The Evening Complex and the Chromatin-Remodeling Factor PICKLE Coordinately Control Seed Dormancy by Directly Repressing DOG1 in Arabidopsis

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ABSTRACT

Primary seed dormancy is acquired during seed development and maturation, which is important for plant fitness and survival. DELAY OF GERMINATION1 (DOG1) plays a critical role in inducing seed dormancy. DOG1 expression increases rapidly during seed development, but the precise mechanism underlying this process remains elusive. In this study, we showed that mutants with a loss or reduced function of the chromatin-remodeling factor PICKLE (PKL) exhibit increased seed dormancy. PKL associates with DOG1 chromatin and inhibits its transcription. We found that PKL physically interacts with LUX ARRHYTHMO (LUX), a member of the evening complex (EC) of the circadian clock. Furthermore, LUX directly binds to a specific coding sequence of DOG1, and DOG1 acts genetically downstream of PKL and LUX. Mutations in either LUX or EARLY FLOWERING3 (ELF3) encoding another member of the EC led to increased DOG1 expression and enhanced seed dormancy. Surprisingly, these phenotypes were abolished when the parent plants were grown under continuous light. In addition, we observed that loss of function of either PKL or LUX decreased H3K27me3 levels at the DOG1 locus. Taken together, our study reveals a regulatory mechanism in which EC proteins coordinate with PKL to transmit circadian signals for directly regulating DOG1 expression and seed dormancy during seed development.

Key words: seed dormancy, chromatin remodeling, clock, DOG1

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INTRODUCTION

Seed dormancy is an important agricultural trait that helps plants survive under unfavorable conditions and prevents preharvest seed sprouting. Primary seed dormancy is induced during maturation and is maintained for a certain period of time in mature seeds. After-ripening or environmental cues trigger the release of dormancy, which leads to germination and the beginning of a new plant life cycle (Bentsink and Koornneef, 2008; Finkelstein et al., 2008; Graeber et al., 2012; Nee et al., 2017; Honogaki, 2019). Seed dormancy is controlled by both endogenous, such as phytohormone signalings, and exogenous factors, such as light (Jiang et al., 2016; Shu et al., 2016; Ravindran and Kumar, 2019).

Early studies have identified many quantitative trait loci (QTL) that contribute to dormancy in Arabidopsis thaliana, rice (Oryza sativa), and wheat (Triticum aestivum) (Alonso-Blanco et al., 2003; Osa et al., 2003; Gu et al., 2006; Bentsink et al., 2010). Further genetic and molecular studies have uncovered many genes involved in regulating the induction and release of seed dormancy and germination (Graeber et al., 2012; Nonogaki, 2014; Shu et al., 2016). DELAY OF GERMINATION1 (DOG1) is a...
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Major QTL that was discovered in a recombinant inbred Arabidopsis population, and variation in DOG1 transcript levels between accessions contributes to natural variation for seed dormancy (Alonso-Blanco et al., 2003; Bentsink et al., 2006). DOG1 encodes a protein with unknown function. It forms a homodimer and the protein level is important for its role in seed dormancy (Nakabayashi et al., 2012, 2015). DOG1 regulates seed germination in part through influencing miR156 and miR172 levels in lettuce (Lactuca sativa) (Huo et al., 2016). DOG1 binds to heme and interacts with the type 2C protein phosphatase ABA HYPERSENSITIVE GERMINATION1, inhibiting its regulation of seed dormancy (Nishimura et al., 2018). DOG1 is subject to alternative polyadenylation, which leads to the production of multiple transcript variants encoding three protein isoforms (Nakabayashi et al., 2015; Cyrek et al., 2016). AtNTR1, a component of the spliceosome, is required for the splicing and expression of DOG1 (Dolata et al., 2015). The noncoding antisense transcript asDOG1 suppresses the expression of the DOG1 sense transcript in cis during seed maturation (Fedak et al., 2016). In turn, asDOG1 transcript levels are controlled by C-TERMINAL PHOSPHATASE-LIKE1-mediated alternative polyadenylation of the sense transcript (Kowalczyk et al., 2017). Two recent studies showed that ETHYLENE RESPONSE FACTOR12 and basic LEUCINE ZIPPER TRANSCRIPTION FACTOR67 either negatively or positively regulate DOG1 expression during seed maturation (Bryant et al., 2019; Li et al., 2019).

