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REVERSAL OF RDO5 1, a Homolog of Rice Seed Dormancy4, Interacts with bHLH57 and Controls ABA Biosynthesis and Seed Dormancy in Arabidopsis[OPEN]

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The control of seed dormancy by abscisic acid (ABA) has been extensively studied, but the underlying mechanism is not fully understood. Here, we report the characterization of two ABA-related seed dormancy regulators in Arabidopsis (*Arabidopsis thaliana*): ODR1 (for reversal of *rdo5*), an ortholog of the rice (*Oryza sativa*) Seed dormancy4 (Sdr4), and the basic helix-loop-helix transcription factor bHLH57. *ODR1*, whose transcript levels are directly suppressed by the transcription factor ABA INSENSITIVE3 (ABI3), negatively regulates seed dormancy by affecting ABA biosynthesis and ABA signaling. By contrast, bHLH57 positively regulates seed dormancy by inducing the expression of the genes *9-CIS-EPOXYCAROTENOID DIOXYGENASE6* (*NCED6*) and *NCED9*, which encode ABA biosynthetic enzymes, and thus leads to higher ABA levels. ODR1 interacts with bHLH57 and inhibits bHLH57-modulated *NCED6* and *NCED9* expression in the nucleus. *bhlh57* loss-of-function alleles can partially counteract the enhanced *NCED6* and *NCED9* expression seen in *odr1* mutants and can therefore rescue their associated hyper-dormancy phenotype. Thus, we identified a novel ABI3-ODR1-bHLH57-NCED6/9 network that provides insights into the regulation of seed dormancy by ABA biosynthesis and signaling.

INTRODUCTION

Dormancy prevents germination when seeds are exposed to short but temporarily favorable periods before the return of adverse conditions and thus delays seedling establishment until the start of the growing season. Dormancy therefore plays a vital role in plant survival and evolution (Linkies et al., 2010; Née et al., 2017b). Seed dormancy is imposed during seed maturation and released during after-ripening or by hydration at specific temperatures. Precise control of seed dormancy in crops results in fast and uniform germination after sowing and prevents preharvest sprouting, which would otherwise negatively impact agricultural production (Gubler et al., 2005).

Seed dormancy is a complex trait influenced by genetic and environmental factors (Graeber et al., 2012; Penfield and MacGregor, 2017). Previous studies revealed that phytohormones, including abscisic acid (ABA), gibberellins (GAs), ethylene, strigolactones, and brassinosteroids, all play important roles in the control of seed dormancy (Seo et al., 2006; Shu et al., 2016a). Among these hormones, ABA and GA have central and antagonistic roles: ABA enhances dormancy, whereas GA stimulates germination. The roles of ABA and GA biosynthesis and signal transduction in the control of seed dormancy and germination have been intensively studied in the past decades (Gubler et al., 2005; Lefebvre et al., 2006; Née et al., 2017b).

During seed maturation, endogenous ABA gradually accumulates to enforce dormancy (Kanno et al., 2010). Cleavage of the ABA precursors 9-*cis*-violaxanthin and 9-*cis*-neoxanthin into the intermediate xanthoxin by 9-*cis*-epoxycarotenoid dioxygenase (NCED) is considered the key rate-limiting step in ABA biosynthesis (Nambara and Marion-Poll, 2005). Of the nine Arabidopsis (*Arabidopsis thaliana*) *NCED* genes, seed-specific *NCED6* and *NCED9* significantly contribute to ABA biosynthesis during seed development. The corresponding *nced6 nced9* double mutant shows a significant decrease of seed ABA content and concomitant reduced seed dormancy (Lefebvre et al., 2006), suggesting that both NCED6 and NCED9 are important for the establishment and maintenance of seed dormancy. A tight control of *NCED6* and *NCED9* expression is therefore vital for seed dormancy. Previous studies have reported that several transcription factors (TFs) control the expression of *NCED6* and *NCED9* during seed development, such as ABSCISIC ACID INSENSITIVE4 (ABI4), DEHYDRATION-RESPONSIVE ELEMENT BINDING FACTOR2C (DREB2C), and MYB96. These TFs activate *NCED6* and *NCED9* expression by binding to their promoters (Je et al., 2014; Lee et al., 2015; Shu et al., 2016b). Aside from biosynthesis, ABA degradation also plays an important role in determining endogenous ABA content and release of dormancy. Among four cytochrome P450 CYP707A family (CYP707A1 to CYP707A4) members in Arabidopsis, CYP707A2 is considered to be the major factor that executes ABA degradation in mature and

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Background: Seed dormancy is established at the end of seed maturation and ensures that seeds sprout under favorable conditions and after they have dispersed away from the mother plant. It is a complex trait influenced by endogenous (phytohormones abscisic acid (ABA) and gibberellins) and environmental factors (including ambient temperature and relative humidity for seed hydration). A tight control of seed dormancy in crops allows uniform germination after sowing and prevents pre-harvest sprouting at the end of the growing season. However, the underlying mechanism of seed dormancy is still not fully understood.

Question: In earlier work, we had isolated the *reduced dormancy5* (*rdo5*) mutant on the basis of its reduced seed dormancy. The *RDO5* gene encode a type 2C protein phosphatase. To better understand the signaling cascade RDO5 participates in, we performed a suppressor mutant screen in the *rdo5-2* background to look for additional factors controlling seed dormancy.

Findings: We identified two ABA-related seed dormancy regulators in Arabidopsis. One was isolated from the suppressor screen (ODR1, for reversal of *rdo5*, an orthologue of the rice Seed dormancy 4 Sdr4). The second protein, the basic Helix-Loop-Helix transcription factor bHLH57, interacts with ODR1. The transcription factor ABA INSENSITIVE 3 (ABI3) directly represses ODR1 transcription, thereby affecting seed dormancy by modulating ABA biosynthesis and ABA signaling. In contrast, bHLH57 imposes seed dormancy by inducing the expression of the genes encoding the ABA biosynthetic enzymes 9-CIS-EPOXYCAROTENOID DIOXYGENASE *NCED6* and *NCED9*, and thus raises ABA levels. ODR1 interacts with bHLH57 and inhibits bHLH57-modulated *NCED6* and *NCED9* expression in the nucleus. Likewise, *bhlh57* loss of function mutants can partially counteract the enhanced *NCED6* and *NCED9* expression seen in *odr1* mutants and can therefore rescue their associated hyper-dormancy phenotype.

Next steps: We plan to uncover more details in the ODR1-dependent pathway, for example how ODR1 controls *DOG1* expression during seed dormancy and germination.

hydrated seeds (Kushiro et al., 2004). The *cyp707a2* mutant over-accumulates ABA and shows stronger seed dormancy (Okamoto et al., 2006).

ABA signal transduction also influences seed dormancy, acting primarily through a cascade that comprises ABA receptors (encoded by *PYRABACTIN RESISTANCE1* [*PYR1*]/*PYR1-like* [*PYL*] *1-13*, also known as *REGULATORY COMPONENTS OF ABA RECEPTOR* [*RCAR*]), type 2C protein phosphatases (PP2Cs), and sucrose non-fermenting 1 related protein kinase (SnRK; Ma et al., 2009; Park et al., 2009; Cutler et al., 2010; Miyakawa et al., 2013). Loss-of-function mutants in ABA signaling show decreased ABA sensitivity, resulting in early release of seed dormancy (Park et al., 2009; Fuchs et al., 2014). ABI3 is a vital ABA-responsive TF that plays a central role in seed maturation and primary dormancy establishment in Arabidopsis. Mutations in *ABI3* lead to reduced dormancy and premature seed germination (Nambara et al., 1995). ABI3 contains a B3 domain that can physically associate with the RY motif found in the promoter of downstream genes, such as *SOMNUS* (*SOM*) and *STAY-GREEN1* (*SGR1*) and *SGR2* (Ezcurra et al., 2000; Mönke et al., 2004; Park et al., 2011; Delmas et al., 2013). In addition, ABI3 interacts with the important ABA-responsive TFs ABI4 and ABI5 to establish seed dormancy (Söderman et al., 2000; Lopez-Molina et al., 2002).

Apart from phytohormones, seed dormancy is also controlled by several genes originally identified as major quantitative trait loci (QTLs) such as *DELAY OF GERMINATION1* (*DOG1*), *DOG18/REDUCED DORMANCY5* (*RDO5*), and *DOG6* (Bentsink et al., 2010). DOG1 is a major seed dormancy factor in Arabidopsis (Bentsink et al., 2006). DOG1 protein levels are tightly correlated with dormancy levels in freshly harvested seeds, with higher DOG1 levels causing delayed germination (Nakabayashi et al., 2012). The basic Leu zipper TFs bZIP67 and Ethylene Response Factor12 (ERF12) bind to the promoter of and control the expression of *DOG1* and seed dormancy in response to cool temperatures and ethylene exposure, respectively (Bryant et al., 2019; Li et al., 2019). *DOG1* transcript levels are also modulated by its antisense transcript *asDOG1* (Fedak et al., 2016). The DOG1 protein interacts with ABA-HYPERSENSITIVE GERMINATION1 (AHG1) and AHG3, which are PP2Cs that belong to the same clade as those interacting with the ABA receptors. Therefore, these PP2Cs are considered a converging point for DOG1- and ABA-dependent dormancy control pathways (Née et al., 2017a; Nishimura et al., 2018). Another seed dormancy factor, RDO5/DOG18, was identified both in a mutagenesis screen and by QTL mapping. RDO5 was shown to control seed dormancy independently of ABA, and a transcriptome analysis suggested that RDO5 does so by controlling the expression of the genes encoding the PUMILIO RNA binding proteins APUM9 and homologs, revealing a posttranscriptional dormancy pathway (Xiang et al., 2014, 2016). RDO5 belongs to the PP2C family of protein phosphatases but is found in a clade distinct from AHG1 and AHG3. In fact, RDO5 functions as a pseudo-phosphatase because it lacks phosphatase activity (Xiang et al., 2016). Interestingly, the RDO5 and DOG1 proteins interact in Arabidopsis seeds (Née et al., 2017a), although the reason or the role of such interaction in seed dormancy is unknown.

