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"Study of the role of the DOF transcription factor DAG1 in the control of seedling development in *Arabidopsis thaliana*"

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PUBLICATION

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ABSTRACT

Seedling development relies on environmental conditions; indeed, once seeds have germinated, they undergo photomorphogenesis or skotomorphogenesis, depending on the presence or absence of light. Photomorphogenesis is a multi-traits process characterised by inhibition of hypocotyl elongation, open and expanded cotyledons, and chloroplast development, whereas skotomorphogenesis is characterised by long hypocotyls and small unfolded cotyledons. Hypocotyl elongation is influenced by both environmental and hormonal cues and it has been extensively studied as a model for cell expansion. Nevertheless, the molecular network underlying this process is not yet fully elucidated.

The *Arabidopsis* Dof protein DAG1 (Dof Affecting Germination1) is a repressor of seed germination, and a key player of the seed-to seedling transition, a crucial developmental phase positively controlled by light, as well as by the phytohormones ABA (abscissic acid) and GA (gibberellins). Indeed, DAG1 controls the ratio of ABA and GA, which play opposite roles, as ABA represses germination whereas GAs promote it.

We have previously shown that inactivation of *DAG1* affects inhibition of hypocotyl elongation. Indeed, light-grown *dag1* mutant seedlings show significant shorter hypocotyls compared to the wild-type, suggesting that DAG1 is a negative component of this light-mediated process. To gain some insight into the molecular network in which DAG1 is

involved, we have analysed the transcriptome profile of both *dag1* and wild-type hypocotyls and seedlings. We have identified more than 250 genes that are differentially expressed in *dag1* hypocotyls, and the analysis of this data suggests that DAG1 is mainly involved in promoting hypocotyl elongation. In addition, a number of the DE genes identified are correlated to the response to ABA stimulus. ABA plays a role in inhibition of hypocotyl elongation, although the molecular mechanism remains unclear.

Therefore, we investigated the effect of ABA on hypocotyl development, and our results showed that ABA negatively controls cell expansion in hypocotyls, by acting on GA metabolism, and repressing auxin biosynthesis. Consistently, addition of exogenous ABA can revert the hypocotyl phenotype of *dag1* mutant seedlings.

In conclusion, our results prove that DAG1 is likely to be an element of a molecular network which controls cell expansion by modulating hormonal response, namely auxin, ABA and GAs.

INTRODUCTION

1 LIGHT

1.1 Photoreceptors

As sessile autotrophic organisms, plants need to continuously adapt to environmental changes to successfully complete their life cycle. Light is the primary source of energy as

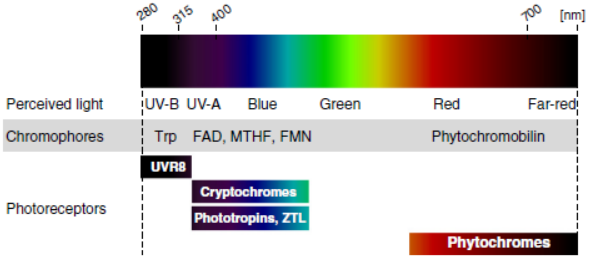


Figure 1. Photoreceptor-mediated light perception in higher plants.
Plant photoreceptors are able to perceive light of different wavelengths. Phytochromes are red/far-red photoreceptors, which bind the plant-specific chromophore phytochromobilin. Phototropins and the Zeitlupe (ZTL) proteins bind FMN (Flavin Mononucleotide) chromophore through their LOV domains. Cryptochromes bind FAD (Flavin Adenine Dinucleotide) and MTHF (methenyltetrahydrofolate) as chromophores. UVR8 perceives UV-B light.

well as a pivotal environmental cue, it controls every physiological process of plant life cycle from seed germination to flowering.

Light is perceived by five classes of photoreceptors: phytochromes, cryptochromes, phototropins, ZTL and UVR8 (Heijde and Ulm 2012) (Figure 1).

Phytochromes perceive red/far-red light, cryptochromes, phototropins and ZTL sense UV-A/blue light, whereas UVR8 perceives UV-B radiation.

Photoreceptors are chromoproteins characterized by the presence of chromophores bound to an apo-protein. Light induces changes in the chromophore leading to structural changes in the apo-protein which turns on the signal transduction pathway. Both the apo-protein and the chromophore determine the characteristic absorption spectra of the photoreceptors (Christie *et al.,* 1998; Imaizumi *et al.*, 2003; Lin *et al.*, 1995; Rockwell *et al.*, 2006).

1.2 Phytochromes

In *Arabidopsis thaliana*, phytochromes are encoded by a small gene family (*PHYA–PHYE*) and play pivotal roles in different developmental processes such as seed germination.

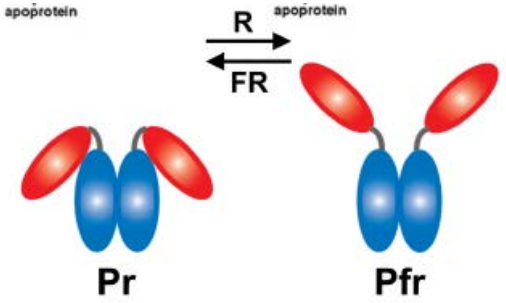


Figure 2. Phytochrome photoconversion.
Red (R) light triggers photoconversion of the phytochromes from the inactive Pr form to the active Pfr form. Conversely, Far Red (FR) light induces the conversion of the active Pfr form into the inactive Pr.

Phytochromes exist in two photoreversible forms *in vivo*: a red light absorbing form (Pr), and a far-red light absorbing form (Pfr). Upon red light absorption, the Pr inactive form is converted to the active Pfr, which in turn can be converted back to the inactive Pr form after absorption of FR light (Mancinelli, 1994; Quail, 1997; Fankhauser, 2001) (Figure 2). Phytochromes are present as homo- or heterodimers, the molecular mass of the

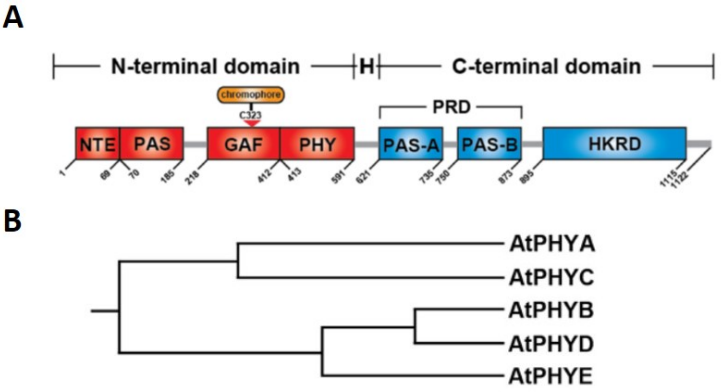


Figure 3: The family of *Arabidopsis* phytochromes.
(A) Structure of a phytochrome chromoprotein: NTE, N-terminal extension; PAS: Per (Period Circadian Protein), Arnt (Ah Receptor Nuclear Translocator Protein), and Sim (Single-Minded Protein); GAF: cGMP-stimulated phosphodiesterase, adenylate cyclases (*Anabaena)* and FhlA *(Escherichia coli)*; PHY: phytochrome; PRD: PAS-Related Domain; HKRD: Histidine Kinase–Related Domain. The chromophore is attached to a conserved cysteine residue in the GAF domain. (B) Phylogenetic tree of the five phytochromes encoding genes in *Arabidopsis thaliana*.

monomeric apoprotein is 125 kDa. These apoproteins are synthesized and autocatalytically assembled with the chromophore in the cytosol.

The N-terminal region (70 kDa) contains four domains: NTE, PAS, GAF and PHY; the C-terminal region (55 kDa) presents two domains: PRD (containing two PAS repeats) and HKRD (Li et al., 2011) (Figure 3A). The GAF domain is covalently bound to the chromophore through a conserved cysteine residue. The PAS domains are used as platforms for protein-protein interaction.

Phytochromes may be divided into two classes, type I and II, on the basis of their light stability. Type I phytochromes are characterised by rapid proteolytic degradation of the Pfr

Table 2. Different Roles of Phytochrome Family Members in Seedling and Early Vegetative Development

Phytochrome Members	Primary Photosensory Activities	Primary Physiological Roles
phyA	VLFRs FR-HIRs	Seed germination under a broad spectrum of light conditions (UV, visible, FR); Seedling de-etiolation under FRc; promoting flowering under LD.
phyB	LFRs R-HIRs EOD-FR (R/FR ratio)	Seed germination under Rc; Seedling de-etiolation under Rc; Shade avoidance response (petiole and internode elongation, flowering).
phyC	R-HIRs	Seedling de-etiolation under Rc.
phyD	EOD-FR (R/FR ratio)	Shade avoidance response (petiole and internode elongation, flowering).
phyE	LFRs EOD-FR (R/FR ratio)	Seed germination; Shade avoidance response (petiole and internode elongation, flowering).

VLFRs: very-low-fluence responses;
LFRs: low-fluence responses;
HIRs: high-irradiance responses;
FR: far-red light;
R: red light;
FRc: continuous far-red light;
Rc: continuous red light;
LD: long day light condition;
EOD-FR: end-of-day far-red light;
R/FR ratio: red/far-red light ratio.

Table 1. Different Roles of the Phytochrome Family Members

form, and control both the Very-Low-Fluence (VLFR) and Far-Red High-Irradiance Responses (FR-HIR). On the contrary, phytochromes of type II are light-stable and control Low Fluence (LFR) and Red light High Irradiance Responses (R-HIR). Analysis of phytochromes deficient mutants revealed that (i) type I phytochromes are encoded by the *PHYA* gene and type II phytochromes by *PHYB–E* genes (Quail, 2002) and that (ii) different members of the family have differential as well as overlapping physiological functions in the control of plant development (Smith *et al.*, 1997; Franklin *et al.*, 2003; Monte *et al.*, 2003).

Phytochromes are translocated inside the nucleus in response to the Pr/Pfr conformational change. The nuclear import of phyB is triggered by continuous red-light exposure, and is counteracted by FR light (Kircher *et al.*, 2002). On the other hand, phyA nuclear translocation is induced by a single brief pulse of any light (FR, R or B) (Hisada *et al.*, 2000; Kim *et al.*, 2000; Kircher *et al.*, 2002) and it occurs within few minutes, whereas phyB nuclear import takes hours (Kircher *et al.*, 1999, Kim *et al.*, 2000; Kircher *et al.*, 2002) (Figure 4).

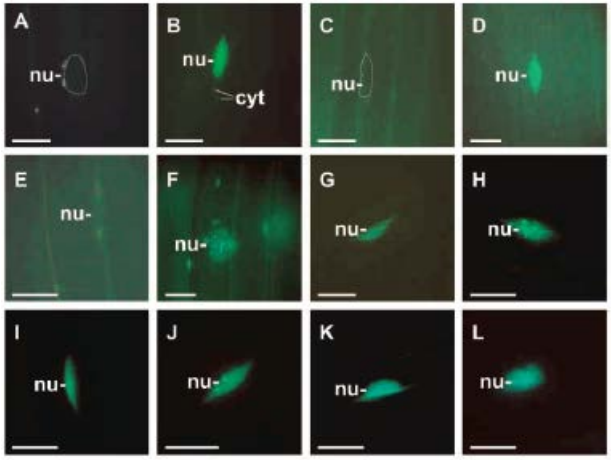


Figure 4. Nucleocytoplasmic Distribution of phyA to phyE:GFP in 7-Day-Old Dark grown Seedlings.
(A) to (L) Fluorescence images of transgenic *Arabidopsis* seedlings expressing phyA:GFP (A to D), phyB:GFP (E and F), phyC:GFP (G and H), phyD:GFP (I and J), and phyE:GFP (K and L) fusion proteins. Fluorescence images of nuclei of dark grown seedlings (A, C, E, G, I, and K) and of seedlings transferred to WL for 10 min (B) or 6 h (F, H, J, and L) or to FR for 6 h (D) are shown (adapted from Kircher *et al*., 2002).

Both phyB and phyA, once imported into the nucleus, localize to discrete subnuclear foci named nuclear bodies (NBs) (Hisada *et al*., 2000; Kircher *et al*., 2002). It has also been demonstrated that phyB NB formation is dependent on the percentage of the Pfr form of phyB, with larger phyB NBs correlated with strong phyB responses (Chen *et al*., 2003).

Several phytochrome signalling components colocalize to NBs, suggesting an important role in phytochrome signalling (Chen, 2008).

After phytochrome activation and transport to the nucleus, these proteins interact with downstream signalling components to control gene expression. PIF/PIF-like

(PHYTOCHROME INTERACTING FACTORS) are transcription factors belonging to basic helix-loop-helix (bHLH) family that directly interact with phytochromes. PIFs regulate gene expression by directly binding a region enriched in G-box (CACGTG) and/or E-box (CACATG and CATGTG) (Leivar and Monte, 2014). Following phyB-PIF interaction, PIFs proteins are phosphorylated and their transcriptional activity is inhibited. After phosphorylation, PIFs proteins are degraded via the ubiquitin-proteasome 26S pathway, PIF7 is the only member of this family that rather being degraded is accumulated in the phosphorylated form (Li *et al*., 2012).

2 LIGHT MEDIATED PROCESSES IN *ARABIDOPSIS*

2.1 Seed germination

Light controls all developmental steps of plant life cycle: seed germination, seedling de-etiolation, gravitropic orientation, shade avoidance, stomatal development, circadian clock, flowering and more.

Seed germination is the first developmental process in plant life and consists in the release of a quiescent embryo, once favourable environmental conditions are met. Germination of seeds is under the control of both environmental and endogenous factors (Koornneef and Karssen, 1994). Although nutrients, oxygen and temperature affect germination, light and water are the most important signal that induce this process. Rupture of both the testa

(seed coat) and the endosperm is the first step of seed germination, followed by radicle (embryonic root) emergence through outer layers.

Seed dormancy is an important adaptative trait which prevents seed germination even under optimal environmental condition. In this respect, dormancy is useful to avoid vivipary while favouring seed dispersion in the environment. The phytohormones gibberellins (GA) and abscisic acid (ABA) control both dormancy and germination of seeds; they function in opposite way: GAs promote seed germination, whereas ABA induces dormancy and represses germination.

2.1.1 Molecular model

Seed germination is triggered mainly by red light (Shropshire *et al.*, 1961), through the photoreceptor phyB, although also continuous far red light, through phyA, can induce it (Shinomura *et al.*, 1996). In the absence of light and/or active phytochromes, seed germination is almost completely abolished (Strasser *et al.*, 2010).

phyB induces seed germination by increasing the biosynthesis of GAs (Yamaguchi *et al.*, 1998; Yamaguchi *et al.*, 2001) and in turn decreasing ABA biosynthesis (Seo *et al.*, 2006; Oh *et al.*, 2007; Sawada *et al.*, 2008). As soon as phyB is activated the expression of the GA biosynthetic genes *GA3ox2* and *GA3ox1* is induced, while the catabolic gene *GA2ox2* is repressed, thus favouring GAs accumulation. On the other hand, ABA accumulation is

reduced by repression of the two biosynthetic genes *NCED6* and *NCED9*, and induction of the catabolic gene *CYP707A2*.

Many transcription factors involved in phyB-mediated seed germination have been described, such as the bHLH transcription factors SPT (SPATULA) and PIL5 (PHYTOCHROME INTERACTING FACTOR 3-LIKE 5) (Oh *et al*., 2004; Penfield *et al*., 2005). After red light exposure, phyB translocates into the nucleus where it directly interacts with PIL5, the master repressor of seed germination, and phosphorylates it, thus inducing its degradation (Huq *et al*., 2004). Consistently, inactivation of *PIL5* partially compensates the reduced germination rate of *phyB* null mutant seeds (Oh *et al*., 2004).

PIL5 controls both GA signalling and metabolism. Indeed, it directly regulates the expression of GAI (GA INSENSITIVE) and RGA (REPRESSOR OF *ga1-3*), two DELLA proteins which are negative regulators of GA-mediated responses (Oh *et al*., 2007); PIL5 does not directly bind any ABA or GA metabolic genes, conversely it controls downstream transcription factors which regulate the metabolism of these hormones, such as SOM (SOMNUS), XERICO and DAG1 (Dof AFFECTING GERMINATION1) (Kim *et al*., 2008; Oh *et al*., 2009; Gabriele *et al*., 2010) (Figure 5).

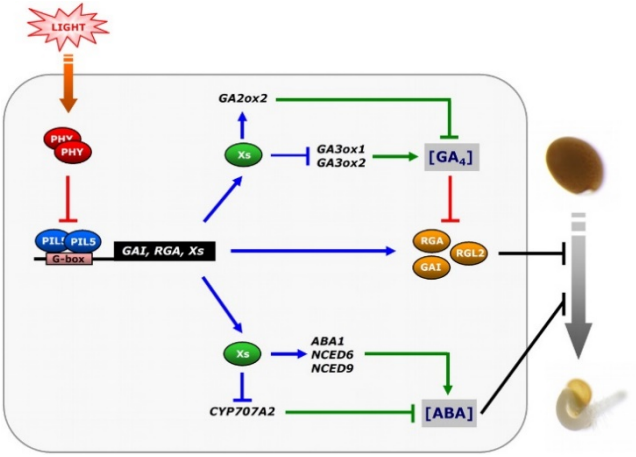


Figure 5: Model of light-dependent seed germination in *Arabidopsis*.
Red lines, events occurring at the protein level; blue lines, events occurring at the transcriptional level; green lines, events occurring via enzymatic activities (adapted from Oh et al., 2007).

2.1.2 Gibberellins

Gibberellins (GAs) are known as a growth-promoting class of hormones, which play a pivotal role throughout plant life cycle. In the early 20th century the "Green Revolution" was enabled by the introduction of dwarfing traits in wheat and rice (Hedden, 2003). The identification of genes leading to dwarfism revealed that they were involved with GAs biosynthesis (Peng *et al.*, 1999). The hydroxylation of the *ent*-gibberellic skeleton is the crucial regulatory step of GA biosynthesis. This last step of bioactive GA biosynthesis is

under the control of GA20ox and GA3ox enzymes, therefore their transcription is tightly regulated (Figure 6).

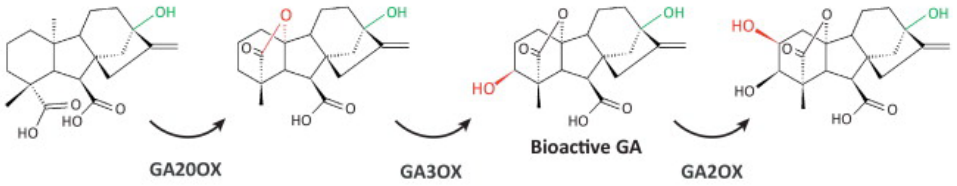


Figure 6. Regulatory steps of GA metabolic pathway.
The final hydroxylation steps of the ent-gibberellene skeleton are the main regulatory steps of GA biosynthesis. These activities result in the biologically active endogenous GAs, GA_1 and GA_4.

GA signalling pathway is under the control of DELLA proteins, which act as negative regulators of GA-mediated responses. The family of DELLA proteins comprises five members: GAI (GA insensitive), RGA (repressor of *ga1-3*), RGL1 (RGA-like 1), RGL2 and RGL3. Following binding of GAs to the receptor GID1 (Griffiths *et al.*, 2006; Nakajima *et al.*, 2006), the GID1–GA complex interacts with the DELLA repressors and undergoes a conformational change, which increases the binding affinity to the F-box SLY1/GID2 subunit of the E3 ligase $SCF^{SLY1/GID2}$ complex (Dill *et al.*, 2004). Subsequently, DELLA proteins are polyubiquitinated and degraded by the 26S proteasome, thus releasing the growth restraint imposed by these repressors.

Although DELLA proteins are transcriptional regulator, they do not directly bind DNA, but rather interact with DNA-binding proteins in a promoter-associated complex, or

alternatively they can interact with transcription factors and prevent the binding to the promoters of their target genes. This mode of action has been demonstrated for both PIF3 and PIF4; DELLA proteins directly interact with the DNA-binding domain of the PIF proteins blocking their transcriptional activity (de Lucas *et al*., 2008, Feng *et al*., 2008) (Figure 7). GAs counteract DELLA-mediated repression thus releasing PIF factors.

