Physiological, genetic and molecular study of the impact of water stress on the germination quality of sunflower seeds

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LIDEA, RAGT2n, SOLTIS, SYNGENTA FRANCE SAS, MAS Seeds

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Résumé substantiel

Ce travail de thèse a été effectué dans le cadre d'un consortium public-privé, composé de cinq partenaires privés (Lidea, RAGT2n, Sotis, Syngenta France SAS, MAS Seeds). Ce consortium a financé les recherches qui ont pu être réalisées dans les laboratoires LIPME INRAE à Toulouse et à Sorbonne Université, dans l'équipe de Biologie des semences du laboratoire de Biologie de Développement de l'Institut Biologie Paris-Seine (IBPS) (UMR 7622-UPMC-CNRS). D'un point de vue fondamental ; il a eu pour objectif principal de mieux comprendre les bases génétiques et moléculaires de la transmission des traits de tolérance au stress, entre la plante mère et la descendance. Au niveau appliqué, la compréhension de ce processus permettra de mieux contrôler l'impact de l'environnement sur la production des semences et leur qualité. Il sera alors possible de développer des biomarqueurs qui caractériseront la qualité et la vigueur des semences.

Ce manuscrit est constitué de 4 parties. La première est une synthèse bibliographique sur l'impact du changement climatique sur l'agriculture. Elle va préciser l'impact du stress sur la biologie des semences et les mécanismes épigénétiques participants à la mémoire de stress chez les plantes. Les 3 parties suivantes sont présentées sous la forme d'articles. Elles concernent : (1) l'étude des effets du stress de la plante mère sur les propriétés physiologiques de la descendance, (2) l'étude de la régulation transcriptionnelle de la tolérance des graines de tournesol aux stress hydriques, et (3) l'étude de la mémoire épigénétique du stress hydrique chez la descendance.

Dans les années à venir, le réchauffement climatique aura des effets délétères sur les cultures. Une réduction des rendements, liée à la fréquence accrue des épisodes de sécheresse est prévue. Le stress hydrique sur les plantes va devenir plus intense et affecter de plus en plus de zones géographiques (Pachauri et al., 2014). La gestion du stress abiotique environnemental va donc devenir un des défis majeurs si l'on veut maintenir, voire augmenter les rendements tout en préservant la qualité des récoltes.

La germination est un préalable essentiel à la croissance et au succès reproductif des plantes, mais elle peut être affectée par divers stress abiotiques. Dans notre premier chapitre, nous avons étudié l'effet du stress de la sécheresse pendant le développement des graines de tournesol sur la germination de la progéniture. Nous avons donc, appliqué différents scénarios de stress, sur des lignées consanguines et des hybrides de tournesol, et évaluer la germination de la descendance. Le stress, dû à une sécheresse, pendant le développement de la graine, a apporté une tolérance aux stress hydriques, à l'hypoxie, au

froid et au sel pendant la germination de la graine et a également induit une dormance des semences plus faible. Nous avons établi que l'induction de ces caractères n'était pas transgénérationnelle mais transmise par la mère et pouvait être reproduite dans les hybrides de tournesol. Le stress hydrique pendant le développement de la graine diminue l'épaisseur du péricarpe et induit une fuite plus importante d'électrolytes solubles du péricarpe, mais il modifie également le métabolisme de l'embryon. Une analyse métabolomique a montré que l'ABA, les oligosaccharides et les polyphénols s'accumulent différemment dans les graines soumises au stress hydrique et pourraient également participer à la tolérance des graines aux conditions de stress abiotique pendant la germination. Dans l'ensemble, ce chapitre révèle un processus d'adaptation qui permet aux plantes de tournesol exposées au stress hydrique pendant leur stade de reproduction de produire des graines plus performantes, plus vigoureuses. En plus d'apporter un nouvel éclairage sur l'adaptation naturelle des populations végétales au changement climatique, ces résultats peuvent avoir des applications dans l'industrie des semences, grâce à la production de semences résistantes à une plus grande variabilité climatique lors de l'établissement de la culture du tournesol.

D’un point de vue appliqué, les producteurs de grandes cultures ne se focalisent plus seulement sur les rendements maximaux en grains, mais ils visent également le maintien des rendements en cas de stress abiotiques, tels que la sécheresse ou les mauvaises conditions de sol (Vear, 2016). Les politiques (Plan écophyto II+) et les dynamiques socioculturelles (marche pour le climat, mouvement écologique…) poussent à s'adapter au changement climatique en développant de nouvelles techniques culturales basées sur la promotion de la tolérance des plantes aux phénomènes de stress climatique. Puisque nous avons démontré qu'une condition de stress hydrique modérée, au cours du développement des graines de tournesol, pouvait induire une plus grande vigueur à la descendance de certains génotypes, nous avons également évalué si ce caractère était inductible. Cette acquisition de tolérance a été étudiée sur des gammes génétiques plus larges d'hybrides de tournesol et dans des conditions non expérimentales de production en champs, chez nos partenaires privés. Enfin, dans certaines expériences réalisées en conditions de production, nous avons estimé l'effet d'un stress hydrique sur la plante mère sur la forme de la plante, du rendement et de la teneur en huile de la descendance, afin d'évaluer la faisabilité économique de cet itinéraire technique. À plus long terme, nos recherches pourraient, en effet, conduire à la conception d'un nouvel itinéraire technique qui devrait permettre d'augmenter la vigueur des graines de tournesol.

Dans ce contexte de dérèglement climatique global, la caractérisation des voies moléculaires impliquées dans la tolérance au stress hydrique chez les plantes est nécessaire pour améliorer leur résilience globale et réduire leur consommation d'eau. Nous proposons, dans le chapitre suivant de ce manuscrit, un modèle transgénérationnel d'acquisition de la tolérance au stress hydrique. Celui-ci est obtenu en appliquant un stress hydrique sur des plantes de tournesol afin d'améliorer la vigueur des graines résultantes. Nos résultats montrent que les graines produites par les plantes stressées présentent des taux de germination améliorés par rapport aux contrôles. Ces mêmes graines présentent également une tolérance au stress salin et à l'hypoxie. En utilisant différentes méthodes de profilage du transcriptome (RNAseq et RT-qPCR), nous avons identifié quelques gènes candidats potentiels qui pourraient expliquer ce type d'acquisition de tolérance. Ces gènes sont liés à trois processus physiologiques différents, à savoir la réponse aux hormones, la réponse aux espèces réactives de l'oxygène (ROS) et les mécanismes épigénétiques. Les profils d'expression observés dans le gène de la 1-Aminocyclopropane-1-Carboxylique Oxidase (ACO), impliqué dans le métabolisme de l'éthylène, et ceux trouvés dans plusieurs récepteurs de l'acide abscissique (ABA) et des gibbérellines (GA) suggèrent un rôle primordial de la régulation hormonale. Enfin, des variantes d'histones et le gène argonaute 1 (AGO1), qui régulent la condensation de l'ADN, semblent également jouer un rôle dans ce processus.

La mémoire transgénérationnelle du stress chez les plantes est une perspective prometteuse pour l'adaptation des plantes aux contraintes climatiques (Molinier et al. 2006). Elle a déjà été observée dans de nombreux cas, notamment chez Arabidopsis, lors de stress hyper osmotiques. Elle est médiée par des mécanismes épigénétiques, comme la (dé)méthylation de l'ADN en réponse au stress (Wibowo et al. 2016). On rappellera que l'implication de la méthylation de l'ADN et du remodelage de la chromatine dans les mécanismes mémoriels du stress avaient déjà été étudiées chez les plantes, pour le stress thermique (Shanker, Bhanu, et Maheswari 2020).

Dans le dernier chapitre de la thèse, le contrôle épigénétique de la régulation des espèces réactives de l'oxygène (ROS) est étudié, en premiers temps. En effet, la régulation des ROS est bien décrite comme étant un facteur physiologique clé, contrôlant la dynamique des graines en germination. La machinerie antioxydante impliquée dans ce processus comprend plusieurs molécules antioxydantes (acide ascorbique, tocophérol, glutathion) ainsi que des enzymes antioxydantes comme l'ascorbate peroxydase (APX), la catalase, la glutathion réductase et la superoxyde dismutase (Noctor et Foyer 1998 ; Apel et Hirt 2004

; Bailly 2019). Dans un deuxième temps, ce dernier chapitre vise à comprendre le contrôle moléculaire de la tolérance acquise au stress observée dans les descendances après un stress hydrique au cours du développement de la graine. Nous nous focalisons ici, sur l'analyse des modifications du méthylome des graines de tournesol provoquées par ce stress parental. Après une caractérisation de ces modifications pendant l'imbibition des semences, nous avons étudié l'interaction entre les modifications épigénétiques de l'ADN et l'expression des gènes pendant la germination. Notre étude a permis l'établissement de la première carte du méthylome chez le tournesol, à un stade de développement clé pour cette culture, à savoir la germination. Nous avons caractérisé une mémoire intergénérationnelle du stress maternel dans la progéniture au niveau du méthylome de l'ADN. Différents types de méthylation de l'ADN existent chez les plantes, la méthylation de la cytosine dans le contexte symétrique CpG et CHG (où H peut être C, A ou T) et dans le contexte asymétrique CHH (Feng, Jacobsen, et Reik 2010). Des différences de méthylation (DMRs) ont été acquises au cours du développement de la graine, Le stress maternel a augmenté les taux de méthylation de CHG et réduit ceux de CHH. Pendant la germination en condition de stress hydrique, les DMRs de CHH étaient particulièrement moins méthylés. Bien qu'aucune relation simple n'ait été identifiée entre la méthylation et le niveau d'expression, les principaux gènes du métabolisme des ROS et de la régulation hormonale ont montré à la fois des differences dans leur expression et dans leur profil de méthylation. Une validation indépendante a confirmé que quatre DMRs étaient également méthylés de manière différentielle dans les graines produites dans des conditions de production en champs. Ces résultats constituent une première étape vers le développement d'un test biologique pour identifier les graines tolérantes. Pour compléter la dimension épigénétique de cette tolérance acquise intergénérationnelle, l'étude d'autres marqueurs épigénétiques reste à faire.

L'ensemble des résultats obtenus dans le cadre de ce travail permet de mieux comprendre quels sont les mécanismes impliqués dans la transmission des traits de tolérance aux stress entre la plante mère et la descendance. Il apporte des solutions pour une application de production en champs.

Dans le chapitre discussion générale, ces données seront mises en perspective et des pistes de recherche pour de futurs travaux seront évoquées.

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Abbreviations

ABA : Abscisic acid

PCA : Principal Component Analysis

RNA : Ribonucleic acid

mRNA : Messenger Ribonucleic Acid

ATP : Triphosphate Acid

Bp : Base pair

CAT : Catalase

CAT1 : Catalase 1

DNA : Deoxyribonucleic acid

ET: Ethylene

GA : Gibbereline

GO : Gene Ontology

GWAS : Genome wide association species

H2O2 : Hydrogen peroxide

HSP : Heat shock protein

LOO : Lipidic radical

LOOH : Lipid peroxide

MDA : Malondialdehyde

MPa : Mega Pascal

MS : Dry matter

NADP : Nicotine amide diphosphate

NADPH : Nicotine amide diphosphate reduced

%germination : Percentage of germination

PEG : Polyethylene glycol

PMG : Thousand kernel weight

QPCR : Quantitative Polymerase Chain Reaction

RNAseq : Ribonucleotide acid sequencing

ROS : Reactive oxygen species

SNP : Single nucleotide polymorphism

SOD : Superoxide dismutase

TFs: : Transcription Factors

α -Toc : α -tocopheryl

α -TocH : α -tocopherol

Introduction

Agriculture must constantly adapt to the many challenges and environmental constraints in order to feed a growing population. As global warming accelerates and phytosanitary regulations are becoming more and more restrictive, farmers have a real need for high-performance seeds with a selection of adapted genetics.

Sunflower (*Helianthus annuus* L.) is one of the most widely grown oilseeds in the world. In 2021, worldwide sunflower production reaches a historical record of 57,3 Mt, with an increase of 8,1 Mt in a single year, clearly linked to an increase in acreage due to a strong demand for sunflower oil. In Europe, sunflower production is the second oilseed production after rapeseed (17 Mt) and reache 10,6 Mt in 2022, which represents an increase of 17% compared to the previous year, according to France Agrimer (France Agrimer, 2022). In France, the national recovery plan for the promotion of plant proteins and a food sovereignty was implemented, the results are encouraging, an increase of 18.1% of production was observed in 2021 (1.9 Mt) and those despite a decrease in cultivated areas from 710 000 to 690 000 h (Terres Inovia, 2021).

This increase in production is important to ensure food security. Indeed, the surface of arable land is decreasing (expansion of residential and industrial areas) or land dependent on irrigation and subject to constant biotic pressure (the appearance of new pathogens). Therefore, farmers need seeds more and more efficient and adapted to the local soil. There are more and more varieties resistant to a specific pathogen due to the gene tolerance existence. For example, new rapeseed varieties created with low sensitivity to phoma (*Pythium*), or wheat with tolerance against some fungi like *Fusarium*. For drought resistance, this trait is more complex to improve. There are different strategies such as the selection of varieties with shorter cycle in order to limit the risks of climatic issues during the flowering period or the harvest. Another strategy is to change cultural practices like the association between plant cover and crop to avoid pesticide and fertilizer uses (SEMAE, 2022). Currently the best adaptation is to mix all these cultural practices. For a better management of drought, it is necessary to develop new strategies by combining agronomic, breeding and biotechnological approaches to promote plant tolerance to climate change.

Academic research laboratories and private companies must work together to resolve this challenge. The SUNRISE project has allowed a great advance in the genetic and climatic understanding of the sunflower crop. This consortium, in addition to the numerous scientific discoveries, has led to this applied thesis. Lidea (Caussade semence), RAGT2n, Sotis, Syngenta France SAS, MAS Seeds and the laboratories LIPME of INRAE Toulouse and Biologie des Semences from Sorbonne Université were all involved in the realization of this

research project. The main objective of this thesis was to better understand the genetic and molecular basis of the transmission of stress tolerance traits between the mother plant and the offspring. At an applied level, the understanding of this process will allow a better control of the impact of the environment on seed quality during their production and the development of biomarkers to characterize seed quality with respect to conditions of production. This manuscript is based on independent articles, with the first one dealing about the induction of abiotic stress tolerance to the progeny at the germination stage. The second article presents a transcriptional study of trans-generational acquired tolerance of germination to various abiotic stresses after drought stress. The last article is a study of the epigenetics aspect of the tolerance acquisition. In particular, it addresses the impact of maternal or germinative stress in DNA methylation, the position, level of methylation and the interaction between methylation and gene expression. All these researches allow us to propose a model of the cellular, genetic and physiological mechanisms involved in the induction of stress tolerance in sunflower seeds during germination by modulating the development conditions of the mother plant. They also led to the promotion of new cultural practices to stimulate tolerance to water stress at the germination stage. The economic and industrial benefits could be very significant for the end use of sunflower seeds because it should lead to a better understanding of the mechanisms of dormancy establishment and vigor according to climatic parameters and should also provide new technical itineraries for the production of high-quality seeds.

Chapter 1:

State of the art and objectives of the study

I) Effect of climate change on agriculture

1.1 Origin and expected effects of global warming

The human activity has profoundly transformed earth biomes on a scale and at rates that are unprecedented. Because of anthropogenic emissions of carbon dioxide, global climate may depart significantly from its natural behaviour for many millennia to come. These changes were so drastic in an extremely short time span that a new geological era has been defined. The term attributed to this present era was "Anthropocene"(Crutzen, 2016; Crutzen and Stoermer, 2021). The major effects of human influence on biotic and abiotic environments were: forest clearance (for land use), emissions of greenhouse gases (CO_2, NO_2 and CH_4) and effects on global temperature. Global warming is a self-maintaining phenomenon, as the clearing of forests has resulted in greenhouse gas emissions and temperature changes on a global scale far greater than those proposed by the industrial area (Ruddiman, 2013). The concentrations of the 2 major greenhouse gases CO_2 and CH_4 began to rise exponentially in atmosphere since 1850 (Fig1). These increases were mostly related to land use, caused by rapid population growth and by the burning of fossil fuels (Raynaud et al., 2003). Atmospheric CO_2 rised from 280 ppm at the beginning of the industrial revolution to the current value of 419 ppm. For atmosphere CH_4 the augmentation was similar, from 800 ppb in 1850 to 1909 ppb in 2021.

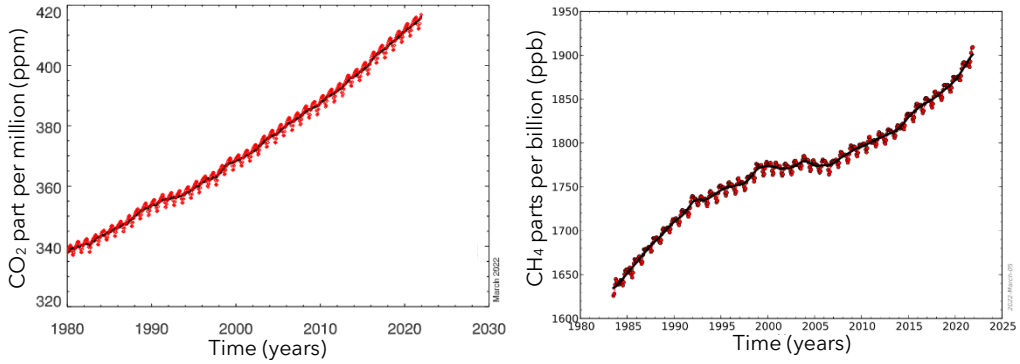

Figure 1 : Increase in atmospheric CO_2 and CH_4 concentrations in last years.
In the figures, the red lines and circles are globally averaged monthly mean values centered on the middle of each month. The black line and squares show the long-term trend (in principle, similar to a 12-month running mean) where the average seasonal cycle has been removed (Dlugokencky, 2022).

The increase of greenhouse gases has been responsible for most of the warming from the middle of the twentieth century ("IPCC," 2022a). The 21st-century global warming

projection far exceeds the natural variability of the past 1000 years and is greater than the best estimate of global temperature change for the last interglacial aera (Crowley, 2000). Different climate models predict increased plant evapotranspiration and lower soil moisture levels in the near future ("IPCC," 2022a). Human-produced greenhouse gases have already trapped enough infrared energy to warm the planet by more than 2°C (Ramanathan and Feng, 2008). In the he last climate prognostic based on the current rate of development, global warming could reach 2.7°C by the end of the century, which is higher than the 1.5°C limit defined by the COP 21 Paris Agreement (Fig 2a). The increase in drought in Europe will be dramatic if the 2°C limit of temperature increase is outreached, with negative impacts on agricultural production, also related to limited water availability (Fig2b).

In the context of global warming, water stress on plants is thus likely to become more intense and to affect more and more geographic areas (Pachauri et al., 2014) and environmental abiotic stress will be a major challenge for the next decades for crop yields (Hatfield et al., 2011). Water stress on plants is likely to become more intense and affect more and more geographic areas (Pachauri et al., 2014).

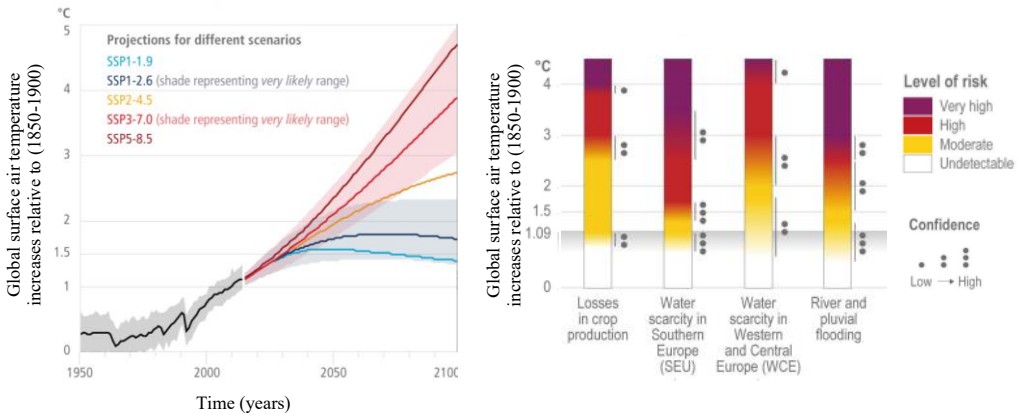

Figure 2 : Climatic model simulations and risks indices.

a. Climatic model simulations of global surface temperature change during the period 1950-2100. b. The risks indices of Intergovernmental Panel on Climate Change (IPCC) for Europe under actual climatic change. The color gradient indicates the level of risk to society and ecosystem as a function of global temperature change (adapted to "IPCC," 2022).

1.2 Impact of abiotic stress on agriculture

Climate change had already an impact on marine and terrestrial biospheres. Both migration and local extinction of populations have occurred. If climate changes proceed as expected (4.3°C increase in global temperature since pre-industrial times), one out of six species

could face extinction (Urban, 2015). Severe ecosystem changes cannot be excluded on any continent. In particular in the boreal temperate ecotone, where heat and drought stresses might lead to large-scale forest die-back (Heyder et al., 2011).

Global warming affects ecosystems at multiple scales, in the organism, populations, or in the species of biodiversity and the species interaction. This impact could change the systemic services that ecosystems provide which support human economies and well-being (Weiskopf et al., 2020). These increases of abrupt changes in ecological systems may make ecosystems more fragile and impact their resilience of system (Zscheischler et al., 2018).

Climate change will have negative consequences on crop production and will impact food security (Chakraborty and Newton, 2011; Schmidhuber and Tubiello, 2007). The different environmental stresses will result in yield reduction. The estimation of each degree-Celsius increase in global mean temperature would, on average, reduce global yields of wheat by 6.0%, rice by 3.2%, maize by 7.4% and soybean by 3.1%. But these estimations are highly heterogeneous across crops and geographical areas. (Zhao et al., 2017). In France the estimated impact on crops is a decrease of yield of −2.6 ± 6.9% per degree Celsius for maize and −6.0 ± 4.2% per degree Celsius for wheat. Drought stress is one of the major limitations to global agricultural production due to the perspective of the water deficit and heat stress.

Climate change have an impact on crop productivity which will affect global food security. For example, demand for agricultural products is estimated to increase by about 50% by 2030 as the global population increases (Coulter, 2004). The consequences of climate change will have effects at different economic, societal and ecological scales. The global food equation will be affected, both on the supply and demand side, and on food systems at the local level (Wheeler and von Braun, 2013). The Food and Agricultural Organization (FAO) estimates that about 2 billion of the global population out of over 7 billion are already food insecure (Von Grebmer et al., 2012).

The climate change will also impact food quality. The contamination of foods and feeds with mycotoxins is a significant problem for food quality and health (Zain, 2011). Mycotoxins like aflatoxins in corn, wheat, ochratoxins in cereal grains (wheat, barley, oats, corn), trichothecenes (in Corn, wheat), zearalenone (in Corn) and ergot alkaloids (in cereals) are the mycotoxins of greatest agro-economic importance. The production of mycotoxins on crops, is highly susceptible to environmental factors, temperature and available moisture, during the pre- and/or post-harvest. Whereas there are many factors involved in mycotoxin contamination, climate is the most important. (Paterson and Lima, 2010)

For oleic cultures, water stress will alter oil content and quality. In peanut plants (*Arachis hypogaea* L.) subjected to drought, linoleic acid and behenic fatty acid contents decreased while stearic and oleic fatty acid contents increased (Dwivedi et al., 1996). In maize, drought stress markedly decreases seed oil content, total tocopherols, flavonoids and oil phenolics(Ali et al., 2012). In soybean (*Glycine max* (L.) Merr.), drought stress can decrease oil content up to 12.4%, and also reduce the oleic acid content (Dornbos Jr. and Mullen, 1992).

Climate change will also affect the amount and nutrient quality of food products. Studies have shown that increased temperature and elevated CO_2 levels can reduce the nutrient density of some staple crops. For example zinc, iron and protein concentrations in wheat, rice and legumes (e.g. field peas, soyabeans) were significantly lower when grown at elevated CO_2 compared to those grown at ambient CO_2 levels (Myers et al., 2014). Some reduction in protein concentrations in other crops, such as barley (14 %), potatoes (6· %), fruit (23 %) and C3 vegetables (17 %) were evaluated under atmospheric CO_2 concentration attributable to anthropogenic CO_2 emissions by 2050 (Medek et al., 2017). Extreme climate phenomena, such as heat waves, storms, droughts and flooding can distrust agricultural productivity due to damage to crops. Moreover some cultural practices, like early seeding or planting of winter wheat increases the risk of flooding during seeding (Howe and White, 2003). Different models of estimation or management of food lost in crop exist (A. Chen et al., 2021; Dutta et al., 2003; Mileti, 1999), and the importance of this problem will increase in the future years.

In addition to the global temperature increase, the occurrence of brief, extremely hot weather, or "heat stress" events raises as well. Heat waves are likely to become more frequent with global warming (Tebaldi et al., 2006). The impacts of this heat stress are multiple: yield reduction associated with pollen sterility, increased pollination failures, increased pests, reduced productivity, decreased fodder quality (Anwar et al., 2013).

Plant pests and diseases could potentially deprive humanity of up to 82% of the attainable yield in the case of cotton and over 50% for other major crops (Oerke, 2006). Each year the estimated losses by plant pathogens impact 10 to 16% of the global harvest (Strange and Scott, 2005).

The period during which the stress occurs determines the extent of the impact on the plant. For *Zea mays,* all vegetative and yield parameters (plant height, leaf area index, grain yield per hectare, as well as the number of spikes per plant, grain yield per strike and 1000 kernel

weight) were significantly affected by water stress (no irrigation) during strike formation stages. If the water stress was applied during vegetative stages, the impact is a decrease of plant height, as well as leaf area development. The duration of the stress is also its important, indeed even short-duration water deficits during the rapid vegetative growth period can cause 28–32% loss of final dry matter weight. This yield loss is more important when the duration time of water stress increases (Boyer and Westgate, 2004; Çakir, 2004).

Drought stress in the early phase of seed filling decreases the subsequent germination percentage (approximately by 9%) of the progeny in soybean (*Glycine max* L.), as compared to control plants (Smiciklas et al., 1992).

1.3 Effect and response to water stress on the whole plant

Plants have evolved a series of mechanisms at the morphological, physiological, biochemical, cellular, and molecular levels to overcome water deficit or drought stress conditions. The drought resistance of plants can be divided into four basic types: drought avoidance, drought tolerance, drought escape and drought recovery (Fang and Xiong, 2015).

Drought is a multiform constraint expressed at different levels of plant organization *via* adaptive responses determined by the characteristics of the genotype. The water balance of the plants can deteriorate, causing situations of deficit. Different adaptive mechanisms are then put into play by the plant to maintain a favorable water status and/or to tolerate water status and/or to tolerate dehydration.

Soil water is used by plants during their development to transport nutrients and produces biomass through the mechanism of photosynthesis. However, plants do not have the capacity to perform photosynthesis without water. Therefore, depending on the species, variety and environmental conditions, or on their water use efficiency, the ratio of CO_2 assimilation or biomass accumulation of water loss varies. There are different processes that allow the plant to maintain the osmotic balance despite drought conditions. For example, the regulation of the stomatal conductance reduces the transpiration by the closure of the stomata to limit the loss of water by evapotranspiration. The reduction of leaf growth and/or the acceleration of the leaf senescence also reduce water losses (Maury et al., 2011). For example, sunflowers and soybeans have been shown to have a water-use efficiency of 54 and 30 $kg.ha^{-1}.cm^{-1}$ respectively (Anderson et al., 2003). Plants lose water through the phenomenon of evapotranspiration. For crops, the evapotranspiration takes into account evaporation of water from the soil surface and the leaves, but also transpiration of free water

into plant tissues through stomata. Evapotranspiration depends on climatic and environmental conditions, such as evaporative demand. The water demand for a crop varies according to the species, the variety and the phenological stage. Maximum evapotranspiration of the crop assesses these plant characteristics and refers to the evaporation demand for a crop growing in large fields, under optimal soil water, which reaches its full production. In addition, plant physiology and crop management factors such as soil salinity, fertilizer application, soil horizon penetrability, disease control, or soil moisture content affect crop development and thus evapotranspiration. Drought stress is perceived by the plant when a significant water deficit occurs because water losses are greater than soil water availability. More importantly, this definition of drought stress does not only depend on environmental conditions such as frequency of precipitation, evaporative demand or amount of water available in the soil, but also on characteristics of the plant species.

At a cellular scale, dought stress decreases CO_2 assimilation rates, reduction in the contents and activities of photosynthetic carbon cycle enzymes. Moreover drought stress also induces generation of active oxygen species (ROS) (Ramachandra Reddy et al., 2004) and the ability of the plant cells to cope with ROS generation is a key factor of plant tolerance to water stress. A set of molecular mechanisms also allow the tolerance of plants to dehydration. They include the accumulation of osmolytes that contribute to maintain an osmotic balance at the cellular level under conditions of dehydration (Bray et al., 2000) and the accumulation of soluble sugars, such as sucrose (Bianchi et al., 1991). During adaptation to drought, plants can accumulate proteins involved in the protection of cellular structures. In particular, the "LEA" (Late-Embryogenesis-Abundant) proteins constitute an important group of proteins that typically accumulate during the late stages of embryogenesis, but more generally in response to cellular dehydration induced by various stresses (Ramanjulu and Bartels, 2002).

As for the whole plants, seeds, either during their formation or during their germination, can also face water stress, but the effects of drought stress on seed biology differ from the ones evidenced for whole plants (Anderson et al., 2003)(Tardieu and Tuberosa, 2010).

II) Effect of water stress on seed biology

2.1 Seed development and structure

Almost all plant cultivations in agriculture and horticulture are based on seeds. The vast majority of our domesticated plants are propagated *via* seeds, which provide most of our caloric intake either directly as food or indirectly as feed for our domestic animals. Seed development in plants begins with a double fertilization of the oosphere and the polar nuclei in the embryo sac. The embryo (2n chromosomes) results from the fertilization of an oosphere by a gamete (Fig 3). The albumen (3n chromosomes) is the fusion of the 2 polar nuclei with the other male gamete. The ovule teguments are at the origin of the seed teguments (2n maternal), and the wall of the ovary gives the pericarp (Goldberg et al., 1994).

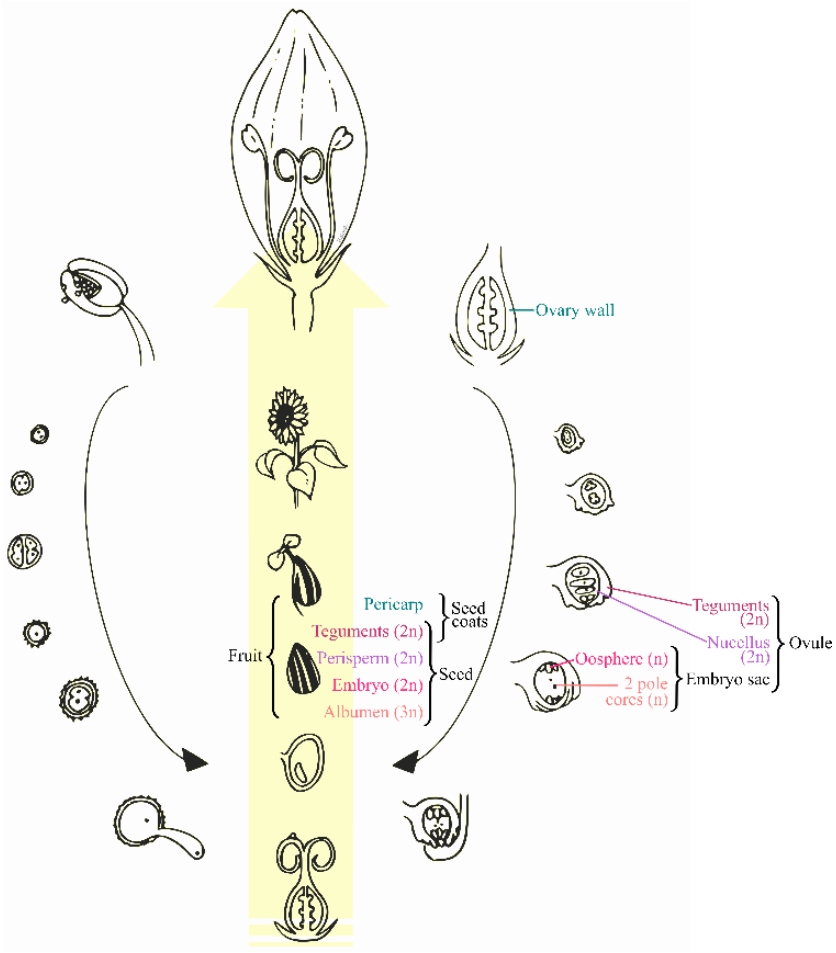

Figure 3 : Origin of the different parts of the seeds and fruits of angiosperms (adapted from Côme et Corbineau, 1998) **Beink©.**

2.2 Seed germination

Germination phase begins when mature dry seeds are imbibed by water and finishes with the emergence of the radicle (embryonic axis). Seed germination includes (Fig 4):

imbibition, germination *sensu stricto* and they grow phases. Imbibition corresponds to the absorption of water during which the metabolism of the seeds is reactivated. Respiratory, metabolic, transcriptional and translational activity resumes. The germination *sensu stricto* is characterised by a stable level of water content in the seed, a reactivation of the synthesis of new mRNAs and the synthesis of new proteins. It ends with the elongation of the radicle through the envelopes. Then the growth phase begins and is characterized by the resumption of water absorption by the seed, mobilization of reserves, cell division and synthesis of new DNA (Bewley, 1997). In oleaginous seeds, such as sunflower, it is mainly triacylglycerol (TAGs) that are hydrolyzed by lipases to release fatty acids providing essential energy to continue the growth (Goepfert and Poirier, 2007) until autotrophy.

Germination term can have different meanings: for seed physiologists, germination is completed when the radicle appears, through the envelopes; for agronomists, germination is completed when the aerial parts emerge, and seedlings change form heterotrophic to autotrophic state. In phases I and II, seed metabolism is reactivated. the activation of respiration and high water uptake leads to the generation of ROS. This can lead to DNA damage. Activation of DNA repair is essential for seed germination to proceed to the next phase. In addition, ROS are also responsible for ABA interaction, with a documented influence on endosperm rupture. ROS, which are part of multiple signaling pathways, act at multiple levels (gray and red boxes in Fig 4), from protein conformation to activation of transcription factors and regulation of gene expression, to cell elongation and endosperm fragility.

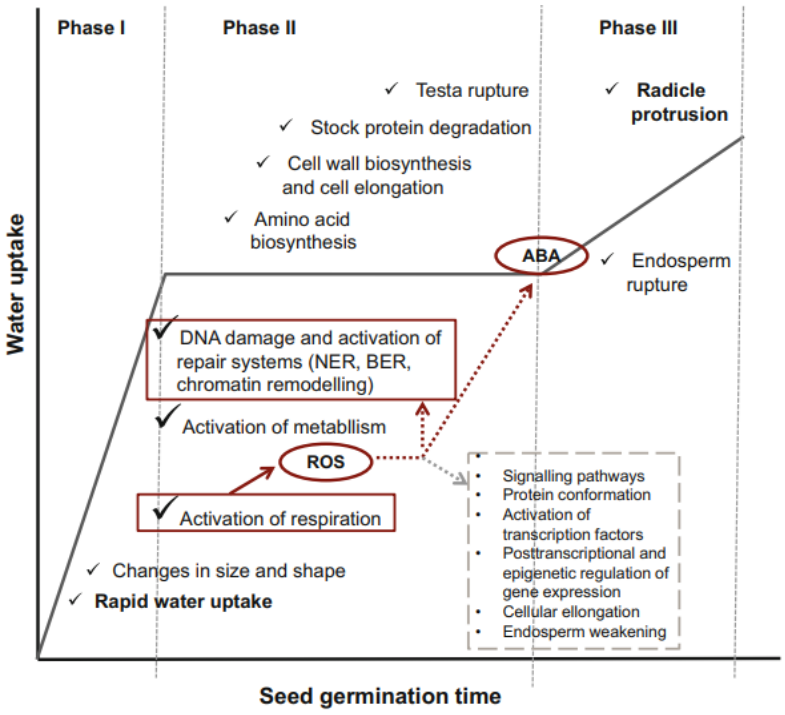

Figure 4 : Theoretical seed imbibition curve showing the evolution of water content according to the three phases of germination. The imbibition (phase I), the germination sensu stricto (phase II) and the growth (phase III) (Macovei et al., 2017)

2.3 Seed vigour

Seed vigour is the ability of a seed to germinate in a wide range of environmental conditions, i.e. optimal and suboptimal conditions, leading to rapid and homogenous seedling emergence and stand establishment (Hampton, 2002). Seed vigour is controlled by genotype, growth conditions of the mother plant (nitrogen apport, environmental), physiological maturity of the seed at harvest, physical alterations of the seed during processing, seed moisture content and temperature during storage (Finch-Savage and Bassel, 2016). Seed vigour can also be altered during seed maturation in the field if the exposition of inappropriate temperatures, moisture fluctuations or pathogens are too important (Dornbos, 1995). High vigour seeds for all crops is justified to ensure an adequate plant population across a wide range of field conditions that occur during emergence (TeKrony and Egli, 1991).

The evolution of seed vigour can be schematically represented like Fig 5. The greatest potential for vigour occurs at physiological maturity when the seed reaches its maximum dry seed weigh and germination potential. During the period between mass maturity and harvest maturity, seeds can be exposed to unfavorable conditions which can directly affect seed vigour. During the storage the unfavorable conditions (high temperatures, excessive

precipitation, seedborne disease infection) can influence the seed capacity to germinate and induce loss of vigor during seed deterioration. This seed deterioration is progressive with time and the consequences manifested in a reduction in performance capability. Deterioration is influenced by the characteristic of seed lot (genetic and physiological composition). Fig 5 show the acquisition of viability, germination and vigour during seed development and maturation and the opposite during seed deterioration (Tekrony, 2003). The environmental conditions of growth the mother plant, the maturity of the seed at harvest, can also influence the seed vigour (Finch-Savage and Bassel, 2016).

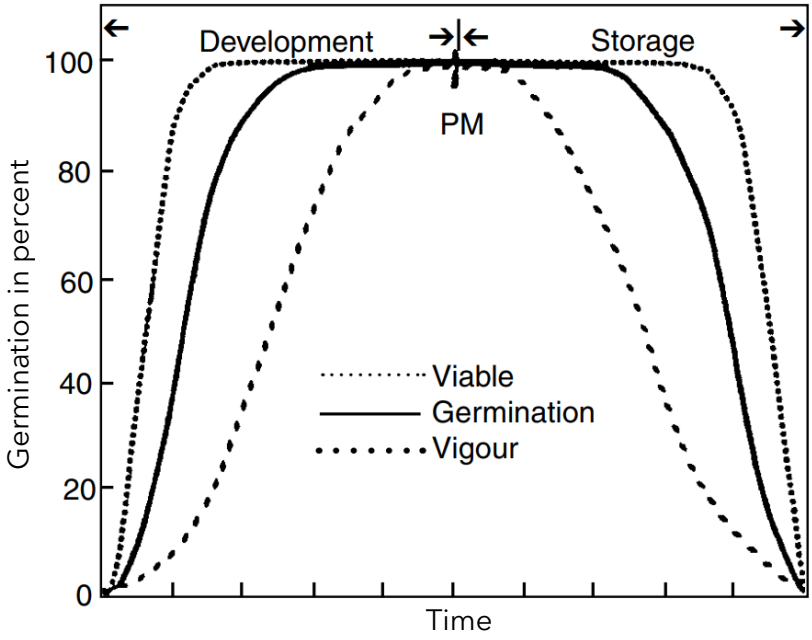

Figure 5 : Evolution of seed viability, germination and vigour.
Proposed increases and decreases in soybean seed viability, germination and vigour during seed development and deterioration, respectively. (PM = maximum dry seed weight, physiological maturity) (Tekrony, 2003).

2.4 Seed Dormancy

Seed dormancy is defined by the inability of a viable seed to complete germination under favorable environmental conditions (for example water, temperature and oxygen) (Baskin and Baskin, 2004; Penfield, 2017a). In some case, dormant seeds can germinate in specific conditions but not fully. In fact, dormant seeds are more sensitive to environmental factors than non-dormant seeds (Baskin and Baskin, 2004; D. Côme and Corbineau, 1998). This is an adaptative mechanism to prevent germination after harvest, in order to avoid seedling development under the non favorable conditions of winter (Née et al., 2017). However, a

deep dormancy can also prevent germination during optimal time and have an impact on cycle duration (shorter cycle for example) (Donohue et al., 2010).

Two types of dormancy have been described (Fig 6). Primary dormancy is established at the end of seed development on the mother plant. Primary dormancy induction is determined by the expression of the DELAY OF GERMINATION 1 (DOG1) during the late stages of seed maturation (Nakabayashi et al., 2012), and the plant hormone ABA prevents precocious germination of embryos (Siraree and Misra, 2020). It is gradually eliminated over time. If the environmental conditions during seed germination are not optimal, a secondary seed dormancy can appear. This dormancy is essentially under the control of environmental conditions (temperature fluctuation, light and oxygen), rather than the environment experienced by the parent plant (Finch-Savage and Footitt, 2017; Finch-Savage and Leubner-Metzger, 2006).

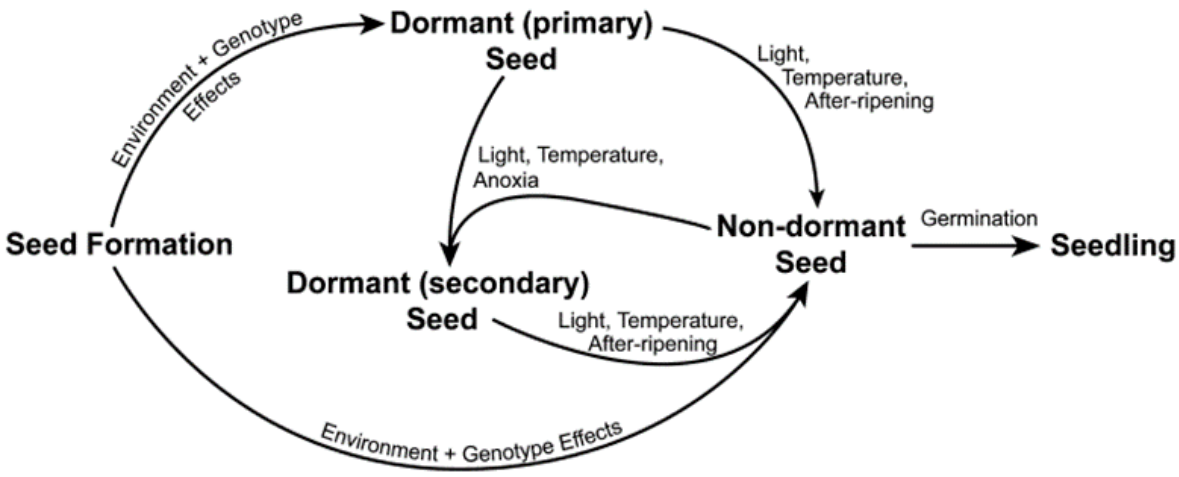

Figure 6 : Main events and factors involved in the establishment of primary and secondary dormancy (Bewley et Black, 1994).

Seed envelops play an important role in dormancy mechanism (Vigliocco et al., 2017). Dormancy can be classified as embryonic dormancy or seed coat dormancy. Seed coat dormancy results from the impermeability of the envelops to water or oxygen (Côme and Corbineau, 1998). In Angiosperm, physiological dormancy, *i.e.* seeds needs to have a treatment like post-maturation or cold stratification to alleviate dormancy, is the most common. The intensity of this phenomenon at harvest can be low, medium or high. Moreover this phenomenon depends on species (Baskin and Baskin, 2004).

2.5 Regulation of seed germination2.5.1 Role of ROS

Reactive oxygen species (ROS) have a capacity to damage macromolecules in cells (lipids, nucleic acids, and proteins) by oxidation, but they also play a role in seed dormancy and germination (Bailly et al., 1996). The level of ROS in seed is a critical factor during germination. If the amount of ROS during imbibition is too low, the seed is not able to germinate but an increase of the cellular level of ROS during seed imbibition results in germination. However, if ROS level is too high, this becomes deleterious and causes cellular oxidative damage that prevent or delay germination. The increase of ROS level can happen when seeds are aged or placed in inappropriate environmental conditions during their imbibition (Bailly, 2004). So, to manage the level of ROS, they are regulated by different antioxidative defense mechanisms (Fig 7 b.). ROS regulation mechanisms can be classified into two types: enzymatic and non-enzymatic antioxidants. The enzymatic one includes superoxide dismutase SOD, catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX) (Apel and Hirt, 2004). The non-enzymatic systems are mainly mediated by antioxidant vitamins like, ascorbic acid (vitamin C, AsA) and α-tocopherol (vitamin E , α-tocH) (Bailly et al., 2008).

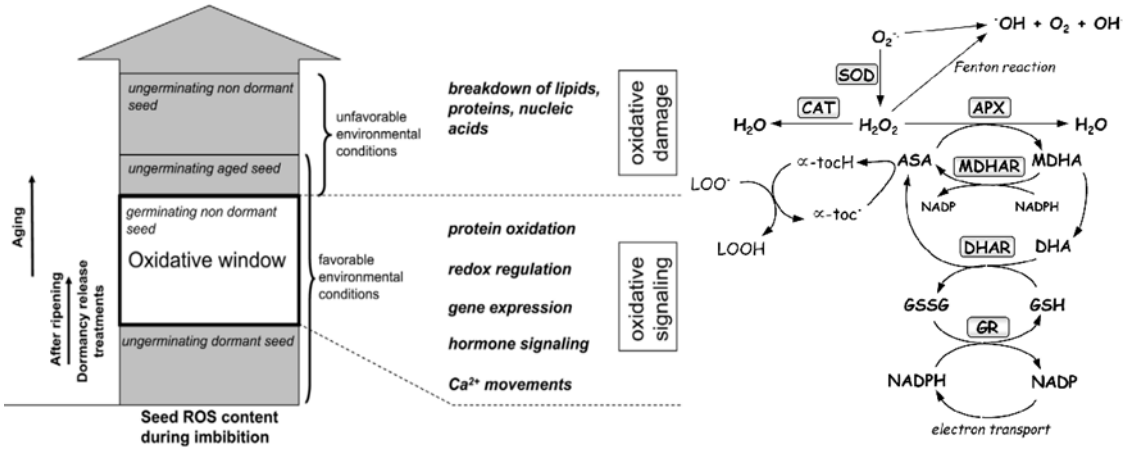

Figure 7 : Role of ROS in seed physiology.

a. The amount of ROS in seeds during their imbibition is represented as boxes. The oxidative box is the only window how ROS content promote seed germination. b. scheme of main detoxifying systems in plants. This system is composed by Catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), ascorbate (ASA), monodehydroascorbate (MDHA), dehydroascorbate (DHA), oxydised glutathione (GSSG), reduced glutathione (GSH), α-tocopherol (α-tocH), α-tocopheryl (α-toc·), lipid peroxide (LOOH), lipids radicals (LOO·) (Bailly, 2004; Bailly et al., 2008).

The accumulation of ROS has been observed during seed germination (Li et al., 2017). They can directly interact with polysaccharides of the cell wall that might promote cell elongation

of the radicle (FRY, 1998). An appropriate level of ROS can decrease seed dormancy and promote seed germination, However, below this ROS threshold can induce seed dormancy. Its molecular mechanisms are poorly understood in most crops (Fig 7.a) (Li et al., 2022). The interaction between ROS and hormones (abscisic acid and gibberellins) plays a role in the regulation of seed dormancy and germination. The accumulation of ROS is affected by hormonal balance. Some study in *Avena fatua* or *Brassica parachinensis* show an increase of ROS by exogenous GA treatments (Cembrowska-Lech et al., 2015; Chen et al., 2021) In *Arabidopsis* ROS homeostasis can be altered by Arabidopsis Abscisic Acid-Insensitive 5 (ABI5) a component of ABA signaling, though affecting CAT expression and his activity (Bi et al., 2017).

2.5.2 Hormonal regulation of seed germination

The transition from seed dormancy to germination is principally controlled by the hormonal balance of abscisic acid (ABA) and gibberellins (GAs) (Bradford and Nonogaki, 2008). ABA promotes dormancy induction and maintenance, whereas GA promotes progression to germination. Environmental signals regulate this balance by modifying the expression of biosynthetic and catabolic enzymes.

ABA is a sesquiterpene compound resulting from the cleavage of carotenoids, it controls storage reserve accumulation and desiccation tolerance of orthodox seeds (seeds which will survive drying and/or freezing during ex-situ conservation) (Nambara et al., 2010). ABA produced by maternal tissues or exogenously is not sufficient to induce dormancy (Finch-Savage and Leubner-Metzger, 2006) and the embryo by itself also plays a role in the induction of dormancy. ABA concentrations are determined principally by the balance between biosynthesis and catabolism. The hormone ABA acts through a very conserved signal transduction pathway in evolution (Sun et al., 2020). This track is composed of a PROTEIN PHOSPHATASE 2C (PP2C), PYRABACTIN RESISTANCE 1-Like (PYL) and SNF1-RELATED PROTEIN KINASE 2 (SnRK2). ABA can be bind to a soluble PYL protein and triggers a conformational change that allows the receptors to bind and inhibit the PP2C that normally represses ABA signaling.(Melcher et al., 2009)

GAs are a family of 136 tetracyclic diterpenes, among them some are bioactive (eg GA4) and stimulate seed germination in a wide range of plant species (Thomas et al., 2005). This hormone promotes the growth of the seed embryo by weakening the barrier tissues of the envelopes (albumen and teguments) through the induction of hydrolytic enzymes (Leubner et al., 1996). GAs also promote the mobilization of seed storage reserves and stimulate

expansion of the embryo (Bewley and Black, 1994b). The notion of hormonal balance is determinant for germination. Indeed (Ali-Rachedi et al., 2004; Bewley, 1997) a decrease in ABA levels is required before GAs level and sensitivity can increase (Jacobsen et al., 2002).

