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# Contribution à la compréhension de l'effet de maturation des graines sur leur qualité physiologique chez les légumineuses

Rubiana Falopa Rossi

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en Horticulture et Semences (IRHS)

THÈSE EN COTUTELLE : Universidade Estadual Paulista  
Júlio de Mesquita Filho (Brésil)

**Contribution à  
la compréhension de l'effet  
de la maturation des graines  
sur leur qualité physiologique  
chez les légumineuses**

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présentée par

## **Rubiana Falopa ROSSI**

Contribution à la compréhension de l'effet de la maturation des graines  
sur leur qualité physiologique chez les légumineuses.

Soutenue à Botucatu (Brésil) le 15 juillet 2016 devant le jury composé de

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PHYSIOLOGICAL SEED QUALITY IN LEGUMES

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Botucatu, 15 de julho de 2016



I dedicate my thesis to my dear parents

João Rossi and Silvia Helena Falopa.

You are everything in my life.





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**Table 15.** Gene Ontology (GO) enrichment analysis of transcripts DEG up-regulated to natural drying. Analysis was performed on the top 833 genes using SoyBase, with annotations for Wm82.a2.v1 (Glyma 2.0). #Genome: Genome Count present in this GO category; # Expressed: Number of submitted genes present in this GO category.

**Table 16.** Gene Ontology (GO) enrichment analysis of transcripts DEG down-regulated to artificial drying. Analysis was performed on the 309 genes using SoyBase, with annotations for Wm82.a2.v1 (Glyma 2.0). #Genome: Genome Count present in this GO category; # Expressed: Number of submitted genes present in this GO category.

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**Table 18.** Gene Ontology (GO) enrichment analysis of transcripts DEG increased during imbibition. Analysis was performed on the 1081 genes using SoyBase, with annotations for Wm82.a2.v1 (Glyma 2.0). #Genome: Genome Count present in this GO category; # Expressed: Number of submitted genes present in this GO category.

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## ABSTRACT

During seed maturation, germination, desiccation tolerance and longevity are acquired sequentially. Seed maturation is terminated by a desiccation phase that brings the embryo to a quiescent state. Seed maturity at harvest influences seed longevity and crop establishment. After harvest, seeds are usually dried to water content compatible with long term storage and post-harvest treatments. However, there is a lack of understanding of how seed longevity is acquired during seed maturation and how premature drying impacts longevity and resumption of cellular activities during imbibition. This was addressed here by comparing transcriptome changes associated with maturation drying and imbibition of seeds of soybean and *Medicago truncatula*, harvested at an immature stage and mature dry stage. The immature stage corresponded to end of seed filling when longevity was not acquired while other vigor traits were acquired. Transcriptome characterization in soybean revealed that enforced drying was not similar to maturation drying in planta, which stimulated degradation of chlorophyll and synthesis of protective chaperones. Eighty-nine % of the differentially expressed genes during a 18h-imbibition period showed a similar pattern between immature and mature seeds, consistent with a comparable germination between stages. An analysis of the 147 transcripts that increased during imbibition of mature seeds but not in immature seeds suggested an activation of processes associated with shoot meristem development and DNA repair. These data were compared with imbibing immature and mature seeds of *Medicago* and revealed an overrepresentation of genes involved in phototropism, seed coat and innate immunity in mature seeds. This work should provide new tools to optimize harvest at maximum seed quality.





## RESUMO

Durante a maturação da semente, a germinação, a tolerância à dessecação e a longevidade são adquiridos sequencialmente. A maturação da semente termina com a fase de dessecação que traz o embrião a um estado de repouso. Na cadeia de produção de sementes, o estágio de maturação no momento da colheita é o primeiro fator que influencia a longevidade das sementes e estabelecimento da cultura. Após a colheita, as sementes são normalmente secas para um teor de água compatível com os tratamentos pós-colheita e armazenamento a longo prazo. No entanto, há uma falta de compreensão de como a longevidade das sementes é adquirida durante a maturação da semente e qual o impacto da secagem prematura na longevidade e na retomada das atividades celulares durante a embebição. Esta questão foi abordada aqui, comparando alterações transcriptoma associados com a secagem maturação e embebição de sementes de soja e *Medicago truncatula*, colhidos em um estágio imaturo e estágio seco maduro. A fase imatura correspondeu final de enchimento de grãos, quando a longevidade não foi adquirida enquanto outros traços de vigor foram adquiridos. A caracterização do transcriptoma de soja revelou que a secagem forçada não era semelhante à maturação de secagem na planta, o que estimulou a degradação da clorofila e síntese de chaperones de proteção. Oitenta e nove % dos genes diferencialmente expressos durante um período de 18 horas de embebição mostrou um padrão similar entre as sementes imaturos e maduros, consistente com uma germinação comparáveis entre os estágios. Analisando os 146 transcritos que aumentam durante a embebição de sementes maduras, mas não em sementes imaturas sugeriu uma activação dos processos associados ao desenvolvimento de meristema e reparação do DNA. Esses dados foram comparados com sementes imaturas e maduras de *Medicago* durante a imbebição e revelou uma sobre-representação de genes envolvidos no fototropismo, revestimento de sementes e imunidade inata em sementes maduras. Este trabalho deve fornecer novas ferramentas para otimizar a colheita de sementes no ponto máximo de qualidade.



## RÉSUMÉ

Pendant la maturation des graines, la germination, tolérance à la dessiccation et longévité sont acquises de manière séquentielle. La maturation s'achève par la dessiccation qui amène l'embryon à l'état de quiescence. La maturité des graines à la récolte est le premier facteur qui influence la longévité et l'établissement de la culture lors du semis. On ne comprend pas comment la longévité est installée pendant la maturation et comment un séchage prématuré influence la longévité et la reprise des activités cellulaires pendant l'imbibition. L'objectif de la thèse était de répondre à ces questions en comparant les transcriptomes de graines immatures et matures de soja et *Medicago truncatula* pendant la dessiccation et l'imbibition. Les graines immatures furent récoltées après le remplissage avant la dessiccation, lorsque la longévité n'est pas encore acquise. Chez le soja, la comparaison des transcriptomes des graines immatures et matures montre que le séchage forcé n'est pas identique à la dessiccation in planta qui se caractérise par la synthèse de protéines chaperones. Plus de 89% des gènes différentiellement exprimés après 18 h d'imbibition présentent des profils d'expression identiques dans les graines immatures et matures, en accord avec la germination comparable de celles-ci. L'analyse des transcrits dont la teneur augmente uniquement pendant l'imbibition des graines mature suggère la mise en place de mécanismes de réparation. La comparaison de ces données avec *Medicago* montre que l'imbibition des graines matures se caractérise par une sur-représentation des gènes liés au phototropisme, à la testa et réponse immunitaire. Ce travail doit permettre le développement d'outil d'analyse de la maturité des graines lors de leur récolte.



## **VERSION ABRÉGÉE EN FRANÇAIS**

### **INTRODUCTION**

Le Soja (*Glycine max*,  $2n = 40$  chromosomes) est une plante, annuelle de la famille des légumineuses, originaire d'Asie de l'Est. Les premières traces de son utilisation datent de 2.838 av. J.-C. en Chine ce qui indique qu'il peut être l'une des plus anciennes espèces cultivées (MORSE, 1950 cité dans BOZATO and BOZATO, 1987). Au Brésil l'introduction du soja a eu lieu en 1882 dans les Etats de Bahia et de São Paulo (D'UTRA, 1882; DAFFERT, 1893, cités dans BOZATO and BOZATO, 1987). Actuellement, le Brésil est le deuxième producteur mondial de soja après les Etats-Unis. Sa production a atteint 100 millions de tonnes et un rendement moyen de  $3\text{kg ha}^{-1}$  pour une surface d'environ 33 millions d'hectares (CONAB, 2016). Les graines sont riches en protéines et traditionnellement utilisées pour la production d'huile et l'alimentation humaine et animale (QIU and CHANG, 2010).

A cause de l'importance croissante de soja pour l'économie brésilienne il y a un effort continu d'augmenter sa production via l'augmentation des rendements. Dans ce contexte, l'utilisation de semences de haute qualité, préalable à l'implantation d'une culture efficace est un levier efficace. Cependant, les mécanismes contrôlant la qualité des graines restent mal compris. Pour la plupart des espèces, la qualité des semences est acquise au cours du développement pendant la phase de maturation (BEWLEY et al., 2013).

Chez le soja la phase finale de maturation est considérée comme une étape importante du développement de la graine; où plusieurs composantes de la qualité, tels que la tolérance à la dessiccation, la vigueur et la longévité sont acquises après la maturité de masse (ZANAKIS et al., 1994). Ces données contrastent avec celles de FRANÇA-NETO, TEKRONY qui montrent que la maturité physiologique coïncide avec la maturité de masse. Chez plusieurs espèces comme le haricot, l'*Arabidopsis*, la tomate, le poivron et le melon, la qualité des semences augmente à la phase de maturation tardive (BEWLEY et al. 2013).

La maturation favorise la transition d'un mode de développement à un programme orienté vers la germination (BEWLEY et al., 1997). A maturité, les graines sèches d'*Arabidopsis* contiennent entre 12000 et 17000 transcrits caractérisés



(WEITBRECHT et al., 2011, ). On ne sait pas si ces transcrits sont tous nécessaires pour la germination. L'identité des ARNm spécifiques et nécessaires pour la germination et quand ils sont synthétisés au cours du développement ne sont pas connus. Une étude récente a montré que, pendant les 3 premières heures d'imbibition des graines d'Arabidopsis il y avait une traduction de certains ARNm associés au développement tels que les LEA et les protéines de stockage (GALLAND et al., 2014). Ceci indique qu'un sous-ensemble d'ARNm stockés dans les graines sèches caractéristiques du développement ne disparaît pas au cours de la phase de dessiccation pendant la maturation). Cependant, il n'est pas connu si une dessiccation artificielle des graines immatures induit un profil des ARN stockés similaire ou totalement différent de celui des graines sèches à maturité. Cette information permettrait d'avoir une idée si ces ARNs sont nécessaires pour la germination et la longévité. Cela nous informera aussi si la desiccation prématurée est identique à celle associée à la maturation. Du point de vue de l'agriculture, ces informations peuvent être utiles pour identifier des indicateurs putatifs de la maturité des graines.

Dans ce contexte, l'objectif de cette étude est de comprendre comment la maturation des graines influe sur les mécanismes moléculaires qui régulent et conduisent à la germination des graines ou à une germination vigoureuse. Pour ce faire, nous avons comparé les transcriptomes de graines de soja à deux stades de maturité : immature (R7.2), correspondant à la fin du remplissage et mature (stade R9) Au cours des dernières années, de nombreuses études ont été menées sur les graines de *Medicago truncatula* (GALLARDO et al., 2003; CHATELAIN et al., 2012; VERDIER et al., 2014; RIGHETTI et al., 2015); une légumineuse modèle dont le génome possède une bonne synténie avec celui du soja (*Glycine max*). C'est pourquoi, les données obtenues sur le soja ont été comparées avec celles obtenues sur *Medicago* en utilisant un dispositif expérimental similaire au cours de la maturation et de l'imbibition.



Version Abrégée en Français

**Table 1.** Caractérisation des stades phénologiques des graines de soja selon la classification originale de Fehr et Caviness (1977) et adaptée dans ce travail

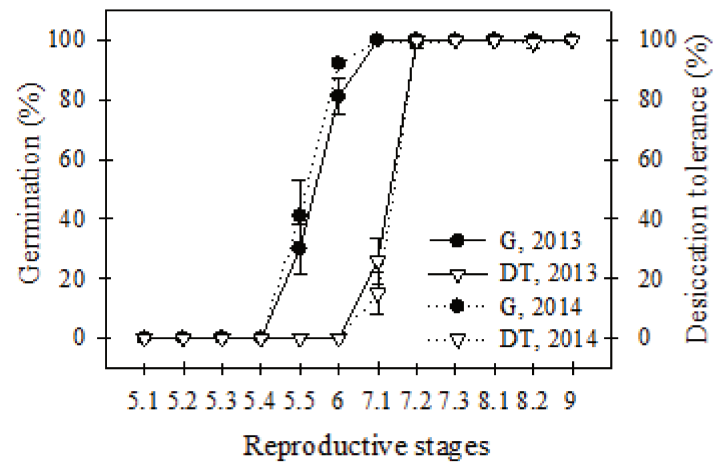
<b>Stades</b>	<b>Dénomination</b>	<b>Description</b>
R <sub>5,1</sub>	Remplissage du grain	Graines perceptibles au toucher au travers de la gousse, jusqu'à 10% de remplissage des grains
R <sub>5,2</sub>	Remplissage du grain	Taux remplissage de 10% à 25%
R <sub>5,3</sub>	Remplissage du grain	Taux de remplissage entre 25% et 50%
R <sub>5,4</sub>	Remplissage du grain	La Plupart des gousses presentent des graines avec un taux de remplissage entre 50% et 75% de remplissage des grains
R <sub>5,5</sub>	Remplissage du grain	Plupart des gousses entre 75% et 100% de remplissage des grains
R <sub>6</sub>	Grains développés	Gousses avec 100% de remplissage des grains et des feuilles vertes
R <sub>7,1</sub>	Maturation précoce	Jusqu'à 50% des feuilles et les gousses sont jaunes
R <sub>7,2</sub>	Maturation précoce	Entre 51% et 75% des feuilles et gousses sont jaunes
R <sub>7,3</sub>	Moitié de la maturation	Plus de 76% des feuilles et des gousses sont jaunes
R <sub>8,1</sub>	Maturation	Jusqu'à 50% de défoliation
R <sub>8,2</sub>	Maturation	Plus de 50% de défoliation avant la récolte
R <sub>9</sub>	Maturité de récolte	Le point de récolte

## **MATERIEL ET METHODES**

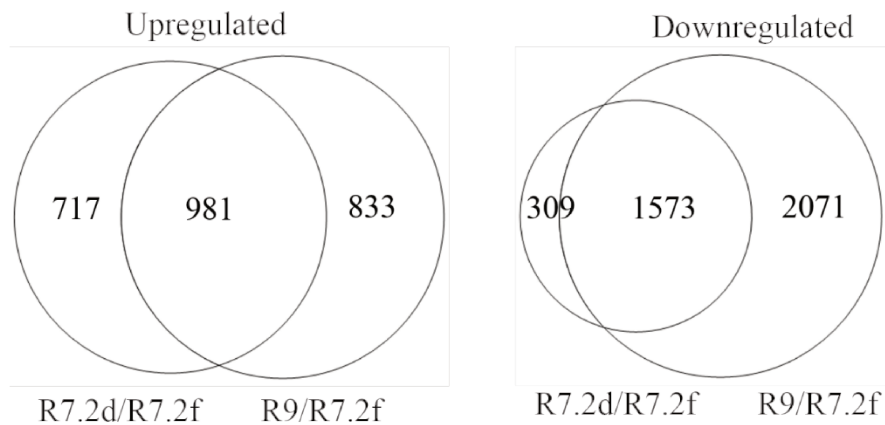
Pour cette étude, les plantes de soja (BRS 284) ont été cultivées à Botucatu (l'Etat de São Paulo au Brésil) en 2013 et 2014. La maturation des graines a été suivie après marquage des fleurs et en tenant compte des stades phénologiques proposées par Fehr et Caviness (1977) avec des modifications (Table 1). Les plantes de Medicago ont été cultivées en chambre de culture et les graines ont été prélevées à 28 jours après pollinisation et à l'abscission selon les protocoles décrits par Chatelain et al. (2012).

Pour évaluer la qualité physiologique durant la maturation de la graine, la germination, la tolérance à la dessiccation et la longévité ont été mesurée après séchage rapide selon la méthode de Chatelain et al. (2012). Pour étudier la longévité, des graines immatures ont été séchées rapidement puis stockées à 75% HR and 35 ° C. Ces conditions représentent un bon compromis entre le vieillissement naturel et le vieillissement accéléré où les hautes températures et l'humidité relative provoquent des effets délétères qui ne sont pas identique au vieillissement naturel. La longévité est déterminée par le nombre de jours de vieillissement pour diminuer la germination en 50%, la période de demi-viabilité, P50 (CHATELAIN et al., 2012). La vigueur germinative ont été mesurée dans des conditions stressantes (15°C ou 100 mM NaCl) en conditions lumineuse ou à l'obscurité.

L'étude du transcriptome a été réalisée par séquençage de l'ARN extrait de graines des stades R7.2 à R9 en utilisant la plate-forme HiSeq2500 (Illumina), dans le laboratoire centre de technologie de haute performances dans les sciences de la vie (LaCTAD), une partie de Universidade Estadual de Campinas (Unicamp). A partir des données générées dans le séquençage, les reads ont été alignés sur le génome de référence de soja 'Williams 82' avec le programme d'alignement Bowtie2. L'estimation de l'expression génique différentielle a été réalisée à l'aide DESeq2. Les données d'expression ont été normalisées La plupart des gènes importants ont été identifiés à 0,5% le taux de fausses découvertes (FDR) calculée en utilisant P-valeur ajustée pour les tests multiples en utilisant la méthode Benjamini-Hochberg. Le transcriptome des graines matures et immatures de Medicago a été réalisée par micro-array NIMBLEGENE selon les méthodes décrites par Righetti et al. (2015).



**Figure 1.** Acquisition de la capacité germinative (G) et de la tolérance à la dessiccation (TD) au cours du développement de la graine du soja cultivé en 2013 et 2014.



**Figure 2.** Diagramme de Venn comparant l'effet du séchage forcé et in planta sur les gènes différentiellement exprimés au stade R7.2 et R9.

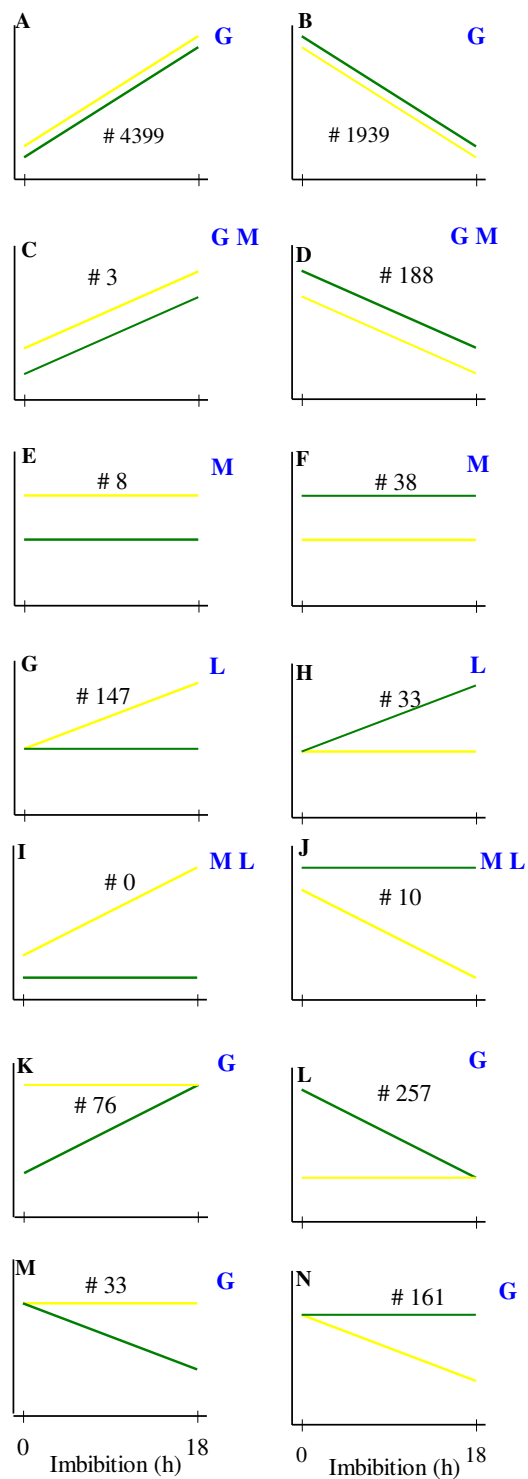
## RESULTATS ET DISCUSSION

Pendant le développement, l'accumulation maximale de masse sèche se produit au stade R7.2. En ce qui concerne l'ABA sa teneur est faible au début de l'embryogenèse. Elle augmente rapidement et atteint un pic de 3,1 µg/graine au stade R6, puis diminue lentement jusqu'en fin de maturation. La capacité à germer est progressivement acquise entre les stades R5.5 et R6 alors que la tolérance à la dessiccation est acquise entre le stade R6 et R7.2, avant le début de la dessiccation qui débute au stade R8.1 et le stade R9 (mature, Figure 1).

Nous avons comparé la vigueur des graines immatures au stade R7.2 avec les graines matures au stade R9 en mesurant la vitesse de germination et la croissance des hypocotyles après 7 j d'imbibition. Aucune différence dans ces caractéristiques ne furent observées. Nous n'avons pas observé de différences lorsque les graines immatures et matures furent mises à germer en présence de NaCl ou à 15°C. En revanche, on observe une différence de longévité entre graines immatures et matures. Ainsi, les graines récoltées au stade R7.2 possède un P50 de 27 jours alors que les graines récoltées au stade R9 ont un P50 de 48 jours.

L'absence de différence en terme de vigueur germinative entre les stades R7.2 et R9 nous permet donc d'examiner s'il existe la proportion d'ARN stockés commun entre les graines immatures ayant subi un séchage forcé rapide et les graines matures ayant subi séchage lent in planta. Par ailleurs, les différences peuvent indiquer des mécanismes impliqués dans la longévité. Dans un premier temps, nous avons essayé de savoir si le séchage prématuré au stade R7.2 induit un changement radical dans le transcriptome comparé à la desiccation de maturation. Dans ce but, les ARN totaux ont été extrait de graines au stade R7.2 lorsque la tolérance à la dessiccation est acquise mais pas la longévité, avant et après séchage et à partir de graines sèches matures récoltés au stade R9. L'analyse de données RNAseq a produit entre 14 et 38 millions de reads selon les stades. Environ 56044 transcrits ont été identifiés en utilisant la base de données Soybase (<http://www.soybase.org/genomeannotation/>). Les variations du niveau d'expression ont été calculées en divisant la moyenne de l'expression normalisée de R9 par celle de R7.2. Les gènes significatifs ont été sélectionnés en utilisant une p- valeur de 0.05 corrigée par la méthode de Benjamini-Hochberg. La comparaison des transcriptomes des graines immatures et matures montre que le séchage forcé comme l'indique la figure 2.

Version Abrégée en Français



**Figure 3.** Profil d'expression des gènes différentiellement exprimés après 18h d'imbibition de graines immatures (jaunes) et mature (vert). Les nombres indiquent la quantité de transcrits. G, germination, L longévité, Maturation.

Une analyse d'enrichissement des catégories GO des 500 gènes les plus différentiellement exprimé montre parmi les gènes surexprimés une sur-représentation des catégories « Response to heat », « protein folding », « response to endoplasmic reticulum stress ». En effet, les transcrits les plus exprimés dans les graines matures encodent des protéines de choc thermique (« HeatShockProteins –HSP »). On trouve également les GO « red signaling pathway », « response to light stimulus ». Peut-être que ces gènes sont nécessaires pour contrôler la photomorphogenèse pendant la levée des semis et leurs transcrits sont déjà synthétisés au cours de la maturation. Cependant, la germination des graines immatures au stade R7.2 ou R9 n'a pas révélé de différences lorsque les graines sont imbibées à la lumière (200 pmol, lumière continue). Une hypothèse alternative est que les graines en développement pourraient réagir à des signaux lumineux tels que la photopériode et le contrôle de la qualité de la lumière du processus de maturation et de l'acquisition de la longévité. Il est connu que la lumière qui pénètre dans les tissus du fruit chez le pois et le soja et qui atteignent l'embryon est principalement la lumière dans la région rouge lointain (ALLEN et al. , 2009). Aussi l'expression des gènes dans les graines en développement chez le soja est contrôlé par l'horloge circadienne avec un phasage spécifique d'organe « the circadianclockwith an organ-specificphasing » (HUDSON et al., 2010). Cependant, dans nos expériences, les graines ont été récolté toujours rigoureusement au même moment de la journée. En outre, parmi les loci génétiques caractérisant les groupes de maturité entre les accessions de soja sont deux gènes paralogues codant photorécepteur PhyA-like impliqué dans la réponse à la photopériode et le contrôle du temps de floraison aux locus de maturité E3 et 4 (LIU et al., 2008). Des travaux supplémentaires sont nécessaires pour comprendre la prévalence des termes GO associés à la perception lumière.

Parmi les 500 gènes les plus réprimés, on trouve les catégories associées à la photosynthèse telles que « generation of precursor metabolites and energy », « photosynthesis light reaction », « PSII ». Ensuite nous avons testé l'hypothèse qu'une approche transcriptome comparant les changements pendant l'imbibition précoce de deux lots de graines différant en longévité pourrait donner accès à des mécanismes de réparation qui seraient synthétisés lors de l'imbibition des graines de l'étape R9 ou, alternativement, aux mécanismes détériorants lors de l'imbibition des graines immatures. Pour ce faire, nous avons obtenu des données RNASeq provenant de graines immatures séchées et matures avant imbibition (0h) et après 18 heures d'imbibition (Figure 3).

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Dans le profil d'expression G, nous avons éidentifié et confirmé par RT-QPCR l'homologue du FACTOR DE RÉPONSE AUXIN 5, ARF5/MONOPTEROS. Sa fonction, en conjonction avec un signal d'auxine est de favoriser la formation du primordium cellule fondateur d'initier des fleurs. De même, un transcrit codant un facteur de transcription de classe de domaine HD, régulé par l'auxine a été trouvé. Si nous supposons que ces facteurs de transcription sont synthétisés comme mesure préventive, alors l'auxine devrait jouer un rôle dans la longévité des graines. Cette hypothèse est également soutenue par un travail récent de Righetti et al (2015) dans lequel ils ont construit un réseau de co-expression de la longévité des semences. Ces auteurs ont indiqué que les gènes présents dans le module de longévité croisée étaient fortement enrichis dans les sites de liaison des facteurs de réponse auxine (ARF) dans leurs promoteurs. Le même argument d'une mesure préventive contre les tissus potentiellement endommagés peut s'appliquer à l'homologue de HSFA9 qui était fortement régulé à la hausse en imbibant des graines mature. Il pourrait faciliter la production de chaperon pour réparer les protéines qui ont été endommagées pendant le stockage. Il est intéressant de noter que le PMIT (PROTEIN-L-ISOASPARTATE METHYLTRANSFERASE 1), une protéine induite par ABA impliquée dans la réparation de protéines dans des graines âgées a été régulée à la baisse pendant l'imbibition. Nos données du profil G fournissent également des preuves de la réparation de l'ADN qui pourrait être induite pendant l'imbibition.

## **CONCLUSIONS**

Ce travail décrit les changements dans le transcriptome associés aux événements se produisant en fin de maturation lorsque les graines se préparent pour le stade sec et à la germination. Au stade R7.2 correspondant la maturité de masse et maturité physiologique d'après la littérature, la plupart des caractéristiques de la vigueur de la graine ont été acquises, à l'exception de la longévité des graines. Par conséquent, ce stade R7.2 ne peut pas être déclaré physiologiquement mature. L'analyse transcriptome montre que des différences importantes entre les graines du stade R7.2 et R9 corroborent l'idée que la maturation ne se termine pas à ce stade.





## INTRODUCTION

Soybean (*Glycine max*,  $2n = 40$  chromosomes) is a native legume from East of Asia and the first reports of its use are from 2838 B.C. in China (MORSE, 1950 according to BOZATO and BOZATO, 1987). In Brazil, the introduction of soybean took place in 1882 in the State of Bahia and the State of Sao Paulo (D'UTRA, 1882; DAFFERT, 1893, according to BOZATO and BOZATO, 1987). Currently, Brazil is the second largest soybean producer in the world, preceded only by the United States and according to the latest information released by CONAB, soybeans are cultivated in an estimated area of 33 million of hectares with an estimated production of 100 million tons and an average yield of  $3037 \text{ kg ha}^{-1}$  (CONAB, 2016). Soybean is a rich source of protein and oil and has been traditionally used for oil production, food and feed (QIU and CHANG, 2010).

With the continued increase in world demand for sources of plant oil and proteins, soybean production has spread rapidly to tropical regions. In Brazil, there is a continuous effort to increase its production, mainly by increasing its yield per area. The use of high quality seeds is a critical factor leading to crop yield. Characteristics of seed quality include genetic purity, physical and physiological quality and seed health (POPINIGS, 1985). Physiological seed quality represents a key trait because it determines the success of plant establishment, and therefore crop yield. It is defined as the sum of the seed physiological properties that leads to homogenous and vigorous germination and seedling establishment and a good longevity during storage.

In many plant species, the physiological quality traits are not acquired in the same time during seed development. The capacity to germinate and desiccation tolerance are acquired prior to maximum dry weight, whereas seed vigor is acquired later during seed maturation. Ideally, the seed harvesting should occur when all the above characteristics reach their maximum levels. However, there is no consensus as to when this occurs during the maturation. On the one hand, agronomy and seed technology studies consider that physiological quality is maximum when the seed filling has ended (so-called mass maturity) and can decrease thereafter during the end of maturation drying or during seed processing (TEKRONY et al., 1979; OBENDORF et al., 1980; FRANÇA NETO et al., 2007; REN et al., 2009). On the other hand, for seed physiologist, physiological maturity refers to the developmental stage at which seeds achieve maximum viability and



vigor, which is not necessarily correlated with seed filling. In many species, further maturation drying to 45% moisture is necessary to achieve maximum germination speed and absence of abnormal seedlings (ELLIS et al., 1987; ZANAKIS et al., 1994; CHATELAIN et al., 2012). Seed longevity, another key factor implicated in physiological quality increase continuously after seed filling until dispersal or harvest (ELLIS et al., 1987; ELLIS et al., 1993; PROBERT et al., 2007; CHATELAIN et al., 2012). For practical reasons, seeds are harvested during the maturation drying, otherwise they would be crushed during the harvest. Therefore, commercial harvest has to be delayed until the seed moisture decreases to levels that are compatible to harmless mechanical handling. During this period, the seeds that remain on the plant are highly prone to deterioration, particularly when humidity and/or temperature remain high, conditions that typically occur in tropical regions.

The production of high quality seeds requires that the maturation and harvesting phases occur under mild temperatures (COSTA et al., 2003; FRANÇA-NETO et al., 2007). In Brazil there are studies analyzing the appropriate regions for the production of high quality soybean seeds (COSTA et al., 1994; PÁDUA et al., 2014). While the late phase of seed development appears to be critical in order to harvest at maximum physiological quality and despite the lack of consensus as to when maximum seed vigor is acquired in soybean, we still lack basic knowledge of the molecular processes occurring during the late phase of maturation after seed filling when seed vigor is acquired. In soybean, transcriptome studies have generated a wealth of data describing seed development, mainly during embryogenesis and filling (HAJDUCH et al., 2005; HUDSON, 2010; JONES et al., 2010, LIBAULT, 2010; SEVERIN et al., 2010; ASAKURA et al., 2012; SHA et al., 2012, SHAMIMUZZAMAM and VODKIN, 2012; AGHAMIRZAIE et al., 2013). However all these transcriptome studies never included developmental stages after seed filling, while 20-40 days can pass between mass maturity and dry mature seeds depending on the environmental conditions during cultivation. In order to optimize harvesting processes to ensure high quality seeds, it is therefore essential to revisit the molecular events occurring during seed maturation in association with the acquisition of various characteristics associated with seed quality.

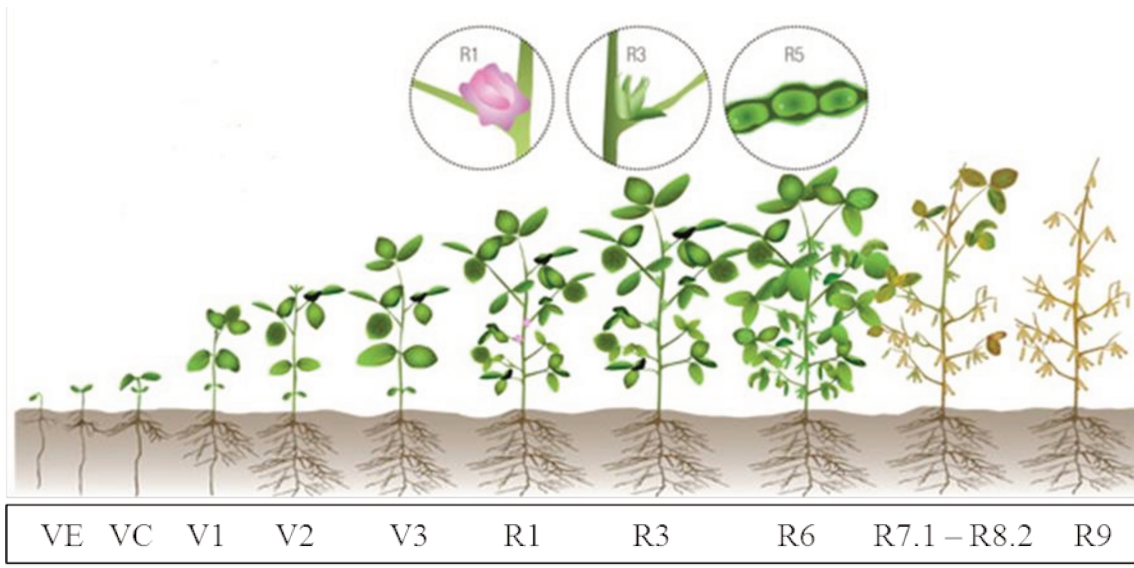
It is generally inferred that desiccation during seed maturation promotes the transition from a developmental mode to a germination-oriented program (BEWLEY and BLACK, 1994). In *Arabidopsis*, De novo transcription is not required for protein



synthesis during early imbibition, suggesting that the initial phase of germination depends only on pre-existing “stored” mRNA, which have accumulated during seed development (RAJJOU et al., 2004). At maturity, dry seeds of *Arabidopsis* contain approximately 12000 transcripts which have been characterized (reviewed in WEITBRECHT et al., 2011). This implies that these transcripts must be synthesized during seed maturation. However, when this occurs has not been investigated. Also, not all stored transcripts are necessary for germination. The identity of the specific mRNAs required for germination and when they are synthesized during development is still not known. A recent study on *Arabidopsis* showed that during the first 3h of imbibition seeds translate some mRNAs that were part of developmental program such as LEAs and storage proteins, indicating that a subset of stored mRNA in dry seeds are characteristic of the maturation program and did not disappear during maturation drying (GALLAND et al., 2014). It is not known whether an artificial drying treatment in immature seeds would induce a similar or entirely different profile of stored mRNA compared to maturation drying. Such information would be useful to provide putative molecular indicators to assess the maturity status of harvested soybean seeds and whether post-harvest drying during seed processing is sufficient to replace natural maturation drying.

In this work, we will show that the acquisition of seed vigor is quite different in soybean from that of *Medicago truncatula*, a model legume used for genomic studies. Also, a parallel work showed that longevity is acquired shortly after seed filling according to a pattern that is different from *M. truncatula* (LIMA, 2016). The soybean genome is approximately 975Mb with 88647 transcripts and 56044 protein-coding loci predicted (SCHMUTZ et al., 2010). According to Mudge et al. (2005), there exist highly syntenic regions in the genomes of soybean and *M. truncatula*, that is, regions of gene content conserved between both species. These authors reported that the up to 75% of soybean genes are colinear with *M. truncatula*. Therefore, we compared changes in transcriptome of developing seeds of both species in order to understand the mechanisms involved in germination and seed vigor in legumes. This was addressed here by comparing transcriptome changes associated with maturation drying and imbibition of seeds of soybean and *M. truncatula*, harvested at an immature stage after seed filling and mature dry stage.

After a literature review and presentation of material and methods, we will first present and discuss the results obtained on the impact of premature drying in



**Figure 4.** Soybean growth stages (available in McWilliams et al., 1999).

developing soybean. Then we will present and discuss the data regarding to changes during imbibed of immature and mature seeds of soybean and *M. truncatula*.

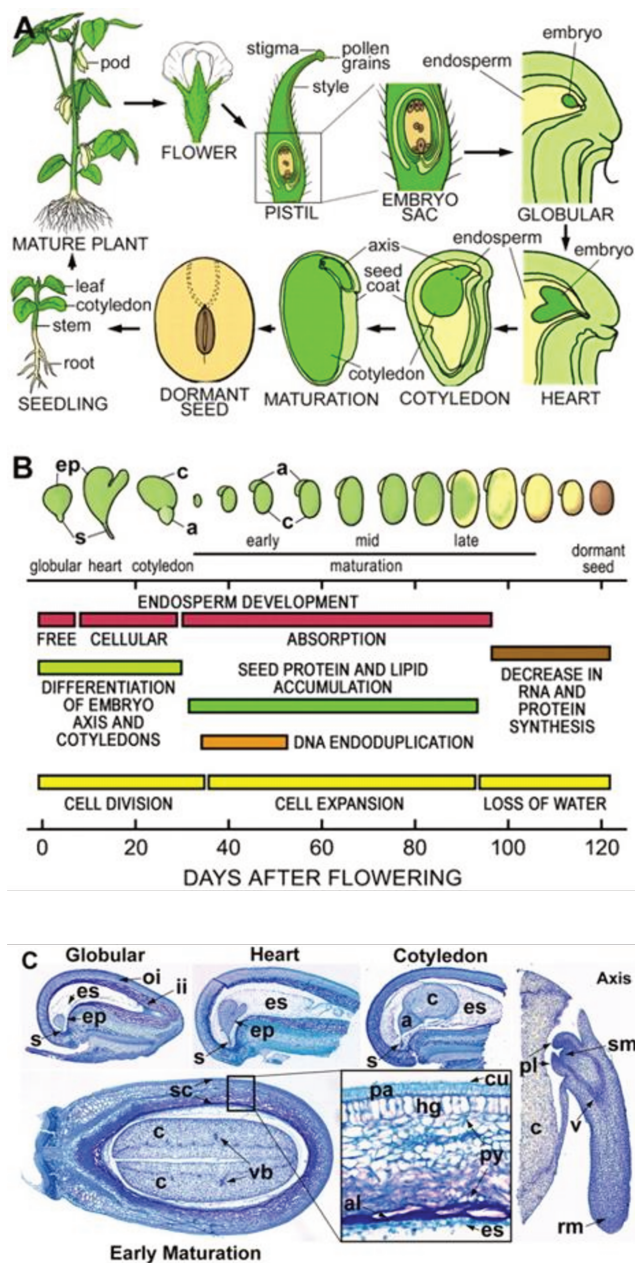
## **CHAPTER I. LITERATURE REVIEW**

### **I.1. Soybean seed production and stages of development**

The proper establishment of a seed production field requires availability of water ranging between 450 and 800 mm per cycle, being higher during germination to emergence and flowering to seed filling (7-8 mm of water per day) and temperatures between 20 and 30 °C. Soil temperature below 10 °C results in delays in seed germination and triggers soil-borne pathogens. For good seedling emergence, the soil should not exceed 85% of available water and not be less than 50% The sowing date is one of the factors that most influence the yield of soybeans. The theoretical best timing is between 30 and 45 days before the summer solstice, this time is considered sufficient for the plant meet its growing season and develop with height and size compatible to high productivity and mechanized harvesting. In general, the varieties adapted to Brazilian conditions are cycled between 90 and 150 days (EMBRAPA, 2011). Other criteria to consider for high yields are the history, the crop rotation and the properties of the soil, such as fertility, drainage and topography and the maturity group, plant density (200–230 thousand plants/ha, according to the genotype).