In order to dissect the RDO5-mediated seed dormancy network, we performed a suppressor mutagenesis screen with the low dormancy mutant *rdo5-2*. Here, we report the identification of one such suppressor (globally called *odr*, for reversal of the *rdo* phenotype): *odr1*. Mutations in *ODR1* cause stronger seed dormancy. Expression of *ODR1* is repressed by ABI3, and ODR1 negatively affects the expression of *NCED6* and *NCED9* and ABA content in freshly harvested seeds. Furthermore, ODR1 interacts with bHLH57 and prevents bHLH57-mediated induction of *NCED6* and *NCED9* expression. In agreement, the *odr1-2 bhlh57*

double mutant decreased the expression of *NCED6* and *NCED9* compared to *bhlh57* single mutants, while rescuing the hyper-dormancy phenotype of *odr1*-2. We therefore discovered a new seed dormancy pathway that includes ABI3, ODR1, bHLH57, NCED6, and NCED9. Because the rice (*Oryza sativa*) ortholog of ODR1 was previously identified as a QTL in rice, our work also provides a molecular link between presprouting research in rice and seed dormancy in Arabidopsis.

RESULTS

The Seed-Specific Protein ODR1 Negatively Controls Seed Dormancy

We had previously reported the cloning and initial characterization of the positive dormancy factor RDO5, which controls seed dormancy without influencing ABA metabolism or signal transduction. Loss-of-function *rdo5* mutant alleles cause strongly reduced seed dormancy (Xiang et al., 2014, 2016). Seeking to uncover the function of RDO5 in seed dormancy regulation, we performed a γ-ray mutagenesis screen with *rdo5-2*, a T-DNA insertion mutant with substantially reduced dormancy duration (Xiang et al., 2014). We identified six mutants that suppressed the *rdo5-2* dormancy phenotype and named them *odr1* to *odr6* (for reversal of the *rdo* phenotype). The *odr1* mutant exerted the most significant effect on seed dormancy and was therefore selected for further characterization. We used bulked segregant analysis–based sequencing to identify mutations that might be responsible for the phenotype and identified an 8-bp deletion in At1g27461 that caused a reading frame shift leading to a premature stop codon (Supplemental Figures 1A and 1B). To confirm the identity of At1g27461 as *ODR1*, we obtained an independent mutant in the gene, the homozygous T-DNA insertion mutant SALK_022729, which we named *odr1-2*. This mutant carried a T-DNA insertion in the single *ODR1* exon and lacked full-length *ODR1* transcript (Supplemental Figures 1C and 1D). The *odr1-2* allele is therefore presumed to be a null allele. Germination assays showed that *odr1-2* had reduced germination compared to the wild-type Columbia (Col-0; Figures 1A and 1B). We also introduced the *odr1-2* T-DNA insertion into *rdo5-2* via crossing and conducted germination assays with the double mutant: it showed lower germination percentage than *rdo5-2*, indicating that *odr1-2* also effectively suppressed the dormancy phenotype seen in *rdo5-2* (Figure 1A). Additionally, to confirm the repressive role of ODR1 in seed dormancy, we generated overexpression lines in the *odr1-2* background by placing a copy of *ODR1* under the control of the cauliflower mosaic virus 35S promoter (Supplemental Figure 1D).

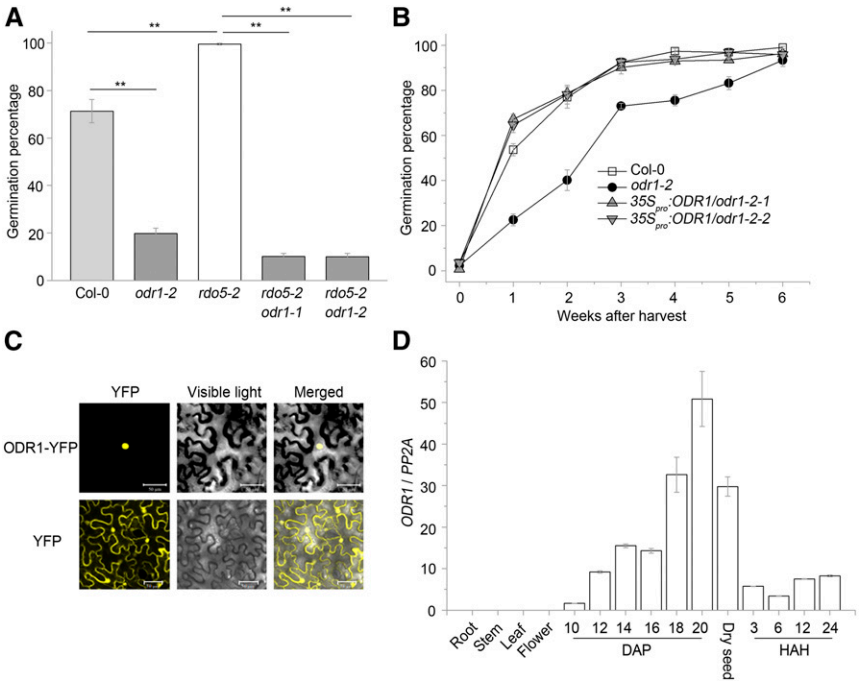

Figure 1. ODR1 Is a Nucleoprotein That Reduces Seed Dormancy and Is Predominantly Expressed in Mature Dry Seeds.

(A) Germination percentages of Col-0, *rdo5-2*, *odr1-1 rdo5-2*, *odr1-2*, and *rdo5-2 odr1-2* seeds after 1 week of storage. Values are means ± SD of three independent batches of seeds per genotype. **P < 0.01 by ANOVA analysis.

(B) Germination percentages after different periods of dry storage of Col-0, odr1-2, and two overexpression lines containing a $35S_{pro}$:*ODR1* insertion in the *odr1-2* background. Values are means ± SD of three independent batches of seeds per genotype.

(C) Subcellular localization of ODR1-YFP in *N. benthamiana* leaves. YFP is used as the control. Bars = 50 μm.

(D) RT-qPCR analysis of *ODR1* transcript levels in various organs and developing siliques and mature seeds in Arabidopsis. The expression values were normalized to *PP2A*. Values are means ± SD of three biological repeats. DAP, days after pollination; HAH, hours after hydration.

Germination assays showed that homozygous *35S$_{pro}$:ODR1/odr1-2* seeds had similar dormancy rates as Col-0 (Figure 1B). These results established that constitutive expression of *ODR1* rescues the enhanced dormancy phenotype of *odr1-2* and that *ODR1* negatively impacts seed dormancy.

The *ODR1* gene consists of a single exon of 1065 bp, encoding a 355–amino acid protein. Protein sequence alignment and phylogenetic analysis demonstrated that ODR1 and its homologues are conserved within angiosperms (Supplemental Figures 2A and 2B). *ODR1* was previously described as *DROUGHT RESPONSIVE GENE*, a seed-specific gene induced under drought conditions (Moon et al., 2016). The *drg* mutant was shown to be more sensitive to a number of abiotic stresses, including osmotic stress, drought, and freezing. The putative rice ortholog of DRG, the zinc finger protein Sdr4, is a positive factor of seed dormancy (Sugimoto et al., 2010). In another study, germination of freshly harvested *drg/SEED DORMANCY4-LIKE* (*Atsdr4L*) mutant seeds were found to be insensitive to exogenous GA (Cao et al., 2020). SDR is already assigned to designate short-chain dehydrogenase reductases in Arabidopsis, while DRG can also stand for developmentally regulated GTP binding proteins. We therefore propose to rename *DRG/AtSDR4* as *ODR1* to avoid confusion and to adopt a single gene identifier representative of its underlying function.

We analyzed the subcellular localization of ODR1 by transient expression in tobacco (*Nicotiana benthamiana*) leaves. The ODR1-yellow fluorescent protein (YFP) fusion protein was exclusively localized to the nucleus (Figure 1C). We next evaluated *ODR1* expression levels in different Arabidopsis tissues by RT-qPCR: *ODR1* transcript was undetectable in roots, stems, leaves, or flowers, but it gradually increased during seed maturation and reached its highest level in seeds 20 d after pollination, while it sharply declined in hydrated seed (Figure 1D). This pattern of expression is consistent with the expression data for *ODR1* in the Arabidopsis eFP Browser (Supplemental Figure 3). Taken together, *ODR1* is specifically expressed in seeds and encodes a nuclear protein that is involved in the control of dormancy.

ODR1 Strongly Impacts ABA-Mediated Dormancy

ABA plays a crucial role in the control of seed dormancy and germination. To test whether the enhanced dormancy of *odr1-2* was associated with ABA, we first measured the ABA content in freshly harvested seeds from different genotypes, including Col-0, *odr1-2*, *35S$_{pro}$:ODR1/odr1-2*, *rdo5-2*, and *rdo5-2 odr1-2*. ABA levels in *odr1-2* freshly harvested seeds were ~40% higher compared to those in Col-0 and the overexpression lines (Figure 2A), which is consistent with the stronger dormancy seen in *odr1-2* seeds. The *rdo5-2 odr1-2* double mutant also showed higher ABA levels compared to the *rdo5-2* single mutant, which itself was similar to Col-0 in freshly harvested seeds (Figure 2B), as previously reported by Xiang et al. (2014). These results suggest that ODR1 might influence ABA metabolism. Next, we evaluated the germination behavior of freshly harvested seeds of Col-0 and *odr1-2* in the presence of the ABA biosynthesis inhibitor fluridone. The *odr1-2* mutant responded more strongly to fluridone compared to Col-0 by exhibiting lower germination percentage, suggestive of heightened ABA sensitivity (Figure 2C). Overall, these results demonstrate that the stronger seed dormancy in *odr1-2* is largely caused by a high level of endogenous ABA in dry seeds and de novo ABA synthesis during seed hydration. Finally, we evaluated the ABA sensitivity of Col-0 and *odr1-2* after-ripened seeds. Seeds of both genotypes germinated fully and synchronously after stratification on half-strength Murashige and Skoog (MS) growth medium in the absence of ABA. However, *odr1-2* seeds were more sensitive to ABA as shown by reduced germination percentage when the medium was supplemented with 0.5 μM ABA. This suggests that loss of function of ODR1 affects ABA sensitivity of seeds (Figure 2D).

Since ABA content was higher in *odr1-2* seeds compared to Col-0, we evaluated the expression levels of pivotal ABA metabolism genes (Supplemental Figure 4), including *NCED*s (*NCED2*, *NCED3*, *NCED5*, *NCED6*, and *NCED9*, involved in ABA biosynthesis) and *CYP707A*s (*CYP707A1* to *CYP707A4*, linked to ABA degradation) in Col-0 and *odr1-2* freshly harvested and in seeds that had been hydrated for 6 h. Nearly all of the *NCED*s (except *NCED5*) and *CYP707A2* were significantly upregulated in dry and/or hydrated seeds in *odr1-2* compared with Col-0 (Figure 2E). Differential expression of *NCED*s could well explain the increased ABA content of *odr1-2* freshly harvested seeds, while the elevated expression levels of *CYP707A2* might indicate a feedback reaction to the increased ABA content in *odr1-2* seeds. However, ODR1 does not activate gene expression by direct binding to promoters, as we failed to detect ODR1 binding ability to any of the *NCED*s and *CYP707A*s promoters in yeast one-hybrid assays (Supplemental Figure 5). We also found that ODR1 indirectly regulated *DREB2C* and *ABI4* gene expression in dry and/or hydrated seeds but did not physically interact with the encoded proteins (Supplemental Figure 6). Taken together, these results suggest that ODR1 controls seed dormancy by affecting the expression of genes involved in ABA metabolism but is unlikely to behave as a TF.