The perception of an ABA signal involves many actor: PYR/PYL/RCAR (PYRABACTIN RESISTANCE PROTEINS/PYR-LIKE PROTEINS/REGULA TORY COMPONENTS OF ABA RECEPTOR), some receptors, PPC (PHOSPHATASE 2C) phosphatases and SnRK2 (SNF1-RELA TED PROTEIN KINASE 2) kinases. The hormone signalization is a controlled by multiple transcription factors. It includes the protein basic leucine zipper transcription factor the ABA Insensitive 5 (ABI5), stabilized by ABA, Able to reduce the accumulation of bioactive GA.

In dormant seeds, the high content of ABA induces an active signaling pathway involved in dormancy maintenance (Fig 8.). In contrast the lower ABA concentration in nondormant seeds would be associated with a higher level of ROS (H_2O_2), which could promote ABA signaling and repress GA signaling pathways (El-Maarouf-Bouteau and Bailly, 2008)

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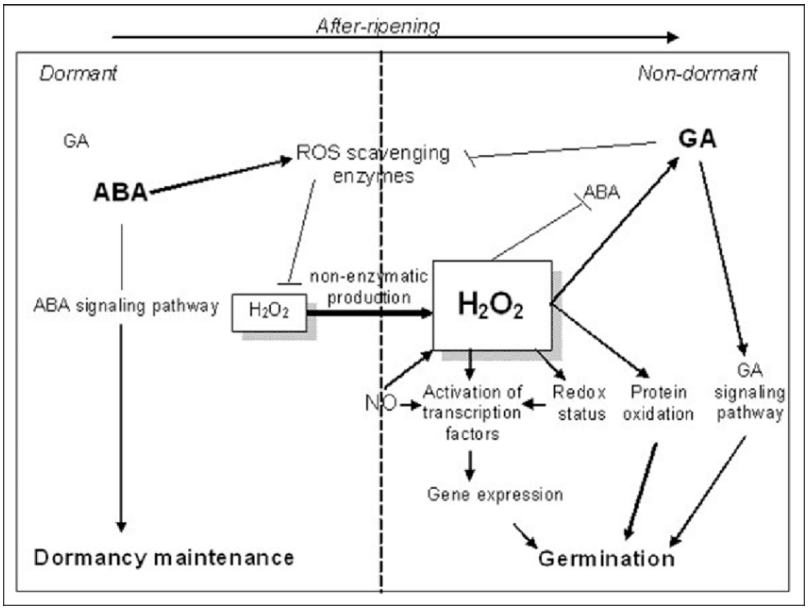

Figure 8 : Scheme of model hormonal balance between abscisic acid (ABA) and gibberellic acid (GA) in dormant or non-dormant seed.
The size of the letters is proportional to the relative amount of the compounds (ABA, GA, H2O2) (El-Maarouf-Bouteau and Bailly, 2008).

ABA regulates several aspects of plant development including seed development, desiccation tolerance of seeds and seed dormancy and plays a crucial role in the plant's response to abiotic (drought, salinity, cold, and hypoxia) and biotic stresses. (Huang et al., 2012) ABA is synthesized mainly in response to drought and high-salinity stress (Shinozaki et al., 2003).

In addition to the ABA/GAs hormonal balance, other hormones are involved in the regulation of germination and dormancy, such as brassinosteroids (Hu et al., 2021) or ethylene. These hormones regulate elongation and cell division in developing plants. They participate in the perception of external factors, allowing the plant to adapt to environmental change (Zhu et al., 2013).

Ethylene is one of the first phytohormone discovered playing a important regulation in plant (Abeles et al., 1992) It plays a key role in seed germination (Corbineau et al., 1990) and cell elongation but also in fertilization, fruit ripening, seed dispersal and defense against pathogens (Ahammed et al., 2020). Ethylene or his precursor 1-aminocyclopropane-1-carboxylic acid (ACC) can stimulate the germination and break the primary dormancy (Corbineau et al., 2014), . The ethylene metabolism is well known, the syntheses require some enzyme to convert the methionine to ethylene The first steps are the conversion in S-adenosylmethionine (SAM) by SAM synthase and in ACC by ACC synthase (ACS), and the last step is the oxidation of ACC by ACC-oxidase (ACO) and is the limiting reaction ethylene synthesis (Houben and Van de Poel, 2019, Xu and Zhang, 2015). Ethylene with other plant hormones like ABA and GA plays an important role during all life steps of the plant life notably during the development stage but also in the environmental stress responses (Van de Poel et al., 2015).

III) Seeds and water stress

3.1 Stress during seed development

The concept of phenotypic plasticity corresponds to the ability of an organism to change in response to stimuli from the environment (West-Eberhard, 2008). This capacity is acquired during the developmental for optimization of responses to external environments. So, the maternal environmental during the seed development is a critical time for offspring. By the influence of the expression of genetic variation for germination and even the genes involved in germination, maternal environmental has an effect in the seed capacity to germinate

(Donohue, 2009a). This effect is named "Maternal environmental effects" and refers to a particular phenomenon in which the external ecological environment of the maternal parent influences the phenotype of its progeny (Fig 9). The plasticity model is shown in figure 9. It shows how plants constantly evolve with their environment. The occurrence of environmental changes, such as biotic or abiotic stresses, determines how parents predict the selective environment for their offspring. If the environment changes randomly, a significant delay between the perception of a change in the parental environment and selection can reduce the similarity between the environment and the adaptation of the offspring. In an environment with random stresses (Fig. 9 b.), parental cues are less accurate predictors of selective environments than offspring cues. This is also the case for in a scenario of gradual environmental change Fig 9c. the offspring are better predictors of the environment. In contrast, in a seasonal environment, (cyclic environment Fig 9d.) parental cues are better predictors of the selective environment than offspring cues (Auge et al., 2017).

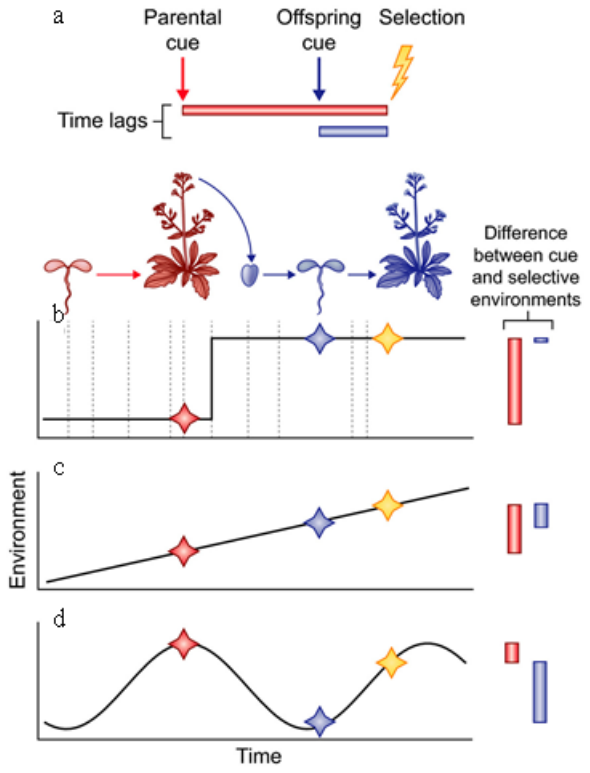

Figure 9 : Scheme of phenotypic plasticity model.

a. The black lines represent the evolution of the environment in which the plant lives over time.
The stars represent a perceived environmental event for the parent in red and the offspring in blue, and the time of selection is represented in yellow. The "difference between the cue and the selective environments" is the time between the perceived change in environment and the natural selection of the offspring. b. Environment with random change. The vertical dashed lines show the possibility of random environmental change, and the black line shows the actual environmental change that occurred. c. Environment changes directionally, d. Environmental change is cyclical.

Plasticity has been reported in response to several biotic and abiotic environmental cues, including temperature (Yakovlev et al., 2010), drought (Herman et al., 2012), shade (Galloway and Etterson, 2007), nutrient availability (Kou et al., 2011), salinity (Boyko et al., 2010), herbivory (Rasmann et al., 2012) or viral infection (Kathiria et al., 2010). For example the water stress on parent or grandparents can influence of traits in offspring. (Herman et al., 2012), the *Polygonum persicaria* stressed by drought stress give progeny with longer root systems. This example shows who the plastic responses to stresses in grandparents or parents can "preadapt" the progeny for same stresses and increase the progeny fitness.

3.2 Stress during the germination

Germination is the most critical phase in the development of crop plants. Early effect of drought in early season leads to an altered and irregular germination causing poor crop stand (Farooq et al., 2012). In *Arabidopsis*, a drought stress lineage shows an increase of seed dormancy, and that persists during one generation removed from stress (Ganguly et al., 2017a).

In laboratory drought stress is most commonly simulated by the use of polyethylene glycol (PEG). PEG contains high molar weight oligomers and polymers and is prepared by ethylenoxide polymerization. PEG causes water stress because it does not cross the membranes and can be used as a water stress simulator (Emmerich and. Hardegree, 1990; Michel and Kaufmann, 1973). This stress obstructs seed germination by reducing water absorption (Channaoui et al., 2017).

Different adaptation mechanisms to limit dehydration of plant cells or repair osmotic stress impacts are active during a water stress. Accumulation of solutes, modification of cell wall properties to avoid dehydration, or use of proteins and protective mechanisms to tolerate reduced water content by preventing or repairing cell damage are strategies used by plant cells to tolerate water stress.

Low water content is unfavorable for germination and seedling survival. Water stress in plants has an impact in seed dormancy (Baskin and Baskin, 1998). A secondary dormancy can be induced by incubating seeds under water stress conditions (Auge et al., 2015). This induction of secondary dormancy is described Fig 10. The curves correspond to the percentage of water on germination of Arabidopsis seeds matured at 14 or 25°C and stored for one or five months and first incubated in different solutions (water stress for the black squares and no water stress for the white squares). Seeds were pre-incubated at different water stress levels from 0 to -1.8 MPa. Dormancy was induced on the pre-incubated seeds

at less than -1.5 MPa. (decrease in germination). This secondary dormancy was not induced when the water potential of the first incubation was too high (above 1.5MPa) (Auge et al., 2015).

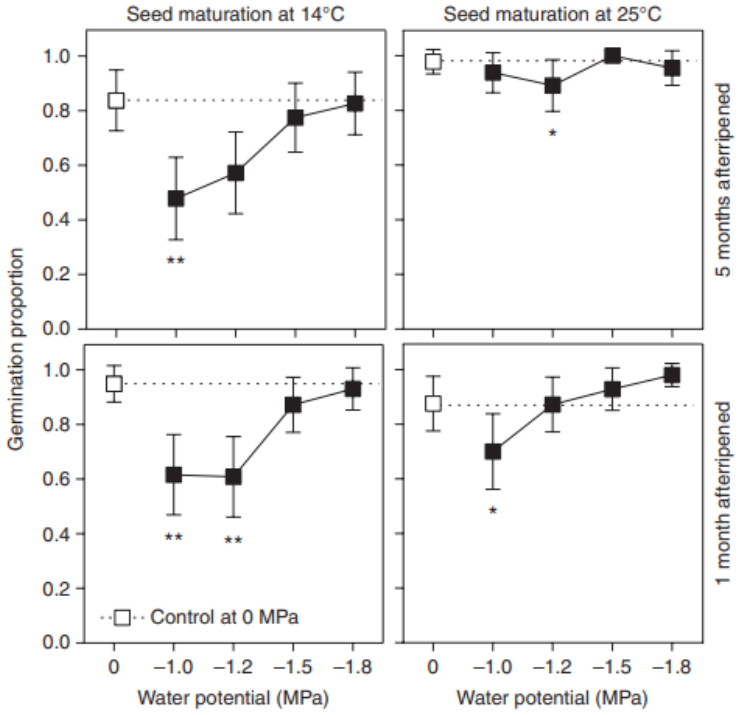

Figure 10 : Effects of water stress on secondary dormancy induction.
Germination percentage (mean ± SE) of Arabidopsis seeds matured at 14° C (a. c.) and 25° C (b. d.). the seeds were stored either one month (a. b.) or 5 months (c. d.), and pre-incubated at different water potentials (from 0a -1.8MPa). Squares indicate control seeds without pre-incubation, and dotted lines indicate the average germination of control seeds. Asterisks indicate significant differences observed between pre-incubated seeds compared to the control treatment without pre-treatment. *p-value=0.05, **p-value=0.01 (Auge et al., 2015).

Some genetic studies about the identification of gene candidates and QTL (Quantitative Trait Loci) promoting tolerant seeds and vigor were done in this last decade. They give some solution for the varietal selection of different crop species, Zea mays L (Trachsel et al., 2016), tomato (Geshnizjani et al., 2020), rice (Selamat and Nadarajah, 2021), sunflower (Davar et al., 2011) . Most QTL studies define the desired phenotype and involve loci with various beneficial effects and high heritability. The use of these genetic markers in varietal selection allows the development of effective and durable resistances. In sunflower Some study identify putative QTLs related for water stress tolerance, like osmotic adjustment (Poormohammad Kiani et al., 2007) photosynthesis parameters (Poormohammad Kiani et al., 2008), water content (Abdi et al., 2013) and chlorophyll concentration (Hervé et al., 2001).

IV) Epigenetic modifications in plants

Plant genomes are modified by epigenetic marks that help regulate different plant functions. We are interested in this phenomenon to better understand the memory of stress in plants and to study the mechanisms behind it.

4.1 Chromatin and genome structure

DNA of eukaryotic cells, is located in the nucleus and organized around histone proteins, this conformation forming the chromatin. This structure can take several states of condensation, open or close (Qiu, 2006). The two states of chromatin conformation are heterochromatin, the condensed form, visible as dense regions, poorly transcribed, and euchromatin, a poorly condensed form allowing transcription. Two subclasses of heterochromatin exist. Constitutive heterochromatin is constitutively condensed and located at the level of regions close to the centromere and telomeres. Facultative heterochromatin adopts a transient and reversible state of compaction depending on gene expression (Eichten et al., 2014).

The nucleosome is the basic unit of chromatin. It consists of an octamer of four histone proteins (H2A, H2B, H3 and H4). The nucleosome allows 147 bp of DNA to be wrapped around it. The nucleosome can have post-translational modifications including histone acetylation, ubiquitination, methylation or phosphorylation. They modify its chemical characteristics and play a role in chromatin compaction. Histone H1 binds to the DNA molecule between nucleosomes. Histone variants and histone modifications have an important role in the dynamics of chromatin compaction and its accessibility to the transcription machinery (Qiu, 2006).

The regulation of chromatin production is a key factor for seed and plant development, but also plays a role in the response to environmental stress. Three epigenetic mechanisms are related to the plant development and response to the environmental regulation: DNA methylation, histone modification and non-coding RNAs (small interfering RNA, siRNAs, microRNAs, miRNAs,long ncRNAs) (Kapazoglou et al., 2018).

4.2 Epigenetics and Heritability

Waddington was the first to introduce the concept of epigenetics by the epigenesis (Waddington, 1942). Holliday has defined epigenetic as the information transition

meiotically heritable and which do not rely on a modification of the DNA sequence (Holliday, 1994, 1987). Then Bird extended the definition (Bird, 2007) to the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states. This definition of epigenetics corresponds to a study of changes in the activity of genes transmitted through cell divisions as a result of changes in the state of chromatin, in the absence of the conditions that gave rise to it, or shortest through changes in gene expression not attributable to nucleotide sequence variation.

4.3 Stress memory: Maternal Effect versus Transgenerational Effect

Maternal effect can be defined as the causal influence of the maternal genotype or phenotype on the offspring phenotype (Wolf and Wad, 2009) but not a transmission of heritable mark during several generations. Maternal effect has an essential role in the environmental adaptation (Mousseau and Fox, 1998a). For example, in *Phaseolus vulgaris L* nitrogen management of maternal plant impacts seed vigour and seedling's establishment (Pereira et al., 2021).

A distinction was made between intergenerational and transgenerational epigenetic inheritance. For intergenerational memory, the epigenetic marks in the germline affect the following generation but are not maintained in the next germline. In contrast the transgenerational memory can be maintained during several generations (at least two generations of offspring) Fig 11. (Bošković and Rando, 2018). Epigenetic changes can be random and sporadic in origin, or induced by the environment (nutrition, stress). In the case of a female mouse exposed to epigenetic modifications, if she is pregnant, the fetus may be affected in utero (F1), as well as the fetal germline (the future F2). These effects are considered as "parental effects" with intergenerational epigenetic efect (red background). It is only at the third generation (F3) that an epigenetic effect can be considered as transgenerational (orange background). In the case of males (F0) with an epigenetic change induced by environmental stress, the first generation (F1) is therefore considered intergenerational (blue background). And it is only from the second generation (F2) that an epigenetic effect can be considered as transgenerational (orange background) (Heard and Martienssen, 2014).

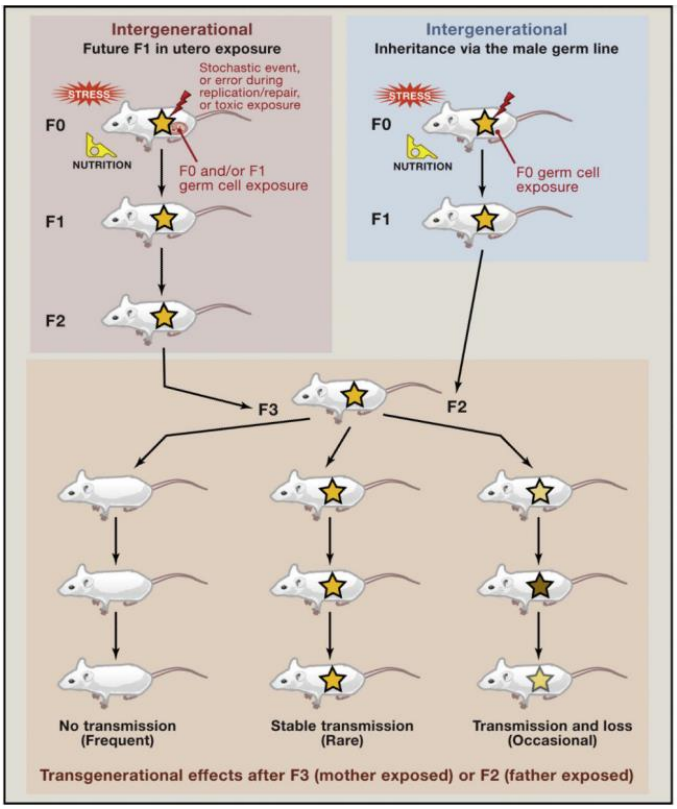

Figure 11 : Difference between transgenerational and intergenerational epigenetic effects.
This effect depends of male or female line. F0 corresponds to the generation undergoing the stress inducing epigenetic changes. The red and blue background correspond to an intergenerational epigenetic transmition. the orange background corresponds to a transgenerational epigenetic transmission (Heard and Martienssen, 2014).

Imprinting is an example of epigenetic inheritance (McGrath and Solter, 1984; Scott et al., 1998), where maternal or paternal origin contributions to the embryonic genome are not equivalent. Some hypothesis on this subject postulate that control of imprinted genes is the result of conflicts between mothers and fathers' fitness. The resource allocation to offspring differs in function of parents, the mother plant investigated more in the offspring the male which only transmits hie genetic. The paternally expressed genes tend to drive increased provisioning to the offspring, whereas maternally expressed genes prevent excessive investment in the progeny to limit to decrease its fitness (Haig, 2004). For example, drought stress has transgenerational effects on soybean seed germination and seedling vigour (Wijewardana et al., 2019).

In animals without DNA methylation ,like *C. elegans*, the mechanisms involved in the acquisition and inheritance of tolerance characters by the environment is often related to small RNAs (Ashe et al., 2012a) and histone modifications (Öst et al., 2014a). In opposite For

mammalians it can be associated with DNA methylation modifications (Baxter and Drake, 2019a; Radford et al., 2014a) .

In plants, the environmental conditions can induce heritable traits mediated by DNA methylation changes (Jiang et al., 2014). For example, plant exposure to stress can create stress imprint effects (Bruce et al., 2007) and modify the stress tolerance of plants. Some memory mechanism exists, like the epigenetic modification caused by environmental stress. One of the most studied has been the vernalization mechanism in which plants remember a cold stress (Sung and Amasino, 2004)(Sani et al., 2013). Transgenerational stress memory has been studied in plants submitted to heat stress, with the implication of DNA methylation and chromatin remodelling (Shanker et al., 2020). The transgenerational memory of stress in plants is an interesting perspective for the plant adaptation to the climate constraint (Molinier et al., 2006). In Arabidopsis, a hyperosmotic stress memory is mediated by epigenetic mechanisms, like DNA demethylation in response to stress (Wibowo et al., 2016a).

4.4 Molecular mechanisms of epigenetic

4.4.1 DNA methylation

DNA methylation is regulated in two ways, de novo methylation and methylation maintenance. De novo methylation inserts methyl CH3 groups on cytosine bases in the genome. It is carried out by the pathway "RNA-dependent DNA Methylation" (RdDM). The main actors of this pathway are the small RNAs. Three types of methylation exist, depending on the context of cytosines, directly followed by a guanine (CpG), with another base before the guanine (CHG) or followed by 2 bases without guanine (CHH) (Matzke and Mosher, 2014). DNA methylation can be maintained across cell divisions during DNA replication. Unlike mammals where DNA methylation is maintained by a single methyltransferase (DNMT1), plants have several methyltransferases that allow different maintenance of cytosines depending on their type (CG, CHG or CHH) (Chen and Riggs, 2011) Fig 12. In *A. Thaliana*, CG symmetric cytosine methylation is mediated by METHYLTRANSFERASE (MET1), MET1 recognizes hemi methylated CG due to replication (Law and Jacobsen, 2010; Stroud et al., 2014). CHG methylation is linked to histone H3 (H3K9me2) methylation and is maintained by the action of an auto reinforcer loop. Histone methyltransferases (KYP, SUPPRESSOR OF VARIEGATION 3-9 HOMOLOGUE PROTEIN 5 & 6 (SUVH4,5&6) bind to CHG methylated sites and catalyze the methylation of lysine nine of histone H3. CMT3 (and

partially CMT2) protein bind to the chromodomain and methylated DNA in return (Du et al., 2015; Stroud et al., 2014). CHH methylation is asymmetric. After DNA replication, therefore, there isn't cytosine to guide the maintenance of methylation. It is maintained by the action of CMT2, primarily for the inner part of long transposable elements (TE) and by the RdDM pathway involving Pol IV DOMAINS REARRANGED METHYLASE 2 & AGO4 for short transposable elements and the ends of long transposable elements (Stroud et al., 2013; Zhang et al., 2018).

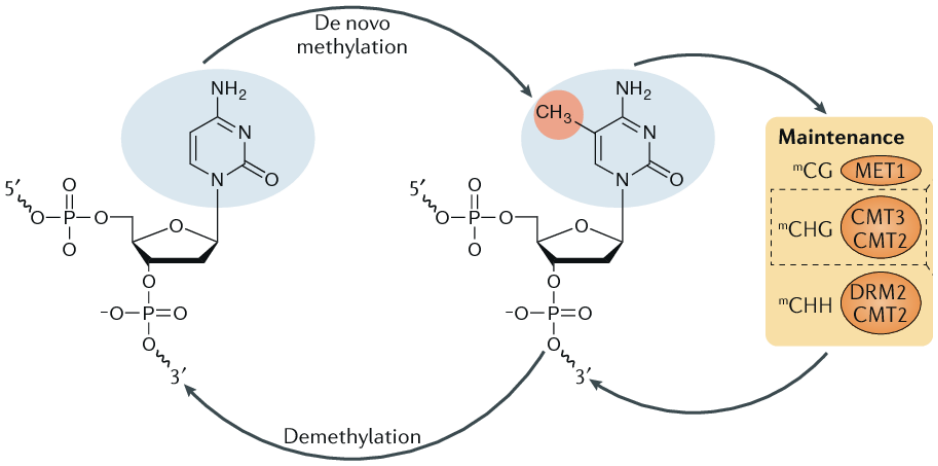

Figure 12 : Dynamic regulation of DNA methylation in plants (Zhang et al., 2018).
CHG methylation is maintained by MET1. CHG methylation is maintained by CHROMOMETHYLASE 3 (CMT3) or CMT2. CHH methylation is maintained by DRM2 via RdDM or by CMT2. DNA methylation can be suppressed by active demethylation (DNA demethylases) or by passive demethylation due to failure to maintain methylation after DNA replication.

In the absence of maintenance of DNA methylation, it disappears passively through DNA replication Fig. 13. Active demethylation also exists. It is ensured by the action of DNA glycosylases. For example, the ROS1 family proteins, including AtROS1, AtDME, AtDML2&3 act throughout plant development to maintain the absence of methylation at genes containing transposable elements. ROS1 and DME are recruited to the target loci by the MBD7-IDM or SWR1 complex and by the facilitates chromatin transactions (FACT) complex, respectively. After they are recruited to the target loci, ROS1/DME cleaves the 5-mC from the DNA backbone. The single-nucleotide gap will be filled with an unmethylated cytosine through the base-excision repair pathway, in which apurinic or apyrimidinic site lyase (ARP, APE1L), zinc finger DNA 3′ phosphatase (ZDP) and DNA ligase I (LIG1) are involved (Liu and Lang, 2020; Parrilla-Doblas et al., 2019).

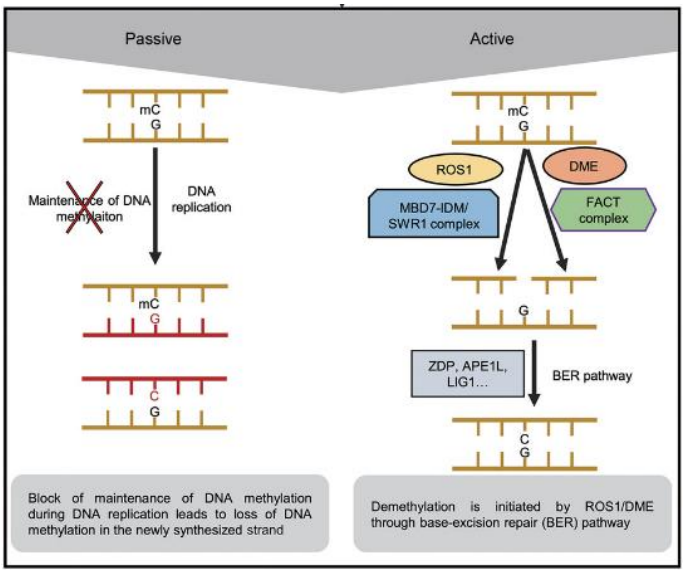

Figure 13 : Mechanisms of demethylation in plants (Liu and Lang, 2020).
DNA demethylation can occur passively during DNA replication when the DNA methylation pathway is not active; as a result, DNA methylation in the newly synthesized DNA will be diluted. Active DNA demethylation is mediated by the plant-specific enzyme family ROS1/DME.

In plants, the extent of germline reprogramming of DNA methylation has been examined in pollen cell types (Calarco et al., 2012). In sperm cells and their microspore progenitors, more than 80% of symmetric DNA methylation are retained (CG or CHG) sequence context, but asymmetric CHH methylation is specifically reduced Fig 14 (Heard and Martienssen, 2014). During the differentiate from somatic cells, the germline has 2;3 stereotypical mitotic divisions after the formation of the haploid microspore (pollen) and megaspore (ovule) (Fig 14. a.). In pollen, symmetric CG CHG methylation is retained in the microspore and sperm cells. But CG methylation is lost from a few hundred imprinted and other genes in the companion vegetative cell nucleus (Fig 14.b.). CHH methylation is sharply reduced in the microspore and sperm cells.

For the whole genome of *A. thaliana*, the study of the methylation level of cytosine of each type shows a high level for the CG methylation type, a medium level for CHG and a lower level for the CHH methylation type (respectively >80%, 20-60% <20%) Fig 15. But this distribution differs according to species (Niederhuth et al., 2016). Despite the mechanics allowing the maintenance of DNA methylation after DNA replication, the 100% methylation level is rarely observed even in the CG context (Quadrana and Colot, 2016).

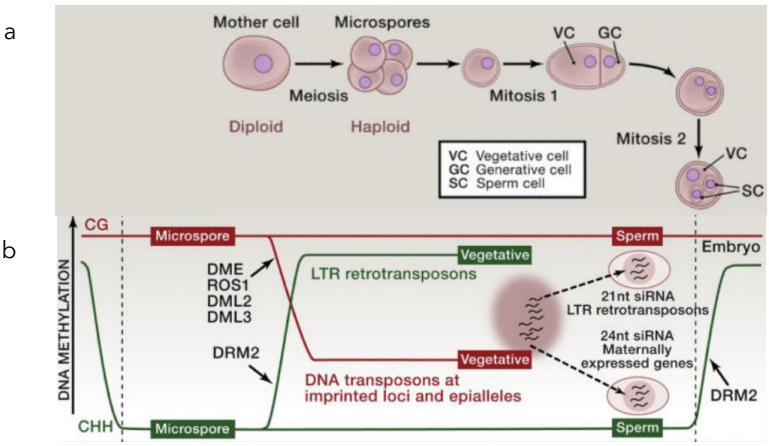

Figure 14 : DNA reprogramming methylation in plants.
a. Schema of germline development. b. Dynamic of Germline reprogramming of DNA methylation in plants (Heard and Martienssen, 2014).

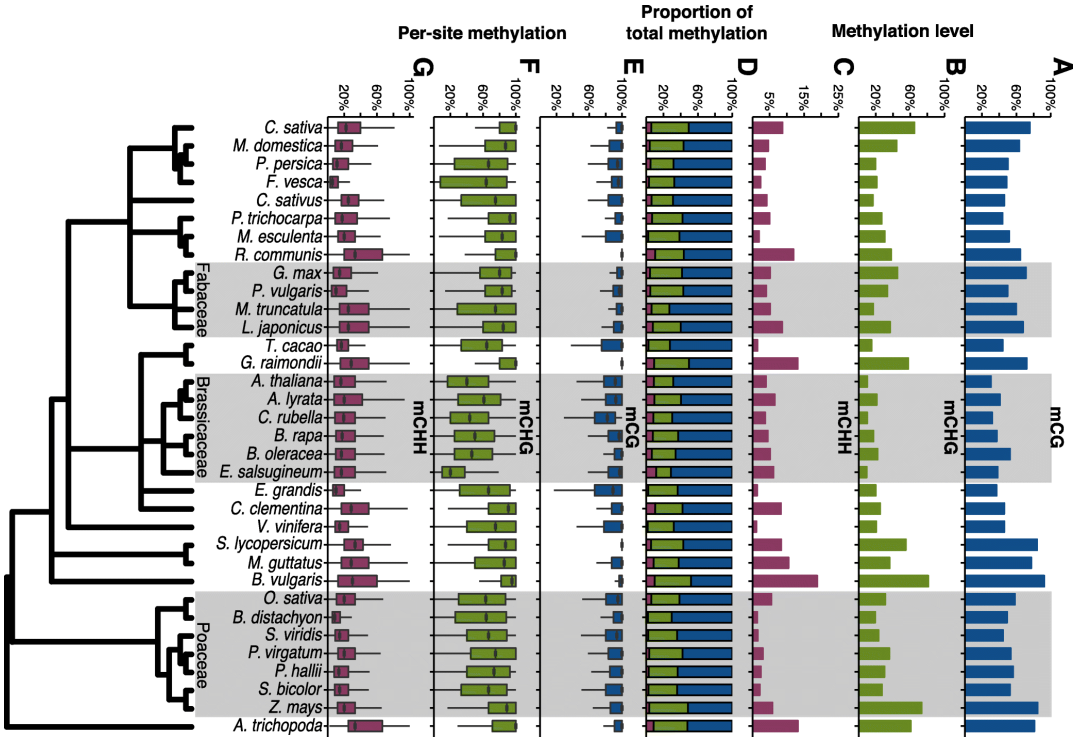

Figure 15 : Genome-wide DNA methylation rates in different angiosperm species angiosperms in the context of a. CG, b.CHG and c. CHH, per-site distribution of methylation levels for CG, f) CHG, and g) CHH. (Niederhuth et al., 2016).

4.4.2 Role of methylation position

The type and the position of methylation have different impacts on gene regulation (Bewick and Schmitz, 2017). For most genes, the typical profile of DNA methylation is characterized by its position in the transcribed regions or gene body only in the CG context, with depletion at the TSS and TTS (Fig 16.). Genes with a methylated gene body contain more exons and evolve more slowly than unmethylated genes and appear to have more important functions than unmethylated genes (Muyle and Gaut, 2019; Takuno and Gaut, 2013).The expression of gene body methylated alleles was consistently and significantly higher than unmethylated alleles (Muyle et al., 2020). An active demethylation plays a role in fruit development, ripening (Giovannoni et al., 2017), and stress response to biotic (López Sánchez et al., 2016) or abiotic stress (Kim et al., 2019). The gene body methylation was very conserved during the evolution (Takuno and Gaut, 2013).

In addition to this general case of genes body methylation (GbM), four other types of methylation profiles within the gene body have been described in Fig 16 (Bewick and Schmitz, 2017). They are divided according to the methylation type and its location: in the promoter Transcription Start Site (TSS), in the gene body and in post Coding DNA Sequence (CDS) sequence Transcriptional Termination Sites TTS) related to their impact on transcription. (Fig. 16) The first group are unmethylated genes, they show no methylation in the different methylation types (CG, CHG or CHH) (Fig. 16 a). The other class are genes with CG methylation only at the TSS (Fig 16 c), this methylation position leading to repression of gene expression (Niederhuth et al., 2016). Genes with CG and CHG methylation, without CHH methylation in the transcribed region are expressed at a lower level compared to genes with GbM (Niederhuth et al., 2016). (Fig. 16 d) The last class is gene with CHH methylation-enriched (and possibly also CG and CHG) (Fig. 16 e), they have repressed expression except in pollen and seed (Bewick and Schmitz, 2017).

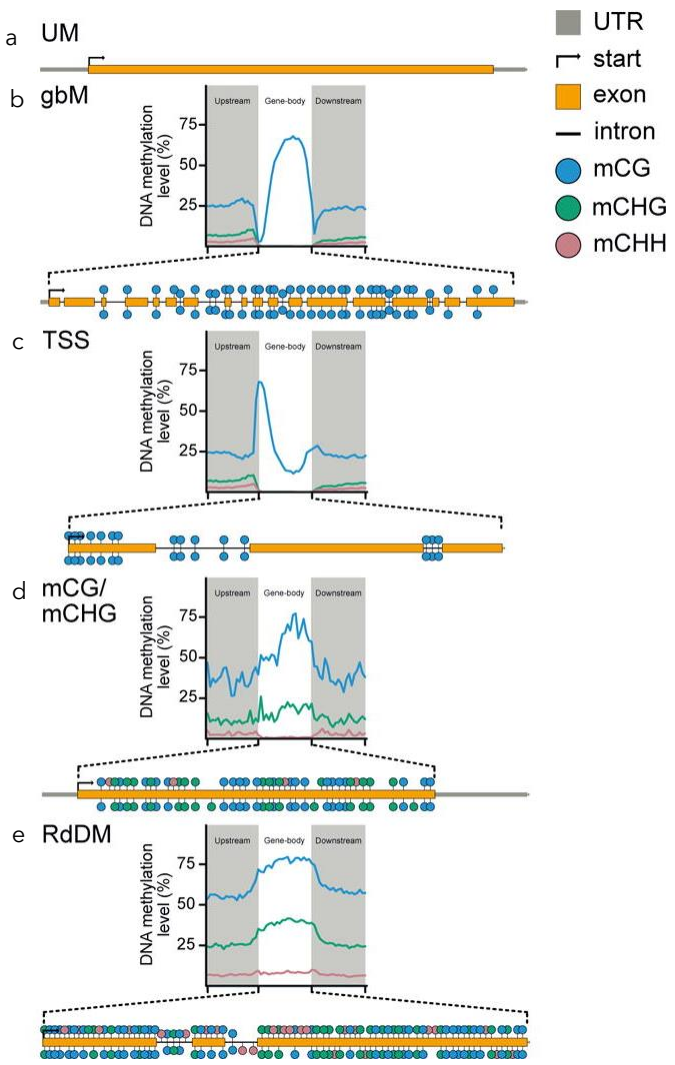

Figure 16 : DNA methylation profiles at the gene level (Bewick and Schmitz, 2017).
a. Gene with no methylation in any context (UM: UMethylated). b. Gene with methylation only in CG context, in the transcribed region and absent from the TSS and TTS (gbM: genes body methylation). c. Presence of CG methylation only at the TSS. d. Presence of CG and CHG methylation in the transcribed region. e. Presence of CHH methylation guided by the RdDM pathway, suggesting the presence of methylation in CG and CHG.

In rice, methylation at the transcriptional terminal region (TTR) represses gene expression more than when it is present at the promoter (Li et al., 2012). In maize seeds the methylation in promoter and post CDS of protein-coding genes is negatively correlated with gene expression. At the opposite methylation in gene bodies was positively correlated with gene expression (Lu et al., 2015).

DNA methylation is also known for regulating the transposon element (TE) activity (Kim and Zilberman, 2014). In fact, the methylation of TE can be considered as a defense of the plant genome against selfish DNA elements and it provides stability to the DNA (Liu and Lang, 2020).

4.4.3 Relation between DNA methylation and water stress

Drought-induced site-specific DNA methylation is associated with drought tolerance in rice (Wang et al., 2011). DNA methylation during seed priming has also been shown to induce stress tolerance, especially for drought (Aswathi et al., 2021). Different adaptation epigenetic mechanism has been described (Table1): hypermethylation, hypomethylation, demethylation, global methylation shift or differential methylation genotype dependent. For example, in tomato (*Solanum lycopersicum*), water deficit promotes a demethylation of about 100 cytosine of the Asr1 gene. This is accompanied by a decrease of histone mark and the induction of the expression of the expression of the gene (González et al., 2011).

Table 1 : DNA methylation modifications in biotic and abiotic stresses in plant (Saraswat et al., 2017).

Gene	Plant	Stress	Methylation status	Reference
Asr1	Tomato	Drought stress	CG hypermethylation and CHH hypomethylation	(González et al. 2011)
Asr2	Tomato	Drought stress	CHH hypomethylation in regulatory region	(González et al. 2013)
NtGPDL	Tobacco	Cold	Hypomethylation	(Choi and Sano 2007)
ZmMI1	Maize	Cold	Root-specific hypomethylation	(Steward et al. 2002)
Glyma11g02400	Soybean	Salinity	Demethylation	(Song et al. 2012)
Glyma16g27950	soybean	Salinity	Hypomethylation	(Song et al. 2012)
Glyma20g30840	soybean	Salinity	Hypomethylation	(Song et al. 2012)
Genome wide	Maize	Cold stress	Global methylation shift	(Tan 2010)
Genome wide	Rice	Drought stress	Genotype-dependent differential methylation	(Wang et al. 2011)

V) Sunrise project: SUNflower Resources to Improve yield Stability in a changing Environment

5.1 Project context

5.1.1 Partnership

SUNRISE is an Investment in Biotechnology and Bioresources project with public and private funding for a total budget of 21M€. Its objective was to develop knowledge, resources and tools in sunflower in order to adapt this crop to the challenges of climate change, in particular by maintaining its productivity under drought conditions. The project links several disciplines: genetics, genomics, physiology, agronomy and social sciences. It brings together research actors (9 INRAE and university laboratories), the technical institute for oilseeds and 6 biotechnology and seed companies with the aim of working not for but with field actors and ensuring a more rapid and efficient transfer of knowledge, methods and resources produced.

5.1.2 Objectives of SUNRISE

The actual challenge of agronomic practice is to improve the plant capacity to grow in climatic conditions more and more stressful (Ainsworth and Ort, 2010; Lippmann et al., 2019). Some alternatives are already possible to adapt the plant to global warming, cultural pratique (modification of plant seedlings date, irrigation), geographic adaptation (news allocation of fields with other species) (Caubel et al., 2018), priming(Hussain et al., 2018a). Another way of action is the selection of more tolerant plants (Alves de Freitas Guedes et al., 2019; Fang and Xiong, 2015; Moschen et al., 2017a).

For crop plants, as sunflower, water stress, in combination with heat stress, could potentially decrease yields from 5 to 20 % in southern parts of Europe in 2030, according to climatic predictions (Debaeke et al., 2017a). In particular, exposure to drought at some specific phenophases like germination, anthesis, and achene filling, etc., is the most critical factor and can cause up to 50% yield reduction for sunflower (Hussain et al., 2008). SUNRISE aimed to decipher the genes and gene networks involved in sunflower oil yield potential and its stability from year to year and location to location, but also to identify alleles in the large *Helianthus annuus* gene pool that would be of interest for sunflower breeding (hybrid or lined). SUNRISE had identified the loci and/or heterosis mechanisms that are most involved in hybrid plant vigour, based on parental alleles, and then construct new gene pools to stabilize yield.

5.1.3 Main results

A major result of SUNRISE is the sequencing of the first sunflower reference genome in 2017 (Badouin et al., 2017). Two other sunflower genomes were then sequenced in SUNRISE. The omic technology developped by SUNRISE for this sequencing has became a standard in genomics and has been widely reused for the sequencing of dozens of plants, animals and microorganisms (rose, Medicago, grapevines, broomrape, human, macaques, bees, etc...). A method for identification of the genetic control of drought tolerance was developed (Gosseau et al., 2019a; Mangin et al., 2017). A combined quantitative genetics and agronomic modelling approach through private partner trials identified sunflower types adapted to different climates in Europe and to future climate scenarios was performed. The integration of these results with the modelling of genetic regulations has highlighted the gene networks involved in drought tolerance. SUNRISE has conceptualized eco-innovations in the field of plant breeding and has studied the barriers to acceptance of these innovations by farmers at the French and European levels. SUNRISE allowed the development of genetic

material incorporating wild genetic diversity. The genetic resources produced within the framework of SUNRISE are unique: the Sunflower Biological Resource Center based at LIPME has developed with seed partners 2437 recombinant lines, 448 introgression lines with wild sunflowers. SUNRISE has enabled the molecular characterization of 3500 lines genotyped at high density, which prepares the exploitation of genetic resources in future projects. The development of high-throughput phenotyping tools (Gosseau et al., 2018) was enabled in Heliaphen platform. It allowed the development of the first high-throughput phenotyping tools on sunflower (Fig 17.). The interest of this platform is to create and control variable water stress conditions and to follow the consequences on the growth of the plants between the vegetative and flowering stages.

Figure 17 : General view of the Heliaphen platform.
a. The Heliaphen platform comprises an area of 650 m^2, containing 1,300 plants spread over 13 blocks consisting of two rows of 50 pots each. b. The Heliaphen robot is able to pick up pots, weigh and water them, and phenotype individual plants.

All these results have allowed the establishment of this Thesis which is funded by 5 companies partners of SUNRISE: Lidea (Caussade semence), RAGT2n, Soltis, Syngenta France SAS, MAS Seeds).

5.2 Sunflower biology

5.2.1 Plant biology

Sunflower (*Helianthus annuus* L.) is originated from North American and was domesticated by the Native American Indians for its edible seeds (Harter et al., 2004). The cultivation of sunflowers to be the main oil crop took place in the second half of the 20th century thanks to two major achievements in breeding. The drastic increase in the percentage of oil in the seeds and the selection of plants with a cytoplasmic male sterility system (Leclercq, 1969) combined with the restoration of fertility by nuclear genes (Kinman, 1970) which allowed the commercial production of hybrid seeds (Fernández-Martínez et al., 2010). The hybrid vigour expression is based on complex interactions between phenotypic components influenced by the environment (Lippman and Zamir, 2007). The theory suggests a linear

relationship between heterosis of a hybrid and the genetic distance between its parents considering all loci underlying the quantitative trait of interest (Hill and Mackay, 2004, p. 200).

Indeed, the use of hybrid plants give an heterosis effect and improves seed and oil yield. Sunflower is a cross-pollinating species and breeders have exploited heterosis for both seed and oil yield. Commercial seeds are issued from hybrids for this reason. For industrial production of a hybrid, the strategy of plant cross is based on the cytoplasmic male sterility and fertility restoration. In sunflower plant sterility on pollen can exist by different causes, nuclear sterility or cytoplasmic male sterility (CMS). For the production of CMS plants, a cross with "maintainer" is done. Maintainer line is with high agronomic value and its pollen is fertile by absence of CMS. The cross between CMS plant and maintainer plant gives "Line A" isogenic to maintainer plants but with sterile pollen (CMS). In final to have a fertile hybrid, line A is crossed with a "restorer" plant characterized by dominant fertility gene Fig 18.

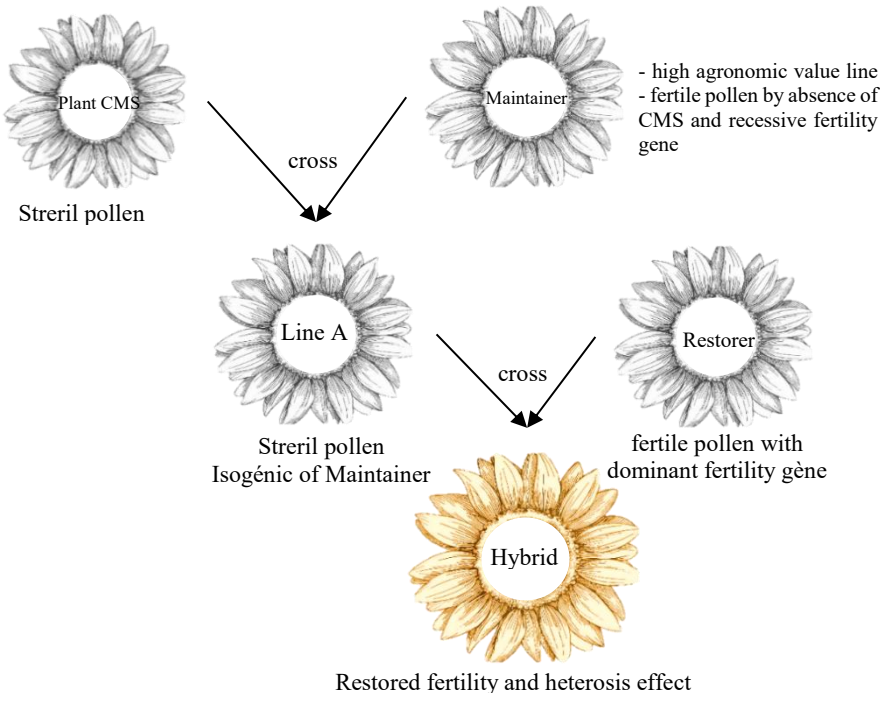

Figure 18: Principe of sunflower hybrid production.
The CMS line has a sterile pollen caused by cytoplasmic sterility. Maintainer plant is high agronomic value line with fertile pollen by absence of CMS and recessive fertility genes. Restorer plant has dominant fertility gene in genome. The arrows correspond to cross between two plants.

5.2.2 Seed biology

Sunflower seeds are achenes arranged in circles on the inflorescence (flower heads of sunflower). Each flower gives an achene. An achene (Fig 19) is an embryo covered by a tegument and a pericarp not welded together. (Côme and Corbineau, 1998). The embryo is composed of two primary organ systems: the axis and cotyledon (Fig. 19).

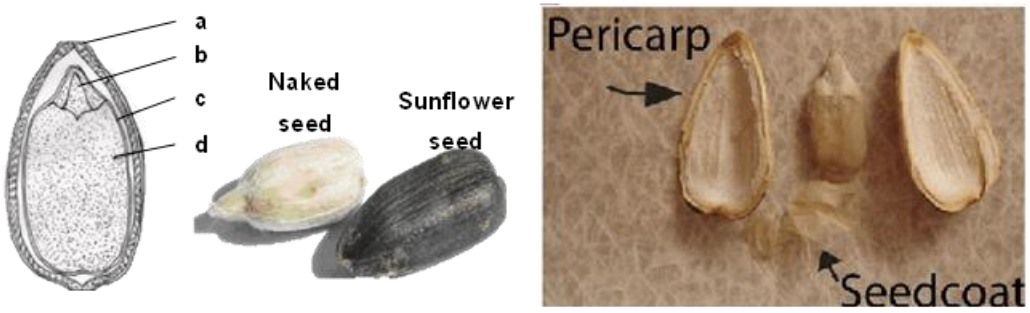

Figure 19 : Anatomy of a sunflower seed.
a. pericarp, b. embryonic axis c. Seed coat d. cotyledon (Biris et al., 2019).

After fertilization, the seed develops in 3 phases. The first phase of histodifferentiation allows the establishment of the embryo (embryogenesis). This process is carried out with a high-water content (70-75% in relation to the mass of fresh matter, MF). The second phase is followed by accumulation of reserves in the albumen and/or the embryo, in particular in the cotyledons. The tolerance of seeds to water loss is acquired during this phase. This process is regulated by ABA and is associated with dehydration of the tissues. The reserve synthesis finished when the water content is around 40-55% of MF. At the same time, the dry matter weight of the seeds increases and then reaches a plateau. The development ends with dehydration or maturation of the seeds. At the end of maturation, the water content is in the order of 8 to 15 %. Physiological maturity is reached at the end of seed filling.

At harvest, sunflower seeds are physiologically dormant (Baskin and Baskin, 2004). This dormancy is transitory, (Fig 20.a.) after 6 months of storage the germination percentage reaches 100% in a large temperature range (10°C to 30°C). Two types of dormancies exist (integumentary and embryonic). The integumentary dormancy is visible for each temperature (Fig 20b). The percentage of germination increases when the pericarp isremoved (naked seed) and even more for the seed coat for high temperature.

