



LEAFY and APETALA1 down-regulate ZINC FINGER PROTEIN 1 and 8 to release their repression on class B and C floral homeotic genes

Tieqiang Hu^a, Xiaohui Li^{b,c}, Liren Du^a, Darren Manuela^a, and Mingli Xu^{a,1}

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Organ initiation from the shoot apical meristem first gives rise to leaves during vegetative development and then flowers during reproductive development. *LEAFY* (*LFY*) is activated after floral induction and together with other factors promotes the floral program. *LFY* functions redundantly with *APETALA1* (*AP1*) to activate the class B genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), the class C gene *AGAMOUS* (*AG*), and the class E gene *SEPALLATA3*, which leads to the specification of stamens and carpels, the reproductive organs of flowers. Molecular and genetic networks that control the activation of *AP3*, *PI*, and *AG* in flowers have been well studied; however, much less is known about how these genes are repressed in leaves and how their repression is lifted in flowers. Here, we showed that two genes encoding *Arabidopsis* C2H2 ZINC FINGER PROTEIN (ZFP) transcription factors, *ZP1* and *ZFP8*, act redundantly to directly repress *AP3*, *PI*, and *AG* in leaves. After *LFY* and *AP1* are activated in floral meristems, they down-regulate *ZP1* and *ZFP8* directly to lift the repression on *AP3*, *PI*, and *AG*. Our results reveal a mechanism for how floral homeotic genes are repressed and derepressed before and after floral induction.

ZP1 | ZFP8 | AP3 | PI | AG

In most angiosperms, the shoot first produces leaves then flowers in their life cycle. The transition from vegetative development to reproductive development is tightly controlled by multiple pathways that respond to both endogenous and environmental cues (1, 2). These pathways converge on a set of floral integrators that activate floral meristem identity (FMI) genes such as *LEAFY* (*LFY*) and *APETALA 1* (*AP1*). *LFY* and *AP1* activate each other and a set of floral homeotic genes to specify floral organ identities in floral meristems (3–5). An *Arabidopsis* flower consists of four whorls of floral organs with four sepals in the first whorl, four petals in the second whorl, six stamens in the third whorl, and two fused carpels in the fourth whorl. These different floral organ identities are specified by different combinations of floral homeotic gene activities as specified in the ABCE model. The outer sepals are specified by class A+E activities and the petals specified by class A+B+E activities. The reproductive organs of a flower, stamen and carpel, are specified by class B+C+E and class C+E activities, respectively (6). *AP1*, a class A gene in addition to its role in FMI, the class B genes *AP3* and *PISTILLATA* (*PI*), the class C gene *AGAMOUS* (*AG*), and the class E gene *SEPALLATA 3* (*SEP3*) are all direct targets of *LFY* activation. *SEP3* is not only an *LFY* direct target, but also a *LFY* cofactor in the direct activation of *AP3*, *PI*, and *AG* (7, 8).

While much work has focused on the activation of *AP3*, *PI*, and *AG* in the floral meristems, little is known about how these genes are silenced in leaves and how this silencing is released in FMs. Indeed, silencing of these genes is important for proper development of a plant (9, 10). *AP3* and *AG* are silenced in vegetative leaves by polycomb repressive complex 2 (PRC2), which catalyzes trimethylation of histone 3 lysine 27 (H3K27me3) on their chromatin (11, 12). Whether transcription factors are involved in the silencing of *AP3*, *PI*, and *AG* and how the silencing is lifted remain unknown. Here, we showed that two zinc finger protein transcription factors, *Arabidopsis* ZINC FINGER PROTEIN 1 (*AtZP1*, *ZP1*) and *ZFP8*, are involved in the repression of *AP3*, *PI*, and *AG* in leaves. It was reported that *ZP1* is involved in the suppression of root hair initiation and elongation, and *ZFP8* is involved in epidermal trichome initiation (13–15). *ZP1* has an Ethylene-responsive element binding factor-associated Amphiphilic Repression (EAR) motif that carries transcriptional repressor activities (13). Here, we found that *ZP1* and *ZFP8* repress *AP3*, *PI*, and *AG* in vegetative leaves and are down-regulated by *LFY* and *AP1* in floral meristems to lift the repression on *AP3*, *PI*, and *AG*. Therefore, *LFY* activates the expression of *AP3*, *PI*, and *AG* both directly and indirectly to ensure robust production of flowers after floral induction (3, 7, 8).

