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Research

A novel role for histone methyltransferase KYP/SUVH4 in the control of *Arabidopsis* primary seed dormancy

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Summary

- Seed dormancy controls germination and plays a crucial role in the life cycle of plants. Chromatin modifications are involved in the regulation of seed dormancy; however, little is known about the underlying mechanism.
- KYP/SUVH4 is required for histone H3 lysine 9 dimethylation. Mutations in this gene cause increased seed dormancy. *KYP/SUVH4*-overexpressing *Arabidopsis* plants show decreased dormancy. *KYP/SUVH4* expression is regulated by abscisic acid (ABA) and gibberellins (GA). The sensitivity of seed germination to ABA and paclobutrazol (PAC) is enhanced slightly in *kryptonite-2* (*kyp-2*) and *suvh4-2/suvh5* mutants, but weakened in *KYP/SUVH4*-overexpressing plants.
- In the *kyp-2* mutant, several dormancy-related genes, including *DOG1* and *ABI3,* show increased expression levels, in agreement with a negative role for *KYP/SUVH4* in gene transcription.
- Genetic analysis showed that *DOG1* and *HUB1* are epistatic to *KYP/SUVH4*, suggesting that these genes regulate seed dormancy in the same genetic pathway.

Introduction

Higher plants must adjust their germination timing to their native habitat so that they can survive and complete their life cycle. Germination is tightly regulated by seed dormancy, which is an ecologically important adaptive trait that has evolved to repress germination under temporary favorable conditions (Bewley, 1997). This property enables plants to delay germination until conditions are optimal for survival of the next generation. In the model plant *Arabidopsis thaliana*, dormancy can be broken after a period of seed after-ripening or on seed stratification, that is, exposure to cold and moist conditions. In the field, low dormancy levels often cause preharvest sprouting in crops, such as wheat, rice and barley, resulting in reduced grain yield and quality.

The molecular and biochemical bases of seed dormancy remain largely unclear; however, notable progress has been achieved at the transcriptomic (Nakabayashi *et al.*, 2005; Cadman *et al.*, 2006; Carrera *et al.*, 2008; Okamoto *et al.*, 2010), proteomic (Chibani *et al.*, 2006) and metabolomic (Fait *et al.*, 2006) levels. These studies have indicated that the induction and release of seed dormancy are associated with changes in the level of gene expression, compounds and proteins. An intricate molecular network in the control of seed dormancy and germination is emerging.

Molecular and genetic analyses have presented evidence that abscisic acid (ABA) is central to the establishment and maintenance of seed dormancy (Finch-Savage & Leubner-Metzger, 2006; Holdsworth *et al.*, 2008; North *et al.*, 2010), whereas gibberellins (GAs) are important for germination (Debeaujon & Koornneef, 2000; Ogawa *et al.*, 2003; Kucera *et al.*, 2005). Mutations impairing ABA biosynthesis reduce seed dormancy, whereas overexpression of biosynthesis genes or mutations in catabolism genes enhance seed dormancy (Finkelstein *et al.*, 2007; Holdsworth *et al.*, 2008). Representative mutants, such as *aba1*, *aba2*, *aba3*, *nced6/nced9* and *cyp707a2*, show altered seed dormancy levels (Koornneef *et al.*, 1982, 1984; Giraudat *et al.*, 1992; Léon-Kloosterziel *et al.*, 1996; Lefebvre *et al.*, 2006; Okamoto *et al.*, 2006). Many genes in the ABA signaling network also regulate the induction and maintenance of seed dormancy. The ABA-supersensitive mutant *era1* confers enhanced seed dormancy (Cutler *et al.*, 1996). *ABI3*, encoding a seed-specific B3 domain-containing protein, plays a crucial role in seed maturation with an additive effect on seed dormancy (Sugliani *et al.*, 2010). Members of the PP2C family, including

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ABI1, ABI2 and HAB1, are key regulators of the ABA signaling pathway and function as negative regulators of seed dormancy (Beaudoin *et al.*, 2000; Nambara *et al.*, 2000; Miyazono *et al.*, 2009). ABA-activated kinases of the SnRK2 family act redundantly as positive regulators of seed dormancy (Nakashima *et al.*, 2009). A recent major breakthrough has been the identification of ABA receptors. The RCAR/PYR type of ABA receptor can bind and inactivate PP2C proteins, allowing SnRK2 to phosphorylate downstream substrates (Ma *et al.*, 2009; Park *et al.*, 2009; Umezawa *et al.*, 2009). Further work is required to define the role of the identified ABA receptors in seed dormancy and germination.

The role of GA in the control of seed germination is antagonistic to ABA (Razem *et al.*, 2006; Weiss & Ori, 2007; Toh *et al.*, 2008). GA-deficient mutants, such as *ga1-3* and *ga2-1*, can delay seed germination (Koornneef & Veen, 1980). GA signaling pathway proteins are also involved in seed germination regulation and, among these, the DELLA protein RGL2 is the main repressor of seed germination (Lee *et al.*, 2002; Peng & Harberd, 2002; Ariizumi & Steber, 2007). Other DELLAs, including RGA, GAI and RGL3, play additional roles in seed germination (Cao *et al.*, 2005; Piskurewicz & Lopez-Molina, 2009). It is widely accepted that the equilibrium between dormancy and germination is regulated by a dynamic hormonal balance between ABA and GA (Gutierrez *et al.*, 2007).

Seed dormancy is a typical quantitative trait controlled by the interplay between environmental signals and endogenous developmental processes. *Arabidopsis* shows natural variation for seed dormancy, and several delay of germination (DOG) quantitative trait loci (QTL) have been identified for this trait (Alonso-Blanco *et al.*, 2003). A transcriptomic study using different near-isogenic lines for DOG QTL revealed largely different gene ontology profiles, indicating the involvement of several independent pathways (Bentsink *et al.*, 2010). The major seed dormancy QTL DOG1 has been cloned (Bentsink *et al.*, 2006). *DOG1* is only expressed in developing and mature seeds and encodes a protein with unknown function. The *dog1* mutant is characterized by the absence of dormancy and does not show any pleiotropic phenotypes, indicating that DOG1 may play a specific role in the onset of seed dormancy. A QTL in rice, Sdr4, contributes to differences in seed dormancy between *japonica* (Nipponbare) and *indica* (Kasalath), and has been identified as a seed dormancy-specific regulator (Sugimoto *et al.*, 2010). *Sdr4* encodes a protein with unknown function that plays a regulatory, rather than a structural or metabolic, role in the promotion of dormancy. The cloning of *Sdr4* provided an opportunity to explore the genetic control and modification of seed dormancy in crops.

Recent studies have provided genetic evidence for the transcriptional control of seed dormancy and germination by chromatin remodeling. The *REDUCED DORMANCY 4* (*RDO4*) locus encodes a C3HC4 RING finger protein with homology to histone-modifying enzymes of yeast Bre1 and human RNF20/RNF40 (Liu *et al.*, 2007). The mutants fail to ubiquitinate histone H2B and the locus was consequently renamed *HISTONE MONOUBIQUITINATION 1* (*HUB1*). Defects in a close homolog, designated *HUB2*, also cause decreased dormancy. Histone H2B monoubiquitination is associated with actively transcribed genes, and the *hub1* mutant shows altered expression levels of several dormancy-related genes. *HDA6* and *HDA19*, encoding histone deacetylases, which involve chromatin remodeling, modulate seed germination by affecting ABA-induced gene expression (Chen & Wu, 2010; Chen *et al.*, 2010). Finally, the *REDUCED DORMANCY 2* (*RDO2*) locus encodes the transcription elongation factor S II (TFIIS; Liu *et al.*, 2011). Plants with RNAi-mediated knockdown of TFIIS expression also show reduced seed dormancy (Grasser *et al.*, 2009). TFIIS factors can enhance elongation by promoting cleavage and reactivation of nascent transcripts, whose elongation is blocked under specific conditions in yeast and mammalian cells (Wind & Reines, 2000). Similar blocks may occur in a drying or dry seed. Taken together, these studies clearly reveal that chromatin modifications and transcription elongation regulate seed dormancy and germination.

