mutants were dormant since germination at 25°C was restricted to 5 % for *rboh DB* seeds, and to 10 % for *rboh D* and *rboh B* seeds, and it was limited to 20 % for Col-0 seeds (Figure 7a). In darkness, seeds of all mutants fully germinated in the presence of GA (1 mM) (Figure 7b) or after stratification (Figure 7c). In addition, seed germination of all mutants was enhanced when incubated in light at 25°C, ranging between 90 and 100 % (Figure 7d). Furthermore, we also determined whether ROS production in response to dormancy breaking treatments could be associated with the stimulation of *RBOH* genes, but this was not the case (Figures 7a,b,c,d). We also observed that H₂O₂ production by treated seeds of *rboh DB* mutants after 24 h of imbibition was 2 times higher than in non-treated dormant ones (Figure 7 i).

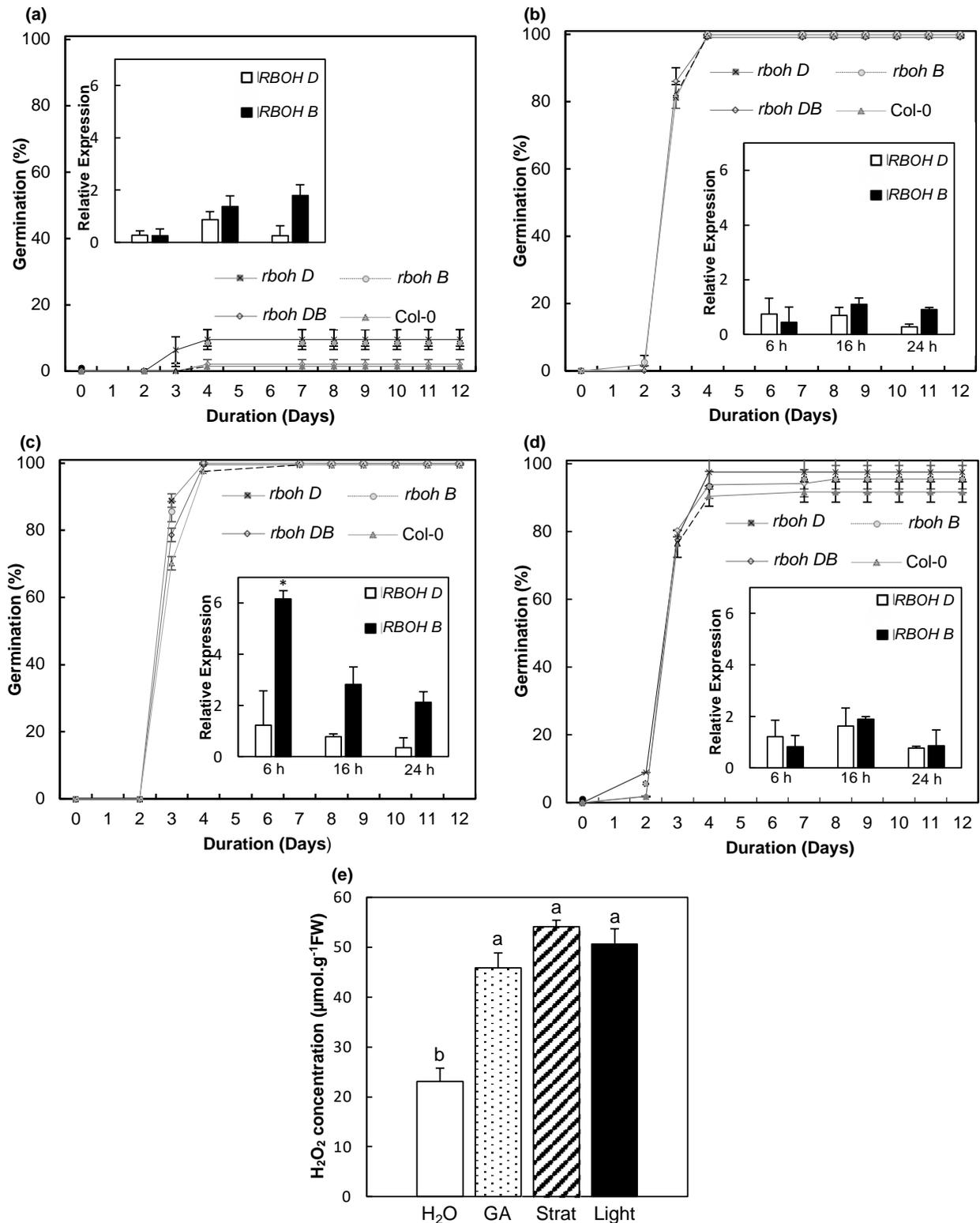


Figure 7. Germination of freshly harvested *Col-0* seeds and of seeds of *rbob D*, *rbob B* and *rbobDB* mutants at 25°C in darkness on water (a), on a solution of gibberellic acid (GA) 1 mM at 25°C (b), after cold-stratification (Strat) for 4 d at 4°C (c) or at 25°C under constant light (d). Inset are shown the transcript abundance of *RBOHD* and *RBOHB* after 6, 16 and 24 h of imbibition in the same conditions. An arbitrary value of 1 was assigned to dry seeds. (e) Hydrogen peroxide content in *rbobDB* seeds imbibed for 24 h in the same conditions. Different letters and asterisks represent significantly different means with $P < 0.05$ according to Kruskal Wallis Means of 3 replicates \pm SD.

To gain further insight on the dynamics of ROS production during seed dormancy release we localized ROS within cells of embryonic axes using DCFH-DA fluorescence. When dormant seeds were imbibed at 25°C on water in the dark, ROS were only produced within mitochondria from 6 h to 24 h of imbibition (see Jurdak *et al.*, 2020). After 6 h of imbibition with dormancy release treatments, ROS mainly localized within mitochondria where they appeared as small green foci as estimated by DCFH-DA fluorescence (Figure 8 a-1, a-4, a-7). Mitochondria were formally identified using the mitochondrial marker Mito-ID Red (Figure 7 (b-1, b-4, b-7)). Merged views of ROS localization and DAPI staining, used to identify nuclei, did not show any specific fluorescence within the nucleus after 6 h of imbibition in the 3 conditions (Figure 8 d-1, d-4, d-7). After 16 h of imbibition, ROS were no longer localized in the mitochondria of seeds imbibed in presence of GA or in the light, as shown by the distinct red foci observed in the merged images (Figure 8 d-2, d-8), while ROS remained localized in the mitochondria of stratified seeds (Figure 8 d-5). Nevertheless, distinct green foci were detectable after 24 h of imbibition for all three treatments implying that ROS localization was no longer mitochondrial however nuclear ROS localization was evident by the overlap of the DCFH-DA dye and DAPI as shown by Figure 8 (d-3, d-6, d-9).

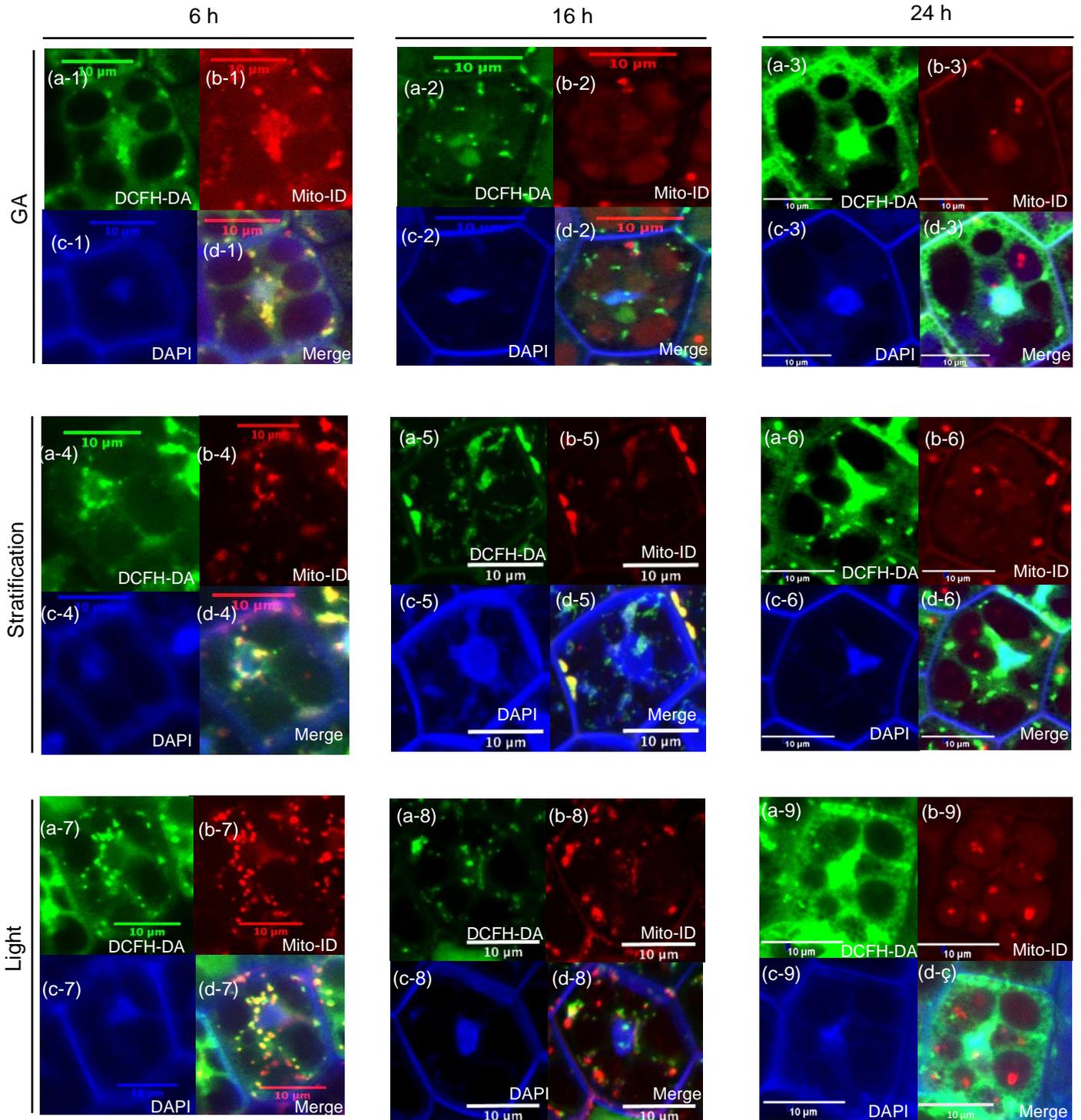


Figure 8- Representative observation of the fluorescence emission obtained by confocal microscopy of embryonic axes of Col-0 seeds incubated for 6 (a1- d 1, a4- d4, a7- d7), 16 (a2- d2, a5- d5, a8- d8) and 24 h (a3- d3, a6- d6, a9- d9) on water at 25°C in darkness in the presence of gibberellic acid (GA) (1 mM), after stratification for 4 days at 4°C or after exposure to constant light, and treated by DCFH-DA (a1- 9), Mito-ID (b 1-9), DAPI (c1-9). (d- 9) merged images.

We used Grx1-roGFP lines to determine the involvement of peroxisomes in ROS production in response to dormancy breaking treatments after 24 h of imbibition at 25°C (Figure 9). Fluorescent foci observed after excitation at 405 nm, which corresponded to the oxidized state of ro-GFP, were used to evaluate the peroxisomal localization of ROS in cells of embryonic axes of dormant and germinating Grx3-roGFP seeds after 24 h at 25°C (Figure 9). The number of fluorescent foci observed at 405 nm increased in treated seeds compared to dormant seeds. One to 3 fluorescent foci were observed in dormant (Figure 9 a) seeds whereas this number ranged between 6 and 8 in germinating seeds (Figures 9 b,c,d).

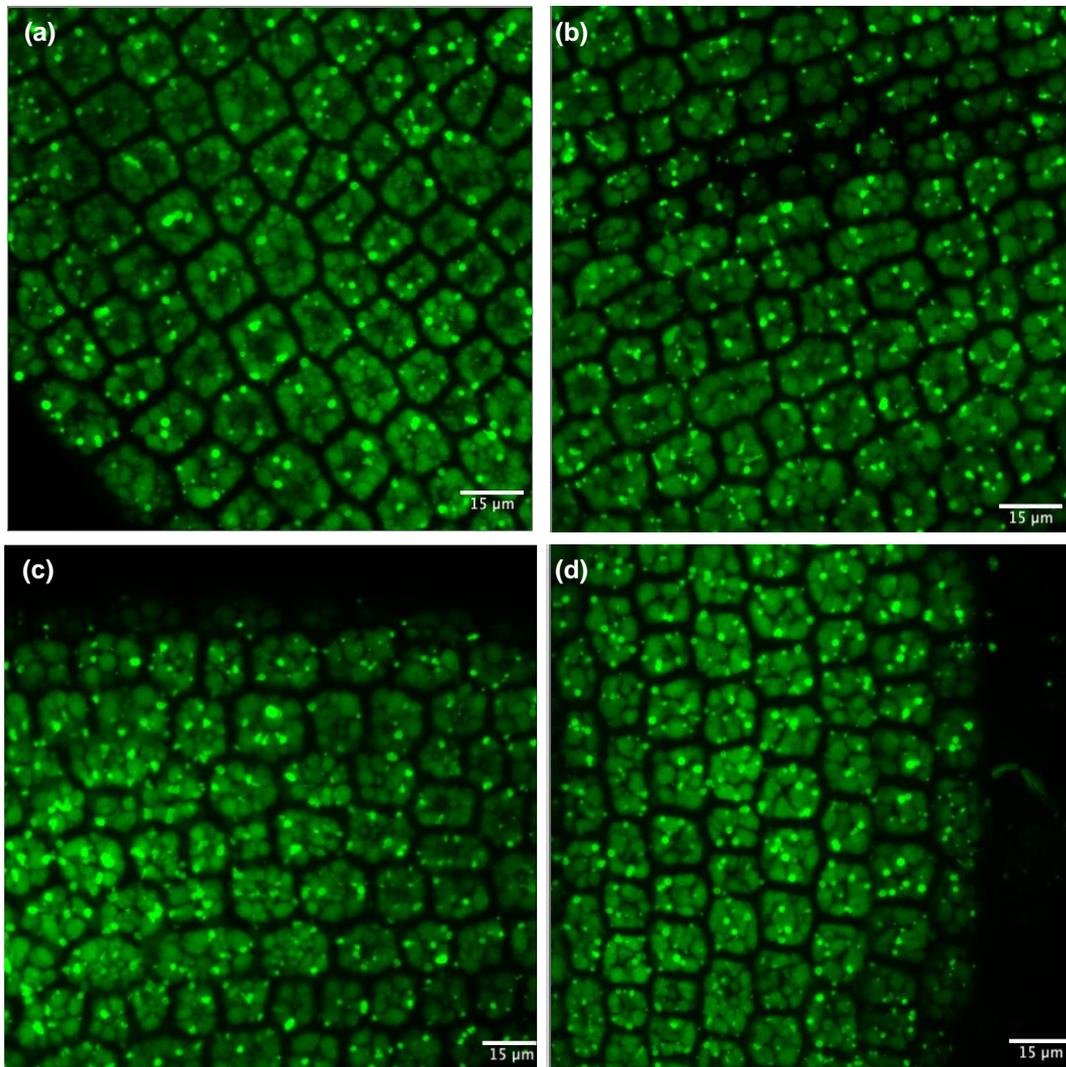


Figure 9- Fluorescence images of embryonic axes of dormant Grx1-ro-GFP seeds incubated on water for 24 h at 25°C in the dark (a) and in presence of gibberellic acid (GA) (1 mM) (b), after stratification for 4 days at 4°C (c) or at 25°C under constant light (d) after excitation at 405nm.

4.4 Reactive oxygen species production by the peroxisome is involved in the response to GA, light and stratification

Cell imaging suggested a potential role of peroxisomes in the production of ROS in germinating seeds. We therefore studied the changes in expression of a subset of genes known for being involved in peroxisome function and metabolism (Figure 10). They included *SO* (codes for sulfite oxidase which catalyzes the conversion of sulfite to sulfate by producing H_2O_2 Corpas et al., 2020), *XDH1* (codes for the Xanthine dehydrogenase 1 a configuration of the Xanthine oxidase, which generates $O_2\cdot-$ directly, and it couples xanthine oxidation to urate, Walker et al., 2018), *GOX2* (codes for the glycolate oxidase involved in photorespiration and is considered as an important source of intracellular H_2O_2 Corpas et al., 2017), *CAT2* (codes for the catalase responsible for the conversion of H_2O_2 to water and oxygen without production of other ROS, Mhamdi et al., 2010), *SOX* (codes for the sarcosine oxidase that catalyzes the oxidative of sarcosine (*N*-methylglycine) to glycine while producing hydrogen peroxide, Fagan & Palfey, 2010) and *PAO4* (codes for the peroxisomal polyamine oxidase responsible for the oxidation of acetyl spermine and acetyl spermidine while generating hydrogen peroxide Floris & Finazzi Agrò, 2013). The relative abundance of all the transcripts studied increased after 6 h of imbibition but it was very significant for the *GOX* transcripts since its abundance increased 20 fold in stratified seeds, 15 fold in seeds imbibed in light and 8 fold in seeds imbibed in GA compared to dry seeds but only 5 times after imbibition on water (Figure 10a). No significant induction of the other genes was observed in any of the tested conditions after 6 h of imbibition (Figure 10a). This tendency was confirmed at the other time points of the experiment (Figures 10b,c) since the most significant changes were also restricted to *GOX* expression. Although *GOX* transcript abundance decreased during seed imbibition, it remained high in germinating seeds and always higher than non-germinating seeds.

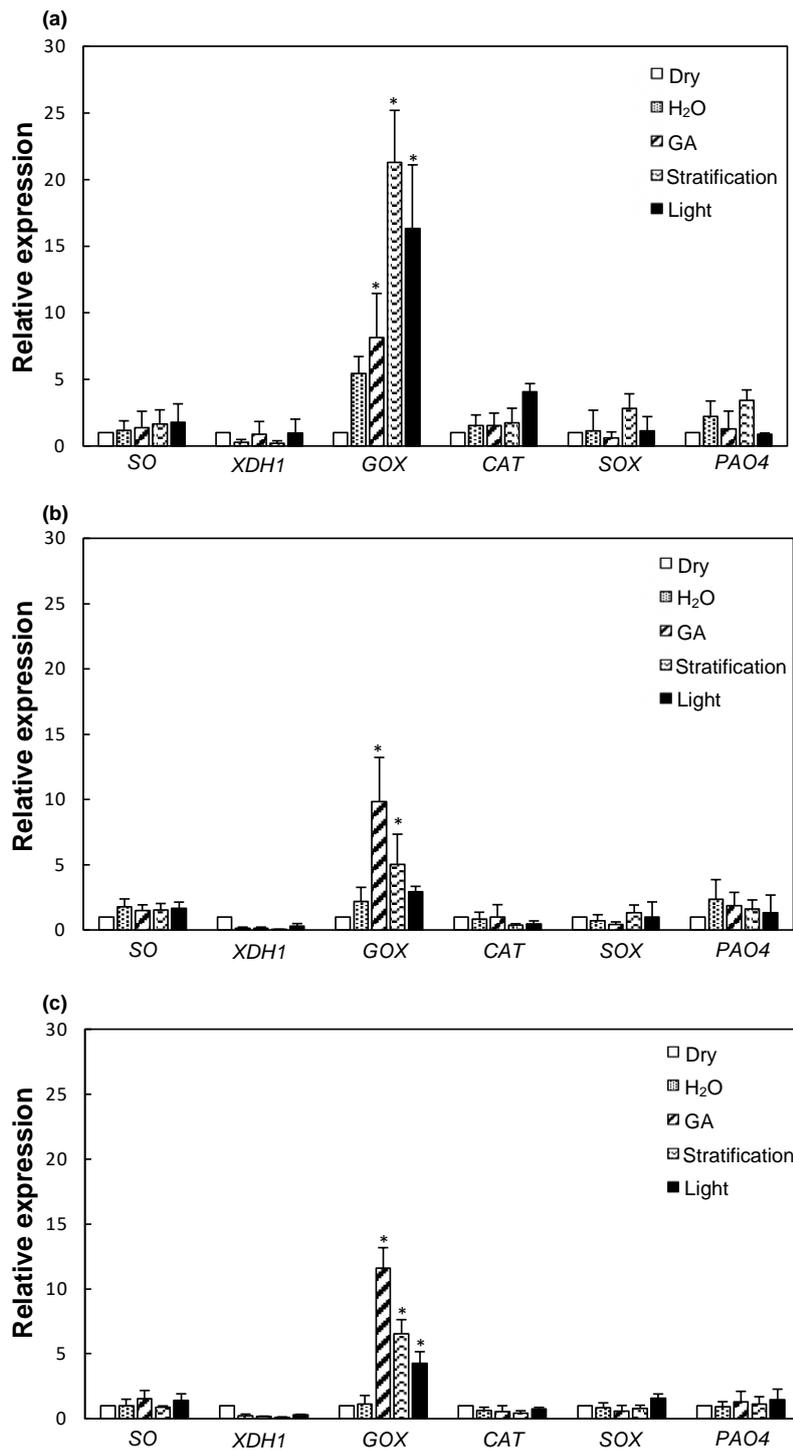


Figure 10- Transcript abundance of *SO*, *XDH1*, *GOX*, *CAT*, *SAX*, *PAO4*, in freshly harvested Col-0 dry seeds or in seeds imbibed for 6 (a, b), 16 (c, d) and 24 h (e, f) on water at 25°C in darkness, in the presence of gibberellic acid (GA) (1 mM), after stratification for 4 days at 4°C or after exposure to constant light. An arbitrary value of 1 was assigned to dry seeds. Data are means of 3 replicates \pm SD. Asterisks represent significantly different means when comparing water to GA, stratification and light with $P < 0.05$ according to Kruskal Wallis.