Significance

Proper repression of the floral homeotic genes *AP3*, *PI*, and *AG* in leaf primordia and activation of them in floral meristems is essential for reproductive fitness. Many studies have revealed molecular details about the activation of *AP3*, *PI*, and *AG* in floral meristems, but relatively less is known about how they are repressed in leaves and how this repression is lifted in floral meristems. Two genes encoding *Arabidopsis* ZINC FINGER PROTEIN (ZFP) transcription factors, *ZP1* and *ZFP8*, are highly expressed in leaf primordia and down-regulated in floral primordia. Here, we show that *ZP1* and *ZFP8* repress these floral homeotic genes in leaves. *LFY* and *AP1* down-regulate *ZP1* and *ZFP8* in floral meristems, lifting the repression on floral homeotic genes.

Author affiliations: ^aDepartment of Biological Sciences, University of South Carolina, Columbia, SC 29208; ^bDepartment of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907; and ^cCenter for Plant Biology, Purdue University, West Lafayette, IN 47907

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The authors declare no competing interest.

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¹To whom correspondence may be addressed. Email: minglixu@sc.edu.

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Results

LFY and AP1 Repress *AtZP1* and *ZFP8* in Developing Flowers. The C2H2 zinc finger protein *AtZP1* (AT4G17810, *ZP1*) is required for root hair initiation and elongation (13), but its roles in the aerial parts of a plant remain unknown. To analyze the role of *ZP1* in *Arabidopsis* shoot development, we cloned a 5.5 kb-DNA fragment upstream of the *ZP1* transcription start site (TSS) and fused it with β -glucuronidase (*ZP1::GUS*). A temporal analysis of *ZP1* expression in the shoot by this construct showed that *ZP1* is highly expressed in developing vegetative leaves and is down-regulated in flowers and fruits (Fig. 1A). Next, we examined vegetative and reproductive development in a *zpl1* T-DNA insertion line (*zpl1-3*, SAIL_24_B09). qRT-PCR analysis on this mutant line showed that there was an approximately 75% reduction of *ZP1* transcripts in this mutant, suggesting that this might be a strong allele (*SI Appendix*, Fig. S1A and B). Further phenotypic analysis on the number of juvenile leaves, adult leaves, cauline leaves, and floral organs showed that there was no significant difference between this mutant and Col-0 (*SI Appendix*, Fig. S1C–E). However, plants overexpressing *ZP1* (*35S::ZP1*) never flowered in long-day (LD) conditions (*SI Appendix*, Fig. S1F). *ZP1* is a member of the C2H2 ZFP transcription factor family that consists of 176 members, and the absence of a phenotype in the loss-of-function mutant might result from genetic redundancy. To identify potential C2H2 ZFPs that might act redundantly with *ZP1*, we performed RNA-seq analysis on 2-wk-old shoot apex and 4-wk-old inflorescence of Col-0. *ZP1* has one zinc finger domain and belongs to the C1-1i subset with other 32 members (*SI Appendix*, Fig. S2). Our results showed that in the C1-1i subset family, *ZP1*, *ZFP4*, *ZFP7*, and *ZFP8* were down-regulated more than two fold in 4-wk-old inflorescence, while *SUPERMAN* (*SUP*), *KNUCKLES* (*KNU*), and *RABBIT EARS* (*RBE*), three ZFPs that were known to function in floral organ development, were up-regulated in 4-wk-old inflorescence. *ZFP8* was reported to function redundantly with *GLABROUS INFLORESCENCE STEMS* (*GIS*) and *GIS2* to initiate epidermal trichomes in leaves, fruits, and stems (14). To confirm the RNA-seq results, we performed qRT-PCR analysis in LD-grown 2-wk-old shoot apex and 4-wk-old Col-0 inflorescence. Floral organs were not visible in 2-wk-old plants but were well developed in 4-wk-old plants, and the floral organ identity ABCE genes (*API*, *AP2*, *AP3*, *PI*, *AG*, and *SEP3*) were highly up-regulated in 4-wk-old tissues (Fig. 1B). *ZP1*, *ZFP4*, *ZFP7*, and *ZFP8* were down-regulated about 60 to 80% from 2-wk to 4-wk-old tissues (Fig. 1B and (*SI Appendix*, Fig. S3A), while *SUP*, *KNU*, and *RBE* were up-regulated 5 to 100 folds (*SI Appendix*, Fig. S3A). We examined the relative expression levels of *ZP1*, *ZFP4*, *ZFP7*, and *ZFP8* in cotyledons; young leaves (petiole have not developed yet); mature leaves; cauline leaves; the 1-wk-old shoot apex; and flowers at stages 4 to 6, stages 7 to 9, and stages 10 to 12 by qRT-PCR. Our results showed that *ZP1* was expressed at higher levels in young leaves than that of cotyledons, it was down-regulated 30 to 40% in mature leaves, cauline leaves, 1-wk-old shoot apex, and it was down-regulated more than 90% in floral organs from stages 4 to 12 (Fig. 1C). Transcripts of *ZFP4*, *ZFP7*, and *ZFP8* were not significantly changed in young leaves and mature leaves, nor shoot apex compared to their levels in cotyledons. However, they were down-regulated 90% in floral organs from stages 4 to 12 (Fig. 1C and (*SI Appendix*, Fig. S3C). As *LFY* and *AP1* promote FMI and floral organogenesis, we wondered whether downregulation of *ZP1*, *ZFP4*, *ZFP7*, and *ZFP8* in Col-0 inflorescence might be regulated by *LFY* and *AP1* (3, 4, 8, 16). To investigate the possible regulation of *ZP1*, *ZFP4*, *ZFP7*, and *ZFP8* by *LFY* and *AP1*, we analyzed the abundance of *ZP1*, *ZFP4*,

