The DAG1 transcription factor negatively regulates the seed-to-seedling transition in Arabidopsis acting on ABA and GA levels

Alessandra Boccaccini, Riccardo Lorrai, Veronica Ruta, Anne Frey, Stephanie Mercy-Boutet, Annie Marion-Poll, Danuše Tarkowská, Miroslav Strnad, Paolo Costantino and Paola Vittorioso

Abstract

Background: In seeds, the transition from dormancy to germination is regulated by abscisic acid (ABA) and gibberellins (GAs), and involves chromatin remodelling. Particularly, the repressive mark H3K27 trimethylation (H3K27me3) has been shown to target many master regulators of this transition. DAG1 (DOF AFFECTING GERMINATION1), is a negative regulator of seed germination in Arabidopsis, and directly represses the GA biosynthetic gene GA3ox1 (gibberellin 3-β-dioxygenase 1). We set to investigate the role of DAG1 in seed dormancy and maturation with respect to epigenetic and hormonal control.

Results: We show that DAG1 expression is controlled at the epigenetic level through the H3K27me3 mark during the seed-to-seedling transition, and that DAG1 directly represses also the ABA catabolic gene CYP707A2; consistently, the ABA level is lower while the GA level is higher in dag1 mutant seeds. Furthermore, both DAG1 expression and protein stability are controlled by GAs.

Conclusions: Our results point to DAG1 as a key player in the control of the developmental switch between seed dormancy and germination.

Keywords: DAG1, Seed development, Chromatin remodelling, GA, ABA, Arabidopsis thaliana, DOF proteins

Background

The transition from a growth-arrested seed to a germinating seed represents a crucial developmental switch in the life cycle of a plant [1]. Seeds of several annuals, including Arabidopsis, develop dormancy during the late stages of their development: although mature, these seeds are not capable of germinating even under favourable environmental conditions. Indeed, seed dormancy has been crucial for adaptation and evolution of seed plants.

Development of the Arabidopsis embryo consists of two phases: embryogenesis (from 0 to 6 days after pollination, DAP), and the embryo growth phase (from 7 to 10 DAP). Subsequently, seed maturation takes place until 21 DAP, when the seed is fully developed. Dormancy is established once embryo development is completed [2], and it is released within few weeks to several months after seed harvest, depending on the ecotype [3, 4].

Abscisic acid (ABA) produced during seed maturation is necessary to induce seed dormancy; gibberellins (GAs) release dormancy and promote germination, thus counteracting the effects of ABA, whereas the role of GAs during seed development is less clear. It has been shown that the increase in ABA level is crucial for proper progression through maturation, and a high ratio of ABA to GAs is the main determinant for the establishment of dormancy [3, 5].

While the importance of the dynamic balance between ABA and GAs is clear, so far the molecular mechanisms underlying seed dormancy induction, maintenance and release remain poorly understood [6].

Genetic analysis allowed the identification of a number of seed dormancy regulatory factors. Among these, DOG1...
we analysed its expression from late-maturation to non-
dormant wild type seeds (developing seeds dissected from
siliques at 13, 16, and 19 days after pollination, DAP, and
dry seeds at 0 and 28 days after harvest, DAH) by means
of RT-qPCR.
This analysis revealed that DAG1 is highly expressed
at 13 DAP, and that its expression subsequently de-
creases (16 DAP) to reach at 19 DAP a steady low level
that is retained during dry storage (Fig. 1a).
De novo RNA synthesis is rapidly induced in non-
dormant seeds following imbibition [17]: we therefore
analysed DAG1 expression in seeds imbibed for 6, 12
and 24 h, compared to dry seeds. As shown in Fig. 1b,
the DAG1 transcript level strongly increased following
imbibition, reaching after 24 h a level almost 10-fold
that of dry seeds.

Results
DAG1 is expressed during seed maturation and dormancy
and is modulated via epigenetic control
We have previously shown that inactivation of DAG1
reduces seed dormancy [12]. To assess whether and
when DAG1 is involved in the establishment of dormancy,
we analysed its expression from late-maturation to non-
dormant specific dormancy, and germination has been recently proposed [10]. Indeed, the DOG1 gene is marked by H3K27me3, a repressive epigenetic trait, and is upregulated upon loss of Polycomb Repressive Complex 2 (PRC2), responsible for this epigenetic mark [11], PRC2 is required for the switch from embryonic to vegetative growth, and seeds lacking a functional PRC2 showed enhanced dormancy and germination defects [11].

