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Nathalie Berger, Bertrand B. Dubreucq, François Roudier, Christian C. Dubos, Loic L. Lepiniec. Transcriptional regulation of Arabidopsis LEAFY COTYLEDON2 involves RLE, a cis-element that regulates Trimethylation of Histone H3 at Lysine-27. *The Plant cell*, 2011, 23 (11), pp.4065-4078. 10.1105/tpc.111.087866 . hal-01000017

HAL Id: hal-01000017

<https://hal.science/hal-01000017>

Submitted on 29 May 2020

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Transcriptional Regulation of *Arabidopsis* *LEAFY COTYLEDON2* Involves *RLE*, a *cis*-Element That Regulates Trimethylation of Histone H3 at Lysine-27 ^W

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***LEAFY COTYLEDON2 (LEC2)* is a master regulator of seed development in *Arabidopsis thaliana*. In vegetative organs, *LEC2* expression is negatively regulated by Polycomb Repressive Complex2 (PRC2) that catalyzes histone H3 Lys 27 trimethylation (H3K27me3) and plays a crucial role in developmental phase transitions. To characterize the *cis*-regulatory elements involved in the transcriptional regulation of *LEC2*, molecular dissections and functional analyses of the promoter region were performed in vitro, both in yeast and in planta. Two *cis*-activating elements and a *cis*-repressing element (*RLE*) that is required for H3K27me3 marking were characterized. Remarkably, insertion of the *RLE cis*-element into *pF3H*, an unrelated promoter, is sufficient for repressing its transcriptional activity in different tissues. Besides improving our understanding of *LEC2* regulation, this study provides important new insights into the mechanisms underlying H3K27me3 deposition and PRC2 recruitment at a specific locus in plants.**

INTRODUCTION

In eukaryotes, complex developmental programs require tightly regulated expression of a specific set of genes at both spatial and temporal levels. Chromatin state dynamics and transcription factors are involved in the underlying molecular mechanisms (Beisel and Paro, 2011). Recent research has emphasized the importance of histone posttranslational modifications, such as acetylation, methylation, ubiquitination, or phosphorylation, in these regulatory processes (Berger, 2007; Kouzarides, 2007; Zhang et al., 2007a; Lee et al., 2010). In plants, genome-wide chromatin and transcriptomic analyses have confirmed the correlation existing between histone posttranslational modifications and transcriptional activity (Pfluger and Wagner, 2007; Roudier et al., 2009, 2011; Lauria and Rossi, 2011). For instance, Histone H3 Lys (residues 4, 9, 27, and 36) methylations are strongly associated with the transcriptional regulation of plant genes (Li et al., 2008; Liu et al., 2010; Lafos et al., 2011; Zheng and Chen, 2011). H3K4me3 and H3K36me2/me3 are predominantly linked with active chromatin, whereas H3K9me1/me2 and H3K27me3 modifications are considered to be repressive. More specifically, dynamic regulation of H3K27me3 marking has been shown to play a crucial role in the developmental regulation of gene

expression in eukaryotes, including plants (Kouzarides, 2007; Zhang et al., 2007a; Schatlowksi et al., 2008; Lafos et al., 2011; Zheng and Chen, 2011). H3K27me3 is extensively distributed over the transcribed and promoter regions of genes. It extends over large intergenic regions in animals, whereas the distribution seems to be more gene centered in plants (Turck et al., 2007; Zhang et al., 2007b; Schatlowksi et al., 2008).

H3K27me3 deposition is catalyzed by Polycomb-group (PcG) proteins with a SET [for Su(var)3-9, Enhancer-of-zeste, Trithorax] domain that belong to Polycomb Repressive Complex2 (PRC2) (Schuettengruber and Cavalli, 2009; Simon and Kingston, 2009; Morey and Helin, 2010; Margueron and Reinberg, 2011). On this line, it has been recently shown that PRC2 activity is necessary for H3K27me3 deposition in plants (Schubert et al., 2006; Bouyer et al., 2011). PcG proteins were first identified in *Drosophila melanogaster* and are evolutionary conserved among higher eukaryotes, including plants (Ringrose et al., 2004; Hennig and Derkacheva, 2009; Chen et al., 2010; Sawarkar and Paro, 2010; Margueron and Reinberg, 2011; Zheng and Chen, 2011). The PRC2 complex is composed of four core proteins, including ENHANCER OF ZESTE [E(Z); a PcG protein with a SET domain and methyltransferase activity], EXTRA SEX COMBS (ESC; a WD40 domain protein), SUPPRESSOR OF ZESTE-12 (a zinc finger protein), and NUCLEOSOME REMODELING FACTOR55. PcG proteins act in establishing and maintaining inactive chromatin states at numerous target genes throughout development (Francis and Kingston, 2001; Schwartz et al., 2006; Schwartz and Pirrotta, 2008; Müller and Verrijzer, 2009; Köhler and Hennig, 2010; Margueron and Reinberg, 2011).

Recruitment of PRC2 at a specific locus likely relies on multiple mechanisms (Margueron and Reinberg, 2011). Genomic

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^WOnline version contains Web-only data.

www.plantcell.org/cgi/doi/10.1105/tpc.111.087866

domains named Polycomb Repressive Element (PRE) have been described mainly in *Drosophila* (Ringrose and Paro, 2007; Schuettengruber et al., 2007). PRE domains are characterized by short DNA motifs that include binding sites for regulatory proteins like PLEIOHOMEOTIC (PHO), ZESTE (Z), and GAGA factors (GAFs) (Schuettengruber and Cavalli, 2009). PHO and GAFs would be directly involved in recruiting PRC2. Recently, PRE-like elements have also been identified in mice and humans (Sing et al., 2009; Woo et al., 2010). Nevertheless, our understanding of PRC2 recruitment is still scarce, and no PRE elements have been identified in plants to date (Schatlowski et al., 2008; Morey and Helin, 2010; Sawarkar and Paro, 2010; Smith and Shilatifard, 2010; Enderle et al., 2011; Margueron and Reinberg, 2011).

In *Arabidopsis thaliana*, the three E(Z) homologs, namely MEDEA (MEA) and the two partially redundant proteins CURLY LEAF (CLF) and SWINGER (SWN), act throughout plant development (Kiyosue et al., 1999; Luo et al., 1999; Pien and Grossniklaus, 2007; Hennig and Derkacheva, 2009; Köhler and Hennig, 2010; Rodrigues et al., 2010; Zheng and Chen, 2011). MEA predominantly represses the MADS box gene *PHERES1* and *MEA* itself in the endosperm (Baroux et al., 2006; Gehring et al., 2006; Jullien et al., 2006). CLF and SWN are required for the repression of floral homeotic genes, such as *AGAMOUS* (*AG*) or *SHOOTMERISTEMLESS* (*STM*) (Goodrich et al., 1997; Katz et al., 2004; Schubert et al., 2006; Farrona et al., 2008). PRC2 activity also represses the transcription of the master regulators of seed development during vegetative and/or endosperm development (Makarevich et al., 2006; Bouyer et al., 2011). These master regulators include LEAFY COTYLEDON1 (*LEC1*), a protein homologous to the HAP3 subunits of the CAAT Box binding Factors, and three B3-domain transcription factors, namely, ABA INSENSITIVE3 (*ABI3*), FUSCA3 (*FUS3*), and *LEC2* (Braybrook and Harada, 2008; Santos-Mendoza et al., 2008). These AFL (for *ABI3*, *FUS3*, and *LEC2*) regulatory proteins control a network of local and redundant gene regulations (Santos Mendoza et al., 2005; To et al., 2006). Among these regulators, *LEC2* is specifically expressed in embryos within a short temporal window, from 4 d after pollination (DAP) to 14 DAP, peaking at 10 DAP, and is not expressed in any other tissues (Stone et al., 2001; Kroj et al., 2003). The ectopic expression of *LEC2* in transgenic plants triggers the formation of somatic embryos as well as other organ-like structures and confers embryonic characteristics to seedlings (Stone et al., 2001).