The *Arabidopsis KYP/SUVH4* gene, encoding a histone methyltransferase, mediates histone H3 lysine 9 dimethylation (Jackson *et al.*, 2002). In this study, we report that KYP/SUVH4 functions as a negative regulator of seed dormancy. The *kryptonite-2* (*kyp-2*) mutant shows increased seed dormancy and sensitivity to ABA, whereas overexpression of *KYP/SUVH4* in seeds leads to reduced dormancy and ABA sensitivity. We also present evidence that KYP/SUVH4 influences gene expression of dormancy-related genes, including *DOG1*, and several genes in the ABA signaling pathway. This is the first report to suggest that *KYP/SUVH4* may play a regulatory role in the control of seed dormancy.

Materials and Methods

Plant materials and growth conditions

The mutant *kyp-2* was obtained by crossing the double mutant *kyp-2/gl1* in the Landsberg *erecta* (L*er*) background, ordered from the Nottingham Arabidopsis Stock Centre (NASC, Nottingham, UK) (http://arabidopsis.info/), with L*er*. The *kyp-2* mutant has been described by Jackson *et al.* (2002). The mutants of *rdo2*, *hub1-2* and *dog1* in the L*er* background have been described by Liu *et al.* (2007, 2011) and Bentsink *et al.* (2006). The *ga1-3* and *abi3-5* mutants are also in the L*er* background. All double mutants were generated by crossing and selection in the F2 generation. Molecular markers for genotyping of the *kyp-2* and *hub1-2* mutations have been described in Jackson *et al.* (2002) and Liu *et al.* (2007), respectively. The single-strand conformational polymorphism (SSCP) marker for the genotyping of *rdo2* was based on the 4-bp deletion in *rdo2*, using the PCR primers RDO2-F (5′-CAAGAAGTGCTGATGAGCCAATG-3′) and RDO2-R (5′-ATCGGAGCCAGAGCATTCTAGG-3′). The simple sequence length polymorphism (SSLP) marker for the genotyping of *dog1* was amplified using primers DOG1-F (5′-TCAGTTTCTCCGCAACATCG-3′) and DOG1-R (5′-CAAATTCAAACCGAACCCAAC-3′).

Seeds were sown in soil and grown in the glasshouse under photoperiodic cycles of 16 h light : 8 h dark at 22°C (day temperature) and 18°C (night temperature). The seeds sown on half-strength Murashige and Skoog (MS) medium were first sterilized with 10% (v/v) NaClO. Plates were kept in the dark at

4°C for 3 d to break dormancy (stratification), before moving into a climate chamber with a photoperiod of 16 h light : 8 h dark at 22°C. The 5-d-old seedlings were transferred from the plates to soil in pots.

Germination tests were performed as described by Alonso-Blanco *et al.* (2003). All germination experiments were performed on filter paper in 6-cm Petri dishes. Each genotype had at least eight replicates (consisting of 80–100 seeds from one individual plant per Petri dish). The average germination percentage was determined after 7 d of incubation in a climate room (25°C, 16 h light with 80–90 μmol m^{-2} s^{-1} light intensity). Filter papers were soaked with either water or solutions of the GA biosynthesis inhibitors paclobutrazol (PAC) or ABA. Seeds for each germination assay were collected from plants of different genotypes grown simultaneously and stored under identical conditions.

Screening of T-DNA insertion lines

T-DNA insertion lines in the Columbia-0 background for *KYP/SUVH4* (At5g13960) and *SUVH5* (At2g35160) were obtained from the Salk collection (*suvh4-2* and *suvh4-3*; http://signal.salk.edu) or GABI-Kat collection (*suvh5*; http://www.gabi-kat.de) with the following seed stock numbers: *suvh4-2*, Salk_130630; *suvh4-3*, Salk_105816; *suvh5*, GABI_263C05. PCR-based screening was used to identify homozygous individuals for T-DNA insertions in *KYP/SUVH4* and *SUVH5*. The gene-specific primers, designed by the SIGNAL T-DNA verification primer design program, were used in combination with T-DNA left border primers. Reverse transcription-polymerase chain reaction (RT-PCR) with RNA isolated from leaves was performed to confirm the homozygous knockout lines. PCR was performed with 25 cycles for *ACTIN2* and 35 cycles for *KYP/SUVH4* and *SUVH5*, with the following gene-specific primers: for *KYP/SUVH4*, P1 (5′-GTACCGACTGAAACGATTGGA-3′), P2 (5′-AGTTCGGTTGACACATTTTGG-3′), P3 (5′-CC CAAGAAAAATAATCGGTGA-3′) and P4 (5′- CCAATCGTT TCAGTCGGTAC-3′); for *SUVH5*, P5 (5′-TAGAGCCAGAG CCAAAGATGC-3′) and P6 (5′-CTCTTTTTATCCAGGGCAACC-3′).

Constructs and plant transformation

For the *pDOG1::KYP/SUVH4* and *p35S::KYP/SUVH4* constructs, total RNA was isolated from L*er* young leaves using the TIANGEN TRNzol-A kit. cDNA fragments encoding the amino acid sequence of At5g13960 and containing attB1 and attB2 sites at the 5′ and 3′ terminals were amplified by RT-PCR using the following primers: 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTATCGATGGCTGGAAAAAGGAAACGAGCTAATGC-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTGTTAGTAAAGGCGTTTCCTACAATTTAGCGCTCCAC-3′. The amplified fragments were cloned into the Gateway entry vector pDONR207 (Invitrogen, http://www.invitrogen.com) by BP reaction (Invitrogen), and then transferred to a destination vector containing the *DOG1* promoter (a gift from Dr Melanie Bartsch, Max Planck Institute for Plant Breeding Research, Cologne, Germany) and Pleela vector (GenBank accession number AF404854) by LR reaction (Invitrogen). The recombinant plasmid was introduced into L*er* wild-type or *kyp-2* mutant plants by infiltration with *Agrobacterium tumefaciens* strain GV3101 or GV3101 pm90RK (Clough & Bent, 1998). Transformed *Arabidopsis* lines were selected on the basis of their ability to survive after being sprayed twice with 150 mg l^{-1} BASTA. The 3 : 1 segregating transformants were selected on MS medium containing 5 μg ml^{-1} DL-phosphinothricin. T3 homozygous transgenic plants were used for phenotypic analysis. *KYP/SUVH4* transcript levels in freshly harvested dry seeds of transgenic plants were checked by quantitative RT-PCR.