The circadian clock plays key roles in integrating multiple environmental signals (such as light and temperature) into endogenous transcriptional reprogramming, which enables plants to anticipate changes and to gate their responses according to the time of day. In Arabidopsis, the circadian oscillator is composed of multiple interlocking loops that function in transcriptional and post-translational regulation (Harmer, 2009; Pruneda-Paz and Kay, 2010; Greenham and McClung, 2015). Three clock proteins, LUX ARRHYTHMO (LUX), EARLY FLOWERING3 (ELF3), and ELF4, comprise the evening complex (EC), a critical component in the regulation of circadian outputs (Nusinow et al., 2011; Mizuno et al., 2014). LUX is an MYB-domain-containing transcription factor that directly binds to DNA through the cognate LUX binding site ([LBS]; GATT/ACG) in its target genes. ELF3 acts as an adaptor linking ELF4 and LUX, which form a ternary transcriptional repression complex (Helfer et al., 2011; Nusinow et al., 2011). However, the questions whether and how the circadian clock directly regulates DOG1 expression and seed dormancy remain unknown.

PICKLE (PKL) is an ATP-dependent chromatin-remodeling factor that affects the levels of trimethylation of histone H3 Lys 27 (H3K27me3) at loci involved in cell elongation (Jing et al., 2013; Zhang et al., 2014). PKL plays essential roles in regulating various developmental processes and environmental responses, including embryonic development, root meristem activity, photomorphogenesis, and thermomorphogenesis (Ogas et al., 1999; Fukaki et al., 2006; Perruc et al., 2007; Aichinger et al., 2011; Jing et al., 2013; Zha et al., 2017). Here, we show that PKL and the EC negatively regulate seed dormancy. Mutations in PKL, LUX, or ELF3 lead to reduced germination of freshly harvested seeds. PKL physically interacts with LUX, which directly binds to the regulatory regions and mediates the circadian regulation of DOG1 expression. Moreover, PKL is recruited to DOG1 chromatin and affects H3K27me3 levels to inhibit its expression. Therefore, EC proteins act together with the chromatin-remodeling factor PKL to prevent seeds from becoming overly dormant by directly controlling DOG1 transcription during seed development.

RESULTS

PKL Inhibits Primary Seed Dormancy

PKL is involved in regulating seed germination (Perruc et al., 2007). The pkl-1 seeds in mature siliques showed a lower level of germination than the wild-type (Columbia-0 [Col-0]) control (Figure 1A), suggesting that PKL likely also plays a role in seed dormancy. To confirm this notion, we grew Col-0 and pkl plants under identical growth conditions and collected seeds at the same developmental stage. Freshly harvested seeds of various pkl mutant alleles, including pkl-1, pkl-10, pkl-11, and pkl-12 (Jing et al., 2013, 2019), exhibited lower germination rates than Col-0 seeds (Figure 1B and 1C). The reduced germination of pkl seeds was not due to developmental defects, as they all fully germinated after cold stratification (Figure 1D). The germination rates of pkl seeds gradually increased with increasing storage period (Figure 1E). The expression of the PKLp:GUS (β-GLUCURONIDASE) reporter gene in transgenic Arabidopsis seeds was clearly observed in embryos and endosperm after 12 h of imbibition (Figure 1F). The phytochrome B (phyB) photoreceptor mediates red/far-red reversible seed germination (Jiang et al., 2016). However, mutations in PKL did not affect light-induced regulation of seed germination (Supplemental Figure 1). These observations suggest that PKL regulates primary seed dormancy.

PKL Associates with the DOG1 Locus and Represses Its Transcription

DOG1, REVEILLE1 (RVE1), and RVE2 are essential for controlling seed dormancy (Jiang et al., 2016). To explore how PKL regulates seed dormancy, we examined DOG1, RVE1, and RVE2 transcript levels in freshly harvested seeds after imbibition. DOG1 transcript levels were approximately 13- and 6-fold higher after 12 and 24 h of imbibition, respectively, in pkl-1 versus Col-0 (Figure 2A), suggesting that downregulation of DOG1 during imbibition is less inhibited in pkl and that PKL inhibits DOG1 expression. However, RVE1 and RVE2 transcript levels did not drastically differ between the pkl mutant and the wild type (Supplemental Figure 2A).

