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The plant *Arabidopsis thaliana* faces the ABA-mediated processes through the DAG1, WRKY6 and WRKY18 proteins, with the control of the Polycomb Repressive Complex 2

Candidate
Veronica Ruta

Supervisor
Prof./Dr Paola Vittorioso

Coordinator
Prof. Marco Tripodi

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Introduction

1. Abiotic stress in plants

Plants, as sessile organisms, have to survive to frequently changing environments presenting adverse or stressful conditions, which directly affect growth and development. The increasing attention towards global climate changes and the concern about the future survival of crops, triggered attempts to deeply analyse how plants cope with abiotic stress, focusing on how plant have evolved different molecular programs to rapidly perceive changes in the environment and adapt properly. Levitt distinguished two strategies that plants evolved in response to stress: stress avoidance and stress tolerance. Stress avoidance involves a multiplicity of protective mechanism that delay or prevent the negative impact of stress factors on a plant, through stable and inherited adaptation. On the other hand, stress tolerance is defined as the potential to acclimate to stressful conditions [1].

Abiotic stresses (such as drought, high salinity and low temperature) cause osmotic stress triggering turgor loss, reduced protein activity, and excess levels of reactive oxygen species (ROS) directly leading to oxidative damage. The cellular disorder resulting from stress causes growth defects, reduced fertility and

premature senescence. The phytohormone abscisic acid (ABA) was shown to be a pivotal regulator of abiotic stress responses in plants, triggering major changes in plant physiology, as ABA-deficient plants have a consistently altered stress response [2-4]. The presence of abiotic stress induces local ABA biosynthesis, ABA is then transported throughout the plant to promote stomatal closure, as well as to induce stress-activated gene transcription, required to mediate the physiological response and metabolic adjustments. In parallel to ABA induction, plants modulate the production and distribution of growth-promoting hormones (gibberellins, GA, brassinosteroids, BR and cytokinins, CK) to allow an optimal response.

The AP2/ERF (APETALA2/ ETHYLENE RESPONSIVE FACTOR) family of plant-specific transcription factors is involved in the stress-response pathway; their expression is low under normal conditions, but is quickly induced or repressed by hormone- and stress-related stimuli [5-11]. AP2/ERFs are characterized by an AP2/ERF domain, which consists of 40-70 conserved amino acids involved in DNA binding[8, 12, 13]. In *Arabidopsis* this family is encoded by 145 loci [12]. Even in the single-cell green alga *Chlamydomonas reinhardtii* is present an *AP2/ERF* encoding-gene [14]. Interestingly, in bacterial and viral endonucleases, sequences homologous to the AP2/ERF domain have been found, suggesting that the AP2/ERF domain was

transferred to plants by a cyanobacterium through endosymbiosis, or by a bacterium or virus through lateral gene transfer events [15]. AP2/ERF factors are classified in four main subfamilies: AP2, RELATED TO ABSCISIC ACID INSENSITIVE3 / VIVIPAROUS1 (RAV), DEHYDRATION - RESPONSIVE ELEMENT BINDING proteins (DREBs) and ERFs [12, 13]. Among the AP2/ERF factors, the DREB family responds to and regulates cold, heat, drought and salt tolerance by directly controlling stress-responsive genes that present in the promoters a Dehydration Responsive Element (DRE) [16-18]. DREBs were first identified using a yeast one-hybrid system, by screening for the trans-acting factors able to bind the DRE element present in a number of drought and cold inducible promoters [19, 20]. In particular, DREB1s (DREB-A1 subgroup) are involved in cold tolerance [21], while DREB2s (DREB-A2 subgroup) in drought and heat tolerance [6]. Lastly, the DREB-A4 sub-family positively regulates drought and salt tolerance [22-25]. Ectopic or selective expression of DREB1a/CBF3 (C-REPEAT BINDING FACTOR3) can significantly enhance plant tolerance to multiple abiotic stresses, like drought, freezing and high salinity [20](Fig. 1). Interestingly, the stress-inducible DREBs are found in phylogenetically divergent species (i.e. oilseed rape, tomato, maize, rice and barley), substantiating the crucial role of these factors in stress response of land plants [26-30] (Fig. 2).

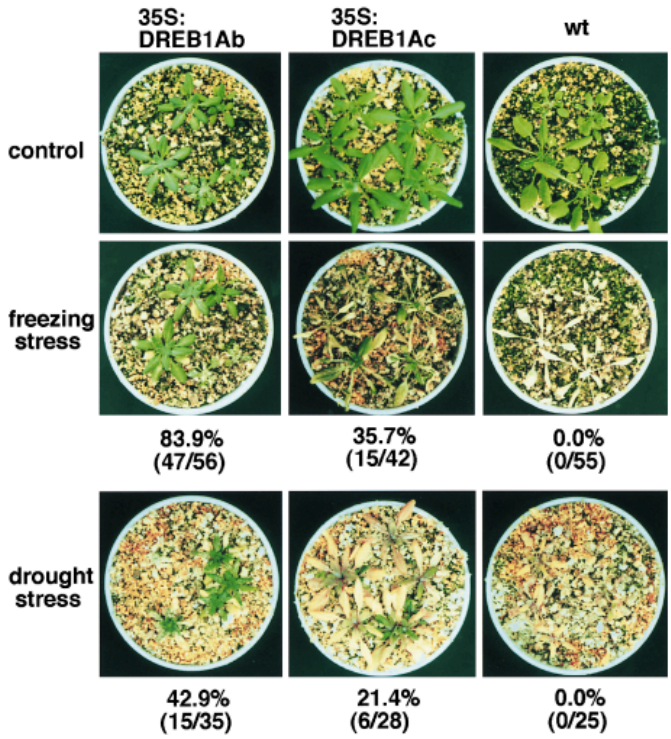


Fig. 1. Tolerance to freezing and drought of the *35S:DREB1Ab* and *35S:DREB1Ac* transgenic plants.

Wild type (wt) and 3 weeks-old transgenic plants growing under normal conditions (control), freezing stress (plants exposed to -6°C for 2 days and returned to 22°C for 5 days); drought stress (water withheld for 2 weeks). Under the images are indicated the percentages of surviving plants and numbers of surviving plants per plant total number (from [20]).

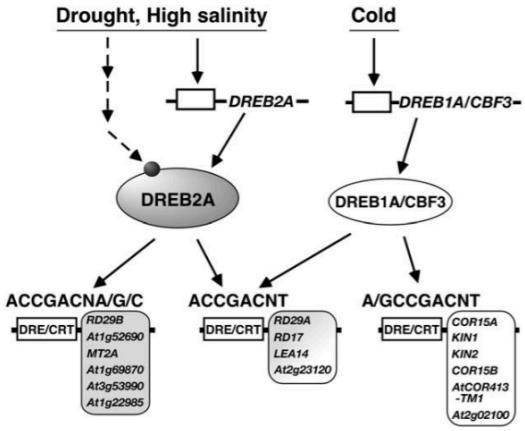


Fig. 2. Molecular model of DREB1A and DREB2A control of stress conditions.

The genes downstream of the DREB proteins are divided into three groups. The middle group contains the downstream targets of both DREBs (from [31]).

Among abiotic stresses, drought is one of the main issues of agriculture yields, due to the complexity of the water-limiting environments. Accordingly, plants have evolved different types of drought resistant strategies. Plant drought resistance can be divided into four basic types: drought avoidance, drought tolerance, drought escape and drought recovery [1, 32]. The transcription factor DREB2A activates genes involved in the drought- and salt-

related stress response, and its function has been identified by generating a constitutively active form of DREB2A (DREB2A-CA), by deletion of the negative regulatory domain (DREB2A-NRD) [20, 31]. Qin and colleagues demonstrated that DREB2A is regulated by the proteasome 26S through two E3 ubiquitin ligases, namely DREB2A-INTERACTING PROTEIN 1 and 2 (DRIP1 and 2), resulting in fine-tuning of DREB2A abundance [33]. Another major factor affecting water supply for global agriculture is represented by water salinity. High water salinity greatly limits root water uptake, damages cell physiology and reduces growth [34, 35]. In the presence of salt stress, the increase in ABA levels and the activation of ABA signaling pathways, trigger salt responses [36]. In addition, salt-stress activates ethylene signaling, which integrates with the ABA pathway on DELLA proteins - negative regulators of gibberellin (GA) responses - hub of this network to finally promote salt tolerance. In salt-treated wild type plants, reduced levels of bioactive GAs result in increased DELLA protein levels and enhanced growth-repressing effects. The consequent slower growth rate extends the duration of the vegetative growth phase. The quadruple-DELLA mutant (lacking GA INSENSITIVE, GAI, REPRESSOR OF *ga1-3*, RGA, RGA-LIKE 1 and 8, RGL1 and RGL2) is less inhibited by salt concentrations than the wild type. The combined effect of the DELLA proteins GAI and RGA play the main role in salt-activated

growth repression. The DELLA-dependent growth restraint may allow a versatile growth response to environmental variability, thus promoting survival [37, 38].

1.1 Cold stress response

Among abiotic stress, low temperature is one of the most relevant factors limiting the distribution of plant species [1, 39]. The response that allows the plants to increase the freezing tolerance is called cold acclimation [1]. To avoid damage due to freezing temperature, plants change membrane composition, accumulate stress-related proteins to prevent dehydration, activate antioxidant enzymes and protect the cold-sensitive photosynthetic machinery [40]. The CBF1-3 transcription factors play a key role in the response to low temperature; the *Arabidopsis CBF* loci map on chromosome 4 in tandem. The *cbf* triple mutant - produced with the CRISPR/Cas9 technology - showed an extreme sensibility to freezing, after cold acclimation, suggesting that the CBFs act as positive regulators of cold acclimation, possibly via the control of cold signaling pathway [41-43] (Fig. 3).

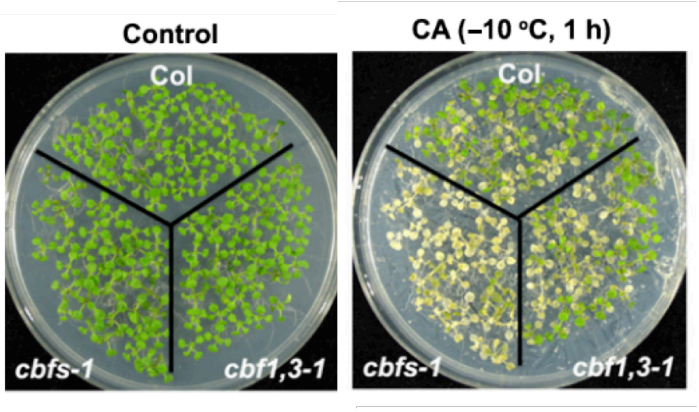


Fig. 3. Freezing tolerance of *cbf1,3-1* and *cbfs-1* mutants.
Two weeks-old seedlings treated in a freezing chamber from 0°C and cooled at -1°C per hour. The seedlings were kept in the chamber until -10°C for 1h (cold-acclimated, CA). The images show the recovery after 3 days at 22°C. Col: wt, *cbf 1,3-1:* double mutant, *cbfs-1:* triple mutant. Adapted from [41].

Interestingly, different *Arabidopsis* accessions show diverse degree of freezing tolerance; indeed, CBF activity in cold climates helps the plant to adapt to low temperature, whereas this function can be deleterious in warmer climates [44-50]. Expression of *CBF* genes is low under normal condition, but it increases transiently within minutes of cold exposure, returning to resting levels after few hours at low temperature [20, 51, 52]. The trascriptomic analysis of the *cbf* triple mutant revealed that lack of CBF proteins affects the cold-regulation of 449 target genes, belonging to the functional clusters of "carbohydrate metabolism", "lipid metabolism" and "cell wall modification", therefore corroborating the involvement

of CBFs in the metabolic and biochemical adjustments to increase freezing tolerance [41, 42].

At low temperature the beta helix-loop-helix (bHLH) transcription factor called INDUCER OF CBF EXPRESSION1 (ICE1) is activated; ICE1 relives the repression of *CBF* genes, mediated by the factor MYELOBLASTOSIS 15 (MYB15), resulting in the activation of CBFs which in turn trigger the cold signaling pathway [21, 53]. Recently, it has been demonstrated that the CALMODULIN-BINDING TRANSCRIPTIONAL ACTIVATORS (CAMTA) has also a role in activating the cold pathway, indeed it binds the *CBF1* and *CBF2* promoters inducing their expression [54, 55].

The activation of CBFs is not only temperature-dependent, since it is also affected by an integrate environmental information about light quality and daylight length, as well as internal signals like the circadian clock and multiple hormonal pathways. Indeed, the photoreceptor phytochrome B (phyB) interacts with PHYTOCHROME INTERACTING FACTOR 4 AND 7 (PIF4 and PIF7), which repress *CBF* expression, mediating the photoperiodic control of *CBF* transcript levels [56]. Among the hormones involved in cold response, ethylene is the negative regulator of the CBF pathway and cold acclimation; it represses the expression of *CBFs* through the accumulation of EIN3 and PIF3, which directly bind the *CBF* promoters [57-59]. On the contrary, brassinosteroids

(BRs) are positive regulators of this pathway; indeed BRASSINAZOLE-RESISTANT1 (BRZ1) and CESTA (CES) directly up-regulate *CBF* expression [60, 61]. In the presence of low temperature GA levels decrease resulting in the stabilization of DELLA proteins and the promotion of cold acclimation by increasing *CBF* transcripts [62] [63]. Despite the hypothesis that the CBF-mediated pathway is independent from ABA, it has recently been shown that, in the presence of exogenous ABA, expression of *CBF* genes is induced; consistently, ABA-insensitive mutants showed a reduced induction of *CBFs* at low temperature. Furthermore, ICE1, ICE2 and CAMTA3 (upstream regulators of *CBF* genes) are regulated by the ABA signaling factors OPEN STOMATA 1 (OST1) and HEPTAHELICAL PROTEIN 1, 2 and 3 (HHP1,2,3) which positively control expression of *CBF* genes [64-66].

It has been proposed that, possibly, some elements of ABA signaling pathway can have an ABA-independent role in the process of cold response [65]. Nevertheless, ABA level increases in the presence of low temperature and, in ABA-deficient mutants, some CBFs targets are less induced [2, 67-69].

Expression of *CBF* genes is also regulated through the epigenetic machinery, namely by histone modifications; lack of the Polycomb Repressor Complex 2 (PRC2), which is responsible of trimethylating histone H3 on K27 (H3K27me3), a repressive

epigenetic mark, results in increased *CBF* mRNA levels. Consistently, a genome wide analysis of histone modification in *Arabidopsis* at warm temperature revealed an increased H3K27me3 level in *CBF* promoters, required to silence these genes under non-inducing conditions [70].

2. ABA and ABA-related processes

ABA is an essential hormone in the control of plant growth and development, and in plant adaptation to environmental challenges (reviewed in [71-73]).

ABA level is the result of a fine balance between biosynthesis and catabolism; the biosynthetic pathway is partly localised in the chloroplast, although the final steps are in the cytoplasm [74-76]. The synthesis reaction is catalysed by chloroplast localized nine-cis-epoxycarotenoid dioxygenases (NCEDs) [77, 78]. As for ABA catabolism, a small group of cytochrome P450-type enzyme, the CYP707As, convert ABA to phaseic acid or neophaseic acid [79-82].

ABA signaling pathway involves five core components: ABA receptors, negative regulators, positive regulators, ABA-responsive transcription factors and ABA-responsive genes [74]. The ABA receptors belong to the pyrabactin resistance 1 (PYR1), PYR1-like (PYLs) and Regulatory Components of ABA Receptors (RCAR)

family [83-85]; in *Arabidopsis* there are 14 PYR/PYLs/RCAR, localized in the cytoplasm, plasma membrane or nucleus [83, 86]. The negative regulators of ABA signaling are a group of protein phosphatase A (PP2c) responsible of dephosphorylating the positive regulator of ABA, SUCROSE NONFERMENTING 1 (SNF1)-related protein kinases 2 (SnRK2). In the absence of ABA, PP2Cs inhibit SnRK2s activity therefore blocking ABA signal transduction. In the presence of ABA, the hormone bound the receptors, and in turn this complex interacts and inactivates PP2C. Consequently, the SnRK2 proteins are able to activate downstream responses, namely specific transcription factors called ABA-responsive element binding factors (AREBs/ ABFs) (Fig. 4). The ABA INSENSITIVE (ABI) factors are key positive regulators of ABA signaling; indeed mutations in *ABI3*, *ABI4* and *ABI5* genes result in ABA insensitive phenotype [87]. In particular, ABI3 is required during seed maturation to establish both desiccation tolerance and seed dormancy. ABI4 directly and positively controls the catabolic GA gene *GA2ox7* and the ABA biosynthetic gene *NCED6* to increase the ABA/GA ratio and promote dormancy [88, 89]. ABI5 plays its role not only during seed maturation and germination but also in the response to abiotic stresses [90-94].

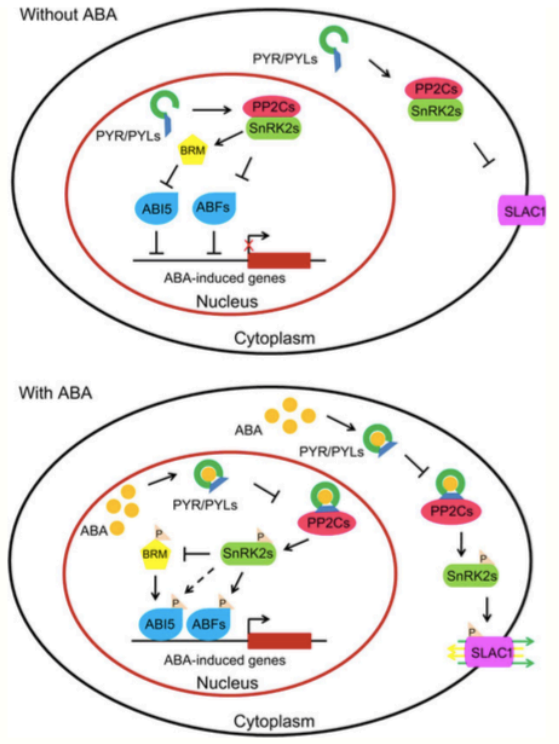


Fig. 4. ABA signaling pathway in *Arabidopsis*.
PYR/PYL family of ABA receptors, PP2Cs negative regulators, SnRK2s positive regulators. In the presence of ABA, the receptors PYR/PYL bind ABA and in turn this complex binds PP2Cs releasing the positive regulators SnRK2s. (From [95]).

2.1 Dormancy and Desiccation tolerance

The progeny of Angiosperms is represented by the seed, defined as a dispersal unit, able to develop into a whole plant when the

environmental conditions are favourable for growth of the next generation [96]. In the model plant *Arabidopsis thaliana,* seed development is divided in two phases: embryogenesis and maturation.

Embryogenesis ends when all embryo structures have been formed and cell division arrests. At this time the seed enters the maturation phase, characterised by a switch from maternal to filial control. This phase ends when storage compounds are accumulated, water level reduced, dormancy and desiccation tolerance are established and ABA levels increased [97, 98].

The maturation stage leads to dormancy, characterised by seed inability to germinate even under favourable conditions. Dormancy is an important evolutionary trait to prevent vivipary. Seed dormancy controls the timing of germination in response to environmental stimuli, preventing germination out of season, and plays an important role in seed plant evolution and adaptation to climatic changes.

The main molecular elements that control both seed maturation and dormancy establishment are FUSCA 3 (FUS3), ABSCISIC ACID INSENSITIVE 3 (ABI3), and LECTIN-LIKE2 (LEC2).

ABI3, FUS3 and LEC2 belong to a family of transcription factors characterized by the presence of a conserved B3-binding domain. The main role of these proteins during dormancy is to increase ABA content; consistently lack of any of these proteins results in

reduced dormancy, faster germination and decreased levels of seed storage proteins compared to the wild type [99-101].

In *Arabidopsis* there are 51 LEA-encoding genes [102]. The LEA proteins have a protective role during water restraint; LEA protein levels raise just before seed desiccation, as well as in vegetative tissues exposed to dehydration, osmotic and low temperature stress [103].

While storage compounds are accumulated and seed dormancy is established, Desiccation Tolerance (DT) is also induced. DT, which is defined as the ability of the seed to germinate after re-dehydration without lethal damage, is fully established before seed dehydration. When seed germination is induced, the seed becomes desiccation sensitive.

The unicellular green algae (Chlorophyta) have acquired DT in order to be able to colonize intertidal zones; this phenomenon has been conserved during evolution and in higher plants is present in spores, pollen and seeds [104, 105].

DT relies on a number of protection mechanisms induced during dehydration, such as accumulation of LEA proteins and metabolism drop [106, 107].

The molecular mechanism underlying the establishment of DT involves ABA signaling, with the activation of the ABA positive regulators ABI3 and ABI5, which induce downstream genes and pathways responsible for the accumulation of LEA proteins, sugar,

osmolytes and aminoacids required for the establishment of DT. LEA proteins and sugar are involved in structural and macromolecular protection; in particular, LEA proteins act as a hydration buffer, sequestering ions and renaturing unfolded protein, whereas sugar maintains the osmotic balance under stress [108].

Since DT depends on water dehydration, the molecular pathways underlying this process are, at least in part, overlapping with signaling pathways related to the response to environmental stress. Indeed, in the presence of a mild osmotic stress (i.e. with polyethylene glycol) or exogenous ABA, the re-establishment of DT in seeds is induced. This has been proved in *Arabidopsis thaliana* as well as in other species [109, 110].

To assess the re-establishment of desiccation tolerance in *Arabidopsis*, Maia [110] defined four distinct developmental stages (Fig. 5):

-stage I: testa rupture

-stage II radicle protrusion

-stage III a primary root of 0.3-0.5 mm in length

-stage IV the appearance of the first root hairs.

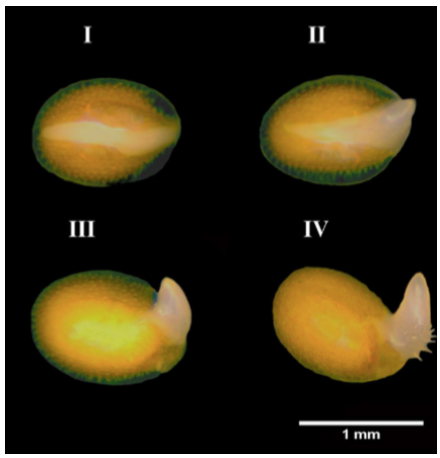


Fig. 5. The four developmental stages of *Arabidopsis* seeds.
I testa rupture, II radical protrusion, III primary root of 0.3 mm length, IV first root hair. (From [110]).

It has been established that during the first three stages the seed is able to re-induce germination after dehydration by PEG- treatment, at stage IV this capacity is lost. Notably, in *Arabidopsis* seeds the degree of DT sensitivity varies in different organs, the cotyledons being the most tolerant while roots are more sensitive.

Recent studies have shown that ABA-insensitive mutant seeds, namely *abi3, abi4,* and *abi5*, are partially unable to re-establish DT compared to the wild type. Indeed, while at the beginning of germination these mutants are still able to recover DT, later this response is lost, consistently with the reduced sensitivity to exogenous ABA. This suggested that the sensitivity to ABA more than ABA content, controls the induction of DT; indeed during the

treatment with PEG the content of ABA does not change, despite the biosynthetic and catabolic ABA genes are misregulated [109] (Fig. 6).