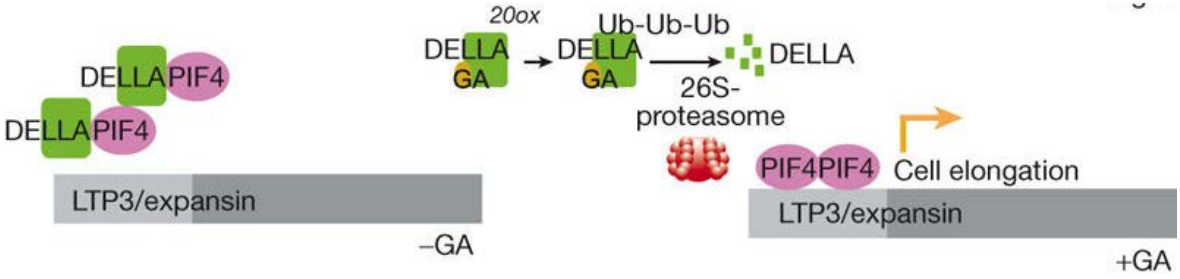


Figure 7. Model for DELLA activity on PIF4.
DELLAs bind PIF4 and repress its ability to bind DNA. GAs induce proteasome-mediated degradation of DELLAs and allow accumulation of PIF4, thus promoting PIF4-activated gene expression (adapted from de Lucas *et al*., 2008).

2.1.3 Abscissic acid

Abscissic acid (ABA) has been shown to regulate many aspects of plant life cycle such as embryo development, seed dormancy and germination, cell division and elongation, response to biotic and abiotic stresses, stomatal closure and floral induction. Conversely to gibberellins, ABA is a growth-inhibiting hormone. Despite this growth-restraint activity, ABA-deficient mutant plants (*aba1-1* mutant) display a stunted phenotype (Figure 8), due

to the impaired control of stomata closure, which results in defective transpiration (Koornneef *et al*., 1982).

Figure 8. Phenotype of wild-type and ABA deficient plants.
Phenotype of wild-type and ABA-deficient plants (*aba1-1*), with or without exogenous ABA.

ABA levels are monitored through constant control of the ratio between synthesis and catabolism. NCED (nine-cis-epoxycarotenoid dioxygenase 6) enzymes are a class of proteins which control the rate-limiting step of ABA biosynthesis, expression of *NCED* genes is thus tightly regulated. Plants exploit extensively catabolism to control ABA content, in particular the ABA-8'-hydroxylases enzymes, encoded by the *CYP707A* family.

ABA is perceived through the PYRs (PYRABACTIN RESISTANT), PYLs (PYR-like) or RCARs REGULATORY COMPONENT OF ABA RECEPTOR) receptors family. Once ABA is bound to the PYR/PYL/RCAR receptors, a complex with PP2Cs (PROTEIN PHOSPHATASE 2Cs) is formed and the phosphatase activity of this protein is repressed. PP2Cs inhibits phosphorylation activity of SnRK2 (SUCROSE NONFERMENTING 1 (SNF1)-RELATED PROTEIN KINASES 2) which in turn activates downstream transcription factors and a number of ABA-responsive targets (Finkelstein, 2013; Yang *et al*., 2017) (Figure 9).

ABI3, ABI4 and ABI5 (ABA INSENSITIVE3, 4, 5) are the key elements of ABA signalling; indeed, inactivation of these ABI transcription factors results in ABA-insensitive phenotype (Koornneef *et al*., 1984). These proteins are abundantly expressed in seeds and regulate many seed-specific genes, they have similar effects on seed development and ABA sensitivity, although mutation in *ABI3* results to be more severe than in *ABI5* and *ABI4* (Parcy *et al*., 1994; Finkelstein *et al*., 1998; Finkelstein and Lynch, 2000).

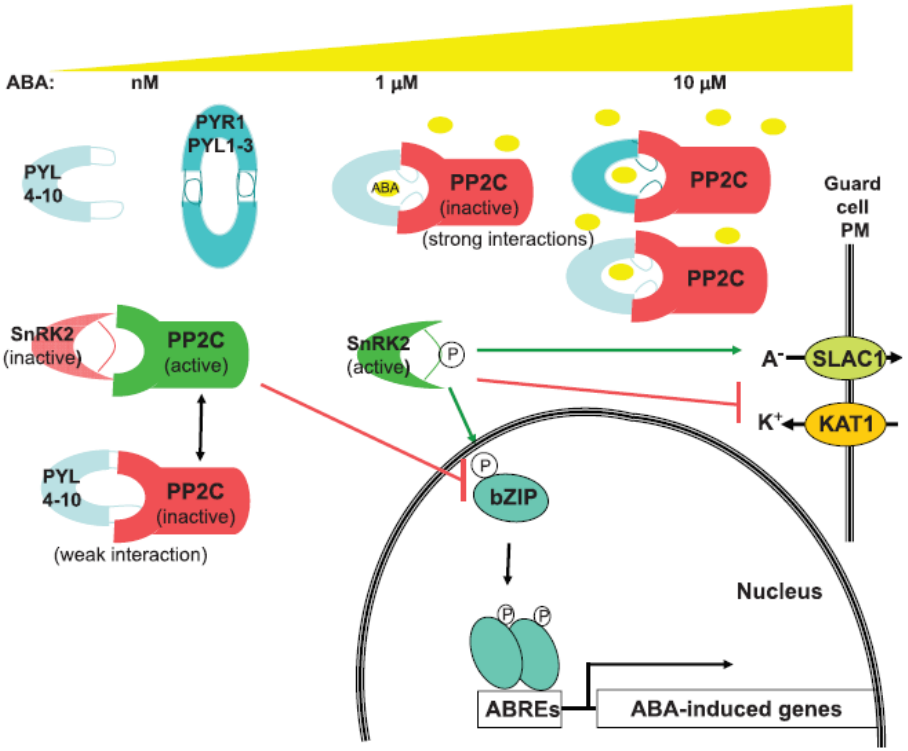


Figure 9. ABA signalling pathway.

PYL4-10, PYR1/PYL1-3 interact with PP2Cs after ABA binding, leading to the inactivation of PP2Cs and de-repression of the SnRK2s, which in turn phosphorylate a number of proteins involved in ABA response. Green arrows indicate activation and red bars indicate repression (adapted from Finkelstein 2013).

2.2 Photomorphogenesis

Once seed germination is accomplished, seedling development relies on environmental conditions; indeed, they undergo photomorphogenesis or skotomorphogenesis, depending on the presence or absence of light.

Photomorphogenesis is a multi-traits process characterized by inhibition of hypocotyl elongation, opening and expansion of cotyledons and chloroplast development. In the absence of light, which is the natural condition of seeds germinating underground, seedlings go through a skotomorphogenic developmental program, characterized by longer hypocotyls, closed and etiolated cotyledons and apical hook formation.

GAs promote etiolated growth; indeed, the GA biosynthetic mutant *ga1-3* displays a constitutive photomorphogenic phenotype when grown in the absence of light (Alabadì *et al*., 2004). This phenotype can be at least partially complemented by *rga* and *gai* null alleles, suggesting that these two DELLA proteins play a pivotal role in the GA-dependent

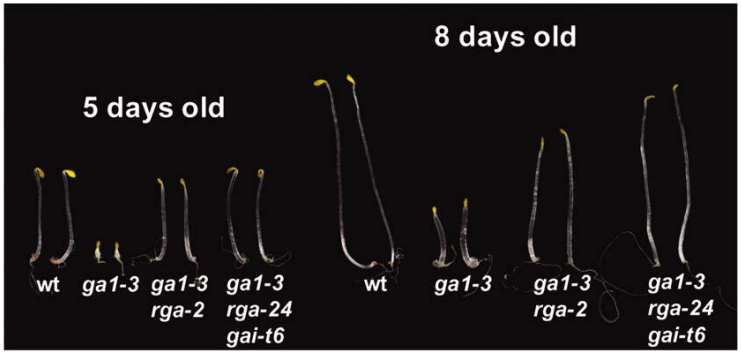


Figure 10. GA biosynthesis and signalling mutants grown in the dark.
Phenotypes of 5- and 8-d-old dark-grown *Arabidopsis* GA biosynthesis and signaling mutants (adapted from Alabadì *et al*., 2004).

repression of photomorphogenesis (Alabadì *et al*., 2004) (Figure 10).

GA metabolism is controlled by light during both seed germination and seedling development. Interestingly, the effect of light on GA metabolism during these two processes is opposite: in fact, light positively controls GA biosynthesis in seeds, whereas in seedlings it induces the expression of the GA catabolic genes, thus resulting in decreased GA content (Achard *et al*., 2007).

GAs and light, which antagonistically control photomorphogenesis, both converge on PIF proteins. Seedlings lacking PIF1, PIF3, PIF4 and PIF5, namely the *pifq* mutant, display constitutively photomorphogenic phenotype when grown in the absence of light, suggesting that these transcription factors act redundantly to positively control

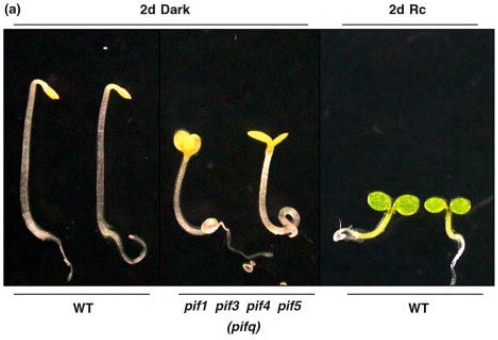


Figure 11. Light inhibits PIF-promoted skotomorphogenesis through phy-mediated degradation of PIFs.

PIF proteins promote skotomorphogenesis. This developmental program is reversed either by light exposure, or in the dark by inactivation of *PIF1*, *PIF3*, *PIF4* and *PIF5,* namely in the *pifq* mutant (adapted by Leivar *et al*., 2010).

skotomorphogenic development (Leivar *et al*., 2009; Leivar and Quail, 2011; Zhang *et al*., 2013) (Figure 11). PIFs factors are sequestered by DELLAs proteins, which inhibit their transcriptional activity thus promoting photomorphogenesis. Recently it has been shown that DELLA proteins also promote degradation of PIF factors through the ubiquitin-proteasome pathway (Li *et al*., 2016). The action of DELLA proteins is counteracted by GAs which induce their degradation allowing PIFs accumulation (de Lucas *et al*., 2008; Feng *et al*., 2008).

Among the genes regulated by PIF proteins, there are many photosynthesis/chloroplast-related genes (Leivar *et al*., 2009). Also hormone-related genes are controlled by PIFs, mainly auxin-related genes (Leivar *et al*., 2009).

2.2.1 Auxin

Auxin is a phytohormone which plays pivotal roles in many plant developmental processes. Auxin levels are monitored through the control of biosynthesis, degradation and polar transport. Auxin also regulates cell elongation, although its action depends on other hormonal and environmental signals; indeed auxin response requires both gibberellins (Chapman *et al*., 2012) and brassinosteroids (Goda *et al*., 2004).

Auxin is perceived by the TIR1 (TRANSPORT INHIBITOR RESPONSE 1) and AFB (AUXIN SIGNALLING F-box) receptors (Dharmasiri *et al*., 2005a; Parry *et al*., 2009), which are F-box

proteins belonging to the SCF (Skp1/Cullin/Fbox) complex. This complex recognizes the Aux/IAAs proteins, which represses auxin-regulated transcription, leading to their proteasome-mediated degradation (Kepinski *et al*., 2005; Dharmasiri *et al*., 2005a, b).

Auxin controls the transcription of several hundred genes (Okushima *et al*., 2005); two transcription factors families mediate auxin response, the ARFs (AUXIN RESPONSE FACTORS) and the Aux/IAA corepressor proteins. ARF factors bind to the Auxin Response Elements (AuxREs), to either activate or repress gene expression. Aux/IAAs affect transcription by interacting with the ARF factors and repressing their activity.

ARF6 and ARF8 are both involved in the control of hypocotyl elongation, indeed they function redundantly to regulate this process (Nagpal *et al*., 2005). Their activity is counteracted by IAA3 which directly recognizes several ARFs, including ARF6 and ARF8 (Vernoux *et al*., 2011). Many target genes of ARF6 are also targeted by PIF4 and BZR1, which directly interact with this ARF factor (Oh *et al*., 2014).

2.2.2 Brassinosteroids

Brassinosteroids (BRs) are steroid hormones which repress photomorphogenesis, indeed mutant seedlings affected in BRs biosynthesis, such as *det2* (*de-etiolated 2*), display a constitutive photomorphogenic phenotype when grown in the dark (Li *et al*., 1996) (Figure

12). BRs are perceived by the BRI1 (BRASSINOSTEROID RECEPTOR 1) receptor, which triggers a phosphorylation cascade leading to the activation of the transcription factor BZR1 (BRASSINAZOL-RESISTANT 1) (Kim *et al*., 2010; Tang *et al*., 2011; Vert *et al*., 2011). BZR1 is phosphorylated and inactivated when BRs levels are low; conversely when BRs levels increase, the dephosphorylated BZR1 is able to bind the target genes and activate brassinosteroid response (Vert *et al*., 2006). Genome-wide analysis revealed that BZR1 binds to the promoter regions of light-regulated genes (Sun *et al*., 2010). Light and BRs signals converge on common targets through the direct interaction of BZR1 with PIF4; consistently, PIF4 and BZR1 share 50% of their targets, which they directly bind on overlapping sites of the promoters (Oh *et al*., 2012). Therefore, these transcription factors cooperate in the control of a number of their target genes, to promote cell elongation and skotomorphogenesis (Oh *et al*., 2012).

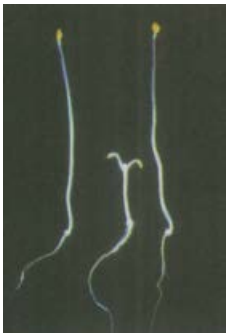


Fig. 12. Complementation of *det2* by the wild-type *DET2* gene.
Dark-grown 10 days-old seedlings. From left to right: wild-type, *det2- 1*, and the complemented line (adapted from Li *et al*., 1996).

2.3 Hypocotyl elongation: a molecular model

Hypocotyl growth is mainly due to cell expansion (Gendreau *et al.*, 1997). Hypocotyl elongation is influenced by both environmental and hormonal cues and it has been extensively studied as a model for cell expansion. Nevertheless, the molecular network underlying this process is not yet fully elucidated. Hypocotyl cell elongation is controlled by a molecular transduction pathway which mediates the hormonal and environmental cues. Indeed, the regulatory factors PIFs, BZR1, ARFs and DELLAs integrate gibberellin, brassinosteroids, auxin and light signals (Figure 13). Light antagonizes gibberellins, auxin and brassinosteroids to repress hypocotyl elongation thus promoting photomorphogenesis.

Cell elongation is synergistically promoted by PIFs, ARF6/8 and BZR1 transcription factors. These proteins interact with each other and cooperate to control expression of common target genes, among which a number of genes encoding factors involved in cell elongation (such as *EXP8*, *PREs*, *HFR1*, and more) and auxin response (*SAURs* and *PINs*) (Oh *et al.*, 2014). This transcriptional control is counteracted by RGA, which directly interacts with the ARF6 domain responsible for the ARF6-PIF4/BZR1 interaction (Oh *et al.*, 2014).

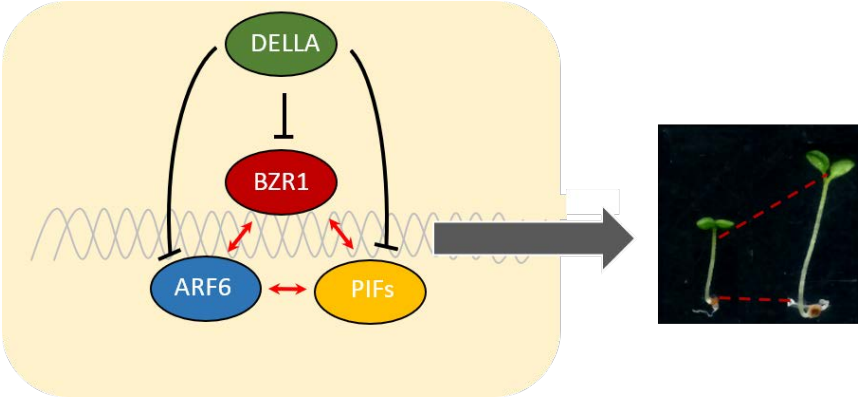


Figure 13. Diagram of the central growth regulation circuit.
The light-regulated PIF4, the auxin-regulated ARF6, and the BR-regulated BZR1 interact in a complex to cooperatively regulate common targets and promote hypocotyl cell elongation. The GA-regulated DELLA proteins interact with these factors and inhibit their DNA binding (adapted from Oh *et al.*, 2014).

3 DOF proteins

3.1 The DOF family of transcription factors

DOF (DNA binding with One Finger) proteins are a family of transcription factors with a single zinc-finger DNA-binding domain. These proteins are present only in plants; the first DOF protein was identified in maize (Yanagisawa *et al.*, 1995), and so far, DOF proteins have been identified in several species, from green unicellular algae to mosses, ferns, gymnosperms and angiosperms. The number of *DOF* genes varies from species to species, in *Arabidopsis* there are 36 genes, while in rice, barley and wheat there are respectively 30, 26 and 31 (Lijavetzky *et al.*, 2003; Moreno-Risueno *et al.*, 2007; Shaw *et al.*, 2009).

DOF transcription factors family are characterised by the DNA-binding domain, which is in the N-terminal region. The DOF domain is a highly conserved region of 52 amino acids containing the $CX_2CX_{21}CX_2C$ motif (Figure 14). Mutations in any of the four cysteines alters the zinc finger structure, abolishing DNA binding both *in vitro* and *in vivo* (Yanagisawa *et al*., 2001).

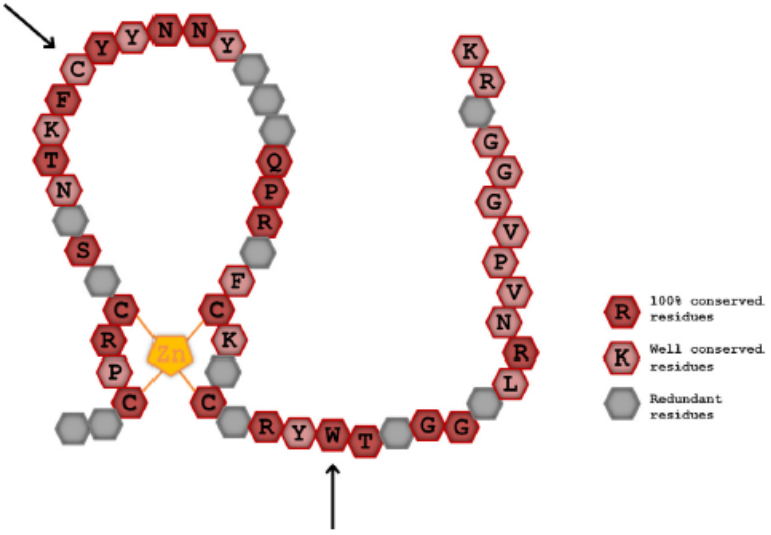


Fig. 14. Schematic representation of the DOF domain.
Conserved amino acid residues are shown in red. Well conserved residues (up to 75%) are shown in pink and less-conserved residues are shown in gray (adapted from Noguero *et al*., 2013).

The DNA consensus sequence recognized by DOF transcription factors is AAAG/CTTT. Promoters containing either a cluster of several binding motifs or a single motif are likely to be recognized by DOF proteins (Yanagisawa *et al*., 2000). Since this DNA binding motif is extremely short, specificity of the DNA binding is usually dependent on the interaction with other transcription factors. This has been demonstrated for several Dof proteins, like DAG1 which interact with the DELLA protein GAI, to directly repress *GA3ox1* (Zhang *et al*., 1995; Yanagisawa *et al*., 1997; Krohn *et al*., 2002; Wei *et al*., 2010; Boccaccini *et al*., 2014b). DOF proteins are known to be involved in many plant developmental processes, such as endosperm and seed development, seed dormancy and germination, vascular development, phytochrome signalling, flowering and more (Figure 15).

