The H3K27me3 Demethylase RELATIVE OF EARLY FLOWERING6 Suppresses Seed Dormancy by Inducing Abscisic Acid Catabolism[1][OPEN]

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Seed dormancy is an adaptive trait that is crucial to plant survival. Abscisic acid (ABA) is the primary phytohormone that induces seed dormancy. However, little is known about how the level of ABA in seeds is determined. Here we show that the Arabidopsis (*Arabidopsis thaliana*) H3K27me3 demethylase RELATIVE OF EARLY FLOWERING6 (REF6) suppresses seed dormancy by inducing ABA catabolism in seeds. Seeds of the *ref6* loss-of-function mutants displayed enhanced dormancy that was associated with increased endogenous ABA content. We further show that the transcripts of two genes key to ABA catabolism, *CYP707A1* and *CYP707A3*, but not genes involved in ABA biosynthesis, were significantly reduced in *ref6* mutants during seed development and germination. In developing siliques, REF6 bound directly to *CYP707A1* and *CYP707A3*, and was responsible for reducing their H3K27me3 levels. Genetic analysis demonstrated that the enhanced seed dormancy and ABA concentration in *ref6* depended mainly on the reduced expression of *CYP707A1* and *CYP707A3*. Conversely, overexpression of *CYP707A1* could offset the enhanced seed dormancy of *ref6*. Taken together, our results revealed an epigenetic regulation mechanism that is involved in the regulation of ABA content in seeds.

Seeds are vital for the survival and distribution of seed-plant species. Seed germination is the beginning of plant development, and the purpose of plant growth is to produce seeds. In mature seeds, the initiation of germination is determined by a period of quiescence, which is known as seed dormancy (Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006; Shu et al., 2013). Seed dormancy is an adaptive trait that ensures seeds germinate only when environmental conditions are favorable for seedling development and reproductive success, and dormant seeds do not germinate even under optimal conditions (Nonogaki, 2014; Shu et al., 2016; Chahtane et al., 2017; Yang et al., 2020). Under optimal conditions, nondormant Arabidopsis (*Arabidopsis thaliana*) seed sets usually finish germination within 48 h after imbibing, while dormant seed sets can germinate only partially or not at all. The speed of seed germination under suitable environments is widely used to evaluate seed dormancy of seed sets

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(Baskin and Baskin, 2004; Okamoto et al., 2006; Matakiadis et al., 2009; Shu et al., 2013). Seed dormancy is mainly induced by abscisic acid (ABA) generated from both zygotic tissues and the mother plant (Karssen et al., 1983; Kanno et al., 2010) and broken by GA during the imbibing stage of early germination (Yamauchi et al., 2004; Finch-Savage and Leubner-Metzger, 2006; Nonogaki, 2014; Shu et al., 2016). In developing Arabidopsis seeds, the endogenous ABA content reaches a maximum level in the middle stage of seed development, when embryo growth and seed filling occur, ~9 to 10 d after flowering (Jo et al., 2019). An additional small peak of ABA accumulation takes place late in development at ~15 to 16 d after flowering, when the seed-coat–browning process is initiated, and seeds (embryos) acquire desiccation tolerance and become quiescent (Okamoto et al., 2006; Kanno et al., 2010). On the other hand, the ABA in dry seeds decreases rapidly during seed imbibition and then maintains a basal level that is correlated with the germination potential of the seed (Okamoto et al., 2006). However, how the level of ABA during seed development and germination is determined is not well understood.

In Arabidopsis, NINE-CIS-EPOXYCAROTENOID DIOXYGENASE2 (AtNCED2), AtNCED5, AtNCED6, AtNCED9, ABA DEFICIENT1, ABA DEFICIENT2, and ALDEHYDE OXIDASE3 (AtAAO3) are key enzymes involved in ABA biosynthesis. The loss of function of genes encoding these enzymes leads to decreased endogenous ABA content and thus reduced seed dormancy (González-Guzmán et al., 2002; Seo and Koshiba, 2002; Tan et al., 2003). Conversely, overexpression of these genes can increase ABA content in seed and enhance seed dormancy and thus delay seed germination (Tan et al., 2003; Kushiro et al., 2004; Chahtane et al., 2017).

ABA is hydroxylated at positions C-7′ and C-8′ in many plant species (Kushiro et al., 2004; Saito et al., 2004; Okamoto et al., 2006), and a 9′-hydroxylation pathway has also been identified in several plants (Zhou et al., 2004). The ABA 8′-hydroxylation pathway is thought to be predominant in many physiological processes and catalyzed by three cytochrome P450 monooxygenases, CYP707A1, CYP707A2, and CYP707A3 (Kushiro et al., 2004; Saito et al., 2004; Okamoto et al., 2006; Matakiadis et al., 2009). Loss-of-function mutations of ABA catabolism genes *CYP707A1*, *CYP707A2*, and *CYP707A3* result in enhanced seed dormancy associated with increased endogenous ABA content in seeds (Kushiro et al., 2004; Okamoto et al., 2006). The *CYP707A1* and *CYP707A3* genes were found to be expressed predominantly during the middle stage of seed development, downregulated during the late maturation stage, and maintained at a low level in dry seed, while the *CYP707A2* transcript levels increase from the late-stage to mature dry seed (Kushiro et al., 2004; Saito et al., 2004; Okamoto et al., 2006; Matakiadis et al., 2009). The transcription of all three *CYP707As* is induced in the early imbibing stage.

Histone methylation and demethylation are essential for transcriptional regulation and play a fundamental role in regulating diverse developmental processes, such as flowering, architecture, and abiotic stress tolerance (for review, please see Liu et al., 2010a). Histone methylation is dynamically regulated by Lys (Lys, K) methyltransferases ("writers"), and demethylases ("erasers"). Methylation occurs mainly at Lys-4 (K4), Lys-9 (K9), Lys-27 (K27), and Lys-36 (K36) of histone H3 (Liu et al., 2010a). Those Lys residues can be augmented with different numbers (1 to 3) of methyl groups, which confer various biological functions. Generally, methylations at H3K9 and H3K27 are associated with gene silencing, whereas methylations at H3K4 and H3K36 are related to gene activation (Berger, 2007). As a repressive mark, H3K27me3 represses gene expression by a conserved mechanism in eukaryotes (Zhang et al., 2007; Hennig and Derkacheva, 2009). In Arabidopsis, H3K27me3 is catalyzed by histone methyltransferases, i.e. CURLY LEAF, MEDEA, or SWINGER, which are essential components of Polycomb Repressive Complex2 (Liu et al., 2010a). The demethylation of H3K27me3 in Arabidopsis is catalyzed by several Jumonji (JMJ) domain-containing proteins, including RELATIVE OF EARLY FLOWERING6 (REF6), EARLY FLOWERING6, and JMJ13, JMJ30, and JMJ32 (Liu et al., 2010a; Lu et al., 2011; Gan et al., 2014; Yan et al., 2018).