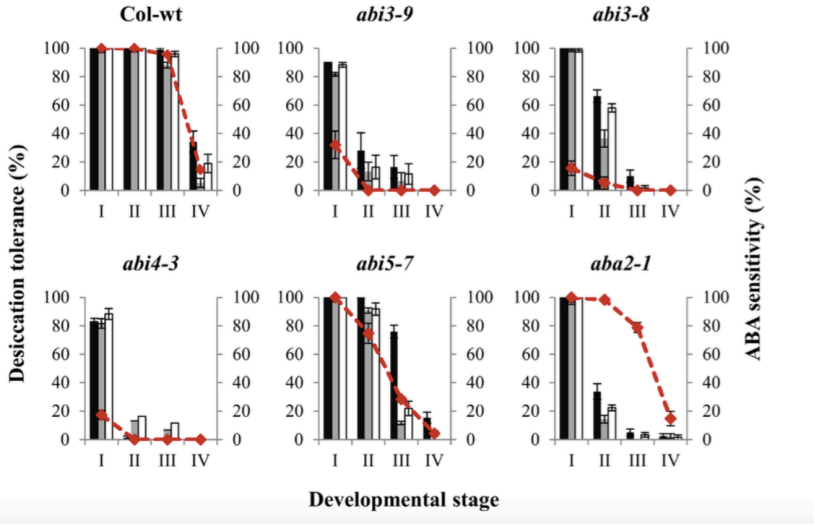


Fig. 6. Re-establishment of DT and ABA-sensitivity in germinated *Arabidopsis* seeds.

Red lines represent ABA sensitivity, Black bars represent survival of cotyledons, grey bars represent primary roots and white bars represent seedlings. (From [109]).

2.2 Hypocotyl development

Germination of seeds leads to the emergence of young seedlings, whose development relies on environmental conditions, light being the most important clue. In the absence of light, which is the natural condition of seeds germinating underground, seedlings

undergo a skotomorphogenic developmental program, characterized by long hypocotyls, small and etiolated cotyledons and by the presence of an apical hook. On the other hand, photomorphogenesis is a multi-traits process characterized by inhibition of hypocotyl elongation, opening and expansion of cotyledons and chloroplast development.

Hypocotyl growth is only dependent on cell expansion [111], thus this process has attracted attention because of the simplicity of the organ, and also because - besides light - a number of plant growth factors affect this process, namely GAs and auxin, as well as ethylene, BRs [112] and ABA [113-115].

GAs and light play an antagonistic role in this process: GAs promote cell elongation and prevent de-etiolation, whereas light inhibits hypocotyl growth through the GA-DELLA signaling mechanism [116]. Consistently, light positively controls the GA catabolic genes [116]. The opposite action of GA and light converges on the PIF proteins, repressors of light-mediated processes [117].

Among PIF proteins, PIF1, PIF3, PIF4 and PIF5 are mostly involved in the repression of photomorphogenesis; indeed, the quadruple *pif* mutant (lacking PIF1, PIF3, PIF4 and PIF5) displays a photomorphogenic phenotype in the dark, suggesting that these PIF factors act redundantly to promote etiolated growth. [118] Li and coauthors proved that DELLA proteins interact with PIF1,

PIF3, PIF4 and PIF5, to trigger degradation of these PIF factors, via the ubiquitin-26S proteasome system. Thus, they proposed a model of how GA and light signals coordinate their action to control plant hypocotyl elongation (Fig. 7) [118].

A molecular network including PIF and DELLA proteins acting through auxin and BRs has been described as the BZR-ARF-PIF/DELLA module, which mediates the control of cell expansion (and hypocotyl elongation) through hormones and light signals during seedling morphogenesis [119, 120]. Auxin (Aux/IAA) promotes cell elongation by integrating the environmental clues with hints from other hormones, namely GAs and BRs [121, 122]. Auxin signaling is repressed - in the absence of IAA - through the Aux/IAA negative regulators, which are degraded via the ubiquitin-proteasome 26S once IAA is present, thus inducing the IAA-responsive genes through the AUXIN RESPONSE FACTOR (ARF) transcription factors [123, 124]. ARF proteins bind the Auxin Response Elements (AuxREs) [125]. The *arf6arf8* double mutant shows shorter hypocotyls than the wild type and the *arf6* and *arf8* single mutants in the dark, suggesting that ARF6 and ARF8 regulate hypocotyl elongation redundantly [126].

As for BRs, similarly to the *pif* quadruple mutant, *Arabidopsis* BR-insensitive or BR-deficient mutant seedlings show a de-etiolated phenotype in the dark [127, 128]. Indeed, the activity of BZR1,

ARF6 and PIF4, which synergistically promote hypocotyl elongation, are inhibited by DELLA proteins [120, 129].

The hormone ethylene also plays a critical role in hypocotyl elongation. Ethylene, through its positive effector EIN3, triggers two distinct pathways: the PIF3-dependent growth-promoting pathway and an ETHYLENE RESPONSIVE FACTOR 1 (ERF1)-mediated growth-inhibiting pathway, to coordinately regulate hypocotyl elongation in response to soil conditions during seedling emergence [130]. The transcription factor ERF72, encoded by a target of EIN3, interacts with BZR1 and ARF6, inhibiting ARF6 transcriptional activity. Liu and co-authors proposed a new model in which light, auxin, BR and ethylene signaling pathways regulate hypocotyl growth. In the dark, ERF72 is localized in the cytoplasm, while ARF6 and BZR1 are in the nucleus to regulate the expression of cell elongation-related genes and promote skotomorphogenic development. After light exposure, ERF72 translocates to the nucleus and interacts with ARF6 and BZR1 to attenuate the transcriptional control of ARF6 and BZR1 target genes. Therefore hypocotyl growth is inhibited and seedlings undergo photomorphogenesis [131] (Fig. 8).

The role of ABA in hypocotyl elongation is still controversial but recently it has been proposed that ABA represses GA biosynthesis, thus stabilizing DELLA proteins, and inhibiting the activity of PIFs, ultimately repressing auxin biosynthesis. Alternatively, ABA

inhibits PIF proteins, which no longer induce both GA and auxin biosynthesis (Fig. 9) [115].

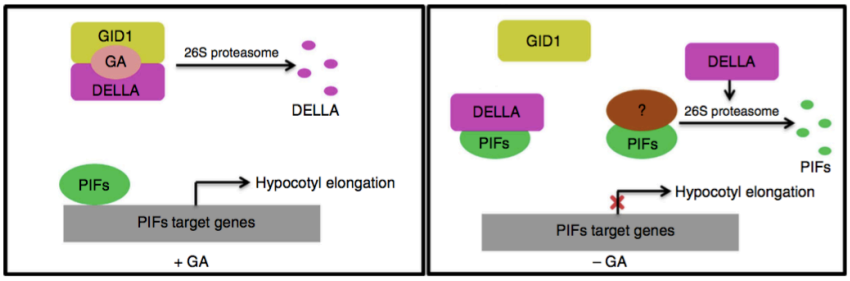


Fig. 7. A model of DELLA activity to inhibit PIF proteins.
On the left, DELLA proteins are degraded in the presence of GAs, thus releasing PIF factors and triggering hypocotyl elongation. On the right, in the absence of GAs, DELLA proteins inhibit PIF activity or by sequestering PIF factors, or by promoting their degradation through the 26S-proteasome, thus preventing binding of PIF factors to their targets, and in turn hindering hypocotyl elongation. (From [118]).

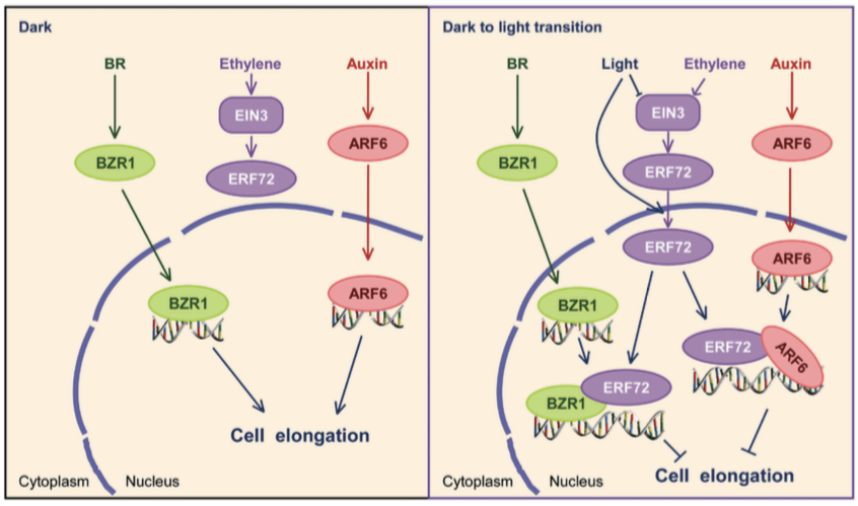


Fig. 8. Crosstalk among IAA, BRs, and ethylene signaling pathways regulates hypocotyl growth and photomorphogenesis in *Arabidopsis* seedlings.

In the model is shown how these pathways, through ARF6 and BZR1promote hypocotyl elongation in the dark, while in the light the interaction of these factors with ERF72 inhibits cell elongation. (From [131]).

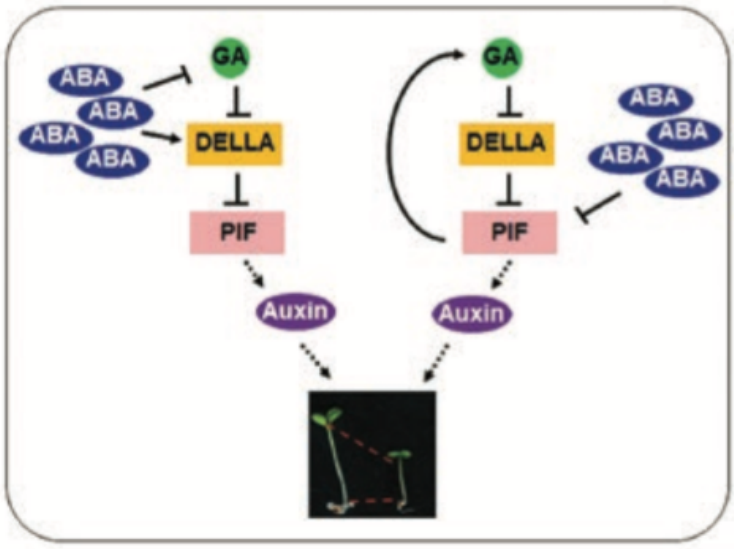


Fig. 9. Schematic model of ABA function in the inhibition of hypocotyl elongation in a DELLA-dependent or -independent pathway.

On the left ABA represses GA biosynthesis, thus DELLA proteins are stabilized, and inhibit the activity of PIF proteins, repressing auxin biosynthesis. On the right ABA inhibits PIF proteins, which no longer induce both GA and auxin biosynthesis. (From [115]).

3. DAG1: a Dof transcription factor involved in the seed-to-seedling transition

3.1 The Dof Family of plant transcription factors

Dof Affecting Germination 1 (DAG1) belongs to the DNA-binding with One Finger (DOF) plant-specific family of transcription factors, characterized by a single zinc-finger DNA-binding domain. The DOF proteins have been identified first in maize [132], and then from green unicellular algae to mosses, ferns gymnosperms and angiosperms [133-135]. These proteins share a conserved 52–amino acid domain containing a single zinc finger ($CX_2CX_{21}CX_2C$) and a downstream basic region localized in the protein N-terminus (DOF domain) (Fig. 10).

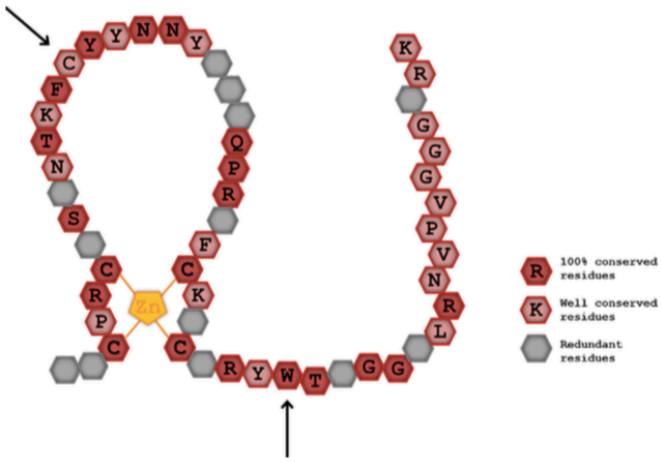


Fig. 10. Schematic representation of the DOF domain.

In red are showed the conserved amino acid residues. The well-conserved residues are pink and in gray are shown the less-conserved residues. (From [136]).

As a result of the highly conserved DNA binding domains, all the Dof proteins recognize similar target sequences, with a CTTT consensus core [137]. Therefore, the action specificity of these factors relies on specific interaction with other regulatory proteins [138-143]. DOF proteins are involved in plant-specific processes, such as maturation, dormancy and germination of seeds, or light-mediated processes and vascular differentiation (Fig. 11).

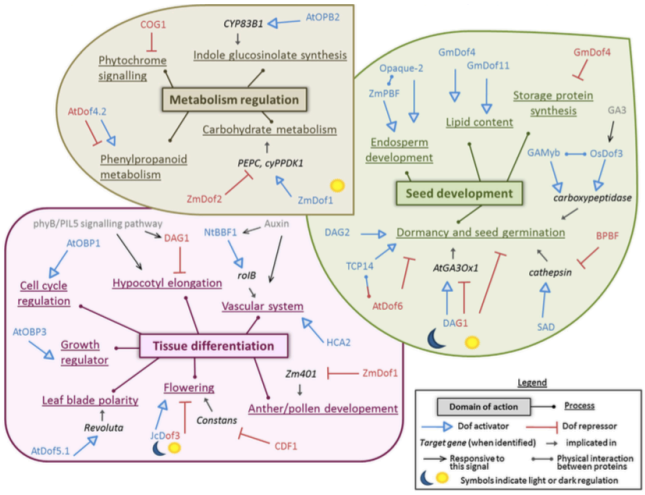


Fig. 11. Processes regulated by DOF proteins.

Examples of processes in which DOF proteins are involved. (From [136]).

3.2 DAG1 and Seed germination

We identified DAG1 as a repressor of light-mediated seed germination, acting downstream of the master repressor of this process PIL5/PIF1 [120], which indirectly controls DAG1 expression in the dark [144]. Indeed, lack of DAG1 results in an increase of germination potential; consistently, *dag1* mutant seeds require less GA and less light to germinate compared to wild type seeds [145, 146].

Recently we proved that DAG1 controls the hormonal balance between GAs and ABA during the seed-to-seedling transition,

playing a key role in the establishment and maintenance of dormancy [147]. Indeed DAG1 directly represses the ABA catabolic gene *CYP707A2*, and the GA biosynthetic gene *GA3ox1* [138, 144, 147] (Fig. 12).

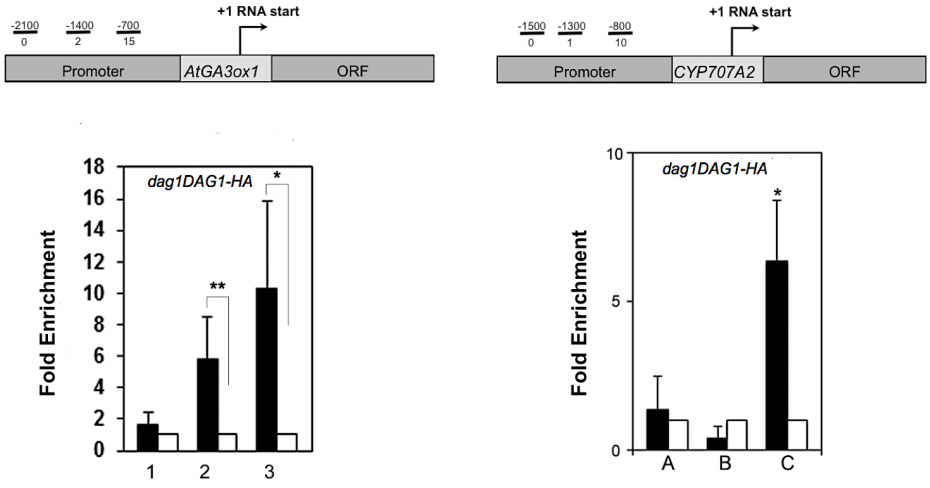


Fig. 12. DAG1 directly binds the promoters of *GA3ox1* and *CYP707A2*.

Top: graphic representation of *GA3ox1* (left) and *CYP707A2* (right) promoters. Underlying thick lines marked by letters (1, 2, 3 and A, B, C respectively) are referred to different promoter fragments used for qPCR. Bottom: chromatin from *dag1DAG1-HA* seeds was immunoprecipitated with anti-HA antibodies (black bars) or without antibodies (white bars), and the amount of DNA was measured by qPCR. The values of fold enrichment were normalized to internal controls (relative to input), and are the average of two biological replicates

presented with SD values. Significant fold enrichments were analyzed by t-test (*P ≤ 0,05). (Adapted from [138, 147]).

In the molecular pathway repressing seed germination in the dark, PIF1 directly activates transcription of the *GAI* and *RGA* genes [148], to increase the ABA/GA ratio. Nevertheless, PIF1 does not directly control ABA and GA metabolism, rather it functions through downstream repressors, such as DAG1 [144].

We have previously demonstrated, by ChIP assay, that GAI cooperates with DAG1 in repressing *GA3ox1*, since lack of GAI in the *dag1gai-t6DAG1-HA* line affects the binding of DAG1 to the DOF sites on the *GA3ox1* promoter. By yeast two-hybrid assay, we also proved that GAI directly interacts with DAG1. In addition, DAG1 and GAI mutually affect their expression [149].

DAG1 is expressed in the vascular system of the mother plant and during embryogenesis from the globular stage to mature embryo [138, 144, 145].

In addition, *DAG1* is expressed in dry and imbibed seeds, its expression is modulated from maturation to germination. Indeed *DAG1* is highly expressed during the late stage of seed maturation (13 DAP, Days After Pollination), then its expression decreases, keeping a steady-state level during dormancy [147]. Similarly to a number of seed-specific genes [150], *DAG1* expression is controlled at the epigenetic level, during the seed-to-seedling

transition. Indeed, by ChIP analysis we proved that, in young seedlings, the *DAG1* locus is enriched in the repressive epigenetic mark H3K27me3, catalysed by the Polycomb Repressive Complex2 (PRC2), whereas it is enriched in the activating epigenetic mark H3K4me3 in dry seeds, consistently with its role during the seed-to-seedling transition [147] (Fig. 13).

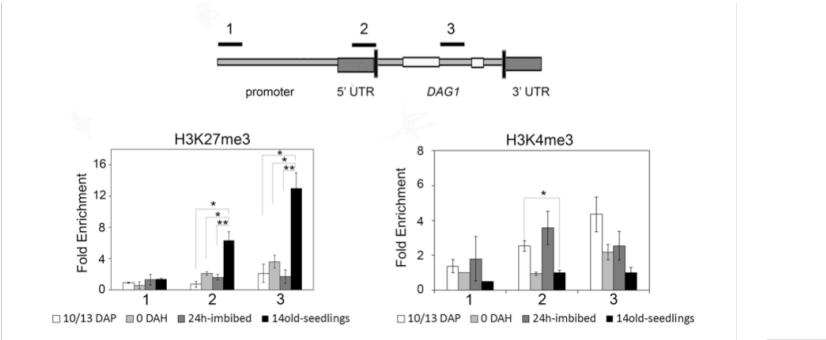


Fig. 13. *DAG1* expression is controlled at the epigenetic level.

Top: graphic representation of the *DAG1* locus. Bottom: Chromatin from wild type developing seeds at 10/13 DAP (days after pollination), 0 DAH (days after harvest), 24h-inbibited seeds and 14 old-seedlings was immunoprecipitated with specific antibodies against the H3K27me3 (left) and H3K4me3 (right). (Adapted From [147]).

It is known that, during dormancy, GA levels must be kept low [151]; our data proved that DAG1 plays a crucial role in maintaining GA biosynthesis low by repressing the expression of *GA3ox1*. Interestingly, we demonstrated that both DAG1 expression and protein stability are controlled by GA, as the *DAG1* transcript level is increased and the protein stabilized by bioactive GAs, suggesting the presence of a feedback loop through which GAs promote DAG1 expression/stabilization to repress their own biosynthesis during seed germination [147].

3.3 DAG1 and Hypocotyl development

It has been previously proposed that DAG1 may be a negative component of the mechanism of light-mediated inhibition of hypocotyl elongation, as lack of *dag1* results in a shorter hypocotyls compared to the wild type [144] (Fig. 14).

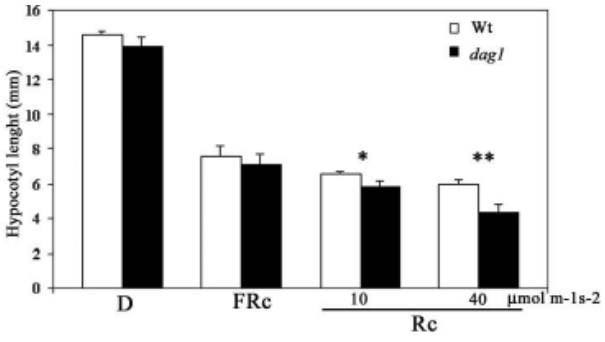


Fig. 14. DAG1 is a negative component of light-mediated inhibition of hypocotyl elongation.

Hypocotyl length of wild type and *dag1* seedlings grown in the dark (D), Far Red continuous (FRc), and Red continuous (Rc). (From [144]).

More recently, we have shown that DAG1 promotes hypocotyl elongation, through the control of ABA, ethylene and auxin signaling in this developmental process [152].

We have analysed the trascriptomic profile of 4 days-old *dag1* and wild type hypocotyls by means of high-throughput RNA-seq. Interestingly, our analysis revealed that "response to abscisic acid", "response to ethylene" and "response to auxin" are among the significantly enriched processes. Consistently, a large number of Differentially Expressed Genes (DEGs), are related to ABA, auxin and ethylene signaling (Tab. 1). In particular we have identified seven WRKY-encoding genes, known to be involved in ABA signaling, and seven *ERF* (*ETHYLENE RESPONSE FACTOR*) genes. Moreover, four *SAUR* (*SMALL AUXIN RESPONSIVE*) genes were down-regulated in *dag1* hypocotyls (Tab. 1). The *SAUR* genes are rapidly induced by auxin, and it was recently

shown that they are light-repressed in hypocotyl, suggesting a positive role of these genes in hypocotyl elongation [152, 153]

Table1:

Categories	**Gene Name**
ABA-related genes	***ABR1***
	RAB18
	WRKY40
Ethylene Responsive Factors genes	***ERF2***
	ERF5
	ERF11
	ERF105
	ERF109
SMALL AUXIN UP RNA genes	***SAUR50***
	SAUR63
	SAUR65
	SAUR67
WRKY-family	***WRKY6***
	WRKY18
	WRKY28
	WRKY33
	WRKY46
	WRKY70

4. WRKY: a family of stress-related transcription factors

The WRKY family is one of the largest transcription factor families in plants; the *Arabidopsis* genome encodes for 72 WRKY proteins [154, 155]. A large number of studies on these proteins revealed that they are related to pathogen responses [154, 156, 157], senescense [158], morphogenesis [159], cold tolerance [160], as well as response to drought and high salinity stresses [161]. The WRKY proteins are characterised by a DNA-binding domain, defined by a conserved WRKYGQK sequence of 60 amino acids at the N-terminal and a zinc-finger-like motif. The WRKY DNA-binding domain recognises the W-box elements containing the TTGAC(C/T) motif, although the flanking sequences of the W-box provide the binding selectivity of these transcription factors. The binding of WRKY proteins to the W-box has been demonstrated by several experiments, both *in vitro* and *in vivo* [156, 157].