The soybean growth stages throughout the culture are shown in figure 4. The cotyledons appear above the soil surface from 7 to 10 days after sowing. New vegetative stages are developed every 5 days under normal temperatures (McWILLIAMS et al.,1999).





**Figure 5.** Soybean seed development (adapted from Le et al., 2007). A, Cartoon depicting soybean life cycle. B, Schematic representation of soybean seed development. Embryo morphologies and developmental events were adapted and modified from Goldberg et al. (1989). C, Paraffin transverse 10- $\mu$ m sections of soybean globular, heart, cotyledon, and early maturation seeds. Inset contains a magnified view (40x) of the seed coat. Axis longitudinal section was obtained from an early maturation seed. a, Axis; al, aleurone; c, cotyledon; cu, cuticle; ep, embryo proper; es, endosperm; hg, hourglass cells; ii, inner integument; oi, outer integument; pa, palisade layer; pl, plumule; py, parenchyma; rm, root meristem; s, suspensor; sc, seed coat; sm, shoot meristem; v, vascular tissues; vb, vascular bundle (LE et al., 2007).

## **I.2. Seed development**

Seed development is a complex process that can be divided into three partially overlapping phases: embryogenesis, seed filling and late maturation phase (BEWLEY and BLACK, 1994). This complex process involves the interplay of a network of many developmental processes and metabolic pathways together with their interactions with the environment (BEWLEY et al., 2013).

Induction of flowering and differentiation of flower parts are considered the starting points for seed formation. In soybean, the start of the flowering occurs after 40 to 70 days after seedlings emergence, depending on the cultivar, sowing date and weather conditions (FEHR and CAVINESS, 1977). According to Ritchie et al. (1982) in soybean seed development, around 3 to 5 days after flowering (DAF) the embryo reaches the globe or globular stage. Then, 8 to 10 DAF, starts to formation of cotyledons during the heart stage. The development continues, around 22 to 24 DAF the cotyledons are developed by taking the total size of the seed and the primordium of the first trifoliolate leaf begins to develop. Figure 5 presents in detail the soybean seed development (LE et al., 2007).

Embryogenesis marked as a 'lag' phase initiated at fertilization and dominated by histodifferentiation and early cell expansion. It is a period of active cell division characterized by a rapid increase in water content with little dry weight accumulation (BEWLEY and BLACK, 1994). Maturation characterized by the 'seed filling', involving a rapid deposition of storage compounds (proteins, lipids and carbohydrates) and cell expansion. These nutritive reserves subsequently support the initial growth during germination and seedling establishment (BAUD et al., 2002). The final phase called late maturation or maturation drying starts when the seed has reached its maximum dry weight and start to undergo maturation drying in preparation for the quiescent and/or dormant state (BEWLEY and BLACK, 1994).



### **I.2.1 Embryogenesis**

The embryogenesis begins with fertilization. The fusion of the male gamete and the egg nuclei produces a diploid zygote that will differentiate and form the embryo. Following fertilization, the zygote undergoes an asymmetrical mitotic division on that gives rise to a small apical cell and a large basal cell. Further divisions lead to the formation at the pre-globular embryo and the suspensor. During the seed development, the suspensor transports nutrients and growth-regulating hormones to the growing embryo, serving as the connection between maternal and endosperm tissue. In the globular stage, the embryo develops radial patterning through a series of cell divisions, with the outer layer of cells differentiating into the protoderm. In the next phase, designated heart stage, start up the development of the cotyledons, which is accompanied by changes in the internal structure beginning the organization of the tissue systems. Bilateral symmetry is apparent from the heart stage. Following, the embryo changes to a heart-shaped appearance in dicots. In monocots, only a single cotyledon is formed. Continued cell division, growth and differentiation lead to the late heart stage and then to the torpedo stage. The shoot meristem differentiates from the apical-central region of the embryo. The ground meristem of the globular stage develops into the storage parenchyma cells of the cotyledons. The procambium forms the vascular conducting tissue within in the mature embryo. The axis tissues in the mature seed are the root axis (radicle), shoot axis (plumule) and hypocotyl (BEWLEY et al., 2013).

The endosperm (triploid) development also occurs during the embryo development. The endosperm division starts to advance and occur faster than the zygote division. This happens because the embryo can use the endospermatic tissue for its development during maturation and subsequent germination/growth stages (MARCOS FILHO, 2005; BEWLEY et al., 2013). In most dicot seeds, the endosperm is almost completely consumed during development of the embryo. In these seeds, the reserves are stored in the cotyledons (MARCOS FILHO, 2005). In soybean seeds, the embryo is formed by two cotyledons and embryo axis, which are responsible for 90% of the soybean seed weight.



## **I.2.2 Seed filing**

The synthesis of stored reserves occurs during the seed growth period after histodifferentiation. The early and mid phases of maturation are dominated by the action of ABA, initially synthesized in the maternal tissues and later in the embryo and endosperm, although to a lower level in the embryo and endosperm (NAMBARA and MARION-POLL, 2003). Subsequently, abscisic acid (ABA) levels decline during late maturation. Most seeds contain large quantities of nutrient reserves, mainly carbohydrates, oils (triacylglycerols), proteins, and small amount of phytin, The reserves are located within the embryo itself or in an alternative storage tissue, namely the endosperm (cereals; castor bean) or the perisperm (sugar beet) (BEWLEY et al., 2013).

The mature soybean seed is composed on average of 37% protein, 17% oil, 26% carbohydrates and 5% minerals, (MEDIC et al., 2014). Seeds also contain secondary metabolites, such as alkaloid and phenolic compounds, including phenolic acid, lignin, and isoflavones; which are associated with plant defense and survival mechanisms under biotic and abiotic stress factors (PELTIER et al., 2009). The storage reserves in the seed are synthesized during seed development. The synthesis occurs in specific cellular compartments for each type of substance: starch and lipid in plastids, and proteins in cytosol and endoplasmic reticulum. The accumulation of carbohydrates precedes the lipids and proteins because the carbohydrates are precursors or participate indirectly in the synthesis of these compounds in the seeds (MARCOS FILHO, 2005). During development of soybean seed, oil accumulation is faster in the early stages, while proteins accumulate in later stages. The content of monosaccharides and disaccharides decreases during the course of development and seed maturation, while raffinose family oligosaccharides accumulate during the last weeks before full maturation (SALDIVAR et al., 2011; MEDIC et al., 2014).

Sucrose is the first source of carbon in the developing of seeds, and amino acids are the first nitrogen source. In some legumes, e.g., soybean and garden pea, the translocated sucrose produced by photosynthesis in the leaves and pods may be stored temporally as starch in the pod prior to remobilization and transfer to the developing seeds (FERREIRA and BORGHETTI, 2004; MARCOS FILHO, 2005; BEWLEY et al., 2013). Although the starch is present in significant amounts in early development soybean seed, it



is degraded to less than 1% at maturity, being considered a transitory (SALDIVAR et al., 2011).

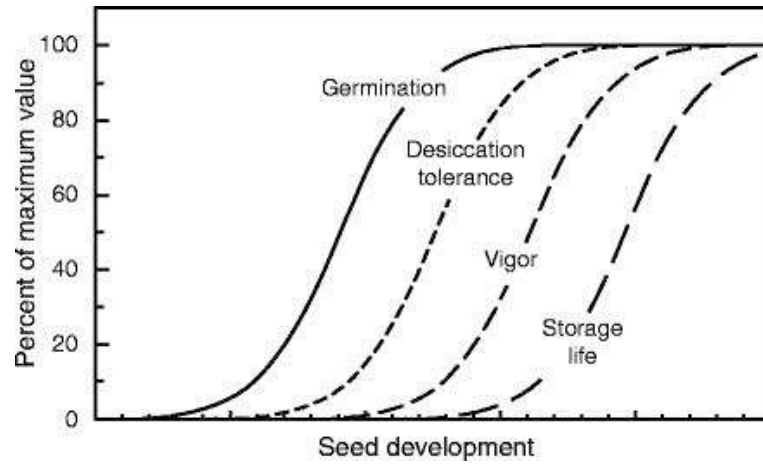
Hexose, sucrose, amino acids and other solutes are transported to the seed via the phloem, through osmotic gradient. The pressure gradient from the font to the drain guides the mass flow in the phloem. Assimilates are translocated from the parental plant via a vascular strand that branches from the vascular tissue running through the pod. This strand passes through the funiculus and into the seed coat. Passage of assimilates through the funiculus, and from the seed coat into the cotyledons is aided by the presence of transfer cells. In soybean seeds, the phloem in the seed coat, may exhibit an extensive reticulate network (MARCOS FILHO, 2005; BEWLEY et al., 2013). There is no direct vascular connection between the embryo within the seed and the mother plant. Nutrients are therefore released into the extracellular space (apoplast) between the parental and filial tissues, and then imported into the liquid endosperme and embryo.

### **I.2.3 Late maturation**

The third phase of seed development is the least characterized. It is mainly represented by the loss of water leading to the quiescent state after seed filling. However, when the seed filling is terminated, seeds of most species do not necessarily proceed directly to final drying, (SANHEWE and ELLIS, 1996). In arabidopsis seed, this period comprising of 1-2 days (BAUD et al., 2002; VICENTE-CARBAJOSA and CARBONERO, 2005; LE et al., 2010), for medicago seeds in around 21 days (CHATELAIN et al., 2012) and in soybean seeds from 20 to 40 days (ZANAKIS et al., 1994).

It is generally inferred that desiccation during seed maturation promotes the transition from a developmental mode to a germination-oriented program. Indeed, slow drying at an immature stage generally induces the ability to germinate. In cereals, this can be at a very early during seed development soon after embryogenesis but in most species, slow drying must occur during seed filling. By analyzing germination of castor bean seeds during development, it was observed that immature seeds that were not subjected to artificial drying, failed to complete germination; only 30-40% of the seeds harvested at 50 DAF reached normal post-germination growth, while seeds harvested at 35 DAF and subjected to drying, showed 100% normal post-germination growth (KERMODE and





**Figure 6.** Pattern of development of seed physiological quality traits (BEWLEY et al., 2013).

BEWLEY, 1985). However, the maturation drying has received little attention in the literature. For example, it is not clear whether it is a passive phenomenon primarily controlled by environmental conditions or a function of the physiological activity of the mother plant or seed.

### **I.3 Acquisition of physiological quality during development**

Seed physiological quality or seed vigor is a collection of properties inherent to a seed lot that allow a high and uniform level of germination, which will produce uniform, vigorous seedlings without defects under various environmental conditions, including stressful conditions. The maintenance of physiological quality during handling after harvest and subsequent storage is also critical. Poor storage conditions and/or the absence of protective mechanisms allowing long term storage will contribute to a fast deterioration of the seed lots and negatively impact germination and seedling emergence. Therefore, physiological quality also include the capacity to tolerate desiccation and the capacity to revive without defects after extended periods of storage in the dry state (i.e. longevity). Seed physiological quality can be influenced by several factors, including nutritional deficiencies in the mother plants, occurrence of pests and diseases, stressful conditions during seed development, drying, processing, storage and transport (CARVALHO and NAKAGAWA, 2000).

During seed development, the different characteristics of physiological quality are not acquired synchronously. The capacity to germinate (germinability) is acquired prior to maximum dry weight. This is followed by the development of desiccation tolerance (in orthodox seeds). Concomitantly, seed vigor is acquired, which is represented by greater speed of germination, uniform seedling establishment and greater tolerance of stressful conditions during germination. Finally, longevity increases in the last stages of development, just prior to or associate with the maturation drying phase, as shown in figure 6 (BEWLEY et al., 2013). The acquisition of these different components will be described in the following sections.

Ideally, seed crops, like soybean, should be harvested when maximum physiological quality is acquired. However, in commercial practices, this is very difficult to achieve for several reasons. Despite text book patterns similar to figure 6, there is no consensus in the literature as to when the maximum quality occurs during seed



development. This is particularly true for soybean, where maturation is further complicated by the existence of several maturity groups and determinate and indeterminate cultivars. Also, the environment during seed development will influence the different seed traits. Finally, the mechanical harvest imposes that seeds are dried to a window moisture content to avoid threshing and mechanical damage.

### **I.3.1 Germinability**

Germinability can be defined as the ability of an immature seed to germinate. In legume seeds, germinability is usually acquired early during the seed filling in the middle of the development phase. According to Zanakis et al. (1994) and Obendorf (1980), germinability in soybean increases between 25 and 40 days after flowering (DAF).

Seeds germination is traditionally described by the “three-phasic pattern of germination”, proposed by Bewley and Black (1978). Phase I: imbibition, characterized by the fast water uptake, resulted from the low matric potential of the dry seed. The water continues to get inside the seeds until all cellular content turns totally hydrated. Phase II: plateau, when the water content is relatively constant and the respiration level is low. Phase II has variable duration according to plant species, tissue composition, and hydric potential of the substrate. Phase II is characterized by preparatory biochemical activities like synthesis or release of more hexoses after initial hydrolysis and synthesis of enzymes and mRNAs. Phase III is characterized by a rapid increase in water uptake that corresponds to embryo growth resumption after visible germination (radicle protrusion). Finally, seedling establishment is initially supported by metabolites produced by hydrolysis and conversion of stored substances in the seed, as proteins, carbohydrates, and oils, until the plant turns photosynthetically active (BEWLEY and BLACK, 1978; BEWLEY et al., 2013).

In dry seeds, the phospholipid bilayer are thought to be in a gel phase. During imbibition, they turn to a crystalline state, resulting in cell damages, with a release of ions and low molecular weight metabolites from the seeds. The repair process of such desiccated membranes and the damages imposed by the quick imbibition visible in phase I starts during the imbibition (HOEKSTRA et al., 1999). Imbibing cotton seeds, and may act to stabilize and enhance cellular compartmentation (SANDOVAL et al., 1995).

Imbibing seeds have a fast resumption of metabolic activity. The essential structures and enzymes to metabolism resumption are inside the dry seeds, having

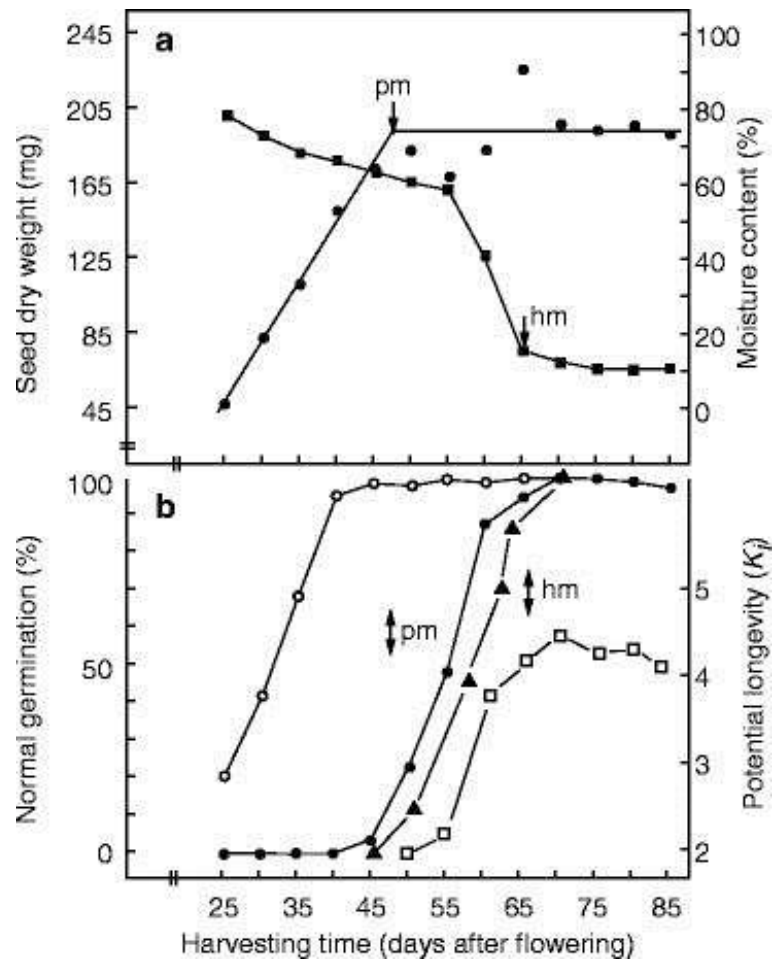


persisted to desiccation and maturation phases. One of the earliest steps to metabolism resumption is respiration, which is detected already a few minutes after imbibition begins. The glycolytic and oxidative pentose phosphate pathways resumes during Phase I, and Krebs' cycle enzymes are activated (BOTHA et al., 1992). These enzymes may be protected in the dry seed by mitochondrion-specific LEA proteins (GRELET et al. 2005).

DNA might suffer from damage during desiccation and rehydration. Therefore, the damaged DNA must be repaired during seed germination. In arabidopsis seeds the inactivation of DNA LIGASE VI, exhibited by *atlig6* mutants, results in a late germination (WATERWORTH et al., 2010). Macovei et al. (2011) observed that formamidopyrimidine-DNA glycosylase (FPG) and 8-oxoguanine DNA glycosylase/lyase (OGG1) are positively regulated during the initial phases of germination in *Medicago truncatula* seeds, suggesting its involvement in DNA repair. Another DNA repair enzymes are the poly (ADP-ribose) polymerases (PARPs) such as NIC2. Hunt and Holdsworth (2007) reported *nic2-1* mutant seeds has high levels of nicotinamide and retarded germination.

The time course of seed germination is related to both sequential and selective mRNA translation, emphasizing a fine regulation of the translational machinery (GALLAND et al., 2014).

At maturity, dry seeds contain around 10.000 transcripts called “stored mRNAs”, which are associated with messenger ribonucleoprotein complexes (mRNPs). Global transcriptome analyses of several species, e.g. Arabidopsis, tomatoes and sunflowers, using high throughput approaches such as microarrays indicate that transcriptional activity is initiated in seeds during the first few hours after the start of imbibition (HAI) (WEITBRECHT et al., 2011; RAJJOU et al., 2012; GALLAND et al., 2014; GALLAND and RAJJOU, 2015). In arabidopsis seeds, the most genes positively regulated around 3 HAI are that one associated to primary metabolism, including pentose phosphate pathway since respiration is one of the earliest cell activities during imbibition. Howell et al. (2011), observing early metabolic and transcriptome changes in rice germination, found that glycolysis and the TCA cycle are activated to support energy demanding processes.



**Figure 7.** Changes in (a) seed dry weight (●) and moisture content (% wet basis, ■) and (b) the capacity of freshly harvested (○) and dried (to 10–11% moisture content, ●) seeds to germinate normally within 14 days (○, ●) or for dried seeds to germinate within 3 days after imbibition (▲). Also shown in bare changes in the potential longevity ( $K_i$ ) of seeds harvested and dried at different times of development and then aged at 12% moisture content and 40°C (□). Modified from Zanakis et al. (1994) in Bewley et al. (2013).

### **I.3.2 Acquisition of desiccation tolerance**

Desiccation tolerance (DT) is the ability of certain organisms to deal with extreme water loss to levels below 0.1g H<sub>2</sub>O per gram dry weight and completely recover metabolic function after rehydration (LEPRINCE and BUITINK, 2010; FARRANT et al., 2007). In orthodox seeds, the acquisition of DT is initiated concomitantly with the accumulation of reserves and is established before the end of seed maturation (PAMMENTER and BERJAK, 1999; BEWLEY et al., 2013). In soybean, Sun and Leopold (1993) and Zanakis et al. (1994) found that the embryonic axis acquire germinability around 34 DAF while the the maximum dry weight was acquired after 48 DAF. However, both studies gave contrasting results regarding the induction of desiccation tolerance. According to Sun and Leopold (1993), it was acquired at physiological maturity (48 DAF) whereas Zanakis et al. (1994) showed it was was acquired at around 66 DAF, after the end of seed filling and after physiological maturity (Figure 7).

Desiccation tolerance is a multifactorial phenomenon in which many components act in synergism (for reviews see HOEKSTRA et al., 2001; LEPRINCE and BUITINK, 2010). They include protective mechanisms that can be grouped in three categories, namely: 1) protection by stabilization of membranes and proteins by non-reducing sugars such as sucrose and raffinose oligosaccharides family, late embryogenesis abundant (LEA) proteins and heat shock protein (HSP). 2) protection against oxidative damage and ROS production by a range of antioxidant compounds including tocopherols, glutathione, together with a coordinated response of metabolism during drying and 3) protection against structural stresses imposed by drying such as cell wall modification, reorganization of endomembranes and cytoskeleton, vacuolization and chromatin condensation.

According to Hoekstra et al. (2001), non-reducing sugars can prevent the harmful effects of desiccation on the cell membranes. They are thought replace water molecules at the phospholipid heads, thereby maintaining the space of lipids, and avoiding the transition of the liquid phase to the gel phase. LEA proteins are grouped in different families families that are either specific to seeds or expressed both during seed development and in vegetative tissues submitted to osmotic stress (WISE, 2003). They are composed by a high proportion of hydrophilic amino acids, resulting in high solubility after heating and highly intrinsically disordered in the hydrated stage (WANG et al., 2003,





TUNNACLIFFE and WISE 2007). The LEA proteins can protect cellular structure, membranes, and proteins. Also they can act as hydration buffer, sequester ions. (reviewed by TUNNACLIFFE and WISE 2007). While most LEA proteins appear during seed maturation, they are not correlated with desiccation tolerance (CHATELAIN et al. 2012; DELAHAIE et al., 2013 and references therein). Instead, they appear to be correlated with seed longevity.

HSP are molecular chaperones that play an important role in protein stabilization during assembling of multi-protein complex, folding or unfolding, during transport sub-cellular level compartments (LINDQUIST and CRAIG, 1988). According Vertucci and Farrant (1995), the role of HSPs is related to conservation and repair of macromolecular structure during dehydration and hydration. Although the HSPs are synthesized in organisms as a response to heat, the balance of synthesized proteins and the relative importance of the HSPs families for stress tolerance are very different among the species (QUEITSCH et al., 2000). The gene expression for HSPs during the development of seeds generally occur at the same time of gene expression for LEAs, and both has a positive response to ABA. In *Arabidopsis thaliana* embryos, HSPs accumulate during maturation (WEHMEYER and VIERLING, 2000).

### **I.3.3 Acquisition of longevity**

During seed development, seed longevity is the last quality traits to be acquired (BEWLEY et al., 2013). Seed longevity is defined as the capacity to remain viable during storage in the dry state (BUIKINK and LEPRINCE 2004; CHATELAIN et al., 2012). It is acquired progressively during the late phase of seed maturation but this has not been characterized extensively. In soybean and *Medicago*, longevity increases steadily during maturation drying and reaches a maximum at around seed abscission. This decline is usually attributed to field weathering, corresponding to wet and hot conditions that prevent seed drying or worse rehydrate seeds (TEKRONY et al., 1980; CHATELAIN et al., 2012). In other species, longevity can increase steadily during maturation until seed abscission after which it remains steady or continues to increase during further drying (PROBERT et al., 2007).

After harvest, seed longevity depends on the moisture content (or ambient relative humidity), oxygen pressure and temperature during storage. High



temperature and high humidity induces a rapid deterioration whereas storage in dry and cold conditions (e.g. 5°C, 15% RH) can prolong the shelf life by a factor 10 to 100 according to the species. This increase is due to the formation of a glassy state, which corresponds to an amorphous matrix where the cytoplasmic viscosity is so high that it resembles a solid (BUITINK and LEPRINCE, 2004). In the glassy state, there is no active metabolism because the high viscosity prevents the formation of ATP. In addition, deleterious reactions are severely slowed down, thereby stabilizing the seeds. Several authors have used so-called accelerated aging treatments as a proxy to determine seed longevity and thereby seed vigor. Typically, seeds are stored at 42°C and 100% RH which induces a combination of progressive rehydration and heat stress which rapidly deteriorates the seeds (TEKRONY et al., 1980, RAJJOU et al., 2008). There are claims that these types of treatments mimic the storage behavior in optimal conditions or represents a good test to assess physiological maturity. However, both genetic and biochemical evidence show that the mechanisms of deterioration are not identical at different water content/temperature combinations.

Seed longevity is attributed to two main strategies: protection and repair (reviewed in SANO et al., 2015; RAJJOU and DEBEAUJON, 2008, BUITINK and LEPRINCE, 2008). Protection is offered by the presence of high concentrations of non-reducing sugars, including sucrose and RFO that are good glass formers. Also, *in vitro* studies have shown that these sugars protect the cellular integrity during desiccation by stabilizing membranes and proteins and can also act as antioxidant. Other protective compounds include LEA proteins (HUNDERTMARK et al., 2011; CHATELAIN et al., 2012), heat shock proteins (PRIETO-DAPENA et al., 2006) and a set of antioxidant defenses against oxidative stress such as glutathione, tocopherols and flavonoids present in the testa (DEBEAUJON et al., 2000; SANO et al., 2015 ). Seed longevity has also been shown to be associated with DNA and protein repair systems such as DNA ligase (WATERWORTH et al., 2010), protein L-isoaspartyl methyltransferase (OGÉ et al., 2008) and methionine sulfoxide reductases (MSRs; CHATELAIN et al., 2013).



### **I.3.4 Acquisiton of physiological maturity**

We mentioned above that seed harvesting must occur when the seed potential is the highest, that which corresponds to the so-called physiological maturity (maximum germinatibility, longevity and vigor). Several works have focused on determining the criteria that define physiological maturity using a range of physiological assays (percentages of germination and desiccation tolerance, seedling growth, various parameters of seed longevity), technological assays (accelerated aging, controlled deterioration, percentage of abnormal seedling) and biochemical assays (electrolyte leakage, chlorophyll content). In soybean, seed maturity was first associated with leaf abscission, and leaf and pod colors. Willard (1925) related the maturity point to yellowing of soybean leaves, changes in pod color, and to a leaf abscission rate of approximately 50%. According to Marcos Filho (2005) the seed physiological maturity was proposed for the first time by Shaw and Loomis (1949), using as criterion the maximum dry weight. This stage was also called “relative maturity” by Aldrich (1943) and “morphological maturity” by Anderson (1955), all of them had as criterion the maximum dry weight (MARCOS FILHO, 2005). However, data from other experiments showed that physiological maturity does not always correspond to maximum dry weight. Oat, beans, rice, and soybean seeds acquired high germination and vigor after maximum dry mass was reached, suggesting that biochemical changes promoting metabolic adjustments after the end of reserve deposition are still happening and can influence seed quality (MARCOS FILHO, 2005). Ellis and Pietra Filho (1992) showed that the maximum physiological potential occurs after physiological maturity and proposed the term “mass maturity” to denominate the point of maximum dry mass, discriminating it from the point of maximum vigor. Zanakis et al. (1994) observed also in soybean seeds that the maximum dry mass was reached 45 DAF (Figure 7a), when the seeds are intolerant to desiccation, and the desiccation tolerance is acquired between 45 and 55 DAF, much later than mass maturity. Also, seed vigor, assessed by the  $K_i$  of the viability equation that is used to assess seed longevity during storage increases after mass maturity (Figure 7b). Increase in seed quality, after the reach maximum dry weight, has been confirmed in other studies (ELLIS et al., 1987; ELLIS et al., 1993; PROBERT, 2007; CHATELAIN et al., 2012)

The quality of soybean seeds is controlled by genetic factors. An example is the difference of soybean genotypes for resistance to mechanical damage which



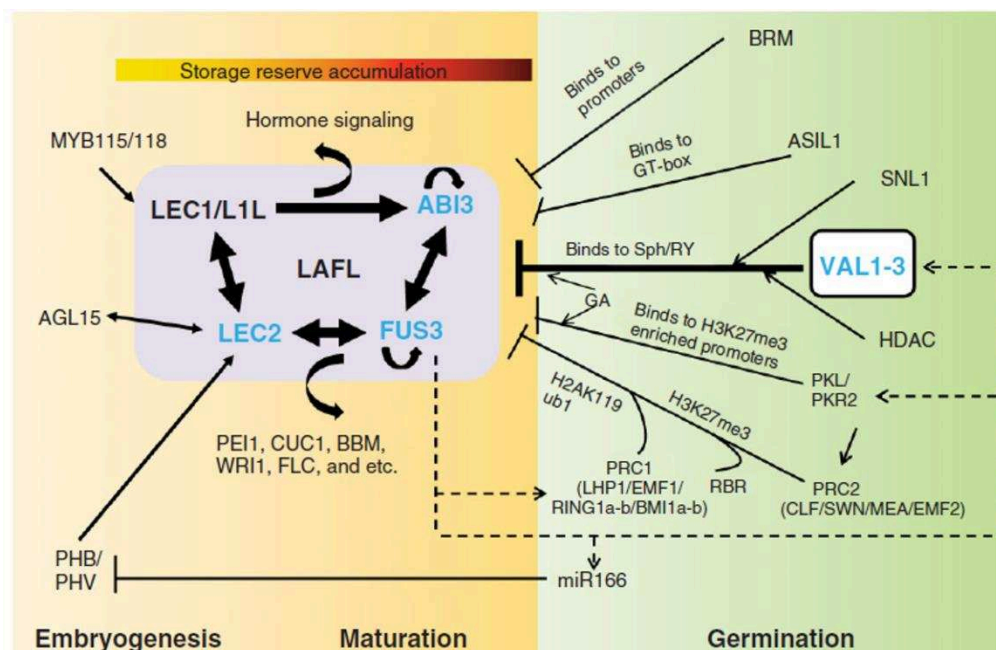
has been related to the higher lignin content in the seed coat (CARBONELL and KRZYZANOWSKI, 1995, CAPELETI et al., 2005). Potential deterioration in the field has also been related to the degree of permeability of the integument (SOUZA and MARCOS FILHO, 2001). The genotype can also influence the intensity of the deterioration process during field weathering.

Poor germination and reduced seed longevity may be associated with response to heat stress during development. Through the proteomic analysis, Ren et al. (2009) were able to identify 20 proteins whose accumulations were changed due to high temperature. The authors stressed that high temperature during seed development results in changes in the vigor and longevity and changes in seed protein expression profiles. The occurrence of green seeds at the end of the maturation process has been reported as another issue for Brazilian soybean growers. The green seed is a problem, because it reduces seed quality and oil quality (GOMES et al., 2003; ZORATTO et al., 2009, PÁDUA et al., 2009; TEIXEIRA et al., 2016). According to Teixeira et al. (2016) the chlorophyll retention is generally associated with pronounced increase in temperature, which leads to a rapid decrease in water content and impairment of the natural degreening.

### **I.3.5 Seed quality is affected by the position of the mother plant**

Seed attributes (weight, shape) and physiological quality appear to be related to the pod position on the mother plant and its position within the pod. Illipronti et al. (2000) analyzed the relationships between the time of pod set and seed position on the mother plant, as stem type (main stem, primary branch or secondary branch), main stem section, branch section and pod section in which of the seeds were derived. It was found that the seeds from the main stem were heavier and larger than seeds from branches. However, there were no difference in seed viability from different main stem sections or different pod sections. In contrast, Prasad et al (2000) observed differences in seed weight but also in the percentages of seed germination, being higher on the main stem. The susceptibility to deterioration during the storage might also be related to the seed position on the plant (SHARMA et al., 2013). Ghassemi-Golezani et al. (2012) stated that soybean seeds originated in the pods at the top of the stem exhibits higher quality, compared with those from the middle and lower part of the plant.





**Figure 8.** LAFL and VAL networks regulate the seed to seedling phase transition. Spatial and temporal patterns of LAFL gene expression are refined by mutual interactions, according to Jia et al. (2014). Important direct targets of LAFL factors include (1) SSP and LEA genes, (2) transcription factor genes that control seed specific processes including PEI1, CUC1, BBM, WRI1 and FLC, and (3) genes that function in major hormone metabolism and signaling pathways. AGL15, PHB/PHV and MYB are proposed to act upstream of LAFL network. The LAFL network is repressed by VAL B3 factors and other repressors during seed germination to enable the transition to seedling development. Black lines with arrows indicate activation, and black lines ending with bars indicate repression. Inferred functions with less experimental evidence are indicated by dashed lines.

**Table 2.** Main phenotypes of mutants in the master regulators of seed maturation. Adapted from To et al. (2006) and Gutierrez et al. (2007).

Phenotype	Gene				
	Wild Type	LEC1	LEC2	FUSCA3	ABI3
Chlorophyll retention in dry seeds	No	Yes	Yes	No	Yes
Expression of storage protein	Normal	Reduced	Reduced	Reduced	Reduced
Oil content	Normal	Reduced	Reduced	Reduced	Reduced
Trichomes	No	Yes	Yes	Yes	No
Desiccation tolerance	Yes	No	Yes	Partial	No

### **I.3.6 Regulation of seed maturation programs**

The transition between the different phase of seed development is coordinated by specific interactions between growth regulators (mainly ABA) and alteration in sucrose/hexose balance that is imported into the developing seeds. (VICENTE-CARBAJOSA et al., 1998; SANTOS-MENDOZA et al., 2005; GUTIERREZ et al., 2007). Seed maturation program in Arabidopsis is dependent on a transcriptional network referred to as LAFL developmental network, according to figure 8 (JIA et al., 2014). This transcriptional network is composed by several master regulators: three members of the B3 family of transcription factors, LEAFY COTYLEDON (LEC2), ABSCISIC ACID-INSENSITIVE 3 (ABI3) and FUSCA 3 (FUS3), and a fourth regulator, (LEC1) a HAP3 subunit of the CCAAT-box binding transcription factor (CBF). The role and importance of these regulators have been discovered based on the severe defects in various maturation programs as in the table 2.

There is a spatial and temporal pattern for expression of LEC1, LEC2, ABI3 and FUS3 during seed development, and their functions overlap in the general control of seed maturation (LE et al., 2010).

Other studies have shown that ABI3, FUS3 and LEC2 can regulate the expression of an oleosin gene (oleosin gene) and the accumulation of lipids (CROWE et al., 2000; SANTOS-MENDOZA et al., 2005). The loss or inactivation of ABI3 function changes the accumulation of reserve compounds and leads to loss of desiccation tolerance, dormancy, ABA sensitivity to germination and chlorophyll degradation (VICENTE-CARBAJOSA and CARBONERO, 2005). The control and accumulation of oil in seeds is also related to the master regulator LEC2 by its regulating activity on WRINKLED 1 (WRI1) (BAUD et al., 2007), which protein product targets fatty acid synthesis genes (BAUD et al., 2002; MAEO et al., 2009). FUS3 is also related to flavonoid biosynthesis, as it represses the expression of TRANSPARENT TESTA GLABRA1 (TTG1) in the protoderm (TSUCHIYA et al., 2004, LEPINIEC et al., 2006).

ABA prevent premature germination of immature seeds before the end of development (ACKERSON, 1984; HSING et al., 1995; MISRA and BEWLEY, 1985; ROSENBERG and RINNE, 1986; TEKRONY et al., 1979). In soybean seeds, as in other crops, there is a clear relationship between the embryo ABA content and the ability of immature embryos to germinate. During the normal course of embryogenesis, ABA



content remains high and thus, precocious germination also do not occur. The maximum level of ABA in soybean seeds occurs between 18 and 21 days after flowering (DAF) (QUEBEDEAUX et al., 1976; ACKERSON, 1984). In developing alfalfa seeds, Xu et al. (1991) noticed that the capacity of embryos and seeds to germinate increased at the same time that content of ABA declined, and reaches maximum values shortly before physiological maturity, fluctuating only between 97% and 100%; however, DT in soybean seeds was not attained until 65 - 70 DAF.

Both the abscisic acid (ABA) as gibberellic acid (GA) are important regulators of germination and dormancy in seeds. Gibberellins are known as growth hormones to act on the extensibility of the cell wall, the enzyme activity and the change in osmotic potential interfering therefore directly on germination, mobilization of sugars and breaking of dormancy (KARSSSEN, 1995).

In barley, it was noted that the ABA blocks the signal cascade activated by GA, before the formation of GAMyb transcription factor through the kinase protein PKABA1, which is induced by ABA (ZENTELLA et al., 2002). As a consequence this inhibiting, the gene transcription of hydrolytic enzymes does not occur and paralyzes the degradation. The balance between the levels of these hormones and their signaling pathways are important (FINKELSTEIN et al., 2008). Finch-Savage et al. (2007) observed that genes involved in GA synthesis, as GA3ox1, have increased expression to break dormancy and germination, while the genes involved in ABA synthesis have decreased expression as NCED4; NCED6; NCED9.

ABA plays also an important role in desiccation tolerance (Table 2). Three different loci for ABA insensitivity, designated *abi1*, *abi2*, and *abi3*, have been described. The *abi1* and *abi2* mutants suffer from water stress in the vegetative stage, whereas the *abi3* mutation specifically affects seed development (KOORNNEEF et al., 1984). The *abi3* mutant seeds exhibit reduced LEA contents and reduced chromatin condensation. In addition, the fact that chlorophyll is retained in dry seeds of *abi3* mutants might be interpreted as a symptom of lack of coordination to metabolic reduction during drying. According to review by Sano et al. (2016), the ABA, mediated by ABI3, regulates pathways in desiccation tolerance and seed longevity. In *Arabidopsis* seed, a central role of seed longevity signaling has been attributed to downstream ABI3 through the expression of heat shock transcription factor A9 (HSFA9; KOTAK et al., 2007

**Table 3.** Chemical characteristic of the soil at 0-0.20 m depth before sowing in 2012 (A) and 2013 (B).

	pH (CaCl <sub>2</sub> ) <sup>†</sup>	SOM <sup>‡</sup> g.dm <sup>-3</sup>	P <sub>resin</sub> mg.dm <sup>-3</sup>	K <sup>+</sup> -----	Ca <sup>2+</sup> mmol <sub>c</sub> .dm <sup>-3</sup>	Mg <sup>2+</sup> -----	H+Al <sup>§</sup> -----	CEC <sup>¶</sup>	BS <sup>#</sup> %
A	5,8	30	41	1,3	38	20	32	91	65
B	5,2	20	31	2,5	42	19	37	100	63

<sup>†</sup> Soil pH measured in calcium chloride solution.

<sup>‡</sup> Soil organic matter.

<sup>§</sup> Potential acidity.

<sup>¶</sup> Cation exchange capacity.

<sup>#</sup> Base saturation.

## CHAPTER II. MATERIAL AND METHODS

### II.1 Plant materials, growth conditions and sampling points

#### II.1.1 Soybean

The study was performed at the São Paulo State University - College of Agricultural Science, UNESP - FCA (Botucatu, SP – Brazil). The soybean cultivar used in this study was BRS 284, fitting to early maturity group with cycle of 120-126 days. The experiment was performed in 2 consecutive years, 2012/13 and 2013/14. The experimental area was located at latitude 22°50' S and longitude 48°25' W, and altitude of 750 m. The climate is a humid subtropical with dry winters and a well-defined dry season between the months of May and September. Mean rainfall averages is about of 1500 mm, while the highest monthly mean temperature is over 23°C and the lowest is below 18°C (CEPAGRI). The soil in the experimental area is classified as a Nitossolo Vermelho (EMBRAPA, 2006). It was collected soil samples before soybean sowing in the years of 2012 and 2013, to evaluate fertility according to Raij et al. (1997).