Consistent with the involvement of proteins with a SET domain, other reports have confirmed that the expression of *LEC2* is controlled, in a developmental manner, by PcG complexes. It has been shown recently that FERTILIZATION INDEPENDENT ENDOSPERM, the *Arabidopsis* ESC homolog of the PRC2 complex, negatively regulates the expression *LEC2* and other AFL genes (Bouyer et al., 2011). PRC1-like ring finger proteins also repressed the expression of the AFL genes (Chen et al., 2010). In addition, PICKLE (PKL), a chromatin remodeler of the CHD3 family, and a closely related homolog (PKR2) are also involved in this regulation (Ogas et al., 1999; Zhang et al., 2008; Aichinger et al., 2009). PKL promotes indirectly H3K27me3 deposition at different loci, including *LEC2*, in vegetative tissues, through the induction of PcG gene expression (e.g., *SWN* or

EMBRYONIC FLOWER2) (Aichinger et al., 2009, 2011). Other mechanisms involved in the regulation of *LEC2* expression have been identified (Zhang and Ogas, 2009). For example, the inhibition of histone deacetylase activity triggers *LEC2* expression upon germination (Tanaka et al., 2008). ASIL1 (for *Arabidopsis* 6b-interacting protein 1-like 1), a plant-specific trihelix factor, represses *LEC2* during both germination and early maturation phase (Gao et al., 2009).

Besides the large amount of data accumulated regarding the regulation of *LEC2* expression, the DNA *cis*-elements involved in the specific expression of *LEC2* during seed development are unknown. More specifically, the means by which the *LEC2* locus is marked by H3K27me3 during vegetative development remain to be determined. In this work, we identified and functionally characterized three *cis*-regulatory elements involved in the developmental regulation of *LEC2* expression. Two of them were found to be essential for the transcriptional activation of *LEC2*. The first one presents some similarities with *CArG* regulatory elements recognized by MADS box transcription factors. The second one is similar to a GAFs box and bound by plant BASIC PENTACYSSTEINE (BPC) proteins, both in yeast and in vitro. The third element, which we named *Repressive LEC2 Element* (*RLE*), is 50 bp long and is necessary for the repression and H3K27 trimethylation of *LEC2* in vegetative tissues. Remarkably, the *RLE cis*-element was also sufficient to regulate the activity of an unrelated promoter (P_{F3H}) and to promote H3K27me3 mark deposition in transgenic plants. Therefore, *RLE* acts as a functional homolog of the PRE element found in *Drosophila*. Taken together, the data presented here shed new light on the regulation of *LEC2*, a master regulator of seed development and bring important new insights into the regulation of H3K27me3 deposition and, thus, PRC2 recruitment in plants.

RESULTS

Identification of the *cis*-Regulatory Elements That Control the Specific Expression of *LEC2*

It has been previously shown that the 2.5-kb sequence located upstream of the *LEC2* open reading frame recapitulates the embryo-specific pattern of *LEC2* expression when fused to the β -glucuronidase (*GUS*) reporter gene (Stone et al., 2001; Kroj et al., 2003). Here, it was shown that a 500-bp-long fragment, hereafter denoted as P_{LEC2} , was sufficient to faithfully confer a similar expression pattern (Figure 1A). In addition, the expression of the *LEC2* cDNA under the control of P_{LEC2} fully complemented the *lec2-4* mutation (see Supplemental Figure 1 online).

To gain further insight into the *cis*-regulatory elements necessary for *LEC2* expression during embryogenesis, a series of 5'-end deletions was performed on P_{LEC2} , and the resulting DNA fragments were fused to the *GUS* reporter gene to test their respective transcriptional activity in transgenic plants (Figure 2A). The analysis of *GUS* activity identified a region between -447 and -400 bp upstream of the first codon that is necessary for the expression of *LEC2* in the embryo. Deletions every 5 bp narrowed this region down to a 5-bp motif (*CATAT*; between -447 and -442 bp). This 5-bp regulatory sequence showed

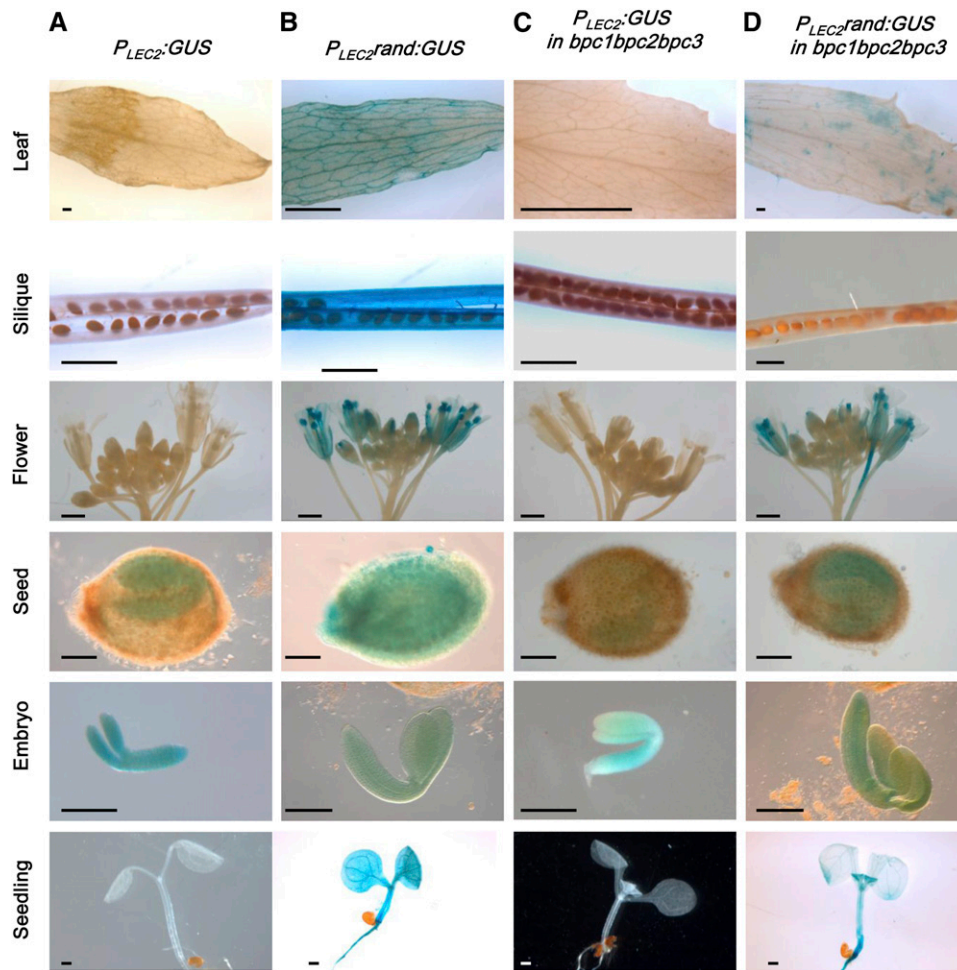


Figure 1. Expression Patterns Using the Wild Type and Mutated *LEC2* Promoter.

Representative GUS patterns are presented for *P_{LEC2}::GUS* (A) and *P_{LEC2rand}::GUS* (B) in the wild-type Columbia-0 background and for *P_{LEC2}::GUS* (C) and *P_{LEC2rand}::GUS* (D) in the *bpc1 bpc2 bpc3* triple mutant background, respectively. Bars = 1 mm in leaf, silique, and flower, and 0.1 mm in seed, embryo, and seedling. Independent transgenic lines ($n > 12$ for each construct) were assayed for GUS activity in leaves, siliques, flowers, seeds, embryos, and seedlings (10 d after sowing).

no similarity to any known consensus *cis*-regulatory element described in public databases.

In parallel, a series of 3'-end deletions of *P_{LEC2}* was performed and fused to a minimal 35S promoter carrying a transcription start site derived from the cauliflower mosaic virus that was placed upstream of the *GUS* reporter gene (Debeaujon et al., 2003). Analysis of the GUS activity in transgenic plants indicated that the 50-bp region upstream of the first codon, which corresponds to the 5'-untranslated region of *LEC2*, was required for the activity of *P_{LEC2}* in this context (Figure 2B). Surprisingly, further deletion of 150 bp resulted in the recovery of GUS expression. In fact, the promoter fragment extending from -500 to -250 bp was sufficient to confer ubiquitous GUS expression throughout plant and seed development. This analysis suggested that the 50-bp domain comprised between -200 and -150 bp contained sufficient information to repress the transcriptional activity of *P_{LEC2}* (Figure 2B).