We had shown previously that RDO5 controls seed dormancy in an ABA-independent manner. However, loss-of-function mutants of *odr1-1* and *odr1-2* both suppressed the weak dormancy phenotype of *rdo5-2* in double mutant combinations, which indicated that the ODR1-mediated ABA metabolism pathway was epistatic to RDO5 for seed dormancy. To further confirm this relationship, we generated the *rdo5-2 cyp707a2* double mutant by crossing. Germination assays showed that the *rdo5-2 cyp707a2* double mutant had a stronger dormancy compared to *rdo5-2* (Figure 2F). This result demonstrated that the *cyp707a2* mutant, which causes an over-accumulation of ABA in seeds, is epistatic to *rdo5*-mediated reduced seed dormancy. Surprisingly, the dormancy level of *rdo5-2 cyp707a2* was even higher than that of the *cyp707a2* single mutant. This might be due to a feedback response to the *rdo5* mutation in order to keep seeds in a dormant status, which becomes noticeable in the *cyp707a2* mutant background. This is supported by increased transcript levels following 6 h of seed hydration in the *rdo5-2* background for two *PYR/PYL/RCAR* genes (At4g17870 and At2g38310) and an *SnRK2* gene (At1g78290) involved in ABA signaling (Xiang et al., 2014). We also found that *DOG1*, the core factor controlling seed dormancy, was upregulated in *odr1-2*, but again without direct interaction between the encoded protein and ODR1. Higher *DOG1* expression might also contribute to the hyper-dormancy phenotype of the *odr1-2* mutant (Supplemental Figure 7).

Overall, ODR1 controls seed dormancy at least partially through modulation of ABA biosynthesis, which acts downstream of the RDO5-mediated dormancy pathway.

ODR1 Is a Direct Target of ABI3

A previous chromatin immunoprecipitation (ChIP) followed by hybridization to tiling arrays (ChIP-chip) and transcriptome analysis predicted that *ODR1* was one of the 98 direct targets of ABI3 (Mönke et al., 2012). Based on this finding, we analyzed the *ODR1* promoter sequence and found two potential RY motifs (CATGCA) known to be binding sites for ABI3 (Figure 3A). To validate the control of *ODR1* expression by ABI3, we performed a yeast one-hybrid assay, whose results indicated that ABI3 specifically binds to the *ODR1* promoter at the proximal RY motif

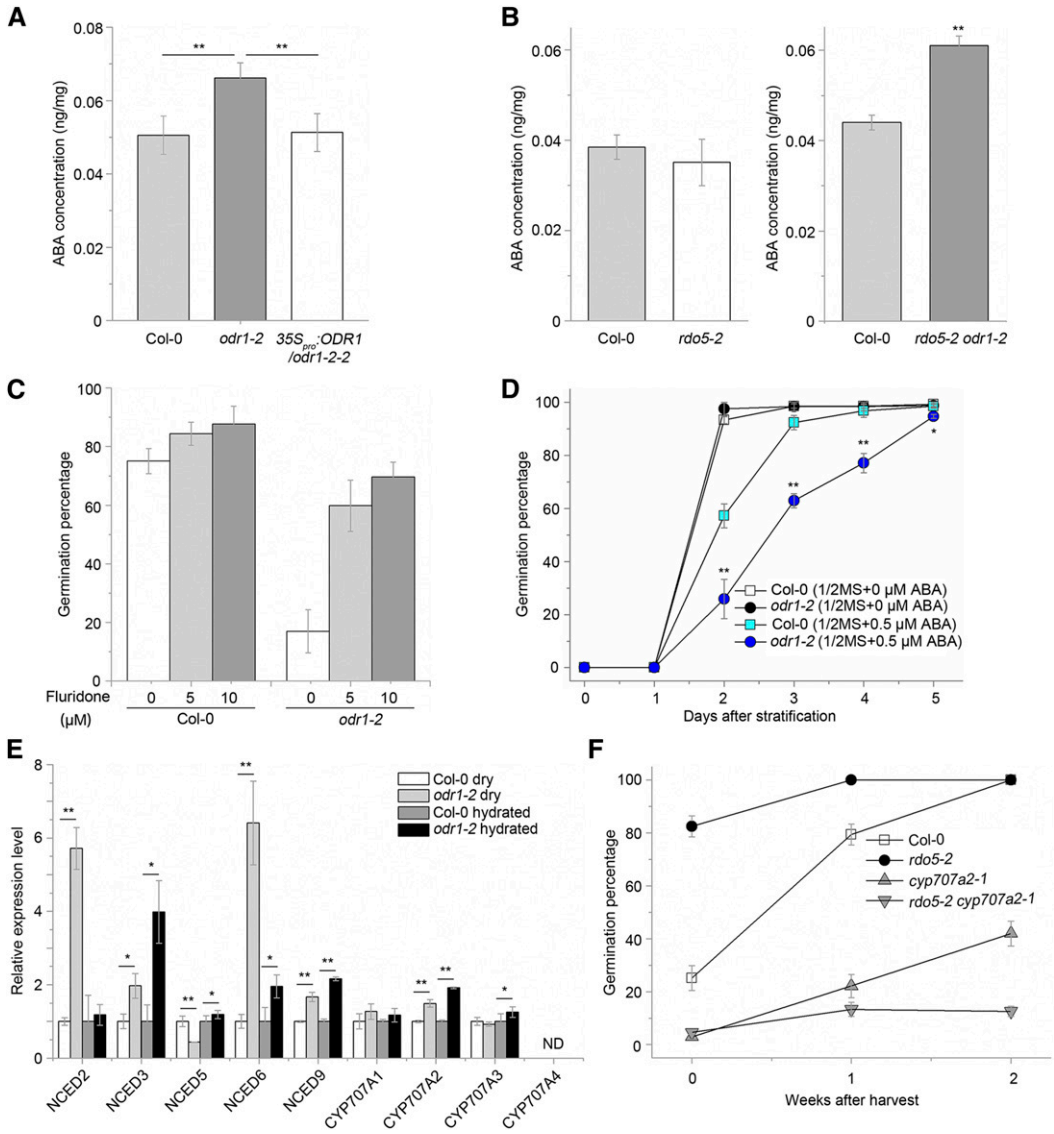

Figure 2. ODR1 Affects ABA Content and Seed ABA Sensitivity.

(A) ABA content in Col-0, *odr1-2*, and *35S$_{pro}$:ODR1/odr1-2-2* freshly harvested dry seeds. Values are means ± SD of three biological repeats. *P < 0.05 or **P < 0.01 by Student's *t* test.

(B) ABA content in Col-0, *rdo5-2*, and *rdo5-2 odr1-2* freshly harvested seeds. Values are means ± SD of three biological repeats. **P < 0.01 by Student's *t* test.

(C) Germination percentages of Col-0 and *odr1-2* seeds after 1 week of storage, tested in the presence of 0.05% ethanol (control) or 0.05% ethanol plus the indicated concentrations of fluridone. Values are means ± SD of three independent batches of seeds per genotype.

(D) Germination percentages of Col-0 and *odr1-2* freshly harvested seeds on 1/2 MS medium supplemented with 0 or 0.5 μM ABA. Values are means ± SD of three independent batches of seeds per genotype. Significance analysis was performed between *odr1-2* and Col-0 with 0 μM ABA treatment, and 0.5 μM ABA treatment. *P < 0.05 or **P < 0.01 by Student's *t* test.

(E) RT-qPCR analysis of *NCED*s and *CYP707A*s transcript levels in Col-0 and *odr1-2* freshly harvested dry seeds and seeds hydrated for 6 h. The expression values were normalized to *PP2A*. Values are means ± SD of three biological repeats. *P < 0.05 or **P < 0.01 by Student's *t* test.

(F) Germination percentages of Col-0, *rdo5-2*, *cyp707a2-1*, and *rdo5-2 cyp707a2-1* freshly harvested seeds. Values are means ± SD of three independent batches of seeds per genotype.

(CATGCA$_{-363}$), but not at the more distal motif (CATGCA$_{-700}$) (Figure 3B). Furthermore, we performed ChIP followed by quantification of immunoprecipitated chromatin by qPCR with chromatin extracted from seedlings overexpressing ABI3 fused to a FLAG tag (*35S$_{pro}$:ABI3-FLAG*; Supplemental Figure 8; Park et al., 2011). We found that the DNA fragment containing the proximal RY motif (CATGCA$_{-363}$) of the *ODR1* promoter was highly enriched compared to other DNA fragments (Figure 3C). To investigate how ABI3 controlled *ODR1* expression, we then performed a transient expression assay using *N. benthamiana* leaves, and it showed that ABI3 repressed the expression of *ODR1* (Figure 3D). In addition, expression levels of *ODR1* in *abi3-1* and *35S$_{pro}$:ABI3-FLAG* seeds demonstrated that overexpression of *ABI3* reduced *ODR1* expression, while the weak allele *abi3-1* showed enhanced *ODR1*