We have previously shown that inactivation of the gene DAG1 (DOF AFFECTING GERMINATION1) reduces seed dormancy [12]. DAG1 is a repressor of the seed germination process in Arabidopsis: dag1 null mutant seeds require lower GAs and red light fluence rates than wild type seeds to germinate [12–14].

We have also demonstrated that DAG1 acts in the seed germination phytochromeB (phyb)-mediated pathway, downstream of PIL5 (PHYTOCHROME INTERACTING FACTOR3 LIKE5), and it negatively regulates the GA biosynthetic gene GA3ox1, by directly binding to its promoter [15, 16]. In addition, inactivation of DAG1 results in an increase of the ABA catabolic gene CYP707A2 in germinating mutant seeds, suggesting that DAG1 may regulate this gene [15].

More recently, we showed that the DELLA protein GAI (GA INSENSITIVE) interacts with DAG1 thus cooperating in repressing GA3ox1 [16].

In the present study, we point to a key role of DAG1 in the developmental switch between seed dormancy and germination, and in the seed-to-seedling transition process. Indeed, DAG1 controls the level of GAs and ABA during seed maturation and dormancy by repressing GA3ox1 and CYP707A2 through direct binding to their promoters. Consistently, in dag1 mutant seeds the ABA level is reduced while the level of GAs is increased. In addition, our data show that GAs control DAG1 expression and DAG1 protein stability during imbibition. Furthermore, we show that the expression profile of DAG1 is controlled at the epigenetic level through the H3K27me3 repressive mark, which is known to target regulatory genes of the seed-to-seedling stage.

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we analysed its expression from late-maturation to non-
previously shown that inactivation of \(DAG1\) affects the expression of both the ABA catabolic gene \(CYP707A2\) and of the GA biosynthetic gene \(GA3ox1\) in germinating seeds [15], we extended the analysis by measuring by RT-qPCR the expression of the main ABA and GA metabolic genes - the catabolic gene \(CYP707A2\) encoding the ABA 8′-hydroxylase, the biosynthetic genes \(NCED6\) and \(NCED9\) encoding the 9-cis-epoxy-carotenoid dioxygenase for ABA, the catabolic gene \(GA2ox1\) encoding GA2-oxidase, the biosynthetic genes \(GA3ox2\) and \(GA3ox1\) encoding GA3-oxidases for GA - in \(dag1\) and wild type developing seeds at 13, 16 and 19 days after pollination (DAP), and in mature dry seeds at 0 and 28 days after harvest (DAH).

As shown in Fig. 2a, of the ABA-related genes only the expression of \(CYP707A2\) was increased in \(dag1\) developing seeds compared to the wild type at 13 and 16 DAP (3.5- and 8-fold, respectively), but not at later maturation stages (19-21 DAP).

As for GA, the expression of \(GA3ox1\) was comparable in \(dag1\) and wild type developing seeds at 13 and 16 DAP, and also at 19 DAP when it dropped sharply in both stages of developing seeds, whereas at 21 DAP it was more than 26 times higher in \(dag1\) than in wild type developing seeds (Fig. 2b).

These results confirm that DAG1 controls ABA and GA pathways in seeds and provide support to the notion that it promotes seed dormancy in mature dry seeds acting on these two hormones.

To verify whether the post-harvest control of DAG1 on dormancy is exerted via the same hormones and genes, we compared the expression of the ABA and GAs metabolic genes in \(dag1\) mutant and wild type dry seeds at 0, 14 and 28 DAH. Interestingly, only the ABA catabolic gene \(CYP707A2\) and the GA biosynthetic gene \(GA3ox1\) were deregulated by \(DAG1\) inactivation: expression of \(CYP707A2\) was increased up to 4-fold at 28 DAH, while \(GA3ox1\) was significantly upregulated at 0, 14 and 28 DAH (Fig. 3a and b).
These results suggest that indeed DAG1 controls dormancy via the regulation of the GA3ox1 and CYP707A2 metabolic genes also after seed maturation and harvest, thus playing a key role in the control of the ABA/GA balance required for the developmental switch between seed dormancy and germination.