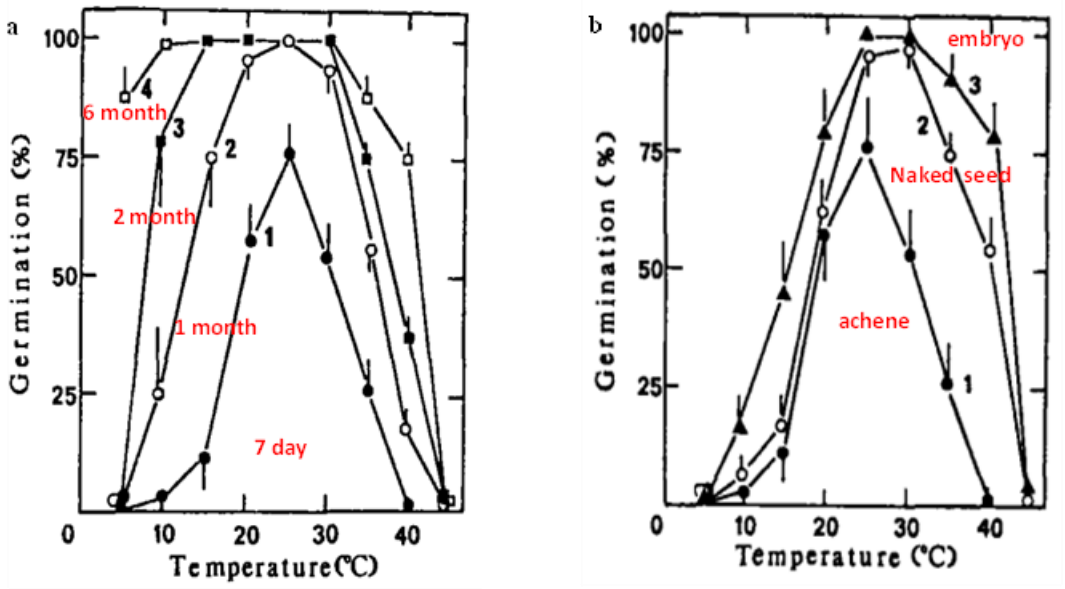

Figure 20 : Germination profile of sunflower seeds
a. Germination profile of sunflower seeds in function of temperature and a. time of storage or b. presence or absent of seed tegument (Corbineau and Côme, 1989).

In sunflower seed, the ROS content increases progressively during the breaking of dormancy in dry conditions (Oracz et al., 2008). But if storage is prolonged, the continued accumulation of ROS can lead to loss of seed viability (Kibinza et al., 2011).

5.3 Impact of water stress on sunflower seed

Sunflower is moderately drought tolerant plant and successfully grows in diversified agro-climatic conditions. Drought stress negatively influences seed germination of sunflower (Midaoui et al., 2001). In fact, water stress during the time of sowing may cause erratic and delayed germination leading to poor seedling development or stand establishment for crop species (Ellis, 1992). Studies about cellular mechanisms involved in the response of seeds to water stress during sunflower seed germination are scarce and often only descriptive (Hegarty, 1978). A recent development of bioinformatics tools such as Pathifier (Ponnaiah et al., 2019) allowed new perspectives of transcriptomic analysis in relation with water stress. Saux et al. (2020b) showed that some pathway were dysregulated during water stress and involved in stress tolerance, such as the pathways in relation of biosynthesize of amino acids (alanine, threonine, putrescine, glycine betaine, proline, spermidine, spermine, arabinose, anthocyanins, diacylglycerols, brassinosteroids) or in relation with ABA. The nucleotide, and secondary metabolisms were also involved in response to water stress during germination.

Some heat shock proteins (HSPs) seemed have a role in germination under water stress (Saux et al., 2020b). They are not specific to one stress (Jacob et al., 2017) and play a role in seed desiccation tolerance and in seed longevity (Vierling, 1991). Specifically, the antioxidant activity of HSPs 70 is thought to play a role in water stress tolerance and to accumulate specifically during germination under stressful conditions. (El-Maarouf-Bouteau et al., 2015; Layat et al., 2014). The metabolism and cell extension mechanisms are also impacted by water stress. Down regulation of genes like tubulin, cellulose synthase, beta galactosidase, and glycosyl hydrolase is associated with a better tolerance to water stress in sunflower seeds (Saux et al., 2020b).Differences in accumulation of reactive oxygen species and antioxidant enzymes activities were observed between germination of sensitive and drought tolerant sunflower seeds (Saux et al., 2020b). Sunflower seed production in warm or cold conditions during seed development induced a higher dormancy (Bodrone et al., 2017) but an acceleration of desiccation during seed maturation on the mother plant was associated with lower dormancy (Lachabrouilli et al., 2021).

VI) Objectives of the Thesis

The germination stage is an absolutely decisive element of final crop yield. For sunflower, emergence losses observed in the South of France can be between 20 and 30% on average and can even exceed 50% in case of irregular emergence (Terres Inovia, 2021) because the target plant population (about 60,000 plants/ha) is often not reached. Germination success is strongly modulated by environmental conditions (temperature, oxygenation, water availability) and intrinsic seed qualities (germinative vigor, dormancy). Environmental constraints such as drought and high temperatures play a major role in sunflower seed germination, leading to irregular stand and ultimately reduced yield (Bradford, 1995). They also impact regulation of dormancy induction in crop plants (Seiler et al., 2011), including sunflower (Corbineau et al., 1990). Seed vigour is multifactorial and results from genetic components, seed development conditions on the parent plant but also from post-harvest operations and storage conditions. This phenotypic trait is therefore difficult to predict for a seed lot and the molecular mechanisms involved in its expression remain poorly understood. In sunflower, the existence of both embryonic and integumentary dormancy can also penalize sowing success, especially for batches from off-season production and sown in France shortly after harvest.

Within the framework of the SUNRISE project, a series of experiments conducted over several years by the LIPME and Sorbonne University teams has highlighted the role of seed

development conditions on the mother plant in seed germination quality. Indeed, a water stress applied on the plant after flowering confers to the seeds produced a better germination under water stress conditions. This effect was clearly identified over several production cycles (4 successive years of experiments) on the SF193 genotype (XRQ maintainer).

This preliminary work demonstrated the influence of production conditions on seed germination quality and highlighted the transmissibility of phenotypic traits between generations in the sunflower. These data should be viewed in the context of a growing number of studies that clearly show the importance of transgenerational environmental effects in seed germination physiology (Auge et al., 2017).

The main objective of this thesis work, conducted in close collaboration between the LIPM and Sorbonne University teams, is therefore to better understand the genetic and molecular basis of the transmission of stress tolerance traits between the mother plant and the progeny seed.

At the applied level, the understanding of this process will allow a better control the impact of the environment during the production of seeds on their quality and to develop biomarkers to characterize the quality of seeds with respect to the conditions of their production. The thesis project is divided into 3 complementary parts covering the physiological, genetic and molecular aspects of the observed phenomenon.

The first part focuses on the physiology component and more especially on the characterization of the effects of drought stress occurring after flowering of the mother plant on the physiological properties of the progeny and identification of the relative roles of embryo and maternal tissues in this process.The second part is dealing with the molecular regulation of this phenomenon, at the transcriptional level.And the last part focuses on the role of the methylome in this transgenerational stress.

Chapter 2 :

Maternal drought stress induces abiotic stress tolerance to the progeny at the germination stage in sunflower

Maternal drought stress induces abiotic stress tolerance to the progeny at the germination stage in sunflower

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Highlights

- Drought stress during sunflower seed development induces seed tolerance to abiotic stresses and lower dormancy
- The modulation of germinative properties by the environment is not transgenerational but maternally transmitted
- Seed pericarp and embryo both play a role in the inheritance of germinative traits

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Abstract

Climate change produces more frequent and intense drought events during seed development that can affect seed quality. Germination is critical to ensure plant growth and reproductive success but it can be impacted by various abiotic stresses. Here, we studied the effect of drought stress during sunflower seed development on the germination of the progeny. We applied different scenarios of drought stress during seed development in sunflower inbred lines and hybrids and assessed seed germination of the progeny. Drought stress during seed development provided tolerance to water, hypoxic, cold and salt stresses during seed germination and also induced lower dormancy. We established that the induction of these traits was not transgenerational but maternally transmitted and could be reproduced in sunflower hybrids. Drought stress during seed development decreased pericarp thickness and induced higher leakage of soluble electrolytes from pericarp but it also modified embryo metabolism. A metabolomics analysis showed that ABA, oligosaccharides and polyphenols accumulated differently in drought stress seeds and could also participate in the seed tolerance to abiotic stress conditions during germination. Altogether our results reveal an adaptative process that allows sunflower plants exposed to drought stress during their reproductive stage to produce seeds with higher fitness. Besides bringing novel insight on natural adaption of plant populations to climate change, these results may have implications for the seed industry through the production of seeds resilient to higher climatic variability during the establishment of sunflower crop.

Keywords: abiotic stress, development, germination, maternal effect, seed, sunflower

I) Introduction

In the context of global warming, water stress on plants is likely to become more intense and to affect more geographic areas (Pachauri et al., 2014). Climate models predict increased plant evapotranspiration and lower soil moisture levels in a nearby future (IPCC, 2019). For crop plants, as sunflower, water stress, in combination with heat stress, could potentially decrease yields from 5 to 20 % in southern parts of Europe in 2030, according to climatic predictions (Debaeke et al., 2017). Besides this effect on whole plant fitness, climate change is also expected to modify the pattern of seed development on the mother plant, which will in turn modify seed germinative properties (seed vigour) and seed dormancy. Both traits (*i.e.* seed vigour and dormancy) control the germination process and drive seedling emergence in the field. Seed vigour is defined as the properties of seeds to germinate rapidly and homogenously in wide environmental conditions (ISTA, 1999), whereas dormancy is an intrinsic seed property that prevents germination in apparently favourable environmental conditions (Bewley, 1997). The dispersal units of sunflower are achenes and they display a physiological dormancy (Baskin and Baskin, 2004) that results from an inhibitory action of the envelopes (pericarp and seed coat) and from the embryo itself (Corbineau et al., 1990). The pericarp-seed coat imposed dormancy mostly affects germination above 20-25°C while embryo dormancy prevents germination below 15°C (Corbineau et al., 1990). Thus the germination process of sunflower seeds is strongly sensitive to a combination of extrinsic factors, such as water, light, oxygen and temperature and intrinsic factors such as dormancy. For example Saux et al. (2020) have shown that lowering water availability had a marked impact on sunflower seed germination. Gay et al. (1991) demonstrated that germination of whole achenes was strongly inhibited when oxygen tension was below 10 %.

Because plants are sessile organisms exposed to variable environments throughout their life or between generations, their phenotypic plasticity has been widely studied (Galloway, 2005).In particular, phenotype of the progeny can be modified by the environment experienced by the mother plant (Roach and Wulff, 1987). For example, in *Arabidopsis thaliana*, cool temperatures during seed maturation induce strong primary dormancy (Chiang et al., 2011; Donohue et al., 2007) while high temperatures reduce dormancy and negatively affect the final seed yield (Huang et al., 2014). In wheat, higher levels of dormancy were obtained in grains from plants grown at lower temperatures (Walker-Simmons and Sesing, 1990). Several studies have also shown that seed vigour

could be positively influenced by abiotic stress during seed development (Finch-Savage and Bassel, 2016). The maternal effect is defined as the causal influence of the maternal genotype or phenotype on the offspring phenotype (Wolf and Wade, 2009). The mechanisms involved in maternal effect in plants on the offspring are not completely understood because they can be mediated by the seed coat, which is a maternal tissue, by the triploid endosperm, with two-thirds of its genotype of maternal origin, or can result from a maternal effect on embryo provisioning during seed development. In addition to maternal effect, which concerns a single generation, the so-called transgenerational effect can occur on the progeny that did not experience any stress during its development (Heard and Martienssen, 2014). This effect is characterized if a phenotypic memory of an initial environmental stress can persist over multiple generations. It is supposed to result from a pre-programming of offspring phenotypes via epigenetic mechanisms such as histone modification or DNA methylation (Bruce et al., 2007; Wolf and Wade, 2009). Transgenerational effect has been demonstrated in the context of various biotic and abiotic stresses in plants, such as drought stress in *Brassica napus* L. (Hatzig et al., 2018). In Arabidopsis, for example, progeny issued from a warm parental environment (heat stress) showed faster germination rates, growth of root elongation, higher leaf biomass and increased seed production in different temperatures compared to seeds from plant grown in cold environment (cold stress) (Blödner et al., 2007). Recently it also been shown that spaceflight could affect the growth of future generations through changes in epigenetic modifications (Xu et al., 2021). Yin et al. (2019) summarized 139 studies about transgenerational effect and suggested possible transgenerational effects in many cases that can have an impact on the responses of plants to changing environments.In sunflower, seed developmental conditions have been shown to have an effect on seed dormancy at harvest. Bodrone et al., (2017) showed that high temperatures during seed development decreased embryo dormancy but increased achene dormancy and Lachabrouilli et al., (2021) showed that faster seed desiccation during late maturation phase induced lower achene dormancy. However, the effect of water stress on sunflower mother plant, a highly probable environmental cue in the context of global warming, on seed vigour and dormancy has not been investigated yet. In addition, nothing is known about possible transgenerational effects of stress for this species. The aim of this work was to characterized the effects of a drought stress occurring after flowering of the mother plant on the physiological properties of the progeny and to identify the relative roles of embryo and maternal tissues (*i.e.* pericarp mainly) in this process.

II) Material and methods

2.1 Seed production

Sunflower (*Helianthus annuus* L.) seeds were obtained from hybrid or line plants cultivated on Phenotoul-Heliaphen platform at Auzeville-Tolosane (INRAe Toulouse, France) (Gosseau et al., 2019). All plants were grown in pots (15 l, Sopparco) filled with soil Proveen PAM2. During the plant development 4 fertilizations were applied with 1 l of Peter's Professional (N-K-P 17-07-27 at 0.8 g/l + Hortilon 0.46 g/l) per plant. Several seed production protocols were set-up as follow.

Plants from XRQ genotype were either fully irrigated till harvest [Fraction of Transpirable Soil Water (FTSW) maintained to a value of 1 (Sinclair et al., 2005), watered plants (W)] or were drought-stressed 2 d before flowering till maturity by holding irrigation until a FTSW value of 0.2 (stressed plants, S), as already described by Gosseau et al. (2019). The value of FTSW for drought stress was chosen accordingly to Gosseau et al. (2019) because it resulted in visible drought stress on sunflower plants but it did not prevented seed formation and development. Drought stress was also applied from flowering to the beginning of maturation [10 days after flowering (daf)] (early stress,E), from the beginning (10 daf) to the end (25 daf) of the maturation phase (Middle stress, M) and from the end of maturation phase (25 daf) to harvest (Late stress, L) (Fig. 1). In all cases water stress corresponded to a FSTW value of 0.2. Seeds resulted from self-fertilization and were all harvested at the same time.

Seeds from different genetic crosses were also produced to study the parental origin of acquired tolerance and the potential impact of cytoplasmic sterility genes. Sunflower XRQ/A (sterile cmsPET1 line) plants were crossed with sunflower PSC8 plants. Each parental plant was subjected to either S or W conditions, as previously described (Fig. 1) and resulting seeds were harvested simultaneously.

To determine the paternal and maternal components in response to drought stress, XRQ/A (sterile cmsPET1 line) plants, stressed or not, were crossed with XRQ/B (fertile cmsPET1 maintainer line) plants, also stressed or not, which gave 4 possibilities of crossing. Seeds were harvested simultaneously.

At last, in order to study the transgenerational effect of drought stress on seed physiology, the same growing and self-fertilizing scheme was repeated for 3 generations, obtaining 3W and 3S seeds from XRQ plants grown either in well-watered (W) or in drought

stress (S) conditions, respectively. At the last generation (*i.e.* the 4th), we applied drought stress to the mother plants of seeds issued from well-watered plants, thus giving 3WS seeds, and reciprocally, thus giving 3SW seeds. The same water regime was also maintained in the last generation to 3W and 3S seeds, giving 4W and 4S seeds, respectively.

After harvest, all seeds were dried at 26°C for 48 h. They were then stored in a cold room at 3°C with a hygrometry of 30 % relative humidity (RH). After 6 months of storage, all seeds were non-dormant.

2.2 Characterization of seed development

Impact of water stress on plants on yield and biomass traits have been determined as a function of the water stress indicator sFTSW, which integrates the fraction of transpirable soil water over time (Gosseau et al., 2019). Number of seeds was determined for each plant and thousand kernel weight (TKW) was calculated by (seed dry weight / seed number)*1000. Plant biomass (stems, leaves, flower head, seeds) was measured after drying during 48h at 80°C. Seed dry weight was measured after seed cleaning and further drying at 80°C during 24h.drying during 24h at 120°C and. The relative root mean square error (rRMSE) was calculated by R for each graph.

2.3 Germination assays

Germination assays were performed with 3 replicates of 25 seeds (achenes) or naked seeds (*i.e.* seeds without pericarp) obtained from 8 plants. Whole achenes or naked seeds were placed at 20°C in darkness in 9 cm Petri dishes one layer of cotton wool moistened seeds with distilled water, with a solution of polyethylene glycol (PEG 8000) giving a defined water potential value indicated in the text (according to Michel and Kaufmann, 1973) or with 300 mM NaCl. PEG and NaCl concentrations were chosen after preliminary experiments showing that these concentrations were decreasing seed germination speed without decreasing markedly final seed germination rates (i.e. remaining above 80 %). Since seed sensivity to PEG varied among the seed batches we also adapted the PEG concentrations to each seed production, since -1.0 MPa, for example, might in some case induce a too strong inhibitory effect on seed germination. Germination in atmospheres with different oxygen tensions was performed as described by Gay et al. (1991).

Naked seeds were also germinated at 20°C on a PEG solution containing extracts of pericarps (obtained after imbibing 60 pericarps in 15 ml of PEG -0.7 MPa for 24 h) or directly on a bed of pericarps extracted from W seeds and placed between the PEG solution and the seeds (bedding assay).

A seed was considered as germinated when the radicle pierced the envelopes (seed coat and pericarp) (whole seeds) or when embryonic axis elongated (naked seeds)

2.4 Electrolyte leakage measurements

Solute leakage of seed pericarp seed was estimated by measurements of electrolyte leakage with 3 replicates of pericarps obtained with 20 seeds. Conductimetry was measured with a microcomputer conductometer (K220, CONSORT Bioblock) after soaking pericarps during 3 h in 10 ml of distilled water at 20°C. The total electrolyte leakage of pericarps was obtained after boiling pericarps in water at 80°C for 20 min and it was used to express relative conductivity (Bailly et al., 1996). Results are expressed in µS by g of dry pericarp or as percentage of total leakage and correspond to the means of 3 replicates ± SD.

2.5 Measurement of phenolic compounds

Phenolic compounds were extracted from 0.1g of pericarp in 3.5ml of ethanol 70 % (v/v). The extracts were mixed with 1ml of Folin-Ciocalteu reagent and 1ml of Na_2CO_3 20 % (w/v) and heated at 40°C for 30min.(Caboni et al., 1997) Absorbance of samples was read at 760 nm by spectrophotometer (Genesys 10S UV_Vis Thermo Scientific). The total phenolic content in each extract was calculated using a standard curve obtained with gallic acid. Results are expressed as milligrams of total phenolic equivalents per g of pericarp. Measurements are means of 3 replicates± SD.

2.6 Microscopy

Transversal cross sections of pericarps were performed in the equatorial area of the seeds using a cryostat Leica CM3050S and sections were observed with a microscope AXIOZOOM V16 ZEISS apotome 2 at a magnification of 258X. Pericarp thicknesses and area of phytomelamin layer were measured with 10 samples using Fiji Image J 1.53c software.

2.7 Metabolomic analysis

Liquid chromatography (LC) MS–MS was performed with 5 replicates of 50 mg of naked seeds. The extraction was done in 800 µl methanol: water = 7:3 and 20 µl of internal standards (d3-Leucine, 13C9-Phenylalanine, d5-Tryptophan, 13C3- Progesterone). The mixture was ground in a Tissue grinder (50 Hz, 5min) followed by ultrasonic treatment with water bath at 4°C for 30min and keep in the refrigerator at -20°C for 1h. Subsequently, the samples were centrifuged at 4°C for 15 min at 14000 rpm, filtered and placed in a vial (1.5ml) for LC-MS analysis. Twenty µl of each sample was mixed into a Quality Check sample to evaluate the repeatability and stability of LC-MS analysis. A LC-MS system consisting of

Waters 2D UPLC (Waters, USA) and Q Exactive high resolution mass spectrometer (Thermo Fisher Scientific, USA) was used for metabolite separation and detection. A Hypersil GOLD aQ column (100*2.1 mm, 1.9 µm, Thermo Fisher Scientific, USA) was used and the mobile phase consisted of solvent A (water + 0.1% formic acid) and solvent B (acetonitrile + 0.1% formic acid). The flow rate was 0.3 ml/min. The column oven was maintained at 40°C. The injection volume was 5 µl. Q Exactive mass spectrometer (Thermo Fisher Scientific, USA) was used to obtain MS1 and MS2 data. The MS scan method was in the range of m/z 150–1500, The MS1 resolution was 70,000, AGC was $1e^6$, and the maximum injection time was 100 ms. According to the precursor ion intensity, Top 3 ions were selected for MS2 analysis, MS2 resolution was 35,000, AGC was 2e5, maximum injection time was 50 ms, and collision energy were set as: 20, 40 and 60 eV. The parameters of ESI were sheath gas of 40 l/min, aux gas of 10 l/min, spray voltage(|KV|) of 3.80 in positive ion mode and of 3.20 in negative ion mode, capillary temperature of 320°C and aux gas heater temperature of 350°C. Raw data collected by LC-MS/MS was imported into Compound Discoverer 3.1 (Thermo Fisher Scientific, USA) for data processing, which mainly included peak extraction, retention time correction within and between groups, adduct ion combination, missing value filling, background peak labeling and metabolite identification. Finally, the molecular weight, retention time, peak area and identification results were derived. Metabolites were identified using BGI Library and mzCloud database.

2.8 Statistics

Statistical analyses were performed using the OriginPro software version 9.1 (Seifert, 2014). Tukey tests with a p.value= 0.05 were used for analyzing germination data, and the number of replicates are indicated within the legends. For the metabolite screening by LC-MS, multivariate statistical analysis and univariate analysis were used to select the groups metabolites between. The used multivariate statistical Analysis methods are PCA (Principal Component Analysis) unsupervised pattern recognition method, and PLS-DA (Discriminant Analysis, Partial Least Squares Method Discriminant Analysis) a supervised pattern recognition method. The used univariate analyses are Fold-Change analysis (FC) and T test (Student's T test). The clustering analysis was conducted for differential metabolites, and the log2 conversion and Zero-mean normalization treatments were used for the data. The clustering algorithm used Hierarchical Cluster and the Euclidian distance was used for distance calculation.

III) Results

3.1 Effect of maternal water stress on the mother plant

Fig. 22 shows the effect of the drought stress scenarios shown Fig. 21 on the pattern of seed development *on planta*. Seed moisture content (MC) decreased continually from flowering to harvest (60 daf). Differences in seed MC caused by the water stress scenarios were visible at the first time-point only, i.e. at 10 daf. At this time-point, seed MC of S (stress during the entire seed development) and E (early stress) seeds was close to 4.5 $gH_2O.gDW^{-1}$ whereas it was close to 6 $gH_2O.gDW^{-1}$ for seeds that developed on non-stressed plants (W), middle- and late-stressed plants (M, L) (Fig. 22a). At the second (25 daf) and last (60 daf) time points, seed MC was roughly similar in all seed lots, i.e. close to 1-2 $gH_2O.gDW^{-1}$ and 0.4 $gH_2O.gDW^{-1}$, respectively (Fig. 22a). Seed fresh weight increased from flowering till ca 30 daf and decreased until harvest in all conditions (Fig. 22b). As expected, during seed development, seed fresh weight was lower in seeds from stressed plants (S and E) than in seeds issued from other categories. At harvest, seed fresh weight was close to 0.8 g for all seed lots. At last, water stress applied on the mother plant at any stage (S, E, M, L) did not modify seed dry weight during development, when compared to well-watered plants (W) (Fig. 22 c).

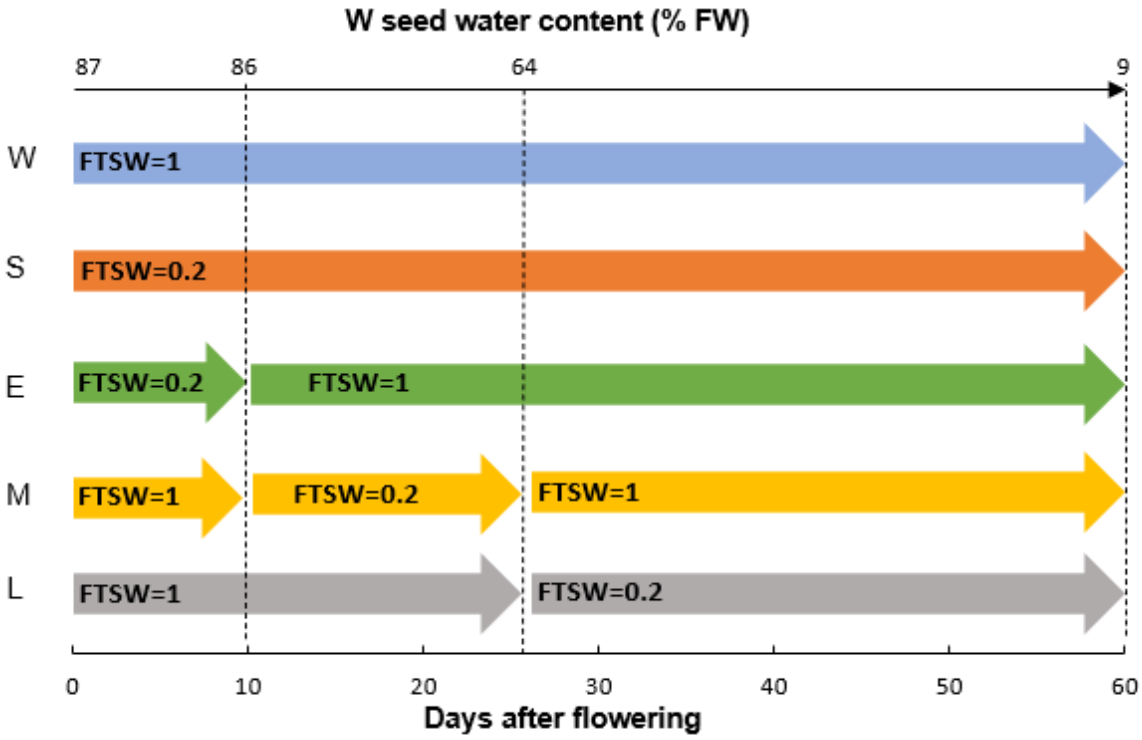


Figure 21 : Experimental design.

Schematic representation of the experimental design showing the different drought stress scenarios applied during sunflower seed development. W, well-watered condition (FTSW=1); S, drought stress (FTSW=0.2) during entire seed development; E (Early), M (Middle), and L (Late) application of drought stress (FTSW=0.2), as indicated by the time scale below the figure.

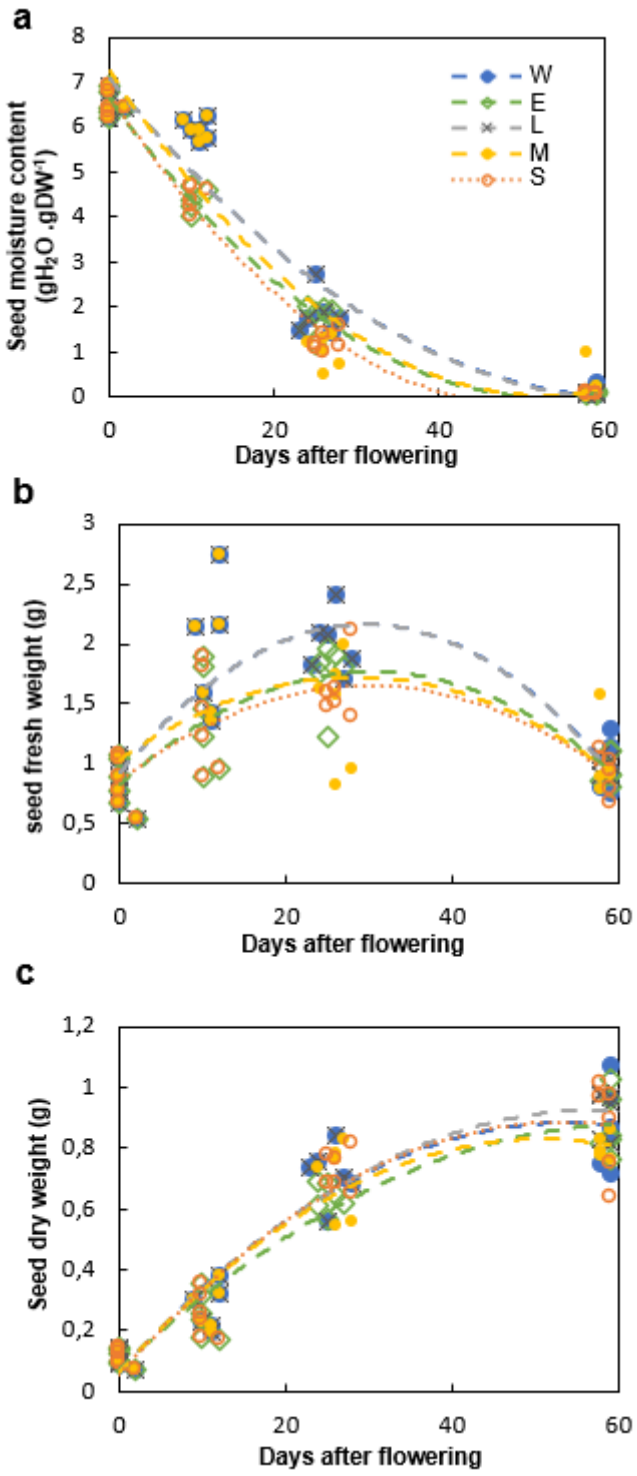


Figure 22 : Agronomic parameter.

Changes in water content (a), fresh weight (b), dry weight (c) of seeds produced under the different scenarios of water stress shown Fig. 1. Mother plants (XRQ genotype) were grown in well-watered condition (W, constant FTSW=1) or under drought stress (FTSW=0.2) during the early (E), middle (M) and late (L) phase of seed maturation and from flowering to harvest (S). (d) Germination at 20°C in the presence of a -0.7 MPa PEG solution of W, E, M, L and S seeds. Means ± SD of 3 replicates. Statistics were made using Tukey's test with alpha=0.05.

We confirmed the impact of drought stresses on seed production using the sum average daily water deficit, expressed as sFTSW (Supplementary Fig. S1). Stressed plants had higher values of sFTSW. The S plants had a sFTSW value around 30 whereas W plants had the lower sFTSW value, close to 11. L plants had a sFTSW value close to the one of W plants, and E and M plants displayed intermediate sFTSW values, close to 20 (Supplementary Fig. S1). The major effects of drought stress were observed on the plant biomass, as previously reported (Gosseau *et al.*, 2019) with a negative impact of stress on the plant dry weight (Supplementary Fig. S1c). In contrast, the impact of water stress on the progeny (thousand seed weight, seed number and seed weight) was less important, highly variable and not statistically significant.

3.2 Effect of maternal environment on seed dormancy and vigour

We first assessed the effect of maternal stress on seed dormancy. Immediately after harvest, W and S seeds were dormant, *i.e.* they poorly germinate at 20° (Fig. 23a). S seeds, however, were less dormant than E, M, L and W seeds, as shown by their higher germination (Fig. 23a). After 6 months of storage in ambient temperature all seeds were no dormant and fully germinated at 20°C (Fig. 23b).

We also investigated the effect of the maternal environment on seed vigour traits, using non-dormant seeds only (*i.e.* stored for 6 months, their germination at 20°C on water is shown in Fig. 23b). When seeds came from well-watered plants (W) their germination was dramatically inhibited by water stress since only *ca* 45 % of W seeds were able to germinate in the presence of PEG -0.7 MPa (Fig. 24a). In contrast, S seeds, produced by drought stressed plants, fully germinated within 5 d in this condition (Fig. 24a). When the drought stress was applied early (E) or lately (L) on the mother plants, it did not significantly modify seed germination when compared to W seeds. However, applying drought stress during seed maturation phase (M) had a beneficial effect on seed germination under water stress, even though it was not as pronounced as for S seeds (Fig. 24a). Because many abiotic stress responses share common physiological and molecular pathways in plants (Ben Rejeb et al., 2014; Koyro et al., 2012), we investigated whether drought stress applied on the mother plant could also improve seed germination in other penalizing conditions, i.e. under cold stress, salt stress and hypoxia. When non-dormant seeds were imbibed for 24 h at 5°C and then germinated at 20°C, germination of W seeds decreased to ca 80 % (Fig. 24b). This negative effect of a cold period on seed germination was cancelled in S, E, L and M seeds (Fig. 24b). In the presence of 300 mM NaCl, germination of W, E, M and L seeds decreased

to ca 70 % whereas S seeds germinated to 84 % (Fig. 24c). Finally, we also assessed seed germination in hypoxia by germinating seeds under 3 and 5 % oxygen (Fig. 24d). Germination of S seeds was only partially inhibited in hypoxia since they germinated to 75 % in 3% oxygen and to 82 % in 5 % oxygen (Fig. 24d). In contrast W seeds only germinated to 16 and 58 % in 3 and 5 % oxygen, respectively (Fig. 24d). When water stress on the mother plant was applied at the early (E) or maturation (M) stages it improved seed germination under hypoxia when compared to W seeds. However the late application of water stress on the mother plant did not confer a better tolerance to hypoxia (Fig. 24d).

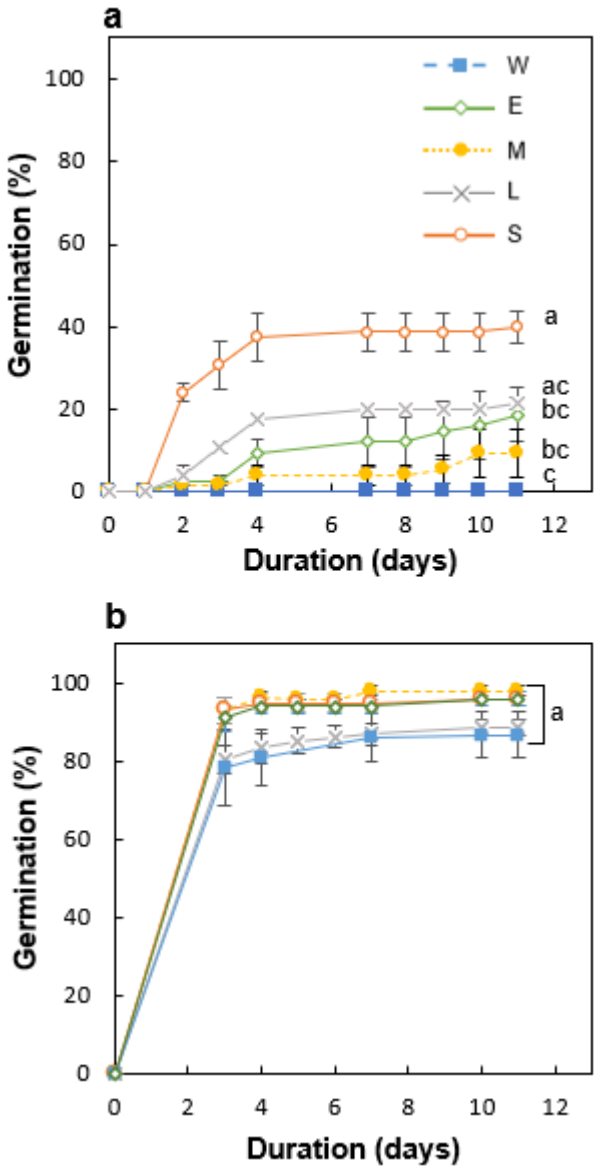


Figure 23 : Kinetics of seed germination.
Germination of XRQ/B dormant whole seeds (a) and non-dormant whole seeds (c) on water at 20°C in darkness. Seeds were obtained from plants grown in well-watered condition (W), in drought stress condition during the early (E), middle (M) and late phase (L) of seed maturation, or from flowering to harvest (S). Data are means of 3 replicates ± SD. Statistics were made using Tukey's test with alpha=0.05.

3.3 Possible involvement of pericarp in seed germination

The effects of hypoxia on seed germination (Fig. 24d) suggested that pericarp could play a role in this response since it is well know that seed envelop structures can modulate oxygen diffusion towards the embryo (Porter and Wareing, 1974; Werker, 1980). We therefore investigated whether the maternal environment could modify seed germinative properties through an effect on pericarp. We addressed this question by germinating W and S naked seeds in the conditions previously described.

Pericarp removal strongly stimulated germination of dormant W seeds at 20°C on water (Fig. 25a), since it reached almost 55 %, vs 0 % for whole seeds (see Fig. 23a). Germination of naked dormant S seeds increased from 40 to 68 % (Fig. 23a and Fig. 25a). Non-dormant naked W and S seeds germinated similarly under water stress (Fig.25b), after cold stress (Fig. 25c), on a NaCl solution (Fig. 25d) or in 3 % oxygen (Fig. 25e), and in all cases at higher levels than whole achenes (cf. Fig. 24). This was particularly spectacular for W seeds, which indicated that their pericarp played an inhibitory role on seed germination.

To understand the possible role of pericarp in seed germination, we investigated the structure of pericarps from S and W seeds by microscopy (Fig. 26a and 26b). Thickness of S pericarp (Fig. 26a) was lower than that of W pericarp (Fig. 26b), i.e. 0.99 µm vs 1.44 µm although the number of cell strata was roughly the same (4-5). The area of the pigmented sub-epidermal layer containing phytoalexin was similar in W and S pericarps but sclerenchyma cells of S pericarps were smaller with thicker cell walls (Fig. 26a and 26b). Further, we measured conductivity of pericarps isolated from S and W seeds (Fig. 26c). Electrolyte leakage of pericarps from W and S increased similarly during their imbibition, from ca 1000 to 1300 $\mu S.g^{-1}$ (Fig. 26c). However, when expressed as a function of total electrolytes, conductivity of pericarps from S seeds was significantly higher than the one of W seeds (Fig. 26c), although total electrolyte leakage was similar in both pericarps and close to 1600 $\mu S.g^{-1}$ (data not shown). After 3 h of imbibition, almost 83 % of electrolytes had leaked from S pericarps when only 75 % leaked from W pericarps (Fig. 26c). Since seed conductivity often results from phenolic compounds we measured total phenol content in pericarps (Fig. 26d). It was significantly higher in S pericarps than in W pericarps, with 5.43 mg vs 2.88 mg of total phenol per g of pericarp, respectively (Fig. 26d). At last we tested whether soluble compounds from pericarp could directly influence germination and therefore play a role of pericarp in tolerance to abiotic stresses. For this, we germinated W naked seeds (in the presence of a -0.7 MPa PEG solution) with macerate of pericarps or using a pericarp bedding assay (see material and methods). As already shown (see Fig. 26b), removing of pericarp allowed W naked seeds to fully germinate under water stress conditions, in contrast to whole W seeds whose germination did not exceed 40 %. However, when naked W seeds were imbibed on a PEG solution containing a macerate of pericarp or when placed on extracted pericarps (pericarp beding assay) then germination declined (Fig. 26d).

We also performed a non-targeted metabolomic analysis with dry naked S and W seeds. The objective of this study was to determine whether drought stress during seed development had only an effect on pericarp properties or could also have an effect on

embryo per itself (Fig. 27). This analysis allowed the identification of 474 metabolites which clustered in 4 groups with 48 and 31 classes (Supplementary Fig. S2 and S3). Out of this, 21 metabolites accumulated differently between S and W seeds. The Heat map Fig. 27 shows metabolites that were specifically accumulated in either S or W seeds. In S seeds, complex sugars such as stachyose, tracheloside, intermediates such as malic, isopropylmalic, quinic and glutamic acids and ω9 insaturated fatty acids like palmitoleic (C16 ω9) and linoleic acid (C18 ω9,12) accumulated. On the contrary, we observed a reduction of some aromatic amino acids (thryptophan, phenylalanine) and precursors (shikimic acid), of furoic, isochlorogenic C acids, of methylcytidine and interestingly of abscisic acid (ABA) in W seeds.

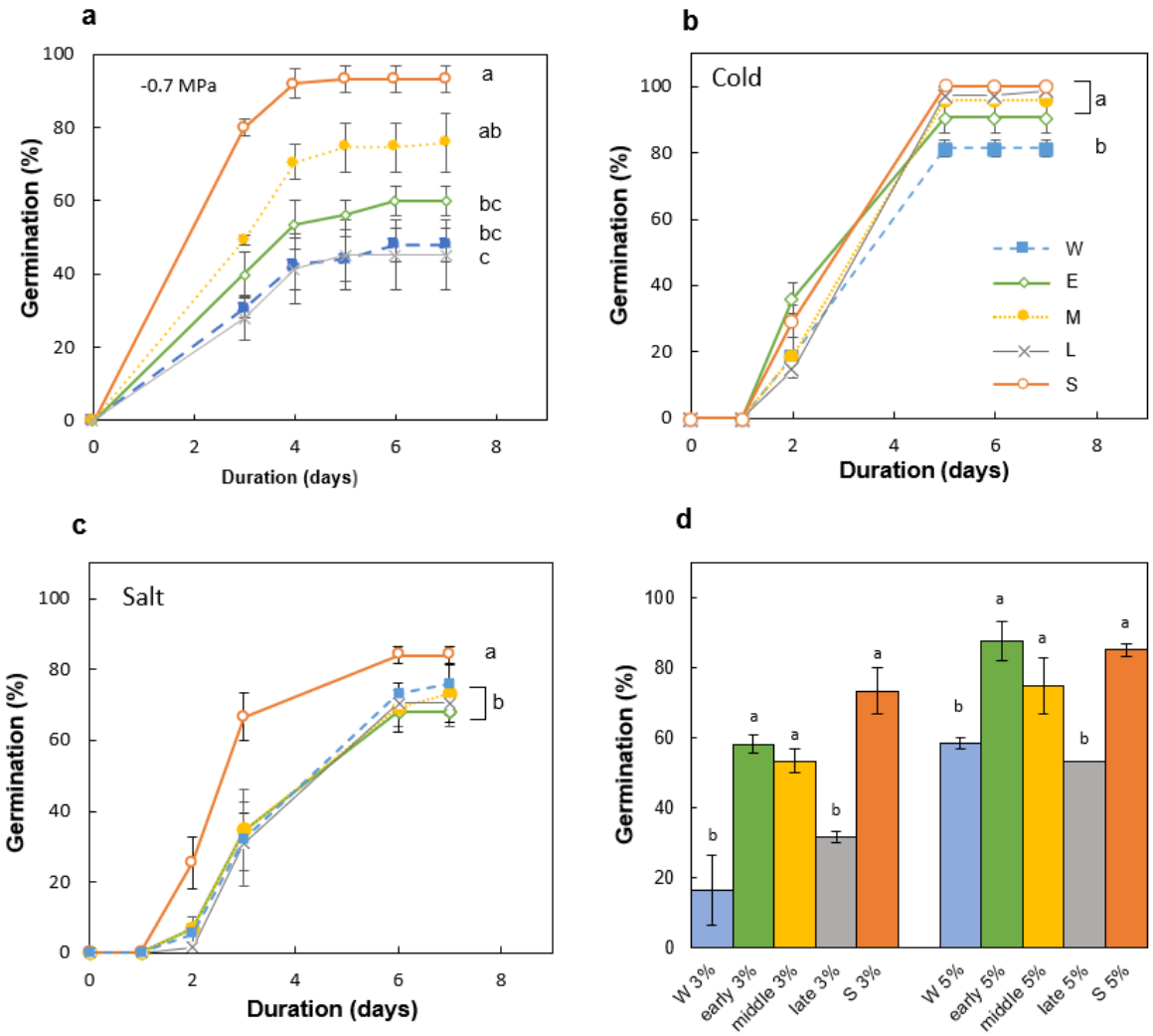


Figure 24 : Tolerance characterization.

Germination at 20°C in darkness of non-dormant seeds produced by plants grown in well-watered condition (W) or under drought stress during the early (E), middle (M) and late (L) phase of seed maturation and from flowering to harvest (S). (a) Germination in the presence of a -0.7 MPa PEG solution. (b) Germination after 24h at 5°C on water. (c) Germination on a 300 mM NaCl solution and (d) germination in the presence of 3 or 5 % oxygen. Data are the mean of 3 replicates ± SD. Statistics were made using Tukey's test with alpha=0.05.

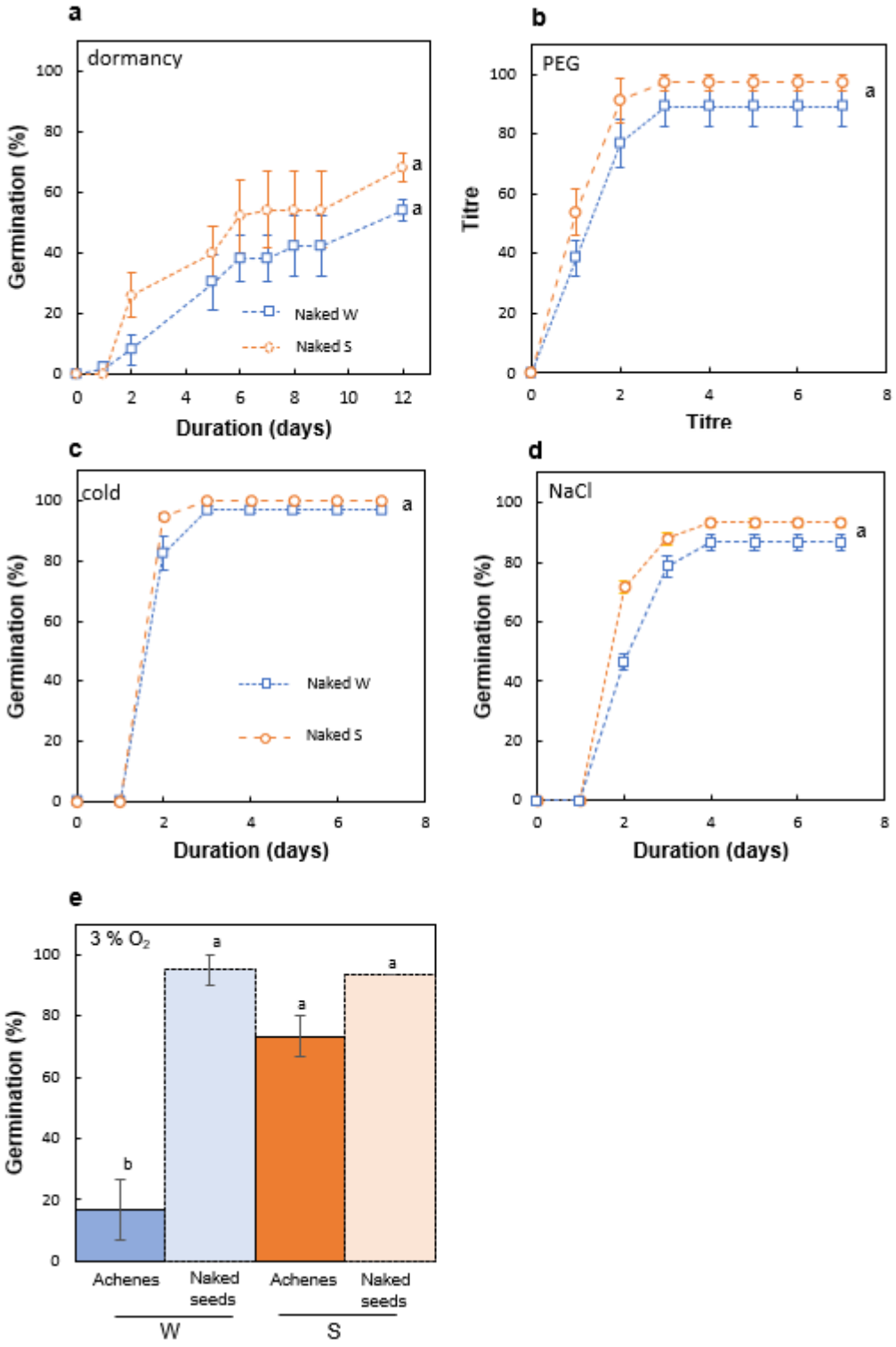
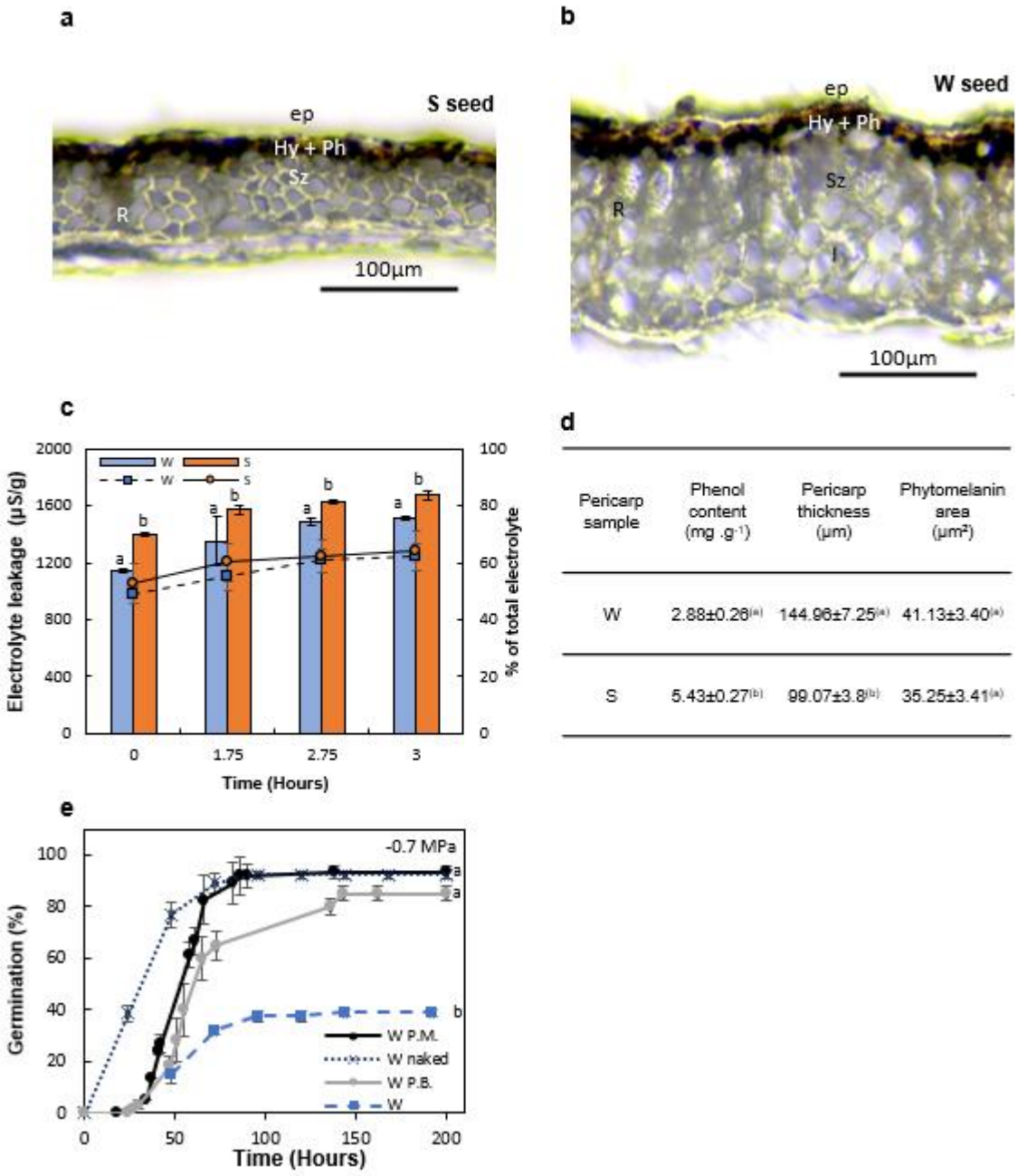


Figure 25 : Impact of periscope in seed tolerance.