Arabidopsis REF6 was first reported as a repressor of *FLOWERING LOCUS C* to promote flowering (Noh et al., 2004; Lu et al., 2011; Gan et al., 2014). The loss-of-function *ref6-1* mutant shows diverse phenotypes, including suppressed leaf senescence and lateral root formation (Wang et al., 2019a, 2019b), defects in cotyledon separation (Cui et al., 2016), and Brassinosteroid-related phenotype (Yu et al., 2008). The deletion of *REF6* leads to the ectopic accumulation of H3K27me3 at hundreds of genes in seedlings (Lu et al., 2011; Li et al., 2016; Cui et al., 2016). Other groups and ours previously showed that REF6 zinc-finger domains could directly target genomic loci containing CTCTGYTY motifs (Y represents C or T; Li et al., 2016, 2018; Qiu et al., 2019; Wang et al., 2019a). In this study, we demonstrate that REF6 is a negative regulator of seed dormancy and is involved in the decrease of ABA content during seed germination. We show that REF6 suppresses seed dormancy via inducing ABA catabolism during seed development and germination. Our results revealed an epigenetic regulation mechanism involved in the regulation of ABA content in seeds.

RESULTS

Loss-of-Function of REF6 Results in Enhanced Seed Dormancy

In a previous work on REF6 (Li et al., 2016), we occasionally observed that the seeds of *ref6-1* germinated later than Columbia (Col) wild type. The expression of *REF6* was observed during seed development, seed storage, and seed germination stages (Supplemental Fig.

S1A). Furthermore, ABA treatment induced the expression of *REF6* during seed germination (Supplemental Fig. S1B). These results imply that REF6 may be involved in the regulation of seed dormancy and germination. To test this hypothesis, we conducted detailed germination experiments using Col and *ref6* mutant seeds that were stored for different time points. Without stratification treatment (ST), we observed that ~70% of freshly harvested (FH) wild-type seeds germinated at 2.5 d after sowing, while no *ref6-1* seeds germinated (Fig. 1A, left, and B). Although the difference in germination ratio between Col and *ref6-1* seeds was reduced after ST compared with no stratification treatment (NST), the germination of *ref6-1* seeds was still obviously delayed compared with that of wild-type Col (Fig. 1A, right). Seeds stored at room temperature for 1 week or 2 months (after-ripened) were further used in the germination assay. The results showed that, in both conditions, the germination of *ref6-1* seeds was substantially slower than that of Col (Fig. 1, C and D). To confirm that the delay of germination was caused by the mutation in *REF6*, the seed germination rate of a complemented transgenic line, *ref6-1 ProREF6:REF6-GFP* (Li et al., 2016), was tested. The result showed that the seed germination rate of *ref6-1 ProREF6:REF6-GFP* was similar to Col (Fig. 1E). Taken together, these results demonstrate that REF6 is involved in suppressing seed dormancy.

The Endogenous ABA Content in Seeds of *ref6-1* Is Significantly Increased

Seed dormancy is antagonistically regulated by ABA and GA (Bewley, 1997; Chahtane et al., 2017). The enhanced seed dormancy in *ref6-1* could be due to reduced GA or increased ABA levels. Therefore, we quantified the endogenous ABA and GA contents in *ref6-1* seeds using a liquid chromatography-tandem mass spectrometry system. In FH dry seeds with NST, the ABA level of *ref6-1* was significantly higher than that of Col (Fig. 2A). After ST, although the ABA contents in seeds of both Col and *ref6-1* were reduced, the ABA content in *ref6-1* was still significantly higher than that in Col (Fig. 2A). In contrast to ABA, the GA level in the FH seed of *ref6-1* was similar to that of Col with ST or NST (Fig. 2B). Thus, the loss of REF6 causes the increase of the ABA level in seeds.

To test if there is a causal relationship between the increased ABA level in *ref6-1* and enhanced seed dormancy, we generated *ref6-1 aba2-1* double mutants by crossing. *aba2-1* is a mutant with substantially decreased ABA content due to the loss of function of

