NILO CESAR QUEIROGA SILVA

SEED GERMINATION IN RESPONSE TO SALT STRESS AND SEED DORMANCY: ON THE ROLES OF HORMONES AND TRANSCRIPTION FACTORS

Thesis submitted to the Plant Physiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Dimas Mendes Ribeiro

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“De tudo, ficaram três coisas: a certeza de que ele estava sempre começando, a certeza de que era preciso continuar e a certeza de que seria interrompido antes de terminar. Fazer da interrupção um caminho novo. Fazer da queda um passo de dança, do medo uma escada, do sonho uma ponte, da procura um encontro”.

(Fernando Sabino)

ABSTRACT

SILVA, Nilo Cesar Queiroga, D.Sc., Universidade Federal de Viçosa, June, 2021. **Seed germination in response to salt stress and seed dormancy: On the roles of hormones and transcription factors**. Adviser: Dimas Mendes Ribeiro.

The formation of a plant population depends on the plasticity of the germination of its seeds. This thesis aims to understand the mechanism of seed germination in response to salt stress and the role of transcription factors in germination. In chapter, it is reviewed the impact of climate change on seeds and the effect of hormonal balance in seeds. In addition, we present a brief review of the epigenetic effects on seeds and their impacts on plant adaptability, which can contribute to new perspectives. In the following chapters, we sought to investigate (i) the role of sodium and pH in hormonal balance during germination in *Stylosanthes humilis.* (ii) the role of transcription factors (TF) in dormancy and germination of *Arabidopsis thaliana* seeds. These studies were carried out in two independent experiments, which comprise chapters II and III presented here. In the second chapter, the following experiments were proceeded: seeds of *S. humilis* were incubated in a growth chamber. Ethylene, abscisic acid (ABA), 1-carboxylic acid-1-aminocyclopropane (ACC), ACC synthase (ACS) and ACC oxidase (ACO) and the primary metabolites were quantified. The results show that saline stress induces ABA synthesis independently of pH, leading to a reduction in the biosynthesis of ethylene, the main hormone involved in the germination of *S. humilis*. The third chapter aimed to understand how the transcription factor *TEOSINTE BRANCHED1 CYCLOIDEA PROLIFERATING CELL FACTORS* (TCP) family regulates the germination of *Arabidopsis thaliana* seeds. To help elucidate the functioning of the TCP8 and TCP14 genes, the TCP mutants were tested during seed germination. RNA-Sequencing data from seeds of TCP mutants were used to identify potential genes regulated by TCPs. The phenotype was evaluated and RT-qPCR (Real Time - quantitative PCR) were performed. In addition, transient gene expression analyses were performed on tobacco leaves. The results of this study show that TCP8 is a negative regulator of breaking dormancy in *Arabidopsis thaliana*. Together, these results suggest that TCP8 and TCP14 (both of class I TCPs) modulate the dormancy break in a contrasting manner. Thus, the role of TCP8 and TCP14 was demonstrated

during the germination of *A. thaliana*. Together, these data indicate the complexity of the germination process.

Keywords: Germination. Dormancy. Seeds. Salt Stress. *Stylosanthes humilis.* Transcription factors. *Arabidopsis thaliana.*

RESUMO

SILVA, Nilo Cesar Queiroga, D.Sc., Universidade Federal de Viçosa, junho de 2021. **Germinação de sementes em resposta ao estresse salino e dormência das sementes: papéis dos hormônios e dos fatores de transcrição**. Orientador: Dimas Mendes Ribeiro.

A formação de uma população de plantas depende da plasticidade da germinação de suas sementes. Esta tese tem como objetivo compreender o mecanismo de germinação de sementes em resposta ao estresse salino e o papel de fatores de transcrição na germinação. No capítulo I, é revisado o impacto das mudanças climáticas nas sementes e o efeito no equilíbrio hormonal. Além disso, fazemos uma breve revisão dos efeitos epigenéticos em sementes e seus impactos na adaptabilidade de plantas, o que pode contribuir para novas perspectivas. Nos capítulos seguintes, buscou-se investigar (i) o papel do sódio e do pH no equilíbrio hormonal durante a germinação de sementes de *Stylosanthes humilis.* (ii) O papel dos fatores de transcrição na dormência e germinação de sementes de *Arabidopsis thaliana*. Esses estudos foram realizados em dois experimentos independentes, que compõem os capítulos II e III aqui apresentados. No segundo capítulo, foram realizados os seguintes procedimentos: sementes de *S. humilis* foram incubadas em solução de NaCl e Na_2CO_3 para a quantificação de etileno, ácido abscísico (ABA), ácido 1-carboxílico-1-aminociclopropano (ACC), ACC oxidase (ACO) e metabólitos primários. Os resultados mostram que o estresse salino induz a síntese de ABA de forma independente do pH, levando a redução na biossíntese do etileno, principal hormônio envolvido na germinação de *S. humilis*. O terceiro capítulo, teve como objetivo compreender como o fator de transcrição da família *TEOSINTE BRANCHED1 CYCLOIDEA PROLIFERATING CELL FACTORS* (TCP) regula a germinação de sementes de *A. thaliana*. Para ajudar a elucidar o funcionamento dos genes TCP8 e TCP14, mutantes TCP foram testados durante a germinação das sementes. Dados de sequenciamento de RNA de sementes de mutantes TCP foram usados para identificar os potenciais genes regulados por TCPs. O fenótipo foi avaliado e RT-qPCR (Real Time-quantitative PCR) foram realizados. Além disso, foram realizadas análises de expressão transiente de genes em folhas de tabaco. Os resultados deste estudo mostram que o TCP8 é um regulador negativo da

quebra de dormência em *A. thaliana*. Juntos, esses resultados sugerem que o TCP8 e o TCP14 (ambos de TCPs de classe I) modulam a quebra de dormência de maneira contrastante. Assim, o papel do TCP8 e do TCP14 foi demonstrado durante a germinação de *A. thaliana*. Além disso, esses dados indicam o complexo processo de germinação.

Palavras-chave: Germinação. Dormência. Stress salino. *Stylosanthes humilis*. Fatores de transcrição. *Arabidopsis thaliana*.

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SUMMARY

GENERAL INTRODUCTION

Plants developed the capacity to adapt and acclimate to distinct environmental conditions. Regulation of growth and development is susceptible to environmental conditions which plants need to cope. This regulation is managed by molecules called hormones. The synthesis, conjugation, degradation of hormones are tightly regulated (Hernández-García et al 2021, Carrera-Castaño et al 2020). Low concentrations of hormones are able to trigger gene transcription and control physiological processes in plants in response to environment. Hence, hormones act as messengers translating environment signals to intern process in plants (Benková 2016). However, it is necessary to note that hormones act combined with other molecules in an interconnected way. This complex interaction with other hormones or molecules is recurrent, occurring multi alternative pathways. This complex network makes the regulation of hormones trick to understand (Aerts et al 2020).

Epigenetic modifications are chemical modifications of chromatin that can reason an alternate in gene expression, except altering the DNA sequence. Recent research has shown that there are considerable epigenetic variations in plant populations (Miryeganeh and Saze 2020). At least some of them may have extensive consequences on phenotypic variation and are heritable throughout generations. Thus, epigenetic variations can contribute to plant survival in a climate change scenario (Miryeganeh and Saze 2020). Efforts have been made to cover the potential roles of DNA modifications in plant due to responses to abiotic environmental stress conditions, such as, cold, heat, CO_2, drought and salinity stress (Kong et al 2018).

Dormancy phenomenon on seeds is an evolutional adaptation to environment, which permits the coordination of seed germination on favourable conditions

(Klupczyńska and Pawłowski 2021). In ecological terms, this property is indispensable for maintaining species. The mechanisms involved in seed dormancy breakage have been widely studied. It is recognized to be managed through several transcription factors (TF) coordinating the growth of the embryo through genetic and environmental signals (Yan and Chen 2020). TF are proteins that include sequences of amino acids that allow them to be in a position to bind to precise DNA sequences, activating or repressing the transcription of genes. Most of these proteins are grouped into families, in accordance to a common DNA binding domain (Carrara and Dornelas 2020). The molecular regulation in germination is extensively studied, and very progress has been made with the identification of TF regulating seed development, dormancy and germination (Nelson et al 2017, Jia et al 2021). However, the mechanisms of functioning and activation of these genes are nonetheless poorly understood.

Due to the scenario described above, (i) firstly we proposed to carry out a review in which an overview of how climate changes can disrupt seed germination is presented. In addition, a brief resume of the role of hormones in seeds and how may epigenetic be an adaptive driver. In the following chapters, we propose to examine (ii) the role of sodium and pH in hormone balance during germination in *Stylosanthes*; and (iii) the role and the interaction of transcription factors in dormancy seed of *Arabidopsis*. In the chapter II, we present a study that shows salt stress upregulates the synthesis of ABA, in a pH-independent way, which inhibits the ACS and ACO and leads to a decrease in ethylene, the major hormone that facilitates the germination of *Stylosanthes*. In the third chapter, we demonstrated the role of TCP8 and TCP14 during the germination of *Arabidopsis*. In overall, this thesis aims to further our understand of the mechanism on seed germination in stress conditions, which may

provide new insights into how germination can be affected in a global climate changes scenario.

REFERENCES

Aerts N, **Mendes MP**, **Van Wees SCM** (2020) Multiple levels of crosstalk in hormone networks regulating plant defense. The Plant Journal. 105: 489-504

Benková E (2016) Plant hormones in interactions with the environment. Plant Molecular Biology. 91: 597

Carrara S, Dornelas MC (2020) TCP genes and the orchestration of plant architecture. Tropical Plant Biology. 14: 1-10

Carrera-Castaño G, Calleja-Cabrera J, Pernas M, Gómez L, Oñate-Sánchez L (2020) An update overview on the regulation of seed germination. Plants. 9: 1-41

Jia C, Zhao S, Bao T, Zhao P, Peng K, Guo Q, Gao X, Qin J (2021) Tomato BZR/BES transcription factor SlBZR1 positively regulates BR signaling and salt stress tolerance in tomato and *Arabidopsis*. Plant Science. 302: 110719

Klupczyńska EA, **Pawłowski TA** (2021) Regulation of seed dormancy and germination mechanisms in a changing Environment. International Journal of Molecular Sciences. 22: 1357

Kong L, Liu Y, Wang X, Chang C (2020) Insight into the role of epigenetic processes in abiotic and biotic stress response in wheat and barley. International Journal Molecular Science. 21:1480.

Nelson Sven K., Ariizumi Tohru, Steber Camille M (2017) Biology in the dry deed: transcriptome changes associated with dry seed dormancy and dormancy loss in the *Arabidopsis* GA-Insensitive sleepy1-2 mutant. Frontiers in Plant Science. 8: 2158

Miryeganeh M, **Saze H** (2020) Epigenetic inheritance and plant evolution. Population Ecology. 62: 17– 27

Yan A, Chen Z (2020) The control of seed dormancy and germination by temperature, light and nitrate. The botanical review. 86: 39-75

CHAPTER 1 - The role of hormones on seed germination in climate change scenario

ABSTRACT

Plants are exposed to a variety of biotic and abiotic stimuli with lack of mobility to escape. Therefore, plants have evolved to adapt their developmental and physiological responses to the environment. Some environmental stresses occur infrequently during the life cycle, but others, such as seasonal drought, heat, salinity and herbivore may be recurrent. Thus, plant responses to these stresses may be transient to provide plants with the tools necessary to survive, which may predispose the plant to a more efficient stress response in the next time a stress occurs. The possibility of transmitting this response to offspring seeds has been recently suggested. Various epigenetic effects such as chromatin remodeling, DNA methylation and histone modifications have been linked to biotic and abiotic stresses. This chapter reviews how epigenetic mechanism may act as an evolution force under climate change scenario. Moreover, we show how complex interactions among hormones regulate seed germination and dormancy. Finally, we look at how the climate change may influence the fate of offspring plants. Also the possible agricultural implications and new challenges posed by climate change.

Keywords: Abscisic acid; Ethylene; Gibberellin; Climate change; Epigenetics; Seed germination

INTRODUCTION

The distribution of plant population depends on the adaptive aspects of seeds to the environment during germination. Germination is a complex and susceptible process in which morphological and physiological changes occur, resulting in embryo growth reactivation and, eventually, new plant development. Seeds show a species-specific response to various environmental conditions such as temperature, light, oxygen, and fire (Miransari and Smith 2014, Luna 2020). Hormonal balance is critical for plant growth, seed development, and germination (Corbineau et al 2014). At the cell level, it seems that different organelles can sense dramatic stress changes promoting an integrated network to respond to abiotic perturbation (Zhu 2017). However, global warming is changing the abiotic parameters in such a faster way that some species might not be able to cope with these abrupt changes. In addition, climate change can disrupt germination and dormancy patterns, causing agricultural productivity loss. Reports showing the impact of climate changes in seed banks and germination patterns from non-model-plants are still scarce (Footitt et al 2018). Plants facing changes in the environment need to respond quickly by altering their metabolism, proteins, hormone balance, and phenotype. Phenology changes can become permanent and be transmitted to the next generation. In this vein, to adapt to the environment, plants can store information to cope with future stress events or even to provide information to the next generation through genetic marks. However, how these mechanisms work remain poorly understood (Schmid et al 2018).

By analyzing the abiotic stresses due to the climatic change scenario, it is possible to suggest alterations in the plant dynamics. Here, we provide new perspectives of the stress responses concerning seed germination in climate change

scenarios. Due to the complexity of the germination mechanism in a scenario of global change, the development of strategies to reduce the impacts of climate change on crops seems compelling. In this review, we summarize the biosynthesis, signaling, and role of hormones in seed dormancy and germination. In addition, we discuss how climate changes can disrupt seed germination. To conclude, we further analyze how the environment can alter the fate of offspring seeds.

CLIMATE CHANGE

New efforts have been made to predict the plant species distribution on geographic and temporal dynamics in the context of climate change (Zait et al 2020). The forecasted changes in climate can disrupt the germination, dormancy, and seed bank patterns of various species, changing distribution and germination time (Cochrane 2016, Aragón-Gastélum et al 2018, Footitt et al 2018, Newton et al 2020). Different plant populations can respond to stressful events in divergent ways, adopting distinct strategies. Recent studies with non-model-plants have shown that global changes can impact the life cycle of plants, and, as a result, the plant community (Yi et al 2019). Another example has been shown with seeds of *Alliaria petiolate*, which require a cold winter for break dormancy and a rapid adaptation to warmer conditions will be a requirement for this population to survive (Footitt et al 2018). Nowadays, the major concern about global warming is how agricultural production will be affected. Recently, the Intergovernmental Panel on Climate Change (IPCC) (2019) has reported that an increase in 1.5 °C can disrupt the climate with an escalation of salinity in agricultural lands and an increase in sea levels. Studies were made to elucidate how abiotic stresses such as high temperature,

salinity, and CO_2 may impact the fitness and yield of the most consumed crops by humans, such as maize, rice, and wheat (Watt et al 2020). Recent models suggested that yields of the main crops would decrease due to warmer temperatures, which would likely impact food security in the coming years (Wang X et al 2020).

Accumulation of salt in soils is a major concern to affect yield in main crops. Recent evidence has shown increased areas of salinization in agricultural lands and many models have been proposed to identify fragile areas susceptible to salinization (Corwin 2020). Strategies have been proposed to cope with salt stress such as the development of resistant cultivars, which may improve seed germination under stress. However, seed germination under salt stress is complex due to the involvement of many physiological mechanisms (Jaarsma et al 2013, Bhattarai et al 2020). Furthermore, alterations in the levels of proteins and metabolites can induce morphological and physiological responses to salinity in the environment (Bhattarai et al 2020). One major physiological response is the increase of abscisic acid (ABA) levels in roots during drought or salt stress (Jia et al 2002). Ethylene is another hormone involved in such conditions. In this context, it has been shown the involvement of ethylene in the root formation of cucumber in saline soils (Yu et al 2019). At the protein level, it has been suggested that CUPIN DOMAIN PROTEIN (CDP3.1) enhances seed germination in rice under salt stress (Xu et al 2017). Using *Arabidopsis* mutants, it was demonstrated the association of the transcription factor NAC (NAM, ATAF, and CUC) in salt stress tolerance during seed germination and plant development (Yao et al 2018). How this complex response network works on germination, it remains to be elucidated.

HORMONES ROLE IN SEEDS

Slight changes in the environment can alter the hormone balance that plays an important role in the regulation of seed development, dormancy, and germination (Li et al 2015, Ozga et al 2017, Xia et al 2018). ABA, ethylene, gibberellin (GA), auxin (IAA), cytokinin, and brassinosteroids (BR) are hormones that can influence seed germination and dormancy in several species (Miransari and Smith 2014, Sall et al 2017, Née et al 2017, Vishal and Kumar 2018, Nemoto et al 2018).

Ethylene is an important hormone that regulates root development, hypocotyl growth, and other processes during plant growth and development, not being restricted to germination. One of the most well-known roles for ethylene happens during the breaking of seed dormancy (Ahammamed et al 2020). Ethylene can interact with other hormones, such as ABA and GA, promoting cell wall expansion and starch degradation (Ogawa et al 2003, Sun et al 2019, Ahammamed et al 2020). The production of ethylene during germination starts immediately after imbibition, tending to accumulate concomitantly with the primary radicle protrusion, with a sharp decline in biosynthesis after *sensu stricto* germination. This characteristic is dependent on the species and was observed in Brassica, *Gossypium spp*. *Arachis hypogaea*, *Xanthium pennsylavanicum*, *Phaseolus vulgaris*, *Helianthuns annus*, *Pisum sativum*, *Arabidopsis* and *Stylosanthes* (Corbineau et al 2014). Interestingly, some species are more vigorous when ethylene production capacity is higher (Xia et al 2018).

The biosynthesis of ethylene in seeds occurs by the same metabolic route as the other plant organs, in which S-Adenosyl-methionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) are the main intermediates. ACC is

produced from the SAM reaction via ACC synthase (ACS). The last step in ethylene production occurs with the oxidation of ACC via ACC oxidase (ACO) activity, which is a regulatory enzyme for ethylene biosynthesis. During the oxidation of the ACC, it is yielded CO_2 and HCN (Corbineau et al 2014, Sun et al 2020, Ahammamed et al 2020). It is observed that environments that inhibit the germination of non-dormant seeds, such as high temperatures, salinity, and hypoxia are attenuated when ethylene is applied exogenously (Corbineau et al 2014). Ethylene has been shown to improve salt tolerance during germination, increasing proline and peroxidase content in *Medicago sativa* (Wang Y et al 2020). In addition, ethylene plays a key role in antagonizing ABA signaling via ETHYLENE RESISTANT2 (ETR2). ETR2 can repress ETHYLENE RESISTANT1 (ETR1) and ETHYLENE INSENSITIVE4 (EIN4) two genes that stimulate the ABA signaling in *Arabidopsis* (Wilson et al 2014, Bakshi et al 2018). Moreover, ethylene may regulate ABA biosynthesis by repressing the genes 9-cis-epoxycarotenoid dioxygenase (NCED2) and CYP707A1 in wheat (Sun et al 2020, Wang Y et al 2020). It seems that ethylene can indirectly cause the destabilization of DELLA proteins, thus promoting GA downstream signaling. GA can also promote protein stability involved in ethylene biosynthesis, as was seen in ACS5 on *Arabidopsis* (Iqbal et al 2017).

ABA is an essential hormone in plants by playing a variety of roles during plant growth. ABA defective plants present anomalies such as withered appearance and reduced vigor, which are restored with exogenous application of this hormone (Chen et al 2020). In mutants of ABA signaling and biosynthesis, the seed dormancy is easily overcome, implying the direct function of ABA in seed dormancy maintenance (Ali et al 2021). In seeds, ABA performs a fundamental role to keep the dormancy in unfavorable environments. During embryo development, maternal ABA performs a

substantial function in *Arabidopsis* and *Nicotiana* repressing germination (Ali et al 2021). ABA is also synthesized in embryos and testa at some stage in embryo development. The presence of ABA in seed during seed development and maturation allows the synthesis and storage of proteins and reserves. It has been shown that ABI5, bZIP67 together with ABI3 and ABI4 may regulate the expression of genes that are involved in ABA-mediated seed storage (Ali et al 2021). The ABA level in tissues is determined by the balance of synthesis, inactivation, conjugation, compartmentation, and transport (Seo and Marion-Poll 2019). The early reactions for ABA synthesis happen in plastids from precursor β-carotene, by which occurs a hydroxylation to form zeaxanthin and, posteriorly, violaxanthin; then, an isomerization in 9-cis-violaxanthin or 9`-cis-neoxanthin occurs (Seo and Marion-Poll 2019). NCED is encoded by a multigene family, in which the expression is tightly regulated by the rate-limiting in ABA synthesis. NCED participates in the cleavage of 9-cis-violaxanthin and 9`-cis-neoxanthin to xanthoxin, the last step of ABA synthesis in plastids (Sall et al 2017). After that, xanthoxin, a compound of 15 carbons, is transported from plastid to cytosol to complete the synthesis of ABA. ABA then can be conjugated with glucose and be stored in the vacuole or be transported by ABCG transporters or be inactivated by 8-hydroxylation by CYP707A in the endoplasm reticulum (Vishal and Kumar 2018, Seo and Marion-Poll 2019). The balance of synthesis and catabolism of ABA can regulate seed dormancy together with other hormones.

Gibberellin, a tetracyclic diterpenoid, is another hormone extensively studied during the seed germination process. ABA and GA are major hormones controlling seed germination and dormancy (Kanno et al 2010). Therefore, the balance between GA/ABA levels in seeds can determine their fate. Moreover, it has been shown that

GA can interact with other hormones during plant growth and development (Tuan et al 2018). The biosynthesis of GA is strictly regulated by genetic and abiotic factors. GA over-expresser or down-expresser mutants have been used to study the pathways of GA biosynthesis and signaling (Hernández-García et al 2021). Here we will briefly discuss the metabolism and importance of GA during seed germination.

The expression of GA biosynthesis and signaling genes is differentially expressed as dependent on species, plant stages, tissues, and cells along the life cycle (Binenbaum et al 2018). The GA biosynthetic pathway occurs in three different cellular compartments, plastid, endoplasmic reticulum, and cytosol. The biosynthesis of GA occurs from geranylgeranyl diphosphate (GGPP) to *ent*-kaurene into the plastid (Hernández-García et al 2021). This compound is transported to endoplasmic reticulum and oxidized to GA_{12} or GA_{53} (Hernández-García et al 2021). All the active GAs are formed in the cytosol through a series of oxidation reactions. Due to the high sensitivity, the active GAs are strictly regulated. The GA regulation may involve the inactivation by 2-oxoglutarate-dependent dioxygenases (2-OGD) enzymes. GA20*ox,* a 2-OGD enzyme, is known to act in *Arabidopsis* decreasing GA_1, GA_3 and GA_4 (active GAs) (Hernández-García et al 2021).

During seed maturation, GA biosynthesis is repressed preventing the early germination with the involvement of DELAY OF GERMINATION (DOG) family. The signaling of ABA and GA is part of a complex network of protein interactions (Ali et al 2021). Moreover, it had been proposed the involvement of 26S proteasome during seed germination. GIBBERELLIN INSENSITIVE DWARF1 (GID1) is a protein found in cytoplasm and nucleus and is the receptor of GA (Hernández-García et al 2021). Protein DELLA, a repressor of TEOSINTE BRANCHED1/CYCLOIDEA/ PROLIFERATING CELL FACTOR (TCP) and PHYTOCHROME INTERACTING

FACTOR (PIF), that inhibits germination, is ubiquitinated after interacting with GID1 and other proteins, forming a multi-complex protein (Hernández-García et al 2021). Therefore, DELLA becomes a target by 26S proteasome and will be degraded. The DELLA degradation will allow PIF and TCP to bind on the target genes (Fig 1.1). These genes targeted by PIF and TCP will promote the mobilization of the seeds reserves that will be used during germination (Oracz and Stawska 2016).