The *pSUVH4::GUS* construct was created by fusing *c.* 2 kb of the *KYP/SUVH4* promoter (− 2049 to − 1 relative to ATG of *KYP/SUVH4*) to the vector pBI101 carrying the *β-glucuronidase* (*GUS*) gene. Primers for PCR were as follows: 5′-AAGCTTAGTGTAACTAACCAATCAAG-3′ and 5′-GTCGACCATCGATCACTCTTTTTCCC-3′. The restriction endonuclease sites *Hin*dIII and *Sal1* were designed at the 5′ and 3′ ends of the *KYP/SUVH4* sequences for subcloning purposes. Plasmids containing the *pSUVH4::GUS* reporter gene were then introduced into the *Arabidopsis* accession Columbia-0 by *A. tumefaciens* (GV3101)-mediated transformation. Transgenic plants were selected on MS medium with 50 μg ml^{-1} kanamycin. Homozygous T3 plants from 3 : 1 segregating T2 lines were selected for GUS assays (Kroj *et al.*, 2003). Developmental patterns of GUS activity were analyzed using a Leica S6D (Bannockburn, IL, USA) equipped with a Nikon SMZ1500 and Nikon DS-Fi1 digital camera (Mississauga, ON, Canada). At least 12 independent lines were examined. All of the constructs used in this study were confirmed by sequencing.

RNA isolation and quantitative RT-PCR analysis

Total RNA was extracted from stems, roots, leaves, flowers and buds of L*er* plants using Trizol (Invitrogen) following the protocol. Total RNA was extracted from imbibed seeds or fresh dry seeds using the RNAqueous kit with plant RNA isolation aid (Ambion), and purified with the Qiagen RNeasy mini kit. cDNA was synthesized with a QuantiTect reverse transcription kit (Qiagen). Quantitative real-time PCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen) and ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. *ACTIN2* was used as an internal standard to normalize the data. The primer sets used for PCR are listed in Supporting Information Table S1. The specificity of the amplifications was verified by analysis of the PCR products on agarose gels and by melting curve analysis. The efficiency of the amplifications was confirmed by analysis of standard curves and ranged from 0.97 to 1.05.

Results

KYP/SUVH4 is a negative regulator of seed dormancy

Recent studies have shown that chromatin remodeling plays a role in the control of seed dormancy and germination (Liu *et al.*, 2007, 2011). Therefore, we screened mutants in genes

controlling chromatin modifications for seed dormancy phenotypes. The *kyp* mutant was selected and further investigated in detail.

KYP/SUVH4 encodes an H3 Lys 9 methyltransferase, required for H3K9 methylation (Jackson *et al.*, 2002). Mutants of this gene were identified in a mutagenesis screen for suppressors of gene silencing at the *Arabidopsis SUPERMAN* locus. Additional morphological defects were not observed for *kyp* in this study. We obtained a *kyp-2* single mutant by crossing the double mutant *kyp-2 gl1* in the L*er* background with the L*er* wild-type. The seed dormancy of the mutant was determined by analyzing the germination rate of seeds stored in dry condition for different periods. The results revealed that the *kyp-2* mutant showed significantly enhanced seed dormancy (Fig. 1a). The *kyp-2* seeds reached up to 100% germination after 5 wk of dry storage, but wild-type L*er* took only 3 wk of dry storage to reach a similar germination level under the same conditions. The *gl1* single mutant obtained from the same crossing showed a similar germination phenotype to the wild-type during seed dry storage (Fig. S1a), indicating that GL1 does not affect seed dormancy. These results imply that KYP/SUVH4 functions in the regulation of seed dormancy release.

To further investigate the effect of *KYP/SUVH4* on seed dormancy, we created transgenic plants expressing *KYP/SUVH4* driven by the *DOG1* promoter, which confers a strong and seed-specific expression, and the 35S promoter, which confers a strong and constitutive expression. *KYP/SUVH4* transcript levels in the transgenic plants were indeed much higher than in the wild-type (Figs 1c, S2b). In contrast with the *kyp-2* mutant, *pDOG1::KYP/SUVH4* and *p35S::KYP/SUVH4* transgenic plants exhibited significantly reduced seed dormancy (Figs 1b, S2a). After 1 wk of dry storage, *pDOG1::KYP/SUVH4* transgenic lines pDS1-9 and pDS4-1 reached 59% and 57% germination, respectively, whereas only 28% of wild-type seeds germinated. Freshly harvested dry seeds from the *p35S::KYP/SUVH4* transgenic plants germinated at 83% and 39%, respectively, whereas only 11% of the wild-type seeds germinated at this time (Fig. S2a). These results confirm that *KYP/SUVH4* plays a negative role in the regulation of seed dormancy.

We studied the expression pattern of *KYP/SUVH4* in transgenic plants containing the GUS reporter gene, driven by a 2-kb region 5′ of the *KYP/SUVH4* gene, and by real-time PCR. GUS signals were detected universally in all tissues of the transgenic plants (Fig. 2a), and RT-PCR showed strongly increased expression of *KYP/SUVH4* in imbibed seeds (Fig. 2b). Information retrieved from the public *Arabidopsis* microarray database (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) also confirmed that *KYP/SUVH4* is strongly upregulated by imbibition (Fig. S3a). These results indicate that *KYP/SUVH4* may function in the transition phase of seed germination.

KYP/SUVH4 reduces the sensitivity of seed germination to ABA and PAC

Mutants with a delayed germination phenotype, such as *era1*, *ahg2* and *ahg4*, have been reported to be hypersensitive to ABA

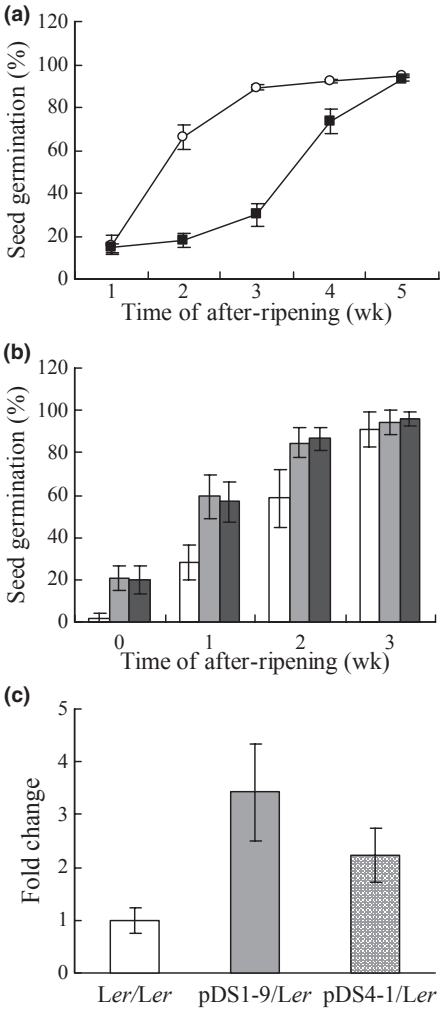

Fig. 1 Seed dormancy of mutant and transgenic *Arabidopsis* plants. (a) Seed germination of L*er* (circles) and *kyp-2* (squares) on water in the light after different periods of after-ripening. Percentages of seed germination are means (± SE) based on at least eight individual plants for each line. (b) Seed germination of L*er* (white bars) and two independent homozygous transformants, pDS1-9 (light gray bars) and pDS 4-1 (dark gray bars), containing the *pDOG1*::*SUVH4* construct in the L*er* background, on water in the light after different periods of dry storage. Percentages of seed germination are means (± SD) based on at least eight individual plants for each line. (c) *KYP/SUVH4* transcript levels in transgenic lines pDS1-9 and pDS4-1. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) was used to check *KYP/SUVH4* expression levels. RNA was extracted from freshly harvested seeds. The expression values were normalized using *ACTIN2* as an internal control. The mean values and SE were from three independent experiments.