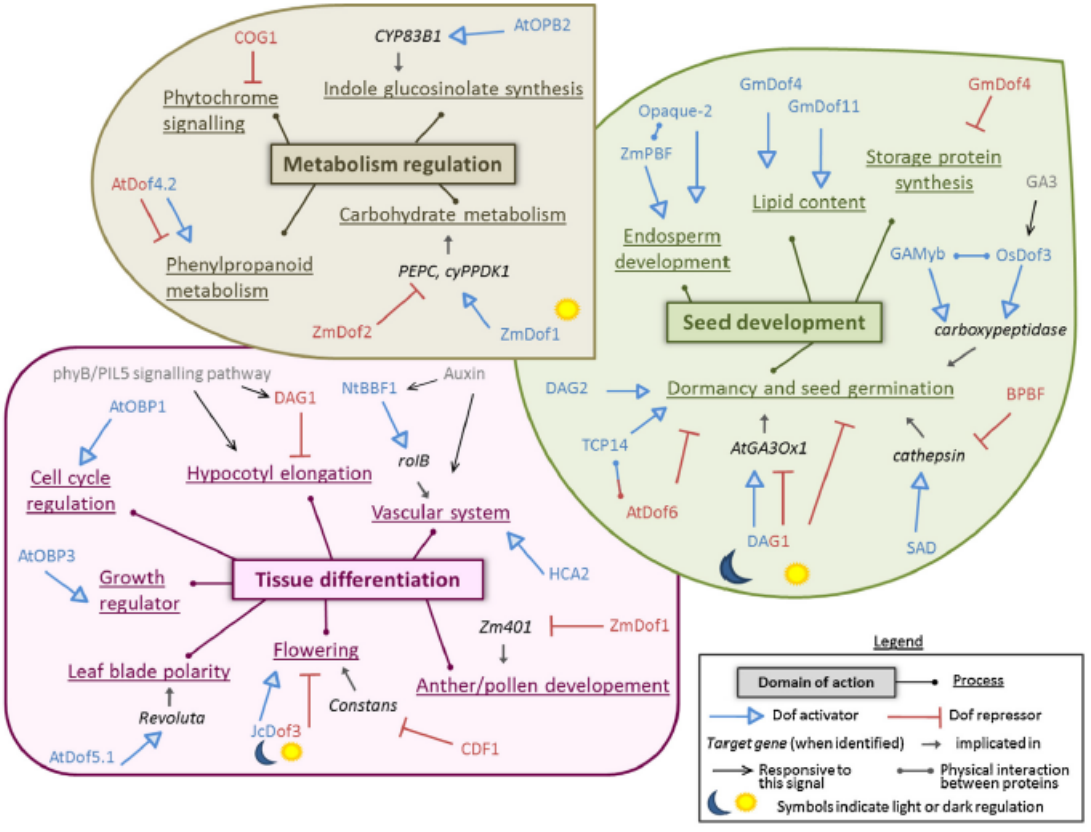


Fig. 15. Processes regulated by DOF proteins.
Examples of some processes in which DOF transcription factors are involved (adapted from Noguero *et al.,* 2013).

3.2 The *Arabidopsis* Dof proteins DAG1 and DAG2

DAG1 and DAG2 (DOF Affecting Germination 1 and 2) are two *Arabidopsis* DOF proteins closely related, showing 77% overall amino acidic identity (Figure 16). *DAG1* maps on chromosome 3 (At3g61850), whereas *DAG2* maps on chromosome 2 (At2g46590).

Analysis of the *pDAG1::GUS* and *pDAG2::GUS* transgenic lines revealed that the two promoters had very similar if not indistinguishable tissue specificities. GUS-specific staining was localized in both cases to the vascular system of the mother plant (Gualberti *et al*., 2002) (Figure 17). In addition, it was recently shown that both *DAG1* and *DAG2* are expressed during embryogenesis. GUS activity in both *pDAG1::GUS* and *pDAG2::GUS* was observed in embryos at the globular, heart, torpedo, and bent cotyledon stages. Interestingly, GUS staining was extended to all cells at the globular stage, whereas from the heart stage on it was restricted to the procambium (Boccaccini *et al*., 2014a; Santopolo *et al*., 2015).

DAG1 is expressed during seed maturation and dormancy and is controlled at the epigenetic level through the H3K27me3 mark during the seed-to-seedling transition. *DAG1* expression reach its maximum during late stage of seed maturation (13 DAP, Days After Pollination) and then its expression decreases reaching a steady low level (19 DAP) that is retained during dry storage (Boccaccini *et al*., 2016).

```
DAG2     1 MDATKWTQGLQEMMNVKPMEQIMIPNNNTHQPNTTSNARPNTILTSNGVSTAGATVSGVSNNNNNTAVVAERKARPQEKL
DAG1     1 MDATKWTQGFQEMINVKPMEQMISSTNN-NTPQ--Q---QPTFIATNTRPNATASNGGSGGNTNNTATMETRKARPQEKV

DAG2    81 NCPRCNSTNTKFCYYNNYSLTQPRYFCKGCRRYWTEGGSLRNVPVGGSSRKNKRSSSSSSSNILQTIPSSLPDLNPPILF
DAG1    75 NCPRCNSTNTKFCYYNNYSLTQPRYFCKGCRRYWTEGGSLRNVPVGGSSRKNKRSSTPLAS---P-SNPKLPDLNPPILF

DAG2   161 SNQIHNKSKGSSQDLNLLSFPVMQDQHHHHVHMSQFLQMPKMEGNGNITHQQQPSSSSSVYGSSSSPVSALELLRTGVNV
DAG1   151 SSQIPNK---SNKDLNLLSFPVMQDHHHH----------------------------------------ALELLRS----

DAG2   241 SSRSGINSSFMPSGSMMDSNTVLYTSSGFPTMVDYKPSNLSFST---DHQGLGHNSNN---RSEALHSDHHQQGRVLFPF
DAG1   184 NGVSSRGMNTFLPGQMMDSNSVLYSSLGFPTMPDYKQSNNNLSFSIDHHQGIGHNTINSNQRAQDNNDDMNGASRVLFPF

DAG2   315 GDQMKELSSSITQEVDHDDNQQQKSHGNNNNNNNSSPNNGYWSGMFSTTGGGSSWZ
DAG1   264 SD-MKELSS--TTQE---------KSHG----------NNTYWNGMFSNTGG-SSWZ
```

Figure 16. DAG1 and DAG2 Proteins Are Very Closely Related and Share an Identical DNA Binding Domain.
Alignment of the amino acid sequences of the proteins encoded by the Dof genes *DAG1* and *DAG2*. Green indicates identical amino acid residues, and red indicates conservative substitutions. The Dof domain is underlined.

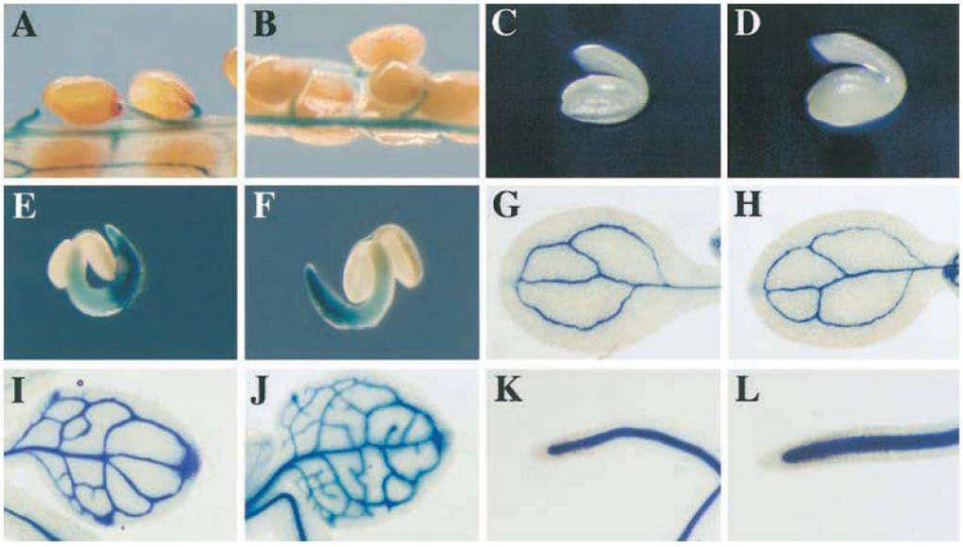


Figure 17. Expression profile of the *DAG1* and *DAG2* genes.
(A), (C), (E), (G), (I), and (K), Activity of the *DAG2::GUS* construct in siliques, mature embryo, 7-day old plantlet, cotyledon, leaf, and primary root, respectively. (B), (D), (F), (H), (J), and (L), Activity of the *DAG1::GUS* construct in siliques, mature embryo, 7-day old plantlet, cotyledon, leaf, and primary root, respectively (Gualberti *et al*., 2002).

3.3 DAG1 and DAG2 play opposite roles in seed germination

We have previously shown that lack of the DOF proteins DAG1 and DAG2 affects in opposite ways seed germination: *dag2* mutant seeds required more light and GA than wild-type seeds to germinate, whereas germination of *dag1* seeds is less dependent on these factors (Papi *et al*., 2000, 2002; Gualberti *et al*., 2002).

DAG1 is a repressor of light-mediated seed germination, acting downstream of the master repressor PIL5/PIF1 (Oh *et al*., 2004). PIL5 directly control the expression of *GAI* and *RGA*, which have been long considered functionally redundant. We have recently proved that these DELLA proteins play distinct roles in seed germination. Indeed, the germination properties of the *dag1rga28* double mutant seeds are different from those of the *dag1* and *rga28* single mutants, suggesting that RGA and DAG1 act in independent branches of the PIL5-controlled germination pathway. Surprisingly, the *dag1gai-t6* double mutant proved embryo-lethal, suggesting an unexpected involvement of (a possible complex between) DAG1 and GAI in embryo development (Boccaccini *et al*., 2014a, b).

More recently we have demonstrated that DAG1 plays a key role in the control of the developmental switch between seed dormancy and germination (Boccaccini *et al*., 2016). Indeed, DAG1 acts on ABA and GA levels to establish and maintain seed dormancy, and to repress seed germination. DAG1 negatively controls the ABA catabolic gene *CYP707A2* and the GA biosynthetic gene *GA3ox1*, through direct binding to their promoters. Consistently,

in *dag1* mutant seeds the ABA level is reduced while the level of GAs is increased (Boccaccini *et al*., 2016). Furthermore, we demonstrated that DAG1 is regulated via the ubiquitin-proteasome 26S pathway, and that GAs promote DAG1 accumulation. This suggests that GAs may stabilize DAG1 to repress their own biosynthesis during key developmental stages (Boccaccini *et al*., 2016).

As for DAG2, we have recently shown that it promotes the expression of the GA biosynthetic genes *GA3ox1* and *GA3ox2*, and it represses the GA catabolic gene *GA2ox2* (Santopolo *et al*., 2015). In addition, *DAG2* expression is repressed by PIL5, and by DAG1, which directly binds the Dof binding motif on the *DAG2* promoter (Santopolo *et al*., 2015).

3.4 *dag1* mutation affects hypocotyl elongation

Previously, we have also shown that light-grown *dag1* mutant seedlings show significant shorter hypocotyls compared to the wild-type, suggesting that DAG1 is a negative component of the light-mediated inhibition of hypocotyl elongation (Gabriele *et al*., 2010). Indeed, *dag1* mutant seedlings grown for five days in continuous red light (10 or 40 µmol m^{-2} s^{-1}) show a significant decrease in hypocotyl elongation in a fluence-dependent manner (Figure 18 A) (Gabriele *et al*., 2010). In addition, under continuous red light (40 µmol m^{-2} s^{-1}) hypocotyl length of *dag1phyB* double mutants were similar to *phyb,* indicating that *dag1* inactivation cannot revert the *phyb* long hypocotyl phenotype (Figure 18 B). This evidence

demonstrates that DAG1 acts downstream phyB in the light-mediated inhibition of hypocotyl elongation.

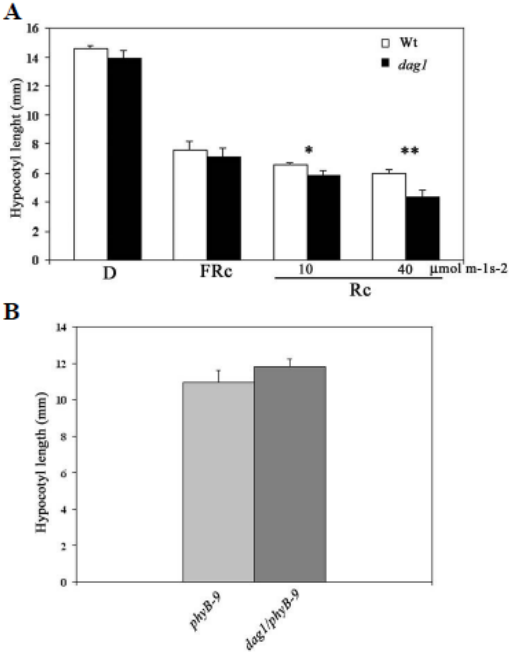


Figure 18. DAG1 is a negative component of phyB-mediated inhibition of hypocotyl elongation.
(A) Hypocotyl length of Wt and *dag1* 5-d old seedlings grown in the dark (D), continuous Far-Red (FRc) and two different intensities of continuous Red (Rc) light. (B), Hypocotyl length of *phyB-9* and *dag1phyB-9* seedlings grown in continuous R light (40 µmol m-2 s-1) (Gabriele *et al*., 2010).

AIM OF THE WORK

The *Arabidopsis* Dof protein DAG1 (Dof Affecting Germination1) is a key player of a number of light-mediated processes in plants, namely seed germination and photomorphogenesis. We have recently elucidated its role during maturation, dormancy and germination of seeds. Indeed, DAG1 controls the ratio of ABA and GA, the two phytohormones that are mainly involved in this developmental phase, with opposite roles. DAG1 acts downstream the master repressor of seed germination PIL5/PIF1, and directly represses the GA biosynthetic gene *GA3ox1* and the ABA catabolic gene *CYP707A2*.

On the other hand, the function of DAG1 in photomorphogenesis, has not yet been elucidated. It has been previously shown that *dag1* mutant seedlings grown under Red light showed shorter hypocotyls compared to the wild-type ones, suggesting that DAG1 may be either a repressor of photomorphogenesis, or a repressor of the light-mediated inhibition of hypocotyl elongation process.

The overarching goal of my PhD research project is to unveil the function of DAG1 during seedling development; in particular it will be assessed whether DAG1 is necessary for the transition from skotomorphogenesis to photomorphogenesis, or conversely, it plays a positive role only on hypocotyl growth, irrespective of light conditions.

RESULTS

1 Lack of DAG1 affects hypocotyl cell expansion

We have previously shown that loss-of-function *dag1* mutant seedlings, grown under continuous Red light, show a significant decrease of hypocotyl length (Gabriele *et al*., 2010). Since *dag1* mutant seeds germinate faster than the wild-type ones (Papi *et al*.,2000), we wondered whether this trait could affect *dag1* hypocotyl length compared to the wild-type. Therefore, we performed an analysis of *dag1* and wild-type hypocotyl growth measuring every day hypocotyl length of seedlings grown under Red light, up to five days. Two days-old *dag1* seedlings showed slightly longer hypocotyls compared to the control, possibly due to early germination of *dag1* seeds. However, at three days, hypocotyl length of *dag1* and wild-type seedlings was comparable; subsequently, at four and five days, *dag1* hypocotyls were significantly shorter than wild-type ones, indicating that the growth of *dag1* hypocotyl was severely reduced compared to the control (Figure 19 A).

It is known that most of hypocotyl cells derive from the embryo, and that hypocotyl growth is mainly due to longitudinal expansion (Gendreau *et al*., 1997). To assess whether *dag1* hypocotyl phenotype was linked to a reduced cell number or to decreased cell elongation, the number of hypocotyl epidermal cells was counted both in wild-type and *dag1* three days-old seedlings grown under Red light. The results of this analysis revealed that *dag1*

mutant hypocotyls have the same number of epidermal cells as the wild-type ones (Figure 19 B), indicating that *DAG1* inactivation affects hypocotyl cell expansion.

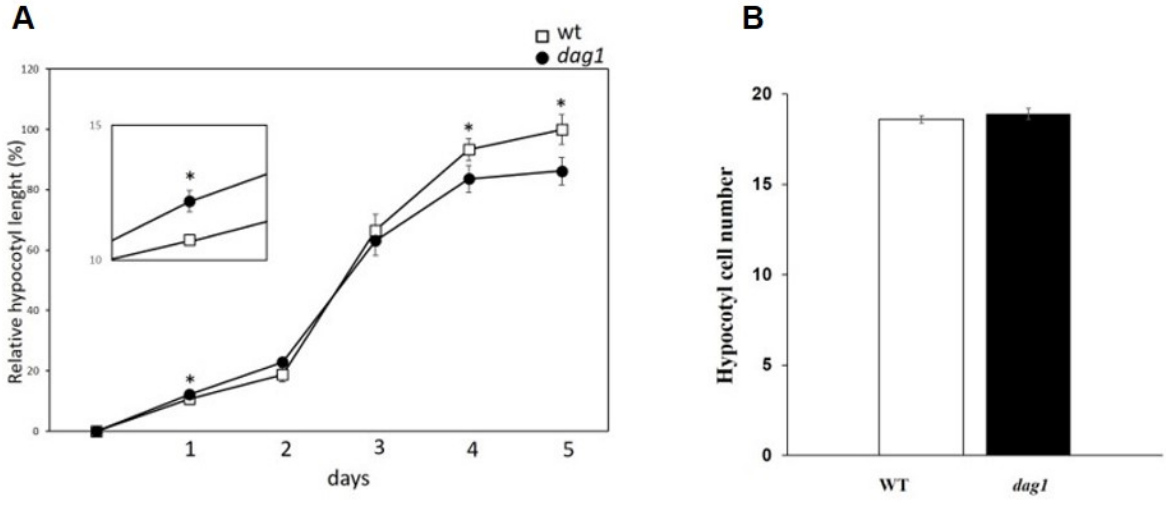


Figure 19. *DAG1* inactivation affects hypocotyl cell expansion.
(A) Hypocotyl growth of *dag1* and wild-type. Hypocotyl length of seedlings grown under Red light was measured every day up to five days. (B) Epidermal cell number of wild-type (white bar) and *dag1* (black bar) hypocotyls of three days-old seedlings. The values are the mean of three biological replicates, presented with SD values. Significant differences were analyzed by *t*-test (*P≤0.05).

2 GA biosynthesis is altered in *dag1* hypocotyls

Since DAG1 controls GA metabolism in seeds (Gabriele *et al*., 2010; Boccaccini *et al*., 2016), we wondered whether it may play a similar role also during seedling development. The action of GAs in this process is synergistic with that of brassinosteroids; therefore, we analysed *dag1* response to these two hormones for hypocotyl elongation. Exogenously applied GAs (1, 10 and 100 µM) or BRs (0.1 or 1 µM) did not complement *dag1* hypocotyl phenotype, as *dag1* hypocotyl length was significantly shorter respect to wild-type in treated or untreated seedlings to the same extent (Figure 20 A-B).