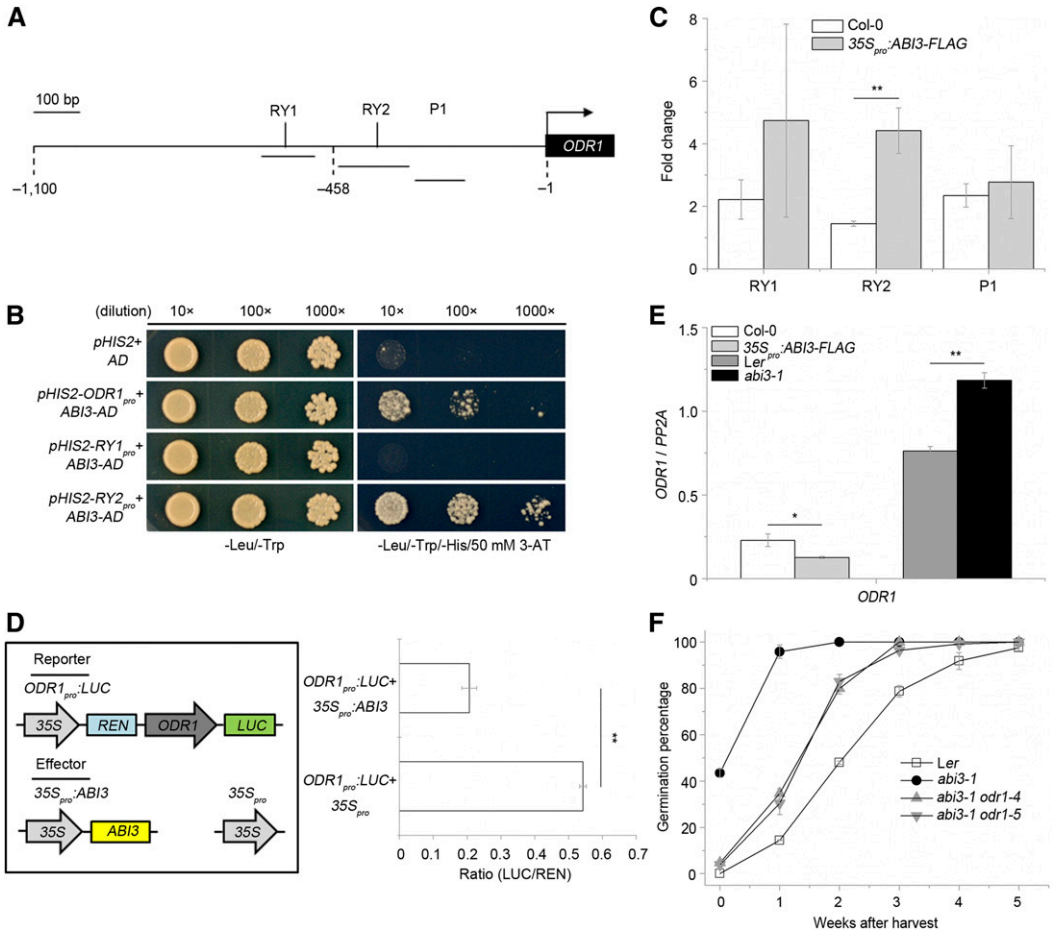

Figure 3. ABI3 Represses *ODR1* Expression.

(A) Schematic diagram of the *ODR1* promoter. Three fragments were amplified for yeast one-hybrid assay: the –1- to –457-bp fragment (containing RY2 element [RY2pro]), the –458- to –1100-bp fragment (containing RY1 element [RY1pro]), and the –1- to –1100-bp fragment (ODR1pro). Three fragments for ChIP-qPCR detection (named RY1, RY2, and P1) are depicted with parallel black lines below the promoter. Bar = 100 bp.

(B) Yeast one-hybrid assay: ABI3 binds to the *ODR1* promoter through the RY2-containing fragment. 3-AT (50 mM) was added in the -Leu/-Trp/-His medium. Dilution 10×, 100×, and 1000× corresponds to an OD_{600} of 0.1, 0.01, and 0.001, respectively.

(C) ChIP-qPCR assay: ABI3 mainly binds to the RY2 motif-containing fragment of the ODR1 promoter. Immunoprecipitation was performed using seeds hydrated for 6 h from Col-0 and *35S$_{pro}$:ABI3-FLAG* with anti-FLAG antibody or anti-IgG. qPCR was performed with specific primers listed in Supplemental Data Set 1. The comparison is between the nonimmune control (anti-IgG) and the immunoprecipitation (anti-FLAG). Values are means ± SD of three biological replicates. **P < 0.01 by Student's *t* test.

(D) Transient expression assay: ABI3 represses *ODR1* expression in *N. benthamiana* leaves. Firefly LUC activity was normalized to REN activity (as an internal control). Schematic representation of various constructs used in the assay is shown on the left panel. Values are means ± SD from three independent transformants for each construct. **P < 0.01 by Student's *t* test.

(E) RT-qPCR analysis of *ODR1* transcript levels in *35S$_{pro}$:ABI3-FLAG* seeds (Col-0 background) and *abi3-1* mutant seeds (*Ler* background). Values represent the *ODR1* expression levels change after 6 h of seed hydration. Values are means ± SD of three biological replicates. *P < 0.05 or **P < 0.01 by Student's *t* test.

(F) Germination percentages after different periods of dry storage of the wild-type *Ler*, *abi3-1*, *abi3-1 odr1-4*, and *abi3-1 odr1-5* seeds. Values are means ± SD of three independent batches of seeds per genotype.

expression during seed hydration compared to the wild-type control (Figure 3E). Finally, we used the genome editing technique clustered regularly interspaced short palindromic repeats and CRISPR-associated nuclease (CRISPR/Cas9) to inactivate *ODR1* in an *abi3-1* background. After genomic PCR verification and Sanger sequencing, three lines with different editing scars in *ODR1* (containing a 1-bp insertion, a 32-bp deletion, and a 55-bp deletion, respectively) were identified and named *odr1-3*, *odr1-4*, and *odr1-5* (Supplemental Figure 9). Germination assays of the wild-type Landsberg *erecta* (L*er*), *abi3-1*, *abi3-1 odr1-4*, and *abi3-1 odr1-5* showed that removal of ODR1 activity could largely suppress the weak dormancy rates of *abi3-1* (Figure 3F), suggesting that *ODR1* genetically acts downstream of *ABI3* and that ABI3 requires functional ODR1 to control seed dormancy. Taken together, we found that ABI3, which is an essential factor of ABA signaling transduction and seed dormancy, represses *ODR1* expression by binding to its promoter.

ODR1 Interacts with the bHLH-Type Protein bHLH57 in the Nucleus

We performed a yeast two-hybrid screen using ODR1 as bait to identify potential interacting partners and identified bHLH57 (At4g01460), a putative bHLH-type TF. The direct physical interaction between ODR1 and bHLH57 was confirmed using a yeast two-hybrid assay using their full-length coding sequences. A yeast strain AH109 (*Saccharomyces cerevisiae*) containing both ODR1 and bHLH57 was able to grow on synthetic medium lacking His, Leu, and Trp and supplemented with 2 mM 3-amino-1,2,4-triazole (3-AT), in contrast to the negative controls (Figure 4A). We also performed bimolecular fluorescence complementation (BiFC) assays by cotransfecting different combinations of proteins fused to fragments of YFP into *N. benthamiana* leaves. Yellow fluorescence signal was detected in the nuclei of *N. benthamiana* cells coexpressing ODR1-YFPn and bHLH57-YFPc, while the expression of either fusion protein alone did not result in measurable YFP signal (Figure 4B). Finally, we confirmed the interaction between ODR1 and bHLH57 with pull-down experiments using total protein extracts from *Escherichia coli* producing bHLH57-glutathione S-transferase (GST) and ODR1-6×His. The bHLH57-GST fusion protein was able to pull down ODR1-6×His, indicating that bHLH57 interacts with ODR1 in vitro (Figure 4C). These results therefore confirm that ODR1 physically interacts with bHLH57, suggesting that they may modulate seed dormancy as a complex.

bHLH57 Imposes *NCED6*- and *NCED9*-Mediated Seed Dormancy

To assess whether bHLH57 controls seed dormancy, we obtained the homozygous T-DNA insertion mutants *bhlh57-1* (SALK_027604) and *bhlh57-2* (SAIL_84_E04). The *bhlh57-1* allele carries the insertion ~50 bp upstream of the ATG and reduced *bHLH57* transcript levels to 10% of Col-0. The T-DNA is inserted within the first exon in *bhlh57-2* and abrogates *bHLH57* transcript accumulation (Figure 5A; Supplemental Figure 10A). Germination assays showed that both mutants had weaker dormancy compared to Col-0, indicating that bHLH57 positively controls seed

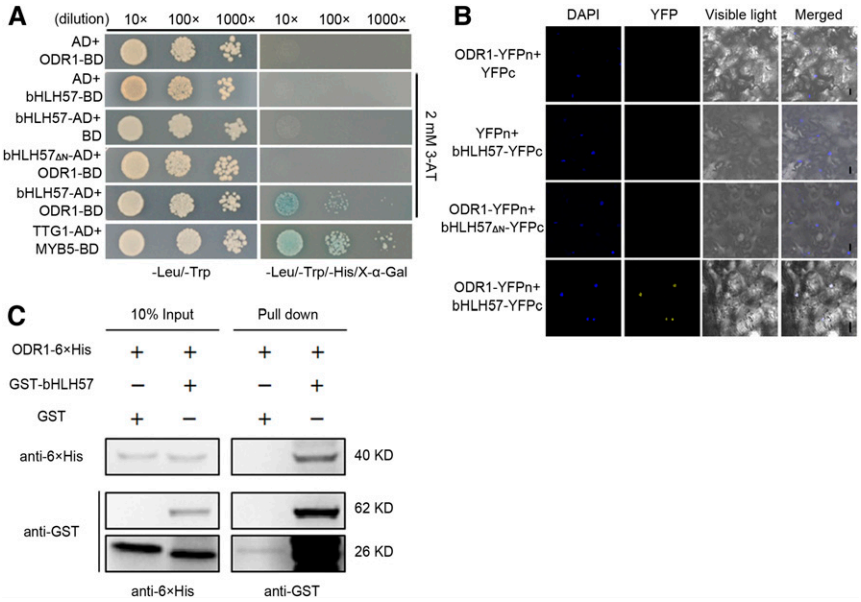

Figure 4. ODR1 Interacts with bHLH57.

(A) Yeast two-hybrid assay: ODR1 interacts with bHLH57. 3-AT (2 mM) was added in the -Leu/-Trp/-His/5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal) medium. Interaction between TTG1 and MYB5 was used as positive control. bHLH57ΔN represents a truncated bHLH57 (N terminus [1 to 111 amino acids] of bHLH57 is removed). Dilution 10×, 100×, and 1000× corresponds to an OD_{600} of 0.1, 0.01, and 0.001, respectively.
(B) BiFC assay: interaction between ODR1 and bHLH57 in *N. benthamiana* leaves. 4′,6-Diamidino-2-phenylindole (DAPI)–labeled nuclei. Bars = 20 μm.
(C) GST-bHLH57 pull-down assays: in vitro interaction between bHLH57 and ODR1. GST and GST-bHLH57 served as bait. ODR1-6×His served as prey.

dormancy (Figure 5B). bHLH57 localizes to the nucleus and is expressed in various plant organs, including roots, stems, and leaves, but is very low in flowers (Figures 5C and 5D). *bHLH57* transcript levels gradually decreased during seed maturation and declined after seed hydration (Figure 5D).