Germination at 20°C in darkness of naked seeds (*i.e.* without pericarp) produced by plants grown in well-watered condition (W) or under drought stress from flowering to harvest (S). Germination of dormant naked seeds (a) and of non-dormant naked seeds in the presence of a -0.7 MPa PEG solution (b), after 24h at 5°C on water (c), on a 300 mM NaCl solution and in the presence of 3 or 5 % oxygen (d) obtained from plants grown in well-watered condition (W), in drought stress condition during the early (E), middle (M) and late phase (L) of seed maturation, or from flowering to harvest (S). Data are the mean of 3 replicates ± SD or of 2 replicates of 30 seeds ± SE (hypoxia experiment). Statistics were made using Tukey's test with alpha=0.05..



Pericarp sample	Phenol content (mg .g^{-1})	Pericarp thickness (µm)	Phytomelanin area (µm²)
W	2.88±0.26$^{(a)}$	144.96±7.25$^{(a)}$	41.13±3.40$^{(a)}$
S	5.43±0.27$^{(b)}$	99.07±3.8$^{(b)}$	35.25±3.41$^{(a)}$

Figure 26 : Composition and role of pericarp in seed germination.

Composition and role of pericarp in seed germination. (a,b) Cross sections of pericarps of S (a) and W (b) seeds imaged by optical microscopy. Ep: epidermis, HY+PH: hypodermis plus phytomelanin layer, Sz: Sclerenchyma zone, I: internal parenchyma, R : parenchyma ray. (c) Electrolyte leakage in µS.g^{-1} (bars) and in percentage of total electrolytes (curves) of pericarps of seeds issued from plants grown in well-watered conditions (W) or under drought stress (S). Data are means of 3 replicates of 25 seeds ± SD. (d) Phenol content and morphological characteristics of pericarps from W and S. Data are the mean of 3 measurements ± SE (phenolics) or 10 measurements ± SE (morphology). (e) Germination of whole (W) and naked W seeds and of naked W seeds in the presence of a pericarp macerate (W P.M.) or on a pericarp bed (W P.B.) at 20°C under water stress (PEG -0.7 MPa). Statistics in c, d and e were made using Tukey's test with alpha=0.05.

3.4 Possible involvement of pericarp in seed germination

Lastly it was important to decipher the genetic bases of the transmission of germinative traits by the mother plant to the offspring. This was achieved by studying the effect of different schemes of crosses on the induction of water stress tolerance only. Following the first observations obtained with seeds of XRQ/B line produced by self-fertilization, we checked whether the maternal effect on inherited tolerance to water stress (Fig. 28a) could also be observed in the context of hydrid production. To test this hypothesis, we crossed male-sterile plants (XRQ/A) with restorer plants (PSC8/R) that were well-watered (W) or stressed (S) or. from flowering to harvest (S). When each parent was stressed, hybrid seeds germinated to 80 % in the presence of -1.0 MPa PEG solution, when only 43 % of seeds produced by well-watered parent plants germinated (Fig. 28a).

We next determined whether the better tolerance to water stress in S seeds was transmitted by the maternal gamete and/or by the paternal gamete. For this, we combined water stresses in parent plants using cmsPET1 XRQ/A female plants and XRQ/B maintainer male plants (Fig. 28b). As observed previously, seeds obtained from stressed male and female plants (XRQ/A S x XRQ/B S) had the highest germination rate on a solution of -1.0 MPa PEG solution (58 %) whereas when parent plants were well watered (XRQ/A W x XRQ/B W) seed germination was lower (Fig. 28b). When male plants only were stressed (XRQ/A W x XRQ/B S) seed germination was the same as for seeds obtained from non-stressed plants (XRQ/A W x XRQ/B W). In contrast, when female plants only were stressed (XRQ/A S x XRQ/B W), resulting seeds displayed a faster germination and an intermediate final germination (Fig. 28b).

Finally, to determine if the induction of water stress tolerance was transgenerational or only resulting from a maternal effect, we grew plants for 3 successive years with or without water stress (at each generation plants issued from W or S seeds were grown without or with water stress, respectively, giving 3W and 3S seeds). The last year the same experimental protocol was applied, giving 4S and 4W seeds but in addition 3W plants were stressed (giving 3WS seeds) and 3S plants were well watered (giving 3SW seeds). Germination of the resulting seeds was assessed on a PEG solution and it showed that seeds were able to fully germinate under water stress only when they were produced by a plant that had been stressed during seed development at the last generation (Fig. 28c).

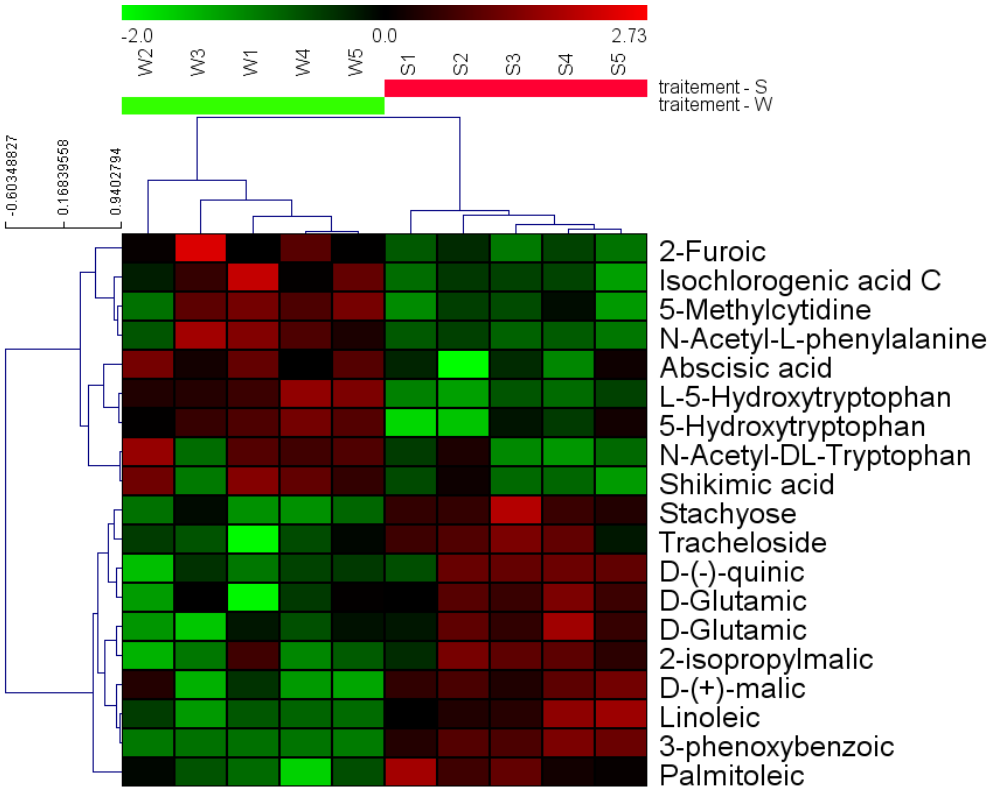


Figure 27 : Heatmap showing the differencially accumulated metabolites in dry seeds obtained from plants grown in well-watered condition (W) or in drought stress condition from flowering to harvest (S). Each row in the figure represents a metabolite and each column represents a sample. Colors ranging from green to red indicate metabolite abundance.

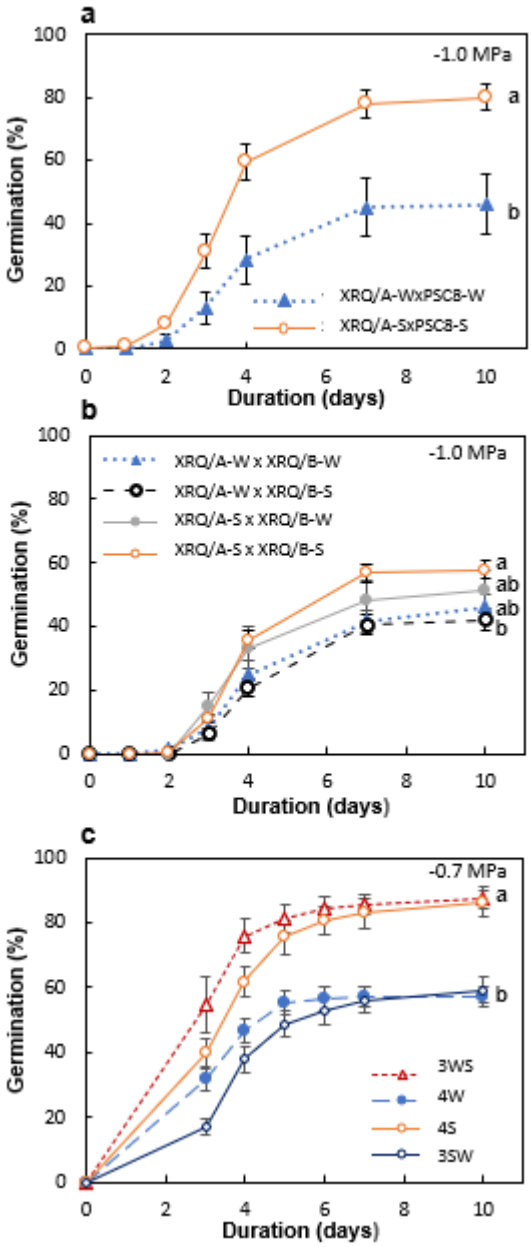


Figure 28 : Impact of maternal stress in seed metabolome.

. Germination at 20°C on a PEG solution of seeds issued from plants grown in well-watered conditions (W) or under drought stress (S). (a) Seeds from the INEDI hybrid obtained from the cross of parental lines XRQ/A and PSC8. (b), seeds issued from different crosses female (XRQ/A) and male (XRQ/B) parental lines. (c), seeds from plants stressed or not for 3 generations (3S and 3W, respectively) before being produced in well-watered condition (3SW and 4W) or not (3WS and 4S) at the fourth generation. Concentrations of PEG solutions are indicated within the figures. Data are the mean of 3 replicates of 25 seeds ± SE. Statistics were made using Tukey's test with alpha=0.05.

IV) Discussion

On the Heliaphen Phenotyping platform, pattern of sunflower seed development reproduced the dynamics of water content and seed dry weight changes already observed for seeds of this species (Rondanini et al., 2006; Lachabrouilli et al., 2021). The time from

flowering to harvest lasted ca 60 days and was associated with a relatively constant rate of water loss (Fig 2a). Achene fresh weight increased rapidly during early grain-filling and peaked at ca 35 daf when seed mass maturity was reached at ca 40 daf (Fig. 2), what is similar to what was observed by Lachabrouilli et al. (2021). Application of water stress on the mother plant had a limited and transient effect on these characteristics and was only visible for E and S seeds at the first time point (10 daf). Water stress (FSTW=20) applied continuously after flowering (i.e. S condition) had a unpredictable effect on the components of the yield at harvest, as estimated by TSW, seed dry weight and seed number per plant when compared to control plants, that was considered as being not significant (Supplementary Fig. S1). In contrast, as already shown by Gosseau et al., (2019), drought stress had a negative effect on whole plant biomass.

As already reported (Donohue, 2009; Fernández Farnocchia et al., 2019; Penfield, 2017), the maternal environment had an effect on seed dormancy. At 20°C, a temperature at which sunflower seed dormancy can be expressed (Gay et al., 1991; Lachabrouilli et al., 2021), germination was not complete, which revealed dormancy (Corbineau et al., 1990). Seeds issued from constantly drought stressed plants (S) were clearly less dormant than seeds from the other stress regimes (Fig. 3a). In particular, early, middle or late drought stress did not induce a phenotype of dormancy compared to well water plants (W). This suggests that faster seed desiccation during seed filling, as shown Fig. 2a, is likely to play an important role in the establishment of sunflower seed dormancy. Interestingly, Lachabrouilli et al. (2021) also demonstrated that rapid sunflower seed desiccation on the mother plant was associated with lower dormancy, which is in agreement with our findings. In *Lolium perenne*, whose seeds also display a physiological dormancy, high temperature during seed filling reduced dormancy (Fernández et al., 2021). In our conditions, after 6 months of dry storage, all seed batches became non-dormant as shown by their full germination at 20°C (Fig. 3b).

A moderate drought stress continuously applied to the mother plants from flowering to harvest (S seeds) induced a dramatic stimulation of germination of non-dormant seeds under water stress, when compared to seeds obtained from well-watered plants (Fig. 4a). The beneficial effect of the drought stress required that it was applied within a proper time window to induce water stress tolerance at the germination stage. This time window, which ran from 10 daf to 25 daf, roughly corresponded to the beginning of the seed filling phase. Earlier or later stresses had no significant effect on the induction of water stress tolerance, which suggests that this trait was acquired during seed filling but not during the late

maturation phase, in contrast to what was shown for seed longevity (Leprince et al., 2017). Interestingly, drought stress on the mother plant also induced tolerance to other abiotic stresses to the offspring. In particular it induced a better germination after cold stress, under salt stress and in hypoxia, if it was applied continuously after flowering (Fig. 4). Indeed, the time-window of drought stress application that induced stress tolerance depended on the nature of the stress. Tolerance to cold stress was acquired during any regime of drought stress, tolerance to salt stress required a constant water stress during seeds filling (S seeds) and tolerance to hypoxia was not induced if drought stress was applied lately (L condition). This demonstrates that the mechanisms and signaling pathways involved in the tolerance to different stresses are specific and are timely induced during the seed developmental program. The relationship between pre-germination cues with post-germination environments has already been shown in various contexts (D'Aguillo et al., 2019). For example, the quantity and quality of light during seed maturation can influence seed germination of the next generation (Vayda et al., 2018). Heat stress, often concomitant to drought stress, can induce thermotolerance in the next generation in wheat seeds (Wang et al., 2016). The effect of drought stress during seed development on seed vigour is already documented but in some cases it has been shown to be detrimental for the progeny (Hatzig et al., 2018; Wijewardana et al., 2019) whereas some other studies have shown an opposite effect (Matzrafi et al., 2021; Van Dooren et al., 2020). Drought stress was also shown to improve germination of the progeny under water stress conditions in rice (Zheng et al., 2017) and *Brassica napus* L (Hatzig et al., 2018). The induction of tolerance to various stresses by a single stress applied on the mother plant, as evidenced here, has already been shown by Lui et al. (2017) in *Brassica rapa* where cold acclimation conferred tolerance to heat stress to the progeny. Similarly, in wheat, seeds collected from drought stressed plants had better tolerance against salt stress (Tabassum et al., 2017). To our knowledge, the effect of maternal environment on seed sensivity to hypoxia has not been demonstrated yet.

Pericarp removal had a dramatic effect on seed germination properties (Fig. 5), and it is worth noting that it generally conferred the phenotype of whole S seeds, i.e. lower dormancy and higher vigour, to naked W seeds. Sunflower seeds display a physiological dormancy (Baskin and Baskin, 2004) that includes an inhibitory action of the envelopes (pericarp and seed coat), which is mostly expressed at 20-25°C, and an embryo dormancy, which generally prevents germination below 15°C (Corbineau et al., 1990). Removal of pericarp increased germination of dormant seeds but it did not exceed 70 %, which suggests that the observed dormancy resulted from the additive effect of both embryo

dormancy and pericarp coat imposed dormancy (Lachabrouilli et al., 2021). This also demonstrates that embryo dormancy can be expressed at high temperature, even though it is generally reported to be expressed at low temperature (Bodrone et al., 2017; Françoise Corbineau et al., 1990). The specular increase in W seeds germination after pericarp removal (Fig. 3a and Fig. 6a) suggests that the inhibitory action of the pericarp was much higher in W seeds than in S seeds, thus bringing a first line of evidence of a direct effect of the maternal environment on pericarp properties. Here we also show that pericarp strongly repressed germination of non-dormant W seeds under stress conditions (water stress, cold, salt stress, hypoxia) and that the maternal environment suppressed this inhibitory effect, as shown by the germination of whole S seeds (Fig. 6). The role of seed covering structures on non-dormant seed germination is also well documented, mostly with regards to water and oxygen diffusion from the imbibition medium to the embryo (Grafi, 2020; Huss and Gierlinger, 2021; Magnée et al., 2020). However the effect of pericarp on seed germination under cold or salt stress is not documented to our knowledge.

Sunflower seed pericarp is a maternal tissue formed by the fusion of the ovary tissues and part of the receptacle (Schneiter et al., 1997). Our results show that the acquisition of stress tolerance and low dormancy in the offspring of drought stressed plants involved the pericarp. Transverse sections of pericarps allowed identifying their histological structures (Fig. 6). Pericarps from S seeds were much thinner than pericarps from W seeds, which resulted from smaller sclerenchyma cells with thick cell walls, but the general organization appeared to be the same, including a phytomelanin pigmented layer, and sclerenchyma areas in between parenchyma rays (Denis et al., 1994; Lindström et al., 2021). This suggested however that drought stress during seed development had a significant effect on sclerenchyma cells enlargement and cell wall deposition. Thickness of seed coats are known to be environmentally responsive in many species (Baskin and Baskin, 1998; Mousseau and Fox, 1998) and the effect of growing environment conditions on sunflower pericarp anatomy has already been demonstrated elsewhere (Lindström et al., 2021). Conductivity measurements revealed that the level of electrolyte leakage was timely similar in both S and W seeds but that a more important fraction of electrolytes leaked from S pericarps during their imbibition (Fig. 6c). This suggests that a rapid removal of chemicals from pericarp of whole S seeds could help their faster germination. Phenolic content of S pericarps was nevertheless higher than the one of W pericarps, when expressed per g FW^{-1} (Fig. 6). However, if one considers the difference of thickness between S and W pericarps (Fig. 6), then the amount of soluble phenolics surrounding the embryo was roughly similar

in pericarps of both categories of seeds. Interestingly the pericarp bedding assay and germination of naked seeds with pericarp extracts (Fig. 6d) confirmed that pericarps of W seeds, in particular, contained compounds that inhibited germination. We therefore propose that pericarps of S seeds would release more rapidly inhibitors of germination in the imbibition media, which would in turn allow a better germination of dormant seeds at 20°C and of non-dormant seeds under various stresses. Alternatively the chemical composition of pericarps of S and W seeds could also differ, S seeds containing lower amounts of inhibitory compounds, and this will have to be further investigated. This is in agreement with the studies of Khadka et al., (2020) and Swetha et al., (2021) who showed that the maternal environment had an effect on chemical composition of pericarps of *Anastatica hierochuntica* and *Brassica juncea*, respectively. Phenolic compounds, present in the pericarp of sunflower seeds, have been frequently cited as being good candidates for inhibiting seed germination (Reigosa Roger et al., 1999). Phenolics are easily oxidized (Devlin and Harris, 1984) and can in turn cause oxygen limitation to the embryo, thus decreasing germination. Other inhibitory compounds cannot be excluded, such as ABA, which was the major component of the pericarp of dry seeds from sunflower plants grown under drought (Andrade et al., 2009).

The balance of maternal versus embryonic control of germination is up to date poorly characterized. If our results unambiguously showed the role of pericarp in the induction of water stress tolerance to the offspring, one could not exclude that this maternal effect might also include a role of maternal provisioning on metabolite and hormone contents, or proteins and transcripts within the embryo. We indeed identified metabolites that specifically accumulated in either W or S embryos during seed development, which demonstrates that water stress on the mother plant had also an effect on the embryo characteristics *per se*. Even though it is too speculative to attribute a direct role to the accumulated metabolites in the germination phenotypes of S and W seeds, the accumulation of some compounds may have a biological sense. For example, stachyose, which accumulated in S seeds, belongs to raffinose-family oligosaccharides (RFOs) which are known to accumulate during seed late maturation (Baud et al., 2002) and have the role to protect biological from desiccation-related damage (Hincha et al., 2003) and in seed longevity (Angelovici et al., 2010). Stachyose has been shown to accumulate in *Coffea arabica* seeds under water deficit, high salt, and heat stress (dos Santos et al., 2011; Santos et al., 2015). The fatty acids palmitoleic and linoleic also accumulated in embryos of S seeds. Specific accumulation of fatty acids in oil crops such as sunflower could be interesting

specific food or industrial usages and will require further investigation. A set of metabolites was also specifically found in embryos of W seeds and among them we noticeably identified ABA (Fig. 7). Sunflower seed dormancy depends on ABA metabolism (Bianco et al., 1996; Bodrone et al., 2017), which is synthetized ca 2 weeks after flowering (LePage-Degivry et al., 1990), and the higher content of ABA in W seeds is in agreement with the higher dormancy phenotype highlighted for these seeds (Fig. 3).

Since a genetic component has already been shown to be involved in inheritance of seed traits (Alvarez et al., 2021; Donohue, 2009), we studied the relative importance of gametes using male sterile, maintainer, and restorer plants. Our results showed that the acquisition of water stress tolerance at the germination stage could be obtained for a commercial hybrid and was not dependent of the cytoplasmic origin (Fig. 8). Beside genetics, we studied whether paternal and/or maternal environments contribute to the acquisition of this tolerance. We carried out reciprocal crosses with male and female plants that were either stressed or not and concluded that the maternal environment was solely controlling the acquired tolerance as unstressed plants never produced water stress tolerant seeds. This is in accordance with the literature since most studies have demonstrated that environment elicits maternal effects (Donohue, 2009; Dyer et al., 2010; Galloway, 2005; Mousseau and Fox, 1998) and that the paternal effect on seed germination was often reported as being not statistically significant (Baskin and Baskin, 2019). We also performed a multigenerational experiment, in which drought stress, or none, was repeated over three generations before plant growing conditions were inverted for an additional generation. This confirmed our previous results and showed that growing plants under drought stress during the last generation was the prerequisite to provide seed tolerance to water stress. This shows that the offspring phenotype resulted from a maternal effect and not from a transgenerational effect, similarly to results obtained in Arabidopsis (Ganguly et al., 2017; Van Dooren et al., 2020).

In conclusion, we demonstrate that water stress during sunflower seed development induced seed tolerance to various abiotic stresses and reduced dormancy in the progeny at the germination stage. Our results show that these phenotypes result from the maternal environment but are not transgenerational because they did not persist over subsequent generations. This mechanism could be an adaptation to improve the offspring fitness and permit seed germination in sub-optimal conditions thus ensuring successful establishment of the next generation. Our data also bring new insights about the relative roles of maternal tissues and embryo physiology in germination tolerance to abiotic stresses. Future studies

will aim to determine how these both components interact together to drive the germination process.

Authors contribution

NL and CB designed and supervised the research. BV performed experiments and analyzed data. NB produced plants and seeds. BV NL and CB wrote the article.

Conflict of interest

The authors declare that they have no conflict of interest

Acknowledgments

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Addendum to Chapter 2:

Effects of maternal drought stress in condition of sunflower production

I) Introduction

In the context of global warming, changes in practices for sustainable agriculture will be necessary (Plan écophyto II+, 2018). Field crop producers are no longer only focusing on maximum grain yields, but they also aim on maintaining yields under abiotic stresses, such as drought or poor soil conditions.(Vear, 2016). This political and socio-cultural dynamic pushes them to adapt to climate change by developing new cultivation techniques based on the promotion of plant tolerance to climatic stress phenomena. Since we demonstrated on Heliaphen platform (previous chapter) that a moderate drought stress condition during sunflower seed development could induce higher vigour to the progeny of some genotypes, we also assessed whether this trait was inducible (i) in a wider range of sunflower hybrids and (ii) in non-experimental conditions of seed production in various sites of our private partners. Lastly, we have estimated the effect of drought stress on the mother plant on plant fitness, yield and oil content of the progeny, in order to evaluate the economic feasibility of this technical itinerary. In the longer term, our research could indeed lead to the design of a novel technical itinerary that should allow an increase of sunflower seed vigor.

II) Material and method

2.1 Seed production on Heliaphen (Experiment 1)

Fifteen different genotypes of sunflower (SF033, SF035, SF063, SF070, SF075, SF076, SF092, SF145, SF169, SF179, SF193, SF210, SF230, SF232, SF233) were grown in 2018 on the Heliaphen platform (INRAE, Toulouse), as previously described, including well watered and drought stress conditions. All the lines were unbranched maintainer lines and seeds were obtained after self-pollination. The genotypes used here represented a large range of sunflower diversity, as shown Fig supplemental data 4. Some lines were phylogenetically close to each other, such as SF092 and SF033, whereas some others differed markedly at the genetic level (Fig supplemental data 4).

2.2 Seed production by private companies (Experiment 2)

Soltis produced 8 sunflower genotypes (SF009, SF145, SF096, SF193, SF170, SF129, SF092, and SF075) in different locations: either in the field in Daux (Haute-Garonne) (sowing on 22/04/18), in Mondonville (Haute-Garonne) (sowing on 25/05/18), in Seysses (Haute-Garonne) (sowing on 04/06/18) or in the greenhouse in Mondonville (sowing on 30/04/18). The seeds were harvested at maturity and dried in industrial gas driers at 32°C. Syngenta

produced 2 genotypes (SF145, SF193) at two growing sites (Mondonville and Daux), and sowing dates were 4/27/2018 and 4/19/2018, respectively. RAGT produced 2 genotypes (SF145, SF193) at Gaillac (Tarn). Sowing was on 05/25/2018. Seeds were harvested at maturity. Mas Seeds produced SF193 sunflower seeds in two production sites Belciugatele (Romania) and Autainville (Centre-Val de Loire). For each partner and production site, plants were produced under well irrigated and non-irrigated conditions.

2.3 Seed production from seeds produced on Heliaphen platform (Experiment 3)

Seeds of SF193 genotype were produced on Heliaphen platform under well watered and drought stress conditions, as previously described and provided to the partners. Seeds were sown in Mondoville (Haute-Garonne) by Soltis, in Montélimar (Auvergne-Rhône-Alpes) by Syngenta and Dnipro (Ukraine) by RAGT on 10 may 2021, 22 april 2021 and 2021, respectively. Plants were grown in irrigated and non-irrigated conditions (stop of watering after flowering). A tunnel was used in Mondoville for the not irrigated condition. Temperature and precipitations occurring during the assays are provide as Fig supplemental data 5.

2.4 Study of plant development and seed production under well watered and water stress conditions (Experiment 4)

Soltis produced plants from 7 genotypes (commercial hybrids F7AX2MJ, F14JF1MJ, SF2615MJ, F8AZ6MJ, and lines SF193, SF092, SF109) in Mondonville (Haute-Garonne)), with and without irrigation. RAGT production site was in Dnipro (Ukraine) where 3 hydrids (RGT-TG-01, 02 and 03) and lines SF193, SF092, SF109 were cultivated, with or without irrigation. Syngenta produced AD40713MH, FR84209, FS71519, FT2603, RW666RM1 (hybrids) and SF193 and SF092 (lines) plants in Montélimar (Auvergne-Rhône-Alpes) according to the two modalities (irrigated and non-irrigated). Each partner measured several parameters at harvest, as indicated Table 3. Temperature and precipitations occurring during the assays are provide as Fig. Supplemental data2.

Table 2 : Effect of maternal drought stress on plant yield parameters.
Fifteen different genotypes of sunflower were cultivated in irrigated or not irrigated conditions by Soltis, Syngenta and RAGT in 2021. Yield parameters correspond to means of 3 or 6 measurements.

Company	Plant treatment	Genotype	Agronomic characteristic	Location	Number of plants on 6m	Bulk line (g)	TSW (g)	oil (%)	Average yield (g per plant)	Plants average Number	Average of gr/head	Average of kln/head	Average of kg/ha	Average of Units/ha
Soltis	irrigated	F7AX2MJ	Hybrid	Mondonville	21.00	1240.00	98.00	41.78	59.05					
	not irrigated	F7AX2MJ	Hybrid		22.00	1360.00	95.00	43.31	61.82					
	irrigated	F14JF1MJ	Hybrid		18.00	460.00	48.00	46.16	25.56					
	not irrigated	F14JF1MJ	Hybrid		22.00	640.00	47.00	50.00	29.09					
	irrigated	SF2615MJ	Hybrid		25.00	940.00	43.00	49.67	37.60					
	not irrigated	SF2615MJ	Hybrid		22.00	960.00	47.00	51.59	43.64					
	irrigated	F8AZ6MJ	Hybrid		20.00	620.00	73.00	41.67	31.00					
	not irrigated	F8AZ6MJ	Hybrid		20.00	720.00	81.00	43.59	36.00					
	irrigated	SF193	line		7.00	260.00	55.00	45.96	37.14					
	not irrigated	SF193	line		10.00	180.00	52.00	47.30	18.00					
	irrigated	SF092	line		19.00	480.00	32.00	44.92	25.26					
	not irrigated	SF092	line		17.00	240.00	30.00	43.63	14.12					
	irrigated	SF109	line		20.00	1180.00	78.00	43.30	59.00					
	not irrigated	SF109	line		22.00	1200.00	72.00	45.23	54.55					
Syngenta	irrigated	AD40713MH	Hybrid	Montélimar						59.00	47.74	674.63	2783.13	262.21
	not irrigated	AD40713MH	Hybrid							60.00	45.62	639.16	2713.34	253.28
	irrigated	FR84209	Hybrid							62.67	49.65	1635.53	3074.43	674.75
	not irrigated	FR84209	Hybrid							62.33	50.10	1575.15	3084.37	646.38
	irrigated	FS71519	Hybrid							61.33	53.16	711.51	3221.19	287.30
	not irrigated	FS71519	Hybrid							58.33	51.07	685.52	2951.41	263.60
	irrigated	RW666RM1	Hybrid							57.33	29.36	1482.69	1659.72	559.22
	not irrigated	RW666RM1	Hybrid							56.67	25.36	1329.03	1418.26	494.82
	irrigated	SF092	line							63.33	42.78	936.99	2676.99	390.63
	not irrigated	SF092	line							61.67	41.44	895.98	2520.89	363.62
	irrigated	SF109	line							57.00	48.38	630.83	2727.72	237.05
	not irrigated	SF109	line							57.67	43.40	556.82	2465.74	210.96
	irrigated	SF193	line							44.00	35.49	612.92	1528.83	175.84
	not irrigated	SF193	line							44.00	27.82	469.15	1184.36	133.12

Company	Plant treatment	Genotype	Agronomic characteristic	Location	Number of plants on 6m	Bulk line (g)	TSW (g)	oil (%)	Average yield (g per plant)	Plants average Number	Average of gr/head	Average of kln/head	Average of kg/ha	Average of Units/ha
RAGT	irrigated	RGT-TG-01	Hybrid	Dnipro (Ukrain)				38.02	17.41					
	not irrigated	RGT-TG-01	Hybrid					39.23	6.01					
	irrigated	RGT-TG-02	Hybrid					45.85	7.82					
	not irrigated	RGT-TG-02	Hybrid					41.98	8.76					
	irrigated	RGT-TG-03	Hybrid					41.32	39.42					
	not irrigated	RGT-TG-03	Hybrid					39.28	28.41					
	irrigated	SF109	line					33.72	21.92					
	not irrigated	SF109	line					33.32	12.39					
	irrigated	SF092	line					45.40	28.14					
	not irrigated	SF092	line					42.17	20.13					
	irrigated	SF193	line					39.16	5.73					
	not irrigated	SF193	line					38.87	7.50					

2.5 Germination assays

Germination assays were performed as previously described. The percentage of germination of a seed batch after 7 days on a PEG solution was normalized by its percentage of germination on water. The beneficial effect of the treatment corresponds to the normalized germination percentage after 7 days of S seeds minus that of W seeds on a PEG solution. Germination speed was evaluated by T10 and T50, which correspond to the time necessary for germination of 10% or 50% of the seeds on a PEG solution, respectively.

2.6 Statistical Analysis

Correlation matrix and statistical Mann-Whitney tests were done using R Studio software.

III) Results

3.1 Seed production on Heliaphen platform (Exp. 1)

Figure 29 a. shows the effects of the maternal treatment on seed germination of various genotypes under water stress conditions (PEG solution -0.8 MPa). A beneficial effect of the maternal drought stress was observed for 2/3 of the genotypes produced on Heliaphen platform. Drought stress of the maternal plant had negative effect on germination of the seeds from genotypes SF210 and SF233 only. In Fig 29 b. and 29 c. we plotted the final germination percentage (Y axis) against the germination kinetics, expressed as T10 and T50 (X axes). However in some case germination of W seed batches (from untressed mother plants) did not reach 50% germination after 7 days, making the calculation of T50 impossible, thus decreasing the number of genotypes showed on the graph. The majority of genotypes were located in the upper left part of Fig. 29 b. suggesting that maternal drought stressed thus induced an increase in germination speed (decrease in the percentage of T10) and a positive impact on germination at 7 days. However, this effect depended on the genotype. For example, seeds of the genotype SF033 responded markedly to the maternal treatment whereas ones of the genotype SF210 did not (Fig. 29 b.c.).

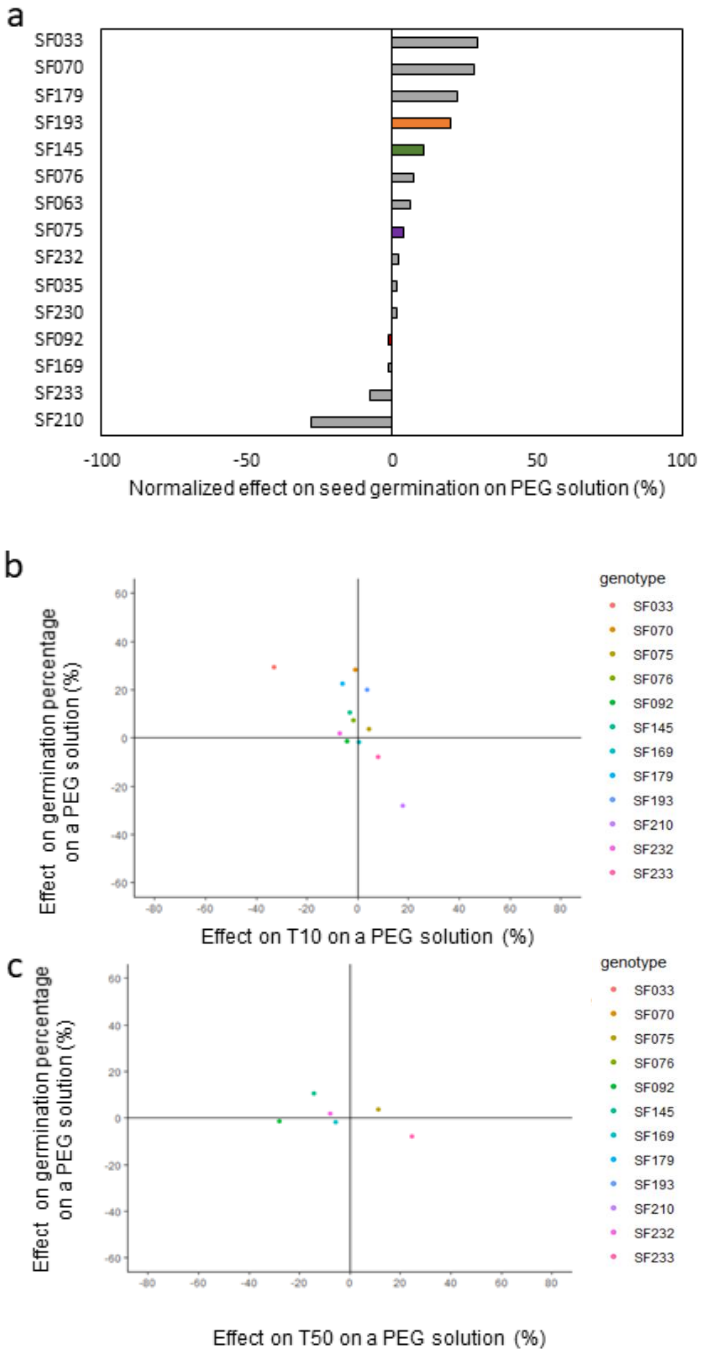

Figure 29 : Effect of maternal drought stress performed on Heliaphen platform on germinability of seeds of various genotypes.

(a) Effect of maternal drought stress on seed germination normalized with reference to germination on water) after 7 day on PEG (-0.8MPa) solution at 20°C. The effect of maternal drought stress on seed germination was calculated by the difference between normalized germination (with regards to germination on water) of S and W seed on a PEG solution. (b,c) Effect of maternal drought stress performed on final seed germination percentage, T10 and T50 values obtained after 7 d seed of imbibition on a PEG solution (-0.8 MPa) at 20°C. Data correspond to the average of 3 germinations assays.

3.2 Seed production by private partners (Exp. 2)

A similar study was performed with seeds of 7 genotypes (SF075, SF092, SF096, SF129, SF145, SF170, SF193) produced by the private partners in experimental fields under well-watered and non-irrigated conditions. Again we show that drought stress on the mother plant induced increased seed vigour for 2/3 of the seed batches studied (Fig. 30 a.). For the genotypes SF009 from Soltis and SF193 from Syngenta only, we observed a negative effect on seed germination under water stress (<10%) on seed germination. For SF193 and SF145 genotypes, the response to the treatment depended on the production site, since we observed beneficial and detrimental effects on seed germination for the various seed batches of these genotypes (Fig. 30a). Fig 30b. and c also show the effect of drought stress on germination speed. Seeds from non-irrigated plants produced by Soltis and Mas Seeds germinated more rapidly and to a higher percentage than control seeds (excepted Soltis SF075). In contrast drought stress on the mother plant decrease germination speed of seeds produced by RAGT.

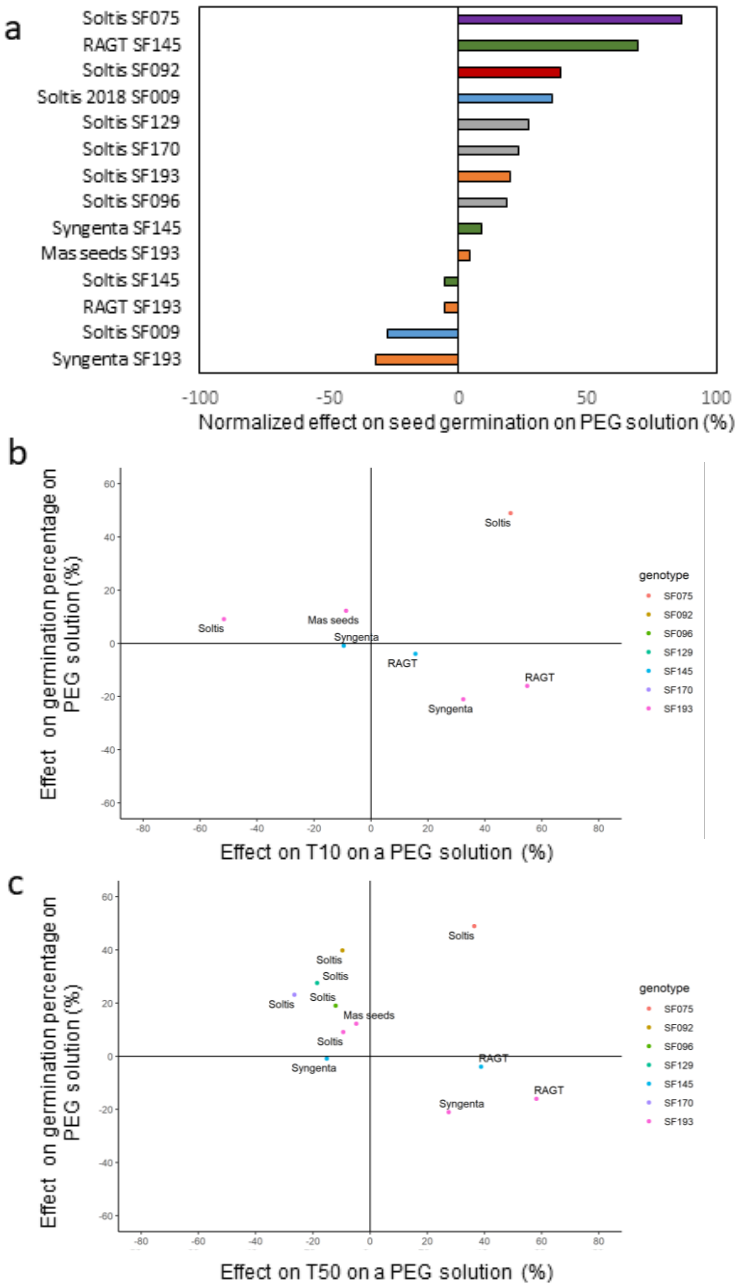

Figure 30 : Effect of maternal drought stress performed by Soltis, Syngenta, RAGT and Mas Seed on germinability of seeds of various genotypes.

(a) Effect of maternal drought stress on seed germination normalized with reference to germination on water) after 7 d on a solution of PEG (-0.8MPa) at 20°C. The effect of maternal drought stress on seed germination was calculated by the difference between normalized germination (with regards to germination on water) of S and W seed on a PEG solution. (b) Effect of maternal drought stress performed on final seed germination percentage, T10 and T50 values obtained after 7 d seed of imbibition on a PEG solution (-0.8 MPa) at 20°C. Data correspond to the average of 3 germinations assays.

3.3 Plant production with seeds from Heliaphen platform (Exp. 3)

Table 2 shows some yield parameters of plants issued from SF193 seeds that were issued from plants grown on the Heliaphen platform under well-watered (W) or drought stress (S)

conditions. Different parameters were followed by each of the partners and statistics were carried out when feasible only (Table 2). Altogether these results show that non-irrigated conditions generally had a negative effect on crop yield parameters. However, the statistical analyses did not reveal significant differences between plants issued from W or S seeds, whatever plants were grown under well-watered or not irrigated conditions (Table 2).

Table 3 : Yield parameters.
Yield parameters of SF 193 plants grown from W and S seeds (produced on Heliaphen ptaform) by Soltis, Syngenta & RAGT in 2021. Plants were grown under irrigated and non-irrigated conditions (see material and methods). Different agronomic parameter at the harvest was studded: number of plants on 6m, Bulk line (g), TSW (Thousand seed weight), oil content (%), average yield (g per plant), Average plants number in plot of 10.1m², Average of gr/head of plant, Average of kln/head of plant, Average of kg/ha, Average of Units/ha. Each parameter was calculated by the average of 3 or 6 plants. Statistical comparison between S and W yield parameters in irrigated or not irrigated conditions was done by Mann-Whitney Test (P.value 0.05). An asterix (*) represents a significative result and (n) represents a non-significative result.

Company	Plant treatment	Genotype SF193	Location	Number of plants on 6m	Bulk line (g)	TSW (g)	oil (%)	Average yield (g per plant)	Average Number plants	Average of gr/head	Average of kln/head	Average of kg/ha	Average of Units/ha
Soltis	irrigated	W	Mondonville	16.00	520.00	53.00	48.14	32.50					
Soltis	irrigated	S	Mondonville	13.00	360.00	54.00	45.09	27.69					
Soltis	not irrigated	W	Mondonville	16.00	400.00	46.00	49.20	25.00					
Soltis	not irrigated	S	Mondonville	16.00	400.00	53.00	46.24	25.00					
Syngenta	irrigated	W	Montélimar						36.00 n	30.56 n	520.50 n	1619.94 n	184.92 n
Syngenta	irrigated	S	Montélimar						37.67 n	33.94 n	531.21 n	1918.35 n	199.20 n
Syngenta	not irrigated	W	Montélimar						34.67 n	27.98 n	466.65 n	1416.98 n	157.72 n
Syngenta	not irrigated	S	Montélimar						36.33 n	25.12 n	405.85 n	1354.07 n	146.23 n
RAGT	irrigated	W	Dnipro				39.46 n	7.80 n					
RAGT	irrigated	S	Dnipro				40.50 n	7.78 n					
RAGT	not irrigated	W	Dnipro				37.58	3.52					
RAGT	not irrigated	S	Dnipro										

3.4 Effect of maternal drought stress on plant yield (Exp. 4)

Raw data of several characteristics of crop yield of the plants grown in irrigated and non-irrigated conditions are presented Tables 3. The effect of the genotype on these parameters is clearly shown Table 3 and very important for oil percentage and TSW values. It is more difficult to draw conclusions from a same genotype cultivated in different locations, since the partners did not measure the same parameters. For each partner we have performed a statistical analysis in order to determine whether maternal drought stress could have an

effect on yield parameters. The correlation matrix (Fig. 31) shows that there were not strong differences of yield parameters between irrigated and non-irrigated conditions.

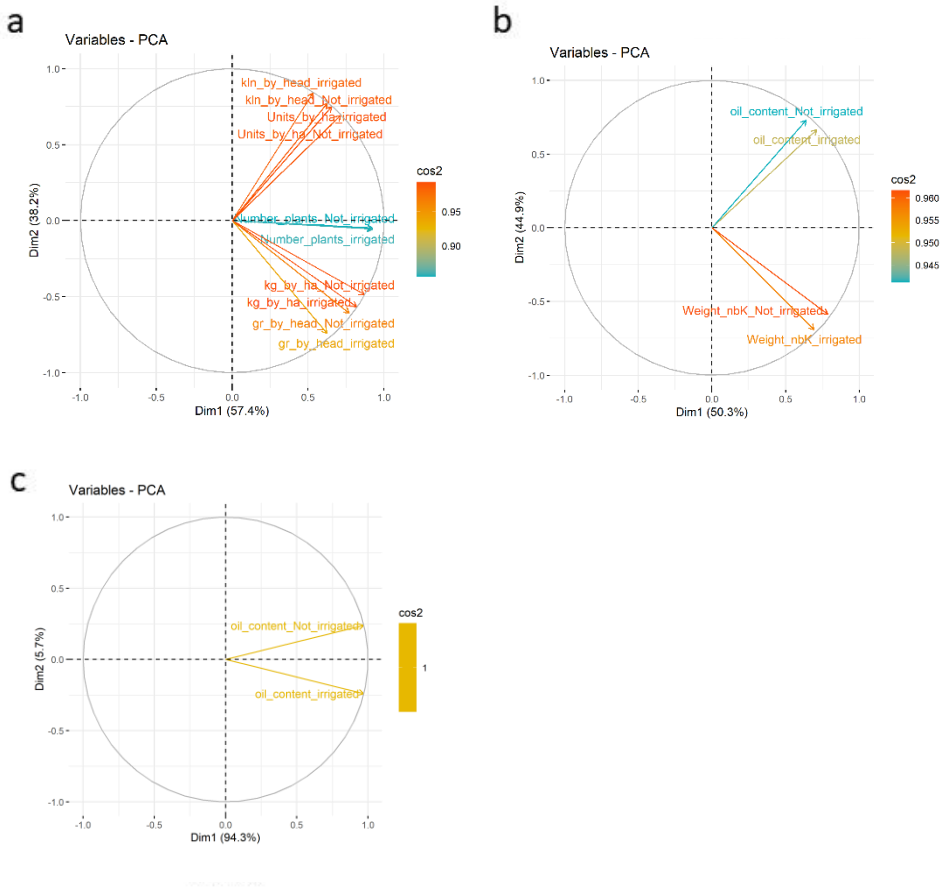

Figure 31 : Correlation matrix.
Correlation matrix of yield parameters of plant produced by Syngenta (a), Soltis (b) and RAGT (c) in irrigated or non-irrigated conditions. See details in Table 3.

IV) Conclusion

The results presented here will help the seed producers to design novel technical itinerary for growing sunflower plants. Our results show that it is possible to induce higher seed vigour (estimated by both germination percentage and speed) of a large panel of genotypes by applying a moderate drought stress to the mother plant under controlled conditions (Heliaphen) (Fig 29). This phenomenon was evidenced with the majority of the genotypes tested, including. maintainer lines (excepted SF210 and SF233, Fig. 29). Maintainers lines are of a special interest for agronomic selection. It allows to maintain the male sterility during the selection of line with high agronomic quality. On a commercial scale, the production of hybrid seeds is realized by a sterile male line with high agronomic quality crossed with a line restoring fertility.

There was however a variability of the stimulation of seed vigour by the maternal environment according to the batches of a same genotype. The environmental and climatic diversity of the production sites (geography, nature of the soil, meteorological phenomena specific to each site) could explain such a difference. Additional data on the environment of each production site are required in order to better understand this effect. A rigorous evaluation of the level of stress applied to the mother plant or the use of reference genotypes would allow a normalization of this method. At last, the possible application of our findings, ie applying a moderate drought stress to the mother plant for increasing seed vigour, requires that the stress does not alter dramatically the final yield. Here we show that growing plants under a moderate water stress did not have a major effect on TSW and oil content, 2 keys parameters of yield in sunflower (Table 2).

Altogether these data suggest that a transfer of our findings to commercial seed production conditions is feasible and will have to be considered properly by the partners in a context of global warming.