5. Discussion

5.1 Reactive oxygen species are produced in germinating seeds in response to GA, light and stratification but only partially result from the mETC

Although *Arabidopsis* seeds are dormant at harvest (Leymarie *et al.*, 2012), it is possible to overcome this blockage of germination using various treatments. Seed dry after-ripening (Leymarie *et al.*, 2012) or treatment of imbibed dormant seeds with gaseous ethylene (Jurdak *et al.*, 2020) both allow seeds to fully germinate in the dark at 25°C. Germination of dormant seeds at 25°C in the presence of GA in the dark or pre-imbibition of dormant seeds at 4°C, *i.e.* cold stratification, also alleviated dormancy, as shown Figure 1. Similarly, imbibition of dormant seeds under continuous light allowed their germination at 25°C (Figure 1). The beneficial effect of these treatments on seed dormancy alleviation is already well documented in *Arabidopsis* and they are commonly used for studying mechanisms associated with this trait (Debeaujon & Koornneef, 2000; Yamauchi *et al.*, 2004; Oh *et al.*, 2006; Chen *et al.*, 2007; Seo *et al.*, 2009; Wang *et al.*, 2018). In agreement with such studies, we checked that the beneficial effect of the treatments resulted from an altered balance of ABA/GA metabolism and signaling (Figure 2). Regarding GA metabolism, the expression of both GA biosynthesis genes, *GA3OX1* and *GA3OX2*, dramatically increased in treated seeds especially in stratified seeds and a decrease in the expression of *GA2OX2*, involved in GA catabolism, was also shown (Figure 2a). It has been previously demonstrated that *de novo* GA biosynthesis after the onset of imbibition is crucial for seed germination (Koornneef & van der Veen, 1980; Nambara *et al.*, 1991; Mitchum *et al.*, 2006; Yamauchi *et al.*, 2007). Gibberellin 3-oxidases, part of the classes of 2-oxoglutarate-dependent dioxygenases (2ODDs), are involved in the final stage of GA biosynthesis and are responsible for the formation of bioactive gibberellins (Hedden & Kamiya, 1997; Olszewski *et al.*, 2002; Yamaguchi, 2006). In *Arabidopsis*, light and stratification has already been shown to markedly promote GA biosynthesis by inducing the expression of *GA3OX1* and *GA3OX2* (Ogawa *et al.*, 2003; Yamauchi *et al.*, 2004). Gibberellic acid treatment stimulated the expression of GA biosynthesis gene since exogenous GA treatment provides GA₃ which is converted to more bioactive isoforms GA₁ and GA₄ through the activity of gibberellin 3-oxidase (Yamauchi *et al.*, 2007). An increase of expression of GA responsive genes was later observed, especially *GASA4* and *EXPA3* after 16 h of germination (Figure 2 c,e). *GAST1* like genes are among the few identified GA-induced genes, and it has been shown that the over-expression of *GASA4* in *Arabidopsis* promoted GA responses including germination

(Rubinovich & Weiss, 2010). Seed germination in response to all 3 treatments also correlated with the induction of genes involved in ABA catabolism (Figure 2 b) and an increase in the expression of *CYP707A* genes was detected. Light, stratification and GA treatment have been shown to induce the expression of *CYP707A* genes in germinating seeds (Matakiadis *et al.*, 2009; Li *et al.*, 2017b; Yan & Chen, 2020b). It is worth saying that the expression of *AAO1* and *ABA1* remained higher in dormant seeds but these genes are involved in ABA biosynthesis (Seo *et al.*, 2004; Barrero *et al.*, 2005) showing again that ABA produced in embryos is crucial for the promotion and prolongation of seed dormancy (Skubacz & Daszkowska-Golec, 2017). Lastly, as expected, the transcript abundance of *ABI4* decreased over time in treated seeds since this key transcription factor in the ABA signaling pathway controls seed dormancy and inhibits germination by downregulation of Arabidopsis response regulators ARR1s (Shu *et al.*, 2013; Huang *et al.*, 2017). Thus the 3 treatments used here release seed dormancy through the canonical regulation of the hormonal balance ABA/GA.

We took advantage of these multiple ways to overcome seed dormancy for addressing the question of the involvement of ROS homeostasis in this process. The measurement of H₂O₂ content in dormant and treated seeds (Figure 3a) showed an increase in its concentration over time, even in dormant seeds, as already shown by Leymarie *et al.*, (2012). Nevertheless, the transition to a germinating state following seed treatment with GA, light or stratification, was associated with an overproduction of ROS, in agreement with previous work that showed that ROS content peaked before radicle protrusion (Bailly *et al.*, 2008a; Oracz *et al.*, 2009; Leymarie *et al.*, 2012; Bailly, 2019). Therefore, whatever the mean of breaking Arabidopsis seed dormancy, it seemed to be always associated with ROS production during the latter phase of seed imbibition (*i.e.* at 24 h in the present case). The dynamics and sources of ROS production are nevertheless far from being known (Bailly, 2019). Jurdak *et al.* (2020) showed that dormancy release induced by ethylene relied first on a ROS production by the mtETC. Here we show that the respiratory inhibitors ROT, AA, SHAM had only a very slight effect on ROS production in response to the dormancy release treatments (Figure 3b), in contrast to what was shown in response to ethylene (Jurdak *et al.*, 2020). In agreement with these findings the germination of treated seeds was only partially prevented by the respiratory inhibitors (Figure 4). Further evidence on the function of mtROS in the germination process were brought using mit-roGFP constructs (Figure 5). The redox status of the mitochondria remained essentially in a reduced state after 24 h of imbibition in treated seeds, thus suggesting a minor mitochondrial ROS production, again

in contrast to what was demonstrated by Jurdak *et al.* (2020) in the presence of ethylene. Indeed, the calculated values of mitochondrial redox potentials in seeds treated with GA, light or stratification remained very close to the one found in dormant seeds imbibed on water (-311 mV, Figure 5i). In the presence of ethylene, the redox potential reached a higher value, close to -275 mV (Jurdak *et al.*, 2020), demonstrating a more oxidized mitochondrial state in the presence of ethylene. Thus mitochondria remained in a relatively reduced state following GA treatment, exposure to light or stratification. Lastly we previously associated mtROS production during seed germination with the activation of mitochondrial retrograde signaling in response to ethylene (Jurdak *et al.*, 2020). Here freshly harvested seeds of the mutants of *aox1a* and *anac013*, two markers of MRR in plants, fully germinated in the presence of GA (10^{-3} M), light and after stratification thus suggesting that the activation of MRR is rather restricted to ethylene (Figure S1). Besides, no significant induction of *AOX1a* or *ANAC013* was observed in any of the 3 treatments in contrast to what has been previously shown in presence of ethylene (Figure 6, Jurdak *et al.*, 2020). Altogether these data strongly suggest mtROS production and subsequent activation of MRR pathway are specific of ethylene and that stimulation of ROS production in response to GA, light and stratification depends on other mechanisms.

5.2 Deciphering the dynamics and localization of ROS production following exposure to light, GA or stratification

Since seed germination in response to GA treatment, exposure to light and stratification positively correlated with an accumulation of ROS, but unexpectedly not with mtETC, we further investigated the mechanisms involved in ROS production in these conditions. This question was first addressed by studying the germination of treated dormant seeds of NADPH oxidases mutants because NADPH oxidases are amongst the most studied ROS-producing enzymes and they have been suggested to play a key role in ROS production during seed germination (Müller *et al.*, 2009; Leymarie *et al.*, 2012; Bailly, 2019). Seed germination of *rboh D*, *rboh B* and *rboh DB* mutants was not impaired in response to the dormancy-alleviation treatments (Figure 7). We also demonstrate that the treatments did not stimulate expression of *RBOH D* and *RBOH B* genes (Figures 7b,c,d) and that H₂O₂ production was not prevented in seeds of the double mutant *rboh DB* in response to the seed dormancy breaking treatments (Figure 7e). Our data therefore demonstrate that seed dormancy breaking was independent of NADPH oxidases generated ROS.

We next investigated the spatiotemporal ROS production in the hypocotyl area of the embryonic axis at different time points of seed imbibition in presence of GA, light and after stratification (Figure 8). After 6 h of imbibition, mitochondria appeared to be the major intracellular source of ROS during imbibition of dormant seeds (see Jurdak *et al.* 2020) and of treated seeds (Figure 8 a-1, a-4, a-7). Although ROS production was magnified by the seed dormancy release treatments (Figure 3), ROS were no longer localized within mitochondria after 16 h of imbibition in seeds imbibed under light or treated with GA (Figures 8 a-2, a-8), but remained partially mitochondrial in stratified seeds (Figure 8 a-5). After 24 h of imbibition, ROS localized within the nucleus whatever the dormancy release treatment (Figures a-3, a-6, a-9). These findings illustrate the complexity of ROS production regulation during seed germination and allow to propose a scenario explaining these mechanisms. As already demonstrated with ethylene treatment (Jurdak *et al.*, 2020), we suggest that mitochondria is the major ROS producing system during early seed imbibition, and that conditions permissive for germination (i.e. dormancy release treatments) stimulate mt-ROS generation. This emphasizes on the pivotal role of mitochondria in the germination process, as already proposed by various authors (Paszkiwicz *et al.*, 2017; Ma *et al.*, 2019; Nonogaki, 2019b; Nietzel *et al.*, 2020; Jurdak *et al.*, 2020), and future studies will have to decipher how positive germination regulators can control mit-ROS production by mtETC. Interestingly, after 24 h of imbibition, ROS localized within the nuclei of cells of germinating seeds only (Figures a-3, a-6, a-9; Jurdak *et al.*, 2020). Eventhough it has been proposed that ROS could be generated in the nucleus (Ashtamker *et al.*, 2007), they are most likely to be transferred from other cellular compartments and contribute to regulation of gene expression (Martins *et al.*, 2018). In accordance with other studies (Leymarie *et al.*, 2012; Jurdak *et al.*, 2020) we demonstrate that ROS generation within the nucleus at the time set of radicle protrusion is a common feature of germinating seeds. Jurdak *et al.* (2020) demonstrated in the same system that accumulation of ROS within the nucleus was associated with transcriptome reprogramming, including changes in expression of genes involved in ABA and GA metabolism and signalling pathways, thus participating in the induction of seed germination. Various studies have shown that ROS, and H₂O₂ in particular, could have a marked effect on gene expression in plants (Willems *et al.*, 2016b), notably through a variety of redox-sensitive transcription factors (Petrov & Van Breusegem, 2012; He *et al.*, 2018). Increasing evidence also suggest that ROS could regulate gene expression trough epigenetic modifications, including post-translational modifications of histone and chemical modifications of DNA(Huang *et al.*, 2019; Lindermayr *et al.*, 2020). Such

mechanisms will have to be closely investigated in order to better understand the role of ROS homeostasis within the nucleus during seed germination.

5.3 A role for the peroxisome in the ROS production in response GA, light and stratification

Since the distinct green foci revealing ROS localization after 16 h of imbibition in treated seeds were relatively close in size to the mitochondria, we hypothesized that peroxisomes could be responsible for the observed ROS production. Peroxisomes are one of the major sites of intracellular hydrogen peroxide production in plant cells and they contain various enzymatic systems involved in H₂O₂ production such as glycolate oxidase (GOX), acyl-CoA oxidase (AOX), urate oxidase (UO), polyamine oxidase, copper amine oxidase (CuAO), sulfite oxidase (SO), sarcosine oxidase (SOX), or superoxide dismutase (SOD) (Schrader & Fahimi, 2006; del Río & López-Huertas, 2016; Corpas *et al.*, 2017, 2020). Here we show that these organelles are likely to be involved in ROS production during seed dormancy breaking at the mid-time of germination completion, ROS being produced by mitochondria during early seed imbibition (Figure 8). Grx1-roGFP constructs (Figure 9) showed that the number of fluorescent foci indicating an oxidative status increased in germinating seeds compared to dormant seeds, thus suggesting a burst in peroxisomal ROS production in response to dormancy alleviation treatments.

Additional evidence on the involvement of ROS production by peroxisomes were provided by studying the expression of known molecular actors of peroxisomal ROS metabolism by qRT-PCR (Figure 10). The expression of *GOX2* was dramatically stimulated by GA, light and stratification. In green tissues glycolate oxidase produces peroxisomal H₂O₂ by oxidizing glycolate during photorespiration (Noctor, 2002; Foyer *et al.*, 2009; Cui *et al.*, 2016). Information concerning the involvement of *GOX* in seed germination is still sparse mainly because photorespiration does not occur during this process, which raises the question of the origin of the substrate of the enzyme in imbibed seeds. Glycolate, the substrate of *GOX*, can also result from the reduction of glyoxylate catalyzed by glyoxylate reductase (*GLYR*) (Simpson *et al.*, 2008). Interestingly it has been demonstrated that the expression of *GLYR* is dramatically stimulated during Arabidopsis seed germination, such as the expression of 3-Ketoacyl-CoA thiolase (*KAT*) and isocitrate lyase (*ICL*), which catalyse the key steps for generating glyoxylate (Dekkers *et al.*, 2013, Figure S2). In contrast, the expression of the other genes involved in ROS production (*SO*, *XDH1*, *SAX*, *PAO4*) or scavenging (*Cat2*) was not markedly modified by any the seed dormancy release treatments

(Figure 9). We therefore propose here that GOX could play a key role in peroxisomal ROS production occurring during seed germination by H₂O₂ generation after oxidation of glycolate. In seeds peroxisome have been up to now reported as being the site of fatty acid β -oxidation which utilizes lipid reserves and serve as fuel during late germination and early steps of seedling growth (Graham 2008; Kao et al 2018). Since peroxisomes are equipped with ROS production systems but also contain enzymes such as catalases, ascorbate peroxidases (APX) and several types of peroxiredoxins which are involved in H₂O₂ scavenging they are considered as being crucial for controlling ROS levels in plant cells and they have been proposed to function as sensors of redox changes induced by environmental issues (Su *et al.*, 2019). Their role in plant developmental process is far less known and to our knowledge this is the first time that their involvement in the germination process as ROS producer is reported. Further work will have to precise how peroxisome adapt ROS generation to exogenous and endogenous stimuli during seed imbibition and what regulatory mechanisms are involved in this process.

6. Conclusion

Our findings allow identifying novel mechanisms of ROS signalling in response to seed dormancy breaking treatments. We demonstrate that mitochondria are the first organelle involved in ROS production during the germination process but that peroxisomes then ensure this function before ROS localize within the nucleus prior to radicle protrusion. This mechanism seems to common in the various treatments of dormancy release but differ from the one involved in ethylene response. Our results open novel fields of investigation in ROS signalling during seed germination and future challenge will be to identify the molecular basis of the crosstalk between ROS generating organelles and to decipher how change in ROS homeostasis with the nucleus can regulate gene expression.

Chapter 4

**Interplay between transcription,
polysomal association and decay
of mRNAs regulates the
alleviation of Arabidopsis seed
dormancy by ethylene**

Interplay between transcription, polysomal association and decay of mRNAs regulates the alleviation of Arabidopsis seed dormancy by ethylene

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NB: Supporting figures for this chapter are provided in the Annex Chapter 4 and the table are available on google drive using the following link: <https://drive.google.com/drive/folders/1wr387PSsQ2iaoT6dzSyZ1shltEAu2Cdn?usp=sharing>

NB: This chapter contains 9 Figures, 2 Supplemental figures and 3 Supplemental tables on Google Drive.

1. Summary

Ethylene is a known positive regulator of seed germination yet its effect on the metabolism of mRNA remains very poorly understood.

Using transcriptomic analysis approaches by RNA-seq we studied the effect of ethylene on gene expression (the transcriptome), the association of mRNA with polysomes (the translatoome) and the stability and degradation of mRNA.

Our findings showed that alleviation of seed dormancy relies on a synchronized and timely regulated participation of either transcription, polysomal association and decay of mRNAs.

Our results demonstrate that selective recruitment of mRNA to polysomes depends on the features of the 5'UTR.

We also show that mRNA decay in response to ethylene does not appear to be related to an overall effect of ethylene on the stability of the transcribed, but rather on 5' to 3' mRNA decay.

Keywords : Arabidopsis, Ethylene, Decay, Transcription, Translation, mRNA

2. Introduction

Seed production and germination play crucial roles in the dispersal and survival of plants. Germination *sensu stricto* follows water uptake by a quiescent dry seed and terminates with radicle protrusion (Bewley, 1997). The progress of a seed towards germination can be regulated by its dormancy status, a process which blocks the ability of a viable seed to germinate under favorable conditions (Bewley & Black, 1994; Bewley, 1997). A lot of effort has been put in the last years to understand this complex phenomenon. To gain more knowledge on the regulation of seed dormancy many treatments, that essentially modify the hormonal balance between abscisic acid (ABA) and the gibberellins (GAs), can be used (Finkelstein *et al.*, 2008; Holdsworth *et al.*, 2008; Nonogaki *et al.*, 2010). Among them ethylene breaks seed dormancy via a complex hormonal signaling network (Arc *et al.*, 2013; Corbineau *et al.*, 2014). The antagonistic relationship between ethylene and ABA has been largely elucidated (Leubner-Metzger *et al.*, 1998; Beaudoin *et al.*, 2000; Arc *et al.*, 2013; Corbineau *et al.*, 2014), as well as the synergic relationship between ethylene and GAs (Ogawa *et al.*, 2003; Siriwitayawan *et al.*, 2003; Corbineau *et al.*, 2014). Reactive oxygen species (ROS) have also been reported to be involved in the transition of Arabidopsis seeds towards a germinating state in the response to ethylene (Bailly, 2019; Jurdak *et al.* 2020).

The molecular aspects involved in the regulation of germination in response to ethylene have been scarcely studied and are poorly known. Wang *et al.* (2018) demonstrated that the alleviation of seed dormancy by ethylene required the activation of the N-end rule pathway. Recently Jurdak *et al.* (2020) demonstrated that ethylene induced a transcriptomic ROS signature and Mitochondrial Retrograde Signaling. Understanding downstream effects of ethylene requires to integrate the multi-level molecular events involved in this process, including transcriptional, post-transcriptional and translational regulation in a comprehensive model. 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 this developmental phase, provided detailed maps of regulatory processes and identified key transcription factors. These genes include seed maturation regulators such as ABSCISIC INSENSITIVE 3 (ABI3), components of 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). Recent 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) or longevity (Verdier *et al.*, 2013).

It becomes clear that the transcriptional level cannot provide a complete and comprehensive understanding of the regulatory mechanisms governing seed 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. In developing seeds, during the preparation for the dry state, mRNA dynamics undergo extensive modification resulting in the accumulation of so-called 'long-lived' or 'stored' mRNA (Bazin *et al.*, 2011). More than 12,000 transcripts are stored in mature dry seeds of Arabidopsis (Nakabayashi *et al.*, 2005), and they serve as templates for *de novo* protein synthesis during the initial phase of germination (Rajjou *et al.*, 2004). In addition, it has been shown that *de novo* transcription upon imbibition is not an essential process for germination (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). It has been demonstrated that germination results from a timely regulated and selective recruitment of mRNAs to polysomes (Layat *et al.*, 2014; Basbous-Serhal *et al.*, 2015). How specific mRNA are translated during seed imbibition remains largely unknown. 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 & 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).

Another less studied post-transcriptional process controlling seed germination is mRNA stability. During germination and seedling establishment, mRNA decay pathways must logically become operational during imbibition to remove transcripts that served for embryonic development and are no longer needed. It has been shown that dormancy alleviation of sunflower seeds during dry after-ripening did not rely on transcriptomic changes but on the oxidation of specific subsets of mRNAs, thus impairing their subsequent translation during seed imbibition (Bazin *et al.*, 2011). Basbouss-Serhal *et al.* (2017) also demonstrated that the 5' to 3' RNA decay machinery participated in the regulation of seed germination by dormancy. Cytoplasmic mRNA degradation generally begins with deadenylation, i.e. shortening or removal of the poly(A) tail and can continue from the 3'-end through the action of the exosome, or it can undergo 5'-to-3' decay (Belostotsky & Sieburth, 2009). The exosome is a multi-subunit complex found in both cytoplasm and nuclear compartments (Lykke-Andersen *et al.*, 2011). Exosome components are not well known in plants but in contrast to human and yeast, plants have two functionally specialized RNA helicases MTR4 and HEN2 that assist the exosome in the degradation of specific nucleolar and nucleoplasmic RNA populations, respectively (Lange *et al.*, 2014). In plants 5'-to-3' decay first requires the removal of the mRNAs 5' cap which is carried out by the decapping complex containing the core components DCP1, DCP2 and VARICOSE (VCS) and localized in the so-called Processing Bodies (PBs). P-bodies are dynamic cytoplasmic complexes that also contain enzymes for 5'-3' decay (Xu & Chua, 2009). Colocalization of DCP1, DCP2, VCS, with the exoribonuclease XRN4 and the helicase DHH1 suggests that P-bodies function in both translational repression and mRNA decapping (Xu & Chua, 2009). RNA decay is known to be involved in the ethylene signaling pathway where degradation of the F-box proteins EBF1 and EBF2 by EIN5 allows the accumulation of EIN 3 protein and triggers the response to ethylene (Olmedo *et al.*, 2006). XRN4 is a component of the signaling cascade for ethylene perception and is also called EIN5 (Olmedo *et al.*, 2006;

Potuschak *et al.*, 2006). Among the different cellular mechanisms that regulate mRNA fate, covalent nucleotide modification has also recently emerged as a powerful modulator of the processing, localization, stability, and translatability of mRNAs (Yue *et al.*, 2019). Methylation of adenosine in the N⁶ position (m⁶A) is the most widespread internal modification present in rRNAs, mRNA, tRNAs, miRNA and long non-coding RNA (Arribas-Hernández & Brodersen, 2020). For instance, m⁶A accounts for almost 80% of all RNA methylation out of which 50% occur in polyadenylated mRNA (Yue *et al.*, 2019). m⁶A is able to modify RNA metabolism at different levels including translation, translation efficiency, 3'-end processing and alternative splicing. In plants, however, little information is available on the regulation of m⁶A especially in the context of seed germination (Liang *et al.*, 2020). At last, small RNAs (sRNAs), which include microRNAs (miRNAs) and small-interfering RNAs (siRNAs), are known to play a key role in gene regulation and are able to repress the expression of specific target genes by base-pairing resulting in RNA cleavage or inhibition of translation (Zeng *et al.*, 2003). Although a role for miRNA in seed germination has been proposed (Liu & El-Kassaby, 2017) its physiological importance in seed dormancy is largely unknown.