Considering the reduced seed dormancy phenotype of *bhlh57* mutants and the interaction between ODR1 and bHLH57, we speculated that the ABA content and ABA sensitivity of *bhlh57-2* seeds might also be affected. Indeed, ABA content in *bhlh57-2* freshly harvested seeds was significantly reduced compared to Col-0 seeds (Figure 5E). ABA sensitivity of *bhlh57-2* seeds was however similar to that of Col-0 (Supplemental Figure 10B), suggesting that the reduced dormancy of *bhlh57-2* seeds was related to ABA metabolism. Next, we analyzed the expression of *NCED*s and *CYP707A*s in *bhlh57-2* freshly harvested seeds. *NCED6* and *NCED9* transcripts were both significantly downregulated

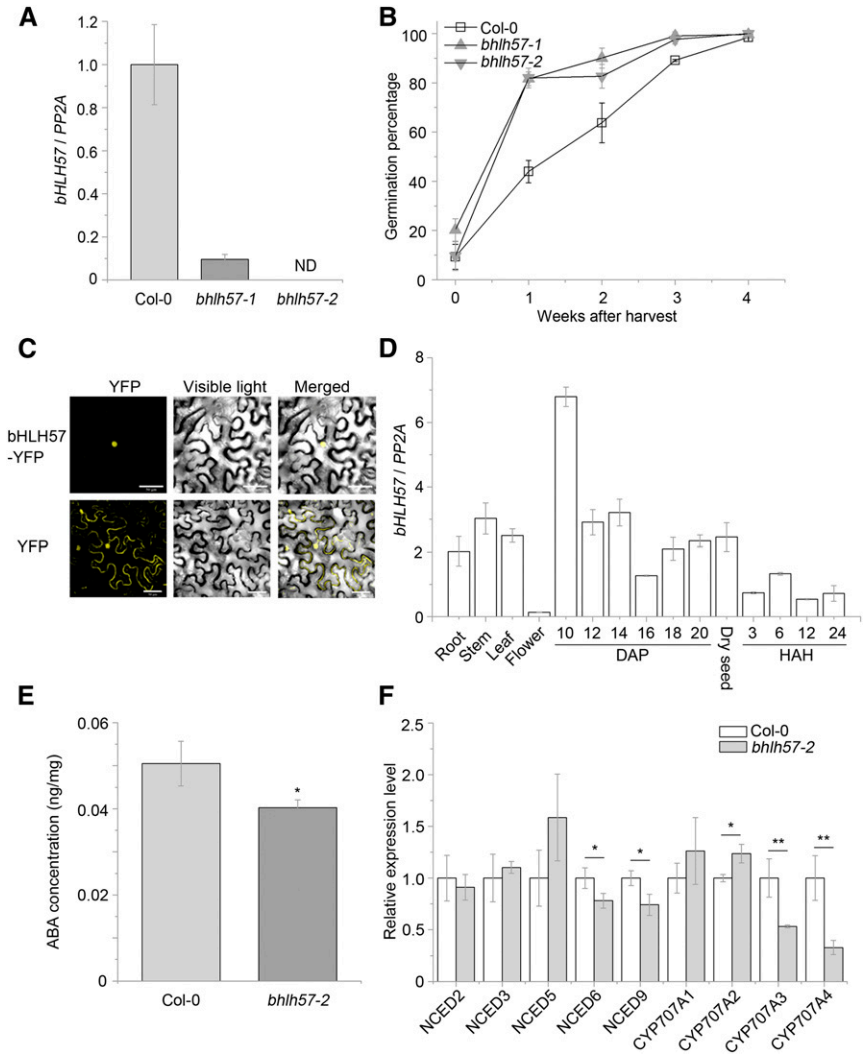

Figure 5. bHLH57 Imposes Seed Dormancy and Induces ABA Biosynthesis.

(A) RT-qPCR analysis of *bHLH57* transcript levels in Col-0, *bhlh57-1*, and *bhlh57-2* freshly harvested seeds. The expression values were normalized to *PP2A*. Values are means ± SD from three biological repeats. ND, not detected.

(B) Germination percentages after different periods of dry storage of Col-0, *bhlh57-1*, and *bhlh57-2* freshly harvested seeds. Values are means ± SD of three independent batches of seeds per genotype.

(C) Subcellular localization of bHLH57-YFP in *N. benthamiana* leaves. Bars = 50 μm.

(D) RT-qPCR analysis of *bHLH57* transcript levels in various organs and developing siliques and mature seeds in Arabidopsis. The expression values were normalized to *PP2A*. Values are means ± SD from three biological repeats. DAP, days after pollination; HAH, hours after hydration.

(E) ABA content in Col-0 and *bhlh57-2* freshly harvested seeds. Values are means ± SD of three independent batches of seeds per genotype. *P < 0.05 by Student's *t* test.

(F) RT-qPCR analysis of *NCED*s and *CYP707A*s transcript levels in Col-0 and *bhlh57-2* freshly harvested dry seeds. The expression values were normalized to *PP2A*. Values are means ± SD from three biological repeats. *P < 0.05 by Student's *t* test.

in *bhlh57-2* compared with Col-0 (Figure 5F), indicating that bHLH57 normally induces the expression of *NCED6* and *NCED9*. In addition, *CYP707A2* expression was elevated in *bhlh57-2*, collectively contributing to the decreased ABA content in mutant seeds. By contrast, both *CYP707A3* and *CYP707A4* were downregulated, which could be due to an ABA metabolic feedback in *bhlh57-2* (Figure 5F). These results, in combination with the interaction between ODR1 and bHLH57 and the altered transcript levels of *NCED6* and *NCED9* in the *odr1-2* mutant, suggest that bHLH57 may directly control *NCED6* and *NCED9* expression.

bHLH TFs generally modulate the expression of their target genes through binding to E-box (CANNTG) motifs in their promoters (Atchley and Fitch 1997). The 1400-bp promoter region upstream of *NCED6* contains five E-box motifs, and an E-box and a G-box motif (CACGTG) were also found to be present in the 600-bp promoter region upstream of *NCED9* (Figure 6A). To determine whether bHLH57 could bind to the promoters of *NCED6* and *NCED9*, we performed yeast one-hybrid and ChIP-qPCR assays. The yeast one-hybrid assays showed that bHLH57 did bind to the *NCED6* and *NCED9* promoters, but it could not bind to the promoters of *NCED2*, *NCED3*, *CYP707A2 to CYP707A4*, or *DOG1* (Figure 6B; Supplemental Figure 11A). Furthermore, we determined that bHLH57 bound to the first, fourth, and fifth E-box motifs in the *NCED6* promoter and to the G-box of the *NCED9* promoter (Supplemental Figure 11B). ChIP-qPCR assays with $35S_{pro}$:bHLH57-GFP transgenic seeds (Supplemental Figure 12) further confirmed the binding between bHLH57 and the promoters of *NCED6* and *NCED9*: the bHLH57 protein preferentially bound to the first, third, fourth, and fifth E-box motifs in the *NCED6* promoter and to both the E-box and G-box motifs in the *NCED9* promoter (Figure 6C). We also performed a transient expression assay in *N. benthamiana* leaves to confirm the positive control of bHLH57 in *NCED6* and *NCED9* expression in plants. bHLH57 activated both *NCED6* and *NCED9* expression relative to negative controls (Figure 6D). Collectively, our data demonstrate that bHLH57 induces *NCED6* and *NCED9* expression through binding to specific E/G-boxes within their promoters, thereby controlling seed dormancy by influencing ABA metabolism.

ODR1 Inhibits the Regulation of *NCED6* and *NCED9* by bHLH57

Our results above demonstrated an interaction between ODR1 and bHLH57. We also documented the opposite phenotypes on seed dormancy, ABA content, and *NCED6* and *NCED9* expression exhibited by the *odr1-2* and *bhlh57-2* mutants. These observations prompted us to investigate whether ODR1 may prevent the induction of *NCED6* and *NCED9* expression by bHLH57. To test this hypothesis, we performed a transient dual-luciferase (LUC) assay by coexpressing $35S_{pro}$:*ODR1* and/or $35S_{pro}$:*bHLH57* with the LUC reporters $NCED6_{pro}$:LUC or $NCED9_{pro}$:LUC in *N. benthamiana* leaves. Coexpression of ODR1 and bHLH57 indeed resulted in a significant decrease in LUC activity compared with expression of bHLH57 alone (Figure 7A), suggesting that the upregulation of *NCED6* and *NCED9* by bHLH57 is blocked by ODR1. Furthermore, we generated the *odr1-2 bhlh57-2* double mutant (Supplemental Figure 13) to evaluate its germination behavior and pattern of *NCED6* and *NCED9* expression. Transcript levels for *NCED6* and *NCED9* were both upregulated in *odr1-2* seeds, but this was largely counteracted when bHLH57 was also inactivated in the *odr1-2 bhlh57-2* double mutant (Figure 7B). Germination percentages indicated that the dormancy level of the *odr1-2 bhlh57-2* double mutant was significantly lower than that of *odr1-2* (Figure 7C), demonstrating that ODR1 and bHLH57 had opposite roles in the control of dormancy and that ODR1 requires bHLH57 for its full function in seed dormancy. Overall, these results suggest that ODR1 inhibits the upregulation of *NCED6* and *NCED9* by bHLH57 in seeds. Interestingly, the *odr1-2 bhlh57-2* double mutant was still more dormant than *bhlh57-2* (Figure 7C) and also showed increased expression of *NCED6* and *NCED9* compared to the wild type (Figure 7B). This indicates that ODR1 also controls *NCED6* and *NCED9* expression through other factors. Collectively, these results demonstrate that ODR1 negatively controls *NCED6* and *NCED9* expression partly through direct interaction and inhibition of bHLH57, which induces *NCED6* and *NCED9* expression.

DISCUSSION

The identification and characterization of seed dormancy genes and understanding their roles in the dormancy mechanism are important for crop genetic improvement. In this study, we isolated two new dormancy factors, ODR1 and bHLH57, and provide genetic, biochemical, and molecular evidence to support the signaling network in which they work alongside ABI3 and NCED6/9. We propose that this network functions in a positive feedback loop with ABA: higher levels of ABA lead to the upregulation of *ABI3* during seed maturation. ABI3 then binds physically to the *ODR1* promoter and represses its expression. In the absence of the ODR1-imposed inhibition, bHLH57 can bind to the promoters of *NCED6* and *NCED9* and elevate their expression levels, resulting in enhanced ABA biosynthesis and stronger seed dormancy. This control may be gradually amplified via a positive feedback loop involving the further induction of ABI3 by ABA (Figure 8).