WRKY proteins contain either one or two WRKY domains. They are classified on the basis of both the number of WRKY domains and the features of their zing-finger-like motif. In the group I are present the transcription factors with two WRKY domains, in the group II the ones with only one WRKY domain. Groups I and II share the same C_2-H_2 finger motif, while a small subset of WRKY proteins (group III) display a C_2-HC motif. The group II were

further divided into IIa, IIb, IIc IId and IIe based on the primary amino acid sequence (Fig. 15) [162].

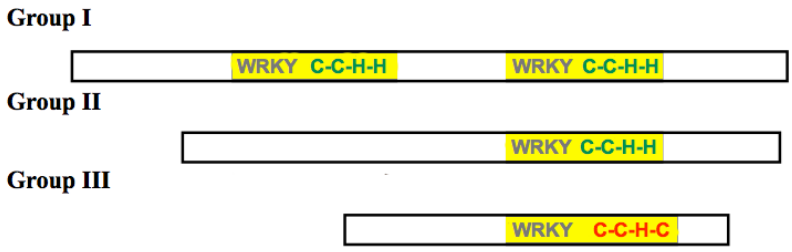


Fig. 15. The consensus WRKY domain for each WRKY group in higher plants.

The yellow boxes represent the WRKY domain, in red and green the two different zinc finger motifs.

A large number of *Arabidopsis WRKY* genes are induced by pathogen infection or SA treatment [163]. Indeed, many plant defence or defence-related genes, including pathogenesis-related and the master regulatory NPR1 (NON-EXPRESSER OF PATHOGENESIS-RELATED GENES 1) encoding gene, contain W-box sequences in their promoters [164]. Several studies in *Arabidopsis* have revealed direct links between specific WRKY proteins and complex plant defence responses; for example, the *wrky70* mutant shows increased plant susceptibility to both biothrophic and necrotrophic pathogens including *Erwinia*

carotovora, Hyaloperonospora parasitica, Erysiphe cichoracearum and *Botrytis cinerea* [165, 166]. On the other hand, lack of WRKY33 results in enhanced susceptibility to necrotrophic fungal pathogens and impaired expression of JA/ET- regulated defence genes [167].

Besides their involvement with the response to biotic stress, WRKY proteins are also involved in plant responses to abiotic stresses. Indeed, a large number of *Arabidopsis WRKY* genes have been shown to be induced by drought, cold or high-salinity stress [168, 169]. The knockout mutant of *WRKY63*, named *abo3*, is hypersensitive to exogenous ABA in seeds and seedlings; conversely, the adult mutant plant shows reduced ABA sensitivity in guard cells, displays more rapid water loss and is more sensitive to drought stress than wild type plants [170]. A similar phenotype has been shown for the *wrky2* mutant, which shows hypersensitivity to ABA response during seed germination. ABA induces WRKY2 protein accumulation in seeds and seedlings; this effect is lost in *abi* and *aba* mutants, suggesting that these ABA-factors are crucial to mediate this ABA action on WRKY2 stabilization [171].

The *Arabidopsis* WRKY18 cooperates with the two related WRKY40 and WRKY60 proteins, in the response to biotic stress with both overlapping and distinct functions [172, 173]. Genetic and molecular analysis of the corresponding mutants proved that

these proteins have also a role in ABA signaling. Indeed, lack of WRKY18 and WRKY60 results in reduced ABA sensitivity for the inhibition of seed germination, as well as increased tolerance to salt and osmotic stress. Conversely, inactivation of *WRKY40* causes an increased ABA sensitivity for germination and a decreased tolerance to salt and osmotic stress [174](Fig. 16).

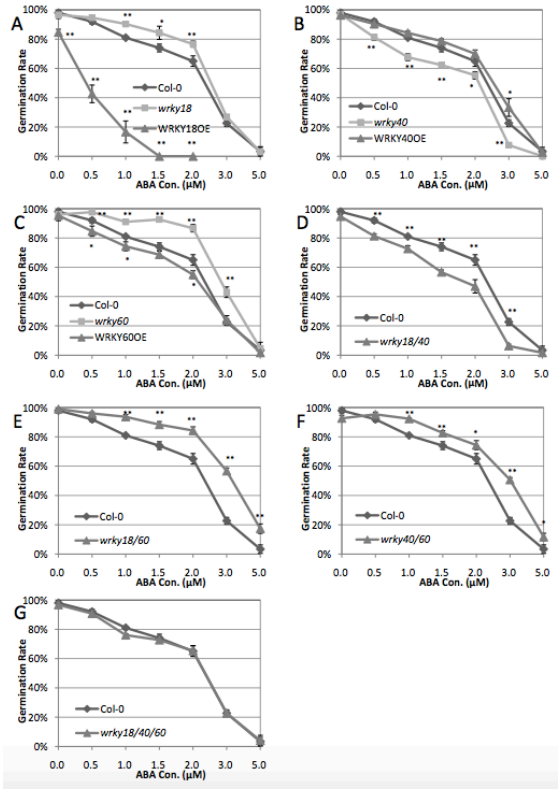


Fig. 16. Germination rates under ABA treatment of *wrky18, wrky40* and *wrky60 mutants*.

Germination of wild type and *wrky18, wrky40* and *wrky60* single, double and triple mutants seeds in the presence of different concentration of ABA. The germination was determinated 120 hours after sowing. (From [174]).

ABA controls the expression of *WRKY18, WRKY40* and *WRKY60*: *WRKY18* and *WRKY40* are rapidly induced upon ABA treatment, whereas ABA-induction of *WRKY60* is delayed and prolonged. In addition, both WRKY18 and WRKY40 are required for ABA-induced *WRKY60* expression [174]. By ChIP analysis, it has been shown that WRKY18 and WRKY60 bind the promoters of the ABA positive regulators-encoding genes *ABI4* and *ABI5*, thus resulting in a complex molecular model in which WRKY18, WRKY40 and WRKY60 alternatively cooperate or play antagonistic roles to control *ABI4* and *ABI5* expression and ABA-related responses [92].

The *Arabidopsis* WRKY6 factor is involved not only in plant pathogen defence but also in ABA-signaling. Studies on *wrky6* knock-out mutants revealed that lack of WRKY6 results in decreased ABA sensibility during seed germination and seedling development, whereas WRKY6 over-expression results in ABA-hypersensitive phenotypes (Fig. 17).

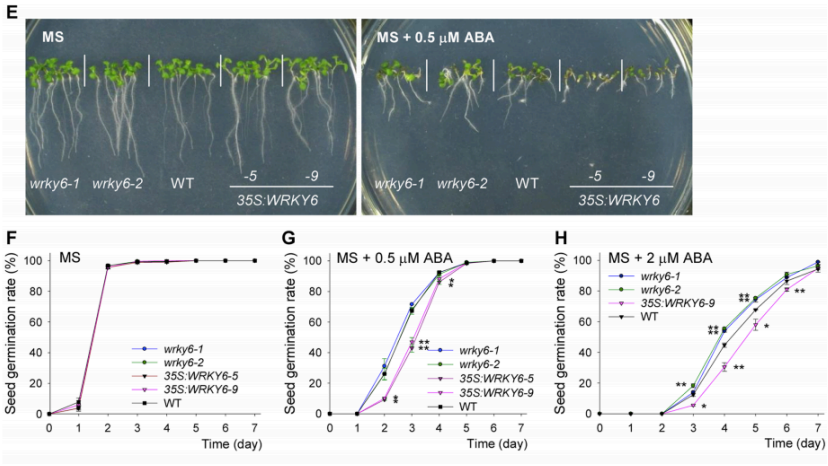


Fig. 17. ABA-sensitivity of *wrky6* mutant and *WRKY6* overexpressing lines. Top: phenotypic comparison between wild type (WT) and mutants in the presence of exogenous ABA (0.5μM). Bottom: Seed germination assay in the absence or presence of ABA. The seed germination rate was calculated at time indicated. (Adapted from [175]).

In the working model proposed, WRKY6 regulates ABA-signaling by directly binding and repressing RAV1, a transcription factor that regulates the expression of *ABIs* during seed germination [93]. RAV1 represses the expression of *ABI3*, *ABI4* and *ABI5* thus promoting seed germination and early seedling development [175].

5. Epigenetics

The term *epigenetics* was introduced in the early 1940s by Conrad Waddington [176], as a refinement of his concept of "epigenetics landscape" [177](Fig. 18). Today epigenetics is defined as “the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence” [178]. The epigenetic modifications comprise histone variants, post-translational modifications of amino acids on the amino-terminal tail of histones, and covalent modifications of DNA bases.

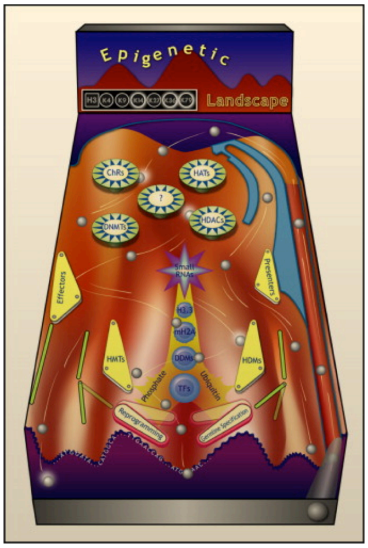


Fig. 18. Modern view of the Epigenetic Molecular Machinery.

H3.3 and macroH2A are shown as histone variants involved in transcriptional activation or repression, respectively. ChR, chromatin remodelers; DNMTs, DNA methyltransferases; HATs, histone acetyltransferases; HDACs, histone deacetylases; HMTs, histone methyltransferases; HDMs, histone demethylases; DDMs, DNA demethylases and TFs, transcription factors [reflecting the genetic component of the epigenetic process. (From [179]).

In plants and animals, the genome size, genome complexity and the ratio of heterochromatin to euchromatin are generally comparable. Plants and mammals make similar use of both DNA methylation and histone post-translational modifications (PTMs) to control gene expression. The comparison of genome organization and epigenetic control in different model systems reveals that there are more common features between plants and mammals than within the animal kingdom itself [180]. Consequently, epigenetic mechanisms discovered in plants or mammals are generally relevant to both systems.

Information content of the genome (DNA sequence) and its expression in response to stress are crucial for the adaptability of a genotype. Developmental and environmental signals can induce epigenetic modifications in the genome, and thus, a single genome in a plant cell gives rise to multiple epigenomes in response to developmental and environmental cues.

Among the enzymes that post-translationally modify histones, the Polycomb group (PcG) proteins are a transcriptional repression

system for the epigenetic control of cell, tissue and organ differentiation, contributing to correctly attain a variety of plant developmental programs. The PcG group proteins were initially identified as master regulators and suppressors of homeotic genes in *Drosophila*. PcG proteins are grouped into two complexes: POLYCOMB REPRESSIVE COMPLEX 1 (PRC1) and 2 (PRC2). PRC2 is responsible for the trimethylation of lysine 27 of histone3 (H3K27me3), which is recognized by PRC1 to establish a silent chromatin conformation by monoubiquitination of histone H2A [181].

PRC2 has four subunits:

- an histone methyltransferase encoded by *ENHANCER OF ZESTE (E(Z))*
- a WD40 domain protein encoded by *EXTRA SEX COMBS (ESC)*
- a Zn-finger protein encoded by *SUPPRESSOR OF ZESTE 12 (SU(Z)12)*
- a nucleosome-remodeling protein encoded by *NUCLEAR REMODELING FACTOR (NURF55*) [182].

In *Arabidopsis*, there are 12 homologs of the Drosophila PRC2 subunits and, in particular, the histone methyltransferase EZ is encoded by three homologs (*CURLY LEAF, MEDEA* and *SWINGER; CLF, MEA* and *SWN*), which share a highly conserved

SET domain, responsible of the catalytic activity [183]. Different combinations of the four subunits result in three PRC2-like complexes: the EMBRYONIC FLOWER (EMF), VERNALIZATION (VRN) and FERTILISATION INDEPENDENT SEED (FIS), which function in different developmental processes, although sharing some target genes [184, 185](Fig. 19).

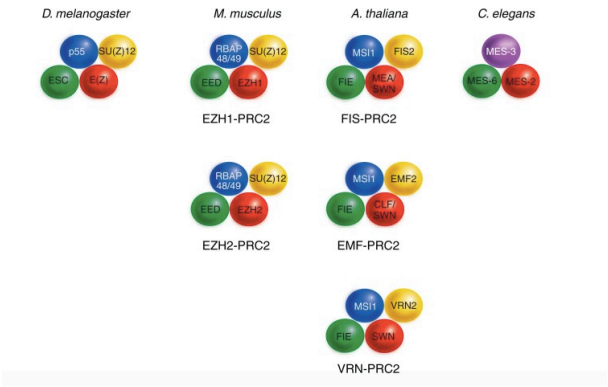


Fig. 19. The conserved core proteins of PRC2 in *Drosophila melanogaster, Mus musculus, Arabidopsis thaliana* and *Caenorhabditis elegants*.
In *Arabidopsis* PRC2 has evolved into three variants with different functions during development. Homologous proteins are indicated with the same color. Notably in *C. elegans* only three proteins form the PRC2 core complex, and the MES-3 subunit is not homologous with any other identified in PRC2 subunits. (From [186]).

In flowering plants, the activity of PRC2 is crucial during endosperm formation as it controls the imprinting of several genes, and mutations in the imprinting machinery lead to embryonic lethality [187]. This has severely hindered studies on the function of PRC2 during seed development (Fig. 20).

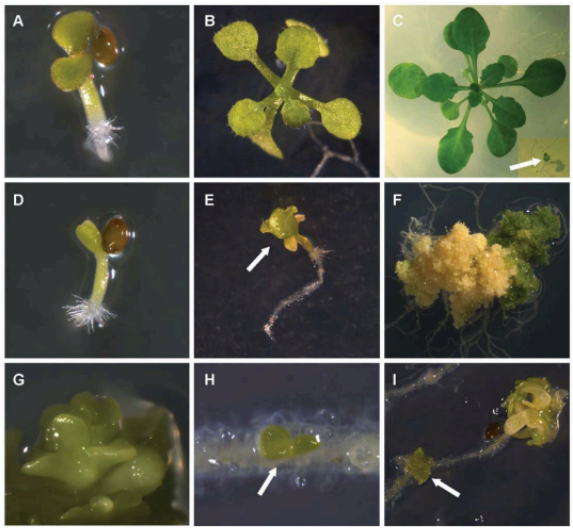


Fig. 20. Phenotypic analysis of post-embryonic development. The development of the wild type (A-C) compared to the *fie* mutant (C-I).
(A and D) 5 days-old wild type and *fie* seedlings. (B and E) 15 days-old wild type and seedlings, the *fie* mutant showing early flowers. (C) 40 days-old wild type seedling is bigger than the *fie* mutant (arrow). (F) 3 months-old *fie* mutant transformed into a callus-like structure. (G-I) Misplaced cells and organs in *fie* mutants, flower-like organs (G), Leaves in root (H) and offshoots (I). (Adapted from [150]).

An exception is represented by the genetic strategy used by Bouyer and collaborators, who were able to bypass the female gametophytic defect of the *fertilization independent endosperm* (*fie*) mutant (Fig. 20), through pollination of heterozygous *fie* mother plants with pollen from a *cdka;1-fie* double heterozygous line. This allowed to generate viable homozygous *fie* mutants, derived from seeds where the endosperm was of uniparental (maternal) origin [150, 188].

PcG silencing is medically relevant as it has often been correlated with human disorders, including cancer, and tissue regeneration, which involve the reprogramming of PcG-controlled target genes. For this reason EZH2 has long been considered an ideal therapeutic target [189].

The first compound described as inhibitor of EZH2 was the 3-deazaneplanocin A (DZNep), which was shown to reduce H3K27me3 levels through depletion of EZH2 protein level, although with a fairly low specificity [190]. Subsequently, efforts in producing selective inhibitors of EZH2 by means of high-throughput screenings have been highly promising [191-194]. Among the compounds identified, the dual inhibitor of EZH2/EZH1, UNC1999, has been shown to be highly effective *in vitro* on both wild type and both gain- and loss-of-function mutant

EZH2. UNC1999 was shown to be able to reduce H3K27me3 levels as well as cell proliferation in a large number of cancer cells, without affecting EZH2 protein level (Xu et al 2015, Konze et al 2013).

AIM OF THE WORK

Plants, as sessile organism, have to survive to changing environmental conditions. The phytohormone abscisic acid (ABA) has a pivotal role in the adaptation to environmental challenges. ABA is also involved in the regulation of plant growth and development, and in particular in the establishment of dormancy, a period of storage necessary to prevent early germination and vivipary.

Dof AFFECTING GERMINATION 1 (DAG1) controls the balance between GA and ABA during dormancy. DAG1 acts through the direct repression of the GA biosynthetic gene *GA3ox1* and the ABA catabolic one *CYP707A2*, thus ensuring the establishment of dormancy and the repression of germination. Recently we proved that DAG1 has also a role during seedling development, indeed *dag1* mutants show a shorter hypocotyl than the wild type. By using high-throughput RNA-seq we proved that DAG1 is involved in the promotion of hypocotyl elongation through the control of ABA, ethylene and auxin signaling.

In this genome-wide analysis on *dag1* hypocotyls, we have identified seven WRKY-encoding genes, known to be involved in the response to abiotic stresses. In particular, we focused our attention on WRKY6 and WRKY18 because they are likely to be

elements of the ABA-mediated developmental processes and stress response.

This project aims to investigate the role of - and the putative interactions between - DAG1 and WRKYs factors in the ABA-related processes and in particular in the response to abiotic stress.

The PRC2 complex has a key role during plant development, in particular during the seed-to-seedling transition. The *DAG1* locus is a target of PRC2, and is marked by H3K27me3 in seeds and seedlings, consistently it had been shown to be up-regulated in mutant plants lacking PRC2.

Mutation on the catalytic subunit of PRC2 results in a severe phenotype, like embyo-lethality in plants or cancer in animals. The effects of different inhibitors of the catalytic subunit of PRC2 have long been tested in animals as a possible anti-cancer therapy.

Taking advantage of the homology of the PCR2 catalytic subunits of animals and plants, the second aim of this PhD project was to develop a pharmacological approach to inhibit the activity of PRC2 in seeds and seedlings of *Arabidopsis thaliana*.

Results:

1. Lack of DAG1 affects expression of ABA-related and *WRKY* genes

We had previously proposed that the *Arabidopsis* DOF transcription factor DAG1 might function as a negative component of the light-mediated inhibition of hypocotyl elongation, as light-grown *dag1* mutant seedlings show significant shorter hypocotyls than the wild type [144]. We investigated the role of DAG1 in hypocotyl elongation by using high-throughput RNA-seq. We compared the trascriptomic profile of *dag1* and wild type hypocotyls and seedlings grown under continuous Red light [152]. This analysis revealed 257 differentially expressed genes (DE genes) in *dag1* hypocotyls. The RNA-seq data suggest that DAG1 is involved in the control of ABA, in hypocotyls. We validated the expression of a number of these hormone-related genes by RT-qPCR on *dag1* and wild type hypocotyls. Of the ABA-related genes, we analysed the expression of RAB GTPASE HOMOLOG B18 (RAB18), a stress-responsive gene involved in ABA and drought response, ABA REPRESSOR1 (ABR1) encoding an APETALA2 (AP2) domain transcription factor known as a repressor of ABA and the ABA-responsive WRKY40 transcription

factor encoding gene Fig. 21 [152]. Among the DE genes we identified 27 transcription factors encoding genes, belonging to 9 different families. The most represented family was the WRKY family [152]. WRKY proteins are transcription factors mainly involved in abiotic-stress response and ABA-mediated processes like dormancy, desiccation tolerance (DT) and seed germination. Since DAG1 controls ABA levels during dormancy and germination playing a key role in the establishment of dormancy [147] we validated these targets through RT-qPCR. The expression of *WRKY6, WRKY18, WRKY28, WRKY33, WRKY40, WRKY46* and *WRKY70* was analysed in 4 days-old hypocotyls grown under continuous Red light. The results of this analysis revealed that expression of *WRKYs* was significantly increased by inactivation of *DAG1* (Fig. 21), thus confirming the data obtained from the RNA-seq analysis.

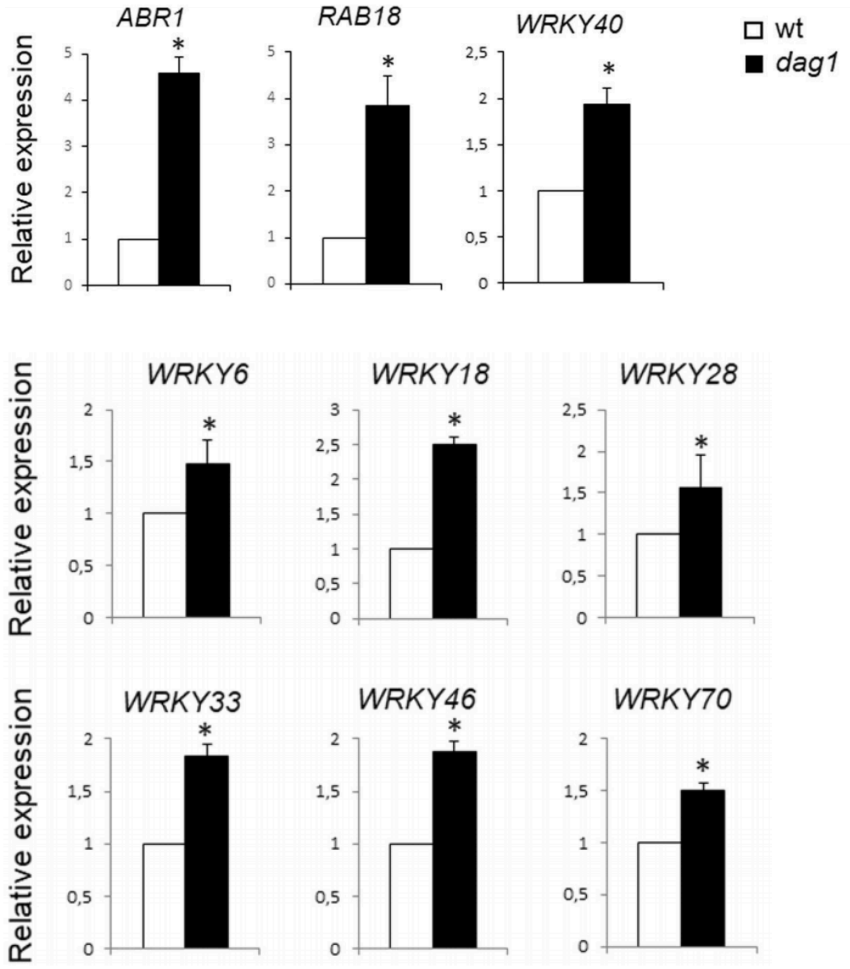

ABR1
RAB18
WRKY40
wt
dag1
Relative expression
WRKY6
WRKY18
WRKY28
WRKY33
WRKY46
WRKY70

Fig. 21. Lack of DAG1 affects the expression of ABA-related and *WRKY* genes under continuous Red light.