The status of seed dormancy is mainly balanced by the antagonistic effects of two phytohormones: gibberellin (GA) and abscisic acid (ABA) (Finkelstein et al., 2008). Indeed, the expression levels of several genes encoding catalytic enzymes of the GA and ABA biosynthesis pathways were altered in pkl-1 compared with Col-0 (Supplemental Figure 3A and S3B). In addition, the transcript levels of several germination-related genes, including LATE EMBRYOGENESIS ABUNDANT1 (LATE), DORMANCY-ASSOCIATED PROTEIN1 (DAP1), ABA HYPERSENSITIVE GERMINATION1 (HSG1), and 1-CYSTEINE PEROXIREDOXIN1 (PRX1) (Finkelstein et al., 2008; Rae et al., 2013), were higher in pkl-1 than in Col-0 (Supplemental Figure 3C), which is consistent with the reduced germination rate of pkl.
To explore the genetic relationship between PKL and DOG1, we generated a pkl dog1 double mutant by crossing pkl-1 with dog1-2. The reduced germination rate of pkl-1 was largely suppressed in the pkl dog1 double mutant (Figure 2B), suggesting that DOG1 acts downstream of PKL. However, the germination rates of pkl rve1 and pkl rve2 were similar to those of the pkl-1 single mutant (Supplemental Figure 2B), indicating that RVE1 and RVE2 do not genetically interact with PKL. Mutations in phyB also lead to increased seed dormancy (Jiang et al., 2016). Seeds of the pkl phyB double mutant had a much lower germination rate than those of the parental single mutant lines (Supplemental Figure 2C), suggesting that PKL and phyB function in parallel to repress seed dormancy.

PKL associates with the chromatin of its target loci (Jing et al., 2013; Zhang et al., 2014). We therefore performed a chromatin immunoprecipitation (ChIP) assay to investigate whether PKL binds to different chromatin regions of DOG1 (Figure 2B). As shown in Figure 2D, the anti-PKL antibody pulled down significantly more chromatin from regions 3, 4, 5, and 6 of DOG1 (spanning the exons and introns of this gene), but not its promoter regions (1 and 2), in Col-0 plants versus the pkl mutant, indicating that PKL associates with the gene body of DOG1.

**PKL Physically Interacts with LUX**

The chromatin-remodeling factor PKL interacts with multiple transcription factors via its central Helicase/ATPase domain (Jing et al., 2013; Zhang et al., 2014). We thus used this ATPase domain as the bait to screen for interacting factors in a yeast two-hybrid assay. The clock component LUX was identified in this screen and subjected to further investigation. To confirm the PKL-LUX interaction, we fused LUX with the B42 activation domain (AD) and full-length PKL or various PKL fragments with the LexA DNA-binding domain (BD) (Figure 3A). As shown in Figure 3B, BD-D6 containing the Helicase/ATPase domain of PKL and BD-D5 containing additional chromo-domains interacted with AD-LUX, as revealed by the presence of blue yeast colonies. BD-D3 and BD-D4 containing the PHD finger and/or chromo-domains did not interact with AD-LUX. Surprisingly, full-length PKL and PKL fragments D1, D8, and D9 failed to interact with AD-LUX. These results suggest that PKL interacts with LUX via its Helicase/ATPase domain and that the N-terminal PHD finger and the C-terminal portion of PKL likely prevent its contact with LUX in yeast cells. However, PKL and its fragments failed to interact with the two remaining EC components, ELF3 and ELF4 (Figure 3B). PKL also weakly interacted with CCA1, but not with LHY or TIMING OF CAB EXPRESSION1 (TOC1), in yeast cells (Supplemental Figure 4).
Next, we performed an in vitro pull-down assay using purified His-D6 (containing the Helicase/ATPase domain of PKL) and GST-LUX or MBP-LUX recombinant proteins. The anti-GST antibody pulled down His-D6 when incubated with GST-LUX, but not with GST alone (Figure 3C). Similarly, the anti-MBP antibody pulled down His-D6 when incubated with MBP-LUX, but not with MBP alone (Figure 3D). We also generated constructs in which PKL was fused with the N terminus of luciferase (nLUC) and LUX was fused with the C terminus of luciferase (cLUC) and carried out a luciferase complementation imaging (LCI) assay in Nicotiana benthamiana leaves. Cotransformation of PKL-nLUC with LUX-cLUC led to strong LUC expression compared with the controls (Figure 3E). Taken together, these results confirm that PKL physically interacts with the EC by directly binding to LUX.