The objective of the present study was to determine whether the effect of ethylene on seed dormancy alleviation could rely on mRNA processing. Using RNAseq we have thus investigated the main feature of mRNA fate in seeds treated by ethylene. We provide a comprehensive sketch of ethylene effect on mRNA metabolism which shows the relative importance in transcription, polysomal association and decay in the regulation of seed dormancy alleviation.

3. Materials and methods

3.1 Plant material

Arabidopsis (*Arabidopsis thaliana*) wild type seeds (Col-0) were used in this study. Seeds were stratified for four days at 4°C prior to sowing in order to enhance their germination. All seeds were sown in soil and placed in monitored growth chambers (21°C) under a photoperiod of 16 h per day with a light intensity of 150 and 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Seeds were harvested at maturity and stored at -20°C to maintain their dormancy until further use.

YFP-DCP1 transgenic lines under the control of their endogenous promoter were provided by the Genomics and Plant Development Laboratory (University of Perpignan, France).

3.2 Germination assays

Arabidopsis seeds (75 seeds x 3 replicates) were sown on a filter paper moistened with 10 mL of deionized water in 9 cm Petri dishes and placed in darkness at 15 or 25 °C. For ethylene treatment, seeds were placed in gas-tight petri dishes in which 0.8 mL of gaseous ethylene (5%, Air Liquide, Paris, France) was injected in order to obtain a final concentration of 100 ppm. Seed germination was followed for 12 days on a daily basis and a seed was considered as germinated when the radicle pierced the seed coat.

3.3 RNA sequencing for the transcriptome

The total RNA was extracted from 50 mg of seeds according to Hellemans *et al.* (2007) using the protocol described by Jurdak *et al.* (2020). The sequencing technology used was an Illumina NextSeq500 (IPS2 POPS Platform). RNA-seq libraries were performed by TruSeq Stranded mRNA Sample Prep Guide_15031047_D protocol (Illumina®, California, U.S.A.). The RNA-seq samples were sequenced in single-end (SE) with a sizing of 260bp and a read length of 75 bases. 21 samples by lane of NextSeq500 using individual bar-coded adapters and giving approximately 20 million of SE reads by sample were generated. The differentially expressed genes (fold change > |2| and bonferroni < 0.05) were then determined using the CLC Genomics Workbench (Qiagen).

3.4 Polysome isolation and RNA extraction

Four hundred mg of *Arabidopsis thaliana* wild-type seeds were ground in liquid nitrogen into a fine powder before adding one volume of the polysome extraction buffer (PEB) which consisted in a mixture of 200 mM Tris-HCl pH 9.0, 200 mM KCl, 25 mM EGTA, 36 mM MgCl₂, 1 % (v/v) octylphenyl-polyethylene glycol (Igepal CA-630), 1 % (v/v) polyoxyethylene (23) lauryl ether (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 µg.ml⁻¹ cycloheximide, 50 µg.ml⁻¹ chloramphenicol, 10 µl. ml⁻¹ protease inhibitor cocktail. The tubes were then centrifuged for 15 min at 14,000 g at 4°C and the supernatant was recovered while paying attention not to disturb the lipidic layer above it before being transferred to a new tube containing a

Miracloth filter. An aliquot of 200 μ L of the supernatant was kept for the isolation of total RNA. Two 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 MgCl₂, 100 mg. ml⁻¹ chloramphenicol and 100 mg. ml⁻¹ cycloheximide) and centrifuged at 180 000 g at 4°C for or 2.5 h using a Beckman (SW41) rotor. The sucrose gradient was fractionated using a density gradient fractionator system with a 254 nm detector and a 2 M sucrose solution as the displacement fluid. The fractions corresponding to the polysomal profile were pooled together prior to the RNA extraction. Total and polysomal RNA were extracted using the same method by adding 300 μ L of 5 % SDS/EDTA (0.2M) and 700 μ L of phenol/chloroform/isoamyl alcohol (25:25:1) (Barkan, 1993). The tubes were centrifuged for 5 min at 13000 RPM at room temperature before adding 1 mL ethanol 100%. The tubes were Centrifuged again for 15 min at 20784 g at room temperature and the pellet was resuspended in 20 μ L of ultrapure water. The quality of the RNA was then verified using a Nanodrop spectrophotometer and the RNA was treated with the TURBO DNA-free™ Kit (ambion, Life sciences) purified using the MACHEREY- NAGEL nucleospin XS purification kit according to manufacturer's instruction.

Relative mRNA abundance in polysomal and total fractions was quantified with the polysomal ratio P/T (%)= (log abundance polysomal RNA / log abundance in total RNA)*100.

3.5 RNA extraction for the degradome analysis

Seeds were imbibed in a solution containing cordycepin at a concentration of 50 μ M for 16 h according to Basbouss-Serhal *et al.*, (2017) (see Supplemental Figure 2) and RNA were extracted as previously described by Zhang *et al.*, (2019). Primer sequences correspond to: AT5G50000-F: AAA-CAG-AGA-CTT-GAG-TGG and AT5G50000-R: AAG-CAA-CTT-CAC-GGC-AAC-ATC-T and AT3G61170-F: GAA-GCT-TAG-AAG-ACG-CGA-AT and AT3G61170-R: CTT-CTA-AGA-GAC-CGT-TTT-TTG-C.

3.6 Bioinformatic analysis

The parallel coordinate plots allow to compare the feature of samples for several quantitative variables that could have completely different ranges and even different units. These plots were obtained using the package "klustR" of the software R Studio by clustering the data according to the K- means. Each vertical bar represents a variable with its own scale. Values are then plotted as series of lines connected across each axis.

The differentially expressed genes for the transcriptomic analysis (fold change $> |2|$ and Bonferroni < 0.05) were determined using the CLC Genomics Workbench (Qiagen) for each condition.

Differentially abundant transcripts in the translome and in the degradome were identified as having a TPM (transcripts per million) value greater than 3.

The pathway based analysis was performed using Pathifier, an algorithm which calculates the pathway deregulation score between control samples (germinating seeds in our study) and other samples (dormant seeds in our study) (Ponnaiah *et al.*, 2019). Data used in this pathway-based analysis consists of the abundance profiles obtained by RNAseq of the transcriptome and the translome. The plant pathways used in this study were retrieved using KEGG (www.genome.jp/kegg/pathway.html). Each pathway consists of a list of genes whose expression data is provided for each sample allowing the calculation of the deviation of a pathway in a given sample compared to the reference. Therefore, the deregulation score corresponds to the extent to which the activity of a pathway the first from the activity of the pathway differs from the activity of the same pathway estimated in seeds undergoing germination.

The Venn diagrams in this study are obtained using the jvenn : an interactive Venn diagram viewer webtool designed by Bardou *et al.*, (2014).

The gene ontology classification was obtained using the ClueGO tool from cystoscope and significantly represented categories having a P value less than 0.05 were visualized. ClueGO combines Gene Ontology (GO) terms as well as KEGG/BioCarta pathways and creates a functionally organized GO/pathway term network (Bindea *et al.*, 2009).

Fasta sequences of 5'UTR of differentially abundant transcripts found in the translome were retrieved using the Bulk Data Retrieval tool in Tair. (<https://www.arabidopsis.org/tools/bulk/sequences/>)

Over-represented motifs in the 5'UTR of differentially abundant transcripts were identified using the MEME site (Multiple EM for motif elicitation, (<http://meme.ncbr.net/meme/>) (Bailey *et al.*, 2009) using the two component mixture with set minimum and maximum widths 6 and 50 bases, respectively. The motif enrichment represents the ratio of the frequency of the over-represented motif in the co-expressed transcripts to the frequency of this motif in the 5'UTR of the whole genome of *Arabidopsis*

thaliana. Additional data concerning uORF was retrieved for differentially abundant transcripts in the transcriptome of dormant and non-dormant seeds. The uORF number, length, GC% were determined using the uORFlight database (Niu *et al.*, 2020).

Arabidopsis miRNA targets were retrieved using a knowledge-based database named Plant miRNA Encyclopedia (PmiREN, <http://www.pmiren.com/>) developed by Guo *et al.*, (2020).

Heatmaps corresponding to differentially abundant transcripts in seeds treated with cordycepin were obtained using the R software by applying a filter that selected significantly abundant transcripts (TPM > 3) whose TPM was lesser than 10 (TPM > 3 and < 10) or lesser than 20 (TPM > 3 and < 20).

3.7 Confocal Microscopy

YFP-DCP1 seeds (provided by Laboratoire Génome et Développement des Plantes, Université Perpignan) were incubated for 24 h in presence or in absence of ethylene at 25°C in darkness. After the removal of the seed coat, the embryos were visualized using an inverted Leica confocal microscope (SP5) with an excitation wavelength of 514 nm and an emission window of 520 to 550 nm. The images were later treated using the ImageJ software. Analysis were carried out on 15 acquisitions per condition. The number of fluorescent cells was counted in every cell and the average was determined using the formula $\text{Mean}_{\text{fluorescent foci}} = \frac{\text{Total number of fluorescent foci}}{\text{Total number of cells}}$.

4. Results

4.1 The beneficial effect of ethylene on seed germination relies on both transcription and association of transcripts with polysomes

As previously observed ethylene treatment broke Arabidopsis seed dormancy (Jurdak *et al.*, 2020, Figure S.1) since ethylene treated seed became able to fully germinated at 25°C in the darkness.

All transcripts identified by RNAseq from freshly harvested seeds were clustered according to their abundance in the transcriptome and in the transcriptome during seed imbibition with or without ethylene. Parallel coordinate plots group together subsets of mRNA displaying similar tendencies of evolution of their abundance within the time samples of seed

imbibition. Figure 1(a) shows clusters whose transcripts followed a similar pattern of abundance in the transcriptome of dry seeds imbibed for 6, 16 and 24 h at 25°C in darkness and in the translome of seeds imbibed for 16 and 24 h at 25°C in darkness. We did not investigate the translome at 6 h of imbibition since at this time point mRNAs are very poorly associated with polysomes (data not shown, (Basbous-Serhal *et al.*, 2015)). Many clusters showed that the abundance of a subset of mRNAs within transcriptome and translome did not vary very importantly between the dry state and the various time points of seed imbibition. In contrast the abundance of a subset of transcripts, grouped together in the dark blue cluster (Number 1 on Figure 1a), in the both the transcriptome and translome increased markedly during seed imbibition reaching a maximum value after 16 h. The expression of transcripts within the pink cluster (Number 2 on Figure 1a) decreased throughout seed imbibition but their abundance within the translome increased markedly after 24 h of imbibition. This representation clearly showed the effect of ethylene on the transcriptome and translome (Figure. 1b). As for imbibition on water a majority of clusters revealed only subtle variations in abundance of transcripts in the transcriptome and the translome. The profile of yellow cluster (Number 3 on Figure 1b) was very specific of ethylene treatment since it contains transcripts whose abundance decreased in the transcriptome but increased in the translome during seed imbibition. The blue cluster (Number 4 on Figure 1b) followed a similar trend but in a lesser extent. Some transcripts were also more abundant in the transcriptome and the translome of imbibed seeds, at all time points of seed imbibition (purple cluster, Number 5 on Figure 1b).

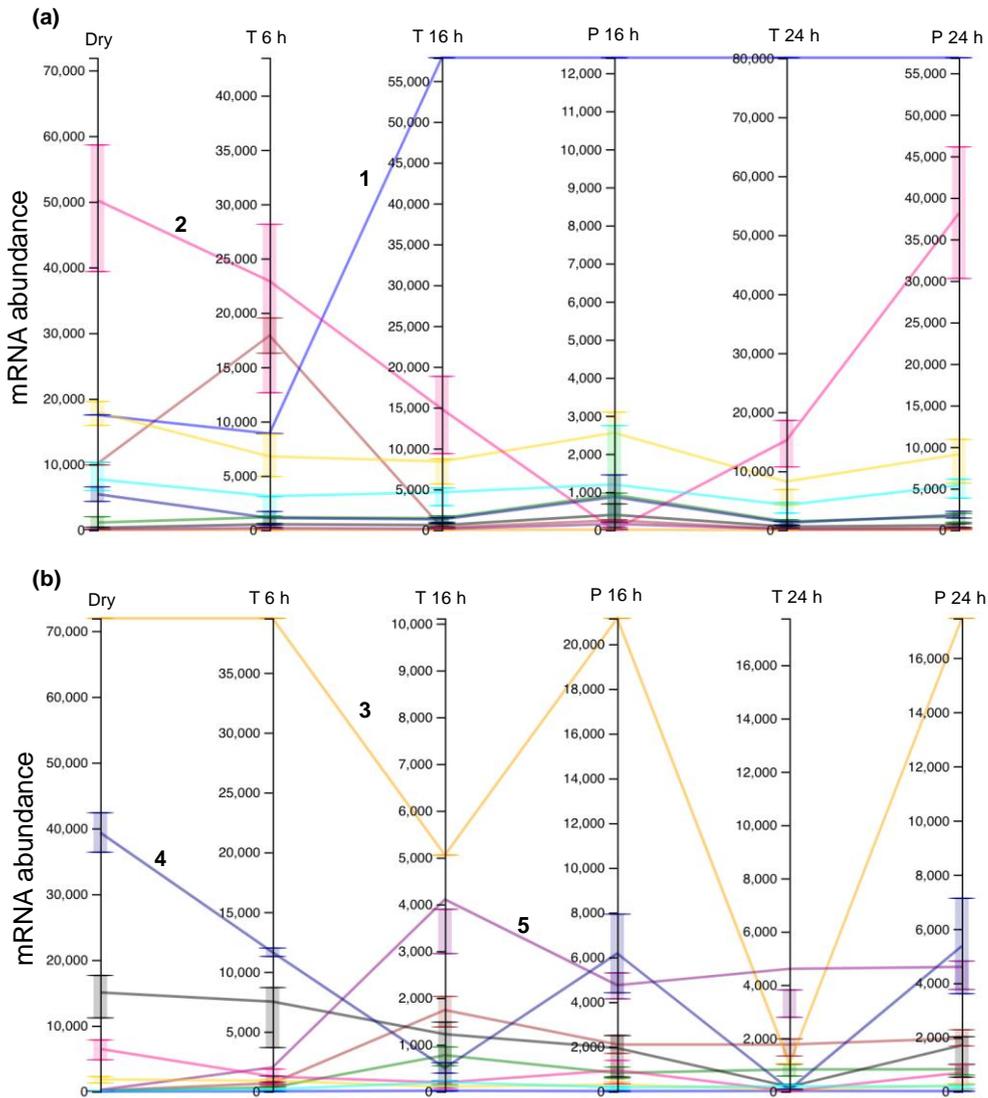


Figure 1. Changes in transcript abundance in the transcriptome (T) and in the translatoome (P) during imbibition of freshly harvested seeds on water (a) or in the presence of ethylene 100 ppm at 25°C in the dark. Transcripts were clustered into parallel coordinate plots according to their abundance in the various samples using “klustR” (R Studio). Clusters with numbers are mentioned in the text.

To further investigate the effect of ethylene on RNA dynamics, we first used Pathifier which is an algorithm that calculates pathway deregulation score (PDS) of a given pathway considering the changes in abundance of the genes belonging to the pathway (Drier *et al.*, 2013). Thus, Pathifier integrates subtle variations of gene expression in a pathway, even the ones considered as non-significant. Pathifier has been successfully adapted to Arabidopsis using 115 annotated KEGG pathways and it allowed the identification of novel actors of seed germination (Ponnaiah *et al.*, 2019). Here PDS values indicated to what extent the activity of a given pathway in ethylene treated seeds might differ from the activity of the same pathway estimated in control seeds. Figure 2 represents PDS values for the 115 KEGG-

derived pathways for the different samples. PDS values obtained in the transcriptome of imbibed seeds differed progressively from the ones found in dry seeds. After 6 h of imbibition PDS values of control and ethylene treated seeds were roughly similar but they progressively differed with prolonged imbibition. After 24 h of imbibition Pathifier clearly revealed that ca 2/3 of the pathways assessed using transcriptomic data were strongly deregulated in ethylene-treated seeds. PDS scores obtained with polysome-associated transcripts differed markedly from the ones obtained with the transcripts only (Figure 2). This allowed the identification of several pathways which were differentially regulated in the translome. They included "mRNA surveillance pathway", "brassinosteroid biosynthesis", "plant hormone signal transduction", "RNA degradation" or "MAPK signaling pathway", for example (Figure 2).

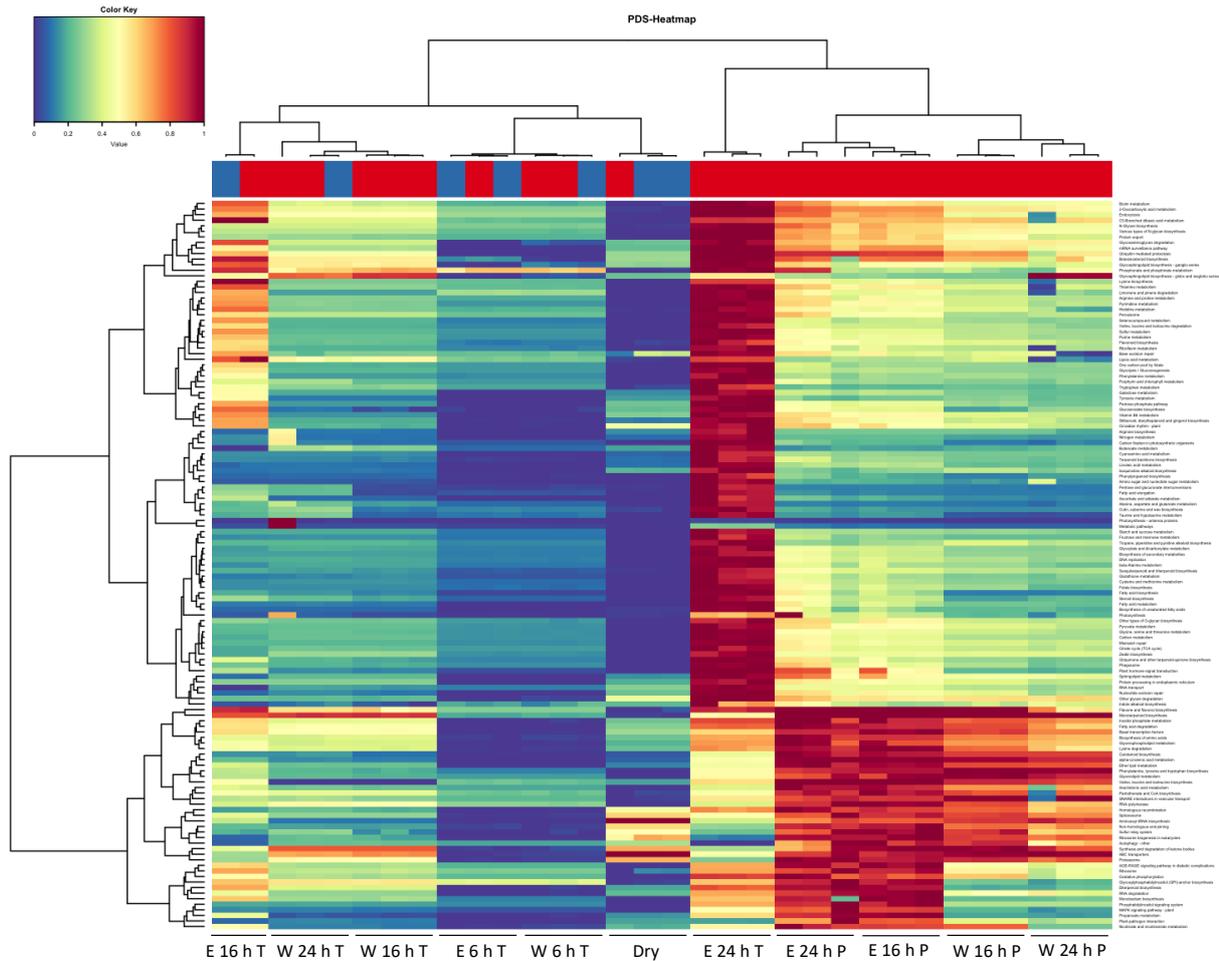


Figure 2. Heatmap showing the clustered pathway deregulation scores (PDS) of all KEGG pathways in the transcriptome (T) and in the translome (P) in freshly harvested dry seeds and in seeds imbibed for 6, 16 and 24 h on water (W) or in the presence of ethylene (E) at 25°C in the dark. Each column corresponds to a sample and each row to a pathway.

4.2 Ethylene involves specific gene expression and selective polysomal association of transcripts

In order to determine whether the germination in response to ethylene depends on a selective recruitment of mRNA to the polysomes and with the aim of investigating the origin of the mRNA bound to the polysomes we performed an integrative analysis of the transcriptome and the translome considering only transcripts displaying significant changes in abundance in the transcriptome (fold change absolute value >2, Bonferroni <0,05) and significant change in abundance in the translome (TPM>3), respectively. Venn diagrams Figures 3 (a-d) show the relationships between differentially abundant transcripts in the transcriptome and in the translome. The Venn diagram in Figure 3a groups together up-regulated genes with differentially abundant transcripts found the translome of dormant seeds imbibed in the absence of ethylene. This shows that a large majority of the

transcripts which are associated with polysomes in these conditions did not correspond to overexpressed transcripts. For example, at 24 h, only 14 transcripts among 390 found in the translome corresponded to up regulated genes at 6, 16 or 24 h (Figure 3a). The same conclusion can be drawn from comparisons between down regulated genes and their abundance in the translome (Figure 3b). It is nevertheless worth noting that transcripts of down regulated genes can be found in the translome at one time point. This is the case at 24 h where 39 transcripts associated with polysomes are also transcriptionally down regulated at this time point (Figure 3b). Ethylene treatment had a marked effect on gene expression and on the association of transcripts with polysomes; as shown on Venn diagrams Figures 3c and 3d, since at each time point, a greater number of differentially abundant transcripts were also detectable in the polysomes compared to dormant seeds (Figures 3a-b). At 24 h, 669 among the 2021 transcripts associated with polysomes corresponded to up-regulated genes at any time point of seed imbibition (Figure 3c). In contrast down-regulated genes in the presence of ethylene were poorly associated with polysomes at any time point of seed imbibition (Figure 3d). At 24 h, for example, only 63 down-regulated genes at any time point of seed imbibition, were also present in the translome among 2021 transcripts (Fig. 3 d). At last, the Venn diagram in Figure 3e allowed the comparison of changes in the translome with and without ethylene during seed imbibition. This Venn diagram shows that, among the 2021 transcripts associated with polysomes after 24 h of ethylene treatment, 1223 were specific of this time point (Figure 3e). This also shows that 548 were constantly addressed to polysomes during seed imbibition (i.e. at 16 and 24 h) with ethylene and that the absence of seed germination, i.e. imbibition on water, also specifically involves the association of 458 transcripts with polysomes, 183 being present at both durations of imbibition (Figure 3e).