(Cutler *et al.*, 1996; Nishimura *et al.*, 2004). To test whether the *kyp-2* mutant and transgenic plants showed altered ABA sensitivity, we examined the seed germination in the presence of increasing concentrations of ABA. Compared with the wild-type, the *kyp-2* mutant was slightly more sensitive to ABA, as its seed germination rate was *c.* 10% lower than that of the wild-type under 0.5 and 1.0 μM ABA in the medium (Fig. 3a), consistent with its increased seed dormancy phenotype. By contrast, *pDOG1::SUVH4* transgenic plants showed *c.* 10% higher germination in the medium containing 0.5 or 1 μM ABA (Fig. 3c).

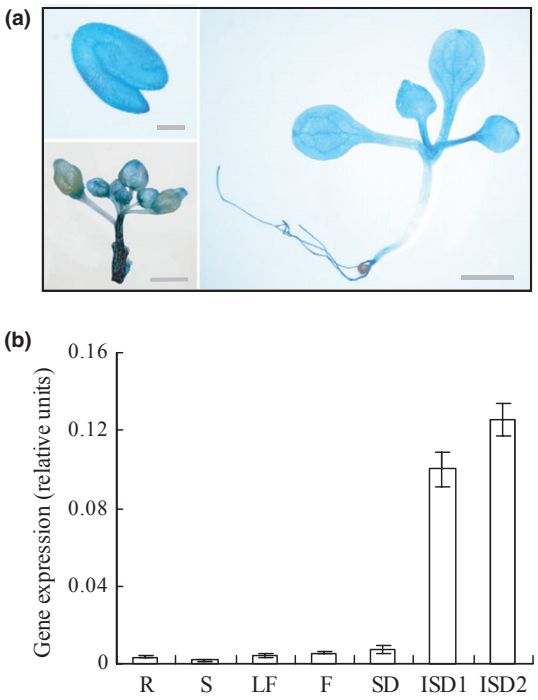

Fig. 2 Expression pattern of *KYP/SUVH4*. (a) β-Glucuronidase (GUS) staining of *pKYP/SUVH4* ::*GUS* transgenic *Arabidopsis* plants reveals GUS signals throughout the entire plant. The top left panel shows an imbibed embryo (bar, 100 μm), the bottom left panel shows an inflorescence (bar, 2 mM) and the right panel shows a 10-d-old plant (bar, 8 mm). (b) Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of *KYP/SUVH4* transcription in different organs of *Arabidopsis* accession L*er*. F, flower and buds; ISD1, imbibed seed 12 h; ISD2, imbibed seed 16 h; LF, leaf; R, root; S, stem; SD, seed. The *ACTIN2* gene was used as an internal control.

These results indicate that KYP/SUVH4 has a weak effect on the ABA signaling pathway, which may also partially explain the altered seed dormancy in *kyp-2* and *pDOG1::SUVH4* transgenic plants.

GAs can promote seed germination and act antagonistically to ABA, indicating that the endogenous GA level affects germination efficiency (Rodríguez-Gacio *et al.*, 2009). We checked the germination efficiency of *kyp-2* and *pDOG1::SUVH4* transgenic plants under various concentrations of PAC, a GA biosynthesis inhibitor. The germination efficiency of *kyp-2* was reduced by *c.* 20% in the presence of 1 or 10 μM PAC in the medium (Fig. 3b). The *pDOG1::SUVH4* transgenic plants showed an enhanced seed germination rate of *c.* 15% under these PAC concentrations (Fig. 3d). As PAC is not highly specific for GA synthesis inhibition (Rademacher, 2000), we analyzed whether the negative influence of PAC could be reversed by supplying GA_{4+7}. Our data clearly showed that the addition of GA completely reversed the PAC effect (Fig. S5). These results indicate that *KYP/SUVH4* can accelerate the release of seed dormancy and promote seed germination by reducing the GA requirement. Overall, although the phenotypes of sensitivity to ABA and PAC are not very strong, they are consistent with the seed dormancy phenotypes of the *kyp-2* mutant and *pDOG1::SUVH4* transgenic lines.

Phenotypes of *KYP/SUVH4* and *SUVH5* knockout mutants

In *Arabidopsis*, another histone methyltransferase domain-containing protein, SUVH5, is required to redundantly catalyze histone H3 Lys 9 dimethylation with KYP/SUVH4 (Ebbs & Bender, 2006; Rajakumara *et al.*, 2011). Therefore, we ordered T-DNA insertion mutant alleles for these genes in the Columbia-0 background (*suvh4-2*, *suvh4-3*, *suvh5*) from the Salk insertion mutant collection and the GABI-Kat collection. The location of these insertions is shown in Fig. 4(a). The homozygous T-DNA insertion lines were identified, as shown by RT-PCR analysis (Fig. 4b), indicating that they are likely to be complete knockout mutants.

We checked the seed dormancy phenotype of the individual *suvh4-2* and *suvh4-3* mutants and found that their germination rates were slightly lower than that of the wild-type, but the difference was not statistically significant (Fig. 5a, *suvh4-3* data not shown). This result is different from that of *kyp-2* (L*er* background), which may be caused by the genetic background. The *suvh5* single mutant showed enhanced seed dormancy (Fig. 5a). In order to verify whether *KYP/SUVH4* and *SUVH5* in the Columbia-0 background have redundant roles in the regulation of seed dormancy, we created the double mutant *suvh4-2 suvh5*. The dormancy level of *suvh4-2 suvh5* was significantly lower than that of *suvh5* and the wild-type (Fig. 5a). After 1 wk of dry storage, the double mutant showed germination of 20%, and *suvh5* and the wild-type 35% and 59%, respectively, indicating the existence of functional redundancy between the two genes. We also tested the germination sensitivity of *suvh4-2 suvh5* to ABA and PAC (Fig. 5b,c). The *suvh4-2 suvh5* double mutant was slightly more sensitive to ABA and PAC than the wild-type, similar to *kyp-2*. These results reveal that *KYP/SUVH4* and *SUVH5* are redundantly involved in the regulation of seed dormancy and germination, partly by influencing the equilibrium between ABA and GA.

KYP/SUVH4 expression is regulated by ABA and GA

The *kyp-2* mutant and *KYP/SUVH4* overexpression lines showed altered seed germination rates in response to ABA and PAC treatment, which indicates that *KYP/SUVH4* could be involved in the ABA and GA pathways. Therefore, we checked the expression of *KYP/SUVH4* in L*er* seeds, imbibed in different concentrations of ABA, GA and PAC. Our results demonstrated that *KYP/SUVH4* was downregulated by ABA and PAC, but upregulated by exogenously applied GA (Fig. 6). We also examined *KYP/SUVH4* expression in the GA-deficient mutant *ga1-3*, which blocks GA biosynthesis (Wilson *et al.*, 1992), and the *abi3-5* mutant, which blocks ABA signaling. *KYP/SUVH4* was weakly expressed in *ga1-3* seeds, and exogenously applied GA could recover *KYP/SUVH4* expression (Fig. 7a), suggesting that GA can promote *KYP/SUVH4* expression in seeds. *KYP/SUVH4* was highly expressed in *abi3-5* seeds (Fig. 7b), indicating that *KYP/SUVH4* is negatively influenced by the ABA signaling pathway.