Chapter 3 :

Transcriptional regulation of maternally inherited tolerance of sunflower seeds to water stress during germination

Transcriptional regulation of maternally inherited tolerance of sunflower seeds to water stress during germination

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I) Introduction

The steep increase of water stress caused by global warming (IPCC 2019) will have negative impacts on crop yields and therefore on food security (Schmidhuber and Tubiello, 2007). In the case of sunflower (*Helianthus annuus* L.), a reduction in yield of up to 20% might be observed in the next 10 years in Southern Europe (Debaeke et al., 2017b). Several agronomic practices can improve the capacity of plants to grow under stressful climatic conditions (Ainsworth and Ort, 2010; Lippmann et al., 2019). They include for instance the modification of the sowing date, crop migration (i.e. changes in the geographical distribution of the crops themselves) (Caubel et al., 2018), and priming (Hussain et al., 2018b). Obviously, breeding for drought tolerance is another powerful tool to cope with climate change (Alves de Freitas Guedes et al., 2019; Fang and Xiong, 2015; Moschen et al., 2017b).

Plants have evolved many mechanisms to cope with water stress. They range from developmental and morphological adaptations to cellular and molecular responses. Cellular adaptation can be achieved for instance through the accumulation of osmolytes, the activation of the antioxidant systems (Laxa et al., 2019), the production of heat-shock proteins and chaperones (Jacob et al., 2017; Sharma et al., 2014), and the regulation of abscisic acid (ABA) signaling (Sah et al., 2016). Drought stress can also alter the reproductive stages of plants, ie seeds, either during their formation on the mother plant or during their germination. In addition, water stress on the mother plant during seed development and subsequent seed germination are not disconnected, and there is an increasing number of studies that have demonstrated the close relationship between these 2 phenological stages(Bruce et al., 2007; Matzrafi et al., 2021; Scott et al., 1998; Wijewardana et al., 2019). Plasticity has been reported in response to several biotic and abiotic environmental cues, including temperature(Yakovlev et al., 2010), drought (Herman et al., 2012), shade (Galloway and Etterson, 2007), nutrient availability (Kou et al., 2011), salinity (Boyko et al., 2010), herbivory (Rasmann et al., 2012) or viral infection (Kathiria et al., 2010). Interestingly, in sunflower, Vanconstenoble et al. (2022) have demonstrated that a moderate drought stress applied on the mother plant from flowering to harvest could confer water stress tolerance to the progeny at the germination stage. These authors have demonstrated that the acquisition of water stress tolerance at the germination stage during seed development was resulting from a maternal effect, and that it was not transmitted to the following generation, ruling out any putative transgenerational effect.

The inheritance of germinative traits from the mother plant to the progeny is particularly crucial in the conditions of a changing climate. Indeed, stand establishment relies on rapid and fast seed germination in a wide range of agroclimatic conditions and thus depends on an intrinsic seed property, namely seed vigour. In particular, high seed vigour confers the ability of a seed to germinate in non-optimal environmental conditions, which is critical for success of seedling emergence and on the long term final crop yield. The conditions of seed development on the mother plant play a critical role on seed vigour (Tekrony, 2003), and they can act in different end even opposite ways. For example in soybean, drought stress during flowering did not affect germination rate or vigour (Smiciklas et al., 1992) but in sunflower, instead, freezing temperatures during seed development decreased vigour (Ahmad, 2002). Despite the agro economic importance of seed vigour, its molecular determinants are poorly understood, probably because this trait is the sum of an array of properties that are affected by genetics, seed development, conditions of harvest and storage, etc… In sunflower, using a large range of genotypes, (Saux et al., 2020a) have shown that vigour of seeds of this species had a strong genetic component.

The molecular mechanisms involved in the modulation of seed vigour by environmental cues during seed development are largely unknown, as well as the molecular mechanisms allowing seed germination under environmental constraints. Seed provisioning by the mother plant, ie the amount and quality of the resources aloocated to seeds has often been mentioned as playing a role in the transmission of maternal effects (Herman and Sultan, 2011). It has also been suggested that the metabolism of abscisic acid (ABA), which induces dormancy and prevents germination might be altered by the seed developmental conditions (Finch-Savage and Footitt, 2017). The epigenetic memory inherited from the mother plant has also often been proposed as playing a role in the transmission of germinative traits to the progeny (Kakoulidou et al., 2021).. Transcriptional regulation mediated by histone modifications is one of the major mechanisms acting during germination and can play a role in plant memory (Zhao et al., 2019). Chromatin conformation is regulated, at least partially, by different histone octamers (H2A, H2B, H3, and H4). The dynamic of variant histones modulates chromatin accessibility (Borg et al., 2021) and some variants are associated to dormancy, like for instance H2B (Liu et al., 2007), or are required for adaptive responses to abiotic stresses, like the H1.3 variant in *Arabidopsis* (Rutowicz et al., 2015). So underspending the histone regulation can participate to induce the adaptation to drought stress (Bourbousse and Barneche, 2019)

The objective of this work was to decipher the molecular mechanisms involved in the acquisition of high vigour seeds by a moderate drought stress applied on the mother plant. Thus we studied changes in the transcriptome of seeds that have been produced during 3 generations under well-watered or drought stress conditions, these former conferring a higher vigour to the progeny. After an in depth analysis of RNA-seq data we have validated the potential interest of a set of candidate genes in various sub-optimal conditions of germination using a microfluidic approach. Our results allow proposing some candidate genes and metabolic pathways that could explain maternel effect on seed germination.

II) Material and methods

2.1 Seed production

A first seed lot ('XRQMLG2011', provided by the INRAE Sunflower Genetic Resource Center) of the cultivated sunflower line XRQ/B (maintainer line of cmsPET1) was used to produce the plants needed for the experiments. The plants were grown as described by Gosseau et al. (2019) and Vancostenoble et al. (2022) using 15 L pots (Soparco, Le Musset, France) filled with Proveen PAM2 potting medium (RHP, 's-Gravenzande, The Netherlands). Four post-stress fertilizations were applied using 1 L of Peters Professional 17-07-27 fertilizer (Everris, Geldermalsen, The Netherlands) at 0.8g/L and Hortrilon fertilizer (Compo, Cesano Maderno, Italy) at 0.46g/L per plant. Until flowering, non-limiting water conditions were applied using a dripping system.

For each condition, the plants were self-fertilized and the seeds obtained were considered as the first generation in 2017. Accordingly, they were named 1W and 1S. After harvest, seeds were dried at 25 °C for 48 hours and then stored at 4 °C and 30% humidity. The same growing and self-fertilizing scheme was repeated for other two generations, obtaining the lines 2W/2S (i.e. second generation) and 3W/3S (i.e. third generation, 2018). A second seed lot of the same genotype was produced independently in 2020 on the Heliaphen platform, and obtained from plants stressed for a generation only, and was used for Microfluidic qPCR analysis.

2.2 Germination assays

Germination assays were performed at 20°C in darkness with 4 replicates of 25 whole achenes placed in 9 cm Petri dishes on a layer of cotton wool. The seeds were moistened with distilled water or with polyethylene glycol (PEG) solutions to obtain water potentials

described in the text (Michel and Kaufmann, 1973). A seed was considered germinated when the radicle had pierced the envelopes (seed coat and pericarp). Germination counts were made daily for 16 days.

2.3 Transcriptome profiling

Embryonic axes from 10 seeds of three independent plants of the third generation of water-stressed (3S) or well-watered (3W) plants were studied in five conditions, namely before germination ('dry condition'), after germination at 16h or 24h in non-stressing conditions ('16h water' and '24h water'), and in PEG-induced stressing conditions ('16h PEG' and '24h PEG' at a water potential of -1.0 MPa).

The tissues were ground in liquid nitrogen as described by (Verwoerd et al., 1989). Total RNAs were extracted using a hot phenol procedure, suspended in 100-µl Milli-Q water after air-drying and then purified with NucleoSpin XS columns (Macherey-Nagel, Allentown, PA, USA). Their concentration was determined using a NanoVue Spectrophotometer (GE Healthcare, Chicago, IL, USA) and their integrity was checked using an Experion Automated Electrophoresis Station (Bio-Rad, Hercules, CA, USA). Finally, the purified total RNAs were sent to the GeT-PlaGe genomic core facility (INRAE Toulouse) for RNA sequencing. The libraries were prepared using a TruSeq Stranded mRNA Prep kit (Illumina, San Diego, CA, USA) and their quality was then assessed with a High-Sensitivity DNA chip run on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The sequencing step was carried out on a HiSeq3000 (Illumina) using a 2x150 bp paired-end sequencing protocol and the Illumina HiSeq3000 sequencing kits.

All the analyses of RNA-seq data were performed using the CLC-Genomics Workbench v10.1 (https://www.qiagenbioinformatics.com/products/clc-genomicsworkbench/). For exploratory purposes, first a principal component analysis (PCA) plot was drawn using all the expression data. Then, the data of the 25 highest and the 25 lowest DE genes were used to create a heatmap corresponding to a clustering analysis based on the Euclidean distance. T-tests with a Bonferroni adjustment (p-value < 0.05) were used to identify differentially expressed genes.

Gene ontology (GO) assignment was done for differentially expressed genes between 3W and 3S in dry seeds imbibed seeds 24H with water or -1MPa PEG and the results were summarized using REVIGO (Supek et al., 2011).

Pathway Deregulation Score (PDS) analysis was performed using Pathifier (Drier et al., 2013) and a total of 478 sunflower pathways extracted through MetExplore2

https://metexplore.toulouse.inra.fr/metexplore2/ (Cottret et al. 2018). Pathifier uses expression values to calculate PDS, corresponding to the deviation of a pathway from a reference. It was initially developed for oncological applications, and then adapted to Arabidopsis and sunflower by (Ponnaiah et al., 2019; Saux et al., 2020b).

2.4 Microfluidic qPCR analysis

Independent batch of XRQ seeds from well-watered or drought stressed plants during one generation (1W/1S), obtained as previously described, were subjected to different germinative stresses: PEG (-0.7 MPa), NaCl (300 mM), hypoxia (3% O_2), or imbibed on water (control) during 24 h.

For RNA extraction, 3 biological samples of 10 embryonic axes of seed were used for each condition and total RNA was extracted using the RNA kit MACHEREY NAGEL 96w. A DNAse control was done before the syntheses of cDNA by the Kit NucleoSpin® RNA Plant.

Primer design was performed using the Primer3 software (https://probes.pw.usda.gov/cgi-bin/batchprimer3/batchprimer3.cgi). The microfluidic qPCR was performed by the Gentyane genomic core facility (INRAE Clermont-Ferrand) running 96.96 Dinamic Arrays on a BioMark (Fluidigm, San Francisco, CA, USA). A quality control step was carried out with the software provided by Fluidigm. The data analysis was then done using Mathworks MATLAB v9.8 and the Statistics and Machine Learning Toolbox v11.7. Gene expression levels were calculated as ΔC_t as described by Livak and Schmittgen (2001) and using the genes *HaS19* and *Ha β-tubulin* as references. Samples for which the expression levels of reference genes were low ($C_{t\ HaS19}$>20 or $C_{t\ \beta\text{-tubulin}}$>19) were discarded. Linear models were fitted to the data using the 'lm' function of R (version XXX) for each of the three germinative stress (PEG, NaCl, hypoxia) and maternal stress as follows:

$$E_{i,m,g} = MS_{i,m} + GS_{i,g} + I_{i,m,g} + \mathrm{e}_{i,m,g}$$

where E is the expression (ΔC_t) of the i^{th} gene, MS the m^{th} maternal stress (drought or well watered plants), GS the g^{th} germination stress (PEG, NaCl, hypoxia or control), I the interaction between maternal and germination stresses and e the residual error.

III) Results

3.1 Effect of multigenerational stress on seed germination and on seed transcriptome

The physiological impact of multigenerational stress on seed germination was investigated under control conditions (i.e. watered) or under water stress obtained by various PEG solutions. Seeds of 2 sunflower lines obtained after 3 generations of self-crossing either under drought stress (i.e. 20% of FTSW, S seeds) or under well-watered conditions (i.e. 100% of FTSW, W seeds) during seed development were used to this aim. The kinetic of germination of the seeds of the first and of the third generation are shown Fig. 32. Under control conditions (i.e. water at 20°C), all of the seeds germinated at 100% regardless their background (S or W) and generation. Germination was delayed and then delayed in the presence of increasing concentrations of PEG solutions (Fig. 32), however S seeds germinated better than W seeds in these conditions at the first and 3rd generations. After 3 generations, 3W seed germination in the presence of PEG solutions of -0.8 and -1.0 MPa did not exceed 70 and 30 %, respectively (Fig. 32c). In contrast, 3S seeds from the 3rd generation germinated to 100 % on PEG solutions of -0.8 MPa and -1.0 MPa, even though this former concentration decreased germination speed (Fig. 32d).

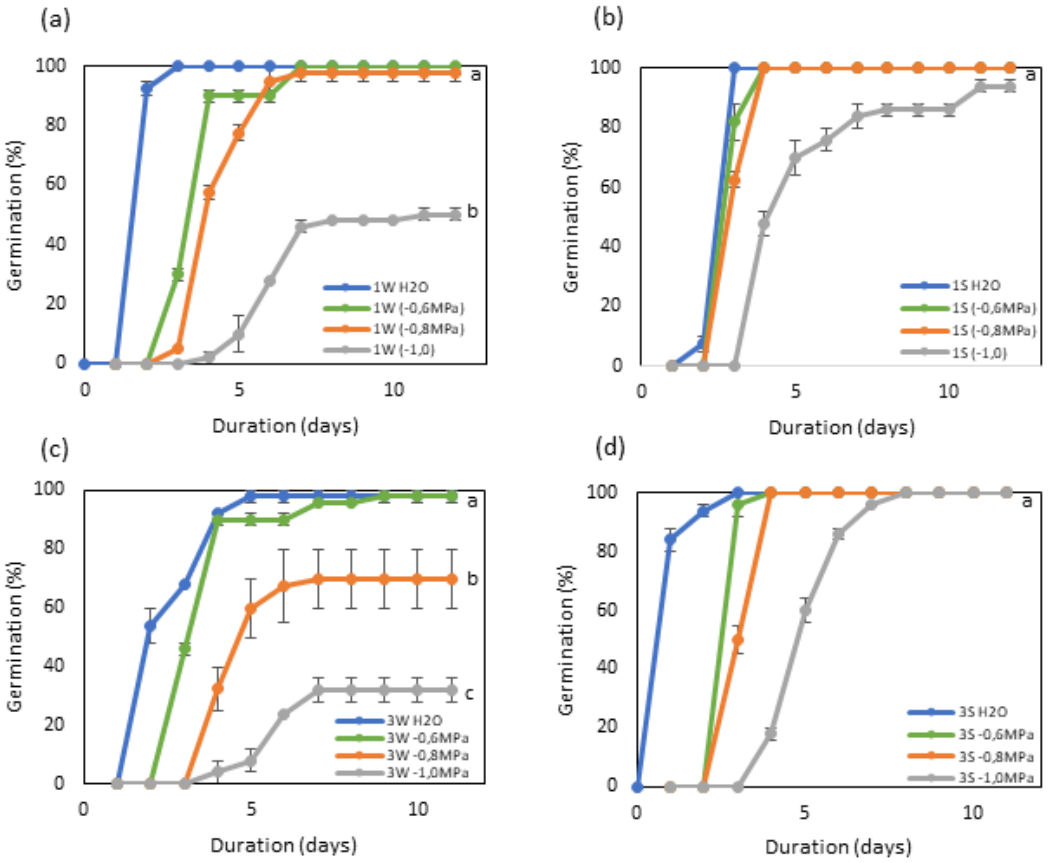

Figure 32 : Germinative kinetic

Germination of XRQ seeds obtained from plants grown during 1 or 3 generations in well-watered condition (respectively 1W and 3W seeds) (a,c) or in water stress condition (respectively 1S and 3S seeds) (b,d). Seeds were germinated at 20°C in darkness on water or on a solution of polyethylene glycol 8000 at -0.6, -0.8, or -1MPa. Data represents the average of 3 germination kinetics ± SE and Mann & Whitney test with alpha = 0.05.

To better understand the processes underlying water stress tolerance acquisition in seeds from sunflower maternal plants exposed to drought stress, RNAseq was carried out with embryonic axes of 3S and 3W seeds during germination at 16 and 24 h under watered conditions or under PEG-induced water stress. Out of the 58,050 genes found in the 'HanXRQr1' version of the sunflower genome (Badouin et al. 2017), 44,176 (i.e. 76%) were expressed in our samples. A principal component analysis (PCA) plot clearly separated germinating seeds from dry ones along the first component (Figure 33). The second component of the plot, instead, differentiated the transcriptomes of 3W and 3S seeds under non-stressing conditions at both 16 h and 24 h. Finally, 3W and 3S transcriptomes under stressing conditions were mainly splited apart by the second component, but the first one contributed significantly to the separation at 16h.

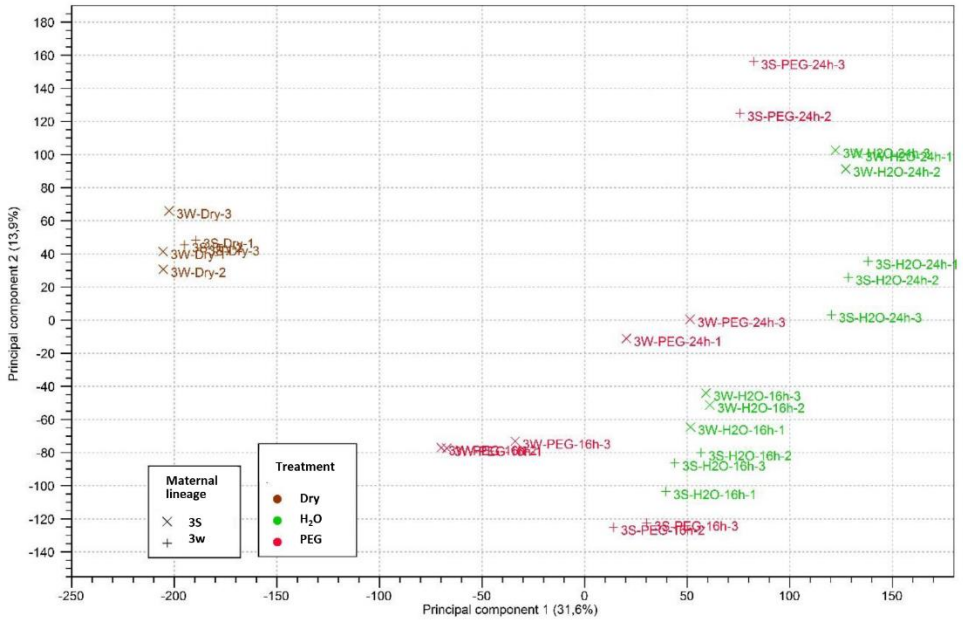

Figure 33 : Principal Component Analysis of RNA-seq

Data was obtained with seeds from plants grown in well-watered conditions (3W) or under drought stress (3S) for 3 generations. Transcriptome analysis was performed in dry seeds and after 16 and 24 h of seed imbibition on water or on a PEG solution (-1.0 MPa) at 20°C in darkness.

3.2 Integrating transcriptomic data into a pathway-based analysis

Pathifier is a pipeline that integrates little variations of gene expression and calculates the deregulation score of a pathway associating several genes (Drier et al 2013) (Fig. 34.). The pathway deregulation score (PDS) analysis carried out with Pathifier identified more than 200 pathways (i.e. circa 40% of those available for sunflower) that were deregulated during germination in control (watered) conditions. These pathways belonged to 11 classes (Fig. 34), and their levels of deregulation increased through time, from dry samples, to 16 h and 24 h of water imbibition. They presented similar patterns in both 3W and 3S dry seeds. All the pathways showed deregulation during germination, with the partial exception of some pathways belonging to the 'Hormone' class and to the 'Secondary metabolism' class. At 16 h on water some pathways of the 'Carbohydrate metabolism class' (e.g. 'UMP biosynthesis' and 'UDP-N-acetyl-D-glucosamine biosynthesis II', Fig supplemental data 6) showed higher deregulation in 3S seeds than in 3W ones.

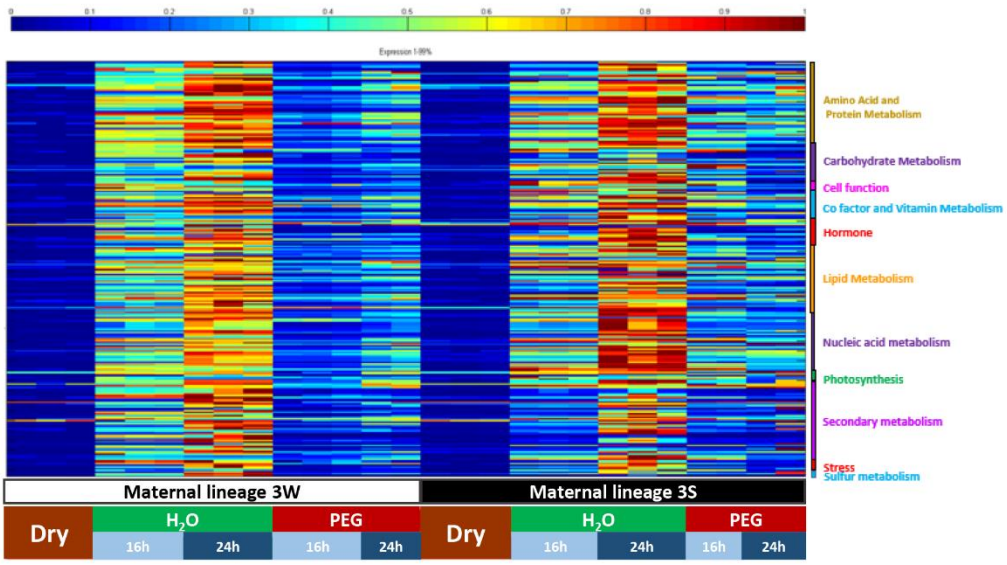

Figure 34 : Pathway analyse.

Heatmap showing the clustered pathway deregulation scores (PDS) of all pathways in sample seeds. Each column corresponds to a sample and each row to a pathway. Treatments are indicated at the bottom of the figure. Seeds were imbibed at 20°C in darkness on water or on a PEG solution (-1.0 MPa).

Overall, the levels of deregulation of the pathways increased through time and were much pronounced under watered conditions than under PEG-induced stress conditions. The transcriptomes of 3S seeds were more reactive under water stress after 16 h than those of 3W seeds (Fig. 34). PEG-treated samples, instead, all exhibited more limited levels of deregulation. At 16 h, all pathways, excepted "secondary metabolism" were more deregulated in 3S seeds than in 3W ones (Fig. 34). Valine, proline , histidine, and homoserine biosynthetic pathways of the 'Amino acid and protein metabolism' class showed the strongest deregulation, together with the 'Starch degradation', 'Xylogalacturonan biosynthesis', the '1,3 beta D glucan biosynthesis' and the 'protein N-glucosylation' pathways of the 'Carbohydrate metabolism' class, for example Fig supplemental data 6 In the case of 'Cofactor-vitamin metabolism' class, the 'Vitamin E biosynthesis', the 'Thio-molybdenum cofactor biosynthesis', the 'Tetrahydrofolate salvage' (Vitamin B9) and the 'L-ascorbate biosynthesis" pathways were also more deregulated in 3S seeds under water stress than in 3W ones at 16 h (Fig supplemental data 6). Lastly, at this time point, pyrimidine related pathways (Fig supplemental data 6 and catabolism of ABA (captured in the abscisic acid glucose ester metabolism pathway Fig supplemental data 6were also strongly deregulated in S seeds. At 24 h on a PEG solution, Pathifier revealed less differences between S and W seed samples (Fig. 34).

3.3 Differential gene expression analysis

Differential analysis of gene expression was performed by fitting a linear model including the maternal background and the germination conditions of the seeds. A total number of 871, 684, and 1,255 differentially expressed genes (DEGs) between 3W and 3S seeds were found in dry conditions, in watered conditions at 24 h and in PEG-induced stressed conditions, respectively (Fig. 35a-c). Only 20 genes were shared among the 3 DEG lists (Fig. 35. b-c.).

Out of the 712 upregulated genes between 3S and 3W in dry seeds, only 18 were also more abundant during seed imbibition on water or on a PEG solution (Fig. 35b). Comparatively, out of the 159 downregulated genes between 3S and 3W in dry seeds, only 2 were shared with watered and PEG stressed conditions (Fig. 35c), thus suggesting typical transcriptomic signatures for each of the seed samples.

When 3S seeds were germinated on PEG, 425 and 753 genes were specifically up- and down-regulated, respectively (Figs. 35 bc), which is much higher than the number of DEGs identified when germinating 3S seeds on water (Figs 35 a, b, c). In other words, water stress during germination induced a distinctive transcriptomic response in S seeds compared to W seeds from irrigated plants, since 94% of the DEGs obtained by comparing 3S and 3W seeds were found only under water stress. These genes included pathways involved in the response to abiotic stresses (abiotic stimulus, salt stress, and osmotic stress), ROS (ROS and flavonoid metabolism) and hormonal pathways (ABA, brassinosteroid) as illustrated by the REVIGO (Supek et al. 2011) analysis (Fig. 35d-i).

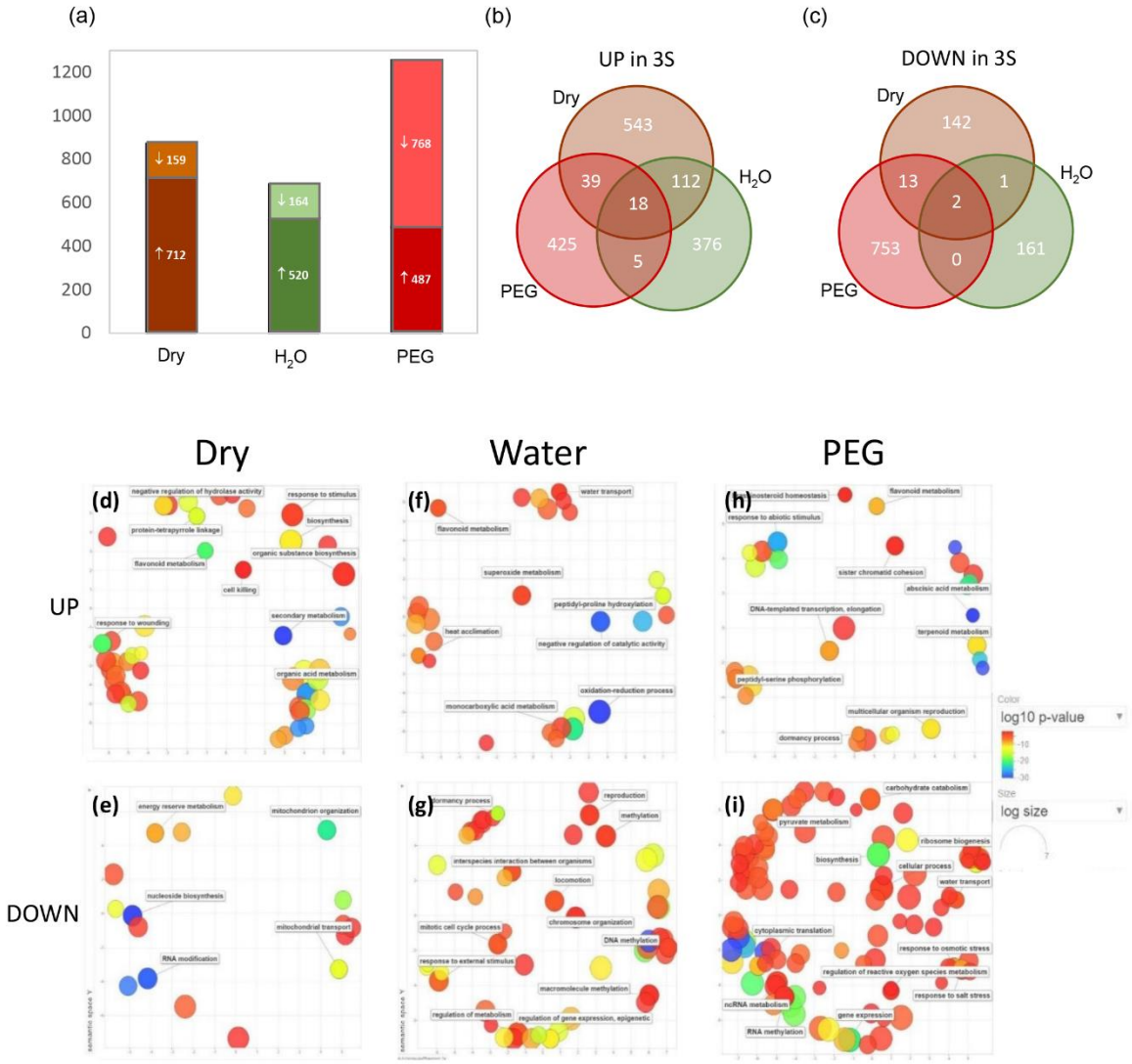

Figure 35 : Transcriptomic study.

Transcriptomic differences between seeds issued from well-watered plants (3W) or drought stressed (3S) plants for 3 generations. (a) Up- (more expressed in 3S) and down-regulated genes in dry seeds, in seeds imbibed for 24 h on water (H_2O) or on a PEG solution (-1.0 MPa) (Fold Change $\geq |2|$; p-value$_{Bonferroni} \leq 0.05$). (b-c) Venn diagrams of up- and down-regulated genes in dry seeds, after 24h in water or in PEG -1MPa. (d-i) REVIGO of Gene Ontology of differentially expressed genes (DEG) between 3S and 3W seeds: (d,f,h) upregulated genes, (e, g, i) downregulated genes in dry seeds (d, e), in seeds imbibed in 24h water (f, g) and in water stress after 24h (h, i).

3.4 Transcriptional footprints of ROS and hormone metabolisms

Since ROS metabolism has been shown to be involved in the regulation of sunflower seed dormancy and germination (Bailly et al., 2008), and that ROS related GO were identified here busing REVIGO, we looked for a possible involvement of ROS in the transmission of vigour traits by the mother plants using the so-called 'ROS wheel' (Willems et al. 2016). ROS wheel defines ROS-related clusters of genes whose expression is known as being modified

by ROS. In all of the 3 conditions (dry, H2O and PEG), 3S seeds showed very similar patterns of downregulation at the level of ROS clusters (Fig. 36a). This was especially the case for genes of 'Cluster III', induced early after light exposure, and for 'Cluster IV', which is related to mitochondrial ATP synthase and H_2O_2 response in cell cultures. On PEG only a subset of 24 genes, mostly belonging to the cluster I -GUN retrograde, were up-regulated (Fig. 36a). Among them, 7 genes were from the cluster III, mitigating the downregulation observed across the three conditions and suggesting differential transcriptomic regulation.

(a)

ROS Clusters	Number of DEG					
	Dry		H_2O		PEG	
	up	down	up	down	up	down
Cluster I - GUN retrograde	1	1	1	1	13	1
Cluster II - HL late	0	1	0	1	2	1
Cluster III - HL early	2	8	1	15	7	11
Cluster IV - ROS CC	0	8	0	4	2	5
Cluster V - ROS	0	0	0	0	0	0
Cluster VI - O2-UVB early	0	3	0	1	0	2
Cluster VII - RBOHF	0	0	0	0	0	0
Cluster VIII - ROS acclimation	0	1	0	1	0	1
Total	3	22	2	23	24	21

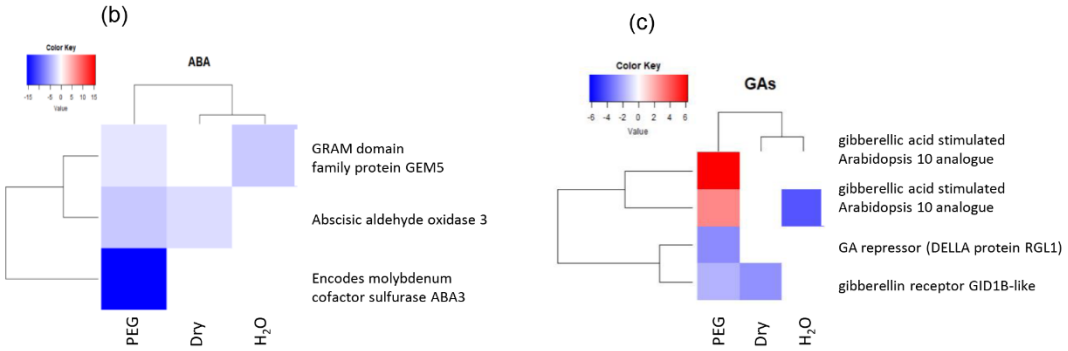

Figure 36 : Transcriptional footprints.

Transcriptional footprints of reactive oxygen species (ROS), ABA and GAs in 3W and 3S seeds. (a) Number of up- or down- differentially expressed genes (DEGs) (fold change absolute value > 2, Bonferroni < 0.05) belonging to the transcriptional footprints of clusters I–VIII (columns) as defined by Willems et al. (2016). (b,c) Heat maps showing DEGs (fold change absolute value > 2, Bonferroni < 0.05) related to abscisic acid (ABA) (b) or gibberellin (GAs) (c).

In 3S seeds, the ABA metabolism appeared downregulated, while gibberellin pathway was activated. The ABA footprint showed under-expressed genes in 3S seeds responsible for ABA synthesis, notably Abscisic aldehyde oxidase 3 (AAO3), the enzyme that catalyzes the last step of ABA biosynthesis (Seo et al., 2004) (Fig. 36b). Under water stress, 3S seeds particularly repressed ABA3 homologue enzyme also involved in the synthesis of ABA (Seo

et al., 2004). The GRAM domain family protein GEM5 is involved of ABA signaling pathway, and could have a function downstream of ABI5 (Mauri et al., 2016).

On the contrary, 3S seeds showed and induction of GA pathway illustrated in figure 36c. by the specific upregulation of two GA regulated genes and the downregulation of RGL1, a DELLA known for participating in the degradation of GA (Davière and Achard, 2016; Sun, 2010). Two analogs of "gibberellic acid stimulated arabidopsis 10" were also more expressed in S seed in PEG imbibition.

3.5 Validation of putative candidate genes of sunflower seed vigour

Three groups of genes were especially represented within the DEG list obtained by comparing 3S and 3W seeds under stress conditions, namely (1) the 'hormone cluster', made up by 35 genes (14 related to ethylene, 7 to ABA, 4 to brassinosteroids and 1 to GA); the 'ROS cluster', consisting in 60 genes known to respond to or to regulate ROS production; (3) the 'epigenetic cluster', including 61 genes involved in epigenetic regulation. These genes are listed in Fig supplemental data 9. The epigenetic cluster was defined based on the chromatin database (http://www.chromdb.org) (Gendler et al. 2008) and the findings obtained about DNA compaction in *Arabidopsis* under drought stress by Bourbousse and Barneche 2019.

The volcano plots Fig. 37 show the distribution of the 3 clusters in response to water stress during seed germination and they highlight differential expression according to the physiological conditions. When comparing W to S seeds in seeds imbibed on a PEG solution it shows that genes belonging the 3 clusters are down regulated in 3W seeds, as expected (Fig. 37a). Fig. 37b compares distribution of the genes in W seeds imbibed on water and on PEG, it shows that genes from the 3 clusters were down regulated on water, in contrast with S seeds where the same genes were up-regulated on PEG (Fig. 37c).

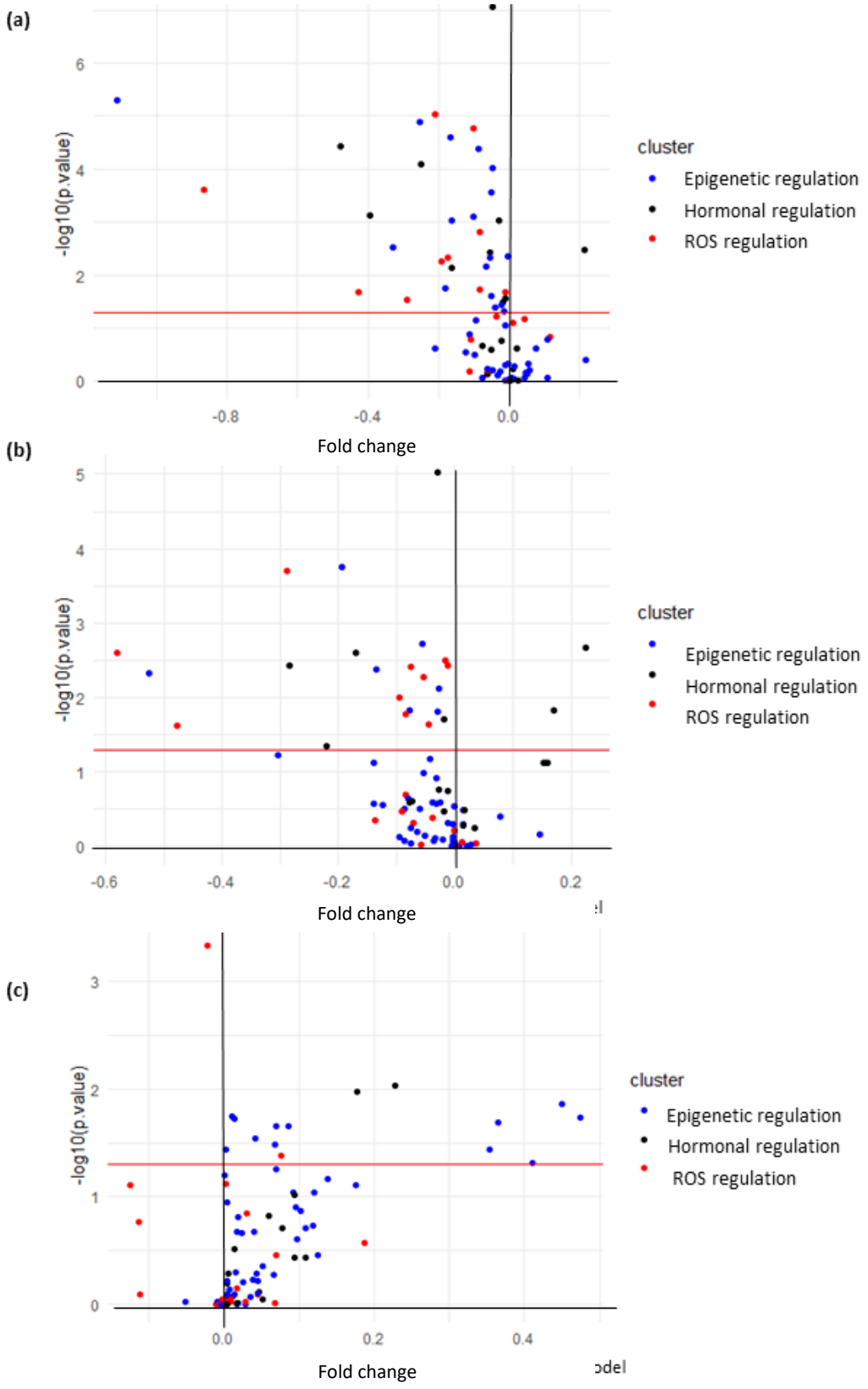

Figure 37 : Volcano plots of candidate gene expression in Fluidingm analysis.

Volcano plots showing genes belonging the clusters Epigenetic, Hormonal or ROS regulations (Fig supplemental data 9.) that are up- (right) or down- (left) regulated. Estimated responses and p-values are from the linear model : $E_{i,m,g} = MS_{i,m} + GS_{i,g} + I_{i,m,g} + e_{i,m,g}$. Estimated effect and p-value of (a) $\Delta E_{i,m=\text{S-W},g=\text{PEG}}$, (S vs W on PEG) (b) $\Delta E_{i,m=\text{W},g=\text{H2O-PEG}}$, (W on water vs W on PEG) (c) $\Delta E_{i,m=\text{S},g=\text{H2O-PEG}}$ (S on water vs S on PEG). Red line corresponds to alpha = 0.05.

We first investigated whether the DEG identified with the seed material produced on Heliaphen platform, and which were involved in response to water stress in 3S seeds, were also identified in S seeds, germinated on a PEG solution, but produced in another year on Heliaphen platform. For this new seed batch, drought stress on the mother plant also induced a better germination in water stress conditions (PEG -0.7 MPa) when compared to seeds from non-stressed plants, although it was much less pronounced than for 3S seeds (Fig supplemental data 12)

We compared changes in gene expression between S and W seeds germinated on PEG for the 2 seed batches (Fig. 39.b c.). Among the 88 genes selected in the 3 clusters only 38 had a p value allowing this comparison (Fig supplemental data 10) and 30 displayed a similar evolution in the 2 experiments. One gene (coding 1-aminocyclopropane-1-carboxylate oxidase) was down expressed in both cases, and 29 genes were over expressed in both Fluidigm and RNAseq analysis. Even though the variation in gene expression of these former genes appeared less pronounced in Fluidigm data, they can be considered as putative good candidates for being involved in the response of seeds to water stress. They include 16 genes in epigenetic cluster,8 genes in relation to hormone cluster and 5 to ROS cluster. In the epigenetic cluster some genes in relation of DNA condensation were found, like histone variants, ARGONAUTE 1 and methyltransferase 13. Three gene in relation with ABA were over expressed in S seeds, the abscisic-aldehyde oxidase-like AAO3, involved in the last step of ABA synthesis, and 2 genes of abscisic acid 8-hydroxylase-2. ABA 8-hydroxylase plays a predominant role in catabolism of ABA and involved in the control of seed dormancy and germination (Kushiro et al., 2004; Okamoto et al., 2006).

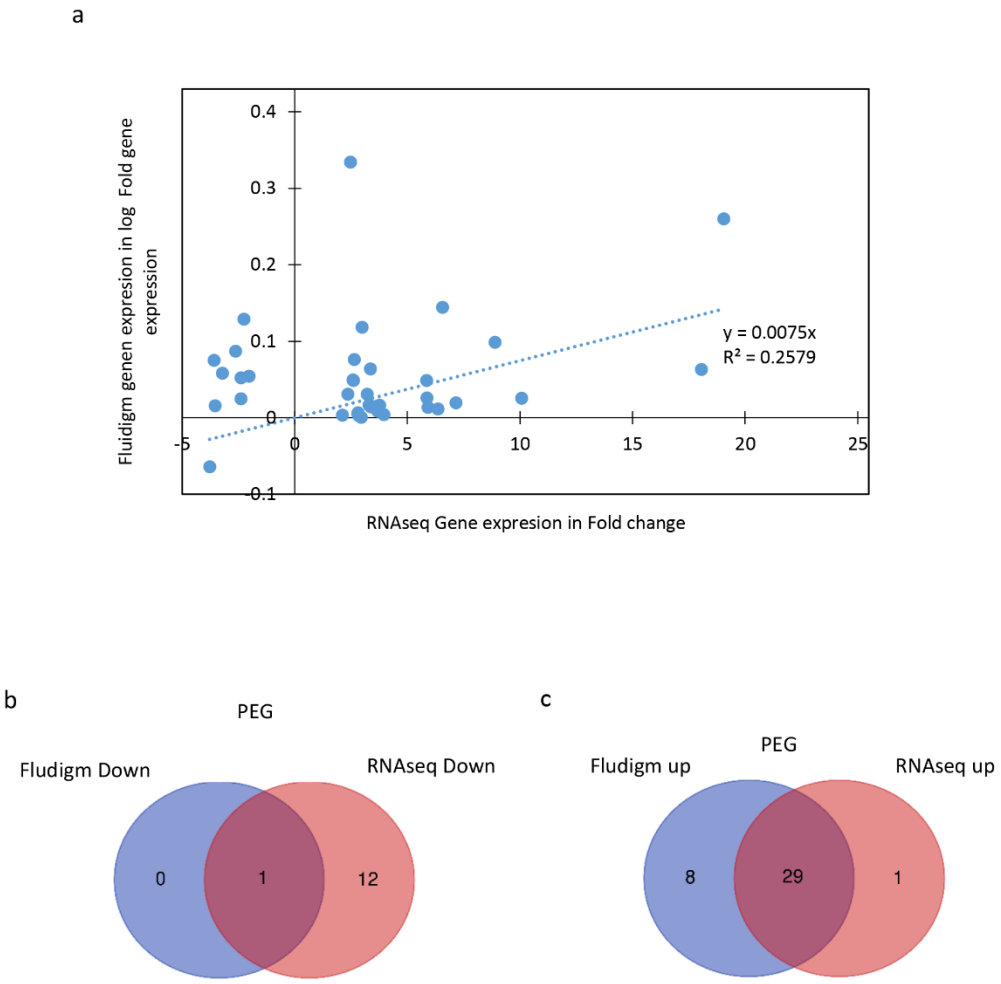

Figure 38 : Comparison between RNAseq and Fluidigm analyses.
a. Correlation between expression of genes candidate in RNAseq of 3S -3W seed (list in table sup data 1) expression with the expression of validate gene Fluidigm analysis of 1S 1W seeds (list in table sup data 2). Gene expression is expressed in fold change for the RNAseq analysis and in log Fold of gene expression in the Fluidigm analysis. Venn diagrams of the number of significantly up (b.) or down-expressed (c.) genes in PEG conditions between 3S/3W seed analyzed in RNAseq and 1S/1W seed analyzed in Fluidigm

The expression of the 89 genes belonging to the 3 clusters was also measured in new embryo axis of seeds (produced in 2020) from 1S and 1W maternal lineages submitted to different abiotic stresses during seed imbibition : 24h imbibition on a PEG solution (-0.7 MPa), saline stress using NaCl at 300 mM, and hypoxia at 3% O_2, all at 20°C in darkness (Vancostenoble et al. 2022). These 3 treatments decreased seed germination of W seeds but not the one of S seeds (see Vancostenoble et al. 2022). After estimating the response to the different contrasts using a linear model, the genes were grouped according to their responses using hierarchical clustering giving seven gene clusters depicted in Figure 39. They include 88 genes that correspond to co-regulated genes involved in ROS response, hormone signaling and epigenetic regulation and involved in acquired tolerance to abiotic stresses at the germination stage.

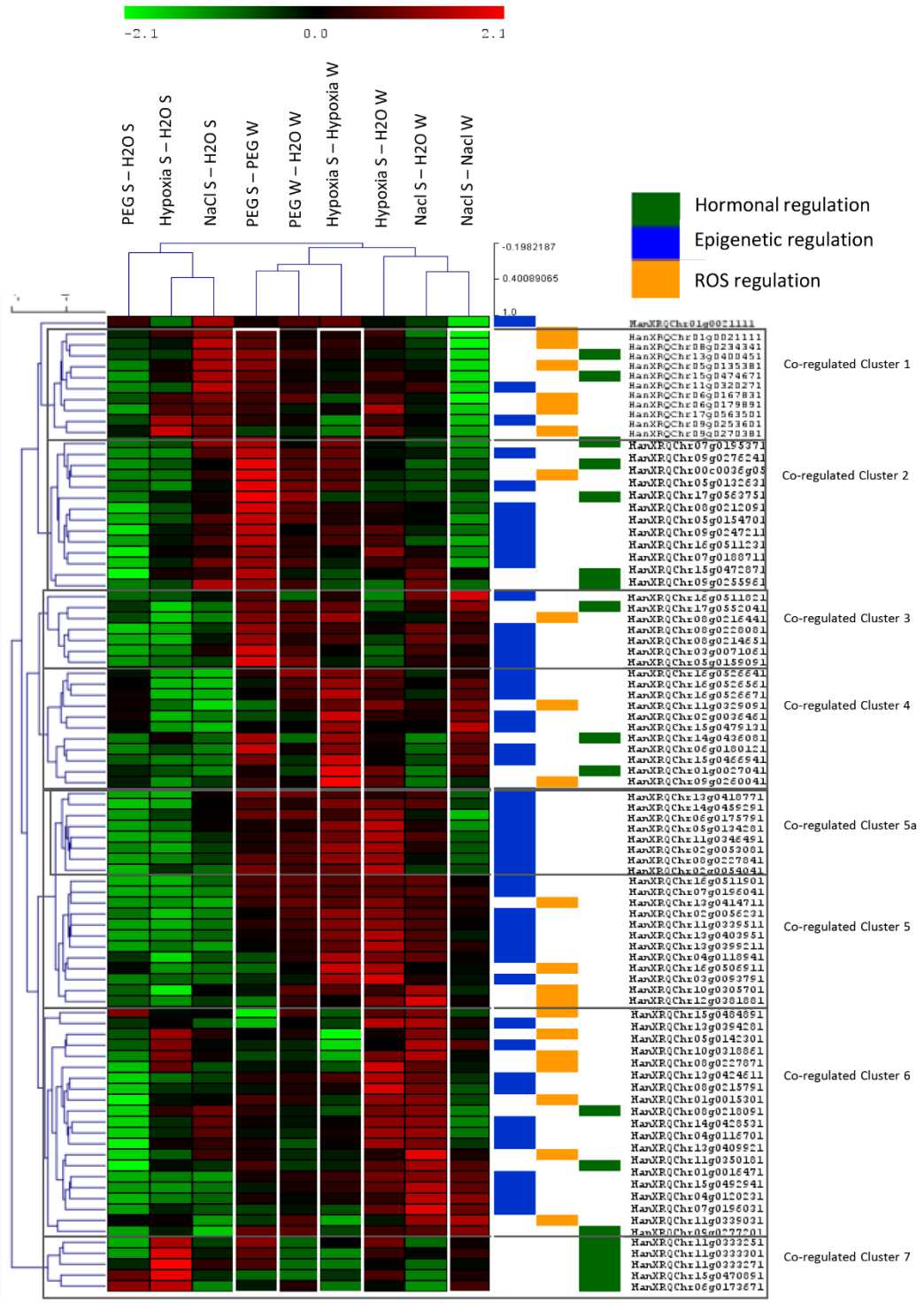

Figure 39 : Heat map and clustering of expression responses of selected DEG.
The candidate gene were involved in epigenetic, hormonal or ROS regulations. Responses are calculated as the effects of the corresponding contrasts in the linear model $E_{i,m,g} = MS_{i,m} + GS_{i,g} + I_{i,m,g} + e_{i,m,g}$. Seeds originate from irrigated plants (W) or drought stressed (S) and germinated in water (H_2O), at –0.7MPa (PEG) in salt at 300mM (NaCl), or under hypoxia at 3% O_2 (Hypoxia). Within the Epigenetic cluster, (histone homologues are labeled with an h).