Top: Relative expression of ABA-related genes in wild type and *dag1* hypocotyls.

Bottom: Relative expression of *WRKY* genes, in wild type and *dag1* hypocotyls. The values are the mean of three independent biological replicates, presented with SD values. Significant differences were analyzed by *t*-test (*P≤0.05). ([152]).

Since it has been recently demonstrated that DAG1 positively controls hypocotyl elongation through cell expansion, we assessed whether the control of DAG1 on the expression of the candidate *WRKY* genes might be independent of light conditions. Therefore, we performed an expression analysis on hypocotyls from dark-grown seedlings. Interestingly, the results revealed that *WRKY* expression was significantly increased by inactivation of *DAG1* regardless of light conditions (Fig. 22).

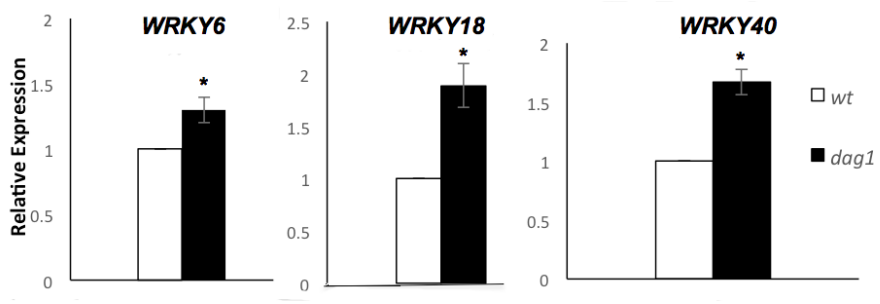


Fig. 22. Lack of DAG1 affects the expression of *WRKY* genes in the dark. The relative expression of *WRKY* genes, in wild type and *dag1* hypocotyls. The values are the mean of three independent biological replicates, presented with SD values. Significant differences were analyzed by *t*-test (*P≤0.05).

2. DAG1 directly binds the *WRKY18* promoter

DAG1, as a Dof transcription factor, binds the CTTT sequence on the promoter of target genes [195, 196]. The presence of a significant number of CTTT binding sites (BS) in the promoter of *WRKY18* was verified by means of Promomer. This *in silico* result was verified by ChIP analysis, using the *dag1DAG1-HA* transgenic line expressing a DAG1-HA functional protein in a *dag1* mutant background [138]. Protein-DNA complexes were precipitated with anti-HA antibodies, or without antibodies as a negative control. As additional negative control, we performed the same assay on untransformed *dag1* seedlings. Three regions of the *WRKY18*

promoter were amplified by qPCR. The relative amount of promoter fragment c (7 Dof BS) precipitated by DAG1-HA was significantly higher compared to the negative controls, whereas fragment b (4 Dof BS), although slightly higher than the control, was not significantly different. As for fragment a (no Dof BS), it was similarly enriched in DAG1-HA and in the negative controls. Therefore, this analysis corroborated the result of the *in silico* analysis, revealing that DAG1 directly binds the *WRKY18* promoter (Fig. 23).

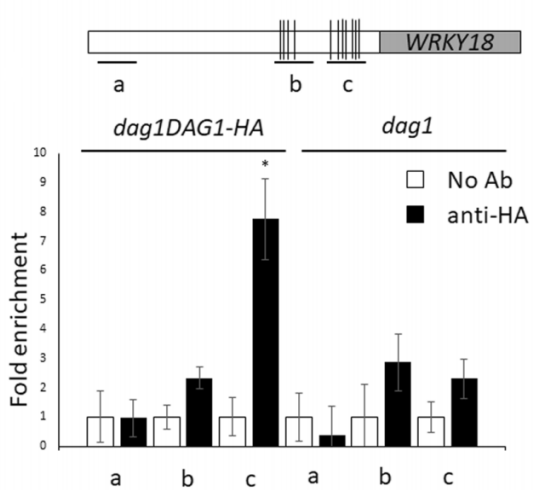


Fig. 23. DAG1 directly binds the promoter of *WRKY18*.

Top: graphic representation of the *WRKY18* promoter. a, b, c are the different promoter fragments used for qPCR containing 0, 4, 7 Dof binding sites

respectively. Bottom: chromatin from *dag1DAG1-HA* (left) and from *dag1* (right) seedlings, as a negative control, was immunoprecipitated with anti-HA antibodies, and the amount of DNA was measured by qPCR for *WRKY18* promoter fragments. The values are the mean of two independent biological replicates, presented with SD values. Significant differences were analyzed by *t*-test (*P≤0.05). ([152]).

3. Phenotypic characterization of the *wrky6* and *wrky18* single mutants

We focused on the WRKY6 and WRKY18 factors for further analysis because they are known to be involved in seed germination and ABA-related processes and signaling [92, 175].
First, we performed a seed germination assay of non-dormant stratified or unstratified seeds, by measuring the germination rate every day up to complete germination. The germination rate was measured as the number of germinating seeds (radicle emerging from the seed coat) on the total number of seeds. Curiously, the *wrky6* and *wrky18* mutations did not affect the germination process. Indeed, in the presence or absence of stratification - a treatment necessary to remove the residual dormancy -, the germination rate of *wrky6* and *wrky18* mutants did not show a significant delay respect to the wild type (Fig. 24).

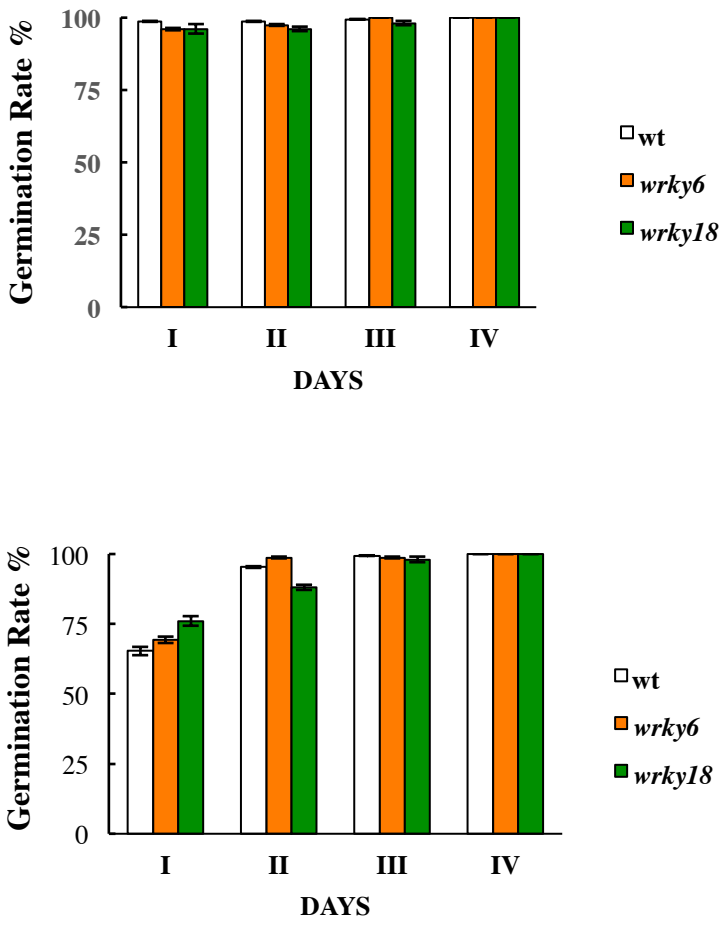
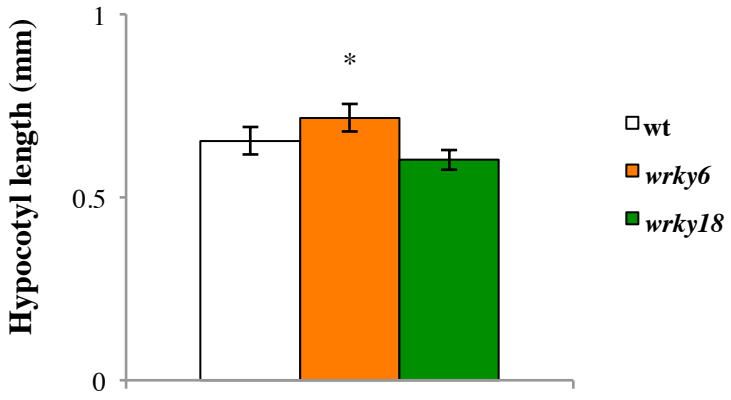


Fig. 24. Germination properties of *wrky6* and *wrky18* mutants.

Top: germination assay with stratified mutant and wild type (wt) seeds; the number of germinated seeds were measured every day after imbibition. Bottom: germination assay with unstratified mutant and wild type (wt) seeds. The values are the mean of two independent biological replicates presented with SD values.

Our genome-wide analysis reveals that the expression of the *WRKY6* and *WRKY18* is affected in *dag1* hypocotyls compared to wild type ones. To verify if these proteins could have a role in hypocotyl elongation, we performed a phenotypic analysis of hypocotyl length of the mutants under two different light conditions: continuous Red light and dark. Interestingly, the inactivation of *WRKY6* results in a significantly increased hypocotyl length than the wild type in both light conditions. Conversely, lack of WRKY18 results in significantly shorter hypocotyls only in dark condition (Fig. 25).



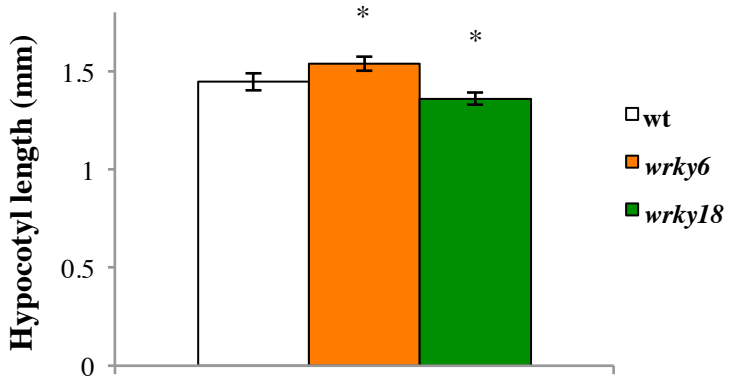


Fig. 25. Hypocotyl length of the *wrky6* and *wrky18* mutants under Red light and in the Dark.

Top: Relative hypocotyl length of 5 days-old mutant and wild type seedlings grown under Red Light. Bottom: Relative hypocotyl length of 5 days-old seedlings grown in the Dark. The values are the mean of three biological independent replicates, presented with SD values. Significant differences were analyzed by *t*-test (*P≤0.05).

4. Phenotypic characterization of the *wrky6dag1* and *wrky18dag1* double mutants in ABA-related processes

ABA has a pivotal role in the response to abiotic stress; given the close relationship between DAG1, WRKYs and ABA we decided to investigate a putative role of these factors in the response to abiotic stresses.

To study the genetic relationship between *DAG1*, *WRKY6* and *WRKY18* in the ABA-mediated processes, we generated the double mutants lines. Since the *dag1* and *wrky* mutant lines were in different ecotypes (Ws-4 and Col-0 respectively), several lines for each genotype (such as double mutants, parental lines and wild type) were selected and analysed to reduce the effect of the ecotype on the phenotype of interest.

4.1 Effect of simultaneous inactivation of *DAG1* and *WRKY6/18* on hypocotyl elongation

4.1.1 Hypocotyl length in different light conditions

Our previous results demonstrated that lack of WRKY6 and WRKY18 resulted in an opposite phenotype: an increased hypocotyl length for the *wrky6* mutant, while the *wrky18* mutant displayed reduced hypocotyl length compared to the wild type. We

proved that DAG1 promotes hypocotyl elongation; therefore, we wondered which was the role of these WRKY proteins in hypocotyl elongation and their relationship with DAG1. We measured the hypocotyl length in the double mutants *wrky6dag1* and *wrky18dag1* respect to the parental lines grown under Red light. As for *wrky6dag1,* this analysis showed that the *wrky6dag1* double mutant had significantly longer hypocotyls than wild type and *dag1*, since its hypocotyl length was comparable to the single *wrky6* mutant, which, as expected, showed significantly longer hypocotyls than *dag1* and the wild type (Fig. 26). Therefore, the *wrky6* mutation is epistatic on the *dag1* mutation, suggesting that WRKY6 could be a negative regulator of hypocotyl elongation. To verify if this effect was light-independent we measured the hypocotyl length of seedlings grown under dark condition. As expected, the *wrky6* single mutant had longer hypocotyls respect to the wild type, while the hypocotyl length of the *dag1* mutant was shorter than the wild type, regardless of light conditions. Interestingly, hypocotyls of the *wrky6dag1* double mutant were shorter than the wild type ones and similar to *dag1* hypocotyls, therefore suggesting a potential role of the light in the regulation of DAG1 on WRKY6 (Fig. 27).

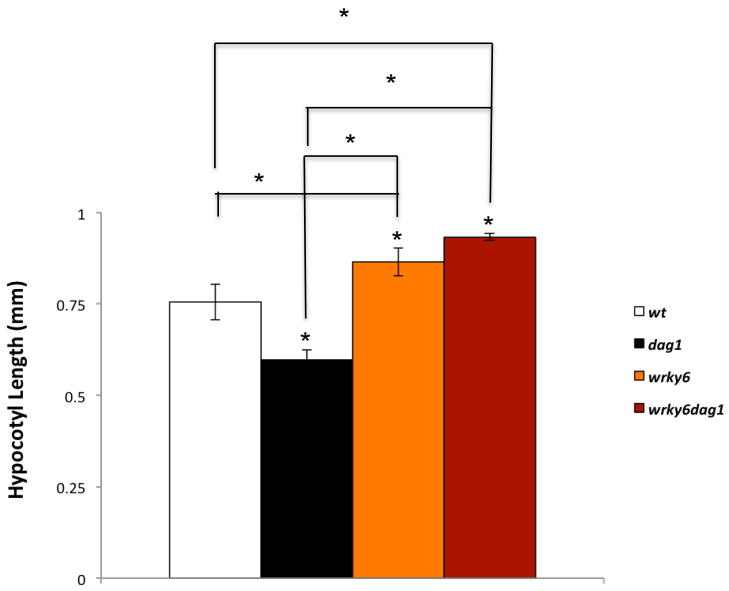


Fig. 26. Hypocotyl length of *wrky6dag1* double mutant under Red light.
Hypocotyl length of 5 days-old double mutant and parental seedlings grown under Red light. The values are the mean of three biological independent replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05).

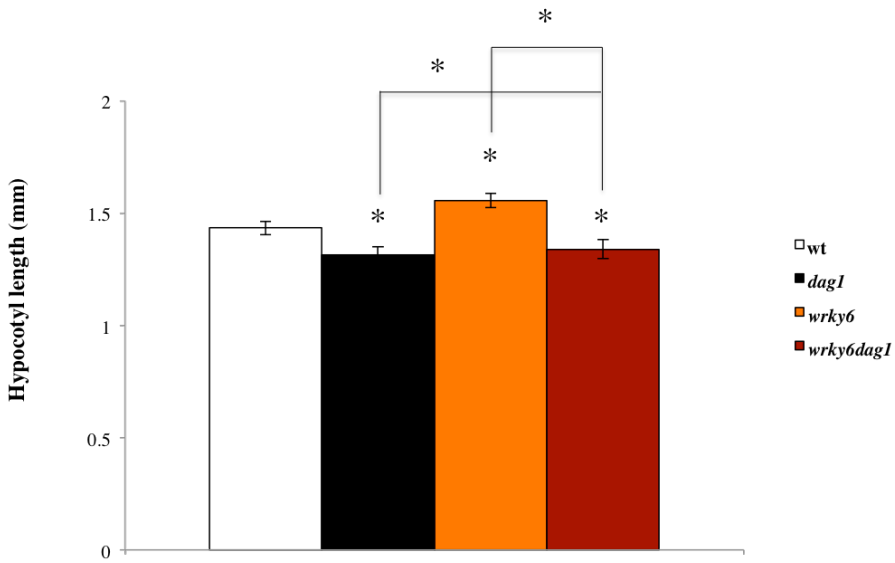


Fig. 27. Hypocotyl length of *wrky6dag1* double mutant in the dark.
Hypocotyl length of 5 days-old double mutant and parental seedlings grown in the dark. The values are the mean of three independent biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05).

As of *wrky18dag1* double mutant, the results of this analysis revealed that inactivation of both *WRKY18* and *DAG1* results in hypocotyls significantly, although slightly, shorter than the wild type, albeit not short as the parental line *dag1*. Consistently with our previous result, the single mutant *wrky18* did not show significant difference with the wild type (Fig. 28).

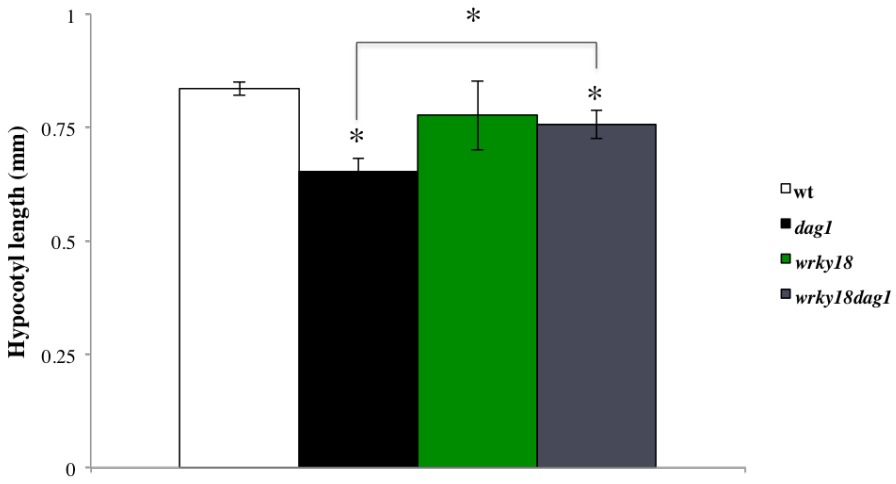


Fig. 28. Hypocotyl length of *wrky18dag1* double mutant under Red light
Hypocotyl length of 5 days-old double mutant and parental seedlings grown under Red light. The values are the mean of three independent biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05).

The *wrky18* single mutant displayed a hypocotyl phenotype only in dark condition (Fig. 25). To assess whether this phenotype was confirmed even in the presence of the *dag1* mutation, we measured hypocotyl length of seedlings grown in the dark. As expected, both the *wrky18* and the *dag1* single mutants showed a reduced

hypocotyl length compared to the wild type. Interestingly, the hypocotyl length of the double mutant *wrky18dag1* was similar to the wild type, indicating that the simultaneous presence of both mutations complemented the hypocotyl shorter phenotype of the single mutants in the dark (Fig. 29).

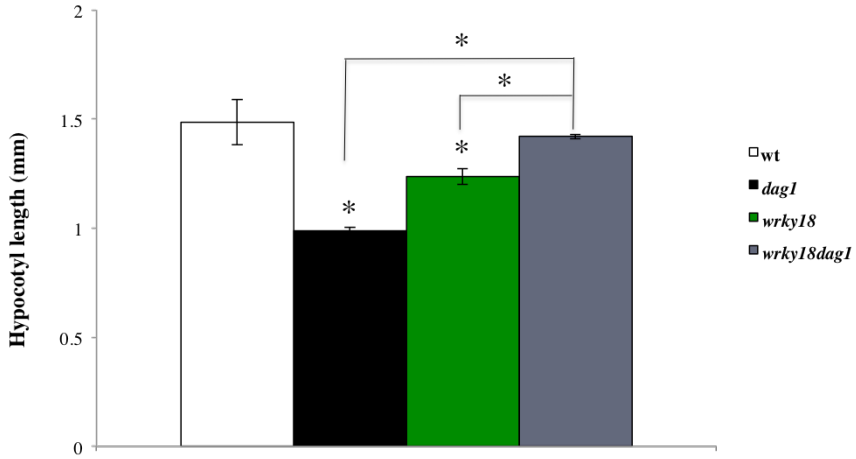


Fig. 29. Hypocotyl length of *wrky18dag1* double mutant in the dark.
Hypocotyl length of 5 days-old double mutant and parental seedlings grown in the dark. The values are the mean of three independent biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05).

4.1.2 ABA effect on hypocotyl elongation

ABA is known as a growth-inhibiting hormone, and it has been shown to repress hypocotyl growth [115]. Since the *wrky* mutants displayed ABA-resistance for seed germination, we wondered whether the WRKY6 and 18 could be involved in ABA-mediated repression of hypocotyl elongation. We measured hypocotyl length in the *wrky6dag1* and *wrky18dag1* double mutant seedlings grown under Red light in the presence of increasing ABA concentrations (0, 1, 10, 100 μM).

In the control (no ABA), the parental double mutant lines showed the opposite phenotype previously described: slightly longer hypocotyls for *wrky6* and *wrky6dag1*, shorter ones for *dag1*. The response to ABA was measured as the ratio of hypocotyl length on ABA respect to the control, for each line.

In the presence of increasing concentrations of ABA, both the *wrky6* and *wrky6dag1* mutant lines showed a reduced sensitivity to ABA, respect to the wild type and similarly to the *dag1* mutant line (Fig. 30).

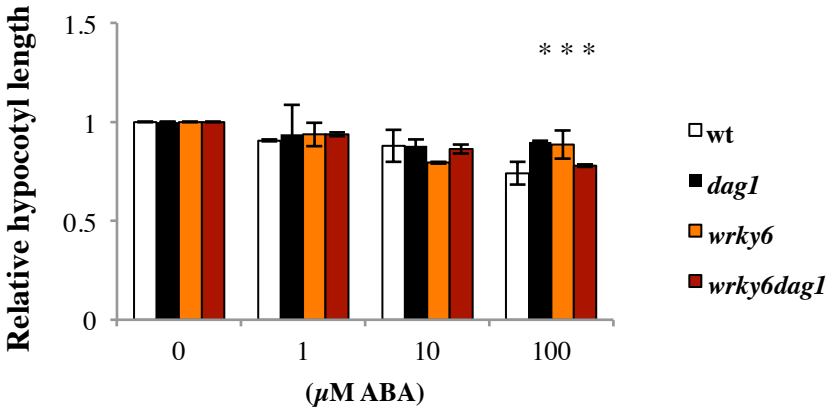


Fig. 30. ABA effect on *wky6dag1* hypocotyl elongation under Red light.
Hypocotyl length of 5 days-old double mutant and parental seedlings grown under Red light, in the presence of increasing ABA concentrations. The values, normalised to the control sample for each line, are the mean of two independent biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05).

The hypocotyl length of both *wrky18* and *wrky18dag1* mutants was not reduced by ABA like the wild type one, similarly to the *dag1* mutant, thus suggesting that inactivation of *WRKY18* results in reduced sensitivity to the inhibitory effect of ABA for hypocotyl growth (Fig. 31).