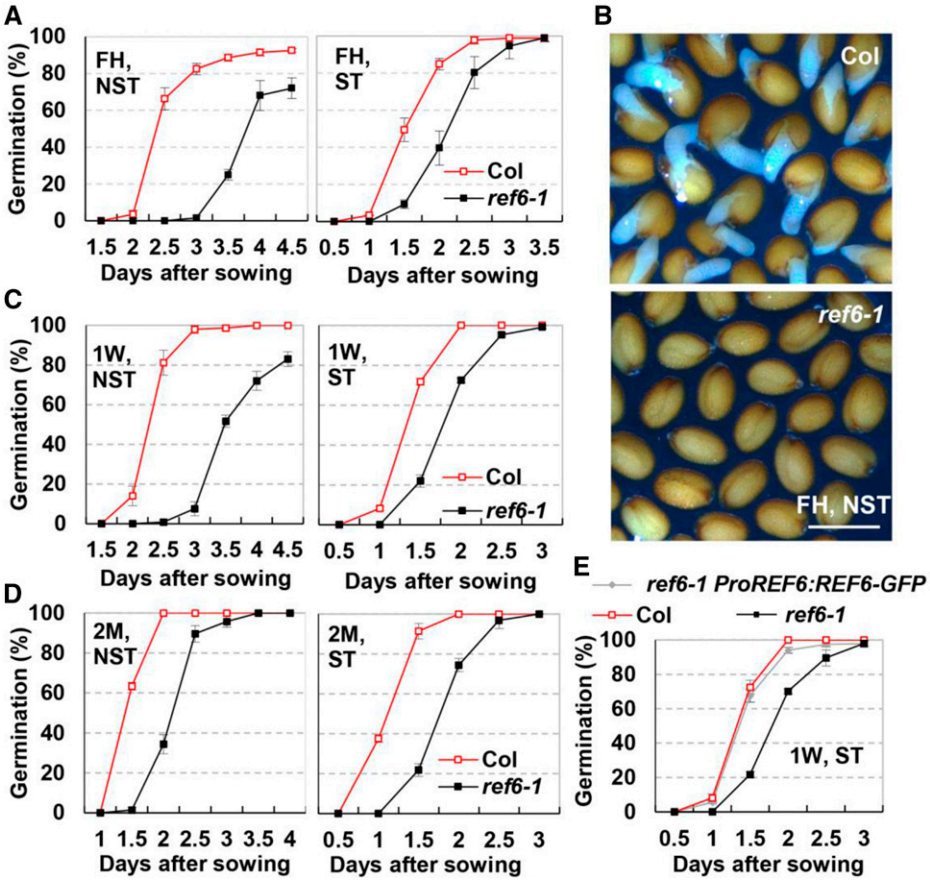

Figure 1. Loss-of-function mutant *ref6-1* displayed enhanced seed dormancy. FH seeds were stored at room temperature for 0 weeks (FH), 1 week (1W), or 2 months (2M) before being subjected to analysis. Seeds were sterilized and stored at 4°C for 0 d (NST) or 4 d (ST) before sowing. A, The germination rate of FH seeds without (left) or with (right) ST. B, Representative pictures showing germinating seeds at 72 h after sowing. FH seeds without ST were sown on one-half strength Murashige and Skoog (1/2 MS). Scale bar = 0.6 mm. C and D, The germination rate of seeds stored for 1W (C), 2M (D) without (left), or with (right) ST on one-half strength MS medium. E, The germination rate of 1-week-stored seeds of Col, *ref6-1*, and *ref6-1 ProREF6:REF6-GFP* with ST on one-half strength MS medium. For A, C, D and E, values (mean ± SD) are from three biological replicates, each consisting of three technical replicates. At least 50 dry mature seeds were used in each technical replication.

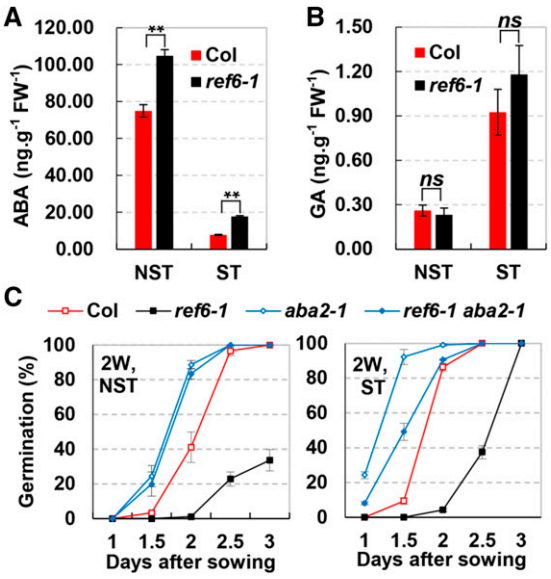

Figure 2. The enhanced seed dormancy of *ref6* depends on the increased endogenous ABA content. A and B, The ABA (A) and GA (B) contents in FH seeds with ST or NST for 4 d. Values (mean ± SD) are from three biological replicates, each consisting of ~100 mg of FH seeds. Asterisks indicate statistical significance of differences determined using Student's *t* test (*$P < 0.05$ and **$P < 0.01$). ns, No significance. C, Loss-of-ABA2 reduced the enhanced seed dormancy of *ref6-1*. The germination rates of 2-week-stored (2W) seeds of various genotypes were indicated. Values are from three biological replicates (mean ± SD), each consisting of three technical replicates. At least 50 dry mature seeds were used in each technical replication.

ABA2, a gene encoding one of the key enzymes in ABA synthesis (González-Guzmán et al., 2002; Léon-Kloosterziel et al., 1996). Two-week-stored seeds were used in the germination assay. Without stratification, the germination rate of *ref6-1 aba2-1* was similar to that of *aba2-1* but substantially higher than that of *ref6-1* (Fig. 2C, left). After stratification, the germination rate of *ref6-1 aba2-1* was also dramatically higher than that of *ref6-1* in the first 2.5 d (Fig. 2C, right). These results indicated that the reduction of ABA content by *aba2* could reduce the seed dormancy of *ref6-1*. Together, these results support a notion that REF6 suppresses seed dormancy by reducing endogenous ABA levels in seeds.

The Transcripts of *CYP707A1* and *CYP707A3* Were Significantly Reduced in *ref6-1* during Seed Development and Germination

ABA content is dynamically regulated by enzymes involved in ABA biosynthesis and catabolism. To identify key factors that are responsible for the increased ABA content in seeds of *ref6-1*, we quantified the transcripts of genes related to ABA biosynthesis (*ABA1*, *ABA2*, *AAO3*, *NCED2*, *NCED5*, *NCED6*, and *NCED9*) and catabolism (*CYP707A1*, *CYP707A2*, and *CYP707A3*) during seed development and germination. The results indicated that, during seed development, there was no significant difference in the transcripts of ABA biosynthesis genes between Col and *ref6* (Supplemental Fig. S2A). On the other hand, the expression levels of two ABA catabolism genes, *CYP707A1* and *CYP707A3*, in *ref6-1* were continuously and significantly lower throughout seed development and early germination stages than they were in Col (Fig. 3, A and B). The transcription of *CYP707A2* in *ref6-1* was substantially lower only at 16 d after pollination (DAP) and dry seed than in Col (Fig. 3C, left). During the early germination, the expression level of *CYP707A2* was similar between *ref6-1* and Col (Fig. 3C, right). It is worth noting that, during the seed development, the expression levels of *CYP707A1* in both Col and *ref6-1* were higher