EPIGENETICS MAY DRIVE PLANT ADAPTATION

The rapid increment of CO_2 in the atmosphere led to a fast increase in temperature in the last century, also expanding the salinity areas (IPCC 2019). Importantly, any change in the environment can reshape plant gene expressions altering their phenotype, regulating the hormonal balance, to adjust to a new environment through epigenetic changes. This change occurs without altering the DNA sequence, however, modifications in chromosome integrity are observed (Schmid et al 2018). The main molecular modification responsible for epigenetic occurs through DNA methylation, a process in which methyl radicals are attached to the DNA; however, the DNA tries to protect its integrity by introducing a high resistance to such alterations. Other modifications such as histone modification and small RNAs alteration have been observed. The action of environmental factors signaling to hormones can induce, change, or suppress the expression of genes, which can alter the ability of the target cell to regulate the corresponding proteins. (Zhang et al 2018). These marks in DNA can be reversible but also inherently stable for generations, thus predisposing plants to mild stress can enhance the response in the next stress event. It was shown that seedlings of *Arabidopsis* exposed to NaCl

(50 mM) alter the transcription profile and physiological responses when adult plants face salt stress again (Sani et al 2013). Potentially, the transfer of this mechanism into the next generation has been observed in *Arabidopsis* (Groot et al 2016). Some authors call the alteration of these inheritance genes of “memory genes” that can keep the information of the environment into the DNA for the next generation (Galviz et al 2020, Shekhawat et al 2021). Some genes such as FORGETTER2 (FGT2) have been suggested to keep information of thermotolerance, however, it is unclear if is transmissible for next generations (Castellanos et al 2020). The stability of epigenetic marks in DNA through the next generations resembles a fast natural selection, holding a fundamental role in ecologic dynamic population to cope with climate change (Thiebaut et al 2019, Wang M et al 2020). In a recent study, it was shown that stresses such as warm and drought can promote different loci methylation, inducing a distinct phenotype in *Hordeum mirinum* (Chano et al 2021). Gene evolution and epigenetic inheritance are also likely to play a key role in genome adaptation. Given the genome is the result of a complex interaction between an organism and its environment, it is fairly reasonable to consider that some of the responses are transmitted to the next generations as shown in *Arabidopsis* (Schmid et al 2018). However, few studies have been done in non-model-plants to exploit the role of epigenetic in the scenario of climate change (Thiebaut et al 2019). Using the epigenetic mechanism as a tool can be an alternative approach to enhance transgenic breeding and improve crop yield worldwide. However, we still need to learn which epigenetic mechanisms are involved and how they are transmitted to offspring seeds in a stress ambient.

CONCLUDING REMARKS

Regardless of our broad information on seed science, numerous significant questions remain open. For instance, the possible climate change scenario modulating the seed metabolism during germination is still poorly understood. Although there are extensive studies with Arabidopsis, most non-model species does not have any information on germination, in climate changes scenario. This might be a new frontier of study permitting a close investigation of models of plant dynamics. Plants are capable to cope with previous stress exposure; consequently, plants can reply faster or more effectively to biotic or abiotic stress conditions. In this regard, the investigation and advancement of how epigenetic adjusts the hormonal biosynthesis and signaling in seeds will bring innovations to agriculture systems. Although molecular mechanisms of epigenetic inheritance are still being elucidated, in the future they will be incorporated in agriculture to make resistant plants to biotic and abiotic stress. However, there are some challenges in the studies of epigenetics inheritance as current usually focus on only one stress element. Therefore, it is difficult to predict their responses in a natural habit, where many stress conditions are present at the same time. In addition, research has to be made to have extra knowledge about non-model plant responses in different conditions. Progress will undoubtedly be made in unraveling the molecular foundation of such stress reminiscence in the coming years. In particular, it will be fascinating to see whether or not one-of-a-kind instances of stress inheritance are encoded by the same mechanisms. A major theme will be to advance beyond correlation by demonstrating gene-specific modifications to the epigenome. Further along, the direct detection of epigenetic marks, using bisulfite sequencing and transcriptome analyses, will enable

to unravel new roles of DNA modification. This will allow the association of these *loci* to candidate genes with specific roles in phenotype and functional responses to stress. In turn, this will highlight key regulatory mechanisms that will enable tailor-made responses to the challenges represented by incoming climate change.

REFERENCES

Ahammamed GJ, Gantait S, Mitra M, Yang Y, Li X (2020) Role of ethylene crosstalk in seed germination and early seedling development: A review. Plant Physiology and Biochemistry. 151: 124-131

Ali F, Qanmber G, Li F, Wang Z (2021) Updated role of ABA in seed maturation, dormancy, and germination. Journal of Advanced Research. DOI: 10.1016/j.jare.2021.03.011

Aragón-Gastélum JL, Flores J, Jurado E, Ramírez-Tobías HM, Robles-Díaz E, Rodas-Ortiz JP, Yáñez-Espinosa L (2018). Potential impact of global warming on seed bank, dormancy and germination of three succulent species from the Chihuahuan Desert. Seed Science Research. 28: 312–318.

Bakshi A, Piya S, Fernandez JC, Chervin C, Hewezi T, Binder BM (2018) Ethylene receptors signal via a noncanonical pathway to regulate abscisic acid responses. Plant physiology. 176: 910–929.

Bhattarai S, Biswas D, Fu YB, Biligetu B (2020) Morphological, physiological, and genetic responses to salt stress in alfafa: a review. Agronomy. 10:577

Binenbaum J, Weinstain R, Shani E (2018) Gibberellin localization and transport in plants. Trend in Plant Sciences. 23: 410-421

Castellanos UR, Friedrich T, Petrovic N, Altmann S, Brzezinka K, Gorka M, Graf A, d Bäurle I (2020) FORGETTER2 protein phosphatase and phospholipase D modulate heat stress memory in *Arabidopsis*. Plant Journal. 104: 7-17.

Chano V, Domínguez-Flores T, Hidalgo-Galvez MD, Rodrigues-Calcerrada J, Pérez-Ramos IM (2021) Epigenetic responses of hare barley (*Hordeum murinum* subsp. *leporinum*) to climate change: an experimental, trait-based approach. Heredity. 126: 748–762

Chen K, Li GJ, Bressan RA, Song CP, Zhu JK, Zhao Y (2020) Abscisic acid dynamics, signaling, and functions in plants. Journal of Integrative Plant Biology. 62: 25-54

Cochrane A (2016) Can sensitivity to temperature during germination help predict global warming vulnerability? Seed Science Research. 26: 14–29

Corbineau F, Xia Q, Bailly C, El-Maarouf-Bouteau H (2014) Ethylene, a key factor in the regulation of seed dormancy. Frontiers in Plant Science. 5: 539

Footitt S, Huang S, Olcer-Footitt H, Clay H, Finch-Savage WE (2018) The impact of global warming on germination and seedling emergence in *Alliaria petiolata*, a woodland species with dormancy loss dependent on low temperature. Plant Biology. 20: 682-690

Galviz YCF, Ribeiro RV, Souza, GM (2020) Yes, plants do have memory. Theoretical Experimental Plant Physiology. 32: 195–202

Groot MP, Kooke R, Knoben N, Vergeer P, Keurentjes JJB, Ouborg NJ, Verhoeven KJF (2016) Effects of multi-generational stress exposure and offspring environment on the expression and persistence of transgenerational effects in *Arabidopsis thaliana*. Plos One. 11: e0151566

Hernández-García J, Briones-Moreno A, Blázquez MA (2021) Origin and evolution of gibberellin signaling and metabolism in plants. Seminars in Cell and Developmental Biology. 109: 46–54

IPCC (2019) Climate Change and Land: an IPCC special report on climate change, desertification, land degradation, sustainable land management, food security, and greenhouse gas fluxes in terrestrial ecosystems [P.R. Shukla, J. Skea, E. Calvo Buendia, V. Masson-Delmotte, H.-O. Pörtner, D. C. Roberts, P. Zhai, R. Slade, S. Connors, R. van Diemen, M. Ferrat, E. Haughey, S. Luz, S. Neogi, M. Pathak, J. Petzold, J. Portugal Pereira, P. Vyas, E. Huntley, K. Kissick, M. Belkacemi, J. Malley, (eds.)]. In press.

Iqbal N, Khan NA, Ferrante A, Trivellini A, Francini A, Khan MIR (2017) Ethylene role in plant growth, development and senescence: interaction with other phytohormones. Frontiers in Plant Sciences. 8: 475

Jaarsma R, de Vries RSM, de Boer AH (2013) Effect of salt stress on growth, Na+ accumulation and proline metabolism in potato (Solanum tuberosum) cultivars. Plos One. 8: e60183

Jia W, Wang Y, Zhang S, Zhang J (2002) Salt-stress-induced ABA accumulation is more sensitively triggered in roots than in shoots. Journal of Experimental Botany. 53: 2201-2206

Kang J, Yim S, Choi H, Kim A, Lee KP, Lopez-Molina L, Martinoia E, Lee Y (2015) Abscisic acid transporters cooperate to control seed germination. Nature Communication. 3: 8113

Kanno Y, Jikumaru Y, Hanada A, Mambara E, Abrams SR, Kamiya Y, Seo M (2010) Comprehensive hormone profiling in developing *Arabidopsis* seeds:

examination of the site of ABA biosynthesis, ABA transport and hormone interactions. Plant and Cell Physiology. 51: 1988-2001

Kong D, Fu X, Jia X, Wang W, Li Y, Li J, Yang X, Ju C (2020) Identification of Quantitative Trait Loci Controlling Ethylene Production in Germinating Seeds in Maize (*Zea mays* L.). Science Reports. 10: 1677

Li W, Khan MA, Yamaguchi S, Liu X (2015) Hormonal and environmental regulation of seed germination in salt cress (*Thellungiella halophila*). Plant Growth Regulation. 76: 41-49

Luna B (2020) Fire and summer temperatures work together breaking physical seed dormancy. Science Reports. 10: 6031

Miransari M, Smith DL (2014) Plant hormones and seed germination. Environmental and Experimental Botany. 99: 110-121

Newton RJ, Hay FR, Ellis RH (2020) Temporal patterns of seed germination in early spring-flowering temperate woodland geophytes are modified by warming. Annals of Botany. 125:1013-1023

Ozga JA, Kaur H, Savada RP, Reinecke DM (2017) Hormonal regulation of reproductive growth under normal and heat-stress conditions in legume and other model crop species. Journal of Experimental Botany. 68: 1885–1894

Sall K, Hendrix D, Sekine T, Katsuragawa, Koyari R, Nonogaki H (2017) Transcriptomics of nine-cis-epoxycarotenoid dioxygenase 6 induction in imbibed seeds reveals feedback mechanisms and long non-coding RNAs. Seed Science Research. 27: 1-11

Sani E, Herzyk P, Perrella G, Colot V, Amtmann A (2013) Hyperosmotic priming of Arabidopsis seedlings establishes a long-term somatic memory accompanied by specific changes of the epigenome. Genome Biology. 14: R59

Schmid MW, Heichinger C, Schmid DC, Guthorl D, Gagliardini V, Bruggmann R, Aluri S, Aquino C, Schmid B, Turbnbull LA, Grossniklaus U (2018) Contribution of epigenetic variation to adaptation in Arabidopsis. Nature Communication. 9: 4446

Seo M, Marion-Poll A (2019) Abscisic acid metabolism and transport. Advances in Botanical Research. 92: 1-49

Shekhawat K, Saad MM, Sheikh A, Mariappan K, Al-Mahmoudi H, Abdulhakim F, Eida AA, Jalal R, Masmoudi K, Hirt H (2021) Root endophyte induced plant thermotolerance by constitutive chromatin modification at heat stress memory gene loci. EMBO Reports. e51049

Sun M, Tuan PA, Izydorczyk MS, Ayele BT (2020) Ethylene regulates post-germination seedling growth in wheat through spatial and temporal modulation of ABA/GA balance. Journal of Experimental Botany. 71: 1985–2004

Thiebaut F, Hemerly AS, Ferreira PCG (2019) A Role for epigenetic regulation in the Adaptation and Stress responses of Non-model Plants. Frontiers in Plant Science. 10: 246.

Tuan PA, Kumar R, Rehal PK, Toora PK, Ayele BT (2018) Molecular Mechanisms Underlying Abscisic Acid/Gibberellin Balance in the Control of Seed Dormancy and Germination in Cereals. Frontiers in Plant Science. 9: 668

Vishal B, Kumar PP (2018) Regulation of Seed Germination and Abiotic Stresses by Gibberellins and Abscisic Acid. Frontiers in Plant Science. 9: 838

Wang MZ, Li HL, Li JM, Yu FH (2020) Correlations between genetic, epigenetic and phenotypic variation of an introduced clonal herb. Heredity. 124: 146–155

Wang X, Zhao C, Müller C, Wang C, Ciais P, Janssens I, Peñuelas J, Asseng S, Li T, Elliott J, Huang Y, Li L, Piao S (2020) Emergent constraint on crop yield response to warmer temperature from field experiments. Nature Sustainability. 3: 908-916

Wang Y, Diao P, Kong L, Yu R, Zhang M, Zuo T, Fan Y, Niu Y, Yan F, Wuriyanghan H (2020) Ethylene enhances seed germination and seedling growth under salinity by reducing oxidative stress and promoting chlorophyll content via ETR2 pathway. Frontiers in Plant Science. 11:1066

Watt C, Zhou G, Li C (2020) Harnessing Transcription Factors as potential tools to enhance grain size under stressful abiotic conditions in cereal crops. Frontiers in Plant Science. 11:1273.

Wilson RL, Kim H, Bakshi A, Binder BM (2014) The ethylene receptors ETHYLENE RESPONSE1 and ETHYLENE RESPONSE2 have contrasting roles in seed germination of Arabidopsis during salt stress. Plant Physiology. 165: 1353-1366.

Xia Q, Saux M, Ponnaiah M, Gilard F, Perreau F, Huguet S, Balzergue S, Langlade N, Bailly C, Meimoun P, Corbineau F, El-Maarouf-Bouteau H (2018) One way to achieve germination: Common molecular mechanism induced by ethylene and after-ripening in sunflower seeds. International Journal of Molecular Sciences. 19: 2464

Xu E, Chen M, He H, Zhang C, Cheng Y, Zhang H, Wang Z (2017) Proteomic analysis reveals proteins involved in seed imbibition under salt stress in rice. Frontiers in Plant Science. 7: 2006

Yi F, Wang Z, Baskin CC, Baskin JM, Ye R, Sun H, Zhang Y, Ye X, Liu G, Yang X, Huang Z (2019) Seed germination responses to seasonal temperature and drought stress are species-specific but not related to seed size in a desert steppe: Implications for effect of climate change on community structure. Ecology and Evolution. 9: 2149–2159.

Yu J, Niu L, Yu J, Liao W, Xie J, Lv J, Feng Z, Hu L, Dawuda MM (2019) The Involvement of Ethylene in Calcium-Induced adventitious root formation in cucumber under salt stress. International Journal of Molecular Science. 20: 1047

Zait Y, Konsens I, Schwartz A (2020) Elucidating the limiting factors for regeneration and successful establishment of the thermophilic tree Ziziphus spina‐christi under a changing climate. Science Reports. 10: 14335

Zhang H, Lang Z, Zhu JK (2018) Dynamics and function of DNA methylation in plants. Nature Reviews Molecular Cell Biology. 19: 489–506

Zhu JK (2016) Abiotic stress signalling and responses in plants. Cell. 167: 313-324

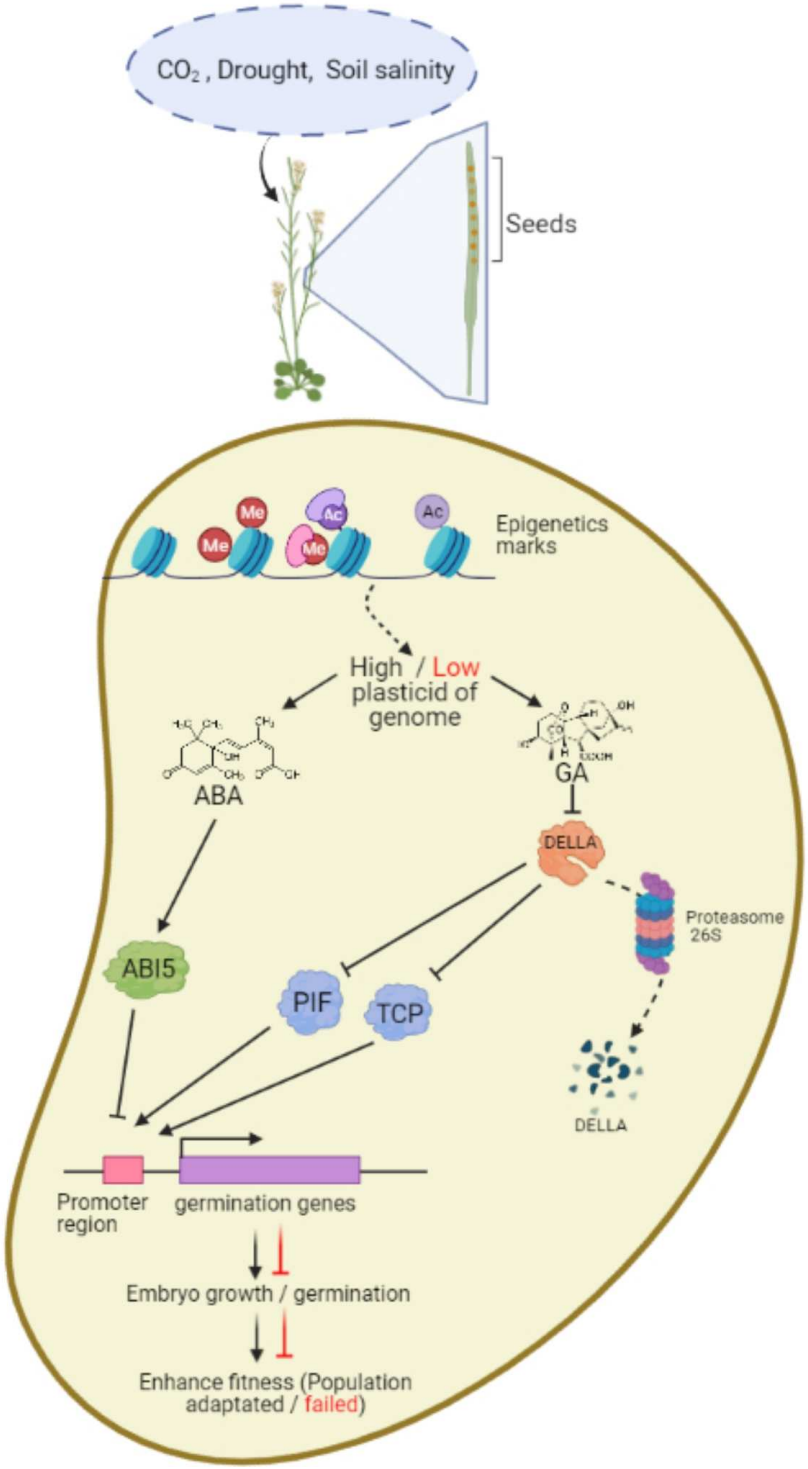

Fig 1.1 Schematic representation of climate change effect in offspring seeds. Climate change increases CO_2, drought, and soil salinity. Epigenetic marks in DNA may regulate the synthesis and signaling of ABA and GA. TCP and PIF may bind to DNA acting in regulation of genes related to germination, after degradation of DELLA repression by proteasome 26. In red low plasticity, germination does not occur. High plasticity favour gene mark to survival to climate change scenario.

CHAPTER 2 - Salt stress inhibits germination of *Stylosanthes humilis* seeds through abscisic acid accumulation and associated changes in ethylene production

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ABSTRACT

In *Stylosanthes humilis*, salt stress tolerance is associated with ethylene production by the seeds, however, how salt stress controls seed germination and ethylene production is poorly understood. Here, we studied the hormonal and metabolic changes triggered by salt stress on germination of *S. humilis* seeds. Salt stress led to decreased seed germination and ethylene production, concomitantly with higher abscisic acid (ABA) production by seeds. Treatment with NaCl and ABA promoted distinct changes in energy metabolism, allowing seeds to adapt to salt stress conditions. Treatment with the ABA biosynthesis inhibitor fluridone or ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) reversed the effects of salt stress on seed germination and ethylene production. Moreover, ethylene concentration was decreased by increasing the pH of the salt solution. High pH, however, did not influence concentration of ABA in seeds under salt stress. We conclude that biosynthesis of ABA and ethylene in response to salt stress constitutes a point of convergence that provides flexibility to regulate energy metabolism and embryo growth potential of *S. humilis* seeds within a given pH condition.

***Keywords**:* Abscisic acid; Ethylene; Primary metabolism; Salt stress; Seed germination

Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, 1-aminocyclopropane-1-carboxylic acid oxidase; ACS, 1-aminocyclopropane-1-carboxylic acid synthase; TCA, tricarboxylic acid

INTRODUCTION

Stylosanthes humilis is a forage legume that occurs naturally in saline regions of South and Central America (Lovato et al., 1999; Costa, 2006). This species has been used by farmers in tropical and subtropical regions for its nitrogen-fixing ability to improve pasture productivity (Gates and Wilson, 1974; Costa, 2006). Along with other *Stylosanthes* species, *S. humilis* is characterized by seed coat imposed physical dormancy, requiring mechanical or chemical scarification to enable germination (Chaves et al., 2017). Seeds of *S. humilis* also present physiological primary dormancy, which is removed after 12-15 months of after-ripening (Vieira and Barros, 1994). In this context, ethylene plays a key role in the dormancy-breakage and germination of *S. humilis* seeds (Ribeiro and Barros, 2006). Moreover, ethylene appears to be associated with tolerance of *S. humilis* seeds to salt stress (Silva et al., 2014).

Salt stress has been proposed to decrease seed germination by regulating the concentration of abscisic acid (ABA) through alterations in *9-cis-epoxycarotenoid dioxygenase3/salt tolerant1* (*NCED/STO1*) expression (Wang et al., 2015). On the other hand, ethylene stimulates germination of seeds of *Arabidopsis* and other species by inhibiting the action of ABA (Linkies et al., 2009; El-Maarouf-Bouteau et al., 2015). The activity of the two key ethylene biosynthesis enzymes, 1-

aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS, EC 4.4.1.14) and ACC oxidase (ACO, EC 1.4.3.3), are regulated by salt stress (Achard et al., 2006; Kazan, 2015). Thus, the balance between ABA and ethylene is likely to play an important role in modulating seed germination under salt stress. Indeed, *Arabidopsis acs7* mutant displayed reduced ethylene production, but a significant increase occurred in seed ABA concentration, which led to a decrease in germination under salt stress (Dong et al., 2011). The importance of ABA for the control of seed germination in saline environments has also been highlighted by the use of *Arabidopsis* mutants with altered ABA biosynthesis (Gonzalez-Guzman et al., 2002). In addition to ethylene and ABA concentrations, carbon and nitrogen metabolism in plants are also sensitive to salt stress (Obata and Fernie, 2012). Salt stress induces changes in the concentrations of tricarboxylic acid (TCA) cycle intermediates, sugars, and amino acids in plants and plays an important role in maintaining metabolic homeostasis under increasing salt concentrations (Richter et al., 2015; Li et al., 2017). However, relatively little is known about the specific role of salt stress in the coordination of primary metabolism during seed germination under salt stress conditions.

Saline soils contain primarily neutral salts such as sodium chloride and sodium sulfate (Rengasamy, 2010). On the other hand, alkaline soils result from continuous increases in the Na_2CO_3 and $NaHCO_3$ concentrations, which leads to a high soil pH (>8.5) (Waskom et al., 2014). Salt stress and alkaline stress inhibit seed germination (Liu et al., 2014; Piovan et al., 2014), yet there is limited understanding of the potential interaction of these stresses, which often occur together in nature. Interestingly, soil pH is crucial to determine germination of *S. humilis* seeds (Ribeiro et al., 2018). In this context, decreases in germination have been attributed to reduced ethylene biosynthesis by seeds incubated in soil with pH above 5.0 (Ribeiro

et al., 2018). Although high environmental salinity inhibits germination of *S. humilis* seeds (Silva et al., 2014), the effects of alkalinity are unclear. It may be that the physiological mechanisms involved in the ability of *S. humilis* seeds to tolerate salt stress are pH dependent.

In this study, we investigated the hypothesis that salt stress increases ABA biosynthesis in *S. humilis* seed to mediate control of ethylene production and primary metabolism during the germination process. Finally, we examined the potential link between salt stress and alkaline stress on seed germination behavior of *S. humilis*.