LUX Directly Binds the DOG1 Chromatin

Multiple putative LBS cis-elements were present in the genomic region of DOG1 (Figure 2C). We reasoned that the LUX transcription factor might directly bind to DOG1. To investigate this possibility, we performed a ChIP assay using anti-GFP antibody to probe 35S:GFP-LUX transgenic lines versus the Col-0 control. Regions 4 and 5 of DOG1 and two positive controls, PSEUDO-RESPONSE REGULATOR7 (PRR7) and PRR9, but not the other regions of DOG1 or the negative control ACT2, were significantly enriched with the anti-GFP antibody in 35S:GFP-LUX plants compared with the wild type (Figure 4A and Supplemental Figure 5). We performed a yeast one-hybrid assay using various DOG1 fragments (P1–P4, Figure 2C) linked to the LacZ reporter gene. The expression of P2:LacZ (position 1–432 bp) and P3:LacZ (459–1182 bp), but not P1:LacZ (1584 to 15 bp) and P4:LacZ (1148–2234 bp), was activated by AD-LUX (Figure 4B). We then synthesized oligonucleotides containing the putative G-box or LBS motifs within regions P2 (P2-1 to P2-4) and P3 (P3-1 to P3-4) and constructed LacZ reporter vectors. As shown in Figure 4B and Supplemental Figure 6, AD-LUX specifically bound to P3-4 (containing GATACT and GATTCT) and activated LacZ expression. Finally, we purified MBP-LUX recombinant fusion proteins from E. coli and performed an electrophoretic mobility shift assay (EMSA). MBP-LUX fusion protein, but not MBP alone, bound to P3-4 oligonucleotides labeled with biotin and caused a mobility shift. The addition of unlabeled nucleotides drastically reduced the mobility shift signal (Figure 4C). These results demonstrate that LUX directly binds to DOG1 at specific sequences.

LUX and ELF3 Inhibit DOG1 Expression and Seed Dormancy

Next, we investigated DOG1 expression and seed dormancy in mutant alleles of lux, elf3, and elf4. DOG1 transcript levels were higher in lux-6 and elf3-1, but not elf4-101, compared with
Col-0 (Figure 4D). Strikingly, transient overexpression of LUX (35S:LUX) inhibited the expression of the DOG1p:LUC reporter gene (driven by the DOG1 promoter and coding sequences) in Arabidopsis protoplasts (Figure 4E). The germination rates of newly harvested lux-6, elf3-1, and elf3-7 seeds were lower than those of the wild type in both light and darkness (Figure 4F and 4G), suggesting that ELF3 and LUX are negative regulators of seed dormancy. However, elf4-101 did not exhibit a distinct seed dormancy phenotype (Figure 4H). After cold stratification, the lux, elf3, and elf4 seeds fully germinated, as did wild-type seeds (Figure 4I). Together, these results suggest that LUX and ELF3 repress DOG1 expression and seed dormancy.

We also generated elf3 dog1 and lux dog1 double mutants and tested their genetic interactions. As shown in Figure 4J and 4K, the germination frequencies of elf3 dog1 and lux dog1 were similar to those of the dog1 single mutant, indicating that DOG1 is epistatic to ELF3 and LUX.

LUX and PKL Regulate H3K27me3 Levels at the DOG1 Locus

To investigate the molecular relevance of the PKL-LUX interaction, we performed a ChiP assay in the pkl-1 and lux-6 mutant backgrounds. The enrichment of PKL at genomic regions 4 and 5 of DOG1 was significantly reduced in lux-6, as well as pkl-1 (Figure 5A), indicating that the association of PKL with DOG1 chromatin depends on LUX. The chromatin-remodeling factor PKL affects H3K27me3 levels of its target genes (Jing et al., 2013; Zhang et al., 2012, 2014). We therefore performed ChiP assays using an anti-H3K27me3 antibody to examine the enrichment of the H3K27me3 histone marker at the DOG1 locus. In the wild type, H3K27me3 was relatively enriched in regions 1, 3, 4, and 5 of DOG1, whereas this enrichment was greatly reduced in pkl and lux (Figure 5B). The germination rates of pkl lux were lower than those of the single mutants (Supplemental Figure 7). These results suggest that PKL and LUX promote the association of H3K27me3 with DOG1 chromatin, which is in agreement with the roles of these proteins in transcriptionally repressing DOG1.