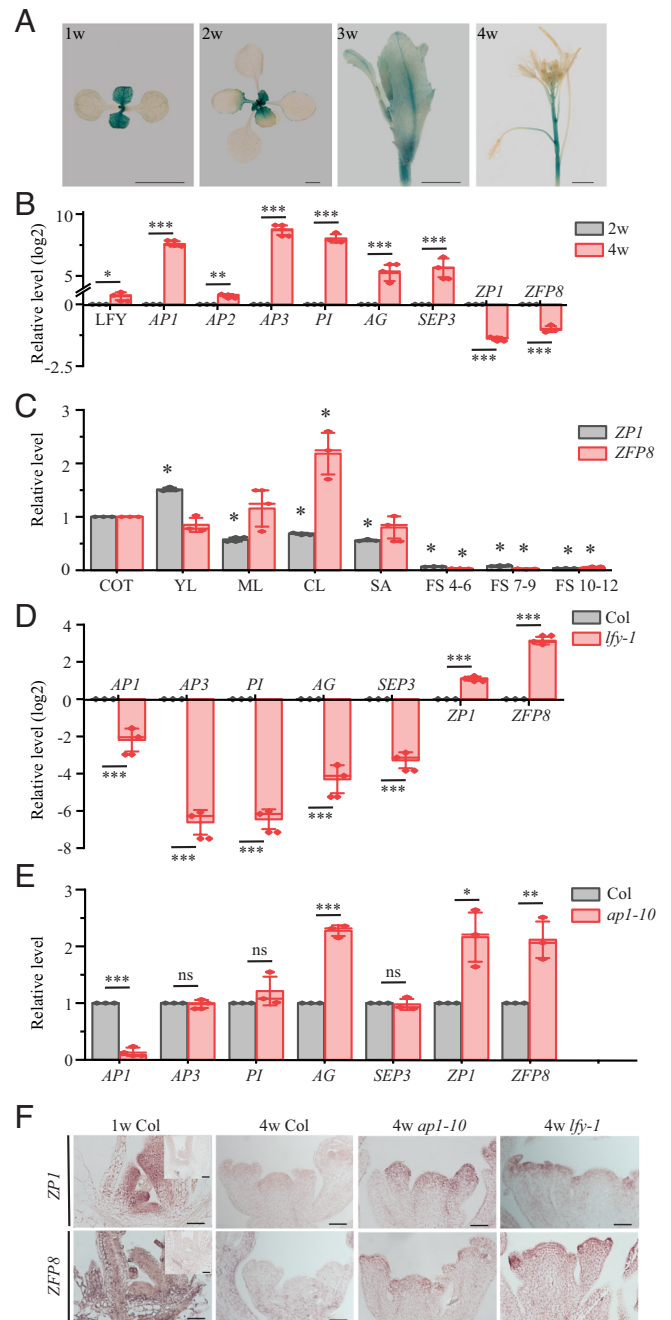


Fig. 1. LFY and AP1 repress *ZP1* and *ZFP8* during floral organogenesis. (A) Expression of *ZP1::GUS* in 1-wk-, 2-wk-, 3-wk-, and 4-wk-old plants grown in LD conditions. (Scale bars: 3 mm.) (B) qRT-PCR analysis of ABCE genes, *ZP1* and *ZFP8* in 2-wk-old Col shoot apex (including the SAM and leaf primordia smaller than 2 mm), and 4-wk-old Col inflorescence (excluding flowers at stage 12 and above). Levels of transcripts of each gene in 2-wk-old shoot apex were set to 1, and the values were log₂ of their fold change. (C) qRT-PCR analysis of *ZP1* and *ZFP8* in different tissues of Col. COT: cotyledons; YL: young leaf; ML: mature leaf; CL: cauline leaf; SA: 1-wk-old shoot apex; FS4-6: flowers at stage 4 to 6; FS7-9: flowers at stage 7 to 9; FS10-12: flowers at stage 10 to 12. Transcripts from *ZP1* or *ZFP8* in cotyledons were set to 1. * Significantly different from the levels in COT, $P < 0.05$, t test. (D and E) qRT-PCR analysis of ABCE genes, *ZP1* and *ZFP8* in 4-wk-old *lfy-1* (D), and *ap1-10* (E). Transcripts from Col were set to 1, and the values in D were log₂ of their fold change. Statistical analysis in B–E: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, $p > 0.05$, one-way ANOVA. (F) In situ hybridization analysis of *ZP1* and *ZFP8* in 1-wk-old Col shoot apices, 4-wk-old Col, *ap1-10*, and *lfy-1* inflorescence apices. Insets are *ZP1* and *ZFP8* in 1-wk *zpl1-3* and *zfp8*, respectively. (Scale bars: 50 μ m.)

ZFP7, and *ZFP8* transcripts in 4-wk-old Col-0, *lfy-1*, and *ap1-10* inflorescence. *API*, *AP3*, *PI*, *AG*, and *SEP3*, direct targets of LFY (8, 16, 17), were down-regulated in *lfy-1*, while *ZP1*, *ZFP4*,

and *ZFP8* were up-regulated in *lfy-1* (Fig. 1D and *SI Appendix, Fig. S3D*). Transcripts of *AP3*, *PI*, and *SEP3* were not affected in *ap1-10*; however, *ZP1*, *ZFP4*, and *ZFP8* were up-regulated in *ap1-10* (Fig. 1E and *SI Appendix, Fig. S3E*).