MATERIALS AND METHODS

Plant material and general conditions

Mature seeds were collected from plants of *S. humilis* growing in a greenhouse in Viçosa (20º 45'S, 42º 15'W), Minas Gerais, Brazil. Seeds were stored in a desiccator at ambient laboratory temperature (25 ± 3 ºC). Seeds were scarified and surface sterilized as described by Silva et al. (2014). Seeds were incubated in 50 ml Erlenmeyer flasks containing two layers of Whatman nº 1 filter paper moistened with 5 ml of test-solution. The Erlenmeyer flasks containing the seeds were placed in a growth chamber at 30 ºC under continuous dark conditions.

Measurement of ethylene, ACC, and in vivo ACO enzyme activity

Ethylene was measured as described by Ribeiro et al. (2010). A gas sample (1 mL) was taken from each Erlenmeyer flask and injected into a gas chromatograph (Hewllett Packard 5890, Series II), and ethylene concentrations were quantified with authentic ethylene standards.

For the ACC assays, seeds were soaked in distilled water, NaCl solution (150 mM) or ABA (10 µM) for 6 h. Subsequently, the seeds were transferred to new Erlenmeyer flasks containing the following solutions after arrow: distilled water→distilled water (control), NaCl→distilled water, NaCl→NaCl, NaCl→NaCl+fluridone, and ABA→ABA for 12 h. At the end of the incubation period, the seeds were washed with distilled water, dried with absorbent paper, weighed and immersed in liquid nitrogen and then stored in a freezer at -80 °C until analysis. ACC concentrations in seeds were measured as described by Bulens et al. (2011).

In vivo ACO enzyme activity were assayed as described by Moya-Léon and John (1994). Fifty seeds were incubated with buffer solution (50 µM $FeSO_4$, 30 mM $NaHCO_3$, 30 mM sodium ascorbate, 0.4 M mannitol, 0.1 M tricine, pH 7.5) alone or also containing NaCl or ABA for 6 h. Afterwards, seeds were through washed with distilled water and transferred to Erlenmeyer flasks with rubber seals and incubated with NaCl, NaCl+Fluridone or ABA in buffer solution alone or containing ACC. Ethylene production by the seeds was determined as described above.

ABA analysis

The levels of ABA in the seeds were determined following the technique described by Müller and Munné-Bosch (2011) with slight modifications. The ABA extraction was carried out in medium containing methanol/isopropanol/acetic acid (20/79/1; v/v/v) and an internal standard ([$^2H_6$] ABA). The samples were injected in the system LC - MS/MS using an Agilent 1200 Infinity Series coupled to a Mass Spectrometry type triple Quadrupole (QqQ), model 6430 (Agilent Technologies, Palo Alto, CA, USA).

Light microscopy

Seeds of *S. humilis* were incubated in distilled water (control), NaCl, NaCl+ACC, NaCl+fluridone, ABA or ABA+ACC for 18 h. Then, seeds were fixed in FAA (formaldehyde/ glacial acetic acid/ 50% ethanol; 1/1/18 v/v/v) for 48 h and stored in 70% ethanol. The seeds samples were dehydrated in ethyl series and embedded in 2-hydroxyethyl methacrylate (Historesin, Leica Instruments, Heidelberg, Germany). Sections (5 µm thick) obtained on a microtome (model RM2155, Leica Microsystems, Deerfield, IL, USA) were stained with 0.05% (w/v) toluidine blue, pH 6.5. Image capture, documentation and analysis were performed as described by de Souza et al. (2018).

Metabolic analysis

The seeds were soaked in distilled water, NaCl (150 mM) or ABA (10 µM) for 6 h. Afterwards, seeds were transferred to new Erlenmeyer flasks containing distilled water, NaCl, NaCl+fluridone or ABA for 12 h. The analysis of metabolite by gas chromatography-mass spectrometry (GC-MS) was performed using 30 mg freeze-dried seed material. Extraction, derivatization and GC-MS analysis were carried out as described by Lisec et al. (2006).

Effect of NaCl and Na_2CO_3 on seed germination

To assess the combined effect of salt stress and pH action on seed germination, seed were treated with distilled water or solutions of NaCl (20, 30, 40, 50 and 60 mM) and Na_2CO_3 (10, 15, 20, 25 and 30 mM). The pH ranges in the solutions of NaCl and Na_2CO_3 were 6.48-6.57 and 10.35-10.65, respectively. The pH,

electrical conductivity and osmotic potential of salt solutions were measured by pH meter (model DM-20, Digimed, São Paulo, SP, Brazil), conductivimeter (model CG-1800, Gehaka, São Paulo, SP, Brazil) and vapour pressure osmometer (model 5500, Wescor, Logan, UT, USA), respectively. Characteristics of NaCl and Na_2CO_3 solutions employed for seed germination experiments are described in Table S1. Seed germination, ethylene production, total ACC and ABA concentrations as well as *in vivo* ACO enzyme activity were measured 18 h from start of seed incubation.

Statistical analysis

The experimental design was completely randomized. The experimental unit of the experiments consisted of 50 seeds per Erlenmeyer flasks with five replicates per treatment. The percent germination results were transformed to arcsin $(\%G/100)^{1/2}$ prior to analysis and all data were checked for normality. Analysis of variance (ANOVA, $P < 0.05$) was performed to determine the effects of the treatments, and then mean values were compared through Tukey test or *t* test at the 5% level of significance. All comparisons were performed with SPSS (Statistical Package for the Social Sciences) 11.0 version.

RESULTS

Regulation of seed germination and ethylene production in response to NaCl and ABA

Seeds treated with NaCl and ABA solution showed an 83% and 95% reduction in germination with a concomitant reduction in ethylene production (73% and 93%, respectively), at the end of the incubation period (Fig. 1A and B). ACC reversed the

inhibition of seed germination promoted by NaCl and ABA solution, a result that is also associated with ethylene production (Fig. 1A and B). In addition, for seeds of *S. humilis* soaked in water (control), ABA+ACC and NaCl+ACC the time required to reach 50% germination (t_{50}) was 9.8, 10.6 and 11.0 h, respectively. Under the action of fluridone, it was verified that the germination of the seeds was 4.6 times greater than as maintained in pure NaCl solution, at the end of 48 h of imbibition (Fig. 1A). Moreover, fluridone solution increased 2.2 times the ethylene production of the seeds maintained in NaCl+fluridone solution, when compared to the pure NaCl solution (Fig. 1B). The results presented in Fig. 1C-N showed that NaCl and ABA reduced the embryo growth potential of *S. humilis* seeds by inhibiting both, cell division and expansion. Importantly, the inhibitory effect of NaCl and ABA on germination of *S. humilis* seeds was reverted when seeds were treated with ACC (Fig. 1I, M). Similarly, inhibition of cell division and expansion by salt stress was overcome by fluridone (Fig. 1H). There was an increased Na concentration in seeds treated with NaCl, NaCl+fluridone or NaCl+ACC, suggesting that fluridone and ACC had no effect on the Na uptake (Fig. S1).

NaCl modulates changes in ABA biosynthesis

To investigate if salt stress triggers an increased ABA concentration modifying ethylene biosynthesis by the seeds, we analyzed the concentrations of ABA and ethylene in seeds treated with NaCl or polyethylene glycol (PEG-6000) at iso-osmotic levels. In contrast with NaCl treatment, PEG-6000 did not inhibit germination and ethylene production by the seeds compared to the control (Fig. 2A and B). There was a significant increase of ABA concentration in seeds treated with NaCl, but not in seeds treated with PEG-6000 as compared with control (Fig. 2B). Moreover, seed

recovered the capacity to germinate after they were transferred from NaCl solution to distilled water (Fig. 3A). Seeds incubated in NaCl solution (150 mM) showed a significant reduction in the concentrations of free ACC (88%) and total ACC (76%) as well as *in vivo* ACO enzyme activity (77%) in relation to control (Fig. 3B-D). However, ACC biosynthesis and ACO enzyme activity of NaCl-treated seeds increased markedly after seeds were transferred to NaCl+fluridone solution or distilled water. After 18 h of incubation of the seeds in ABA solution, significant inhibition of seed germination was observed (Fig. 3A). Similar to the effects of the NaCl solution, the concentrations of free and total ACC as well as *in vivo* ACO enzyme activity in *S. humilis* seeds submitted to the ABA solution were significantly reduced as compared to control seeds (Fig. 3B-D). Moreover, seeds soaked in NaCl solution (150 mM) showed a 7.2-fold increase in ABA production at the end of the incubation period as compared to control seeds (Fig. 3E). On the other hand, NaCl effects on the induction of ABA biosynthesis were inhibited after seeds were transferred to fluridone or distilled water (Fig. 3E).

Changes in metabolite profiles in S. humilis seeds in response to NaCl and ABA

In order to understand the action of NaCl on the primary metabolism of seeds, the metabolic profile was analyzed using gas chromatography coupled to a mass spectrometer (GC-MS). The concentrations of sucrose, trehalose, cysteine, glycine, lysine, ornithine, serine, threonine, tyrosine, histidine, valine and pyruvate in seeds treated with NaCl→NaCl, NaCl→water, NaCl→NaCl+fluridone or ABA→ABA were similar to those observed in seeds incubated with distilled water (control) (Fig. 4). Glucose and myo-inositol were unaltered in seeds treated with NaCl→water,

NaCl→NaCl+fluridone and ABA→ABA, but NaCl→NaCl led to an increase of glucose and myo-inositol concentrations. Increases in concentrations of arginine, asparagine, aspartate, glutamine, glutamate, phenylalanine, proline and tryptophan were only observed in seeds treated with NaCl→NaCl and ABA→ABA (Fig. 4). Moreover, citrate, isocitrate, 2-oxoglutarate (2-OG) and succinate concentrations were significantly reduced in seeds treated with NaCl→NaCl and ABA→ABA, but not in seeds treated with NaCl→water and NaCl→NaCl+fluridone. The concentrations of fructose, 3-P-glycerate, γ-aminobutyric acid (GABA) and methionine were significantly increased in seeds treated with NaCl→NaCl, NaCl→NaCl+fluridone and ABA→ABA while remaining stable in seeds treated with NaCl→water as compared to control (Fig. 4). Clearly, there was a significant increase in alanine concentration in seeds treated with NaCl→NaCl, NaCl→water and ABA→ABA, but not in seeds incubated with NaCl→NaCl+fluridone. Malate and fumarate concentrations were significantly reduced in seeds treated with NaCl→NaCl and ABA→ABA, while they increased in seeds treated with NaCl→NaCl+fluridone as compared with control. On the other hand, there were no significant differences in malate and fumarate concentrations in seeds treated with NaCl→water as compared with control (Fig. 4).

Changes in germination of S. humilis seeds are interlinked with sodium concentration and pH of the medium

Given that salt stress and alkaline stress often occur together in nature, we asked whether alkaline condition might affect the seed germination behavior of *S. humilis* under salt stress. At the same Na^+ concentrations, germination and ethylene production were higher in seeds incubated in NaCl solutions (range pH 6.48-6.57)

than in Na_2CO_3 solutions (range pH 10.35-10.65) (Fig. 5A and B). Na_2CO_3 caused a significant inhibition in seed germination at Na^+ concentration as low as 20 mM, while germination of *S. humilis* seeds treated with NaCl did not differ between 0 and 50 mM Na^+ (Fig. 5A). At 20 mM Na^+ concentration, ethylene production decreased by 15% and 43% in seeds incubated in NaCl and Na_2CO_3, respectively, compared to the control (distilled water) (Fig. 5B). Decreases in germination of seeds treated with NaCl was only observed at 60 mM Na^+ (Fig. 5A). At this concentration, germination was decreased by 35% and 90% in seeds incubated in NaCl and Na_2CO_3, respectively, which caused 42% and 91% inhibition of ethylene production compared to control. Interestingly, NaCl and Na_2CO_3 promoted an increase in ABA concentration as compared with control (distilled water) (Fig. 5C). However, there were no differences in ABA concentration in *S. humilis* seeds between treatments. It is important to note that the seeds recovered the normal rate of germination when they were transferred from NaCl and Na_2CO_3 solutions to distilled water (Fig. 5D). Our results also revealed that the decrease in the total ACC concentration and ACO enzyme activity were greater in seeds incubated in Na_2CO_3 (pH 10.42) than in NaCl (pH 6.48) at the same Na^+ concentration (Fig. 5E and F).

DISCUSSION

The plant hormone ethylene is involved in controlling the germination of *Stylosanthes* seeds under salt stress conditions (Silva et al., 2014). Salt-induced inhibition of *Arabidopsis* and *Glycine max* seed germination may be attributed to ABA biosynthesis and signaling (Wang et al., 2015; Shu et al., 2017). However, the coordination of salt stress with ABA and ethylene production remains poorly defined,

particularly in tropical seed species. In the present study, we found that salt stress increased ABA biosynthesis in *S. humilis* seeds, and this effect was independent of pH (Figs. 2 and 5). The increase in ABA concentration was closely associated with decreases in both ethylene biosynthesis and seed germination (Figs 1 and 2). However, fluridone application to NaCl-treated seeds rescued its germination and ethylene production (Fig. 1). Together, these results indicate that ABA is an important factor controlling the role of ethylene in germination of *S. humilis* seeds during salt stress. This finding is further supported by the fact that the inhibitory effect of NaCl on seed germination was overcome when seeds were treated with ACC (Fig. 1). *S. humilis* occurs naturally in saline soils in Brazil, and natural populations display variation in salt tolerance during germination (Lovato et al., 1999; Silva et al., 2014). It is interesting to note that ABA concentration of seeds treated with NaCl was decreased upon their transfer to water, allowing seed germination (Fig 3). Hence, ABA concentration at germination can play a role as a driver of physiological alterations that allow the successful establishment of *S. humilis* populations in saline environments, particularly after high precipitation when soil salinity is usually reduced due to leaching.

Germination of *S. humilis* is affected by specific ionic effects rather than by osmotic stress (Silva et al., 2014). In this context, ABA concentration was increased in seeds by application of NaCl, but not by PEG at iso-osmotic levels (Fig. 2). Moreover, germination and ethylene concentration were higher in seeds incubated in PEG than in NaCl solution. This suggests that ABA biosynthesis is the causal factor inhibiting germination of *S. humilis* under saline conditions. ABA leads to a decrease in the expression of *ACO* genes in seeds of *Lepidium sativum* and *Arabidopsis*, but it does not affect the expression of *ACS* (Linkies et al., 2009; Linkies and Leubner-

Metzger, 2012). Thus, ACO activity appears to be a limiting step in ethylene biosynthesis during germination of those seeds (Linkies et al., 2009). Our results indicate that NaCl-mediated ABA biosynthesis promoted inhibition in ACC production as well as ACO activity (Fig. 3), resulting in the inhibition of embryo growth potential of *S. humilis* seeds. Interestingly, the effects of the salt stress on seed germination and ethylene production mimicked those of ABA treatment (Figs 1 and 3), suggesting that the regulatory role of ABA in seed germination under salt stress is associated with function of both ACS and ACO. Of note, the inhibitory effect of NaCl on seed germination and ethylene production was overcome after seed transfer to distilled water. Collectively, NaCl inhibits germination and ethylene production by enhancing ABA concentration can be considered as a part of an adaptive mechanism to saline environment.

Salt stress induces rapid changes in cellular primary metabolism (Richter et al., 2015; Chojak-Koźniewska et al., 2018). The increased levels of glutamine, glutamate, aspartate, asparagine and arginine observed in seeds treated with NaCl indicate that those amino acids are being utilized more slowly when germination of *S. humilis* seeds is inhibited by the ABA (Fig. 4). In agreement with this hypothesis, these five amino acids accumulated consistently in seeds treated with ABA (Fig. 4). Moreover, the concentrations of glutamine, glutamate, aspartate, asparagine and arginine of seeds treated with NaCl and afterwards transferred to water or fluridone were similar to those observed in the control, which led to an increase on germination of *S. humilis* seeds. In addition, it was found that ABA treatment increased concentration of tryptophan and phenylalanine (Fig. 4). Since seeds treated with NaCl also showed an increase in the concentrations of tryptophan and phenylalanine, it is reasonable to assume that a modified relationship between aromatic amino acids and germination

in seeds with high ABA production. 3-P-Glycerate is involved in the regulation of membrane/phospholipid biosynthesis during seed germination (Kazmi et al., 2017). We found that the concentration of 3-P-glycerate were higher in seeds treated with NaCl than in seeds imbibed in NaCl and then transferred to water or fluridone (Fig. 4). Given that ABA was increased by NaCl, it is likely that ABA altered the dependence of embryo growth potential on 3-P-glycerate availability (Galland et al., 2017). It was also found that ABA treatment triggers an enhanced 3-P-glycerate concentration in *S. humilis* seeds, emphasizing the role of 3-P-glycerate in coupling embryo growth potential and metabolism. The TCA cycle provides energy and carbon skeletons for a range of biosynthetic processes during seed germination (Rosental et al., 2014; Xu et al., 2016). In this context, ABA treatment negatively affected the accumulation of citrate, isocitrate, 2-OG, succinate, fumarate and malate in *S. humilis* seeds (Fig. 4). Furthermore, the reduction of concentration of citrate, isocitrate, 2-OG, succinate, fumarate and malate in seeds treated with NaCl→NaCl, along with the increase of concentration of TCA cycle intermediates in seeds treated with NaCl→water or NaCl→Fluridone, indicates a different respiration intensity and/or a metabolic remodeling to anaplerosis. Consistent with this hypothesis, seeds imbibed in NaCl and then transferred to water or fluridone showed a fast increase in the germination with a corresponding decrease in ABA concentration compared to seed treated with NaCl solution only (Figs 3). Taken together, our results indicate that the concentrations of ABA and ethylene play an important role in maintaining metabolic function of seed under salt stress conditions.

In addition to salt stress, seed germination is also sensitive to alkaline stress (Piovan et al., 2014; Zang et al., 2018). Since soil pH can alter germination responses of *S. humilis* seeds (Ribeiro et al., 2018), the pH of salt solution could play

an important role in seed germination. In light of this, the greater reduction in seed germination under Na_2CO_3 (range pH 10.35-10.65) treatment compared with NaCl (range pH 6.48-6.57) suggest that under salt stress pH may determine the response to Na^+ concentration in *S. humilis* seeds (Fig. 5). Moreover, there was a significant reduction in the ethylene production in seeds treated with NaCl and Na_2CO_3, but seeds treated with NaCl maintained a higher capacity of ethylene production compared with seeds incubated with Na_2CO_3 (Fig. 5). A reduced ethylene concentration, as found in seeds treated with alkaline salt may result from an impact of the high pH on ethylene biosynthesis pathway. Consistent with this view, inhibition of ACC biosynthesis and ACO activity was much higher in seed incubated in Na_2CO_3 (pH 10.42) than in NaCl (pH 6.48) at the same Na^+ concentration (Fig. 5). Moreover, the decrease in germination and ethylene production in seeds incubated in NaCl solutions at pH 9.0 was greater than in NaCl at pH 7.0 (Fig. S2). Thus, the decrease in ethylene production in seeds treated with Na_2CO_3 can be considered as a way of minimizing damage under salt and alkaline stresses. The significant increase in germination after seeds were transferred from Na_2CO_3 to distilled water is consistent with this (Fig. 5). High pH has been proposed to be involved in the inhibition of seed germination and seedling growth by affecting ABA transport (Degenhardt et al., 2000). Our study indicates that seeds treated with Na_2CO_3 and NaCl showed an increase in ABA concentration in parallel with an increase in neutral and alkaline salt concentrations indicating specific effects of Na^+ concentration in the ABA biosynthesis pathway (Fig. 5).

The observation that seeds incubated with ABA+ACC germinate with a similar time-course as the seeds incubated with NaCl+ACC (Fig. 1) suggests that the mechanism underlying the inhibition of germination of both NaCl- and ABA-treated

seeds is likely the same. In this model, salt stress constitutes a signal that increases ABA concentration, and this increase does not dependent of environment pH (Fig. 6). However, important questions remain about the regulation of ABA biosynthesis during seed germination under salt stress. For example, which genes of the ABA biosynthesis pathway are regulated by salt stress in seeds? Rapid alteration of the ABA biosynthetic genes *9-CIS-EPOXYCAROTENOID DIOXIGENASE3/SALT TOLERANT1* (*NCED3/STOP1*) in response to salt stress have been shown for *Arabidopsis* seeds (Wang et al., 2015). It seems possible, therefore, that regulation of the transcript levels of *NCEDs* occurs in seed of *S. humilis* in response to salt stress. The role of ABA is to inhibit ACS and ACO activity to reduce ethylene biosynthesis and inhibit embryo growth potential of *S. humilis* seeds (Fig. 6). Interestingly, ACC biosynthesis, ACO activity and ethylene production were also decreased by increasing pH (Fig. 6), suggesting that the effect of salt stress on seed germination is not merely the result of excess Na^+. The effect of ABA concentration was not limited to reducing ethylene biosynthesis; it also modified primary metabolism of *S. humilis* seeds (Fig. 4). Thus, the biosynthesis and signaling of ethylene and ABA in *S. humilis* seeds may constitute points of convergence that permit a flexible and appropriated modulation of energy metabolism and embryo growth potential of *S. humilis* seeds within a given pH condition (Fig. 6). Indeed, ABA regulates germination of *Arabidopsis* seeds during salt stress by modulating transcription of ethylene receptors, which in turn is affected by the ethylene receptors (Wilson et al., 2014; Bakshi et al., 2018), indicating that the ethylene pathway is highly flexible to stress adaptation.

CONCLUSIONS

We have presented evidence that salt stress acts as a signal that regulates ABA biosynthesis in *S. humilis* seeds, which leads to a decrease in ethylene production and seed germination. Despite seeds displaying similar concentration of ABA under alkaline pH, ethylene production displayed marked reductions. Thus hormonal balance between ABA and ethylene allows the alterations in energy metabolism and enables seed to adapt to salt stress condition within a given pH condition.

REFERENCES

Achard P, Cheng H, Grauwe LD, Decat J, Schoutteten H, Moritz T, Straeten DVD, Peng J, Harberd NP (2006) Integration of plant responses to environmentally activated phytohormonal signals. Science. 31: 91-94.

Bakshi A, Piya S, Fernandez JC, Chervin C, Hewezi T, Binder BM (2018) Ethylene receptors signal via a noncanonical pathway to regulate abscisic acid responses. Plant Physiology. 176: 910-929.

Bulens I, Van de Poel B, Hertog ML, de Proft MP, Geeraerd AH Nicolai BM (2011) Protocol: An updated integrated methodology for analysis of metabolites and enzyme activities of ethylene biosynthesis. Plant Methods. 7: 17

Chaves IS, Silva NCQ, Ribeiro DM (2017) Effect of the seed coat on dormancy and germination in *Stylosanthes humilis* H.B.K. seeds. Journal of Seed Science. 39: 114-122.

Chojak-Koźniewska J, Kuźniak E, Linkiewicz A, Sowa S (2018) Primary carbon metabolism related changes in cucumber exposed to single and sequential treatments with salt stress and bacterial infection. Plant Physiolgy and Biochemestry. 123: 160-169.

Costa N (2006) Revisão do Gênero Stylosanthes. DPhil Thesis. Universidade Técnica de Lisboa, Lisboa.

Degenhardt B, Gimmler H, Hose E, Hartung W (2000) Effect of alkaline and saline substrates on ABA contents, distribution and transport in plant roots. Plant Soil. 225: 83-94.

de Souza GA, Dias DCFS, Pimenta TM, Almeida AL, Picoli EAT, de Pádua Alvarenga A, da Silva JCF (2018) Sugar metabolism and developmental stages of rubber tree (*Hevea brasiliensis* L.) seeds. Physiology of Plant. 162: 495-505.

Dong H, Zhen Z, Peng J, Chang L, Gong Q, Wang, NN (2011) Loss of ACS7 confers abiotic stress tolerance by modulating ABA sensitivity and accumulation in *Arabidopsis*. Journal of Experimental Botany. 62: 4875-4887.

El-Maarouf-Bouteau H, Sajjad Y, Bazin J, Langlade N, Cristescu SM, Balzergue S, Baudouin, E, Bailly C (2015) Reactive oxygen species, abscisic acid and ethylene interact to regulate sunflower seed germination. Plant, Cell & Environment. 38: 364-374.

Gates CT, Wilson JR (1974) The interaction of nitrogen and phosphorus on the growth, nutrient status and nodulation of *Stylosanthes humilis* H.B.K. (Townsville stylo). Plant Soil. 41: 325-333.

González-Guzmán M, Apostolova N, Bellés JM, Barrero JM, Piqueras P, Ponce MR, Micol JL, Serrano R, Rodríguez PL (2002) The short-chain alcohol dehydrogenase ABA2 catalyzes the conversion of xanthoxin into abscisic aldehyde. Plant Cell. 14: 1833-1846.