***LEC2* Expression Relies on a CT-Rich Element Bound by BPC Proteins**

In silico analysis of the 50-bp sequence located upstream of the first codon revealed a GAGA box motif (inverted CT repeats; Figure 3A) previously found in different plant promoters (Meister et al., 2004; Monfared et al., 2011; Wanke et al., 2011). The conversion by site-directed mutagenesis of CT to CA repeats (*mCA*) did not affect the transcriptional activity of *P_{LEC2}* in planta, whereas changing CT to AT repeats (*mAT*) fully abolished the activity of *P_{LEC2}* (Figure 3A). Previous analyses have shown the positive effect of adding CT repeats on promoter activity (Pauli et al., 2004). Here, the addition of three CT repeats to the GAGA box led to the ectopic activation of *P_{LEC2}* in pollen grains (see Supplemental Figure 2 online). These data indicated that the GAGA box made of eight C-T (or A) dinucleotide repeats is essential for the correct expression of *LEC2*.

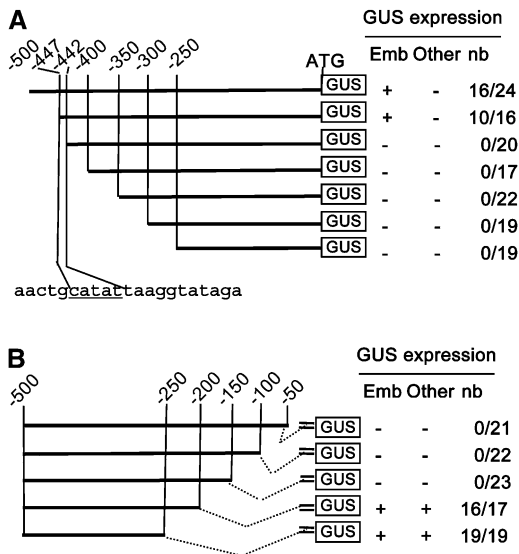


Figure 2. Molecular Dissection Analysis of the *LEC2* Promoter.

Schematic representation of the deletion series of the *LEC2* promoter (P_{LEC2}) fused to the *GUS* reporter gene. The presence or lack of *GUS* expression is indicated by (+) or (-), respectively. The ratio (nb) indicates the number of transgenic lines expressing *GUS* (numerator) and the total number of transgenic lines analyzed (denominator). *GUS* expression was tested in embryos (emb), valves of siliques, flowers, rosettes, cauline leaves, and stems (other).

(A) The 5'-end deletions. The nucleotide sequence underlined is essential for P_{LEC2} activity.

(B) The 3'-end deletions were fused to a minimal promoter derived from the 35S of the cauliflower mosaic virus (=).

In plants, GAGA motifs are bound by BPC proteins (Meister et al., 2004; Monfared et al., 2011; Wanke et al., 2011). In *Arabidopsis*, the BPC family contains seven members grouped into three classes. The accumulation of BPC mRNA in embryos shown in the databases (<http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi>) was confirmed by RT-PCR experiments (Figure 3B). All BPC genes but *BPC5* (which likely is a pseudogene) (Meister et al., 2004; Monfared et al., 2011) were expressed in 10-d-old embryos.

Then, the ability of BPC proteins to interact with P_{LEC2} was tested in yeast one-hybrid experiments (Figure 3C). A strong interaction was detected for the three BPCs of class I (BPC1, 2, and 3), whereas a weak interaction was detected for BPC proteins that belong to class II (BPC4) and III (BPC7). To define the ability of BPC to bind directly to the GAGA motif found in P_{LEC2} , in vitro electrophoretic mobility shift assays (EMSAs) were performed with recombinant BPC2 (class I) and BPC4 (class II) proteins. Both proteins BPC2 and BPC4 proteins were able to interact with the poly-CT double-stranded probe (Figure 3D). Competition experiments with wild-type and mutated (*mAT*) probes demonstrated the specificity of these interactions. Finally, the accumulation of *LEC2* mRNA was measured in seeds of available *bpc* mutants (i.e., *bpc1*, *bpc2*, and *bpc3* single or the corresponding multiple mutants). No significant differences were observed in comparison to wild-type seed mRNA level (see Supplemental Figure 3 online).

Taken together, these results showed that *LEC2* expression relies on a GAGA box and that BPC proteins are probably involved in the transcriptional regulation of *LEC2*. Further analyses of additional *bpc* mutants are required to determine which of the BPC proteins are involved. Interestingly, the functional analysis of a modified *LEC2* promoter, active in vegetative tissues (see the results presented below), strongly supported the role of the BPC proteins in the control of *LEC2* expression.

Characterization of a *cis*-Regulatory Element (*RLE*) That Restricts *LEC2* Expression to the Embryo

To further characterize the domain located between 200 and 150 bp upstream of the first codon (Figure 2B), a new series of deletions fused to the 35S minimal promoter upstream of the *GUS* reporter gene was constructed and tested in transgenic plants (Figure 4). This experiment suggested that at least the sequences between -185/-178 and -168/-160bp were necessary to repress P_{LEC2} transcriptional activity in seed and vegetative tissues, respectively (Figure 4A). To supplement this deletion analysis, mutagenesis experiments were performed using the entire P_{LEC2} promoter. The 50-bp element associated with the repression activity was replaced by a random 50-bp nucleotide sequence, with no similarity within the *Arabidopsis* genome, providing the $P_{LEC2}rand$ promoter. In comparison to the embryo-specific *GUS* staining observed with $P_{LEC2}:GUS$ lines (Figure 1A), the $P_{LEC2}rand:GUS$ lines showed ectopic expression in leaves, stems, flowers, pollen, siliques, and seed coat (Figures 1B and 4B). Similar results were obtained in the Columbia-0 background (see Supplemental Figure 4 online), and these results confirmed the role of the repressive element in the developmental regulation of *LEC2* expression. Thus, we named this element *RLE*. In addition, site-directed mutagenesis was performed to support and refine the structure of the repressive *cis*-regulatory element. Some of the mutations (*m1* and *m3*) did not modify the pattern of *GUS* activity, whereas other mutations (*m2* and *m4*) led to the ectopic activity of *GUS* in pollen (Figure 4B).

Taken together, these results indicated that the 50-bp element located between -150 and -200 bp upstream of the first codon has a negative effect on the transcriptional activity of P_{LEC2} in planta. In addition, they suggested that *RLE* likely contains different functional motifs that are necessary to confer this repressive activity.

Finally, to test if the BPC proteins are involved in the ectopic activity of P_{LEC2} when *RLE* is mutated, $P_{LEC2}rand:GUS$ was introduced into the triple *bpc1 bpc2 bpc3* mutant by transformation. In this genetic background, *GUS* activity was completely lost in siliques and seed integument and was strongly down-regulated in leaves and cotyledons, in comparison to the wild type (Figure 1C). These results strongly suggested that class I BPCs were involved in the regulation of *LEC2* expression.