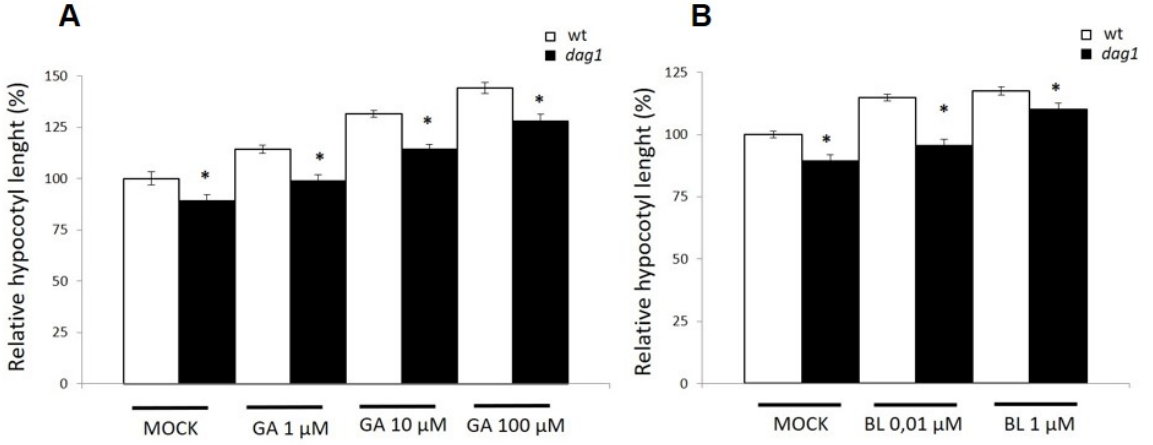


Figure 20. *DAG1* inactivation does not affect the response to exogenous GAs and BRs.
Hypocotyl length of *dag1* (black bar) and wild-type (white bar) seedlings grown under Red light for five days. (A) Seedlings treated with increasing concentration of GA (0, 1, 10 and 100 µM). (B) Seedlings treated with increasing concentration of BR (0, 0.01 and 1 µM). The values are the mean of three biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05).

Since inactivation of *DAG1* also affects GAs sensitivity in seeds (Gualberti *et al*., 2002), we measured *dag1* hypocotyl length of seedlings treated with paclobutrazol (PAC), an inhibitor of GAs biosynthesis, or with paclobutrazol and increasing GAs concentrations. Seedlings were treated with increasing concentrations of PAC (0.05, 0.25 and 0.5 µM), after a white light pulse, to prevent inhibition of germination. The differences in *dag1* hypocotyl length compared to the wild-type control, were significant only at 0.05 µM PAC, while *dag1* and wild-type hypocotyl length was similar at the highest PAC concentrations, suggesting that inhibition of GAs biosynthesis complements *dag1* hypocotyl phenotype (Figure 21A). On the other hand, when exogenous GAs was supplied, both mutant and wild-type seedlings

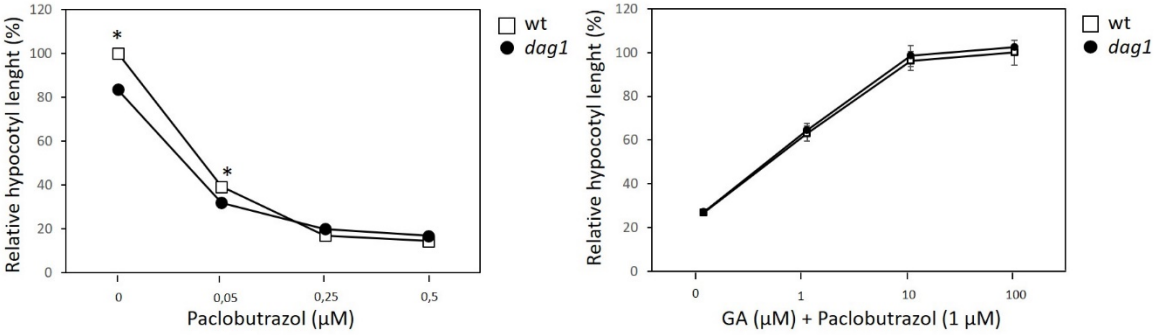


Figure 21. *dag1* mutant phenotype is complemented by the absence of endogenous GAs.
Hypocotyl length of *dag1* (black dots) and wild-type (white square) seedlings grown under Red light for five days. (A) Seedlings treated with increasing concentrations of paclobutrazol (PAC 0, 0.05, 0.25 and 0.5 µM). (B) Seedlings treated with 1 µM PAC and supplied with increasing GA concentrations (0, 1, 10 and 100 µM). The values are the mean of three biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05).

were responsive to GAs to the same extent, indicating that GAs signaling is not altered (Figure 21B). Therefore we focused our attention on GAs metabolism and we analysed the expression of GAs metabolic genes in *dag1* dissected hypocotyls of Red light-grown seedlings. In agreement with *dag1* hypocotyl phenotype, we observed a decreased expression of the GA biosynthetic gene *GA3ox1* and an increased expression in the catabolic gene *GA2ox2*, thus suggesting that GA levels are lower with respect to wild-type hypocotyls (Figure 22).

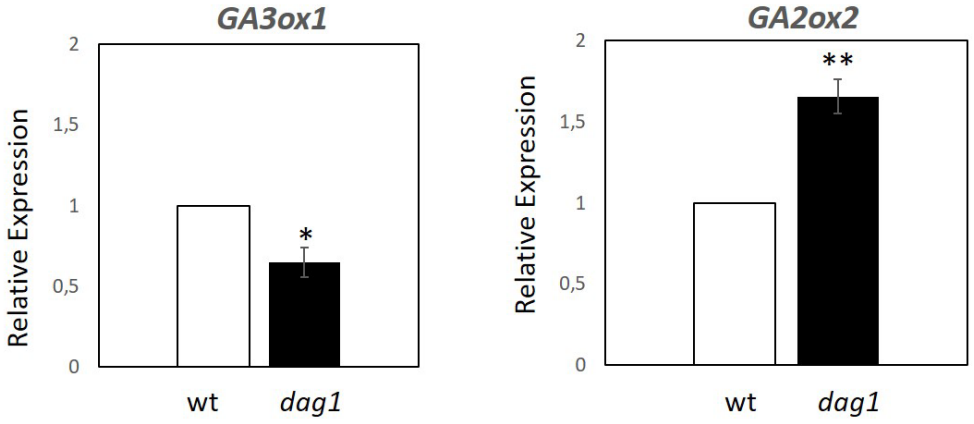


Figure 22. *DAG1* inactivation affects the expression of GA metabolic genes.
Relative expression level of the GA metabolic genes *GA3ox1* and *GA2ox2* in wild-type (white bar) and *dag1* (black bar) dissected hypocotyls of five days-old seedlings grown in Red light. The values are the mean of three biological replicates, presented with SD values. Significant differences were analyzed by *t*-test (*P≤0.05; **P≤0.01).

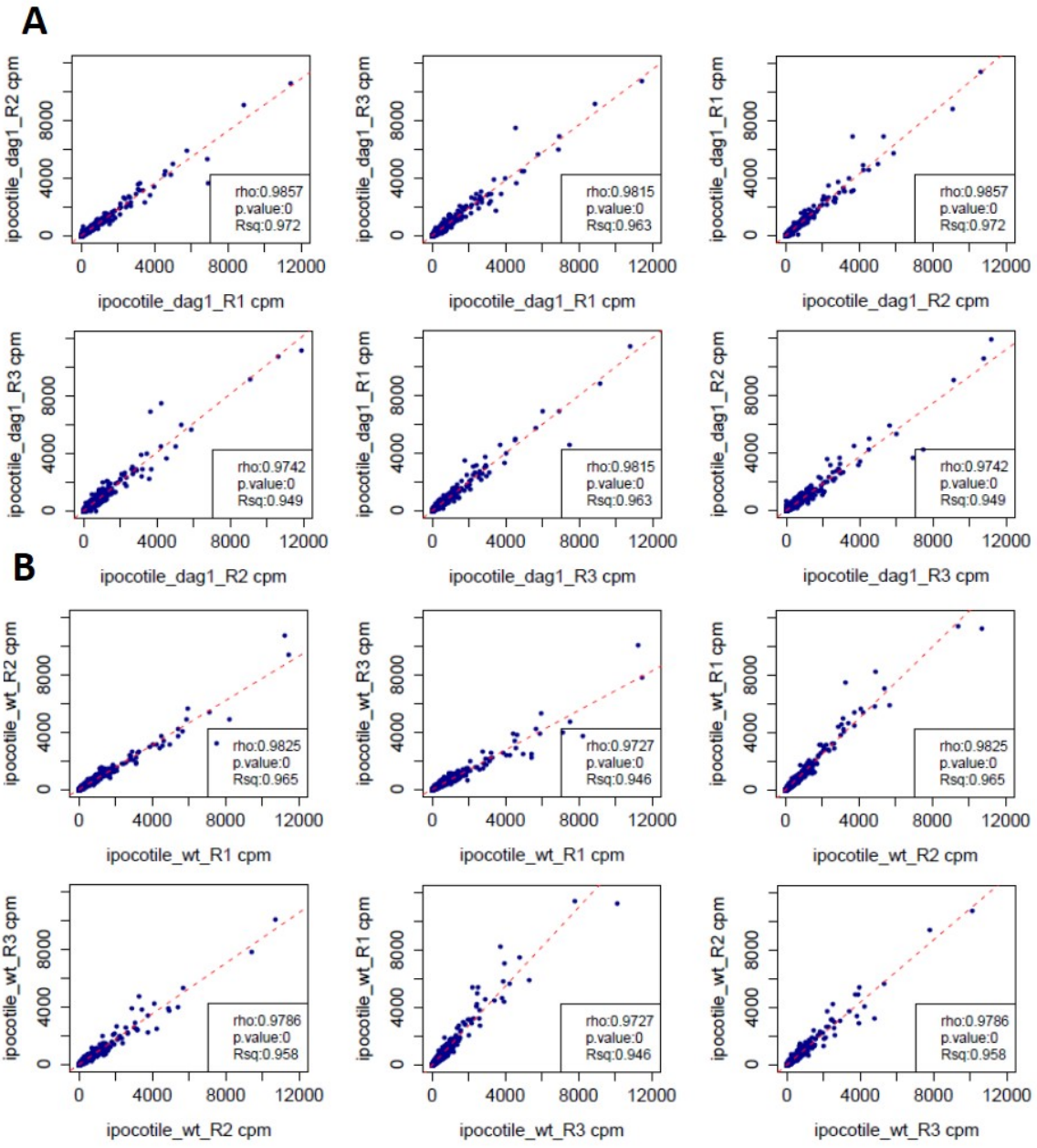
3 Lack of DAG1 severely alters the expression profile in hypocotyls

Although we have previously shown that *DAG1* inactivation does not affect cotyledon expansion (Gabriele *et al*., 2010), we wondered whether DAG1 might be involved in other molecular mechanisms underlying photomorphogenesis.

Therefore, to further elucidate the role of DAG1 in the control of hypocotyl growth, and possibly in other photomorphogenic processes, a transcriptome-wide differential gene expression analysis has been performed by high-throughput RNA sequencing. We performed RNA-seq analysis of 4 days-old *dag1* and wild-type hypocotyls and whole seedlings, grown under continuous Red light. Three biological replicates of each sample were sequenced using the Illumina Hi-seq platform. For each sample, more than 90% of reads successfully mapped to unique regions of the *Arabidopsis thaliana* genome (TAIR10) (Table 2). A very high positive correlation (Pearson correlation coefficient > 0.95) was observed among the replicates of each sample (Figure 23 A-D).

Sample	Tot Reads	% Uniquely Mapped	% Multi Mapped	Unmapped
wt 1 (pt)	18967605	92	7,25	0,75
dag1 1 (pt)	27654782	91	7,9	1,08
wt 1.2 (pt)	25388619	91,25	7,88	0,87
dag1 1.2 (pt)	29184780	91,06	7,74	1,2
wt 1.3 (pt)	21427524	91,98	6,96	1,05
dag1 1.3 (pt)	24563037	91,92	6,84	1,24
wt 1 (hp)	19885967	90,04	5,82	4,05
dag1 1 (hp)	19617630	92,35	6,24	1,4
wt 1.2 (hp)	31727863	93,48	5,42	1,1
dag1 1.2 (hp)	31503405	93,22	5,43	1,36
wt 1.3 (hp)	24355880	92,7	6,52	0,78
dag1 1.3 (hp)	20956766	92,72	5,8	1,47

Table 2. Reads mapped to unique regions of the *Arabidopsis thaliana* genome.


A
ipocotile_dag1_R2 cpm
ipocotile_dag1_R1 cpm
rho:0.9857
p.value:0
Rsq:0.972
ipocotile_dag1_R3 cpm
ipocotile_dag1_R1 cpm
rho:0.9815
p.value:0
Rsq:0.963
ipocotile_dag1_R1 cpm
ipocotile_dag1_R2 cpm
rho:0.9857
p.value:0
Rsq:0.972
ipocotile_dag1_R3 cpm
ipocotile_dag1_R2 cpm
rho:0.9742
p.value:0
Rsq:0.949
ipocotile_dag1_R1 cpm
ipocotile_dag1_R3 cpm
rho:0.9815
p.value:0
Rsq:0.963
ipocotile_dag1_R2 cpm
ipocotile_dag1_R3 cpm
rho:0.9742
p.value:0
Rsq:0.949
B
ipocotile_wt_R2 cpm
ipocotile_wt_R1 cpm
rho:0.9825
p.value:0
Rsq:0.965
ipocotile_wt_R3 cpm
ipocotile_wt_R1 cpm
rho:0.9727
p.value:0
Rsq:0.946
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ipocotile_wt_R2 cpm
rho:0.9825
p.value:0
Rsq:0.965
ipocotile_wt_R3 cpm
ipocotile_wt_R2 cpm
rho:0.9786
p.value:0
Rsq:0.958
ipocotile_wt_R1 cpm
ipocotile_wt_R3 cpm
rho:0.9727
p.value:0
Rsq:0.946
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ipocotile_wt_R3 cpm
rho:0.9786
p.value:0
Rsq:0.958
0
4000
8000
12000

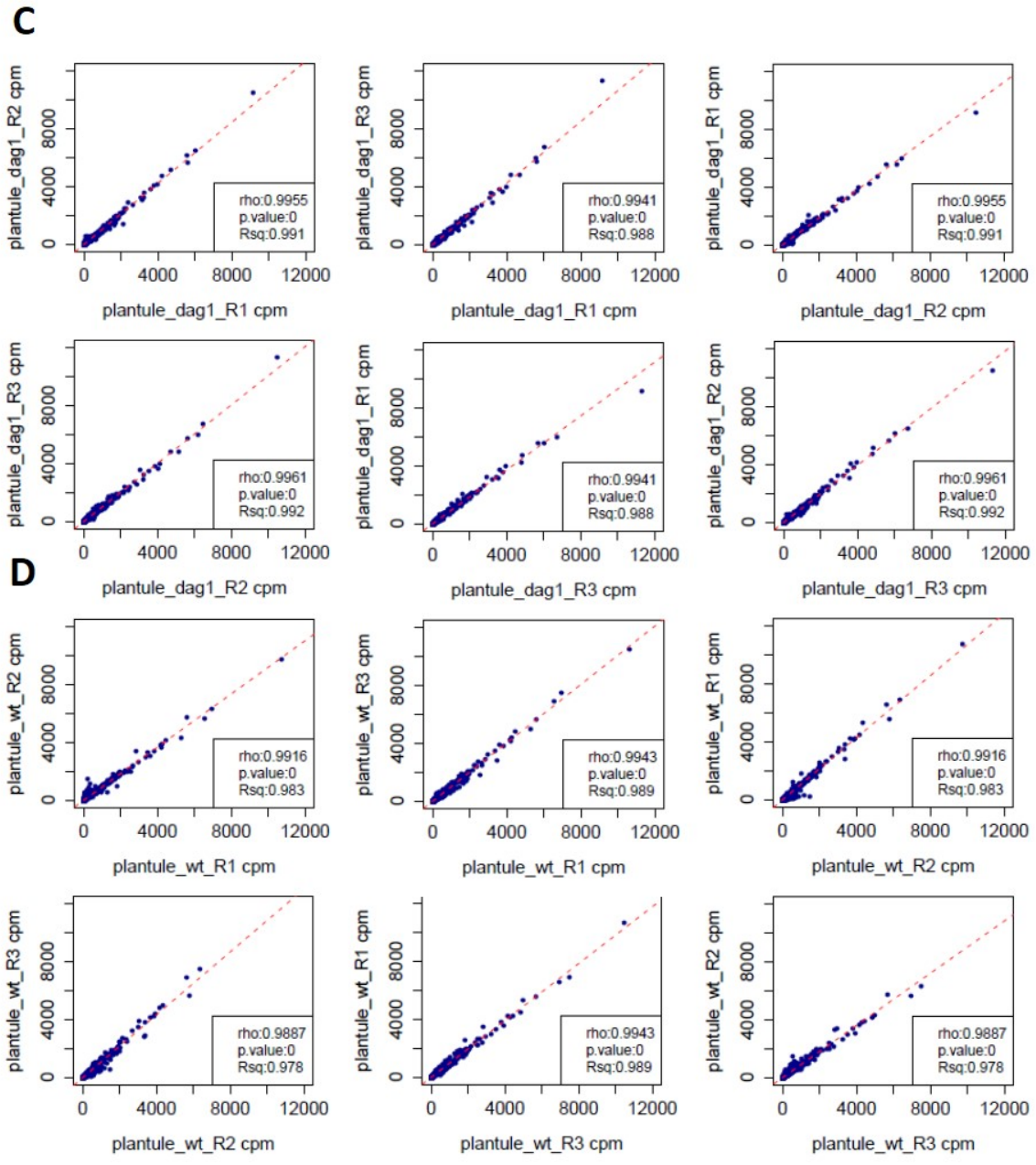
C
plantule_dag1_R2 cpm
plantule_dag1_R1 cpm
rho:0.9955
p.value:0
Rsq:0.991
plantule_dag1_R3 cpm
plantule_dag1_R1 cpm
rho:0.9941
p.value:0
Rsq:0.988
plantule_dag1_R1 cpm
plantule_dag1_R2 cpm
rho:0.9955
p.value:0
Rsq:0.991
plantule_dag1_R3 cpm
plantule_dag1_R2 cpm
rho:0.9961
p.value:0
Rsq:0.992
plantule_dag1_R1 cpm
plantule_dag1_R3 cpm
rho:0.9941
p.value:0
Rsq:0.988
plantule_dag1_R2 cpm
plantule_dag1_R3 cpm
rho:0.9961
p.value:0
Rsq:0.992
D
plantule_wt_R2 cpm
plantule_wt_R1 cpm
rho:0.9916
p.value:0
Rsq:0.983
plantule_wt_R3 cpm
plantule_wt_R1 cpm
rho:0.9943
p.value:0
Rsq:0.989
plantule_wt_R1 cpm
plantule_wt_R2 cpm
rho:0.9916
p.value:0
Rsq:0.983
plantule_wt_R3 cpm
plantule_wt_R2 cpm
rho:0.9887
p.value:0
Rsq:0.978
plantule_wt_R1 cpm
plantule_wt_R3 cpm
rho:0.9943
p.value:0
Rsq:0.989
plantule_wt_R2 cpm
plantule_wt_R3 cpm
rho:0.9887
p.value:0
Rsq:0.978

Figure 23. Correlation among the three biological replicates.
The Scatter plot shows a strong linear correlation between biological replicates of *dag1* (A, C) and wild-type (B, D) samples from dissected hypocotyls (A, B) and seedlings (C-D).

Four comparison groups were constructed: i) wild-type hypocotyls vs wild-type plantlets (WT hp/pt), ii) *dag1* hypocotyls vs dag1 plantlets (*dag1* hp/pt), iii) *dag1* vs wild-type hypocotyls (hp *dag1*/WT), and iv) *dag1* vs wild-type plantlets (pt *dag1*/WT). Results of differential expression analysis are shown in Figure 24A, where genes are reported if they show significant expression change in at least one comparison group.

The comparison of wild-type hypocotyls and plantlets (WT hp/pt) revealed 8724 differentially expressed genes. Of these, 4308 were up-regulated and 4416 were down-regulated in hypocotyls compared to plantlets. In the comparison group of the mutated counterparts (*dag1 hp/pt*), the number of differentially expressed genes (DEGs) increases to 9021, evenly distributed between up- (4456) and down-regulated genes (4565).

As for the comparison of *dag1* and wild-type hypocotyls (hp *dag1*/WT), 257 DEGs were identified. Of these, the majority (225) shows up-regulated expression, consistently with the known function of DAG1 as a repressor (Gabriele *et al*., 2010; Boccaccini *et al*., 2014, 2016). Instead, an opposite trend is observed in plantlets (pt *dag1*/WT) where most of the DEGs (105 out of 149) shows down-regulated expression (Figure 24B), suggesting that DAG1 activity is primarily involved in hypocotyl elongation, consistently with the *dag1* mutant phenotype.