To examine how LFY and AP1 repress *ZP1* and *ZFP8* spatially, we performed in situ hybridization analysis. *ZP1* and *ZFP8* could be detected in shoot apical meristems (SAM) and leaf primordia from 1-wk-old Col-0 (Fig. 1F). In reproductive tissues, *ZP1* and *ZFP8* mRNA could only be slightly detected in the inflorescence and stage 1 to stage 4 floral meristems. However, they could be detected at higher levels in *lfy-1* and *ap1-10* stage 1 to 4 floral meristems (Fig. 1F). Together, the qRT-PCR and in situ hybridization results showed that *ZP1* and *ZFP8* are highly expressed in vegetative tissues and were down-regulated in floral meristems by LFY and AP1 when floral organ identity genes are activated.

LFY and AP1 Bind to *ZP1* and *ZFP8* Directly. The downregulation of *ZP1* and *ZFP8* by LFY and AP1 prompted us to examine whether LFY and AP1 directly regulate *ZP1* and *ZFP8* expression. We made a *LFY::GFP-LFY* construct (18), transformed it into *lfy-1* heterozygotes, and selected *LFY::GFP-LFY lfy-1* that complemented *lfy-1* mutant (*SI Appendix, Fig. S4*). Flower development is synchronized in *35S::AP1-GR ap1 cal* plant, and floral organ development could be activated by dexamethasone (Dex) treatment (17). We collected inflorescence for chromatin immunoprecipitation qPCR (ChIP-qPCR) analysis 24 h after mock or Dex treatment. Potential LFY or AP1 binding sites upstream of *ZP1* and *ZFP8* were selected for ChIP-qPCR analysis based on previous genome-wide ChIP seq analysis that showed peaks for LFY and AP1 association (17, 19). Our ChIP-qPCR

analysis showed that LFY and AP1 bind to the same regions of the *ZFP8* promoter, and LFY has a broader binding region than that of AP1 at *ZP1* promoter (Fig. 2).