The chemical characteristics of the soil in the experimental area are presented in table 3. According to the results of soil chemical analyses and recommendations for soybean crop (RAIJ et al., 1997), it was applied 250 kg ha<sup>-1</sup> of the formulate 02-20-20 (N-P-K) just below and to the side of the seeds, to provide 5 kg ha<sup>-1</sup> of N, 22 kg ha<sup>-1</sup> of P and 42 kg ha<sup>-1</sup> of K.

Seeds were treated with fungicide Carboxin and Thiran (Vitavax-Thiran 200 SC) at a dose of 2.5 mL kg<sup>-1</sup> of commercial product (c.p) per seeds and insecticide Thiametoxan (Cruiser 700 WS), at a dose of 2,0 mL kg<sup>-1</sup> of c.p. per seeds. At the day of sowing, the seeds were inoculated with the bacteria Bradyrhizobium, using the c.p. Biomax-Biosoja, at a dose of 2 mL kg<sup>-1</sup> of seeds. The seeds were sown on December 4th 2012 and December 6th 2013 respectively. There were used 0.45 m between rows and was maintained a population of 17 plants m<sup>-1</sup>. All the phytosanitary controls were performed according to EMBRAPA (2011). The BRS284 genotype used in this experiment was selected because it is recommended for the climatic conditions of the Botucatu region.

**Table 4.** Characterization of the phenological stages of soybean plants at the main stem proposed by Fehr and Caviness (1977) and Ritchie et al. (1982).

<b>Reproductive Stages</b>	<b>Nomination</b>	<b>Description</b>
R1	Beginning flowering	One open flower at any node.
R2	Full flowering	Open flower at one of the two uppermost nodes on the main stem with a fully developed leaf
R3	Beginning pod	Pod with 5mm long at one of the four uppermost nodes on the main stem with a fully developed leaf
R4	Full pod	Pod with 2cm long at one of the four uppermost nodes on the main stem with a fully developed leaf
R5	Beginning seed	Seed with 3 mm long in the pod at the for uppermost nodes of the main stem with a fully developed leaf
R5.1	~ 10% filling	Seeds ~ 5mm long in the pod (perceptible to touch)
R5.2	~ 11 - 25% filling	Seeds ~ 7mm long in the pod
R5.3	~ 26 - 50% filling	Seeds ~ 8mm long in the pod
R5.4	~ 51 - 75% filling	Seeds ~ 10mm long in the pod
R5.5	~ 76 - 100% filling	Seeds ~ 11mm long in the pod
R6	Full seed	Pod containing a green seed that fills the pod cavity at one of the four uppermost nodes on the main stem with a fully developed leaf (plant with green leaves)
R7	Begin of maturation	One normal pod on the main stem that has reached its mature pod color
R7.1		~ 50% yellowing of leaves and pods
R7.2		~ 51 and 75 leaves and yellow pods
R7.3		~ 75 pods and yellow leaves
R8	Full maturation	~ 95% of the pods have reached their mature pod color
R8.1		Beginning at 50% defoliation
R8.2		More than 50% defoliation at harvest
R 9	Harvest maturity	Dry seeds and pods

Flowers in the main stem were tagged. During development, pods were manually removed from more than 200 plants at each stage. Three seeds were extracted and sorted into homogeneous lots based a phenological scale (Table 4) and days after flowering (Table 5).

### **II.1.2 Medicago**

Plants of *Medicago truncatula* were grown in a sterile mix of vermiculite and soil in a growth chamber at 23°C with a 16 hours photoperiod at 200  $\mu\text{mol m}^{-2} \text{s}^{-2}$  light intensity and 60–70% relative humidity. The development was well monitored; flowers were tagged and developing seeds were removed from the pods at 28 days after pollination and at final abscission, corresponding to mature dry seeds. Immature seeds were rapidly dried according to Chatelain et al (2012).

### **II.2 Fresh weight (FW) and dry weight (DW)**

The fresh and dry weights of the seeds were evaluated by using precision analytical balance. Fresh weight and subsequent dry weight were determined in an oven at 60° C for 72 hours or more until constant weight. Four replications of 20 seed each were used and the results were expressed in mg per seed.

### **II.3 Water content determination**

Water content was determined by oven drying at  $105 \pm 3^\circ \text{C}$  for 24 hours (BRASIL, 2009), with four replicates of 20 seeds. The water content of the seeds was expressed as gram of water per gram of dry weight (g H<sub>2</sub>O per g DW).

### **II.4 Seed quality assesment**

Physiological quality of seeds was assessed as described below:

**Germination** – germination test was evaluated by using four replicates of 25 soybean seeds harvested in different reproductive stages (Table 4), using paper rolls, imbibed in water at 25°C in the dark. The immature dry seeds at the stage R7.2 and mature



**Table 5.** Days after flowering (DAF) at different reproductive stages of soybean seed cultivated in the 2013/2014.

Reproductive stages (R)	Days after flowering (DAF)
R5.1	25
R5.2	25
R5.3	27
R5.4	27
R5.5	36
R6	47
R7.1	57
R7.2	63
R7.3	69
R8.1	71
R8.2	73
R9	77

seeds at stage R9 were also evaluated by using paper rolls, imbibed in water at 15°C or imbibed in salt solution (75 mM or 100 mM NaCl) at 25°C, both condition in dark. Or imbibed in water but in light condition (200  $\mu\text{mol m}^{-2} \text{s}^{-2}$ ). For *Medicago truncatula*, three replicates of 50 seeds of each stage were imbibed in water at 20°C in the dark; in cold condition at 10°C in the dark and in salt condition. For both seeds, soybean and *Medicago*, the germination percentage was scored daily by counting radicle protrusion, when the radicle has more than 2 mm length (BEWLEY and BLACK, 1994).

**Normal seedlings** – normal seedling formation was scored after eight days of imbibition. The seedlings were considered normal when presented all the essential structures to ensure the development of a plant. In other words presence of primary and secondary root well developed, hypocotyl, cotyledon, epicotyl and plumule healthy (BRASIL, 2009).

**Desiccation tolerance** - desiccation tolerance (DT) was evaluated by scoring germination (radicle protrusion and normal and abnormal seedlings) after rapid drying of the seeds over an airflow of 42% relative humidity (RH), generated by a saturated salt solution of  $\text{K}_2\text{CO}_3$ , according to Chatelain et al. (2012). The seeds were dried to approximately 0,10 g  $\text{H}_2\text{O}$  per gram dry weight; independently of the reproductive stage. For this, the drying time varied from 24 to 72 hours. Seeds were considered desiccation tolerant when they germinated.

**Hypocotyl length** – the length of 10 hypocotyls was measured after 48 hours of imbibition in water, at 25°C in the dark or in the light.

**Hypocotyl dry weight** – hypocotyls were placed to dry in an oven with air circulation at 80°C for 36 hours.

**Longevity** – Immature dried seeds at stage R7.2 and mature seeds at stage R9 were kept over a saturated solution of NaCl (75% RH) at 35°C in hermetically sealed plastic boxes (CHATELAIN et al., 2012). At different times during storage, seeds were imbibed as described above and germination was recorded. Longevity was expressed as P50, defined as the time (days) for which the seeds lose 50% of its viability during storage.

**Table 6.** Reproductive stages of soybean seeds used in the transcriptome analysis.

<b>Reproductive stages</b>		<b>Hours after imbibition</b>
Immature seeds	R7.2 fresh	0h
	R7.2 dried	0h
	R7.2 dried	18h
Mature seeds	R9	0h
	R9	18h

## **II.5 Abscisic acid (ABA) quantification**

Quantification of abscisic acid (ABA) biosynthesis was performed using High Performance Liquid Chromatography (HPLC). Three replications of 15 embryos (cotyledons and embryonic axis) of fresh soybean seeds were lyophilized for 72h. They were frozen in liquid nitrogen and ground to a powder. ABA extraction was done as proposed by Ma et al. (2013), using 150 mg of embryos for each repetition. Extraction solution were composed of methanol, acetonitrile, Milli-Q water and acetic acid (v: v: v: v, 40: 40: 20: 1).

The chromatographic separation was performed on HPLC Shimadzu model Prominence comprised of degasser mobile phase DGU-20A, the quaternary pumping system comprised of pump LC-20AD, sampler model SIL-20ACHT, controller CBM-20A and oven model columns CTO-20AC. The column used was a Sinergi 2.5 Hydro RP-100A 50x4.6mm, maintained at 40°C during quantification. The column effluent was introduced into the mass spectrometer type triple quadrupole (MS/MS) brand AB Sciex model 4500 equipped with ionization source type ESI (electrospray) at the interface. The results were expressed in nanograms per seeds (ng per seed).

## **II.6 Statistical analysis for seed quality assessment**

An ANOVA test was performed on seed quality and gene expression data at the level of 5% ( $p \leq 0.05$ ). The averages were compared by T-test at 5% probability level.

## **II.7 Transcriptome analysis**

For the transcriptome studies, two reproductive stages of soybean seeds were selected and analyzed before and after 18 h of imbibition 9 (Table 6).



## **II.8 RNA extraction and sequencing (RNAseq)**

In the crop year 2013/2014, seeds were harvested from more than 200 plants at each stage and stored - 80°C in deep freeze. Then, the seeds were homogenized and frozen. Total RNA extraction was performed from two independent duplicates using the NucleoSpin® RNA Plant kit (Macherey-Nagel); using immature seeds (stage R7.2) and mature seeds at stage R9. RNA integrity was assessed by analysis on a 1% agarose gel and concentration was additionally determined by UV spectrophotometry (260 nm, NanoDrop 1000, Thermo Fischer Scientific). RNA integrity number (RIN) was measured using a Bioanalyzer (Agilent Technologies). Those samples exhibiting a RIN values ranging between 8.9–10.0 were used for further analysis.

High quality of total RNA samples were used for library preparation and mRNA sequencing at the Laboratório Central de Tecnologias de Alto Desempenho em Ciências da Vida (LaCTAD) in Campinas, SP – Brazil. cDNA libraries were generated using the TruSeq RNA sample preparation kit (Illumina) to obtain single-end 100-bp long RNA-seq. The libraries were subjected to enzymatic fragmentation by connecting terminal adapters to these fragments and deposited on a flowcell containing 3 lanes via robotic instrument cBOT and sequenced using a HiSeq 2500( Illumina). On the flowcell surface there are oligonucleotides that are complementary to the adapters of libraries. The flowcell was placed in a HiSeq2500 where occurred the incorporation of nucleotides containing fluorescently labeled dideoxy terminators on the fragments linked to the sequencing primer. Upon the occurrence of the incorporation of a nucleotide, the fluorescence was excited with a number of lasers and captured by cameras.

## **II.9 Bioinformatics analysis**

Transcriptome analysis was performed at the Research Institute of Horticulture and Seeds (IRHS, Institut de Recherche en Horticulture et Semence) in Angers – France. in three steps. The first step was a filtering process, The sequences obtained were filtered using parameters of purity provided by Illumina, for the removal of primers, vectors, adapters and long repeated sequences of bases that were not of interest or, can affect the analysis. Then, the alignment program Bowtie2 (LANGMEAD et al., 2009) was used to align reads to the 'Williams 82' soybean reference genome, assembly Glycine

**Table 7.** Primers sequences used as target and reference genes used in RT-qPCR measurements.

<b>Target genes</b>	<b>Primers</b>	
Class I KNOX homeobox transcription factor - Glyma.14G047000	Forward	CAACCTAGATTCTCAAGCTGAA
	Reverse	CTTCTTGAGGCTCCCTAGATA
WRKY transcription factor Glyma 03G224700	Forward	CTTTGGCAGCAGCAATCT
	Reverse	TGCGGTGAACCTGGAATA
ABI3L domain class transcription factor - Glyma 02G200500	Forward	GCCATACCATCACCAACAA
	Reverse	CGAACTCGAACTAGAACTGC
Heat stress transcription factor A3 Glyma 10G066100	Forward	AGCCATGTCTTCTGAAACTC
	Reverse	AAGGTCTTCTGCACCTACTA
Auxin response factor Glyma.14G217700	Forward	CAGGTTCTGTAGGAAGATCAAT
	Reverse	GTGTCATTCAGCAGACCAT
Proteasome subunit beta type Glyma.06G078500	Forward	TCACACAGCTCACTGATAATG
	Reverse	GAAGGAAGTAGCGCACATAG
40S ribosomal protein S20-2 Glyma.03G142300	Forward	CTGTGGTGAAGGTACCAA
	Reverse	CTCCACACCAGGTTCAAT
Polyadenylate-binding protein Glyma.14G084500	Forward	GACGCAGACGGTAAATCAA
	Reverse	TTCCAACATAACCACTCCTTATC
Importin beta-2 subunit family protein Glyma.20G106300	Forward	GATAATAAGCGGGTCCAAGAG
	Reverse	GTCATCAGGTGCTTCAGTATAA

max Wm82.a2.v1 (SCHMUTZ et al., 2010) as a reference ([www.phytozome.net/soybean](http://www.phytozome.net/soybean)). Estimation of differential gene expression was performed using DESeq2, v1.11.21 (LOVE et al., 2014) available as a R/ Bioconductor package (GENTLEMAN et al., 2004). This package is well adapted to perform differential analysis of count data from small replicate numbers and large dynamic range. Following the procedures described in the Deseq2 documentation, read count tables were loaded into R, normalized using the default method for Deseq2. (LOVE et al., 2014). Most significant genes were identified at 0.05 false discovery rate (FDR) calculated using p-value adjusted for multiple testing using Benjamini-Hochberg method. Relative expression data was calculated by dividing the mean gene expression value obtained in the immature stage (R7.2) by the value obtained in mature stage (R9). i.e., the immature stage was considered as the control for the calculation of expression relative to mature (treatment). The ratio of expression between both stages was considered through the logarithmic division ( $\text{Log}_2 [\text{R7.2} : \text{R9}]$ ).

## **II.10 Gene Ontology Analysis**

GO enrichment analysis of differentially expressed genes it was performed using Soybase GO-enrichment tool ([http://www.soybase.org/goslimgraphic\\_v2/dashboard.php](http://www.soybase.org/goslimgraphic_v2/dashboard.php), MORALES et al., 2013) using GO annotation terms from genome version Glyma 2.0. Over- and under-represented GO terms were identified by comparing the GO term enrichment within differentially expressed genes and the entire soybean genome using Fisher's exact test with a Bonferroni correction. Also performed the Gene ontology enrichment analysis using AgriGO (<http://bioinfo.cau.edu.cn/agriGO/analysis.php>, DU et al., 2010), using Arabidopsis thaliana genome annotation (TAIR 10) as genome model and data was corrected for multiple testing error (FDR). For both database the significant level of 0.05 ( $p\text{-value} \leq 0.05$ ) was used for filtering.

## **II.11 cDNA synthesis and quantitative RT-qPCR**

The RNA used for gene expression analysis by RT-qPCR was extracted from the soybean developing stages presented at table 7. Three replicates were used for RNA extraction as previously described. cDNA synthesis was performed by using the iScript™ Reverse Transcription Supermix for RT-qPCR according to the manufacturer's protocol (Bio-Rad).





For a reaction of 20  $\mu\text{L}$ , was used 4  $\mu\text{L}$  of iScript RT Supermix and 1  $\mu\text{g}$  RNA) and completing the volume with Nuclease-free water. The reactions were incubated in a thermocycler at 25°C for five minutes for the initial activation of the enzyme and at 42°C for 30 minutes for cDNA synthesis, followed by inactivation of the enzyme at 85°C for five minutes and concluding the cycle at constant temperature of 12°C. For the expression of candidate genes (Glyma.14G047000, Glyma.03G224700, Glyma.02G200500, Glyma.10G066100 and Glyma.14G217700) was used LuminoCt SYBR Green qPCR ReadyMix (Sigma Life Science), and the volume of the reactions in a total solution of 12.0  $\mu\text{L}$  corresponding to: 6  $\mu\text{L}$  (1x) of SYBRgreen; 0,25  $\mu\text{L}$  (10mM) of forward and 0,25  $\mu\text{L}$  (10mM) of reverse; 3  $\mu\text{L}$  (5ng/  $\mu\text{L}$ ) of cDNA and 2,5  $\mu\text{L}$  of water.

Primer design was performed by using the Software PrimerQuest Tool (<http://www.idtdna.com/Primerquest/Home/Index>). The primers used were designed in the 5'-3' end of the transcript. The  $T_m$  of the primers was between 54 and 58°C; amplicon of 75 - 100 base pairs (Table 7). Primer efficiency was calculated, after the RT-qPCR, through the LinRegPCR program (RUIJTER et al., 2013).

Amplification was performed in a thermocycler Eco Real-Time (Illumina) with consisted an initial step of incubation at 2 minutes at 50°C, 3 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 30 seconds at 60°C for 30 seconds. At the end of the reactions was performed a melting curve to assess specify which consisted the following steps: 15 seconds at 95°C, 55°C and 95°C respectively. The data were analyzed in EcoStudy program version 5.0 of Illumina. Ct values obtained from the Eco Real-Time software (Illumina) were analyzed in an excel sheet by the  $\Delta\Delta\text{Ct}$  method (LIVAK and SCHMITTGEN, 2001). RT-qPCR data of each gene of interest were normalized against the average of the three reference genes used. The gene expression data obtained was analyzed using the REST® program that performs the comparative quantification method of the "Pair-Wise Fixed Reallocation Randomization Test" (PFAFFL et al., 2002). Correlation between the expression values detected by RNA-Seq and qRT-PCR for the 5 genes tested in both stages, R7.2 and R9, with 0h and 18h of imbibition were analyzed by Pearson's correlation.

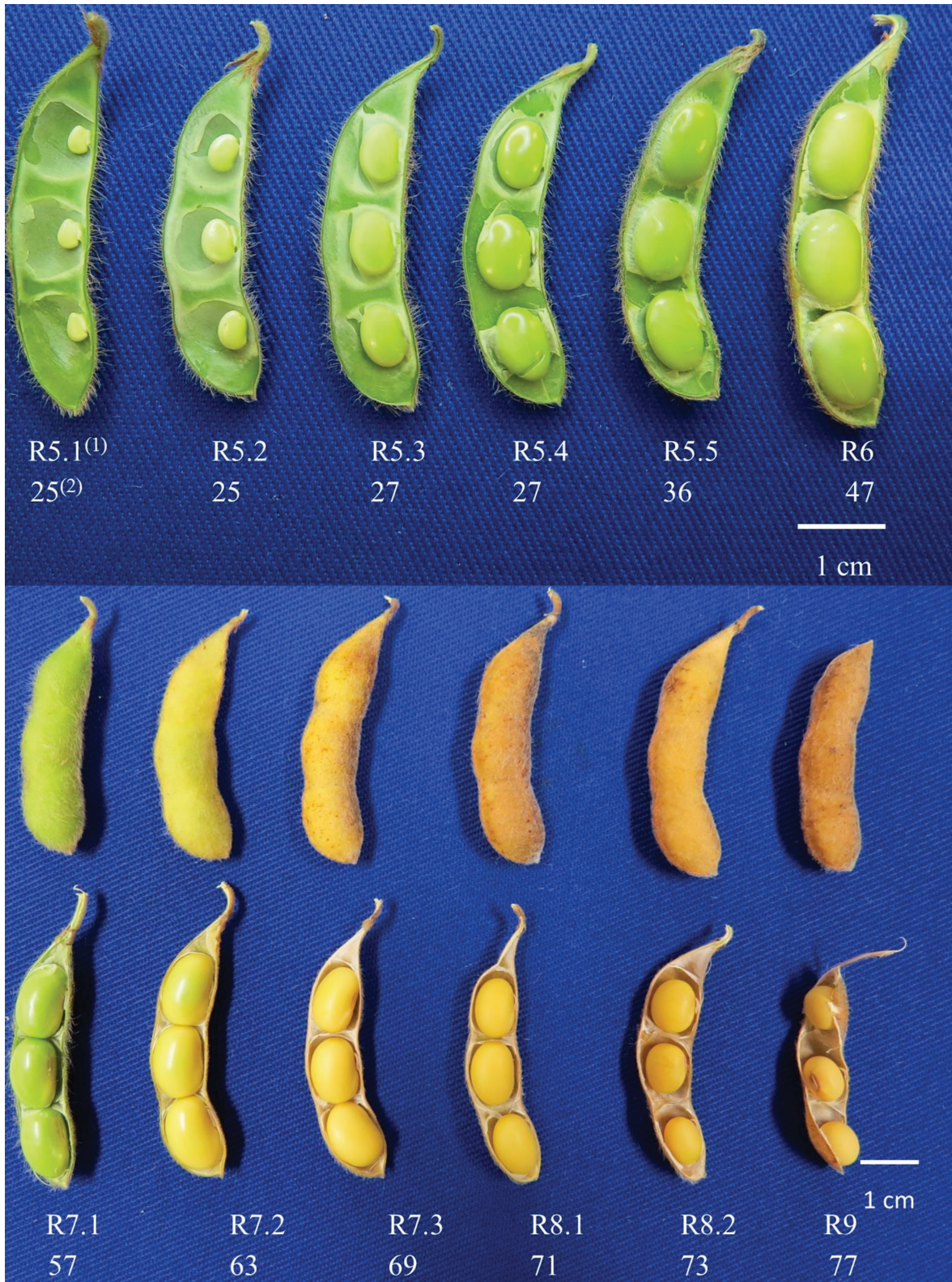


## **II.12 Medicago truncatula: RNA extraction microarray, data extraction and normalization**

For transcriptomic analysis of *Medicago truncatula* three replicates of 50 immature seeds fresh, immature seeds dried and mature seeds, before and after 6h and 24 of imbibition were rapidly frozen in liquid nitrogen and stored at -80°C prior to RNA isolation. Total RNA extraction was performed according to the NucleoSpin® RNA Plant kit (Macherey-Nagel); RNA integrity was assessed by analysis on a 1% agarose gel; quantified and evaluated for purity using the Bioanalyzer 2100 (Agilent).

Nimblegen Microarray, data extraction, normalization and transcriptomic data analysis of seed development during imbibition were performed according to the methodology described in Verdier et al (2013).

For the gene ontology enrichment analysis using AGRIGO (DU et al., 2010), using *Arabidopsis thaliana* genome annotation (TAIR 10) as genome model and data was corrected for multiple testing error (FDR). For both database the significant level of 0.05 ( $p\text{-value} \leq 0.05$ ) was used for filtering.



**Figure 9.** Development of soybean pods and seeds. The characterization of the reproductive stages based on phenological scale (FEHR and CAVINESS, 1977; RITCHIE et al., 1982). <sup>(1)</sup>Phenological scale; <sup>(2)</sup>Days after flowering.

## **CHAPTER III. RESULTS**

### **III.1 Characterization of acquisition of seed quality and abscisic acid quantification during soybean seed development**

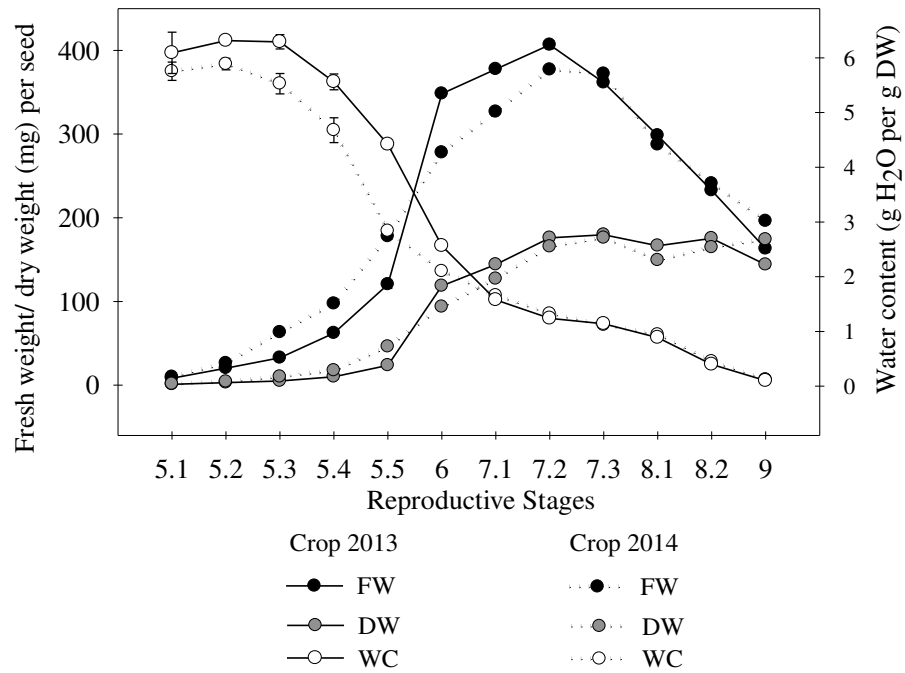
Physiological quality seed characteristics include the ability to germinate, tolerate desiccation and remain viable during storage. Ideally, the seed harvest should occur when all the characteristics reach their maximum levels. However, there is no consensus during the maturation process of soybean seeds about when this occurs. Thus, we analyzed the development phases considering the phenological scale and days after pollination, observing the morphology characteristics of the pods and seeds. We also analyzed the change in water content, fresh and dry weight during seed development. In addition, we performed physiological assessment (germination, desiccation tolerance, vigor and longevity) and ABA quantification.

#### **III.1.1 Characterization of seed maturation stage**

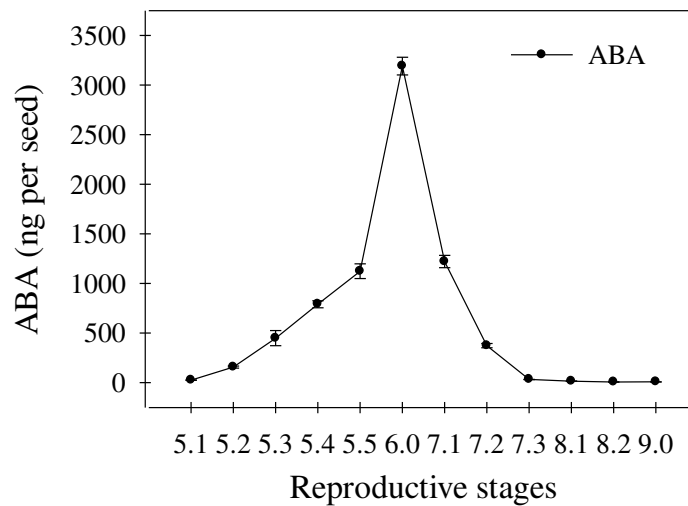
Soybean seed maturation was characterized using phenological stages presented in Figure 9, and by monitoring changes in seed weight and water content. Seed filling started at stage R5.2. Fresh and dry weights increased steadily onwards until stage R7.2. Thereafter, dry weight was stable, indicating the end of the filling phase.

Between R7.1 and R7.2, pod and developing seeds turned yellow as a result of chlorophyll degradation. However, a central part of the cotyledons remained green. Water content varied during the reproductive stages (Figure 10). Between stages R5.3 and R7.2, water content decreased steadily due to the replacement of the water by DW. At stage R7.2, the seed water content was  $1,32 \pm 0,02$  g H<sub>2</sub>O/g DW. Thereafter, it decreased steadily until stage R9, corresponding to the post-abscission stage where water content was 0,12. Therefore, the maturation drying was considered to be from stage R7.2 to R9 since DW did not change significantly.

Next, we determined changes in ABA contents during development. ABA levels increased from stage R5.1 until R6, corresponding to a maximum (Figure 11). Thereafter, it declined progressively. At stage R7.2, the amount was still about  $372,42 \pm 21,85$  (ng per seed) whereas it became undetectable at stage R7.3.



**Figure 10.** Characterization of the soybean seed development. Changes in water content (WC, g H<sub>2</sub>O/ g DW), in fresh weight (mg/seed, FW) and dry weight (DW, mg/seed) and water content (g H<sub>2</sub>O per g DW) at indicated reproductive stages. Data are the average of four replications using 20 seeds. Error bars indicate standard errors.



**Figure 11.** Changes in abscisic acid content during seed maturation at indicated reproductive stages. Data are expressed a seed basis and correspond to the average of three replications using 150 mg of embryos. Error bars indicate standard errors.



### **III.1.2 Acquisition of physiological quality during seed maturation**

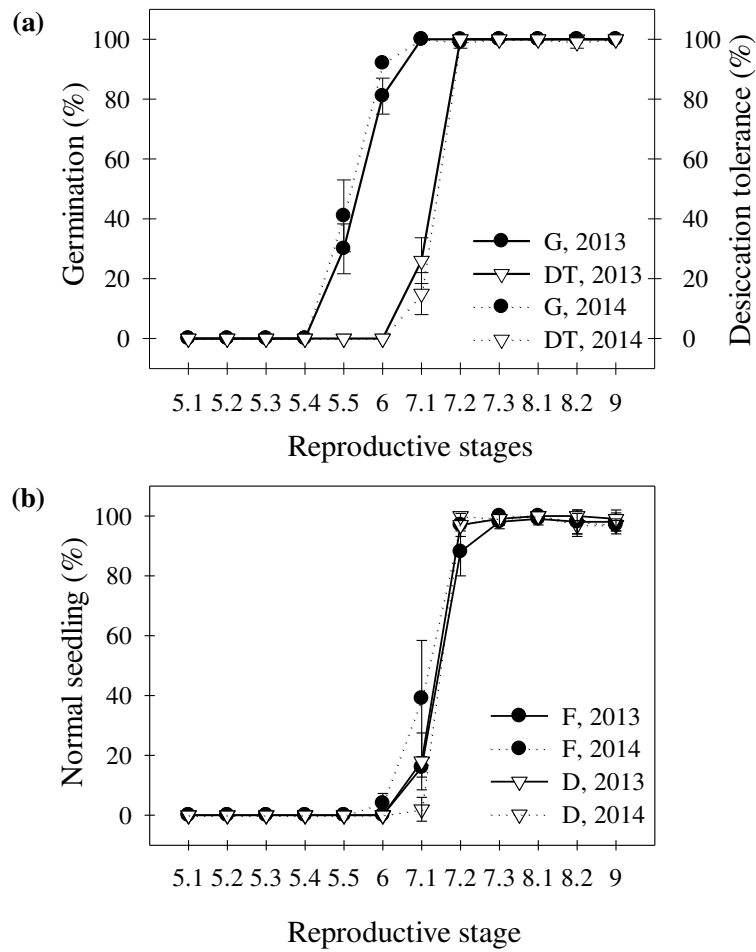
The capacity to germinate was progressively acquired during early seed filling, between stages R5.5 and R6 (Figure 12a). Desiccation tolerance (DT), defined as the ability to germinate after rapid drying to 0,10 g water/g DW was acquired between stage R6 and stage R7.2. Acquisition of normal seedling establishment occurred also between stages R7.1 and R7.2, concomitantly with DT (Figure 12b). No effect of drying was observed, indicating that physiological maturity was obtained at R7.2.

To further assess physiological maturity, we tested when germination in stressful conditions was acquired as a proxy for a vigor test. Two stressful conditions were applied: salt stress using 100 mM NaCl and cold (15°C). Germination and seedling emergence of seeds from stages R7.2 and R9 were tested, since these represent the extremes of maturity (Figure 13a). There was no difference in the germination speed or final percentage of germination and seedling emergence between stages R7.2 and R9 in these different conditions, suggesting that seed vigor is already acquired at stage R7.2. Next, we tested whether there existed difference in hypocotyl elongation between mature and immature stages. For this purpose, hypocotyl length and dry weigh were measured after 48h of imbibition in optimal conditions. Figure 13 (b) shows that no significant difference in both parameters was found between the different stages, thereby confirming that the capacity to establish a normal seedling is fully acquired at R7.2 in our laboratory conditions.

### **III.1.3 Characterization of longevity in immature (stage R7.2) and mature (stage R9) seeds**

During soybean seed maturation no physiological differences regarding germination were observed between immature and mature seeds. However, developing seeds need also to acquire longevity (i.e. the capacity to survive in the dry state for extended periods of time during storage, CHATELAIN et al., 2012). Here longevity was tested for seeds harvested and dried at stage R7.2 and for mature seeds R9. Storage conditions were 75% RH and 35°C which is supposed to induce a moderate ageing (CHATELAIN et al., 2012). During storage, seeds of stage 7.2 started to lose their viability





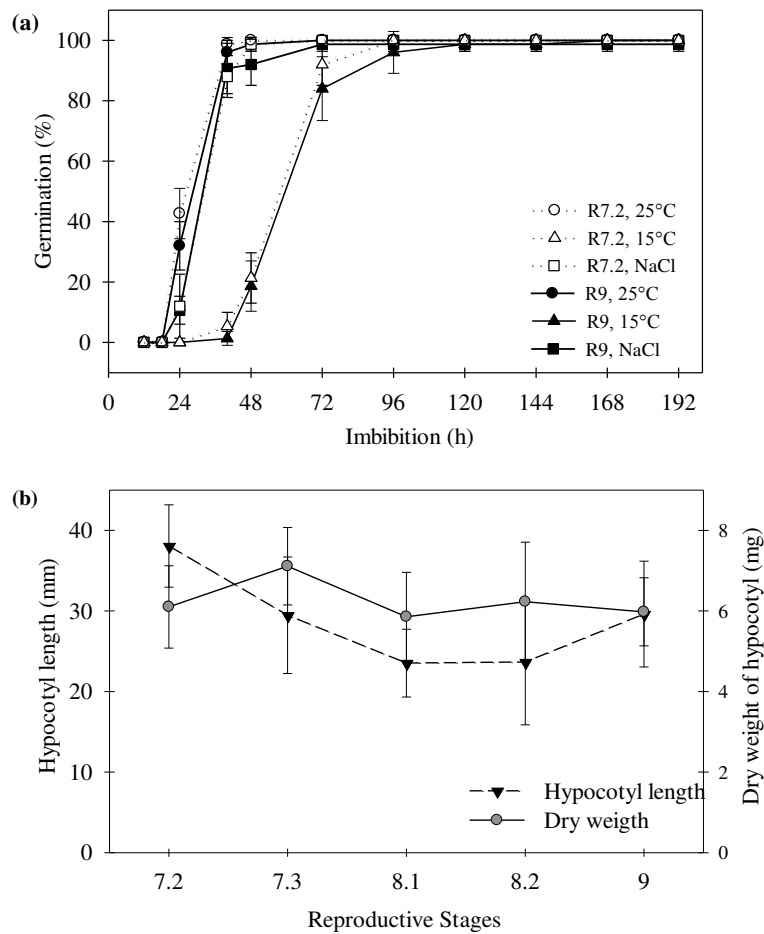
**Figure 12.** Acquisition of physiological quality during development. **(a)** Changes in percentage of germination (G) and desiccation tolerance (DT) of seeds harvested at different reproductive stages. **(b)** Changes in percentage of normal seedlings of seeds harvested at different reproductive stages before (F) and after an enforced rapid drying. Data points are average of four replications of 25 seeds. Error bars indicate standard errors.

after 21 days of storage (Figure 14). Half the population was dead after 27 days as calculated by the fitting curve. In contrast, it took 48 days to kill 50% of mature seeds.

### **III.2 Transcriptome analysis during soybean seed maturation**

Our physiological characterization shows that there is no phenotypic difference in germination, seedling emergence and seed vigor between stages R7.2 and R9, suggesting that physiological maturity in terms of seed emergence was reached at R7.2. Nevertheless, the developing seeds need to mature further to acquire full longevity and therefore its full agronomic potential. Seeds from stage R7.2 contains  $1,32 \pm 0,02$  g H<sub>2</sub>O/g DW. Thus the progress in seed maturation occurs during maturation drying. All of the physiological experiments were performed using immature seeds that were submitted to an artificial drying treatment and compared with R9. Thus we can hypothesize that during maturation drying, the seeds will accumulate all mRNAs that will be stored in the dry quiescent state and used during imbibition to ensure germination (RAJJOU, 2004). However, it is not known whether an artificial drying treatment of immature seeds at R7.2 that lasts for 2-3 days would induce a similar or entirely different profile of stored RNA compared to maturation drying that lasts around 14 days. Such information would be useful to have an idea of those mRNA that are necessary for germination and longevity. It will also give some insights into whether artificial drying is identical to a maturation drying. For an agricultural point of view, such information could be useful to provide putative molecular indicators to assess whether post-harvest drying during seed processing was sufficient to replace natural maturation drying.

To assess changes in transcriptome, a high throughput sequencing on total RNA was performed on two reproductive stages of soybean seeds, immature R7.2, fresh and dried (enforced fast drying), and stage R9 (in planta drying), with two biological replicates per stage. Illumina RNAseq produced between 14 and 66 million reads per library, all libraries having close to or above 90% mapped reads (Table 8). Mapped reads were aligned to 88.647 soybean gene models determined by the Soybean Genome Project available on Phytozome, using the program Bowtie 2 and R packages. Annotation was performed using the Soybase database (<http://www.soybase.org/genomeannotation/>) and lead to the identification of 56.044 transcripts. After annotation, differentially expressed genes (DEG) were considered significant when the BH value was < 0.05.



**Figure 13.** Seed vigor is already acquired at stage R7.2. (a) Germination of immature (stage R7.2) dried and mature (stage R9) seeds in optimal (25°C) and stressful conditions (15°C or 100mM NaCl). Data points are average of four replicates of 25 seeds; error bars indicate standard deviation. (b) Hypocotyl length and dry weight of 48 h imbibed seedlings obtained at indicated reproductive stages. Data are the average of 10 hypocotyls. Error bars indicate standard deviation.

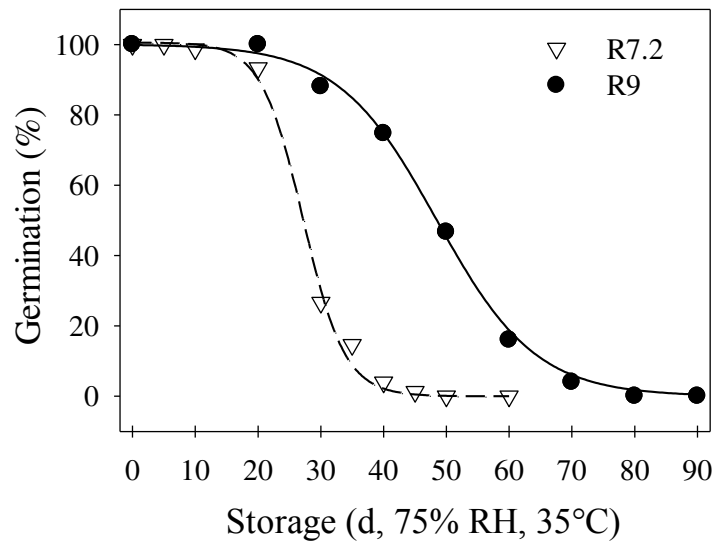
### III.2.1 Transcripts associated with seed longevity

Considering that seed longevity doubled in dried seeds between R7.2 and R9 stages, we first considered DEG genes associated with this process. Table 9 shows there were only 742 DEG transcript being up-regulated at least 2-fold whereas 1525 were down-regulated.