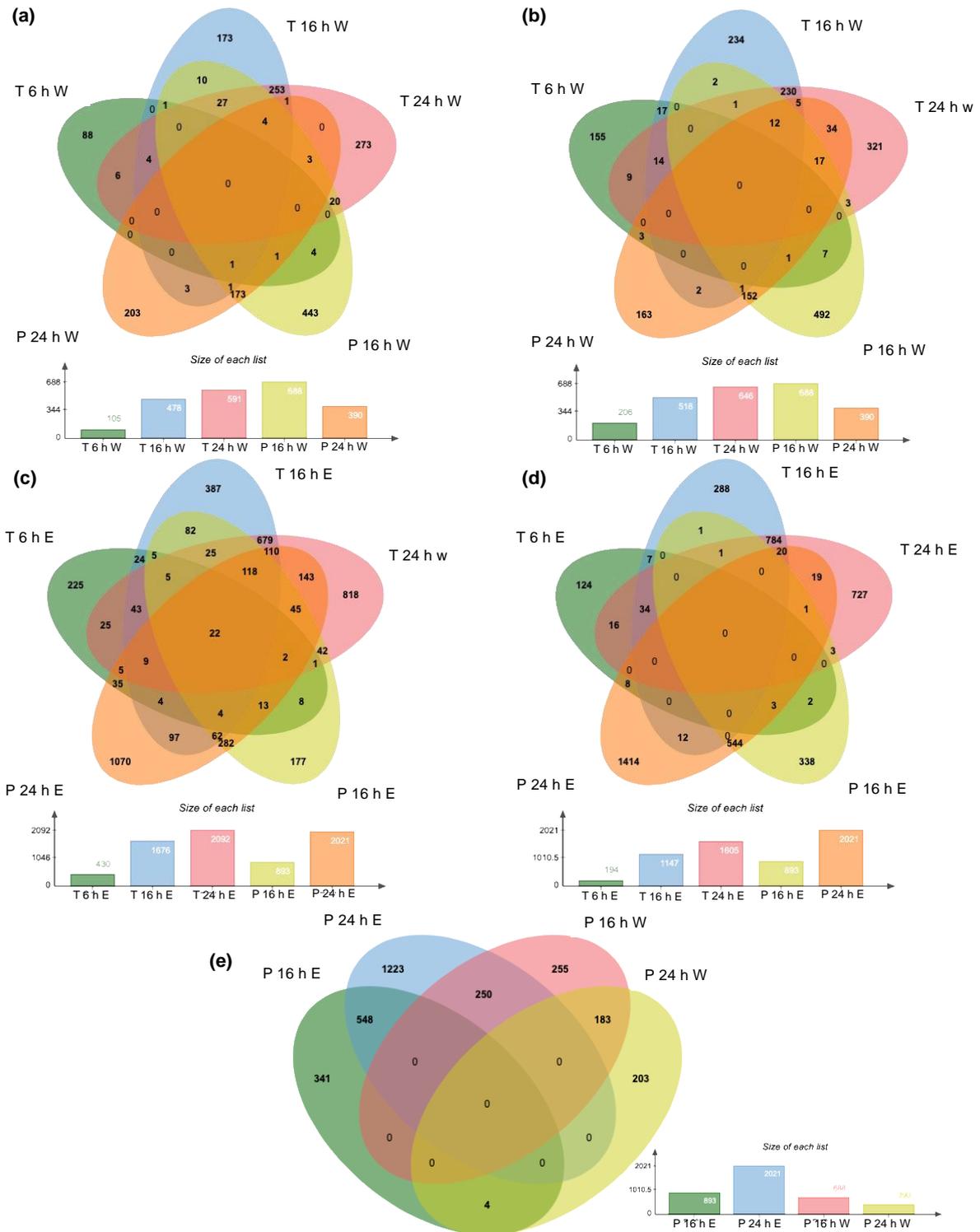


Figure 3. Venn diagrams showing the number of transcripts displaying significant increase (a,c) or decrease (b,d) in abundance in the transcriptome (T) (fold change absolute value >2, Bonferroni <0,05) and in the translatoome (P) (TPM>3) in freshly harvested seeds imbibed for 6, 16 and 24 h on water (a,b) or in the presence of ethylene 100 ppm (c,d). Venn diagram (e) showing the number of significantly abundant transcripts (TPM>3) in the translatoome (P) of freshly harvested seeds imbibed for 16 and 24 h on water or in the presence of ethylene 100 ppm. No value is indicated at 6 h for translatoome since at this duration transcripts were not associated with polysomes (see text). Number of transcripts in each category is indicated below the Venn diagrams

In order to investigate the possible involvement of the translation in the seed dormancy breaking by ethylene, we paid a close attention to the transcripts found as being associated with polysomes at both 16 and 24 h of imbibition in absence and presence of ethylene (Figure 3e) (Supplemental Table 1).

Putative functions were assigned to these transcripts using gene ontology (GO) categorization and they were visualized as functional clusters using clueGO (Figure 4). The 183 transcripts associated with polysomes in non-germinating seeds fell essentially into the categories: "seed dormancy process", "sugar transmembrane transporter activity", and "quercetin 3-O- glucosyltransferase activity" (Figure 4a). More GO categories were identified for the 548 polysome-associated transcripts identified in the presence of ethylene (Figure 4b). Among them we can notice "gibberellic acid mediated signaling pathway", "nucleoside monophosphate biosynthetic process", "response to brassinosteroids", "response to ethylene" or "regulation of carbohydrate metabolic process", for example. Putative roles of some genes belonging to these categories are further detailed in the discussion.

Chapter 4: Interplay between transcription, polysomal association and decay of mRNAs regulates the alleviation of Arabidopsis seed dormancy by ethylene

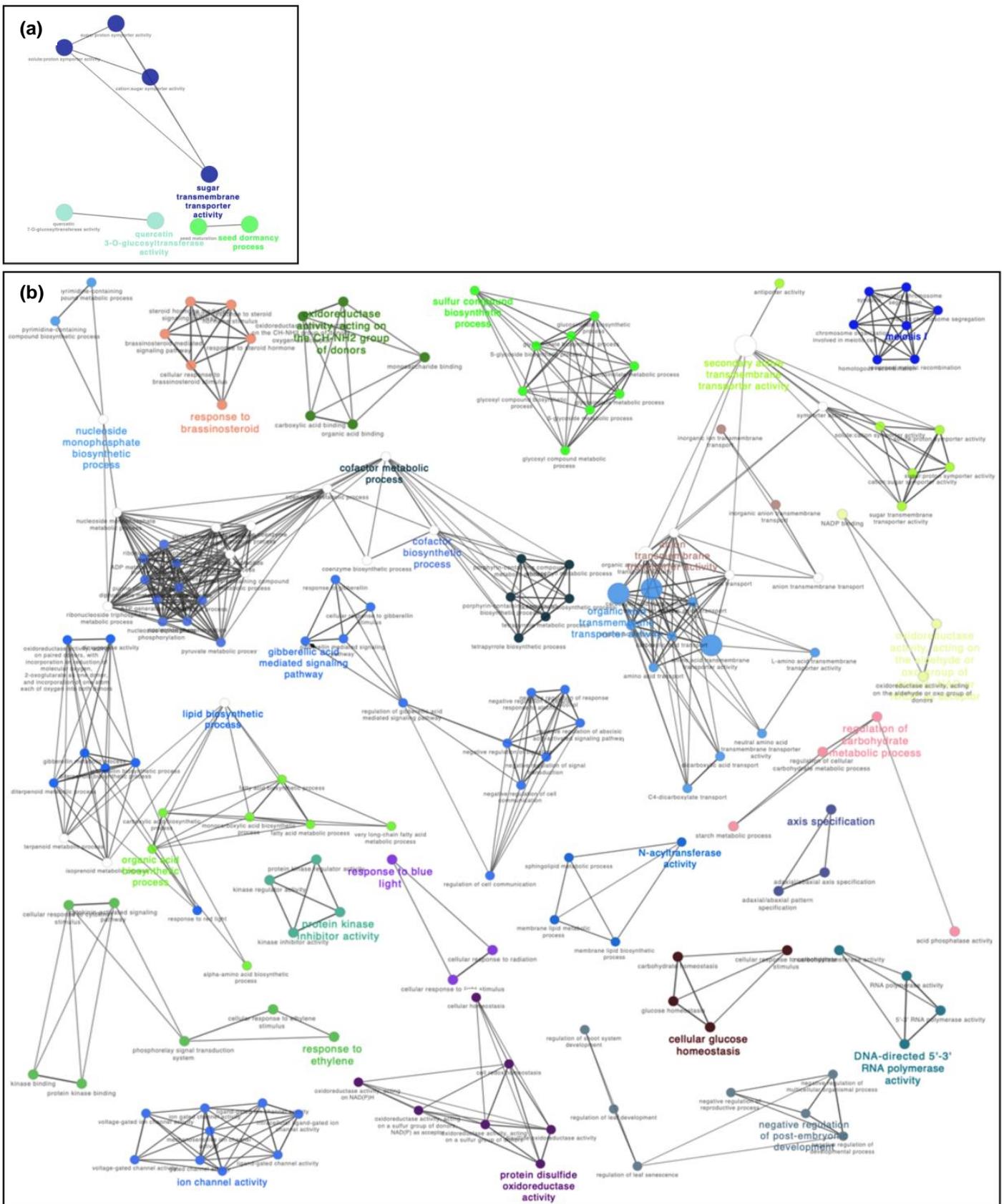
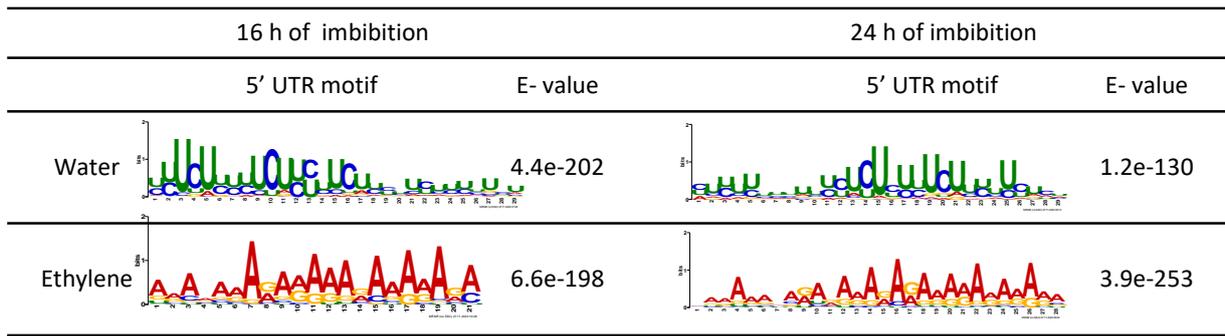


Figure 4. Gene Ontology (GO) classification of in common significantly abundant transcripts (TPM > 3, *P*-value < 0.05) found in the translatoome after 16 and 24 h of imbibition of freshly harvested seeds on water (a) or in the presence of ethylene 100 ppm (b) at 25°C in the dark. GO classifications were obtained using the ClueGO tool in Cytoscape.

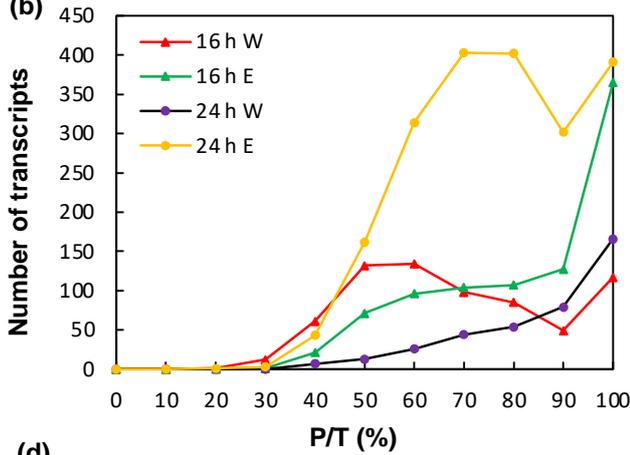
4.3 Are 5'UTR features likely to play a role in selective recruitment of transcripts by ribosomes?

We next investigated whether the association of transcripts with polysomes could rely on specific features of the 5'UTR. Over-represented motifs in the 5'UTR of differentially abundant transcripts in presence or in absence of ethylene were determined using the unsupervised multiple expectation maximization for motif elicitation (MEME) algorithm (Bailey *et al.*, 2009) (Figure 5a). GA-rich motifs were identified in presence of ethylene while CU-rich motifs were identified in dormant seeds, respectively. The polysomal ratio P/T corresponds to the proportion of individual mRNA species in polysomes, it was determined for the differentially abundant transcripts detected in the translome. No relationship between GC content in 5'UTR and P/T value was detected (data not shown). The distribution of polysomal transcripts in Figure 5b shows that a higher number of transcripts with a high polysomal ratio (> 60 %) was found in ethylene treated seeds than in dormant seeds. This was clearly visible after 24 h of imbibition (Figure 5b). The transcripts that 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%). The length of 5'UTR had a similar effect on P/T ratio, in dormant or germinating seeds since its value reached a maximal at around 180-200 nt but decreased when the length was shorter or longer (Figure 5c). We determined the uORF percentage in the 5'UTR of differentially abundant transcripts found in the translome in presence or in absence of ethylene (Figure 5d). More than 50 % of the genes studied had uORFs in 5'UTR (Figure 5d) but this percentage was higher in the presence of ethylene at both durations. The polysomal ratio linearly decreased with the number of uORF/gene (Figure 5e) and with the length of uORF (Figure 5f), but in an identical way in dormant or ethylene treated seeds.

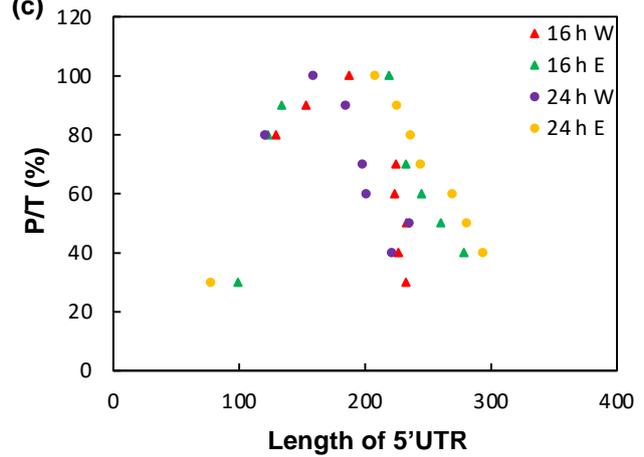
(a)



(b)



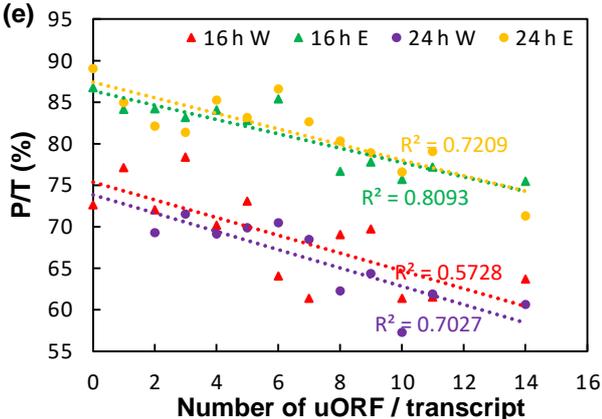
(c)



(d)

Condition	Number of transcripts with uORFs	% of transcripts with uORFs	Number of transcripts without uORFs
16 h water	385	55%	303
16 h ethylene	583	65%	310
24 h water	202	51%	188
24 h ethylene	1278	63%	743

(e)



(f)

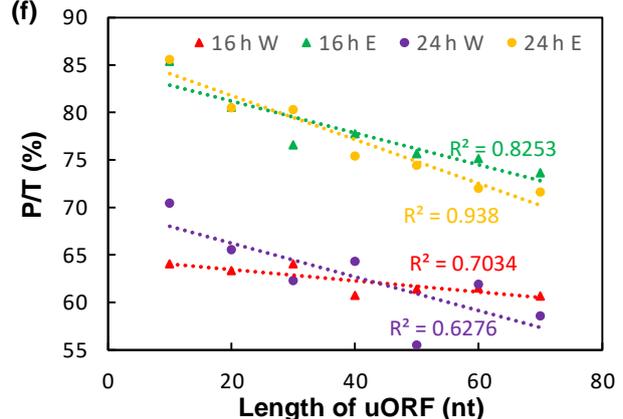
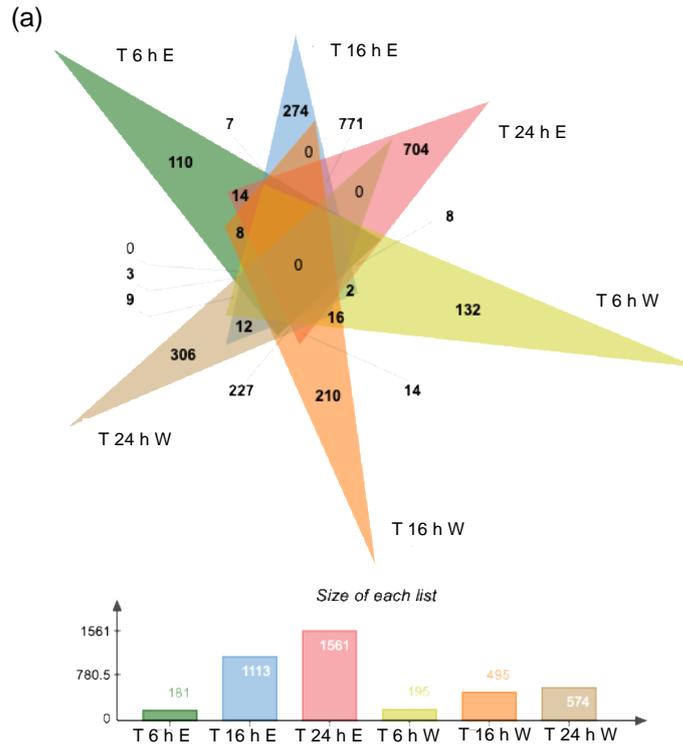


Figure 5. Main features of 5'UTR found in the differentially abundant transcripts in the translatoome of freshly harvested seeds imbibed for 16 and 24 h on water or in the presence of ethylene 100 ppm at 25°C in the dark. **(a)** Consensus sequences of motifs over-represented in the 5'UTR. E-value represents the statistical significance of the motif **(b)** Distribution of the transcripts according to their polysomal ratio (P/T) and **(c)** relationship between the length of the 5'UTR and the polysomal ratio. **(d)** Number and percentage of transcripts possessing Upstream Open Reading Frames (uORF). **(e)** Relationship between the number of uORFs per transcript and the polysomal ratio. **(f)** Relationship between the length of uORFs and the polysomal ratio.

4.4 Breaking of dormancy by ethylene is also associated with RNA decay

We also paid attention to the transcripts whose abundance decreased during seed imbibition on water or during seed treatment with ethylene. Figure 6 shows the numbers of transcripts whose abundance decreased at each time point. It clearly shows that in the presence of ethylene the abundance of many transcripts decreased, when compared to imbibition on water only. At 24 h more than 1500 genes were down regulated in the presence of ethylene, whereas the abundance of only 574 transcripts decreased in the presence of water at this time point (Figure 6). The full list of these genes is shown in Supplemental table 2 and their role is further discussed. Since miRNAs are known to be involved in the repression of gene expression, we investigated whether the down regulated genes identified here could be targets of miRNA (Figure 6b). Only a small percentage of down regulated genes appeared as being targets of miRNAs, nevertheless at 24 h we identified 193 miRNAs targets among the genes down regulated by ethylene. Complete list of these genes is provided Supplemental Table 3. Putative functions were assigned to the down regulated transcripts identified in presence of ethylene.



(b)

Condition	Number of miRNA targets in the transcriptome
6 h H ₂ O	29
16 h H ₂ O	81
24 h H ₂ O	83
6 h C ₂ H ₄	22
16 h C ₂ H ₄	144
24 h C ₂ H ₄	193

Figure 6. Venn diagram showing the number of transcripts significantly decreasing in abundance (fold change absolute value >2, Bonferroni <0,05) in the transcriptome of freshly harvested seeds imbibed for 16 and 24 h on water or in the presence of ethylene 100 ppm at 25°C in the dark. Number of transcripts in each category is indicated below the Venn diagram (a). Number of miRNA targets identified among transcripts displaying significant decrease in the same conditions (b).

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The GO categories essentially fell under the categories “regulation of cellular processes”, “regulation of biological processes” and “response to stress” (Figure 7).