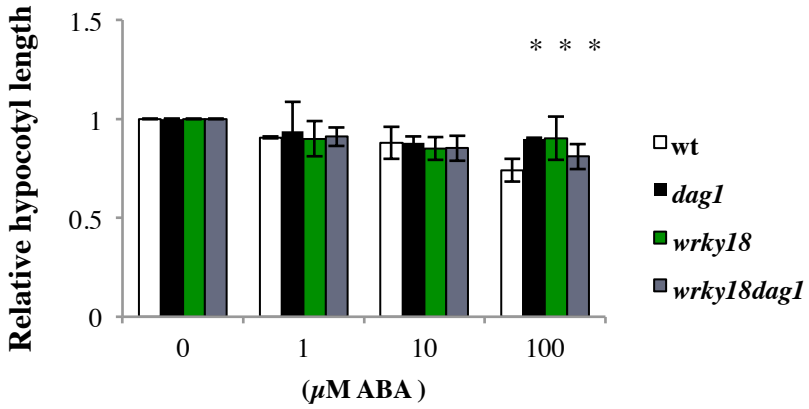


Fig. 31. ABA effect on *wky18dag1* hypocotyl elongation under Red light.
Hypocotyl length of 5 days-old double mutant and parental seedlings grown under Red light, in the presence of increasing ABA concentrations. The values, normalised to the control sample for each line, the mean of two independent biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05).

To evaluate if light could play a role in the resistance to ABA inhibition of hypocotyl length, we measured hypocotyl length of the *wrky6dag1* and *wrky18dag1* double mutant seedlings grown in dark in the presence of increasing ABA concentrations (0, 1, 10, 100 μM). The response to ABA was measured as the ratio of hypocotyl length on ABA respect to the control, for each line (Figs. 32-33).

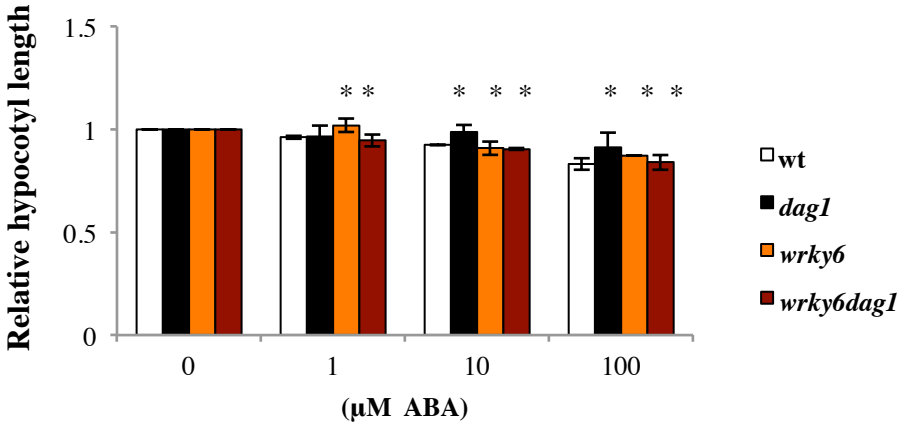


Fig. 32. ABA effect on *wky6dag1* hypocotyl elongation in the dark.
Hypocotyl length of 5 days-old double mutant and parental seedlings grown in the dark, in the presence of increasing ABA concentrations. The values, normalised to the control sample for each line, are the mean of two independent biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05).

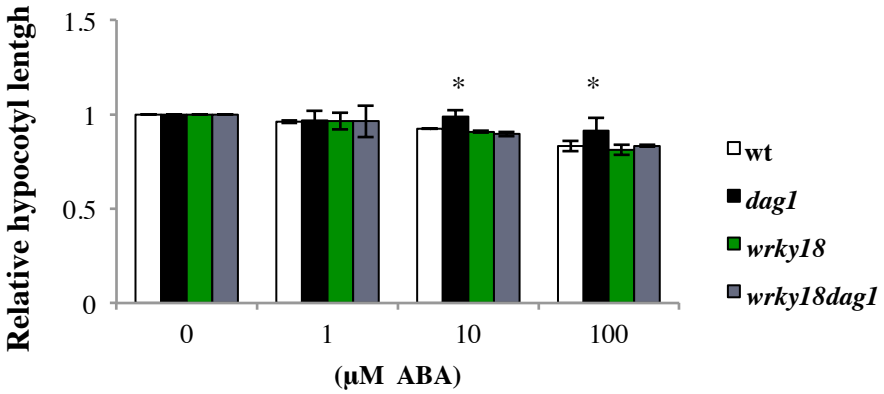


Fig 33. ABA response in *wky18dag1* and elongation in the dark.

Hypocotyl length of 5 days-old double mutant and parental seedlings grown in the dark, with increasing concentration of ABA. The values, normalised to the control sample for each line, are the mean of two independent biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05).

4.2 Desiccation tolerance

Desiccation Tolerance (DT), defined as the ability of the seed to re-induce germination after dehydration without lethal damage, is restricted to the early stages of the seed germination process [110]. Since DAG1 is involved in seed dormancy and germination and is necessary to control ABA levels in seeds, we verified whether it may play a role even in DT establishment. To this end, we set-up a protocol to assess the capacity to re-establish DT, by applying a mild osmotic stress with a PEG solution; following this treatment, we measured the germination rate for four days. The seeds were treated at stages II-III of germination (see Fig. 5), when the radicle protrusion begins. We proved that *dag1* mutant seeds partially lost the capacity to re-induce germination after dehydration, as shown in figure 34. Indeed, the germination rate of *dag1* seeds was significantly lower than the wild type, consistent with the reduced ABA content of *dag1* dry seeds respect to the wild type [147]. These data suggest that the reduction of ABA in dry seeds could trigger a decrease of the capacity to re-induce germination after exposure to drought stress in *dag1* seeds.

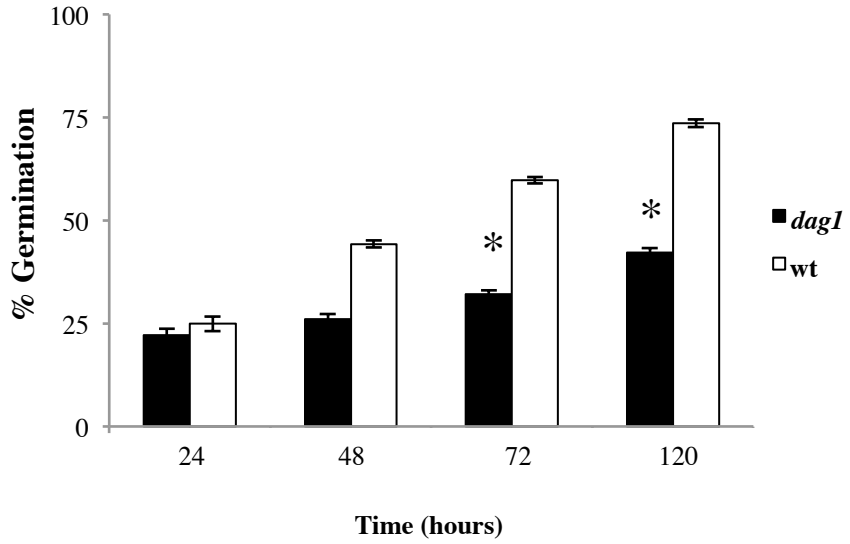


Fig. 34. Re-establishment of DT in *dag1* seeds.

Germination rate after three days of PEG-treatment. The values are the mean of three independent biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05).

Consistently, analysis of *pDAG1::GUS* transgenic line revealed an increase activity of the *DAG1* promoter during the treatment, suggesting an involvement of DAG1 in the re-establishment of DT (Fig. 35).

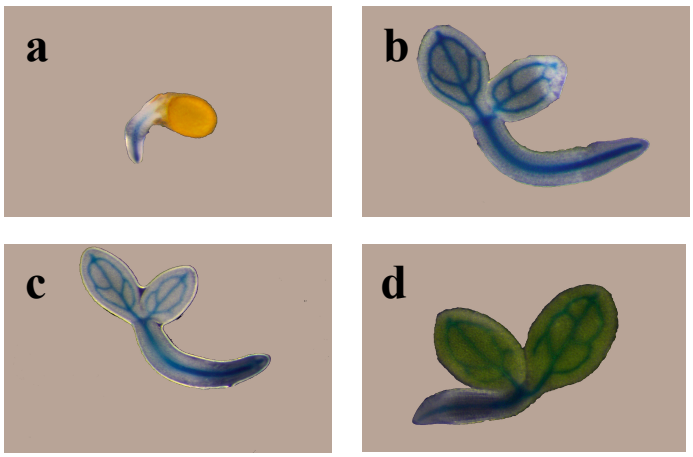


Fig. 35. Expression of *pDAG1::GUS* during the principal steps of re-establishment of DT.

Histochemical analysis of *pDAG1::GUS* seeds and seedlings. Activity of *pDAG1::GUS* (a) at stage III, (b) 3 days in a PEG solution, (c) 24 hours under the hood and (d) 2 days after imbibition.

4.2.1 Effect of simultaneous inactivation of *DAG1* and *WRKY6/18* on the re-establishment of DT

ABA plays a pivotal role in the Desiccation Tolerance process, as ABA signalling mutants, like ABA INSENSITIVE 4, 5 (ABI4, 5), are more tolerant to dehydration after germination; they germinate even after dehydration, whereas the wild type is no longer able to germinate under the same conditions. Considering the close relationship between WRKY6 and 18 with the ABA signaling pathways, we wondered if inactivation of *wrky6/18* could re-

establish the capacity to germinate after dehydration. Our analysis revealed that *wrky6* seeds were less tolerant to dehydration respect to the wild type; consistently, the *wrky6dag1* double mutant showed a more severe phenotype, as *wrky6dag1* seeds were more sensitive to the treatment than the *wrky6* single mutant seeds (Fig. 36). Conversely, *wrky18* mutant recovered the capacity to germinate like wild type seeds; a similar phenotype was displayed by the double mutants, since there was no significant difference in the percentage of germinated seed respect to the control, suggesting that lack of WRKY18 in a dag1 mutant background can restore to ability to germinate after dehydration (Fig. 37).

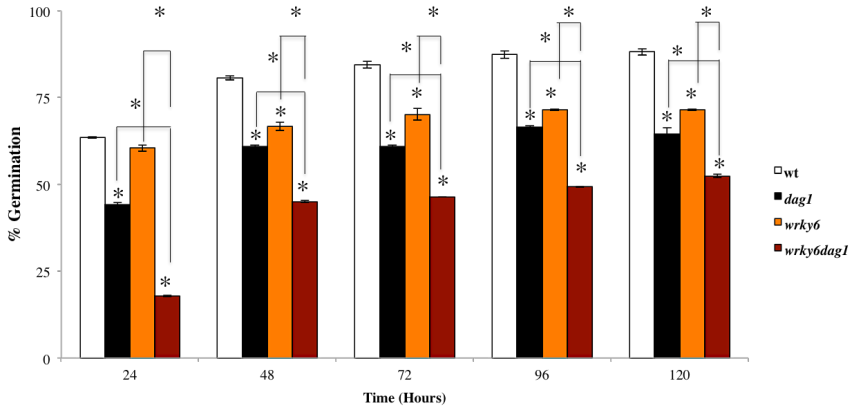


Fig. 36. Re-establishment of DT in *wrky6* and *wrky6dag1* mutant seeds.
Germination rate after three days of PEG-treatment. The values are the mean of three independent biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05).

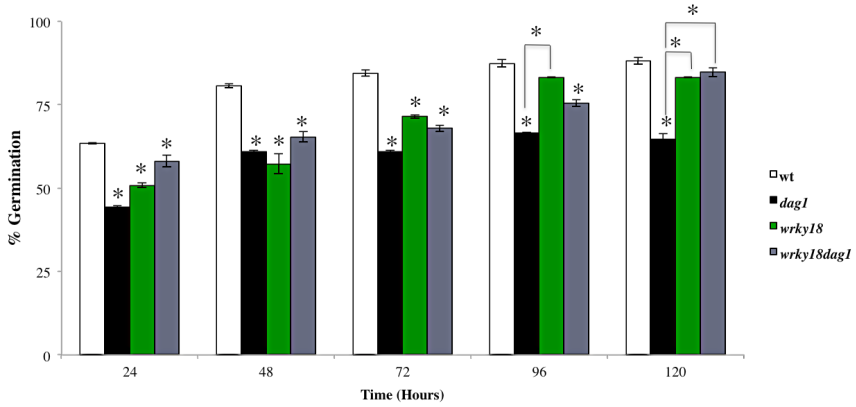


Fig. 37. Re-establishment of DT in *wrky18* and *wrky18dag1* mutant seeds.
Germination rate after three days of PEG-treatment. The values are the mean of three independent biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05).

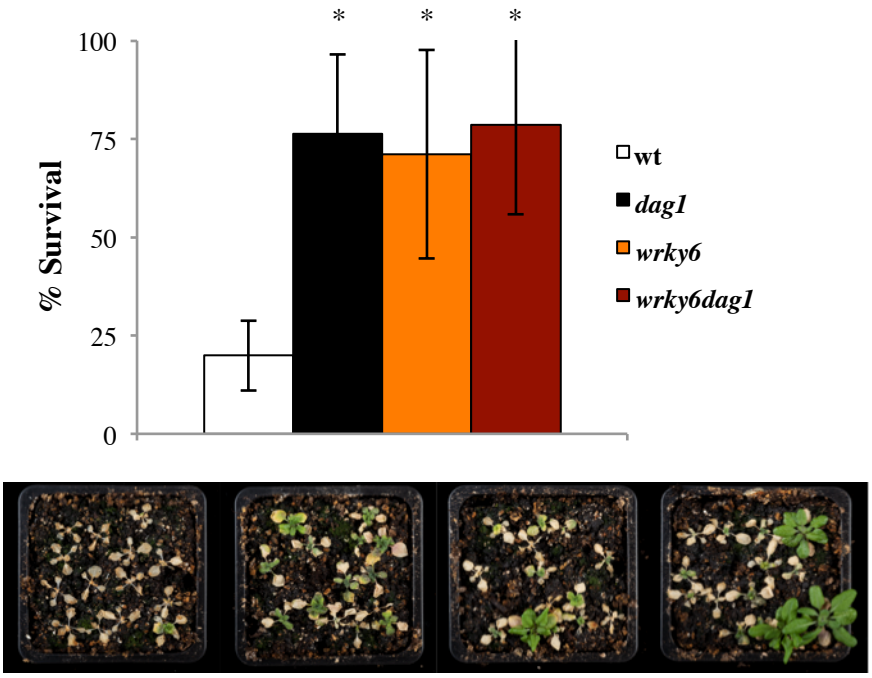
4.3 Cold response

To investigate the response of DAG1, WRKY6 and 18 in the response to cold, drought and salt we have collaborated with Prof. Julio Salinas, who has long experience in this field. These experiments have been performed in his laboratories, taking advantages of the facilities, which are available in those laboratories.

4.3.1 Cold response: phenotypes

To investigate the ability to cold acclimate of the *dag1, wrky6* and *18* mutants, we performed cold acclimation (CA) and non-acclimation (NA) experiments on 14 days-old plants both in *vitro* (plates) and in pots. Usually plants are not tolerant to freezing, however acclimation (exposure of seedlings to 4°C for 7 days) makes seedlings more tolerant to freezing. Double mutant and parental seedlings were transferred at 4°C for 7 days to acclimate (CA Cold-Acclimated condition). As a control the same lines were directly transferred to freezing temperature (NA Non-Acclimated condition). Subsequently, the seedlings were exposed to a freezing temperature (-6°C for NA and -10°C for CA); then the survival rate was measured after 10 days of recovery under control conditions. Interestingly, acclimated *dag1, wrky6* and *wrky6dag1*

mutant seedlings were significantly more tolerant to freezing temperature than the wild type; on the contrary, the same mutants, under non-acclimated conditions, did not show significant differences respect to the wild type (Figs. 38-39).



Wt ***dag1*** ***wrky6*** ***wky6dag1***

Fig. 38. *dag1* and *wrky6* seedlings are more tolerant to acclimated freezing. Top: Double mutant and parental 14 days-old acclimated seedlings were transferred at -10°C for 6 hours. The survival percentage was measured after ten days. The values are the mean of three independent biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05).

Bottom: Representative cold-acclimated plants 7 d after being exposed to -10°C for 6 h.

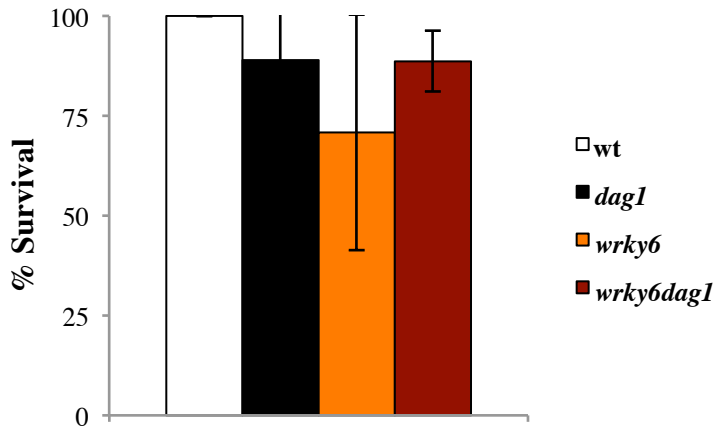


Fig. 39. *dag1* and *wrky6* seedlings do not show increased tolerance to non-acclimated freezing.

Double mutant and parental 14 days-old non-acclimated seedlings were transferred at -6°C for 6 hours. The survival percentage was measured after ten days. The values are the mean of three independent biological replicates, presented with SD values.

As for the *wrky18* and *wrky18dag1* mutant seedlings, although the single mutant was not tolerant to freezing in both conditions (CA and NA), the double mutant acclimated seedlings displayed a more tolerant phenotype, pointing out that lack of DAG1 affects the response to cold acclimated freezing (Fig. 40). On the other hand, we did not observe significant differences in the tolerance to freezing in non-acclimated seedlings (Fig. 41).

These results suggest a negative role of DAG1 in the freezing tolerance, and a possible involvement of WRKY6 in the same process, at least in our experimental conditions.

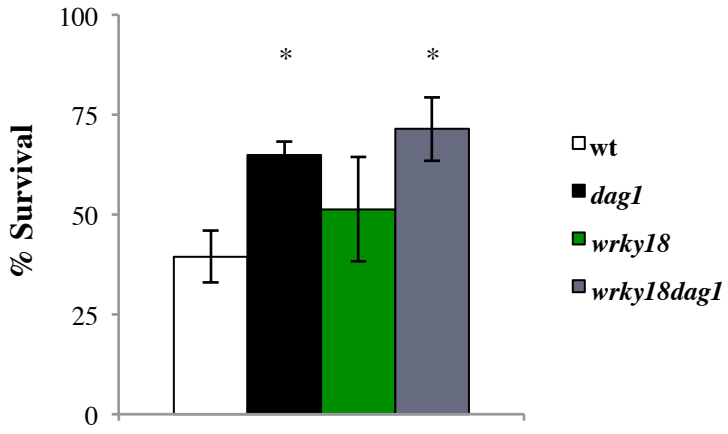


Fig. 40. *wrky18dag1* mutant seedlings are more tolerant to acclimated freezing.

Double mutant and parental 14 days-old acclimated seedlings were transferred at -10°C for 6 hours. The survival percentage was measured after ten days. The

values are the mean of three independent biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05).

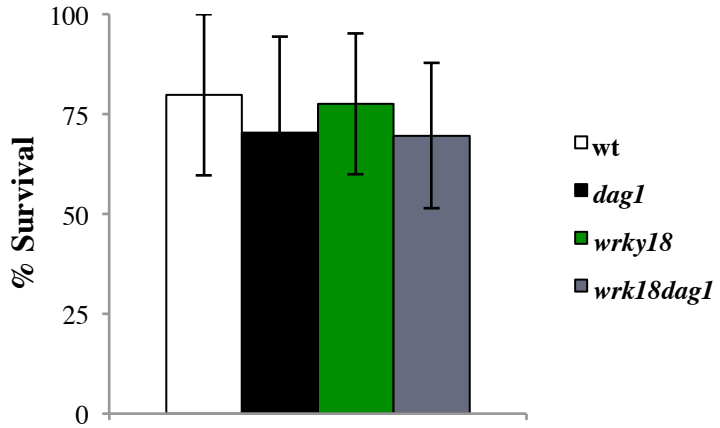


Fig. 41. *dag1* and *wrky18* seedlings do not show increased tolerance to non-acclimated freezing.

Double mutant and parental 14 days-old non-acclimated seedlings were transferred at -6°C for 6 hours. The survival percentage was measured after ten days. The values are the mean of three independent biological replicates, presented with SD values.

Similar results were obtained also with freezing tolerance assays performed in plates (Fig. 42-43).

Fig. 42. *DAG1* inactivation *results* in increased freezing tolerance *in vitro*.
14 days-old seedlings were acclimated at 4°C for four days before freezing at -10 °C for six hours. Wild type on the left, *dag1* mutant seedlings on the right.

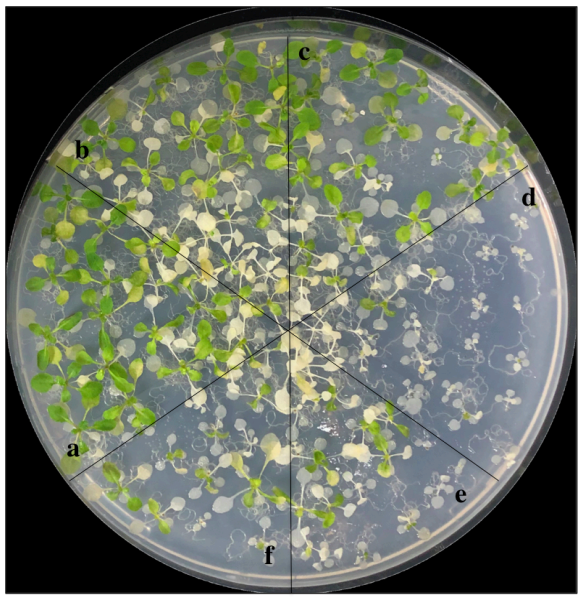


Fig. 43. Acclimated *dag1* and *wrky6* showed increased freezing tolerance *in vitro*.

14 days-old seedlings were acclimated at 4°C for four days before freezing at -10 °C for six hours. (a) *dag1*, (b) *wrky6dag1*, (c) *wrky6*, (d) wild type, (e) *wrky18*, (f) *wrky18dag1*.

4.3.2 Cold response: whole seedling expression analysis

To investigate if the expression of *DAG1* and *WRKYs* is modulated after cold exposure, we performed a RT-qPCR analysis to measure the expression levels. The expression of *DAG1* and *WRKY6* and *18* was analysed in wild type 11 days-old seedlings transferred at 4°C for 3, 8 and 24 hours. *DAG1* expression level was sharply increased after 8 hours of cold treatment and was still induced after 24 hours (3.5 fold) respect to the control condition (Fig.44). A very similar induction after 8 and 24 hours was observed for *WRKY6* expression level (Fig. 45). The *WRKY18* gene, although the *wrky18* mutation does not affect freezing tolerance, showed an increased expression; indeed, *WRKY18* transcript level was 3-fold higher than the control even after 3 hours treatment, and showed a continuous increased expression up to 24 hours (6.5- and 14.5-fold after 8 and 24 hours, respectively) (Fig. 46).