LUX and ELF3 Affect the Circadian Output to Seeds

According to the Arabidopsis eFP browser database, LUX, ELF3, and ELF4 transcript levels gradually increase during seed development and are sharply elevated in dry seeds (Winter et al., 2007). However, PKL expression was maintained at a stable level during seed development (Supplemental Figure 8). Intriguingly, DOG1
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Transcript levels strongly increased during seed development and peaked at the curled cotyledon stage but decreased thereafter and remained low in dry seeds (Supplemental Figure 8). These data suggest that transcription of EC components and DOG1 is temporally controlled during seed development and maturation.

Because the EC transmits circadian signals to regulate plant growth and responses (Nusinow et al., 2011; Hsu and Harmer, 2014), we grew seedlings under 12 h light/12 h dark cycles for 6 d and then transferred to continuous light (CL). DOG1 expression displayed a circadian pattern and peaked at zeitgeber time 28 (ZT28) and then dropped afterward in Col-0 wild type; however, this expression pattern was disrupted in lux and lux pkl mutants (Figure 6A).

Next, we examined the expression pattern of DOG1 in the siliques of Col-0 and lux elf3 grown under long-day (LD) conditions (16 h light/8 h dark). The developing siliques (5 d after pollination) were harvested every 4 h starting from the onset of light (ZT0). We found that the DOG1 expression levels had peaks at ZT8 and ZT20 in Col-0 wild-type siliques, and were drastically increased in lux elf3 and peaked at ZT4 and ZT16 (end of the day) (Figure 6B).
values denote average SD of three biological replicates.

suggesting that DOG1 transcription exhibits photoperiodic regulation. Moreover, we investigated the effects of LUX and ELF3 on seed dormancy in response to circadian changes. We grew plants under LD conditions until flowering and maintained them under LD or transferred them to CL, and analyzed the mature dry seeds. The germination rates were much lower for lux-6 and elf3-7 seeds than for Col-0 seeds under LD conditions, whereas they were similar under CL conditions (Figure 6C).

Finally, we isolated mRNA from the developing siliques of plants grown under both conditions. As expected, DOG1 transcript levels were much higher in lux-6 and elf3-7 compared with Col-0 under LD, but only slightly higher in the mutants under CL (Figure 6D). These results suggest that LUX and ELF3 play important roles in gating circadian signals into seeds during development.

**DISCUSSION**

Many components that regulate the establishment of primary seed dormancy have been identified (Nonogaki, 2014; Shu et al., 2016). DOG1 is a pivotal regulator of the dormancy state of seeds, and DOG1 activity is negatively correlated with the seed germination rate (Nee et al., 2017). Therefore, DOG1 expression must be precisely controlled during seed development and maturation. Here, by performing ChIP analysis, yeast one-hybrid assays, and EMSA, we demonstrated that the LUX transcription factor, a component of the EC, physically binds to a specific region of DOG1 (Figure 4A–4C). Surprisingly, the core binding site of LUX is two atypical LBS motifs located in the second exon, but not in the promoter region, of DOG1. Consistently, the DOG1 locus shows sequence variation in coding region among accessions (Bentsink et al., 2006). This binding site is close to the transcriptional start site of the noncoding antisense asDOG1 sequence, suggesting that it is likely that LUX also directly regulates the expression of asDOG1 (Fedak et al., 2016). In agreement with this notion, asDOG1 transcription was downregulated in the pkl-1 mutant (Supplemental Figure 9). A genome-wide binding study using ChIP coupled to sequencing also showed that DOG1 is a target of EC components (Ezer et al., 2017). Hence, LUX and the EC are the direct upstream regulators of DOG1, which controls seed dormancy.

Several clock proteins, including CIRCADIAN CLOCK ASSOCIATED1, GIGANTEA, and TOC1, help integrate environmental signaling to mediate dormancy release, likely via the indirect regulation of ABA and GA biosynthesis (Penfield and Hall, 2009). Two recent studies showed that functional alleles of PSEUDO-RESPONSE REGULATOR7 and TOC1 are required for the induction of seed germination in response to daily temperature cycles (Aranas et al., 2017). Furthermore, temperature-induced dormancy occurred more rapidly when the morning loop was compromised and delayed versus when the evening loop was compromised (Footitt et al., 2017). However, the precise role of the circadian clock in regulating seed dormancy has been unknown.