The lack of DAG1 alters both ABA and GA levels
To confirm that DAG1 actually controls the levels of ABA and GAs, we measured the content of these hormones in *dag1* and wild type seeds. The amount of ABA in mature dry *dag1* seeds was significantly lower (Fig. 4a) than in wild type seeds, in agreement with the overexpression of the CYP707A2 ABA catabolic gene. As expected, after 24 h imbibition the ABA content strongly decreased in wild type seeds [20]; interestingly, *dag1* mutant seeds showed ABA levels comparable to wild type, suggesting that the role of DAG1 in the accumulation of ABA is restricted to mature dry seeds (Fig. 4a).

Next, we measured GAs after 24 h imbibition, as in dry seeds GAs are undetectable. Consistent with the increased expression level of the GA biosynthetic gene GA3ox1, the amount of all bioactive GAs was significantly higher in *dag1* than in wild type seeds (Fig. 4b).

These results provide further support to the notion that DAG1 controls the levels of ABA and GAs in Arabidopsis seeds, and that the dormancy phenotype of *dag1* mutant seeds depends on alterations of both ABA and GA levels.

DAG1 directly regulates the ABA catabolic gene CYP707A2
We had shown that DAG1 negatively regulates the GA biosynthetic gene GA3ox1 by binding to its promoter [15, 16].

To assess whether DAG1 regulates also the CYP707A2 ABA catabolic gene by directly binding to its promoter in vivo, we performed ChIP assays, using the dag1-DAG1-HA line overexpressing the DAG1-HA chimeric protein in a *dag1* mutant background [15, 16, 21]. Cross-linked and sonicated protein–DNA complexes were precipitated with anti-HA antibodies, or without antibodies as a negative control. As additional negative control, we performed the same assays on *dag1* mutant seeds (Fig. 5, bottom left). Three regions of the
CYP707A2 promoter, one with no DOF binding sites (fragment A), one with two (fragment B), and one with ten (fragment C) were amplified by qPCR (Fig. 5, top).

The relative amount of promoter fragment C precipitated by DAG1-HA was significantly higher than the negative control, whereas the enrichment of precipitated promoter fragments A and B was very low in DAG1-HA and in the negative control (Fig. 5, bottom right), thus confirming that DAG1 directly binds to the CYP707A2 promoter in seeds.

GAs control DAG1 expression and DAG1 protein stability

Since the levels of bioactive GAs increase during seed imbibition as does the level of expression of DAG1, and DAG1 controls GA and ABA levels, we wondered whether DAG1 expression in seeds might be regulated by these hormones. RT-qPCR analysis performed on seeds imbibed 24 h in the presence of GA 4+7, or ABA, showed that the DAG1 transcript level was significantly induced by GAs (up to 4-fold), but not by ABA. Accordingly, in the presence of paclobutrazol (PAC), an inhibitor of GA biosynthesis, DAG1 expression level was comparable to control seeds imbibed with water (Fig. 6a).

Since we have shown above that DAG1 is epigenetically regulated, we verified whether its induction by GAs was mediated by variations in the H3K27me3 and/or H3K4me3 epigenetic marks. This analysis, performed with wild type seeds imbibed 24 h in the presence of GA 4+7 or with water, revealed enrichment of the H3K4me3 activating mark in GA-imbibed seeds, suggesting that DAG1 expression is induced by GAs through chromatin remodelling (Fig. 6b and c).

To investigate whether the stability of the DAG1 protein would be affected by ABA or GAs, we utilized the dag1 mutant line overexpressing a 35S:DAG1-HA translational fusion [15], whose transcription is not induced by either ABA or GAs (Additional file 2: Figure S2).

We performed an immunoblot analysis on DAG1-HA seeds imbibed for 24 and 48 h in the presence of ABA, GA 4+7 or PAC compared to water-imbibed controls. Addition of exogenous GAs increased the level of DAG1-HA, at 24 and 48 h (1.7 and 1.6-fold, respectively); consistently, the presence of PAC reduced the amount of the chimeric protein to the level of the corresponding control (Fig. 7a). Interestingly, the amount of DAG1-HA increased during imbibition up to 48 h, as a consequence of the increase of the endogenous GA levels. In contrast, the level of DAG1-HA was not affected by ABA (Fig. 7a). To gain insight on the molecular mechanism underlying this GA-mediated control of the DAG1 protein, we performed an immunoblot analysis of DAG1-HA seeds imbibed for 48 h then treated for 4/8 h with cycloheximide.
(CHX), to inhibit protein synthesis, or with CHX and GAs. As shown in Fig. 7b, the increase of DAG1-HA during imbibition is mainly due to new synthesis of the protein, since in the presence of CHX the amount of DAG1-HA was drastically reduced. Interestingly, addition of exogenous GAs resulted in an increase of the protein level, suggesting that GAs stabilize DAG1-HA by increasing its half-life (Fig. 7b).