The *RLE* Element Identified in P_{LEC2} Is Necessary for H3K27me3 Deposition

Previous reports have shown that PRC2, which catalyzes H3K27 trimethylation, is involved in the transcriptional repression of

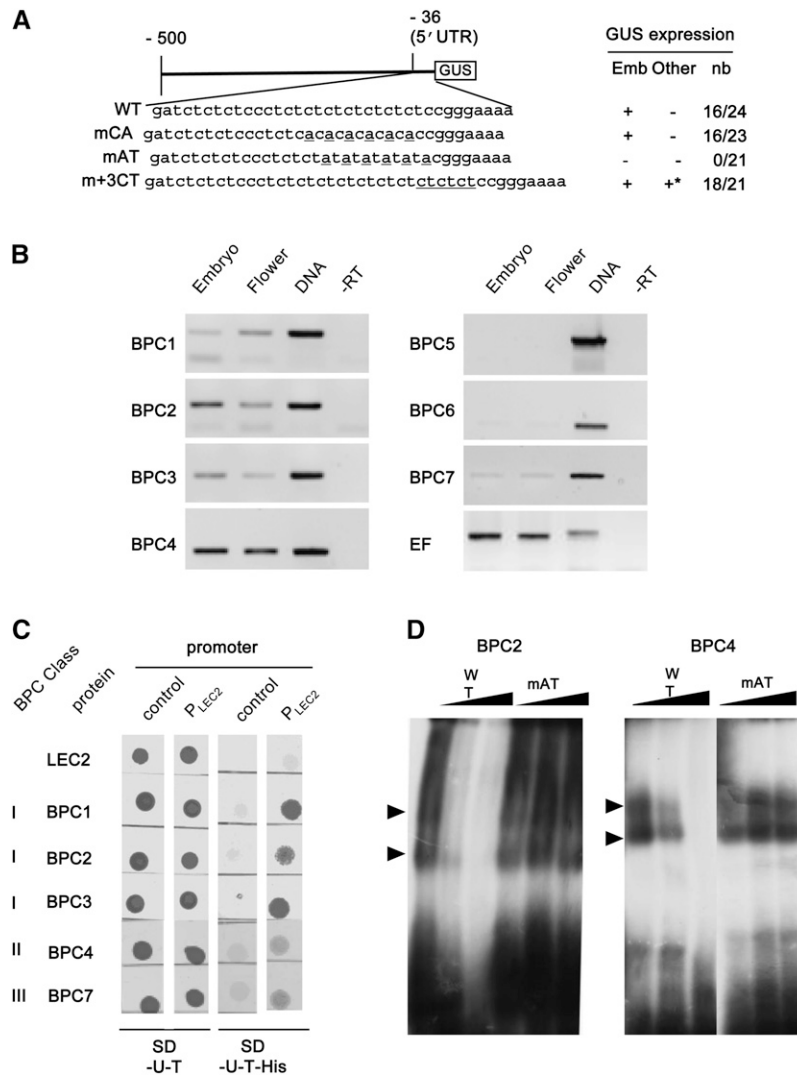


Figure 3. The Promoter of *LEC2* Contains a CT-Rich Repeat Bound by BPC Proteins.

(A) The wild-type (WT) *P_{LEC2}* sequence is shown in the top row (WT). Mutated nucleotides are underlined in the next three rows (mCA, mAT, and m+3CT). GUS activity in pollen is indicated with an asterisk. Presence or lack of GUS expression is indicated by (+) or (-), respectively. The ratio (nb) indicates the number of transgenic lines expressing GUS (numerator) and the total number of transgenic lines analyzed (denominator). GUS expression was tested in embryos (emb), valves of siliques, flowers, rosettes, cauline leaves, and stems (other). UTR, untranslated region.

(B) Analysis of BPC expression in embryos and flowers. Genomic DNA, embryo RNA untreated by the superscript II enzyme (-RT), and *EF1αA4* (EF) were used as control.

(C) One-hybrid analysis. The interactions of the different full-length BPC proteins, fused to GAL4, with *P_{LEC2}* were tested. The ability to grow in the absence of His indicates an interaction.

(D) EMSA competition assays. BPC2 and BPC4 proteins were tested with labeled wild-type CT repeat probe, and competitions were performed with wild-type or mutated CT repeat (mAT) unlabeled probes. Shifts are indicated with arrowheads.

LEC2 in seedlings (Makarevich et al., 2006; Chen et al., 2010; Bouyer et al., 2011). To determine if the *RLE* element identified in *P_{LEC2}* was involved in this chromatin-based regulatory process, H3K27me3 enrichment was investigated by chromatin immunoprecipitation (ChIP) and subsequently analyzed by quantitative PCR. The H3K27me3 mark was detected over the *LEC2* locus, in the promoter and more strongly right downstream of the first

codon. These results are consistent with the data obtained by genome-wide analyses (Zhang et al., 2007b; Roudier et al., 2011).

Then, the level of H3K27me3 at the *P_{LEC2}:GUS* and *P_{LEC2}rand*: *GUS* loci was investigated in 10-d-old seedlings of transgenic plants. The distribution of H3K27me3 at the *P_{LEC2}:GUS* loci was similar to the pattern found at the endogenous *LEC2* locus

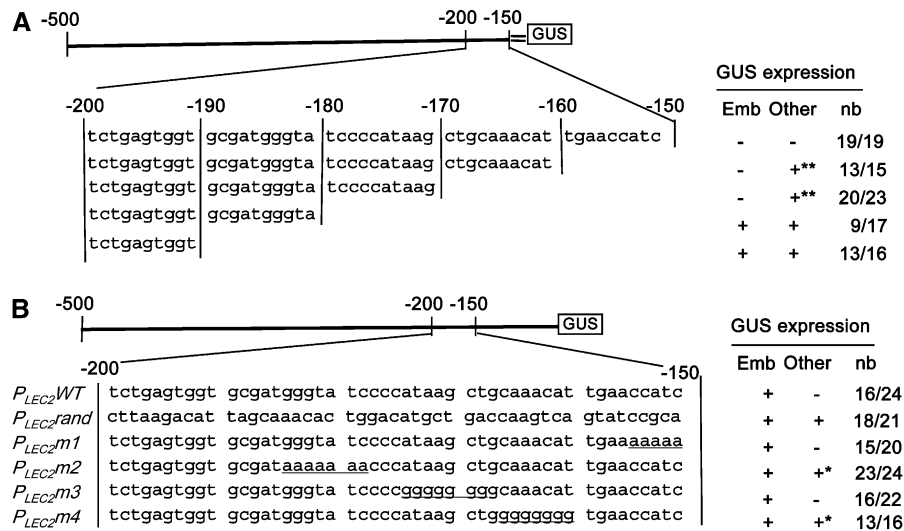


Figure 4. Molecular Dissection Analysis of the *RLE* Domain.

Presence or absence of *GUS* activity is indicated by (+) or (–), respectively. The ratio to the right (nb) indicates the number of transgenic lines that display *GUS* activity (numerator) and the total number of transgenic lines analyzed (denominator). *GUS* activity was tested in embryos (emb), valves of siliques, flowers, rosettes, cauline leaves, and stems (other). The detection of *GUS* activity in pollen (+*) or in all organs with the exception of the seed coat and embryo (+**) is indicated.

(A) Analysis using 3'-end deletion constructs fused to the minimal 35S promoter (=) and to the *GUS* reporter gene.

(B) Analysis using modifications of the *RLE* domain by replacement with a random 50-bp sequence (*P_{LEC2}rand*) or by site-directed mutagenesis (*P_{LEC2}m1*, *m2*, *m3*, and *m4*).

(Figure 5A). Importantly, the level of H3K27me3 over the *P_{LEC2}rand*:*GUS* locus was significantly lower than over *P_{LEC2}*:*GUS* locus (at $P < 0.002$, according to a Student's unpaired *t* test). The level was between 45- and 85-fold lower, depending on the probes considered (Figure 5B). The analyses were performed using several ($n > 10$) independent transgenic lines for each construct. Therefore, the result indicated that the difference between *P_{LEC2}rand*:*GUS* and *P_{LEC2}*:*GUS* loci was strictly dependent on the presence of *RLE cis*-element, independent of the transgene insertion sites within the genome. These observations therefore demonstrated that H3K27me3 enrichment over *LEC2* depends on the *RLE* sequence. Taken together, these data and the ectopic *GUS* activity detected in the *P_{LEC2}rand*:*GUS* transgenic plants (Figure 1B) strongly suggest that the *RLE cis*-element is a major determinant of PRC2-mediated repression of *LEC2* outside of embryogenesis (Figure 1B).