Cao et al. (2020) recently reported a role for *ODR1*/*AtSdr4L* in seed dormancy. Based on a physiological and genetic analysis, the authors concluded that this role was mainly mediated by the GA biosynthesis and signaling pathways. Indeed, *odr1*/*atsdr4l* loss of function was associated with higher expression of the GA biosynthesis genes *GA20-OXIDASE1* (*GA20OX1*) and *GA20OX2* (Cao et al., 2020). Taken together, we propose that ODR1/AtSdr4L, a homolog of OsSdr4, plays a negative role in seed dormancy by adjusting the ABA and GA balance during seed maturation and germination (Figure 8).

ODR1 Interacts with bHLH57 and Inhibits bHLH57 Upregulation of *NCED6* and *NCED9* Expression

We identified ODR1 as a suppressor of the low dormant *rdo5-2* mutant during a γ-ray mutagenesis screen. Considering the germination behavior and ABA content of *rdo5-2*, *odr1-2* single mutants, and *rdo5-2 odr1-2* double mutant, we hypothesized that ODR1 controls seed dormancy by modulating ABA biosynthesis, which is independent of RDO5-mediated seed dormancy. ODR1

interacts with bHLH57 and represses the induction of *NCED6* and *NCED9* expression and ABA biosynthesis directly (Figures 4 to 7). Therefore, ODR1 and bHLH57 control ABA metabolism and dormancy in opposite directions. The mechanisms underlying these control pathways are consistent with the reported role of ABA in seed dormancy (Lefebvre et al., 2006; Seo et al., 2006). In Arabidopsis, *NCED6* and *NCED9* are the main contributors to ABA biosynthesis during seed maturation; in agreement, the *nced6*

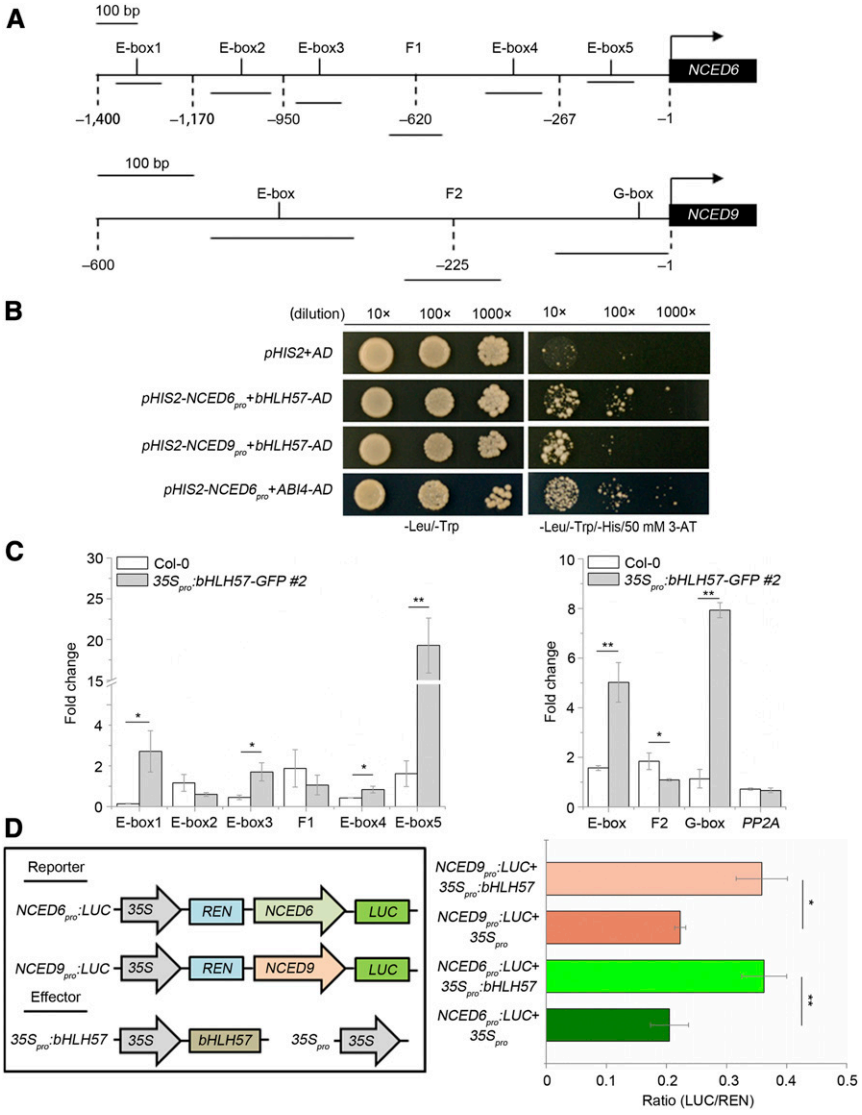

Figure 6. bHLH57 Binds to the *NCED6* and *NCED9* Promoters and Induces Their Expression.

(A) Schematic diagrams of the *NCED6* and *NCED9* promoters. Fragments (from –1 to –266 bp [E-box5$_{pro}$], –267 to –619 bp [E-box4$_{pro}$], –620 to –949 bp [E-box3$_{pro}$], –950 to –1169 bp [E-box2$_{pro}$], and –1170 to –1400 bp [E-box1$_{pro}$]) of *NCED6* promoter and fragments (from –1 to –224 bp [G-box$_{pro}$] and –225 to –600 bp [E-box$_{pro}$]) of the *NCED9* promoter were amplified for a yeast one-hybrid assay. The relative positions of the ChIP-qPCR-amplified fragments in the *NCED6* promoter (named E-box1, E-box2, E-box3, F1, E-box4, E-box5) and in the *NCED9* promoter (named E-box, F2, G-box) are depicted with parallel black lines below the promoter. Bars = 100 bp.

(B) Yeast one-hybrid assay: direct binding of bHLH57 to the *NCED6* and *NCED9* promoters. 3-AT (50 mM) was added in the -Leu/-Trp/-His medium. ABI4 binding to *NCED6* promoter was used as a positive control. Dilution 10×, 100×, and 1000× corresponds to an OD_{600} of 0.1, 0.01, and 0.001, respectively.

(C) ChIP-qPCR assay: bHLH57 binds to the E/G-box motifs of the *NCED6* (left) and *NCED9* (right) promoters. Immunoprecipitation was performed using seeds hydrated for 6 h from Col-0 and *35S$_{pro}$:bHLH57-GFP* with anti-GFP or anti-IgG antibody. qPCR was performed with specific primers listed in Supplemental Data Set 1. The comparison is between the nonimmune control (anti-IgG) and the immunoprecipitation (anti-GFP). Values are means ± SD of three biological replicates. *P < 0.05 or **P < 0.01 by Student's *t* test.

(D) Transient expression assay: bHLH57 induces the expression of *NCED6$_{pro}$:LUC* and *NCED9$_{pro}$:LUC* in *N. benthamiana* leaves. The pGREENII 62-SK empty vector (*35S$_{pro}$*) was used as control. LUC activity was normalized to REN activity. Schematic representation of various constructs used in assay is shown on the left panel. Values are means ± SD of three independent transformants for each construct. *P < 0.05 or **P < 0.01 by Student's *t* test.

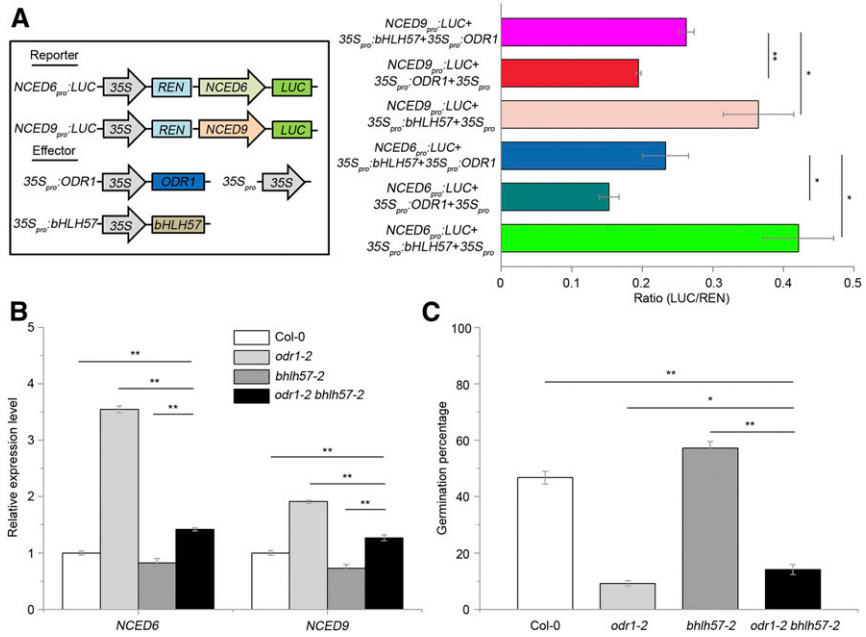

Figure 7. ODR1 Prevents the Induction of *NCED6* and *NCED9* Expression by bHLH57.

(A) Transient expression assay: ODR1 represses the promotion of bHLH57 on luciferase activity driven by the *NCED6* and *NCED9* promoters in *N. benthamiana* leaves. The pGREENII 62-SK empty vector ($35S_{pro}$) was used as the control. LUC activity was normalized to REN activity. A schematic representation of various constructs used in assay is listed on the left panel. Values are means ± SD of three independent transformants for each construct. *P < 0.05 or **P < 0.01 by Student's *t* test.

(B) RT-qPCR analysis of *NCED6* and *NCED9* transcript levels in Col-0, *odr1-2*, *bhlh57-2*, and *odr1-2 bhlh57-2* freshly harvested seeds. The expression values were normalized to *PP2A*. Values are means ± SD of three biological repeats. **P < 0.01 by Student's *t* test.

(C) Germination percentages of Col-0, *odr1-2*, *bhlh57-2*, and *odr1-2 bhlh57-2* after-ripened seeds after 1 week of storage. Values are means ± SD of three independent batches of seeds per genotype. *P < 0.05 or **P < 0.01 by Student's *t* test.

nced9 double mutant has significantly reduced ABA levels and weaker seed dormancy (Lefebvre et al., 2006). By contrast, induction of *NCED6* during seed development increased seed dormancy (Martínez-Andújar et al., 2011).

Studies have reported that the expression of *NCED6* and *NCED9* is directly promoted by TFs, including ABI4, DREB2C, and MYB96 (Je et al., 2014; Lee et al., 2015; Shu et al., 2016b). Therefore, it is interesting to note that our data also support an indirect function for ODR1 on ABA biosynthesis by decreasing the transcription of *ABI4* and *DREB2*, as well as that of the core dormancy gene *DOG1* (Supplemental Figures 6 and 7). This indicates that there are still other pathways in ODR1-mediated dormancy that need further investigation.