We have also investigated whether the post-translational control of DAG1 by GAs is mediated by the 26S proteasome. We performed the analysis on seedlings grown for five days, then treated with MG132, a specific inhibitor of the proteasome, or DMSO as a control [22]. As shown in Fig. 7c, addition of MG132 prompted the over-accumulation of the DAG1-HA chimeric protein, suggesting that DAG1 may be negatively regulated through the 26S proteasome (Fig. 7c).

**Discussion**

We had previously demonstrated that the DAG1 transcription factor is a repressor of seed germination [12, 14] acting downstream of PIL5 and negatively regulating GA biosynthesis [15, 16]. As we have also previously shown that inactivation of DAG1 reduces seed dormancy [12], we have investigated the role of DAG1 from developing seeds to germinating seedlings.

DAG1 controls the dynamic balance between ABA and GA

The results presented in this work suggest that DAG1 plays a key role in the establishment and maintenance of dormancy via the control of the dynamic balance of the levels of the hormones GAs and ABA.

Indeed, during seed maturation DAG1 positively regulates the ABA level by directly repressing the catabolic gene CYP707A2. It has been shown that CYP707A2 is a key enzyme involved in the regulation of ABA level during seed dormancy induction; in particular, expression of CYP707A2 increases from late-maturation stage (16 DAP) to dormant seeds [20], and, consistently, cyp707a2 null mutant seeds accumulate more ABA than wild type seeds at these stages, showing an hyperdormant phenotype [23]. These results are compatible with a role of DAG1 in promoting the establishment of dormancy during seed maturation via the negative regulation of CYP707A2, thus allowing an increase of ABA level before seed desiccation. Indeed, we have previously reported that in germinating seeds CYP707A2 is upregulated in the loss-of-function dag1 mutant [15]; consistently, DAG1
and CYP707A2 are both expressed in the provascular tissue of the embryo [20, 21].

It was previously shown that during dry seed storage GA levels must be maintained low to ensure seed dormancy [19, 24]; our data point to DAG1 playing a crucial role in maintaining GA biosynthesis low by repressing the expression of GA3ox1 [15] (this work). In turn, both DAG1 expression and protein stability are controlled by GA, as the DAG1 transcript level is increased and the protein is stabilized by bioactive GAs. This suggests that DAG1 might play a pivotal role in fine-tuning GA levels in seed germination. It has been shown that the transcript level of the GA biosynthetic gene GA3ox1 is under feedback control by GAs [25–27]: possibly, this occurs via the increase in DAG1 level, which directly represses GA3ox1.

In addition, GAs promote DELLA protein degradation, and in turn they promote transcription of DELLA genes [28]. Interestingly, the DELLA protein GAI cooperates with DAG1 in negatively regulating GA3ox1, and it directly interacts with DAG1 [16]. Indeed, GAI is degraded in the presence of GAs, but it is in turn actively de novo synthesised in order to ensure GA3ox1 repression through interaction with DAG1. In addition, DAG1 and GAI mutually affect their expression [16]. This regulatory loop, where GAs promote both the transcriptional induction and stabilization of a GA biosynthesis repressor (DAG1), as well as the induction of a GA signalling repressor (GAI) helps preventing early germination and/or vivipary, as well as germination under unfavorable conditions.

Similar results have been recently described in the case of the transcription factor ABI4, which controls seed dormancy by regulating the ABA and GA biosynthesis [29]. ABI4 inactivation affects different ABA and GA metabolic genes - notably NCED2 and NCED3 and GA3ox1 and GA2ox8 respectively - and it has been shown to directly regulate the ABA catabolic genes CYP707A1 and CYP707A2 [29]. However, the effect of (the lack of) ABI4 on the expression of these genes seems to be restricted to the first six hours of seed imbibition, whereas inactivation of DAG1 results in upregulation of CYP707A2 and GA3ox1 during seed maturation, storage and in seeds imbibed 12 and 24 h [15] (this work), suggesting that these two transcription factors do not function jointly. In addition, abi4 seeds do not show reduced dormancy since the germination rate of wild type and mutant seeds were similar, although abi4 seeds germinated more quickly than wild type and, differently from dag1 seeds, they did not show increased sensitivity to cold treatment [29], suggesting that DAG1 and ABI4 do not function in the same signaling pathway.
Our results point to DAG1 as a key regulator in the control of the developmental switch between seed dormancy and germination, acting on the balance between ABA and GAs.