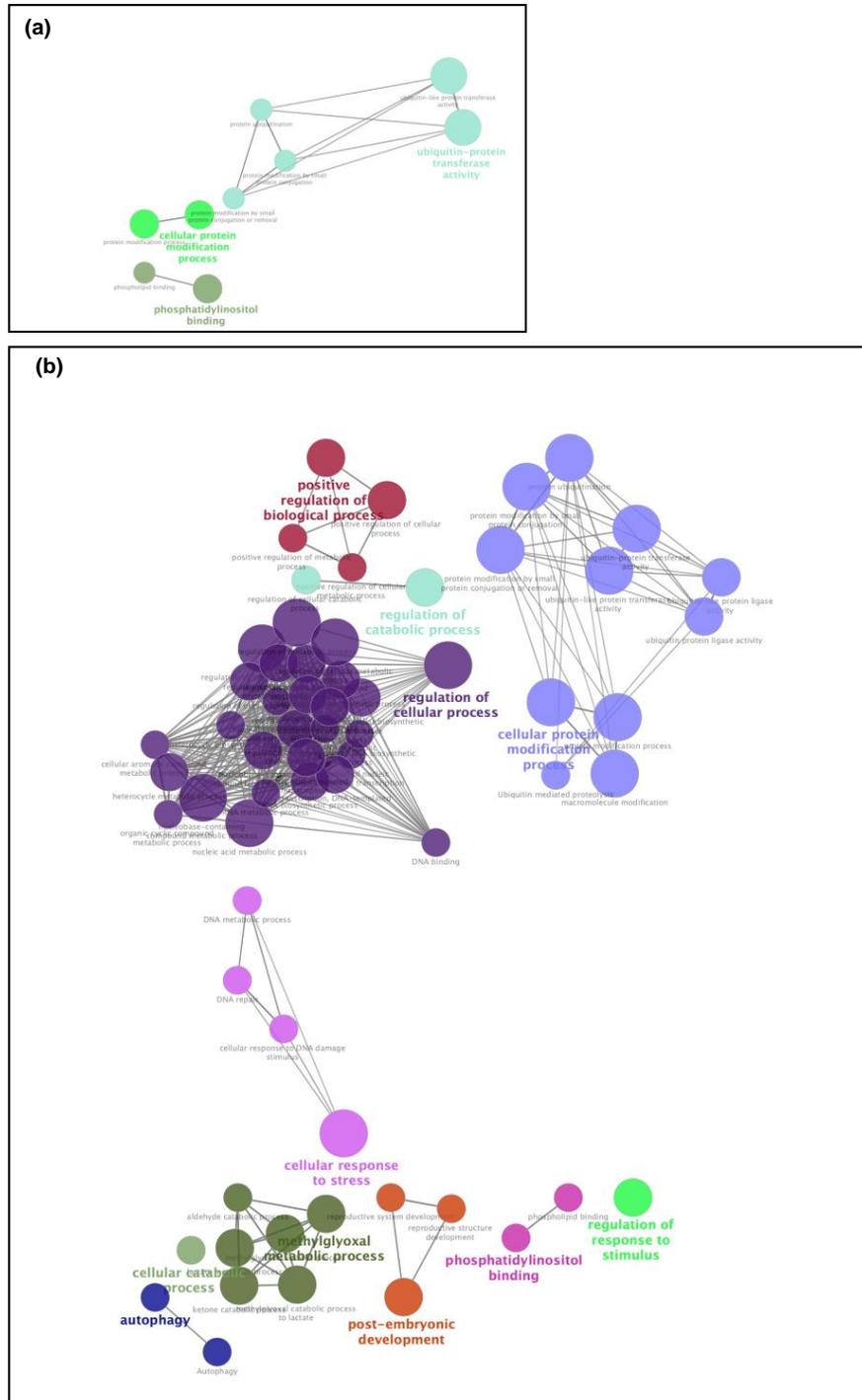


Figure 7. Gene Ontology (GO) classification of transcripts displaying significant decrease in abundance (fold change absolute value >2, Bonferroni <0,05) in the transcriptome of freshly harvested seeds imbibed for 16 (a) and 24 h (b) at 25 °C in the presence of ethylene 100 ppm (b) at 25°C in the dark. GO classifications were obtained using the ClueGO tool in Cytoscape .

We also used cordycepin, an inhibitor of transcription (Müller *et al.*, 1977, see Supplemental Figure 2) previously used by Basbouss-Serhal *et al.*, (2017) in the same context, to determine whether ethylene could alter mRNA stability. Thus, dormant seeds were imbibed for 16 h on a cordycepin solution and we compared the abundance of all transcripts in control and ethylene treated seeds (Figure 8). We verified that cordycepin did not alter seed germination on water neither dormancy breaking by ethylene (Figure S.2a). We also verified that cordycepin effectively inhibited transcription by following the expression of 2 genes known to be up-regulated during imbibition in the transcriptome of seeds imbibed in presence of ethylene (Figure S.2b). The heat map (Figure 8a) shows the differences in the abundance of transcripts (TPM ≥ 3 and ≤ 20 ; i.e. detection threshold for 5832 transcripts) between ethylene-treated and dormant seeds imbibed on cordycepin. In order to visually emphasize on the differences between those two conditions, a second filter (TPM ≥ 3 and ≤ 10 , i.e. detection threshold for 2371 transcripts) (Figure 8b) was applied on the list identified in Figure 8a. The results some transcripts are specifically degraded in dormant or in germinated seeds.

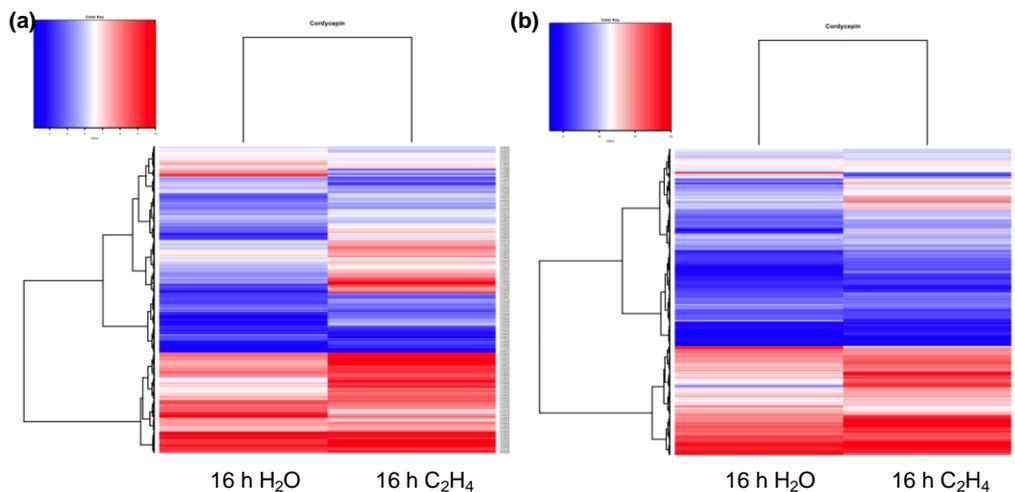


Figure 8. Heat maps showing significantly abundant transcripts (TPM>3) with a TPM < 20 **(a)** and a TPM < 10 **(b)** in the transcriptome of freshly harvested seeds imbibed for 16 h in the presence of cordycepin $50 \cdot 10^{-6}$ M in presence or in absence of ethylene 100 ppm at 25°C in the dark.

At last since mRNA decapping is a critical step in the 5' to 3' mRNA decay pathway, we investigated whether ethylene could induce the expression of the decapping protein AtDCP1, which participate to the 5'-3' mRNA decay (Iwasaki *et al.*, 2007; Buchan *et al.*, 2010; Maldonado-Bonilla, 2014). This was achieved using YFP-DCP1 transgenic seeds that we observed by confocal microscopy. After 24 h of imbibition at 25°C in presence and in absence of ethylene we detected fluorescent foci within the cells of embryonic axis, indicating localization of DCP1 proteins (Figure 9). In average, 2 fluorescent granules per cell were observed in dormant seeds (Figure 9a) compared to 5 fluorescent granules per cel observed following ethylene treatment (Figure 9b).

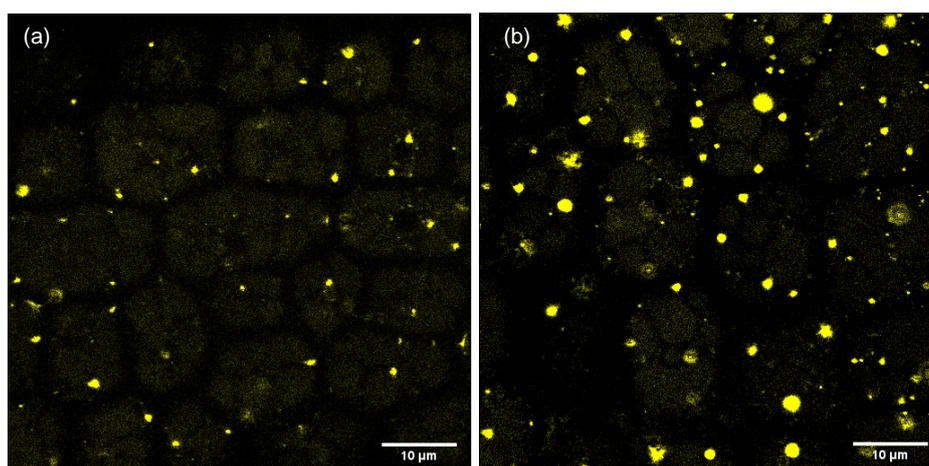


Figure 9. Fluorescence images of embryonic axes of freshly harvested YFP-DCP1 seeds imbibed on water for 24 h at 25°C in the dark in absence **(a)** or in presence **(b)** of ethylene (100 ppm).

5. Discussion

5.1 Ethylene effect on seed dormancy breaking is associated with a specific transcriptional and post transcriptional signature

In a previous study we demonstrated that the upstream response to ethylene in dormant Arabidopsis seeds was associated with ROS production and the activation of MRR (Jurdak *et al.*, 2020). Here combining transcriptomic and post-transcriptomic approaches we have deciphered some components of the downstream responses of dormant seeds to

ethylene. We first show that the breaking of seed dormancy by ethylene is associated with a specific response at both transcriptional and post transcriptional levels, as shown in Figure 1. Clustering showed that up and down regulation of genes expression coexisted with lower and higher abundance of transcripts associated with polysomes, whether seeds were able to germinate or not. It highlighted a clear discrepancy between the overall picture of the transcriptome and the translome in treated and untreated seeds (Figure 1). It is well known that developmental processes in plants does not only rely on the activation on the translational machinery but that they can also involve many different regulators, ranging from chromatin modifications to mRNA processing stability and even post-translational modifications, in order to provide a rapid and adaptive response to environmental signals, for example. In the context of seed germination, it has been demonstrated that selective translation (Layat *et al.*, 2014; Basbouss-Serhal *et al.*, 2015; Bai *et al.*, 2017) or decay (Basbouss-Serhal *et al.*, 2017) was likely to play a role in the completion of this process. Here we show that ethylene affected transcription and decay (as shown by increase and decrease of transcripts) and that the transcript association with polysomes occurred in a highly specific manner (Figure 1). This suggests that the beneficial effect of ethylene on seed germination certainly requires a synchronized action of these regulatory mechanisms. Paying a close attention to the differentially abundant transcripts in the transcriptome and in the translome gave more insights about these mechanisms (Figure 3). Ethylene significantly increased the number of transcripts either more or less abundant in the transcriptome after 16 and 24 h, when compared to water. We also show that ethylene stimulated the association of transcripts with polysomes, which became clearly visible after 24 h of imbibition, which is confirmed when considering the polysomal ratio values which increased in the presence of ethylene (Figures 3c-d). Besides, we demonstrate that the maintenance of dormancy, when seeds were imbibed on water, was associated with both an overexpression and a down regulation of a set of genes (approx. the same number) but also with a significant association of transcripts with polysomes (688 after 16 h, 390 after 24 h of imbibition, Figures 3a-b), thus revealing that translational activity may be involved in the maintenance of dormancy, as already proposed by (Basbouss-Serhal *et al.*, 2015). It is worth noting that a very large proportion of polysome-associated transcripts did not appear to result from overexpressed genes (Figure 3a) which shows that transcriptional and translational activities were probably disconnected in non-germinating seeds. This suggests that, in this context, seed stored mRNA, and even transcript whose abundance is decreasing, participated in dormancy maintenance by being potentially translated into proteins that negatively regulate germination. Long lived mRNAs (i.e. stored mRNAs) have

been identified long time ago in seeds (Dure & Waters, 1965; Waters & Dure, 1966) and their role as templates for protein synthesis during the initial phase of germination has been confirmed later (Rajjou *et al.*, 2004; Sano *et al.*, 2015).

In contrast, in germinating seeds, there was a better correlation between transcript abundance in the transcriptome and in the translome. After 24 h of ethylene treatment, for example, almost one-third of the transcripts associated with polysomes were also found as being more abundant in the transcriptome (Figure 3c). Although the relative importance of transcription is shown in ethylene response, one must keep in mind that two-third of the transcripts found in the polysome were not differentially abundant and thus issued from stored mRNAs. This points out again that transcriptomics studies may bring an uncompleted and sometime erroneous comprehension of the molecular mechanisms driving seed germination. This results are in accordance with Basbouss-Serhal *et al.*, (2015) and Bai *et al.*, (2020) which recently showed that a proportion of seed-stored mRNAs, associated with ribonucleic protein complexes, are translationally up-regulated during early seed germination.

To better understand the specificity of polysomal association we paid attention to the putative mechanisms of selective translation associated with 5'UTR (Kawaguchi & Bailey-Serres, 2005; Merchante *et al.*, 2017). As previously shown by Basbouss-Serhal *et al.* (2015), we identified a U-rich motif in transcripts addressed to polysomes in non-germinated seeds whereas polysome associated transcripts from ethylene treated seeds displayed a GA-rich motif (Figure 5a). In *Arabidopsis* plants it has been reported that adenine (A) content was positively correlated with ribosome loading (Kawaguchi & Bailey-Serres, 2005), which is in agreement with the results obtained here. Other effects of 5'UTR on association of transcripts to polysomes follow the canonical rules evidenced in other studies (Kawaguchi & Bailey-Serres, 2005; Basbouss-Serhal *et al.*, 2015; Bai *et al.*, 2017), but they do not permit to identify the mechanism of selective translation associated with dormancy and germination. Thus only UC and GA-rich motifs appeared as being potentially involved in selective association with polysome in the context of seed germination. This will have to be confirmed with other dormancy release treatment and attention will also have to be paid at the nucleotide sequence in the near environment of start codon AUG.

5.2 Decreased abundance of a subset of transcripts also translate the effect of ethylene on seed dormancy

Interestingly the beneficial effect ethylene on dormancy alleviation was also associated with the decrease of a subset of transcripts (Figure 1 Figures 3d and Figure 6). Although the abundance of some transcripts decreased during imbibition of dormant seeds (Figure 3b and Figure 6), in the presence of ethylene the whole imbibition process from 0 to 24 h led to the decrease in abundance of ca 3000 transcripts (Figure 6). This could suggest that ethylene has a general effect on mRNA stability. To address this point that we have evaluated the abundance of the transcripts after 16 h of imbibition in the presence of cordycepin, an inhibitor of transcription (Müller *et al.*, 1977; Basbouss-Serhal *et al.*, 2017). Indeed, preventing the RNA turnover gives a good indication about RNA stability (Narsai *et al.*, 2007; Chiba *et al.*, 2013), but it can also disrupt mRNA decay mechanisms if these former require an active transcription. Here we did not identify a general and marked decrease in transcript abundance between 0 and 16 h of imbibition, with or without ethylene, showing a high stability of the long-lived mRNAs stored in seeds (Figure 8). The heat maps in Figure 8 indeed show that mRNA degradation only concerned few transcripts but that the nature of the degraded transcripts depends upon the seed treatment, i.e. some transcripts are specifically degraded in dormant or in germinated seeds. This led us to exclude a global destabilisation effect of ethylene on mRNA decay but claim for a regulated mechanism of mRNA decay, as already proposed in other studies (Olmedo *et al.*, 2006; Potuschak *et al.*, 2006). Cell imaging approaches, using a YFP-DCP1 construct, showed that ethylene effect on seed dormancy was associated with an accumulation of DCP1 protein in structures that can be considered as P-bodies (Motomura *et al.*, 2015) (Figure 9). DCP1 is known to localize in P-bodies (Xu & Chua, 2011b) together with VARICOSE (VCS) and DCP2, a component of the decapping complex which removes mRNA cap prior to their digestion by 5' to 3' exoribonucleases (Nagarajan *et al.*, 2013). Here we propose that ethylene induced the decapping of a specific subset of transcripts thus leading to their 5' to 3' degradation. Interestingly the 5' to 3' exoribonuclease XRN4 also localizes in P-bodies (Xu & Chua, 2011b) and has been shown to be a component of the signaling pathway of ethylene (Potuschak *et al.*, 2006). These results are in agreement with the one of (Basbouss-Serhal *et al.*, 2017) who demonstrated the role of 5' to 3' mRNA decay in seed germination and with the ones of Wawer *et al.* (2018) who suggested that seeds lacking decapping activity were more sensitive to ABA and germinated poorly. We also investigated whether miRNA could play a role in mRNA decay during germination or dormancy maintenance. Although we identified

miRNA targets in the down regulated transcripts, they represented only a small proportion among them (Figure 6a, Supplemental Table 2), which allows ruling-out a strong involvement of this mechanism in the response of dormant seeds to ethylene.

5.3 Data-mining reveals molecular regulators of ethylene effect on seed dormancy alleviation

The RNAseq analysis enabled the identification of many different molecular players that are likely to play a role in the effect of ethylene on seed dormancy, and below we will mostly pay attention to the transcripts which were either associated with polysomes or degraded. Only the most significant findings will be discussed but the full list of transcripts either more abundant, degraded or associated with polysomes in the context of dormancy maintenance or alleviation are available (Supplemental tables 1,2 Figures 3-4-6-7). Pathifier confirmed the effect of ethylene on mRNA dynamics during seed imbibition and permitted the identification of several pathways involved in the response to ethylene. When considering polysome-associated transcripts, the pathway "plant signal transduction" was identified as deregulated. This pathway includes auxine, cytokinine, giggerellin, ethylene and brassinosteroids signalling pathways. It is worth noting that "Brassinosteroid biosynthesis" pathway also appeared deregulated in ethylene-treated seeds, and that interestingly the GO category "response to brassinosteroids" was identified when considering differentially abundant transcripts present in the translome of ethylene treated seeds (Figure 4). The beneficial role of brassinosteroids (BRs) on seed germination has long been cited (Steber & McCourt, 2001) but BRs effect on dormancy remains poorly investigated even though BRs have been proposed to overcome the inhibitory effect of ABA on germination (Steber & McCourt, 2001; Hu & Yu, 2014). The crosstalk between ethylene and BRs has been highlighted in other plant systems (Jiroutova *et al.*, 2018). Interestingly it has been shown that the cross-regulation between BRs, ethylene and ROS was involved in controlling root growth in *Arabidopsis*, but this interaction is not yet documented during seed germination (Lv *et al.*, 2018). As expected actors of the ethylene and gibberellins signaling pathways and biosynthesis were also found in the translome of ethylene treated seeds e.g. ERF9, ERF11 and CRF4 for ethylene or GAox2 and GA3ox1 for GAs (Figure 2, Figure 4, Supplemental table 1). Figures 2 and Figure 4 also revealed that the ethylene-induced association of transcripts with polysomes triggered a shift of the cellular metabolism towards the biosynthesis of various biomolecules such as lipids, nucleosides, cofactors, organic acids, sulfur compounds or carbohydrates. Among these pathways, purine and pyrimidine nucleotides are involved in nucleic acid synthesis and participate in

energetic processes and in the synthesis of carbohydrates, lipids, peptides and secondary metabolites (Stasolla *et al.*, 2003). The role of purine and pyrimidine metabolism in seed germination has already been implicated in various studies (Stasolla *et al.*, 2003; Saux *et al.*, 2020) but is not documented in the context of seed dormancy. The selective activation of various metabolic pathways is without any doubt a prerequisite to support the mechanisms involved in cell elongation that characterize radicle protrusion. The abundance in the transcriptome of germinating seeds of known actors of cytoskeleton and cell wall reorganisation such as SPIRAL1-2 (AT1G69230), EB1A (AT3G47690), EB1B (AT5G62500), MAP70 (AT1G68060) or cellulose synthase, PME1 (AT1G53840), respectively, confirms that the late steps of seed germination consist in preparing cell elongation. Yan *et al.* (2020) also demonstrated that the release of dormancy was associated with the expression and translation of a subset of microtubule and cell wall weakening regulators. In contrast we demonstrate that seed imbibition on water correlated with the association of dormancy-maintainers transcripts with polysomes, as already proposed by (Basbouss-Serhal *et al.*, 2015) and as shown in Figure 4a. Among them, one can identify DOG1(AT5G45830), a central player of dormancy maintenance (Carrillo-Barral *et al.*, 2020, p. 1), and NCED9 (AT1G78390), involved in ABA synthesis (Lefebvre *et al.*, 2006), PP2C (AT3G02750), PYL7 (AT4G01026) and PYL9 (AT1G01360), involved in ABA signalling pathway (Zhao *et al.*, 2016).

We also show that the complete sketch of ethylene effect on mRNA dynamics included timely down-regulation and decay of subsets of mRNAs (Figure 6, Supplemental table 2). GO clustering (Figure 7) showed that the abundance of transcripts belonging to major general cellular functions such as "regulation of cellular processes", "biological processes" or "responses to stress" decreased in germinating seeds. This denotes that mRNA decay unquestionably participates in a deep reorganization in cellular processes when seeds are undergoing germination. Going deeper in these GO categories allowed the identification of known actors of dormancy maintenance and release. For example, the abundance of NCED9 (AT1G78390), NCED6 (AT3G24220), AAO3 (AT2G27150), all involved in ABA biosynthesis (Seo *et al.*, 2000; Lefebvre *et al.*, 2006), and of ABF4 (AT3G19290), PP2C (AT4G11040) and PYL13 (AT4G18620), involved in ABA signalling decreased after 16 and 24 h in the presence of ethylene, thus confirming again the antagonist relationship between these 2 hormones. We also reveal unknown actors of the dormancy release process. This is the case for genes belonging to the GO category "phosphatidyl inositol binding" identified after 16 and 24 h of ethylene treatment. To our

knowledge only a very recent study (Coulon *et al.* (2020)) refers to a possible relationship between phosphoinositides and ABA signaling in the control of seed germination. This analysis also identified many GO terms related to ubiquitination (Figure 7) after 16 and 24 h of imbibition with ethylene (Figure 6) which is rather surprising since it is often mentioned that the ubiquitin proteasome system (UPS) degrades proteins, mostly related to ABA signaling pathway, that promote dormancy to allow germination (Chiu *et al.*, 2012). However, the overall analysis of the present data, including transcriptome data, indicates that ethylene induced a dramatic shift towards biosynthesis to the detriment of catabolic activities at the mid-term to germination, which could explain the late down regulation of ubiquitination related processes.

6. Conclusion

Our results show that mRNA metabolism plays a crucial role in the seed germination in response to ethylene. We show that alleviation of seed dormancy relies on a synchronized and timely regulated participation of either transcription, polysomal association and decay of mRNAs. This work allowed identifying many molecular actors involved in the process of germination, whose effect on germination is depending on their mRNA fate. The bases of such tightly regulated molecular mechanisms however remain unclear and further work will have to understand better how selective translation and decay can occur, as well as to determine how they are involved in other seed dormancy release mechanism.