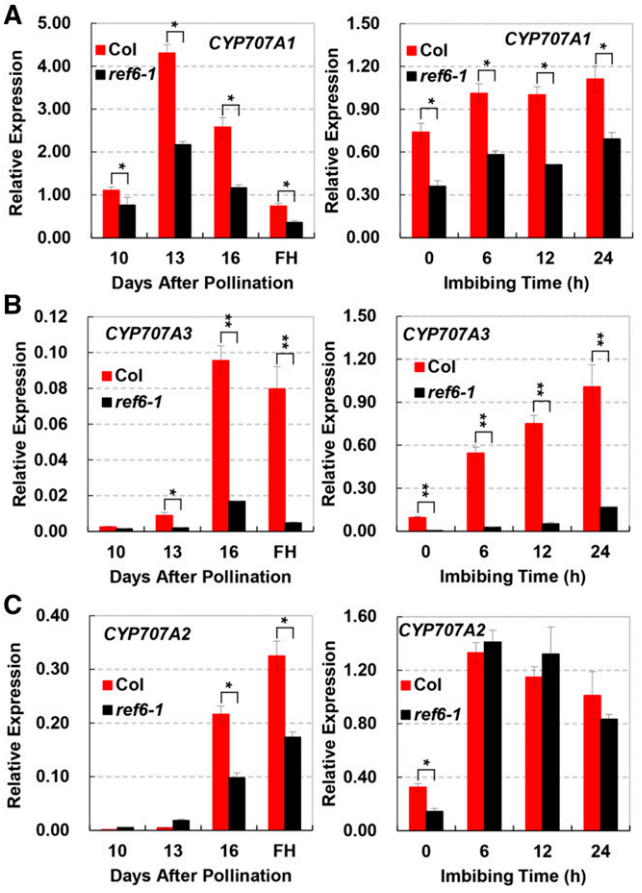

Figure 3. Suppressed ABA catabolism genes in seeds of *ref6-1*. A to C, The relative transcript levels of *CYP707A1* (A), *CYP707A3* (B), and *CYP707A2* (C) in seeds of *ref6-1* and Col during seed development and germination. The developing siliques (10, 13, and 16 DAP) and FH seeds imbibed for different times (0, 6, 12, and 24 h) under dark conditions at 22°C were used in the assay. The FH seeds were the same as the seeds imbibed for 0 h. The gene expression was relative to *ACTIN2*. Values (mean ± SD) represent three biological replicates, each consisting of three technical replicates. Asterisks indicate statistical significance of differences determined using Student's *t* test (*$P < 0.05$ and **$P < 0.01$).

than those of *CYP707A2* and *CYP707A3*, while during seed germination, the highest expressed gene was *CYP707A2* (Supplemental Fig. S2B). These results indicate that the increased ABA concentration in *ref6-1* is associated with the reduced expression of genes involved in the catabolism of ABA, but not with those involved in ABA biosynthesis.

REF6 Directly Binds to The *CYP707A1* and *CYP707A3* Genes during Seed Development

We investigated whether the involvement of REF6 in *CYP707A1* and *CYP707A3* expression during seed development is direct. So, we used chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) to detect the binding of REF6 to *CYP707A1* and *CYP707A3* in developing siliques (11 to 15 DAP) of *ref6-1 ProREF6:REF6-GFP*. The results displayed that REF6 was substantially enriched at the second intron of *CYP707A1* and *CYP707A3* (Fig. 4A; Supplemental Fig. S3A). No REF6 binding signal was found in *CYP707A2* (Supplemental Fig. S3B). The REF6 binding peak was centered on the four CTCTGYTY motifs in *CYP707A1* and *CYP707A3*, respectively, whereas *CYP707A2* had no detectable CTCTGYTY motif. These results suggest that REF6 directly binds to the *CYP707A1* and *CYP707A3* but not *CYP707A2* in developing siliques. We further examined the levels of H3K27me3 at the *CYP707A1*, *CYP707A2*, and *CYP707A3* genes in siliques of Col and *ref6-1* by ChIP-qPCR. Consistent with the results above, the H3K27me3 levels at the *CYP707A1* and *CYP707A3* genes in *ref6* were remarkably higher than those in Col (Fig. 4B; Supplemental Fig. S3B), whereas there was no noticeable increase of H3K27me3 at *CYP707A2* (Supplemental Fig. S3D). Together, these data support the idea that REF6 directly promotes the expression of *CYP707A1* and *CYP707A3* genes during seed development by reducing their H3K27me3 levels.

The Enhanced Seed Dormancy and Endogenous ABA Concentration of *ref6-1* Depend on The Reduced Expression of *CYP707A1* and *CYP707A3*

As described above, the transcription levels of the *CYP707A1* and *CYP707A3* in *ref6-1* were significantly reduced during seed development and germination (Fig. 3, A and B). To explore the causal relationship between ABA catabolism genes and the enhanced seed dormancy of *ref6-1*, we generated double and triple mutants by crossing *ref6-1* with loss-of-function mutants of *CYP707A1* (*cyp707a1-1*), *CYP707A2* (*cyp707a2-1*), and *CYP707A3* (*cyp707a3-1*). FH harvested seeds were used in the seed germination assays. With NST, the seed germination rate of double mutant *ref6-1 cyp707a3-1* was substantially lower than that of single mutant *ref6-1* or *cyp707a3-1* (Fig. 5A, left). After ST,

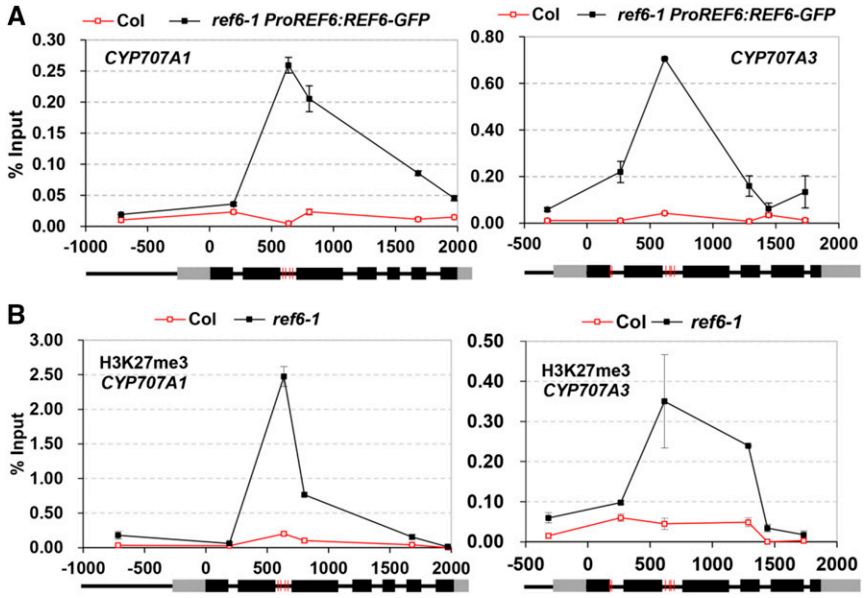

Figure 4. REF6 binds to *CYP707A1* and *CYP707A3* to reduce the level of H3K27me3 in developing seeds. A, ChIP-qPCR showing that REF6 binds to the *CYP707A1* and *CYP707A3* genes during seed development. Developing siliques (10 to 15 DAP) of Col and *ref6-1 ProREF6:REF6-GFP* were used in this assay. B, ChIP-qPCR results showing the level of H3K27me3 at *CYP707A1* and *CYP707A3* in Col and *ref6-1* during seed development. Developing siliques (13 DAP) of Col and *ref6-1* were used in this assay. ChIP signals are displayed as the percentage of input DNA. Col plants were used as the negative control sample. Values (mean ± SD) represent three biological replicates, each consisting of three technical replicates. Schematic representation of the *CYP707A1* and *CYP707A3* genomic loci are shown underneath. Black and gray boxes represent exons and untranslated regions, respectively. The red vertical lines indicate the CTCTGYTY motifs in *CYP707A1* and *CYP707A3*.