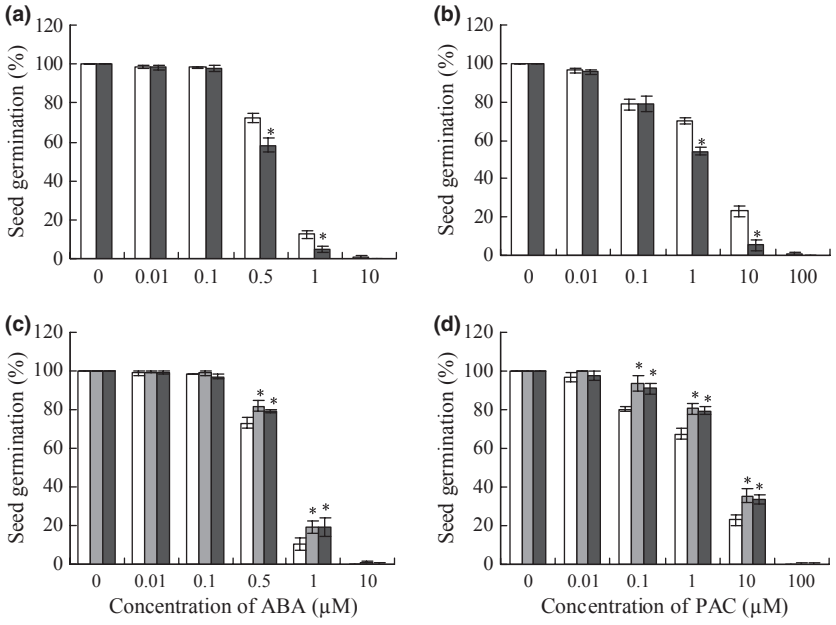

Fig. 3 KYP/SUVH4 affects seed germination sensitivity to abscisic acid (ABA) and paclobutrazol (PAC). Seed germination efficiency of wild-type (*Ler*; white bars) and *kyp-2* (gray bars) (a, b), and wild-type (*Ler*; white bars), pDS1-9 (light gray bars) and pDS4-1 (dark gray bars) (c, d) in the presence of increasing concentrations of ABA (a, c) or PAC (b, d), an inhibitor of gibberellin (GA) biosynthesis. Percentages of seed germination are means (± SD) based on at least eight individual plants for each line. Asterisks indicate a significant difference between the wild-type and the mutant, based on Student's *t*-test ($P < 0.01$).

Transcript levels of dormancy-related genes are altered in the *kyp-2* mutant

Based on the molecular function of KYP/SUVH4 and the seed dormancy phenotypes of its mutant and overexpression lines, we assumed that *KYP/SUVH4* influences seed dormancy by H3K9 methylation, leading to changes in the expression of dormancy-related genes. We analyzed the expression of several seed dormancy-related genes in 24-h imbibed seeds by quantitative RT-PCR. The genes *DOG1*, *ABI3*, *ABI4*, *NCED6*, *NCED9*, *SPT*, *PER1*, *HUB1*, *RDO2* and *ATS2* were selected for this purpose. *DOG1* encodes a protein with unknown function that is essential for dormancy (Bentsink *et al.*, 2006). *ABI3* and *ABI4* are two components of ABA signal transduction. *ABI3* encodes a B3 domain protein, which plays a key role in seed maturation (Sugliani *et al.*, 2010). *ABI4* encodes an APETALA2 domain protein (Finkelstein *et al.*, 1998). *NCED6* and *NCED9* are required for ABA biosynthesis in seeds (Lefebvre *et al.*, 2006). *SPT* is a basic helix–loop–helix transcription factor that represses seed germination and mediates the germination response to temperature (Penfield *et al.*, 2005). *HUB1* and *RDO2* encode a histone monoubiquitination E3 ligase (Liu *et al.*, 2007) and a TFIIS transcription elongation factor (Liu *et al.*, 2011), respectively; both are involved in the control of seed dormancy. *ATS2* encodes a caleosin-like protein (Toorop *et al.*, 2005) and *PER1* shows similarity to the peroxiredoxin family of antioxidants (Haslekås *et al.*, 1998); both are associated with seed dormancy establishment. The expression of *DOG1*, *ABI3*, *ABI4*, *ATS2* and *PER1* in the more dormant *kyp-2* seeds was much higher than in wild-type L*er* seeds (Fig. 8), and showed increases of 12.1, 2.2, 5.3, 3.0 and 3.3 times, respectively, compared with the wild-type. The genes *NCED6*, *NCED9*, *HUB1* and *RDO2* did not show significant expression differences between the two samples (Fig. 8). The gene *SPT* showed slightly less expression in *kyp-2* mutant seeds. Moreover, we also found that *DOG1* and *ABI3* transcript levels were downregulated in *pDOG::KYP/SUVH4*

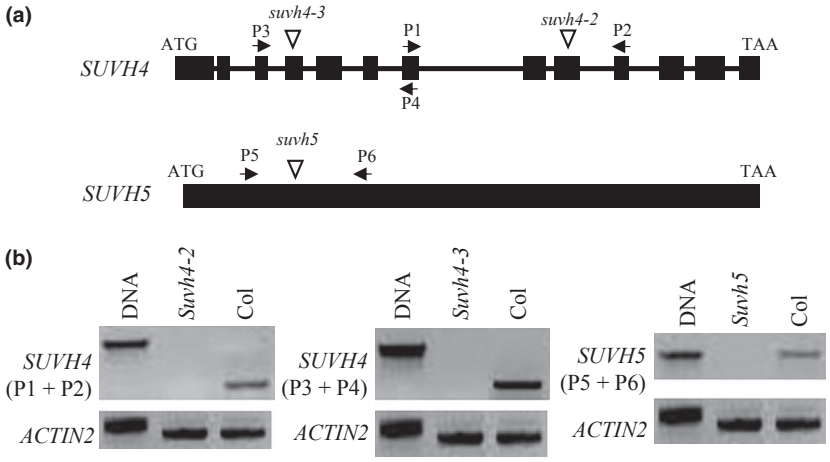

Fig. 4 Genotypic characterization of *SUVH4* and *SUVH5* T-DNA insertion lines. (a) Schematic illustration of the gene structure of *SUVH4* and *SUVH5* with the positions of the T-DNA insertions. The positions of the primers used for reverse transcription-polymerase chain reaction (RT-PCR) analysis in (b) are indicated on top of the structures. Exons are shown as black boxes and introns as lines. (b) RT-PCR analysis of the *SUVH4-2*, *SUVH4-3* and *SUVH5* transcripts in leaves of wild-type and T-DNA insertion mutants. *ACTIN2* was used as control gene.

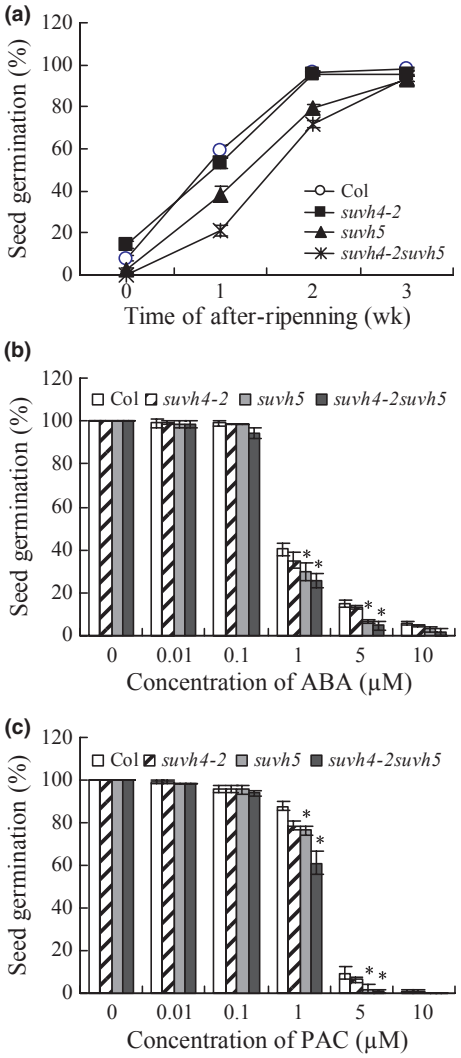

Fig. 5 Seed dormancy and sensitivity to abscisic acid (ABA) and gibberellins (GA) of *suvh4-2*, *suvh5* and the *suvh4-2 suvh5* double mutant. (a) Seed germination on water in the light after different periods of after-ripening. (b) Seed germination in the presence of increasing concentrations of ABA. (c) Seed germination in the presence of increasing concentrations of paclobutrazol (PAC). Percentages of seed germination are means (± SD) based on the seeds from eight individual plants for each line. Asterisks indicate a significant difference between the wild-type and the mutant, based on Student's *t*-test ($P < 0.01$).