As expected, *DAG1* is among the severely down-regulated genes in the *dag1* transcriptome of both hypocotyls and plantlets compared to the wild-type corresponding controls, in agreement with the fact that *dag1* is a knock out mutant (Papi *et al.*, 2000).

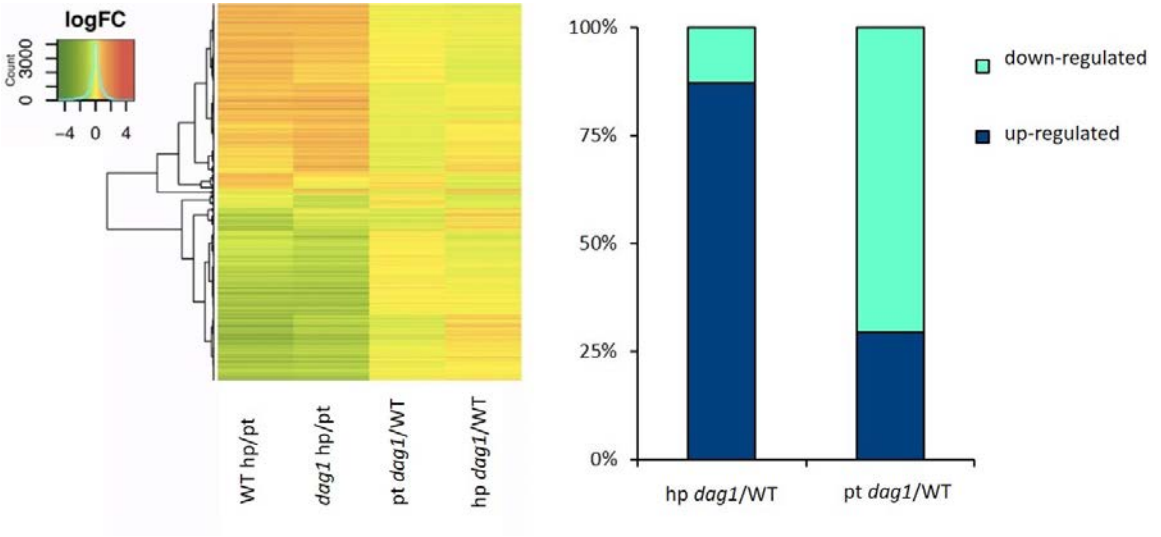

Figure 24. Differentially expressed genes in *dag1* and wild-type hypocotyls and seedlings. (**A**) Heatmap of DE genes in at least one comparison group, Red: up-regulated genes, green: down-regulated gene. log2 fold change reported for statistically significant DE genes. (**B**) Up- and down-regulated genes in the hp *dag1*/WT and pt *dag1*/WT comparison groups.

4 Gene Ontology (GO) enrichment analysis of DE genes

To verify whether DAG1 is primarily involved in hypocotyl elongation, we performed a Gene Ontology (GO) enrichment analysis on the DE genes of the four comparison groups by means of the AgriGO method. The results of this analysis are reported in a heatmap ordered for the p-value of the hp *dag1*/WT group, and clearly show that the most significant differences are in this comparison group, therefore suggesting that DAG1 is mainly involved in hypocotyl development (Figure 25). Therefore to further elucidate the function of DAG1 in the hypocotyl elongation process, we performed a Gene Ontology (GO) enrichment analysis on the DE genes of the hp *dag1*/WT comparison group. This analysis revealed that 21% of DEGs belong to the GO category of hormone stimulus, which comprises genes involved in abscisic acid, auxin, ethylene and jasmonic acid response (Figure 26). Among DE genes belonging to this category, 32% of targets are exclusively involved in response to ABA stimulus, 20% to ethylene stimulus, 14% to auxin stimulus and at last 18% to jasmonic acid stimulus. 16% of the remaining targets are responsive to more hormones (Figure 26).

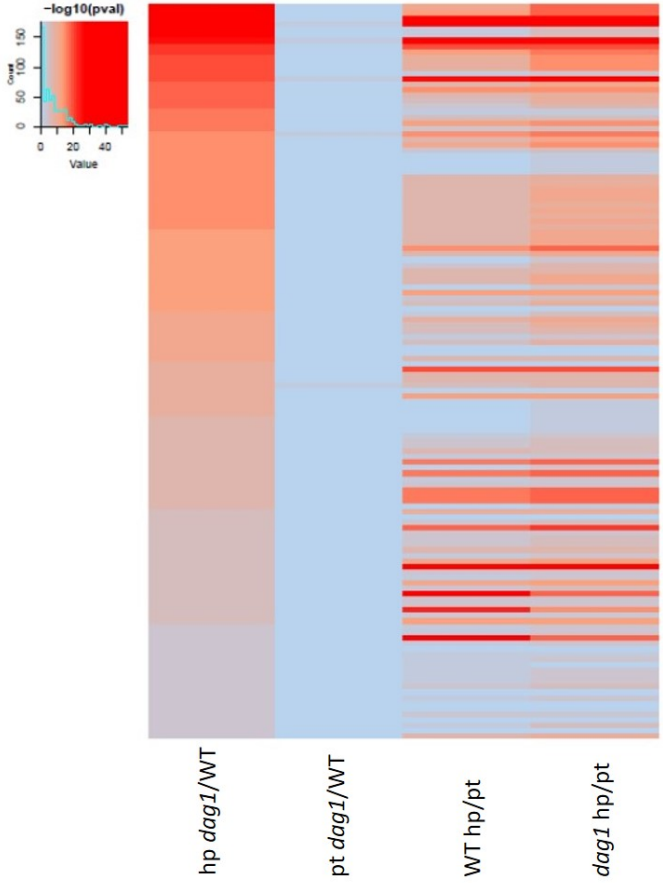

Figure 25. GO

Heatmap of GO enrichment analysis, based on the statically significant categories in at least one comparison group. Red: statistical significant enriched category, the intensity reflects the statistical significance.

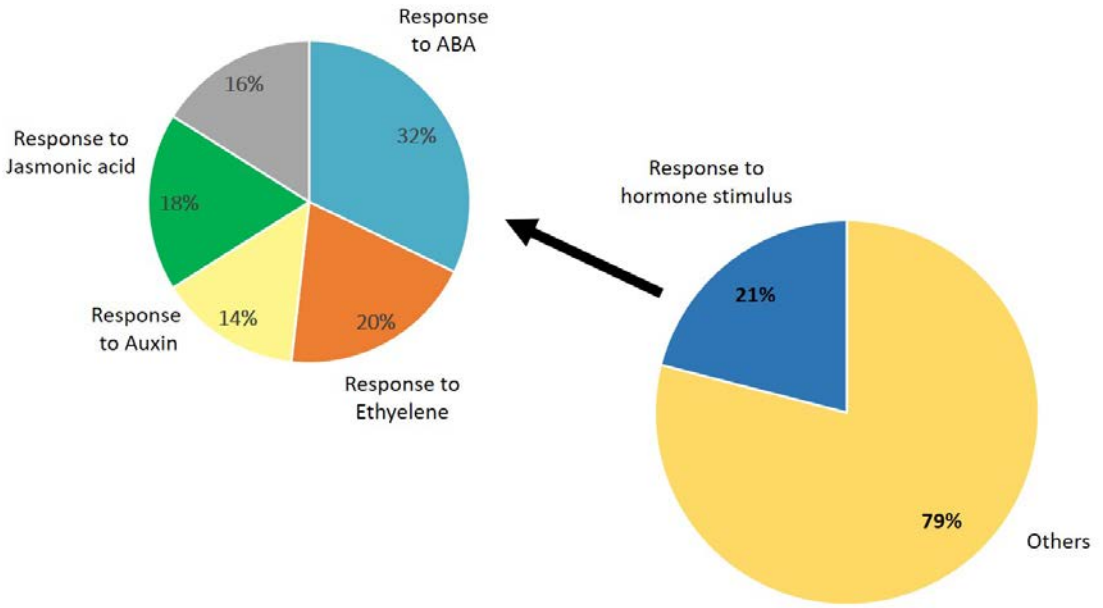

Figure 26. GO term enrichment analysis.

GO enrichment analysis was performed with AgriGO tool. In the two-coloured pie chart (on the right) is reported the percentage of targets falling in the GO category "response to hormone stimulus" (blue). In the five-coloured pie chart (on the left) is shown the percentage of targets belonging to ABA (light blue), ethylene (orange), auxin (yellow) or jasmonic acid (green) response, in grey is shown the percentage of targets responding to more than one of these hormones.

5 Analysis of DEGs showing opposite gene expression changes across conditions/groups

Our transcriptome analysis revealed some clusters of genes showing a significant expression change in different comparison groups as well as a different direction in their change (Fig. 24B).

As it can be observed in Figure 27A, several DEGs are shared between wild-type and *dag1* hp/pt comparison groups. Most of them exhibit a similar behaviour, i.e. they are either up- (3468 genes) or down-regulated (3614 genes) in both WT and *dag1* hp/pt groups. Instead, 21 DEGs are up-regulated in *dag1* hp/pt and down-regulated in the wild-type counterpart. Among these, eight are seed-specific genes (*M17*, *EM6*, At2g23110, At3g17520, At3g53040, *PAP85*, *CRA1* and *SESA5*); *M17, EM6,* At2g23110, At3g17520 and At3g53040 encode for Late Embryogenesis Abundant Proteins (Dure *et al*., 1981), while *PAP85*, *CRA1* and *SESA5* are known as seed storage proteins coding genes (North *et al*., 2010). On the other hand, 6 DE genes showed the opposite trend, being down-regulated in *dag1* hp/pt and up-regulated in the wild-type group and, as expected, *DAG1* was among these genes.

A similar analysis on the hp *dag1*/WT and pt *dag1*/WT groups revealed a set of DE genes shared between the two groups. In particular, 9 genes are up- and 4 down-regulated in both cases (Figure 27B). In addition, 7 DEGs result to be up-regulated in hp *dag1*/WT while they are down-regulated in pt *dag1*/WT. Consistent with the previous analysis, *M17* and

CRA1 were among these genes. On the other hand, only the *microRNA167* (*miR167*) gene was down in the hp *dag1*/WT and up-regulated in the pt *dag1*/WT groups.

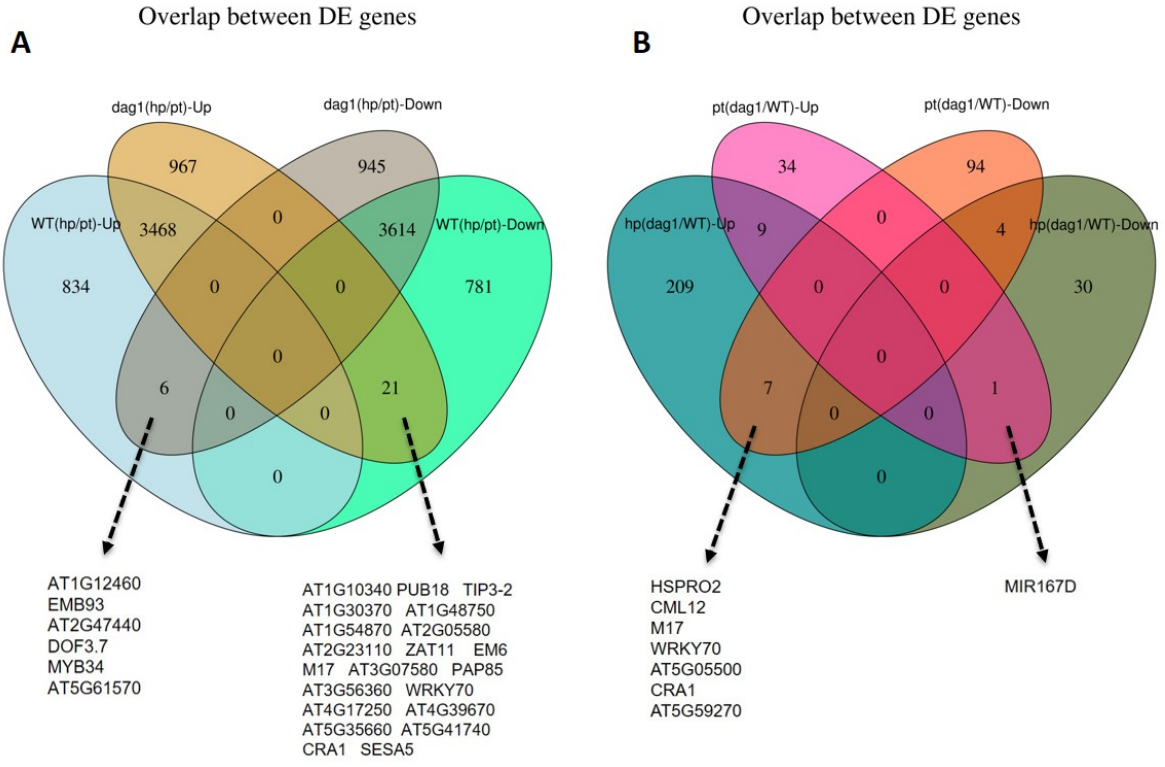

Figure 27. Venn diagram for the up- and down-regulated genes.
(A) Venn diagram of the *dag1* and WT hp/pt comparison groups. (B) Venn diagram of the *dag1*/WT hp and *dag1*/WT pt comparison groups.

6 DAG1 positively regulates the *SAUR* genes

Among the DE genes deregulated in *dag1* compared to wild-type hypocotyls, we have identified a number of genes that are known to be involved in cell elongation.

In particular, light-grown *dag1* hypocotyls showed a decreased expression level of a number of *Arabidopsis Small Auxin Up RNA* (*SAUR*) genes - namely *SAUR50*, *SAUR63*, *SAUR65*, and *SAUR67* – which have been shown to promote hypocotyl elongation (Chae *et al*., 2012; Sun *et al*., 2016). To validate this data we performed RT-qPCR analysis on RNA extracted from *dag1* and wild-type hypocotyls obtained in the same conditions of the RNA-seq experiments. The expression analysis confirmed the data of the RNA-seq, as the transcript level of the *SAUR* genes was significantly decreased in *dag1* hypocotyls compared to the control (Figure 28), thus proving that DAG1 promotes hypocotyl growth also through induction of these *SAUR* genes.

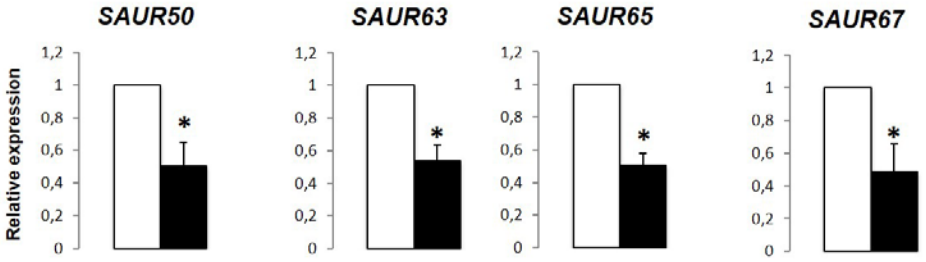

Figure 28. *DAG1* inactivation affects expression of the *SAUR* genes.
Relative expression level of *SAUR* genes in wild-type (white bar) and *dag1* (black bar) dissected hypocotyls of four days-old seedlings grown in Red light. The values are the mean of three biological replicates, presented with SD values. Significant differences were analyzed by *t*-test (*P≤0.05).

7 DAG1 negatively controls seed-specific genes during vegetative development

We have previously shown that DAG1 is a key component of the molecular network controlling the seed-to-seedling transition (Boccaccini *et al*., 2016). Interestingly, our genome-wide analysis revealed a number of DE genes in the hp *dag1*/WT comparison group (7%) whose function is associated with seed dormancy, seed storage, as well as with ABA response in seeds.

Since inactivation of *DAG1* also affects embryo development (Boccaccini *et al*., 2014), we have validated the results on the genes encoding LEA proteins, namely *EM6*, *LEA18*, *M10*, *M17*, At2g23110, At3g17520 and At3g53040. The expression analysis, performed by RT-qPCR on *dag1* and wild-type hypocotyls obtained in the same conditions of the RNA-seq experiments, confirmed that DAG1 is necessary to repress the expression of these LEA encoding genes, as in *dag1* mutant hypocotyls their expression was significantly and sharply increased (Figure 29).

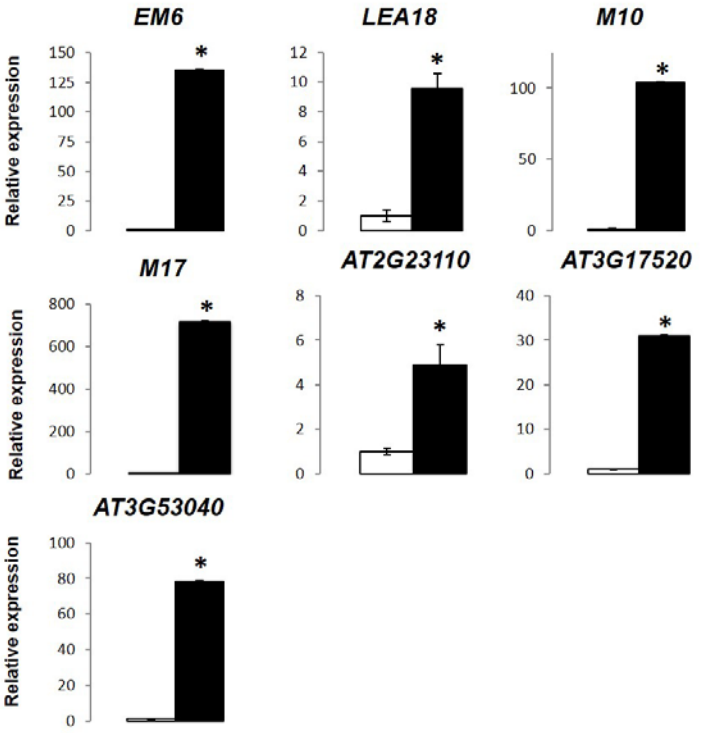

Figure 29. *DAG1* inactivation affects the expression of *LEA* genes.

Relative expression level of *LEA* genes in wild-type (white bar) and *dag1* (black bar) dissected hypocotyls of four days-old seedlings grown in Red light. The values are the mean of three biological replicates, presented with SD values. Significant differences were analyzed by *t*-test (*P≤0.05).

8 Lack of DAG1 affects expression of WRKY transcription factors

The genes differentially expressed in the absence of DAG1 are probably due both to direct and indirect effect of DAG1. Among the DE genes in the *dag1*/WT hp we have identified 27 transcription factors encoding genes, belonging to 9 different families (Table 3).

Name	Locus
WRKY 6	AT1G62300
WRKY18	AT4G31800
WRKY28	AT4G18170
WRKY33	AT2G38470
WRKY40	AT1G80840
WRKY46	AT2G46400
WRKY49	AT5G43290
WRKY70	AT3G56400
PLATZ	AT2G12646
PLATZ	At5g46710
bHLH38	AT3G56970
bHLH39	AT3G56980
bHLH100	AT2G41240
HSFC1	AT3G24520
HSFA4A	AT4G18880
ANAC062	AT3G49530
ERF2	AT5G47220
ERF5	AT5G47230
ERF6	AT4G17490
ERF104	AT5G61600
ERF105	AT5G51190
ERF109	AT4G34410
ABR1	AT5G64750
DREB1C	AT4G25470
DREB1B	AT4G25490
ZAT12	AT5G59820

Table 3. DE transcription factors encoding genes.
List of transcription factors identified in DE genes in the hp *dag1*/WT comparison group.

The most represented transcription factors belong to the WRKY and the ERF (Ethylene Response Factor) families (8 and 7 members respectively).

In particular, the WRKY transcription factors have been shown to be key regulators of many plant processes, including the responses to abiotic stresses, ABA-mediated responses as well as dormancy and germination of seeds (Rushton et al., 2012). Since DAG1 plays a pivotal role in the establishment of seed dormancy and in repressing seed germination, by modulating both ABA and GA level, we set to validate these results by RT-qPCR assays. We analysed the expression of *WRKY6*, *WRKY18*, *WRKY33*, *WRKY40*, *WRKY46* and *WRKY70* in *dag1* and wild-type hypocotyls obtained in the same conditions of the RNA-seq experiments. These results confirmed that lack of DAG1 results in a significant increased expression of these *WRKY* genes (Figure 30).