Clusters 1 and 7 include genes from the 3 categories (Fig 39.) both with genes from ROS wheel III category but cluster 6 includes 7 histone homologues whereas cluster 1 has none.

Cluster 1 includes 2 genes involved in hormonal signaling, an ABA-hydrolase and ABR1 an ethylene responsive factor repressing ABA responses. On the other hand, cluster 6 includes 3 ethylene-responsive genes including EIN2 that mediated histone acetylation in response to ethylene (Zhang et al., 2017) and ERF073 mediating ethylene-mediated hypoxia response in Arabidopsis (Hess et al., 2011). Cluster 2 includes GAI1 homologues that integrates ethylene and ABA signaling that are also represented in this cluster with two homologues of EIN2, an ABA oxidase (ALDO3) and an ABA hydrolase (ABAH2) involved in ABA catalysis and whose mutants show impaired germination phenotypes (Okamoto et al., 2006). Cluster 3 includes a catalase CAT1 involved in ABI5 regulation of germination (Bi et al., 2017) and a homologue to ERF118 and CYTOKININ RESPONSE FACTOR 4 also involved in germination response to cold in Brassica napus (Luo et al., 2021). The gene *HanXRQChr08g0214651* coding for the S-adenosylmethionine decarboxylase key gene of synthesis of polyamines in plants (Pegg, 2009) was strongly differentially express in different imbibition. In cluster 4, we found a homologue to VIN3 like protein 1 involved in chromatin remodeling during vernalization in Arabidopsis (Kim et al., 2010) together with five other genes involved in epigenetic regulation. Cluster 5 groups most of the histone coding genes selected here (14 out of 20 members, indicated "h" in Fig. 39). This coregulation of histones, validates the clustering methodology. A subgroup of cluster 5 (labeled 5a on Fig. 8) is solely composed of histones and of a cytosine methyl transferase (CMT1 homologue). We also find The gene *HanXRQChr02g0053081* coding for histone H3-like centromeric CSE4 a key molecular marker that differentiates the special centromeric chromatin structures from bulk nucleosomes (Cheng et al., 2017) appears to be a gene specific to hypoxia tolerance Fig 39. The cluster 6 show some gene whit the same expression profile during the different stress imbibition. For example, the gene *HanXRQChr05g0142301* coding cytochrome b-c1 complex subunit 7-2 useful for mitochondrial electron transport, ubiquinol to cytochrome c. It was less express during stress imbibition and all the more so for the s seed during abiotic stress. We find also some gene in relation of chromatin compaction like: *HanXRQCihr14g0428531* coding for histone H4, HanXRQChr04g0116701 coding for methyltransferase 13 and *HanXRQChr04g0120231* coding for histone-lysine N-methyltransferase ATX4-like isoform X1. Finally cluster 7, is only composed of genes responding to brassinosteroids (BRU1-like) and involved in ethylene signaling (RAP2-3) and production (EFE). This last gene is down-regulated by brassinosteroids via BZR1 signaling in Arabidopsis seedlings (Moon et al., 2020)

It was also interesting the effect of maternal stress on the patterns of gene expression in various stress conditions (ie hypoxia S/W, PEG S/W, NaCl S/W; see the white frame in Fig. 39). The heatmap shows that the transcriptional response was specific to a single stress, ie that there was almost no overlap between changes in gene expression in water stress, hypoxia and salt stress (Fig. 39). A few genes were however over-expressed in the 3 conditions such as endoribonuclease Dicer homolog 2, ethylene-responsive transcription factor ERF118-like and VIN3 (Fig. 39 and Fig supplemental data 10).

IV) Discussion

Germination is a critical phase for the crop implantation and is often subject to abiotic stresses such as drought, cold and salinity. It is crucial for plants to prepare their progeny in order to guarantee high stress tolerance, hence rapid germination in a wide range of agroclimatic conditions.

As previously observed by Vancostenoble et al. (2022), a moderate drought stress on the mother plant applied during seed development conferred higher vigour to the progeny, as shown by the ability of S seeds to fully germinate in the presence of a -1.0 MPa PEG solution (Fig. 32). The property of seeds to better germinate under water stress conditions was acquired at the first generation (1S seeds) and was not dramatically improved after 3 generations of drought stress (3S seeds) on the mother plant (Fig. 32). This is in accordance with the previous results of Vancostenoble et al. (2022) who demonstrated that the induction of water stress tolerance resulted from a maternal effect and was not transgenerational. Similar findings were obtained with other species, such as Arabidopsis (Ganguly et al., 2017b; Van Dooren et al., 2020a), highlighting the key role of the maternal environment in the acquisition of seed vigour.

The non-targeted transcriptomic analysis performed here brings a comprehensive outlook on the putative mechanisms involved in the inheritance of seed vigour traits by the progeny. The PCA Fig 33 provided a general view of the changes of expression of 44176 genes as modulated by both stress on the mother plant and by stress during germination. Strikingly it shows that the transcriptomic response to water stress at the germination stage was induced by the stress itself since in dry seeds and at 16 and 24 h of imbibition on water transcriptomes of 3W and 3S seeds were relatively close to each other, but they markedly differed when seeds were imbibed on a PEG solution (Fig. 33a). This is an interesting finding because it suggests that the maternal environment conferred the progeny the ability to

adapt transcriptional regulation only when appropriate, ie when seeds were germinated in sub-optimal conditions. To our knowledge this is the first time that one can evidence a "conditional transcriptional memory" induced by the maternal environment and transmitted to the progeny. This is opening novel fields of investigation in the understanding of transgenerational inheritance, and signaling and molecular events involved in this mechanism will have to be addressed properly.

Pathifier analysis demonstrates that, as already shown by (Ponnaiah et al., 2019; Saux et al., 2020b), seed germination (ie imbibition on water), was associated with a dramatic deregulation of an impressive number of pathways, resulting without any doubt from the metabolism resumption that occurs when dry seeds get imbibed (Han and Yang, 2015) (Waterworth et al., 2015). Under water stress pathifier revealed a clear transcriptional discrepancy between 3W and 3S seeds at 16 h of imbibition, but not at 24 h. The analysis of the pathways deregulated at 16 h on PEG in 3S seeds brings insights on the mechanisms specifically involved in water stress response at the germinative stage. Most of them shared similarities with pathways known to be involved in the whole plant response to water stress, despite any issue related to photosynthetic activity. For example, several pathways related to aminoacid metabolism were identified (Fig supplemental data6) and such metabolites are known as compatible osmolytes in response to water stress (Hildebrandt, 2018; Batista Silva et al., 2019). Vitamin E and ascorbate pathways are also deregulated at this stage (Fig supplemental data6). They are both major antioxidant in plants (Bailly et al., 2000; Sies, 1997) and this suggest that, as proposed by (Saux et al., 2020b) with seeds of the same species, that the seed antioxidant potential plays a key role in response to water constraints. The change in many metabolites related to carbohydrate metabolism (Fig supplemental data 6), including starch degradation, probably reflects a progression towards the germination process, through energetic processes and breakdown of reserves for preparing radicle elongation (J. D. Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006). The protein N-glucosylation pathway was also more deregulated in 3S seed, this pathway is known as being involved in environmental response to stress, like a down regulation of this pathway under flooding stress in soybean (Mustafa and Komatsu, 2014). (Kang et al., 2008) showed the necessary of the N-glycosylated proteins maturation in the Golgi apparatus for salt tolerance in *Arabidopsis thaliana*.Interestingly the role of pyrimidine metabolism in response to water stress during seed germination had also been pointed out by (Saux et al., 2020b). Lastly Pathifier also identified ABA catabolism pathway (Fig supplemental data 6) as being deregulated in 3S seeds germinated on PEG, in accordance

with the results of Vancostenoble et al (2022) who showed that ABA content was lower in S seeds and with the REVIGO analyse also performed here (Fig. 35) who identified the GO "abscisic acid metabolic process" among the pathways involved in response. Although ABA is playing a key role in the activation of water stress response in whole plants (Bulgakov et al., 2019), in seeds it inhibits germination (Nambara et al., 2010), and thus it appears that during water stress at the germination stage, ABA catabolism is favored in order to favorize a rapid germination. Measurements of seed ABA content during germination would be necessary to confirm the transcriptomic data.

Analysis of DEG in dry seeds, in seeds imbibed on water and in seeds imbibed on a PEG solution gave additional information about the molecular regulation of seed vigour inheritance. This shows that, among the 44176 genes whose expression varied in our samples, only several of them were either up or down regulated in 3S seeds, ie were influenced by the maternal environment (Fig. 35). This relatively small set of genes is nevertheless in accordance with the one reported by (Bray, 2004) in response to water stress in Arabidopsis. Importantly our results also show that the ability of 3S seeds to better germinate in water stress condition was not directly acquired during seed development but resulted from the regulation of expression of a specific subset of genes when seeds were germination under water stress (Fig. 35). Namely 425 and 753 genes were specifically up- or down- regulated, respectively, during 3S seed imbibition on PEG. This is an interesting finding which suggests that the maternal environment did not directly induced molecular mechanisms of water stress tolerance to the progeny, but that it transmitted to the offspring the ability to trigger a molecular adaptive response to stress only when required. In Arabidopsis leaves it has been proposed that a transcriptional memory can be maintained after dehydration stress/recovery cycles and that it can influence subsequent transcriptional responses (Ding et al., 2012).

Since tolerance of S seeds to water stress during germination was associated with both up and down regulation of genes, we paid attention to these categories which were clustered using REVIGO (Fig. 35), that allowed identifying classical GO associated with either stress tolerance or seed germination (Fig supplemental data 8.). The role of various hormones, notably ABA, GAs, brassinosteroid, and ethylene appeared as being important thus confirming our previous data. We propose that the maternal environment had an effect on the hormonal balance ABA/GAs during germination under water stress in the benefit of GAs. This is also underlined by the transcriptional footprints Fig 36. which suggest that genes belonging to ABA biosynthetic pathway, such as AAO3 (Barrero et al., 2006), ABA3

homologue enzyme also involved in the synthesis of ABA (Seo et al., 2004), and GRAM domain family protein GEM5 (ABA signaling pathway mediator) were down regulated in 3S seeds. While in the same time others involved in repression of GAs signaling, such as RGL1 and GID1 (Davière and Achard, 2016; Sun, 2010). were also repressed. Metabolism of ROS was also identified by REVIGO (Fig. 36), and it role was confirmed when using the ROS wheel (Fig.36). In the presence of PEG 50 genes related to ROS metabolism were up or down regulated in S seeds. They include for example: ethylene-responsive transcription factor 5-like (ERF5) in ROS cluster 6 known to respond to extracellular signals (Fujimoto et al., 2000; Hao et al., 1998). In ROS cluster 3 we found peroxidase 21 which was down regulated in 3S. Its role in removal of H_2O_2 and ROS regulation could be associated with the difference of germination between 3S and 3W seeds (Ranieri et al., 2000). The gene AT1G56220 coding for Auxin-repressed kDa was also donw regulated in 3S seeds, this gene is associated to dormancy associated family protein (AT1G56220 gene in TAIR, www.arabi dopsis.org). Lastly we also identified a large set of genes in relation with epigenetics and DNA conformation whose expression was modified in 3S seeds on PEG (Fig supplemental data 9) Among them the gene coding for the Histone H1-3 variant was up-regulated and this histone is specialized in adaptive response of water deficiently (Rutowicz et al., 2015). The chromatin remodeling has a role in the regulation of seed dormancy (Liu et al., 2007) and the regulation of histone variant H2B (down regulated in 3S seed) (Fig supplemental data 9) can also play a role in the difference of germination between S and W seeds.

Based on these findings we selected some specific genes candidate for water stress tolerance, in the ROS, Hormone and Epigenetic clusters in order to assess their role in the tolerance of S seeds to various stresses such as salt stress and hypoxia (see Vancostenoble et al., 2022) using a micro-fluidigm qPCR approach. The Volcano plots (Fig. 37) confirmed our previous observations showing that tolerance of 3S seeds to water stress resulted from a down regulation of these genes when compared to 3W seeds (Fig 37a) and that, in contrast, they were over expressed IN 3S on PEG, when compared to water (Fig 37c). They also show that the genes belonging to the 3 clusters behave similarly and that there was not a specific regulation of one cluster (Fig. 37).

The cross-comparison of the DEG by RNAseq and Fluidigm analyses using 2 different seed batches allowed the identification of genes that were likely to be associated with the inheritance of better germination under water stress (Fig. 39, Fig supplemental data 9.). Thirty genes were found to be up-regulated in response to both drought stress on the mother plant and water stress during seed germination (Fig supplemental data 10.) and

they are very good candidates for explaining this phenomenon but also as molecular markers of this trait. Among these genes many were related to ethylene signaling and biosynthesis (ie Ethylene-insensitive 2, ethylene-responsive transcription factor ABR1-like isoform X2, ethylene-responsive transcription factor ERF118-like, ethylene-responsive transcription factor RAP2-12-like). Besides its effect on seed dormancy alleviation, including for sunflower (Corbineau et al., 2014), ethylene has also been proposed to play a role in the germination of non-dormant seeds in stressful conditions (Bailly et al, 2022). Ethylene-insensitive 2 and ERFs, for example, have been shown to be involved in response to salt stress in Arabidopsis (Wilson et al., 2014), but this the first time that ethylene is proposed as playing a role in germination of sunflower seeds under water stress. Two Abscisic acid 8 -hydroxylase 2, involved in ABA degradation, and abscisic-aldehyde oxidase-like, involved in ABA synthesis, were also found as being up-regulated. This is rather contradictory but previous data obtained by Vanconstenoble et al. (2022) have shown that ABA content tended to decrease in embryos of S seeds, which suggests that the balance between synthesis and degradation would be in favour of degradation. The presence of components of DNA-dependent RNA polymerase II and III among the up-regulated genes (ie DNA-directed RNA polymerase III subunit RPC5 isoform X1, DNA replication regulator dpb11, mediator of RNA polymerase II transcription subunit 16 isoform X1) highlighted that an active transcription was associated with seed germination under water stress. In addition the presence of several genes belonging to the "Epigenetic" cluster also suggests that the transcriptional memory may have an epigenetic component, as it has already been proposed for Arabidopsis (Avramova, 2015; Friedrich et al., 2021) or for other models such as yeast (Kundu and Peterson, 2010; Ng et al., 2003).

Clustering of microfluidic qPCR results allowed the identification of 7 clusters that grouped together co-expressed genes (Fig. 39), but it was difficult to attribute a specific biological role for these different clusters. Some clusters displayed a strong tendency for including genes from the same clusters designed after RNA seq experiment, for example clusters 3, 5, 5a which mostly contains genes belonging to the "epigentic" clusters. Cluster 7 contained co expressed genes belonging to the ROS cluster, showing the importance of ROS signaling in response to stress.

Lastly, we also investigated whether maternal stress could induce expression of universal genes of seed vigour, ie genes that were over expressed in various stresses. Our analyze showed that the transcriptional response was mostly specific for each stress, thus ruling-out a common molecular response to different environmental issues. Some genes were

however were found were over expressed in each condition and can be considered as good potential markers of seed vigour. Among them is the ethylene-responsive transcription factor ERF118-like, which has been mentioned previously here, and which reinforces the possible role of ethylene metabolism in sunflower seed germination under constraints. *VIN3* is upregulated by vernalization and is a repressor of *FLC* expression in the flowering pathway (Gendall et al., 2001). Interestingly it has also been shown to reduce germination of Arabidopsis seeds from vernalized plants (Auge et al., 2017). Our results suggest that *VIN3* would therefore play a key role in the transmittance of germinative traits from the mother plant to the progeny.

V) Conclusion

In summary, after having demonstrated that a physiological memory could be induced by drought stress on sunflower mother plant (Vancostenoble et al., 2022), we show here that the induction of water stress tolerance at the germination stage is associated with a transcriptional memory. Interestingly our results show that the transcriptional adaptative response is timely triggered in response to stress only, and that this response is stress-specific. This emphasizes again the complexity of seed vigor traits in sunflower seeds, as already proposed by (Saux et al., 2020a). We demonstrate that the adaptation to water stress has strong hormonal and redox components and that epigenetic mechanisms are also likely to play a role in the seed response to the maternal environment. Future studies will have to address properly this finding. At last, at a practical level; this study provides a set of candidate genes that are good markers of maternal effect in sunflower seeds and that can also be used as markers of seed vigor.

Chapter 4 :

Regulation of the DNA methylome of sunflower seeds by maternal drought stress

I) Introduction

Plant exposure to stress can create stress imprint effects (Bruce et al., 2007). Some stress memory mechanism exists, like the epigenetic modification caused by environmental stress. One of the most studied has been the vernalization mechanism in which the plant can remember a cold stress (Sung and Amasino, 2004). This can be accompanied by a specified change of epigenome and participate in long-term somatic memory (Sani et al., 2013). The modifications in the chromatin architecture govern gene expression by regulating the accessibility of genes to transcriptional machinery (Kouzarides, 2007). Three epigenetic mechanisms have been shown to play a role in plant development and response to the environment, ie DNA methylation , histone modification and noncoding RNA (small interfering RNA, siRNAs, microRNAs, miRNAs,long ncRNAs) (Kapazoglou et al., 2018). In animals without DNA methylation, the mechanisms involved in the acquisition and inheritance of characters by the environment are mostly related to small RNAs (Ashe et al., 2012b) and histone modifications (Öst et al., 2014b). In mammals, they can also be associated with DNA methylation (Baxter and Drake, 2019b; Radford et al., 2014b). In plants, DNA methylation is an active process (Liu and Lang, 2020), occurring notably during the endosperm gene imprinting (Zhang et al., 2021), but demethylation can be a passive or an active phenomenon. Active demethylation plays a role in fruit development and ripening (Giovannoni et al., 2017) and in response to biotic (López Sánchez et al., 2016) or abiotic stresses (Kim et al., 2019). Several DNA methylation types exist in plant, they include methylation of cytosine CpG, CHG and CHH, where H can be C, A or T) (Feng et al., 2010). The types and the positions of methylations have different impacts on gene regulation (Bewick and Schmitz, 2017). For example, some correlation exists between gene body CpG methylation and constitutive gene expression (Muyle and Gaut, 2019) but if the CpG methylation is in upstream regions of genes, their expression will be repressed. When CpG is combined with CHG methylation in the gene body, the expression can be promoted, in contrast with the sole CpG methylation in the gene body, which decreases gene expression (Niederhuth et al., 2016). CHH enrichment has also been found to be correlated to gene silencing (Schmitz et al., 2013). DNA methylation is known to regulate transposon element (TE) activity (Kim and Zilberman, 2014). Arabidopsis genome is characterized by high levels of methylation in heterochromatin, enriched with transposable elements (Henderson and Jacobsen, 2007) and methylation of TE is a defense mechanism of the plant genome against selfish DNA elements which provides DNA stability (Li et al., 2020).

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DNA methylation has a role in the modification of the chromatin conformation and it impacts gene transcription (Berger, 2007; Bouyer et al., 2017; Zhang et al., 2018). Schematically, open chromatin is necessary for the gene to be accessible to the transcription machinery and expression is more repressed when the chromatin conformation is compact (Kouzarides, 2007). For plants, DNA methylation changes have been associated with environmental conditions leading to modification of heritable traits (Jiang et al., 2014). Drought-induced site-specific DNA methylation is for example associated with drought tolerance in rice (Wang et al., 2011) and DNA methylation modifications during seed priming has been shown to induce stress tolerance, especially for drought (Aswathi et al., 2021). Transgenerational stress memory in plants has also been shown to be related with DNA methylation and chromatin remodeling (Shanker et al., 2020). The transgenerational memory of stress in plants is a promising perspective for plant adaptation to climatic constraints (Molinier et al., 2006). In Arabidopsis a hyperosmotic stress memory is mediated by epigenetic mechanisms, like DNA (de)methylation in response to stress (Wibowo et al., 2016b) and this phenomenon could be heritable to the progeny.

In 2 previous studies, Vancostenoble et al. (2022a,b) have demonstrated that applying a moderate drought stress on sunflower plants during seed development was conferring tolerance to water stress at the germination stage to the progeny. Besides this physiological memory these authors have also demonstrated the occurrence of a transcriptional memory and they have identified a subset of genes which were involved in this phenomenon. Among them Vancostenoble et al (2022b) identified a specific transcriptional footprint for epigenetic mechanisms. The present study was therefore carried out in order to determine whether DNA methylation could play a role in the inheritance of seed vigour traits to the offspring by the mother plant. We have therefore characterized the methylome of sunflower seeds using sodium bisulfite-treatment and DNA sequencing in order to identify methylation differences in specific genomic region (Differentially methylated regions, DMR). The DMR identified have been validated using McrBC qPCR and we also have investigated their relationship with changes in expression that are associated with acquisition of seed vigour.

II) Materials & methods

2.1 Seed production

The experiment was performed using seeds of sunflower (XRQ/B genotype, seed lot XRQMLG2011) cultivated during three consecutive generations of self-fertilisation at the Heliaphen platform (INRAE, Toulouse). The plants were grown in 15 L pots (Soparco, Le Musset, France) with Terreau Proveen PAM2. We applied four fertilisations post-stress with 1 L of Peter's Professional 17-07-27 (0,8g/L.) + Hortilon (0,46g/L.) by plant. Two irrigation conditions were applied on a subset of the plants (24 per condition): "W" for the well-watered condition with an optimal Fraction of Transpirable Soil Water was maintained (FTSW = 1), and "S" for the drought stress condition, with reduced FTSW (FTSW=0.2). The application of stress began after flowering and was repeated for 3 successive generations giving 3 S seeds (Vancostenoble et al., 2022b). Seeds produced in well-watered conditions for 3 successive generations were noted 3W seeds. After harvest, seeds were dried in an oven at 25 °C for 48 hours and stored in a cold room at 4 ° C at 30% RH. After 6 months of storage, seeds of both lineages were non-dormant.

2.2 RNA Extraction & sequencing analysis

The methodology is described in Vancostenoble et al. (2022b). The XRQr1 reference genome (Badouin et al., 2017) was indexed with the novoindex tool (http://www.novocraft.com/main/index.php) using the bisulfite option to create an index including two references: a "CT" reference, where the Cs on the forward ("Watson") strand are converted in Ts and a "GA" reference which correspond to the complementary (Crick) strand after conversion.

2.3 Sodium bisulfite-treatment library generation and sequencing.

One microgram of genomic DNA was fragmented by acoustic shearing to target 250bp using a Covaris S220 (Covaris, Woburn, MA), according to the manufacturer recommendations. Then DNA fragments underwent an NGS library preparation procedure consisting of end repair, methylated adaptor ligation (Roche, Basel), and DNA size selection using the KAPA HTP kit (Roche, Basel). Individual index sequences were added to each library for identifying reads and sorting them according to their initial origin. The resulting NGS libraries were assessed by capillary electrophoresis using a Bioanalyzer DNA High-Sensitivity Chip (Agilent, Santa Clara, CA). NGS libraries were then treated with sodium bisulfite using the EZ DNA Methylation Lightning kit (Zymo Research, Irvine, CA). DNA fragments carrying both adapters after sodium bisulfite conversion was enriched by 12

cycles of PCR using the KAPA Hifi HotStart Uracil Ready mix (Roche, Basel). Primer dimers were subsequently removed using one round of purification using AMPure beads (Beckman Coulter, Indianapolis, IN). The resulting NGS libraries were assessed by capillary electrophoresis using a Bioanalyzer DNA High-Sensitivity Chip (Agilent, Santa Clara, CA) and quantified using the Quant-It PicoGreen (Life Technologies, Carlsbad, CA). Equimolar pools of 3 libraries were produced and quantified with a qPCR assessment using the kapa Library Quantification Kit Illumina SYBR Fast ABI Prism-1 (Roche, Basel). Each pool of 3 libraries were sequenced with Illumina paired end sequencing (2x100nt) strategy. 100Gb was produced per pool.

2.4 Bisulfite Conversion Efficiency

To assess the efficiency of the conversion process, we tested the number of methylated cytosines on the chloroplast sequence. As the chloroplast is known to be unmethylated we expected to find very few unconverted cytosine compared to the nuclear genome. We obtained less than 0.02% of unconverted cytosine for all samples, which correspond to conversion efficiency above 99.8%.

2.4.1 Read Mapping and Methylation Status

The BS treated reads were aligned against the references using novoalign V3.04.01 with plant adapted stringent parameters. Each read and its reverse complement sequence was compared to both CT and GA indexes. Alignment resulting file was separated in two files according to the mapping strand (CT or GA) and converted into bam compressed files with samtools 1.2 (Li et al., 2009). After mapping, bam files were analyzed with novo-methyl 1.02 according to protocol from novocraft for methylation mapping (http://www.novocraft.com/main/page.php?s= novomethyl) and each bearing C or G position was examined to estimate the percentage of methylation. We then used an in-house perl script to extract C/G positions filtered on consensus calling quality and to format them in Methpipe (Song et al., 2013) compatible files. Measure obtained from each replicate was then merged with merge-methcounts from Methpipe suite.

2.4.2 DMR identification between parents and calculation of methylation levels

DMR identification was performed between pairs of samples from the 2nd and 3rd generation using the methpipe 3.3.1 protocol in each of the 3 cytosine contexts (CpG, CHG and CHH). DMR were filtered to include at least 5 significant C by region.

For CHH DMR analysis, due to the very low level of methylation in this context, a preliminary inversion of methylation rates (as recommended by the authors) has been done on the observed values (1- value) to allow Methpipe to detect the DMR.

The DMR list has then been used to measure the mean methylation rate in each region for every sample using the roimethstat tool from the same Methpipe suite.

2.4.3 Gene ontology and functional category enrichment analysis

GO terms and functional category enrichment analysis were performed using Top GO R analysis. GO term enrichment analysis was conducted for genes included in each cluster using the REVIGO software (Supek et al., 2011).

2.5 McrBC qPCR validation protocol.

XRQ seeds were produced by MAS Seeds without irrigation in Belciugatele (Romania) and with irrigation in Haut-Mauco (France). We considered seeds from Romanian production site as drought stress seeds (S seed) and seeds from Haut-Mauco as well-watered seeds (W seed).

Three replicates of 25 seeds were imbibed during 24h on water and on a PEG solution (-0.7 MPa) at 20°C. The dissected seed embryonic axis was grounded in liquid nitrogen for DNA extraction. Total DNA was isolated using the DNeasy® Mini Kit (Qiagen), following the manufacturer's recommendations. To cleave DNA containing methylcytosine (CpG or CHG or CHH), digestion was carried out overnight at 37°C with 200 ng of genomic DNA and 10 units of the McrBC endonuclease enzyme (New England Biolabs). Primers of 17 candidate DMRs were designed by BatchPrimer3, with an optimal Tm at 60°C (except for HanXRQChr03g0074691, Tm=56°C). Quantitative PCR amplifications were performed on a Light Cycler 480 II (Roche) in a total volume of 10 µl containing 5 µl of 2X no ROX Takyon MasterMix (Eurogentec), 0.2 µM of each primer and 2 ng of digested and undigested DNA. The amplification protocol consisted of an initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 10s, annealing at 60 °C for 20s and extension of 72 °C for 30s. Relative methylation levels were expressed as $\Delta Ct = 2^{-Ct(Target\_DIG)} / 2^{-Ct(Target\_NONDIG)}$

2.6 Statistical analysis

For McrBC qPCR, outliers defined as elements more than three scaled MAD from the median, were removed using *isoutlier* function in Matlab Statistics and Machine Learning Toolbox Version 11.7. We tested the difference in methylation in the 15 DMR validated technically using a linear model (*fitlm* function) with the following model :

$$Meth_{i,r,m,g} = R_{i,r} + MS_{i,m} + GS_{i,g} + I_{i,m,g} + e_{i,r,m,g}$$

where $Meth$ is the methylation ratio (ΔC_t) of the i^{th} gene, R the r^{th} replicate, MS the m^{th} maternal stress (drought or well-watered plants), GS the g^{th} germination stress (PEG or control), I the interaction between maternal and germination stresses and e the residual

error. A Fisher test for performed to determine the DMR enrichment localization in specific parts of the genome : promoters, gene body, post CDS sequence or TE using R with p-value 0.05.

III) Results

3.1 Characterization of sunflower seed methylome

DNA methylation was studied after bisulfite treatment and High-Throughput Sequencing. Three types of DNA methylation: CpG, CHG and CHH were identified (where H can be A, C or T) and we determined differential methylated regions (DMRs) of DNA between 3W and 3S dry seeds and between seeds after 24 h of imbibition at 20°C on water or on a –1 MPa PEG solution.

Overall numbers of DMRs were not markedly modified during seed imbibition suggesting that DNA methylation was acquired during seed development. (Fig. 40). CHH methylation was the more prevalent type of methylation with 126,760 DMRs in dry seeds and 136,039 DMRs in PEG condition (Fig 40b). CpG DMR was the less represented type of methylation with 1,940 DMRs in dry seeds and 2,170 DMRs in PEG condition (Fig 40a). For CHG methylation (Fig 40a) the number of DMRs was between 3,910 and 4,217 (7.3% of difference) in PEG and dry condition, respectively. The most important difference (10.6 % of difference) was for CpG methylation with 3,910 DMRs for PEG and 4,217 for dry seeds (Fig. 40a).

The number of DMRs on each chromosome (Fig supplemental data 1. a., b., c.) and the number of DMR normalised by chromosome size on each chromosome (Fig supplemental data 13. d., e., f.). The preferential localisation of a specific DMR on one chromosome was not clearly established (Fig supplemental data 13.). The chromosome 6 has a smaller number of DMR (only 126 DMR CpG in dry seeds). In comparison, chromosome 13 displayed the higher number of DMR (365 DRM CHG in dry seeds). However, after normalisation, the ratio of CHG DMRs in chromosome 6 and chromosome 13 were comparable, respectively $1.21.10^{-6}$ and $1.85.10^{-6}$. In conclusion, we did not observe effects of maternal stress and seed imbibition on the number of DMRs in specific chromosomes or genome-wide.

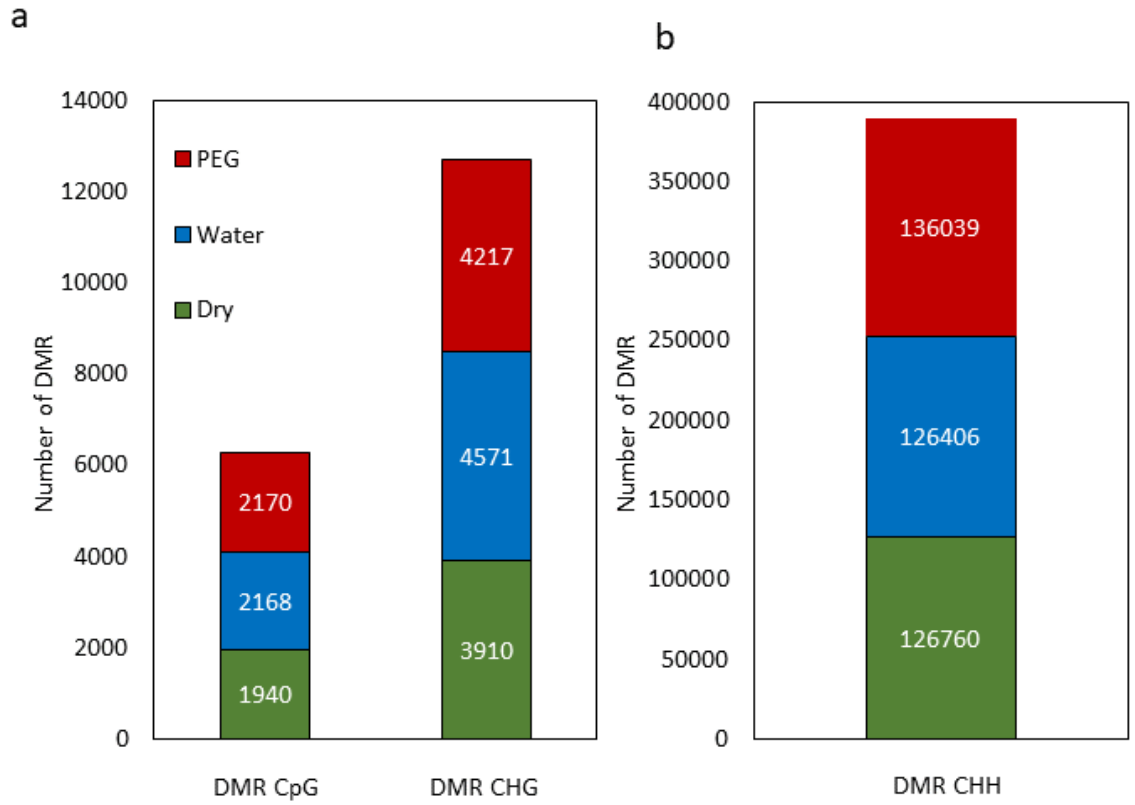


Figure 40 : Number of Differentially Methylated Regions

Number of Differentially Methylated Regions (DMRs) between W & S in dry seeds (in green), in seeds after 24 h of imbibition at 20°C on water (in blue) or -1.0 MPa PEG solution (in red). The 3 type of DMR are represented a. CpG & CHG and b. CHH.

3.2 CpG Methylation

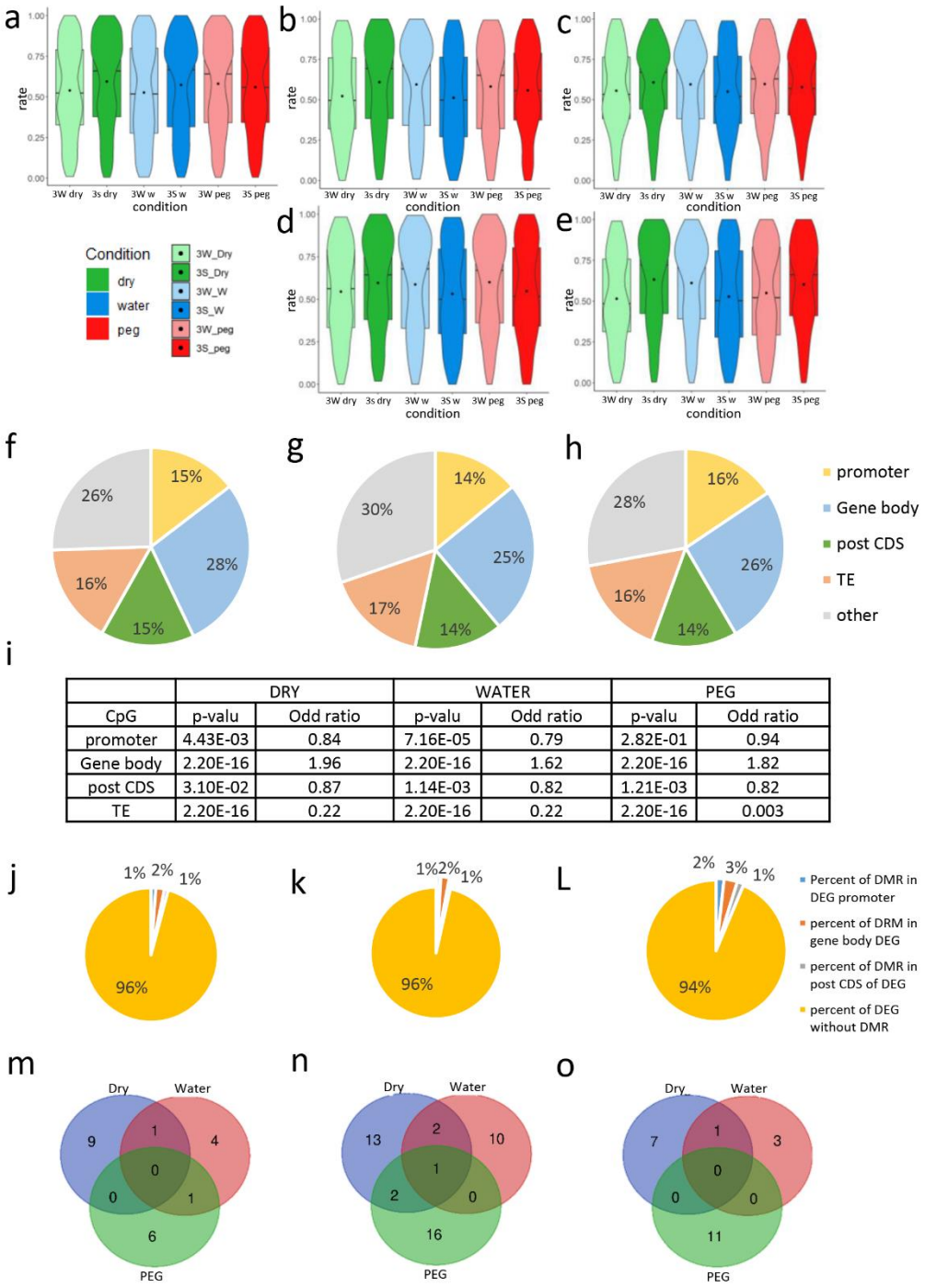
Figures 41.a. to e. show the rates of CpG methylation on DNA in W and S seeds in the different genomic compartments. Maternal drought stress induced an increase in methylation rates of CpG DMRs in dry seeds (Fig. 41a). This trend was observed in all genomic compartments: promoter i.e. 2000bp upstream CDS, gene body, post CDS i.e. 2000bp downstream CDS, and transposable elements) as shown in green on Fig. 41 b-e.

Although methylation rates of CpG DMRs remained higher in 3S seeds after their imbibition in water when considering the entire genome, the opposite was evidenced when looking at specific genomic regions (Fig. 41a-e in blue). This suggests an important methylation pattern modification, with demethylation of these regions and higher methylation of DMR situated in intergenic regions. In water stress during germination, methylation rates of DMRs were less plastic when considered together, with a decrease in post CDS regions in 3S seeds (Fig. 41d).

CpG DMRs were mostly situated within gene body (ca 25 %) and had similar distributions (ca 15 %) in the promoter region, the gene body, the post CDS and TE, whatever the

condition (Fig. 41f-h). The sunflower genome is not homogeneously distributed between coding and non-coding regions and the large majority of the genome is non-coding, more than three quarters of the genome being composed of repeated retrotransposons (LTR-RTs) (Badouin et al., 2017; Staton et al., 2012). Therefore, we tested specific accumulations of methylation in the different genomic compartments. The Fisher enrichment tests (Fig 41.i.) showed an enrichment of CpG DMRs in the gene body (whatever the seed imbibition condition: dry, H_2O or PEG) and lower representation in TE, consistent with a constitutive repression of TE through methylation that was not impacted by maternal lineage.

To identify common regulations of gene methylation and transcription, we estimated the number of genes differentially expressed between 3S and 3W lineages and having CpG DMRs in promoters, coding and post-CDS regions (Fig 41.j-l). The percentage of CpG DMR in DEG was very low, i.e. below 4-6%, representing 21, 44 and 22 genes according to conditions. Venn diagrams (Fig 41. m-o) show very few overlap between conditions, consistent with independent processes.



	DRY		WATER		PEG	
CpG	p-valu	Odd ratio	p-valu	Odd ratio	p-valu	Odd ratio
promoter	4.43E-03	0.84	7.16E-05	0.79	2.82E-01	0.94
Gene body	2.20E-16	1.96	2.20E-16	1.62	2.20E-16	1.82
post CDS	3.10E-02	0.87	1.14E-03	0.82	1.21E-03	0.82
TE	2.20E-16	0.22	2.20E-16	0.22	2.20E-16	0.003

Figure 41 : Study of CpG differentially methylated regions.

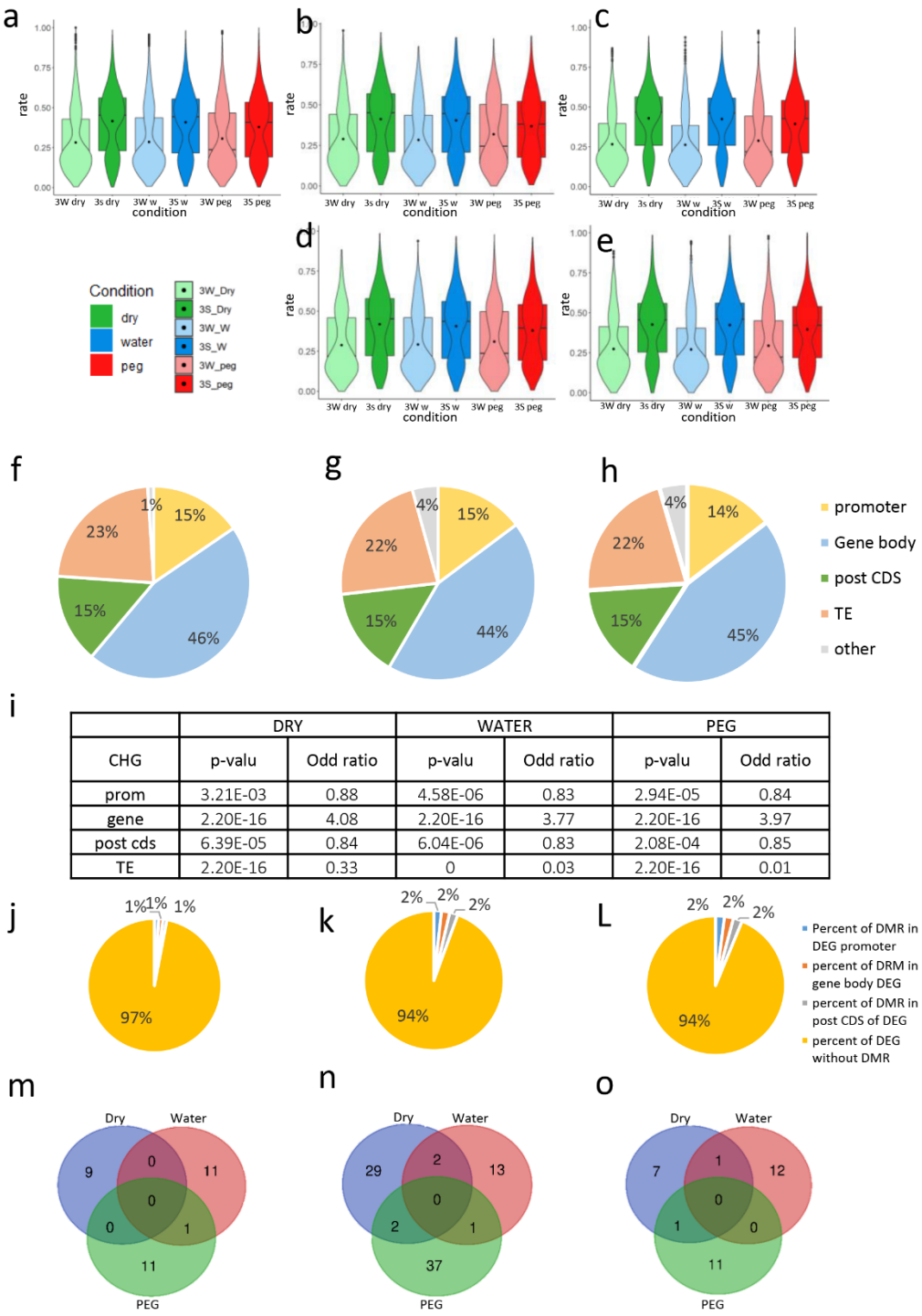
Study of CpG differentially methylated regions (DMR) between W & S maternal lineages. (a) Violon plot representations of DMR methylation rate in the entire genome, (b) in promoter, (c) in gene body, (d) in post CDS, and (e) in transposable elements. Proportion of DMRs according to the different genomic regions in different conditions : (f) dry seeds, (g) water imbibition, and (h) water stress (-1MPa PEG imbibition). (i) Fisher test results for DMR enrichment in different part of genome. Proportion of DEG with DMR in the different genomic regions and conditions: (j) in dry seeds, (k) water imbibition, and (l) water stress (PEG -1.0 MPa). Venn diagrams of CpG DMRs in promoter, gene body or post CDS of DEG in different conditions: (m) dry seeds, (n) water imbibition, and (o) water stress (PEG -1.0 MPa).

3.3 CHG methylation

We always identified a higher methylation rate of CHG DMRs in 3S seeds than in 3W seeds, on the whole genome (Fig. 42a) and in each different part of the genome studied here (Fig. 42b-e). CHG DMRs are mainly located within genes (42-45%) and in their vicinity (31%) Fig 42.f-h. The statistical study of CHG DMR distribution was done in Fig 42.i. The Fisher enrichment tests showed the enrichment of CHG DMRs in the gene body (whatever the germination condition: dry, H_2O or PEG), and a depletion in TE consistent with a constitutive silencing of TE.

Presence of CHG DMRs around differentially expressed genes between 3S and 3W was verified in Fig 42.j to l. The percentage of DMR CHG in DEG was low, around 1-2%.

When comparing the three conditions, extremely low numbers of DEG with CHG DMR were shared between the conditions when considering each compartment: promoter, gene body and post CDS as shown in the Venn diagram in Fig. 42 m-o. This is consistent with independence between CHG methylation and gene regulation in response to maternal stress.



	DRY		WATER		PEG	
CHG	p-valu	Odd ratio	p-valu	Odd ratio	p-valu	Odd ratio
prom	3.21E-03	0.88	4.58E-06	0.83	2.94E-05	0.84
gene	2.20E-16	4.08	2.20E-16	3.77	2.20E-16	3.97
post cds	6.39E-05	0.84	6.04E-06	0.83	2.08E-04	0.85
TE	2.20E-16	0.33	0	0.03	2.20E-16	0.01

Figure 42 : Study of CHG differentially methylated regions.

Study of CHG differentially methylated regions (DMR) between W & S maternal lineages. (a) Violon plot representations of DMR methylation rate in the entire genome, (b) in promoter, (c) in gene body, (d) in post CDS, and (e) in transposable elements. Proportion of DMRs according to the different genomic regions in different conditions : (f) dry seeds, (g) water imbibition, and (h) water stress (PEG -1.0 MPa). (i) Fisher test results for DMR enrichment in different part of genome. Proportion of DEG with DMR in the different genomic regions and conditions: (j) in dry seeds, (k) water imbibition, and (l) water stress (PEG -1.0 MPa). Venn diagrams of CpG DMRs in promoter, gene body or post CDS of DEG in different conditions: (m) dry seeds, (n) water imbibition, and (o) water stress (-1MPa PEG imbibition).

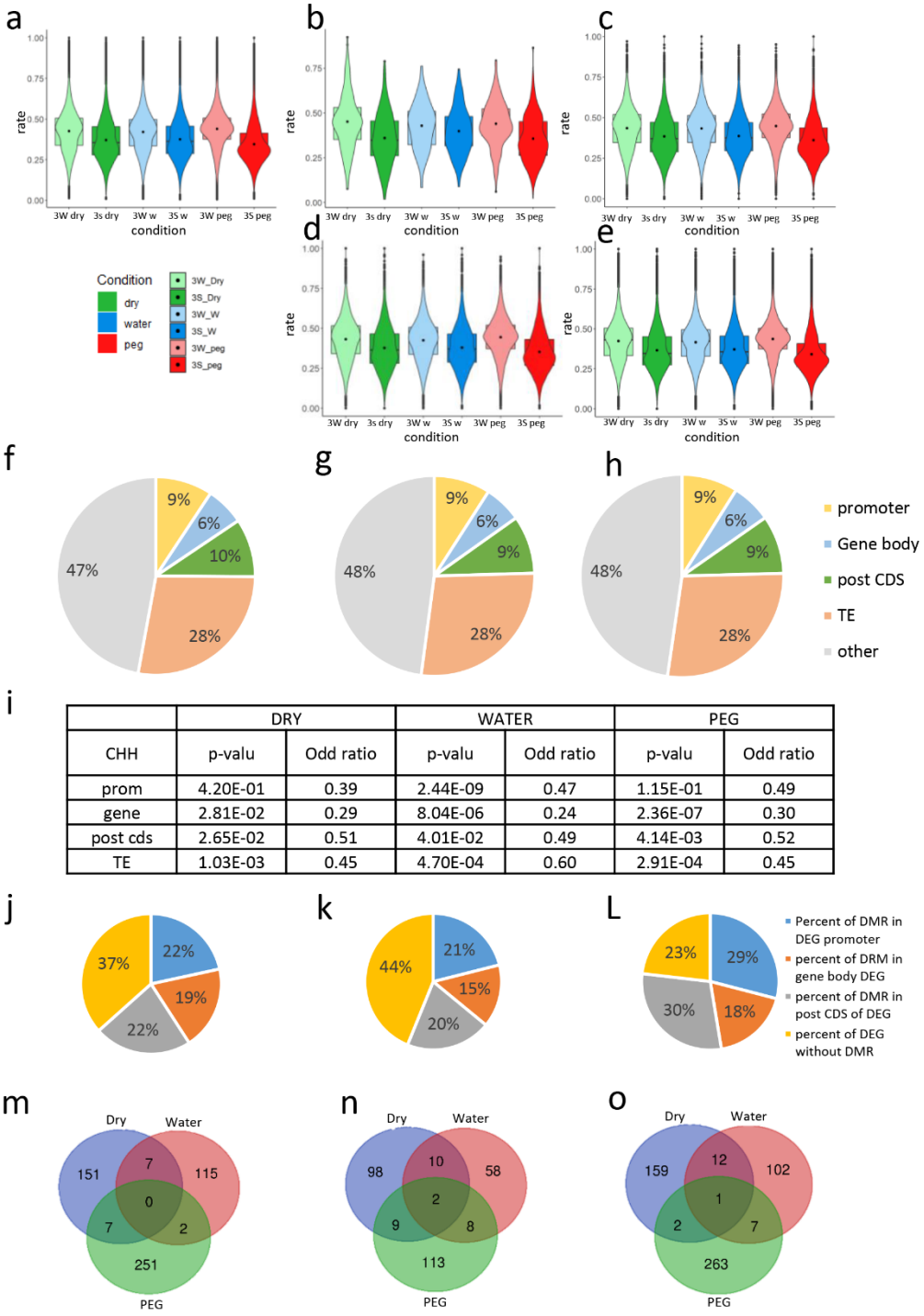
3.4 Study of CHH methylation.

Figure 43 shows the impact of maternal stress in methylation rates in DMR between 3W 3S maternal lineages in the three germination conditions. On the opposite to CpG and CHG methylation, the methylation rate of DMR CHH decreased in stressed maternal lineages. In water conditions, germination did not modify the methylation rates of CHH DMRs. However, CHH DMR had even lower methylation rates in 3S seeds subjected to water stress during their germination. This could be observed in every genomic region.