Insertion of *RLE* in pF3H, an Unrelated Promoter, Is Sufficient to Repress Its Transcriptional Activity in Seed and Can Trigger H3K27me3 Deposition

The repressive function and the role of *RLE* in the initiation of H3K27me3 marking were tested by a gain-of-function experiment with an unrelated promoter. The *RLE* sequence or a mutated version (random) were introduced at similar position (i.e., 150 bp upstream of the first codon) into the promoter of the *FLAVONONE 3-HYDROXYLASE* (*F3H*) gene to generate *P_{F3H}rle*:*GUS* and *P_{F3H}rand*:*GUS* constructs, respectively. The *F3H* promoter was chosen for two reasons. First the *cis*-regulating

elements that control its activity have been described, and no motifs were located at the insertion site (Hartmann et al., 2005). Furthermore, no H3K27me3 was detectable in ChIP-quantitative PCR analyses (Figure 5C), in agreement with previous analyses (Zhang et al., 2007b). Similar analyses were performed to establish the H3K27me3 landscape at the *P_{F3H}rle*:*GUS* and *P_{F3H}rand*:*GUS* loci in transgenic seedlings. It revealed a weak enrichment of H3K27me3 at *P_{F3H}rle*:*GUS* loci when compared with *P_{F3H}rand*:*GUS* (Figure 5D).

The effect of *RLE* insertion on *P_{F3H}* transcriptional activity was investigated. The endogenous *F3H* promoter was fused to the *GUS* reporter gene (*P_{F3H}*:*GUS*) and introduced into *Arabidopsis* as a control. *GUS* activity was analyzed in transgenic plants carrying one of the three different constructs, namely, *P_{F3H}*:*GUS*, *P_{F3H}rle*:*GUS*, and *P_{F3H}rand*:*GUS*. For each construct, 24 independent transformants were analyzed. Whereas a strong *GUS* activity was detected in seeds of 19 transgenic lines carrying *P_{F3H}*:*GUS* and 17 *P_{F3H}rand*:*GUS* lines, it was undetectable in seeds of the 14 *P_{F3H}rle*:*GUS* transgenic lines showing *GUS* activities in other tissues (Figure 6). These results demonstrated that the insertion of *RLE* is sufficient to repress *P_{F3H}* transcriptional activity in the seed (embryo and seed coat). On the contrary, similar *GUS* activity was found in vegetative tissues for the three constructs, suggesting that H3K27me3 marking was not sufficient to fully inhibit gene expression in these tissues. Taken together, these observations demonstrate that the *RLE cis*-element is sufficient to trigger H3K27me3 deposition and control transcriptional activity in a developmentally regulated manner.

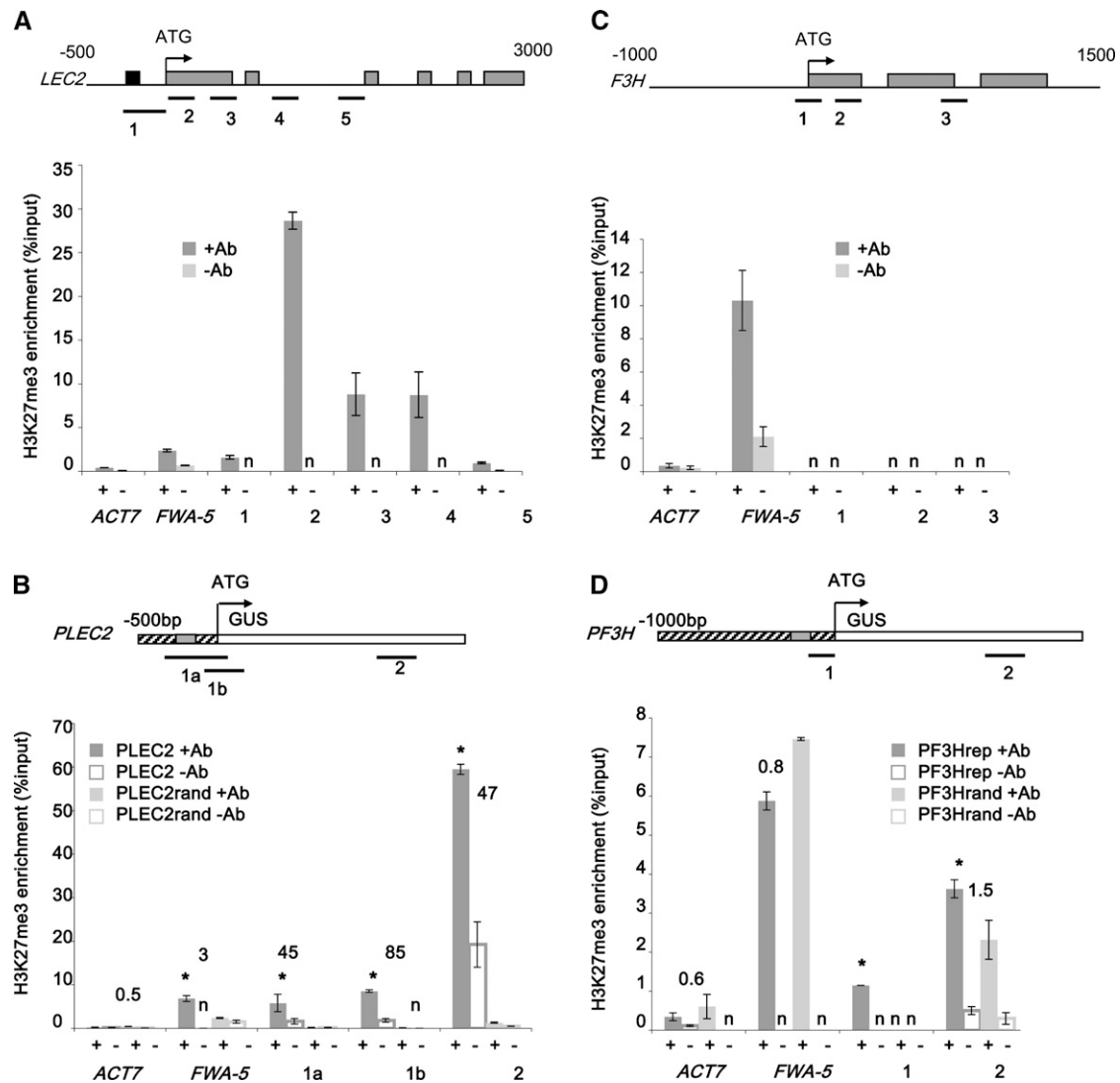


Figure 5. ChIP-PCR Analysis of H3K27me3 Enrichment over the Endogenous and Transgenic *LEC2* and *F3H* Loci.

The H3K27me3 enrichments in 10-d-old seedlings at *LEC2* (A), *P_{LEC2}:GUS* (B), *F3H* (C), and *P_{F3H}:GUS* (D) loci are presented. The enrichment is expressed relative to chromatin input (%input). All the experiments were repeated at least twice on a pool of five independent transgenic lines. *ACT7* and *FWA* genes were used as negative and positive controls, respectively. Bars denote SE, and n = not detectable. For (A) and (C), the positions of the primers used (thick lines), *RLE* element (black box), introns (thin line), and exons (dark boxes) are indicated on the top. For (B) and (D), the positions of the primers (thick lines), *LEC2* promoter (striped box), *RLE* element (dark box), and *GUS* coding sequence (white box) are indicated on the top. The numbers above the histogram bars represent the ratio of the H3K27me3 enrichment when comparing constructs with and without the *RLE* element (i.e., *P_{LEC2}* with *P_{LEC2}Rand* [B] and *P_{F3H}* with *P_{F3H}Rand* [D]). The asterisks indicate that the difference (with or without *RLE*) is significant at P < 0.002 according to Student's unpaired t test.

DISCUSSION

Both a *CArG* and a CT-Rich Activating *cis*-Element Are Essential for *P_{LEC2}* Activity

The molecular and functional dissection of the *LEC2* promoter in planta revealed two essential activating sequences that are necessary for embryo-specific expression (Figure 7). The first *cis*-regulatory element is made of five nucleotides (5'-CATAT-3')

and located 447 bp upstream of the translational start codon. This very short motif did not present any obvious similarities with known *cis*-regulatory sequences. Nevertheless, when a wider frame is considered (i.e., GCATATTAAGG), a similarity was found with the consensus *CArG* motif [i.e., CC(A/T)₆GG] (Riechmann and Meyerowitz, 1997). The *CArG* motifs are known to be recognized by MADS box proteins, a family of transcription factors that control multiple developmental processes, ranging from root to floral or embryo development (Becker and Theissen, 2003).