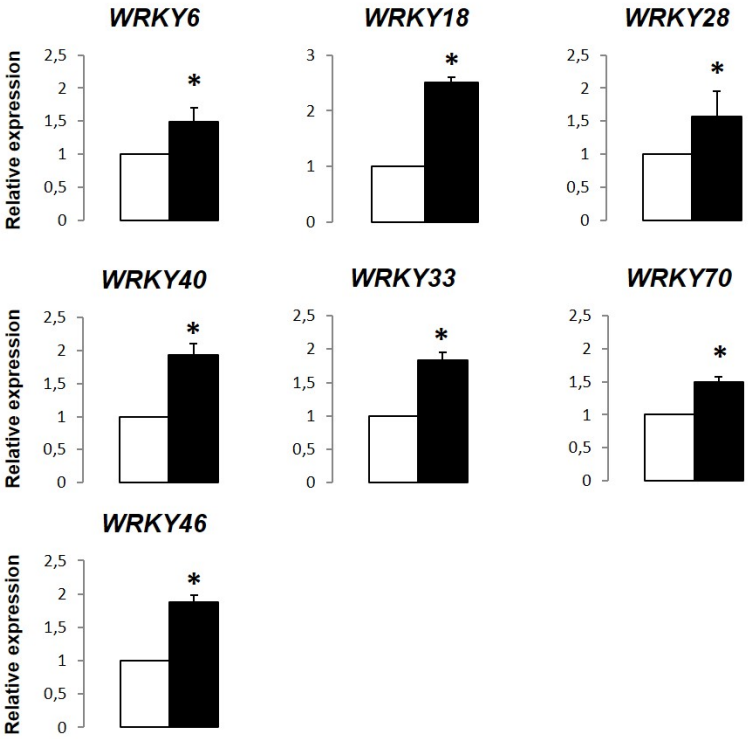

Figure 30. ***DAG1*** **inactivation affects the expression of** ***WRKY*** **genes.**
Relative expression level of *WRKY* genes in wild-type (white bar) and *dag1* (black bar) dissected hypocotyls of five days old seedlings grown in Red light. The values are the mean of three biological replicates, presented with SD values. Significant differences were analyzed by *t*-test (*P≤0.05).

9 The promoter of DE genes is significantly enriched in G-box

DAG1, a Dof transcription factor, is known to bind the CTTT sequence on the promoter of target genes (De Paolis *et al*., 1996; Gabriele *et al*., 2010; Boccaccini *et al*., 2016). Therefore, we analysed whether the promoters of DE genes in the hp *dag1*/WT comparison group, were enriched in the DOF binding sites. Unfortunately, since the Dof binding site is very short, it was difficult to assess the putative direct targets of DAG1. Therefore, we analysed, by means of Promomer (http://www.bar.utoronto.ca/ntools/cgi-bin/BAR_Promomer.cgi), the promoters of the DE genes we have previously validated. This analysis revealed that the promoters of *WRKY18*, *SAUR67* and *M17* showed a significant number of Dof binding sites, suggesting that they are likely to be direct targets of DAG1.

To investigate the function of DAG1 in hypocotyl development and to gain some insight into the molecular network in which DAG1 is involved, we searched for the presence of any sequence significantly over-represented in the upstream regions (500 bp) of the 257 DE genes in the hp *dag1*/WT comparison group, by using the MEME tool (Bailey *et al*., 2009). Two sequences were identified, and subsequent analysis with the Tomtom Motif Comparison Tool (Gupta *et al*., 2007) matched only the MYC/PIF binding motif (CACGTG) (Figure 31). The MYC/PIF binding site is present in 84 targets, among which *SAUR*, *LEA*, *ERF* and other targets.

Interestingly, the *MYC2*, *MYC3* and *MYC4* genes were among the DE genes up-regulated in the *dag1* hp/pt group, suggesting a molecular relationship between DAG1 and these transcription factors in hypocotyl development.

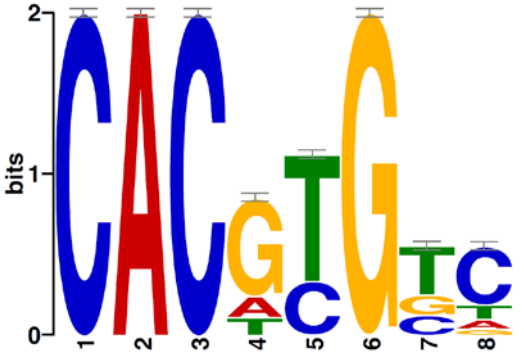

Figure 31. PIF binding motif identified with MEME tool.
Meme motif search identified PIF/MYC-binding motif, defined as G-box (CACGTG).

10 A number of Dof transcription factors are differentially expressed in the hypocotyl

DAG1, similarly to the majority of the *Arabidopsis* DOF proteins, is expressed in the vascular tissue (Papi *et al.*, 2002; Le Hir and Bellini, 2013). To assess whether any DOF protein was differentially expressed in hypocotyl, we analysed DE genes in both the *dag1* and WT hp/pt group, searching for DOF proteins. Fifteen of the 36 *Arabidopsis DOF* genes were deregulated in wild-type hypocotyls respect to seedlings. Ten of these were up-regulated, while five were down-regulated (Table 4).

It should be noted that *DAG1* was among the DE genes up-regulated, according with *DAG1* expression profile previously shown (Gualberti *et al*., 2002). The same analysis performed on the *dag1* hp/pt group revealed that two additional DOF genes, which are phylogenetically related (http://www.pantherdb.org/treeViewer/treeViewer.) were up-regulated in the absence of DAG1 (At1g64620 and At5g60200).

Ip/Pl WT		Ip/Pl *dag1*	
Dof	**Locus/Name**	**Dof**	**Locus/Name**
Dof1.4	At1g28310 ↑	**Dof1.6**	At1g47655 ↑
Dof1.6	At1g47655 ↑	**Dof1.7**	At1g51700 ↑
Dof1.7	At1g51700 ↑	**Dof1.8**	At1g64620 ↑
Dof2.4	At2g37590 ↑	**Dof2.4**	At2g37590 ↑
Dof3.1	At3g21270 ↑	**Dof3.4**	At3g50410/OBP1 ↑
Dof3.4	At3g50410/OBP1 ↑	**Dof4.6**	At4g24060 ↑
Dof3.7	At3g61850/DAG1 ↑	**Dof5.3**	At5g60200 ↑
Dof4.6	At4g24060 ↑	**Dof5.4**	At5g60850/OBP4 ↑
Dof5.4	At5g60850/OBP4 ↑	**Dof5.6**	At5g62940 ↑
Dof5.6	At5g62940 ↑	**Dof1.1**	At1g07640/OBP2 ↓
Dof1.1	At1g07640/OBP2 ↓	**Dof2.2**	At2g28810 ↓
Dof2.2	At2g28810 ↓	**Dof3.7**	At3g61850 / DAG1 ↓
Dof4.1	At4g00940 ↓	**Dof4.1**	At4g00940 ↓
Dof5.1	At5g02460 ↓	**Dof5.1**	At5g02460 ↓
Dof5.7	At5g65590 ↓	**Dof5.7**	At5g65590 ↓

Table 4. DOF genes differentially expressed in hypocotyls.
List of DOF transcription factors in hp/pl comparison group in wt and *dag1* samples. Red = up-regulated, blue = down-regulated.

11 ABA inhibits hypocotyl cell expansion in etiolated and de-etiolated seedlings

DAG1 was shown to repress seed germination through the control of ABA metabolism (Boccaccini *et al*., 2016); since the genome-wide analysis of *dag1* hypocotyls and seedlings, revealed a significant enrichment in GO terms related to ABA response, we wondered whether ABA could play a role also during seedling development. In agreement with our genome-wide results, it has been previously shown that - within 60 hours of light exposure of seeds (induction of germination)- application of ABA arrests seedling growth, suggesting that ABA restrains growth also after the germination process (Lopez-Molina *et al*., 2001).

As schematized in Figure 32A, to investigate the effect of ABA on hypocotyl elongation we measured hypocotyl length of six days-old-seedling supplied with ABA 48 and 72 hours after induction of germination. In the former case, seedlings were grown in the presence of increasing ABA concentrations (1, 10, 100 µM) for four days, and we observed a strong decrease in hypocotyl length - corresponding to 60, 51 and 27% of mock treated controls - as well as clear signs of growth arrest, as shown in Figures 32B. In the latter case, seedlings were grown in the presence of ABA (1, 10, 100 µM) for 3 days, and we observed a significant reduction in hypocotyl elongation - corresponding to 73, 62 and 55% of mock-treated controls - as shown in Figure 32A. This slightly lower inhibition of hypocotyl elongation is possibly a consequence of the longer ABA-free growth (24 hours) of the seedlings as well as the fact that no growth arrest was observed.

To avoid overlap of the effect of ABA on growth arrest with its specific effect on hypocotyl growth, all subsequent assays were performed adding ABA 60 hours after the induction of germination. As shown in Figure 32C, hypocotyl length of red light-grown (photomorphogenesis) seedling decreased, respectively, to 84, 62 and 32% that of controls at 1, 10 and 100 µM ABA, respectively. As shown in Figure 32D, also dark-grown (skotomorphogensis) seedlings were responsive to ABA, as hypocotyl length resulted 80, 74 and 60% that of controls at the three ABA concentrations. To further substantiate the effect of ABA on hypocotyl growth, we compared the hypocotyl length of dark-grown wild-type and ABA biosynthetic mutant *aba2-1* (Leon-Kloosterziel *et al*., 1996). This analysis showed that the hypocotyls of the *aba2-1* mutant were substantially longer (121%) than the controls (Figure 32E), thus corroborating the notion that ABA inhibits hypocotyl growth. As shown in Figure 32F, *aba2-1* mutant hypocotyls showed the same number of epidermal cells as wild-type ones, indicating that ABA inhibits cell expansion and not cell division (Figure 32F).

To rule out that the hypocotyl phenotype of the *aba2-1* mutant was not due to earlier germination, we measured the respective germination rates of *aba2-1* and wild-type seeds up to five days. The germination assay clearly revealed that under our conditions there was not a significant difference between *aba2-1* and wild-type germination rates (Figure 32G).

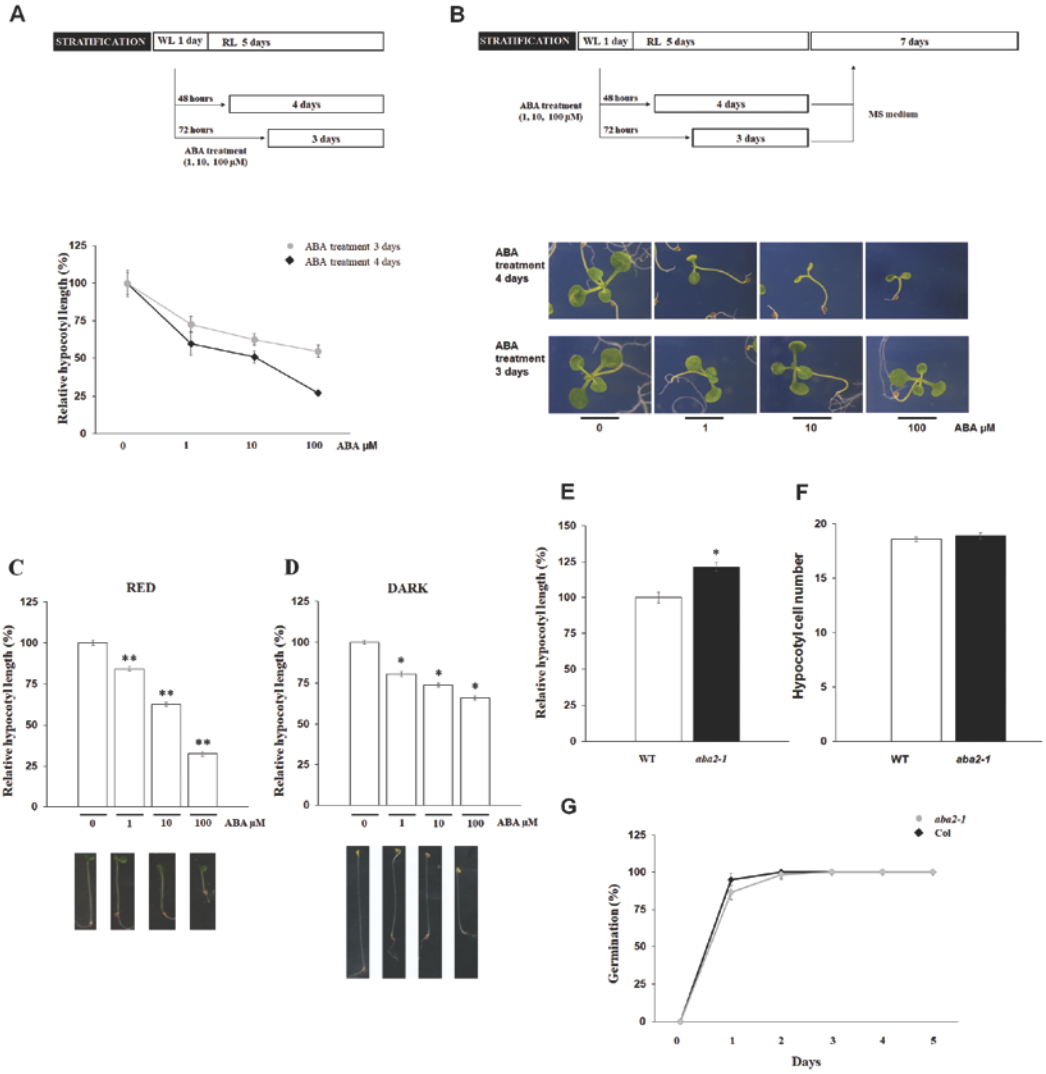
A
STRATIFICATION
WL 1 day
RL 5 days
48 hours
4 days
72 hours
3 days
ABA treatment
(1, 10, 100 µM)
Relative hypocotyl length (%)
ABA treatment 3 days
ABA treatment 4 days
ABA µM
B
STRATIFICATION
WL 1 day
RL 5 days
7 days
ABA treatment
(1, 10, 100 µM)
48 hours
4 days
72 hours
3 days
MS medium
ABA treatment 4 days
ABA treatment 3 days
ABA µM
C
RED
Relative hypocotyl length (%)
ABA µM
D
DARK
Relative hypocotyl length (%)
ABA µM
E
Relative hypocotyl length (%)
WT
aba2-1
F
Hypocotyl cell number
WT
aba2-1
G
aba2-1
Col
Germination (%)
Days

Figure 32. ABA represses cell expansion in hypocotyls.

Schematic representation of ABA treatment after the induction of germination (top), and relative hypocotyl length of six days-old wild-type seedlings transferred on increasing ABA concentrations (1, 10 and 100 μM) 48 or 72 h after induction of germination (AIG), respectively (bottom). (B) Schematic representation of ABA treatment and subsequent recovery on MS medium (top), and images of the seedlings after ABA treatment (bottom). (C, D) Relative hypocotyl length of six days-old wild-type seedlings grown under Red light (C) or in the dark (D), in the presence of increasing ABA concentrations. Top: relative hypocotyl length. Bottom: images of the seedlings in the different conditions. (E) Hypocotyl length of untreated six days-old wild-type (WT, white bar) and *aba2-1* (black bar) mutant seedlings grown in the dark. (F) Epidermal cell number of wild-type (white bar) and *aba2-1* (black bar) hypocotyls of three days-old seedlings. (G) Germination assay of *aba2-1* and wild-type stratified seeds. The values are the mean of three biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05; **P≤0.01).

12 ABA inhibits cotyledon expansion and opening

Given its effect on hypocotyls, we examined whether ABA would inhibit also cotyledon expansion. Measurement of wild-type six days-old seedlings grown under Red light in the presence of ABA revealed a much reduced cotyledon expansion, with an average cotyledon area corresponding to 25% of that of mock-treated controls (Figure 33A). Consistently, as shown in Figure 33B, the area of *aba2-1* mutant cotyledons was significantly larger than normal (138% of that of wild-type cotyledons). Cotyledon opening was also inhibited by ABA treatment: the angle between hypocotyl and petiole increased from 99° in mock treated seedlings to, respectively, 130°, 142° and 156° at 1, 10 and 100 µM ABA, as shown in Figure 33C.

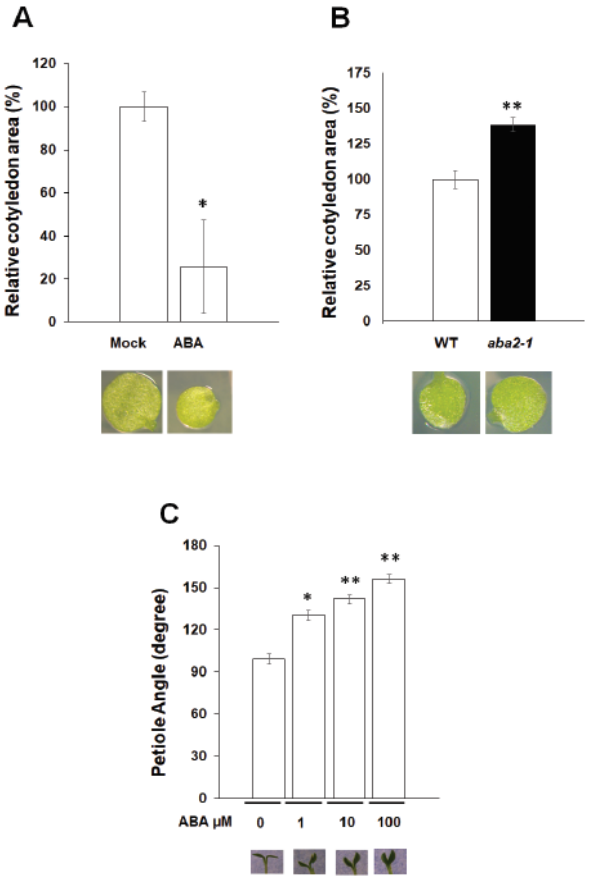

Figure 33. ABA affects cotyledon expansion and opening.

(A) Relative cotyledon area of wild-type mock- or ABA-treated (10 µM) six days-old seedlings. (B) Relative cotyledon area of untreated wild-type (white bar) and *aba2-1* (black bar) mutant six days-old seedlings. Top: relative cotyledon area. Bottom: images of the cotyledons in the different conditions. (C) Petiole angle of six days-old wild-type seedlings in the presence of increasing ABA concentrations (0, 1, 10 and 100 µM). Top: relative petiole angle. Bottom: images of the cotyledons in the different conditions. The values are the mean of three biological replicates, presented with SD values. Significant differences were analyzed by *t*-test (*P≤0.05; **P≤0.01).

13 ABA treatments do not affect *CAB2* expression or protein accumulation

To assess whether ABA inhibits hypocotyl elongation and cotyledon expansion by affecting the photomorphogenesis process, we examined the expression of the light-regulated genes *RbcS1b* (*RIBULOSE BISPHOSPHATE CARBOXYLASE small Subunit1b*) and *CAB2* (*CHLOROPHYLL A/B BINDING PROTEIN 2*) by performing RT–qPCR in seedlings grown in Red light in the presence of ABA. This analysis clearly showed that exogenous ABA did not increase expression of these genes (Figure 34A, B).

To establish whether ABA has an effect at the protein level, we performed an immunoblot analysis for CAB2 on wild-type seedlings treated with ABA, which showed (Figure 34 C) that the amount of this photomorphogenic protein is not affected by ABA. These results indicate that ABA does not affect photomorphogenesis.