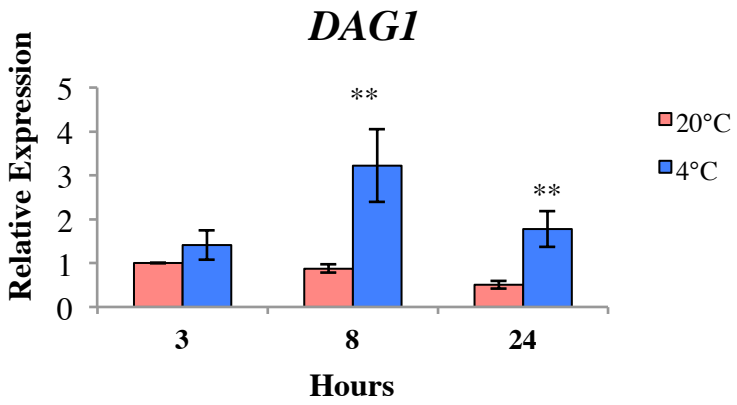


Fig. 44. *DAG1* transcript level is increased by cold treatment.

The relative expression of *DAG1* was measured in 11 days-old seedlings treated at 4°C for 3, 8 and 24 hours. The values are the mean of three independent biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05).

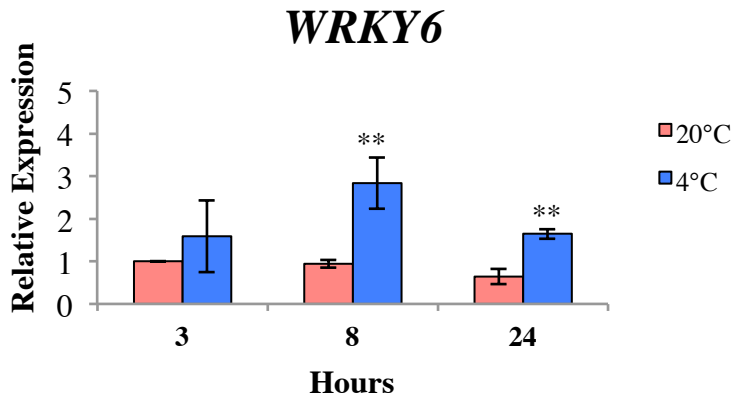


Fig. 45. *WRKY6* transcript level is increased by cold treatment.

The relative expression of *WRKY6* was measured in 11 days-old seedlings treated at 4°C for 3, 8 and 24 hours. The values are the mean of three independent biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05).

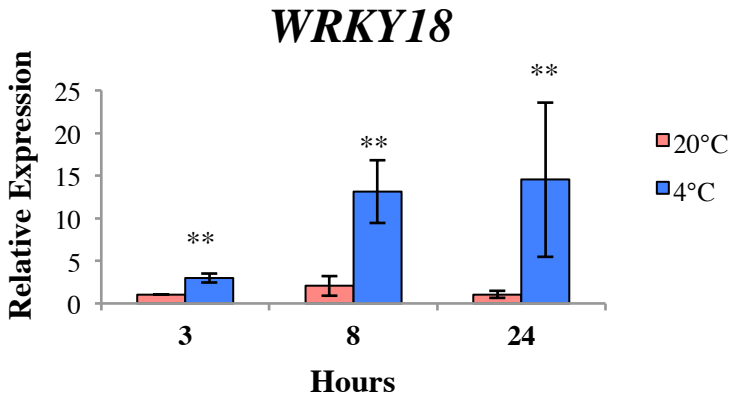


Fig. 46. *WRKY18* transcript level is increased by cold treatment.

The relative expression of *WRKY18* was measured in 11 days-old seedlings treated at 4°C for 3, 8 and 24 hours. The values are the mean of three independent biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05).

4.3.3 Cold response: tissue-specific expression analysis

An *in silico* analysis through the eFP Browser database (bar.toronto.ca) confirmed that *DAG1* expression level increased in response to low temperature; in particular this analysis revealed an increase of *DAG1* transcript levels localised in roots following cold treatment. To substantiate these data we performed a RT-qPCR analysis of 11 days-old cotyledons and roots from cold-treated seedlings. *DAG1* transcript level was significantly increased in roots after 3 and 8 hours cold treatment (Fig. 47). This kinetics was consistent with the expression results on the whole seedlings which showed an increase of *DAG1* expression at 8 and 24 hours of cold treatment, suggesting a root-specific role of DAG1 in response to cold stress. Conversely *DAG1* expression was not increased in cotyledons, thus corroborating the *in silico* analysis (Fig. 48).

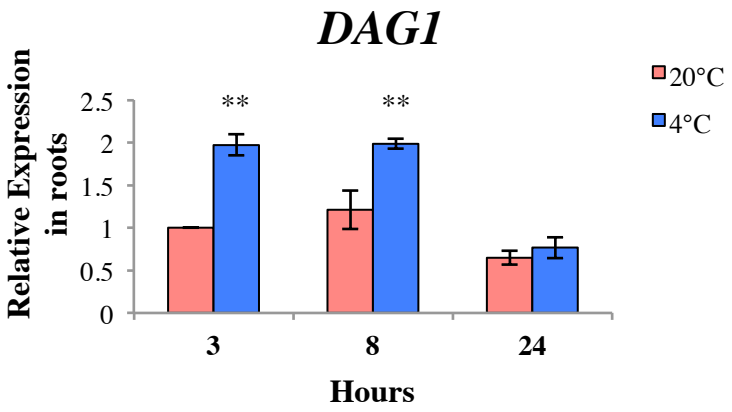


Fig. 47. *DAG1* transcript level is increased by cold treatment in roots.
DAG1 relative expression was measured in roots of 11 days-old wild type seedlings treated at 4°C for 3, 8 and 24 hours. The values are the mean of three independent biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05).

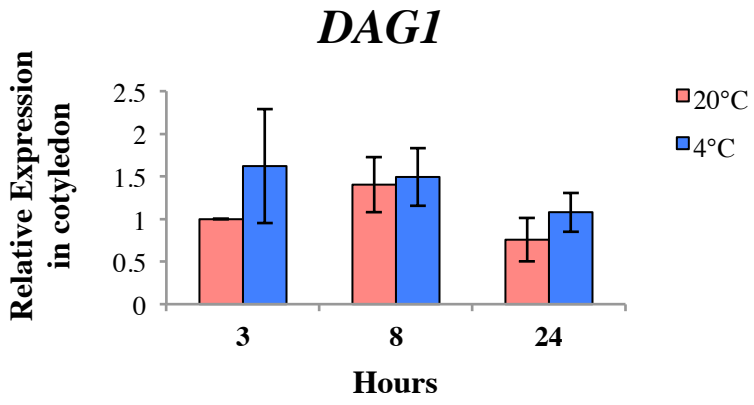


Fig. 48. *DAG1* transcript level is not increased by cold treatment in cotyledons.

DAG1 relative expression was measured in cotyledons of 11 days-old wild type seedlings treated at 4°C for 3, 8 and 24 hours. The values are the mean of three independent biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05).

4.3.4 Cold response: DAG1 targets in roots

Since DAG1 directly controls expression of *WRKY18* and *SAUR67* genes [152], we wondered whether DAG1 might regulate these genes also in the response to cold stress. Therefore, we performed an expression analysis of *WRKY18* and *SAUR67* in *dag1* mutant roots following treatment at 4°C for 3, 8 and 24 hours. Interestingly, expression of the *WRKY18* gene was significantly increased in *dag1* mutant roots after 8 hours of cold treatment (Fig. 49).

Expression of the *SAUR67* gene decreased in *dag1* roots even in normal conditions (20°C), In addition, *SAUR67* was early induced in wild type roots under cold treatment (4-fold), whereas in *dag1* roots the expression level did not increase, clearly indicating that DAG1 is necessary to induce *SAUR67* expression in response to cold treatment (Fig. 50).

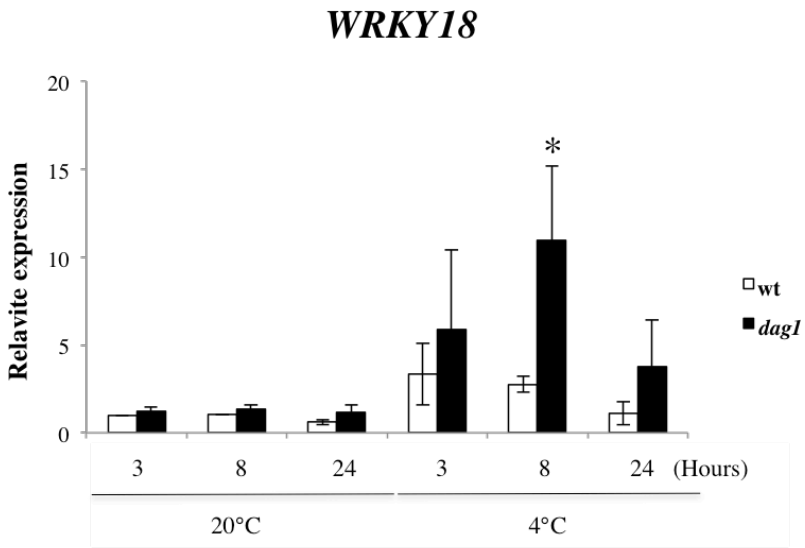


Fig. 49. DAG1 negatively controls expression of *WRKY18* under cold treatment in roots.

The relative expression of *WRKY18* was measured in roots of 11 days-old wild type and *dag1* seedlings treated at 4°C for 3, 8 and 24 hours. The values are the mean of two independent biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05).

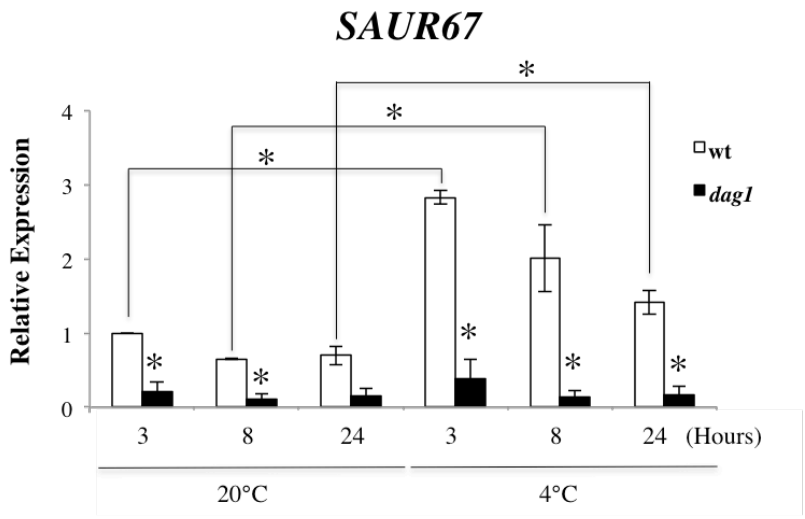


Fig. 50. DAG1 positively controls expression of *SAUR67* under cold treatment in roots.

The relative expression of *SAUR67* was measured in roots of 11 days-old wild type and *dag1* seedlings treated at 4°C for 3, 8 and 24 hours. The values are the mean of two independent biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05).

4.3.5 Cold response: *DAG1* promoter activity (*pDAG1::GUS*) and DAG1 stability

To investigate *DAG1* promoter activity in response to low temperature, we used the transgenic line *pDAG1::GUS* for histochemical analyses. This analysis was performed on 14 days-

old seedlings, treated at 4°C for 3, 8 and 24 hours. The histochemical qualitative assay revealed that the *DAG1* promoter is induced after 8 hours under low temperature, thus corroborating the expression analysis. In addition, it revealed that GUS staining was not confined in roots, but was present in the vascular tissue of the whole seedlings (Fig. 51).

Fig. 51. The *DAG1* promoter is induced by cold treatment.
Histochemical analysis of *pDAG1::GUS* 14 days-old seedlings, following treatment at 4°C for 8 hours. Activity of *pDAG1::GUS* at 20°C (left) and at 4°C (right).

It was previously shown that the DAG1 protein in seeds is controlled by GA, through the proteasome 26S [147]. Therefore,

by using the functional transgenic line *dag1DAG1-HA* [138], we assessed whether DAG1 was regulated at post-translational level in response to cold treatment, DAG1 protein level did not show any significant difference at 4°C respect to 20°C, thus suggesting that the DAG1 protein stability is likely not to be controlled under low temperature (data not shown).

To deeply investigate this new role of DAG1 in response to cold stress, we performed a genome-wide analysis, through RNA-seq assay, by comparing the transcriptome of *dag1* cold-treated plants with untreated *dag1* plants. These samples will be compared to cold-treated and untreated wild type plants.

5. Inhibition of polycomb repressive complex 2 activity reduces trimethylation of H3K27 and affects development in *Arabidopsis* seedlings. [197]

5.1 Treatment of seeds with the RDS3434 inhibitor reduces H3K27me3 levels in *Arabidopsis* seedlings

The RDS 3434 inhibitor has been shown to be specifically active against EZH2 in human leukemia cells, where it induced heavy cell death in a dose-dependent manner [198]. To assess the efficacy of the RDS 3434 inhibitor on *Arabidopsis* seeds, we grew wild type seeds on a medium supplied with increasing concentrations of RDS 3434 (30, 60, 120 μM), or with its solvent DMSO (control), for 5 days. Immunoblot analysis of total proteins of RDS 3434- or DMSO-treated 5 days-old seedlings was performed with specific antibodies against H3K27me3. Measurement of the amount of proteins marked by H3K27me3 showed that the RDS 3434 inhibitor was effective in a dose-dependent manner: while with 30 μM RDS 3434 the slight decrease (16%) of H3K27me3 marked proteins compared to the control was not significant, at 60 and 120 μM they were reduced by, respectively, 45 and 62% (Fig. 52).

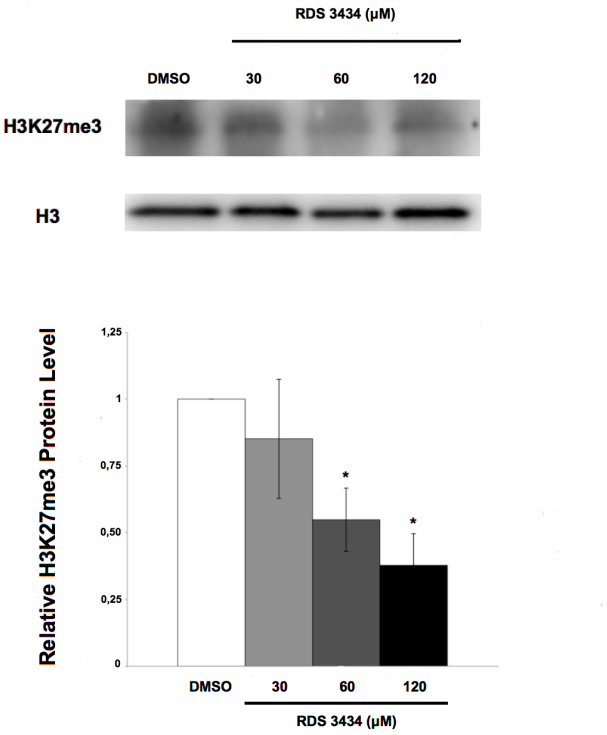


Fig. 52. Treatment with RDS 3434 results in a dose-dependent decrease of the total amount of H3K27me3 marked proteins.

Immunoblot of 5 days old wild type (Ws-4) seedlings directly grown with increasing concentrations (30, 60, 120 µM) of RDS 3434 or DMSO as control. Total proteins were probed with H3K27me3 specific antibodies, and H3 was used as loading control. Western blot (top) and densitometric analysis (bottom).

The protein levels are the mean of three biological replicates, presented with SD values. Significant differences were analyzed by t-test (*P ≤ 0.05, **P ≤ 0.01).

CLF is one of the two EZH2 enzymes that play a crucial role during *Arabidopsis* seedling development; therefore we wondered whether the addition of the inhibitor could further affect H3K27me3 levels in a *clf* mutant. An immunoblot of DMSO- and RDS 3434 (120 μM)-treated *clf-29* mutant seedlings compared to the DMSO- and RDS 3434-treated wild type (Col) was performed. This analysis revealed that treatment with the inhibitor reduced by 30% the amount of proteins marked by H3K27me3 RDS 3434-treated *clf* seedlings (Fig. 53a), thus corroborating our results. In addition, the ratio of DMSO-treated *clf-29*/WT H3K27me3 protein level confirmed the decrease of H3K27me3 level in *clf-29* compared to the wild type (Fig. 53b).

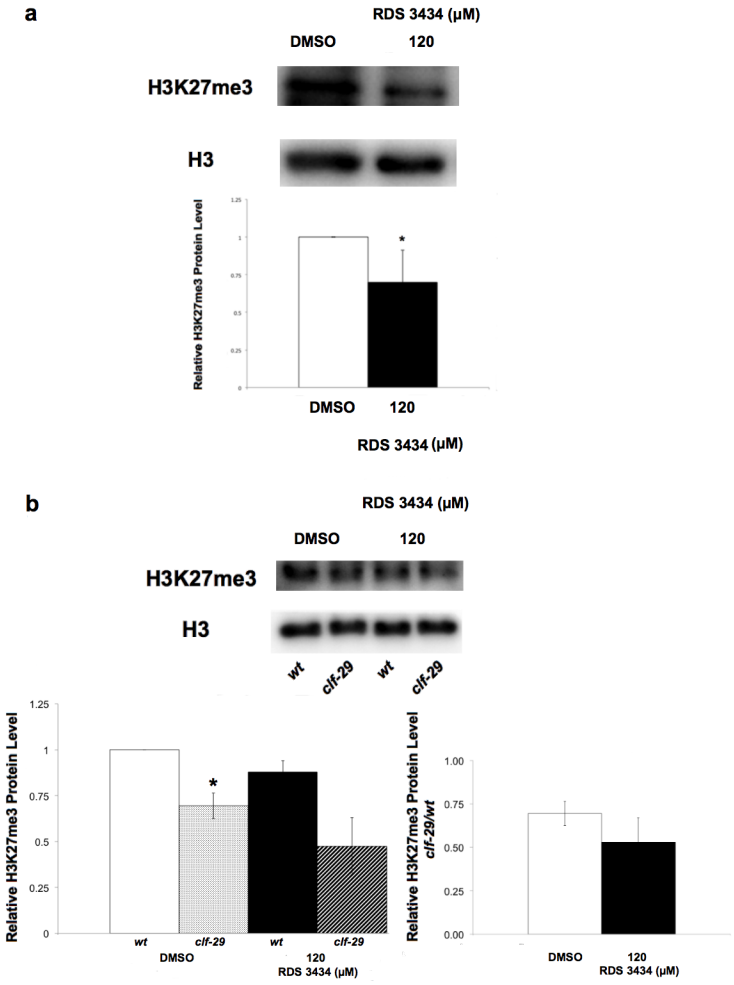


Fig. 53. Treatment with RDS 3434 reduces H3K27me3 protein level in the *curly leaf-29* mutant.

a, b Immunoblot of 5 days-old *clf-29* seedlings directly grown with RDS 3434 (120 μM) or DMSO as control (a), and of DMSO- or RDS 3434-treated wild type (Col) and *clf-29* seedlings (b). Total proteins were probed with H3K27me3 specific antibodies, and H3 was used as loading control. Western blot (top) and densitometric analysis (bottom). In (b) is shown the relative H3K27me3 protein

level (bottom left), and the ratio of DMSO- and 120 μM RDS 3434-treated *clf-29*/WT (bottom right). Results were obtained from two independent replicates with SD values. Significant differences were analyzed by t-test (*P ≤ 0.05).

5.2 The RDS 3434 inhibitor is selectively active against the PRC2 (EZH2) complex

It is known that, in analogy with animal systems, in *Arabidopsis* the function of the PcG complex is counteracted by the Trithorax Group (TrxG) complex, which catalyzes the trimethylation of lysine 4 of histone 3 (H3K4me3) [199]. We thus verified whether treatment with RDS 3434 caused not only a reduction of the H3K27me3 repressive mark, but also an increase of the H3K4me3 activator mark. Immunoblot analysis with specific antibodies against H3K4me3 revealed that treatment with RDS 3434 produced, compared to the control, a small but significant increase in the total amount of H3K4me3 marked proteins at 30 and 120 μM inhibitor (Fig. 54a), consistent with the notion that these antagonistic marks are, in small part, mutually exclusive [200, 201]. To demonstrate that RDS 3434 inhibition was specific for the PRC2 (EZH2) complex over other methyltransferases, we performed an immunoblot analysis with antibodies against H3K36me3, an activating epigenetic mark catalyzed by the SET DOMAIN GROUP 8 (SDG8) [202]. This analysis showed that the

H3K36me3 total protein level was not significantly affected even at the highest concentration of RDS 3434 (Fig. 54b), thus suggesting that this inhibitor functions only on the EZH2 metyltransferase.

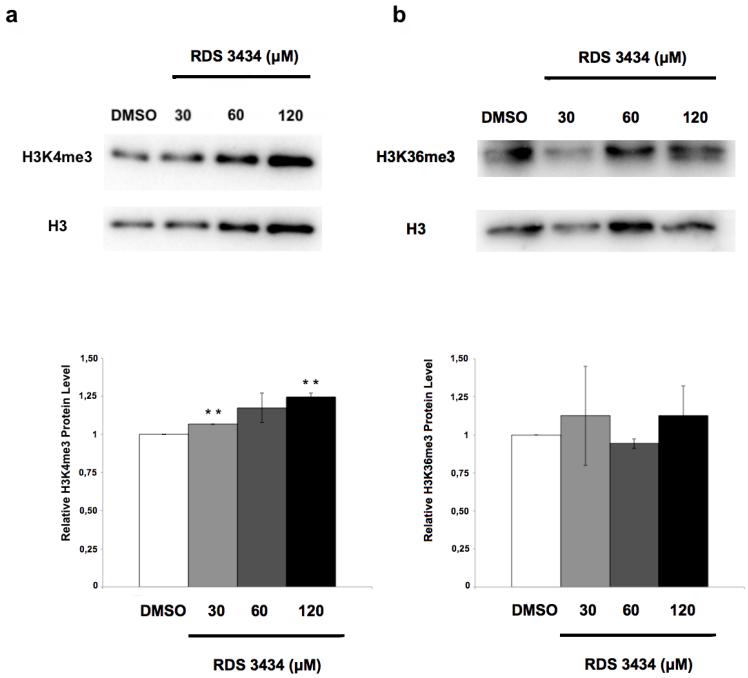


Fig. 54. Reduction of the H3K27me3 mark causes an increase of the antagonistic mark H3K4me3.

a, b Immunoblot of 5 days-old wild type (Ws-4) seedlings directly grown for 5 days in the presence of increasing concentrations (30, 60, 120 µM) of RDS 3434 or DMSO as control. Total proteins were probed with: H3K4me3 (a) or H3K36me3 (b) specific antibodies. H3 was used as loading control. Western

blot (top) and densitometric analysis (bottom). The protein levels are the mean of two biological replicates, presented with SD values. Significant differences were analyzed by t-test (*P ≤ 0.05, **P ≤ 0.01).