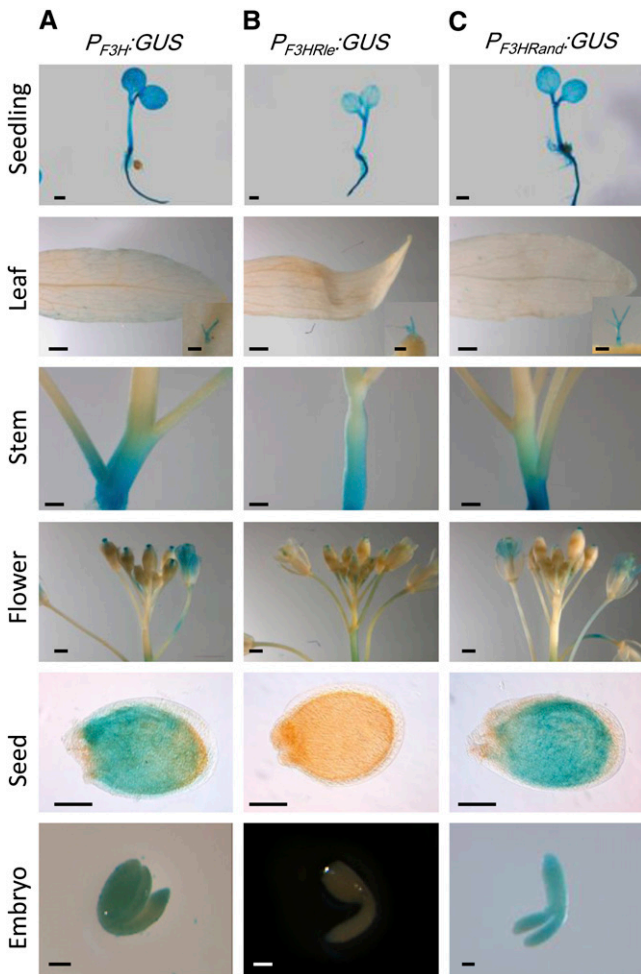


Figure 6. Insertion of the *RLE* Element Inhibits *P*_{F_{3H}} Activity in Seed.

Representative patterns of GUS staining are presented for *P*_{F_{3H}};*GUS* (A), *P*_{F_{3H}}^{Rle};*GUS* (B), and *P*_{F_{3H}}^{Rand};*GUS* (C). Independent transgenic lines ($n = 24$ for each construct) were analyzed for GUS activity in seedling, leaf, trichome, stem, flower, seed, and embryo. Bars = 1 mm for seedling, leaf, and flower and 0.1 mm for trichome, seed, and embryo.

Interestingly, the AGAMOUS-like15 (AGL15) MADS box protein preferentially binds *CArG* motifs with longer *A/T*-rich region, such as *C(A/T-rich)₈G* and *C(A/T-rich)₇GG* sequences (Tang and Perry, 2003; Hill et al., 2008; Zheng et al., 2009). This later motif is similar to the *CArG* sequence found in *P*_{LEC2} [i.e., *C(A/T)₇GG*]. In addition, genetic and genome-wide ChIP analyses have demonstrated that *LEC2* is a direct target of AGL15 (Zheng et al., 2009). Taken together, these results strongly suggested that the *C(A/T-rich)₇gg* motif found in *P*_{LEC2} is one of the AGL15 binding sites involved in the regulation of *LEC2* expression. Furthermore, AGL15 can interact with histone deacetylase HDA19 (Hill et al., 2008) and thus could be involved in histone deacetylase recruitment at the *LEC2* locus.

The second *cis*-regulatory element essential for *LEC2* expression is a *CT*-rich domain (containing eight *CT* repeats) located in

the 5'-untranslated region of *LEC2* (Figure 3A). This domain displays strong similarities with *GAGA* elements (inverted *CT* repeats). Consistent with functional data previously obtained (Meister et al., 2004; Kooiker et al., 2005), the results presented here demonstrated that the *CT*-rich element of *P*_{LEC2} is bound by BPC proteins (preferentially of class I), both in yeast and in vitro. The quantitative differences in DNA binding observed between the BPC in vitro and yeast (Figures 3C and 3D) may be related to the target sequences used [i.e., the entire 500-bp-long promoter for the one-hybrid experiment and a short repetition of three *CT* motifs, $3 \times (CT)_8$, in vitro] (see Supplemental Table 1 online). It has been shown that, in vitro, BPC1 binding was affected by modification of the stretch of purine residues (i.e., by introducing any pyrimidine residue) (Kooiker et al., 2005). Our results suggested that, in planta, the replacement of cytosine by adenine residues strongly affected *P*_{LEC2} activity (Figure 3A). This suggested that the strong base pairing between complementary C-G nucleotides is important for the function of the *CT* element. Interestingly, the addition of three extra *CT* repeats was sufficient to trigger ectopic *P*_{LEC2};*GUS* activity in pollen grains (see Supplemental Figure 2 online). This result was consistent with evidence indicating that *CT* repeats have an enhancer effect on promoter activity (Pauli et al., 2004). The three additional *CT* repeats inserted in *P*_{LEC2} may increase transcriptional activation, up to the threshold of detection of GUS activity, in pollen. A second nonexclusive possibility is that the addition of these repeats increased the affinity for another BPC protein expressed in pollen. This later hypothesis is supported by the fact that the promoter of *BPC2*, *BPC3*, *BPC4*, and *BPC7* are all active in pollen (Monfared et al., 2011).

The *BPC* mRNAs of class I (*BPC1*, 2, and 3) together with *BPC4* (class II) were the most abundant in embryo (Figure 3). Other *BPC* mRNAs were also detected (with the exception of *BPC5*) but at very low levels. These expression profiles together with the results of DNA binding assays suggested that BPC factors are likely to regulate *LEC2* through binding to the *GAGA cis*-element found in *P*_{LEC2}. Consistent with this hypothesis, the ectopic GUS activity driven by *P*_{LEC2}^{Rand};*GUS* was fully abolished in siliques and seeds integuments and was strongly affected in leaves and cotyledons of the triple class I mutant (Figure 1C).

Altogether, these results suggested that BPC proteins that belong to class II or III act redundantly with class I BPCs to activate *LEC2* expression through its specific *GAGA cis*-regulatory motif. This hypothesis is in agreement with the high genetic redundancy recently reported for BPCs (Monfared et al., 2011). Moreover, it has been shown that BPCs of group II can form homodimers or heterodimers with group I BPCs (Wanke et al., 2011). Therefore, obtaining new *bpc* single and multiple mutants of the different classes will be necessary to unravel which of the BPC proteins are involved in the regulation of *LEC2* expression.