It was previously reported that two bHLH TFs, SPATULA (SPT) and PHYTOCHROME INTERACTING FACTOR1 (PIF1), control seed germination and *GA3OX* expression in response to light and temperature signaling in Arabidopsis (Penfield et al., 2005; Oh et al., 2006). bHLH TFs also function in seed dormancy in other plant species (Gao et al., 2018; Zhao et al., 2019). These findings collectively demonstrate the conservation and diversity of the *bHLH* gene family in the control of dormancy. Our study extends a role for the TF bHLH57 in seed dormancy by promoting *NCED6* and *NCED9* expression.

It is noteworthy that our genetic analysis demonstrated that the loss of bHLH57 function in the *odr1-2* background was only partially able to counteract the higher *NCED6* and *NCED9* expression levels and associated hyper-dormancy phenotype brought on by the loss of ODR1 (Figures 7B and 7C). These results indicate that ODR1 probably also controls *NCED6/9* by factors other than bHLH57, like the above-mentioned ABI4 and DREB2C. Furthermore, other dormancy factors such as GA and DOG1 have recently also been reported to be involved in ODR1/AtSdr4L-mediated seed dormancy (Figure 8; Cao et al., 2020).

ODR1 Is Negatively Controlled by ABI3 during Seed Dormancy Establishment

ABI3 is a vital ABA-responsive TF that directly binds to the RY motif in the promoter of its target genes via a B3 domain, and it plays a central role in seed maturation and the establishment of primary seed dormancy (Ezcurra et al., 2000; Mönke et al., 2004). Mutations in ABI3 lead to reduced dormancy and premature seeds germination (Nambara et al., 1995). ChIP-chip and transcriptome analysis in a previous study identified a set of 98 genes (including *ODR1*) involved in seed development and seed protein and lipid accumulation as direct targets of ABI3 (Mönke et al., 2012). In our study, we confirmed the binding activity of ABI3 to the proximal RY motif in the promoter of *ODR1* by yeast one-hybrid and ChIP-qPCR assays (Figures 3B and 3C). Furthermore, we demonstrated that expression of *ODR1* is repressed by ABI3 using gene

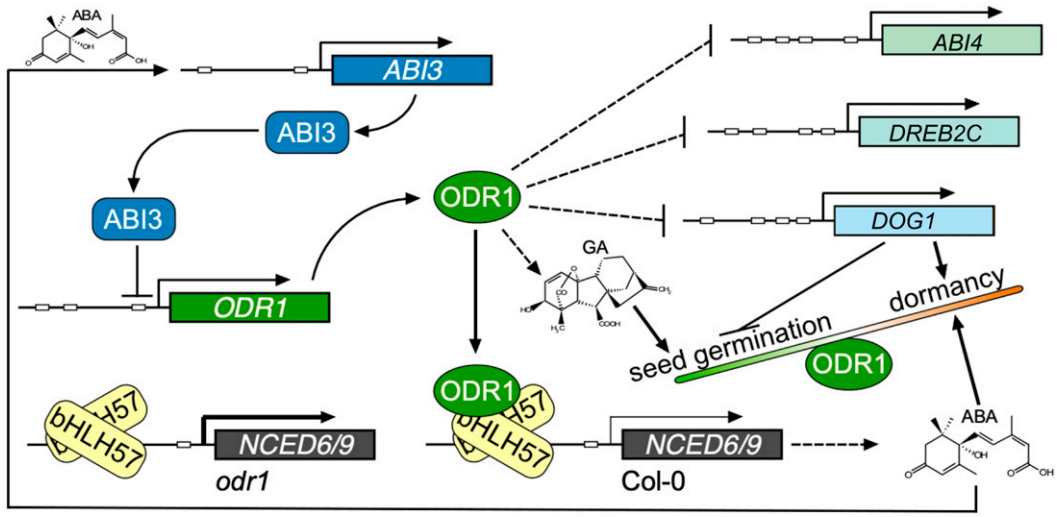

Figure 8. Proposed Working Model for the Control of Seed Dormancy and Germination by ODR1/AtSdr4L.

Over the course of seed maturation, increasing levels of ABA lead to the upregulation of ABI3. ABI3 binds to the promoter of *ODR1* and represses its expression, resulting in the alleviation of ODR1-imposed inhibition of bHLH57. bHLH57 will then bind to the promoter of *NCED6* and *NCED9* and elevate their expression levels, leading to higher ABA biosynthesis and seed dormancy. This regulation may be amplified by the stimulation of ABI3 by ABA. Recently, Cao et al. (2020) demonstrated that ODR1/AtSdr4L also controls seed germination through GA biosynthesis in hydrated seeds. Taken together, we propose that ODR1/AtSdr4L plays a negative role in seed dormancy by adjusting the ABA and GA balance during seed maturation and germination. Our data also support an indirect function for ODR1 on ABA biosynthesis by decreasing the transcription of *ABI4* and *DREB2C* as well as that of the core dormancy gene *DOG1*. This indicates that there are still other pathways in ODR1-mediated dormancy that need further investigation.

expression analysis and transient expression assays (Figures 3D and 3E). Finally, inactivation of *ODR1* in the *abi3-1* background by CRISPR/Cas9 genome editing supported the position of ABI3 upstream of ODR1 in the control of seed dormancy (Figure 3F).

AtODR1 and Its Rice Homolog OsSdr4 Have Opposite Roles in Seed Dormancy

Seed dormancy is a key decision point in plant development. Hence, maintaining suitable levels of dormancy is of great importance and has been under strong natural selection during plant evolution. In our study, we found that ODR1 and its homologs are conserved in angiosperms (Supplemental Figure 2), indicating that they might have a conserved function in seed dormancy. Sdr4 is a putative ODR1 ortholog in rice, with ~35% amino acid identity (and 49% similarity). However, Sdr4 positively affects seed dormancy in several rice cultivars (Sugimoto et al., 2010), which is the opposite of ODR1 in Arabidopsis. We speculate that such divergence in function might be related to their associated ABA-responsive factors: ABI3 and OsVP1. In rice, OsVP1 induces the expression of *Sdr4* during seed development and maturation (Sugimoto et al., 2010), whereas ABI3 represses *ODR1* expression in Arabidopsis seeds. These opposite effects confer a deeper dormancy in *odr1-2* and preharvest sprouting and nondormant phenotypes in *sdr4*. Furthermore, the expression levels of the two closest rice homologues of Arabidopsis *DOG1* were lower in the rice *sdr4* mutant compared to the wild type, while our results showed that *DOG1* was upregulated in *odr1-2* freshly harvested and hydrated Arabidopsis seeds (Supplemental Figure 7). The different expression behavior of *DOG1* and its rice homologues may contribute to the distinct mutant phenotypes. We hypothesize that the relationship between ODR1 and DOG1 might be conserved between dicots and monocots. Given the opposite modes of action displayed by ABI3 and OsVP1 on *ODR1* and *Sdr4* in seed dormancy, it will be of interest to assess the effects of Sdr4 on ABA metabolism and dormancy in rice. Moreover, considering the relatively low identity between ODR1 and Sdr4, we speculate that the opposite dormancy phenotypes of *ODR1* and *sdr4* in dormancy may also be attributed to sequence variation in their coding sequences, which could lead to a divergence in protein function.

METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) genotypes used in this study were either in the Col-0 or L*er* background. T-DNA insertion lines *odr1-2* (SALK_022729), *bhlh57-1* (SALK_027604), *bhlh57-2* (SAIL_84_E04), and *cyp707a2-1* (SALK_072410) were ordered from Arabidopsis Biological Resource Center stock center and are in Col-0. Homozygous lines were confirmed by PCR with gene-specific primers (Supplemental Data Set 1). *abi3-1* is in L*er* and was a kind gift from Yongxiu Liu. $35S_{pro}$:*ABI3-FLAG* is in the Col-0 background and was a kind gift from Giltsu Choi (Park et al., 2011).

Seeds were sown in soil in soil mixture (potting soil:vermiculite [v/v] = 3:1) and grown in the greenhouse or growth chamber with 16-h-light, under 80 to 90 μmol m^{-2} s^{-1} white light intensity/8-h-dark cycle at 24°C/22°C. For germination assays on medium, seeds were sterilized with 10% (v/v) NaClO and 75% (v/v) ethanol and then washed with sterile water and sown on plates containing half-strength MS medium containing 1% (w/v) Suc

and 0.8% (w/v) plant agar. Plates were kept in the dark at 4°C for 3 d before transfer to a growth chamber.

Mapping of *ODR1*

To clone the *ODR1* gene, we followed a next-generation sequencing–based bulked-segregant analysis approach. The *odr-1* was backcrossed with its wild-type *rdo5-2* four times to minimize background effects. Next, a segregating population was produced by outcrossing *ODR1-1* with L*er*. F2 progeny with a homozygous *rdo5-2* background were selected for phenotyping. Bulk DNA was in two groups based on their dormant or nondormant phenotype. After sequencing of bulk DNA, we performed linkage analysis based on whole genome–distributed single-nucleotide polymorphism markers and detailed genome sequence analyses in the candidate region.

Constructs and Plant Transformation

To generate $35S_{pro}$:*ODR1/odr1-2* plants, the full-length coding sequence (CDS) of *ODR1* was amplified and cloned into the pFAST-R01 vector (Shimada et al., 2010) and transformed into *odr1-2* via *Agrobacterium* (*Agrobacterium tumefasciens*) strain GV3101. To generate $35S_{pro}$:*bHLH57-GFP*–overexpressing plants, the *bHLH57* CDS was amplified and cloned into the pFAST-R05 vector (Shimada et al., 2010) and transformed into Col-0 with *Agrobacterium* strain GV3101. Single copy insertions and homozygous lines were identified based on red fluorescence screening (the red fluorescent protein being encoded by the binary vector).

The *ODR1* genome-edited lines in the *abi3-1* background were generated using the p*YAO*-based CRISPR/Cas9 system according to the instructions from Yan et al. (2015). Briefly, two 20-nucleotide single guide RNAs (sgRNA; 5′-CAAGCGAGGCCGAGGCGGGA-3′ and 5′-AAGAGA CCAAAGTCGTCATG-3′) with specific recognition for *ODR1* were synthesized and cloned into the vector pAtU6-26-sgRNA-SK and digested with *Bsa*I. The fragment harboring AtU6-26-sgRNA^{ODR1} was digested with *Spe*I and cloned into the pCAMBIA1300-pYAO-Cas9 vector. Finally, the plasmid pCAMBIA1300-p*YAO*-Cas9-AtU6-26-sgRNA^{ODR1} was transformed into *abi3-1* with *Agrobacterium* strain GV3101. Homozygous lines were identified by genome PCR and sequencing.