Here, we showed that LUX and ELF3 repress DOG1 expression and seed dormancy and that DOG1 acts downstream of LUX and ELF3 (Figure 4), suggesting that the EC controls seed dormancy mainly through directly regulating DOG1 expression. ELF4 itself might play a minor role in this process. Consistently, LUX acts as a transcriptional repressor to inhibit the expression of downstream genes (Helfer et al., 2011). However, lux-2 and lux-5 did not exhibit altered seed germination in a previous study (Penfield and Hall, 2009). This discrepancy might be due to the different mutant alleles and/or growth environments used in the two studies. Indeed, mutation of any member of the EC leads to arrhythmic cycles in plants (Helfer et al., 2011; Nagel and Kay, 2012).

Strikingly, we found that lux and elf3 mutant seeds displayed high levels of DOG1 expression and strong dormancy when the parent plants were grown under LD conditions, whereas this phenotype was largely diminished when the plants were grown under CL (Figure 6B and 6C), indicating that the exogenous photoperiod and/or endogenous circadian rhythms affect the establishment of primary seed dormancy and that this process is dependent (at least in part) on LUX and ELF3. Consistent with this notion, LUX, ELF3, and ELF4 transcript levels were negatively correlated with that of DOG1 during seed development (Supplemental Figure 8). We previously demonstrated that the
photoreceptor phytochrome B (phyB) plays a key role in regulating DOG1 expression and seed dormancy (Jiang et al., 2016). A genome-wide ChIP sequencing study suggested that the EC plays a central role in coordinating endogenous and environmental signals (Ezer et al., 2017). It is likely that, at least, phyB and the clock oscillator integrate both environmental (light and temperature) and circadian signaling to seeds during the growth of the parent plant and seed development. Therefore, EC components play essential roles in transmitting the seasonal and photoperiodic signals that suppress seed dormancy through regulating DOG1 transcriptional activity. We do not exclude the possibility that other clock component(s) might also contribute the circadian gating of seed dormancy. This mechanism might help plants maintain a relatively low seed dormancy state that confers proper germination for the next generation.

Transcriptional regulation of seed dormancy is also associated with chromatin restructuring (Nee et al., 2017). Chromatin-remodeling factors alter DNA histone contacts and the accessibility of genomic regions to the transcriptional machinery or transcription factors, thus playing crucial roles in regulating gene expression (Ho and Crabtree, 2010; Ho et al., 2013; Han et al., 2015). Our study demonstrated that LUX physically interacts with the chromatin-remodeling factor, PKL, and that recruitment of PKL to the chromatin region of DOG1 depends on LUX (Figure 5). H3K27me3 levels at specific DOG1 chromatin regions were greatly reduced in the lux and pkl mutants compared with the wild type (Figure 5C). Other enzymes involved in histone methylation, demethylation, or deacetylation also affect seed dormancy. For instance, mutants of the H3K4 histone methyltransferase ARABIDOPSIS TRITHORAXRELATED7 exhibit reduced seed dormancy, whereas mutants of the H3K9 histone methyltransferase SUVH4 show upregulation of DOG1 and increased seed dormancy (Liu et al., 2007; Zheng et al., 2012). Consistently, double mutations in the H3K4 histone demethylases LYSINE SPECIFIC DEMETHYLASE-LIKE1 and 2 led to elevated H3K27me3 levels on DOG1 chromatin, thereby repressing its transcription and leading to reduced seed dormancy. Arrow indicates positive regulation and bar denotes negative regulation.
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METHODS

Plant Materials and Growth Conditions

The pki-1, pki-10, pki-11, pki-12, dog1-2, elf3-1, elf3-7, lux-6, elf4-101, rve1-2, rve2-1, and phyB-9 mutants, as well as the PKLp::GUS and 3SS:GFP-LUX transgenic lines, were described previously (Hicks et al., 2001; Khanna et al., 2003; Jing et al., 2013; Jiang et al., 2016; Zha et al., 2017; Zhang et al., 2018). All mutants and transgenic lines are of the Col-0 ecotype. Double mutant and transgenic plants were generated by genetic crossing, and homozygous lines were verified by PCR genotyping, antibiotic selection, and/or sequencing. Adult plants were grown side-by-side in soil in a growth chamber with regular irrigation at 22 °C ± 2°C and 60%–70% humidity, under LD (16 h light/8 h dark, 100 μmol m⁻² s⁻¹) conditions. Far-red, red, and white light was supplied by light-emitting diodes.