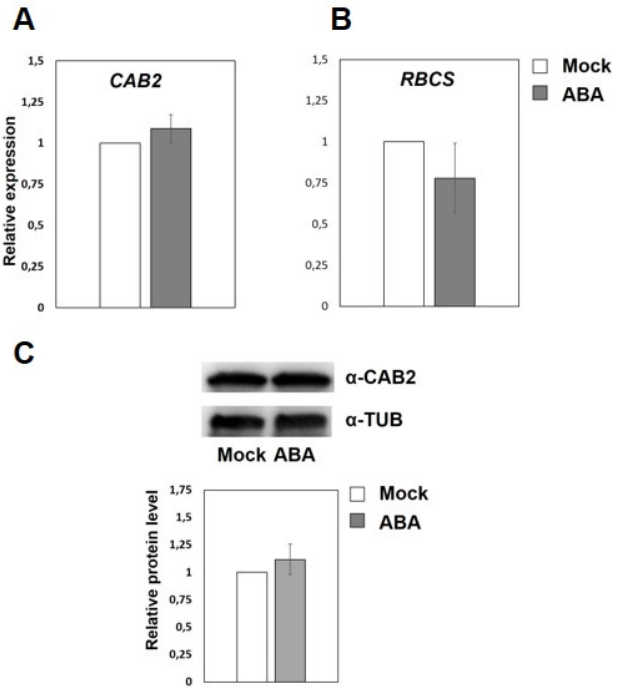

Figure 34. Photomorphogenesis is not altered by ABA.

(A, B) Expression level of *CAB* (A) and *Rbcs* (B) genes in 5 days-old wild-type seedlings mock- or ABA-treated (4 h; 100 µM) (white and grey bars, respectively). Expression levels were normalized with that of the *UBQ10* (*At4g05320*) gene. (C) CAB protein level in 5 days-old wild-type seedlings mock- or ABA-treated (100 µM) (white and grey bars, respectively). Western blot (top) and densitometric analysis (bottom). TUB used as loading control. The values are the mean of three biological replicates, presented with SD values. Significant differences were analyzed by *t*-test.

14 ABA represses GA biosynthesis and induces GA degradation

GA and ABA regulate in opposite manner hypocotyl elongation (Alabadì *et al*., 2004), to verify whether GA might complement the effect of ABA, we measured hypocotyl length of Red light-grown seedling treated with 10 µM ABA and increasing amounts of GA (1, 10 and 100 µM). This revealed that 10µM GA was sufficient to revert, although not completely, the effect of ABA (Figure 35A).

To assess whether ABA inhibits hypocotyl elongation acting on GA metabolism, we measured by RT-qPCR the transcription of a number of GA metabolic genes on four days-old seedlings treated for four hours with ABA compared to mock-treated controls. The genes analysed were the GA biosynthetic genes encoding *ent*-kaurenoic acid oxidase (*KAO1*), GA20-oxidase1 and GA20-oxidase2 (*AtGA20ox1* and *2*), GA3-oxidase1 and GA3-oxidase2 (*AtGA3ox1* and *2*); and the GA catabolic genes encoding GA2-oxidase2 and GA2-oxidase4 (*AtGA2ox2* and *4*).

All the biosynthetic genes were significantly downregulated after ABA treatment: the relative expression levels of *AtGA3ox1*, *AtGA3ox2*, *AtGA20ox1 AtGA20ox2* and *KAO1* were, respectively, 12.5-, 2.5-, 4.3-, 1.5- and 1.7-fold lower than in mock-treated controls (Figure 35B). Conversely, the catabolic genes *AtGA2ox2* and *AtGA2ox4* showed an increased relative expression level, corresponding to 2.1- and 1.3-fold that of mock-treated controls (Figure 35C).

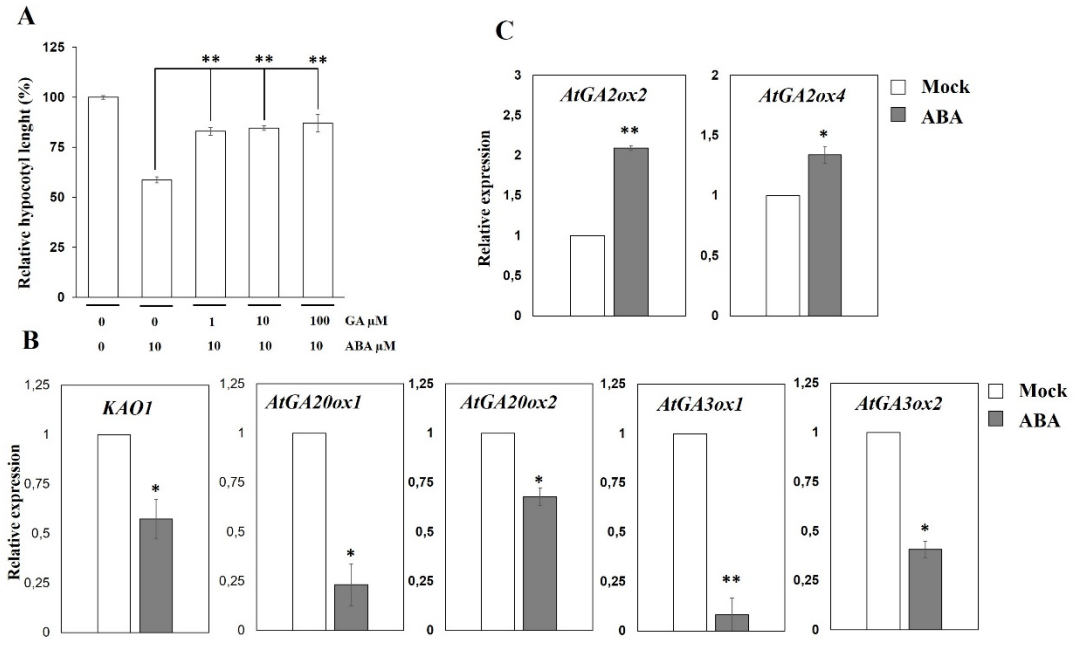

Figure 35. ABA represses GA biosynthesis.

(A) Relative hypocotyl length of six days-old wild-type seedlings transferred on ABA (10 µM), plus increasing GA concentrations (0, 1, 10 and 100 µM). (B) Relative expression level of the GAs biosynthetic genes: *KAO1*, *AtGA20ox1, AtGA20ox2, AtGA3ox1*, *AtGA3ox2*. (C) Relative expression level of the GAs catabolic genes *AtGA2ox2*, *AtGA2ox4*. RNA from four days-old wild-type seedlings mock- or ABA-treated (4 h; 100 µM) (white and grey bars, respectively). Expression levels were normalized with that of the *UBQ10* (*At4g05320*) gene. The values are the mean of three biological replicates, presented with SD values. Significant differences were analyzed by *t*-test (*P≤0.05; **P≤0.01).

15 ABA stabilizes DELLA proteins

The DELLA proteins GA INSENSITIVE (GAI) and REPRESSOR OF *ga1-3* (RGA) both inhibit hypocotyl elongation (de Lucas *et al*., 2008; Feng *et al*., 2008). GAs trigger proteasome-mediated degradation of these DELLA proteins, thus relieving their inhibitory effect on hypocotyl elongation. As we observed that ABA affects GA metabolism, we verified

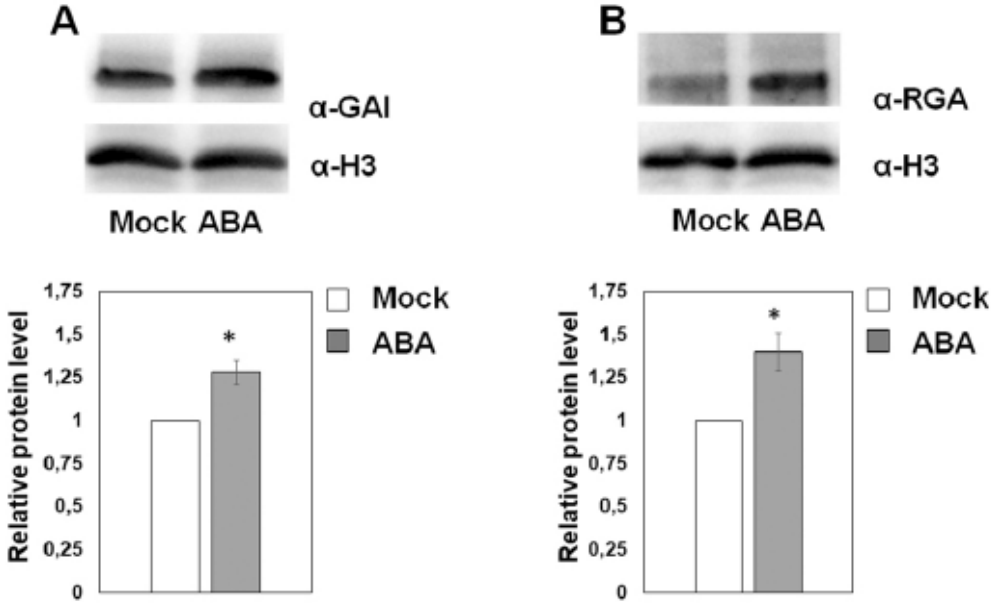

Figure 36. ABA represses GA signalling.
(A, B) Protein level of GAI (A) and RGA (B) in four days-old wild-type seedlings mock- or ABA-treated (4 h; 100 µM) (white and grey bars, respectively). H3 used as loading control. Western blot (top) and densitometric analysis (bottom). Protein levels are the mean of three biological replicates, presented with SD values. Significant differences were analyzed by *t*-test (*P≤0.05; **P≤0.01).

whether ABA treatment leads to the stabilization of GAI and RGA. We performed an immunoblot analysis on seedlings treated for four hours with ABA, using anti-GAI and anti-RGA specific antibodies. Addition of ABA significantly increased the level of both GAI and RGA proteins to, respectively, 1.24- and 1.4-fold compared to mock-treated seedlings (Figures 36 A, B).

To assess whether the ABA inhibition of hypocotyl elongation was, at least in part, dependent on stabilization of DELLA proteins, we performed immunoblot analysis on seedlings treated with ABA for different times (12, 24 and 48 hours), using an anti-GAI antibody. The results confirmed that GAI was stabilised by ABA-treatment at least up to 48 hours (Figure 37). These results suggest that ABA inhibits cell expansion in hypocotyl elongation by reducing GA level and consequently increasing the level of the GAI and RGA DELLA proteins.

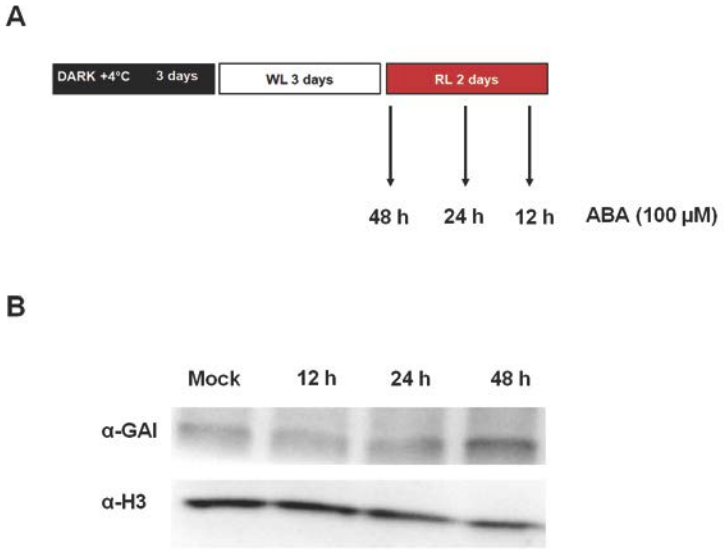

Figure 37. ABA represses GA signalling.
(A) Schematic representation of ABA treatment after the induction of germination. **(B)** Immunoblot analysis of GAI in five days-old wild-type seedlings mock- or ABA-treated (12, 24, 48h; 100 µM). H3 used as loading control. Protein levels are the mean of three biological replicates, presented with SD values. Significant differences were analyzed by t-test (*P≤0.05; **P≤0.01).

16 ABA response is altered in *della* and in *pif* multiple mutants

It has been shown that the increase in hypocotyl length caused by GA treatment is similar to that observed in the quintuple mutant lacking all five DELLA proteins (Feng *et al*., 2008). According to the conclusions drawn in the previous paragraph, the hypocotyl response to ABA of this multiple *della* mutant should be impaired. As shown in Figure 38A, hypocotyl of quintuple *della* mutant seedlings grown under red light in the presence of ABA, were less sensitive to ABA inhibition than controls. While hypocotyls of mock-treated *della* mutants were as expected significantly longer compared to the wild-type (181%), ABA-treated mutant seedlings were less sensitive to the hormone.

DELLA proteins directly repress the transcriptional activity of PIF proteins (de Lucas *et al*., 2008; Feng *et al*., 2008), and promote their degradation (Li *et al*., 2016). PIFs in turn repress photomorphogenesis, as the quadruple *pif1pif3pif4pif5* mutant (*pifq*) shows a constitutive photomorphogenic phenotype (Leivar *et al*., 2008). As shown in Figure 38B hypocotyl elongation in the quadruple *pif* mutant is insensitive to ABA, as hypocotyl length does not show differences between mock and ABA treated (1, 10 and 100 µM) seedlings. This suggests that ABA exerts its function via the PIF signalling pathway.

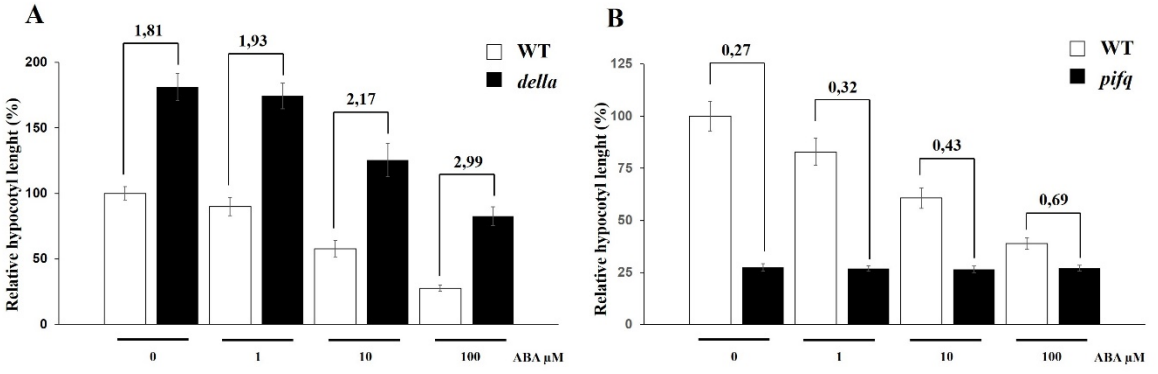

Figure 38. ABA response in *della* and *pif* multiple mutants.
(A, B) Ratio of the hypocotyl length of pentuple *della* mutant (A) or of quadruple *pif* mutant (B) to wild-type seedlings. Seedlings were grown six days under Red light, in the presence of increasing ABA concentrations (0, 1, 10 and 100 µM). The values are the mean of three biological replicates, presented with SD values. Significant differences were analyzed by *t*-test (*P≤0.05).

17 ABA response is altered in *dag1* seedlings

Our results show that ABA represses hypocotyl elongation by affecting GA signalling and biosynthesis. Two GA metabolic genes, which we have shown to be ABA-responsive, have reduced transcript levels in *dag1* hypocotyls. Therefore we wondered whether DAG1 could be involved in ABA-mediated repression of hypocotyl elongation. We measured hypocotyl length in *dag1* seedling grown under red light in the presence of increasing ABA concentrations (1, 10 and 100 µM). As shown in Figure 39, although at the lowest ABA concentrations (1 µM) *dag1* hypocotyl length was shorter than the wild-type, the

difference was reduced compared to the mock-treated control, suggesting that *dag1* responds to ABA to a lesser extent than the control. Consistently, at the higher ABA concentrations (10 and 100 µM) hypocotyl length of *dag1* and wild-type seedlings was comparable, thus indicating that addition of exogenous ABA can revert the hypocotyl phenotype of *dag1* mutant seedlings.

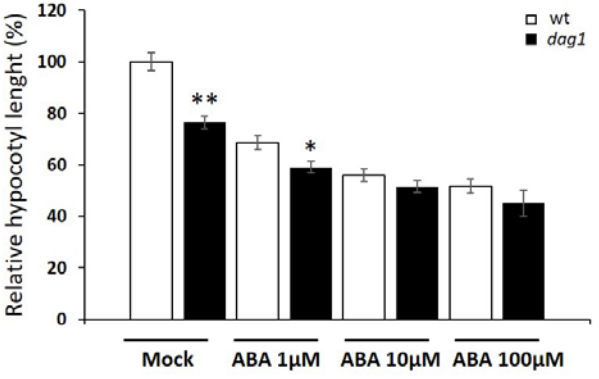

Figure 39. ABA response in *dag1* mutant seedlings.
Hypocotyl length of *dag1* (black bar) and wild-type (white bar) seedlings grown under Red light for five days, with increasing concentration of ABA (0, 1, 10 and 100 µM). The values are the mean of three biological replicates, presented with SD values. Significant differences were analyzed by *t*-test (*P≤0.05; **P≤0.01).

18 ABA represses auxin biosynthesis

PIF proteins positively regulate hypocotyl elongation by directly inducing the expression of *YUCCA* (*YUC*) genes encoding the auxin biosynthetic enzymes flavin monooxygenases (Hornitschek *et al*., 2012; Sun *et al*., 2012). Auxin is synthesized in cotyledons and transported in hypocotyls where it promotes elongation (Tanaka *et al*., 2002; Tao *et al*., 2008; Procko *et al*., 2014); to assess whether ABA inhibits hypocotyl elongation acting on auxin, we analysed the activity of the synthetic auxin response reporter *DR5::GUS* after ABA treatment. As shown in Figure 40A, GUS activity is reduced in ABA-treated seedlings (bottom panels), suggesting that ABA lowers auxin level. We then analysed the expression of three *YUC* genes, *YUC3*, *YUC5* and *YUC6* upon ABA treatment. As shown in Figure 40B *YUC3*, *YUC5* and *YUC6* were significantly downregulated by ABA in whole seedlings, the expression levels being, respectively, 2.3-, 20- and 4.1-fold lower than in mock-treated controls.

These results suggest that in inhibiting hypocotyl elongation ABA ultimately acts on auxin biosynthesis pathway.

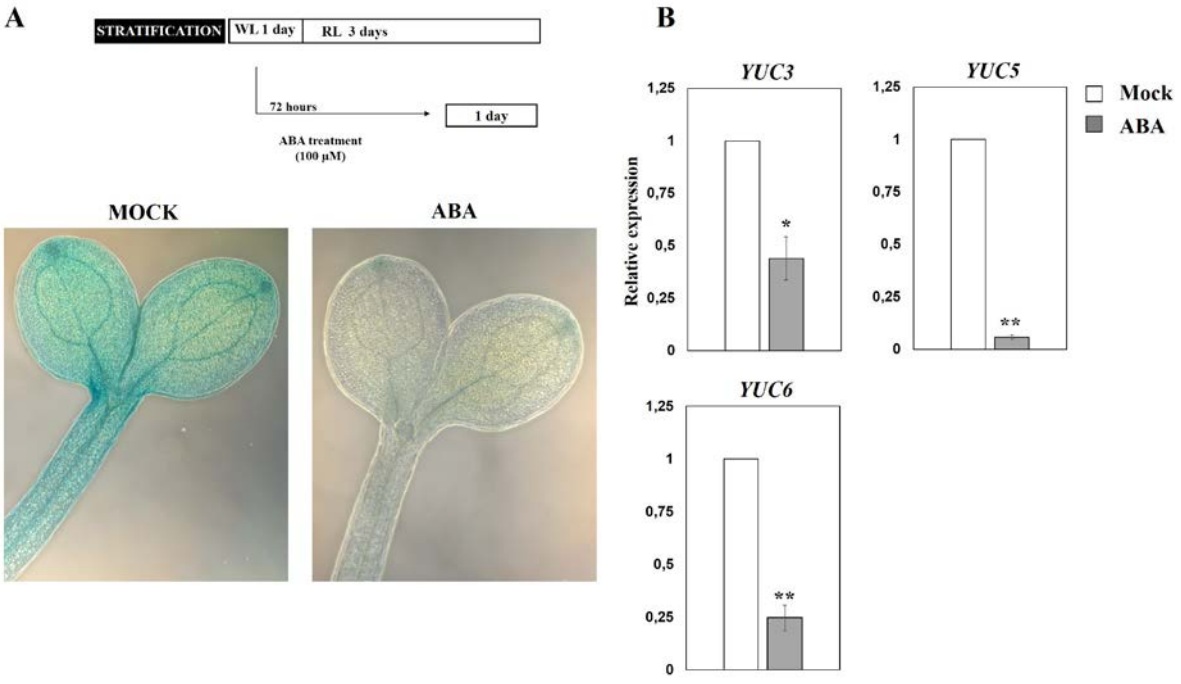

Figure 40. Auxin biosynthesis is downregulated by ABA.