Next, a GO enrichment analysis of the top 500 DEG was performed. Results are shown in Table 10 and revealed a significant over-representation of terms associated with the synthesis of HSP (response to heat, protein folding, response to ER stress; Table 10).

A high number of categories associated with response to light were also over-represented such as “response to high light intensity”, “far-red light photoreceptor activity”, “response to very low fluence”, “red light stimulus”, “photoreceptor activity” (Table 10). A closer inspection of genes belonging to these categories revealed the presence of phytochrome A and PIF homologues (PIF7, Photoreceptor interacting factors), suggesting a role for red light as an environmental cue during seed maturation. Thus we hypothesized that light might play a role in germination. To test this, we imbibed seeds in complete darkness and in the light at 30°C. The speed and percentage of germination were monitored (Figure 15) together with the root and hypocotyl length after during imbibition (Table 11). Regardless of the light conditions, both immature and mature seeds germinated at the same time and to the same level. No significant differences in the root and hypocotyl lengths were observed between R7.2 and R9, suggesting that the skotomorphogenesis and de-etiolation processes were similar between stage R7.2 and R9 (Table 11).

The main GO categories that were over-represented in the top 500 DEG with transcript levels that decreased between stage R7.2 and stage R9 were associated with photosynthesis (Table 10, Supplementary Table 2) suggesting that photosynthesis would be detrimental for longevity. Some transcripts corresponded to homologues encoding chloroplastic genes such as proteins D1 (psbA), D2 (psbD), A1 (psaA) and A2 (psaB), subunits of photosystem II and I.



**Figure 14.** Changes in germination percentages of seeds at stages R7.2 and R9 during storage at 35°C and 75% relative humidity (RH). Third-order sigmoid curves were fitted to the data to obtain the P<sub>50</sub> values, corresponding to the time of storage (days) at which the dried seed lot reached 50% germination.

**Table 8.** Mapping of single-end reads of developing seeds to the soybean genome. # reads: number of reads following trimming of the libraries for quality; # mapped reads number of reads that unambiguously mapped to the soybean genome; # unmapped number of reads that could not be mapped to a single location in the soybean genome. Data of two replicates (rep1 and rep2), in freshly harvested seeds (F) or rapidly dried seeds (d), at 0h and 18h of imbibition, for seeds harvested at the stage R7.2 and R9.

Stage/Replicates	# reads	# Mapped reads	% Mapped reads	#Unmapped reads	%Unmapped reads
R7.2 F rep 1	38 088 839	34 971 702	91,8	3 117 137	8,2
R7.2 F rep 2	34 381 008	31 799 644	92,5	2 581 364	7,5
R7.2 d 0h rep 1	14 259 082	12 881 662	90,3	1 377 420	9,7
R7.2 d 0h rep 2	66 764 127	60 626 937	90,8	6 137 190	9,2
R7.2 d 18h rep 1	23 345 963	21 399 094	91,7	1 946 869	8,3
R7.2 d 18h rep 2	28 353 184	26 028 156	91,8	2 325 028	8,2
R9 0h rep 1	28 824 583	26 457 357	91,8	2 367 226	8,2
R9 0h rep 2	23 064 604	21 097 336	91,5	1 967 268	8,5
R9 18h rep 1	25 774 186	23 183 699	89,9	2 590 487	10,1
R9 18h rep 2	33 725 757	30 287 347	89,8	3 438 410	10,2

#### **IV.2.1 Impact of enforced premature drying on differentially expressed genes**

To investigate whether premature drying induced changes in the transcriptome, we first compared the transcriptome response of immature dried seeds (stage R7.2d) with that of mature seeds (stage R9), so-called “natural drying” with that of the fresh immature seeds fresh (stage R7.2f) vs artificially dried seeds (R 7.2d), so called “enforced drying” (stage R9; Table 12). First, we compared the number of DEG ranked according to the level of expression. Here, a large number of DEG was found to be up and down-regulated with approximately the same intensity (Table 13). Using these values, we calculated from each intensity categories the ratio between the numbers of DEG being up-regulated and that of DEG being down-regulated for both artificial and enforced drying. Results are presented in Figure 16.

While the ratio were around 1 for immature seeds that were dried artificially, the values were much lower for naturally dried seeds, particularly for those transcripts that were highly abundant (Figure 16). This suggests that RNA metabolism is not identical during drying, with the natural drying favoring a degradation of RNA.

Venn diagrams were constructed for genes that were differentially up-regulated or down-regulated) between enforced drying and natural drying (Figure 17). For the up-regulated DEG, there was more or less an equal distribution between transcripts that were common to natural and artificial drying (39% of both transcriptomes) and those specific to each drying treatment. A total of 42% of DEG were specific to artificial drying of immature seeds whereas 46% were specific to natural drying. However, for the down-regulated genes, there was a strong overlap between both transcriptome, with only 16 % being specific to artificial drying. Also, there was a large number of DEG that specifically decreased during natural drying representing 57% of the transcriptome. This confirms the idea that there is an increased degradation of transcripts during natural drying.

To gain further knowledge of genes that were specifically up- and down-regulated by natural and artificial drying treatments, a GO enrichment analysis was performed on each category. The transcriptome containing the 717 transcripts whose level was specifically increased during enforced drying of immature seeds were associated a significant over-representation of terms associated with photosynthesis, such as

**Table 9.** Number of differential transcripts between immature dried seeds ranked according to the level of expression calculated on a Log<sub>2</sub> ratio.

Comparison	# Log <sub>2</sub> (ratio) transcripts							
	> 1	> 2	> 3	> 4	< -1	< - 2	< - 3	< - 4
R9 /R7.2d	742	60	9	1	1525	272	20	0

**Table 9.** Gene Ontology (GO) enrichment analysis of transcripts corresponding to DEG up-regulated between R7.2d and R9. Analysis was performed on the top 500 genes using SoyBase, with annotations for Wm82.a2.v1 (Glyma 2.0). #Genome: Genome Count present in this GO category; # Expressed: Number of submitted genes present in this GO category.

GO_id	#Genome	#Expressed	p-value	GO_description
GO:0009408	659	51	2,57E-18	response to heat
GO:0006457	779	49	6,79E-14	protein folding
GO:0042542	511	42	8,43E-16	response to hydrogen peroxide
GO:0009644	582	41	4,61E-13	response to high light intensity
GO:0034976	483	30	7,19E-08	response to endoplasmic reticulum stress
GO:0009220	315	22	2,49E-06	pyrimidine ribonucleotide biosynthetic process
GO:0006334	128	14	6,22E-06	nucleosome assembly
GO:0006626	235	14	9,85E-03	protein targeting to mitochondrion
GO:0031516	6	5	5,70E-07	far-red light photoreceptor activity
GO:0009883	6	5	5,70E-07	red or far-red light photoreceptor activity
GO:0008020	8	5	5,20E-06	G-protein coupled photoreceptor activity
GO:0010161	23	5	1,28E-02	red light signaling pathway
GO:0004673	41	5	4,90E-02	protein histidine kinase activity
GO:0010203	4	3	8,42E-03	response to very low fluence red light stimulus
GO:0009881	6	3	1,14E-02	photoreceptor activity

“photosynthesis”, “photosynthetic electron transport chain”, “chlorophyll binding”, “photosystem II assembly”(Table 14).

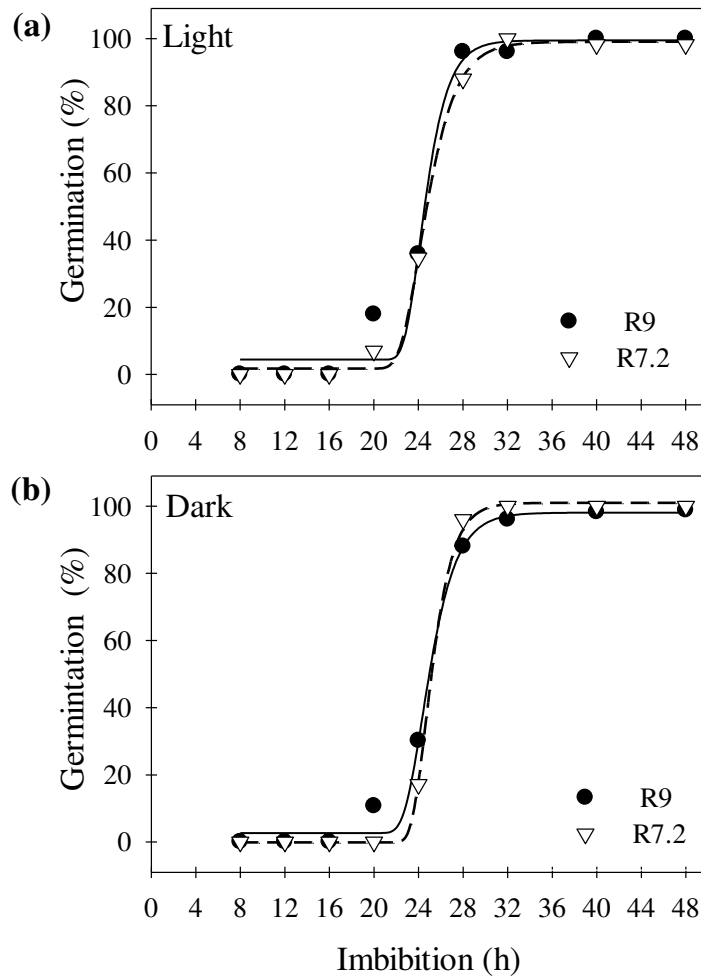
A closer look at the most abundant transcripts (Supplementary Table 3) in immature dried seeds (R7.2d) revealed the presence of “Pectinesterase”, “Hydroxyproline-rich glycoprotein-like protein”, “Protein PsbN”, “Ring finger protein”, “E3 ubiquitin-protein ligase”, among proteins related to abiotic stress responses. Also, we found transcripts encoding a glutathione  $\gamma$ -glutamylcysteinyltransferase-3 and glutathione peroxidase, two enzymes involved in glutathione mediated protection against oxidative damage and maintaining redox homeostasis (MAY et al., 1998; POTTERS et al. 2002).

The transcriptome containing the 833 transcripts whose level was specifically increased during natural maturation drying of immature seeds were associated a significant over-representation of genes involved in protein folding, including DNAj family, chaperones and heat shock proteins (Table 15). Among the mostly up-regulated genes, we noticed a “Heat shock transcription factor A6b” (HSFA6b), “Thioredoxin-like 4A” and “Asparagine synthetase”. Also, it was noted the presence of genes related with proteins degradation and recycling (Ubiquitin-conjugating enzyme), detoxification of secondary metabolites (MATE efflux family protein) and biotic stress.

For the 309 genes that were down-regulated in artificially dried immature seeds, the enrichment analysis revealed only 1 GO containing only 3 genes, suggesting that the down-regulation does not affect a particular biological process (Table 16). However, when we looked at the top down-regulated genes, we noticed several transcripts involved in ethylene signaling, including a homologue of ESR1 (ethylene response sensor 1), 2 homologues of EIN3-binding F box protein 1. The presence of several transcription factors (AP2 transcription factor, NAC, MYBR domain class, TIFY) was also noticed.

The 2071 down-regulated genes associated with the natural drying process were associated with cell wall categories ("cellulose synthase activity", "primary cell wall biogenesis" and "cellulose synthase (UDP-forming) activity"), and secondary metabolism ("acetyl-CoA metabolic process"; Table 17).





**Figure 15.** Germination of seeds harvested at the indicated reproductive stages (R7.2 and R9) exposed to be continued light ( $200 \mu\text{mol m}^{-2} \text{s}^{-2}$ ) or dark conditions. Data points are average of two replications of 28 seeds.

**Table 10.** Root and hypocotyl length measured after 120 h of imbibition of immature dried seeds (stage R7.2) and mature (stage R9) in continuous light ( $200 \mu\text{mol m}^{-2} \text{s}^{-2}$ ) or dark conditions. Data are the average  $\pm$  standard error of 25 seedlings.

	Stages of maturation	
	R7.2	R9
	----- Length (mm) -----	
	<b>Light</b>	
Root	$15,78 \pm 0,54$	$15,90 \pm 0,67$
Hypocotyl	$5,90 \pm 0,25$	$6,13 \pm 0,22$
	<b>Dark</b>	
Root	$15,06 \pm 0,45$	$15,30 \pm 0,77$
Hypocotyl	$9,37 \pm 0,49$	$9,35 \pm 0,63$

### **III.3 Transcriptome comparisons of immature seeds dried (stage R7.2d) and mature seeds (stage R9) during imbibition**

In the section III.2 our transcriptome data suggest that during the late stage of maturation, seeds are able to synthesize mRNA in preparation for the dry state. It is estimated that 12,000 and 13,500 transcripts are stored in the dry seeds of *Arabidopsis* (NAKABAYASHI et al., 2005) and our soybean DEG are in the same range. These stored mRNAs are thought to be used during seed imbibition to ensure germination. The proof of this hypothesis comes from the observation that blocking transcription with  $\alpha$ -amanitin does not prevent radicle emergence during imbibition in *Arabidopsis* (RAJJOU et al., 2004); indicating that newly synthesized proteins are translated from stored mRNA. However, complex changes in the stored mRNA have been reported during imbibition prior to radicle emergence. Some are rapidly degraded whereas others are maintained to high levels (GALLAND and RAJJOU, 2015). Using ribosome profiling techniques that monitor translation, it was possible to describe which transcripts were engaged into translation in dormant seeds where germination is blocked compared to non-dormant seeds (BASBOUS-SERHAL et al., 2015). In non-dormant seeds like soybean, it is not clear which mRNAs are important for germination and which one can be degraded because they are no longer necessary to ensure germination while they were necessary for maturation and remained highly abundant in dry seeds. Molecular biology experiments to address this issue are difficult to perform because measuring the rate of transcript degradation is very complex (BAZIN et al. 2011). Also, the use of  $\alpha$ -amanitin should be avoided because it introduces artefacts at the transcriptome levels when used in cell culture. An additional layer of complexity comes from the observation that maturation-specific genes can be expressed after germination upon osmotic treatment that blocks radicle growth and re-induces desiccation tolerance (BUITINK et al., 2006). Therefore, the molecular comparison between stages R7.2 and R9, where no germination difference is observed, represents an interesting physiological system to study changes in transcript levels that occur during early imbibition before germination of seeds that stored different sets of mRNAs as shown previously.

**Table 11.** Gene Ontology (GO) enrichment analysis of transcripts corresponding to DEG down-regulated between R7.2d and R9. Analysis was performed on the top 500 genes using SoyBase, with annotations for Wm82.a2.v1 (Glyma 2.0). #Genome: Genome Count present in this GO category; # Expressed: Number of submitted genes present in this GO category.

GO_id	#Genome	# Expressed	p-value	GO_description
GO:0015979	452	62	1,93E-39	photosynthesis
GO:0006091	116	51	1,10E-60	generation of precursor metabolites and energy
GO:0006354	225	51	1,29E-43	DNA-dependent transcription, elongation
GO:0019684	320	35	2,09E-18	photosynthesis, light reaction
GO:0010207	372	24	2,46E-07	photosystem II assembly
GO:0045333	53	12	2,94E-09	cellular respiration
GO:0009772	28	11	2,39E-11	photosynthetic electron transport in photosystem II
GO:0015986	58	7	7,09E-03	ATP synthesis coupled proton transport
GO:0009767	39	6	7,15E-03	photosynthetic electron transport chain
GO:0009769	12	4	8,11E-03	photosynthesis, light harvesting in photosystem II
GO:0006176	5	3	1,31E-02	dATP biosynthetic process from ADP

**Table 12.** Number of differential transcripts between artificial drying (R7.2 d/R7.2 f) and natural drying (R9/R7.2f) in soybean seeds. Only transcripts differently expressed with BH-value < 0.05 were considered.

Comparison	# Log <sub>2</sub> (ratio)							
	> 1	> 2	> 3	> 4	< -1	< - 2	< - 3	< - 4
Enforced drying (R7.2d/ R7.2f)	4554	1698	569	199	4218	1882	639	198
Natural drying (R9/ R7.2f)	5475	1814	614	188	6919	3644	1459	521

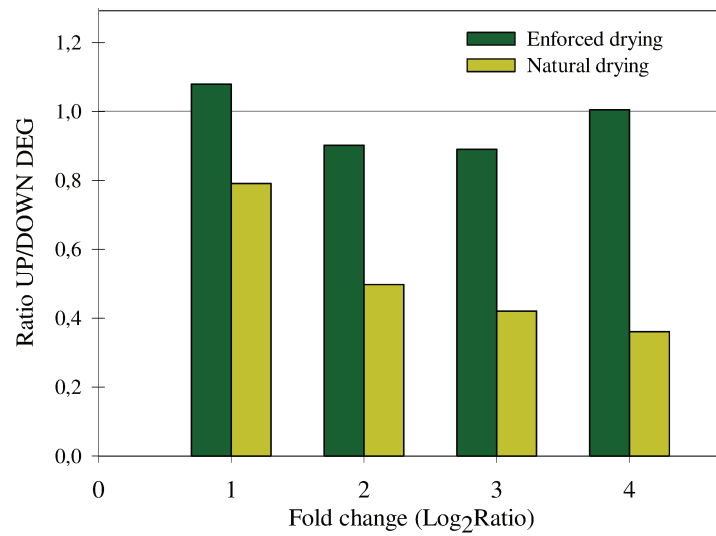
As explained below, major differences occurred during seed imbibition of R7.2 compared to R9 stages. Therefore, the next question was to assess how these differences could be attributed to the level of maturity of the seed lots and highlight those biological processes that are (un)necessary for germination. Also, the only phenotypic difference between harvested seeds at stage R7.2 and mature seeds is related to an increased longevity. Thus, we hypothesize that a transcriptome approach comparing changes during early imbibition of two seeds lots differing in longevity could give access to repair mechanisms that would be synthesized during imbibition of seeds from stage R9 or, alternatively to deteriorative mechanisms taking place during imbibition of immature seeds. To assess changes in transcriptome, we obtained RNASeq data sets from entire dry seeds from stage R7.2 (enforced fast drying) and stage R9 (in planta drying) before at imbibition (0h) and after 18 hours of imbibition, a time point when seeds were fully hydrated but had not germinated. Below we discuss the results found.

### **III.3.1 Profiles of differentially expressed genes (DEG) during imbibition**

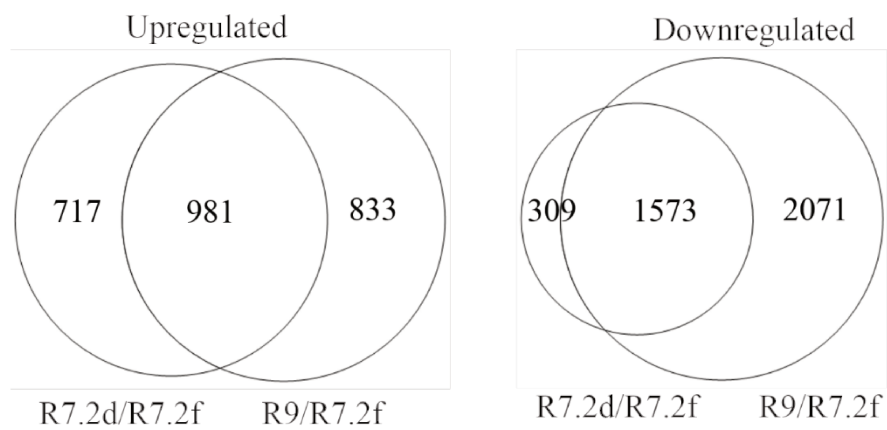
After normalization and background removal, data was filtered to remove transcripts with variance < 1 among stages. From this extended data set, 13.477 transcripts were selected as differentially expressed genes (DEG) between the different stages. Next, a clustering analysis was performed to describe the different profiles of differentially expressed genes during imbibition. Transcripts clustered into 13 patterns of expression that are shown in Figure 18. These patterns suggest three categories of expression: genes associated with germination (letter G), maturation (M) or longevity (L).

1. The pattern G corresponds to genes that up or down regulated during imbibition (profiles A, B) regardless of the maturity of seeds. It also include genes that up or down regulated after 18 h of imbibition in R7.2 seeds to reach similar levels of expression as in stage R9 (profiles K-N).

2. Maturation associated genes (M) correspond to transcript that are always significantly different between stages R7.2 and R9 regardless of the imbibition time or their profile during imbibition. Transcripts of the M category could be putative markers of the level of maturity reached by the seeds since their level of expression is always higher or lower in stage R9 compared to stage R7.2 during the first 18h of imbibition.



**Figure 16.** Ratio between the numbers of UP and DOWN differentially expressed genes (DEG) for each intensity categories. Value of 1 means an equal amount of DEG being up or down regulated.



**Figure 17.** Venn diagrams of DEGs that are up-regulated and down-regulated during enforced drying (R7.2d/R7.2f) and natural drying (R9/R7.2f). Only transcripts with  $2 < \text{Log}_2 \text{ratio} > 2$  were taken into account.

3. Genes were declared to be associated with longevity based on a putative repair function. Thus expression levels L genes could correspond to repair mechanisms where the genes could act as an activator (profiles G, I) or repressor (H-J). The amount of genes varies greatly between the different profiles. The vast majority of them behaved the same way, with transcripts increasing (profiles A, C) or decreasing (profiles B, D) during imbibition and were associated with germination.

The fact that different profiles can be found during seed imbibition implies a complex mRNA metabolism where transcripts are selectively recognized from immature seeds and being degraded. In the following sections, we will describe the following patterns according the relevance of the objectives of this work and to the amount of transcript. Therefore, we will focus on profiles A, B, G, N, and L.

### **III.3.2 Transcriptome changes during seed imbibition (Profile A and B)**

Firstly, we verified whether the pattern of differentially expressed genes (DEG) were similar during imbibition between stage R7.2 and R9 using the expression profile that were linked to germination (Figure 19, A-B). The number of up- and -down DEG after 0 and 18h were counted and ranked in four categories based on their level of expression ( $\log_2$  ratio from 1 to 4 for lowly and highly differentially expressed transcripts).

Figure 19 shows that the overall pattern of expressions was similar for both up- and down- genes in both stages, suggesting no gross difference in RNA metabolism between both stages. However, slight differences were observed. We observed a higher amount of differentially up-regulated genes in stage R9, particularly for those that are highly expressed during imbibition. Indeed, compared to R7.2, they were 50% more transcripts that increased 16 fold ( $\log_2$  4) during imbibition of seeds from stage R9. An inverse trend was found for transcripts that decreased during imbibition. Compared to R7.2, they were 40% less transcripts found at 18h of imbibition of stage R9 seeds that had 16 fold-decreased level. This suggests that RNA degradation is likely to be more important in mature seeds. It is noteworthy that these differences do not mean that the expression profile of those genes were radically different during imbibition. Indeed, when we counted the amount of DEG at 18h between stage R7.2 and R9, none were found to be more than two fold ( $\log_2$  ratio) differentially up-regulated.

**Table 13.** Gene Ontology (GO) enrichment analysis of transcripts DEG up-regulated to artificial drying. Analysis was performed on the top 717 genes using SoyBase, with annotations for Wm82.a2.v1 (Glyma 2.0). #Genome: Genome Count present in this GO category; # Expressed: Number of submitted genes present in this GO category.

GO_id	#Genome	# Expressed	p-value	GO_description
<b>Overrepresented</b>				
GO:0015979	452	80	1,75E-55	photosynthesis
GO:0010207	372	22	7,13E-05	photosystem II assembly
GO:0019684	320	45	3,49E-26	photosynthesis, light reaction
GO:0006354	225	66	5,24E-61	DNA-dependent transcription, elongation
GO:0006091	116	66	6,97E-85	generation of precursor metabolites and energy
GO:0008137	58	15	1,86E-12	NADH dehydrogenase (ubiquinone) activity
GO:0016168	58	11	2,66E-07	chlorophyll binding
GO:0045333	53	13	7,21E-10	cellular respiration
GO:0080044	50	6	2,57E-02	quercetin 7-O-glucosyltransferase activity
GO:0016651	40	11	3,47E-09	oxidoreductase activity, acting on NAD(P)H
GO:0009767	39	10	1,76E-07	photosynthetic electron transport chain
GO:0080046	29	5	1,66E-02	quercetin 4'-O-glucosyltransferase activity
GO:0009772	28	9	1,43E-07	photosynthetic electron transport in photosystem II
GO:0042773	24	8	1,03E-06	ATP synthesis coupled electron transport
GO:0045156	19	7	1,86E-06	electron transporter, transferring electrons within the cyclic electron transport pathway of photosynthesis activity
GO:0080045	16	4	1,97E-02	quercetin 3'-O-glucosyltransferase activity
GO:0003959	14	8	1,81E-09	NADPH dehydrogenase activity
GO:0009769	12	7	8,38E-08	photosynthesis, light harvesting in photosystem II
GO:0045157	7	3	2,76E-02	electron transporter, transferring electrons within the noncyclic electron transport pathway of photosynthesis activity
GO:0006176	5	3	1,94E-02	dATP biosynthetic process from ADP
<b>Underrepresented</b>				
GO:0006952	1116	3	2,54E-02	defense response

To unravel the molecular processes occurring during early imbibition, we obtained the expression profiles of DEG that increased and decreased during maturation, regardless of their maturity stage. This represented respectively 4399 and 1939 being up and down-regulated transcripts (Figure 19). Next, we performed a GO enrichment analysis on both profiles using the expression data from soybase.org as a background (Table 18). To increase the significance of the analysis, we limited the number of input genes to 1081 transcripts, corresponding to a minimum of 8-fold increase during imbibition. Results are shown in Table 18.

Biological processes that were more over-represented during imbibition were associated with growth of different tissues (root tip, root hair, meristem, cell wall elongation). Also, genes involved in lipid reserve mobilization appears to be activated, as highlighted by the categories “Very long chain fatty acid metabolic process” and “acetyl-CoA metabolic process”. The GO “regulation of hormone levels” did not contain genes whose function are related to a particular hormone but exhibited genes that had overlapping GO annotation with other categories such as “sterol biosynthesis process”, “anthocyanin accumulation”. Interestingly, the 5 genes representing the GO “ethylene binding” corresponded to probes encoding five different ethylene receptors, including an Arabidopsis homologue of ESR1.

GO enrichment analysis of DEGs that were down-regulated reveals a high number of categories associated with myo-inositol and inositol-phosphate (Table 19). In seeds, inositol serves a matrix to store phosphate in the form of phytate. Many genes have been characterized and were shown to be expressed during seed development. The DEG found here represents biosynthetic genes. Thus, during imbibition they represent sets of transcripts that are no longer necessary and being degraded. The GO “protein folding” and “response to ABA” was also highly significant. These categories contained genes involved in ABA metabolism and signaling pathway such as homologues of ATAF1 involved in attenuating ABA signaling, OST1 (OPEN STOMATA 1), a member of ABA activated SNF1-related protein kinases (SnRK2) involved in ABA signaling, ABI5 (ABA INSENSITIVE 5), APF2, a bZIP factor interacting with ABI5, KEG (KEEP ON GOING) a, RING E3 ligase involved in abscisic acid signaling by degrading ABI5, PP2CA (PROTEIN PHOSPHATASE 2CA), a negative regulator of ABA, ABA1 involved in ABA synthesis, CYP707A2; (+)-abscisic acid 8'-hydroxylase involved in ABA degradation. Interestingly PMIT (PROTEIN-L-ISOASPARTATE METHYLTRANSFERASE 1), an



**Table 14.** Gene Ontology (GO) enrichment analysis of transcripts DEG up-regulated to natural drying. Analysis was performed on the top 833 genes using SoyBase, with annotations for Wm82.a2.v1 (Glyma 2.0). #Genome: Genome Count present in this GO category; # Expressed: Number of submitted genes present in this GO category.

GO_id	#Genome	# Expressed	p-value	GO_description
GO:0006457	779	31	4,64E-02	protein folding
GO:0009644	582	29	1,05E-03	response to high light intensity
GO:0042542	511	27	7,94E-04	response to hydrogen peroxide
GO:0006334	128	13	4,90E-04	nucleosome assembly

**Table 15.** Gene Ontology (GO) enrichment analysis of transcripts DEG down-regulated to artificial drying. Analysis was performed on the 309 genes using SoyBase, with annotations for Wm82.a2.v1 (Glyma 2.0). #Genome: Genome Count present in this GO category; # Expressed: Number of submitted genes present in this GO category.

GO_id	#Genome	# Expressed	p-value	GO_description
GO:0030643	7	3	1,26E-02	cellular phosphate ion homeostasis

**Table 16.** Gene Ontology (GO) enrichment analysis of transcripts DEG down-regulated to natural drying. Analysis was performed on the 2071 genes using SoyBase, with annotations for Wm82.a2.v1 (Glyma 2.0). #Genome: Genome Count present in this GO category; # Expressed: Number of submitted genes present in this GO category.

GO_id	#Genome	# Expressed	p-value	GO_description
GO:0016126	363	39	2,09E-03	sterol biosynthetic process
GO:0006084	178	22	3,31E-02	acetyl-CoA metabolic process
GO:0016759	74	13	1,62E-02	cellulose synthase activity
GO:0016760	56	11	2,30E-02	cellulose synthase (UDP-forming) activity
GO:0004579	10	6	9,78E-04	dolichyl-diphosphooligosaccharide-protein glycotransferase activity
GO:0009833	13	6	1,42E-02	primary cell wall biogenesis

ABA induced protein involved in repairing protein in aged seeds was also present in this category.

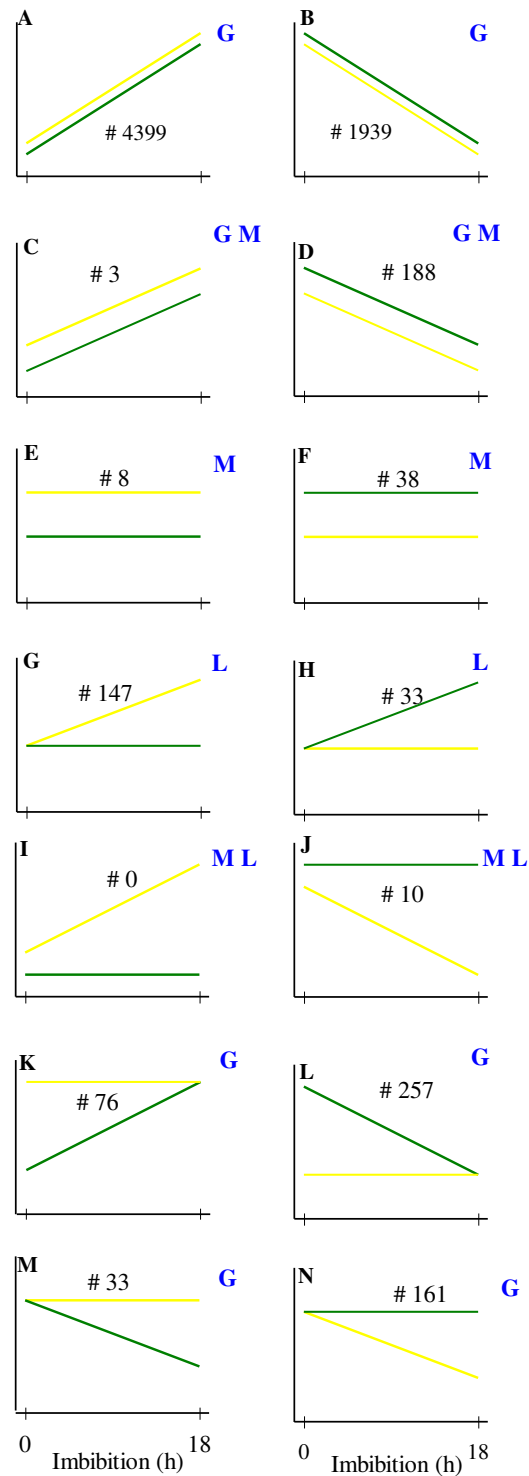
A close inspection of the transcriptome also highlighted many homolog genes responsible for a cross talk between light and ABA/GAI signaling such FRS11 (FAR1-related sequence 11), LZFI (LIGHT-REGULATED ZINC FINGER PROTEIN 1), FRS6 (FAR1-related sequence 6); PIF4 (phytochrome interacting factor 4), PAT1 (phytochrome A signal transduction 1).

The GO category “chlorophyll oxygenase” represents genes involved in chlorophyll degradation. Chlorophyll breakdown occurs during the late phase of seed development at around stage R7.2. Since the transcripts disappeared both in R7.2 and R9 imbibing seeds, this suggests that during maturation, they were not degraded during maturation. Chlorophyll retention in dry mature soybean seeds represents a major agronomic problem that leads to economic losses as green seeds have poor nutritional and physiological quality. They were also transcripts involved in photosynthesis.

We also looked at the top 30 transcripts that were mostly down-regulated during imbibition to have an idea about those transcripts that were unstable during imbibition. Many of them corresponded to genes having protective functions during stress such as the LEA gene Seed Maturation protein PM39, HSPs, DNAj and other less characterized genes with chaperone functions (Bcl2-associated anthanogen-like protein (Table 20).

### **III.3.3 Analysis of transcription factors during imbibition**

We investigated whether the patterns of transcription factors (TF), whose expression varied during imbibition between immature (stage R7.2) and mature (stage R9). Only transcripts with BH-value < 0.05 and similar profiles for immature and mature seeds were considered. We first calculated the % of enrichment of TF in the different profiles (Table 21). For this, we assumed that there were no specific enrichment in the expression profile A and B, that is DEG up and down regulated during seed imbibition of both stages. TF represented 4.5% of the total amount in both UP and DOWN DEG.

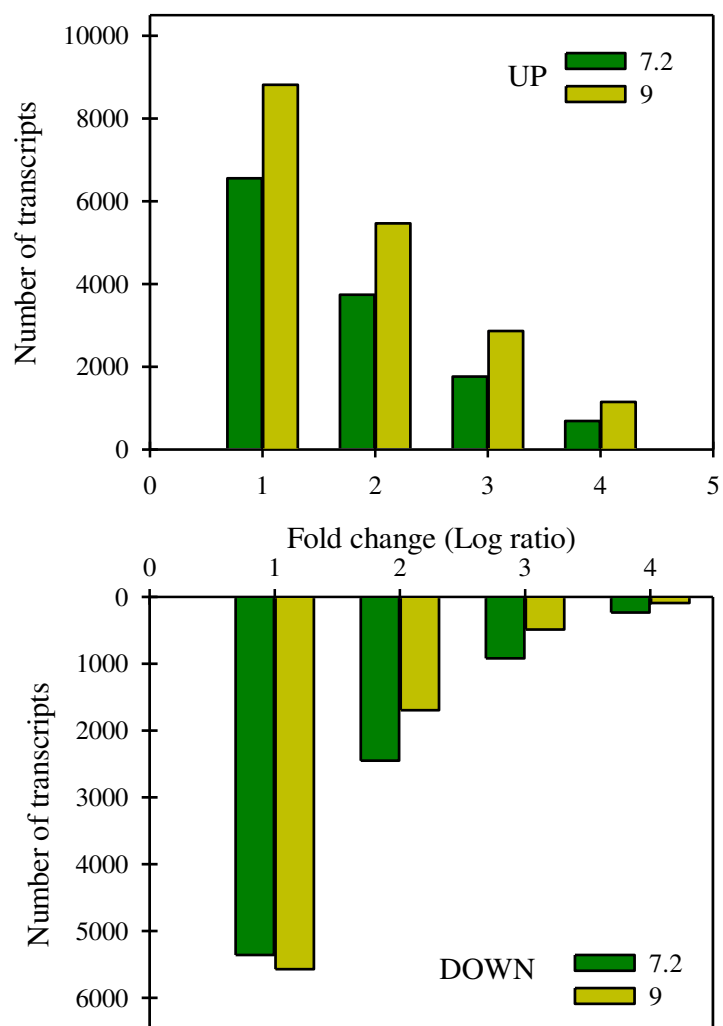


**Figure 18.** Profiles of expression of differentially expressed genes in dried seeds from R7.2 (green) and R9 stages (yellow), before (0h) and after 18 h of imbibition in optimal conditions. Numbers on the right indicate the amount of transcripts exhibiting the profile and letters (blue) indicate the type of pattern: G, germination associated genes, M maturity genes, L longevity associated genes.

Therefore, we inspected the list of the 13 transcription factors from category G (Table 22) The mostly up-regulated DEG was a Class I KNOX box homeobox factor whose homolog in Arabidopsis is BUM1. This TF is required for shoot apical meristem (SAM) formation during embryogenesis and for SAM function throughout the lifetime of the plant. We also noted the presence of GRAS-type TF, whose homologue is SGR7, (SHOOT GRAVITROPISM 7) and is essential for normal shoot gravitropism. There is also an ABI3-like transcript. In Arabidopsis, this AP2/B3-like transcriptional factor family is apparently not related to ABA signaling. Expressed in the lateral organ boundary region, it operates in a network of regulatory genes controlling leaf serration.

### **III.3.4 Down regulated transcripts during seed imbibition of stage R7.2 only (Profile L)**

Next, we investigated which genes were down regulated during imbibition of R7.2 genes but were already down regulated in stage R9 before imbibition. With 257 transcripts, this profile (L, Figure 18) represents the second largest one. We reasoned that these represent transcripts are likely to represent these transcripts that were not degraded progressively during enforced drying. Nevertheless, they can decrease in two different cellular contexts: seed drying during maturing and seed imbibition prior to germination. The GO enrichment analysis (Table 23) revealed a few number of significant categories that are closely related. Indeed, both contained mainly chloroplastic genes involved in transcription and elongation with the chloroplasts. These include ribosomal genes, genes encoding proteins functioning in the photosystem1 and electron transport chain. Paradoxically, while chloroplasts will be resynthesized later after germination during the greening process, transcripts are first degraded even if they could be reused later. A closer inspection of the transcriptome did not reveal any transcript that could be conspicuously linked to degradative processes that could occur as a result of damage incurred during enforced drying.



**Figure 19.** Number of differential transcripts between 0h and 18h of immature (stage R7.2) and mature (stage R9) seeds. Significant differences between transcript levels were assessed using a BH-value  $< 0.05$ .

### **III.3.5 Decreased transcript during imbibition of mature seeds only (Profile N)**

Next, we examined a third largest category (Figure 18, profile N) representing transcripts that decreased during imbibition of stage R9 seeds but remained high in immature seeds. This category is interesting because shows that degradation mechanisms are distinct between mature and immature seeds. Although this category contained a higher number of transcripts (161), the GO enrichment analysis did not reveal processes that are of significance in our study (Table 24).

### **III.3.6 Increased transcripts during imbibition of mature seeds (Profile G)**

Profile G is characterized by transcripts increasing during imbibition of stage R9 seeds but remaining low and steady in stage R7.2 seeds. It could contain genes that encode repair processes since, according to CHEN et al. (2013) they can be activated even if symptoms of loss of viability are not visible. To check this, we screened the annotation of all 147 probes for a known repair processes and found two probes. First, DNA2, a member of DNA replication helicase family protein (Glyma.06G310500) whose Arabidopsis homologue was recently found to be associated with DNA repair. The second probe is (Glyma.18G194300), the Arabidopsis homolog of a Werner syndrome-like exonuclease whose expression was significantly upregulated in stage 9 during imbibition but not stage R7.2 (Figure 20).