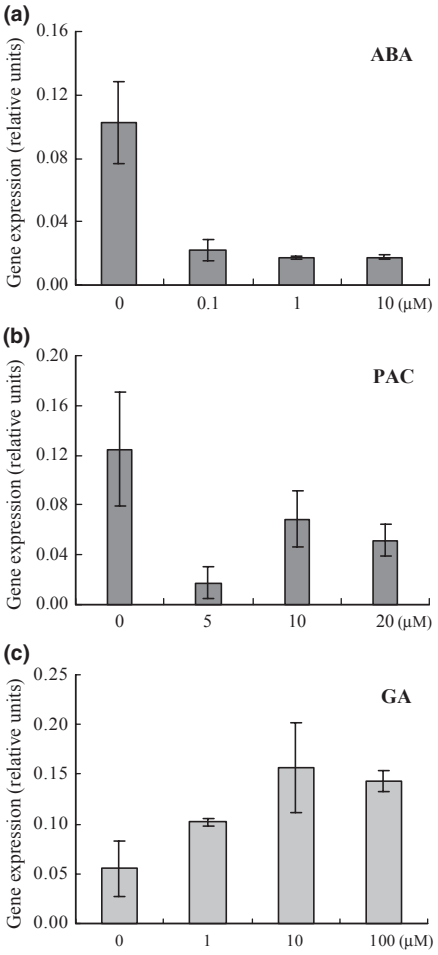

Fig. 6 *KYP/SUVH4* transcript level is regulated in response to abscisic acid (ABA) and gibberellins (GA). *KYP/SUVH4* expression determined by quantitative real-time PCR in germinating seeds of L*er* treated with different concentrations of ABA (a), paclobutrazol (PAC) (b) and GA (c). All seeds were collected 16 h after imbibition in the light. Error bars denote SD from three independent experiments. All the RNAs were extracted from *c*. 40 mg of treated seeds.

transgenic plants (Fig. S4b), indicating that KYP/SUVH4 could be a repressor of *DOG1* and *ABI3* transcription. Overall, our data show a significant change in expression levels of seed dormancy-related genes and ABA signaling pathway genes in *kyp-2* mutant seeds and transgenic lines, indicating a role of chromatin modification carried out by KYP/SUVH4 in the establishment and maintenance of seed dormancy.

Relationship of *kyp/suvh4* with other seed dormancy mutants

The *kyp-2* mutant shows increased seed dormancy, and we were interested in the influence of the *kyp-2* mutation on mutants with decreased dormancy levels. Therefore, the *kyp-2* mutant was crossed with the *hub1-2*, *rdo2* and *dog1* mutants, which all showed reduced seed dormancy. The double mutants *hub1-2 kyp-2*, *rdo2 kyp-2* and *dog1 kyp-2* were selected by molecular markers. Seeds from the double mutant *hub1-2 kyp-2* and *dog1 kyp-2* plants were completely nondormant, similar to the *hub1-2* and *dog1* single mutants, indicating that the *hub1* and *dog1* mutants are epistatic to *kyp/suvh4* (Fig. 9a,b). This suggests that *KYP/SUVH4* probably regulates seed dormancy through the same pathway as *DOG1* and *HUB1*. However, seeds from the double mutant *rdo2 kyp-2* plants showed an intermediate dormancy level (Fig. 9c). We conclude that *RDO2* and *KYP/SUVH4* regulate seed dormancy through independent genetic pathways. These results confirm the existence of a complex molecular network in the control of seed dormancy.

Discussion

KYP/SUVH4 belongs to the family of SU(VAR)3-9-like proteins, which function in histone methylation and are

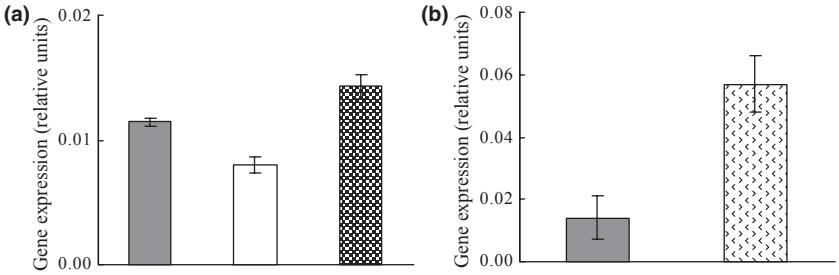

Fig. 7 *KYP/SUVH4* expression in *ga1-3* and *abi3-5* backgrounds. (a) Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of *KYP/SUVH4* transcripts in L*er* (gray bar) and *ga1-3* seeds imbibed for 16 h with water (white bar) or GA_{4+7} (10 μM; stippled bar). The expression values were normalized using *ACTIN2* as an internal standard. The mean values and SE were calculated from three independent experiments. (b) Quantitative RT-PCR analysis of *KYP/SUVH4* transcripts in L*er* (gray bar) and *abi3-5* (stippled bar) freshly harvested seeds. The expression values were normalized using *ACTIN2* as an internal control. The mean values ± SE were calculated from three independent experiments.