Expression of *ZP1* and *ZFP8* under the Control of LFY and AP1 Promoters Prevents Activation of Class B and C Genes. To examine the biological relevance of the downregulation of *ZP1*, *ZFP4*, *ZFP7*, and *ZFP8* by LFY and AP1, we expressed the coding region of *ZP1*, *ZFP4*, *ZFP7*, and *ZFP8* behind the promoters of *LFY* (18) and *AP1* (3.5 kb). *LFY::ZP1*, *LFY::ZFP4*, *LFY::ZFP7*, *LFY::ZFP8*, *AP1::ZP1*, *AP1::ZFP4*, *AP1::ZFP7*, and *AP1::ZFP8* were transformed into Col-0, and vegetative and reproductive development was analyzed in these plants. The number of rosette leaves was not significantly changed in these transgenic plants, except that there was a small reduction of rosette leaves in *AP1::ZFP8* (*SI Appendix, Fig. S5A*). Inflorescences from *LFY::ZP1* have many visible leaves, and there were large bracts subtending flowers as the inflorescence elongated (Fig. 3C). The basal flowers from *LFY::ZP1* are fertile, with reduced number of petals and stamens (Fig. 3I). Many of the later-arising floral meristems from *LFY::ZP1* were arrested at early stages such that petals, stamens, and carpels were not visible (Fig. 3I, *Inset*). *LFY::ZFP8* plant produced more secondary inflorescences than those of Col-0 (*SI Appendix, Fig. S5B*), and flowers from *LFY::ZFP8* were subtended with small bracts, had no petals, occasionally had 1 or 2 stamens (*SI Appendix, Fig. S5C*), and had carpels that were not properly fused (Fig. 3J). Flowers from *AP1::ZP1* and *AP1::ZFP8* were very much like flowers from *LFY::ZFP8*, and they all look like flowers lack of class B and C activities (Fig. 3K and L). Floral organ identities in *LFY::ZFP4*, *LFY::ZFP7*, *AP1::ZFP4*,