Next, GO enrichment analysis was performed (Table 25). Categories that were over-represented were related to organ formation, in particular in relation to light (red, far-red and blue), and meristem activity, such as flower morphogenesis, organ morphogenesis, cotyledon morphogenesis. Many of these transcripts were related to auxin signaling and auxin.

**Table 17.** Gene Ontology (GO) enrichment analysis of transcripts DEG increased during imbibition. Analysis was performed on the 1081 genes using SoyBase, with annotations for Wm82.a2.v1 (Glyma 2.0). #Genome: Genome Count present in this GO category; #Expressed: Number of submitted genes present in this GO category.

GO_id	#Genome	#Expressed	p-value	GO description
<b>Over-represented</b>				
GO:0000271	274	35	1,37E-11	polysaccharide biosynthetic process
GO:0010075	450	41	7,04E-09	regulation of meristem growth
GO:0006084	178	24	5,31E-08	acetyl-CoA metabolic process
GO:0010817	212	26	7,46E-08	regulation of hormone levels
GO:0048767	504	41	2,28E-07	root hair elongation
GO:0016126	363	33	7,51E-07	sterol biosynthetic process
GO:0004553	546	42	2,26E-06	hydrolase activity, hydrolyzing O-glycosyl compounds
GO:0009932	248	26	2,26E-06	cell tip growth
GO:0009825	265	26	9,06E-06	multidimensional cell growth
GO:0043481	310	27	5,79E-05	anthocyanin accumulation in tissues in response to UV light
GO:0042335	131	15	1,58E-03	cuticle development
GO:0005975	911	49	2,62E-03	carbohydrate metabolic process
GO:0000038	104	13	2,81E-03	very long-chain fatty acid metabolic process
GO:0071555	448	30	3,01E-03	cell wall organization
GO:0051740	14	5	1,14E-02	ethylene binding
<b>Under-represented</b>				
GO:0003735	733	3	1,73E-03	structural constituent of ribosome
GO:0006412	763	3	4,83E-03	Translation

### III.4. Validation of the RNAseq data

As well discussed in this work, the only physiological difference found between stage R7.2 and R9 was in relation to seed longevity, wherein R9 is increased. Thus, study the changes in transcript levels, during imbibition of the seed these stage, sparked further more our interest. The validation of the RNAseq data was performed by using RT-qPCR. Therefore, five interesting genes belonging to the profile G; profile characterized by increasing transcripts during imbibition at stage of R9, but remaining low and steady at stage R7.2 (Figure 18). The expression level ( $\text{Log}_2$  fold change) obtained by of RNA-Seq analysis are shows in the Table 26.

We validated the expression of Class I KNOX homeobox transcription factor. These transcripts are important regulators in maintaining the shoot apical meristem and patterning adequate of organ initiation (HAKE et al., 2004). KNOX I was strongly expressed by RT-qPCR for mature seed was observed increased 18.2 fold change during imbibition (Figure 22 a). WRKY transcription factor it was also validated. This family is one of the largest families of transcriptional regulators in plants, some of them have been related to response to biotic and abiotic stress (RUSHTON et al 2010) and responsible for regulated phosphate homeostasis in *Arabidopsis* seeds (SU et al., 2015). Recently the WRK3 was related to seed longevity of *M. truncatula* (RIGHETTI et al 2015), with then an important transcription factor found in our data.

Longevity genes in *M. truncatula* were found to be enriched in binding sites for auxin-binding factors (RIGHETTI et al. 2015), implying that auxin also may have a role in seed longevity. Consistent with these observations, we found Auxin Responsive Factor family being up-regulate in our data by RNAseq analysis. So, we analysis its expression by RT-qPCR and confirmed its expression, with 5 fold change more for stage R9 during imbibition (Figure 22 d).

Our transcriptome revealed also the presence of HSF, a family of transcription factors that are considered important for longevity seed (PRIETO-DAPENA et al., 2006; ALMOGUERA et al., 2009; PERSONAT et al., 2014).

The correlation between expression value by RNAseq and quantitative real-time PCR (RT-qPCR) is present in Figure 21.



**Table 18.** Gene Ontology (GO) enrichment analysis of transcripts DEG decreased during imbibition. Analysis was performed on the 1939 genes using SoyBase, with annotations for Wm82.a2.v1 (Glyma 2.0). #Genome: Genome Count present in this GO category; # Expressed: Number of submitted genes present in this GO category.

GO_id	#Genome	# Expressed	p-value	GO_description
<b>Overrepresented</b>				
GO:0009737	1254	82	4,17E-03	response to abscisic acid stimulus
GO:0006457	779	60	5,09E-04	protein folding
GO:0009414	888	60	3,25E-02	response to water deprivation
GO:0010264	140	20	4,57E-04	myo-inositol hexakisphosphate biosynthetic process
GO:0003954	40	11	1,02E-04	NADH dehydrogenase activity
GO:0045333	53	10	2,90E-02	cellular respiration
GO:0016857	14	6	3,60E-03	racemase and epimerase activity, acting on carbohydrates and derivatives
GO:0052726	10	5	8,83E-03	inositol-1,3,4-trisphosphate 5-kinase activity
GO:0047325	10	5	8,83E-03	inositol tetrakisphosphate 1-kinase activity
GO:0052725	11	5	1,57E-02	inositol-1,3,4-trisphosphate 6-kinase activity
GO:0010277	12	5	2,60E-02	chlorophyllide a oxygenase [overall] activity
GO:0009268	6	4	2,98E-02	response to pH
<b>Underrepresented</b>				
GO:0016788	372	1	8,25E-03	hydrolase activity, acting on ester bonds

**Table 19.** Number of transcription factors (TF) in different profiles of genes, and p values after comparing these values with profile A and B. NS not significant, NT not tested.

Profiles	# genes	# TF	p Value
C	3	0	NS
D	188	1	NS
E	8	2	NS
F	38	0	NT
<b>G</b>	<b>147</b>	<b>13</b>	<b>0.014</b>
H	33	1	NS
I	0	0	NT
J	10	0	NT
K	76	5	NS
L	257	8	NS
M	33	2	NS
N	161	4	NS

### **III.5 Comparative analysis between soybean and *Medicago truncatula* to identify genes related to seed vigor acquisition during maturation**

During imbibition, immature soybean seeds exhibiting a lesser longevity that mature seeds exhibited many changes in the transcriptome that were specific to the maturity level. However, during imbibition, many genes are expressed that are not only related to seed(ling) performance, making it difficult to separate gene expression related to seed vigor from genes involved in other functions. Therefore, to understand which mechanisms that might be related to seed quality, we compared our transcriptome data with those of developing seeds of *Medicago truncatula*. Thus, a comparative analysis between the two legume species is expected to eliminate a large number of genes that are no specific.

#### **III.5.1 Acquisition of seed vigor during maturation of *Medicago truncatula***

First, we selected a stage during *Medicago* development that exhibited the same characteristics than those of immature soybean at stage R7.2. This stage corresponded to seeds of 28 days after pollination, corresponding to the end of seed filling (CHATELAIN et al., 2012). The water content of the seeds at 28 DAP was 0,52 g H<sub>2</sub>O g DW<sup>-1</sup>. After harvest, immature seeds were rapidly dried back at 43% RH. Both dried and mature seeds were equilibrated at this same RH prior to the physiological experiments. Thereafter, the water content drying was 0,091 ± 0,002 and 0,11 (±0,002) g H<sub>2</sub>O g DW<sup>-1</sup> for immature and mature seed batches, respectively. Next, we compared the different characteristics of seed physiological quality. Seeds at 28 DAP germinated to 100%, albeit slower than fully mature seeds (Figure 23).

Cold imbibition at 10°C decreased the speed of germination, but no difference was found in the delay or percentage of germination between the two stages (Table 27). However, the percentage of normal seedlings under cold imbibition was significantly lower in the immature seed lot compared to the mature seed lot: 43% of the immature seeds developed in abnormal seedlings (Figure 24). No difference was observed germination when seeds were imbibed in salt (data not shown).

**Table 20.** List of the top 30 transcripts whose level decrease the most during imbibition.

<b>Gmax 2.0 ID</b>	<b>Tax Hit</b>	<b>p-value</b>	<b>Name</b>
Glyma.12G237200	Q1SKZ9_MEDTR	4,00E-43	Zinc finger, CCHC-type
Glyma.12G085000	E6ZE62_BETVM	7,00E-13	ATP synthase subunit alpha
Glyma.03G193400	G7L603_MEDTR	0	Xylem serine proteinase
Glyma.01G116600	G7KS96_MEDTR	7,00E-67	Alcohol dehydrogenase-like protein
Glyma.16G184100	D7LKY7_ARALL	1,00E-24	Protease inhibitor/seed storage/lipid transfer protein family protein
Glyma.17G034400	Q2HSK9_MEDTR	7,00E-100	Ubiquitin-conjugating enzyme, E2
Glyma.08G324100	O24101_MEDTR	3,00E-27	MtN5 protein
Glyma.15G242700	B9I8W9_POPTR	1,00E-96	Purine permease family protein
Glyma.08G156600	B4YYB2_THEHA	5,00E-09	ST63-2
Glyma.16G192200	Q9SWB3_SOYBN	1,00E-93	Seed maturation protein PM39 (Fragment)
Glyma.16G192000	Q9SWB3_SOYBN	6,00E-126	Seed maturation protein PM39 (Fragment)
Glyma.13G173200	Q9LK41_ARATH	4,00E-172	Beta-1,3-glucanase
Glyma.16G189900	Q9SWB3_SOYBN	0	Seed maturation protein PM39 (Fragment)
Glyma.16G185900	Q9SWB3_SOYBN	2,00E-147	Seed maturation protein PM39 (Fragment)
Glyma.07G030800	Q9SSQ8-2	7,00E-33	-2 Isoform 2 of 26.5 kDa heat shock protein, mitochondrial
Glyma.16G192700	Q9SWB3_SOYBN	3,00E-81	Seed maturation protein PM39 (Fragment)
Glyma.16G193200	Q9SWB3_SOYBN	4,00E-144	Seed maturation protein PM39 (Fragment)
Glyma.07G043600	G7L932_MEDTR	7,00E-72	17.4 kDa class III heat shock protein
Glyma.16G190100	Q9SWB3_SOYBN	3,00E-96	Seed maturation protein PM39 (Fragment)
Glyma.12G130000	G7JDS4_MEDTR	1,00E-177	Chaperone protein dnaJ
Glyma.18G284900	G7KUT0_MEDTR	3,00E-59	Bcl-2-associated athanogene-like protein
Glyma.09G139600	Q9SWB3_SOYBN	0	Seed maturation protein PM39 (Fragment)
Glyma.U039700	Q9SWB3_SOYBN	4,00E-98	Seed maturation protein PM39 (Fragment)
Glyma.08G071000	G7JCQ8_MEDTR	0	White-brown-complex ABC transporter family
Glyma.08G177500	W0TQL8_ACAMN	0	Dihydropyrimidinase
Glyma.05G092300	ATPAM_PHAVU	3,00E-169	ATP synthase subunit alpha, mitochondrial
Glyma.15G083900	G7ZXJ0_MEDTR	2,00E-90	Transcription factor TGA5
Glyma.17G074500	FH13_ARATH	0	Formin-like protein 13
Glyma.08G056600	U5FRT1_POPTR	2,00E-159	Hydrolase family protein
Glyma.17G074500	FH13_ARATH	0	Formin-like protein 13

Like in soybean, the major difference between 28 DAP seeds and mature seeds was their seed longevity. During storage in conditions similar to that used for soybean (75% RH and 35°C), germination decreased almost directly upon incubation (Figure 25). Half of the seed population had died after 7 days of storage. In contrast, further maturation led to an increase in seed storability. Mature seeds could be stored for two weeks before a decrease in viability and half of the seed population died after only 25 days of storage (Figure 25). Longevity was increased by almost four-fold in mature seeds compared to immature seeds. These findings are similar to those observed for soybean (Figure 14). Immature seeds of stage R7.2 started to lose their viability after 21 days of storage (Figure 14). Half the population was dead after 27 days. In contrast, it took 48 days to kill 50% of mature seeds.

### **III.5.2 Characterization of genes that are differentially expressed in immature vs mature seeds of legumes**

Transcriptomes were obtained from immature (28DAP) dried and mature seeds. Total RNA was isolated in three biological replicates and used to hybridize the Affymetrix *M. truncatula* GeneChip containing 102,121 probe sets. After statistical analysis, differentially expressed genes (DEG) were considered significant when the p-value was  $< 0,01$ . Genes differential expression being up-regulated corresponding the expression level of  $\text{Log}_2 > 1$ ; and being down-regulated corresponding the expression level of  $\text{Log}_2 < -1$  (Table 28).

A comparative analysis was performed between the homologues of *Medicago* and soybean using the *Arabidopsis* AGI number, since no database exists that provides a correct and complete comparison of the two legume species. Considering the seed longevity increases in both soybean and *M. truncatula*, after filling while the seeds are losing water, we first analyzed DEG related with longevity.

In *M. truncatula* seeds, there were only 640 DEG transcripts being up-regulated at least 2-fold, whereas 1788 were down-regulated, between immature seed dried (28 DAP) and mature. This result was similar when analyzing the transcripts expressed in soybean seeds between immature seed dried (R7.2) and mature (R9); wherein 742 DEG transcript being up-regulated and 1525 were down-regulated.

**Table 21.** Annotation of the transcription factors expressed corresponding to Profile G.

<b>Gmax 2.0 ID</b>	<b>Tax Hit</b>	<b>p-value</b>	<b>Name</b>	<b>R9 18h/ R7.2d 18h</b>	<b>R9 18h/ R9 0h</b>
Glyma.14G047000	A5Y4H1_MEDTR	2,00E-161	Class I KNOX homeobox transcription factor	1,09	5,04
Glyma.02G026300	Q0PJI4_SOYBN	0,00E+00	MYB transcription factor MYB138	1,08	4,18
Glyma.08G137900	G7LAC7_MEDTR	0,00E+00	BZIP transcription factor bZIP133	1,32	3,08
Glyma.05G105600	K7NCY5_QUESU	0,00E+00	GRAS family transcription factor	1,05	2,60
Glyma.06G125100	G7J693_MEDTR	2,00E-124	Ethylene responsive transcription factor 1a	2,10	2,46
Glyma.03G224700	G7KUM9_MEDTR	9,00E-155	WRKY transcription factor	1,30	2,44
Glyma.02G200500	D9ZIL2_MALDO	3,00E-71	ABI3L domain class transcription factor	1,08	2,41
Glyma.10G066100	D1M7W9_SOLLC	9,00E-120	HSF Heat stress transcription factor A3	1,97	1,85
Glyma.13G100500	D9ZJ13_MALDO	1,00E-155	HD domain class transcription factor	1,07	1,70
Glyma.05G157000	Q0GPG2_SOYBN	6,00E-115	BZIP transcription factor bZIP111	1,24	1,63
Glyma.13G249800	G7IK44_MEDTR	2,00E-167	Transcription factor bHLH112	1,03	1,59
Glyma.14G217700	I1MC56_SOYBN	0,00E+00	Auxin response factor	1,06	2,92
Glyma.09G052400	Q948W7_PEA	2,00E-123	Zinc-binding protein	1,03	1,43

Among UP-regulated transcripts, only 7%, corresponding to 36 DEG, were found to be common in both species. A closer look at those transcripts (Table 29) revealed genes encoding MALATE SYNTHASE, PHY A, PHYB, NFXlike 1, ABI-1-like 1, and SCARECROW-like 14.

When we compared the 1788 DEG transcripts being down-regulated in *Medicago* during seed maturation with the 1525 were down-regulated for soybean also during maturation, 223 transcripts were found in common for both species. This corresponds to 20% DEG transcripts expressed during maturation. The GO enrichment analysis corresponding at these transcripts is shown in Table 30. GO categories were related to processes associated with response to stimulus, metabolism and defense.

### **III.5.3 Characterization of genes that are differentially expressed in immature and mature legume seed during imbibition**

The next step was to understand which mechanisms might be underlying the differences in seed quality. During imbibition, many genes are expressed that are not only related to seed(ling) performance, making it difficult to separate gene expression related to seed vigor from genes involved in other functions. A comparative analysis between the two legume species is expected to eliminate a large number of genes that are not specific. Therefore, changes in transcriptome of 28 DAP and mature seed were monitored during different times of imbibition: 0h, 6h and 24h. The number of differential transcripts expressed, being up-regulated and down-regulated are shown in the table 31.

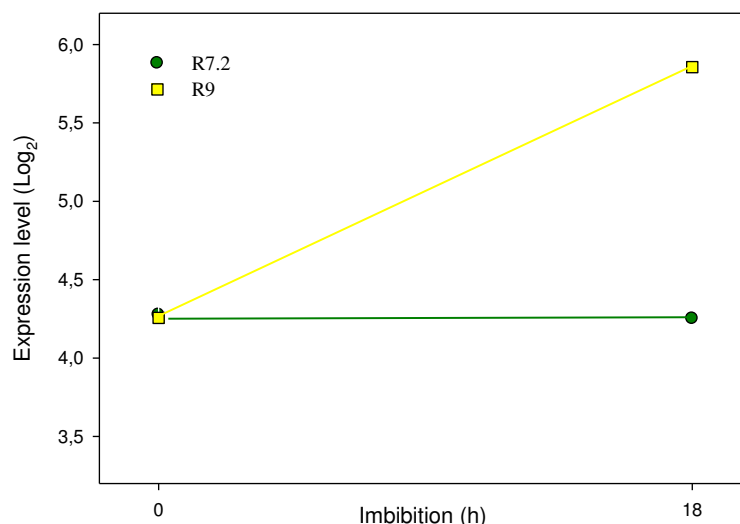
During the 24h of imbibition, the total number of up- and down-DEG in mature seeds were 2.06-fold and 1.2 fold higher compared to immature seeds. The comparison between mature and immature at each time point of imbibition also indicated that a higher number of DEG in mature seeds compared to immature seeds. This difference was observed already in dry seeds and increased steadily during imbibition for up-regulated transcripts (Table 31). For the down regulated DEG, the number of transcripts decreased mostly between 6 and 24h. Like in soybean this suggests that the RNA metabolism during imbibition appears to be dependent on the maturity stage.

**Table 22.** Gene Ontology (GO) enrichment analysis of transcripts DEG that decreased during imbibition of R7.2 genes but were already degraded in stage R9 before imbibition (profile L of Figure 18). Analysis was performed on the 257 genes using SoyBase, with annotations for Wm82.a2.v1 (Glyma 2.0). #Genome: Genome Count present in this GO category; # Expressed: Number of submitted genes present in this GO category.

GO_id	#Genome	# Expressed	p-value	GO_description
GO:0015979	452	14	7,30E-03	photosynthesis
GO:0006091	116	12	6,71E-08	generation of precursor metabolites and energy
GO:0006354	225	12	1,16E-04	DNA-dependent transcription, elongation

**Table 23.** Gene Ontology (GO) enrichment analysis of transcripts DEG that decreased during imbibition of R9 seeds but remained high in stage R7.2 during imbibition (profile N of figure 18) .Analysis was performed on the 161 genes using SoyBase, with annotations for Wm82.a2.v1 (Glyma 2.0). #Genome: Genome Count present in this GO category; # Expressed: Number of submitted genes present in this GO category.

GO_id	#Genome	#Expressed	p-value	GO_description
GO:0006878	17	3	2,67E-02	cellular copper ion homeostasis
GO:0016048	4	2	4,45E-02	detection of temperature stimulus



**Figure 20.** Changes in expression of probe Glyma.18G194300 encoding a 3'-5' exonuclease whose closest homologue in Arabidopsis is a Werner syndrome-like exonuclease. Data are expressed as normalized read counts/ millions and are the mean of two replicate samples.

To understand which mechanisms are associated with germination in both legumes, we built Venn diagrams using the genes that were differentially up-regulated or down-regulated in soybean seed at 18h of imbibition and in Medicago seed at 6h and 18h (Figure 26). From the 373 genes that had higher transcript levels in mature imbibed soybean seeds compared to immature seeds, 61 (16%) genes were also upregulated in mature compared to immature Medicago seeds (Figure 23 a). Transcripts of 35 genes were already differential after 6h of imbibition, out of which 14 were no longer differential at 24h of imbibition. For 26 genes, transcript levels became differential only after 24h of imbibition. Next, we examined the genes down-regulated during imbibition. From the 740 down-regulated genes between immature and mature imbibed soybean seeds, 129 (17%) genes were also downregulated in mature compared to immature Medicago seeds (Figure 26 b). Transcripts of 110 genes were already differential after 6h of imbibition, out of which 77 were no longer differential at 24h of imbibition. For 19 genes, transcript levels became differential only after 24h of imbibition. Since we are interested in genes that remain differential during imbibition between mature and immature legume seeds, further analyses were performed on the 47 genes with higher transcript levels in mature vs immature seeds, and the 52 genes with lower transcripts in mature compared to immature seeds. Their gene function is further analyzed in the following sections.

### **III.5.3.1 Analysis of genes with higher transcript levels in mature legume seeds during imbibition versus immature.**

We were interested in DEG genes that remain differential during imbibition between mature and immature legume seeds. We first analyzed DEG with higher transcript levels both in soybean and Medicago. shows 47 genes with higher transcript levels in mature vs immature seeds (Supplementary Table 5). For most of these genes, their expression level was not yet differential in dry seeds, but increased during imbibition. Analyses were performed using PLantGSEA (YI et al., 2013). For the GO cellular component, 18 out of the 47 genes were assigned to the plasma membrane (enrichment with FDR of  $1.35e^{-4}$ ) and 5 to the apoplast (FDR  $1.93e^{-4}$ ). For the GO biological function (Table 32), gene involved in phototropism were overrepresented, as well as genes involved in seed coat n in relation to pigmentation and anthocyanin accumulation and in innate immune response and ion transport. A second analysis was



**Table 24.** Gene Ontology (GO) enrichment analysis of transcripts DEG that increased during imbibition of R9 seeds but remained low in stage R7.2 during imbibition (profile G of figure 18). Analysis was performed on the 147 genes using SoyBase, with annotations for Wm82.a2.v1 (Glyma 2.0). #Genome: Genome Count present in this GO category; #Expressed: Number of submitted genes present in this GO category.

GO_id	#Genome	# Expressed	p-value	GO_description
GO:0048519	139	12	4,59E-07	negative regulation of biological process
GO:0048439	191	13	1,74E-06	flower morphogenesis
GO:0010051	193	12	1,85E-05	xylem and phloem pattern formation
GO:0009887	289	14	3,30E-05	organ morphogenesis
GO:0010358	19	5	1,13E-04	leaf shaping
GO:0009855	326	14	1,42E-04	determination of bilateral symmetry
GO:0009944	203	10	1,96E-03	polarity specification of adaxial/abaxial axis
GO:0009886	79	6	1,41E-02	post-embryonic morphogenesis
GO:0048507	119	7	1,67E-02	meristem development
GO:0048826	12	3	3,72E-02	cotyledon morphogenesis

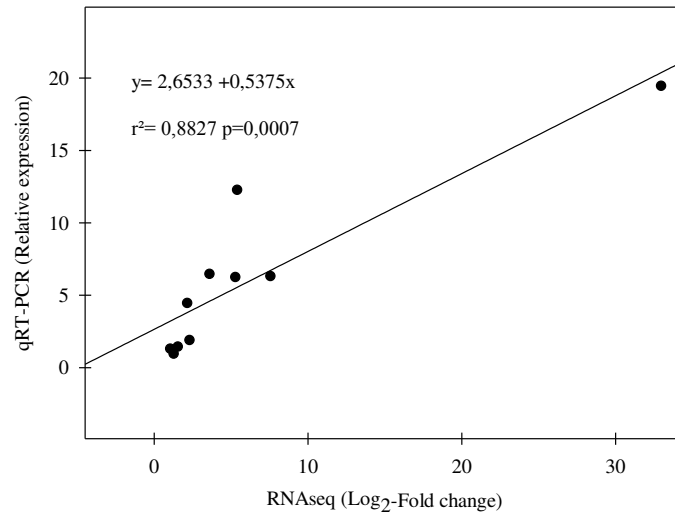
**Table 25.** Relative expression by RNA seq (Log<sub>2</sub>-Fold change) transcription factors of selected for validation.

Id G. max	Name (Soybase)	Log <sub>2</sub> R7.2 0h	Log <sub>2</sub> R7.2 18h	Log <sub>2</sub> R9 0h	Log <sub>2</sub> R9 18h
Glyma.14G047000	Class I KNOX homeobox transcription factor	2,41	3,53	1,80	6,84
Glyma.03G224700	WRKY transcription factor	4,24	4,34	4,26	6,70
Glyma.10G066100	HSF Heat stress transcription factor A3	6,06	6,70	7,58	9,44
Glyma.14G217700	Auxin response factor	2,52	3,74	3,16	6,08
Glyma.02G200500	ABI3L domain class transcription factor	5,57	5,93	5,51	7,91

performed using the curated gene sets. This revealed a significant overrepresentation of genes that were detected as down-regulated by light in hypocotyl, cotyledon and roots of seedlings. The GO enrichment analysis also showed 18 genes associated with seed coat development and 113 genes associated with seed coat pigmentation (Table 33).

### **III.5.3.2 Analysis of genes with lower transcript levels in mature legume seeds during imbibition versus immature.**

There are 52 DEG transcripts down-regulated during legume seed imbibition (Supplementary Table 6). The detailed analysis of this list revealed four members of the "Cupin family protein"; two members "HSP20-like chaperones superfamily protein"; one "Raffinose synthase family protein" The Table 33 shows the main GO terms and includes "Raffinose family oligosaccharide biosynthetic process, myo-inositol transport" and secondary metabolism "Phenylpropanoid metabolic process"; "Response to wounding", "flavonoid biosynthetic process". Also GA metabolism appear to be significantly over-represented.

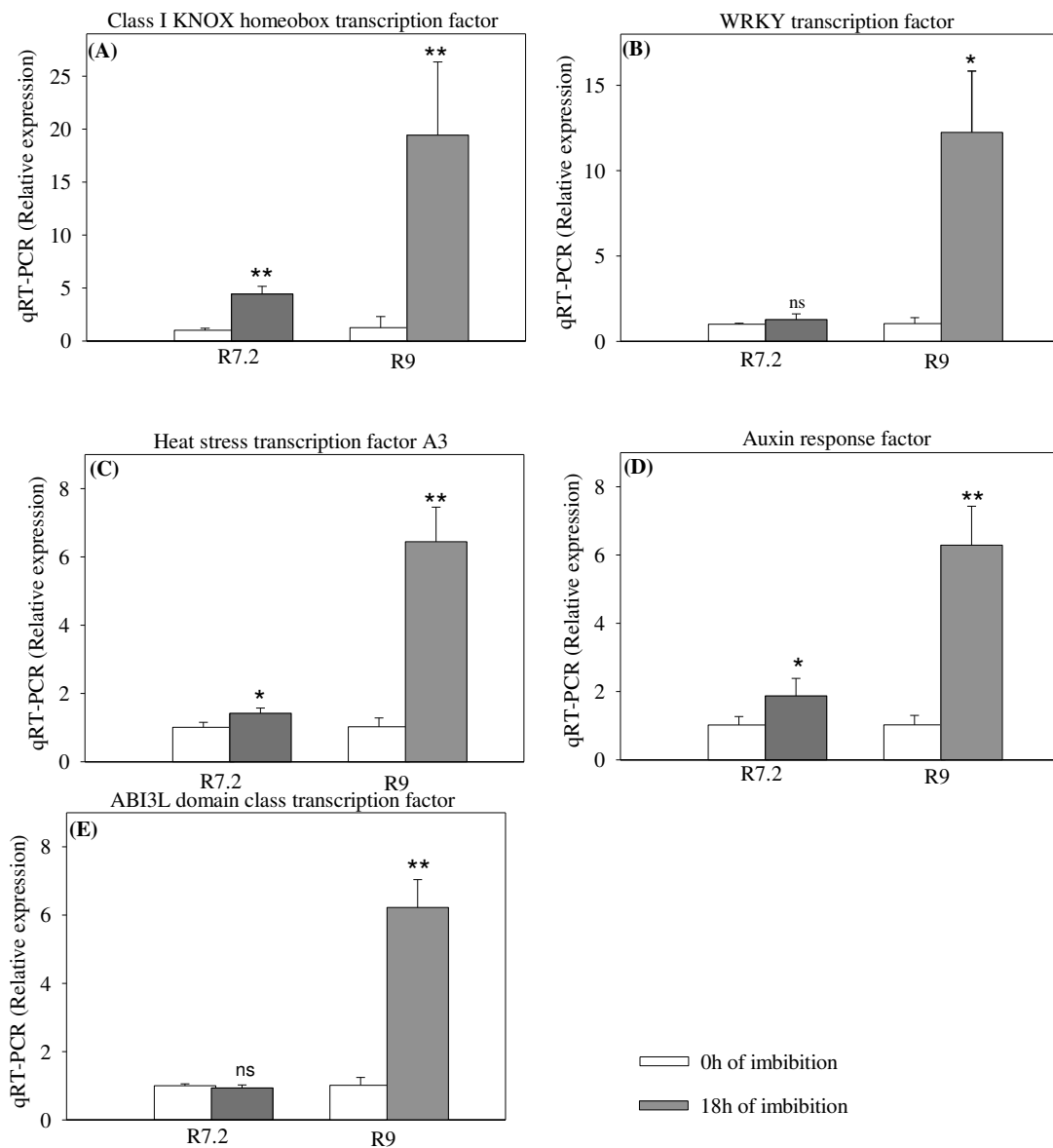


**Figure 21.** Correlation between RNaseq and quantitative real-time PCR (RT-qPCR). Comparison of Log<sub>2</sub> fold change of 5 genes obtained by RNA-seq and RT-qPCR. Real-time PCR was performed using three biological repetitions in duplicate cDNA from each RNA-seq sample. Statistically significant Pearson correlation is shown between the expression levels measured using real-time PCR and RNA-seq.

### **III.5.4 Abscisic acid and Gibberellin signaling and metabolism during legume imbibition**

During immature and mature seeds imbibition, we found genes related to signaling and metabolism of ABA and GA in our transcriptome. In relation to ABA, we found a higher number of transcript associated with signaling than for the metabolism. However, two abscisic acid 8'-hydroxylase associated with ABA metabolism were highly expressed in both stages (mature and immature seeds). The major ABA catabolic pathway is catalysed by ABA 8'-hydroxylase. This enzyme was shown to be a cytochrome P450 CYP707A family. In plants, P450s participate in numerous metabolic processes including phytohormone biosynthesis and catabolism, and secondary metabolism (SCHULER, 1996). In addition, our transcriptome data showed five transcripts that were down regulated and are involved in ABA metabolism. Among those there Zeaxanthin epoxidase were detected. This gene encodes for an enzyme that catalyzes the conversion of zeaxanthin to violaxanthin, a key reaction for ABA biosynthesis. In plants, the level of abscisic acid is controlled by the balance between its biosynthesis and catabolism. Thus, our data indicated that ABA levels in soybean seed during maturation is controlled by ABA catabolism as well as absence or reduction of its synthesis due to the down regulation of genes associated with ABA synthesis.

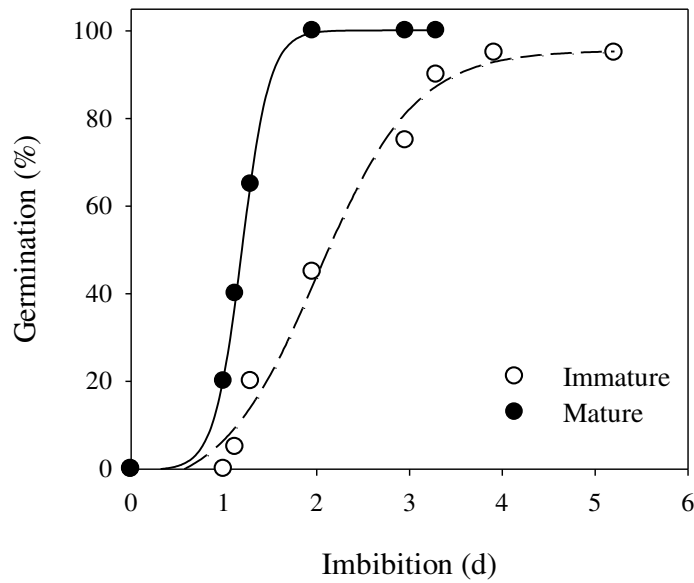
For ABA signaling six transcripts were up regulated referred to as abscisic acid response element binding (AREB3) or ABA-responsive promoter element(ABRE) binding factor, which controls response to ABA in seeds(FINKELSTEIN and LYNCH, 2002). The ABA responsive element found in this work interacts with BZIP transcription factor described for *Medicago truncatula* and *Arabidopsis thaliana* as an import group of transcription factor involved in regulation of processes including pathogen defense, light and stress signaling, seed maturation and flower development (JACOBY et al. 2002). On the other hand, a group of protein phosphatase type 2C is down regulated. The protein phosphatase is important in the phosphorylation of transcription factor such as BZIP transcription factor allowing response of the seed to ABA. Thus, the lower expression of this enzyme may have an effect on the stress response that is expected in soybean seed during seed maturation.



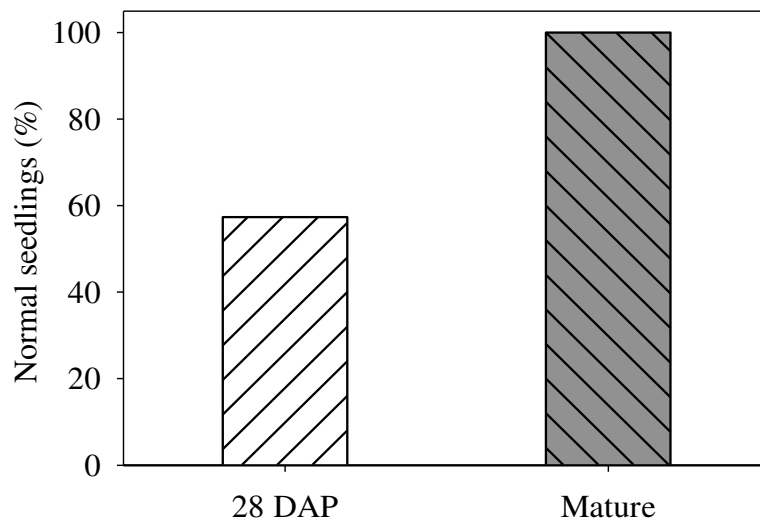
**Figure 22.** Relative expression levels (RT-qPCR) of indicated transcription factors being upregulated during imbibition. Gene identification number: (A) Glyma.14G047000; (B) Glyma.03G224700; (C) Glyma.10G066100; (D) Glyma.14G217700; (E) Glyma.02G200500. Samples were collected of stage R7.2 and R9. From control was used seeds not imbibition at each stage. Each column represents the average data with standard errors from three biological repetitions. The data were analyzed using the Relative expression software tool (REST 2009 –Qiagen) and differences were considered: ns, no significant; \*\* significant p-value < 0.01 and \* significant p-value < 0.05.

For the GA metabolism one Gibberellin 20-oxidase 2 and two Gibberellin 3-oxidase 1 were highly expressed in both mature and immature soybean seeds. These transcripts encode for two major enzymes involved in GA biosynthesis. The GA 20 oxidase is involved in the biosynthesis of GA<sub>20</sub> and GA 3-oxidase is associated with the synthesis of GA<sub>1</sub>. The GA<sub>1</sub> is considered the biologically active form of GA in plants that control plant growth. However, we also found two Gibberellin 2-beta-dioxygenase and two Gibberellin 2-oxidase that were down regulated in immature and mature soybean seeds. These transcripts are associated with the inactivation of GA<sub>1</sub>. Thus, our results indicate that GA metabolism is very active during maturation of soybean seeds by activating and inactivating GA synthesis and its levels in the cells. For GA signaling two DELLA proteins were up regulated and another two were down regulated. DELLA proteins act as nuclear transcription regulators, acting as repressors of gibberellin signaling, by blocking the promoter region of GA-responsive genes. Their removal is necessary for GA to induce the promoter (BEWLEY et al. 2013). In addition, four GAMYB1 transcripts were found to be up regulated. The GAMYB1 is a GA-regulated MYB transcriptional regulator that plays an important role in GA signaling in cereal aleurone cells (GUBLER et al., 1995). Thus, this transcript may be associated with activation of hydrolytic enzymes.

Our next step was to analyze in detail the list of genes highly expressed of mature seeds in *Medicago truncatula* (#genes 7038) and immature seeds (# 4441) during imbibition. We noticed that for *Medicago* seeds the genes related to signaling and metabolism are present, mostly in mature seeds (Table 37), i.e., in mature seed there are genes involved in ABA metabolism such as zeaxanthin epoxidase, AREB3 and HAI2.



**Figure 23.** Germination of *Medicago truncatula* dried seeds harvested at 28DAP (immature) and mature. Data are the means of 3 replicates of 50 seeds of each stage.



**Figure 24.** Changes in percentage of normal seedlings of *Medicago truncatula* seeds harvested at 28 DAP (immature) and mature imbibed for 7d at 10°C in the dark. Data represents the average of three replications of 50 seeds. Add standard errors

## **CHAPTER IV. DISCUSSION**

### **IV.1 Characterization of acquisition of seed quality and abscisic acid quantification during soybean seed development**

The first work to characterize phenological scale of soybean plant development was proposed by Fehr and Caviness (1977). This phenological scale contains vegetative stages) and eight reproductive stages (R1, R2, R3...R8). Then an updated version was published by the same group of researchers (RITCHIE et al., 1982). In this new publication, the stage R7 was divided into R7.1 (50% yellowing of leaves and pods); R7.2 (between 50 and 75%) and R7.3 (above 75%). The R9 stage was proposed to describe the point of field maturation or harvest maturity, which happens when the seeds water content reaches approximately 12%. Since the scale proposed by Ritchie et al (1982) mostly consider changes in the plant, leaf and pod (Table 4) we further improved this scale by adding seed trait information like seed size and of the pod, changes in color of the cotyledons and embryonic axis, as well as changes of the hilum and pod color (Figure 6).