***RLE* Is Sufficient to Repress Transcriptional Activity and Is Necessary to Trigger H3K27me3 Deposition**

The molecular and functional dissection of *P*_{LEC2} presented here has identified a new and negative regulatory *cis*-element (*RLE*) involved in restricting the expression of *LEC2* to the seed (Figure 7). This 50-bp-long *cis*-acting sequence located 150 bp

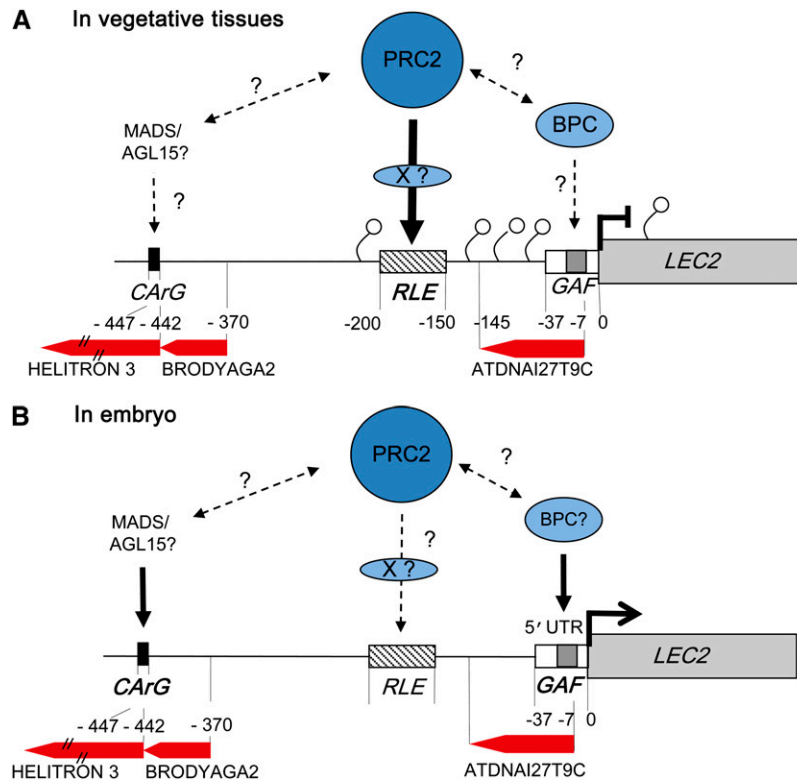


Figure 7. Schematic Structure and Regulation of the *LEC2* Promoter.

Putative model of regulation in vegetative tissues (A) and in the embryo (B). The *CARG* box, *GAF* box, and *RLE* element are represented as well as the transposon marks (*MuDR* and *HEL:Helitron*) and putative functional interactions between the proteins. Small circles represent H3K27me3 marks.

upstream of the *LEC2* first codon is required for repressing *LEC2* expression in leaves, stems, flowers, pollen, seed coat, and siliques (Figures 1 and 2B). Additional experiments (i.e., deletions of 10 bp and site-directed mutagenesis approaches) confirmed these observations and suggested that *RLE* is made of different functional stretches of nucleotides that are necessary to confer this repressive activity (Figure 4B).

Remarkably, the presence of *RLE* in *P_{LEC2}:GUS* is sufficient to trigger H3K27me3 deposition in transgenic seedlings (Figures 5C and 5D). In addition, the insertion of *RLE* into the *F3H* promoter (*P_{F3H}RLE*) was sufficient to inhibit its transcriptional activity in both the seed coat and embryo but not in vegetative tissues after germination (Figure 6B). The detection of GUS activity in vegetative tissues is not totally unexpected. Indeed, it may be related to the environmental regulation of *F3H* (Pelletier and Shirley, 1996; Blödner et al., 2007; Dubos et al., 2008; Zhang et al., 2011) and to counteracting chromatin-based regulation (Liu et al., 2010; Schmitges et al., 2011). Consistent with this hypothesis, genome-wide analyses showed that in seedlings the *F3H* endogenous locus is largely enriched in H3K4me3, a mark usually associated with transcriptionally active chromatin (Roudier et al., 2011). In conclusion, the *RLE cis*-element is necessary to trigger H3K27me3 enrichment and concomitant inhibition of transcriptional activity, although additional factors are likely involved in maintaining or alleviating this repression.

RLE Functions as PREs Identified in *Drosophila*

It has been shown that PcG complexes that control the H3K27me3 level are involved in the transcriptional repression of *LEC2* in seedlings (Makarevich et al., 2006; Chen et al., 2010; Bouyer et al., 2011). Our results demonstrated that the *RLE* element found in *P_{LEC2}* is sufficient to initiate H3K27me3 deposition at the *LEC2* locus. In addition, some PREs have been found in the proximal promoter of target genes in *Drosophila* (Morey and Helin, 2010; Enderle et al., 2011), as *RLE* in *LEC2*. Last, it has been demonstrated recently that H3K27me3 deposition is abolished in *fie* mutant lacking PRC2 activity (Bouyer et al., 2011). These results suggest that the *RLE* element functions as *Drosophila* PRE. Nevertheless, the weak effect of *RLE* on H3K27me3 deposition at *pF3H* suggests that it may not be sufficient in any genomic contexts.

PREs contain a few short transcription factor binding sites, including a GAGA box (Hagstrom et al., 1997; Horard et al., 2000). Similarly, *RLE* is associated with a CT-rich element in *P_{LEC2}*. In addition, some other plant promoters that are regulated by PcG also display CT-rich elements and are regulated by BPC proteins. For instance, *SEEDSTICK* and *INNER NO OUTER (INO)* are two targets of BPC1 and BPC2, respectively (Villanueva et al., 1999; Meister et al., 2004; Kooiker et al., 2005). Both loci are associated with strong H3K27me3 enrichment (Zhang et al., 2007b; Roudier et al., 2011). GA repeats can be found in the

regulatory sequences of many other PcG targets, such as *AG*, *FLOWERING LOCUS C (FLC)*, and *STM* (Schatlowski et al., 2008). In *Drosophila*, the *GAGA* factors were shown to be involved in the regulation of gene expression in relation with chromatin packaging (Adkins et al., 2006; Nakayama et al., 2007). Therefore, it is very tempting to speculate that there is an interaction between BPC and PcG silencing in plants. The availability of new and multiple *bpc* mutants will give the opportunity to further investigate this hypothesis, by looking at the chromatin landscape, at different loci containing both type of elements, such as *LEC2*, but also *INO*, *STM*, *FLC*, or *STM*.

No sequences showing similarity with *RLE* were detected genome-wide. This is not surprising considering that *PREs* are made of different short transcription factor binding sites. These motifs are usually small, degenerate, and difficult to detect by simple DNA sequence comparison (Ward and Bussemaker, 2008; Badis et al., 2009; Moyroud et al., 2011). Additional functional analyses, similar to those presented in this report, will be necessary to establish a model allowing a more systematic identification of putative *RLE* elements in plant genomes. Sequence analysis of *P_{LEC2}* revealed the presence of three transposable elements (Figure 7). The transcription start site and the *GAGA* box are included in a class II transposable element sequence of the MuDR superfamily (Lisch, 2002). The *CArG* motif is located at the beginning of a HELITRON element (Kapitonov and Jurka, 2007; Yang and Bennetzen, 2009), which is itself juxtaposed with another MuDR sequence. Finally, *RLE* is flanked by the two MuDR sequences. It can be reasonably speculated that insertion of these transposable elements likely shaped the specific expression pattern of *LEC2*. It has been shown that transposons can be involved in transcriptional regulation of nearby genes, including *PRE*, although the precise mechanisms remain unknown and seem to be rather gene specific (Valenzuela and Kamakaka, 2006; Weil and Martienssen, 2008; Comet et al., 2011). In addition, selected transposons are specifically targeted by the PCR2 complex in *Arabidopsis* endosperm (Weinhofer et al., 2010). However, the hypothesis that transposons have a general function in recruiting or regulating PcG activity in plants requires further experimental data.

To conclude, the data presented here demonstrate that the developmental regulation of *LEC2* expression results from a combination of activating and repressive mechanisms involving histone H3 methylation. This regulation involves at least three distinct *cis*-regulatory elements located within the proximal *P_{LEC2}* promoter, including repressive *RLE* element. Remarkably, *RLE* is sufficient to initiate H3K27me₃ deposition and to repress transcription in a developmentally regulated manner. This study provides important new insights into the mechanism by which PRC2 might be recruited at specific target loci and paves the way for a more comprehensive understanding of PcG-mediated gene silencing in plants.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana, accession Wassilewskija, was used for plant transformation. The mutant lines *bpc1* (SALK_072966.43.30.x), *bpc2*

(SALK_090810C), and *bpc3* (N89719) were obtained from the Nottingham Arabidopsis Stock Centre (Scholl et al., 2000) and have all been described by Monfared et al. (2011). Plants were grown, transformed, and selected as previously described (Harscoët et al., 2010).