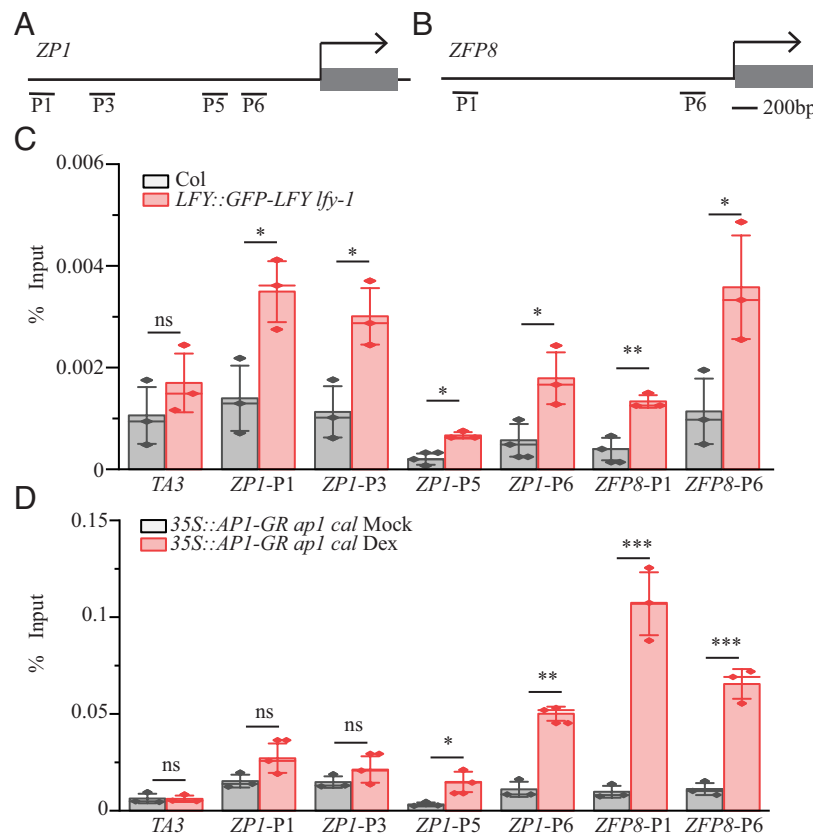


Fig. 2. LFY and AP1 bind to *ZP1* and *ZFP8* directly. (A and B) Schematic structure of *ZP1* (A) and *ZFP8* (B) and localization of potential LFY binding sites. Arrow indicates TSS, and gray box represents the first exon. (C) ChIP-qPCR analysis of *ZP1* and *ZFP8* fragments in Col and *LFY::GFP-LFY lfy-1*. (D) ChIP-qPCR analysis of *ZP1* and *ZFP8* fragments in *35S::AP1-GR ap1 cal* plant treated by mock or Dex. ns denotes not significantly different groups, $P > 0.05$. Stars denote significantly different groups, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, $p > 0.05$, one-way ANOVA. The *TA3* retrotransposon (AT1G37110) served as a negative control locus for ChIP-qPCR.

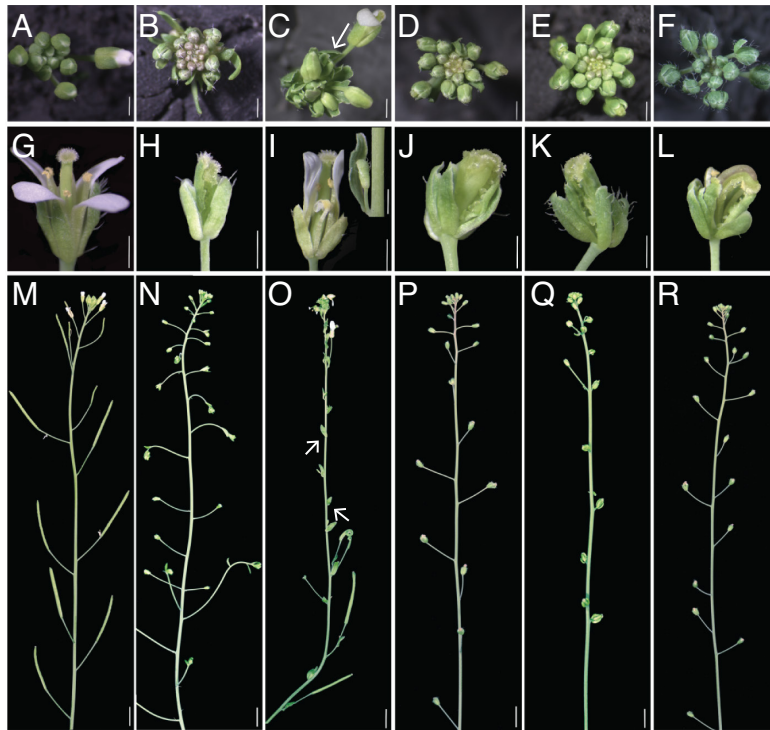


Fig. 3. Expression of *ZP1* and *ZFP8* driven by *LFY* and *AP1* promoter, respectively, resulted in loss of floral organ identity. (A–F) Top view of inflorescences from Col (A), *lfy-1* (B), *LFY::ZP1* (C), *LFY::ZFP8* (D), *AP1::ZP1* (E), and *AP1::ZFP8* (F). (G–L) Flowers from Col (G), *lfy-1* (H), *LFY::ZP1* (I), *LFY::ZFP8* (J), *AP1::ZP1* (K), and *AP1::ZFP8* (L). In *LFY::ZP1*, basal flowers had reduced number of petals and stamens and apical flowers were arrested at earlier development stages that petals and stamens were not visible (Inset), and bracts developed with each flower (arrows). In *LFY::ZFP8*, *AP1::ZP1*, and *AP1::ZFP8*, petals and stamens were not properly developed, and carpels were not properly fused, resembling *lfy-1* flowers. (M–R) Inflorescences from Col (M), *lfy-1* (N), *LFY::ZP1* (O), *LFY::ZFP8* (P), *AP1::ZP1* (Q), and *AP1::ZFP8* (R). (Scale bars in top two rows: 1 mm, scale bars in bottom row: 5 mm.)