The position of CHH DMR in each part of the genome was not a random phenomenon: 75-76% of CHH DMR were found in non-coding regions, 9% in promoters, 6% in gene bodies and 9-10% in post CDS. We observed these ratios in all dry, water and water stress conditions. The Fisher enrichment tests (Fig. 43i.) showed CHH DMR were enriched in the coding part of the genome and in promoters.

DEG between 3S and 3W seeds co-localized with CHH DMR in 63% in dry seeds, 66% during germinating seeds in water and in 77% of cases during germination in water stress.

The Venn plot in Fig 43.m.n.o, shown the number of DMR in promoter (m), gene body (n), region post CDS (o) in the different germinative stress. Whatever the genome position, the majority of CHH DMRs colocalizing with DEG are specific to seed conditions.



	DRY		WATER		PEG	
CHH	p-valu	Odd ratio	p-valu	Odd ratio	p-valu	Odd ratio
prom	4.20E-01	0.39	2.44E-09	0.47	1.15E-01	0.49
gene	2.81E-02	0.29	8.04E-06	0.24	2.36E-07	0.30
post cds	2.65E-02	0.51	4.01E-02	0.49	4.14E-03	0.52
TE	1.03E-03	0.45	4.70E-04	0.60	2.91E-04	0.45

Figure 43 : Study of CHH differentially methylated regions.

Study of CHH differentially methylated regions (DMR) between W & S maternal lineages. (a) Violon plot representations of DMR methylation rate in the entire genome, (b) in promoter, (c) in gene body, (d) in post CDS, and (e) in transposable elements. Proportion of DMRs according to the different genomic regions in different conditions : (f) dry seeds, (g) water imbibition, and (h) water stress (-1MPa PEG imbibition). (i) Fisher test results for DMR enrichment in different part of genome. Proportion of DEG with DMR in the different genomic regions and conditions: (j) in dry seeds, (k) water imbibition, and (l) water stress PEG -1.0 MPa). Venn diagrams of CpG DMRs in promoter, gene body or post CDS of DEG in different conditions: (m) dry seeds, (n) water imbibition, and (o) water stress (PEG -1.0 MPa).

3.5 Looking for types of DMR controlling maternally acquired tolerance to water stress

Correlations between gene expression differences between 3S and 3W and DMR methylation rates according to their positions (promoter, gene body or post terminal sequence) allowed to study whether some specific DNA modification could account for gene expression response to maternal stress.

We analyzed potential correlations using biplot graphs from PCA (Fig. 44). When taking all DMR types and all genomic regions (promoter, gene body and termination) in the PCA, we observed a negative correlation of terminal CHG DMR with gene expression (Fig. 44). In other conditions, no correlation was observed, since expression did not participate in the first two PCA axis. When looking at methylation types separately, we did not observe any consistent patterns over the different conditions. CpG DMR and gene expression were negatively correlated in terminal regions in dry seeds, in gene bodies in water conditions, and in promoters in water stress condition, but positively correlated in terminal regions in water stress conditions (Fig. 44). For CHG, the correlation was positive in gene body, and promoter in dry seeds and water conditions respectively and negative in promoters in water stress condition (Fig. 44). For CHH it was negative in gene body in dry seeds and in promoter in water conditions (Fig. 44). Overall, we were not able to identify a role of a specific methylation types and of particular gene regions in the control of gene expression following maternal stress.

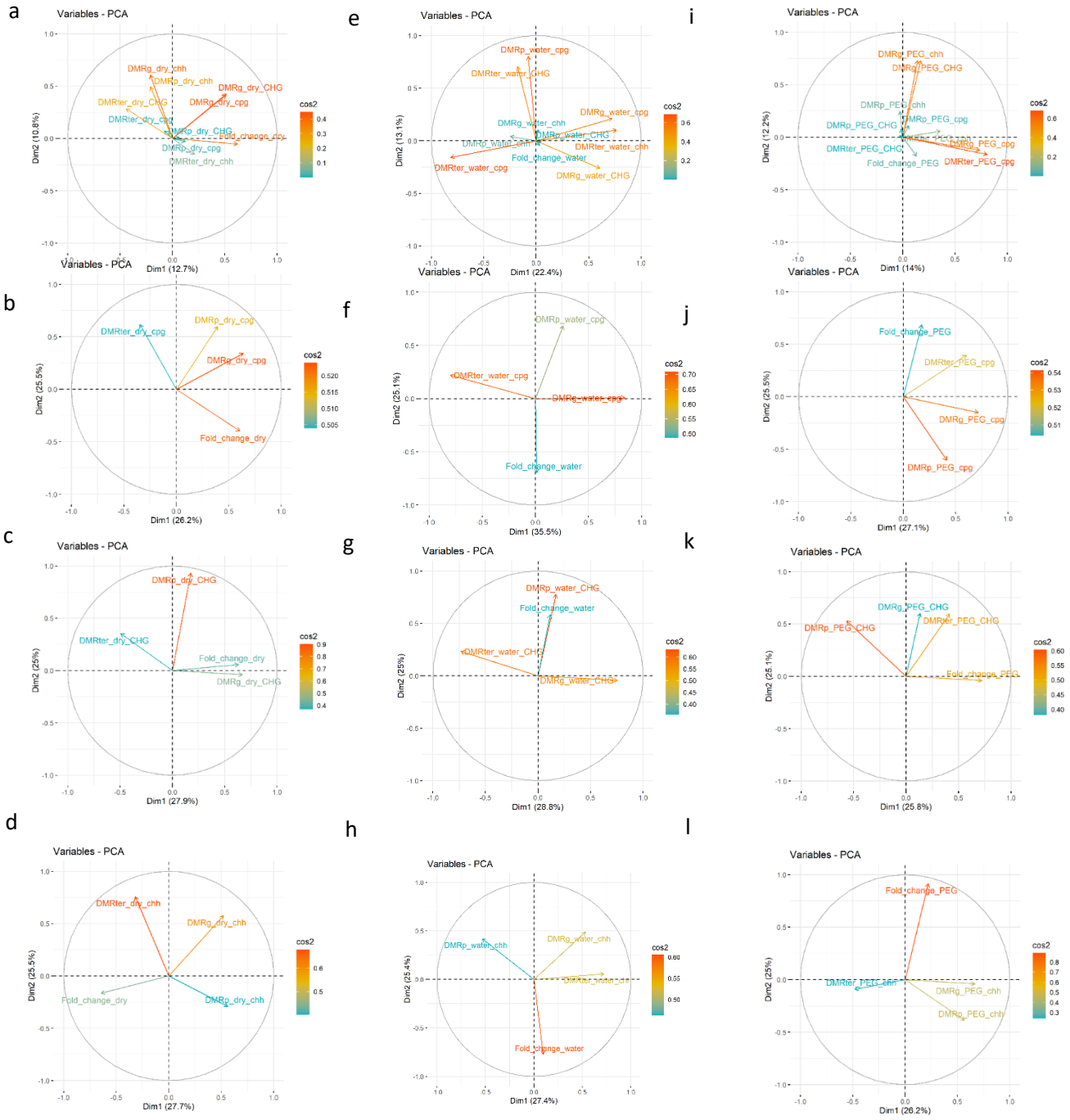


Figure 44 : Correlation study. Biplot graphs including the gene expression differences between 3S and 3W (Fold change) and methylation rate differences in the different genomic regions: promoter (labeled p), gene body (labeled g) and post CDS (labeled ter). Seeds in the following conditions: (a, d, g, j) dry seeds, germinating seeds in water (b, e, h, k), and water stress PEG -1MPa (c, f, i, l). Methylation types considered: (a-c) all types, (d-f) CpG only, (g-i) CHG only, and (j-l) CHH only.

3.6 Selection and validation of candidate DMR by McrBC qPCR

To validate DMRs using an independent technique and on samples produced in agronomical conditions, we selected 17 DMRs (Table 4) associated to maternal lineages, that colocalized with DEG in water stress conditions after 24 h. Selected DEG belong to different cellular mechanisms involved in stress tolerance, like hormone pathways (ABA, GA and ethylene), and ROS regulation. These DEG were up or down regulated during PEG imbibition. For example probable phosphatase 2C 38 was more expressed 30.25 times in S seed than in W seeds. The DMR were chosen to be hypo- or hyper-methylated, for example probable phosphatase 2C 38 was hypo methylated in its promoter for CHG methylation for 3W seeds and hyper methylated for CHH methylation type.

Before genetic analysis we validated the seed acquired tolerance of S seeds (Supp. Fig 13). The S seeds produced in Romania under water stress condition had a better tolerance to water stress during germination (PEG -0.7MPa) than the XRQ seeds product in France in well-watered condition.

3.7 Validation of DMR candidate by McrBC qPCR

We used the endonuclease McrBC (restriction enzymes from Escherichia coli K12 chromosome) which has the the ability to cleave DNA with 5-hydroxymethylcytosine, 5-methylcytosine or 4-methylcytosine preceded by a purine thus allowing the possibility to check the presence or absence of DNA methylation in a selected locus (Fouse et al., 2010; Raleigh, 1992, p. 12).

The McrBC qPCR protocol on sunflower seeds was technically validated in 15 out of 17 candidate DMR candidate (Table 4) by removing non-specific amplification and higher amplification of digested samples. For the 15 other candidates, we tested if the methylation rate was controlled by the seed origin and or the germination stress using a linear model.

Table 4 : Liste of candidate DMR

Liste of candidate DMR used for McBc QPCR validation. a. The DMR candidate was all localized in promoter of differential gene expression (DEG) during PEG imbibition. b. the level of methylation and the diferance of proportion ax C methylated in in region selected. c. Summary of results of McrBc qPCR

expression DEG cadidate in PEG imbibition				
Name	Gene function	AT	Fold change (3Wseed -3S seed)	P.value
HanXRQChr01g0027041	probable phosphatase 2C 38	AT5G66080	-30.25	8.37E-14
HanXRQChr03g0074691	Auxin repressed kDa	AT1G56220	2.02	8.82E-08
HanXRQChr03g0061291	ABC transporter B family member 8	NA	2.93	1.05E-07
HanXRQChr05g0156881	ABC transporter F family member 4-like isoform X3	NA	-2.58	4.35E-12
HanXRQChr06g0173671	Ethylene responsive transcription factor RAP2-3	AT3G16770	-4.62	0

HanXRQChr08g0212091	GA repressor DELLA	AT1G66350	2.8	1.95E-08
HanXRQChr11g0350181	Ethylene insensitive 2-like	NA	2.99	5.07E-11
HanXRQChr13g0416691	phosphatase 2C 29	NA	2.53	5.57E-08
HanXRQChr14g0427441	catalase	AT4G35090	2.52	4.51E-11
HanXRQChr15g0470891	1 aminocyclopropane 1 carboxylate oxidase	AT1G05010	-3.78	8.70E-07
HanXRQChr15g0483901	L-ascorbate peroxidase 3, peroxisomal like	NA	-3.28	9.84E-14
HanXRQChr15g0463031	glutaredoxin	NA	2.42	6.11E-09
HanXRQChr15g0484421	alcohol dehydrogenase 1	AT1G77120	-2.13	9.60E-08

b

Methylation in DMR candidate						
Name	Gene function	AT	Methylation type	3W methylation rate	3S methylation rate	DMR (W-S)
HanXRQChr01g0027041	probable phosphatase 2C 38	AT5G66080	CHG	0,089	0,813	-0,724
HanXRQChr01g0027041	probable phosphatase 2C 38	AT5G66080	CHH	0,457	0,222	0,235
HanXRQChr03g0074691	Auxin repressed kDa	AT1G56220	CpG	0,75	0,151	0,601
HanXRQChr03g0061291	ABC transporter B family member 8	NA	CHH	0,52	0,634	-0,113
HanXRQChr05g0156881	ABC transporter F family member 4-like isoform X3	NA	CHH	0,60	0,322	0,280
HanXRQChr05g0156881	ABC transporter F family member 4-like isoform X3	NA	CpG	0,43	0,962	-0,528
HanXRQChr06g0173671	Ethylene responsive transcription factor RAP2-3	AT3G16770	CHH	0,51	0,357	0,152
HanXRQChr08g0212091	GA repressor DELLA	AT1G66350	CHH	0,43	0,316	0,110
HanXRQChr11g0350181	Ethylene insensitive 2-like	NA	CHH	0,39	0,209	0,178
HanXRQChr13g0416691	phosphatase 2C 29	NA	CHH	0,34	0,466	-0,129
HanXRQChr13g0416691	phosphatase 2C 29	NA	CHH	0,27	0,476	-0,202
HanXRQChr14g0427441	catalase	AT4G35090	CHH	0,31	0,602	-0,297
HanXRQChr15g0470891	1 aminocyclopropane 1 carboxylate oxidase	AT1G05010	CHH	0,65	0,503	0,152
HanXRQChr15g0483901	L-ascorbate peroxidase 3, peroxisomal like	NA	CHH	0,42	0,128	0,295
HanXRQChr15g0463031	glutaredoxin	NA	CHH	0,53	0,088	0,444
HanXRQChr15g0484421	alcohol dehydrogenase 1	AT1G77120	CHH	0,31	0,441	-0,133

C

Validation by McBc QPCR				
Name	Gene function	AT	Technical Validation by McBcr QPCR	Biological validation by McBc QPCR
HanXRQChr01g0027041	probable phosphatase 2C 38	AT5G66080	Yes	Yes
HanXRQChr01g0027041	probable phosphatase 2C 38	AT5G66080	No	No
HanXRQChr03g0074691	Auxin repressed kDa	AT1G56220	Yes	No
HanXRQChr03g0061291	ABC transporter B family member 8	NA	Yes	No
HanXRQChr05g0156881	ABC transporter F family member 4-like isoform X3	NA	Yes	No
HanXRQChr05g0156881	ABC transporter F family member 4-like isoform X3	NA	Yes	No
HanXRQChr06g0173671	Ethylene responsive transcription factor RAP2-3	AT3G16770	Yes	No
HanXRQChr08g0212091	GA repressor DELLA	AT1G66350	Yes	No
HanXRQChr11g0350181	Ethylene insensitive 2-like	NA	Yes	No
HanXRQChr13g0416691	phosphatase 2C 29	NA	Yes	Yes
HanXRQChr13g0416691	phosphatase 2C 29	NA	Yes	Yes
HanXRQChr14g0427441	catalase	AT4G35090	Yes	No
HanXRQChr15g0470891	1 aminocyclopropane 1 carboxylate oxidase	AT1G05010	Yes	No
HanXRQChr15g0483901	L-ascorbate peroxidase 3, peroxisomal like	NA	Yes	No
HanXRQChr15g0463031	glutaredoxin	NA	No	No
HanXRQChr15g0484421	alcohol dehydrogenase 1	AT1G77120	Yes	No

We confirmed 3 significantly different methylated regions in seeds produced in contrasting fields and another only significantly differentially methylated according to germination

conditions (Fig. 45). The DMR linked to *HanXRQChr01g002704* (phosphatase 2C 38) in chromosome 1 between 141,046,209 and 141046500bp was significantly un-methylated in S seeds during imbibition on PEG.

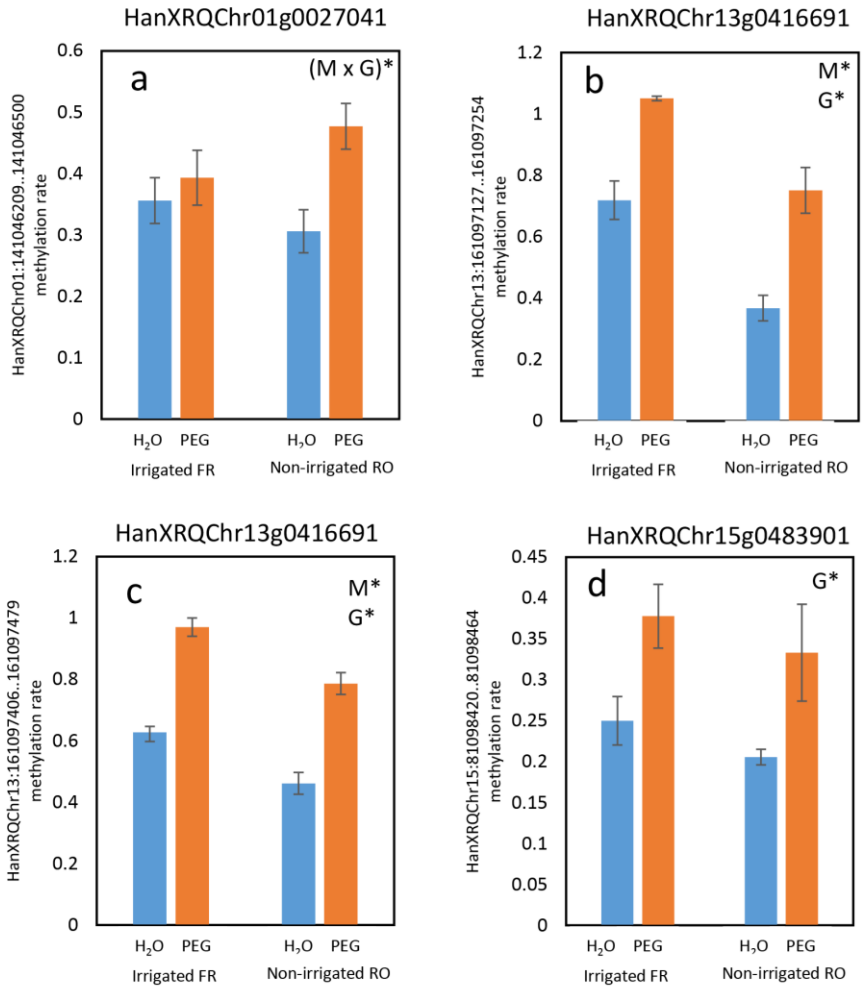


Figure 45 : Un-methylation rates of four DMR validated.

Un-methylation rates of four DMR in germinating seeds produced in agronomical conditions using McrBC qPCR. Test results using a linear model for the maternal lineage effect (M), the germination condition effect (G) and their interaction (MxG) are presented when significant. Means and standard errors of 3 replicates of 30 seeds.

In the promoter of *HanXRQChr13g0416691* (phosphatase 2C 29), 2 DMR could be tested: one CHH DMR in chromosome 13 between 161,097,127 and 161,097,254bp and the other CHH DMR between 161,097,406 and 161,097,479bp. They were both hypomethylated during germination in water stress but maternal stress induced higher methylation rates for both (Fig 45 b and c).

CHH DMR in the promoter of *HanXRQChr15g0483901* (L-ascorbate peroxidase 3) in the chromosome 15 between 81,098,420 and 81,098,464bp did not show significant hypo-methylation in S seeds, like observed in the trancriptomic study on Heliaphen seed lots and

like Phosphatase 2C 29 homologue. Indeed, water stress at the germination stage induced a reduction of methylation (Fig 45c).

IV) Discussion

The epigenetic variation caused by the environment can be transmitted to the progeny in plants and mammals (Heard and Martienssen, 2014; Noshay and Springer, 2021), but the interaction of stress tolerance acquisition and epigenetic mark do not make a consensus (Nestler, 2016). In Arabidopsis, transgenerational effects are not a general response to all abiotic stress (Pecinka et al., 2009). But the study of chromatin mechanisms allows to understand their role in adaptation of plants to environmental stress including a mechanism of stress memory (Lämke and Bäurle, 2017). Here, we aimed at explaining the role of epigenetic modifications and specifically of DNA methylation in the abiotic stress tolerance at the germination stage acquired via exposition to drought in the maternal plant during seed development.

4.1 Methylation of specific genomic regions according to maternal stress history

Seed imbibition did not influence the number, the position or the methylation rate of DMR on a global level. This stability of the methylome under water stress has already been described in rice by (W. Wang et al., 2016). Our result concludes that the presence of CHG DMRs was mostly in the coding part of the genome, whereas the CpG DMRs were more homogeneous distributed. This observation is related to the very low proportion of coding sequence in the genome, in fact, three quarters of the sunflower genome being composed of terminal repeat retrotransposons (LTR-RT) (Badouin et al., 2017).

The specificity of DMR CHG and CpG localization close to the coding sequences can be hypothesized to influence gene expression as in *Heterobasidion parviporum* where an increase of CHG methylation reduces TE expression (Zeng et al., 2019).

Water stress during germination did not clearly change the global rate of DMR methylation in our study. This is in accordance with the global stability of plant methylome observed in Arabidopsis even if transgenerational drought stress memory can be observed (Ganguly et al., 2017b). This methylome stability can be explained to maintain genome integrity and TE silencing.

4.2 Relation between Gene Expression and Methylation

Environmental stress, like drought, can influence the DNA methylation patterns in plants (Eichten and Springer, 2015). In our study, 3 generation of maternal drought stress induced numerous DMRs of the three types of methylation. Globally, we observed higher CHG methylation after maternal stress and lower rates of CHH methylation. This CHH pattern is consistent with observation in oak (Dubin et al., 2015; Gugger et al., 2016), where maximal temperature in a climatic gradient in California drove CpG and CHG methylation reductions and drought CHH reduction.

Dubin et al. (2015) showed a global modification of CHH methylation rates when Arabidopsis where grown at 10°C or 16°C, and this was likely linked to CMT2 DNA methylase activity. Similarly, we observed a reduction of CMT2 homologue (*HanXRQChr15g0479131*) expression in drought stress progeny together with a global reduction of CHH methylation in DMRs. Out of the 10 co-regulated genes in our transcriptomic study (Vancostenoble et al., 2022b), we found 3 homologues to O-methyltransferases that were also down-regulated, suggesting that these enzymes could be involved in the reduction of CHH methylation after drought stress in parental plants. In addition, the VRN5 gene homolog (*HanXRQChr16g0526641*), known to be part of the PCR2 complex involved in vernalization was also present in our results. In Arabidopsis this complex is well described as a regulator of histone H3K27me3 modification in FLC gene locus silencing. (Baulcombe and Dean, 2014). In this cluster, we found a Phosphatase 2C homologue (*HanChr01g0027041*) similar to PP2C6 in Arabidopsis that interacts with the histone acetyltransferase GCN5. This suggests a joint regulation of DNA and histone methylation and acetylation to participate in chromatin remodeling necessary for the expression modifications observed between drought-stressed or irrigated lineages.

Our results also confirmed previous results of a lack of clear correlation between the DNA methylation rate of DMRs for any methylation type and gene expression differences according to maternal lineages as in (Van Dooren et al., 2020b). This can be explained because it was limited to DNA methylation and we did not characterize other epigenetic factors such as histone methylation and acetylation, lncRNA, si RNA, therefore, lacking components of the gene expression regulatory system.

4.3 Towards a biomarker based on DNA methylation to characterize a posteriori seed lineage

We confirmed using an independent technique (McrBC-qPCR) and on seed lots produced by breeders in agronomical conditions as a first step towards a biomarker to characterize the drought stress during seed development and therefore the abiotic stress tolerance of germination in seed lots.

The 3 DMRs with maternal lineage differential methylation were used to characterize the tolerance to water stress. They were associated with 3 genes. The Phosphatase 2C homologue (*HanXRQChr01g0027041*), up regulated in 3S , showed with DMR CHG hyper methylated in promoter, as an analogue of FORGETTER2 (FGT2) gene in *Arabidopsis* , which encodes a type of protein 2C phosphatase (PP2C) D-clade (Urrea Castellanos et al., 2020), this gene is likely involved maintaining stress memory, in our biological system. The other Phosphatase 2C homologue (*HanXRQChr13g0416691*) coding phosphatase 2C 29 where to CHH DMR candidate was validated in McrBC qPCR. It is an interesting result because the PP2C was well known and was a kay player on plant abscisic acid (ABA) signal transduction (Fuchs et al., 2013; Rodriguez, 1998). ABA promote dormancy so this up regulation in 3W seed during stress imbibition can explain one part of the tolerance and the better germination of progeny from stressed plants. The last validated DMR was a CHH methylation type in the promoter of *HanXRQChr15g0483901*, genes coding peroxidase like, and the more precise gene annotation indicate analogue of L-ascorbate peroxidase 3. The ascorbate peroxidases (APX), with his various isoforms are found in different plant cells compartments and play an essential role in the control of intracellular reactive oxygen species (ROS) levels. (Ribeiro et al., 2012). APXs have a central role in the hydrogen peroxide detoxifying system in plant cells. APX can use ascorbic acid as the electron donor to detoxifying ROS (H_2O_2) (Shigeoka et al., 2002). Deficiencies of cytosolic APX, in Arabidopsis, can lead to oxidative damages during abiotic stress (Davletova et al., 2005). In Arabidopsis ascorbate peroxidase 3, peroxisomal membrane-bound antioxidants, is dispensable for growth and development (Narendra et al., 2006), and the over expression of Arabidopsis APX3 leads to higher fruit number and seed mass in transgenic tobacco plants (*Nicotiana tabacum L)* under water deficit stress (Yan et al., 2003). This APX3 mediated tolerance could also participate in the higher tolerance in stressed lineages observed in our study.

These DMRs and co-localizing genes, because of their cross-species and cross-environment robustness, constitute high candidates for the development of biomarkers. However, testing other DMRs, connected to tolerance acquisition, and validating them in other seed

batches should be conducted to develop a more robust tool. As various abiotic stresses, aside drought, can promote stress memory or somatic memory through modification of chromatin conformation (Bäurle and Trindade, 2020), it would be interesting to explore the methylation status of these DMRs after heat, cold or nutritive stresses occurring during seed development.

Finally, our results consist in the regulation of TE by methylation. The proportion of CpG & CHG DMRs found in TE are low, when compared to other genomic regions, i.e. these methylation types were statistically more accumulated in gene bodies. This is in agreement with literature where TE methylation down-regulate the transposase expression to limit their expansion in the genome, and maintain genome stability (Bewick and Schmitz, 2017; Seymour and Becker, 2017).

DNA methylation is not the only chromatin conformation regulating mechanism that plays a role in stress memory (Friedrich et al., 2019). As suggested by the transcriptomic analysis, histone modification (methylation and acetylation) should also act synergistically with DNA modification to induce the abiotic stress tolerance in sunflower seeds.

V) Conclusion

Our study reported the first methylome map in sunflower and at a key development stage for this crop, i.e. germination. We characterized an intergenerational memory of the maternal stress in the progeny at the DNA methylome level. This impact was present in dry seeds up to germination in different conditions. This difference of methylation was acquired during the seed development and at a global level drought increased CHG and reduced CHH methylation rates and during germination in water stressing condition CHH DMRs were particularly less methylated. Although no simple relation was identified between methylation and expression level, major genes in epigenetic imprinting, ROS metabolism and hormonal regulation showed both differential expression and different methylation patterns induced by maternal stress. Independent validation confirmed 4 DMRs also differentially methylated in seeds produced in field conditions. This constitutes a first step toward the development of a biological test to identify tolerant seeds. To complete the epigenetic dimension of this intergenerational acquired tolerance, the study of other epigenetic markers remains to be done.

Chapter 5 :

General Discussion

The data obtained in this study provide new knowledge on the physiological, molecular and epigenetic mechanisms involved in the expression of tolerance of germination to abiotic stresses acquired by intergenerational stress memory in sunflower seeds. This study also allowed the development and validation of molecular and epigenetic screening methods and provided a number of candidate biomarkers of tolerance.

From a physiological point of view, we have characterized a broad tolerance to abiotic stresses as well as the parameters of the induction of this tolerance. We have defined the induction window of the acquisition of this germline tolerance and its mechanism of transmission to the progeny. We showed that the application of a stress on the mother plant during the seed maturation phase induces, by inter- and not transgenerational effect, a germline tolerance to abiotic stresses.

Using a range of physiological and molecular approaches we have identified different biological mechanisms explaining this phenomenon. The origin of tolerance to abiotic stresses can be either related to pericarp or embryonic. Indeed, we have proved the primordial role of the pericarp. A complementary study of its metabolic composition should allow us to determine whether some compounds could play a role in oxygen permeability of the pericarp. A study of the oxidative status of the seeds, as regulated by oxygen diffusion through the pericarpwould provide answers on the role of ROS in this mechanism. The role of the pericarp in the regulation of gene expression in the embryo will be to address properly which will that require further transcriptomics studies either focusing on candidate genes using microfluidics or by RNA sequencing. This will particularly allow us to understand the influence of oxygen permeability and embryonic redox status on regulation of gene expression.

The metabolic study on embryos have shown an impact of maternal stress on seed biochemical composition. In fact, we have identified 22 metabolites as putative seed vigour markers, including some polyphenols, amino acids and oligosaccharides that can be used to develop biomarkers to predict inter-generationally acquired tolerance to abiotic stress.

In embryos we have shown that the impact of maternal stress on the progeny relied on a transcriptional memory. The validation of tolerance to different abiotic stresses using micro-fluidic qPCR alos allowed to identify a subset of genes that were involved in the inheritance of water stress tolerance. These results appeared to be consistent to previous results reported in other species such as Arabidopsis because these markers belonged to 3 gene classes, which are hormonal regulation, ROS and epigenetic regulation. The epigenetic

regulation of the transmission of the tolerance was further studied by DNA methylation sequencing. Despite the small amount of differentially methylated and differentially expressed genes, we have validated in agronomic conditions a method for identifying methylation. Four epigenetic markers characterizing abiotic stress tolerance in seeds were identified and they can be promising markers to predict tolerance to abiotic stresses in seeds. The near future prospects of these techniques bring new solutions for the screening of genetic and epigenetic markers to abiotic stresses in routine, and furthermore this method can be used to select other markers for other abiotic stresses (hypoxia, salt, cold, accelerated aging).

The perspectives offered by these results are the study of other epigenetic mechanisms underlying drought stress memory. In particular it will be interesting to study the role of chromatin compaction and of histone variants. An in-depth study of transcription factors would also bring additional insights to this work. These molecular mechanisms could explain the expression of key tolerance genes and improve our predictive capacities.

Further studies dealing with the cellular signaling of ROS and hormones would be interesting perspectives to refine the understanding of this phenomenon. We could determine the content of major antioxidants (thiols, tocopherols, ascorbate) in seeds of the different samples of the study. Additional measurements of NAD and NADP, in the reduced and oxidized states, would also allow us to determine the role of the redox state and pyridine nucleotides in seed vigor. From a genetic point of view, the study of the impact of stress in the mother plant on a larger population should be developed to verify the diversity of lines likely to acquire tolerance. The SAM core-collection population created by USDA, UGA Athens and UBC Vancouver, sequenced and genotyped with 271 lines (maintainer and restorer lines) could be tested. The identification of genomic regions controlling the transmission of the trait (GWAS) would be feasible in collaboration with UGA Athens who has developed the biostatistical pipelines for this population.

From an applied point of view, the perspectives of this thesis work are to continue the transfer of knowledge for seed production in the fields of private companies. The validation of the phenomenon on numerous genotypes and on hybrid plants is very encouraging. Moreover, the prospect of testing the vigor of the seeds produced in the field would be a complementary way to validate the technical itinerary. Extending the diversity of tested lines of high agronomic interest and refining the technical itinerary of seed production would be a feasible short-term perspective. In the longer term, the different genetic and epigenetic markers could help seed companies to breed varieties more tolerant to abiotic stresses and

the molecular markers could be used for coating or priming tests. All together, this would participate to the development of more resilient crop production and maintain agricultural practices and services in the context of climate change.

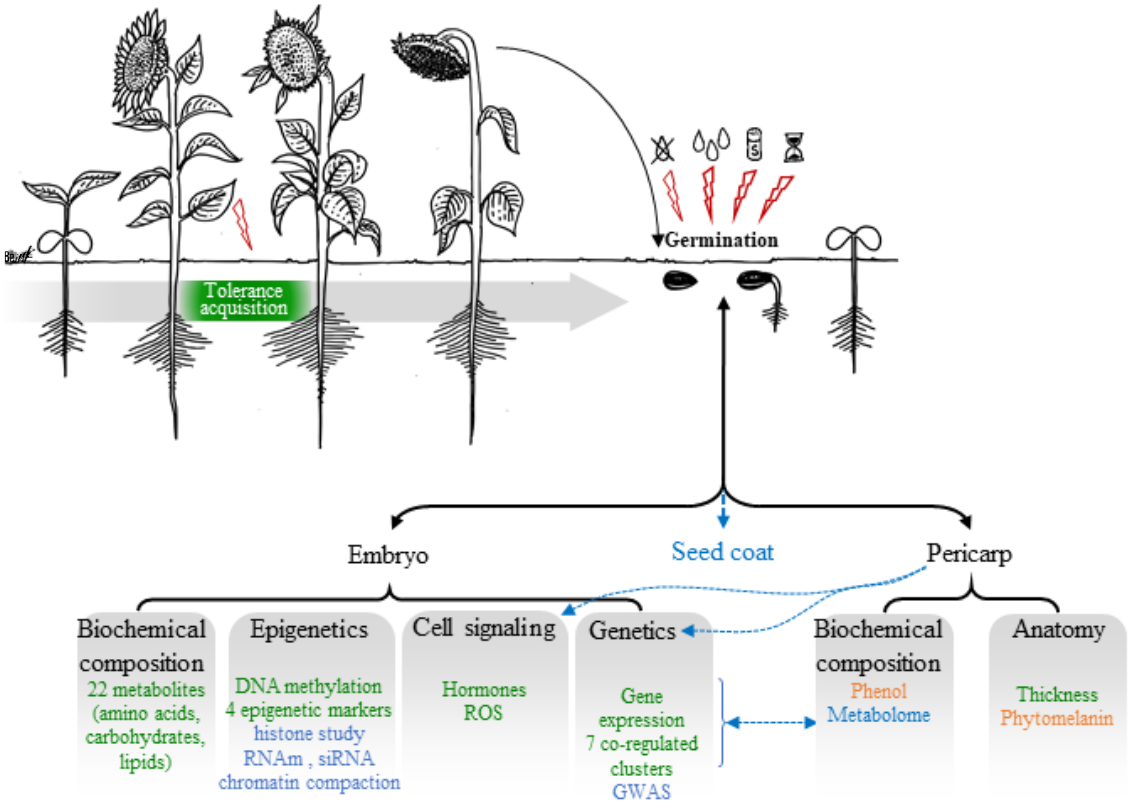


Figure 46: Scheme of Inter-generational tolerance acquisition in sunflower seeds to abiotic stress stress and seed aging) during germination. The validated hypothesis are indicated in green, the refuted ones in orange and the perspectives in blue. (*Beink* ©)

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Annexes

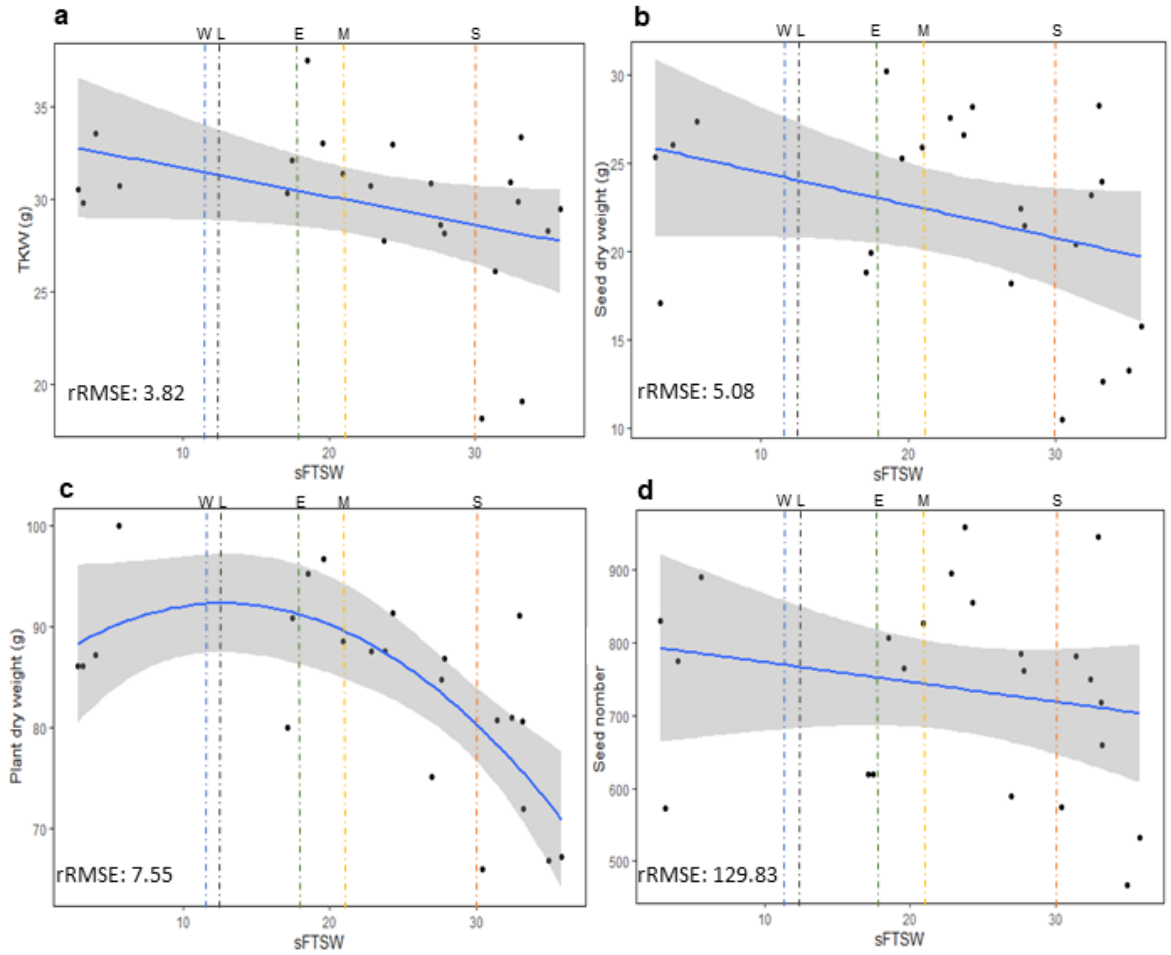

Fig supplemental data 1 : Agronomic results.

Effect of water stress (expressed in SFTSW) on (a) Thousand-kernel weight (TKW), (b) Seed weight (per plant), (c) Plant biomass and (d) seed number per plant. W, well-watered condition; S, drought stress during entire seed development; E (Early), M (Middle), and L (Late) application of drought stress are indicated by the dotted lines on the graphs. Each dot represents an individual plant. rRMSE, relative root means square error of the polynomial ratio model

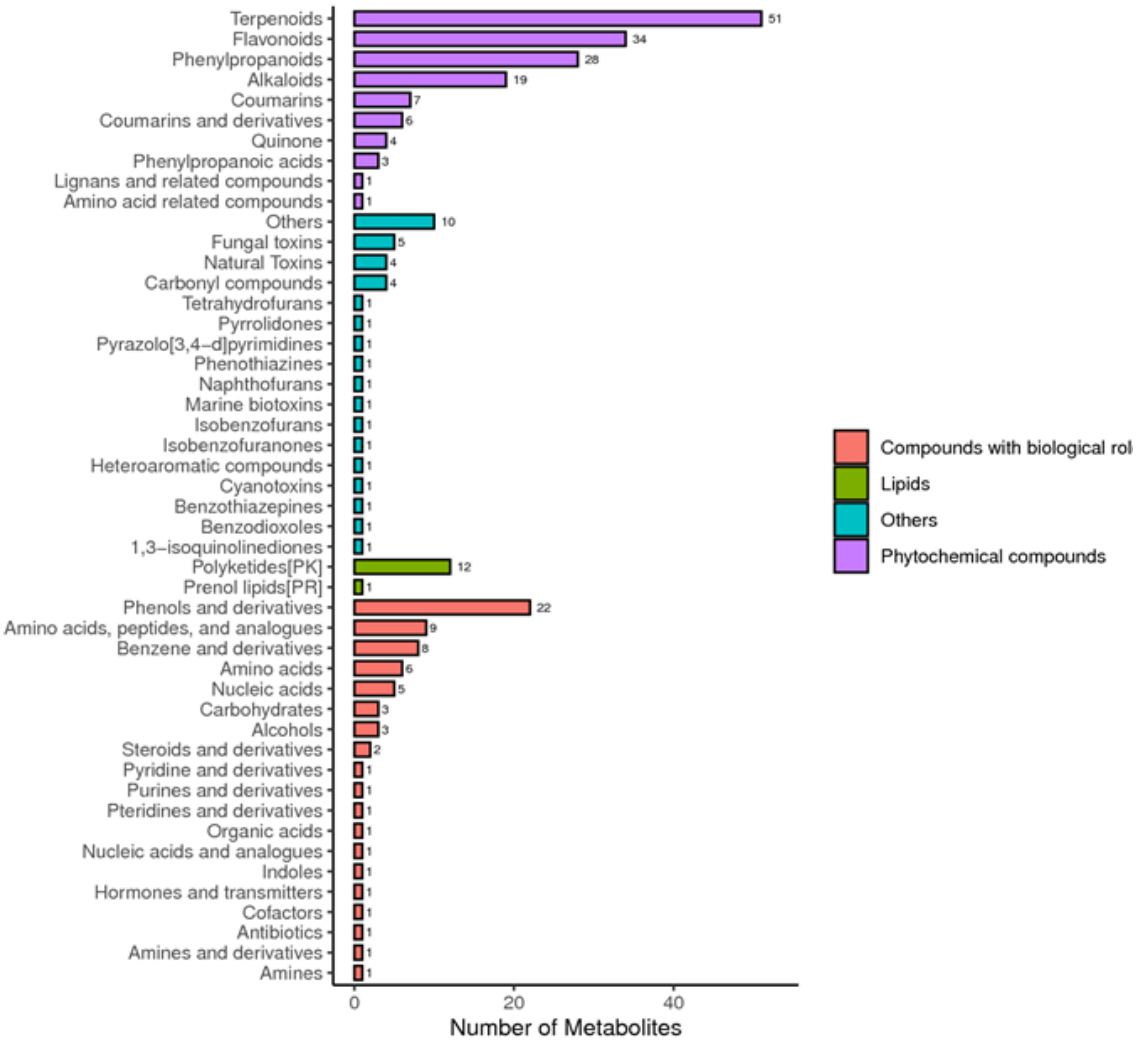

Fig supplemental data 2 : Clustering of metabolites identified using positive ion mode.

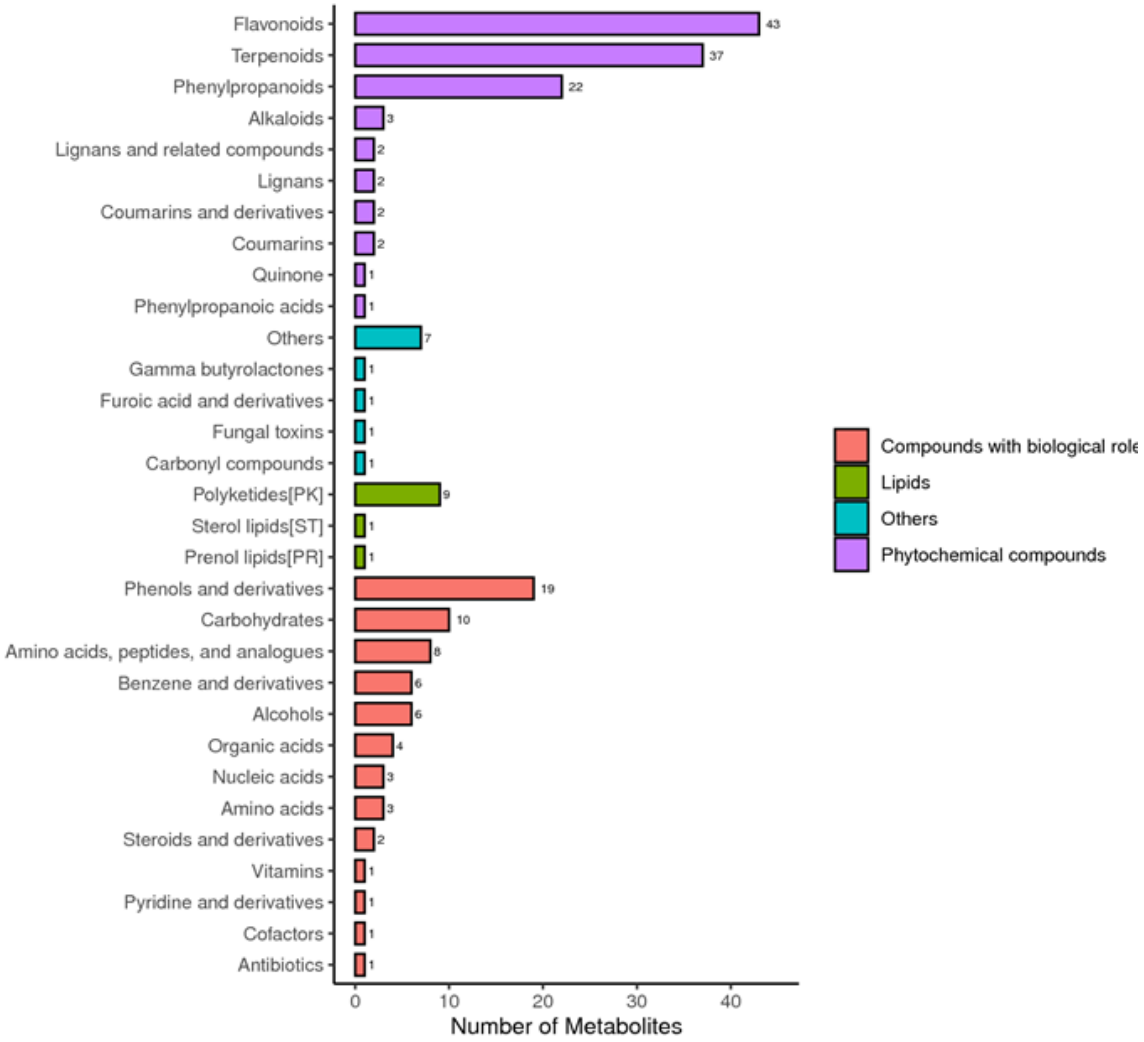

Fig supplemental data 3 : Clustering of metabolites identified using negative ion mode.

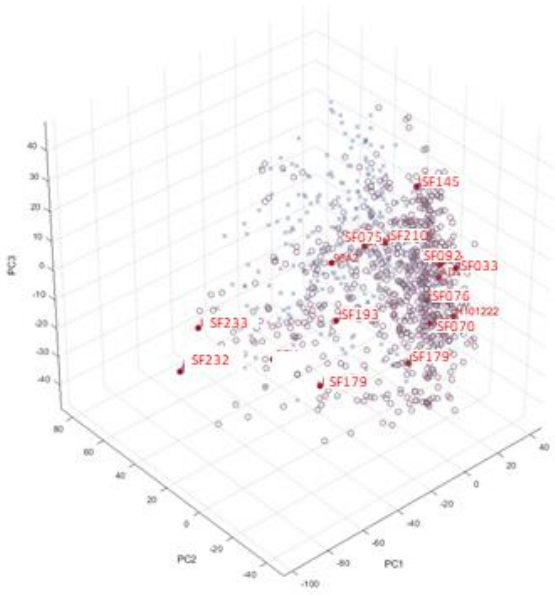

Fig supplemental data 4 : Genetic diversity of Sunflower.

PCA of genetic diversity in sunflower presnet in the database AXIOM50K with includes 1054 line of INRAe. Each point represents a sunflower line; cross symbols are restorer lines and circles are maintainer lines. The lines studied in 2018 at Heliaphen platform are shown in red. The phylogenetic difference between genotypes is represented by the distance between two points.

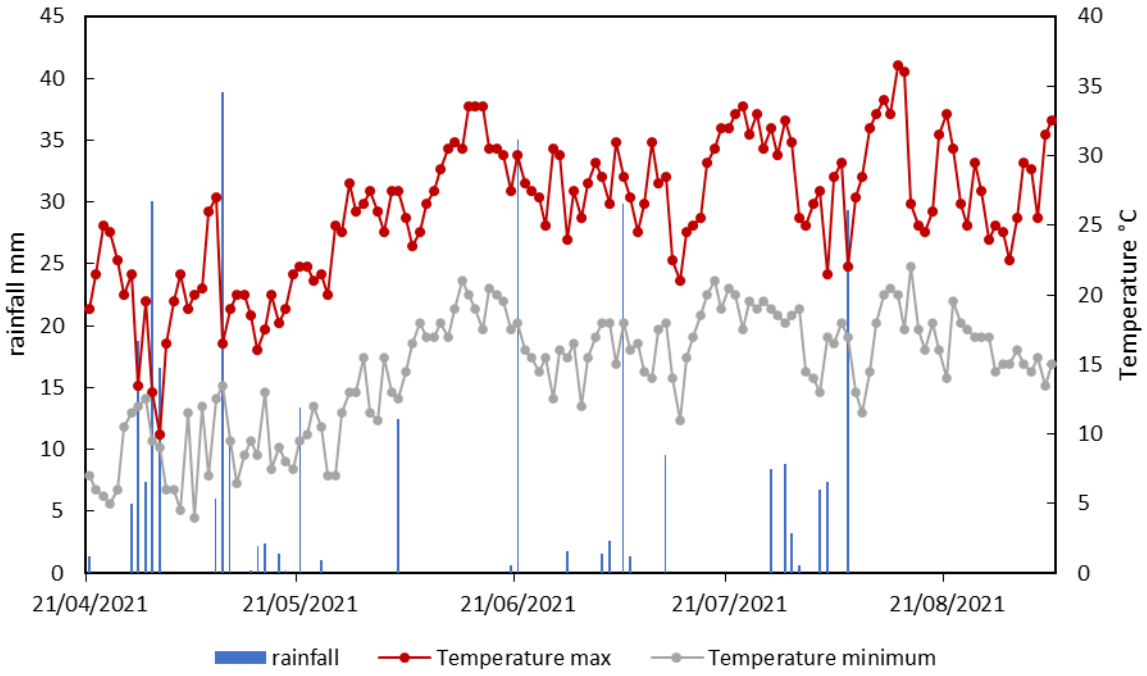

Fig supplemental data 5 : Climatic data.
Meteorological record of the Syngenta production site in Montélimar. Rainfall, maximum and minimum temperatures per day are shown.

