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 unfavorable 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, Sdr of seed dormancy between Arabidopsis (L. eretica) 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 Brel1 and human RNF20/RNF40 (Liu et al., 2007). The mutants fail to ubiquitinate histone H2B and the locus was consequently renamed HISTONE MONOUBQUITINATION 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 (TFIIIS; Liu et al., 2011). Plants with RNAi-mediated knockdown of TFIIS expression also show reduced seed dormancy (Grasser et al., 2009). TFIIIS 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/g1l in the Landsberg erecta (Ler) background, ordered from the Nottingham Arabidopsis Stock Centre (NASC, Nottingham, UK) (http://arabidopsis.info/), with Ler. The kyp-2 mutant has been described by Jackson et al. (2002). The mutants of rdo2, hub1-2 and dog1 in the Ler 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 Ler 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’-TACGATTCTCCGCAAATCG-3’) and DOG1-R (5’-CAAATTCAACCGAACCAC-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

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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 (sub4-2 and sub4-3; http://signal.salk.edu) or GABI-Kat collection (sub5; http://www.gabi-kat.de) with the following seed stock numbers: sub4-2, Salk_130630; sub4-3, Salk_105816; sub5, 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’-TACCGGACTAAGGAGTTTGA-3’) , P2 (5’-ATGTCGAGTTCGAGTTTGA-3’), P3 (5’-CC CAAGAAAAATCTTTT-3’), and P4 (5’-ACATTTTATCGTGTAATAAGGG-3’); for SUVH5, P5 (5’-TAGAGCCAGACAATGGAATG-3’) and P6 (5’-CTCTTTTTTATCCAGGCGAACC-3’).

Constructs and plant transformation

For the pDOG1::KYP/SUVH4 and p35S::KYP/SUVH4 constructs, total RNA was isolated from Ler 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’-GGGGACACGGTTTAGAAGAAAGACAGGTTATATGC-3’ and 5’-GGGACACGGTTTAGAAGAAAGACAGGTTATATGC-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 Ler 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 tri-phosphinotrichin. 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 pHBI101 carrying the β-glucuronidase (GUS) gene. Primers for PCR were as follows: 5’-AGGTCTAGTGGTAACTACAATCAAAG-3’ and 5’-GTCGACCATC-GATCCTTTTTCGCC-3’. The restriction endonuclease sites HindIII and SalI 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 Ler 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 Ler background with the Ler 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 Ler 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 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 imibed 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 (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 ~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 ~10% higher germination in the medium containing 0.5 or 1 μM ABA (Fig. 3c).
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 (suhb4-2, suhb4-3, suhb5) 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 suhb4-2 and suhb4-3 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, suhb4-3 data not shown). This result is different from that of kyp-2 (Ler background), which may be caused by the genetic background. The suhb5 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 suhb4-2 suhb5.

The dormancy level of suhb4-2 suhb5 was significantly lower than that of suhb5 and the wild-type (Fig. 5a). After 1 wk of dry storage, the double mutant showed germination of 20%, and suhb5 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 suhb4-2 suhb5 to ABA and PAC (Fig. 5b,c). The suhb4-2 suhb5 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 Ler 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.
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 TFIIH 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 (Haslekás 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 Ler 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 insertion mutants.

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).

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.
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...
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. 7](image-url) **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 Ler (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 Ler (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](image-url) **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 Ler (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.
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 seeding.

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. 5b), 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. 6a) and TAGGIT workflow (Fig. 6b) 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., 2011).
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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References

contains at least 29 active genes encoding SET domain proteins that can be assigned to four evolutionarily conserved classes. *Nucleic Acids Research* 29: 4319–4333.


Supporting Information

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

Fig. S1 Seed dormancy of g1l 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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