Kazan K (2015) Diverse roles of jasmonates and ethylene in abiotic stress tolerance. Trends Plant Science. 20: 219–229.

Kazmi RH, Willems LAJ, Joosen RVL, Khan N, Ligterink W, Hilhorst HWM (2017) Metabolomic analysis of tomato seed germination. Metabolomics. 13: 145.

Li M, Guo R, Jiao Y, Jin X, Zhang, H, Shi L (2017) Comparison of Salt Tolerance in Soja Based on Metabolomics of Seedling Roots. Frontiers Plant Science. 8: 1101.

Linkies A, Müller K, Morris K, Tureckova V, Wenk M, Cadman CSC, Corbineau F, Strnad M, Lynn JR, Finch-Savage WE, Leubner-Metzger G (2009) Ethylene interacts with abscisic acid to regulate endosperm rupture during germination: a comparative approach using Lepidium sativum and *Arabidopsis thaliana*. Plant Cell. 21: 3803-3822.

Linkies A, Leubner-Metzger G (2012) Beyond gibberellins and abscisic acid: how ethylene and jasmonates control seed germination. Plant Cell Reports. 31: 253-270.

Lisec J, Schauer N, Kopka J, Willmitzer L, Fernie AR (2006) Gas chromatography mass spectrometry-based metabolite profiling in plants. Nature Protocols. 1: 387-396.

Liu Y, Wang Q, Zhang Y, Cui J, Chen G, Xie B, Wu C, Liu H (2014) Synergistic and antagonistic effects of salinity and pH on germination in switchgrass (*Panicum virgatum* L.). PLoS One. 9: e85282.

Lovato MB, Lemos Filho JP, Martins OS (1999) Growth responses of *Stylosanthes humilis* (Fabaceae) populations to saline stress. Environmental and Experimental Botany. 41: 145-153.

Moya-León MA, John P (1994) Activity of 1-aminocyclopropane-1-carboxylate (ACC) oxidase (ethylene-forming enzyme) in the pulp and peel of ripening bananas. Journal of Horticutural Science. 69: 243-250.

Müller M, Munné-Bosch S (2011) Rapid and sensitive hormonal profiling of complex plant samples by liquid chromatography coupled to electrospray ionization tandem mass spectrometry. Plant Methods. 7: 37.

Obata T, Fernie AR (2012) The use of metabolomics to dissect plant responses to abiotic stresses. Cellular and Molecular Life Science. 69: 3225-3243.

Piovan MJ, Zapperi GM, Pratolongo PD (2014) Seed germination of *Atriplex undulata* under saline and alkaline conditions. Seed Science and Technology. 42: 286-292.

Qu XX, Huang ZY, Baskin JM, Baskin CC (2008) Effect of temperature, light and salinity on seed germination and radicle growth of the geographically-widespread halophyte shrub *Halocnemum strobilaceum*. Annal of Botany. 101: 293-299.

Rengasamy P (2010) Soil processes affecting crop production in salt-affected soils. Aust. Journal of Soil Research. 37: 613-620.

Ribeiro DM, Barros RS (2006) Sensitivity to ethylene as a major component in the germination of seeds of *Stylosanthes humilis*. Seed Science Research. 16: 37-45.

Ribeiro DM, Mapeli AM, Carnelossi MAG, Delatorre CA, Barros RS (2010) Dormancy breakage of *Stylosanthes humilis* seeds by aluminium. Seed Science Research. 20: 145-152.

Ribeiro RP, Costa CC, Medina EF, Araújo WL, Zsögön A, Ribeiro DM (2018) Ethylene coordinates seed germination behavior in response to low soil pH in *Stylosanthes humilis*. Plant Soil. 425: 87-100.

Richter JA, Erban A, Kopka J, Zorb C (2015) Metabolic contribution to salt stress in two maize hybrids with contrasting resistance. Plant Science. 233: 107-115.

Rosental L, Nonogaki H, Fait A (2014) Activation and regulation of primary metabolism during seed germination. Seed Science Research. 24: 1-15.

Silva PO, Medina EF, Barros RS, Ribeiro DM (2014) Germination of salt-stressed seeds as related to the ethylene biosynthesis ability in three *Stylosanthes* species. Journal of Plant Physiology. 171: 14-22.

Shu K, Qi Y, Chen F, Meng Y, Luo X, Shuai H, Zhou W, Ding J, Du J, Liu J, Yang F, Wang Q, Liu W, Yang T, Wang X, Feng Y, Yang W (2017) Salt stress represses

soybean seed germination by negatively regulating GA biosynthesis while positively mediating ABA biosynthesis. Frontiers of Plant Science. 8: 1372.

Vieira HD, Barros RS (1994) Response of seed of *Stylosanthes humilis* to germination regulators. Physiology of Plants. 92: 17-20.

Wang T, Tohge T, Ivakov AA, Mueller-Roeber B, Fernie AR, Mutwil M, Schippers JH, Persson S (2015) Salt-Related MYB1 (SRM1) coordinates abscisic acid biosynthesis and signaling during salt stress in *Arabidopsis*. Plant Physiology. 169: 1027-1041.

Waskom RM, Bauder JG, Andales AA (2014) Diagnosing saline and sodic soil problems. http://www.ext.colostate.edu (accessed 24 March 2018).

Wilson RL, Kim H, Bakshi A, Binder BM (2014) The ethylene receptors ETHYLENE RESPONSE1 and ETHYLENE RESPONSE2 have contrasting roles in seed germination of Arabidopsis during salt stress. Plant Physiology. 165: 1353-1366.

Xu HH, Song SH, Wang RX, Wang WQ, Song SQ (2016) Proteomics analysis reveals distinct involvement of embryo and endosperm proteins during seed germination in dormant and non-dormant rice seeds. Plant Physiology and Biochemistry. 103, 219-242.

Zhang H, Tian Y, Guan B, Zhou D, Sun Z, Baskin CC (2018) The best salt solution parameter to describe seed/seedling responses to saline and sodic salts. Plant Soil 426: 313-325.

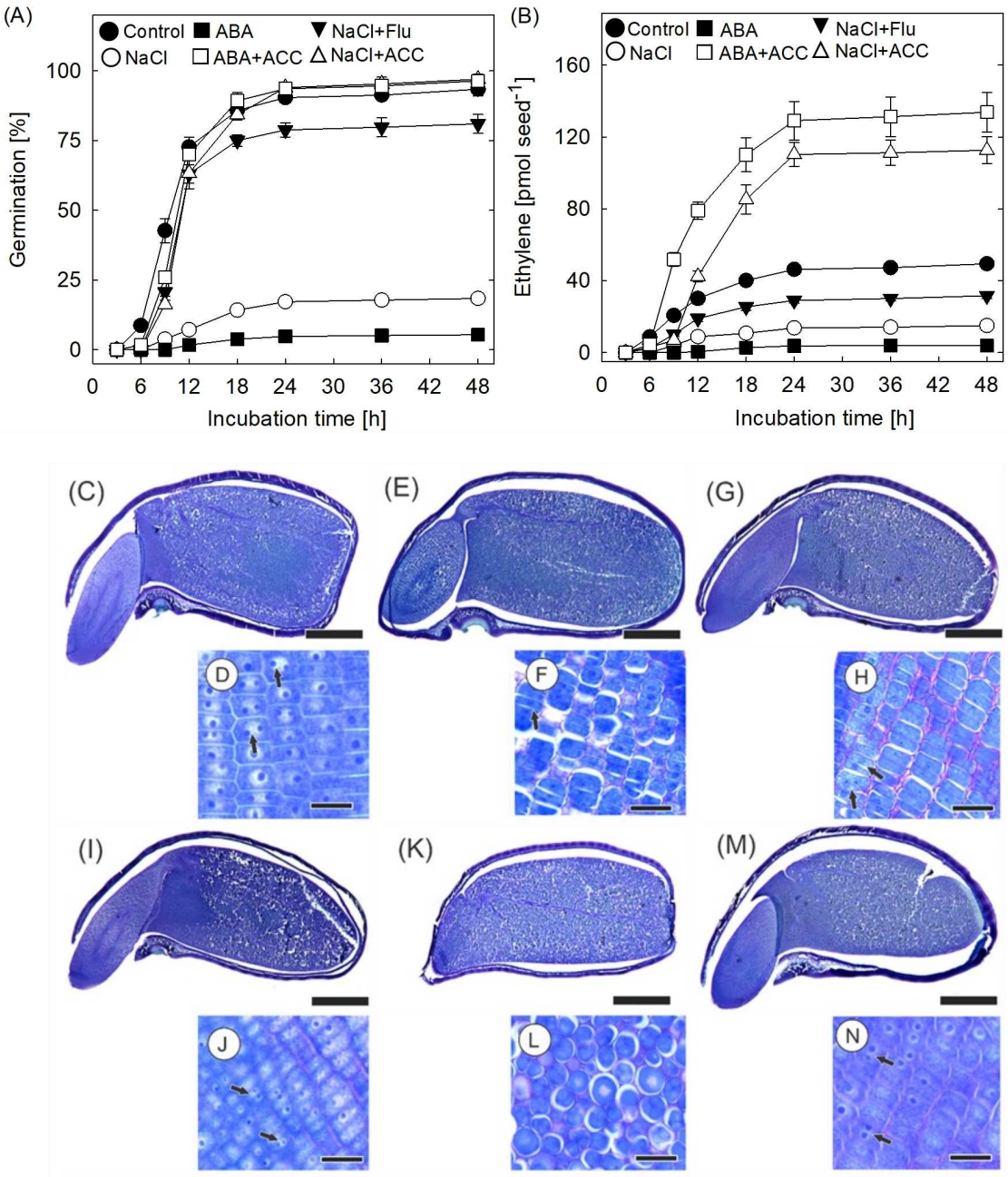

Fig 2.1 Effects of NaCl, NaCl+ACC, NaCl+fluridone, ABA or ABA+ACC on the time course of germination (A) and on the ethylene production (B) of *S. humilis* seeds. Photomicrographs of median longitudinal section of *S. humilis* seeds imbibed for 18 h in distilled water (C, D); NaCl (E, F), NaCl+fluridone (G-H), NaCl+ACC (I, J), ABA (K, L) or ABA+ACC (M, N). Detail of embryonic axis tissues from *S. humilis* seeds (D, F, H, J, L, N). The arrows indicate regions with cell division. Bars = 800 µm (C, E, G, I, K, M) 25 µm (D, F, H, J, L, N). Data are means ± standard error of three separate experiments, with five replicates each.

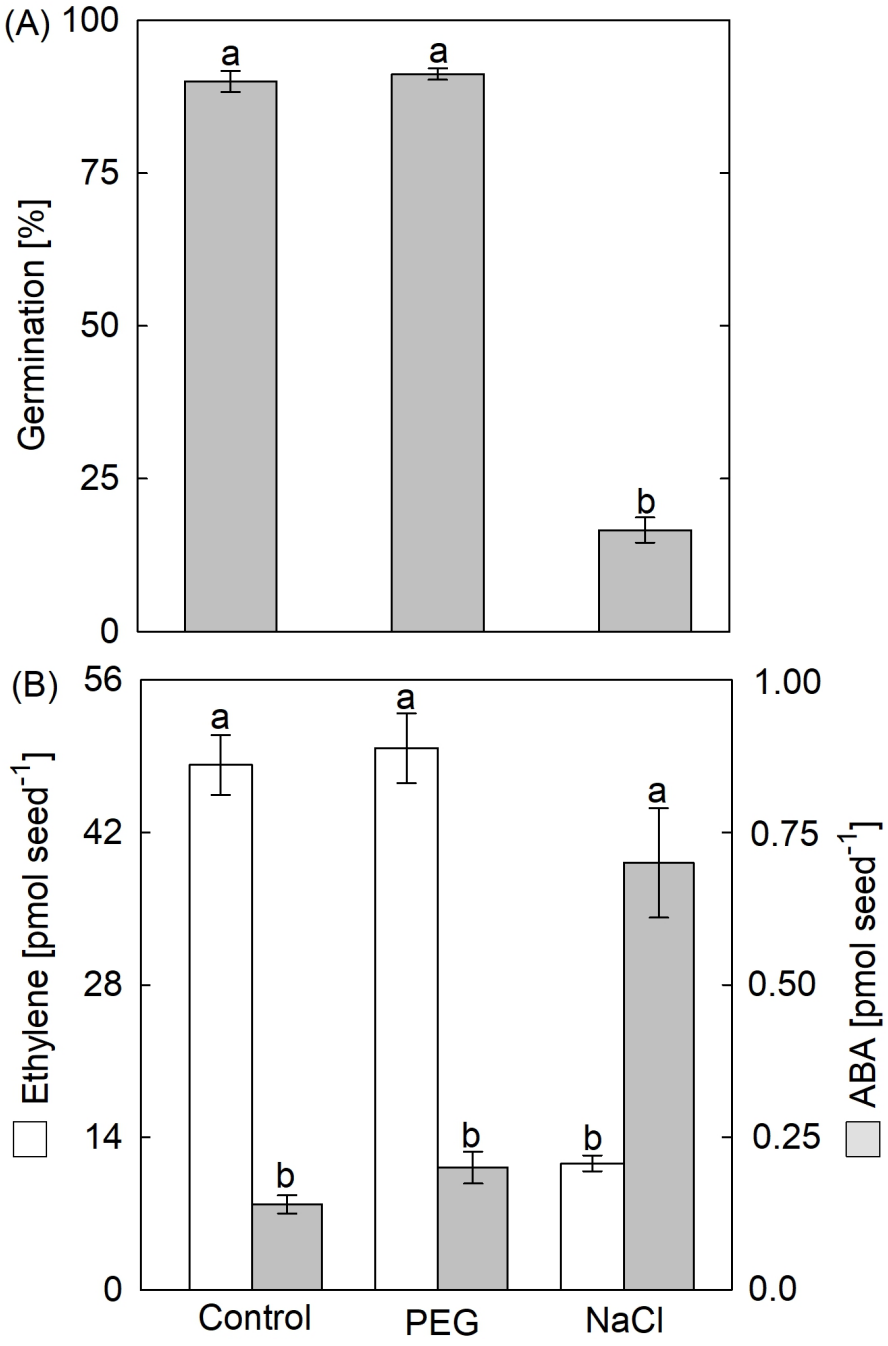

Fig 2.2 Comparison of the germination response as well as concentrations of ABA and ethylene in *S. humilis* seeds treated with iso-osmotic solution of NaCl and PEG. Osmotic potential of solutions was -0.68 MPa [150 mM NaCl; 21% (w/v) PEG]. Germination (A), ethylene production and ABA concentration (B) were measured 18 h from start of seed incubation. Bars followed by the same letters do not differ statistically at the 5% level by Tukey test. Data are means ± standard error of three separate experiments, with five replicates each.

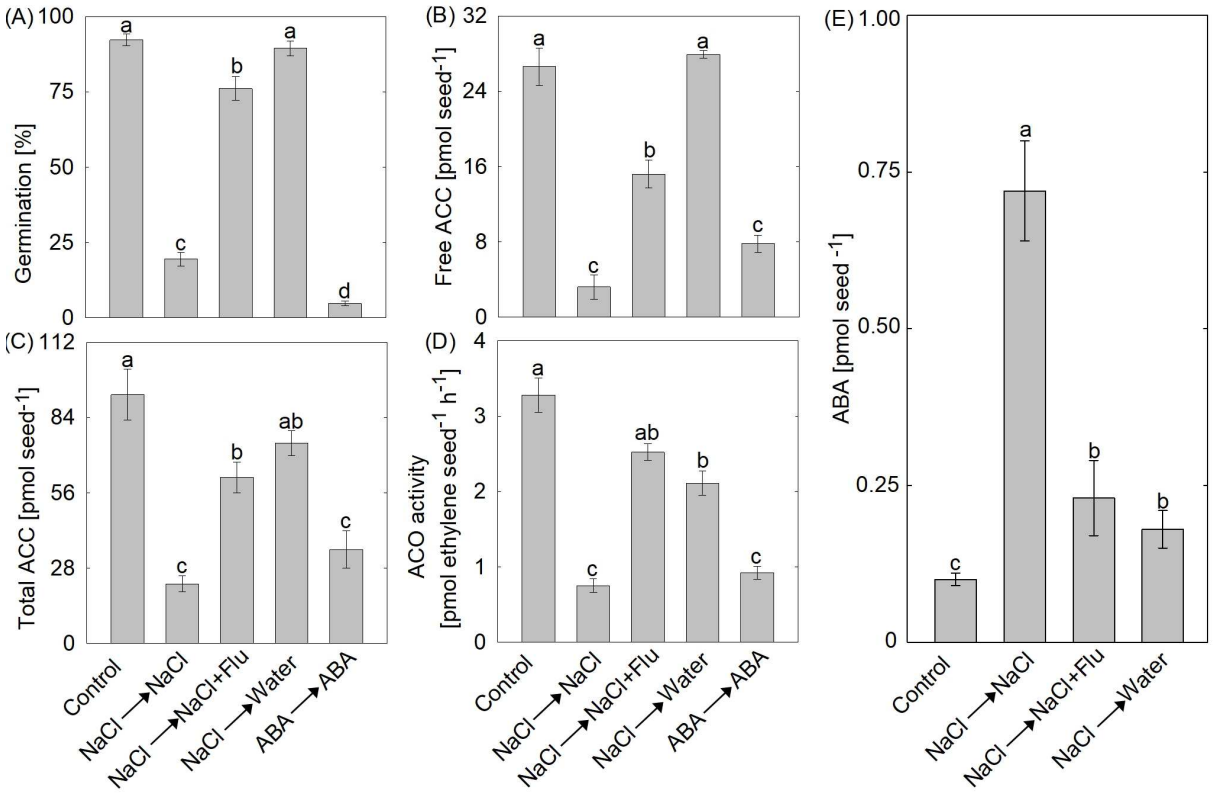

Fig 2.3 Germination, ACO enzyme activity, ACC and ABA concentrations in *S. humilis* seeds. NaCl (150 mM) or ABA (10 µM) was provided to seeds in distilled water for 6 h and, then seeds were transferred to distilled water, NaCl, NaCl+fluridone (50 µM) or ABA for an additional 12 h (as indicated by arrow). Germination (A), free ACC (B), total ACC (C), *in vivo* ACO enzyme activity (D) and ABA (E) were measured 18 h from start of seed incubation. Bars followed by the same letters do not differ statistically at the 5% level by Tukey test. Data are means ± standard error of three separate experiments, with five replicates each.

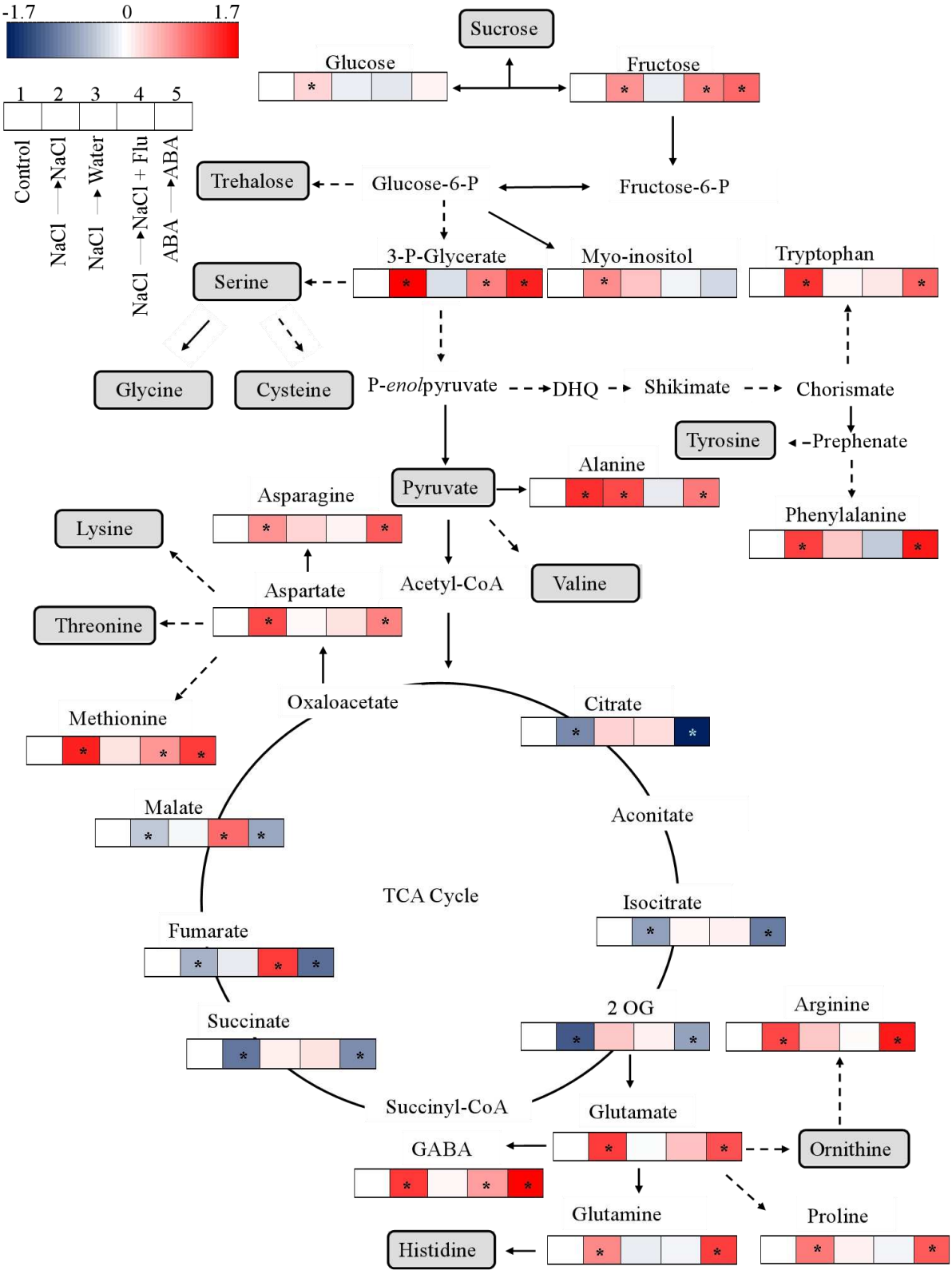

Fig 2.4 Changes in metabolite profiles in *S. humilis* seeds maintained in distilled water, NaCl (150 mM) or ABA (10 µM) for 6h and then transferred to distilled water, NaCl, NaCl+fluridone (50 µM) or ABA for a further 12 h. Continuous arrows indicate a

one-step reaction, and broken arrows indicate a series of biochemical reactions. Data are normalized with respect to mean response calculated for the control (water→water) treatment and $\log_2$ transformed. Red and blue colors indicate that the metabolite content is increased or decreased, respectively. Asterisks indicate values determined by the Student's *t*-test to be significantly different from the control ($P < 0.05$). Values are presented as means of three separate experiments, with five replicates each. DHQ, 3-dehydroquinate; 2-OG, 2-oxoglutarate; GABA, γ-aminobutyric acid. The full dataset from the metabolite profiling study is available as Table S2.