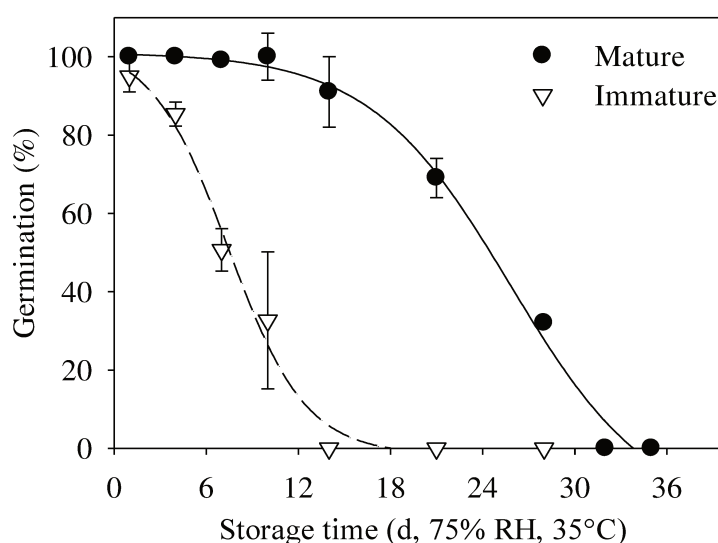
During the development, germinability was acquired early, between R5.5 and R6. At stage R7.2, the seed water content decreased steadily until stage R9. Therefore, the maturation drying was considered to be from stage R7.2 to R9 since dry weight did not change significantly. The slow dehydration during the maturation process is necessary to produce viable soybean seeds, to result in chlorophyll degradation, and to allow genome changes to produce specific enzymes that act in germination process (ADAMS et al., 1982).

The abscisic acid was also quantified and we found a peak at stage R6, shortly before the induction of desiccation tolerance. However, whether this peak is necessary to induce desiccation tolerance remains to be investigated. Similarly, a decrease in the rate of dry weight accumulation was associated with a sharp decline in ABA concentration. Abscisic acid acts at different stages along the formation and maturation of the seed. In the early embryogenesis stage, the ABA is produced by the maternal tissue and prevents vivipary (BEWLEY et al., 2013). During maturation, this hormone is produced by the embryonic tissue allowing protein and lipid synthesis (KERMODE 1990, GUTIERREZ et al 2007). Endogenous content in the seed undergoes considerable reduction after reserve accumulation and the beginning of the dehydration of the seed



**Table 26.** Speed of germination (T50, days) of immature (28DAP) and mature *Medicago truncatula* seeds during imbibition at 10°C and 20°C, in the dark.

Temperature	T50 (d)	
	Immature (28DAP)	Mature
10°C	5,0	2,9
20°C	2,1	1,2
10°C /20°C	2,4	2,4



**Figure 25.** Survival curves of immature (28 DAP) and mature *Medicago truncatula* seeds showing a decrease in percentage of germination during storage at 35°C and 75% relative humidity (RH). Germination was tested at different storage time intervals using three replicates of 50 seeds. Error bars represent standard deviation.

**Table 27.** Number of differential transcripts between immature dried and mature seeds of *M. truncatula* ranked according to the level of expression calculated on a  $\text{Log}_2$  ratio. Mat: for mature seeds; Imm d: for immature dried seeds.

	$\text{Log}_2(\text{ratio})$							
	> 1	> 2	> 3	> 4	< -1	< -2	< -3	< -4
Mat 0h/ Imm d 0h	640	72	7	1	1788	272	20	0

(BEWLEY and BLACK 1994). Thus, it is clear the importance and participation of ABA in seed formation. In addition to inhibiting germination, ABA also plays a role on desiccation tolerance and on the synthesis of late maturation proteins. According to Schussler et al. (1984), ABA may be involved in the stimulation of rapid unloading of sucrose into the testa of soybean and ABA in the cotyledons may enhance sucrose uptake by the cotyledons. Liu et al. (2010) reported that ABA concentration in seed was positively correlated with seed-filling rate.

Interestingly, we found that the point where soybeans reach highest longevity occurred after the point of physiological maturity. We observed that soybean seeds need a step of further drying before harvest to achieve high longevity . longevity is defined as the total time-span during which seeds remain viable, therefore, longevity is taken into account to define seed vigor. The time between the seed harvest and subsequent sowing may be a few days or extend over several months, according to cultivar, place of production, the prevailing environmental conditions and technology production. Therefore, it is extremely important for soybean producers have the assurance that the maximum longevity was acquired.

The physiological characterization shows that there is no phenotypic difference in germination, seedling emergence and seed vigor between stages R7.2 and R9, suggesting that physiological maturity in terms of seedling emergence was reached at R7.2 and not at stage R7, as previously proposed. Thus, the stage R7 is not sufficient to describe the acquisition of germination, desiccation tolerance and vigor. Our results are consistent with those proposed by Zanakis et al. (1994), confirmed that the late maturation and dehydration phase, after physiological maturity are important phases of soybean seed development when several quality components are acquired such as desiccation tolerance, vigor and longevity.

The identification of physiological maturity point during seed development remains controversial among researchers, by having two schools of thoughts. The 'physiological maturity' has been defined by some as the point during development when seed dry weight has reached its maximum value (TEKRONY and EGLY, 1997) and which no longer occur significant increases in dry weight of the seeds (ELLIS and PIETA FILHO, 1992). Ellis and Pietá Filho (1992), reinforce that the term 'physiological maturity' must be used to refer the point of maximum physiological quality, while that for the maximum dry weight must be used as 'mass maturity'. Whereas that for some species, the

**Table 28.** List of d up-regulated DEG in soybean and Medicago truncatula during seed maturation. NA, not applicable

Probe_id	Seq_id Mt4.0v1	Arabidopsis	p-value	id Gmax	Tax Hit	p-value	Name Arabidopsis gene
Medtr_v1_002024	AC233577_31_1	AT5G03860	0,00E+00	Glyma.17G128000	I1MUM7_SOYBN	0	malate synthase
Medtr_v1_006650	MEDTR1G085160_1	AT1G09570	0,00E+00	Glyma.10G141400	B5U9F5_SOYBN	0	phytochrome A
Medtr_v1_006650	MEDTR1G085160_1	AT1G09570	0,00E+00	Glyma.19G224200	C1PHB8_SOYBN	0	phytochrome A
Medtr_v1_006650	MEDTR1G085160_1	AT1G09570	0,00E+00	Glyma.20G090000	B4YB07_SOYBN	0	phytochrome A
Medtr_v1_077941	IMGAlcontig_68627_1.1	AT5G27950	0,00E+00	Glyma.20G229600	U7E2V5_POPTR	0	P-loop containing nucleoside triphosphate hydrolases superfamily protein
Medtr_v1_004695	MEDTR1G019890_1	AT4G24620	3,60E-01	Glyma.02G212600	I1JGY5_SOYBN	0	phosphoglucose isomerase 1
Medtr_v1_006290	MEDTR1G079370_1	AT2G02360	4,00E-67	Glyma.10G169300	T2DNH1_PHAVU	6,00E-113	phloem protein 2-B10
Medtr_v1_018521	MEDTR3G085440_1	AT1G27920	0,00E+00	Glyma.11G249900	G7JC01_MEDTR	0	microtubule-associated protein 65-8
Medtr_v1_017850	MEDTR3G072830_1	AT2G41705	1,00E-119	Glyma.04G252000	G7J3J9_MEDTR	0	camphor resistance CrcB family protein
Medtr_v1_071574	IMGAlcontig_8315_1.1	AT1G53210	0,00E+00	Glyma.12G228000	E5GBG6_CUCME	1,00E-153	sodium/calcium exchanger family protein / calcium-binding EF hand family protein
Medtr_v1_071574	IMGAlcontig_8315_1.1	AT1G53210	0,00E+00	Glyma.13G272000	E5GBG6_CUCME	0	sodium/calcium exchanger family protein / calcium-binding EF hand family protein
Medtr_v1_077815	IMGAlcontig_58561_1.1	AT2G39970	1,00E-110	Glyma.11G227400	G7KBX5_MEDTR	6,00E-169	Mitochondrial substrate carrier family protein
Medtr_v1_024414	MEDTR4G062100_1	AT1G07710	0,00E+00	Glyma.U033200	G7JSN3_MEDTR	0	Ankyrin repeat family protein
Medtr_v1_024414	MEDTR4G062100_1	AT1G07710	0,00E+00	Glyma.12G074900	G7JSN3_MEDTR	0	Ankyrin repeat family protein
Medtr_v1_046651	MEDTR7G111060_1	AT1G02305	1,00E-154	Glyma.19G223300	T2DLX7_PHAVU	0	Cysteine proteinases superfamily protein
Medtr_v1_072024	IMGAlcontig_11361_1.1	AT5G05250	1,00E-40	Glyma.06G142900	C7IVU8_9POAL	2,00E-25	unknown protein
Medtr_v1_072024	IMGAlcontig_11361_1.1	AT5G05250	1,00E-40	Glyma.04G222500	C7IVU8_9POAL	2,00E-25	unknown protein
Medtr_v1_073748	IMGAlcontig_51360_2.1	AT2G30580	1,00E-102	Glyma.10G162800	B9H8H8_POPTR	3,00E-132	DREB2A-interacting protein 2
Medtr_v1_073371	NA	AT2G46225	1,00E-100	Glyma.16G030200	G7L308_MEDTR	9,00E-148	ABI-1-like 1
Medtr_v1_073371	NA	AT2G46225	1,00E-100	Glyma.07G062200	G7L308_MEDTR	2,33E-156	ABI-1-like 1
Medtr_v1_020457	MEDTR3G114840_1	AT4G35790	0,00E+00	Glyma.11G081500	I1LI58_SOYBN	0	phospholipase D delta
Medtr_v1_011512	MEDTR2G048720_1	AT1G30220	0,00E+00	Glyma.05G142400	G7IGM5_MEDTR	0	inositol transporter 2
Medtr_v1_046086	MEDTR7G100110_1	AT1G07670	0,00E+00	Glyma.03G175200	F9W2W4_MEDTR	0	endomembrane-type CA-ATPase 4

highest physiological quality is achieved after maximum dry weight, i.e, is acquired progressively and later during development.

Between mass maturity and harvest maturity the longevity almost doubled, since P50 increased from 27 days at R 7.2 to 48 days at R9 (Figure 14). Therefore, our results confirm that seed quality characteristics are acquired sequentially when the seeds lose water and are still in the mother plant (ELLIS et al. 1987; ELLIS et al. 1993; DEBEAUJON, 2000; PROBERT, 2007; CHATELAIN et al. 2012; VERDIER et al. 2013). The increase in physiological quality after physiological maturity is observed in beans, *Brassica napus*, *arabidopsis*, tomatoes, peppers, and melons (BEWLEY et al., 2013).

An important model in the studies of seed traits is the legume species *Medicago truncatula* (GALLARDO et al., 2003; CHATELAIN et al., 2012; VERDIER et al., 2013). *M. truncatula* is closely related to economically relevant legumes like alfalfa (*Medicago sativa*), and pea (*Pisum sativum*) and can be used as a platform for physiological and molecular studies in legume species. Chatelain et al. (2012) observed that longevity (P50) in *M. truncatula* increased over 30-fold between 28DAP, corresponding to the end of seed filling and 44 DAP point of abscission, that corresponding to harvest maturity.

## **IV.2 Transcriptome analysis during soybean seed maturation**

In soybean, transcriptome studies have generated a wealth of data describing seed development, mainly during embryogenesis and filling (HAJDUCH et al., 2005; HUDSON, 2010; JONES et al., 2010, LIBAULT, 2010; SEVERIN et al., 2010; ASAKURA et al., 2012; SHA et al., 2012, SHAMIMUZZAMAM and VODKIN, 2012; AGHAMIRZAIE et al., 2013).

However, transcriptome changes associated with the acquisition of seed physiological quality remains unknown. Furthermore, how maturation drying influences the transcriptome in preparation for the quiescent dry state is unknown. Using RNAseq, we compared two late maturation stages during which seed longevity doubled whereas no other physiological traits were acquired. This revealed three main processes associated with longevity: an increase in HSP transcripts and transcripts associated with light signaling and a decrease in transcripts associated with photosynthesis. Artificial drying at

**Table 29.** Continuation...

Probe_id	Seq_id Mt4.0v1	Arabidopsis	p-value	id Gmax	Tax Hit	p-value	Arabidopsis gene Name
Medtr_v1_004109	MEDTR1G009360_1	AT4G32690	3,00E-18	Glyma.14G140400	A2TDC3_9ROSI	2,00E-104	hemoglobin 3
Medtr_v1_089457	IMGAlcontig_64154_1.1	AT4G28300	3,00E-51	Glyma.13G297200	B9IQU2_POPTTR	3,00E-153	Protein of unknown function (DUF1421)
Medtr_v1_084563	IMGAlcontig_74723_1.1	AT1G63850	0,00E+00	Glyma.08G016800	Q7X711_ORYSJ	0	BTB/POZ domain-containing protein
Medtr_v1_022917	MEDTR4G023030_1	AT2G44500	0,00E+00	Glyma.09G003300	G7JVS2_MEDTR	0	O-fucosyltransferase family protein
Medtr_v1_051083	MEDTR8G079710_1	AT5G67150	1,00E-74	Glyma.17G152600	G8A1G0_MEDTR	2,00E-161	HXXXD-type acyl-transferase family protein
Medtr_v1_010757	MEDTR2G034040_1	AT2G18790	0,00E+00	Glyma.15G140000	I1MGE5_SOYBN	0	phytochrome B
Medtr_v1_010757	MEDTR2G034040_1	AT2G18790	0,00E+00	Glyma.09G035500	B4YB10_SOYBN	0	phytochrome B
Medtr_v1_099183	NA	AT2G35110	1,00E-66	Glyma.20G019300	E3UV65_MEDTR	0	Gwarled, NCK-associated protein 1 involved in actin nucleation
Medtr_v1_023121	MEDTR4G027040_1	AT1G10170	0,00E+00	Glyma.08G055900	G7JH33_MEDTR	0	NF-X-like 1
Medtr_v1_018957	MEDTR3G091570_1	AT5G24300	0,00E+00	Glyma.06G129400	Q75T80_PHAVU	0	starch synthase 1
Medtr_v1_050784	MEDTR8G075190_1	AT4G35930	2,00E-72	Glyma.16G123900	G7LB69_MEDTR	4,00E-128	F-box family protein
Medtr_v1_077006	NA	AT2G46550	1,00E-62	Glyma.03G258600	-	NA -	unknown protein
Medtr_v1_024497	MEDTR4G064150_1	AT1G07530	1,00E-168	Glyma.11G138400	G7JUT6_MEDTR	0	SCARECROW-like 14
Medtr_v1_001224	AC225530_1_1	AT3G21090	1,00E-86	Glyma.12G020300	G7JRM5_MEDTR	0	ABC-2 type transporter family protein
Medtr_v1_093809	IMGAlcontig_163394_1.1	AT3G56640	0,00E+00	Glyma.16G084100	B9H8R1_POPTTR	0	exocyst complex component sec15A
Medtr_v1_066683	NA	AT4G17080	1,00E-116	Glyma.05G067200	B8XY96_BRARP	8,00E-49	Histone H3 K4-specific methyltransferase SET7/9 family protein
Medtr_v1_017942	MEDTR3G076820_1	AT1G23870	7,00E-17	Glyma.02G033500	G8XR07_GOSAR	0	trehalose-phosphatase/synthase 9
Medtr_v1_071318	IMGAlcontig_60598_1.1	AT4G00430	1,00E-145	Glyma.01G220600	G7KC21_MEDTR	0	plasma membrane intrinsic protein 1;4
Medtr_v1_071318	IMGAlcontig_60598_1.1	AT4G00430	1,00E-145	Glyma.11G023200	G7KC21_MEDTR	0	plasma membrane intrinsic protein 1;4
Medtr_v1_030138	MEDTR5G013480_1	AT5G41150	0,00E+00	Glyma.01G204500	G7JYL5_MEDTR	2,00E-125	Restriction endonuclease, type II-like superfamily protein
Medtr_v1_050847	MEDTR8G075940_1	AT3G13080	5,00E-91	Glyma.08G101500	M7YF87_TRIUA	0	multidrug resistance-associated protein 3

stage R7.2 was compared with natural maturation drying to check whether premature drying induced changes in the transcriptome.

In orthodox seeds, such as soybean, desiccation tolerance and maintenance of a quiescent state are associated with the presence of particular proteins such as the LEA proteins, seed storage proteins and heat shock proteins (HSPs) (RAJJOU and DEBEAUJON, 2008). Our data shows, in association with the increase in longevity, an over-representation of terms associated with the synthesis of HSP (response to heat, protein-folding, response to ER stress). Indeed, 10 transcripts encoding small HSP were found in the top 30 highly expressed genes in dry seeds (Supplementary Table S1). This result is in agreement with previous transcriptome studies on soybean (JONES et al., 2010), *Medicago truncatula* (VERDIER et al., 2013) and *Arabidopsis thaliana* (KOTAK et al., 2007) in which late seed maturation was shown to be accompanied by an increase in HSP transcript levels. Using a gene co-expression network in developing *Medicago* seeds, Verdier et al (2013) found a module in which HSP were associated with longevity. It is likely that the proteins encoded by these genes are also synthesized because small HSP were found in the proteome in developing *Arabidopsis* seeds during late seed maturation (WEHMEYER and VIERLING, 2000). The role of HSP in protecting seeds against seed ageing was also demonstrated by Tejedor-Cano et al. (2010) who showed that a drastic reduction of small HSPs in transgenic tobacco seeds led to decreased stability during storage. Kaur et al (2015) revealed that small HSPs (OsHSP18.2 class II cytosolic HSP) have the ability to improve seed vigor and longevity by reducing deleterious ROS accumulation in *Arabidopsis* seeds. Interestingly Bettey et al (1988) reported that the amount of HSP correlated with seed germination performance in *Brassica*. Therefore, it would be interesting to evaluate whether the amount of HSP could be a marker for seed longevity in soybean.

Our transcriptome revealed also the presence of HSF, a family of transcription factors known to modulate the expression of HSP coding genes, such as two homologues of HSFA2 and one of HSFA6B. Seed-specific overexpression of the sunflower (*Helianthus annuus*) HSF HaHSFA9 in tobacco (*Nicotiana tabacum*) led to increased stability against accelerated aging of seeds demonstrating the importance of such factors in seed longevity (PRIETO-DAPENA et al., 2006; PERSONAT et al., 2014). This transcription factor also interacts with the sunflower drought-responsive factor HaDREB2 (DEHYDRATION RESPONSIVE ELEMENT BINDING PROTEIN B2) in a seed-

**Table 29.** Gene Ontology (GO) enrichment analysis of transcripts DEG down- regulated during legume seed maturation. Analysis was performed on the 223 genes using PlantGSEA, with annotations for Arabidopsis AGI number.

Gene Set Name	# genes in genome	GO_Description	Genes in Overlap	p-value	FDR
Response to stimulus	6222	GO:0050896	103	1.83e-18	3.74e-15
Response to stress	4037	GO:0006950	78	1.01e-18	2.76e-15
Response to chemical stimulus	3953	GO:0042221	74	1.36e-16	1.39e-13
Response to abiotic stimulus	2615	GO:0009628	56	4.41e-16	4.01e-13
Response to organic substance	2739	GO:0010033	52	7.58e-12	3.45e-09
Response to biotic stimulus	1675	GO:0009607	37	2.83e-11	9.64e-09
Cellular response to stimulus	2302	GO:0051716	37	4.88e-06	6.31e-4
Defense response	1644	GO:0006952	35	5.18e-10	1.46e-07
Multi-organism process	1804	GO:0051704	35	1.96e-08	4.23e-06
Response to hormone stimulus	1364	GO:0009725	34	9.39e-13	4.8e-10
Response to other organism	1411	GO:0051707	32	2.84e-10	8.6e-08
Cellular response to chemical stimulus	1403	GO:0070887	27	1.48e-06	2.34e-4
Response to temperature stimulus	955	GO:0009266	26	2.45e-11	8.7e-09
Cellular nitrogen compound metabolic process	1369	GO:0034641	26	3.47e-06	4.78e-4
Response to abscisic acid stimulus	618	GO:0009737	23	9.45e-16	7.74e-13
Response to carbohydrate stimulus	811	GO:0009743	22	1.24e-09	3.18e-07
Amine metabolic process	1030	GO:0009308	21	7.95e-06	9.72e-4
Response to fungus	497	GO:0009620	17	1.63e-10	5.14e-08
Response to chitin	421	GO:0010200	16	2.11e-11	7.85e-09
Response to jasmonic acid stimulus	471	GO:0009753	15	1.74e-08	4.07e-06
Hormone-mediated signaling pathway	589	GO:0009755	15	3.5e-06	4.78e-4
Response to wounding	340	GO:0009611	14	4.17e-11	1.37e-08
Hyperosmotic response	250	GO:0006972	12	1.6e-11	6.23e-09
Response to heat	303	GO:0009408	12	3.93e-09	9.75e-07
Response to ethylene stimulus	353	GO:0009723	12	1.59e-07	3.1e-05
Defense response to fungus	342	GO:0050832	11	1.82e-06	2.81e-4

specific manner to enhance stability against accelerated aging (ALMOGUERA et al., 2009). Likewise in the legume *M. truncatula*, several members of the AP2/EREBP family were co-expressed with HSF in the longevity module (VERDIER et al. 2013). Our soybean transcriptome also showed several members of the AP/EREBP being differentially up and down-regulated indicating that different members of this family might be required to regulate gene expression during soybean seed development. In *Arabidopsis*, ABI3 induces HSFA9 (KOTAK et al., 2007). Here, ABA was found to peak much mid-way through seed development shortly before the induction of desiccation tolerance. However, whether this peak is necessary to induce HSF remains to be investigated. More work is needed to understand the signaling pathways inducing HSF and longevity during maturation

Although the role of ABA in seed longevity is supported by numerous studies (OOMS et al. 1993; PARCY et al. 1994; MAO and SUN 2015) the implication of other hormones is less well documented. Auxin acts downstream of ABI3 to enhance seed longevity by alleviating HaIAA27-mediated HaHSFA9 repression in sunflower (CARRANCO et al. 2010). Longevity genes in *M. truncatula* were found to be enriched in binding sites for auxin-binding factors (RIGHETTI et al. 2015), implying that auxin also may have a role in seed longevity. Consistent with these observations, we found three members of the Auxin Responsive Factor family being up-regulated (homologues of *Arabidopsis* ARF19, ARF4 and ARF8) while none was found to be significantly down-regulated. Also, all members of AUX/IAA transcription factors were down-regulated or no significantly differentially expressed during the increase in seed longevity.

Besides the synthesis of HSP and the putative role of HSFs, our transcriptome raises the question whether red light influences seed longevity. We observed a high number of categories associated to “far-red light photoreceptor activity”, to “response to very low fluence”, to “red light stimulus”, to “photoreceptor activity” were up-regulated during the increase in longevity. A closer inspection of genes belonging to these categories revealed the presence of phytochrome A and PIF homologues (PIF7, Photoreceptor interacting factors). In our data, when mature and immature soybean seeds were imbibed in light and dark conditions, no difference in seed germination and hypocotyl elongation was found between mature and immature seeds (Figure 15) suggesting that regulatory factors controlling skotomorphogenesis and de-etiolation via the phytochromes are identical between stage R7.2 and R9. Flowering and maturity is highly controlled by



**Table 30.** Continuation ...

Gene Set Name	# genes in genome	GO_Description	Genes in Overlap	p-value	FDR
Two-component signal transduction system (phosphorelay)	169	GO:0000160	10	3.12e-12	1.5e-09
Abscisic acid mediated signaling pathway	252	GO:0009738	10	1.05e-07	2.1e-05
Cellular response to abscisic acid stimulus	267	GO:0071215	10	3.52e-07	6.54e-05
Intracellular signal transduction	222	GO:0035556	9	3.69e-07	6.71e-05
Jasmonic acid biosynthetic process	135	GO:0009695	7	1.91e-07	3.63e-05
Chlorophyll catabolic process	58	GO:0015996	4	1.08e-05	1.25e-3

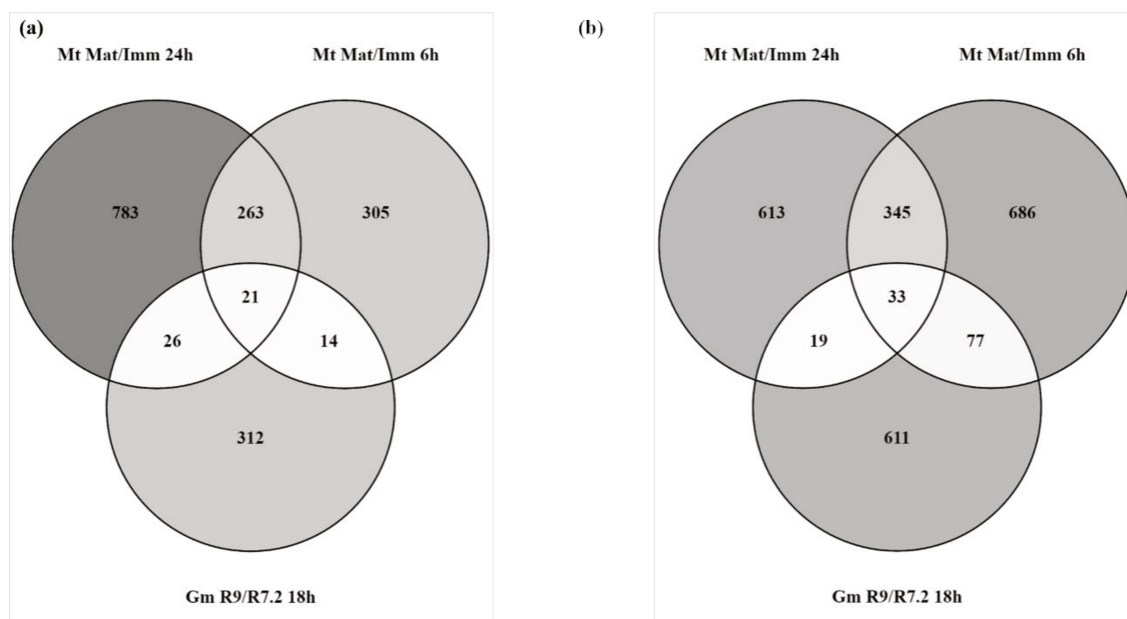
**Table 30.** Number of differential transcripts between immature dried (Imm d) and mature (Mat) seeds ranked according to the level of expression calculated on a Log<sub>2</sub> ratio. Time of imbibition: 0, 6 and 24 h in water at 20° C in the dark.

	Log <sub>2</sub> (ratio)							
	> 1	> 2	> 3	> 4	< -1	< - 2	< - 3	< -4
Mat 0h/ Imm d 0h	640	72	7	1	1788	272	20	0
Mat 6h/ Imm d 6h	811	109	16	1	1502	271	25	6
Mat 24h/ Imm d 24h	1428	352	58	2	1375	116	6	0
Mat 24h/ Mat 0h	7038	3629	1473	351	5267	1710	440	59
Im d 24h/ Imm d 0h	4441	1161	366	90	4690	1307	318	59

major genes, in soybean two of them correspond two paralogous genes encoding a PhyA-like photoreceptor and strongly controls flowering time and time to physiological maturity according to the photoperiod (LIU et al., 2008). Therefore, our transcriptome could be showing a remnant of such behavior. Gene expression in developing soybean seeds is also controlled by the circadian clock with an organ-specific phasing, which could influence the expression of light receptors (HUDSON et al., 2010). However, in our experiments, the seed material was always harvested rigorously at the same time of the day to avoid artifacts due to the operation of the circadian clock.

Developing seeds might respond to light cues such as photoperiod and light quality differently according to the stage of maturation. It is known that the light penetrating the fruit tissues in pea and soybean that reach the embryo is predominantly in the far red region (ALLEN et al., 2009). Interestingly, in lettuce seed longevity as assessed by accelerated aging was higher in seeds obtained from parent plants grown under a higher proportion of far-red light, indicating that seeds did respond to light and that red light was detrimental to longevity (CONTRERAS et al., 2009). Reports on *Arabidopsis* showed that transferring plants from different light environments during seed development strongly influenced seed germination (HAYES and KLEIN, 1974). A transfer before seed maturation had no significant effect, whereas a transfer during seed maturation induced a decrease in the germination in the dark, from 78 to 45 % when the transfer was performed when seeds started to dehydrate and from 78% to 0% when seed were dry. Further work is necessary to understand the role of light perception during maturation and a putative role of PhyA in the maturation of seeds.

Approximately 68% of the transcripts expressed decreased during the increase in seed longevity. The main categories that were over-represented are associated with photosynthesis (Table 11). This might be explained by the fact that chlorophyll degradation takes place in stage R7.2 and is fully degraded by stage R9 (Figure 6). In most species, chlorophyll degradation occurs at the final of seed maturation before the seed reaches the dry state (NAKAJIMA et al., 2012; TEIXEIRA et al., 2016). Chlorophyll retention in dry oily seeds appears to be detrimental to seed longevity. Seeds of the *Arabidopsis* green-seeded (*grs*) mutant contained twice more chlorophyll than wild-type seeds and exhibited reduced storability (CLERKX et al., 2003). Likewise, seeds of the non-yellow coloring1 (*nyc1*)/*nyc1*-like (*nol*) double mutant that are affected in the conversion of chlorophyll b to chlorophyll a, the first step of chlorophyll degradation,



**Figure 26.** Venn diagrams of genes that are upregulated (A) or downregulated (B) in *Glycine max* and *Medicago truncatula* during imbibition. Gm R9/R7.2 18h: genes that are expressed significantly between immature (R7.2) and mature (R9) soybean seeds during 18h of imbibition. Mt Mat/Imm 6h or 24h: genes that are expressed significantly between immature (28DAP) and mature *M. truncatula* seeds during 6h and 24h of imbibition.

**Table 31.** Gene Ontology (GO) enrichment analysis of transcripts DEG with higher transcript levels in mature vs immature imbibed legume seeds. Analysis was performed on the 47 genes using PlantGSEA, with annotations for Arabidopsis AGI number.

Gene Set Name	# genes in genome	GO_Description	Genes in Overlap	p-value	FDR
Phototropism	13	GO:0009638	2	3.57E-21	1.77E-17
Seed coat development	18	GO:0010214	2	4.13E-16	3.42E-13
Pigmentation	113	GO:0043473	3	9.92E-08	1.70E-05
Anthocyanin accumulation in tissues in response to uv light	113	GO:0043481	3	9.92E-08	1.70E-05
Response to blue light	126	GO:0009637	3	5.52E-07	8.85E-05
Defense response	1644	GO:0006952	10	1.96E-05	2.46E-03
Tropism	166	GO:0009606	3	2.11E-05	2.56E-03
Innate immune response	926	GO:0045087	7	4.02E-05	4.65E-03
Ion transport	1016	GO:0006811	7	1.38E-04	1.32E-02

contained 10-fold more chlorophyll than wild-type and had a strongly reduced longevity (NAKAJIMA et al., 2012). However, the cause-effect relationship between longevity and chlorophyll remains elusive. It could be that the photosynthesis machinery needs to be degraded to avoid the potential damage imposed by the ROS produced by the photosynthetic electron transport chains during drying.

Although there are no differences in germination percentage between stage R7.2 and R9, it was possible to observe a variation of the transcripts according to drying process. Premature drying showed that an increase in transcripts negatively correlated with seed longevity, such as photosynthesis.

### **IV.3 Transcriptome comparisons of immature seeds dried (stage R7.2d) and mature seeds (stage R9) during imbibition**

#### **IV.3.1 Increased transcripts during imbibition**

Germination is a complex process during which the seed must quickly recover from maturation drying, resume metabolism and complete essential cellular events to allow the embryo to emerge, and prepare for subsequent seedling growth (NONOGAKI et al., 2010). In soybean seeds, the molecular events leading to these processes are far from being completely known. It has been shown that during late maturation, seeds are able to synthesize and store mRNAs. These stored mRNAs are thought to be used during seed imbibition to ensure germination (RAJJOU et al., 2004; GALLAND and RAIJOU, 2015). In our study, immature dried seeds and mature seeds did not differ in germination (Figure 12). Consistent with these observations, we found that the vast majority of transcripts in stages R7.2 and R9, representing 82% of the total DEG during imbibition, followed the same trend in both stages during imbibition, suggesting that the main factors regulating germination are probably already set in seeds at R7.2.

When imbibing seeds were compared to dried seeds and we observed an over-representation of biological processes associated with growth of different tissues (root tip, root hair, meristem, cell wall elongation; Table17). This probably reflects the activation of genes that are necessary for radicle growth during imbibition. Transcripts of these genes were very low in dried seeds regardless of the maturation stages and were not differentially expressed during drying. Therefore, it is difficult to reconcile these

**Table 33.** Gene Ontology (GO) enrichment analysis of transcripts DEG with lower transcript levels in mature vs. immature imbibed legume seeds. Analysis was performed on the 52 genes using PlantGSEA, with annotations for Arabidopsis AGI number.

Gene name	# genes in genome	GO_Description	N <sup>o</sup> . Genes in Overlap	p-value	FDR
Regulation of secondary metabolic process	157	GO:0043455	5	5.59e-15	1.68e-12
Flavonoid metabolic process	250	GO:0009812	6	9.06e-14	2.14e-11
Phenylpropanoid metabolic process	400	GO:0009698	7	1.17e-11	2.29e-09
Myo-inositol transpor	2	GO:0015798	1	1.7e-11	2.96e-09
Low affinity nitrate transport	2	GO:0080055	1	1.7e-11	2.96e-09
Flavonoid biosynthetic process	225	GO:0009813	5	1.85e-10	2.78e-08
Response to wounding	340	GO:0009611	5	7.44e-07	7.24e-05
Rna transport	5	GO:0051029	1	2.52e-06	2.24e-4
Rna export from nucleus	5	GO:0006407	1	2.52e-06	2.24e-4
Small molecule biosynthetic process	1859	GO:0044283	12	4.7e-06	3.89e-4
Dna-dependent transcription, elongation	133	GO:0006354	3	4.95e-06	4.00e-04
Gibberellin biosynthetic process	54	GO:0009686	2	1.03e-05	7.96e-4
Response to abiotic stimulus	2615	GO:0009628	14	2.42e-05	1.7e-3
Response to abscisic acid stimulus	618	GO:0009737	6	3.02e-05	2.08e-3
Polyamine metabolic process	60	GO:0006595	2	3.19e-05	2.16e-3
Gibberellin metabolic process	61	GO:0009685	2	3.78e-05	2.5e-3
Small molecule metabolic process	4053	GO:0044281	18	5.17e-05	3.02e-3
Response to temperature stimulus	955	GO:0009266	7	2.31e-4	0.0122
Generation of precursor metabolites and energy	730	GO:0006091	6	2.41e-4	0.0124
Gibberellic acid mediated signaling pathway	81	GO:0009740	2	4.69e-4	0.021
Gibberellin mediated signaling pathway	82	GO:0010476	2	5.16e-4	0.0225

observations with the idea that mRNAs are stored in the dry state to prepare the seeds for germination and, because of that there is no need for transcriptional activity. In *Arabidopsis*, 500  $\mu$ M  $\alpha$ -amanitin did not inhibit the percentages of germination (RAJJOU et al., 2004). However, it decreased the speed of germination by 22% in wild-type seeds and about 350% in several testa mutants in *Arabidopsis*. Also, treated seeds originated radicles that were not vigorous compared to untreated seeds (RAJJOU et al., 2004). There is increasing evidence that the seed coat is essential for *Arabidopsis* germination by regulating ABA and GA signaling pathway in the embryo. Many reports showed that the endosperm layer is responsible for most, if not all, of the germination-repressive activity (BETHKE et al., 2007; LEE et al., 2012; KANG, 2015). Altogether, these data on *Arabidopsis* suggest that gene activation in the envelope is necessary for germination. In soybean, the testa is dead after maturation drying (RANATHUNGE et al., 2010). Therefore, regulation of seed germination must occur within the embryo. Because of the differences we observed between the transcript profile observed in imbibing seeds and the transcripts stored in dry seeds, we speculate that in contrast to *Arabidopsis*, gene activation is necessary for soybean seed germination.

Genes involved in lipid reserve mobilization appear to be activated early during imbibition in immature seeds (R7.2) and mature seeds (stage R9). Among the top differentially expressed transcripts during imbibition (Supplementary Table 4), we found transcripts encoding Phosphoenolpyruvate carboxykinase, two probes encoding an isocitrate lyase and several probes encoding lipases whose levels increased 50-100 fold in 18h imbibed seeds. In oily seeds, ATP-dependent phosphoenolpyruvate carboxykinase plays a fundamental role in the provision of sucrose during germination and early post-germinative seedling growth. This enzyme is important because it catalyzes the conversion of oxaloacetate to phosphoenolpyruvate in the gluconeogenic production of sugars from storage oil in germinating oilseeds (RYLOTT et al 2003).

In a more detailed analysis of GO enrichment (Table 18) five probes encoding five different ethylene receptors, including an *Arabidopsis* homologue of ESR1 were found to be up-regulated. Further inspection of the transcriptome revealed the many more transcripts involved in the ethylene-signaling pathway such as several homologues of ETHYLENE-RESPONSIVE TRANSCRIPTION FACTORS (including RAP2-3), ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 3 (EREBF3), EREBF1, ETHYLENE-OVERPRODUCTION PROTEIN 1, ETHYLENE OVERPRODUCER-LIKE

**Table 33.** Continuation...

<b>Gene name</b>	<b># genes in genome</b>	<b>GO_Description</b>	<b>N° Genes in Overlap</b>	<b>p-value</b>	<b>FDR</b>
Cellular response to gibberellin stimulus	83	GO:0071370	2	5.66e-4	0.0243
Raffinose family oligosaccharide biosynthetic process	10	GO:0010325	1	6.44e-4	0.027
Glycoside biosynthetic process	215	GO:0016138	3	7.37e-4	0.0305
Response to high light intensity	224	GO:0009644	3	1.03e-3	0.041
Response to hormone stimulus	1364	GO:0009725	8	1.05e-3	0.0415

PROTEIN (ETO1 LIKE-1), a negative regulator of ethylene synthesis in Arabidopsis. We also found that several probes of ACC SYNTHASE and ACC OXIDASE, two genes involved in the biosynthesis of ethylene were also strongly up-regulated, suggesting that ethylene was produced during germination in imbibing soybean seeds. Ethylene participates in the germination of seeds, for example, lettuce and chick-pea (MATILLA and MATILLA-VAZQUEZ, 2008). Since studies using inhibitors of ethylene biosynthesis or of ethylene action and analysis of mutant lines altered in genes involved in the ethylene signaling pathway (*etr1*, *ein2*, *ain1*, *etr1*, and *erf1*) demonstrate the involvement of ethylene in the regulation of germination and dormancy (reviewed in CORBINEAU et al. 2014). Ishibashi et al (2013) confirmed that soybean seed germination was regulated through ethylene production in response to reactive oxygen species (ROS). However, apart for several transcripts encoding a L-ascorbate oxidase-like protein, our transcriptome did not reveal that seeds were submitted to ROS. Our data suggest that ethylene plays an important role in soybean seed germination.