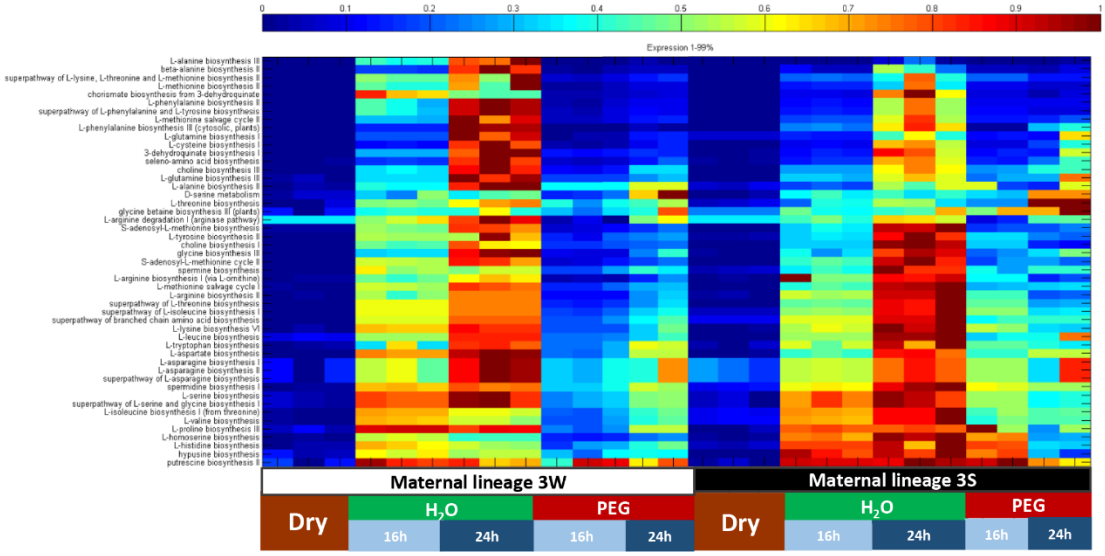

Fig supplemental data 6 : Pathifier result.

Effect of water imbibition and water stress on pathway deregulation. Pathways are specifically deregulated by water imbibition or water stress (Peg imbibition −0.1 MPa) after 16h or 24H of imbibition at 20°C in seeds of 3W and 3S. The control condition is the dry condition. Pathifier heat map of The Amino acid and Protein Metabolism a.

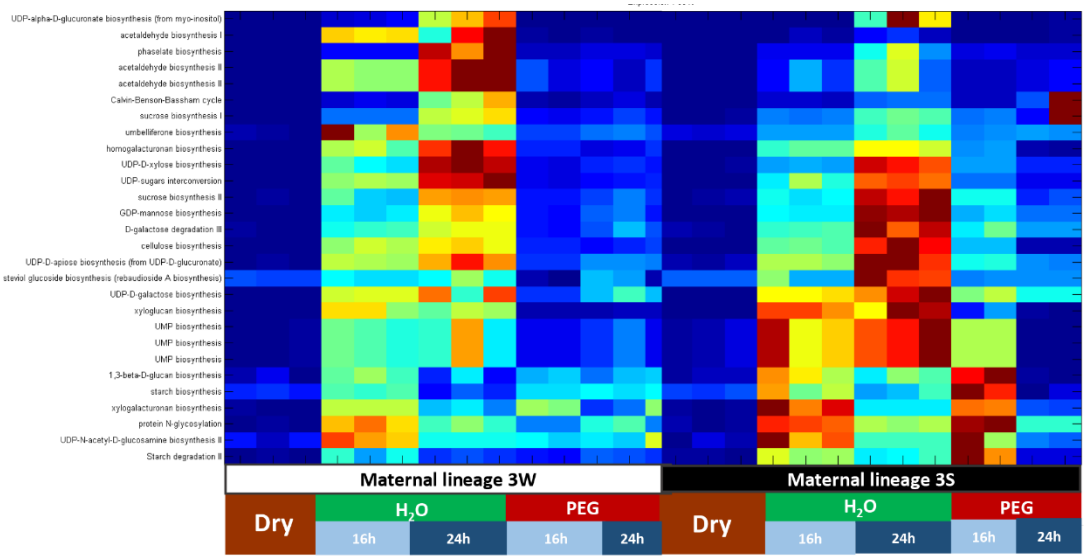

Fig supplemental data 6 b.Pathifier heat map of Carbohydrate Metabolism

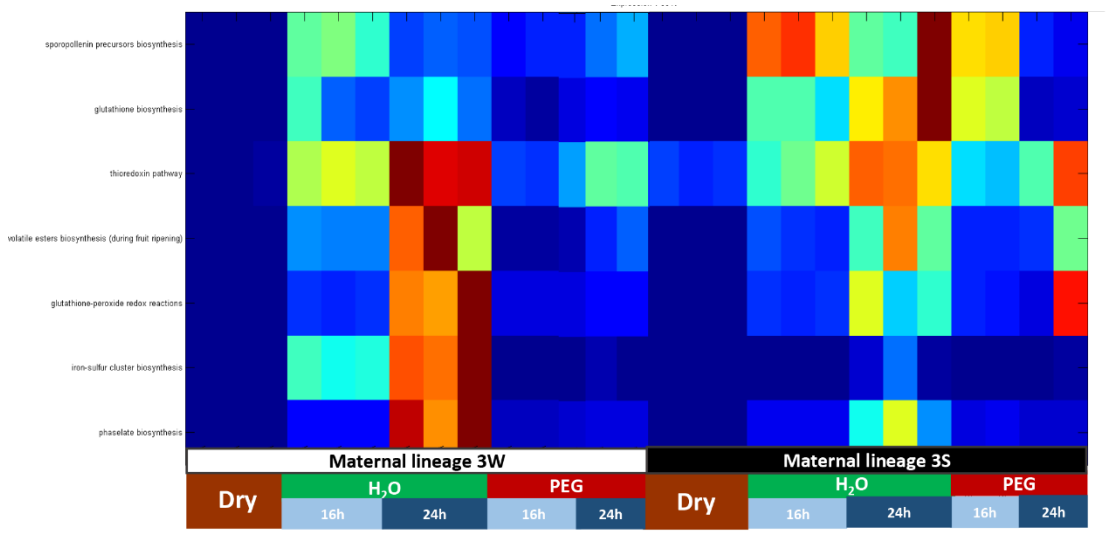

Fig supplemental data 6 c. Pathifier heat map of cell function and Sulfur Metabolism

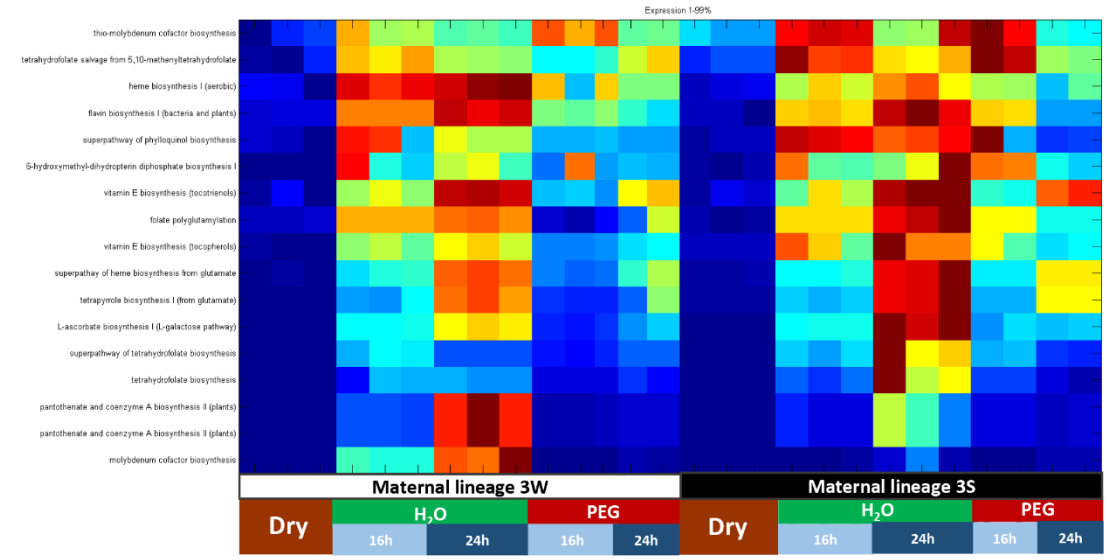

Fig supplemental data 6 d. Pathifier heat map of Cofactor-Vitamin Metabolism

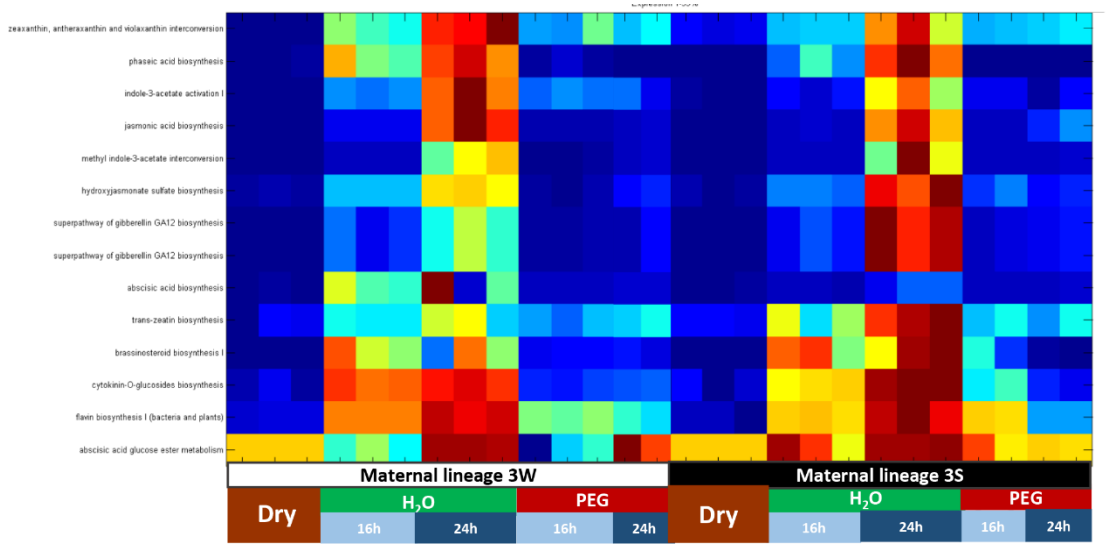

Fig supplemental data 6 e. Pathifier heat map of Cofactor-Vitamin Metabolism

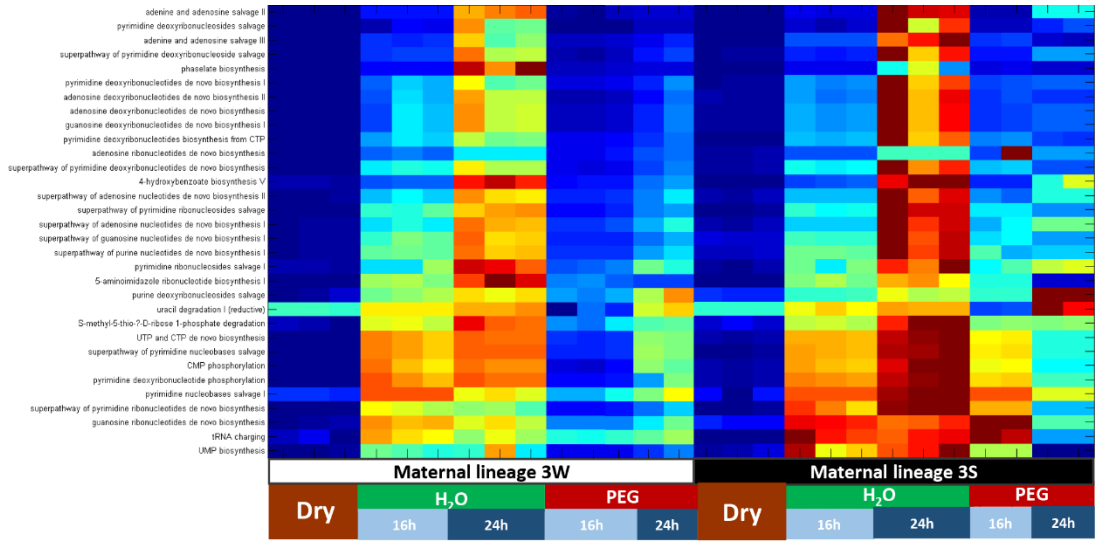

Fig supplemental data 6 f Pathifier heat map Pathifier heat map Lipid Metabolism

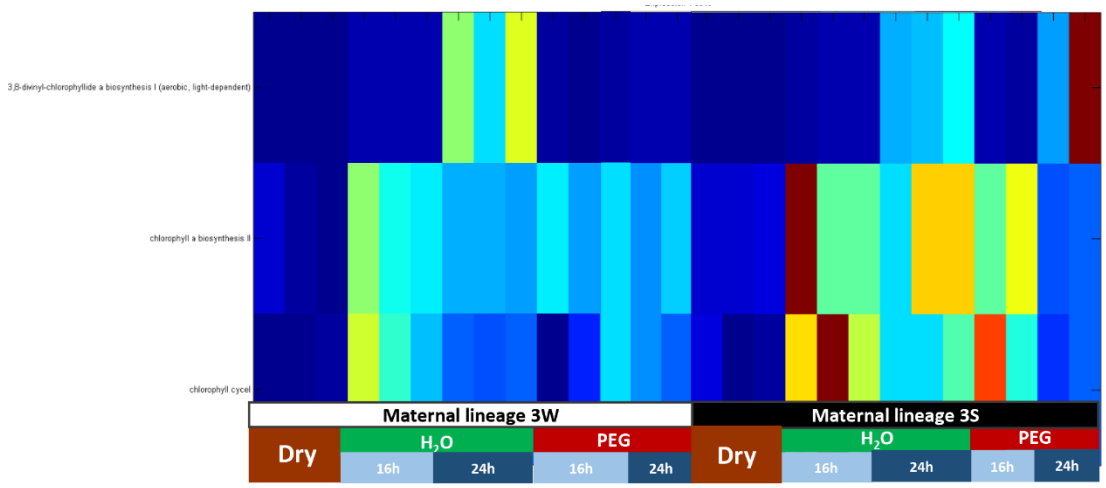

Fig supplemental data 6 gPathifier heat map Pathifier heat map Nucleic acid Metabolism

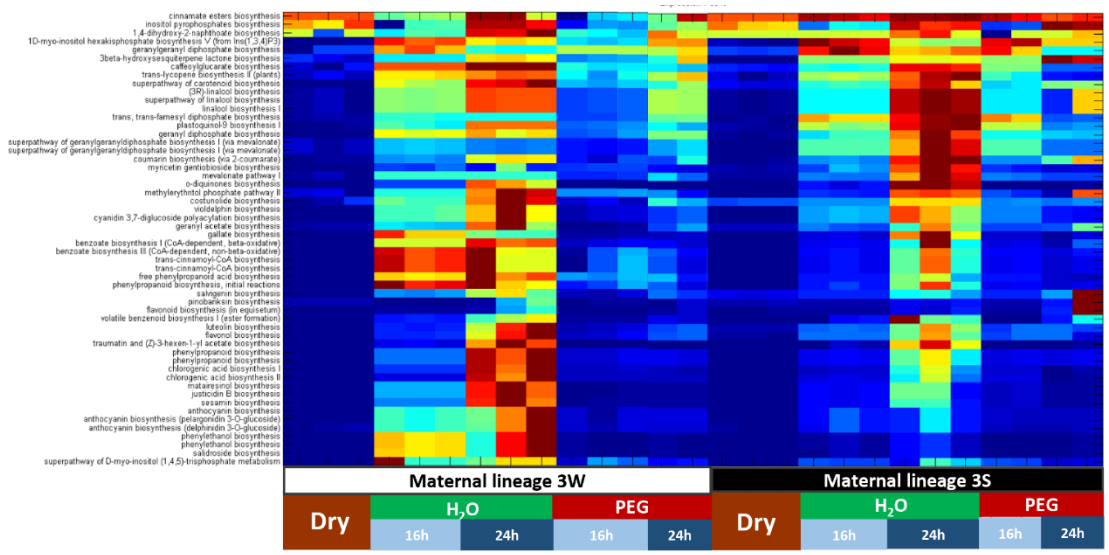

Fig supplemental data 6 i Pathifier heat map Patifier heat map orf Secondary Metabolism

GO name	Description of GO
GO:0043086	"negative regulation of catalytic activity"
GO:0051248	"negative regulation of protein metabolic process"
GO:0032787	"monocarboxylic acid metabolic process"
GO:0030162	"regulation of proteolysis"
GO:0006082	"organic acid metabolic process"
GO:0019511	"peptidyl-proline hydroxylation"
GO:0018126	"protein hydroxylation"
GO:0034599	"cellular response to oxidative stress"
GO:0015749	"monosaccharide transmembrane transport"
GO:0010286	"heat acclimation"
GO:0042221	"response to chemical"
GO:0009581	"detection of external stimulus"
GO:0006063	"uronic acid metabolic process"
GO:0052696	"flavonoid glucuronidation"
GO:0008643	"carbohydrate transport"
GO:0009410	"response to xenobiotic stimulus"
GO:0009812	"flavonoid metabolic process"
GO:0010035	"response to inorganic substance"
GO:0017006	"protein-tetrapyrrole linkage"
GO:0006801	"superoxide metabolic process"
GO:0006833	"water transport"
GO:0042044	"fluid transport"
GO:0015695	"organic cation transport"
GO:0015791	"polyol transport"
GO:0045338	"farnesyl diphosphate metabolic process"
GO:0009638	"phototropism"

Fig supplemental data 7 : REVIGO of DEG up xpressed in PEG imbibition
DEG of 3W 3S seeds calculater by RNAseq

TermID	Name
GO:1901566	"organonitrogen compound biosynthetic process"
GO:0043603	"cellular amide metabolic process"
GO:0006412	"translation"
GO:0044271	"cellular nitrogen compound biosynthetic process"
GO:0010467	"gene expression"
GO:0044249	"cellular biosynthetic process"
GO:0009058	"biosynthetic process"
GO:0009059	"macromolecule biosynthetic process"
GO:0034641	"cellular nitrogen compound metabolic process"
GO:0042254	"ribosome biogenesis"
GO:0044085	"cellular component biogenesis"
GO:0071840	"cellular component organization or biogenesis"
GO:0044267	"cellular protein metabolic process"
GO:1901564	"organonitrogen compound metabolic process"
GO:0002181	"cytoplasmic translation"
GO:0019538	"protein metabolic process"
GO:0006970	"response to osmotic stress"
GO:0009651	"response to salt stress"
GO:0043933	"protein-containing complex subunit organization"
GO:0009853	"photorespiration"
GO:0016072	"rRNA metabolic process"
GO:0006090	"pyruvate metabolic process"
GO:0034622	"cellular protein-containing complex assembly"
GO:0046939	"nucleotide phosphorylation"
GO:0043094	"cellular metabolic compound salvage"
GO:0009132	"nucleoside diphosphate metabolic process"
GO:0042866	"pyruvate biosynthetic process"
GO:0006096	"glycolytic process"
GO:0016052	"carbohydrate catabolic process"
GO:0007030	"Golgi organization"
GO:0009166	"nucleotide catabolic process"
GO:0046490	"isopentenyl diphosphate metabolic process"
GO:0044260	"cellular macromolecule metabolic process"
GO:0019359	"nicotinamide nucleotide biosynthetic process"
GO:0030490	"maturation of SSU-rRNA"
GO:0044237	"cellular metabolic process"
GO:0010411	"xyloglucan metabolic process"
GO:0072524	"pyridine-containing compound metabolic process"
GO:0006833	"water transport"
GO:0042044	"fluid transport"
GO:0009201	"ribonucleoside triphosphate biosynthetic process"
GO:0046434	"organophosphate catabolic process"
GO:0010256	"endomembrane system organization"
GO:0034404	"nucleobase-containing small molecule biosynthetic process"
GO:0090407	"organophosphate biosynthetic process"
GO:0045727	"positive regulation of translation"
GO:0034250	"positive regulation of cellular amide metabolic process"
GO:0046034	"ATP metabolic process"
GO:0009141	"nucleoside triphosphate metabolic process"
GO:0006091	"generation of precursor metabolites and energy"
GO:0008283	"cell population proliferation"
GO:0044238	"primary metabolic process"
GO:0006534	"cysteine metabolic process"
GO:0034660	"ncRNA metabolic process"
GO:0031507	"heterochromatin assembly"
GO:0006807	"nitrogen compound metabolic process"
GO:0009126	"purine nucleoside monophosphate metabolic process"

Fig supplemental data 8 : REVIGO of DEG down expressed in PEG imbibition
DEG of 3W 3S seeds calculater by RNAseq

TermID	Name
GO:0009628	"response to abiotic stimulus"
GO:0019637	"organophosphate metabolic process"
GO:0009069	"serine family amino acid metabolic process"
GO:0008535	"respiratory chain complex IV assembly"
GO:0009987	"cellular process"
GO:0043170	"macromolecule metabolic process"
GO:0018198	"peptidyl-cysteine modification"
GO:0006452	"translational frameshifting"
GO:0043243	"positive regulation of protein-containing complex disassembly"
GO:0000096	"sulfur amino acid metabolic process"
GO:0009658	"chloroplast organization"
GO:0046394	"carboxylic acid biosynthetic process"
GO:0072521	"purine-containing compound metabolic process"
GO:2000377	"regulation of reactive oxygen species metabolic process"
GO:0006081	"cellular aldehyde metabolic process"
GO:1901605	"alpha-amino acid metabolic process"
GO:0080129	"proteasome core complex assembly"
GO:0006094	"gluconeogenesis"
GO:0019755	"one-carbon compound transport"
GO:0009657	"plastid organization"
GO:0006720	"isoprenoid metabolic process"
GO:0051788	"response to misfolded protein"
GO:0006448	"regulation of translational elongation"
GO:0071704	"organic substance metabolic process"
GO:0000079	"regulation of cyclin-dependent protein serine/threonine kinase activity"
GO:0055086	"nucleobase-containing small molecule metabolic process"
GO:0043244	"regulation of protein-containing complex disassembly"
GO:0051644	"plastid localization"
GO:0006624	"vacuolar protein processing"
GO:0006529	"asparagine biosynthetic process"
GO:0006528	"asparagine metabolic process"
GO:0001510	"RNA methylation"
GO:0044283	"small molecule biosynthetic process"
GO:0034248	"regulation of cellular amide metabolic process"
GO:0022411	"cellular component disassembly"
GO:0072488	"ammonium transmembrane transport"
GO:0031125	"rRNA 3'-end processing"
GO:0010628	"positive regulation of gene expression"
GO:0010038	"response to metal ion"
GO:0001666	"response to hypoxia"
GO:0070482	"response to oxygen levels"
GO:0006805	"xenobiotic metabolic process"
GO:0042793	"plastid transcription"
GO:0006450	"regulation of translational fidelity"
GO:0010207	"photosystem II assembly"
GO:0032787	"monocarboxylic acid metabolic process"
GO:0010440	"stomatal lineage progression"
GO:0001708	"cell fate specification"
GO:0000469	"cleavage involved in rRNA processing"
GO:0010310	"regulation of hydrogen peroxide metabolic process"
GO:0009862	"systemic acquired resistance salicylic acid mediated signaling pathway"
GO:0040029	"regulation of gene expression epigenetic"
GO:0045892	"negative regulation of transcription DNA-templated"
GO:0000463	"maturation of LSU-rRNA from tricistronic rRNA transcript (SSU-rRNA 5.8S rRNA LSU-rRNA)"

Fig supplemental data 8 : REVIGO of DEG down expressed in PEG imbibition
DEG of 3W 3S seeds calculater by RNAseq

Name	Fold change in PEG	Description	Hormone cluster	ROS cluster	Epigenet cluster
HanXRQChr08g0227841	-184.67	histone H2A			histone H2A
HanXRQChr11g0339031	-133.41	oxygen-evolving enhancer 3-2, chloroplastic-like			photosystem II assembly/stability (ROS wheel 1)
HanXRQChr13g0414711	-50.91	fructose-bisphosphate aldolase 1, chloroplastic-like			glycolysis and gluconeogenesis (ROS wheel 1)
HanXRQChr08g0228081	-47.55	histone H2A			histone H2A
HanXRQChr16g0511901	-36.77	fusion of histone 2A and enhanced yellow fluorescence			histone 2A
HanXRQChr08g0227831	-35.41	histone H2A			histone H2A
HanXRQChr01g0027041	-30.25	probable phosphatase 2C 38	hormone		
HanXRQChr02g0053081	-27.75	histone H3-like centromeric CSE4			histone H3
HanXRQChr02g0056231	-27.21	histone			histone
HanXRQChr08g0227871	-25.42	Histone H2A			Histone H2A
HanXRQChr15g0464041	-25.2	chlorophyll a-b binding , chloroplastic		ROS wheel 1	
HanXRQChr03g0093791	-19.46	histone			histone
HanXRQChr16g0506911	-19.09	cold and drought-regulated CORA-like		CORA-like	
HanXRQChr08g0223721	-18.51	histone			histone
HanXRQChr13g0394281	-18.25	histone			histone
HanXRQChr07g0196031	-16.39	histone H2A-like			histone H2Alike
HanXRQChr16g0526641	-16.32	orcinol O-methyltransferase			DNA methylation
HanXRQChr13g0424611	-15.69	histone			histone
HanXRQChr13g0403951	-14.66	histone			histone
HanXRQChr01g0016471	-14.5	DNA topoisomerase 2-like			DNA topoisomerase 2like
HanXRQChr02g0054041	-13.54	DNA (cytosine-5)-methyltransferase CMT1			DNA methylation
HanXRQChr11g0339511	-12.95	fusion of histone 2A and enhanced yellow fluorescence			histone 2A
HanXRQChr01g0021111	-11.75	histone			histone
HanXRQChr11g0346491	-11.51	core histone H2A H2B H3 H4			core histone H2A H2B H3 H4
HanXRQChr13g0404011	-10.02	histone			histone
HanXRQChr13g0418771	-9.38	histone			histone
HanXRQChr14g0436081	-8.98	brassinosteroid-regulated BRU1-like	brassinosteroid		
HanXRQChr15g0466941	-8.34	probable histone H2A variant 1			probable histone H2A variant 1
HanXRQChr13g0399211	-8.15	histone			histone
HanXRQChr11g0333251	-7.91	brassinosteroid-regulated BRU1-like	brassinosteroid		
HanXRQChr11g0333271	-7.79	brassinosteroid-regulated BRU1-like	brassinosteroid		
HanXRQChr07g0196041	-7.3	histone H2A-like			histone H2Alike
HanXRQChr11g0329091	-6.68	vignain-like			Hydrolase cysteine-type endopeptidase activity
HanXRQChr08g0218091	-5.52	histone H2B			histone H2B
HanXRQChr09g0260041	-5.18	protochlorophyllide reductase, chloroplastic		ROS wheel 1	
HanXRQChr11g0333301	-5.02	brassinosteroid-regulated BRU1-like	brassinosteroid		
HanXRQChr06g0173671	-4.62	ethylene-responsive transcription factor RAP2-3	ethylene		
HanXRQChr15g0492941	-4.4	O-methyltransferase OMT4			DNA methylation
HanXRQChr16g0526671	-4.17	orcinol O-methyltransferase			DNA methylation
HanXRQChr09g0277201	-4.05	ethylene-responsive transcription factor RAP2-12-like	ethylene		
HanXRQChr15g0470891	-3.78	1-aminocyclopropane-1-carboxylate oxidase	ethylene		
HanXRQChr01g0015301	-3.59	ethylene-responsive transcription factor At4g13040	ethylene		
HanXRQChr09g0253601	-3.54	ubiquitin-conjugating enzyme E2 2-like			UBC2
HanXRQChr04g0118941	-3.36	40S ribosomal S18			ribosome
HanXRQChr02g0036461	-3.35	40S ribosomal S26-1-like			ribosome

Fig supplemental data 9 : List of gene candidate selectioned by RNAseq.
The Fold change of RNAseq in Seeds originate from irrigated plants (W) or drought stressed (S) imbibed in water (H2O) and PEG (-1MPa) .

Name	Fold change in PEG	Description	Hormone cluster	ROS cluster	Epigenet cluster
HanXRQChr10g0305701	-3.22	heme-binding 2		ROS wheel (1)	
HanXRQChr08g0216441	-3.14	catalase isozyme 1		catalase	
HanXRQChr06g0175791	-3.09	histone H2A-like			histone H2Alike
HanXRQChr06g0179891	-2.9	cytochrome b-c1 complex subunit 7-2		ROS wheel (3)	
HanXRQChr16g0526561	-2.67	trans-resveratrol di-O-methyltransferase-like			DNA methylation
HanXRQChr15g0479131	-2.65	DNA (cytosine-5)-methyltransferase CMT3			CMT1 (DNA methylation)
HanXRQChr08g0234341	-2.63	stem-specific TSJT1-like		ROS wheel (2)	
HanXRQChr05g0134281	-2.53	histone H2A-like			histone H2Alike
HanXRQChr17g0563501	-2.39	transcription factor bHLH130-like		AKS3,FBH4 (bHLH family)	
HanXRQChr10g0318861	-2.39	profilin		ROS wheel (3)	
HanXRQChr12g0381881	-2.36	probable NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12		ROS wheel (3)	
HanXRQChr09g0270381	-2.31	SPIRAL1-like 1		ROS wheel (1)	
HanXRQChr05g0142301	-2.26	cytochrome b-c1 complex subunit 7-2		ROS wheel (3)	
HanXRQChr14g0459291	-2.2	histone H2A			histone H2A
HanXRQChr14g0428531	-2.03	histone H4			histone H4
HanXRQChr07g0195371	2.12	Ethylene-insensitive 2	ethylene		
HanXRQChr16g0511231	2.35	mediator of RNA polymerase II transcription subunit 16 isoform X1			SFR6
HanXRQChr08g0214651	2.47	S-adenosylmethionine decarboxylase			MBD6
HanXRQChr01g0027351	2.61	ethylene-insensitive 2	ethylene		
HanXRQChr08g0212091	2.8	GA repressor DELLA	hormone		
HanXRQChr16g0511821	2.81	endoribonuclease Dicer homolog 2			DNA methylation
HanXRQChr15g0484891	2.84	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein		ROS wheel 3	
HanXRQChr17g0563751	2.95	probable histone-lysine N-methyltransferase ATXR3			probable histonelysine Nmethyltransferase ATXR3
HanXRQChr11g0350181	2.99	ethylene-insensitive 2-like	ethylene		
HanXRQChr05g0132631	3.21	heat shock cognate 70 kDa 2-like		ROS wheel 3	
HanXRQChr00c0036g0571221	3.3	abscisic-aldehyde oxidase-like	ABA		
HanXRQChr06g0183501	3.36	argonaute 1			AGO1
HanXRQChr09g0276241	3.37	RNA polymerase II-associated factor 1 homolog			histone modification
HanXRQChr09g0247211	3.6	probable ATP-dependent DNA helicase CHR12			helicase CHR12
HanXRQChr05g0135381	3.68	abscisic acid 8 -hydroxylase 2	ABA		
HanXRQChr13g0409921	3.72	heat shock factor HSF30			HSFA2 (TF family HSF) ROS wheel 3
HanXRQChr05g0154701	3.76	PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1			PIE1
HanXRQChr06g0180121	3.96	VIN3 1			VRN5
HanXRQChr17g0552041	4.59	ethylene-responsive transcription factor ERF118-like	ethylene		
HanXRQChr05g0159091	5.8	DNA polymerase I B, chloroplastic mitochondrial-like			DNA polymerase I
HanXRQChr04g0120231	5.86	histone-lysine N-methyltransferase ATX4-like isoform X1			histonelysine Nmethyltransferase ATX4
HanXRQChr15g0472871	5.87	DNA ligase 1-like			DNA ligase
HanXRQChr06g0178991	5.92	Abscisic acid 8 -hydroxylase 2	ABA		
HanXRQChr06g0167831	6.36	DNA replication regulator dpb11,			DNA replication regulator
HanXRQChr11g0320271	6.56	ethylene-responsive transcription factor ABR1-like isoform X2	ethylene ABA		
HanXRQChr07g0188711	7.16	DNA-directed RNA polymerase III subunit RPC5 isoform X1			DNAdirected RNA polymerase III
HanXRQChr04g0116701	8.89	methyltransferase 13			methyltransferase
HanXRQChr08g0215791	10.07	peroxidase 3		peroxidase	
HanXRQChr15g0474671	18.06	lysine histidine transporter 1-like		ROS wheel 3	
HanXRQChr13g0400451	19.05	UDP-glycosyltransferase 73C3-like		ROS wheel 3	

Fig supplemental data 9 : b. List of gene candidate selectioned by RNAseq.
The Fold change of RNAseq in Seeds originate from irrigated plants (W) or drought stressed (S) imbibed in water (H2O) and PEG (-1MPa) .

Gene Name	RNA seq Fold change	PEG log_2 ddCT	Hypoxia log_2 ddCT	Nacl log_2 delta.dCT	Description	cluster
HanXRQChr13g0400451	19.05	0.260		-0.343	UDP-glycosyltransferase 73C3-like	ROS_cluster
HanXRQChr15g0474671	18.06	0.063		-0.051	lysine histidine transporter 1-like	ROS_cluster
HanXRQChr08g0215791	10.07	0.026			peroxidase 3	hormone_cluster
HanXRQChr04g0116701	8.89	0.099			methyltransferase 13	hormone_cluster
HanXRQChr07g0188711	7.16	0.020			DNA-directed RNA polymerase III subunit RPC5 isoform X1	hormone_cluster
HanXRQChr11g0320271	6.56	0.144			ethylene-responsive transcription factor ABR1-like isoform X2	ROS_cluster
HanXRQChr06g0167831	6.36	0.012	0.009		DNA replication regulator dpb11.	hormone_cluster
HanXRQChr06g0178991	5.92	0.014			Abscisic acid 8 -hydroxylase 2	Epigenet_cluster
HanXRQChr15g0472871	5.87	0.026			DNA ligase 1-like	Epigenet_cluster
HanXRQChr04g0120231	5.86	0.049			histone-lysine N-methyltransferase ATX4-like isoform X1	Epigenet_cluster
HanXRQChr17g0552041	4.59		0.007		ethylene-responsive transcription factor ERF118-like	hormone_cluster
HanXRQChr06g0180121	3.96	0.004			VIN3 1	Epigenet_cluster
HanXRQChr05g0154701	3.76	0.017			PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1	ROS_cluster
HanXRQChr05g0135381	3.68	0.009		-0.007	abscisic acid 8 -hydroxylase 2	hormone_cluster
HanXRQChr09g0247211	3.6	0.015			probable ATP-dependent DNA helicase CHR12	hormone_cluster
HanXRQChr09g0276241	3.37	0.014	0.010		RNA polymerase II-associated factor 1 homolog	Epigenet_cluster
HanXRQChr06g0183501	3.36	0.064			argonaute 1	Epigenet_cluster
HanXRQChr00c0036g0571221	3.3	0.017			abscisic-aldehyde oxidase-like	Epigenet_cluster
HanXRQChr05g0132631	3.21	0.031	0.014		heat shock cognate 70 kDa 2-like	ROS_cluster
HanXRQChr11g0350181	2.99	0.119			ethylene-insensitive 2-like	Epigenet_cluster
HanXRQChr17g0563751	2.95	0.001			probable histone-lysine N-methyltransferase ATXR3	Epigenet_cluster
HanXRQChr15g0484891	2.84	0.003			S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	hormone_cluster
HanXRQChr16g0511821	2.81	0.007			endoribonuclease Dicer homolog 2	hormone_cluster
HanXRQChr08g0212091	2.8	0.005			GA repressor DELLA	Epigenet_cluster
HanXRQChr09g0255961	2.64	0.076			transcription factor GTE8-like isoform X1	Epigenet_cluster
HanXRQChr01g0027351	2.61	0.049			ethylene-insensitive 2	Epigenet_cluster
HanXRQChr03g0071061	2.58	0.050			transcription factor GTE7-like	Epigenet_cluster
HanXRQChr08g0214651	2.47	0.334	0.210		S-adenosylmethionine decarboxylase	ROS_cluster
HanXRQChr16g0511231	2.35	0.031			mediator of RNA polymerase II transcription subunit 16 isoform X1	Epigenet_cluster
HanXRQChr07g0195371	2.12	0.004			Ethylene-insensitive 2	Epigenet_cluster
HanXRQChr14g0428531	-2.03	0.055			histone H4	Epigenet_cluster
HanXRQChr05g0142301	-2.26	0.129			cytochrome b-c1 complex subunit 7-2	Epigenet_cluster
HanXRQChr10g0318861	-2.39	0.052			transcription factor bHLH130-like	ROS_cluster
HanXRQChr17g0563501	-2.39	0.025			profilin	hormone_cluster
HanXRQChr05g0134281	-2.53		0.089		histone H2A-like	ROS_cluster
HanXRQChr08g0234341	-2.63	0.087		-0.135	stem-specific TSJT1-like	ROS_cluster
HanXRQChr10g0305701	-3.22	0.058	0.085		heme-binding 2	Epigenet_cluster
HanXRQChr09g0253601	-3.54	0.016			ubiquitin-conjugating enzyme E2 2-like	Epigenet_cluster
HanXRQChr01g0015301	-3.59	0.075			ethylene-responsive transcription factor At4g13040	hormone_cluster
HanXRQChr15g0470891	-3.78	-0.064			1-aminocyclopropane-1-carboxylate oxidase	ROS_cluster
HanXRQChr09g0277201	-4.05			0.030	ethylene-responsive transcription factor RAP2-12-like	hormone_cluster
HanXRQChr01g0021111	-11.75			-0.155	histone	Epigenet_cluster
HanXRQChr07g0196031	-16.39			0.003	histone H2A-like	Epigenet_cluster

Fig supplemental data 10 : List of validated gene by Fluidigm.

Significative genes deferentially expressed (P.value < 0.05) in different stress condition (-0.7 Mpa PEG, Hypoxia and 300mMol NaCl, imbibed 24h). The differential expression was calculated in log_2 Fold change et log_2 Delta dCt for the fluidigm analysis. Seeds originate from irrigated plants (W) or drought stressed (S). The color red et bleu correspond to up or down expressed gene.

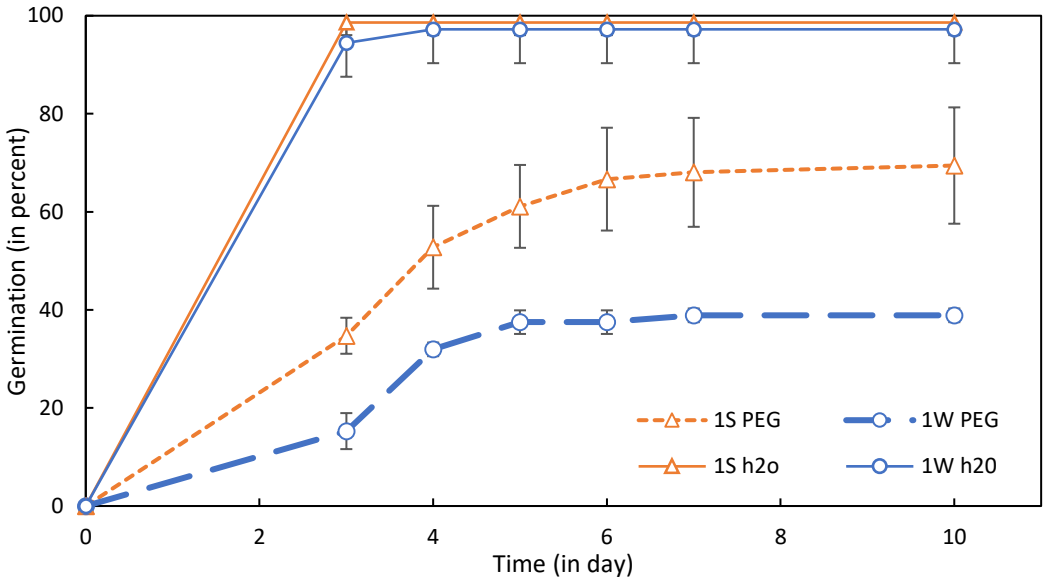

Fig supplemental data 11 : Germination kinetic of 1S or 1W
Germination kinetic of 1S or 1W seed imbibed seed in water or PEG (-0,7MPa) during 10 day. This seed was used for Fluidigm experimentation.

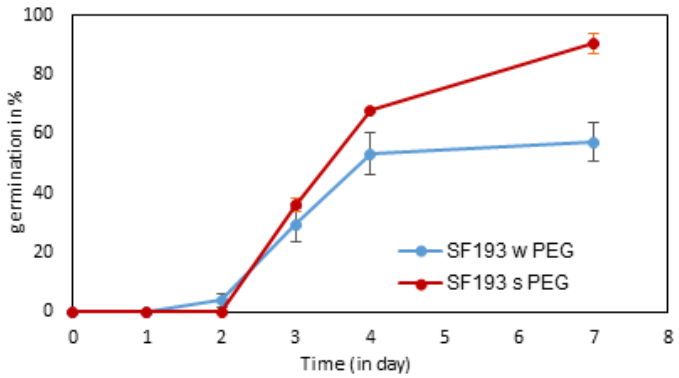

Fig supplemental data 12 :Seed germinative kinetics.
Seed germinative kinetic of XRQ seed produced in fiel in PEG condition (-0,7MPa). This seed was used for McBc QPCR.

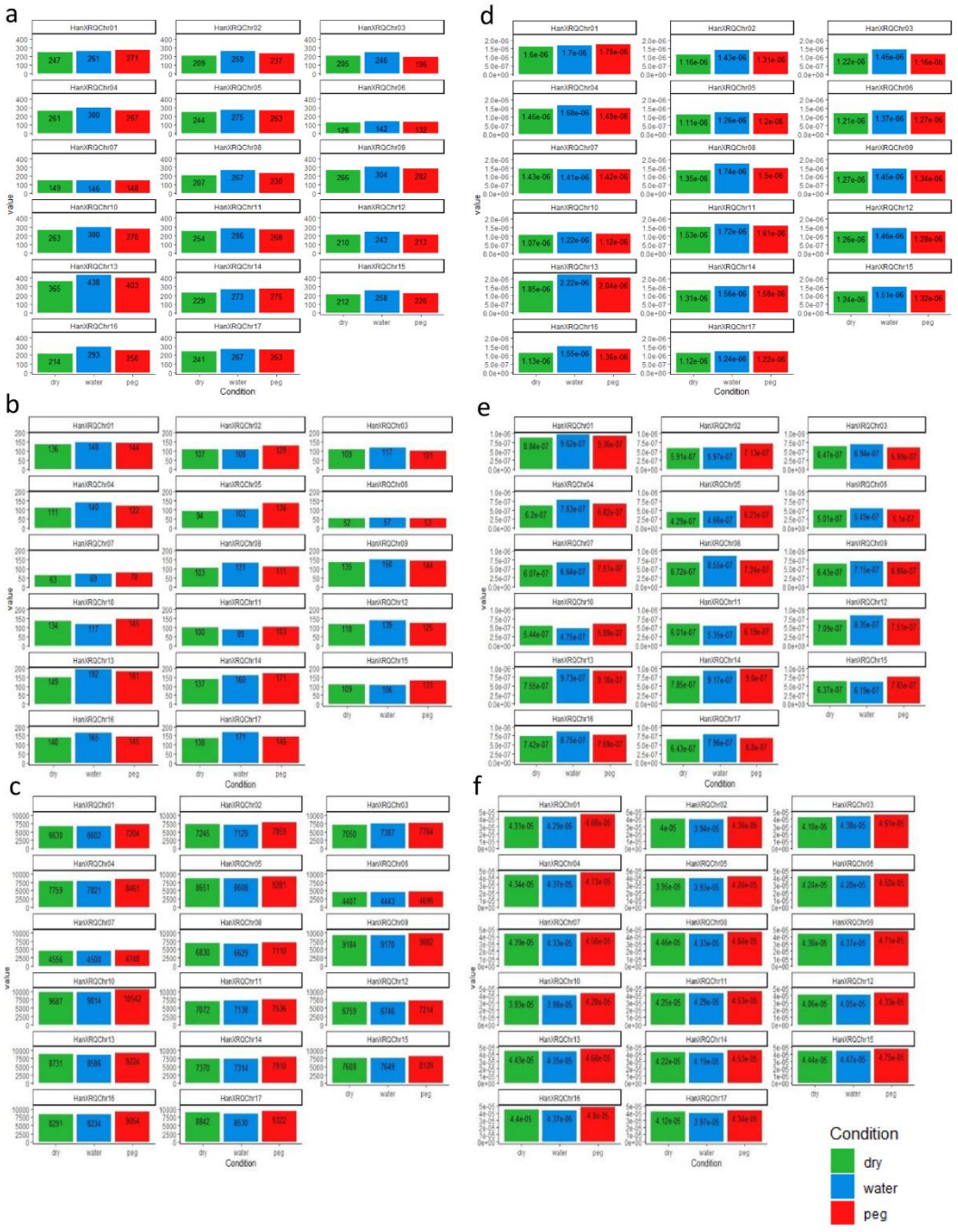

Fig supplemental data 13 : Number of Differentially Methylated Regions (DMR).

Number of Differentially Methylated Regions (DMR) between 3W & 3S seeds for each chromosome in dry seeds (green), seeds after imbibition in water (blue), and in water stress condition (PEG at -1MPa, in red). The tree types of DMR are presented (a) CHG, (b) CpH, and (c) CHH. (a.b.c) represents the DMR count and d.e.f. represents the DMR ratio (count/chromosome size).

Résumé/Abstract

Etude physiologique, génétique et moléculaire de l'impact d'un stress hydrique sur la qualité germinative des semences de tournesol

La tolérance des plantes cultivées au stress hydrique est un problème majeur du changement climatique et les futures conditions environnementales risquent de réduire drastiquement les rendements agricoles dans un avenir proche. La germination rapide et homogène des semences est une composante majeure du rendement final des cultures, mais elle peut être considérablement altérée par les conditions environnementales lors du semis. De plus, les conditions de l'environnement maternel pendant le développement des semences peuvent avoir un effet marqué sur la germination des semences de la génération suivante. En menant des expériences en conditions contrôlées sur la plate-forme de phénotypage Heliaphen (INRAE Toulouse), nous avons montré qu'un stress hydrique modéré appliqué sur les plantes mères de tournesol après leur floraison induit une meilleure germination, malgré différents stress appliqués lors de la germination, suggérant ainsi un effet maternel. Le traitement maternel semble influencer également le niveau de dormance des semences des deux générations suivantes, ce qui suggère l'existence de mécanismes de mémoire du stress maternel (effet transgénérationnel) sur la descendance. Nous avons recherché les gènes potentiellement impliqués dans la tolérance aux stress abiotiques, grâce à une étude transcriptomique et une validation par qPCR en micro-fluidigm qui nous ont permis de mettre en évidence une différence d'expression de certains marqueurs génétiques en fonction des conditions de stress maternels. D'autre part, nous avons déterminé qu'une composante épigénétique est associée à l'induction de la tolérance au stress hydrique par l'environnement maternel sur les semences. Pour ce faire, nous avons soumis les plantes mères à un stress hydrique et avons ainsi mis en évidence un effet sur le méthylome et le transcriptome des semences produites. Nous avons pu caractériser le méthylome des semences de tournesol grâce à un séquençage haut débit de l'ADN après traitement au bisulfite. Nous avons pu montrer que les conditions de développement des semences ont eu une influence directe sur le nombre et le niveau de méthylations des régions différentiellement méthylées (DMR). En revanche la germination n'a pas eu d'effet majeur sur le profil des DMR ce qui suggère que les modifications de la méthylation de l'ADN se sont produites pendant le développement des graines. Des DMR ont été identifiées dans la séquence codante, dans le promoteur ou dans la région de la séquence post-codante d'un ensemble de gènes dont l'expression a été modifiée par le stress hydrique appliqué sur la plante mère. La comparaison de la méthylation de l'ADN avec les changements d'expression des gènes induits par l'environnement maternel de la graine nous a permis de proposer un modèle de transmission et d'hérédité de la tolérance au stress au stade de la germination.

Keywords: abiotic stress, development, germination, maternal effect, seed, sunflower, epigenetic, tolerance.

Physiological, genetic and molecular study of the impact of water stress on the germination quality of sunflower seeds

Crop tolerance to water stress is a major climate change issue and future environmental conditions may drastically reduce crop yields in the near future. Rapid and uniform seed germination is a major component of final crop yield, but it can be significantly altered by environmental conditions during sowing. In addition, the conditions of the maternal environment during seed development can have a marked effect on seed germination in the next generation. By conducting experiments under controlled conditions on the Heliaphen phenotyping platform (INRAE Toulouse), we showed that moderate water stress applied to sunflower mother plants after flowering induced better germination, despite different stresses applied during germination, suggesting a maternal effect. The maternal treatment also seems to influence the level of seed dormancy in the two following generations, suggesting the existence of memory mechanisms of the maternal stress (transgenerational effect) on the progeny. We searched for genes potentially involved in tolerance to abiotic stresses, thanks to a transcriptomic study and a validation by qPCR in micro-fluidigm, which allowed us to highlight a difference in the expression of certain genetic markers according to the maternal stress conditions. On the other hand, we determined that an epigenetic component is associated with the induction of water stress tolerance by the maternal environment on seeds. To do so, we subjected the mother plants to water stress and thus demonstrated an effect on the methylome and transcriptome of the seeds produced. We were able to characterize the methylome of sunflower seeds using high-throughput DNA sequencing after bisulfite treatment. We were able to show that seed development conditions had a direct influence on the number and level of methylations of differentially methylated regions (DMRs). In contrast, germination did not have a major effect on the DMR profile suggesting that the changes in DNA methylation occurred during seed development. DMRs were identified in the coding sequence, promoter, or post-coding sequence region of a set of genes whose expression was altered by water stress applied to the parent plant. Comparison of DNA methylation with gene expression changes induced by the maternal seed environment allowed us to propose a model of transmission and inheritance of stress tolerance at the germination stage.

Keywords: abiotic stress, development, germination, maternal effect, seed, sunflower, epigenetic, tolerance.