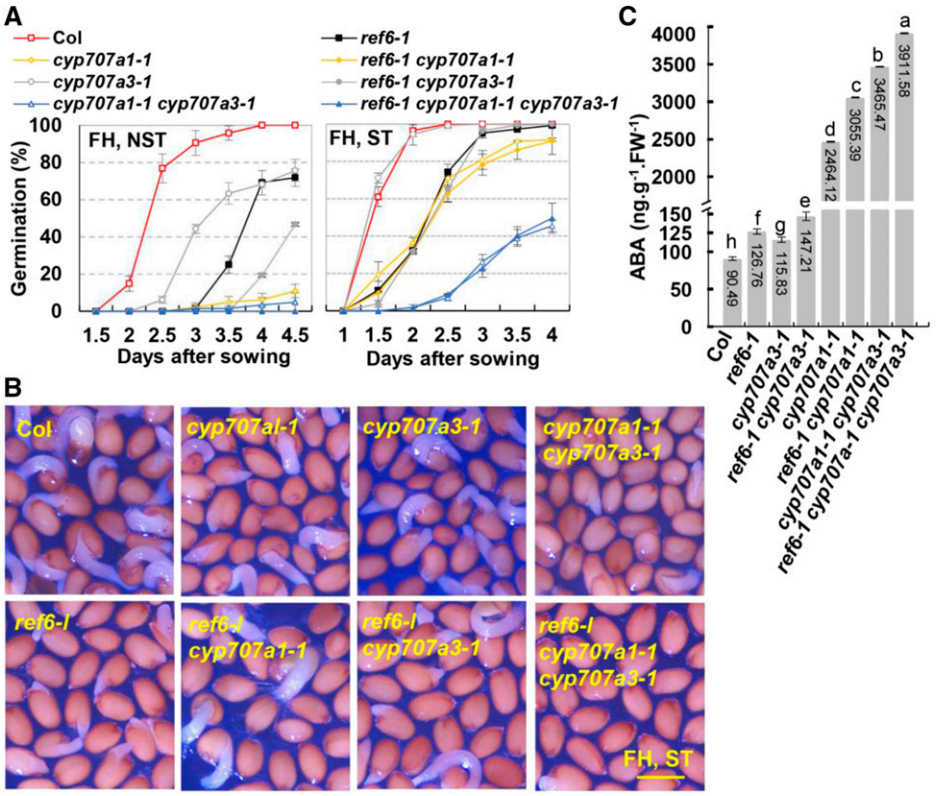

Figure 5. The enhanced seed dormancy and endogenous ABA concentration of *ref6-1* depend on the reduced expression of *CYP707A1* and *CYP707A3*. A, The seed germination rates of Col and various mutants. FH seeds with or without ST were used in the experiments. Values (mean ± SD) are from three biological replicates, each consisting of three technical replicates. At least 50 dry mature seeds were used in each technical replication. B, Representative pictures showing germinating seeds at 2.5 d after sowing. FH seeds with ST were sown on one-half strength MS. Scale bar = 0.6 mm. C, The endogenous ABA concentration in FH seeds of Col and various mutants. Values (mean ± SD) are from three biological replicates, each consisting of ~100 mg of FH seeds. Lowercase letters indicate statistical differences determined using one-way ANOVA with Duncan's multiple range test (for FH-NST-4D, the *F* value = 373,910 and degrees of freedom = 13).

the seed germination rate of *ref6-1 cyp707a3-1* was lower than that of *cyp707a3-1* in the first 2.5 d, but similar to that of *ref6-1* (Fig. 5A, right, and B; Table 1). With NST, after 4 d, the seed germination rates of *ref6-1* and *cyp707a1-1* were ~69% and 6%, respectively, while the seeds of *ref6-1 cyp707a1-1* failed to germinate (Fig. 5A, left; Table 1). After ST, the seed germination rate of *ref6-1 cyp707a1-1* was very close to that of *cyp707a1-1* (Fig. 5A, right and B; Table 1). The results of germination assays of triple mutant *ref6-1 cyp707a1-1 cyp707a3-1* demonstrated that, despite ST, the seed germination rate of *ref6-1 cyp707a1-1 cyp707a3-1* were similar with that of double mutant *cyp707a1-1 cyp707a3-1* (Fig. 5, A and B; Table 1). With regard to the *cyp707a2-1*, in contrast, we found that the seed germination percentages of double or triple mutants that contain *ref6-1* were lower than that of single or double mutants that do not contain *ref6-1*, respectively (Table 1; Supplemental Fig. S4A). Furthermore, the quantification of ABA in FH seeds demonstrated that the increased endogenous ABA contents of various mutants well-corresponded with the seed germination results (Fig. 5C; Supplemental Fig. S4B). These results suggest that the enhanced seed dormancy of *ref6-1* was predominantly dependent on the reduction of *CYP707A1* and *CYP707A3* but not dependent on *CYP707A2*.

Overexpression of *CYP707A1* Offset the Enhanced Seed Dormancy of *ref6-1*

As described above, the decreased expression of *CYP707A1* is mainly responsible for the enhanced seed dormancy of *ref6-1*. To further verify this, *CYP707A1* was overexpressed in *ref6-1* through generating transgenic plants *ref6-1 Pro35S:CYP707A1*. Three independent transgenic lines that showed overexpressed *CYP707A1* were obtained (Fig. 6A). Seed germination analysis with the transgenic seeds exhibited that the germination rates of the three *ref6-1 Pro35S:CYP707A1* lines were similar to that of Col, and were substantially higher than that of *ref6-1* (Fig. 6, B and C). These results showed that overexpression of *CYP707A1* could rescue the dormancy phenotype of *ref6-1*.