(A) Schematic representation of ABA treatment (top) Histochemical staining of four days-old *DR5::GUS* mock- or ABA-treated (100 µM) seedlings (bottom). (B) Relative expression level of the IAA biosynthetic genes: *YUC3, YUC5* and *YUC6.* RNA from four days-old wild-type seedlings mock- or ABA-treated (4 h; 100 µM) (white and grey bars, respectively). Expression levels were normalized with that of the *UBQ10* (*At4g05320*) gene. The values are the mean of three biological replicates, presented with SD values. Significant differences were analysed by *t*-test (*P≤0.05; **P≤0.01).

DISCUSSION

The *Arabidopsis* transcription factor DAG1 plays a pivotal role during maturation, dormancy and germination of seeds, by monitoring the ABA/GA ratio, thus preventing premature seed germination and controlling the seed-to seedling transition.

We have previously demonstrated that DAG1 is also involved in the repression of the light-mediated inhibition of hypocotyl elongation (Gabriele *et al*., 2010). However, we have not yet elucidated how DAG1 controls this process; indeed, DAG1 may be either a repressor of photomorphogenesis, or a repressor of the light-mediated inhibition of hypocotyl elongation.

Among plant growth processes, hypocotyl elongation has attracted much attention because of the simplicity of this organ, and because numerous plant growth factors affect this process acting on cell expansion, namely GAs, auxin, ethylene, and brassinosteroids (Vandenbussche *et al*., 2005).

Here we show that DAG1 controls hypocotyl elongation by promoting cell expansion rather than cell division. Indeed, epidermal cells number in the hypocotyl are the same among wild-type and *dag1* seedlings, suggesting that the difference in hypocotyl length is due to a reduction in cell expansion.

Cell expansion is controlled by many environmental and endogenous factors such as light, GA and auxin. Interestingly, the expression levels of the *GA3ox1* and *GA2ox2* genes are

decreased in *dag1* mutant hypocotyls, consistent with *dag1* hypocotyl phenotype, since lower GA levels imply a decrease in hypocotyl elongation. Thus we can state that DAG1 controls cell expansion at least in part through the positive control of GA metabolism.

On the other hand, *DAG1* inactivation results in upregulation of *GA3ox1* in seeds, and consistently *dag1* mutant seeds shows an increased level of bioactive GAs (Gabriele *et al*., 2010; Boccaccini *et al*., 2016). GAs levels are controlled by light: indeed, GA biosynthesis is triggered by light in seeds, whereas it is repressed in seedlings where the effect of GAs is antagonistic to that of light as for photomorphogenesis. DAG1 functions as a repressor of light-mediated processes, namely seed germination and photomorphogenesis, consistently, it controls GA levels in opposite way during these two developmental processes.

To assess whether the function of DAG1 is restricted to hypocotyl growth or affects also other photomorphogenic traits, we took advantage of a genome-wide approach. We studied the *dag1* and wild-type transcriptome of both whole seedlings and dissected hypocotyls, and the GO analysis on the DE genes of the four comparison groups revealed that the most significant differences are in the hp *dag1*/WT, therefore suggesting that DAG1 is mainly involved in hypocotyl development.

We have previously shown that DAG1 plays a key role in the control of the seed-to seedling transition in *Arabidopsis* (Boccaccini *et al*., 2016). Interestingly, a quite high number of DE

genes in the hp *dag1*/WT comparison group are related to seed-specific functions; in particular, several LEA proteins encoding genes are upregulated in *dag1* hypocotyls compared to the control. LEA proteins are a ubiquitous group of polypeptides that were first described to accumulate during plant seed dehydration, at the later stages of embryogenesis. *LEA* genes have been classified according to their seed-specific expression profile, although it has been shown that some of them are induced in vegetative tissues in response to water, cold or saline stress, making their seed-specificity uncertain. In addition, LEA synthesis has long been considered to be regulated by ABA (Hughes and Galau, 1991; Parcy *et al*., 1994). It should be noted that the *LEA* genes which are upregulated in *dag1* hypocotyls are mainly expressed in seeds, suggesting that DAG1 is required to repress their expression during the seed-to-seedling transition. It should also be noted that at least two of the *LEA* genes upregulated in *dag1* hypocotyls, are also induced by ABA (*EM6*, At3g17520) (Bies-Ethève *et al*., 2008).

The WRKY transcription factors are known as key components of ABA signalling, and a number of them are involved in dormancy and germination of seeds, as well as in ABA-mediated abiotic stress response (Rushton *et al*., 2012). Interestingly, the *WRKY* genes are the most represented transcription factors-encoding gene family, among the DE genes of our transcriptome analysis. Recently, it has been shown that *WRKY6* -which is upregulated in *dag1* hypocotyls- is repressed during seed germination and early seedling development,

and induced by exogenous ABA, suggesting that it is involved in ABA signaling during seed germination and early seedling development (Huang *et al*., 2016).

In addition, a further GO analysis on the hp *dag1*/WT revealed that *DAG1* inactivation primarily affects the hormonal response in hypocotyls. Indeed, about 20% of DE genes in hypocotyls belong to this category. Among these DE genes, the *SAUR* genes, known to be auxin-responsive genes involved in hypocotyl elongation (Chae *et al*., 2012; Oh *et al*., 2014; Sun *et al*., 2016). In addition, most of the DE genes belonging to the hormonal response category, are involved in ABA response.

An inhibiting effect of ABA on hypocotyl growth under conditions of skotomorphogenesis has been recorded (Finkelstein *et al*., 2013) but the mechanism has not been elucidated. Recently, a genome-wide analysis of the shade avoidance response in *Arabidopsis* has shown that ABA-responsive genes are down-regulated in elongated hypocotyls and upregulated in non-expanded cotyledons (Kohnen *et al*., 2016).

By analyzing skoto- and photomorphogenic responses in *Arabidopsis*, we show here that ABA is capable of inhibiting cell expansion in hypocotyls and cotyledons of both light- and dark-grown seedlings. The observation that *aba2-1* seedlings, mutant of the *ABA2* gene – encoding an ABA biosynthetic enzyme that converts xanthoxin to ABA-aldehyde - with severely reduced endogenous ABA content (23% of wild-type level, Nambara *et al*., 1998), have hypocotyls longer than the wild-type, corroborates the notion that ABA inhibits

hypocotyl growth. It has also been reported that lack of ABA2 results in reduced dormancy (Nambara *et al*., 1998). On the other hand, our germination assay shows that *aba2-1* mutant seeds do not germinate earlier than wild-type seeds, further supporting our results on hypocotyl elongation. ABA and GA are known to play opposite function in a number of plant developmental processes (Razem *et al*., 2006). GA metabolism is controlled by ABA in seeds, since both the expression of GA biosynthetic genes and GA levels were increased in *aba2* mutant seeds (Seo *et al*., 2006). Indeed, the *aba2-1* mutant has been isolated through its reduced sensitivity to the GA inhibitor paclobutrazol (Leon-Kloosterziel *et al*., 1996).

Our results indicate that ABA negatively controls GA metabolism also in seedlings. Consistently, addition of exogenous GA complements the ABA-mediated inhibition of hypocotyl elongation.

It has been shown that ABA can determine a developmental arrest in *Arabidopsis* seedlings if applied within 60h from induction of germination (Lopez-Molina *et al*., 2001). The effect we report here of ABA on the inhibition of cell expansion of hypocotyls and cotyledons occurs up to 72 hours after stratification, indicating that it is not due to this developmental arrest.

In the dark, GAs repress photomorphogenesis (Alabadì *et al*., 2004). DELLA proteins, GA-signalling repressors, are degraded in the presence of GAs; conversely, in the light the

amount of DELLA proteins raises as a consequence of reduced GAs levels (Achard *et al*., 2007). The molecular network underlying the switch between skoto- and photomorphogenesis also includes the PIF proteins PIF3 and PIF4, which negatively control photomorphogenesis (de Lucas *et al*., 2008; Feng *et al*., 2008). It has been shown that DELLA proteins inhibit the transcriptional activity of PIF proteins by interacting with them and masking their DNA-binding domain (de Lucas *et al*., 2008; Feng *et al*., 2008). More recently, it has been shown that DELLA proteins also promote degradation of the PIF proteins via the ubiquitin-proteasome pathway (Li *et al*., 2016). It has also been shown that the AUXIN RESPONSE FACTORS (ARFs) ARF6 and ARF8 cooperate with PIF4, through direct interaction, to positively control hypocotyl elongation (Oh *et al*., 2014). PIF4 also induces the expression of auxin biosynthetic genes *YUC8*, *TAA1*, and *CYB79B2* (Hornitschek *et al*., 2012; Sun *et al*., 2012), thus increasing auxin levels. On the other hand, the DELLA protein RGA counteracts the effect of the ARF6-PIF4 complex by directly interacting with ARF6 (Oh *et al*., 2014).

Interestingly, inactivation of *DAG1* affects the expression of *miR167D* both in hypocotyls and seedlings, in opposite way. miR167D belongs to a family of microRNAs which target both *ARF6* and *ARF8* (Wu *et al*., 2006).

Here, we show that ABA represses GA biosynthesis, induces GA degradation, and stabilizes the DELLA proteins GAI and RGA. In addition, our data suggests that ABA may act via the

PIF proteins, as hinted by the significantly decreased ABA sensitivity of the *pif* multiple mutant. Finally, we show that IAA biosynthesis is downregulated by ABA treatment.

Our results suggest a model whereby in hypocotyl elongation (and cotyledon expansion) ABA inhibits cell expansion by negatively regulating GAs, thus causing an increased level of DELLA proteins; this, possibly via a decreased activity of PIF proteins, would result in a downregulation of auxin biosynthetic genes and therefore inhibition of cell expansion (Figure 41). It has been recently demonstrated that hypocotyl cell elongation is controlled by major hormonal and environmental pathways through a central circuit of interacting transcription regulators (Oh *et al*., 2014). Therefore, it is tempting to speculate that DAG1 might be an element of a molecular network which controls cell expansion by modulating hormonal response, namely auxin, ABA and GAs, through the ARF-PIF/DELLA module.

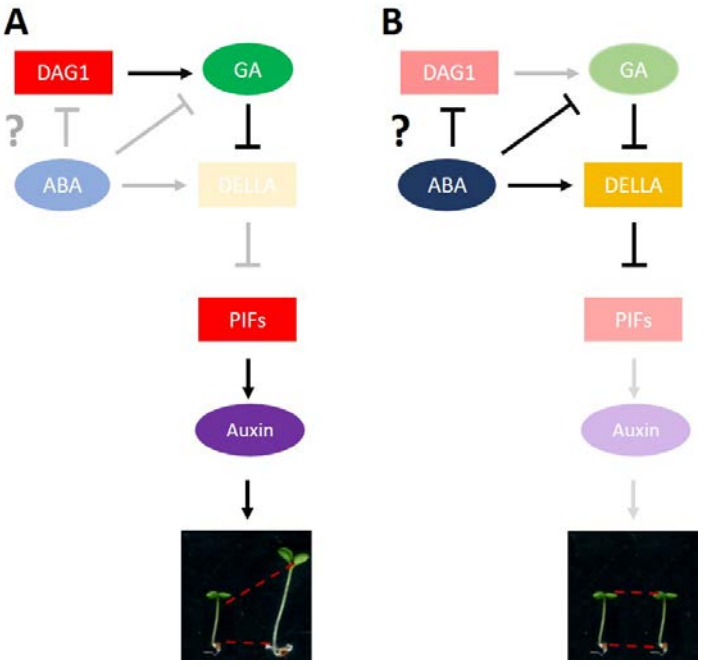

Figure 41. Model of the ABA-mediated hypocotyl elongation process.
Hypocotyl elongation is controlled: (A) by GAs accumulation which destabilize DELLA proteins and allow PIFs mediated cell elongation, ultimately acting on auxin biosynthesis; (B) by ABA which inhibits GA biosynthesis thus stabilizing DELLA proteins, inhibiting the activity of PIFs. DAG1 functions downstream of ABA, by increasing GA level, thus promoting hypocotyl elongation.

MATERIALS AND METHODS

Plant material and growth conditions

All *Arabidopsis thaliana* lines used in this work 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 (Papi *et al*., 2000), 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 *aba2-1* and pentuple *della* mutant were kindly provided by Dr. L.L. Molina and by Prof. X.W. Deng, respectively. The *pif* quadruple mutant was obtained from the European *Arabidopsis* Stock Centre (NASC). The corresponding wild-type lines were used (Columbia for *aba2-1* and *pif* quadruple mutant and Landsberg for the pentuple *della* mutant). The *DR5::GUS* line is the one described in Ulmasov *et al*., (1997).

Phenotypic analysis

For hypocotyl elongation, cotyledon expansion and bending analysis, 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. Hypocotyl lenght and cotyledon area were measured after six days. For hypocotyl elongation in darkness the samples were first grown in white light for 24 hours, then wrapped in several

aluminum sheets for four days. For ABA and GA treatments, seeds were sown on MS agar (halfstrength MS, 0.8% agar, pH 5.7) with one layer of filter paper 595 (Schleicher & Schüll, Dassel, Germany), then, at different times (48, 60, 72 hours) after light exposure, seedlings were transferred to plates containing different ABA and GA concentrations (Duchefa 0941). Hypocotyl length, cotyledon area and bending were measured using IMAGEJ software. For cell number measurement, three days-old wild-type and *aba2-1* seedlings, grown on orizzontal plates under Red light (intensity 100 µmol/m^{-2} s^{-1}) with a 16h light/ 8h dark photoperiod, were fixed in a ethanol/acetic acid mixture (6:1). The samples were cleared as previously described (Weigel and Glazebrook, 2002). Seedlings were mounted in chloral hydrate mixture and images of the samples were taken with a Nikon coolpix 990 camera mounted on Zeiss Axioskop 2 plus microscope equipped with DIC optics. For each sample, the number of cells in an epidermal cell file without stomata was counted.

For the germination assay, wild-type and *aba2-1* mutant seeds were stratified 3 days at 4°C, then grow in a growth chamber at 22°C. 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.

The values are the mean of three biological replicates presented with SD values. Significant differences were analyzed by t-test (*P ≤ 0,05; **P ≤ 0,01).

GUS analysis

Analysis of DR5::GUS 3 days-old seedlings mock- or ABA-treated (100 µM) was performed under an Axioskop 2 plus microscope (Zeiss). Histochemical staining and microscopic analysis were carried out according to Capone et al., (1991), except that seedlings were incubated at 37°C for 12 hours. Stained seedlings (after washing in 70% ethanol) were analysed and photographed under an Axioskop 2 plus microscope (Zeiss).

Transcript analysis

Total RNA from four days-old seedlings – respectively treated 4, 12, 24 or 48 hours with ABA (100 µM) in liquid medium, or mock-treated as a control, under monocromatic red light - was extracted and purified according to Vittorioso et al., (1998). RT-qPCR assays were performed according to Gabriele *et al.*, (2010). Relative expression levels were normalized with *UBQ10* (*At4g05320*) reference gene. The primers used are listed in Table S1 (Supplementary Tab. S1). The values of relative expression levels are the mean of three biological replicates presented with SD values. Significant differences were analyzed by t-test (*$P \leq 0,05$; **$P \leq 0,01$).

Specific primer sets used in the qRT-PCR:

	Forward	Reverse
AtGA3ox1	GCTTAAGTCTGCTCGGTCGG	AGTGCGATACGAGCGACG
AtGA3ox2	ACGTCGGTGACTTGCTCCA	GTTAACCCTGGCTCGGTGAA
AtGA2ox2	TCCGACCCGAACTCATGACT	CGGCCCGGTTTTTAAGAGAC
AtGA2ox4	CTCTTTCGGCGATGGTTATG	AAACGGCTATCCTCAAGTCG
KAO1	TCGACCCTGAAGTCTTTCCA	TCGACCCTGAAGTCTTTCCA
AtGA20ox1	AGCGAGAGGAAATCACTTGC	AGCGAGAGGAAATCACTTGC
AtGA20ox2	TGCCAAACACCAGATCTCAC	TGCCAAACACCAGATCTCAC
UBQ10	GGCCTTGTATAATCCTGATGAATAA	GGCCTTGTATAATCCTGATGAATAAG
YUC3	GAAGGCAGCGACATTTTCTC	TACCCCTTCACGTTTCAAGC
YUC5	GGGTTAACGGTCCTGTAATCGT	TCTGCTCTCTCCAATACCACAAAG
YUC6	AGGTCCACTCGAGCTCAAAA	CCTTCTTATCCCCGAACACA

Immunoblot analysis

Total proteins were extracted from seedlings treated with ABA (100 µM) or ethanol (mock treatment) according to Gabriele *et al*., (2010). Seedlings were grown in white light condition for 3 days before ABA treatment, to allow GAI and RGA accumulation. Then seedlings were moved in monocromatic red light condition with or without ABA (100 µM),

for 4 hours in liquid medium, or up to 48 hours in solid medium. A total of 30-40 µg of protein extract was separated on SDS-polyacrylamide gel and blotted on a PVDF Immobilon-P Transfer membrane (Millipore). Detection of proteins was performed with anti-GAI or anti-RGA antibodies (Agrisera, Vännäs, Sweden) as primary antibody and peroxidase-conjugated anti-rabbit as secondary antibody (Sigma, St. Louis, USA). H3 was detected using an anti-H3 antibody (Biorbyt, Cambridge, United Kingdom). The values are the mean of three biological replicates presented with SD values. Significant differences were analyzed by t-test (*P ≤ 0,05; **P ≤ 0,01).

RNA-seq

Four days-old seedlings grown under Red light were collected and frozen in liquid nitrogen in the dark. For hypocotyls ~1000 seedlings grown under Red light have been dissected, collected and frozen in liquid nitrogen in the dark.

Total RNA from four days-old seedlings, grown under monocromatic red light, was extracted and purified according to Vittorioso *et al.* (1998). Any contaminating genomic DNA was removed using on column DNAse digestion. The quality of the RNA was verified on gel and with Agilent Bioanalyzer 2100.

RNA-seq data processing and detection of differentially expressed genes

RNA-seq reads were mapped to the *A.thaliana* Tair10 genome assembly using STAR2 (Dobin *et al*., 2013) with default parameters. The gene and transcript annotation from the Ensembl Plant database (http://plants.ensembl.org) was provided during the alignment step. After filtering for uniquely mapped reads, gene-level read counts were obtained using the HTSeq-count algorithm (Anders *et al*., 2014) and then processed using the edgeR package (Robinson *et al*., 2010). For each sample, raw gene counts were first converted into CPM (counts per million) and those having CPM<1 in at least three samples (the minimum number of samples in a group) were filtered out in order to remove genes expressed at very low levels. Gene expression levels and fold-changes were estimated after TMM (**T**rimmed-**M**ean of **M** values) normalization. Both common (all genes in all samples) and separate (tag-wise) dispersion parameters were estimated using the Cox-Reid model (Robinson *et al*., 2010) and integrated into a Negative Binomial generalized linear model (NB-GLM). Statistical significance of differential expression was assessed using a GLM-likelihood ratio test and the 'Benjamini-Hochberg' correction for multiple testing. A FDR adjusted p-value ≤ 0.05 was used to define differentially expressed genes.

Gene ontology analysis

Functional annotation analysis of DE genes was performed using the Singular Enrichment Analysis tool (SEA) from the agriGO ontology database

(http://bioinfo.cau.edu.cn/agriGO/analysis.php, Du *et al*., 2010). The Fisher's exact test and a q-value significance threshold of 10^{-4} was used to identify functional enriched categories.

Statistical analysis

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

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