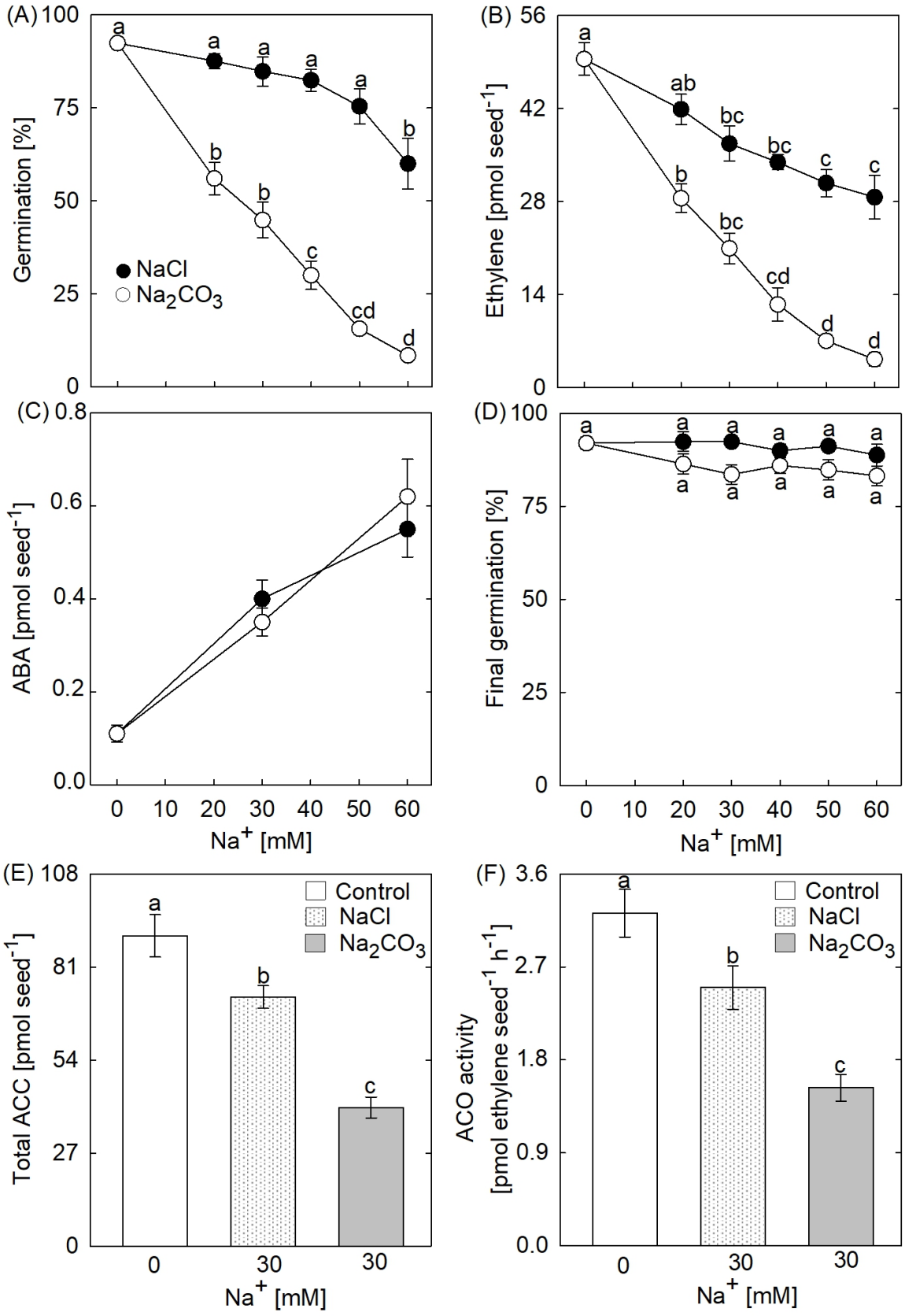

Fig 2.5 Effects of NaCl and Na_2CO_3 on germination, ethylene production, ACO enzyme activity and on concentrations of ACC and ABA of *S. humilis* seeds.

Germination (A), concentrations of ethylene (B) and ABA (C) of *S. humilis* seeds imbibed in NaCl (range pH 6.48-6.57) and Na_2CO_3 (range pH 10.35-10.65) solutions were assayed 18 h from start of seed incubation. The final germination (D) was calculated as (A/B) x 100 (Qu et al., 2008), where A is the number of seeds germinated in the salt solutions after 18 h incubation, plus those that recovered to germinate in the distilled water after another 18 h incubation, and B is total number of seed tested. Total ACC (E) and *in vivo* ACO enzyme activity (F) of seeds treated with NaCl (pH 6.48) and Na_2CO_3 (pH 10.42) at the same Na^+ concentration. Characteristics of NaCl and Na_2CO_3 solutions employed for seed germination experiments are described in Table S1. Values with the same letters do not differ statistically at the 5% level by Tukey test. Data are means ± standard error of three separate experiments, with five replicates each.

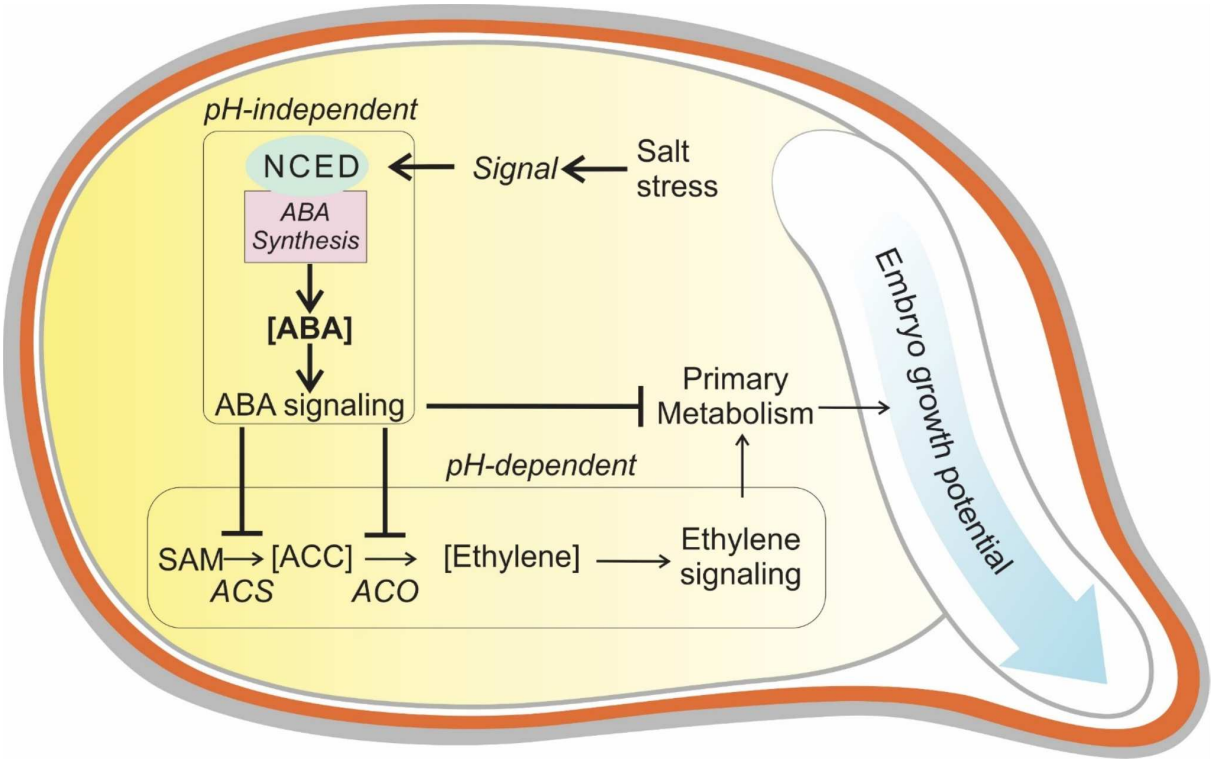

Fig 2.6 Scheme summarizing the effect of salt stress on germination of *S. humilis* seeds. Salt stress causes increased ABA biosynthesis. Thus, the activation of the ABA synthesis and signaling components inhibits ethylene biosynthesis by a decrease in enzyme activities of ACS and ACO. In addition, ABA and ethylene signaling pathways regulate primary metabolism, which affects the embryo growth potential. Interestingly, alkaline pH also inhibits ethylene production, but not ABA synthesis. Hence, seed germination occurs at a rate determined by the ethylene and ABA concentrations in response to salt stress and pH of the medium. The width of the lines denotes relative signalling strength.

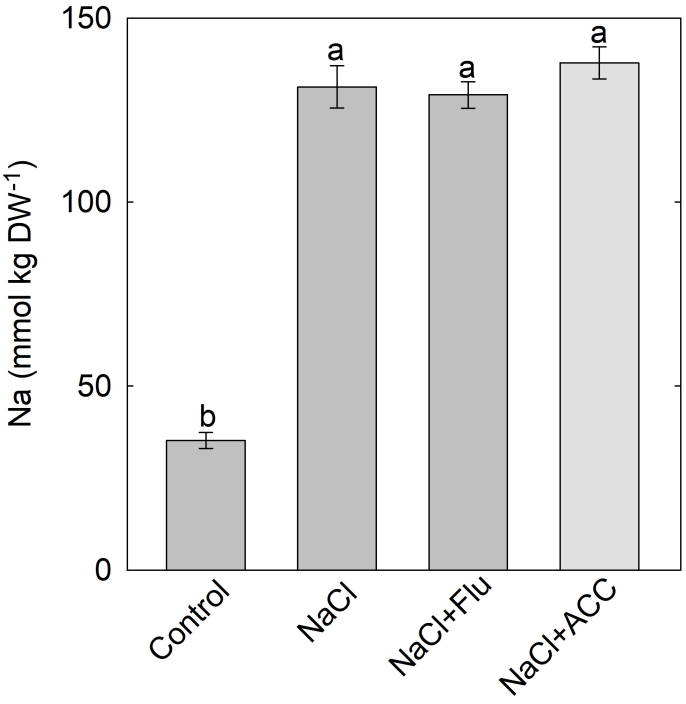

Fig S 2.1 Concentrations of sodium in *S. humilis* seeds incubated with distilled water (control) NaCl, NaCl+fluridone or NaCl+ACC for 18 h. The dried seeds samples were digested with $HNO_3/HClO_4$ (2:1; v/v). Na was determined by using an inductively coupled plasma optical emission spectrometer (ICP-OES, Perkin Elmer Optima 3000 XL). Bars followed by the same letters do not differ statistically at the 5% level by Tukey test. Data are means ± standard error of three separate experiments, with five replicates each. DW, dry weight.

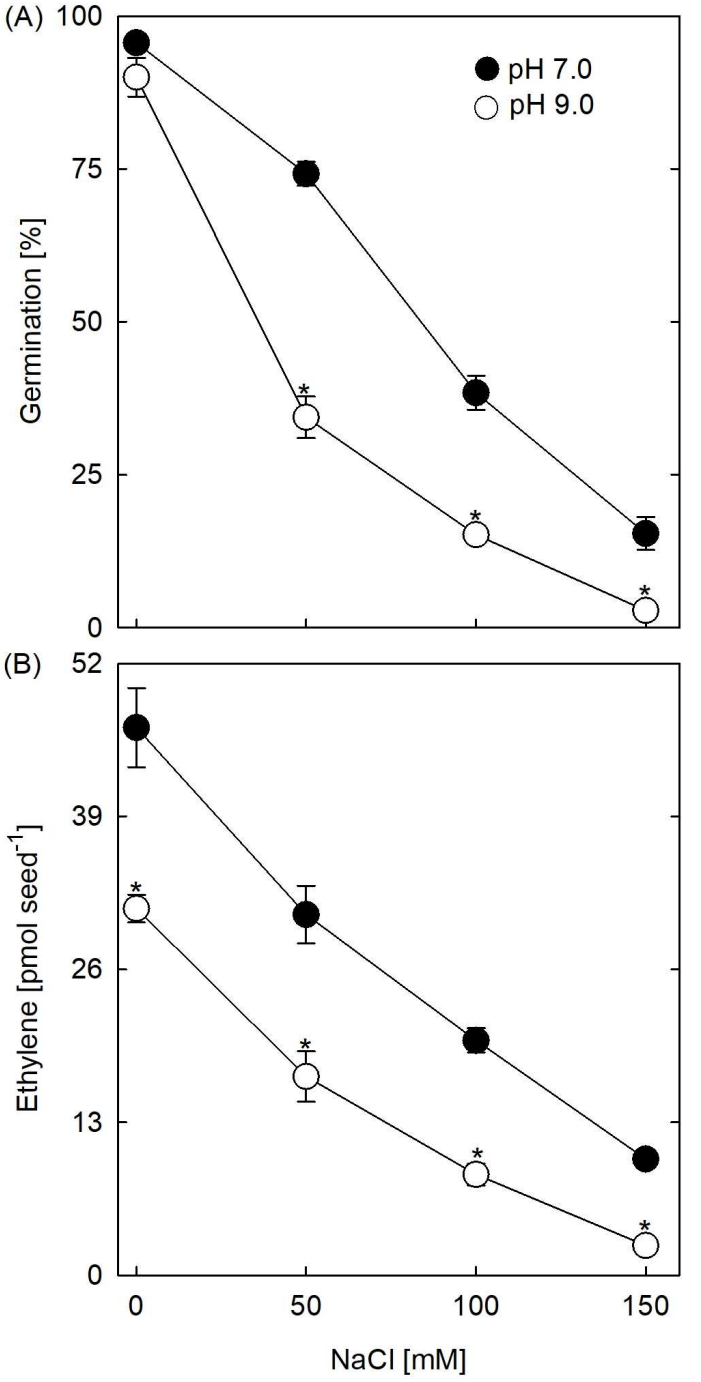

Fig S 2.2 Effects of NaCl supplied in buffer solution at pH 7.0 and 9.0 on germination and ethylene production of S. *humilis* seeds. Seeds were incubated in 10 mM Trizma Base buffer solution at pH 7.0 and 9.0 alone or containing NaCl. Seed germination (A) and ethylene production (B) were determined 18 h from start of seed incubation. Asterisks indicate values determined by the Student's *t*-test to be significantly different from the NaCl solutions at pH 7.0 ($P < 0.05$). Data are means ± standard error of three separate experiments, with five replicates each.

Tab S 2.1 Salt concentration (C), sodium ion concentration (Na^+), pH, osmotic potential (π) and electrical conductivity (EC) of the distilled water and with addition of NaCl or Na_2CO_3.

Treatment	C [mM]	Na^+ [mM]	pH	π [MPa]	EC [dS m^{-1}]
NaCl	20	20	6.51	-0.08	3.41
	30	30	6.48	-0.12	3.71
	40	40	6.55	-0.18	4.55
	50	50	6.57	-0.24	5.72
	60	60	6.50	-0.32	6.63
Na_2CO_3	10	20	10.35	-0.06	2.50
	15	30	10.42	-0.11	3.78
	20	40	10.53	-0.14	4.92
	25	50	10.61	-0.19	5.99
	30	60	10.65	-0.22	6.98
Distilled water	0	0	6.40	0	0.01

Tab S 2.2 Changes in metabolite profiles in S. humilis seeds maintained in distilled water, NaCl (150 mM) or ABA (10 µM) for 6h and then transferred to distilled water, NaCl, NaCl+fluridone or ABA for a further 12 h. Data are normalized with respect to mean response calculated for the control (water→water) treatment. Values in boldface were determined by the Student's t-test to be significantly different from control ($P < 0.05$). Values are presented as means ± standard error of three separate experiments, with five replicates each.

Metabolite	Relative value				
	Control	NaCl→NaCl	NaCl→water	NaCl→NaCl+Flu	ABA→ABA
Sugares and sugares derivates					
Fructose	1.00±0.08	**1.43±0.06**	0.91±0.12	**1.50±0.12**	**1.65±0.09**
Glucose	1.00±0.04	**1.15±0.03**	0.92±0.10	0.89±0.05	1.04±0.16
3-P-Glycerate	1.00±0.10	**2.32±0.14**	0.85±0.07	**1.52±0.13**	**2.09±0.10**
Myo-inositol	1.00±0.04	**1.46±0.09**	1.22±0.10	0.94±0.05	0.82±0.06
Sucrose	1.00±0.14	1.15±0.11	1.14±0.05	0.87±0.12	0.84±0.11
Trehalose	1.00±0.07	1.20±0.08	0.82±0.08	0.91±0.10	1.05±0.05
Amino acids					
Alanine	1.00±0.11	**1.99±0.10**	**1.82±0.08**	0.90±0.08	**1.55±0.09**
Arginine	1.00±0.03	**1.84±0.08**	1.20±0.11	1.01±0.05	**2.13±0.07**
Asparagine	1.00±0.10	**1.43±0.05**	1.15±0.07	1.04±0.09	**1.71±0.10**
Aspartate	1.00±0.05	**1.82±0.09**	1.02±0.15	1.10±0.17	**1.51±0.06**
Cysteina	1.00±0.11	1.02±0.12	1.07±0.06	0.91±0.12	1.17±0.11
GABA	1.00±0.08	**1.94±0.07**	1.03±0.12	**1.38±0.11**	**2.31±0.09**
Glutamate	1.00±0.07	**1.91±0.08**	0.99±0.05	1.23±0.15	**1.78±0.12**
Glutamine	1.00±0.09	**1.48±0.10**	0.91±0.08	0.95±0.09	**1.89±0.08**
Glycina	1.00±0.11	0.98±0.15	1.23±0.14	0.84±0.10	1.02±0.07
Histidine	1.00±0.14	0.79±0.11	1.02±0.09	0.92±0.12	1.14±0.10
Lysine	1.00±0.05	1.02±0.06	1.21±0.14	0.79±0.07	1.15±0.13
Methionine	1.00±0.08	**2.09±0.09**	1.10±0.09	**1.41±0.09**	**1.91±0.06**
Ornithine	1.00±0.12	0.88±0.15	1.12±0.15	0.90±0.07	0.91±0.04
Phenylalanine	1.00±0.09	**1.88±0.02**	1.20±0.10	0.76±0.10	**2.15±0.09**
Proline	1.00±0.14	**1.56±0.08**	1.05±0.06	0.95±0.06	**1.72±0.11**
Serine	1.00±0.05	1.00±0.09	0.82±0.12	0.93±0.09	1.11±0.13
Threonine	1.00±0.10	0.99±0.10	0.94±0.18	1.04±0.11	1.02±0.06
Tryptophan	1.00±0.06	**1.97±0.05**	1.03±0.03	1.08±0.04	**1.67±0.05**
Tyrosine	1.00±0.11	1.16±0.12	1.20±0.18	0.96±0.06	0.91±0.10
Valine	1.00±0.09	0.87±0.10	0.97±0.11	0.97±0.07	1.05±0.08
Organic acids					
Citrate	1.00±0.09	**0.54±0.07**	1.16±0.13	1.12±0.15	**0.31±0.06**
Fumarate	1.00±0.07	**0.67±0.05**	0.90±0.09	**1.92±0.10**	**0.47±0.08**
Isocitrate	1.00±0.06	**0.61±0.08**	1.03±0.05	1.05±0.06	**0.50±0.09**
Malate	1.00±0.07	**0.75±0.14**	0.96±0.08	**1.64±0.07**	**0.63±0.04**
2 OG	1.00±0.08	**0.42±0.09**	1.20±0.14	1.05±0.12	**0.62±0.07**
Pyruvate	1.00±0.10	0.76±0.10	0.91±0.17	1.18±0.09	1.09±0.12
Succinate	1.00±0.09	**0.49±0.06**	1.06±0.05	1.10±0.11	**0.55±0.05**

CHAPTER 3 - TCP8 transcription factors regulate negatively *A. thaliana* seed germination

ABSTRACT

The proper manage of dormancy release and germination is imperative for accomplishment plant establishment. TEOSINTE BRANCHED1/CYCLOIDEA/ PROLIFERATING CELL FACTOR (TCP), a transcription factor (TF), have an evolutionary conserved motif in a range of plant species. Investigations in model plants confirmed the participation of TCP in a variety of roles during plant growth and development. However, the identification of how TCP and the upstream and downstream mechanism works regulating dormancy and germination continue unclear. In this work, we characterised mutants of TCP8 and TCP14 in *Arabidopsis thaliana*. TCP8 and TCP14 seeds presented a contrasted germination pattern at harvest. We recognized TCP14 and TCP8 promoting and inhibiting germination, respectively. The contrast among WT, tcp8 and tcp14 transcriptomes evidenced reasonable transcriptomic adjustments after imbibition with a sustained expression of genes associated to seed germination. Here we showed that TCP8 negatively regulated genes related to the initial phase of germination. Also, TCP regulated *EXPA* gene members that is associated with GA during germination. Transcriptional activity was assayed in transiently transformed *Nicotiana benthamiana* under the control of a synthetic promoter as a reporter. TCP8 and TCP14 binds to GASA4 and MCM2 promoter regions activating the transcription. As a whole, this study advance our understanding as the function of TCP8 in seed germination in *Arabidopsis thaliana*.

Keywords: Seed; Dormancy, Germination; *Arabidopsis thaliana;* Transcription Factor; Teosinte Branched1/Cycloidea/Proliferating cell factor.

INTRODUCTION

The establishment of a plant populations depends on the adaptive aspects of seed propagation and germination. Seed germination is a vulnerable stage in the plant life cycle and is tightly regulated by environmental and genetic factors (Hamala et al 2017, Kadereit et al 2017). Seed dormancy is a mechanism to prevent seed germination in unfavourable conditions. Seeds adopted different dormancy mechanisms to overcome this stage such as physical, physiological, morphological and morphophysiological responses (Finch-Savage and Leubner-Metzger 2006, Jaganathan et al 2019). It has been pointed out the importance of temperature during seed germination and how it can affect hormones that regulate the dormancy and germination (Basbouss-Serhal et al 2016). It is also well established the involvement of the acid abscisic (ABA) and gibberellin (GA) working antagonistically in the control of dormancy and germination (Shu et al 2018, Tuan et al 2018, Cao et al 2020).

Transcription factors (TFs) are proteins that can work alone or in a complex protein activator, binding to DNA, regulating the transcription process of DNA to RNA (Danisman 2016). Most of these proteins are grouped into families, according to the domain of DNA binding. TFs can regulate positively or negatively a large number of functional genes, as a response to stress conditions, cell division, also modulate grain size and plant development (Watt et al 2020). Some TF have a basic region called helix-loop-helix (bHLH) family. This basic region interacts directly with the DNA followed by two alpha helices connected by a disordered region that can dimerize

(Toledo-Ortiz et al 2003). Teosinte Branched1/Cycloidea/ Proliferating cell factor (TCP) are a family of transcription factors, that owns a bHLH region, and were conserved in plant species and control a broad range of roles in plant growth, development, and pathogen resistance (Li 2015, Li et al 2018, Van ES et al 2019, Ferrero et al 2019). The TCP DNA binding domain contains a sequence of amino acids that is very similar between the 45 family members found in *Arabidopsis* and *Oryza sativa.* However, in no-binding-motif region, TCPs have no significant similarity to each other (Yao et al 2007). According to the conservation of specific amino acid residues, the gene family of TCP was subdivided into two classes (Carrara and Dornelas 2020). Besides that, TCP present a protein interaction domain, which allowing their dimerization, a form by which they become biologically active. Studies on possible dimerization have shown that TCP proteins form dimers more efficiently with proteins of the same class, and more often in form heterodimers (Danisman 2016, Kubota et al 2017). TCP protein bind to a highly conserved DNA domain commonly a Helix-Loop-Helix region promoting or repressing cell proliferation by class I or II, respectively (Liu et al 2019). The two classes of TCP family have been postulated to work antagonistically modulating the expression of gibberellin, salicylic acid, auxin, and by being sensitivity to ABA (Liu 2015, Resentini et al 2015, Wang et al 2015, Ding et al 2019, Ferrero et al 2019). In addition, TCP14/15 have been pointed out as regulators of the GA biosynthesis by targeting the gene GA20ox1 during plant development (Ferrero et al 2019). Moreover, TCP14 regulates the GA biosynthesis and responsive genes, leading to seed germination, through embryo growth stimulation (Tatematsu et al 2008, Zhang et al 2019).

TCP8 and TCP14 belongs to the class I family due to the homology of residues (Li 2015). TCP8 have been described as a regulator of leaf development

(Aguilar-Martinez and Sinha 2013). Additionally, the role of TCP8 in delaying flowering and participating in plant-pathogen interaction has been also demonstrated (Li et al 2018, Wang et al 2019, Spears et al 2019). Adjust control of dormancy and germination is an important adaptive feature. Furthermore, it is a mechanism that has been receiving extremely attention from an agronomic point of view. It is known that members of the TCP family are vital TF that regulate molecular mechanisms by interfering in plant growth and development (Liu et al 2019). Due TCPs class I are yet poorly studied during seed germination, sounds promising to understand how these TF can leads to the improvement of seed germination. The more details of the functions and regulatory mechanism of TCPs in the seed germination and dormancy are presented, the better is the theoretical foundation that may allow us to efficiently exploit it in agrosystems. However, up to now, there are remaining questions concerning the mechanism of seed germination.