DISCUSSION

ABA is a crucial hormone for seed development, dormancy, germination, and abiotic stress responses (Chen et al., 2020b). However, how the level of ABA is regulated in seeds is far from clear. In this study, we found that histone H3K27 demethylase REF6 is involved in the reduction of ABA content during seed development and germination (Fig. 2A). Seed dormancy is conferred by ABA in dry seed (Finch-Savage and Leubner-Metzger, 2006; Shu et al., 2013) and released by GA generated during seed germination (Bewley, 1997; Chahtane et al., 2017). In the dry seeds, the ABA content of *ref6-1* was significantly higher than that of Col (Fig. 2A), while no apparent difference in GA levels between them was observed (Fig. 2B), indicating that REF6 is involved in reducing ABA content in seeds. The loss of function of REF6 enhances seed

Table 1. *The seed germination percentages of each genotype at 4 or 2.5 d after sowing*

Lowercase letters indicate statistically significant differences, which were determined with the software SAS (https://welcome.oda.sas.com/login) using one-way ANOVA and Duncan's multiple range test (for FH-NST-4 days, the *F* value = 536.51 and degrees of freedom = 13; for FH-ST-2.5 days, the *F* value = 260.08 and degrees of freedom = 13).

Genotype	Germination (%) FH NST (4 d)	Germination (%) FH ST (2.5 d)
Col	100 a	100 a
ref6-1	69.20 ± 6.51 b	74.37 ± 4.11 c
cyp707a1-1	6.22 ± 3.46 e	70.91 ± 4.49 c,d
cyp707a2-1	29.14 ± 2.68 c	89.39 ± 7.56 b
cyp707a3-1	68.22 ± 1.68 b	99.21 ± 1.37 a
cyp707a1-1 cyp707a2-1	0 f	3.86 ± 3.19 i,j
cyp707a1-1 cyp707a3-1	4.05 ± 3.3 e,f	7.04 ± 1.75 h,i,j
cyp707a2-1 cyp707a3-1	5.80 ± 1.78 e	36.52 ± 2.83 f
ref6-1 cyp707a1-1	0 f	62.76 ± 4.27 e
ref6-1 cyp707a2-1	0 f	19.95 ± 5.24 g
ref6-1 cyp707a3-1	19.39 ± 2.71 d	65.18 ± 7.02 d,e
ref6-1 cyp707a1-1 cyp707a2-1	0 f	0 j
ref6-1 cyp707a1-1 cyp707a3-1	0 f	8.82 ± 0.49 h,i
ref6-1 cyp707a2-1 cyp707a3-1	0 f	12.17 ± 4.01 h

dormancy and reduces seed germination (Fig. 1). Further genetic experiments support the notion that the increased ABA level is responsible for the enhanced seed dormancy of *ref6-1* because *aba2-1 ref6-1* double mutants reduced the seed dormancy of *ref6-1* (Fig. 2C). Due to the reduced expression of *CYP707A1* and *CYP707A3* in developing *ref6-1* seeds (Fig. 3, A and B), ABA content in the FH *ref6-1* seeds was significantly higher than that in Col (Fig. 2A). During seed imbibing, the significantly reduced expression of *CYP707A1* and *CYP707A3* in *ref6-1* will lead to reduced efficiency of ABA catabolism during the ST in *ref6-1* seeds compared to Col. Together, the higher amount of ABA in FH *ref6-1* seeds as well as the reduced catabolism of ABA during the stratification in *ref6-1* seeds would result in higher ABA level in stratified *ref6* seeds. Consistent with this, after ST, ABA concentration in *ref6-1* seeds was still obviously higher than that in Col (Fig. 2A), while the seed germination rate of *ref6-1* was lower than that of Col (Fig. 1A, right). After ST for 4 d, the ABA levels in both Col and *ref6-1* were dramatically decreased, but the difference in ABA content between Col and *ref6-1* became smaller (Fig. 2A). In addition, after ST, the GA content of *ref6-1* was slightly higher than that of Col (Fig. 2B). These may be responsible for the smaller difference in seed germination rate between *ref6* and Col after ST (Fig. 1A).

Both the biosynthesis and catabolic pathways of ABA in plants have been uncovered (Kushiro et al., 2004; Saito et al., 2004; Okamoto et al., 2006; Matakiadis et al.,

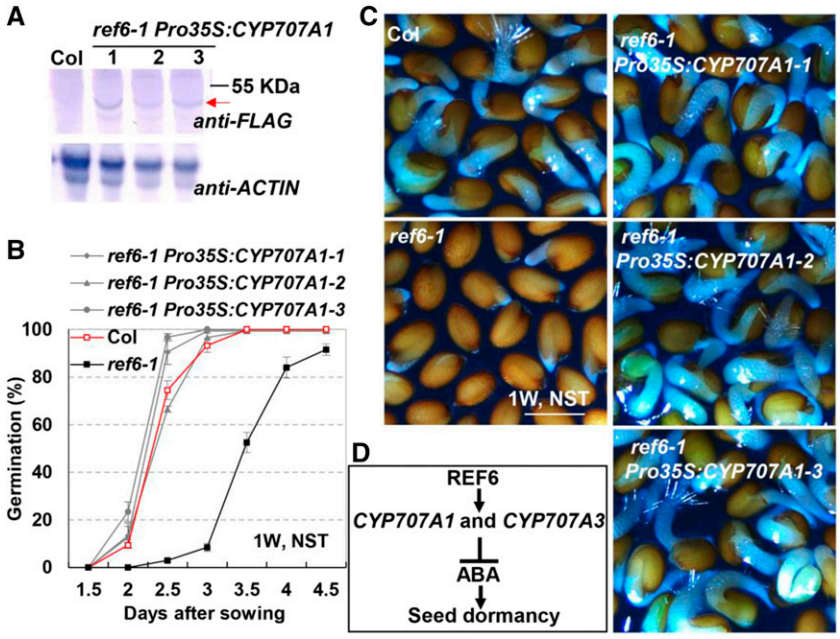

Figure 6. Overexpression of *CYP707A1* reduced the seed dormancy of *ref6-1*. A, Western-blotting analysis of three independent *ref6-1 Pro35S:CYP707A1* transgenic lines. Seven-day-old seedlings were used to extract protein. ACTIN was used as the loading control. The red arrow indicates the recombinant protein CYP707A1-4XFLAG. Experiments were repeated twice, and the graph shows the results of one of the experiments. B, Seed germination rates of Col, *ref6-1* and *ref6-1 Pro35S:CYP707A1* lines. One-week-stored seeds without ST were subjected to analysis. Values (mean ± SD) are from three biological replicates, each consisting of three technical replicates. At least 50 dry mature seeds were used in each technical replication. C, Representative pictures showing germinated seeds at 72 h after sowing. One-week–stored seeds without ST were sown on one-half strength MS medium. Scale bar = 0.6 mm. D, A working model showing the molecular mechanism by which REF6 suppresses seed dormancy.