**DAG1 is controlled at the epigenetic level during seed development and early seedling growth**

Our results show that trimethylation of histone H3 lysine 27 (H3K27me3), an important epigenetic mark, targets the **DAG1** locus. This chromatin repressive state is catalyzed by the Polycomb Repressive Complex 2 (PRC2), which has been shown to control the transition from seed to seedling in Arabidopsis [11]. Indeed, several seed developmental regulatory genes like **DOG1**, **ABSCISIC ACID INSENSITIVE 3 (ABI3)** and **SOMNUS (SOM)** have been shown to be targets of PRC2 and marked by H3K27me3 during this developmental switch. In addition, Bouyer et al. [11] performed a genome-wide analysis of the chromatin state of fie (fertilization independent endosperm) mutant plants lacking PRC2, and found that **DAG1** expression is upregulated, suggesting that also **DAG1** may be a target of PRC2.

Consistently, our ChIP assays indicate that the transcribed region of **DAG1** is significantly enriched in the H3K27me3 repressive mark in seedlings, pointing to **DAG1** as a seed developmental gene.

Similarly to **DAG1**, expression of **SOM**, encoding a CCCH-type zinc finger protein, is up-regulated in fie mutants [11]. Interestingly, SOM down-regulates GA and upregulates ABA levels [30] to repress seed germination at high temperature [30, 31]. Our findings that **DAG1** is marked by H3K27me3 during the transition from dormant seeds to vegetative growth, further substantiate the idea that PRC2 functions during seed development to sustain the opposite action of ABA and GA as previously suggested by Bouyer et al. [11].

By means of a **pDAG1::GUS** line, we have previously shown that the **DAG1** promoter is active during embryogenesis, from globular stage to mature embryo [21]. The expression analysis we presented in this
paper, performed by RT-qPCR, clearly revealed that DAG1 is finely modulated during seed maturation and dormancy. The DAG1 transcript level, is high at 13 DAP, it progressively decreases during maturation and dormancy and raises again in non-dormant seeds following imbibition. In addition, DAG1 expression is induced by exogenous GAs in imbibed seeds, as the treatment with PAC results in DAG1 expression level comparable to the control imbibed with water. Consistently, the DAG1 locus is enriched in the H3K4me3 activating mark, as revealed by the ChIP assay performed with H3K4me3 antibodies, suggesting this epigenetic mark is necessary to ensure an active transcriptional state of the DAG1 locus.

It was recently shown that other seed development regulatory genes such as ABI3 and DOG1 display a similar expression profile as DAG1, in that they are switched from an active to a repressive chromatin state during the transition from seed to seedling [32]. It will be interesting to assess whether all these regulatory genes work in the same or in different but possibly cross-talking regulatory networks in controlling the seed-to-seedling transition.

Conclusions
While the importance of the balance between ABA and GAs during seed germination is well established, evidence on the molecular mechanisms underlying the transition from seed to germinating seedling is scanty. This work identifies a key component of the molecular network controlling the seed-to-seedling transition. Indeed, our work provides convincing evidence that DAG1 plays a crucial role in the establishment and maintenance of dormancy by controlling the balance of the levels of the hormones GA and ABA, acting on their biosynthesis and catabolism, respectively.

Methods
Plant material and growth conditions
dag1 is the allele described in Papi et al. [12] in Ws-4 ecotype, dag1DAG1-HA is the transgenic line described in Gabriele et al. [15]. All Arabidopsis thaliana lines used in this work were grown in a growth chamber at 24/21 °C with 16/8-h day/night cycles and light intensity of 300 μmol/m² s⁻¹ as previously described [12].

Seed germination assay
All seeds used for germination tests were harvested from mature plants grown at the same time, in the same conditions, and stored for 4–5 weeks in the dark under dry conditions at room temperature. For seed germination assays, triplicate sets of 60–100 non-sterilized seeds for each genotype were sown on five layers of filter paper 595 (Schleicher & Schüll, Dassel, Germany), soaked with 5 ml water, under dim-green safe light. All germination assays have been performed with different seed batches.