This study focused on comprehend how TF from class I TCP family regulates *Arabidopsis thaliana* seed germination. In order to help elucidate the functional of TCP8 and TCP14 genes, we analysed the physiological germination behaviour in *tcp8* and *tcp14* mutants and in TCP8 and TCP14 overexpressor lines. The transcriptional activation by TCP8 and TCP14 of two genes were carried out by transactivation assay in *Nicotiana Benthamiana* leaves. In order to have a more precise idea of the function of these factors in germination and to know which genes they could regulate we studied the global expression of genes by RNA sequencing in the wild type and the two mutants, *tcp8* and *tcp14* during seed imbibition. We also analysed the transcription expression of genes involved in germination by qRT-PCR. Altogether, our finds suggest, that TCP8 and TCP14 (both from class I TCPs) modulate germination in a contrasting manner.

MATERIAL AND METHODS

Plant Materials

Arabidopsis thaliana ecotype Columbia (Col-0) was used as wild type (WT), mutant lines *tcp8*, *tcp14*, *ptcp8:tcp8-GFP*, *35S:tcp8-GFP*, *ptcp14:tcp14-GFP* were grown in substrate inside a growth chamber with controlled temperature (22 ± 2 °C), under a photoperiod of 16 h light / 8 h dark and light intensity of 100 $\mu E/m^{2} \cdot s^{-1}$. Seeds were harvest and stored in Falcon tubes in -20°C freezer until the use.

Germination assays

Seeds were incubated in Petri dishes of 90 mm containing a layer of cotton with a Whatman paper soaked with 15 mL of distilled water or test solution. The Petri dish was placed in a growth chamber in darkness at a controlled temperature of 15, 20, or 25 °C. The seed germination was considerate when the radicle emerged through testa. Germination was scored every 24 hours.

Genotyping of tcp8.1, tcp14.4, ptcp8:tcp8-GFP, 35S:tcp8-GFP and ptcp14:tcp14-GFP mutant

Plants from F1 and F2 seeds were genotyped at the rosette stage to isolate homozygous plants from mutated alleles. Plant genomic DNA was extracted from leaves using the following protocol: leaf tissue was ground in 750 µL of extraction buffer (100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, pH 8.0, 2 % CTAB, 0.2 % β-mercaptoethanol) in 1,5 mL Eppendorf. The sample was incubated 30 min at 60 °C with 750 µL of chloroform-isoamyl alcohol (24:1), then the extract was vortexed and centrifuged at 5000 rpm, 15 min, at 4°C. One volume of supernatant (500 µL) was precipitated with one volume of isopropanol. After homogenization, the mixture

was centrifuged at 15000 rpm for 40 min at 4°C and DNA pellet was solubilized in 50 µL milliQ H_2O.

Two pairs of primers were used for genotyping *TCP8* and *TCP14* alleles. LBb Sail (T- DNA left border primer) and *TCP8* or *TCP14* (downstream the T-DNA) were used to amplify specifically *tcp8* or t*cp14* allele. The primers used for genotyping are indicated in Tab. S3.1.

PCR reactions were performed in a final volume of 20 µL including 1 µL of DNA, 2 µL of 10X Dream Taq Green buffer, 0.4 µL of 10 mM dNTPs, 0.2 µL of 10 µM each primer, and 0.5 unit of Dream Taq DNA polymerase (Thermo Scientific).

Amplifications were carried out in a thermocycler (Eppendorf, Mastercycler gradient) with the following program: an initial step of denaturation at 95 °C for 5 min then forty cycles were performed with a step of denaturation at 95 °C for 30 s, a step of annealing at 56 °C for 30 s, a step of extension at 72 °C for 1 min 30 s, and a final extension was performed at 72 °C for 15 min. Then the PCR products were run on 1 % agarose gel electrophoresis containing 1/10000 (v/v) SYBR® Safe (Thermo Scientific). The fragments were separated for 60 ± 10 min at 90 mV and detected under UV on a gel imaging system (Genius3, Syngene).

RNA extraction, cDNA synthesis and RT-qPCR

Total RNAs were isolated from 50 mg seeds (dry or imbibed for 24 h) and were ground in liquid nitrogen by pestle and mortar and transferred to a 2 mL Eppendorf tube, according to Chang et al (1993). Total RNA concentration of the samples was determined using a Nanodrop (Eppendorf). RNA quality was verified by electrophoresis on a 1 % agarose gel containing 1:10000 SYBR Safe (v/v) and separated for 60 ± 10 min at 90 mV, before observation under UV on a gel imaging

system (Genius3, Syngene). DNase treatment was performed with Turbo DNA-free DNase (ThermoFisher, Waltham, MA, USA) in a final volume of 40 µL with 25 µL of RNA extract. DNase was inactivated for 5 min at room temperature with 10 µL of DNase Inactivation Reagent (Thermo Scientific). Mix was then centrifuged for 2 min, at 10000 rpm and 50 µL of the upper phase were collected. RNAs were subsequently purified on Nucleospin XS columns (Macherey-Nagel, Hoerdt, France) according to manufacturer's instructions. Complementary DNA (cDNA) was synthesized from total RNA (2 µg) with RevertAid reverse transcriptase (ThermoFisher). Real-time PCR amplification was performed in Mastercycler epgradients Realplex2 (Eppendorf, Hambourg, Germany), using maxima SYBR Green/quantitative PCR (qPCR) Master Mix (ThermoFisher) as described by the manufacturer. The qPCR program was as follow: 95 °C / 10 min followed by 30 cycles (95 °C / 30 s; 56 °C / 30 s; 72 °C / 30 s). Primer pair efficiencies and critical thresholds were calculated using Realplex2 software (Eppendorf). The results of three biological replicates, each with at least three technical replicates were normalized using three genes (AT4G34270, AT5G53560, AT4G26410) exhibiting a steady-state level. The relative quantification of transcription was an arbitrary value of 100% was assigned to the WT seeds (dry seed or 24 h imbibition) for the normalization of the relative transcript abundance and calculated based on the $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001). Primers used are listed in Table S3.1.

LUC activity assay

For transactivation experiments, the reporter construct was a pGreenII 0800-LUC vector (Hellens et al 2005) in which LUCIFERASE (LUC) reporter gene was expressed under the control of a synthetic promoter, which comprised of six repeats

of the class I TCP consensus binding site (GTGGGCCCAC) upstream of a minimal 35S promoter, as previously described elsewhere (Resentini et al 2015). *Agrobacterium tumefaciens* strain GV3101 was grown overnight in liquid LB medium supplemented with appropriate antibiotic at 30 °C, 150 rpm. When culture reached an OD600 nm 0.5-0.7, *Agrobacterium* were centrifuged for 15 min, at 9600 g, 4°C. Pellet was washed with pre-cooled 10% glycerol successively with 1/1, 0.5/1, 0.25/1 and 0.02/1 (v/v) of the initial culture volume. *Agrobacterium* were resuspended in 1 mL of 10% of glycerol, aliquoted and stored at -80 °C.

Transformations (1,8 kV, 5.5 ms) were performed using 100 µL of GV3101 electrocompetent cells supplemented with 2-3 ng of plasmid using a MicroPulserTM electroporator (BioRAD). Bacteria were resuspended in 1 mL of LB medium and incubated at 30 °C, 150 rpm, for 4 h. Agrobacterium were plated on LB agar supplemented with appropriate antibiotics and incubated for 48 h at 30°C. Positives clones were identified by PCR and cultured for plasmid extraction (GeneJet Plasmid miniprep Kit, Thermo Scientific) following the manufacturer's instructions.

Agrobacterium GV3101 (50 mL) were grown in liquid LB medium overnight at 30°C, 150 rpm. Bacteria were collected by centrifugation at 4000 g, 20 min, 4 °C and resuspended in AIB buffer (10 mM $MgCl_2$, 10 mM MES-KOH, pH 7.5, 150 µM acetosyringone) to a final O.D. = 0.6. Bacteria suspensions were incubated at room temperature for 3 hours and subsequently diluted to a final OD = 0.2. GV3101 cells harboring the different constructs were subsequently mixed and bacterial mix was infiltrated into the abaxial epidermis of 4-week-old tobacco (*Nicotiana benthamiana*) leaves using a 1 mL syringe without a needle.

N. benthamiana plants were grown in chamber 22 ± 2 ºC, 16/8 hours light/dark, for 4 weeks. All constructs used have been obtained as described for Zhang et al

(2019). Leaves of 4-week-old *N. benthamiana* were infiltrated with *Agrobacterium* with 1:4 ratio of cells with reporter and effector constructs, respectively. Protein was extracted from 2 cm diameter disks of each infiltrated leaf 72 hours after infiltration, following by centrifugation at 13000 g for 5 min at 4 ºC. To measure luciferase activity, 40 µL of Dual-Glo® luciferase substrate (Promega) was deposited in a 96-well plate and 1 µL of each protein extract was added to a single well and incubated for 10 min in darkness. Firefly luciferase luminescence was then measured (TriStar2 S LB 942 Multimode Microplate Reader, Berthold). Subsequently, 40 µL of Dual-Glo® Stop&Glo® substrate (Promega,) was added to each well and incubated for 10 min in darkness. Renilla luciferase luminescence was measured. The extracted was load in a 96-well plate. LUC activities Firefly and the control Renilla were assayed with the Dual-Glo Luciferase Assay System (Promega) according to manufacturer's instructions and quantified with TriStar2 S LB 942 Multimode Microplate Reader (Berthold, Thoiry, France). Activity was calculated as a ratio between Firefly and Renilla luminescence. The ratio of each combination was then normalized towards the activity measured for the reporter construct alone.

Transcriptome studies

Total RNA was extracted from 50 mg of seeds from three independent experiments using RNEAsy kit (Qiagen, Hilden, Germany) according to the supplier's instructions. RNA-seq libraries were generated following the TruSeq Stranded protocol (Illumina, California, USA) with a sizing of 260 bp. RNA-seq libraries were then sequenced in paired-end (PE) and a read length of 75 bases on an Illumina Nex-Seq500 (IPS2 POPS platform). Eighteen samples per lane of Next-Seq500 were pooled using individual barcoded adapters, which generated approximately 20 million

of single reads per sample. All steps of the experiment, from growth conditions to bioinformatics analyses, were saved in CATdb database according to the international MINSEQE standard ‘minimum information about a high-throughput sequencing experiment’.

RESULTS

Physiological and molecular characterization of tcp8 and tcp14 mutants

Germination phenotyping of *tcp8.1* and *tcp8.2* mutants (SAIL_656_F11, Aguilar Martinez and Sinha 2013) and *tcp14.4* (Kieffer et al 2011) was performed. To test seed viability and germination independently of dormancy effect, germination tests were performed firstly at 15 °C on freshly harvested seeds. At 15 °C *tcp8* mutant seed germinated slightly rapidly than Col-0 and *tcp14.4* seeds, and all the lines fully germinated after 5 days of imbibition, indicating a perfect viability of seeds (Fig. 3.1a). On the other hand, the germination rate of *tcp8* seeds at 25 °C was significantly higher when compared with Col-0 and *tcp14.4,* after 4 days of imbibition. After 7 days it was observed the values of 34% and 4% in the germination rate for *tcp8* and Col-0, respectively (Fig. 3.1c). We therefore assayed germination rate at an intermediate temperature (20 °C). Not surprisingly, seeds germination was recovered when assayed at 20 °C, showing an intermediate germination rate between 15 °C and 25 °C for Col-0 and *tcp14.4*. On the other hand, about 95 % *tcp8* seeds germinated at 20 °C (Fig. 3.1b). At 20°C, the germination rate was decreased for both lines with a final germination ranging from 80% to 95%, indicating that seeds were dormant at harvest (Fig. 3.1b). Nevertheless, *tcp8* mutant seed presented a significantly higher germination as compared to Col-0 after 7 days (95 and 80%,

respectively). This evidences therefore that *tcp8* mutant seeds were less dormant (Fig. 3.1b). This phenotype was confirmed at 25°C, with *tcp8* mutant seeds germinating more than Col-0 seeds after 7 days (34 and 4%, respectively) (Fig. 3.1c). To further characterize *tcp8* mutant phenotypes, a potential link with GA metabolism and/or response was investigated, in two independent *tcp8* lines (*tcp8.1* and *tcp8.2*). In the first step, the effect of exogenous GA treatment on Col-0 and *tcp8* seeds was compared (Fig. 3.1d). In these experiments, we focused on *tcp8* mutant that provided the most eminent phenotype. As observed in Fig. 3.1c, GA absent treatment, the rate of germination at 25°C was very low in Col-0 and *tcp8* seeds. When GA was applied at 100 µM, the germination rate was stimulated in both Col-0 and mutant seeds after 3 days of imbibition. Nevertheless, the stimulation was significantly higher in *tcp8* seeds than in Col-0. When treated with 100 µM GA, no significant difference was observed between *tcp8* and Col-0 seeds.

To confirm the opposite role of TCP8 and TCP14 in germination we constructed TCP8 and TCP14 mutant lines under their native promoter or 35S promoter. The lines used were *ptcp8:tcp8-GFP*, *35S:tcp8-GFP*, *ptcp14:14-GFP*. Consistent with the germination test of *tcp8* and *tcp14* mutants, the Col-0 seeds displayed an intermediary germination rate as compared to *ptcp14:tcp14-GFP* and *ptcp8:tcp8-GFP,* except the line 12.11, which the germination percentage decreased as compared to Col-0 (Fig 3.2). The germination rate of *ptcp14:tcp14-GFP* were 40% and 58% for lines 2.5 and 1.8, respectively. The Col-0 reached 37% after 7 days of the test. The lines of *tcp8* mutants, under the native and 35S promoters reached 18% and 6% germination rate, respectively. After 7 days of imbibition the line *ptcp14:tcp14* 1.8 showed more than 2-fold the germination percentage as compared to *ptcp8:tcp8-GFP,* and at least 9-folds more when compared to *35S:tcp8-GFP* (Fig 3.2). These

results suggest contrasting roles of TCP8 and TCP14 during seed germination (Fig 3.2). The expression of TCP8 and TCP14 transcripts abundance was analysed during imbibition in freshly harvested seeds by quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) in Col-0, *tcp8.1, ptcp8:tcp8, 35S:tcp8* and *ptcp14:tcp14* seeds (Fig 3.3). TCP8 transcript abundance decreased 24 hours after imbibition for all the lines (Fig 3.3a). Although the profiles were slightly different in TCP8 mutants, TCP8 transcripts were detected in dry and imbibed seeds. TCP8 transcripts were maximum 20% on *tcp8.1* and increased 2-fold in *35S:tcp8* seeds, compared to Col-0 (Fig 3.3a). TCP14 transcript abundance increased 24 hours after imbibition, except to the line *ptcp14:tcp14 12.11*. Although the relative transcripts were different among TCP14 mutants. TCP14 transcripts were increased at least 5-fold in *ptpc14:tcp14* lines compared to Col-0 (Fig 3.3b).

Molecular response of tcp8 mutant to GA

To further establish the link between TCP8 and GA, the expression of GA synthesis, catabolism and response genes were analysed (Fig. 3.4a). The data obtained from the comparison of *tcp14* and Col-0 seed transcriptome, indicated that 17.4% of the 947 genes that were downregulated in *tcp14* seeds after 24 hours imbibition were reported as GA-dependent (Ogawa et al 2003). In general, genes involved in GA synthesis and response were repressed in *tcp14* mutant when those involved in GA catabolism were stimulated. Part of the genes that presented a marked downregulation in *tcp14* seeds was selected and further transcript level was analysed in *tcp8* mutant seeds.

In dry seeds, no significant difference was observed between Col-0 and *tcp8* seeds. Indeed, transcript abundance of genes involved in GA synthesis, (i.e.

GA3ox2), and in GA response (i.e. *GASA4*, *GASA14, EXPA3 and CP1*), were very weak, when compared to the absence of GA in dry seeds (Fig. 3.4a). Otherwise, the level of a main GA catobolism-related gene (*GA2ox2*) was high, which may contribute to the maintenance of a low rate of GA in dry seeds. After 24 hours imbibition, the level of GA synthesis and response genes was markedly increased in Col-0 seeds. In *tcp8* seeds, transcript abundance also increased to a significantly higher level than in Col-0 seeds (Fig. 3.4a). *GA3ox2* transcripts were two folds as abundant in *tpc8* seeds as compared to Col-0, with a similar behaviour for *GASA14, EXPA3* and *CP1* (1.6 to 3.4-folds). On the other hand, no difference was observed for *GA2ox2* transcript abundance between dry and imbibed seeds, and also between Col-0 and mutant seeds (Fig. 3.4a).

Analysis of replication genes in tcp8 mutant seeds

Transcriptomic analyses showed that a large number of genes related to the cell cycle and DNA replication especially associated with the transition from G1 to S phase, were downregulated in *tcp14* imbibed seeds (Zhang et al 2019). We also examined genes related to DNA replication such as *MINI CHROMOSOME MAINTENANCE* (MCMs), *PROLIFERATING CELL NUCLEAR ANTIGEN1* (PCNA1) and *DNA POLYMERASE ALPHA 2* (POLA2) that were downregulated in *tcp14* mutant (Zhang et al 2019). All these genes are over-expressed in *tcp8* seeds compared to Col-0 (Fig 3.4b). As we observed, the transcripts of the three replicative helicases (*MCM2*, *MCM3* and *MCM7*), *POLA2,* required for the initiation of replication, and *PCNA1* were hardly detectable in dry seeds, in both Col-0 and *tcp8* (Fig 3.4b). On the other hand, these transcripts strongly accumulated during imbibition in Col-0

seeds (between 1.4- to 3-fold) (Fig. 3.4b). These results indicate that TCP8 negatively regulated genes related to the initial phase of germination.

Analysis of GASA4 and ABA genes in tcp8 and tcp14 mutant seeds

We examined the expression of *GASA4* in TCP8 mutants lines under the control of they native promoter (*pTCP8:TCP8-GFP*) and an enhancer promoter (*35S:TCP8-GFP*). The GASA4 transcripts decrease of approximately 90% and 60% under the lines *35S:TCP8-GFP* and *pTCP8:TCP8-GFP*, after 24 hours of imbibition, respectively (Fig 3.5a). In dry seeds all the lines presented low GASA4 transcription.

We also analysed the expression of genes related to ABA signaling gene (ABI5) and synthesis (NCED) in *pTCP:TCP4-GFP* lines, after 24 hours of imbibition. It was observed a moderated decrease and rise of ABI5 transcripts in pTCP14:TCP14 1.8 and pTCP14:TCP14 12.11, respectively (Fig 3.5b). However, for genes related to ABA synthesis was observed a sharp increase of NCED3, NCED6 and NCED9 transcripts, reaching almost 400% more transcripts on pTCP14:TCP14 12.11. The exception was for NCED3 transcripts in pTCP14:TCP14 1.8 line were a slight reduction was observed (Fig 3.5b).

Half of the genes regulated by TCP8 are regulated in an opposite way by TCP14 during imbibition

RNA sequencing approach was used to analyse differentially abundant transcripts of *tcp8*, *tcp14* and Col-0 in dry and 24h imbibed seeds. As shown in Figure 3.6 a,b,c, a small number (298, 16, 288) of total differentially expressed genes (DEGs) were observed by comparing Col-0/*tcp14* and Col-0/*tcp8* in dry seeds. In contrast, dramatic changes in total DEGs (>1500) are observed in 24 hours imbibed

seeds comparing with dry seeds. Interestingly, *tcp8* seeds had fewer DEGs as compared to the Col-0 (Fig 3.6b,d). On the other hand, *tcp14* presented a higher DEGs. Nonetheless, a low number of DEGs was observed between *tcp8* seed and Col-0 imbibed (Fig 3.6e). Transcript level of 91 genes increase in *tcp8* compared to Col-0 at 24 hours. Among them, 53 are oppositely regulated in *tcp8* and *tcp14*, indeed they are repressed in *tcp14* mutant at 24 hours of imbibition. The transcript level of 26 genes decreased at 24 hours compared to Col-0 and seven genes among them are oppositely regulated in *tcp14* mutant. Globally, 50% of genes regulated by TCP8 at 24 hours of imbibition are regulated oppositely by TCP14.

TCP8 is able to active GASA4 transcription and TCP14 activates MCM2 transcription

Direct target gene candidates of TCP8 and TCP14 were examined. To investigate the ability of TCP4 and TCP8 to regulate the transcriptional activity, effector plasmids were constructed (Fig 3.7a). Transiently transcriptional activity was assayed in transformed tobacco (*N. benthamiana*) leaves with the reporter constructed, which is Firefly Luciferase (LUC) gene under the control of a promoter containing 6 repeats TCP binding motif from class I TCP and a combination of effectors. After the effectors and reporter were transiently expressed in tobacco leaves, luciferase activity was measured. LUC activity was significantly increased by the expression of TCP8 and TCP14 (11 and 7-folds, respectively) fused with the transcriptional activator VP16 (Fig 3.7b). Afterwards, we investigate whether TCP8 and TCP14 were able to bind and activate GASA4 and MCM2 promoter regions. TCP8 was able to increase the LUC activity within GASA4 promoter enhancing almost 3-fold (Fig 3.7c). On the other hand, in the same promoter, TCP14 fused with

VP16 increased the LUC activity by 1.5 fold (Fig 3.7c). When used MCM2 promoter region, TCP8 and TCP14 were able to increase the LUC activity by 1.9 and 3.5-folds, respectively (Fig 3.7d).

DISCUSSION

TCP family can regulate several processes in plant growth and development (Danisman 2016). Although it has been already documented that TCP can participate in germination event (Tatematsu et al 2008, Resentini et al 2015, Zhang et al 2019, Xu et al 2020), how the interplay among TCPs from the same class acts on germination remains unclear. Here we identified that TCP8 and TCP14, both from class I of the TCP family, act as germination regulators. Our results also revealed that TCP8 and TCP14 present an opposite role during germination, although the knowledge on the interplay between TCP8 and TCP14 is unknown. These findings are supported by the greater difference in germination rate and different genes expression. As fully germination was observed only in 15 °C, but not at 25 °C (Fig 3.1), it is likely that TCP8 participates maintaining dormancy. It is interestingly to note that the opposite behaviour was shown by *tcp14* seeds, with a higher level of dormancy in freshly seeds as compared to Col-0. These corroborate with the previously finds in *Arabidopsis thaliana* (Zhang et al 2019). So far, TCP8 is associated with delayed flowering in *Arabidopsis,* regulating *FLOWERING LOCUS C* (*FLC)* (Wang et al 2019), and in plant defense via EF-Tu Receptor (EFR) promoter (Spears et al 2019). Interestingly, few genes have been identified as specific in acting to keep dormancy such as *DELAY OF GERMINATION* (DOG) family. This gene family have been reported to interact with ABA enhancing the dormancy after the TF bZIP67 binding to DOG promoter (Carrilo-Barral et al 2020). However, based on our

results from RNA-seq, there is no regulation of DOG (Fig 3.6), suggesting that other genes may act as key markers to control dormancy breaking. Moreover, NCED5 and ABA4, genes related to ABA biosynthesis, were not regulated in *tcp14* seeds after 24 hours. However, *EXPA14*, gene related to expansion of cell wall and loosing were down-regulated. Expansion is a key step due the elongation of cells during seed germination, increasing the growth potential and causing the rupture of seed coat (Steinbrecher and Leubner-Metzger 2018). In this context, the regulation of genes associate with hormones is a key aspect during germination. This corroborate with the regulation of *EXPA* genes by GA (Yan and Chen 2020). Our data indicates that Col-0 and *tcp8* mutant seeds exhibited a high sensitivity to GA (Fig 3.1). However, ABA4 and NCED, genes that encodes proteins required during ABA biosynthesis have not the transcripts altered (Tab S3.2). As a whole, these data evidences that *tcp8.1* mutant seeds were significantly less dormant than Col-0 at harvest. Together, these results indicate that seeds are viable and *tcp8.1* mutants are sensitive to GA and are less dormant at harvest than Col-0.

As TCP14 and TCP8 are a class I TCP we expected similar role during germination (Danisman 2016). However, our results clear demonstrated TCP8 and TCP14 contrasted expression patterns. These suggests that may TCP8 and TCP14 participates unalike temporal expression pattern, performing different function during seed germination. TCP14 elevated expression level just prior to *strictu* germination, these corroborate as shown in other study which TCP14 may be involved in the promotion of cell expansion through the control of *EXPA9* gene (Xu et al 2020), the final step of germination which culminate with radicle protrusion.