2009); however, how the key genes in the two pathways are transcriptionally regulated is still largely unknown. ABI4 has been found to inhibit the transcription of *CYP707A1* and *CYP707A2* and thus reduces the catabolism of ABA (Shu et al., 2013). Reactive oxygen species (ROS) were reported to promote the transcription of *CYP707As* and thus decrease the ABA content in seeds (Bailly and Bailly, 2004; Liu et al., 2010b; Chen et al., 2020a). We show here that REF6 is involved in the regulation of ABA catabolism genes (Fig. 3), but not of biosynthesis genes in seeds (Supplemental Fig. S2A). REF6 is an H3K27me3 demethylase that directly binds to CTCTGYTY motifs in its target loci (Lu et al., 2011, 2016, 2018; Cui et al., 2016; Qiu et al., 2019; Wang et al., 2019a). We found that REF6 directly bound to (Fig. 4A) and promoted the expression of *CYP707A1* and *CYP707A3* during seed development and germination (Fig. 3, A and B). Loss of REF6 led to the reduced transcript levels of *CYP707A1* and *CYP707A3* during seed development (Fig. 3A) that were accompanied by the increased H3K27me3 levels at the two genes (Fig. 4B). During seed development, the expression of *REF6* was increased from 10 DAP to 13 DAP (Supplemental Fig. S1A). Therefore, it can be inferred that the H3K27me3 demethylation activity of REF6 should be stronger at 13 DAP than that at 10 DAP, and thus the expression of *CYP707A1* and *CYP707A3* at 13 DAP would be higher than that at 10 DAP. Consistent with this speculation, our results showed that the expression of *CYP707A1* and *CYP707A3* at 13 DAP was higher than that at 10 DAP (Fig. 3, A and B). The expression of *REF6* also well corresponded to that of *CYP707A1* and *CYP707A3* during seed imbibing (Fig. 3, A and B; Supplemental Fig. S1A). These observations support the notion that the H3K27me3 demethylation activity of REF6 is likely coupled with the regulation of *CYP707A1* and *CYP707A3*. It would be useful to generate a REF6 that only lacks the demethylation activity in *ref6-1* mutant, and then measure whether the expression of *CYP707A1* and *CYP707A3* in this material could be recovered. Interestingly, REF6 did not bind to *CYP707A2* (Supplemental Fig. S3B), indicating that the expression of *CYP707A2* in the dry seed is likely indirectly regulated by REF6. The different regulation mechanisms between *CYP707A1*/*CYP707A3* and *CYP707A2* by REF6 may be responsible for the different spatiotemporal expression patterns observed for *CYP707A2* and *CYP707A1*/*CYP70A3* (Fig. 3; Kushiro et al., 2004; Okamoto et al., 2006).

All three CYP707As can be responsible for ABA catabolism; however, each CYP707 gene plays a different role during seed development and germination. CYP707A2 plays a major role in the rapid decrease of ABA levels during early seed imbibition (Kushiro et al., 2004; Matakiadis et al., 2009), whereas CYP707A1 and CYP707A3 are mainly involved in reducing the ABA content in developing seeds (Kushiro et al., 2004; Okamoto et al., 2006). A similar level of expression of *CYP707A2* in imbibing seeds between Col and *ref6-1* (Fig. 3C) argued against the involvement of *CYP707A2* in REF6-regulated ABA catabolism (Fig. 2A). Consistent with this argument, the ABA content in the double mutant *ref6-1 cyp707a2-1* was significantly higher than that of the single mutant *ref6-1* or *cyp707a2-1* (Supplemental Fig. S4B). Moreover, introduction of *ref6-1* into *cyp707a2-1 cyp707a3-1* and *cyp707a1-1 cyp707a2-1* enhanced ABA content in both double mutants. As a result, the seed germination rate of double mutant *ref6-1 cyp707a2-1* was substantially lower than that of single mutant *ref6-1* or *cyp707a2-1* (Table 1; Supplemental Fig. S4A). The seed germination rates of triple mutants *ref6-1 cyp707a2-1 cyp707a3-1* and *ref6-1 cyp707a1-1 cyp707a2-1* were also lower than those of *cyp707a2-1 cyp707a3-1* and *cyp707a1-1 cyp707a2-1*, respectively (Supplemental Fig. S4A).

Previous studies have reported that CYP707A1 is the primary enzyme for ABA 8′-hydroxylation during the midmaturation stage of seed (Okamoto et al., 2006). We show here that the significantly reduced transcription of *CYP707A1* and *CYP707A3* (Fig. 3, A and B) in *ref6-1* is responsible for the higher ABA content of *ref6-1* (Fig. 2A). Consistently, the seed germination of triple mutant *ref6-1 cyp707a1-1 cyp707a3-1* was similar to those of double mutant *cyp707a1-1 cyp707a3-1* (Fig. 5A; Table 1). However, the germination rate of double mutant *ref6-1 cyp707a1-1* was similar to that of *cyp707a1-1*, while the seed germination of double mutant *ref6-1 cyp707a3-1* was similar to that of *ref6-1* (Fig. 5A; Table 1). These results suggest that the enhanced seed dormancy of *ref6-1* is predominantly dependent on the reduction of *CYP707A1* and, to a lesser extent, of *CYP707A3*. Consistent with this, *CYP707A1* had the highest expression level in developing seeds among the three *CYP707As* (Supplemental Fig. S2B). Moreover, overexpression of *CYP707A1* in *ref6-1* could completely offset the enhanced seed dormancy (Fig. 6).