Seed Germination Assays

Seed germination assays were performed as previously described by Xiang et al. (2014) and Née et al. (2017). For ABA response assays, freshly harvested seeds were sterilized and plated on half-strength MS medium containing 1% (w/v) Suc and 0.8% (w/v) plant agar supplemented with 0 or 0.5 μM ABA. After 3 d of stratification at 4°C in the dark, plates were moved into a growth chamber at 25°C/20°C (day/night) under a 12-h-light/12-h-dark photoperiod. Germination percentages were calculated every day. For fluridone treatments, freshly harvested seeds were plated onto a filter paper soaked with 0, 5, or 10 μM fluridone in Petri dishes and incubated in a growth chamber at 25°C/20°C (day/night) under a 12-h-light/12-h-dark photoperiod. Germination percentages were calculated after 7 d. Stock solutions of ABA and fluridone were dissolved in ethanol.

Subcellular Localization and BiFC Assay

To generate constructs for subcellular localization analysis, the CDSs of *ODR1* and *bHLH57* were amplified and cloned in frame with enhanced YFP driven by the 35S promoter in pEarleyGate101(Earley et al., 2006). Vectors were transiently transfected into *Nicotiana benthamiana* leaves as previously described by Liu et al. (2010). Yellow fluorescence signal was detected using a confocal laser scanning microscope (Zeiss) at the excitation wavelength of 513 nm. For BiFC assays, the CDSs of *ODR1* and *bHLH57* and truncated *bHLH57* (*bHLH57*ΔN) were amplified and inserted into pBatTL-B sYFPn (YFPn) and pBatTL-B sYFPc (YFPc) vectors, respectively. The BiFC assay was performed as described by Née et al. (2017a).

RT-qPCR Assay

Total RNA was extracted from various organs using RNAqueous columns and RNA Isolation Aid (Invitrogen) as previously described by Kushiro et al. (2004). First-strand cDNA was synthesized using All-In-One RT MasterMix (Applied Biological Materials) according to the product instructions. qPCR was then performed with PowerUp SYBR Green Master Mix (Life Technologies) with gene-specific primers (Supplemental Data Set 1). *PP2A* (At1g69960) was used as the internal control. Three independent biological replicates with three technological repeats each were performed.

Yeast Two-Hybrid and One-Hybrid Assays

The yeast two-hybrid library screen was performed with the Arabidopsis Mate and Plate Library (Clontech) according to the user's manual. For confirmation of interaction between ODR1 and bHLH57, *bHLH57* CDS was amplified and inserted into the pGADT7 vector and cotransformed into yeast strain AH109 harboring pGBKT7-ODR1 and confirmed with the method described previously by Liu et al., 2016). Interaction between TTG1 and MYB5 was used as positive control (Gonzalez et al., 2009). Yeast one-hybrid assays were performed as described previously by Li et al. (2011). The respective promoter fragments (http://www.cbs.dtu.dk/services/Promoter/) for *ODR1* (1100 bp upstream of ATG), *NCED*s (*NCED2* [1800 bp upstream of ATG], *NCED3* [1000 bp upstream of ATG], *NCED5* [1100 bp upstream of ATG], *NCED6* [1400 bp upstream of ATG], *NCED9* [600 bp upstream of ATG]), and *CYP707A*s (*CYP707A1* [1800 bp upstream of ATG], *CYP707A2* [1800 bp upstream of ATG], *CYP707A3* [2000 bp upstream of ATG], and *CYP707A4* [1800 bp upstream of ATG]) were amplified and inserted into the pHIS2 vector digested with *Eco*RI and *Sac*I individually. The CDSs of *ABI3*, *ODR1*, *bHLH57*, and *ABI4* were amplified and individually fused in frame with the GAL4 activation domain in the pGADT7-Rec2 vector digested with *Sma*I. The various pairs of recombined pHIS2 and pGADT7-Rec2 plasmids were cotransformed into the yeast strain AH109 individually and grown on synthetic defined (SD)/-Leu/-Trp/-His/-Ade medium with 50 mM 3-AT. Binding of ABI4 to the promoters of *NCED6* and *CYP707A1* was used as positive controls (Shu et al., 2016b).

GST Pull-Down Assay

To generate constructs for GST pull-down assays, the CDS of *ODR1* and *bHLH57* were amplified and fused in frame with a His tag or GST in the vectors pET-28a and modified pGEX, to generate vectors expressing ODR1-6×His and GST-bHLH57 proteins in *Escherichia coli* (BL21). GST pull-down assays were performed with the Pierce GST Protein Interaction Pull-Down Kit (Thermo Fisher Scientific) according to the user's manual. Briefly, isolated bait lysate GST or GST-bHLH57 was incubated with glutathione agarose resin in Tris-buffered saline (TBS) solution (25 mM Tris-HCl and 150 mM NaCl, pH 7.2) for 1 h at 4°C with gentle rocking. After immobilization of the bait protein, the prey lysate was incubated with the glutathione agarose resin-bait protein complex in TBS solution for 1 h at 4°C. The resin was washed several times with wash solution (TBS solution: lysis buffer [v/v] =1:1), and proteins bound to the resin were eluted with 10 mM glutathione elution buffer, pH 8.0, and analyzed by immunoblotting using anti-GST (at a 1:1000 dilution; catalog no. BE2013, EASYBIO) and anti-6×His (at a 1:10,000 dilution; catalog no. ab184607, Abcam).

Transient Expression Assay

To generate plasmids for the transient expression assays, the respective promoters of *ODR1*, *NCED6*, and *NCED9* (same promoter fragments used

in yeast one-hybrid assays) were amplified and inserted into pGreenII-0800-LUC vector to produce *ODR1*$_{pro}$:*LUC*, *NCED6*$_{pro}$:*LUC*, and *NCED9*$_{pro}$:*LUC*. The coding sequences of *ABI3*, *bHLH57*, and *ODR1* were amplified and inserted into pGreenII 62-SK vector to generate *35S*$_{pro}$:*ABI3*, *35S*$_{pro}$:*bHLH57*, and *35S*$_{pro}$:*ODR1*, respectively The various pairs of reporter and effector plasmids were cotransfected into 4-week-old *N. benthamiana* leaves with *Agrobacterium* strain GV3101. Transfected plants were maintained under continuous white light for 4 d. Firefly LUC and *Renilla* luciferase (REN) activity were assayed using the Dual-Luciferase assay reagents (Promega) and a GLOMAX 20/20 luminometer (Ye et al., 2017). Three biological replicates were measured for each sample.

ChIP-qPCR Assay

ChIP assays were performed following the procedure described previously (Wang et al., 2016). Briefly, ~1 g of hydrated seeds was cross-linked with 1% (w/v) formaldehyde solution for 15 min under vacuum, and the fixation reaction was then terminated by adding 2 M Gly to a final concentration of 0.125 M. After grinding all the samples into a powder, chromatin was isolated and sheared by sonication to 300 to 1000 bp. The genomic DNA fragments were immunoprecipitated by the addition of anti-FLAG (catalog no. F1804, Sigma-Aldrich) or anti-GFP (catalog no. ab290, Abcam) antibodies. The precipitated DNA was recovered with the EpiQuik Plant ChIP Kit (Epigentek, P-2014-24) and analyzed by qPCR with specific primers (Supplemental Data Set 1). Three biological replicates were measured for each sample.

Quantification of ABA

The measurement of ABA was performed as described previously, with small modifications (Müller and Munné-Bosch 2011), Briefly, 20 mg of seeds was homogenized in a precooled methanol:isopropanol (20:80 [v/v]) solution containing 0.2% (v/v) formic acid using a TissueLyser (JX-24) with zirconia beads for 3 min at 30 Hz. ABA was extracted at –20°C overnight. The supernatant was collected after 14,000*g* centrifugation at 4°C for 15 min and dried with a flow of nitrogen. The residue was dissolved with 100 μL of cold methanol solution containing internal standard d6-ABA (CDN Isotopes). Quantification of ABA was performed with an ultra-performance liquid chromatography–tandem mass spectrometry system consisting of an ultra-performance liquid chromatography system (Waters) and a triple quadruple tandem mass spectrometer (AB Sciex). Three independent biological replicates were performed for each sample.

Accession Numbers

Sequence data from this investigation can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *ODR1* (At1g27461), *ABI3* (At3g24650), *bHLH57* (At4g01460), *NCED2* (At4g18350) *NCED3* (At3g14440), *NCED5* (At1g30100), *NCED6* (At3g24220), *NCED9* (At1g78390), *CYP707A1* (At4g19230), *CYP707A2* (At2g29090), *CYP707A3* (At5g45340), *CYP707A4* (At3g19270), *ABI4* (At2g40220), *DREB2C* (At2g40340), *TTG1* (At5g24520), *MYB5* (At3g13540), *DOG1* (At5g45830), *RDO5* (At4g11040), and *PP2A* (At1g13320).

Supplemental Data

Supplemental Figure 1. Mapping of *ODR1* and Identification of *ODR1* mutants.

Supplemental Figure 2. Sequence alignments and phylogenetic analysis of ODR1 and ODR1-related proteins.

Supplemental Figure 3. Expression profile of *ODR1*.

Supplemental Figure 4. Expression profiles of *NCED*s and *CYP707A*s in seed.

Supplemental Figure 5. Yeast one-hybrid assay between ODR1 and predicted promoters of *NCED*s and *CYP707A*s.

Supplemental Figure 6. The relation of ODR1 with DREB2C and ABI4.

Supplemental Figure 7. The relation of ODR1 with DOG1.

Supplemental Figure 8. Expression levels of *ABI3* in freshly harvested dry and imbibed seeds of *35S*$_{pro}$:*ABI3-FLAG* overexpression lines.

Supplemental Figure 9. Construction of *ODR1* knock-out lines in the *abi3-1* background through CRISPR-Cas9 genome editing.

Supplemental Figure 10. Identification of *bhlh57* and sensitivity of *bhlh57-2* to exogenous ABA treatment.

Supplemental Figure 11. Yeast one-hybrid between bHLH57 and predicted promoters of *NCED*s, *CYP707A*s and *DOG1*.

Supplemental Figure 12. Expression levels of *bHLH57* in *35S*$_{pro}$:*bHLH57-GFP* overexpression lines.

Supplemental Figure 13. Identification of the *odr1-2 bhlh57-2* double mutant with genomic PCR.

Supplemental Data Set 1. Primers used in this study.

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AUTHOR CONTRIBUTIONS

Y.X. and F.L. designed the experiments; F.L., Y.X., L.D., and H.Z. performed the experiments; F.L. analyzed the data; F.L., Y.X., and W.J.J.S. wrote the article.

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