General conclusion and perspectives

Numerous studies have been carried out in recent years with the aim of identifying the molecular mechanisms involved in the regulation of seed dormancy, a phenomenon that is directly responsible for the growth and dynamics of plants within ecosystems. Nevertheless, these mechanisms remain largely misunderstood. The ability of a seed to germinate appears to result from a fine interaction between inhibitory hormones (ABA) and stimulators (GAs, ethylene) of germination. These hormonal mechanisms are also integrated into a more complex network of cellular signaling involving the homeostasis of reactive oxygen species (Oracz *et al.*, 2007, 2009; Leymarie *et al.*, 2012). It has been shown for example that ethylene stimulated the production of ROS in sunflower seeds (El-Maarouf-Bouteau *et al.*, 2014). Ethylene and Reactive Oxygen Species (ROS) are players of seed dormancy alleviation, but the molecular basis of their action and crosstalk remains largely unknown.

We took advantage of the beneficial effect of ethylene on the germination of dormant *Arabidopsis thaliana* seeds for deciphering the mechanisms of ROS production and its effects during germination (Figure 16). Although previous studies demonstrated that ROS and ethylene would act synergistically to promote germination (Oracz *et al.*, 2007, 2009; El-Maarouf-Bouteau & Bailly, 2008b; El-Maarouf-Bouteau *et al.*, 2013), this is the first time that this putative relationship was addressed in *Arabidopsis* seeds. Contrary to what is claimed in many studies (see Bailly (2019) for review) it appeared that rbohS were not playing a major role in ROS production in response to ethylene or even in response to other methods of dormancy alleviation (light, stratification, GAs). Nevertheless our results all clearly showed that germination was only possible when ROS were produced by the seed tissues during their imbibition, thereby confirming old hypotheses (Bailly, 2004; Bailly *et al.*, 2008a). The finding that rboh activity is not required for seed germination is rather new and unexpected, which led us to study more in detail the contribution of mitochondria in ROS production. By combining cell imaging approaches, measurements of ROS and redox status, use of inhibitors and mutants we have decrypted the mechanism of action of ethylene on ROS production. We show that the mt-ETC was clearly the first site of ROS production during seed imbibition in the presence of ethylene but also in stratified seeds, GA-treated seeds and seeds exposed to continuous light. This is an important outcome of this work which allows us to propose that mitochondria and the respiration process do not only serve to supply energy but would also be involved in the regulation of a developmental process such as germination. To our knowledge only one study (Ma *et al.* 2019) recently pointed out that mitochondria could be also involve in the sensing of environmental factors permissive

for germination. Interestingly a recent review (Nonogaki, 2019a) also hypothesized that a loop of intracellular signaling by the nucleus, plastid, and mitochondrion could play a major role in seed dormancy regulation, but without any experimental evidence. This study is the very first to demonstrate that MRR can be involved in the regulation of seed germination, in that case in response to ethylene. Very interestingly also recent work by Merendino *et al.* (2020) demonstrated the involvement of mitochondrial signals in *Arabidopsis* skotomorphogenesis, a process which is controlled by ethylene, gibberellins and auxins, suggesting that MRR may also play a key role in hormone signaling networks. Future studies will have to investigate properly and revisit the role of mitochondria in seed germination. One also should consider the effect of anterograde signals from the nucleus to the mitochondria, which include for example AOX1a. More importantly the study of other seed dormancy release systems revealed that this MRR is not ubiquitous, even though the production of ROS in early imbibition is common to all systems. Unexpectedly we show that peroxisomes could play a role in ROS production during seed germination and this is also the very first time that such a role is proposed for these organelles. Thus, it will be interesting to properly investigate the dynamics of ROS production by these organelles and to determine whether they can exert significant effects on the redox status of mitochondria and on retrograde signaling. Further studies will also aim to identify the nature of the message involved in oxidative signaling from organelles to nucleus and if the nucleus can generate by itself an oxidative burst. Indeed, in all cases studied here we show that the germination processes ends with an accumulation of ROS within the nucleus. ROS are known to modify gene expression but through unclear mechanisms. Indeed, it is not known how simple compounds, such as H_2O_2 , can induce a wide variety of specific transcriptomic responses. Increasing evidences indicate that ROS could directly influence the epigenome landscape (R. M. *et al.*, 2020) but their link to programmed development remain to be investigated. It is known that germination is associated with wide changes of the embryo DNA methylome (Kawakatsu *et al.*, 2017; Narsai *et al.*, 2017) and higher order chromatin organization (van Zanten *et al.*, 2012). It has also been demonstrated that the *Arabidopsis* COMPASS-like complex a major actor in the deposition of the H3K4me3 histone mark, directly regulates the *DOG1* master regulatory gene and seed dormancy (Fiorucci *et al.*, 2019). We propose that nuclear ROS accumulation has an effect on chromatin compaction and thus modify gene expression for allowing seed germination. We are currently assessing this hypothesis.

Beside deciphering the dynamics of ROS production during seed germination, we also paid attention to the dynamics of RNA metabolism in the same process. We show that a synchronized activation of transcription, association of transcripts with polysomes and mRNA decay is associated with the beneficial effect of ethylene on seed germination. This allows us to provide a comprehensive scheme of ethylene on mRNA fate, also including the role of long-lived RNAs. This is to our knowledge the first time that a study integrates these different levels in a single model, since most of the studies dealing with dormancy and germination regulation were carried out at the transcriptional level only. This emphasises that the study of post-transcriptional mechanisms is of a paramount importance for having a good picture of the molecular regulation of this developmental step. The RNAseq data provide the identification of many regulators of this process. We demonstrate that the shift of the hormonal balance ABA/GA towards GA signalling and germination requires a multilevel and concerted effect on all levels of mRNA fate, from transcription to decay. Further work will have to focus on better understanding the causes of such a regulation. We will pursue deep mining of RNAseq data in order to identify possible regulators of translation or decay of mRNAs. The sequence around the AUG codon, the 3' end, the co-translational decay, the possible involvement of antisens or lncRNAs will have to be investigated. We also expect to investigate the roles of methylation (m⁶A) and oxidation (formation of 8-OHG) of mRNAs in this process. Indeed, such mechanisms are likely to regulate mRNA translation and decay but there are poorly explored up to date, mainly because of technical limitations. Thus we have initiated the study of mRNA methylation using Oxford Nanopore technology, which allows direct RNA sequencing (Parker *et al.*, 2020). The adaptation the data processing on Arabidopsis is actually in progress in order to identify RNA modifications and we expect to provide novel insights in our model of dormancy regulation very soon.

General conclusion and perspectives

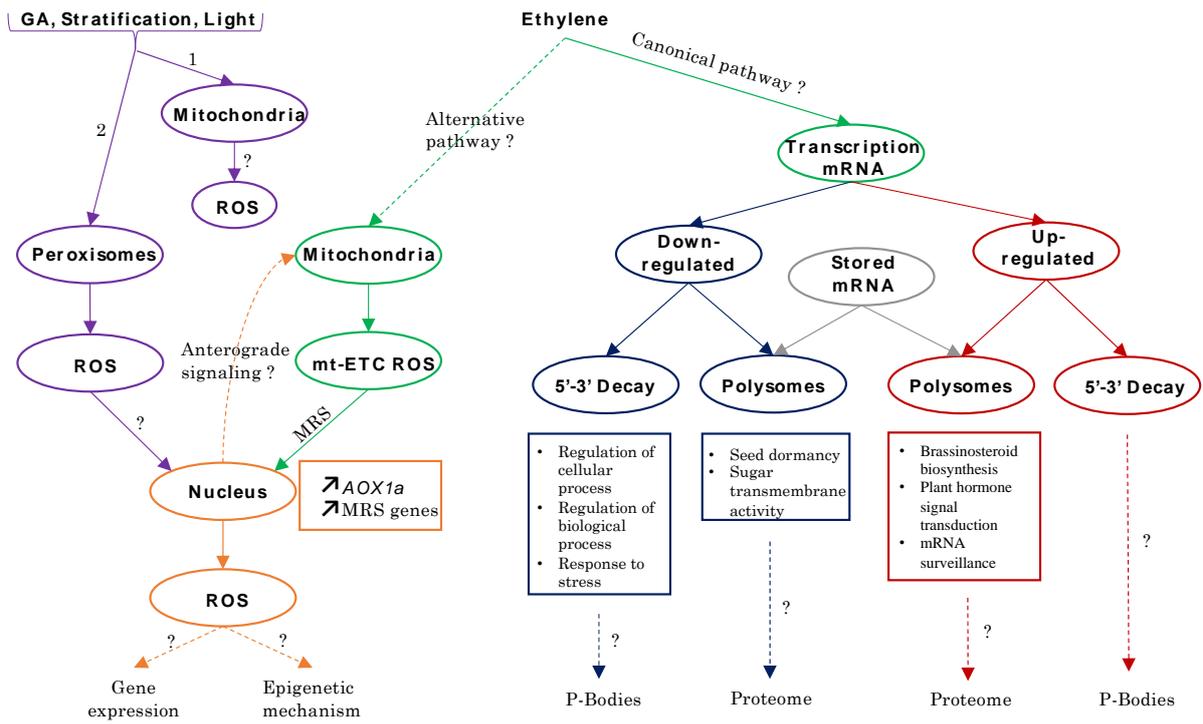


Figure 16-Schematic representation of the changes involved in oxidative signaling and mRNA metabolism in the response of *Arabidopsis thaliana* seeds to ethylene.

Annex

Annex Chapter 2

Fig. S1 Hydrogen peroxide production in Col-0 seeds incubated on water at 25°C on water and in presence of AA ($5 \cdot 10^{-6}M$) ROT ($5 \cdot 10^{-5}M$) and SHAM ($10^{-4}M$). Data are means of 3 replicates \pm SD. Different letters represent significantly different means with $P < 0.05$ according to Kruskal Wallis.

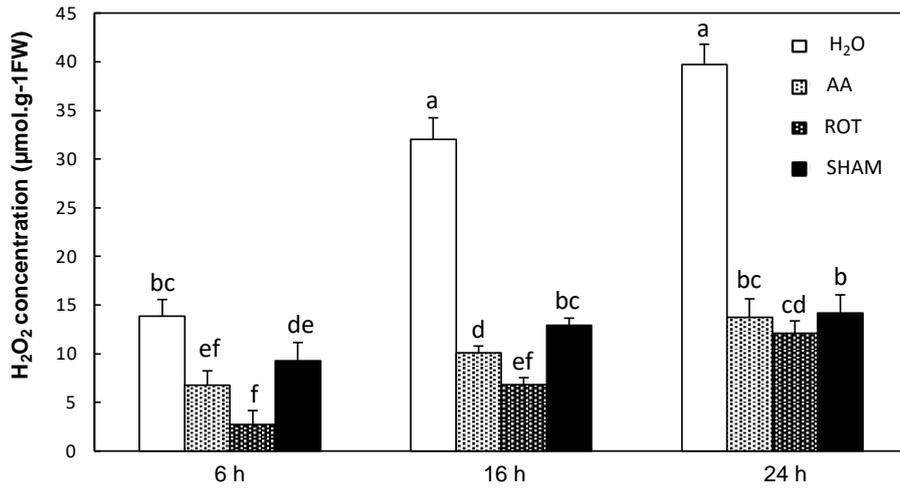


Fig. S2 Germination of freshly harvested seeds of Col-0, *rbohDB*, *mao* and *ein2* on water and in the dark at 25°C in absence and presence of ethylene. Data are means of 3 replicates \pm SD.

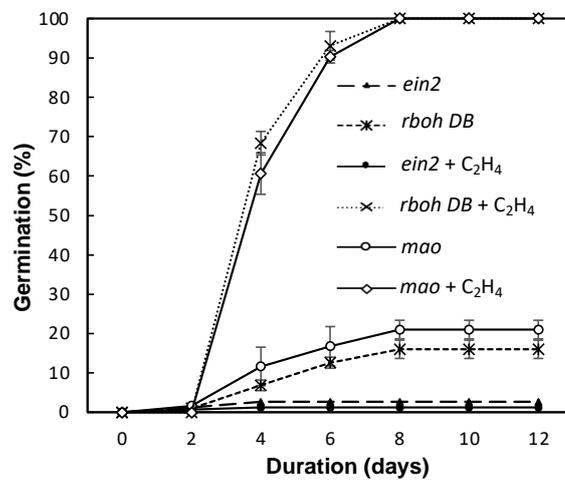


Fig. S3 Fluorescence images of embryonic axes of dormant mitochondrial ro-GFP seeds incubated on water in the dark at 25°C for 6 h (a, b, e, f) and 16 h (c, d, g, h) at 25 ° C in absence (a, b, c, d) or in presence (e, f, g, h) of ethylene 100 ppm. Excitation at 405 nm (a, c, e, g) or 488 nm (b, d, f, g).

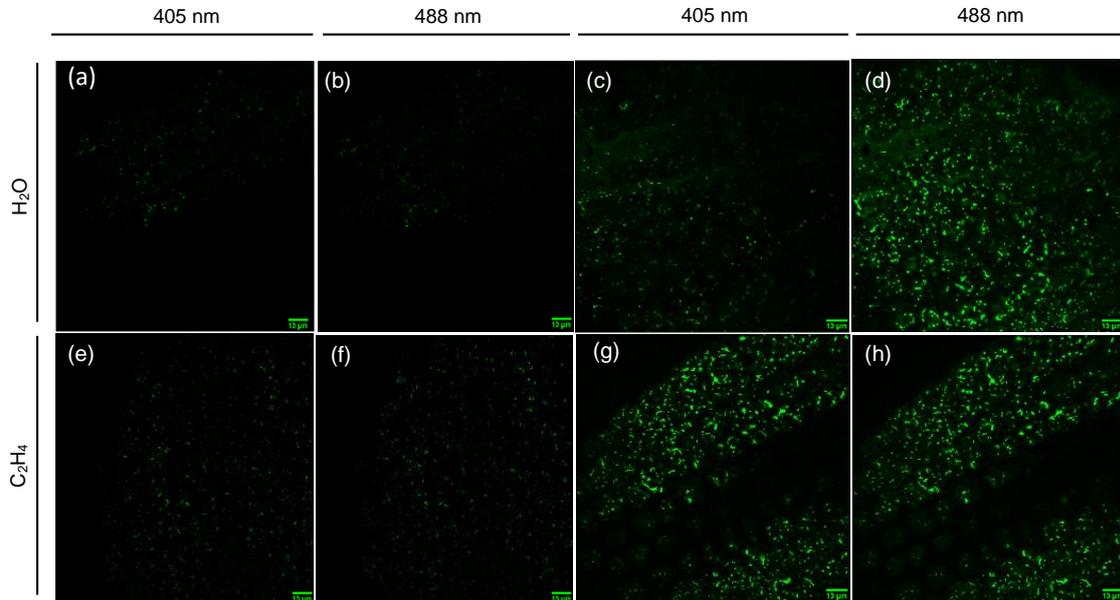


Fig. S4 Representative observation of autofluorescence emission obtained by confocal microscopy of cells of embryonic axes of dormant Col-0 seeds incubated at 25 ° C in the presence of 100 ppm ethylene after excitation at 488 nm (a), 561 nm (b) and 405 nm (c).

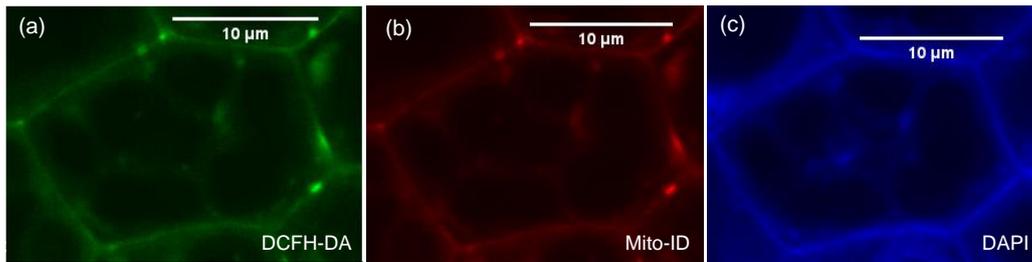


Fig. S5 Inhibitors of the mitochondrial electron transport chain diminish the nuclear localization of ROS. Fluorescence images of cells of embryonic axis of dormant Col-0 seeds incubated for 16 h on water at 25°C in the dark in presence of ethylene and treated by $5 \cdot 10^{-5}$ M ROT (a, b, c), 10^{-4} M SHAM (d, e, f) or $5 \cdot 10^{-6}$ AA (g, h, i) and stained with DCFH-DA (a, d, g), DAPI (b, e, f). (c, f, i) merged images.

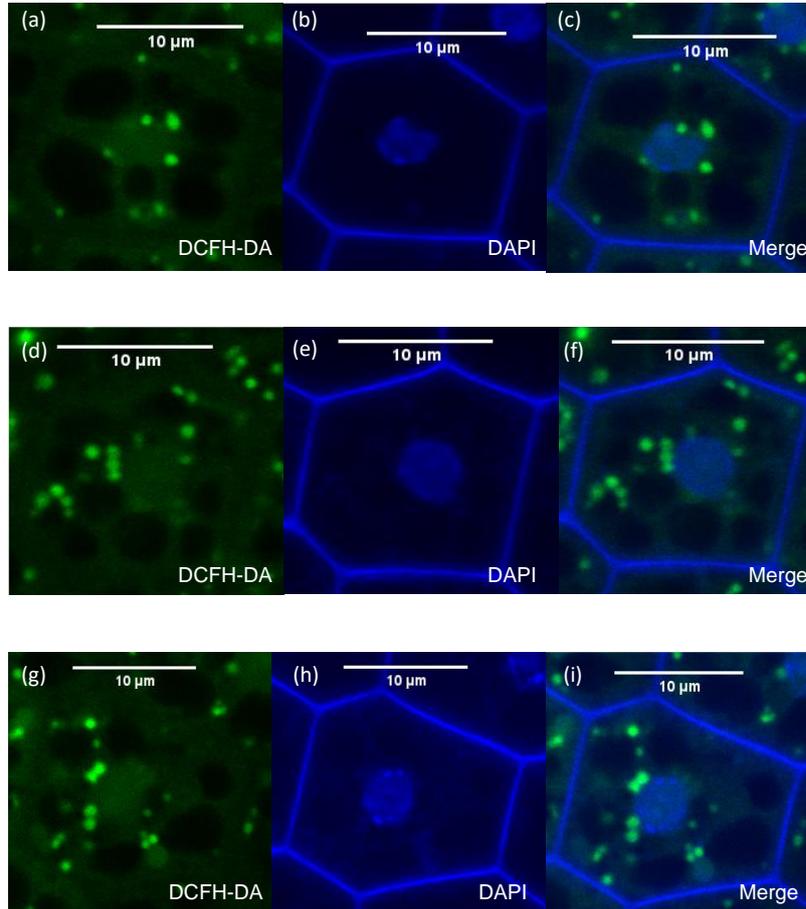


Fig. S6 Inhibitors of the mitochondrial electron transport chain diminish the nuclear localization of ROS. Fluorescence images of cells of embryonic axis of dormant Col-0 seeds incubated for 24 h on water at 25°C in the dark in presence of ethylene and treated by $5 \cdot 10^{-5}$ M ROT (a, b, c), 10^{-4} M SHAM (d, e, f) or $5 \cdot 10^{-6}$ AA (g, h, i) and stained with DCFH-DA (a, d, g), DAPI (b, e, f). (c, f, i) merged images.

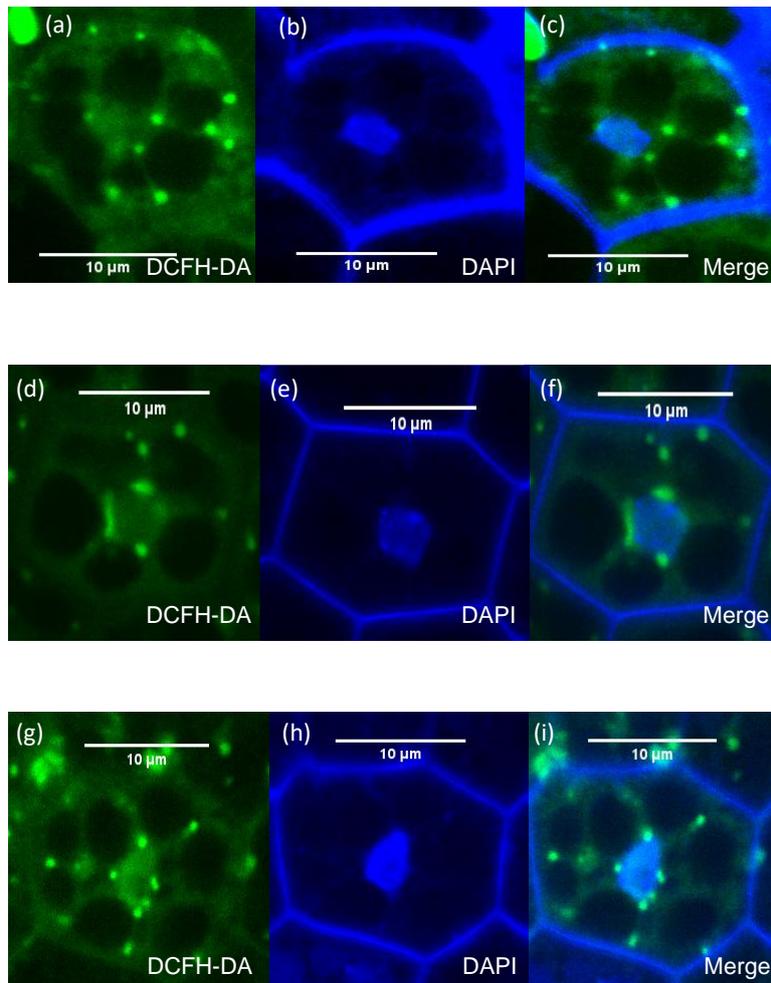


Table S1.

List of seeds used for the germination assays. The AGI of the genes were determined using TAIR. T-DNA insertion lines were ordered from the Nottingham Arabidopsis Stock Centre (NASC).