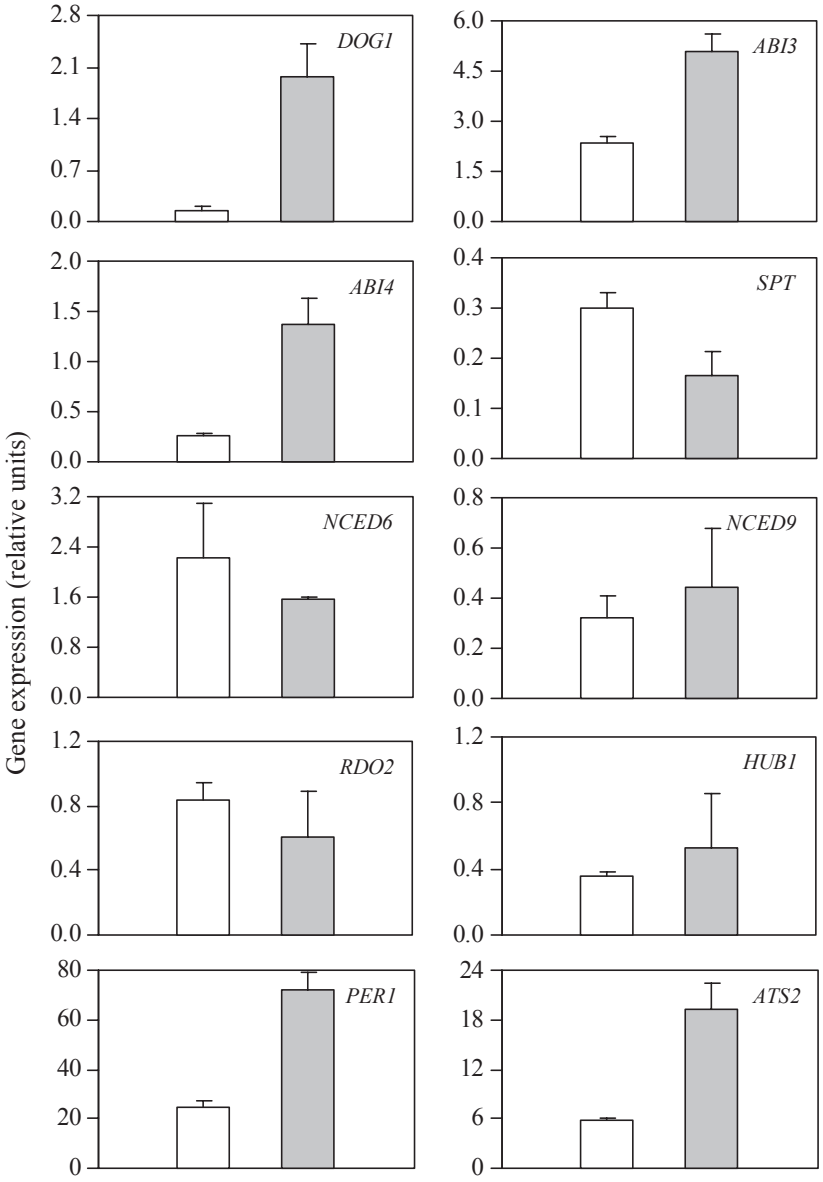

Fig. 8 The expression levels of seed dormancy-related genes are altered in *kyp-2*. Transcript levels of *DOG1*, *ABI3*, *ABI4*, *SPT*, *NCED6*, *NCED9*, *RDO2*, *HUB1*, *PER1* and *ATS2* were determined by quantitative reverse transcription-polymerase chain reaction (RT-PCR). cDNA was generated from 24-h imbibed freshly harvested seeds from wild-type L*er* (open bars) and *kyp-2* (closed bars). The expression values of the individual genes were normalized using *ACTIN2* as an internal standard. The mean expression values (+SD) were calculated from the results of three independent experiments.

characterized by the presence of conserved SET domains (Jackson *et al.*, 2002). The proteins encoded by SU(VAR)3-9 in *Drosophila* and its yeast (CLR4), human (SUV39H1) and mouse (SUV39H1) homologs have a key function in heterochromatin packaging and are required for the transfer of a methyl group to histone H3K9 (Tschiersch *et al.*, 1994; Ivanova *et al.*, 1998). In

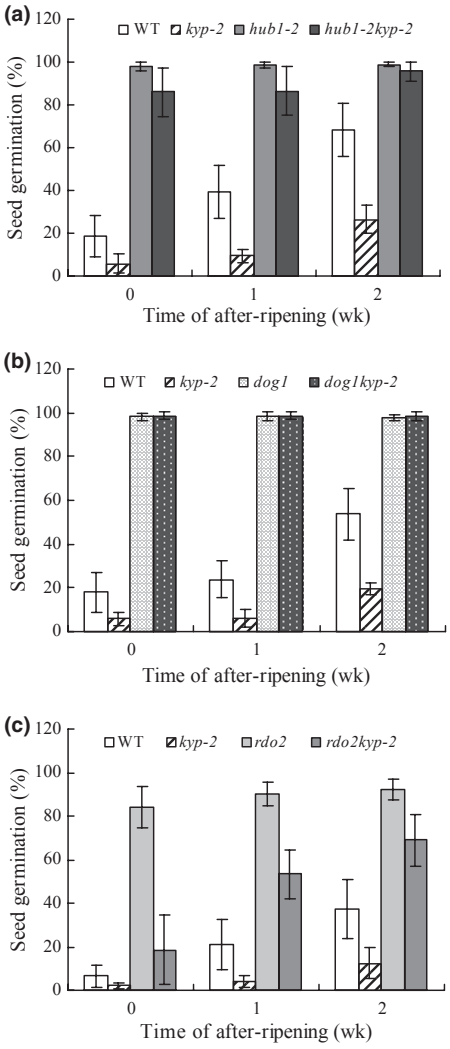

Fig. 9 Seed dormancy levels of *kyp-2* in the *hub1-2*, *dog1* and *rdo2* mutant backgrounds. Germination rates on water in the light after different periods of after-ripening are shown for seeds of L*er*, *kyp-2*, *hub1-2* and *hub1-2 kyp-2* (a), L*er*, *kyp-2*, *dog1* and *dog1 kyp-2* (b) and L*er*, *kyp-2*, *rdo2* and *rdo2 kyp-2* (c). Percentages of seed germination are means (± SD) based on the seeds from eight individual plants.

contrast with animals and fungi, which have one or two Su(var)3-9 homologues, *Arabidopsis* has ten SUVH genes encoding SU(VAR)3-9 homologous proteins, including *KYP/SUVH4* and *SUVH5* (Baumbusch *et al.*, 2001). KYP/SUVH4 is the major H3K9 methyltransferase contributing to histone H3 lysine 9 dimethylation genome-wide. The *suvh4* mutant displays a global loss of H3K9me2 as assessed by immunoblot analysis and immunocytology (Jackson *et al.*, 2002, 2004). The SUVH5 protein also has histone methyltransferase activity *in vitro* and contributes to the maintenance of H3 mK9 *in vivo* (Ebbs & Bender, 2006; Rajakumara *et al.*, 2011). The *kyp* mutants in the L*er* background did not exhibit morphological defects, even after extensive inbreeding (Jackson *et al.*, 2002). The single *suvh5* and double *suvh4 suvh5* mutants in the Columbia-0 background also displayed no morphological defects in our study. However, *clr4* mutants in yeast show mild phenotypes, such as a marked increase in iodine staining and sporulation frequency, and the overexpression of Clr4 causes a cell division defect phenotype under starvation conditions (Ivanova *et al.*, 1998). In *Drosophila*, SU(VAR)3-9 has distinct effects on position effect variegation (Tschiersch *et al.*, 1994). Therefore, we hypothesized that mutations in *Arabidopsis* SUVHs might cause developmental phenotypes that had not been observed previously. In this article, we show that *KYP/SUVH4* is expressed in all tissues, but the highest levels are detected in imbibed seeds (Fig. 2b). The analysis of mutant and transgenic plants showed that KYP/SUVH4 plays a role in seed dormancy (Figs1a,b, S2a).

Histone modifications are involved in the transition phases of plant development because of their essential role in the regulation of gene expression and the maintenance of genome stability (Ahmad *et al.*, 2010; He *et al.*, 2011; Jiang *et al.*, 2011). The *kyp* mutants were identified by screening *clark kent-stable* (*clk-st*) suppressors for their ability to recover the defects of *clk-st* in the number of floral organs (Jackson *et al.*, 2002), suggesting that KYP/SUVH4 is involved in reproductive organ formation during the transition from vegetative growth to reproductive growth. We have identified an increased dormancy phenotype for the *kyp* mutant, which is independent of *clk-st*. This indicates that KYP/SUVH4 is also involved in the transition from seed to seedling.