In summary, we have revealed an epigenetic mechanism involved in the regulation of ABA concentration in seeds (Fig. 6D). We show that during seed development, REF6 directly upregulates the expression of *CYP707A1* and *CYP707A3* to promote the catabolism of ABA, leading to a relatively low level of ABA content, and thus a suppression of seed dormancy. As a result, the *ref6* mutant seeds display an enhanced seed dormancy phenotype. Given the widespread conservation of the H3K27me3 demethylases in crops, including rice (*Oryza sativa*; Cheng et al., 2018) and maize (*Zea mays*; Qian et al., 2019), we anticipate our findings will prove informative for understanding the mechanisms governing the ABA level in diverse crops. Revealing how plants regulate the ABA level would represent a key step toward our understanding of the action of ABA in plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*; ecotype Columbia-0 [Col]) was used as wild type. Seeds were sterilized with 10% (v/v) NaClO and stratified at 4°C for 0 d (NST) or 4 d (ST) before being sown on one-half strength MS (Duchefa) medium. Seven-day-old seedlings were transferred to soil and grown in a

greenhouse under 23°C ± 1°C with a 16-h photoperiod/8-h dark. For all experiments, plants were grown under identical conditions, and the seeds were harvested from different plants at the same time and pooled. The mutants *ref6-1* and *aba2-1* were obtained from the Arabidopsis Biological Resource Center at Ohio State University; https://abrc.osu.edu/). The mutants, *cyp707a1-1*, *cyp707a2-1*, and *cyp707a3-1*, were kindly provided by Eiji Nambara. The complemented line *ref6-1 ProREF6:REF6-GFP* was generated in our previous report (Li et al., 2016). Multiple mutants were generated by crossing the *ref6-1* with *aba2-1*, *cyp707a1-1*, *cyp707a2-1*, and *cyp707a3-1*, respectively.

Seed Germination Assay

Plants of each genotype were grown under the same condition, and seeds were harvested at the same time. FH seeds stored for different times were used in seed germination assays. Radicle protrusion was regarded as the indicator of a germinated seed. The seed germination experiments were carried out three times, and each consists of three technical replicates. At least 50 dry mature seeds were used in each technical replication for each genotype.

Generation of Transgenic Plants

The open reading frame of *CYP707A1* was amplified by PCR using the gene-specific primers listed in Supplemental Table S1, digested by *Kpn*I and *Sal*I, and subcloned into the *pCAMBIA1306* vector, which drives the expression of *CYP707A1-4×FLAG* under the *CaMV35S* promoter. Resultant binary constructs were introduced into *Agrobacterium tumefaciens* strain GV3101, which was used to transform the *ref6-1* mutant plants using the floral dip method (Clough and Bent, 1998). The transformed seeds were selected by hygromycin to get transgenic plants *ref6-1 Pro35S:CYP707A1*, which can be detected by an anti-FLAG antibody in an immunoblotting analysis.

RNA Extraction and Gene Expression Analysis

Developing siliques and seeds of each genotype were used to extract total RNA. Total RNA was extracted and reverse-transcribed as described in Chen et al. (2020a). The relative gene expression was determined by quantitative real-time PCR, which was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems) using the SYBR Green Realtime PCR Master Mix (TOYOBO). Gene-specific primers are listed in Supplemental Table S1. Gene expression was relative to *ACTIN2*. Data represent three biological replicates, each consisting of three technical replicates.

Protein Extraction and Immunoblotting

One-hundred milligrams of 7-d-old seedlings were used to extract total protein. Seedlings were ground in liquid nitrogen and then incubated in 100 μL of protein extraction buffer (20 mM of Tris-HCl at pH7.5, 150 mM of NaCl, 0.5% [v/v] Tween 20, 1 mM of EDTA, and 1 mM of dithiothreitol) containing a protease inhibitor cocktail ("Complete," catalog no. 04693116001; Roche). After gentle shaking for 30 min at 4°C, the supernatant was collected by two centrifugations (10 min, 16,000*g*, 4°C).

Immunoblotting analysis was performed with the specific antibodies anti-FLAG (catalog no. M20008; Abmart) and anti-ACTIN (catalog no. M20009; Abmart) according to Chen et al. (2018).

Quantification of ABA and GA

Plants of each genotype were grown under the same condition, and seeds were harvested from different plants at the same time. Approximately 100 mg of FH seeds were used to quantitate the endogenous ABA and GA concentrations. Seeds were frozen by liquid nitrogen, and then the ABA and GA contents were determined by liquid chromatography-tandem mass spectrometry (8030 Plus; Shimadzu) as previously described for ABA (Zhou et al., 2017) or GA (Yang et al., 2012). The experiments were repeated three times (three biological replicates, each consisting of three technical replicates), and similar results were obtained.

ChIP-qPCR

ChIP experiments were conducted as described in the literature (Li et al., 2016, 2018; Chen et al., 2017; Yu et al., 2020), with minor modifications. Briefly, 2 g of developing siliques (10 to 15 DAP) were collected and ground into fine powder in liquid nitrogen and then well homogenized with extraction buffer 1 (2 M of Suc, 1 M of Tris-HCl at pH 8.0, 1 M of $MgCl_2$, 14.3 M of β-mercaptopropionate, and 0.1 M of phenylmethylsulfonyl fluoride [protease inhibitor cocktail]). After that, 37% (v/v) formaldehyde was added immediately to the homogenized buffer until the final concentration was 1%, and cross linked on a shaker for 15 min at 4°C and stopped by 0.125 M of Gly. Chromatin was isolated and sheared into 500- to 1,000-bp fragments by sonication. The sonicated chromatin was incubated with 4 μL of antibody to GFP (catalog no. ab290; Abcam) or H3K27me3 (catalog no. 07-449; Millipore) overnight at 4°C. Precipitated DNA was then dissolved with sterilized water. ChIP-qPCR was performed with three technical replicates, and results were calculated as the percentage of input DNA according to the Champion ChIP-qPCR user manual (SABioscience). Values are from three biological replicates. Sequences for the primers used in the ChIP-qPCR are listed in Supplemental Table S1.

Statistical Analysis

Statistical significance of differences was determined using Student's *t* test and one-way ANOVA with Duncan's multiple range test.

Accession Numbers

Genes referenced in this article can be found in The Arabidopsis Information Resource (https://www.arabidopsis.org/) under the following accession numbers: REF6 (At3g48430), CYP707A1 (At4g19230), CYP707A2 (At2g29090), CYP707A3 (At5g45340), NCED6 (At3g24220), NCED9 (At1g78390), NCED2 (At4g18350), NCED5 (At1g30100), ABA1 (At5g67030), ABA2 (At1g52340), and AAO3 (At2g27150).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. The expression pattern of *REF6* in seeds in different stages or treated by ABA.

Supplemental Figure S2. The transcription levels of ABA biosynthesis and catabolism genes in siliques or seeds of *ref6-1* and Col.

Supplemental Figure S3. REF6 does not bind *CYP707A2*.

Supplemental Figure S4. CYP707A2 does not contribute to the enhanced seed dormancy of *ref6-1*.

Supplemental Table S1. Primer sequences used in this study.

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