Ethylene is known to regulate germination and dormancy by controlling GA and ABA signaling pathway (reviewed in CORBINEAU et al. 2014). GA promotes germination whereas ABA inhibits it. Consistent with this, we found several transcripts associated with GA signaling and GA biosynthesis being upregulated and ABA signaling and synthesis being down-regulated. GA-related transcripts encoded several GA-regulated proteins of unknown function, GA-20 OXIDASE and GA-3 OXIDASE and the GA receptor GID1. Down-regulated ABA related transcripts were homologues of the signaling pathway (OST1, a member of ABA activated SNF1-related protein kinases (SnRK2), a PP2CA (PROTEIN PHOSPHATASE 2CA), ABI5, APF2, a bZIP factor interacting with ABI5, and KEG involved in degrading ABI5. Further work to determine the existence of a cross-talk between ethylene, GA and ABA in the regulation of soybean seed germination are of interest. We also found several Arabidopsis homologues of the ABA signaling pathway being up-regulated during imbibition, such as several receptors of ABA (including RCAR10, REGULATORY COMPONENTS OF ABA RECEPTOR 10, PYR1, PYR1-like), ABA signaling proteins such as ABI2 and ABI5-like and an enzyme involved in ABA synthesis (ABA4). Although the ABA signaling pathway bears the same characteristics between seeds and vegetative tissues, the components are different. Therefore, we speculate that during imbibition, soybean seeds replace its seed-specific ABA signaling pathway by a vegetative-specific signaling pathways. This is supported in



**Table 32.** List of up-regulated DEG involved in metabolism and signaling of ABA and GA during imbibition of soybean seeds. Immature seeds (I), mature seeds (M) or both stages (B).

Process involved	G.max 2.0 ID	Tax Hit	p-value	Soybase gene name	Arabidopsis	Arabidopsis gene name	p-value	Presence
ABA metabolism	Glyma.16G076600	Q0H212_PHAVU	0	Abscisic acid 8'-hydroxylase	AT4g19230	CYP707A1	0	B
	Glyma.01G153300	B0L642_SOYBN	0	Abscisic acid 8'-hydroxylase	AT4g19230	CYP707A1	0	B
ABA signalling	Glyma.19G122800	Q2HUH2_MEDTR	3,00E-143	AREB3	AT3g56850	AREB3	1,00E-102	B
	Glyma.03G003400	Q2HUH2_MEDTR	2,00E-141	AREB3	AT3g56850	AREB3	2,00E-103	M
	Glyma.05G079800	Q2HUH2_MEDTR	2,00E-124	AREB3	AT3g56850	AREB3	4,00E-90	M
	Glyma.19G067900	Q2HUH2_MEDTR	7,00E-127	AREB3	AT3g56850	AREB3	8,00E-98	M
	Glyma.20G049200	G7K7P6_MEDTR	9,00E-113	AREB3	AT3g56850	AREB3	1,00E-59	B
	Glyma.06G314400	D9ZIQ3_MALDO	7,00E-166	AREB3	AT3g56850	AREB3	1,00E-91	M
GA metabolism	Glyma.09G149200	O04280_PHAVU	0	Gibberellin 20-oxidase 2	AT4g25420	GA20OX2	0	B
	Glyma.13G361700	G3OX_PEA	0	Gibberellin 3-oxidase 1	AT1g15550	GA3OX1	1,00E-149	B
	Glyma.15G012100	G3OX_PEA	0	Gibberellin 3-oxidase 1	AT1G15550	GA3OX1	6,00E-150	B
GA signaling	Glyma.04G150500	Q0HA68_MALDO	0	DELLA protein	AT1g14920	GAI	3,00E-158	M
	Glyma.11G216500	A5HVE5_PHAVU	0	DELLA protein	AT3g03450	RGA-Like2	0	B
	Glyma.06G312900	I6LZP2_SOYBN	0	GAMYB1 protein	AT3g11440	MYB65	4,00E-102	B
	Glyma.04G125700	I6LZP2_SOYBN	0	GAMYB1 protein	AT5g06100	MYB33	2,00E-102	B
	Glyma.13G187500	I6LZP2_SOYBN	0	GAMYB1 protein	AT5g06100	MYB33	9,00E-120	B
	Glyma.15G225300	I6LZP2_SOYBN	0	GAMYB1 protein	AT5g06100	MYB33	3,00E-118	B

our transcriptome by the upregulation of several CALCIUM-DEPENDENT PROTEIN KINASE (CPK2, CPK13, CDPK-RELATED KINASE 1).

Another feature of the transcriptome of germinating soybean seeds is the over-representation of processes related to organ formation, in particular in relation to light (red, far-red and blue), and meristem activity, such as flower morphogenesis, organ morphogenesis, cotyledon morphogenesis. Many of these transcripts were related to auxin signaling and auxin transport such as AUXIN RESPONSE FACTOR 5 (ARF5, Monopteros, involved in reprogramming cell identity during embryonic development, ARF8, auxin efflux carriers (PIN1 and PIN3). It also contained a homologue of HB-3, a WUS type homeodomain protein that is required for meristem growth and development and acts through positive regulation of WUS. This is intriguing, considering that embryogenesis has terminated before stage R5. Also, whole seeds were used for RNAseq, with the effect of strongly diluting transcripts that are specific to meristems. Therefore, these genes may play additional auxin-related roles as suggested by the Arabidopsis TAIR annotation. Auxin by itself is not necessary for seed germination but may participate to the fine-tuning different aspects of cellular growth necessary for germination in relation with environmental cues such as light, gravitropism. For example, in Arabidopsis, AUXIN RESPONSE FACTOR10 (LIU et al., 2013) and ARF 13 regulate germination by controlling the expression of ABI3 during imbibition. Whether the identified genes in soybean play similar role remains to be determined.

Intriguingly, we found several transcription factors involved in similar process related to organ formation and meristem activity, which were significantly up-regulated in mature seeds but not in immature seeds during imbibition. This is the only profiles containing a significant enrichment of TF (Table 21). This includes a Class I KNOX box homeobox factor whose homolog in Arabidopsis is STM/BUM1. This TF is required for shoot apical meristem (SAM) formation during embryogenesis and for SAM function throughout the lifetime of the plant. We also noted the presence of GRAS-type TF, whose homologue is SGR7, (SHOOT GRAVITROPISM 7) and is essential for normal shoot gravitropism. There was also a transcript annotated as ABI3-like. In Arabidopsis, this AP2/B3-like transcriptional factor family is apparently not related to ABA signaling. Expressed in the lateral organ boundary region, it operates in a network of regulatory genes controlling leaf serration being all these processes regulated by auxin fluxes.

**Table 33.** List of down-regulated DEG involved in metabolism and signaling of ABA and GA during imbibition of soybean seeds. Immature seeds (I), mature seeds (M) or both stages (B).

Process involved	G.max 2.0 ID	Tax Hit	p-value	Soybase gene name	Arabidopsis	Arabidopsis gene name	p-value	Presence
ABA metabolism	Glyma.01G186200	I1LHE5_SOYBN	0	Zeaxanthin epoxidase, chloroplastic	At5g67030		0	B
	Glyma.11G055700	I1LHE5_SOYBN	0	Zeaxanthin epoxidase, chloroplastic	At5g67030		0	B
	Glyma.17G174500	I1MVX7_SOYBN	0	Zeaxanthin epoxidase, chloroplastic	At5g67030		0	I
	Glyma.15G250100	NCED1_PHAVU	0	9-cis-epoxycarotenoid dioxygenase NCED1	At3g14440	NCED3	0	I
	Glyma.09G282900	H2BIS8_CITSI	0	ABA 8'-hydroxylase	At2g29090	CYP707A2	0	B
ABA signaling	Glyma.14G162100	Q9ZPL9_LOTJA	7,00E-173	Protein phosphatase type 2C	At2g29380	Highly ABA induced PP2C gene3	5,00E-84	B
	Glyma.01G225100	G7KAL2_MEDTR	9,00E-144	Protein phosphatase 2C	At2t29380		7,00E-83	B
	Glyma.11G018000	G7KAL2_MEDTR	2,00E-148	Protein phosphatase 2C	At2g29380		7,00E-81	B
	Glyma.19G069200	Q9ZPL9_LOTJA	4,00E-164	Protein phosphatase type 2C	At1g07430	ABA-induced PP2C gene2, HON, HONSU	3,00E-85	I
GA signaling	Glyma.10G190200	Q0HA68_MALDO	0	DELLA protein	At1g14920	GAI	8,00E-151	B
	Glyma.20G200500	Q0HA68_MALDO	0	DELLA protein	At1g14920	GAI	2,00E-137	B
GA metabolism	Glyma.12G029800	G7K109_MEDTR	2,00E-120	Gibberellin 2-beta-dioxygenase	At4g21200	GA2OX8	3,00E-119	B
	Glyma.13G218200	G2OX_PHACN	0	Gibberellin 2-beta-dioxygenase	At1g30040	GA2OX2	2,00E-153	I
	Glyma.15G093900	G2OX_PHACN	0	Gibberellin 2-oxidase	At1g30040	GA2OX2	1,00E-154	B
	Glyma.13G259400	Q4W8C3_PHAAN	0	Gibberellin 2-oxidase	At1g78440	GA2OX1	6,00E-130	B

### **IV.3.2 Decreased transcripts during imbibition**

Transcript associated with phytate synthesis disappeared rapidly during imbibition. The phytic acid (myo-inositol-1-phosphate) is the main source of phosphorus stored in seeds during development (RABOY, 1987). In soybean seeds, phytic acid is deposited in protein bodies as a complex of chelated minerals and proteins known as phytin (PRATTLEY and STANLEY, 1982). Phytin represents approximately 1% of the total weight in dry seeds (HEGEMAN and GRABAU, 2001). Adequate levels of phosphorus are critical to the growth and development of all organisms for a range of functions such as macromolecular structure, energy generation, and metabolic regulation. While the demand for phosphorus increases dramatically during periods of rapid cell growth and division, such as seed germination (HEGEMAN and GRABAU, 2001), we found no evidence in our transcriptome of an activation of genes involved in phytin degradation. Probably, this occurs later after germination.

In the next step, we analyzed differential expression profiles during imbibition. In a more detailed inspection in profile L (Figure 18, Table 23) we noted that this profile contained mainly chloroplastic genes involved in transcription and elongation with the chloroplasts. These include ribosomal genes, genes encoding proteins functioning in the photosystem1 and electron transport chain. This shows that these transcripts were probably not degraded during maturation drying. Paradoxically, while chloroplasts will be resynthesized later after germination during the greening process, left-over transcripts associated with photosynthesis are first degraded even if they could be reused later. This is stressed by the up-regulation of transcripts encoding photosynthesis genes during imbibition of R9 seeds (such as PHOTOSYSTEM II SUBUNIT Q-2, PHOTOSYSTEM II REACTION CENTER PSB29 PROTEIN, PHOTOSYSTEM I LIGHT HARVESTING COMPLEX GENE 3 PHOTOSYSTEM I SUBUNIT PSAD). The same line of argument applies for transcripts encoding LEA and HSP proteins that are dramatically decreasing during imbibition, while it is known that osmotic stress reducing desiccation tolerance or priming induces the accumulation of these proteins (BOUDET et al., 2006, BUITINK et al., 2006).

**Table 34.** List of up-regulated DEG involved in metabolism and signaling of ABA and GA during imbibition of *Medicago truncatula* seeds. Immature seeds (I) or mature seeds (M).

Process involved	Probe_id	Seq_id Mt 4.0 v1	Arabidopsis	Arabidopsis gene Name	p-value	Presence
ABA metabolism	Medtr_v1_031175	MEDTR5G025610_1	AT4G19230	CYP707A10	0	M
	Medtr_v1_050546	MEDTR8G072260_1	AT4G19230	CYP707A1;	0	M
	Medtr_v1_031145	MEDTR5G025270_1	AT4G19170	nine-cis-epoxycarotenoid dioxygenase 4	0	I
ABA signaling	Medtr_v1_030457	MEDTR5G017350_1	AT5G67030	ABA1.; zeaxanthin epoxidase	1,00E-130	M
	Medtr_v1_030456	MEDTR5G017330_1	AT5G67030	ABA1,) (ABA1)	1,00E-124	M
	Medtr_v1_030459	MEDTR5G017370_1	AT5G67030	ABA1,LOS6,NPQ2,ATABA1,ZEP,IBS3,ATZEP; zeaxanthin epoxidase (ZEP) (ABA1)	9,00E-15	M
	Medtr_v1_045419	MEDTR7G088090_1	AT3G56850	AREB3,DPBF3; ABA-responsive element binding protein 3	2,00E-81	M
	Medtr_v1_015247	MEDTR3G010660_1	AT3G56850	AREB3,DPBF3; ABA-responsive element binding protein 3	3,00E-71	M
	Medtr_v1_035019	MEDTR5G080680_1	AT1G07430	HAI2; highly ABA-induced PP2C gene 2	1,00E-118	M
GA metabolism	Medtr_v1_089118	IMGAlcontig_52215_1.1	AT1G14920	GAI,RGA2; DELLA protein	0	M
	Medtr_v1_019312	MEDTR3G096500_1	AT5G51810	GA20OX2, gibberellin 20 oxidase 2	1,00E-115	M
	Medtr_v1_088394	IMGAlcontig_55897_1.1	AT1G14920	GAI,RGA2; DELLA protein	1,00E-133	I
	Medtr_v1_006731	MEDTR1G086550_1	AT1G02400	Gibberellin 2-oxidase 6	1,00E-106	M
	Medtr_v1_086002	IMGAlcontig_71636_1.1	AT1G78440	Gibberellin 2-oxidase 1	5,00E-88	I
	Medtr_v1_083540	IMGAlcontig_91823_1.1	AT1G78440	GA2OX1; gibberellin 2-oxidase 1	1,00E-109	I
	Medtr_v1_088591	IMGAlcontig_165928_1.1	AT5G51810	Gibberellin 20 oxidase 2	1,00E-130	I
	Medtr_v1_089034	NA	AT4G25420	Gibberellin 20-oxidase	1,00E-150	M
	Medtr_v1_026223	MEDTR4G096840_1	AT1G30040	Gibberellin 2-oxidase	1,00E-113	I
	Medtr_v1_086002	IMGAlcontig_71636_1.1	AT1G78440	Gibberellin 2-oxidase 1	5,00E-88	I
Ga signaling	Medtr_v1_013745	MEDTR2G102570_1	AT1G15550	GA3OX1; gibberellin 3-oxidase 1	1,00E-119	M
	Medtr_v1_004175	MEDTR1G011580_1	AT1G15550	GA4,ATGA3OX1,GA3OX1; gibberellin 3- oxidase 1	2,00E-88	M
	Medtr_v1_009951	MEDTR2G019370_1	AT1G30040	GA2OX2; gibberellin 2-oxidase	1,00E-123	M
	Medtr_v1_092485	IMGAlcontig_247863_1.1	AT1G47990	ATGA2OX4,GA2OX4; gibberellin 2-oxidase 4	2,00E-59	M
	Medtr_v1_029438	MEDTR5G005570_1	AT4G21200	ATGA2OX8,GA2OX8; gibberellin 2-oxidase 8	1,00E-126	M
	Medtr_v1_010774	MEDTR2G034280_1	AT3G03450	RGA-like 2	4,00E-51	I
	Medtr_v1_010772	MEDTR2G034260_1	AT3G03450	RGA-like 2	1,00E-52	I
	Medtr_v1_083540	IMGAlcontig_91823_1.1	AT1G78440	GA2OX1; gibberellin 2-oxidase 1	1,00E-109	I

### IV.3.3 Are repair processes associated with seed longevity?

The only phenotypic difference between harvested seeds at stage R7.2 and mature seeds, stage R9 is related to a reduced storability that was found in seeds harvest in stage R7.2. So, we hypothesized that a transcriptome approach comparing changes during early imbibition of two seeds lots differing in longevity could give access to repair mechanisms that would be synthesized during imbibition of seeds from stage R9. We previously mentioned that genes involved in meristem development and morphogenesis were highly expressed in imbibed seeds at stage R9 but not in immature seeds. For example, STM/BUM1 I KNOX KNOTTED1-like homeobox factor was strongly upregulated as confirmed by RT-qPCR. This gene is an important and conserved transcriptional mediator of meristematic potential (reviewed in SCOFIELD and MURRAY, 2006). It is essential for initial shoot meristem formation and maintenance of SAM organization. Since it is associated with the regulation of stem cell niches, we could speculate that inducing the expression of such regulator could be a preemptive measure to ensure proper organ formation during germination in case the meristem would be damaged. Indeed, the expression of such genes could play an essential role in the regeneration of organs that could have suffered from deterioration during storage. Consistent with this, we also identified and confirmed by RT-QPCR the upregulation of the homologue of the AUXIN RESPONSE FACTOR 5, ARF5/MONOPTEROS. Its function, in conjunction with an auxin signal is to promote primordium founder cell fate to initiate flowers. Likewise, a transcript encoding a HD domain class transcription factor, which homologue in Arabidopsis play a similar role to KNOTTED-1 and is regulated by auxin was found. If we assume that these transcription factors are synthesized as a preemptive measure, then auxin should play a role in seed longevity. Evidence for this hypothesis comes from a recent work by Righetti et al (2015) in which they constructed a co-expression network of seed longevity. These authors reported that genes, which are present in the cross-species longevity module were highly enriched in binding sites for auxin response factors (ARFs) in their promoters. Instead of an expected 6% of genes, 60% of the *M. truncatula* genes in the longevity module contained the ARFAT cis-element (RIGHETTI et al 2015).

The same argument of a pre-emptive measure against potentially damaged tissues applied for the homologue of HSFA9 that was strongly upregulated in



imbibing mature seeds (Figure 19). It could facilitate the production of chaperone to repair proteins that were damaged during storage. Interestingly PMIT (PROTEIN-L-ISOASPARTATE METHYLTRANSFERASE 1), an ABA induced protein involved in repairing protein in aged seeds was down-regulated during imbibition

Our data from profile G also provides evidence of DNA repair that could be induced during imbibition. First, DNA2, a member of DNA replication helicase family protein (Glyma.06G310500), which Arabidopsis homologue was recently found to be associated with DNA repair and maintenance of the meristem. During DNA break repair, DNA2 mediates 5'-end resection of DNA by cleaving the 5'-single stranded DNA. The second probe is Glyma.18G194300, the Arabidopsis homolog of a Werner syndrome-like exonuclease whose expression was significantly up-regulated in R9 during imbibition but not stage R7.2 (Figure 16). Only a limited amount of information on this type of exonuclease is available in plants. This enzyme is thought to channel DNA double strand-break repair processes into non-recombinogenic pathways, thus facilitating genomic stability. Consistent with these observations, our profile also contained a DEG encoding a MMS19 nucleotide excision repair protein-like protein (Glyma.13G354600).

#### **IV.4 Comparative analysis between soybean and *Medicago truncatula* to identify genes related to seed vigor acquisition during maturation**

High seed quality is crucial for seedling establishment and yield. Seed quality characteristics such as germinability and longevity are acquired during seed maturation, in both soybean and *Medicago*. Our data on soybean revealed that complex transcriptomic features governing seed longevity and RNA metabolism during seed imbibition were dependent on the maturity stage. However, a cause effect relationship between transcriptome changes and expression of seed vigor in relation with the level of maturation was not secured. This was addressed here by a comparative analysis between soybean and *Medicago truncatula* at the same physiological stages

The first similarity that caught our attention was the presence of NFXL-1 that was up-regulated for both, soybean and *medicago* during maturation. Recently, Righetti et al. (2015) discovered the presence of NFXL-1 in an expression profile during seed maturation of both *Medicago* and *Arabidopsis*. This gene was part of the longevity module and a mutant affected in NFXL-1 displayed decreased longevity (RIGHETTI et





al.,2015). Our analysis corroborate this observation and suggest that the function of this gene in regulating seed longevity is conserved between species. This gene, also present in humans encodes a NFX-1-type nuclear Zn-finger transcriptional repressor that binds a conserved cis-acting element, the X-box in promoters of class II MCH genes. Little is known regarding its function. In Arabidopsis, it promotes growth under salt and osmotic stress by activating the expression of ABA stress responsive genes such as LEA genes and H<sub>2</sub>O<sub>2</sub> signaling (LISSO et al., 2006). Further work to understand the role of NFXL-1 in seed vigor would be of interest.

As mentioned above, during the late stage of maturation, seeds are able to synthesize mRNA in preparation for the dry state. These stored mRNAs are thought to be used during seed imbibition to ensure germination (RAIJOU et al., 2004; GALLAND and RAIJOU, 2015). To test whether this is also the case in soybean we compared the transcriptome of soybean imbibing seeds with results found in the analysis of medicago seeds. Analysis of transcripts corresponding to DEG up-regulated during legume seed imbibition revealed genes highly overrepresented that are involved in phototropism. Two genes were identified in the overrepresented group related to phototropism: the phototropic-responsive NPH3 gene, which is a highly conserved gene between Arabidopsis (AT5G64330), Medicago truncatula (two genes: Medtr2g009340.1 and Medtr4g093850.5) and soybean (Glyma.07G180900), and a Phototropin 1/NPH1 gene (AT3G45780, Medtr4g061610, Glyma.12G074100), which is known to interact with the blue light photoreceptor NPH1. The NPH1/NPH3 signaling pathway stimulates the shoot ward auxin flux by modifying the subcellular targeting of PIN2 in the root apex transition zone, leading to blue-light-induced root phototropism (WAN et al., 2012). Considering that PHYA and PHYB were also revealed by our species comparison, it would be interesting to further investigate the link between light photoreception activity and seed vigor

Seed germination and innate immunity response both play a significant role in the plant life. Since it is necessary to provide defense mechanisms against various possible tensions that the seed/seedling can experience in the production field. Our data show an over-representation of GO terms involved in innate immune response in imbibing seeds. Seven genes that represent this GO were found. Among them it is worth mentioning the SnRK1 gene, which plays an important role in plant growth as a key regulator of sugar signaling, stress, and seedling growth (XUE-FEI et al.; 2012).



Raffinose synthase Glyma.06G179200 was in the list of the 52 DEG transcripts that were down-regulated during legume seed imbibition, The content of RFO increased during soybean seed maturation and has been traditionally associated with desiccation tolerance (OBENDORF et al., 2009; VERDIER et al., 2013). RFOs are suggested to protect cellular integrity during desiccation by stabilizing membranes during dehydration. Whether RFO are an important source of energy during seed germination is unclear. The inhibition of the breakdown of RFOs caused significant delay in germination (BLÖCHL et al., 2007). However, according to DIERKING and BILYEU (2009) the reduced amount of raffinose and also stachyose, did not reduce or delay soybean seed germination. It has been mentioned here the importance of the ABA, as a regulator of growth and development of plants, that participates in the induction and maintenance of seed dormancy (RAJJOU et al., 2012). ABA is required for the seed maturation program, acting via the master regulator TFs to control storage and late embryogenesis accumulating (LEA) protein deposition and the acquisition of desiccation tolerance (VERDIER et al., 2013). The decrease in ABA concentration after seed imbibition allows the progress of germination (NONOGAKI et al., 2010). Thus, during germination, the seed must establish a specific catabolism, reducing their sensitivity and performing the biosynthesis of inhibitors to reduce ABA activity levels (RAJJOU et al., 2012; ARC et al., 2013). The ABA catabolism may be performed by oxidation, or conjugation with glucose. The main catabolic route is through 8'-hydroxylation by cytochrome P450 monooxygenase subfamily of CYP707A (ARC et al., 2013). Some genes are directly related to the ABA synthesis, such as ABA 1, 9-cis-epoxycarotenoid dioxygenase (NCEDs) and ABA2/G1N1/SDR1 (BENTSINK et al., 2006; HOLDSWORTH et al.; 2008), which mutants exhibits low ABA concentrations during seed development, resulting in no dormancy seeds (HOLDSWORTH et al.; 2008).

Two genes are related to seed coat development: the homologues of At5g49360, and At5g67360. The first gene encodes a BETA-XYLOSIDASE 1, a bifunctional {beta}-D-xylosidase/{alpha}-L-arabinofuranosidase that is required for pectic arabinan modification in Arabidopsis seed coats. This gene is expressed specifically in tissues undergoing secondary wall thickening (Arsovski et al., 2009). At5g67360 encode a subtilisin-like serine protease 1.7, which is essential for mucilage release from seed coats in Arabidopsis (RAUTENGARTEN et al., 2008). This suggest that seed coat regulation is important for seed vigor.



## CHAPTER V. CONCLUSIONS AND PERSPECTIVES

This works describe changes in transcriptome associated with the the late seed maturation events in legume when the developing seeds prepare for the dry stage and germination. At stage R7.2, most of the seed vigor traits were acquired except for seed longevity, which increased until the final mature stage. Therefore, stage R7.2 can not be declared as physiologically mature. The transcriptome analysis shows that important differences between seeds of stage R7.2 and R9 which corroborate the idea that maturation does not end at this stage. Thus late maturation of soybean seeds is accompanied by a a significant over-representation of genes related to response to heat, protein-folding, response to ER stress and genes related to light. This suggest that protective factors related to protein aggregation is probably a key to the induction of longevity but not necessary for seed germination. To confirm this, it would be interesting to verify whether we can associate the genetic variation in seed longevity with different levels of transcripts involved in the protection against protein aggregation. Our transcriptome raises the question whether red light influences seed longevity. Our soybean cultivar is not known to be light sensitive. However, light perception through the seed coat and pod might be perceived by the embryo as an environmental cue. It would interesting to test whether the canopy around the pod influence the progress of maturation. Also, it would be valuable to check whether the level of transcripts that are supposed to be regulated by light are also related to the level of seed maturity in various genotypes from different maturity group. From a technological point of view, it would be interesting to see whether the levels of certain transcripts could be used as an indicateur of the level of maturity.

The GO term “Seed coat development” and innate immune response appeared to be associated with maturity and longevity in both *Medicago truncatula* and soybean. Susceptibility to mechanical damage is associated to its lignin content, while longevity and potential deterioration in the field have been related to the degree of permeability of the integument (SOUZA and MARCOS FILHO, 2001). The integument black coloring soybeans exhibit slower imbibition, increased resistance to deterioration in field, greater thickness antifungal properties, and higher lignin content compared to light-colored seed coats (CHACHALIS and SMITH 2000; SANTOS et al., 2007; MERTZ et al., 2009; DELLAGOSTIN et al., 2011). Therefore, seed coat and resistance against pathogens might be key factors controlling seed vigor and this deserve further attention. It would be



interesting to describe changes in the acquisition of seed vigor in various genotypes that exhibit different seed colors and/or different coat permeability.

Dry seeds contain several thousands of stored mRNA that are essential for germination. Therefore, preparation for the dry state must involve the synthesis of these transcripts. However, this work suggests that the decrease in gene expression is probably equally important. In this regard, our work suggests that the decrease in transcripts associated with photosynthesis might be a factor leading to seed vigor and longevity. The fact that during imbibition of immature seeds chloroplast-encoded transcripts that did not disappear during maturation are rapidly degraded underscores this hypothesis. The incidence of green seeds in soybean seed lots is a problem characterized by the retention of chlorophyll in mature seeds, which is associated with the occurrence of extreme temperatures and periods of drought during the final stage of maturation. Therefore, how genes associated with photosynthesis and chlorophyll degradation are related to the acquisition of seed vigor and longevity deserve further studies, both during maturation and imbibition.

Finally, our work highlighted that hormone homeostasis and signaling are important for seed maturation and germination. Next to changes in transcripts associated with ABA and GA metabolism/signaling, our work suggests that two additional hormones might play an important role, namely ethylene and auxins. This gives support to the recent work of Samarah et al. (2016) showing that rates in ethylene release decreased as developing seeds progressed in maturity. Another feature of the transcriptome of imbibing soybean seeds is the over-representation of processes related to auxin activity and transport through organ formation, and meristem activity and cotyledon morphogenesis. Auxin by itself is not necessary for seed germination but may participate to the fine-tuning of different aspects of cellular growth necessary for germination. For example, auxin might be implicated in inducing radicle growth competence through early transcriptional regulation. Transcripts associated with ABA-induced stress response, Chl degradation, phytate synthesis and chaperone function disappear rapidly during imbibition. Imbibition-induced transcriptome profile associated with longevity highlights a putative DNA repair.





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## ANNEXES

**Supplementary Table 1.** List of the top 30 transcripts whose level increase the most during maturation.

<b>Gmax 2.0 ID</b>	<b>Tax Hit</b>	<b>p-value</b>	<b>Name</b>	<b>R90h/ R7.2d 0h</b>	<b>BH-value</b>
Glyma.06G142900	C7IVU8_9POAL	2,00E-25	Sorghum bicolor protein targeted either to mitochondria or chloroplast proteins T50848	4,11	1,06E-35
Glyma.17G224900	HSP21_SOYBN	3,00E-114	17.9 kDa class II heat shock protein	3,73	7,81E-15
Glyma.14G099900	HSP21_SOYBN	1,00E-93	17.9 kDa class II heat shock protein	3,69	3,70E-33
Glyma.17G077500	Q39889_SOYBN	0	Heat shock protein	3,51	1,01E-12
Glyma.14G222700	B9GQ52_POPTR	0	Prolyl oligopeptidase family protein	3,34	9,65E-10
Glyma.08G106700	G5ELZ2_TOBAC	0	FK506-binding protein	3,27	1,84E-15
Glyma.06G235100	-	-	-	3,25	4,59E-09
Glyma.12G200600	-	-	-	3,13	2,81E-16
Glyma.04G222500	C7IVU8_9POAL	2,00E-25	Sorghum bicolor protein targeted either to mitochondria or chloroplast proteins T50848	3,05	7,23E-21
Glyma.13G176200	HSP14_SOYBN	5,00E-106	17.5 kDa class I heat shock protein	2,90	7,22E-19
Glyma.09G040400	E9KT46_9FABA	7,00E-86	PR10.13.36 (Fragment)	2,88	6,30E-05
Glyma.18G061100	K7MQ84_SOYBN	0	Asparagine synthetase	2,82	4,74E-21
Glyma.07G267800	OPT3_ARATH	0	Oligopeptide transporter 3	2,79	6,70E-19
Glyma.06G134900	A9QVH3_9FABA	2,00E-125	Heat shock protein	2,79	2,32E-17
Glyma.12G200400	-	-	-	2,63	7,19E-10

**Supplementary Table 1.** Continuation ...

<b>Gmax 2.0 ID</b>	<b>Tax Hit</b>	<b>p-value</b>	<b>Name</b>	<b>R90h/ R7.2d 0h</b>	<b>BH-value</b>
Glyma.14G140400	A2TDC3_9ROSI	2,00E-104	Truncated hemoglobin	2,63	1,86E-07
Glyma.09G181200	G7KML0_MEDTR	0	Kinesin-like protein KIF3A	2,63	4,14E-12
Glyma.10G093100	E4MXB6_THEHA	2,00E-86	mRNA, clone: RTFL01-22-A04	2,63	7,59E-10
Glyma.07G080400	G7L1G0_MEDTR	0	Phospholipase D	2,56	1,90E-09
Glyma.18G259700	G7L1Y9_MEDTR	0	Cell division protease ftsH-like protein	2,50	1,88E-08
Glyma.12G073100	Q76LA6_SOYBN	2,00E-180	Cytosolic ascorbate peroxidase 2	2,46	2,39E-15
Glyma.09G283400	G7IBD4_MEDTR	0	Chaperone protein dnaJ	2,45	5,81E-10
Glyma.03G068100	D4N5G1_SOYBN	0	Rubisco activase	2,45	1,08E-07
Glyma.07G200200	HSP16_SOYBN	7,00E-110	18.5 kDa class I heat shock protein	2,44	2,81E-16
Glyma.06G114600	Q9XI02_ARATH	0	F8K7.18 protein	2,44	8,68E-06
Glyma.20G213900	HSP41_SOYBN	2,00E-136	22.0 kDa class IV heat shock protein	2,43	8,38E-16
Glyma.10G176800	B7FAD8_ORYSJ	2,00E-117	cDNA, clone: J100065N04, full insert sequence	2,41	3,21E-10
Glyma.03G189900	Q8H0I7_PETHY	0	Cytochrome P450	2,40	3,08E-09
Glyma.09G246300	G7KSH3_MEDTR	2,00E-134	Hydroxyproline-rich glycoprotein-like protein	2,37	1,19E-06
Glyma.16G178900	B9MX43_POPTR	8,00E-121	Glutamine cyclotransferase family protein	2,36	1,05E-07

**Supplementary Table 2.** .List of the top 30 transcripts whose level decrease the most during maturation.

<b>Gmax 2.0 ID</b>	<b>Tax Hit</b>	<b>p-value</b>	<b>Name</b>	<b>R9 0h/ R7.2d 0h</b>	<b>BH-value</b>
Glyma.01G130200	I1J7M1_SOYBN	1,00E-76	Non-specific lipid-transfer protein	-3,76	6,16E-14
Glyma.02G061600	G7K9G6_MEDTR	3,00E-27	Protein disulfide-isomerase	-3,47	7,84E-11
Glyma.05G204800	P21_SOYBN	6,00E-128	Protein P21	-3,43	5,28E-20
Glyma.01G034700	TBL27_ARATH	6,00E-119	Protein ALTERED XYLOGLUCAN 4	-3,33	3,26E-07
Glyma.13G133000	G7L0M5_MEDTR	1,00E-49	Zinc finger protein	-3,32	5,53E-19
Glyma.10G033500	F4INW4_ARATH	0	Histone H1flk-like protein	-3,32	3,36E-07
Glyma.16G208900	Q43709_SOYBN	2,00E-64	Bowman-Birk proteinase isoinhibitor D-II	-3,30	3,58E-09
Glyma.18G127200	G7K9P5_MEDTR	0	Peptide transporter PTR1	-3,30	8,85E-25
Glyma.10G058200	I1L905_SOYBN	0	Phenylalanine ammonia-lyase	-3,30	6,77E-24
Glyma.19G052900	-	-	-	-3,21	2,45E-07
Glyma.06G160300	Q39894_SOYBN	1,00E-42	Seed-specific low molecular weight sulfur-rich protein	-3,19	8,39E-08
Glyma.08G250300	Q69F92_PHAVU	3,00E-51	B12D-like protein	-3,14	1,99E-07
Glyma.13G309900	A2Q4H0_MEDTR	1,00E-154	Mitogen-activated protein kinase kinase kinase A	-3,12	6,64E-08
Glyma.02G000500	K7K5Q4_SOYBN	2,00E-138	Auxin-induced protein	-3,11	7,94E-07
Glyma.05G236900	I1K5N0_SOYBN	0	Pectinesterase	-3,10	8,54E-06



**Supplementary Table 2.** Continuation...

<b>Gmax 2.0 ID</b>	<b>Tax Hit</b>	<b>p-value</b>	<b>Name</b>	<b>R9 0h/ R7.2d 0h</b>	<b>BH-value</b>
Glyma.15G274900	G7JEB0_MEDTR	1,00E-27	Ycf68	-3,09	1,79E-19
Glyma.12G090600	S4Z2Z0_TRIMO	2,00E-26	Hypothetical chloroplast RF68	-3,08	1,43E-24
Glyma.U040600	Q8S8Z5_SOYBN	0	Syringolide-induced protein B13-1-1 (Fragment)	-3,04	4,87E-07
Glyma.11G114700	H6T057_9POAL	5,00E-67	Photosystem II CP43 chlorophyll apoprotein (Fragment)	-3,02	1,86E-13
Glyma.08G244500	G7L593_MEDTR	0	UDP-glucosyltransferase HRA25	-3,01	4,29E-11
Glyma.14G158500	-	-	-	-3,00	4,07E-28
Glyma.06G186200	Q9AUH7_PEA	2,00E-17	UVI1	-2,99	7,48E-12
Glyma.03G176300	T2DNZ6_PHAVU	4,00E-126	In2-1 protein	-2,98	1,15E-08
Glyma.18G228200	C1E094_MICSR	7,00E-60	Vacuolar iron family transporter	-2,97	1,75E-06
Glyma.20G054100	I1NE44_SOYBN	0	Lipoxygenase	-2,97	2,46E-06
Glyma.13G194400	G3FGW7_SOYBN	5,00E-97	Leginsulin 1	-2,94	7,90E-08
Glyma.14G132100	H8PI40_GLYSO	2,00E-76	Maturase K	-2,93	7,90E-08
Glyma.10G173200	I1LBY5_SOYBN	2,00E-73	Protein yippee-like	-2,93	3,40E-06
Glyma.05G057600	Q9SXE7_ARATH	1,00E-37	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	-2,90	5,57E-11
Glyma.08G241600	G7L453_MEDTR	0	Cyclic nucleotide-gated ion channel	-2,89	7,93E-08

**Supplementary Table 3.** List of the top 30 transcripts whose level increase the most during artificial drying.

<b>Gmax 2.0 ID</b>	<b>Tax Hit</b>	<b>p-value</b>	<b>Name</b>	<b>R7.2d/ R7.2f</b>	<b>BH-value</b>
Glyma.05G236800	I1K5N1_SOYBN	0	Pectinesterase	5,40	9,5E-17
Glyma.10G206000	F4I9C1_ARATH	2,00E-07	Hydroxyproline-rich glycoprotein-like protein	5,21	3,6E-08
Glyma.11G068100	-	-	-	5,06	2,5E-20
Glyma.11G187000	PCS3_LOTJA	3,00E-120	Glutathione glutamylcysteinyltransferase 3	5,06	4,2E-09
Glyma.15G188400	PSBN_SOYBN	4,00E-22	Protein PsbN	5,02	1,4E-17
Glyma.08G229200	-	-	-	4,89	1,6E-17
Glyma.20G184500	S4WWG9_GLYSO	1,00E-139	Hydroxyproline-rich glycoprotein family protein (Fragment)	4,74	7,5E-09
Glyma.12G151700	ATL44_ARATH	4,00E-10	RING-H2 finger protein ATL44	4,73	1,2E-06
Glyma.05G236900	I1K5N0_SOYBN	0	Pectinesterase	4,69	6,0E-08
Glyma.09G171400	V9P892_9ROSI	3,00E-11	Photosystem II protein M	4,55	1,5E-09
Glyma.15G208300	PSBM_POPTR	2,00E-13	Photosystem II reaction center protein M	4,49	4,8E-14
Glyma.03G181000	-	-	-	4,45	3,8E-22
Glyma.07G079300	-	-	-	4,40	6,2E-42
Glyma.01G105100	-	-	-	4,35	7,4E-06
Glyma.17G245200	Q0PJI9_SOYBN	1,00E-79	MYB transcription factor MYB124	4,34	6,1E-07

**Supplementary Table 3.** Continuation...

<b>Gmax 2.0 ID</b>	<b>Tax Hit</b>	<b>p-value</b>	<b>Name</b>	<b>R7.2d/ R7.2f</b>	<b>BH-value</b>
Glyma.09G171300	C1K530_9ASTR	8,00E-08	Cytochrome b6-f complex subunit 8	4,29	4,7E-13
Glyma.16G038100	-	-	-	4,24	8,3E-11
Glyma.13G313100	Q2HSI2_MEDTR	0	Protein kinase	4,09	1,0E-04
Glyma.06G186200	Q9AUH7_PEA	2,00E-17	UVI1	4,08	1,1E-19
Glyma.18G132800	R9ZV93_GLYTO	7,00E-33	Protein Ycf2	4,07	2,5E-05
Glyma.07G143800	V9Z7G6_9ROSI	6,00E-57	Photosystem I P700 apoprotein A1 (Fragment)	4,07	2,9E-14
Glyma.11G114700	H6T057_9POAL	5,00E-67	Photosystem II CP43 chlorophyll apoprotein (Fragment)	4,03	4,2E-22
Glyma.04G095000	A0AS46_MEDVI	9,00E-96	Photosystem II D2 protein (Fragment)	4,01	8,5E-22
Glyma.05G074900	Q66VR0_9ERIC	5,00E-29	PsbC (Fragment)	3,95	6,4E-15
Glyma.03G176300	T2DNZ6_PHAVU	4,00E-126	In2-1 protein	3,95	8,8E-13
Glyma.09G185400	-	-	-	3,94	2,6E-08
Glyma.06G224500	K4HGK5_CAMSI	2,00E-54	Photosystem I P700 apoprotein A1 (Fragment)	3,92	3,3E-13
Glyma.13G068600	NU3C_SOYBN	2,00E-75	NAD(P)H-quinone oxidoreductase subunit 3, chloroplastic	3,87	8,1E-19
Glyma.U013900	-	-	-	3,87	9,8E-05
Glyma.06G171100	-	-	-	3,86	3,2E-18

**Supplementary Table 4.** List of the top 30 transcripts whose level increase the most during imbibition for both stages (profile A).