Several experiments have demonstrated that H3K9 methylation by KYP/SUVH4 and SUVH5 acts genetically upstream of DNA methylation by CMT3 (Jackson *et al.*, 2002; Ebbs & Bender, 2006; Rajakumara *et al.*, 2011). H3mK9, mediated by the Su(var)3-9 homologues SUVH4/KYP and SUVH5 histone methyltransferase, is required for the maintenance of CNG methylation by the CMT3 DNA methyltransferase. However, an analysis of the seed dormancy phenotype of the *cmt3-7* mutant indicates that this gene is not involved in seed dormancy (Fig. S1b), suggesting that KYP/SUVH4 and CMT3 play different roles in the control of seed germination. The influence of KYP/SUVH4 on dormancy may not involve CMT3 and DNA methylation. We downloaded the public microarray data for genome-wide expression analysis in *kyp* and *cmt3* mutants from GSE22957 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE22957), and reanalyzed the data using the CyberT (http://cybert.microarray.ics.uci.edu/) method (Baldi & Long, 2001) and TAGGIT approach (Carrera *et al.*, 2007). The results revealed that *cmt3* showed quite different genome-wide expression patterns (Fig. S6a) and TAGGIT workflow (Fig. S6b) when compared with the *kyp* mutant. Many more genes were influenced in *cmt3*, although the overlap between these two mutants was significant. This indicates that KYP/SUVH4 and CMT3 may have different effects on plant development and growth, including seed germination.

It has been found recently that chromatin remodeling is crucial for the induction and maintenance of seed dormancy. HDA6 and HDA19, two histone deacetylases, have been shown to influence seed dormancy and germination by affecting seed maturation and the ABA signaling pathway (Chen & Wu, 2010; Chen *et al.*, 2010). *HUB1*, encoding a C3HC4 RING finger protein that functions as an E3 ligase in histone H2B monoubiquitination, also plays a role in the control of seed dormancy (Liu *et al.*,

2007). The loss of H2B monoubiquitination causes changes in the expression of dormancy-related genes and results in a reduced seed dormancy phenotype. Moreover, mutations in *SUP32/UBP26*, encoding a ubiquitin-specific protease executing H2B deubiquitination, cause a dramatic increase in monoubiquitinated H2B (Sridhar *et al.*, 2007). The *ubp26* mutant shows enhanced seed dormancy (Y. Liu, unpublished). H2B deubiquitination by SUP32/UBP26 is required for heterochromatic histone H3 methylation and DNA methylation, and is likely to be an early and crucial event in heterochromatin formation (Sridhar *et al.*, 2007). Thus, H2B monoubiquitination may possibly influence seed dormancy via histone H3K9 methylation and DNA methylation. In this article, we show evidence that KYP/SUVH4 and SUVH5, which are involved in heterochromatic histone H3K9 methylation (Johnson *et al.*, 2002; Rajakumara *et al.*, 2011), play a role in seed dormancy and germination (Figs. 1, 5 and Fig. S2). Mutations in *KYP/SUVH4* and *SUVH5*, and overexpression of *KYP/SUVH4*, significantly alter seed dormancy.

Mutations in *KYP/SUVH4* cause a reduction in methylated histone H3 lysine 9, a loss of DNA methylation and reduced gene silencing (Johnson *et al.*, 2002; Jackson *et al.*, 2004). Therefore, we checked the expression level of several genes regulating dormancy and ABA pathways by quantitative RT-PCR. Increased expression of dormancy-related genes, including *DOG1*, *ABI3*, *ABI4*, *ATS2* and *PER1*, was found in the *kyp-2* mutant (Fig. 8). These genes could be directly or indirectly regulated by histone methylation. *DOG1* is only expressed in the seed and is absolutely required for the induction of seed dormancy. Expression differences of *DOG1* between accessions are correlated with dormancy levels (Bentsink *et al.*, 2006). Overexpression of *DOG1* confers increased seed dormancy in *Arabidopsis* (M. Bartsch, unpublished; Max Planck Institute for Plant Breeding Research, Cologne, Germany). These studies provide solid evidence that differences in *DOG1* expression level can lead to altered seed dormancy. *ABI3* and *ABI4* are two important components in the ABA signaling pathway, and overexpression or mutations of *ABI3* and *ABI4* in *Arabidopsis* confer altered sensitivity to ABA and/or altered seed dormancy (Giraudat *et al.*, 1992; Finkelstein *et al.*, 1998). The *kyp-2* mutant only showed minor changes in the expression of *NCED6* and *NCED9*, which are involved in ABA biosynthesis. This indicates that the ABA level may not be altered in *kyp-2*. *DOG1* expression is regulated by ABA or ABA signaling factors, such as ABI3 and ABI5 (Graeber *et al.*, 2010), and the *dog1 ga1-3* double mutant shows a low GA requirement for germination (Bentsink *et al.*, 2006). These results indicate that *DOG1* may be involved in the balance of ABA and GA signaling in the germination process. In our research, *KYP/SUVH4* expression was also found to be regulated by ABA and GA (Figs 6, 7). KYP/SUVH4 plays a role in the balance of the ABA and GA signaling pathways, which could indirectly cause the gene expression change of *DOG1*, *ABI3* and other genes. The levels of *DOG1*, *ABI3* and *ABI4* transcripts were significantly higher in *kyp-2* seeds than in the wild-type (Fig. 8), which is consistent with the increased seed dormancy and ABA sensitivity. Overall, our data suggest that KYP/SUVH4 influences seed dormancy and ABA/GA sensitivity by decreasing the expression of dormancy- and ABA-related genes.

Seed dormancy and germination are regulated by various endogenous and environmental factors, including hormones, nutrients, seed coat, temperature and light. A complex molecular network regulates the induction and maintenance of seed dormancy (Finkelstein *et al.*, 2007; Holdsworth *et al.*, 2008). Our genetic analysis has shown that *DOG1* and *HUB1* are epistatic to *KYP/SUVH4*, and *RDO2* behaves additively (Fig. 9). This indicates that *KYP/SUVH4* could regulate seed dormancy through the same genetic pathway as *DOG1* and *HUB1*, but in a parallel pathway with *RDO2*. *RDO2* encodes a transcription elongation factor TFIIS protein which can act in seed dormancy (Grasser *et al.*, 2009; Liu *et al.*, 2011). KYP/SUVH4 plays a role in the transcriptional activation as a repressor. *KYP/SUVH4* may act physiologically upstream of *DOG1* because the *kyp* mutation causes an increase in *DOG1* expression levels (Fig. 8). *HUB1* also acts upstream of *DOG1* (Liu *et al.*, 2007). Therefore, *DOG1* may be a main cross-link point of *KYP/SUVH4* and *HUB1* in the regulation of seed dormancy. It would be interesting to identify the molecular mechanisms connecting H3K9 methylation and H2B ubiquitination, and to investigate their direct influence on gene transcription and seed dormancy.

Our data suggest that KYP/SUVH4 can influence the transcription of seed dormancy-related and ABA signaling pathway genes, such as *DOG1*, *ABI3* and *ABI4*, explaining the enhanced seed dormancy of *kyp-2* mutants. Overexpression of *KYP/SUVH4* results in reduced seed dormancy. A genetic analysis showed that *HUB1* is epistatic to *KYP/SUVH4*. In addition, we have shown that interactions between chromatin modifications are likely to play an important role in regulating the transition from seed to seedling.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Seed dormancy of *gl1* and *cmt3-7* mutant.

Fig. S2 Seed dormancy of *p35S::KYP/SUVH4* transgenic plants.

Fig. S3 Information on *KYP/SUVH4* expression retrieved from the public *Arabidopsis* microarray database.

Fig. S4 Analysis of *pDOG1::KYP/SUVH4* transgenic lines.

Fig. S5 Seed germination of *kyp-2* in response to paclobutrazol (PAC) and gibberellins (GAs).

Fig. S6 Transcriptome reanalysis of *kyp* and *cmt3*.

Table S1 List of primers used in reverse transcription-polymerase chain reaction (RT-PCR)

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