Seed Dormancy Test

To investigate seed dormancy, mature seeds at the same developmental stage were harvested, surface sterilized, plated on 0.6% agar (pH 5.7) under light, and then transferred to darkness or white light (80 μmol m⁻² s⁻¹) for 4 d. Seeds with protruded radicals were considered to be germinated, and the germination frequency was used to determine the degree of seed dormancy. The viability of seeds in each batch was tested after cold stratification at 4°C for 3 d.

For the phyB-dependent germination assay, seeds were harvested and stored dry at room temperature for up to 5 months. After sterilization and plating (within 1 h), the seeds were irradiated with far-red light (3.5 μmol m⁻² s⁻¹) for 5 min to inactivate phyB (phyB-off), followed by 5 min of red light (10 μmol m⁻² s⁻¹) to activate phyB (phyB-on) (Jiang et al., 2016). All seeds were then incubated in the dark for 4 d and the germination frequency was determined. At least 100 seeds were used for each genotype per experiment, and three replicates were performed for statistical analysis.

GUS Staining

Seeds from PKLp:GUS (Jing et al., 2013) transgenic plants were imbibed for 12 h and incubated in the solution provided with the GUS Histochemical Kit (Real-Times) at 37°C for 6 h following the manufacturer’s instructions. The embryos and endosperm were dissected and photographed under a dissecting microscope (Olympus).

Plasmid Construction

The regulatory or coding sequences or fragments of PKL, ELF3, ELF4, and LUX were amplified from Col-0 genomic DNA or cDNA using High-fidelity Pfu DNA Polymerase (Invitrogen). All primers used for cloning with the appropriate restriction sites are listed in Supplemental Table 1. The genes were cloned into the pEASY-Blunt vector and verified by sequencing. Various plasmids and vectors were digested with the corresponding restriction enzymes, followed by ligation and transformation into E. coli strain Trans-T1. The PKL, D8, and D9 fragments were inserted into pLexA (Clontech) to generate LexA-D8/D9. Full-length ELF3, ELF4, and LUX were cloned into yeast vectors pB42AD and pGBK7(TK) (Clontech), generating AD-ELF3/ELF4/LUX and GBD-ELF3/ELF4/LUX, respectively. The coding sequences of ELF3, ELF4, and LUX were also cloned into pUC19-cLUC (Chen et al., 2008), resulting in ELF3/ELF4-cLUC/LUX. LUX was cloned into pMAL-c5X-1 to produce MBP-LUX. The full-length PKL sequence was inserted into pUC19-nLUC (Chen et al., 2008) to generate PKL-nLUC. Various DOG1 fragments, including P1 to P4, P2-1 to P2-4, and P3-1 to P3-4 were cloned into pLacZ-2p (Lin et al., 2007), generating the corresponding LacZ reporter constructs. The promoter and coding sequences of DOG1 were ligated into pCAMBIA1302-LUC, resulting in DOG1p:LUC.

The binary construct was introduced into Agrobacterium tumefaciens strain GV3101 by electroporation and transformed into wild-type Arabidopsis via the floral dip method. Transgenic plants were selected on MS plates in the presence of 50 mg/l kanamycin or hygromycin. Homozygous lines were used in all experiments.

Transcriptional Analysis

Freshly harvested seeds were imbibed for 12 or 24 h as described in the figure legends. Total RNA was isolated from plants using a Universal Plant Total RNA Extraction Kit (BioTeke), and first-strand cDNA was synthesized from the RNA using reverse transcriptase (Invitrogen). The cDNA templates were amplified using an SYBR Premix Ex Taq Kit (Takara) in a LightCycler 480 (Roche) following the manufacturer’s instructions with primers listed in Supplemental Table 1. Three technical replicates were performed per sample, and relative expression levels were normalized to IPP2. Each experiment was performed at least three times with similar results, and representative data from a single experiment are shown.

ChIP Assay

For the ChIP reactions, procedures for crosslinking, chromatin isolation, sonication, and immunoprecipitation were performed as described (Bowler et al., 2004). In brief, 1.5 g of tissue from 5-d-old seedlings was harvested and fixed for 5 min in 1% formaldehyde under a vacuum. Chromatin was isolated and sonicated to produce ~500-bp DNA fragments. The chromatin samples were immunoprecipitated with anti-PKL (Jing et al., 2013), anti-GFP (Abcam, ab1218), or anti-H3K27me3 (Millipore, 07-449) antibodies. The precipitated DNA was subjected to phenol/chloroform extraction, precipitated in ethanol, and dissolved in water. The relative enrichment of each fragment was determined using the precipitated DNA samples by qPCR using SYBR Green PCR Master Mix. The ChIP assays were performed with three biological replicates. The primer pairs used for the ChIP assays are listed in Supplemental Table 1.