5.3 Treatment with RDS 3434 increases the expression level of PRC2 target genes

Since PRC2 is a transcriptional repression system, inhibition of EZH2 and consequent decrease of H3K27me3 levels should result in the transcriptional derepression of PRC2 target genes. Thus, we assessed whether treatment with the RDS 3434 inhibitor would actually affect the expression of two independent *Arabidopsis* PRC2 target genes: DOF AFFECTING GERMINATION 1 (DAG1), and WRKY70, respectively encoding a Dof and a WRKY transcription factor (TF). DAG1 encodes a negative regulator of seed germination [138, 144, 203], which is marked by H3K27me3 in seeds and seedlings [147] and had been shown to be upregulated in mutant plants lacking PRC2 [150]. WRKY70 encodes a TF involved in the cross-talk between salicylic acid- and jasmonic acid-dependent defense signaling, and has been reported to be a target of both PRC2 and Trithorax (Trx) [204, 205]. We performed an expression analysis (RT-qPCR) on RNA extracted from RDS 3434-treated (30, 60,120 μM) and DMSO-treated 5 days-old seedlings. As shown in Fig. 55a-b, the expression level of both

DAG1 and WRKY70 significantly increased upon treatment with RDS 3434 in a dose-dependent fashion - respectively 1.9- and 2.3-fold the level of the control for DAG1 (60 and 120 μM RDS 3434), and 4.2-, 5.8- and 12.1-fold for WRKY70 (30, 60,120 μM RDS 3434). Under the same experimental conditions, the expression level of SMALL AUXIN UP RNA 14 (SAUR14), which is not a PRC2 target gene [206], was not affected by treatment with RDS 3434, thus confirming the efficacy of this inhibitor only for PRC2 (Fig. 55c). We then assessed whether treatment with the inhibitor would actually result in loss of the H3K27me3 repressive mark in the PRC2 target loci DAG1 and WRKY70. To this end, we performed chromatin immunoprecipitation (ChIP) assays with H3K27me3-specific antibodies, or without antibodies as negative control, on samples derived from RDS 3434 (120 μM)-treated and DMSO-treated 5 days-old seedlings. We measured the enrichment of H3K27me3 by amplification, through quantitative (qPCR), of one region in the body of both DAG1 and WRKY70 genes, because the H3K27me3 epigenetic mark is usually restricted to the transcribed regions of target genes [207, 208]. Interestingly, in samples derived from RDS 3434 (120 μM)-treated seedlings, the level of H3K27me3 in the DAG1 and WRKY70 genes was significantly decreased (Fig. 55 d-e), consistently with their increased expression levels.

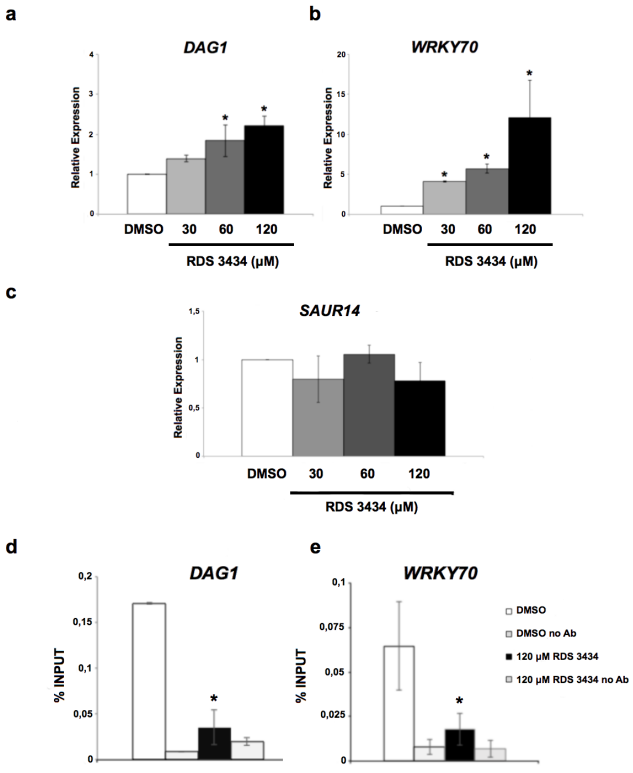


Fig. 55. Inhibition of EZH2 results in an increased expression of two PRC2 target genes.

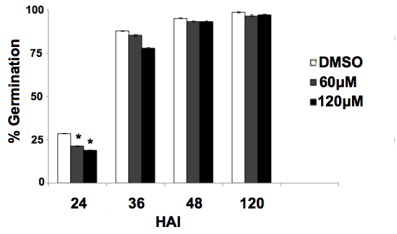
a, c Relative expression level of the PRC2 target genes, *DAG1* (a) and *WRKY70* (b), and of the non-target gene *SAUR14* (c), in wild type (Ws-4) seedlings directly grown for 5 days in the presence of increasing concentrations (30, 60, 120 μM) of RDS 3434 or DMSO as control. Relative expression levels were normalized with the *GAPDHa* (At3g26650) gene, and are presented by the ratio

of the corresponding mRNA level in the control, which was set to 1. d, e Chromatin from samples derived from 5 days-old seedlings grown in the presence of 120 μM RDS 3434 or DMSO as control, immunoprecipitated with antiH3K27me3 antibodies, or without antibodies as a negative control. The amount of DNA for *DAG1* (d) or *WRKY70* (e) was measured by qPCR. The values of fold enrichment were normalized to input. All the primers used are listed in Table 1. The results were obtained from two independent replicates with SD values. Significant differences were analyzed by t-test (*P ≤ 0.05, **P ≤ 0.01).

5.4 Reduction of the H3K27me3 mark affects seed germination and root development

The transcriptional control mediated by PRC2 is crucial during seed germination, as it silences seed specific genes thus allowing proper seedling growth and development [150, 199]. Therefore, we assessed whether treatment with the inhibitor RDS 3434 would affect germination of seeds. As shown in Fig. 56 a, treatment with the inhibitor caused a significant reduction of the germination rate at 24 h after imbibition (HAI) - 26.5 and 34.3%, at 60 and 120 μM RDS 3434, respectively. On the other hand, seedling growth of treated and untreated samples was very similar (Fig. 56 b), ruling out the possibility that the observed reduction of H3K27me3 upon treatment with RDS 3434, may pleiotropically affect seedling growth and development.

a



b

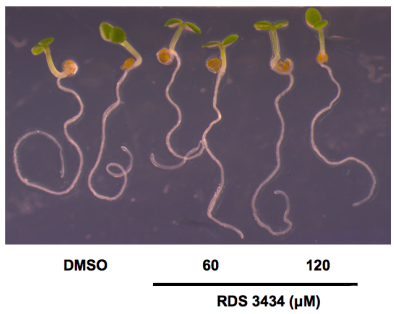


Fig. 56. Inhibition of EZH2 results in delayed seed germination.

a. Seed germination assays of wild type (Ws-4) seeds imbibed in the presence of RDS 3434 (60, 120 µM) or DMSO as control. Germination rate was scored at 24, 36, 48 and 120 HAI (Hours After Imbibition). Data represent the mean of two independent biological replicates each performed in duplicate (25 seeds per replica). Significant differences were analyzed by t-test (*P ≤ 0.05, **P ≤ 0.01).

b. 5 days-old wild type (Ws-4) seedlings directly grown for 5 days in the presence of increasing concentrations (60, 120) of RDS 3434 or DMSO as control.

Similarly, 5 days-old seedlings of *clf* or *swn* single mutants do not show severe developmental defects, since only *clf* adult plants are characterized by dwarfism and early flowering, whereas *swn* mutants display very weak phenotypes [209, 210]. Since treatment with RDS 3434 further reduces H3K27me3 level in *clf-29* seedlings, we assessed whether seed germination of mutant seeds would be affected by treatment with RDS 3434 (120 μM). Interestingly, *clf-29* mutant seeds treated with the inhibitor showed a 50% reduction of the germination rate at 24 HAI (Fig. 57). However, treatment with the inhibitor did not result in more severe phenotypes during seedling development (Fig. 57b). It has been proposed that PRC2 controls primary root growth, since lack of the EZ catalytic subunits CLF and SWN results in short meristem and decreased primary root length [211].

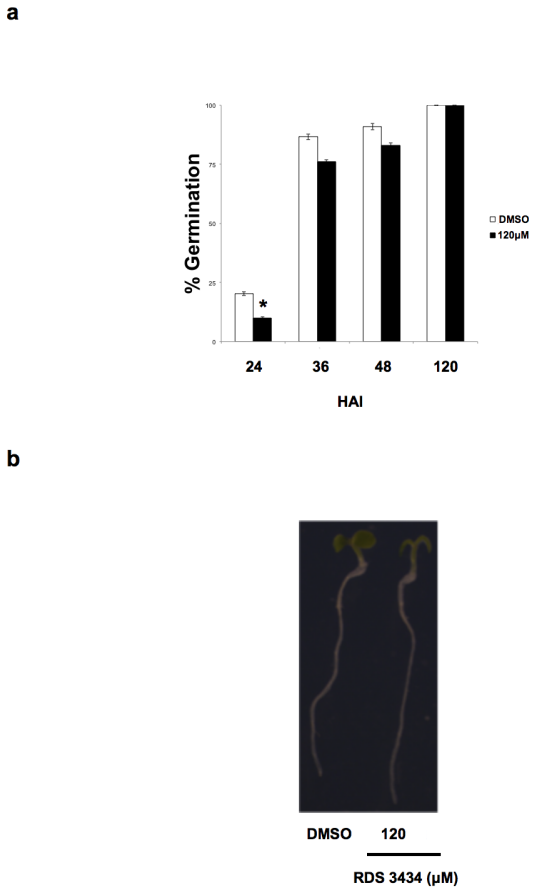


Fig. 57. Treatment with RDS3434 of *clf-29* mutant seeds affects seed germination.

a. Seed germination assays of *clf-29* mutant seeds imbibed in the presence of RDS 3434 (120 µM) or DMSO as control. Germination rate was scored at 24, 36, 48 and 120 HAI (Hours After Imbibition). Data represent the mean of two independent biological replicates each performed in duplicate (25 seeds per replica). Significant differences were analyzed by t-test (*P ≤ 0.05, **P ≤ 0.01).

b. 5 days-old *clf-29* mutant seedlings directly grown for 5 days in the presence of RDS 3434 (120 μM) or DMSO as control.

Therefore, we assessed whether treatment with the RDS 3434 inhibitor results in root developmental defects. Although treatment with 60 and 120 μM RDS 3434 inhibitor did not affect root growth (data not shown), a higher dose (240 μM) resulted in a reduced number of root meristematic cells. Consistently, the expression domain of ROOT CLAVATA HOMOLOG1 (RCH1), a gene specifically marking the root meristematic zone [212], is reduced in RDS 3434-treated plants compared to the control, as visualized by a RCH1- GFP transcriptional fusion (Fig. 58 a-b). A decrease in meristem size can be caused by a reduced division rate or by a more rapid elongation/differentiation (i.e. exit from the meristem) of meristematic cells. To distinguish between these two possibilities, we first visualized root meristem cells in the G2–M phase in RDS 3434-treated and untreated plants harboring the D-Box CYCB::GUS construct, a marker of the G2-M transition [213]: no difference in the cell division potential could be detected (Fig. 58 c). To detect possible variation in cell elongation/differentiation, we measured the length of the first

elongated and of the fully differentiated cells in both treated and untreated plants. Whereas the length of fully elongated cells was unvaried, the first elongated cells were longer in RDS 3434-treated plants (Fig. 58 d-e), indicating that RDS 3434 affects root meristem activities controlling the elongation/differentiation potential.

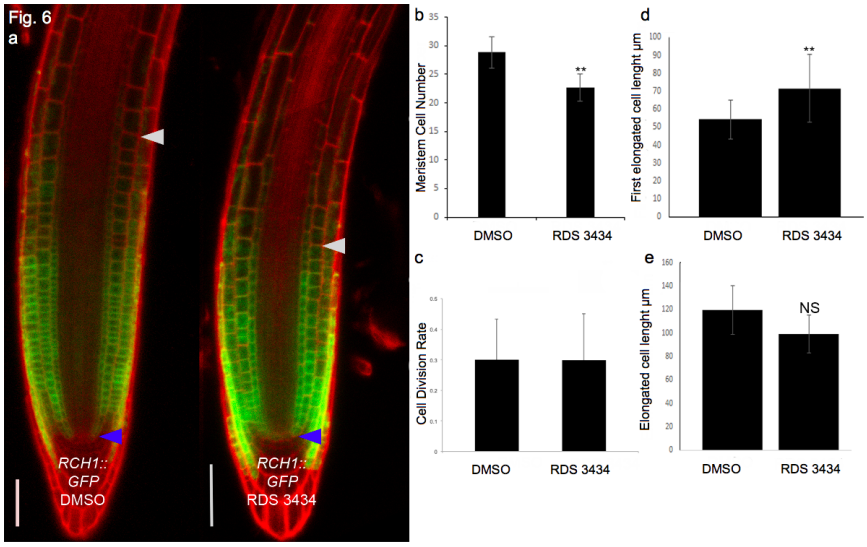


Fig 58. Inhibition of EZH2 affects root development.

a. Confocal microscopy images of RCH1::GFP roots from 5 days-old seedlings grown in the presence of RDS 3434 (240 µM) or DMSO as control. Blue and white arrowheads indicate the Quiescent Center (QC) and the cortex Transition Boundary (TB), respectively. b. Root meristem cell number. c. Quantification of

GUS spots per meristem in treated and untreated of *CYCLINB1;1pro:CDB-GUS* roots. d, e. Length of the first elongated cell (d), and of the differentiated cell (e) (n = 30). Data represent the mean of two independent biological replicates, presented with SD values. Significant differences were analyzed by t-test (*P ≤ 0.05, **P ≤ 0.01).

DISCUSSION

DOF affecting germination 1 (DAG1) is a plant-specific transcription factor that, during the seed-to-seedling transition, controls the hormonal balance between the phytohormones GAs and ABA, to induce dormancy and repress germination of seeds [147]. DAG1 acts through direct repression of the GA biosynthetic gene *GA3ox1* and the AB*A* catabolic gene *CYP707A2* [138, 144, 147].

DAG1 plays a role also in the control of hypocotyl elongation: lack of DAG1 results in shorter hypocotyls compared to the wild type. Recently, through a genome-wide approach, we proved that DAG1 promotes hypocotyl elongation acting on the ABA, ethylene and auxin signaling pathways [152]. Indeed, our analysis reveals that the relative expression of a number of *SMALL AUXIN RESPONSIVE (SAUR)* genes, known as positive regulators of hypocotyl elongation [153], decreases in *dag1* mutant hypocotyls, coherently with the positive role of DAG1 in hypocotyl elongation. It is known that ABA, a growth-inhibiting hormone, represses hypocotyl elongation by acting on gibberellin and auxin pathways [115]. Consistently, DAG1 acts through a negative control of ABA signaling: in *dag1* hypocotyls the expression of genes involved in this pathway is increased [152].

Remarkably, we have also identified seven WRKY-encoding genes which are up-regulated in *dag1* hypocotyls. Our findings show that DAG1 represses the expression of these *WRKY* genes in hypocotyls independently of light conditions. The WRKY are plant specific transcription factors known for their pivotal role in biotic stress response, and recently their role in abiotic stress response is also emerging in literature [154, 163, 166]; among the seven WRKY encoding genes identified, we have focused our attention on WRKY6 and WRKY18 for their strong correlation with ABA-signaling and ABA-mediated processes [174, 175].

Since ABA is the main phytohormone involved in plant response to abiotic stress, we decided to further investigate a potential involvement of DAG1 in ABA-mediated processes and in the molecular mechanisms of stress response. In addition, we have investigated the relations between DAG1, WRKY6 and 18 in these processes and molecular pathways.

DAG1 and WRKY6/18 cooperate to control light-dependent hypocotyl growth

Our recent studies revealed that DAG1 promotes hypocotyl growth regardless of light conditions; indeed *dag1* hypocotyl length is reduced compared to the wild type in Red light as well as in the dark.

The results presented here on WRKY6, which is known as a positive regulator of ABA-signaling during seed germination and early seedling development [175], suggest a new putative role of WRKY6 in hypocotyl elongation, as the absence of this protein results in longer hypocotyls, regardless of light conditions. *Wrky6dag1* seedlings grown under red light, display *wrky6*-like hypocotyls, longer than the wild type. Conversely, in the absence of light, *wrky6dag1* hypocotyls are shorter than the wild type, and similar to *dag1*, thus suggesting that WRKY6 activity might be controlled by a light-dependent upstream factor.

Interestingly, it has been recently shown that WRKY36 is also involved in hypocotyl elongation [214]. WRKY36 is likely to promote hypocotyl elongation by directly repressing the expression of *HY5*, encoding a key transcription factor, which positively controls photomorfogenesis [215]. UVB light, a photomorphogenic signal which controls photomorphogenesis, triggers the nuclear localization of UV RESISTANT LOCUS 8 (UVR8), which, once in the nucleus, interacts with WRKY36 to prevent it from binding DNA, thus releasing WKY36 repression on *HY5* promoter. As a result, UVB promotes *HY5* transcription, therefore inhibiting hypocotyl elongation [214].

As for the relationship between DAG1 and WRKY18, although we have proved that DAG1 directly represses *WRKY18* expression, we cannot draw a conclusion on the epistatic relationship between

DAG1 and WRKY18. It is noteworthy to point up that WRKY18 interacts with the two closely related WRKY40 and WRKY60 proteins, to alternatively cooperate or play antagonistic roles in the control of the *ABA INSENSITIVE 4* and *5* genes, encoding two main positive regulators of ABA signaling [92]. The WRKY60 encoding genes is not among the Differentially Expressed Genes in *dag1* hypocotyls, therefore DAG1 is likely to act directly on the *WRKY18* gene, and possibly on the *WRKY40* ([152]; this thesis). This could imply that the action of DAG1 on *WRKY18* is partially masked by the effect of at least the WRKY60 protein. More phenotypic and molecular analysis on multiple mutants, lacking also WRKY 40 and WRKY60, will be necessary to unveil the molecular mechanism underlying their function on hypocotyl elongation.

Consistent with previous results on *dag1* hypocotyl response to the growth inhibitory effect of ABA [115], our results revealed a reduced sensitivity to ABA treatment for hypocotyl growth of the *wrky6dag1* and *wrky18dag1* double mutants.

Similarly to DAG1, the Dof CDF5 protein has been recently demonstrated to promote hypocotyl growth. CDF5 is the target of both the PSEUDO-RESPONSE REGULATORS 9/7/5 (PRR9/7/5) and PIF 3/4/5 proteins, which antagonistically regulate the promoter of *CDF5* [216]. The PRR repressors, which have a key role in the regulation of the clock-mediated processes [217], are

also negative regulators of hypocotyl elongation [218]; indeed PRR proteins target growth-related genes, which are also directly induced by the PIF proteins. CDF5, once induced by the PIF factors, promotes expression of a number of growth-promoting genes.

In the dark, *DAG1* expression is induced by PIF1 in seeds, therefore it will be interesting to further analyse the relation between DAG1 and CDF5, and a putative control of DAG1 through the circadian clock.

DAG1 is more sensitive to Desiccation Tolerance

The effects of DT are confined in a narrow temporal window delimited by the radical protrusion; at the appearance of the first root hairs, the seeds become sensitive to dehydration and lose the ability to re-establish DT [109, 110]. To study this process, we set up a protocol to measure the capacity of the seeds to re-induce germination after a mild osmotic stress through PEG treatment. DAG1 induces dormancy by controlling the ABA/GA ratio, and given the close relationship between dormancy and DT, we investigated the effect of DT on *dag1*. Our results clearly establish that the *dag1* mutants are significantly less tolerant to desiccation, and these finding are consistent with the lower ABA levels in *dag1* dry seeds compared to the wild type [147]. The analysis of *wrky6* reveals that lack of WRKY6 protein results in a significantly lower

capacity to re-induce germination after dehydration in germinated seeds, and the simultaneous absence of DAG1 and WRKY6 proteins in the double mutant results in an additive phenotype compared to the single mutants phenotype, thus suggesting that DAG1 and WRKY6 possibly act in parallel branches of the molecular pathway controlling this process. Conversely, *wrky18* mutant recovers the capacity to germinate, although mutant seeds germinate slower than the wild type control; a similar phenotype is displayed by the double mutant, which, after 120 hours, do not show significant difference in the percentage of germinated seed respect to the control, that lack of WRKY18 in a *dag1* mutant background can restore to ability to germinate after dehydration.

DAG1 is a new element of the molecular pathway of the Cold Acclimation response

Plants have to frequently cope with changing environmental conditions. ABA also plays a pivotal role in the stress response by triggering mayor changes in gene expression and adaptive physiological responses. Among the ABA-related stresses that were studied in this thesis project, the most notable is the cold response. Low temperature is one of the most relevant factors limiting the distribution of plant species [1, 39], and as such, cold acclimation plays a major role in plant survival [1]. The principal actors in the freezing tolerance are the C-repeat Binding Factors1-3

(CBF1-3) proteins, which act as positive regulators of cold acclimation; the *CBF* genes are rapidly and transiently induced after exposure to cold[41, 47, 55].

Interestingly, our results prove that *dag1* mutant plants are significantly more tolerant to freezing temperatures respect to the wild type. This phenotype is dependent on the cold acclimation treatment, because non-acclimated *dag1* mutant plants are sensitive to freezing as the wild type, thus corroborating the specificity of the putative involvement of DAG1 in this process. Consistently, the steady-state level of *DAG1* messenger is increased by low temperature, and this increase of *DAG1* transcript level is likely to be mainly in roots, although not strictly root-specific.

Interestingly, the sub-family of CYCLING DOF FACTOR (CDF1-5) transcriptional repressors, has been involved in freezing tolerance, downstream of the GIGANTEA (GI) protein, which plays a crucial role in a number of diverse signaling pathways, besides clock regulation, like light signaling and stress responses. Although *cdf* mutants do not show a phenotype following cold treatment, it was shown that the increased stability of CDF proteins in a *gi* mutant background, led to increased tolerance to cold stress [219].

Besides other hormones, namely ABA, Ethylene and GAs, recently it has been shown of BRs in the regulation of cold tolerance. In particular, it has been proposed that the BZR1 transcription factor

positively regulates the cold response in two different ways: one CBF-dependent pathway, with BZR1 directly binding the *CBF* promoters, and the second CBF-independent. Interestingly, it was suggested that BZR1 could modulate the expression of *WRKY6,* which the Authors proposed as a positive regulator of the process [61].

Our results demonstrate that the *wrky6* mutant is more tolerant to freezing compared to the control, thus suggesting a negative role for WRKY6 in cold tolerance. This apparent discrepancy could be due to the different experimental conditions: indeed, while our plants were grown in soil and have been treated at 4°C for 1 week, Li and collaborators performed all their experiment with seedlings grown *in vitro*. It has been already shown that different experimental conditions - soil or plates - could result in different cold responses [59].

As for WRKY18, our results did not show an evident role of this protein in the response to freezing, not even under CA conditions. Nevertheless, the expression analysis of the DAG1 direct targets *WRKY18* and *SAUR67* in *dag1* mutant roots following cold treatment, revealed that *WRKY18* is induced by cold treatment and its transcript level is negatively controlled by DAG1 under this condition. Surprisingly, our results show that SAUR67 is required in the cold response, since *SAUR67* was early induced in wild type

roots under cold treatment, and DAG1 is necessary for its induction, as in *dag1* roots the expression level did not increase.
Taken together these results suggest a negative role of DAG1 in the cold tolerance, specifically in the CA pathway.
To deeply investigate this function of DAG1, we performed a genome wide analysis, through RNA-seq assay, by comparing the transcriptome of *dag1* cold-treated plants with untreated *dag1* plants. These samples will be compared to cold-treated and untreated wild type plants. The results of this analysis will allow to unveil new molecular elements of the CA pathway of the response to cold; in addition it will also elucidate the role of DAG1 in this pathway with respect to the CBF factors.