It has been suggested that TCP8 acts as part of a large network of TCPs, however, may work interdependently during the responses to biotic stress (Spears et

al 2019). Elsewhere, it was demonstrated that TCPs from class I can regulate the expression of GA-related genes during heat stress (Ferrero et al 2019), although, during other phases it is completely unknown. Here, we show that genes related to GA biosynthesis (*GA3ox2*) and response (*GASA4* and *GASA14*) are upregulated in *tcp8* seeds (FIG 3.4). In addition, *GASA4* has been strongly associated with seed germination events (Ibarra et al 2013), suggesting that GA may regulated by TCP8 during germination. *EXPA3* is involved in the expansion of the cell wall and the final event of radicle protrusion, comprising thus, the germination in different lines (Ibarra et al 2013). Consistent with other study, here we demonstrated the participation of TCP regulating *EXPA* gene member (Xu et al 2020). Our results from RNA-seq suggests that TCP8 and TCP14 act regulating half of genes in an opposite way. It has been suggested that TCP8 and TCP14 plays opposite roles during branching (Van Es et al 2019). Moreover, although a complex interaction between the TCPs in plant development has been demonstrated, it remains unclear how they interact (Van ES et al 2019).

To better understand such events, the patterns of TCP8 and TCP14 could be observed during imbibition such as kinetic protein abundance of these two TCPs and thus identify the underlying response in seed dormancy. Noteworthy, using the transgenic lines *pTCP8:TCP8-GFP* and *pTCP14:TCP14-GFP* it is possible to perform Confocal Microscopy analyses. It is reasonable to expect that TCP8 and TCP14 will be present in the nucleus as shown in other plant tissues (Mazur et al 2017). The remaining questions need to be investigated to unravel the localization of TCP8 and TCP14 protein in seeds tissues and cell compartments. In this study, we highlighted that dormancy breaking and germination, at least in part, are regulated by TCP8 and TCP14. Complex regulation through these TCPs may occur during imbibition to fine-

tune the germination time. Further research are consequently required to establish if there is TCP8 and TC14 interaction during seed germination.

We further showed TCP8 and TCP14, expressed in tobacco leaves, differentially active *GASA4* and *MCM2* by directly binding to the promoters of genes encoding *GASA4* and *MCM2* genes, which positively regulates seed germination. This is consistent with evidences that shows *GASA4* overexpression promoting seed germination by modulating response to GA (Nelson and Steber 2017 Cheng et al 2019). The activation of TCP14, by VP16, activation domain, a powerful activator transcription was expected, meaning that TCP14 was able to bind to the promoter region but not activate it without VP16 (Fig 4.7b). These data may suggests that TCP14 may require cooperative effort of multiple proteins to activate a transcription. In previously study was demonstrated that TCP14 is activated by MPK8 and further regulates positively germination (Zhang et al 2019). These observations rises question for the existence of other regulatory mechanisms in TCP8. One possible explanation is the post-translational modification of TCPs by Small Ubiquitin-like Modifier (SUMO) proteins as shown in *Escherichia coli* (Mazur et al 2017). It will be interesting to evaluate if MPK8 may affect TCP8 activity. Also, in future assays should be worthy analyse via Electrophoretic Mobility Shift Assay (EMSA) and protein-protein interaction to reveal if TCP8 and TCP14 works regulating which other and enhancing or inhibiting binding the on *MCM2* and *GASA4*.

Previous studies on genes functions showed that simple mutants do not normally present distinctive phenotypes (Danisman et al 2013). Moreover, the pattern of gene expression from the same class is quite similar, suggesting that these genes have redundant functions. In addition, it has been suggested that TCPs from the same class may work redundantly (Danisman et al 2013). However, *tcp8* and *tcp14*

simple mutants shows singular phenotype. In addition, our results suggests that TCP8 and TCP14 may works independently and in opposite way during germination. These finds may open a new frontier of study of TCP class I on seed germination.

CONCLUSIONS AND FUTURE PERSPECTIVES

Here we identified, the transcription factors, TCP8 and TCP14, as key participants to germination and dormancy in *Arabidopsis thaliana*. We have shown that two TCP class I transcription factors (TCP14 and TCP8), have opposing roles during germination. TCP14 and TCP8 induce and inhibit germination, respectively. TCP8 and TCP14, expressed in tobacco leaves, differentially activated *GASA4* and *MCM2* by directly binding to the promoters regions, which positively regulated seed germination. In addition, TCP regulated *EXPA* gene members. Genes related to germination and dormancy are controlled by TCP8 and TC14 during germination. The germination genes controlled by TCP8 and TCP14 makes them an encouraging target to genetic engineer in many crops. Genes regulated oppositely by both TF should be careful analysed to unveil the unique molecular pathways involved. Although, some issues were partially clarified, further study will be necessary to reveal the underline mechanism.

Firstly, as discussed previously, the quantitative of TCP8 and TCP14 in dry and imbibed seeds needs to be measured. One way to cope this is doing the extraction of proteins and analyses by Western Blot. The second improvement should be carried out concerns to localization of the TF studied. It was observed in preliminary analyses, that TCP8 and TCP14 are expressed in seed. In the future, therefore, it will be necessary to perform a Confocal Fluorescence Microscopy. This

will allow us clarify in which tissues (endosperm, embryo) are transcription factors and where they are at sub-cellular level during the imbibition of seed. Third, it is a key point to understand how this TF works along GA and ABA. To clarify the link between transcription factors and the hormones ABA and GA, the quantification of the hormones should be performed in the seeds. Moreover, should be interestingly to know if MPK8 is able to interact and phosphorylate TCP8, as it occurs with TCP14. This knowledge will be fundamental for understanding the molecular mechanisms involved in the control of germination in higher plants.

REFERENCES

Aguilar-Martinez JA, Sinha N (2013) Analysis of the role of *Arabidopsis* class I TCP genes AtTCP7, AtTCP8, AtTCP22, and AtTCP23 in leaf development. Frontiers in Plant Science. 4: 406.

Basbouss-Serhal I, Leymarie J, Bailly C (2016) Fluctuation of Arabidopsis seed dormancy with relative humidity and temperature during dry storage. Journal of Experimental Botany. 67: 119–130.

Cao H, Han I, Li J, Ding M, Li Y, Li X, Chen F, Soppe WJJ, Liu Y (2020) *Arabidopsis thaliana* SEED DORMANCY 4-LIKE regulates dormancy and germination by mediating the gibberellin pathway. Journal of Experimental Botany. 71: 919–933

Carrara S, Dornelas MC (2020) TCP Genes and the orchestration of plant architecture. Tropical Plant Biology. 14: 1-10

Carrillo-Barral N, Rodrigues-Gacio MDC, Matilla AJ (2020) Delay of germination-1 (DOG): a key to understand dormancy. Plants 9: 480

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Reporter. 11: 113–116

Cheng X, Wang S, Xu D, Liu X, Li X,Xiao W, Cao J, Jiang H, Min X, Wang J, Zhang H, Chang C, Lu J, Ma C (2019) Identification and analysis of the GASR Gene family in common wheat (*Triticum aestivum* L.) and characterization of TaGASR34, a gene associated with seed dormancy and germination. Frontiers in Genetics. 10: 980.

Danisman S (2016) TCP Transcription Factors at the interface between environmental challenges and the plant's growth responses. Frontiers in plant science. 21: 1930.

Ding S, Cai Z, Du H, Wang H (2019) Genome-Wide Analysis of TCP Family Genes in Zea mays L. Identified a Role for ZmTCP42 in Drought Tolerance. International Journal of Molecular Science. 20: 2762

Ferrero LV, Viola IL, Ariel FD, Gonzalez DH (2019) Class I TCP transcription factors target the gibberellin biosynthesis gene GA20ox1 and the growth promoting genes HBI1 and PRE6 during thermomorphogenic growth in *Arabidopsis.* Plant cell Physiology. 60: 1633-1645

Finch-Savage WE, Leubner-Metzger G (2006) Seed dormancy and the control of germination. New Phytologist.171: 501-523

Hamala T, Mattila TM, Leinonen PH, Kuittinen H, Savolainen (2017) Role of seed germination in adaptation and reproductive isolation in *Arabidopsis lyrata*. Molecular Ecology. 26: 3484-3496.

Ibarra SE, Auge G, Sánchez RA, Botto JF (2013) Transcriptional programs related to phytochrome a function in *Arabidopsis* seed germination. Molecular Plant. 6: 1261-1273

Jaganathan G, Li J, Biddick M, Han K, Song D, Yang Y, Han Y, Liu B (2019) Mechanisms underpinning the onset of seed coat impermeability and dormancy-break in Astragalus adsurgens. Scientific Reporters. 9: 9695

Kadereit G, Newton RJ, Vandelook F (2017) Evolutionary ecology of fast seed germination—A case study in Amaranthaceae / Chenopodiaceae. Perspectives in Plant Ecology Evolution and systematics. 29: 1-11

Kieffer M, Master V, Waites R, Davies B (2011) TCP14 and TCP15 affect internode length and leaf shape in Arabidopsis. Plant Journal. 68: 147-158.

Kubota A, Ito S, Shim JS, Jonhson RS, Song YH, Breton G, Goralogia GS, Kwon MS. Cintron DL, Koyama T, Takagi MO, Paz JLP, Kay SA, MacCoss MJ, Imaizumi T (2017) TCP4-dependent induction of CONSTANS transcription requires GIGANTEA in photoperiodic flowering in Arabidopsis. PLoS Genetic. 13: e1006856

Lei N, Yu X, Li S, Zeng C, Zou L, Liao W, Peng W (2017) Phylogeny and expression pattern analysis of TCP transcription factors in cassava seedlings exposed to cold and/or drought stress. Scientific Reporter. 7: 10016

Li Shutian (2015) The Arabidopsis thaliana TCP transcription factors: A broadening horizon beyond development. Plant Signalling and Behaviour. 10: 7

Li M, Chen H, Chen J, Chang M, Palmer IA, Gassmann W, Liu F, Fu ZQ (2018) TCP Transcription Factors Interact With NPR1 and Contribute Redundantly to Systemic Acquired Resistance. Frontiers in Plant Science. 9: 1153

Liu MM, Wang MM, Yang J, Wen J, Guo PC, Wu YW, Ke YZ, Li PF, Li JN, Du H (2019) Evolutionary and Comparative Expression Analyses of TCP Transcription Factor Gene Family in Land Plants. International Journal of Molecular Science. 20: 3591

Mazur MJ, Spears BJ, Djajasaputra A, van der Gragt M, Vlachakis G, Beerens B, Gassmann W, van den Burg HA (2017) Arabidopsis TCP Transcription Factors Interact with the SUMO Conjugating Machinery in Nuclear Foci. Frontiers in Plant Science. 8: 2043.

Nelson SK, Steber CM (2017) Transcriptional mechanisms associated with seed dormancy and dormancy loss in the gibberellininsensitive sly1-2 mutant of *Arabidopsis thaliana.* PLoS ONE. 12: e0179143

Resentini F, Felipo-Benavent A, Colombo L, Blazquez MA, Alabadi D, Masiero S. (2015) TCP14 and TCP15 mediate the promotion of seed germination by gibberellins in *Arabidopsis thaliana*. Molecular Plant. 8: 482-485.

Shu K, Zhou W, Chen F, Luo X, Yang W (2018) Abscisic Acid and Gibberellins Antagonistically Mediate Plant Development and Abiotic Stress Responses. Frontiers in Plant Science. 9: 416

Spears BJ, Howton TC, Gao Fei, Garner CM, Mukhtar MS, Gassmann W (2019) Direct regulation of the EFR-Dependent immune response by *Arabidopsis* TCP transcription factors. MPMI. 32: 540-549

Steinbrecher T, Leubner-Metzger G (2018) Tissue and cellular mechanism of seeds. Current Opinion in Genetics and Develpment. 51: 1-10

Tatematsu K, Nakabayashi K, Kamiya Y, Nambara E. (2008)Transcription factor AtTCP14 regulates embryonic growth potential during seed germination in Arabidopsis thaliana. Plant Journal. 5: 42-52

Toledo-Ortiz G, Huq E, Quail PH (2003) The *Arabidopsis* basic/helix-loop-helix transcription factor family. Plant Cell. 15: 1749-70

Tuan PA, Kumar R, Rehal PK, Toora PK, Ayele BT (2018) Molecular mechanisms underlying abscisic acid / gibberellin balance in the control of seed dormancy and germination in cereals. Frontiers in Plant Science 9: 668

Van Es SW, van der Auweraert EB, Silveira SR, Angenent GC, van Dijk AD, Immink RG (2019) Comprehensive phenotyping reveals interactions and functions of Arabidopsis thaliana TCP genes in yield determination. Plant Journal. 99: 316-328.

Xu H, Lantzouni O, Bruggink T, Benjamins R, Lanfermeijer F, Denby K, Schwechheimer C, Bassel GW (2020) A Molecular signal integration network underpinning *Arabidopsis* seed germination. Currently Biology. 30: 1-10

Wang X, Xu X, Mo X, Zhong L, Zhang J, Mo B, Kuai B (2019) Overexpression of TCP8 delays *Arabidopsis* flowering through a FLOWERING LOCUS C dependent pathway. BMC Plant Biology. 19: 534

Wang X, Gao J, Zhu Z, Dong X, Wang X, Ren G, Zhou X, Kuai B (2015) TCP transcription factors are critical for the coordinated regulation of ISOCHORISMATE SYNTHASE 1 expression in Arabidopsis thaliana. The Plant Journal. 82: 151-162

Watt C, Zhou G and Li C (2020) Harnessing Transcription Factors as potential tools to enhance grain size under stressful abiotic conditions in cereal crops. Frontiers in Plant Science. 11: 1273.

Yan A, Chen Z (2020) The control of seed dormancy and germination by temperature, light and nitrate. The botanical review. 86: 39-75

Yao X, Mal H, Wang J, Zhang D (2007) Genome Wide Comparative Analysis and expression pattern of TCP Gene Families in *Arabidopsis thaliana* and *Oryza sativa.* Journal of Integrative Plant Biology. 49: 885−897.

Zhang W, Cochet F, Ponnaiah M, Lebreton S, Matheron L, Pionneau C, Boudsocq M, Resentini F, Huguet S, Blázquez MA, Bailly C, Puyaubert J, Baudouin E (2019) The MPK8-TCP14 pathway promotes seed germination in *Arabidopsis*. The Plant Journal. 100: 677-692

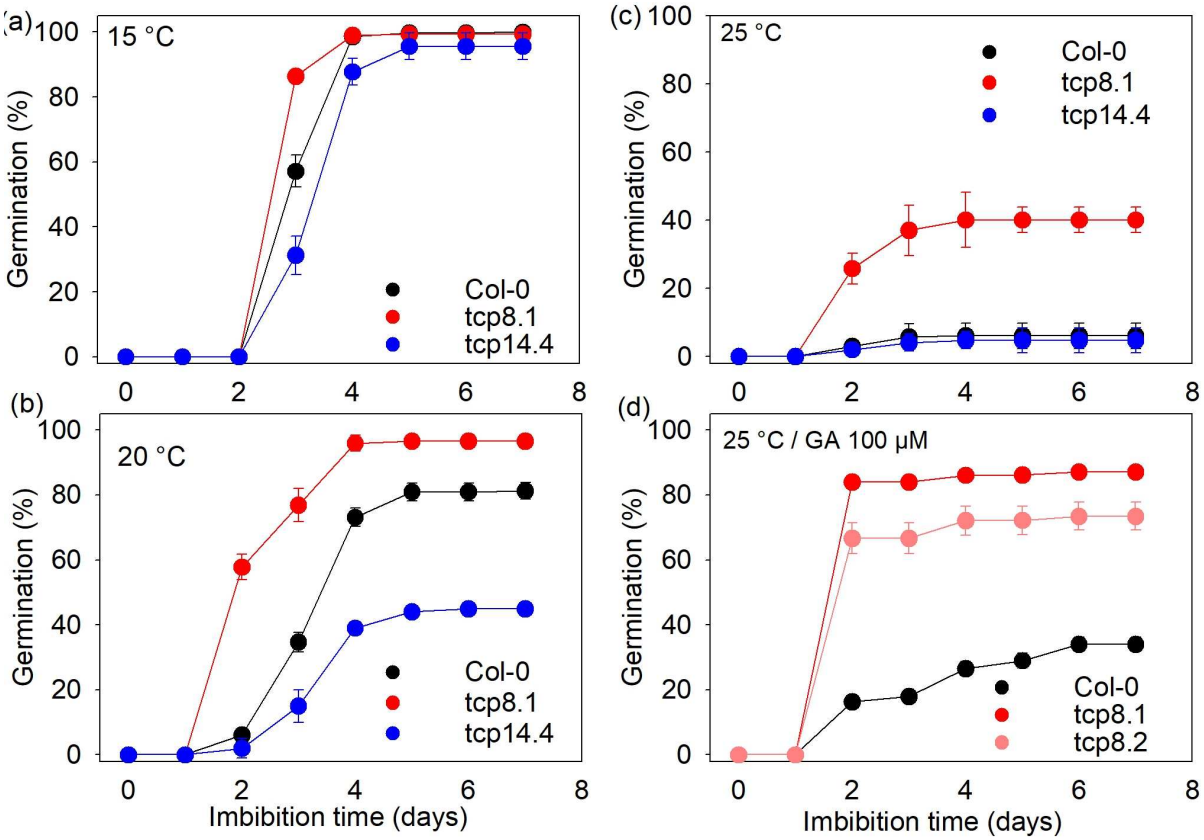

Fig 3.1 Germination of Col-0, *tcp8* and *tcp14.4* freshly harvested seeds. The seeds were imbibed in 15 mL of distilled water at (a) 15°C, (b) 20°C or (c) 25°C (d) 25°C + GA 100 µM in darkness for 7 days. Germination was scored daily. Values represent germination means ± standard error of six replicates.

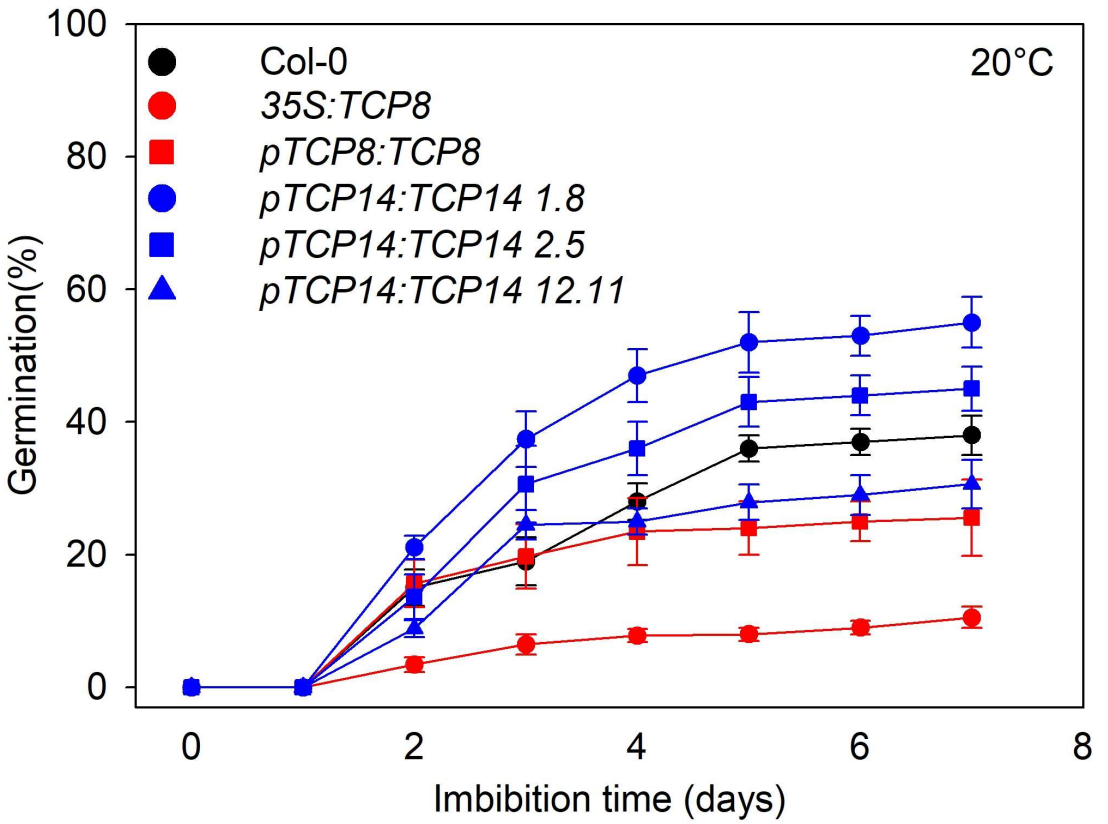

Fig 3.2 Germination of Col-0, *35S:TCP8*, *pTCP8:TCP8* and *pTCP14:TCP14* (lines 1.8, 2.5, 12.11) freshly harvested seeds. The seeds were imbibed with 15 mL of distilled water 20°C in darkness for 7 days. Germination was scored daily. Values represent germination means ± standard error of six replicates.

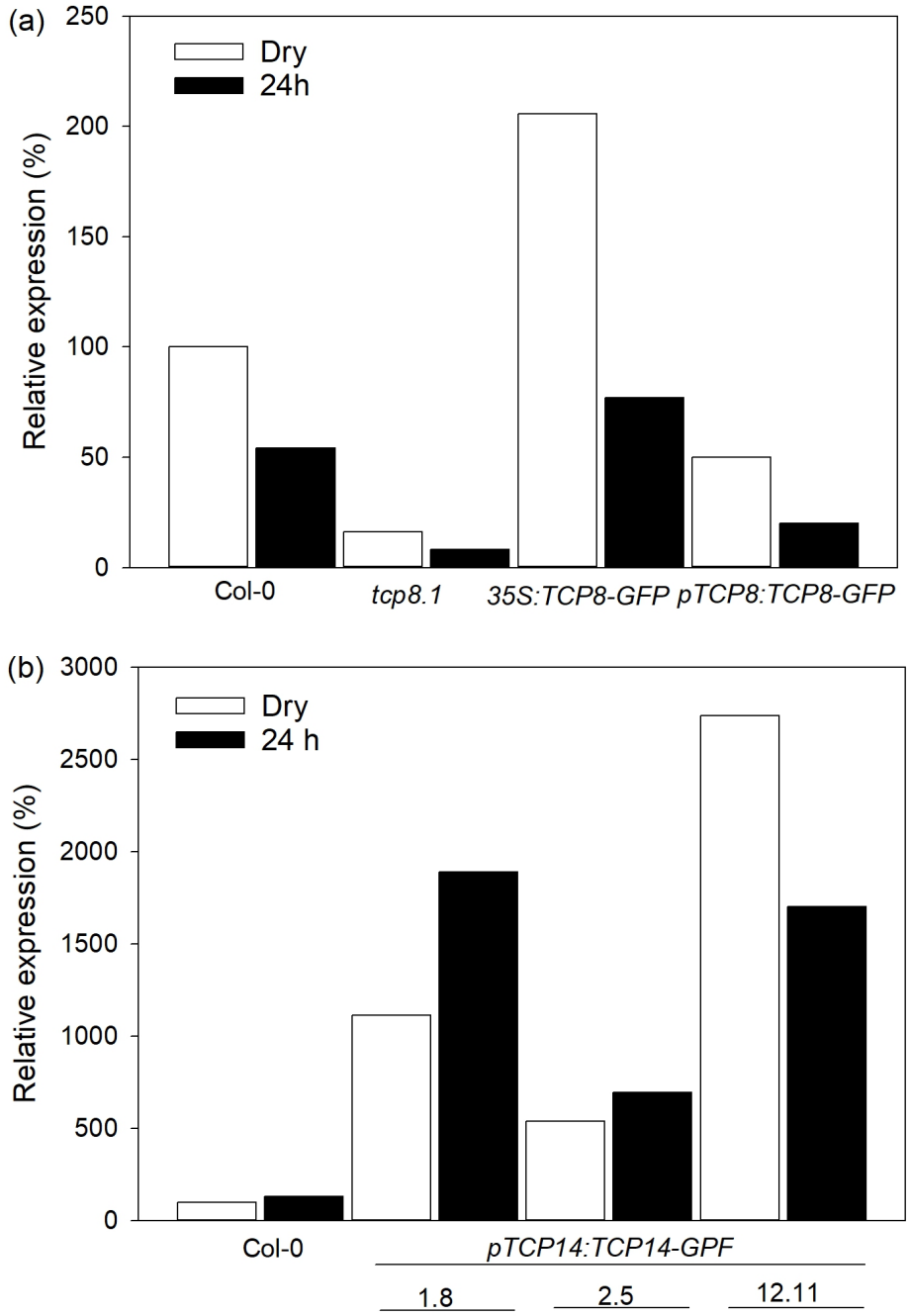

Fig 3.3 Transcript abundance of TCPs in seeds. Transcript levels were compared by RT-qPCR in dry and 24 h imbibed seeds at 20°C in darkness. (a) The percentage expression of *TCP8* (AT1G58100). (b) The percentage expression of *TCP14* (AT3G47620). Expression was normalized in relation to three housekeeping genes (AT4G34270, AT4G26410, AT5G53560). Results from one biological experiment are expressed relative to transcript levels in Col-0 imbibed seeds and are representative of three biological repeats.