Constructs and Plant Transformation

All the primers used in this work are described in Supplemental Table 1 online. For 5'-end deletions of the *LEC2* promoter (*P_{LEC2}*), genomic DNA was PCR amplified with the Phusion DNA polymerase (Finnzymes) using primers carrying B1 and B2 GATEWAY recombination sites (Invitrogen). PCR products were cloned by BP recombination (according to the manufacturer's recommendations) into the entry vector pDONR207 and then LR recombined into the destination vector pBI101R1R2 for plant transformation (Baudry et al., 2004). Modified CT repeat constructs were performed similarly using 3' primers as follows: AttB2mCA, AttB2mAT, and AttB2m+3CT.

The 3'-end deletion series constructs were PCR amplified using 5' and 3' oligonucleotides carrying *KpnI* and *XhoI* restriction sites, respectively. The 5' primer used was P500*KpnI*, and the 3' primers were P250*XhoI*, P200*XhoI*, P190*XhoI*, P180*XhoI*, P170*XhoI*, P160*XhoI*, P150*XhoI*, P100*XhoI*, and P50*XhoI*. PCR products were digested with *KpnI-XhoI* and cloned into *KpnI-XhoI*-digested pBS35sminGUS (Debeaujon et al., 2003). *P_{LEC2}* fragments fused to the minimum 35S promoter and the *UidA* reporter gene were transferred into pCAMBIA1300 linearized by *HindIII-KpnI* digestion and ligated with T4 DNA ligase (New England Biolabs).

For the *P_{LEC2}rand* construct, the fragment P500-200 was amplified with the Phusion DNA polymerase using the 5' primer P500*KpnI* and the 3' primer P200rand*XhoI*. The sequence (5'-CTTAAGACATTAGCAAA-CACTGGACATGCTGACCAAGTCAGTATCCGCAT-3') was randomly chosen, and in silico analysis was performed to ensure that no known *cis*-regulatory elements were found in the sequence. The PCR product was digested with *KpnI-XhoI* and cloned into *KpnI-XhoI*-digested pBS35sminGUS (Debeaujon et al., 2003). The minimum 35S promoter was discarded after *XhoI-NcoI* digestion of the p200rand-pBS35sminGUS and replaced by the PCR amplified fragment between -150 and the translational start site, with P150*XhoI* and PATGNcoI 5' and 3' primers, respectively. The *F3H* promoter sequence was PCR amplified with Phusion DNA polymerase and primers AttB1PF3H and AttB2PF3H carrying B1 and B2 GATEWAY recombination sites. *P_{F3H}ire* and *P_{F3H}rand* were amplified with three successive PCRs using AttB1PF3H/PF3Hrev1, AttB1PF3H/PF3Hrev2, and AttB1PF3H/AttB2PF3H and were cloned using the approach described for wild-type *PF3H*. All these promoter fragments were recombined in the entry vector pDONR207 and then LR recombined into pBI101R1R2 for plant transformation, as described above.

One-Hybrid Assays

Reporter constructs containing the different promoters were made in the pHIS-i plasmid (Clontech), which carries the *URA3* selection marker and the minimal *HIS3* promoter upstream of the *HIS3* reporter gene (Li and Herskowitz, 1993). The 500-bp-long *LEC2* promoter (*P_{LEC2}*) was PCR amplified with the Phusion DNA polymerase with P500*KpnI* and PATGNcoI 5' and 3' primers, respectively, and blunt cloned into pHIS-i digested with *SmaI*. The control (-500 to -250 fragment of the *LEC2* promoter) was cloned using the same method with 5' primer P500*KpnI* and the 3' primer P250*LEC2* (see Supplemental Table 1 online). *LEC2* and *BPCs* cDNAs were amplified with the Phusion DNA polymerase using primers carrying B1 and B2 GATEWAY recombination sites. PCR products were BP recombined according to the manufacturer's (Invitrogen) recommendations into pDONR207 and then LR recombined into pDEST22 vector that allows fusion with the GAL4-activating domain. pHIS-i constructs

were stably transformed into the EGY 48 yeast (*Saccharomyces cerevisiae*) strain and selected on SD-Uracil medium prior to transformation with pDEST22 vectors for protein expression. The interactions were tested on SD-Uracil-Trp-His medium. To increase the stringency of the test, various concentrations of 3-aminotriazol were used, from 5 to 50 mM.

EMSA Experiments

The cDNAs obtained for one-hybrid experiments were introduced into pDEST15 (Invitrogen), which produces an N-terminal glutathione S-transferase tag fusion. BPCs proteins were produced in *Escherichia coli* and purified with the Glutathione Sepharose 4B system (Amersham Biosciences) according to the manufacturer's instructions. One hundred nanograms of both forward and reverse primers (EMSACT/mAT forw and rev) were boiled for 5 min and annealed at room temperature. Double-stranded probes were ³²P labeled after Klenow treatment (Invitrogen). Binding conditions have been previously described (Kooiker et al., 2005).

Histochemical GUS Assays

Arabidopsis tissues were immersed in GUS staining solution (100 mM sodium phosphate buffer, pH 7.2, 10 mM EDTA, 0.1% Triton, and 1 mM X-GlcA) and then placed under vacuum for 1 h. After incubation at 37°C overnight, the staining solution was removed and samples were cleared by sequential changes of 75 and 95% (v/v) ethanol.

ChIP Experiments

The experiments were performed as previously described (Gendrel et al., 2002). Briefly, all the experiments have been duplicated and performed using pools of five independent transformants. Relative enrichment was measured by comparison between input and ChIP value. *ACT7* was used as a negative control (H3K27me3 modification is barely detectable over the *ACT7* locus), and *FWA* was used as a positive control for H3K27me3 enrichment (Roudier et al., 2011). Primers used in this analysis are described in Supplemental Table 1 online.

Analysis of Gene Expression

RNA extractions were performed using the total mammalian RNA kit (Sigma-Aldrich) according to the manufacturer's instructions. cDNAs were synthesized using the SuperScriptII first-strand synthesis system for RT-PCR (Invitrogen) and dT oligonucleotides. The PCR experiments were conducted as previously described (Baud et al., 2005), with specific primers described in Supplemental Table 1 online. The data presented are the results of three biological and three technical repeats.

Accession Numbers

Sequence and mutant data from this article can be found in the GenBank/EMBL data libraries or the Arabidopsis Genome Initiative database under the following accession numbers: *bpc1* (SALK_072966.43.30), *bpc2* (SALK_090810C), *bpc3* (N89719), *BPC1* (At2g01930), *BPC2* (At1g14685), *BPC3* (At1g68120), *BPC4* (At2g21240), *BPC5* (At4g38910), *BPC6* (At5g42520), *BPC7* (At2g35550), *LEC2* (At1g28300), *ACT7* (At5g09810), *FWA-5* (At4g25530), and *EF1α4* (AT5G60390).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Complementation of the *lec2-4* Mutation by *P_{LEC2}:LEC2*.

Supplemental Figure 2. Activation of *PLEC2* in Pollen by Adding 3CT Repeats in the GAF Box of *P_{LEC2}*.

Supplemental Figure 3. *LEC2* Expression Is Not Modified in *bpc* Mutants.

Supplemental Figure 4. Expression Patterns of *PLEC2:GUS* and *PLEC2rand:GUS* in the Col-0 Ecotype Background.

Supplemental Table 1. List of Primers Used in This Study.

ACKNOWLEDGMENTS

Part of this work was supported by grants from the Agence Nationale de la Recherche to L.L., namely, TF-code (BLAN-07-185566) and CERES (BLAN-10-123801). We thank Charles S. Gasser for providing *bpc* mutant seeds. Our laboratory belongs to the Laboratoire d'Excellence Saclay Plant Sciences (<https://www6.inra.fr/saclay-plant-sciences>).

AUTHOR CONTRIBUTIONS

N.B., B.D., and C.D. performed the experiments. All the authors designed the research, analyzed data, and wrote the article.

Received May 31, 2011; revised October 11, 2011; accepted October 30, 2011; published November 11, 2011.

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Transcriptional Regulation of *Arabidopsis* *LEAFY COTYLEDON2* Involves *RLE*, a *cis*-Element That Regulates Trimethylation of Histone H3 at Lysine-27

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Plant Cell 2011;23;4065-4078; originally published online November 11, 2011;
DOI 10.1105/tpc.111.087866

This information is current as of May 2, 2013

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