Gene	Mutant	AGI	T-DNA insertion
<i>MONOAMINE OXIDASE</i>	<i>mao</i>	AT4G37110	SALK_054748
<i>ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 13</i>	<i>anac013</i>	AT1G32870	SALK_096150
<i>ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 17</i>	<i>anac017</i>	AT1G34190	SALK_044777
<i>TRANSLOCASE INNER MEMBRANE SUBUNIT 23-3</i>	<i>tim23-3</i>	AT1G72750	SALK_008072
<i>PEROXIREDOXIN II F</i>	<i>prxII F</i>	AT3G06050	SALK_043925
<i>ALTERNATIVE OXIDASE 1A</i>	<i>aox1a</i>	AT3G22370	SALK_084897
<i>ETHYLENE INSENSITIVE 2</i>	<i>ein2</i>	AT5G03280	N3071

TableS2. List of primers used for the qRT-PCR essays. The AGI of the genes were determined using TAIR and the sequences using the Vector NTI align X and Oligo 7 software.

Gene	AGI	Primers Sequences
<i>ABO6</i>	AT5G04895	F: TCACATTCCTGGCTTCACACAT R: AAGAGGTCAGCTTATATCCCGTTA
<i>ANAC017</i>	AT1G34190	F: TTCCTCAGGTGAATAGTGAAGA R: CTGTTGTATTGCTGACCGTTT
<i>NDUFS4</i>	AT5G67590	F: ACTCTGCTCTTGCTTTTGATTC R: TTGTCCGAGTAAGACTTGACC
<i>AOX1A</i>	AT3G22370	F: CCATGGGAAACGTATAAAGCTGAT R: CCATATCTCCTCTGGAAGAACA
<i>ANAC013</i>	AT1G32870	F: GGAATTACCTGAGAAAGCGTTGTA R: TGCTTTCCAATAGCCACGTT
<i>PRXII F</i>	AT3G06050	F: TTCTCCACCACGCCATTGTCA R: CCGTGTAAGCCCCAGGAAGACCAA
<i>ABI4</i>	AT2G40220	F: AAGTGGCTTGGTACTTTTCGC R: AGGAGACGAAGGGGTTAAGTTG
<i>TIM23-2</i>	AT1G72750	F: TACGAAGTCCCAATCAACAAATCT R: ATCTACGTTGCCTTAAAGCC
<i>MSD1</i>	AT3G10920	F: ATCAAGCTGTGAACAAGGGA R: GAATGGTTGACATGACCTCCGCCG
<i>CB5-E</i>	AT5G53560	F: TGAAGAAGTTTCAAAGCACAACA R: TCATCCATGAATGGAGTCACA
<i>TIP41</i>	AT4G34270	F: GTGAAAACCTGTTGGAGAGAAGCAA R: TCAACTGGATACCCTTTCGCA
<i>RHIP1</i>	AT4G26410	F: GAGCTGAAGTGGCTTCCATGAC R: GGTCCGACATACCCATGATCC

Annex Chapter 3

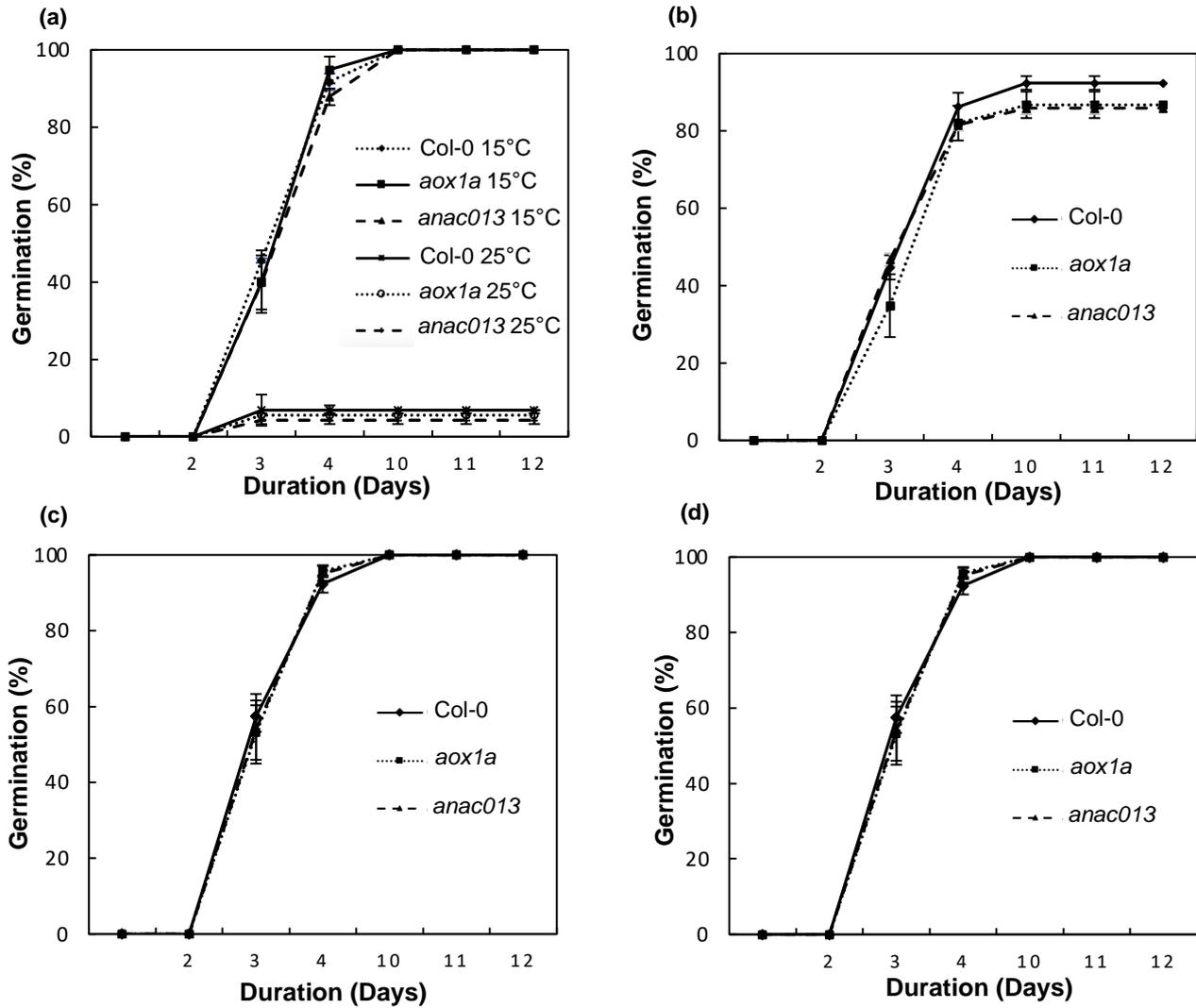


Figure S1- Germination of freshly harvested seeds of the mutants *aox1a*, *anac013* at 15°C and 25°C in darkness on water (a), at 25°C in darkness on a solution of gibberellic acid (GA) 1 mM (b), after cold-stratification for 4 d at 4°C (c) or at 25°C under constant light (d). Means of 3 replicates \pm SD.

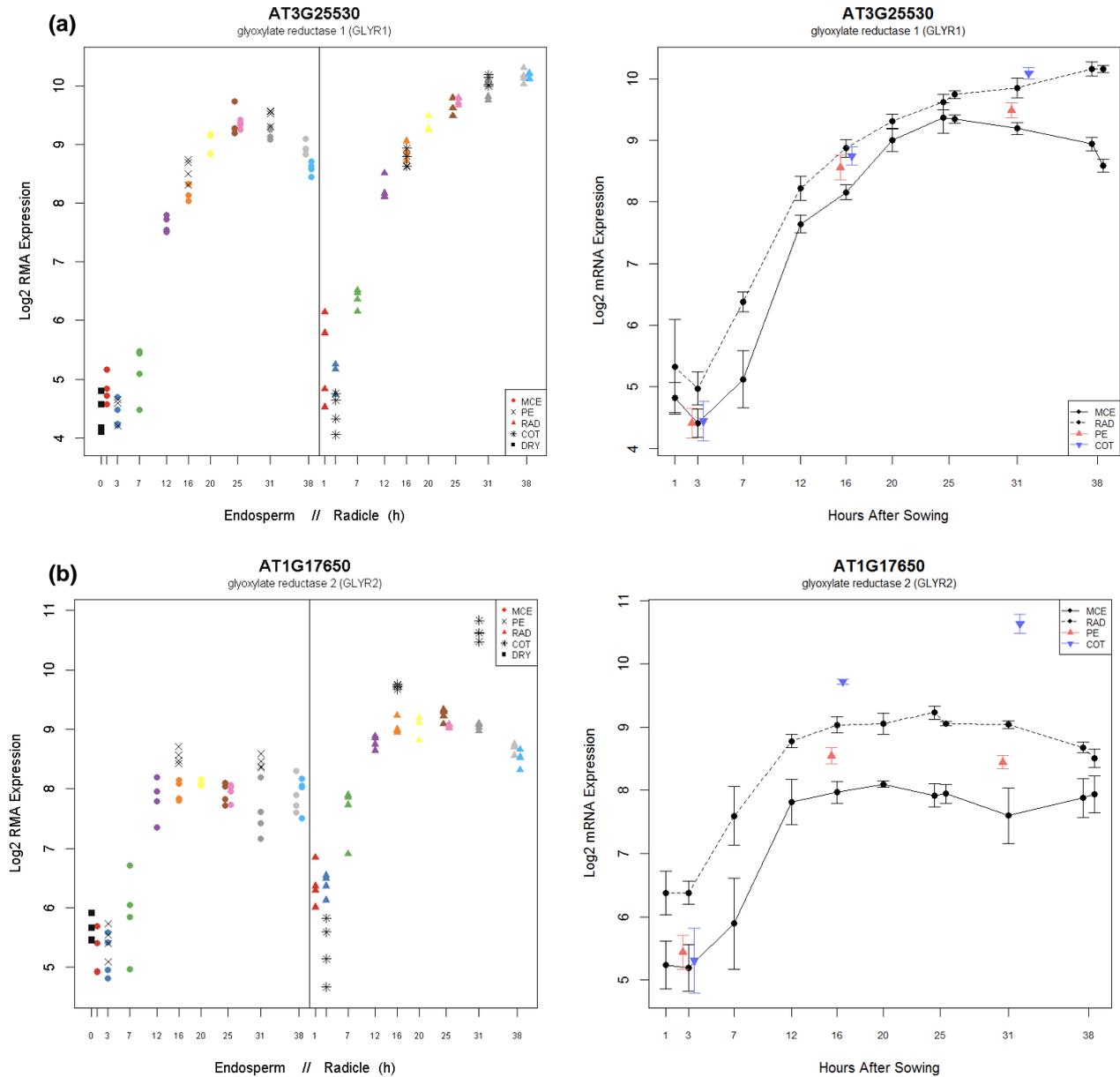


Figure S2- Levels of RNA expression for *GLYR1* (a) and *GLYR2* (b) in Endosperm and Embryo during germination. On the left, each replicate is represented by an individual symbol while on the right graph, the means are plotted with standard deviations by Dekkers *et al.*, (2013), courtesy of SeedNet Nottingham (<http://vseed.nottingham.ac.uk/>). Key: MCE = Micropylar and Chalazal Endosperm, RAD = Radicle, PE = Rest of the endosperm, COT = Cotyledons, DRY = Whole dry seeds.

Table S1- List of seeds used for the germination essays. The AGI of the genes were determined using TAIR. T-DNA insertion lines were ordered from the Nottingham Arabidopsis Stock Centre (NASC).

Gene	Mutant	AGI	T-DNA insertion
<i>ALTERNATIVE OXIDASE 1A</i>	<i>aox1a</i>	AT3G22370	SALK_084897
<i>ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 13</i>	<i>anac013</i>	AT1G32870	SALK_096150
<i>RESPIRATORY BURST OXIDASE HOMOLOG D</i>	<i>rboh D</i>	AT5G47910	SALK_070610
<i>RESPIRATORY BURST OXIDASE HOMOLOG B</i>	<i>rboh B</i>	AT1G09090	SALK_048130

Table S2- List of primers used for the qRT-PCR essays. The AGI of the genes were determined using TAIR and the sequences using the Vector NTI align X and Oligo 7 software.

Gene	AGI	Primers Sequences
<i>AOX1A</i>	AT3G22370	F: CCATGGGAAACGTATAAAGCTGAT R: CCATATCTCCTCTGGAAGAACA
<i>ANAC013</i>	AT1G32870	F: GGAATTACCTGAGAAAGCGTTGTA R: TGCTTTCCAATAGCCACGTT
<i>RBOH B</i>	AT1G09090	F: GACATTGCATAGGAATGGTTGA R: ACTATTGCCTGTGATCTGCT
<i>RBOH D</i>	AT5G47910	F: GTCTCAAGTTCATTGCCACC R: TTATTCATTCTAAGCATTCC
<i>CAT</i>	AT4G35090	F: AACGTGAGAGGTGCATTATTGA R: TGCCTCTCTGGTGTAAGGT
<i>GOX</i>	AT3G14415	F: ATCAAGAACAGATTCACTTTGC R: GTCATTGGCCTCGTCCAT
<i>XDH1</i>	AT4G34890	F: AAGAAATCAAAGAGATGAACTT R: TCTTTCCAGAGCTGATGCAAT
<i>SO</i>	AT3G01910	F: CTGGAAAGGTAAGTATCAAAGG R: TACTGCTTTCCTGGTTCCT
<i>SAX</i>	AT2G24580	F: TCAAACATCAAACCAAGCCTCT R: CCATAACTCCTGCTCCAACGA
<i>PAO4</i>	AT1G65840	F: CAGAACTAAGGCAAGAAGGGAT R: TCGCATCTACAGCAAACCAC
<i>GA3OX1</i>	AT1G15550	F: ACATCACCTCAACTACTGCGAT R: GTCTTCTTCGCTGACCCCAA
<i>GA3OX2</i>	AT1G80340	F: CCAGCCACCACCTCAAATACTGTG R: CTCCCAGTGAACCTAATGCGAACC
<i>GA2OX2</i>	AT1G30040	F: ATGGTGAAGGTGGGGTTTGG R: CTCCCGTTAGTCATAACCTGAAG
<i>EXPA3</i>	AT2G37640	F: CTTAACCGCAACAAACGCCA R: ACAAGTTCCTGACCCACAC
<i>GASA4</i>	AT5G15230	F: ATGTGAAGTGGAGCCAGAAACG R: GCAAGCCTTGTGGTACTGTGTC
<i>GASA14</i>	AT5G14920	F: ACTCGGATAGATTGCGTGCC R: ACGTGTCTTCATGTTGGCGT
<i>AAO1</i>	AT5G20960	F: TCCTTCCACCACACTCGTCGATTC R: AGCGTGAGACACGAGCTAATTGTGA

<i>ABA1</i>	AT5G67030	F: GGCATTTGGTCTAAGGTGAGAA R: CAGACTCGATATCCGCTGGTA
<i>CYP707A2</i>	AT2G29090	F: AGGATCAAAAACGCAACCGG R: AGCCGCCTCTGGACTACTTTA
<i>CYP707A3</i>	AT5G45340	F: CATGCCTTTTGGTAGTGGGATTCAT R: AGCGTGAGACACCGAGCTAATTGTGA
<i>ABI5</i>	AT2G36270	F: AACATGCATTGGGCGGAGTTG R: GCAATTCGGTTGTGCCCTT
<i>ABI4</i>	AT2G40220	F: AAGTGGCTTGGTACTTTTCGC R: AGGAGACGAAGGGGTTAAGTTG
<i>CB5-E</i>	AT5G53560	F: TGAAGAAGTTTCAAAGCACAACA R: TCATCCATGAATGGAGTCACA
<i>TIP41</i>	AT4G34270	F: GTGAAAACCTGTTGGAGAGAAGCAA R: TCAACTGGATACCCTTTTCGCA
<i>RHIP1</i>	AT4G26410	F: GAGCTGAAGTGGCTTCCATGAC R: GGTCCGACATACCCATGATCC

Annex Chapter 4

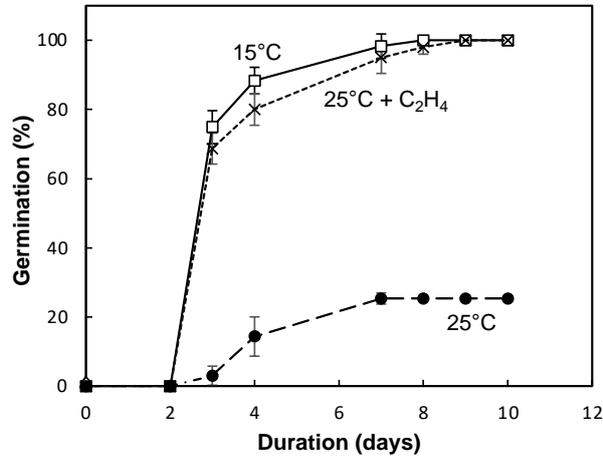


Figure S.1. Germination of freshly harvested seeds on water at 15°C and at 25°C in the dark and in absence or in presence of ethylene (100ppm).

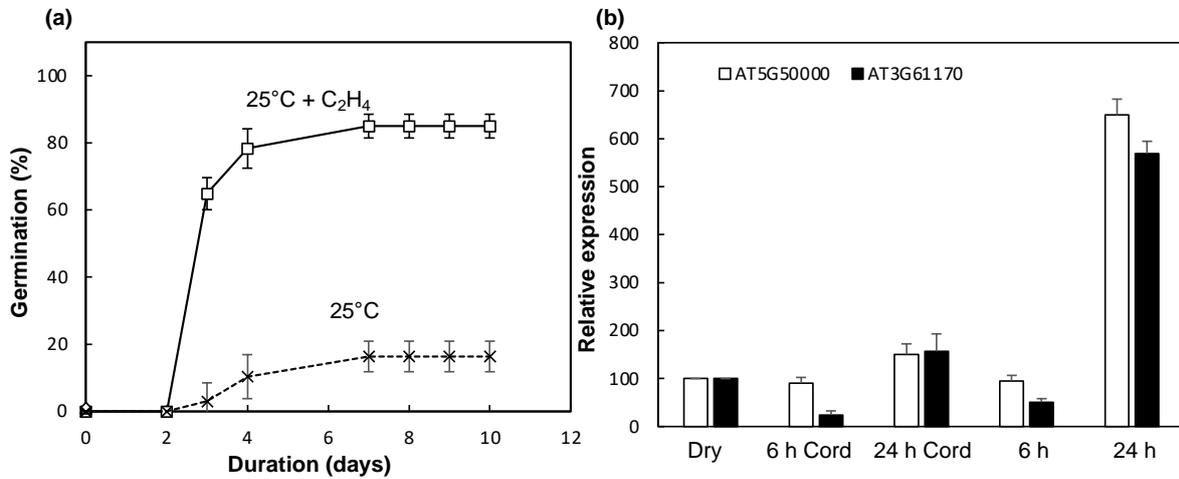


Figure S.2. (a) Germination of freshly harvested seeds on a solution of cordycepin 50.10^{-6} M at 25°C in the dark in the absence or in the presence of ethylene (100ppm) **(b)** Expression level determined by qRT-PCR of *AT5G50000* and *AT3G61170* in seeds treated with 100 ppm ethylene in presence or in absence of cordycepin 50.10^{-6} M (Cord) at 25°C in the dark.

Oxford Nanopore Sequencing

Introduction

Epigenetic modifications play crucial and dynamic roles in different processes including embryo development, stem cell fate determination, floral transition, stress responses, fruit ripening, and root development (Liang et al., 2020). These reversible modifications allow a rapid response to external cues (Hewezi, 2018). The methylation of adenosine in the N6 position (m6A) is the most understood and widespread internal modification present in rRNAs, mRNA, tRNAs, miRNA and long non-coding RNA (Arribas-Hernández & Brodersen, 2020). Nevertheless, its role in seed germination remains very poorly understood as it is limited to data generated by conventional NGS techniques following m6A Immunoprecipitation (see Luo et al. (2014) for reference) which might lead to bias in the identification of m6A sites. Unlike traditional RNA-Seq techniques, long-read nanopore RNA sequencing allows accurate quantification and complete, full-length characterization of native RNA while enabling the also the identification of base modifications alongside nucleotide sequence (Soneson et al., 2019). Our aim was to apply this very recent sequencing technology on Arabidopsis seeds in order to see whether m6A modifications might occur during seed germination in response to ethylene. The following section will detail the workflow that was successfully adapted to Arabidopsis that will enable the identification of m6A modifications.

Plant Material

This essay was conducted on wildtype Arabidopsis Col-0 seeds and the *mta* (mRNA ADENOSINE METHYLASE) mutant (SALK_114710), whose catalytic activity in the writer complex is altered, imbibed for 24 h at 25°C in presence of ethylene (100 ppm).

RNA-extraction and purification

Total RNA were extracted out of 700 mg of Arabidopsis Col-0 seeds and seeds from the *mta* mutant according to Zhang *et al.*, (2019). DNase treatment carried out using the TURBO DNA-free™ Kit. 28 µL of RNA extract, 5 µL of 10X TURBO DNase™ buffer and 1 U of DNase were added in a total volume of 40 µL. The mixture was then heated for 30 minutes at 37°C. 5 µL of DNase inactivation reagent were added in order to inhibit the activity of

the DNase and the mixture was then centrifuged for 2 minutes at 10,000 RPM and 50 μ L of the supernatant were retrieved.

RNA was purified on columns using the MACHEREY-NAGEL nucleospin RNA Clean-up purification kit according to the manufacturer's instructions. The quality and quantity of the extracted RNA were checked using a nanodrop spectrophotometer.

mRNA Purification

mRNA purification was carried out using the Dynabeads™ mRNA Purification Kit according to the manufacturer's instructions. Samples were heated for at 65°C for 2 minutes in order to disrupt secondary structures and placed directly on ice afterwards. 1mg of resuspended Dynabeads™ magnetic beads (per 75 μ g of total RNA) were transferred to a tube which was then placed on the magnet for 30 seconds until the Dynabeads™ magnetic beads were adhered to the wall. The supernatant was removed and an adjusted volume of Binding Buffer (100 μ L per 75 μ g of total RNA) has been added. The tube was placed on the magnet and the same volume of Binding Buffer is added. The total RNA was added to the Dynabeads™/Binding buffer suspension and the mixture was placed on the rotating wheel for 10 minutes at room temperature. The tubes were placed on the magnet and the supernatant was discarded. The complex was washed twice with the Washing Buffer B (200 μ L per 75 μ g of total RNA) while taking care to remove all traces of supernatant between each step. 13 μ L of 10 mM Tris-HCl, pH = 7.5 were added to elute the mRNA and the same step is repeated using 10 μ L to control the efficiency of the first elution. The samples were placed at 65 °C for 2 min and immediately on the magnets and the supernatants, now containing the purified mRNA, was transferred to new RNase-free tubes. The quality and quantity of the purified mRNA were checked using a nanodrop spectrophotometer.