A tool to inhibit PRC2 activity and study its effect on developmental and stress-mediated responses

Epigenetic regulation of gene expression, mediated by post-translational modification of histones or DNA methylation, has a crucial role in genome defence but also in the control of developmental processes as well as in the response of organisms to environmental cues. Polycomb group proteins (PcG) are key epigenetic regulators of development; these proteins, assembled in large complexes, establish and maintain gene repression [220]. PcG proteins are grouped into two complexes: POLYCOMB REPRESSIVE COMPLEX 1 (PRC1) and 2 (PRC2).

In *Arabidopsis*, there are 12 homologs of the Drosophila PRC2 subunits and, in particular, the histone methyltransferase *EZ* is encoded by three homologs (*CURLY LEAF, MEDEA* and *SWINGER; CLF, MEA* and *SWN*), which share a highly conserved SET domain, responsible of the catalytic activity [183]. Different combinations of the four subunits result in three PRC2-like complexes: the EMBRYONIC FLOWER (EMF), VERNALIZATION (VRN) and FERTILISATION INDEPENDENT SEED (FIS), which function in different developmental processes and responses to environmental stimuli, although sharing some target genes [184, 185].

PRC2, which represses its targets by trimethylation of histone 3 lysine 27 (H3K27me3), has a key role in the control of stress response; indeed, loss of CLF results in a reduced resistance to drought [221] suggesting that different PRC2 members have distinct functions in regulating stress responses. It has been demonstrated that CLF interacts with the plant-specific coiled-coiled protein named BLISTER (BLI) [222], which promotes the resistance to cold stress [223]. Kleinmanns and collegues proposed a model in which BLI, associated with PCR2, regulates stress-responsive genes in *Arabidospsis*; indeed, it has been shown that BLI negatively controls ABA-responsive PRC2 target genes to promote resistance to cold and drought stresses [220].

PRC2 also plays a crucial role in the embryo-to-seedling [150, 224, 225] and vegetative-to-reproductive developmental phase transitions [226, 227]. PRC2 is essential for endosperm formation, since it controls the parent-of-origin specific expression of a number of genes: lack of the maternal PRC2 function results in derepression of target genes, causing endosperm over-proliferation and eventually seed abortion [187, 228]. This has severely hampered studies on the function of PRC2 during seed development. In mammals, studies on PRC2 function have benefited of the development and use of inhibitors of the catalytic subunit EZH2, among which RDS 3434 whose effectiveness has been proven on the oncogenic human monocyte cell line U937 [198]. Since the catalytic subunit EZH2 is highly conserved between mammals and plants, we tested the effectiveness of RDS 3434 in *Arabidopsis* and found that indeed it inhibits PRC2-mediated H3K27me3 methylation also in this organism.

Previous genome-wide analyses comparing the global H3K27me3 profile in *clf-28* or *clf-29* mutant seedlings, revealed a decreased level of H3K27me3 in the mutant lines compared to the wild type [229-231]. Consistently, our immunoblot analysis shows a decrease of H3K27me3 levels in *clf-29* compared to the wild type, and reveals that treatment with RDS 3434 further reduces the amount of proteins marked by H3K27me3 in the *clf-29* mutant background. It has already been previously demonstrated that the

lack of both the catalytic CLF and SWN subunits results in delayed germination: *clf-28 swn-4* double mutant seeds germinate within 4 DAI (Days After Imbibition), while wild type seeds germinate within 2 DAI [150]. Similarly, seeds of the double mutant *atmib1a atmib1b*, which lacks the E3 ubiquitin ligase subunit BMI of the PRC1 complex [232, 233], reach full germination at 6 DAI whereas the corresponding wild type seeds germinated at 3 DAI [199]. Additionally, treatment with RDS 3434 affects seed germination in a dose-dependent fashion, as wild type seeds treated with RDS 3434 show a significant reduction of the germination rate within the first 36 h compared to untreated seeds; this phenotype is even more pronounced in the *clf-29* mutant background, thus corroborating the effectiveness of RDS 3434 as an inhibitor. It need to be pointed out that the *clf-29* single mutant has been previously characterised for early flowering phenotype, as well as for the curly leaves and dwarf adult plants [234, 235]. In addition, the null *clf-50* allele, regardless of the different genetic background (Ws), displayed similar enhanced phenotypes with the *swn-1* weak allele [226]. Furthermore, it has been shown that *clf-29* display an increase in the number of meristematic root cells [211, 236], conversely to the *swn-7* allele which has a shorter root with no difference in meristem size [211]. The *clf-28 swn-7* double mutant lacking both EZH2 subunits [211], and the *fertilization independent endosperm 2* (*fie*) mutant, which lacks

the *Arabidopsis* homolog of the DROSOPHILA EXTRA SEX COMBS (ESC) PRC2 subunit [150], have shorter roots and smaller meristems with fewer meristematic cells than wild type. Seedlings treated with RDS 3434 display a decrease of both root meristem size and meristematic cell number, due to an effect of the inhibitor on the elongation/differentiation potential of meristematic cells. In *Drosophila* and in animal stem cells the function of the PRC2 complex is counteracted by the Trithorax Group (TrxG) proteins, which catalyse the trimethylation of lysine 4 of histone 3 (H3K4me3) that acts as a transcriptional activator epigenetic mark [237-241]. In Drosophila and mammals, the silencing effect of H3K27me3 is counteracted by the inductive action of H3K4me3 [239, 240]. In *Arabidopsis*, genome-wide analysis of H3K4me3 and H3K27me3 reveals that only a number of genes are targets of both these antagonistic chromatin marks [207, 208]. Among these genes, key regulators of flower development in the vegetative-to-reproductive transition have been shown to be transcriptionally regulated by H3K4me3 and H3K27me3 [242]. As for the seed-to-seedling transition, a switch from an activated to a repressed state associated to H3K4me3 and H3K27me3, respectively, has been reported for a number of seed developmental genes during germination and early seedling development [147, 199]. However, the antagonism between these two epigenetic marks in *Arabidopsis* is still controversial [243] and it has been recently proposed that

Trx proteins cooperate with the PRC2 proteins to repress seed-specific genes during germination and seedling development [244]. In addition, comparative analysis of wild type and *fie* mutant seedlings lacking a functional PRC2 reveals a genome-wide absence of the H3K27me3 mark in the *fie* mutant; of the H3K27me3 mark-free PRC2 target genes in *fie* seedlings, only a limited number are transcriptionally induced and associated with the H3K4me3 activating mark [150]. In agreement with these results, we show that removal of the repressive mark H3K27me3 involves the establishment of the activating mark H3K4me3 only to a certain degree, since RDS 3434-treated seedlings show only a slight, although significant, increase in the level of H3K4me3-marked proteins. Besides being effective in *Arabidopsis*, we also show that the RDS 3434 inhibitor functions only on the H3K27me3 epigenetic mark; indeed, this compound does not inhibit the methyltransferases of the SET DOMAIN GROUP 8 (SDG8), which catalyses trimethylation of H3K36 [245], as the entire bulk of H3K36me3 is not significantly different in treated samples compared to the untreated control. In addition, expression of the non-PRC2 target gene *SAUR14* (see below) is unchanged in treated samples compared to the control, thus corroborating the selectivity of this inhibitor.

We proved that the use of a pharmacological approach in plants is efficient to inhibit PRC2; although we focused our studies on the

seed-to-seedlings transition, RDS 3434 could represent a powerful tool to further investigate the effects of the transcriptional control mediated by PRC2 in plants, even in the response to abiotic stresses.

Materials and Methods:

Plant material and growth conditions

All *Arabidopsis thaliana* lines used in this thesis were usually grown in a growth chamber at 22 °C with 16/8-h day/night cycles and light intensity of 300 μmol/m^{-2} s^{-1} as previously described [203], unless otherwise noted. Seeds were surface sterilized and plated on MS agar (halfstrength MS, 0.8% agar, pH 5.7) and stratified at 4°C for three days in the dark. The wild type lines (Ws-4 and Col-0) *wrky6-2* (SALK_012997C) and *wrky18-1* (SALK_093916C) have been obtained from the European Arabidopsis Stock Centre (arabidopsis.info). The *wrky6dag1* and *wrky18dag1* double mutants were generated by crossing *wrky6/18* and *dag1* single mutants, and homozygous lines were confirmed by PCR amplification.

The *clf-29* mutant line is in Col-0 (SALK_ N521003), and was kindly provided by Dr. Miguel de Lucas. The marker lines *RCH1:: GFP* and *CYCLINB1; 1pro:CDB-GUS* are in Col-0 ecotype and

were previously described [246, 247]. As for the treatment with RDS 3434, wild type seeds were sown on medium supplied with increasing concentrations of RDS 3434 (30, 60, 120 μM), or with an equal volume of its solvent DMSO (Dimethyl sulfoxide), as control.

Germination assay

For the germination assay wild type and mutant seeds were sown directly on five layers of filter paper 595 (Schleicher & Schüll, Dassel, Germany), soaked with 5 ml water. Seeds were stratified 3 days at 4°C, unless otherwise noted, then grow in a growth chamber at 22°C.

For germination assays in the presence pf RDS 3434, triplicate sets of 25 seeds were surface sterilized and plated on agar (0.8%) with increasing concentrations of inhibitor (60, 120 μM) or with an equal volume of its solvent DMSO as control.

Germination rate was scored based on the number of seeds showing radicle emergence. Seeds were harvested from mature plants grown at the same time, in the same conditions, and stored 4 weeks. All germination assays have been performed with at least two seed batches.

Phenotypic analysis

For hypocotyl elongation, the samples were first grown in white light for 24 hours, then exposed to continous monocromatic Red light (660nM) (mounting Heliospectra LX60 lamp) in a growth chamber at 22°C, or wrapped in several aluminium sheets for four days (Dark condition). Hypocotyl length was measured after four days. For ABA treatment, seeds were sown on MS agar with one layer of filter paper 595, then, 48 hours after light exposure, seedlings were transferred to plates containing different ABA concentrations (0,1,10,100 μM)(Duchefa 0941). Hypocotyl length, were measured using IMAGEJ software.

Root analyses were performed on roots from five days old seedlings, grown on MS agar supplemented with 0.5% sucrose for 3 days, then transferred to the same medium in the presence of RDS 3434 (240 μM) or with an equal volume of its solvent DMSO as control, for 2 days. For light DIC microscopy, samples were mounted on a media containing chloral hydrate (SigmaAldrich): 3 parts glycerol: 1 parts water. Images were acquired utilizing Nomarski optics under a Zeiss Axio Imager.A2 microscope with a dry 40X objective. For all the analyses, at least 30 samples were analyzed and statistically treated. Root meristem size was measured based on the number of cortex cells in a file extending from the quiescent center to the first elongated cortex cell excluded as previously described [247]. Images were obtained using a confocal laser scanning microscope (Zeiss LSM 780). The length

of both the first elongated cell and differentiated cell was measured using image J.

The re-establishment of Desiccation Tolerance assay

50 seeds of each genotype were sown directly on five layers of filter paper 595(Schleicher & Schüll, Dassel, Germany), soaked with 5 ml water. The seeds were stratified for 2 days at 4°C in the dark. The plates were transferred in a growth chamber under standard conditions. Seeds are at stage II (radicle protrusion) [109] were transferred in a PEG solution and kept in the dark for three days. Seeds were rinsed with sterile water under a hood in the dark, then sown on a Petri dishes on 5 dry filters, and left for 24 hours under a hood flow to allow complete dehydration. Subsequently, seeds were soaked with 5 ml of sterile water and transferred under standard conditions. Germination was checked every day up to fourth days. Three independent biological replicas are performed.

Abiotic stress treatments - Cold treatment

(According to [248])

Seeds were surface-sterilized, germinated and grown under standard conditions (20 °C under long-day photoperiods [16 h light, of cool-white fluorescent light, photon flux of 90 μmol m^{-2} s^{-1}]) in pots containing a mixture of organic substrate and

vermiculite (3/1, v/v) or in Petri dishes containing Murashige and Skoog medium supplemented with 1% sucrose (GM) and solidified with 0.9% (w/v) plant agar. Low-temperature treatments were performed by transferring plants growing in pots or Petri dishes under standard conditions to a growth chamber set at 4 °C for 1 week under a long-day photoperiod (16 h of cool-white fluorescent light, photon flux of 40 μmol m^{-2} s^{-1}). For freezing tolerance assays, seeds from the different genotypes, each one coming from the same parent and collected at the same time, were sown in soil-containing pots and allowed to develop for 2 weeks under control conditions. Afterwards, several plants from each pot were removed in order to have a similar number (≈15) of individuals uniformly developed and distributed in all pots. Constitutive freezing tolerance was assessed by exposing plants to 4 °C for 30 min in darkness and subsequently decreasing the temperature at a rate of –1 °C per 30 min until reaching –6 °C. Six hours later, temperature was increased to 4 °C at the same rate and thawing was allowed for 12 h before returning plants to control conditions under long-day light regime for recovering. To determine freezing tolerance after cold acclimation, plants were acclimated at 4 °C for 7 d under long-day photoperiod and subsequently subjected to freezing in the dark by progressively decreasing the temperature (–1 °C per 30 min) until attaining the desired freezing temperature. After exposing plants to the appropriate freezing temperature for 6h,

temperature was gradually increased to 4 °C (+1 °C per 30 min). Twelve hours later, plants were transferred to 20 °C under long-day light regime for recovering. In all cases, survival rate was evaluated after 10 d of recovering.

GUS analysis

Histochemical staining of *pDAG1::GUS* seeds during the re-establishment of DT assay, or of *pDAG1::GUS* in 14 days-old seedlings treated at 4°C for 8 hours was performed according to [249] except that seedlings were incubated at 37°C for 12 hours. Microscopic analyses were carried out under an Axioskop 2 plus microscope (Zeiss).

Expression analysis

(According to [152])

For RNA extraction, four days-old wild type and mutant seedlings, grown under monochromatic Red light or in the dark, were harvested and immediately frozen in liquid nitrogen. For hypocotyls about 1000 seedlings grown in this condition have been dissected, then hypocotyls were frozen in liquid nitrogen in the dark. For expression analysis in the presence of the inhibitor, five days-old seedlings, grown in the presence of increasing concentrations of RDS 3434 (30, 60, 120 μM) or with an equal

volume of its solvent DMSO as control, were frozen and grinded with liquid nitrogen.

Total RNA was isolated by grinding the tissues in liquid nitrogen. The samples were then vortexed for 3min in the presence of an extraction bufer (0.1 MLiCl, 0.1M Tris-HCl [pH 8], 0.01M EDTA, 1% sodium dodecyl sulfate-phenol-chloroform mixture (1:1:1). Tree phenol-chloroform extractions were then performed. RNA was precipitated overnight at 4 °C with 1 volume of 4M LiCl, followed by a second precipitation with 0.1 volume of sodium acetate, pH 5.2. RT-qPCR assays were performed with SYRgreen I master using the LightCycler® 480 instrument (Roche, http://www.roche.com). A total of 1 µl of the diluted cDNA was used, along with the specific primers, listed in Table1. Relative expression levels were normalized with PP2A (At1g69960) reference gene, or with the GAPDH (At3g26650) reference gene, which is not marked by H3K27me3.

For RNA extraction of cold-treated samples, whole seedlings, roots or cotyledons from 11 days-old mutant and wild type seedlings were collected at 3, 8, 24 hours after the cold exposure. The total RNA was obtained using Purezol reagent (Bio-Rad) according to the manufacturer's instructions. RNA samples were treated with DNase I (Roche) and quantified with a Nanodrop spectrophotometer (Thermo Scientific). cDNA was synthesized from each sample with the iScript cDNA synthesis kit (Bio-Rad),

and qPCRs were performed with SsoFast EvaGreen Supermix (Bio-Rad) in a Bio-Rad iQ2 thermocycler. In all cases, the relative expression values were calculated using the *At4g24610* gene as a reference. All reactions were carried out in triplicate employing three independent RNA samples (According to [248]).

Chromatin Immunoprecipitation (ChIP) assay

(According to [152])

ChIP assay was performed with 5 days old-seedlings of the transgenic line overexpressing the DAG1-HA chimeric protein in a *dag1* mutant background and with the *dag1* mutant as a negative control, or wild type seedlings, grown in the presence of RDS 3434 120 μM or with an equal volume of its solvent DMSO as control. Seedlings (about 1gr) were washed with water, then resuspended with 3ml extraction buffer 1 (0.4M sucrose, 0.01M Tris-HCl [pH 8], 5 mM β-mercaptoethanol, 1mM PMSF, 1x protease inhibitors) and treated with 37% formaldehyde for 10min under vacuum. The reaction was stopped with glycine 0.125M. Samples were then harvested with a miracloth membrane and immediately frozen and ground in liquid nitrogen. Extraction buffer was added to the samples (30 ml) then filtered on a miracloth membrane. After a centrifugation (4000 g, 20 min), the pellet was resuspended in 1ml extraction buffer 2 (0.25M sucrose, 0.01M Tris-HCl [pH 8], 10mM MgCl2, 1%Triton x-100, 5mM β-mercaptoethanol, 1mM PMSF,

1x protease inhibitors). After 10min on ice, samples were centrifuged (12000g, 10min, 4°C). The pellet was resuspended in 0.3ml extraction buffer 3 (1.7M sucrose, 0.01M Tris-HCl [pH 8], 2mM MgCl2, 0.15%Triton x-100, 5mM β-mercaptoethanol, 1 mM PMSF, 1x protease inhibitors), then samples were centrifuged again (1 h, 16000 g, 4 °C). The chromatin pellet was resuspended in 0.3 ml lysis buffer (0.05M Tris-HCl [pH 8], 0.01 MEDTA, 1% SDS, 1 mM PMSF, 1x protease inhibitors). Chromatin was sheared by sonication. To an aliquot of each sample (0.1ml) was added 0.9ml ChIP buffer (1.1% Triton, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8], 167 mM NaCl, 1 mM PMSF, 1x protease inhibitors). The immunoprecipitation was performed using HA-probe antibody (Y-11, sc-805 Santa Cruz), or against H3K27me3 (Millipore #07–449), or without antibodies as negative control, overnight at 4°C. After reverse cross-linking, the enriched DNA levels were quantifed by qPCR using specific primer sets (Supplementary Table 2). The Fold enrichment of a specific region was calculated respect to the negative control without antibody.

Protein extraction and Immunoblot analysis

(According to [197])

Five days-old seedlings, grown in the presence of increasing concentrations of RDS 3434 (30, 60, 120 μM), or with an equal volume of its solvent DMSO as controls, were grinded with liquid

nitrogen and dissolved in Chromatin Buffer Extraction (Sucrose 0,4 M; Tris Hcl pH 8 10 mM; β-mercaptoethanol 5 mM; PMSF 0,1 mM; Protease inhibitor cocktail 1X, Sigma-Aldrich P9599). The nuclei were pelleted at 4000 rpm for 20 min, at 4 °C, and dissolved in 800 µl ddH2O. The proteins were precipitated with 150 µl of NaOH/ β-mercaptoethanol (138, 7 µl 2 N NaOH and 11,25 µl β-mercaptoethanol) and then with 55% TCA solution, for 15 min in ice. Following centrifugation for 20 min at 14000 rpm, 4 °C, the pellet was dissolved in HU Buffer (Urea 8 M; SDS 5%; TrisHCl pH 6,8200 mM; EDTA 0,1 mM; DTT 100 mM; bromophenol blu) and boiled for 10 min at 65 °C. Proteins were separated on a 12% SDS-polyacrylamide gel (Bio-Rad) and blotted on a PVDF Immobilon-P Transfer membrane (Millipore). Detection of proteins was performed with specific antibodies against H3K27me3 (Millipore #07–449), H3K4me3 (Abcam- ab8580) or H3K36me3 (Abcam-ab9050), and against histone H3 (Biorbyt orb10805) as a loading control. The anti-rabbit IgG conjugated to peroxidase was used as a secondary antibody and the signal was detected with ECL system. The values are the average of two biological replicates (except the immunoblot for H3K27me3 that was performed with three biological replicates), presented with SD values. Significant fold enrichments were analyzed by t-test (*$P \leq 0,05$).

Statistical analysis

Two-tailed Student's t-test was used to evaluate statistical significance.

Table 2. Primers used in this study

RT-qPCR	Forward	Reverse
GADPH	GCTGAGGAAGTCAACGCTGC	CGGACACTAGTGGCTCATCG
UBQ10	GGCCTTGTATAATCCTGATGAATAAG	GGCCTTGTATAATCCTGATGAATAAG
AT4G26410	GAGCTGAAGTGGCTTCcATGAC	GGTCCGACATACCCATGATCC
DAG1	TTGTCGAAGGTATTGGACCGA	CCGACTGGGACGTTACGAAG
DAG1(Spain)	CTACAGTCTCACGCAACCAAG	CCGACTGGGACGTTACGAAG
RAB18	CGATGGGCGGCATGGGAT	CACCACCGGGAAGCTTTTCCTT
ABR1	TCCGTTGCGTTTCGGTCACA	CTGATTCTGGTGGCGGCTGAA
WRKY6	TCTCGGGGCTGCAGTTCTCT	GGTCAGCTGTGAGTGCCGTT
WRKY18	GGGATGCTACAAGCCAGGGTG	TCCCTTTCGTTTTTCTCCAACGCT
WRKY18 (Spain)	CGGACACAAGCTTGACAGTTAAAGATGG	GCATCTAAAGTAAGCTCTAGGTGACGGG
WRKY28	GCAGACCTCATGACTCCAAGAAGC	ATAATCCAAAGCCGCCGCCA
WRKY33	ACCATCGGTTGTCCAGTGAGGA	AACCGCTACCACGAGCTGC
WRKY40	GCTTGACTGTGCCGGTGACT	GGAAGAAGCCATTTGCTCCACCA
WRKY46	CTGCACCTGCTGCTGTTGAGAA	CGACCACAACCAATCCTGTCCG
WRKY70	CAGGCCAGTTACGTCAATGGGAAAA	GAAATCGCCGCCACCTCCA
SAUR14	GTAGTTCCGGTTTCGTACTTGGACC	CTGCAAGGGATTGTGAGGCCA
SAUR67	CAGAAAAGGCTCTGTTGATGTC	GTTGAGTACTCTGTTCTTGCTG
Primer for ChIP	**Forward**	**Reverse**
DAG1 ChIP	CGCAACAACAACCAACATTC	GCCGTGTTGTTGGTATTTCC
WRKY18 c	CAGCTTTCTATGTCTTGTTGTGC	AAAGCTGTCTTCTATAAAGTCCAG
WRKY18 b	CTGGACTTTATAGAAGACAGC	AGATAGGTTTTGGGATATGTTAGG
WRKY18 a	CGACAGTAAATTTAACCCATGCAT	ACACTGATTGGCTAAAAATATGGG
Primer for screening	**Forward**	**Reverse**
WRKY6	TGTGGACGTGTCATAATTTGG	GAACGTATTAGCCAATCACGC
wrky 6	TGTGGACGTGTCATAATTTGG	TGGTTCACGTAGTGGGCCATCG (T-DNA)
WRKY18	GACAGTAAATTTAACCCATGC	CGCTCTGTTCATTACCACC
wrky18	GCTTGCTGCAACTCTCTCAG (T-DNA)	TCATAGAAATTGAAGGGATACAAAAA

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