Expression assays were performed with 5 ml water, under dim-green safe light. All germination assays have been performed with different seed batches.

Expression analysis
RNA was extracted from developing seeds dissected from silique at 13, 16, 19 and 21 days after pollination (DAP), dry seeds at 0, 14 and 28 days after harvest (DAH) dry seeds or imbibed seeds. The seeds were imbibed for 6, 12 or 24 h on five layers of filter paper, soaked with 5 ml water and exposed to light. For hormone treatments, seeds were imbibed for 24 h in the presence of 100 μm GA 4 + 7 (Duchefa) or 100 μm PAC (Duchefa) or 3 μm ABA (Duchefa) and exposed to light.

RNA extraction and RT-qPCR were performed according to Gabriele et al. [15]. Relative expression levels were normalized with appropriated reference genes. The primers used are listed in (Additional file 3: Table S1). The values of relative expression levels are the mean of three biological replicates presented with SD values. Significative differences were analyzed by t-test (*P ≤ 0.05; **P ≤ 0.01).

Chromatin Immunoprecipitation (ChIP) assay
ChIP assay was performed according to Gabriele et al. [15]. To study the binding of DAG1 to CYP707A2, ChIP assay was performed with the transgenic line overexpressing the DAG1-HA chimeric protein in a dag1 mutant background and with the dag1 mutant as a negative control. The immunoprecipitation was performed using HA-probe antibody (Y-11, sc-805 Santa Cruz). To analyse the epigenetic profile of the DAG1 locus, chromatin was immunoprecipitated overnight using antibodies against H3K27me3 (Millipore #07-449), H3K4me3 (Abcam ab8580), or without antibodies as negative control. After reverse cross-linking, the enriched DNA levels were quantified by qPCR using specific primer sets (Additional file 3: Table S1). The Fold enrichment of a regulatory genes such as ABI3 and DOG1 display a similar expression profile as DAG1, in that they are switched from an active to a repressive chromatin state during the transition from seed to seedling [32]. It will be interesting to assess whether all these regulatory genes work in the same or in different but possibly cross-talking regulatory networks in controlling the seed-to-seedling transition.

ABA and GA dosages
Samples were analysed for GA content according to [33] with some modifications. Seed samples (20–30 mg dry weight) were homogenized in 2 ml polypropylene tubes with 1 ml of 80 % (v/v) acetonitrile containing 5 % (v/v) formic acid and 19 internal GA standards ([2H2]GA1, [2H2]GA3, [2H2]GA4, [2H2]GA5, [2H2]GA6, [2H2]GA7, [2H2]GA8, [2H2]GA9, [2H2]GA12, [2H2]GA13, [2H2]GA15, [2H2]GA19, [2H2]GA20, [2H2]GA24].
The homogenates were centrifuged for 10 min at 4 °C. Supernatants were further purified using mixed-mode anion exchange cartridges (Waters, http://www.waters.com) and analysed by ultra-high performance chromatography (Acuity UPLC System; Waters) coupled to triple-stage quadrupole mass spectrometer (Xevo® TQ MS; Waters) equipped with an electrospray ionization (ESI) interface. GAs were detected using the multiple-reaction monitoring mode based on transition of the precursor ion [M-H]− to the appropriate product ion. Data were acquired and processed by Masslynx 4.1 software (Waters) and GA levels were calculated using the standard isotope-dilution method [34]. For ABA dosage, seeds were frozen in liquid nitrogen and freeze-dried. The dried seeds (10 mg) were ground in 1.6 ml of extraction solvent (acetonitrile/water/acidic acid, 80/19/1, v/v/v), in which 2 ng of [3H2]ABA ((−)-5, 8′, 8′-d4 ABA purchased from Irina Zaharia, Plant Biotechnology Institute, National Research Council Canada, http://www.nrc-cnrc.gc.ca) was added as an internal standard. Samples were centrifuged and the supernatant recovered, the pellet was then re-suspended in 0.1 ml of chromatography mobile phase by sonication, re-centrifuged and the supernatants combined. The extraction solvent was then evaporated and the residue re-suspended by sonication in 0.5 ml of chromatography mobile phase (acetonitrile/water, 50/50/0.05, v/v/v) and filtered through a 1.6 μm GFA filter (Whatman, http://www.whatman.com/). ABA was quantified using a LC-ESI-MS-MS system (Quattro LC, Waters, http://www.waters.com) in a positive ion mode by multiple reaction monitoring (MRM). The results are means of two (ABA dosages) or three (GA dosages) biological replicates and are presented with SD values. Significant differences were analyzed by t-test (*P ≤ 0.05).