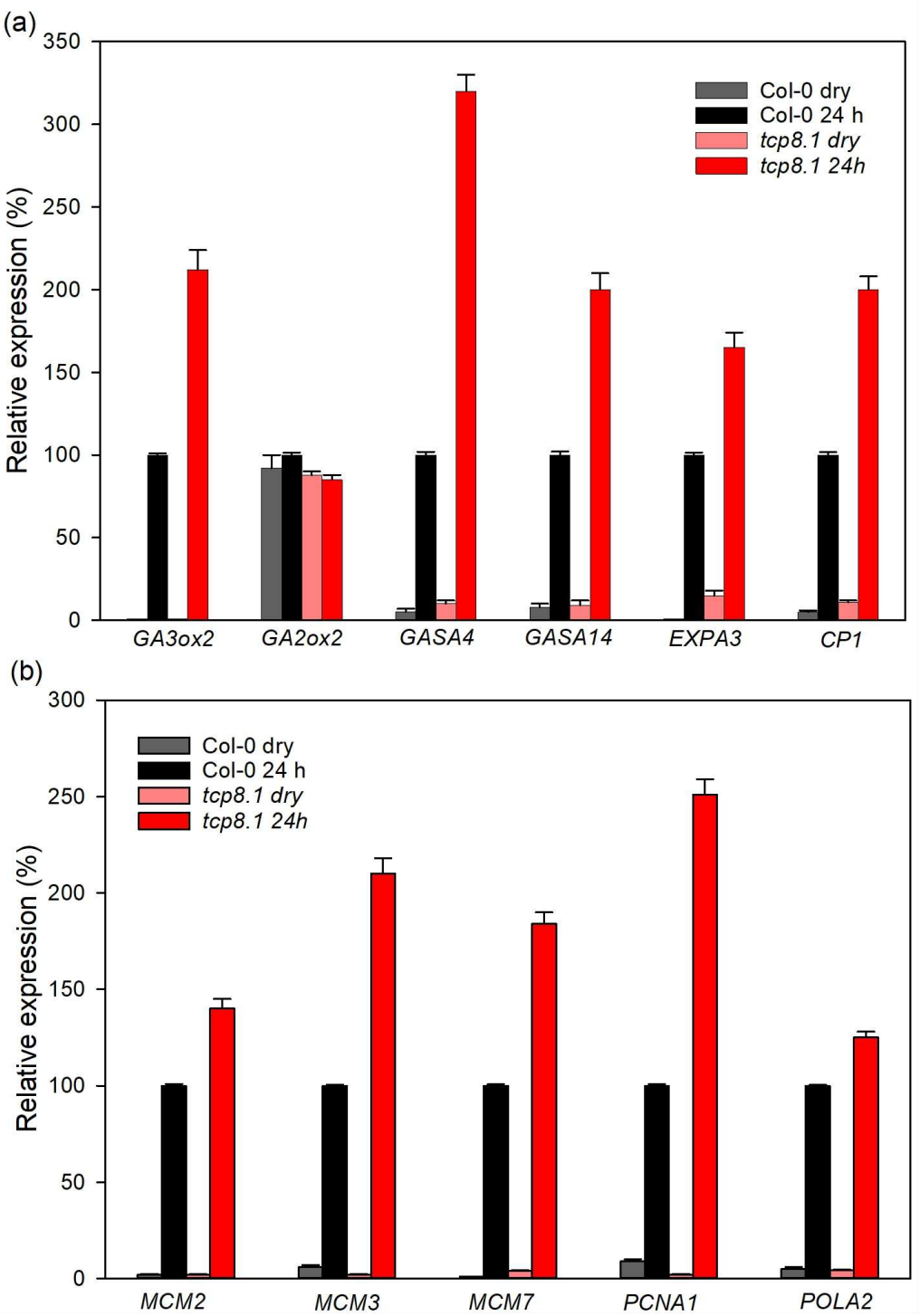

Fig 3.4 Transcript abundance of GA signalling genes in Col-0 and *tcp8.1* seed. Transcript levels were compared by RT-qPCR in Col-0 and *tcp8.1* dry and 24 h imbibed seeds at 20°C in darkness. (a) The percentage expression of *GA3ox2* (AT1G80340), *GA2ox2* (AT1G30040), *GASA4* (AT5G15230), *GASA14* (AT5G14920), *EXPA3* (AT2G37640) and *CP1* (AT4G36880), is presented. (b) The percentage of expression of *MCM2* (AT1G44900), *MCM3* (AT5G46280), *MCM7* (AT4G02060),

PCNA1 (AT1G07370) and *POLA2* (AT1G67630) is presented. Expression was normalized in relation to three housekeeping genes (AT4G34270, AT4G26410, AT5G53560). Results from one biological experiment are expressed relative to transcript levels in Col-0 imbibed seeds and are representative of three biological repeats.

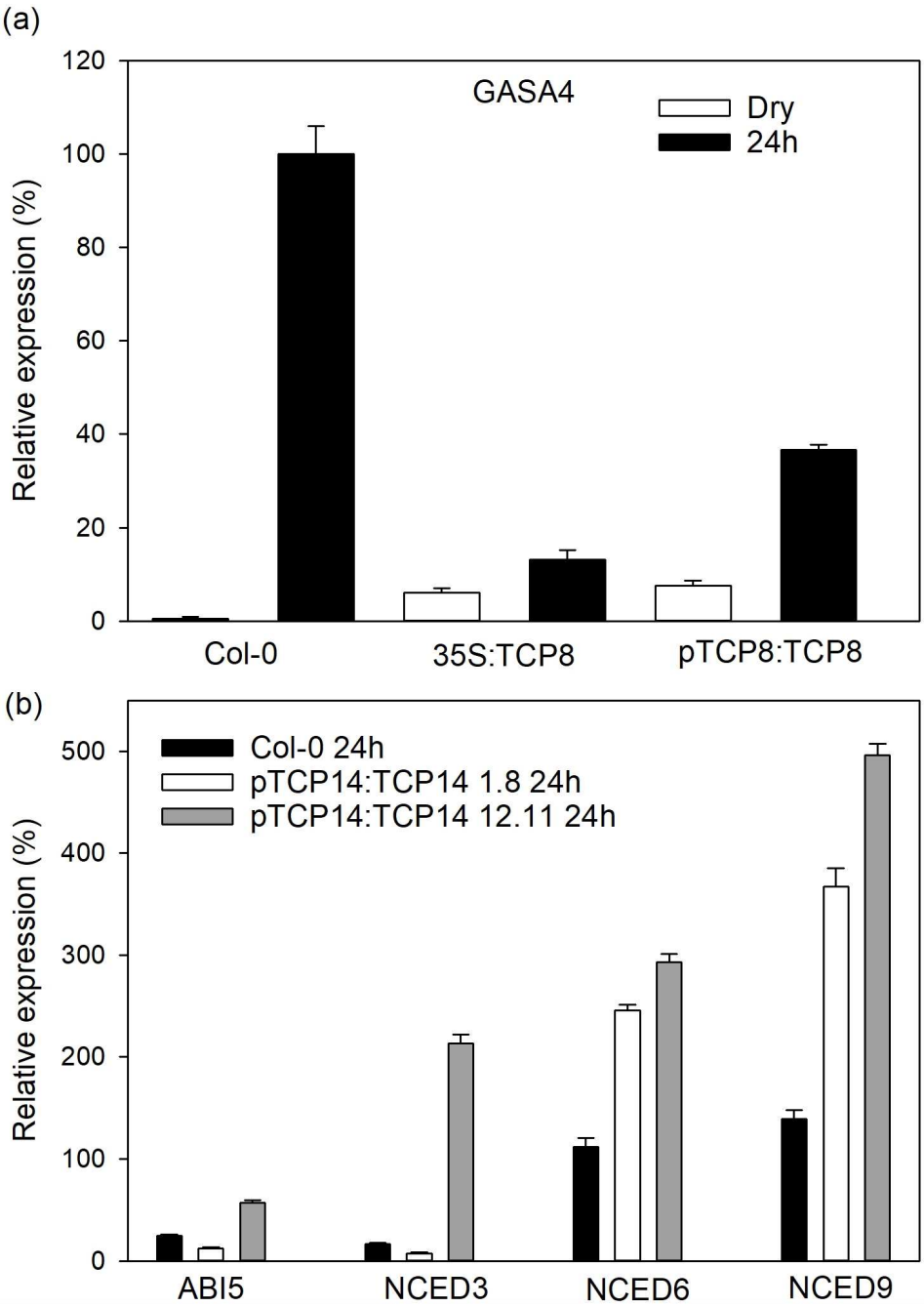

Fig 3.5 Transcript abundance in imbibed seeds at 20°C in darkness (a) The percentage of expression of *GASA4* (AT1G30040) in TCP8 mutants, is presented. (b) ABA genes (signalling gene ABI5 and ABA synthesis) transcript levels were compared by RT-qPCR in Col-0 and pTCP14:TCP14. the percentage expression, ABI5 (AT2G36270), *NCED3* (AT3G14440), *NCED6* (AT3G24220), *NCED9* (AT1G78390) is presented. Expression was normalized in relation to three housekeeping genes (AT4G34270, AT4G26410, AT5G53560). Results from one biological experiment are expressed relative to transcript levels in Col-0 imbibed seeds and are representative of three biological repeats.

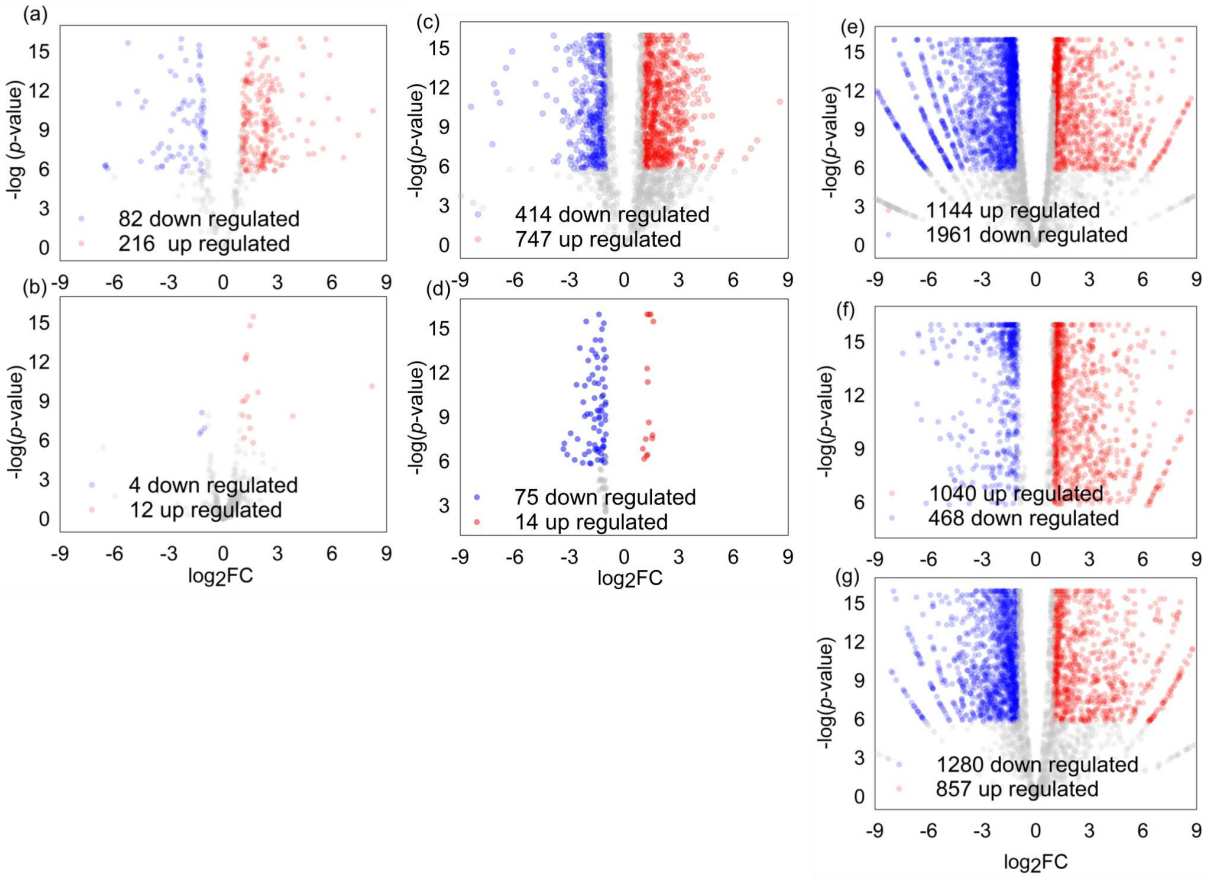

Fig 3.6 Differential Expressed genes between seeds *Arabidopsis* lines. The vertical axis (y-axis) corresponds to the mean expression value of $\log_{10}$ (*p*-value), and the horizontal axis (x-axis) displays the $\log_2$ fold change value. The red, blue and grey dots represent the up-regulated, down-regulated and not changed expressed transcripts, respectively. Positive x-values represent up-regulation and negative x-values represent down-regulation. Higher values of $\log_{10}$ (*p*-value), represent confidence. Each dot represents a gene. Transcripts were analysed at 0 (dry) or 24 h of imbibition. (a) Col-0 dry / tcp14 dry (b) Col-0 dry / tcp8 dry (c) Col-0 24 h / tcp 14 24 h (d) Col-0 24 h / tcp 8 24 h (e) Col-0 dry / Col-0 24 h (f) tcp14 dry / tcp14 24 h (g) tcp8 dry / tcp 24 h.

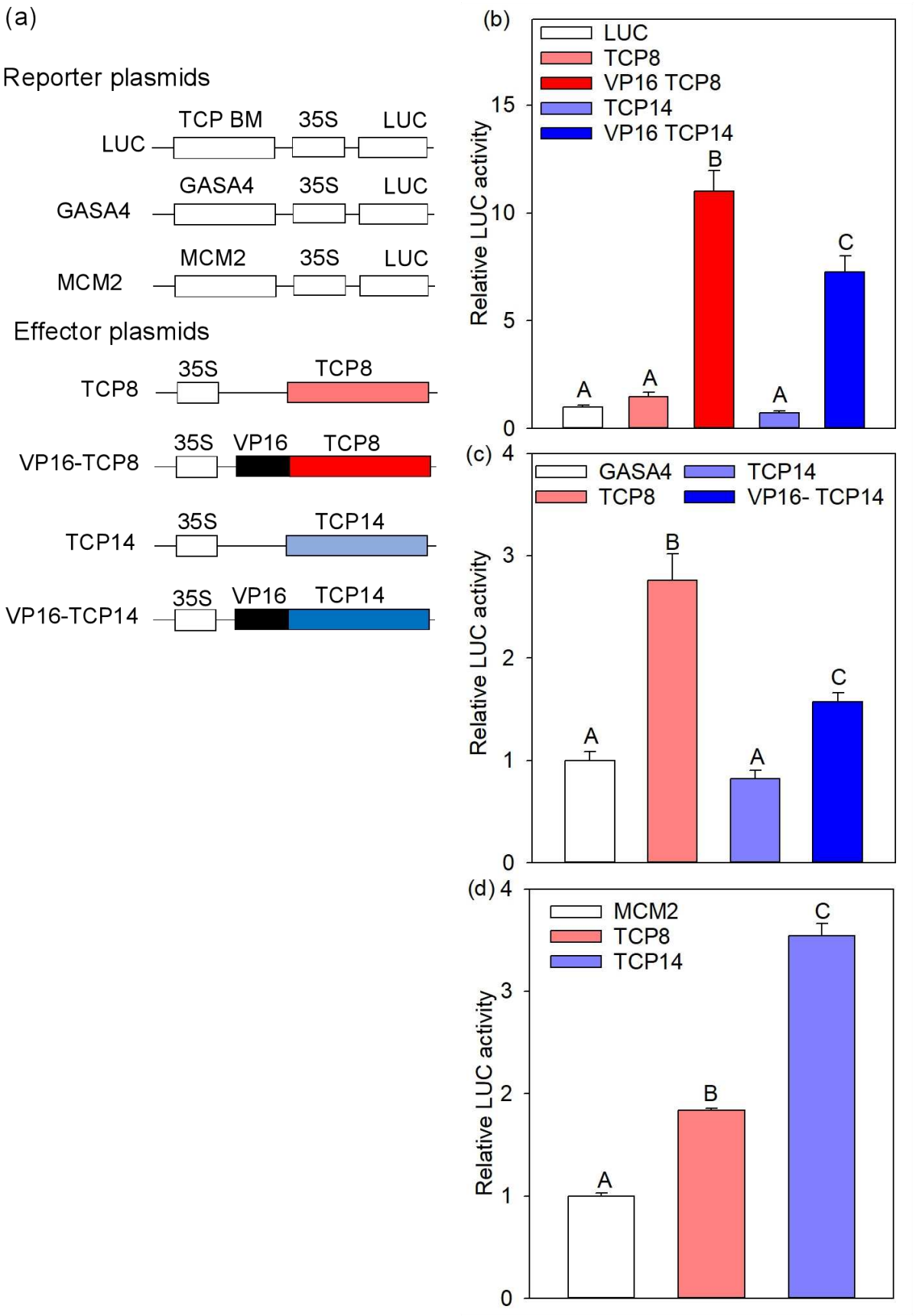
(a)
Reporter plasmids
TCP BM
35S
LUC
LUC
GASA4
35S
LUC
GASA4
MCM2
35S
LUC
MCM2
Effector plasmids
35S
TCP8
TCP8
35S
VP16
TCP8
VP16-TCP8
35S
TCP14
TCP14
35S
VP16
TCP14
VP16-TCP14
(b)
LUC
TCP8
VP16 TCP8
TCP14
VP16 TCP14
Relative LUC activity
0
5
10
15
A
A
B
A
C
(c)
GASA4
TCP8
TCP14
VP16- TCP14
Relative LUC activity
0
1
2
3
4
A
B
A
C
(d)
MCM2
TCP8
TCP14
Relative LUC activity
0
1
2
3
4
A
B
C

Fig 3.7 Transcriptional activity of TCP8 and TCP14. (a) Schematic diagram of the effector and reporter constructs. TCP BM: consensus Class I TCP binding motif. (b) TCP8 and TCP14 has transcriptional activation activity (c) GASA4 is activated by TCP8 (d) MCM2 is activated by TCP14. Means ± SE (n = 30~50 infiltrated leaves from three biological repeats) are represented, and different letters indicate significantly different means (Test Dunn's, $P \leq 0.05$ expressed relative to transcript levels in respective reporter imbibed seeds).

Tab S 3.1 List of primers used.

Primers used for genotyping

Gene ID	Primer name	Sequence 5'-3'
AT1G18150	F1	ACTCAATGTGAAAGCAAAACTGT
	R1	AAATCGGTCTACCCCACTGC
AT3G47620	F2	GACGACAACCATCAACAACAACCTTC
	R2	GTTGTTGATGATGATGTCTCTGTG
	LBb	GCGTGGACCGCTTGCTGCAACT
	R3	CATTTTATAATAACGCTGCGGACATCTAC

Primers used for qPCR experiments

Gene ID	Primer name	Sequence 5'-3'
AT1G15550	GA3ox1-F	ACATCACCTCAACTACTGCGAT
	GA3ox1-R	GTCTTCTTCGCTGACCCCAA
AT1G80340	GA3ox2-F	CCAGCCACCACCTCAAATACTGTG
	GA3ox2-R	CTCCCAGTGAACCTAATGCGAACC
AT1G30040	GA2ox2-F	ATGGTGAAGGTGGGGTTTGG
	GA2ox2-R	CTCCCGTTAGTCATAACCTGAAG
AT4G36880	CP1-F	GGCAAGGAGGTTCCAGAGAC
	CP1-R	CTGCAGTAGTCGAAAACGCC
AT2G37640	EXPA3-F	CTTAACCGCAACAAACGCCA
	EXPA3-R	ACAAGTTCCCGTACCCACAC
AT5G15230	GASA4-F	ATGTGAAGTGGAGCCAGAAACG
	GASA4-R	GCAAGCCTTGTGGTACTGTGTC
AT5G14920	GASA14-F	ACTCGGATAGATTGCGTGCC
	GASA14-R	ACGTGTCTTCATGTTGGCGT
AT1g44900	MCM2-F	TTGCAATCTGGCTTGCGGAT
	MCM2-R	TTGCTGCAGCTGAGGAAAGA
AT5G46280	MCM3-F	GGTCCTCGCTGATAAAGGTATCGTG
	MCM3-R	TCTTCTCTCGCGTAAGGCAAGCTC
AT4G02060	MCM7-F	GCCCTTCTTTCAAGATTCGATCTGC
	MCM7-R	CTTCTTGCCTGATGCTGGAATAAGC
AT1G07370	PCNA1-F	GTGACACAGTTGTGATCTCTG
	PCNA1-R	ATCACAATTGCATCTTCCGG
AT1G67630	DNAPOL2-F	ATTCCCACAGCCTCCTTTCG
	DNAPOL2-R	ATCCACCGTGCAGCAACTTA
AT1G58100	TCP8-F	CTCGGGATGTTAGCCGCTTT
	TCP8-R	ACCGCATTGTTCGCTTGTTC
AT3G47620	TCP14-F	TCCTTCTCATTTCCGCTCCG
	TCP14-R	TTGTTGTTGAAGCATCGCCG
AT4G34270	AT4G34270-F	GTGAAAACTGTTGGAGAGAAGCAA
	AT4G34270-R	TCAACTGGATACCCTTTCGCA
AT4G26410	AT4G26410-F	GAGCTGAAGTGGCTTCCATGAC
	AT4G26410-R	GGTCCGACATACCCATGATCC
AT5G53560	Cyb5-F	TGAAGAAGTTTCAAAGCACAACA
	Cyb5-R	TCATCCATGAATGGAGTCACA

Primers used for cloning

Gene ID	Primer name	Sequence 5'-3'
AT3G47620	TCP14-BIFC-F	CGGATCCATGCAAAAGCCAA
	TCP14-BIFC-R	AGGGTACCATCTTGCTGATC
S102 mutation	TCP14-S102A-F	GTTATTACAAACGCAAGAGGAAGCTGCGGTGGTGG
	TCP14-S102A-R	TCTTTTTGCGTTGTCTGCGGTTGTGACTTTTGC
T5 S6 S7 mutation	TCP14-T5AS6AS7A-F	GGTGGAGACAGCGTCGGAGGAGGAG
	TCP14-T5AS6AS7A-R	GTCCATTATGACATTTAAGATAGCTGCTGCTGGCTTTTGCAT

GENERAL CONCLUSION

This thesis attempted to widen the knowledge of seed dormancy and germination. This study is preliminary and certainly do not aimed to reveal the complete mechanism of seed germination. On the other hand, this research offers a new theoretical foundation of how salt stress and pH may affect ethylene and ABA in seeds of *Stylosanthes humilis.* Also, had aggregated knowledge about the TCP family during *Arabidopsis* germination. The comprehensive of this aspect may light the plant dynamics population on future scenario of climate change. The understand of the molecular mechanisms behind TF and hormones are key steps for reveal the complex network during seed germination. Moreover, the analyses with *Arabidopsis* demonstrated for the first time two TCPs from the same class acting in opposite manner during seed germination. However, more analyses need to be done for reveal the interaction that may occurs among theses TF. This thesis will contribute to direction the future research. Possible proposed work can be done studying the complexity of many signals during germination interfering in TCPs. Moreover, the quantity and the interaction of TCP14 and TCP8 is a topic that worth to be investigate. Furthermore, the quantification of hormones such GA and ABA in *tcp8* and *tcp14* seeds is necessary to understand the cascade of signalling during seed germination. Also, the correlation of epigenetic modulating in TF. The advance of genome editing may be a tool that allow us the study further seed germination in non-model plants.