Yeast Two-Hybrid and One-Hybrid Assays

For the yeast two-hybrid assay, individual BD-fusion constructs were co-transformed with LexAop:LacZ (Clontech) reporter plasmids into yeast strain EGY48, and the AD-fusion constructs were transformed into yeast strain Ym4271. After mating, the transformants were grown on SD-/Trp-Ura-His dropout plates containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for color development. For the yeast one-hybrid assay, AD-LUX or AD control plasmids were cotransformed with various LacZ reporter constructs into yeast strain EGY48. The transformants were grown on SD-/Trp-Ura dropout plates with X-gal for color development. Blue yeast colonies indicated protein–protein or protein–DNA interactions.

Pull-Down Assay

The GST-LUX, MBP-LUX, and D6-Has recombinant fusion proteins or GST and MBP controls were expressed in E. coli strain BL21 (DE3) and purified using Glutathione Sepharose 4B beads (for GST fusion, GE Healthcare), Dextrin Sepharose (for MBP fusion; GE Healthcare), or Ni-NTA Agarose (for His fusion; Qiagen), respectively. Approximately 2 μg of purified bait proteins (GST-LUX or GST, MBP-LUX or MBP) and 2 μg of D6-Has prey proteins were incubated in binding buffer (50 mM Tris–HCl [pH 7.5], 100 mM NaCl, and 0.6% Triton X-100) for 2 h at 4°C. Following the addition of Glutathione Sepharose 4B or Dextrin Sepharose beads, the samples were incubated for 1 h. After washing with binding buffer, the precipitated proteins were eluted by heating the beads at 70°C for 5 min in 10 μl 10× SDS–PAGE loading buffer. The proteins were size-fractioned on a 10% gel and analyzed by immunoblotting with anti-GST (Abcam, ab19256), anti-MBP (Abcam, ab9084), or anti-Has (TransGen, HT501) antibodies.

LCI Assay

The LCI experiments were carried out as described previously (Chen et al., 2008). The nLUC/cLUC fusion plasmids and conjugative P19 plasmid were introduced into Agrobacterium strains GV3101 and EHA105,
respectively. A single colony was transferred to Luria-Bertani medium and cultured overnight to OD<sub>600</sub> = 0.6–0.8. The culture was pelleted, washed twice with transformation buffer (10 mM MES [pH 5.6], and 10 mM MgCl<sub>2</sub>) and resuspended to a final OD<sub>600</sub> of 1.5. Various nLUC/cLUC fusion constructs were mixed with an equal volume of P19. The bacteria were supplemented with 200 mM acetylsyringone and incubated at 28°C for 3–5 h without shaking. The bacterial suspensions were infiltrated into fully expanded young <i>N. benthamiana</i> leaves with a needleless syringe. The plants were grown for 2 d under LD conditions. The infiltrated leaves were sprayed with 2 μl luciferase (dissolved in 0.02% Triton X-100) and incubated in the dark for 10 min before imaging. Luminescence was captured using a NightSHADE LB985 plant imaging system equipped with a CCD camera (Berthold Technologies). The experiments were repeated at least three times.

**EMSA**

MBP-LUX or MBP recombinant proteins were expressed in E. coli BL21 (DE3) cells and purified using Dextrin Sepharose. EMSA was performed using a LightShift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer’s instructions. The two complementary oligonucleotides were annealed, labeled with biotin, and incubated with recombinant proteins in the absence or presence of excess amounts of unlabelled wild-type oligonucleotides. The protein–DNA samples were separated on 5% polyacrylamide gels and the signals captured with a Chemiluminescence Imaging System (biostep).

**Luciferase Transient Expression Assay**

The LUX effector, DOG1p:LUC reporter, and 35S:GUS control plasmids were cotransformed into <i>Arabidopsis</i> protoplasts, and LUC and GUS activity assays were performed as described previously (Tang et al., 2012). The relative reporter expression level was calculated as the LUC/GUS ratio.

**SUPPLEMENTAL INFORMATION**

Supplemental Information is available at Plant Communications Online.

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**AUTHOR CONTRIBUTIONS**

P.Z. and R.L. designed the research. P.Z., S.L., Y.L., L.Y., Y.J., and T.M. performed the research and analyzed the data. R.L. wrote the paper.

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