Direct mRNA Nanopore sequencing

Nanopore libraries were prepared from 1 μ g poly(A) mRNA using the nanopore DRS Kit (SQK-RNA002, Oxford Nanopore Technologies) according to manufacturer's instructions. Libraries were loaded onto A MinION Flow Cell (R9.4.1) Flow Cells (Oxford Nanopore Technologies) and sequenced using the MinION Sequencer (Oxford Nanopore Technologies) and the Fast5 reads were collected.

Processing of nanopore DRS data

Reads were basecalled with Guppy version 1.1.6 (Oxford Nanopore Technologies) using default RNA parameters on the Galaxy work platform. Guppy enables the transformation of Fast5 raw data (electric signals) to fastQ data as shown below.

Example of a FastQ sequence obtained using Guppy:

```
@32a21458-8cb5-4416-91c1-c0539d35a69e runid=52c3a649e09e4d80bb9d26ee606a8770e29cd27c sampleid=m6a-ETH_A2020 read=676 ch=155 start_time=2020-06-09T15:00:51Z
CUGUGGAACACAGAAAGGUAAGAGGGAACGGCAUAGACAUUUCACGUAGCAAUCCAAGGUCCUCCUUGGUCUUAUAGUCC
UCCUCCUUAUAGUUUCCUGAGCUUUGGUUCAUCAUAGCGCCACCAGAUCCUGGACUCGCCGUCUGCUCGUGCCGUCGCUC
CAGACCUACGUGGUUACGGUGACUCCGAUGCUCCCGUGAGAUUCACCACCCUUACACAUGCUUUAUCGUUGGUGACUUAUC
GCCGUCAUCCGCCUUUAACGCCUCAGAGGAUGAGAAGGUCUUUGUGGUUGGACCAUGAUUGGGGAGCUCUCAUCGUUUGGU
AUUUAUG
```

The next step consisted of trimming the first 5 and last 3 bases, usually of a reduced sequencing quality, using NanoFilt (version 0.1.0). It is important to note an additional step enabled the conversion of "U" (uracil) for were converted into "T" (thymine) during the NanoFilt run (see sequence below)

Example of a FastQ sequence obtained using NanoFilt:

```
@ba6f9a37-05ec-4dcc-a8d6-3f4fea4c4fbb runid=52c3a649e09e4d80bb9d26ee606a8770e29cd27c sampleid=m6a-ETH_A2020 read=906 ch=477 start_time=2020-06-09T15:02:21Z
TTGAAAAAATAATCAAGAAATGGCGATGTTTCAGGAAAATACGTGGCGGAAGTACCGTGAAGGGTCCGGCGGAGAAACTACAGG
CGATGGAGGAGCCAAAACAATCTCTTTCCCGGACGCCATCGGCCACCATGTCCAAGGCGTCATGTCCACGATGGCGACTGGGACTCCC
ACGGTCCATCAATCTGGAACACTACACACTTGATGGGAAGCCGGAAGTCATCAAGGAGAAAGAGAGATAGACGACGAGAAG
```

Reads were mapped to the TAIR10 Arabidopsis thaliana reference genome (The Arabidopsis Genome Initiative, 2000) using minimap2 version 2.17-r941 while specifying a k-mer size of 14. Aligned Bam reads were then visualized using the IGB Viewer. As a next step, Nanopolish version 0.13.2 so that reads were indexed, aligned to transcripts instead of the genome and ready for the differential RNA modifications analysis using xPore (version 0.5.6). Data required for xPore consisted of: Fast5 files (raw data), FastQ files (obtained by Guppy), bamtx file (aligned bam files obtained from minimap2) and the Nanopolish output file.

These steps were successfully accomplished and modified to run on Arabidopsis data and we are working on validating our results with data obtained by Parker et al. (2020) where they used an error rate-based analysis via machine learning.

The determination of differentially modified positions m6A sites will consist of filtering the output file of xPore (shown in Table 1 below) while searching for NNANN

sequences (where N could be any base) while respecting a p-value < 0.001 and a differential modification rate (DMR) > 0.005 as described by (Pratanwanich et al., 2020).

Table 1-Extract of the data obtained using xPore that enables the identification of m6A positions (DMR represents the calculated differential modification rate and pval represents the calculated p-value)

Chromosome id	Position	k-mer	DMR_MET_vs_WT	pval_MET_vs_WT
1	25579702	AATGG	0.08822928	0.05216111
1	25579880	CAATT	-0.1635855	0.00516692
1	28472632	GTCCG	0.06776398	0.10631566
1	28472376	ATCGA	0.07304784	0.0357452
1	25579536	TACCT	0.46738097	7.75E-09
1	25579549	CCATG	0.2688305	0.01141191
1	25580040	ATCCA	-0.2806639	0.00017867
1	25580064	TTATC	-0.1995669	0.00544729
1	25579921	AAAGA	0.31960483	0.00466042
1	28472880	AACTT	-0.0700436	0.04081041
1	25579738	TCTAA	-0.0579019	0.11711971
1	28472721	CAGCT	-0.0899753	0.07026081
1	25579443	CCTTT	0.2234041	0.0101108
1	25579899	TAGGA	-0.0091671	0.83856796
1	28472436	TTCTT	0.16120946	0.00376829
1	25579529	GCCTC	0.08142449	0.13668496
1	28472601	AGAAG	-0.0088416	0.81175939
1	25579780	AAAGG	0.08783076	0.08929478
1	28472744	TTCCC	0.03628239	0.37317703
1	28472715	TCTTC	-0.1303591	4.05E-08
1	25579458	TTTGA	-0.0301196	0.27814571
1	25579971	GGATT	-0.2232027	0.00435881
1	25579673	AGAAC	0.1707304	0.01295813

In summary, the analysis of Nanopore sequencing data was successfully adapted to fit our model and enable the identification of differentially methylated position and our next goal is to use this technology to study the correlation between mRNA methylation and the data obtained in chapter 4.

Detailed Materials and Methods

Plant Material

Arabidopsis thaliana wild-type seeds used in this study came from the genetic background (Col-0). Mutants used throughout this thesis came from the same background. roGFP-expressing lines were in Col-0 background and have been described by Jiang *et al.* (2006).

Seeds were stratified for four days at 4 °C before being sown in soil and placed in growth chambers under controlled conditions with a temperature of (21°C) and a photoperiod of 16 hours per day with the light intensity of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Mature seeds were harvested and stored at - 20 °C to preserve their dormancy until further use.

Germination assays

Approximately 75 seeds per replicate were sown on filter papers in 9 cm Petri dishes. Filter papers were moistened with deionized water or gibberellic acid (10^{-3}M), Rotenone (ROT, $5 \cdot 10^{-5} \text{M}$), antimycin A ($5 \cdot 10^{-6} \text{M}$) or salicylhydroxamic acid (SHAM, 10^{-4}M) and placed in the darkness at 15°C or 25°C. Respiratory inhibitor solutions were prepared by first dissolving the powder in 2mL dimethylsulfoxide and the volume was completed to 100 mL with deionized water. 100 ppm of gaseous ethylene was applied to seeds placed into gastight Petri dishes (Corbineau *et al.*, 2014). Stratified seeds were moistened and placed in petri dishes for 4 days at 4°C in darkness before being transferred to 25°C according to the tested condition. Germination was continuously monitored for 12 days and seeds were considered as germinated following radical protrusion. Tests were carried out in triplicates.

RNA extraction

Total RNA was extracted out of 50 milligrams of *Arabidopsis thaliana* seeds using the protocol developed by (Hellemans *et al.*, 2007) and adapted by (Zhang *et al.*, 2019). 1 mL of CTAB buffer (2% hexadecyl trimethyl-ammonium bromide, 2 % Polyvinylpyrrolidone, 100 mM Tris-HCl pH = 8, 25 mM EDTA pH = 8, 2 M NaCl, 2 % 2-mercaptoethanol) was added to the fine powder previously grinded in liquid nitrogen. The mix was vortexed for 2 minutes and heated for 15 minutes at 70 °C. Total RNA was extracted three times following the addition of 1 mL of chloroform isoamyl alcohol 24:1. The two phases were separated by

centrifuging the tubes at 11,000 RPM for 15 minutes at room temperature and the supernatant was recovered after each round. RNA was left to precipitate overnight by adding 1/4 of the total volume in LiCl (10M). The next day, the tubes were centrifuged for 30 minutes at 13,000 RPM at 4 °C in order to recover the pellet which was resuspended in 600 µL of SSTE buffer (1 M NaCl, 0.5 % sodium dodecyl sulfate, 10 mM Tris-HCl pH = 8, 0.5 M EDTA pH = 8). The same step consisting of the addition of 1 mL of chloroform isoamyl alcohol 24:1 was also repeated three times. A volume of ethanol 96 % equivalent to 2.5 times the volume of the recovered supernatant was added and RNA was left to precipitate for two hours at - 80 °C. The tubes were then centrifuged for 30 minutes at 13,000 RPM And the recovered pallet was left to dry for 20 minutes before being resuspended in 30 µL of ultrapure water. The quality and quantity of the extracted RNA were checked using a nanodrop spectrophotometer.

DNase treatment and RNA purification

DNase treatment carried out using the TURBO DNA-free™ Kit. 28 µL of RNA extract, 5 µL of 10X TURBO DNase™ buffer and 1 U of DNase were added in a total volume of 40 µL. The mixture was then heated for 30 minutes at 37°C. 5 µL of DNase inactivation reagent where added in order to inhibit the activity of the DNase and the mixture was then centrifuged for 2 minutes at 10,000 RPM and 50 µL of the supernatant were retrieved.

RNA was purified on columns using the MACHEREY-NAGEL nucleospin RNA Clean-up purification kit according to the manufacturer's instructions. The quality and quantity of the extracted RNA were checked using a nanodrop spectrophotometer.

mRNA Purification

mRNA purification was carried out using the Dynabeads™ mRNA Purification Kit according to. the manufacturer's instructions. Samples were heated for at 65°C for 2 minutes in order to disrupt secondary structures and placed directly on ice afterwards. 1mg of resuspended Dynabeads™ magnetic beads(per 75 µg of total RNA) were transferred to a tube which was then placed on the magnet for 30 seconds until the Dynabeads™ magnetic beads were adhered to the wall. The supernatant was removed and an adjusted volume of Binding Buffer (100 mL per 75 µg of total RNA) has been added. The tube was placed on the magnet and the same volume of Binding Buffer is added. The total RNA was added to the Dynabeads™/Binding buffer suspension and the mixture was placed on the rotating wheel for 10 minutes at room temperature. The tubes were placed on the magnet and the

supernatant was discarded. The complex was washed twice with the Washing Buffer B (200 μL per 75 μg of total RNA) while taking care to remove all traces of supernatant between each step. 13 μL of 10 mM Tris-HCl, pH = 7.5 were added to elute the mRNA and the same step is repeated using 10 μL to control the efficiency of the first elution. The samples were placed at 65 °C for 2 min and immediately on the magnets and the supernatants, now containing the purified mRNA, was transferred to new RNase-free tubes. The quality and quantity of the purified mRNA were checked using a nanodrop spectrophotometer.

Reverse transcription of RNA

Reverse transcription was carried out following the addition of 2 μg of RNA, 4 μL of 5X reaction buffer, 4 μL of 100 μM Oligo dT, 2 μL of 10 mM dNTP, 0.5 μL of Ribolock RNase and 1 μL of Reverse transcriptase (200 U. μL^{-1}). The mix was heated for 1 hour at 42°C and the Reverse transcriptase was inactivated by heating the mix at 70 °C for 10 minutes.

Quantitative RealTime -PCR

7.5 μL of 2X Maxima SYBER Green / ROX PCR Mix (Thermofisher), 0.35 μL of Forward and Reverse primers (10 μM) and 5 μL of cDNA (1/30) were mixed in a total volume of 10 μL . 3 replicates per sample were added in a 96 well PCR plate (Thermofisher). The PCR program consisting of a 10 denaturation step at 95°C and 30 cycles alternating between denaturation at 95°C, annealing at 56 °C and extension at 72°C was run using a Realplex2 thermocycler (Eppendorf). Relative was calculated according to (Helleman et al., 2007) with AT5G53560, AT4G34270 and AT4G26410 as reference genes. Primer sequences are listed in supplemental table 2.

Polysome isolation

400 mg of *Arabidopsis thaliana* wild-type seeds were ground in liquid nitrogen into a fine powder before adding one volume of the polysome extraction buffer (PEB) which consists of a mixture of 200 mM Tris-HCl pH 9.0, 200 mM KCl, 25 mM EGTA, 36 mM MgCl₂, 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 $\mu\text{g}\cdot\text{ml}^{-1}$ cycloheximide, 50 $\mu\text{g}\cdot\text{ml}^{-1}$ chloramphenicol, 10 $\mu\text{L}\cdot\text{ml}^{-1}$ protease inhibitor cocktail. The tubes were then centrifuged for 15 minutes at 14,000

g at 4°C and the supernatant was recovered while paying attention not to disturb the lipidic layer above it before being transferred to a new tube containing a Miracloth filter. An aliquot of 200 µL of the supernatant was kept for the isolation of total RNA. 2 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 MgCl₂, 100 mg. ml⁻¹ chloramphenicol and 100 mg. ml⁻¹ cycloheximide) and centrifuged at 180 000g (38 400 RPM) at 4°C for or 2.5 hours using a Beckman (SW41) rotor. The sucrose gradient was fraction using a density gradient fractionator system with a 254 nm detector and a 2 M sucrose solution as a displacement fluid. The fractions corresponding to the polysomal profile were pooled together prior to the RNA extraction. The total and polysomal RNA were extracted using the same method by adding 300 µL of 5 % SDS/EDTA (0.2M) and 700 µL of phenol/chloroform/isoamyl alcohol (25:25:1) (Barkan, 1993). The tubes were centrifuged for 5 minutes at 13000 RPM at room temperature before adding 1 mL ethanol 100%. The tubes were Centrifuged again for 15 minutes at 13,000 RPM at room temperature and the pellet was resuspended in 20 µL of ultrapure water. The quality of the RNA was then verified using a Nanodrop spectrophotometer and the RNA was treated with the TURBO DNA-free™ Kit (ambion, Life sciences) purified using the MACHEREY- NAGEL nucleospin XS purification kit according to manufacturer's instruction.

Reactive oxygen species quantification

Reactive Oxygen species were extracted out of 30 mg of seeds ground in 2 mL of perchloric acid (0.2N). The resulting liquid was recovered and centrifuged 15 minutes at 13,000 RPM at 4°C. The pH of the supernatant was equilibrated at 7-7.5 with KOH (4N) and perchloric acid (0.2N). The solution was then centrifuged 1 minute (13,000 RPM, 4°C). The supernatant was transferred into a tube and stored in the ice for dosing. The standard curve was prepared following the dilution of hydrogen peroxide solution (10 M) with respective concentrations of 0; 0.1; 0.5; 1; 5 and 10 µM.

The quantification of ROS was done using a spectrophotometer. The mixture used for quantification consisted of 950 µL of water, 400 µL of DMAB (3-dimethylaminobenzoic acid) (10 mM) 80 µL of MBTH (3-methyl-2-benzothiazolinone hydrazone) (1mM), 50 µL of the sample and 4 U of peroxidase VI (Sigma). hydrogen peroxide (H₂O₂) reacts with MBTH and DMAB in presence of peroxidase (POX) to give a blue compound detectable at 590 nm after 5 minutes.

Confocal microscopy

Fluorescence of mit-roGFP seeds, Grx1-GFP seed, DCP1-YFP and various fluorochromes of Col-0 seeds employed in this study was envisioned by confocal microscopy employing a Leica TCS SP5 confocal microscope. Altogether, seeds were imbibed according to conditions detailed in the text and the seed coat was gently removed under a binocular prior cell imaging.

Decoated mit-roGFP seeds were excited at 405 nm (oxidized roGFP) and 488 nm (reduced roGFP) and the resulting fluorescence was detected in a 492-560 nm window. 10 mM Dithiothreitol (DTT) and 10 mM hydrogen peroxide were used in order to determine the total amount of reduced and oxidized roGFP, respectively. These values were used for the quantification of the oxidation degree and the redox potential. Fluorescence was quantified using ImageJ software.

The degree of oxidation of the ro-GFP was calculated according to (Schwarzländer et al., 2008) using the following formula:

$$\text{OXDroGFP} = \frac{R - R_{\text{red}}}{\frac{I_{488\text{ox}}}{I_{488\text{red}}}(R_{\text{ox}} - R) + (R - R_{\text{red}})}$$

Where: R = fluorescence at 405 nm/fluorescence at 488 nm for the sample

R_{red} = fluorescence at 405 nm/fluorescence at 488 nm in the presence of DTT

R_{ox} = fluorescence at 405 nm/fluorescence at 488 nm in the presence of hydrogen peroxide

I_{488ox} = fluorescence measured at 488 nm in the presence of 10 mM hydrogen peroxide

I_{488red} = fluorescence measured at 488 nm in the presence of 10 DTT

And the redox potential was determined from the Nernst formula, as below:

$$E = E_0' - \frac{RT}{zF} \ln\left(\frac{1 - \text{OXDroGFP}}{\text{OXDroGFP}}\right)$$

where E₀' = midpoint redox potential for roGFP1

R = gas constant (8.315 J K⁻¹ mol⁻¹)

T = absolute temperature (298.15 K)

z = number of electrons transferred (2)

F = Faraday constant (9.648104C mol⁻¹)

DCFH-DA (2',7'-Dichlorodihydrofluorescein diacetate, 25 μ M) was used for ROS localization (Leymarie et al., 2012). Seeds were incubated for 30 min at 20°C in 20 mM potassium buffer (pH =6) containing DCFH-DA (25 μ M) and washed twice for 20 min at 20 °C in potassium phosphate buffer (25 mM, pH 6). As for the mitochondrial staining, seeds were then incubated for 20 min at 20 °C in the MITO-ID (ENZO) dye and washed twice for 20 min at 20 °C in the MITO-ID buffer. Regarding nuclear staining, seeds were placed on the slide for 10 min in darkness in presence of 4',6-diamidino-2-phenylindole DAPI (1 μ g/ μ L). In order to visualize the fluorescence emitted by seeds treated with DCFH-DA, embryos were excited at 488 nm and the fluorescence was detected in a 498-550 nm window. Embryos were simultaneously excited at 561 nm to for the mitochondrial staining and the fluorescence was detected in a 571-800 nm window. Lastly, the same embryos were excited at 405 nm in order to visualize the nucleus and the fluorescence was detected in a 415-470 nm window. Image treatment was performed using ImageJ software.

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Abstract

Role of oxidative signaling and the metabolism of mRNA in the response of *Arabidopsis thaliana* seeds to ethylene

Seed dormancy is a phenomenon that is directly responsible for the growth and dynamics of plants within ecosystems. However, the molecular mechanisms involved in its regulation remain poorly understood. We took advantage of the beneficial effect of ethylene on the germination of dormant *Arabidopsis thaliana* seeds for deciphering the mechanisms of ROS production and its effects during germination. We show that mitochondria are the primary site of ROS production in germinating seeds and that this ROS production triggers a crosstalk between the mitochondria and the nucleus, as confirmed by the induction of mitochondrial retrograde signaling marker genes. We demonstrate that the response to ethylene differed from other dormancy release treatments such as treatment with gibberellic acid, cold stratification and exposure to light where ROS were detectable in the mitochondria in the early hours of imbibition before localizing in peroxisomes. In all treatments, ROS were detected in the nucleus pointing to an important role of nuclear ROS in the progress towards germination. We also paid attention to the dynamics of RNA metabolism in the same process. We show that a synchronized activation of transcription, association of transcripts with polysomes and mRNA decay is associated with the beneficial effect of ethylene on seed germination. The RNAseq data enabled the identification of many regulators of this process. We demonstrate that the shift of the hormonal balance ABA/GA towards GA signalling and germination requires a multilevel and concerted effect on all levels of mRNA fate, from transcription to decay.

Keywords: Arabidopsis, germination, dormancy, ethylene, reactive oxygen species, mitochondrial Retrograde Signaling, mRNA metabolism.

Résumé

Rôle de la signalisation oxydative et du métabolisme de l'ARNm dans la réponse des semences d'*Arabidopsis thaliana* à l'éthylène.

La dormance est un phénomène qui est directement responsable de la dynamique des populations végétales dans les écosystèmes. Cependant, les mécanismes moléculaires impliqués dans sa régulation restent mal compris. Nous avons utilisé l'effet bénéfique de l'éthylène sur la germination des graines d'*Arabidopsis thaliana* pour décrypter les mécanismes de production d'espèces réactives de l'oxygène (ROS) pendant la germination. Nous montrons que les mitochondries sont le principal site de production de ROS dans la germination des graines en réponse à l'éthylène et qu'elles sont à l'origine d'une signalisation rétrograde avec le noyau, comme le confirme l'étude de l'expression de gènes marqueurs de ce phénomène. Cette réponse est spécifique à l'éthylène puisque la levée de dormance par l'acide gibbérellique, la stratification au froid ou la lumière montre que les peroxysomes sont dans ces cas impliqués dans la production de ROS. Nous avons également étudié la dynamique du métabolisme des ARNm durant le même processus. Nous montrons qu'une activation synchronisée de la transcription, l'association des transcrits avec des polysomes et la dégradation d'ARNm sont associées à l'effet bénéfique de l'éthylène sur la germination des graines. Des données de RNAseq ont permis d'identifier de nombreux régulateurs de ce processus. Nous démontrons que le changement de l'équilibre hormonal ABA/GA permettant la germination requière la participation de tous les niveaux du métabolisme des ARNm de leur transcription à leur dégradation.

Mots-clés : Arabidopsis, germination, dormance, éthylène, espèces réactives de l'oxygène, signalisation rétrograde mitochondriale, métabolisme des ARNm.