Gmax 2.0 ID	Tax Hit	p-value	Name	R918h/ R90h	BH-value	R7.2d18h/ R7.2d0h	BH-value
Glyma.01G019200	Q94LX7_FLAPR	0	Phosphoenolpyruvate carboxykinase	8,12	1,95E-49	6,01	2,63E-37
Glyma.18G210900	C6SWY4_SOYBN	9,00E-79	Non-specific lipid-transfer protein	8,08	3,53E-44	7,75	9,18E-31
Glyma.14G191700	Q0PJL5_SOYBN	0	MYB transcription factor MYB57	8,03	4,86E-31	8,22	7,20E-23
Glyma.02G028100	B6CAM2_SOYBN	0	Matrix metalloproteinase	7,85	2,82E-32	9,18	2,54E-117
Glyma.02G028000	B6CAM2_SOYBN	0	Matrix metalloproteinase	7,81	5,97E-18	6,45	1,30E-15
Glyma.17G019300	I1MRA7_SOYBN	0	Amine oxidase	7,79	5,39E-40	8,71	2,94E-23
Glyma.17G063900	G7JVH0_MEDTR	0	Mitochondrial chaperone BCS1	7,63	8,18E-17	6,95	1,01E-18
Glyma.06G193800	M1GM36_GOSHI	2,00E-49	GA-stimulated transcript-like protein 2	7,58	2,83E-27	8,04	1,39E-21
Glyma.20G248100	G7IB14_MEDTR	2,00E-84	Transcription factor bHLH128	7,49	2,34E-66	8,39	1,23E-40
Glyma.14G141000	B2ZPK6_SOYBN	0	BURP domain-containing protein	7,47	3,72E-21	6,51	3,53E-15
Glyma.17G012300	G7JJ92_MEDTR	0	L-ascorbate oxidase-like protein	7,46	6,06E-16	7,39	2,56E-13
Glyma.07G096000	Q5N7Q6_ORYSJ	9,00E-24	Receptor-like protein kinase-like	7,45	7,14E-16	6,41	6,51E-15
Glyma.02G036500	E2IXG6_MEDTR	0	Leucine-rich repeat receptor-like kinase	7,38	2,66E-30	7,16	2,27E-22
Glyma.19G219500	G7KTQ1_MEDTR	0	Boron transporter	7,35	1,15E-19	5,94	3,72E-10
Glyma.03G202400	D7L4L9_ARALL	0	Protein binding protein	7,34	5,89E-30	7,13	3,89E-32

**Supplementary Table 4.** Continuation...

<b>Gmax 2.0 ID</b>	<b>Tax Hit</b>	<b>p-value</b>	<b>Name</b>	<b>R918h/ R90h</b>	<b>BH-value</b>	<b>R7.2d18h/ R7.2d0h</b>	<b>BH-value</b>
Glyma.17G015400	G7JI12_MEDTR	0	Cytochrome P450	7,17	3,32E-53	8,22	2,37E-32
Glyma.13G112200	U5G769_POPTR	3,00E-80	Senescence-associated protein family	7,16	4,50E-68	7,26	6,60E-44
Glyma.02G099500	G7JY94_MEDTR	0	AP2 domain-containing transcription factor	7,12	4,28E-14	3,68	8,40E-11
Glyma.13G347700	B3TDK7_SOYBN	0	Lipoxygenase	7,07	4,55E-43	6,50	7,42E-18
Glyma.10G172200	B2LUN7_SOYBN	0	Granule bound starch synthase Ia	7,06	2,64E-62	6,69	5,15E-86
Glyma.13G069900	G7KIU9_MEDTR	9,00E-31	Gibberellin regulated protein	7,05	2,72E-18	5,84	1,57E-14
Glyma.02G224900	Q0PJL5_SOYBN	0	MYB transcription factor MYB57	6,86	6,56E-34	7,85	3,86E-25
Glyma.04G023900	T2DNJ3_PHAVU	0	Tubulin beta-1 chain-like protein	6,85	4,51E-17	6,15	3,19E-13
Glyma.14G036900	G7KCU0_MEDTR	2,00E-113	Early nodulin-like protein	6,85	2,54E-21	6,39	1,36E-21
Glyma.15G142400	F8SMB8_SOYBN	0	Acidic glucanase	6,83	5,72E-17	5,41	1,96E-13
Glyma.13G248000	D9ZIW8_MALDO	3,00E-33	COL domain class transcription factor	6,82	8,55E-25	8,32	2,91E-18
Glyma.10G047100	G7L0N3_MEDTR	5,00E-171	Protein ALUMINUM SENSITIVE	6,79	1,80E-12	4,48	1,66E-12
Glyma.06G027700	T2DN49_PHAVU	0	Cysteine proteinase RD19a-like protein	6,79	1,47E-36	6,06	6,26E-26
Glyma.13G304400	T2DP41_PHAVU	0	Xyloglucan endotransglucosylase/hydrolase protein 9-like protein	6,76	8,89E-25	8,50	2,38E-19
Glyma.20G033900	Q8L5G4_CICAR	3,00E-168	Alpha-expansin 4	6,75	4,23E-35	5,17	1,44E-21

**Supplementary Table 5. Genes up-regulated in mature legume seeds during imbibition versus immature.**

Probe_id	Seq_id Mt4.0v1	Arabidopsis	p-value	id Gmax	Tax Hit	p-value	Arabidopsis	Name Arabdopsis gene	p-value
Medtr_v1_080740	Medtr3g024510.1	AT1G29670.1	1,00E-150	Glyma.13G223100	G7J1P3_MEDTR	1,00E-134	AT1G29670.1	GDSL-like superfamily protein Lipase/Acylhydrolase	2,00E-103
Medtr_v1_072741	Medtr4g011630.1	AT1G15520.1	0,00E+00	Glyma.13G361900	G7IMF4_MEDTR	0,00E+00	AT1G15520.1	pleiotropic drug resistance 12	0,00E+00
Medtr_v1_072741	Medtr4g011630.1	AT1G15520.1	0,00E+00	Glyma.15G011900	G7IMF2_MEDTR	0,00E+00	AT1G15520.1	pleiotropic drug resistance 12	0,00E+00
Medtr_v1_072741	Medtr4g011630.1	AT1G15520.1	0,00E+00	Glyma.15G012000	G7IMF2_MEDTR	0,00E+00	AT1G15520.1	pleiotropic drug resistance 12	0,00E+00
Medtr_v1_087003	Medtr0565s0010.1	AT1G29670.1	1,00E-155	Glyma.13G223100	G7J1P3_MEDTR	1,00E-134	AT1G29670.1	GDSL-like superfamily protein Lipase/Acylhydrolase	2,00E-103
Medtr_v1_087027	Medtr4g062510.1	AT5G06900.1	1,00E-114	Glyma.19G146800	C93A1_SOYBN	0,00E+00	AT5G06900.1	cytochrome P450, family 93, subfamily D, polypeptide 1	0,00E+00
Medtr_v1_010809	Medtr2g034720.1	AT5G49360.1	0,00E+00	Glyma.15G143700	G7IMV1_MEDTR	0,00E+00	AT5G49360.1	beta-xylosidase 1	0,00E+00
Medtr_v1_092529	Medtr4g062500.1	AT5G06900.1	1,00E-119	Glyma.03G143700	C93A1_SOYBN	0,00E+00	AT5G06900.1	cytochrome P450, family 93, subfamily D, polypeptide 1	0,00E+00
Medtr_v1_016378	Medtr3g034290.1	AT3G63470.1	0,00E+00	Glyma.09G226700	G7IZJ6_MEDTR	0,00E+00	AT3G63470.1	serine carboxypeptidase-like 40	0,00E+00
Medtr_v1_029654	Medtr5g008010.1	AT4G33270.1	0,00E+00	Glyma.08G228100	U5FR10_POPTR	0,00E+00	AT4G33270.1	Transducin family protein / WD-40 repeat family protein	0,00E+00
Medtr_v1_076047	#N/D	AT2G27740.1	5,00E-54	Glyma.12G086500	-	-	AT2G27740.1	Family of unknown function (DUF662)	4,00E-77
Medtr_v1_034144	Medtr5g069310.1	AT3G47340.1	1,00E-52	Glyma.11G171400	I1LLM2_SOYBN	0,00E+00	AT3G47340.1	glutamine-dependent asparagine synthase 1	0,00E+00
Medtr_v1_028182	Medtr4g133750.1	AT3G26300.1	1,00E-111	Glyma.01G179400	G7KAT1_MEDTR	0,00E+00	AT3G26300.1	cytochrome P450, family 71, subfamily B, polypeptide 34	2,00E-133
Medtr_v1_074514	Medtr3g073730.1	AT5G10180.1	0,00E+00	Glyma.14G169300	G7KA22_MEDTR	0,00E+00	AT5G10180.1	slufate transporter 2;1	0,00E+00
Medtr_v1_048319	Medtr8g006270.1	AT4G01070.1	1,00E-109	Glyma.05G181100	I2BH27_LINUS	2,00E-172	AT4G01070.1	UDP-Glycosyltransferase superfamily protein	1,00E-133
Medtr_v1_034662	Medtr5g076140.1	AT5G37010.1	3,00E-61	Glyma.18G048000	D0ABF7_9ORYZ	5,00E-61	AT5G37010.1	unknown protein; FUNCTIONS IN: molecular_function unknown; INVOLVED IN: N-terminal protein myristoylation; EXPR	2,00E-69
Medtr_v1_018211	Medtr3g080990.1	AT4G10850.1	2,00E-77	Glyma.13G002700	M7Z837_TRIUA	3,00E-96	AT4G10850.1	Nodulin MtN3 family protein	1,00E-93
Medtr_v1_078735	Medtr6g073013.1	AT1G14440.2	4,00E-34	Glyma.17G002200	B0LK13_SARHE	3,00E-95	AT1G14440.2	homeobox protein 31	9,00E-72
Medtr_v1_048729	Medtr8g018130.1	AT5G17680.1	1,00E-131	Glyma.08G190600	G7JUR1_MEDTR	0,00E+00	AT5G17680.1	disease resistance protein (TIR-NBS-LRR class), putative	3,00E-103
Medtr_v1_001536	Medtr3g102810.1	AT5G67360.1	5,00E-30	Glyma.06G045100	D3YBD3_TRIRP	0,00E+00	AT5G67360.1	Subtilase family protein	0,00E+00
Medtr_v1_032102	Medtr5g037080.1	AT1G70000.2	5,00E-62	Glyma.02G026300	Q0PII4_SOYBN	0,00E+00	AT1G70000.2	myb-like transcription factor family protein	4,00E-77
Medtr_v1_092069	Medtr6g033675.1	AT3G16520.3	1,00E-111	Glyma.16G175400	C6ZJB3_PUEML	0,00E+00	AT3G16520.3	UDP-glucosyl transferase 88A1	2,00E-124

**Supplementary Table 5.** Continuation...

Probe_id	Seq_id Mt4.0v1	Arabidopsis	p-value	id Gmax	Tax Hit	p-value	Arabidopsis	Name Arabdopsis gene	p-value
Medtr_v1_069967	Medtr4g005360.1	AT3G20270.3	3,00E-73	Glyma.08G224700	B6U171_MAIZE	2,00E-80	AT3G20270.3	lipid-binding serum glycoprotein family protein	5,00E-77
Medtr_v1_031602	Medtr5g030950.1	AT4G37930.1	0,00E+00	Glyma.09G202000	I1L4U2_SOYBN	0,00E+00	AT4G37930.1	serine transhydroxymethyltransferase 1	0,00E+00
Medtr_v1_045883	Medtr7g093830.1	AT5G42500.1	1,00E-42	Glyma.03G147700	G7KWC3_MEDTR	2,00E-110	AT5G42500.1	Disease resistance-responsive (dirigent-like protein) family protein	3,00E-52
Medtr_v1_048315	Medtr8g006220.1	AT4G01070.1	1,00E-111	Glyma.05G181100	I2BH27_LINUS	2,00E-172	AT4G01070.1	UDP-Glycosyltransferase superfamily protein	1,00E-133
Medtr_v1_027024	Medtr6g465230.1	AT5G22990.1	2,00E-17	Glyma.10G295200	G7JE49_MEDTR	4,00E-19	AT5G22990.1	C2H2-like zinc finger protein	5,00E-22
Medtr_v1_051967	Medtr8g098360.1	AT5G12250.1	0,00E+00	Glyma.05G207500	G7LHC6_MEDTR	0,00E+00	AT5G12250.1	beta-6 tubulin	0,00E+00
Medtr_v1_051967	Medtr8g098360.1	AT5G12250.1	0,00E+00	Glyma.08G014200	TBB1_SOYBN	0,00E+00	AT5G12250.1	beta-6 tubulin	0,00E+00
Medtr_v1_076300	#N/D	AT3G16520.3	1,00E-106	Glyma.16G175400	C6ZJB3_PUEML	0,00E+00	AT3G16520.3	UDP-glucosyl transferase 88A1	2,00E-124
Medtr_v1_035118	Medtr5g081860.1	AT2G38090.1	1,00E-69	Glyma.03G078000	Q0PJE0_SOYBN	1,00E-160	AT2G38090.1	Duplicated homeodomain-like superfamily protein	3,00E-120
Medtr_v1_072909	Medtr6g033740.1	AT4G28000.1	0,00E+00	Glyma.09G127000	U5FLT2_POPTR	0,00E+00	AT4G28000.1	P-loop containing nucleoside triphosphate hydrolases superfamily protein	0,00E+00
Medtr_v1_099143	Medtr5g017210.1	AT3G50330.1	4,00E-42	Glyma.06G040800	Q00M67_SOYBN	0,00E+00	AT3G50330.1	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	5,00E-45
Medtr_v1_025533	Medtr4g082820.1	AT4G01070.1	3,00E-50	Glyma.05G181100	I2BH27_LINUS	2,00E-172	AT4G01070.1	UDP-Glycosyltransferase superfamily protein	1,00E-133
Medtr_v1_084463	Medtr0021s0360.1	AT1G68710.1	0,00E+00	Glyma.01G092900	ALA10_ARATH	0,00E+00	AT1G68710.1	ATPase E1-E2 type family protein / haloacid dehalogenase-like hydrolase	0,00E+00
Medtr_v1_067075	#N/D	AT5G33320.1	1,00E-135	Glyma.17G088500	P93390_TOBAC	0,00E+00	AT5G33320.1	Glucose-6-phosphate/phosphate translocator-related	1,00E-171
Medtr_v1_027067	Medtr4g115360.2	AT2G10940.2	2,00E-43	Glyma.13G160400	B6TLL4_MAIZE	4,00E-43	AT2G10940.2	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	9,00E-50
Medtr_v1_025449	Medtr4g081230.1	AT5G17680.1	1,00E-126	Glyma.08G190600	G7JUR1_MEDTR	0,00E+00	AT5G17680.1	disease resistance protein (TIR-NBS-LRR class), putative	3,00E-103
Medtr_v1_084574	Medtr3g465430.1	AT3G51770.1	0,00E+00	Glyma.18G059700	F4J4I9_ARATH	0,00E+00	AT3G51770.2	tetratricopeptide repeat (TPR)-containing protein	0,00E+00
Medtr_v1_070121	Medtr7g102070.1	AT2G36910.1	0,00E+00	Glyma.19G184300	U5GAD2_POPTR	0,00E+00	AT2G36910.1	ATP binding cassette subfamily B1	0,00E+00
Medtr_v1_019895	Medtr3g105610.1	AT2G29120.1	0,00E+00	Glyma.13G233300	G7IK74_MEDTR	0,00E+00	AT2G29120.1	glutamate receptor 2.7	8,00E-127
Medtr_v1_027512	Medtr4g123880.1	AT1G56720.3	0,00E+00	Glyma.03G228800	B9N417_POPTR	0,00E+00	AT1G56720.3	Protein kinase superfamily protein	0,00E+00
Medtr_v1_052567	Medtr8g107510.1	AT1G70300.1	0,00E+00	Glyma.01G031800	I1J5A7_SOYBN	0,00E+00	AT1G70300.1	K+ uptake permease 6	0,00E+00

**Supplementary Table 5.** Continuation...

Probe_id	Seq_id Mt4.0v1	Arabidopsis	p-value	id Gmax	Tax Hit	p-value	Arabidopsis	Name Arabdopsis gene	p-value
Medtr_v1_087263	Medtr0451s0010.1	AT3G26300.1	1,00E-110	Glyma.01G179400	G7KAT1_MEDTR	0,00E+00	AT3G26300.1	cytochrome P450, family 71, subfamily B, polypeptide 34	2,00E-133
Medtr_v1_036075	Medtr5g092990.1	AT5G17680.1	2,00E-33	Glyma.08G190600	G7JUR1_MEDTR	0,00E+00	AT5G17680.1	disease resistance protein (TIR-NBS-LRR class), putative	3,00E-103
Medtr_v1_010071	Medtr2g020710.1	AT5G18840.1	0,00E+00	Glyma.15G099500	G7IFL9_MEDTR	0,00E+00	AT5G18840.1	Major facilitator superfamily protein	0,00E+00
Medtr_v1_015300	Medtr3g011390.1	AT3G07040.1	1,00E-127	Glyma.18G093300	G7J1I4_MEDTR	0,00E+00	AT3G07040.1	NB-ARC domain-containing disease resistance protein	4,00E-100
Medtr_v1_077258	Medtr4g056520.1	AT2G27920.1	1,00E-167	Glyma.11G191200	SCP51_ARATH	0,00E+00	AT2G27920.1	serine carboxypeptidase-like 51	0,00E+00
Medtr_v1_074584	Medtr3g464580.1	AT3G47340.1	0,00E+00	Glyma.11G171400	I1LLM2_SOYBN	0,00E+00	AT3G47340.1	glutamine-dependent asparagine synthase 1	0,00E+00
Medtr_v1_024375	Medtr4g061610.1	AT3G45780.2	1,30E-01	Glyma.12G074100	Q5DW44_PHAVU	0,00E+00	AT3G45780.2	phototropin 1	0,00E+00
Medtr_v1_020096	Medtr3g107980.1	AT5G25510.1	0,00E+00	Glyma.04G071700	G7JBQ2_MEDTR	0,00E+00	AT5G25510.1	Protein phosphatase 2A regulatory B subunit family protein	0,00E+00
Medtr_v1_027626	Medtr4g125180.1	AT2G05920.1	0,00E+00	Glyma.09G070000	G7IGE1_MEDTR	0,00E+00	AT2G05920.1	Subtilase family protein	0,00E+00
Medtr_v1_077859	Medtr4g126270.1	AT5G56890.1	0,00E+00	Glyma.13G104300	G7III2_MEDTR	0,00E+00	AT5G56890.1	Protein kinase superfamily protein	0,00E+00
Medtr_v1_040475	Medtr6g086115.1	AT3G24480.1	1,00E-159	Glyma.05G232000	LRX4_ARATH	0,00E+00	AT3G24480.1	Leucine-rich repeat (LRR) family protein	0,00E+00
Medtr_v1_040475	Medtr6g086115.1	AT3G24480.1	1,00E-159	Glyma.08G039400	LRX4_ARATH	0,00E+00	AT3G24480.1	Leucine-rich repeat (LRR) family protein	0,00E+00
Medtr_v1_025546	Medtr4g082950.1	AT3G29320.1	3,00E-49	Glyma.20G026700	K7N0Z8_SOYBN	0,00E+00	AT3G29320.1	Glycosyl transferase, family 35	0,00E+00
Medtr_v1_046321	Medtr7g104050.1	AT3G52910.1	4,00E-71	Glyma.19G192700	G7L5Z0_MEDTR	3,00E-157	AT3G52910.1	growth-regulating factor 4	9,00E-89
Medtr_v1_026135	Medtr4g093850.5	AT5G64330.1	0,00E+00	Glyma.07G180900	G7JV64_MEDTR	0,00E+00	AT5G64330.1	Phototropic-responsive NPH3 family protein	0,00E+00
Medtr_v1_085553	#N/D	AT4G13870.1	2,00E-15	Glyma.18G194300	E5GBP0_CUCME	2,00E-57	AT4G13870.2	Werner syndrome-like exonuclease	2,00E-23
Medtr_v1_048739	Medtr8g018260.1	AT5G17680.1	1,00E-144	Glyma.08G190600	G7JUR1_MEDTR	0,00E+00	AT5G17680.1	disease resistance protein (TIR-NBS-LRR class), putative	3,00E-103
Medtr_v1_048740	Medtr8g018270.1	AT5G17680.1	1,00E-124	Glyma.08G190600	G7JUR1_MEDTR	0,00E+00	AT5G17680.1	disease resistance protein (TIR-NBS-LRR class), putative	3,00E-103
Medtr_v1_009287	Medtr2g009340.1	AT5G64330.1	0,00E+00	Glyma.07G180900	G7JV64_MEDTR	0,00E+00	AT5G64330.1	Phototropic-responsive NPH3 family protein	0,00E+00
Medtr_v1_033153	Medtr5g055310.1	AT1G25390.1	1,00E-121	Glyma.10G271400	G7IC81_MEDTR	0,00E+00	AT1G25390.1	Protein kinase superfamily protein	2,00E-156
Medtr_v1_049375	Medtr4g043630.2	AT5G17680.1	0,00E+00	Glyma.08G190600	G7JUR1_MEDTR	0,00E+00	AT5G17680.1	disease resistance protein (TIR-NBS-LRR class), putative	3,00E-103



**Supplementary Table 6.** Genes down-regulated in mature legume seeds during imbibition versus immature.

Probe_id	Seq_id Mt4.0v1	Arabidopsis	e-value	id Gmax	Tax Hit	p-value	Arabidopsis	Name Arabdopsis gene	p-value
Medtr_v1_031642	Medtr5g031430.1	AT4G39330.1	1,00E-117	Glyma.05G187700	F8SMC1_SOYBN	0,00E+00	AT4G39330.1	cinnamyl alcohol dehydrogenase 9	0,00E+00
Medtr_v1_086340	#N/D	AT4G25640.2	6,00E-11	Glyma.10G232200	I1LDN0_SOYBN	0,00E+00	AT4G25640.1	detoxifying efflux carrier 35	0,00E+00
Medtr_v1_101150	Medtr7g101425.1	AT2G37040.1	7,00E-77	Glyma.10G058200	I1L905_SOYBN	0,00E+00	AT2G37040.1	PHE ammonia lyase 1	0,00E+00
Medtr_v1_046955	Medtr7g114990.1	AT1G60710.1	1,00E-113	Glyma.03G248700	AKR1_SOYBN	3,00E-141	AT1G60710.1	NAD(P)-linked oxidoreductase superfamily protein	2,00E-94
Medtr_v1_042249	Medtr7g016820.1	AT5G13930.1	0	Glyma.01G228700	CHS7_SOYBN	0,00E+00	AT5G13930.1	Chalcone and stilbene synthase family protein	0,00E+00
Medtr_v1_001801	Medtr1g040875.1	AT3G23000.1	1,00E-136	Glyma.10G001700	C6F1T7_SOYBN	0,00E+00	AT3G23000.1	CBL-interacting protein kinase 7	3,00E-167
Medtr_v1_050434	Medtr8g068870.1	AT5G54160.1	6,00E-98	Glyma.04G227700	F8SMC0_SOYBN	0,00E+00	AT5G54160.1	O-methyltransferase 1	0,00E+00
Medtr_v1_086822	Medtr7g096990.1	AT5G44120.3	8,00E-23	Glyma.19G164800	Q9FEC5_SOYBN	0,00E+00	AT5G44120.3	RmlC-like cupins superfamily protein	3,00E-50
Medtr_v1_068697	Medtr8g096910.1	AT4G11650.1	7,00E-49	Glyma.05G204800	P21_SOYBN	6,00E-128	AT4G11650.1	osmotin 34	3,00E-111
Medtr_v1_044768	Medtr7g079820.1	AT3G22640.1	6,00E-68	Glyma.10G028300	SBP_SOYBN	0,00E+00	AT3G22640.1	cupin family protein	6,00E-48
Medtr_v1_087438	Medtr1g103400.1	AT3G22640.1	1,00E-61	Glyma.20G148300	Q94LX2_SOYBN	0,00E+00	AT3G22640.1	cupin family protein	2,00E-65
Medtr_v1_078904	Medtr4g025670.1	AT3G60730.1	1,00E-139	Glyma.03G028900	I1JKR6_SOYBN	0,00E+00	AT3G60730.1	Plant invertase/pectin inhibitor superfamily methylesterase	0,00E+00
Medtr_v1_013494	Medtr2g099570.1	AT1G55020.1	0	Glyma.13G347600	B3TDK4_SOYBN	0,00E+00	AT1G55020.1	lipoxigenase 1	0,00E+00
Medtr_v1_044759	Medtr7g079730.1	AT3G22640.1	3,00E-64	Glyma.10G246500	Q948Y0_SOYBN	0,00E+00	AT3G22640.1	cupin family protein	6,00E-62
Medtr_v1_033736	Medtr5g064060.1	AT1G07400.1	5,00E-57	Glyma.12G058200	G8DZS0_9ROSI	1,00E-39	AT1G07400.1	HSP20-like chaperones superfamily protein	5,00E-26
Medtr_v1_006089	Medtr1g072600.1	AT5G44120.3	5,00E-47	Glyma.10G037100	Q9SB11_SOYBN	0,00E+00	AT5G44120.3	RmlC-like cupins superfamily protein	7,00E-54

**Supplementary Table 6.** Continuation...

Probe_id	Seq_id Mt4.0v1	Arabidopsis	e-value	id Gmax	Tax Hit	p-value	Arabidopsis	Name Arabdopsis gene	p-value
Medtr_v1_005824	Medtr1g061760.1	AT5G03795.1	1,00E-167	Glyma.03G189100	T2DLF4_PHAVU	0,00E+00	AT5G03795.1	Exostosin family protein	0,00E+00
Medtr_v1_032184	Medtr5g038060.1	AT1G69870.1	0	Glyma.18G127200	G7K9P5_MEDTR	0,00E+00	AT1G69870.1	nitrate transporter 1.7	0,00E+00
Medtr_v1_046844	Medtr7g113790.1	AT1G03220.1	9,00E-80	Glyma.03G239700	7SB1_SOYBN	0,00E+00	AT1G03220.1	Eukaryotic aspartyl protease family protein	4,00E-84
Medtr_v1_035086	Medtr5g081530.1	AT1G53540.1	1,00E-60	Glyma.02G076600	Q9XET1_SOYBN	7,00E-108	AT1G53540.1	HSP20-like chaperones superfamily protein	2,00E-47
Medtr_v1_074228	Medtr4g056590.1	AT2G27940.1	3,00E-23	Glyma.11G191800	ATL57_ARATH	2,00E-18	AT2G27940.1	RING/U-box superfamily protein	3,00E-21
Medtr_v1_044473	Medtr7g076320.1	AT2G01770.1	6,00E-18	Glyma.18G228200	C1E094_MICSR	7,00E-60	AT2G01770.1	vacuolar iron transporter 1	6,00E-20
Medtr_v1_019599	Medtr3g100650.1	AT4G24660.1	1,00E-55	Glyma.09G170500	Q5IR71_SOYBN	3,00E-96	AT4G24660.1	homeobox protein 22	2,00E-41
Medtr_v1_029725	Medtr5g008850.1	AT4G10020.1	1,00E-140	Glyma.11G015100	G7K984_MEDTR	0,00E+00	AT4G10020.1	hydroxysteroid dehydrogenase 5	1,00E-177
Medtr_v1_095133	#N/D	AT2G20770.1	0,37	Glyma.12G111000	G7KIT8_MEDTR	0,00E+00	AT2G20770.1	GCR2-like 2	0,00E+00
Medtr_v1_039918	Medtr2g008120.1	AT1G43710.1	1,00E-122	Glyma.15G050600	G7IL92_MEDTR	0,00E+00	AT1G43710.1	Pyridoxal phosphate (PLP)-dependent transferases superfamily protein	0,00E+00
Medtr_v1_000534	Medtr8g016520.1	ATCG00170.1	1,00E-124	Glyma.02G221400	RPOC2_SOYBN	3,00E-84	ATCG00170.1	DNA-directed RNA polymerase family protein	1,00E-73
Medtr_v1_023449	Medtr4g051110.1	ATCG00160.1	1,00E-110	Glyma.03G031100	RR2_SOYBN	1,00E-83	ATCG00160.1	ribosomal protein S2	3,00E-70
Medtr_v1_017981	Medtr3g077280.1	AT5G40390.1	0	Glyma.06G179200	G7J4U7_MEDTR	0,00E+00	AT5G40390.1	Raffinose synthase family protein	0,00E+00
Medtr_v1_004965	Medtr1g025490.1	AT1G75810.1	1,00E-24	Glyma.17G258800	Q9ZQY8_ARATH	1,00E-15	AT1G75810.1	unknown protein	1,00E-34
Medtr_v1_086153	#N/D	AT1G61070.1	0,000001	Glyma.13G278000	Q2KM12_9FABA	1,00E-42	AT1G61070.1	low-molecular-weight cysteine-rich 66	2,00E-08
Medtr_v1_012500	Medtr2g081590.1	AT5G25610.1	1,00E-24	Glyma.12G217400	O24482_SOYBN	0,00E+00	AT5G25610.1	BURP domain-containing protein	9,00E-32
Medtr_v1_027908	Medtr4g130540.1	AT1G16030.1	0	Glyma.17G072400	HSP70_SOYBN	0,00E+00	AT1G16030.1	heat shock protein 70B	0,00E+00

**Supplementary Table 6.** Continuation...

Probe_id	Seq_id Mt4.0v1	Arabidopsis	e-value	id Gmax	Tax Hit	p-value	Arabidopsis	Name Arabdopsis gene	p-value
Medtr_v1_085808	#N/D	AT5G03850.1	2,00E-16	Glyma.03G205800	G7KLD3_MEDTR	6,00E-37	AT5G03850.1	Nucleic acid-binding, OB-fold-like protein	9,00E-34
Medtr_v1_066901	Medtr2g090605.1	AT1G03790.1	2,00E-71	Glyma.12G116900	U5FMC6_POPTR	2,00E-100	AT1G03790.1	Zinc finger C-x8-C-x5-C-x3-H type family protein	2,00E-73
Medtr_v1_044763	Medtr7g079770.1	AT3G22640.1	2,00E-68	Glyma.20G148400	Q94LX2_SOYBN	0,00E+00	AT3G22640.1	cupin family protein	2,00E-65
Medtr_v1_030731	Medtr5g020630.1	AT5G48330.1	1,00E-143	Glyma.01G173400	G7KEX6_MEDTR	0,00E+00	AT5G48330.1	Regulator of chromosome condensation (RCC1) family protein	1,00E-163
Medtr_v1_035904	Medtr5g090990.1	AT1G06570.1	1,00E-175	Glyma.02G284600	A5Z1N7_SOYBN	0,00E+00	AT1G06570.2	phytoene desaturation 1	4,00E-159
Medtr_v1_051080	Medtr8g079680.1	AT5G66860.1	1,00E-84	Glyma.05G081300	G7LGD1_MEDTR	1,00E-137	AT5G66860.1	Ribosomal protein L25/Gln-tRNA synthetase, anti-codon-binding domain	9,00E-106
Medtr_v1_034784	Medtr5g077580.1	AT1G30220.1	0	Glyma.09G087400	G7IGM5_MEDTR	0,00E+00	AT1G30220.1	inositol transporter 2	0,00E+00
Medtr_v1_027404	Medtr4g121940.1	AT3G16990.1	2,00E-79	Glyma.17G032700	PM36_SOYBN	6,00E-169	AT3G16990.1	Haem oxygenase-like, multi-helical	2,00E-98
Medtr_v1_031594	Medtr5g030870.1	AT2G32210.1	0,0001	Glyma.08G171400	-	-	AT2G32210.1	unknown protein	2,00E-15
Medtr_v1_007348	Medtr1g098250.1	AT4G09830.1	3,00E-52	Glyma.08G144000	K0A0U9_ELAGV	2,00E-79	AT4G09830.1	Uncharacterised conserved protein UCP009193	2,00E-57
Medtr_v1_031272	Medtr5g026780.1	AT1G31830.1	0	Glyma.20G106200	D7KGN1_ARALL	0,00E+00	AT1G31830.2	Amino acid permease family protein	0,00E+00
Medtr_v1_026593	Medtr4g104210.1	AT3G27160.1	6,00E-21	Glyma.05G044800	G7JMN6_MEDTR	7,00E-34	AT3G27160.1	Ribosomal protein S21 family protein	2,00E-35
Medtr_v1_068329	Medtr4g037465.1	AT3G06240.1	1,00E-20	Glyma.17G017600	D3YBB5_TRIRP	1,00E-95	AT3G06240.1	F-box family protein	8,00E-23
Medtr_v1_013048	Medtr2g089580.1	ATCG00020.1	1,00E-56	Glyma.11G162200	Q85V85_9POAL	7,00E-133	ATCG00020.1	photosystem II reaction center protein A	7,00E-135
Medtr_v1_044022	Medtr7g070020.1	AT4G32520.2	5,00E-27	Glyma.13G222300	C6ZJY9_SOYBN	0,00E+00	AT4G32520.2	serine hydroxymethyltransferase 3	0,00E+00
Medtr_v1_074217	Medtr8g033390.1	AT2G45630.2	2,00E-90	Glyma.07G082000	E6NU30_JATCU	1,00E-127	AT2G45630.2	D-isomer specific 2-hydroxyacid dehydrogenase family protein	2,00E-115
Medtr_v1_009590	Medtr2g013460.1	AT4G14440.1	2,00E-79	Glyma.10G007900	G7IPS1_MEDTR	6,00E-93	AT4G14440.1	3-hydroxyacyl-CoA dehydratase 1	2,00E-90

**Supplementary Table 6.** Continuation...

Probe_id	Seq_id Mt4.0v1	Arabidopsis	e-value	id Gmax	Tax Hit	p-value	Arabidopsis	Name Arabdopsis gene	p-value
Medtr_v1_068217	#N/D	AT1G71190.1	9,00E-79	Glyma.08G131300	K7V5D8_MAIZE	7,00E-104	AT1G71190.1	senescence associated gene 18	2,00E-111
Medtr_v1_089600	Medtr3g113270.1	AT3G27230.1	1,00E-100	Glyma.04G025700	D7MJ22_ARALL	0,00E+00	AT3G27230.1	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	0,00E+00
Medtr_v1_010187	Medtr2g025050.1	AT4G04020.1	1,00E-103	Glyma.09G008100	Q2HTH4_MEDTR	7,00E-149	AT4G04020.1	fibrillin	6,00E-131
Medtr_v1_101070	#N/D	AT1G55850.1	9,00E-15	Glyma.08G330600	G7IV14_MEDTR	0,00E+00	AT1G55850.1	cellulose synthase like E1	0,00E+00
Medtr_v1_050686	Medtr8g074040.1	AT2G17650.1	0	Glyma.02G042200	G7L9H4_MEDTR	0,00E+00	AT2G17650.1	AMP-dependent synthetase and ligase family protein	0,00E+00
Medtr_v1_077620	#N/D	AT3G20390.1	5,00E-64	Glyma.08G316200	B4UWE2_ARAHY	5,00E-104	AT3G20390.1	endoribonuclease L-PSP family protein	4,00E-79

## RÉSUMÉ

Contribution à la compréhension de l'effet de la maturation des graines sur leur qualité physiologique chez les légumineuses

Pendant la maturation des graines, la germination, tolérance à la dessiccation et longévité sont acquises de manière séquentielle. La maturation s'achève par la dessiccation qui amène l'embryon à l'état de quiescence. La maturité des graines à la récolte est le premier facteur qui influence la longévité et l'établissement de la culture lors du semis. On ne comprend pas comment la longévité est installée pendant la maturation et comment un séchage prématuré influence la longévité et la reprise des activités cellulaires pendant l'imbibition. L'objectif de la thèse était de répondre à ces questions en comparant les transcriptomes de graines immatures et matures de soja et *Medicago truncatula* pendant la dessiccation et l'imbibition. Les graines immatures furent récoltées après le remplissage avant la dessiccation, lorsque la longévité n'est pas encore acquise. Chez le soja, la comparaison des transcriptomes des graines immatures et matures montre que le séchage forcé n'est pas identique à la dessiccation *in planta* qui se caractérise par la synthèse de protéines chaperones. Plus de 89% des gènes différemment exprimés après 18 h d'imbibition présentent des profils d'expression identiques dans les graines immatures et matures, en accord avec la germination comparable de celles-ci. L'analyse des transcrits dont la teneur augmente uniquement pendant l'imbibition des graines mature suggère la mise en place de mécanismes de réparation. La comparaison de ces données avec *Medicago* montre que l'imbibition des graines matures se caractérise par une sur-représentation des gènes liés au phototropisme, à la testa et réponse immunitaire. Ce travail doit permettre le développement d'outil d'analyse de la maturité des graines lors de leur récolte.

## ABSTRACT

*Towards understanding the influence of seed maturation on physiological seed quality in legumes*

*During seed maturation, germination, desiccation tolerance and longevity are acquired sequentially. Seed maturation is terminated by a desiccation phase that brings the embryo to a quiescent state. Seed maturity at harvest influences seed longevity and crop establishment. After harvest, seeds are usually dried to water content compatible with long term storage and post-harvest treatments. However, there is a lack of understanding of how seed longevity is acquired during seed maturation and how premature drying impacts longevity and resumption of cellular activities during imbibition. This was addressed here by comparing transcriptome changes associated with maturation drying and imbibition of seeds of soybean and *Medicago truncatula*, harvested at an immature stage and mature dry stage. The immature stage corresponded to end of seed filling when longevity was not acquired while other vigor traits were acquired. Transcriptome characterization in soybean revealed that enforced drying was not similar to maturation drying in planta, which stimulated degradation of chlorophyll and synthesis of protective chaperones. Eighty-nine % of the differentially expressed genes during a 18h-imbibition period showed a similar pattern between immature and mature seeds, consistent with a comparable germination between stages. An analysis of the 147 transcripts that increased during imbibition of mature seeds but not in immature seeds suggested an activation of processes associated with shoot meristem development and DNA repair. These data were compared with imbibing immature and mature seeds of *Medicago* and revealed an overrepresentation of genes involved in phototropism, seed coat and innate immunity in mature seeds. This work should provide new tools to optimize harvest at maximum seed quality.*



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