Immunoblot analysis

A total of 25 μg of protein was extracted according to Øh et al. [35] and separated on a 12 % SDS-polyacrylamide gel (Bio-Rad) and blotted on a PVDF Immobilon-P Transfer membrane (Millipore). Detection of chimeric proteins was performed with anti-HA antibodies (Santa Cruz, Santa Cruz, CA, USA) as primary antibody and AP-conjugated anti-mouse as secondary antibody (Sigma, St. Louis, U.S.A.). Total proteins revealed by stain-free technology (Bio-Rad), or tubulin levels detected using an anti-tubulin antibody (Sigma, St. Louis, U.S.A.) were used as loading control. For MG132 treatment, 5-days-old dark grown seedlings were treated 4 h with MG132 (50 μm) or DMSO in dark or R light. For GA, Paclobutrazol (PAC) or ABA treatments, 50 μl seeds were imbibed 24 or 48 h, in the presence of 100 μm GA 4+7 (Duchefa) or 100 μm PAC (Duchefa) or 3 μm ABA (Duchefa). For CHX treatment, 50 μl seeds were imbibed 24 h in the presence of GA, or 48 h with water, then transferred on CHX (50 μm) for 4 or 8 h. The protein levels are the mean of three biological replicates, presented with SD values. Significant differences were analyzed by t-test (*P ≤ 0.05).

Additional files

Additional file 1: Figure S1. Negative controls of the ChIP assays. Chromatin from WT embryos at 10/13 DAP, 0 DAH, 24 h-imibed seeds, and 14 days-old seedlings was immunoprecipitated without antibody as a negative control. The amount of DNA was measured by qPCR. The values of fold enrichment were normalized to internal controls (relative to input and to PP2A), and are the average of three independent experiments presented with SD values. (PDF 265 kb)

Additional file 2: Figure S2. Relative expression level of 35S::DAG1-HA in imbibed seeds. Relative expression level of DAG1-HA, under the control of the 35S CaMV promoter, in dag10DAG1-HA seeds, imibed 24 or 48 h. The values of relative expression levels are the mean of three biological replicates, presented with SD values. Expression levels were normalized with that of the UBO10 (AH053230) gene. (PDF 275 kb)

Additional file 3: Table S1. Primers used in this study. (PDF 554 kb)

Abbreviations

ABA: Abscisic Acid; GA: Gibberellins; H3K27me3: Histone H3 at lysine 27 trimethylated; H3K4me3: Histone H3 at lysine 4 trimethylated; DAG1: DOF AFFECTING GERMINATION1; GA3ox1: Gibberellin 3-β-dioxgenase 1; DOG1: DELAY OF GERMINATION1; QTL: Quantitative Trait Loci; PRC2: Polycomb Repressive Complex 2; phyB: Phytochrome B; PIL5: PHYTOCHROME INTERACTING FACTOR3 likes; GAI: GA INSENSITIVE; DAP: Days after pollination; DAH: Days after harvest; CHX: Chlamydoma Mutimn Precipitation; RT-qPCR: Quantitative reverse transcriptase-polymerase chain reaction; qPCR: Quantitative PCR; PAC: Paclobutrazol; CHX: Cycloheximide; DMSO: Dimethyl sulfoxide; fie: Fertilization independent endosperm; ABI3: ABSICISIC ACID INSENSITIVE 3; SOM: SOMNUS

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Availability of data and materials

The data sets supporting the results of this article are included within the article and its additional files.

Authors’ contributions

PV, AB conceived the strategy. PV, AB, AMP, MS designed the experiments. AB, RI, VR, AF, SM, DT performed the experiments. AB, AMP, PC, PV
contributed to data analysis and interpretation. PV and PC wrote the manuscript. AB, AMP, MS, DT revised the manuscript. All Authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable

Ethics approval and consent to participate
Not applicable

Author details
1Istituto Pasteur Italia - Fondazione Cenci Bolognetti, Rome, Italy.
2Istituto Pasteur Italia - Fondazione Cenci Bolognetti, Rome, Italy.

Feedback

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