and *AP1::ZFP7*, however, were not affected (SI Appendix, Fig. S5 C and D).

qRT-PCR analyses on these transgenic plants showed that *LFY* was slightly down-regulated (about 10 to 15%) in *LFY::ZP1* and *AP1::ZFP8* and was not changed in *LFY::ZFP8* nor in *AP1::ZP1*. Interestingly, transcripts of *AP1* were up-regulated (about 1.5-fold to twofold), and transcripts of *AP2* were not changed or slightly down-regulated (Fig. 4 A–D). The class B genes *AP3* and *PI* and the class C gene *AG* were down-regulated in all the backgrounds. In *LFY::ZP1* which produces some fertile flowers, transcripts of *AP3*, *PI*, and *AG* were down-regulated 60 to 70%. In *LFY::ZFP8*, *AP1::ZP1*, and *AP1::ZFP8*, which have fewer fertile flowers than those of *LFY::ZP1*, transcripts of *AP3*, *PI*, and *AG* were down-regulated 70 to 90% (Fig. 4 A–D). Transcripts of *SEP3* were not significantly changed in any of them (Fig. 4 A–D), suggesting that the downregulation of *AP3*, *PI*, and *AG* in the transgenic plants was not caused by reduced activities of *LFY*. Upregulation of *AP1* may have resulted from reduced activities of *AG*, which acts antagonistically to *AP1* (20, 21).

Next, we examined the spatial expression of *LFY*, *AP1*, *AP3*, *PI*, and *AG* in these transgenic plants by in situ hybridization. *LFY* and *AP1* were detected in stage 1 and stage 2 floral meristems in the transgenic plants, comparable to their expression in Col-0 (Fig. 4E). In Col-0, *AP3* and *PI* were easily detected in stage 3 and stage 4 floral meristems in the second and third whorls where petal and stamen would be initiated. *AP3* and *PI* were absent or much reduced in the second and third whorls of *LFY::ZP1*, *LFY::ZFP8*, *AP1::ZP1*, and *AP1::ZFP8* or only detected at the base of the third whorl close to its boundary with the fourth whorl (Fig. 4E). In Col-0, *AG* was expressed in the center of the floral meristem that would give rise to stamens and carpels. However, *AG* expression was dramatically reduced or present in a narrower domain in these

transgenic plants (Fig. 4E). Together, the qRT-PCR and in situ hybridization analysis showed that *AP3*, *PI*, and *AG* were significantly down-regulated in these transgenic plants, while *LFY* and *AP1* were maintained at similar levels as they were in Col-0. Our results suggest that downregulation of *ZP1* and *ZFP8* in the *LFY* and *AP1* expression domain is essential for upregulation of *AP3*, *PI*, *AG*, and proper development of petal, stamen, and carpel identities.

Rescue of Petal Development in *ap1-10* and *lfy-1* by Loss-of-Function *zfp1 zfp8*. *LFY* and *AP1* activate each other, and they function redundantly to activate the expression of *AP3* and *PI* (3–5, 16, 17). To examine whether downregulation of *ZP1* and *ZFP8* is required for floral organ development, we crossed *zfp1-3* and *zfp8* (14) (SI Appendix, Fig. S6I) to *ap1-10* and *lfy-1*. We first analyzed floral organ development in *zfp1-3* and *zfp8* single mutants and the *zfp1-3 zfp8* double mutant. Our results showed that mutations in *ZP1* and *ZFP8* did not affect flowering time, FMI, and floral organ development (SI Appendix, Fig. S6). The *ap1-10* mutant produces secondary flowers in the axils of the first whorl leaf-like organs. The primary flower usually does not contain petals, although secondary flowers may produce some petals. *ap1-10 zfp1-3* and *ap1-10 zfp8* double mutants sometimes produced three petals per flower in their secondary flowers. The *ap1-10 zfp1-3 zfp8* triple mutant produced two, three, or four petals per flower in their secondary flowers more often, significantly more than the average number of petals per flower in the double mutants (Fig. 5 A and C). Most *lfy-1* flowers do not produce petals either, with one to two petals being produced occasionally. More *lfy-1 zfp1-3* flowers had two petals, but the average number of petals per flower is not significantly different from *lfy-1*. More *lfy-1 zfp1-3 zfp8* triple mutant flowers produced

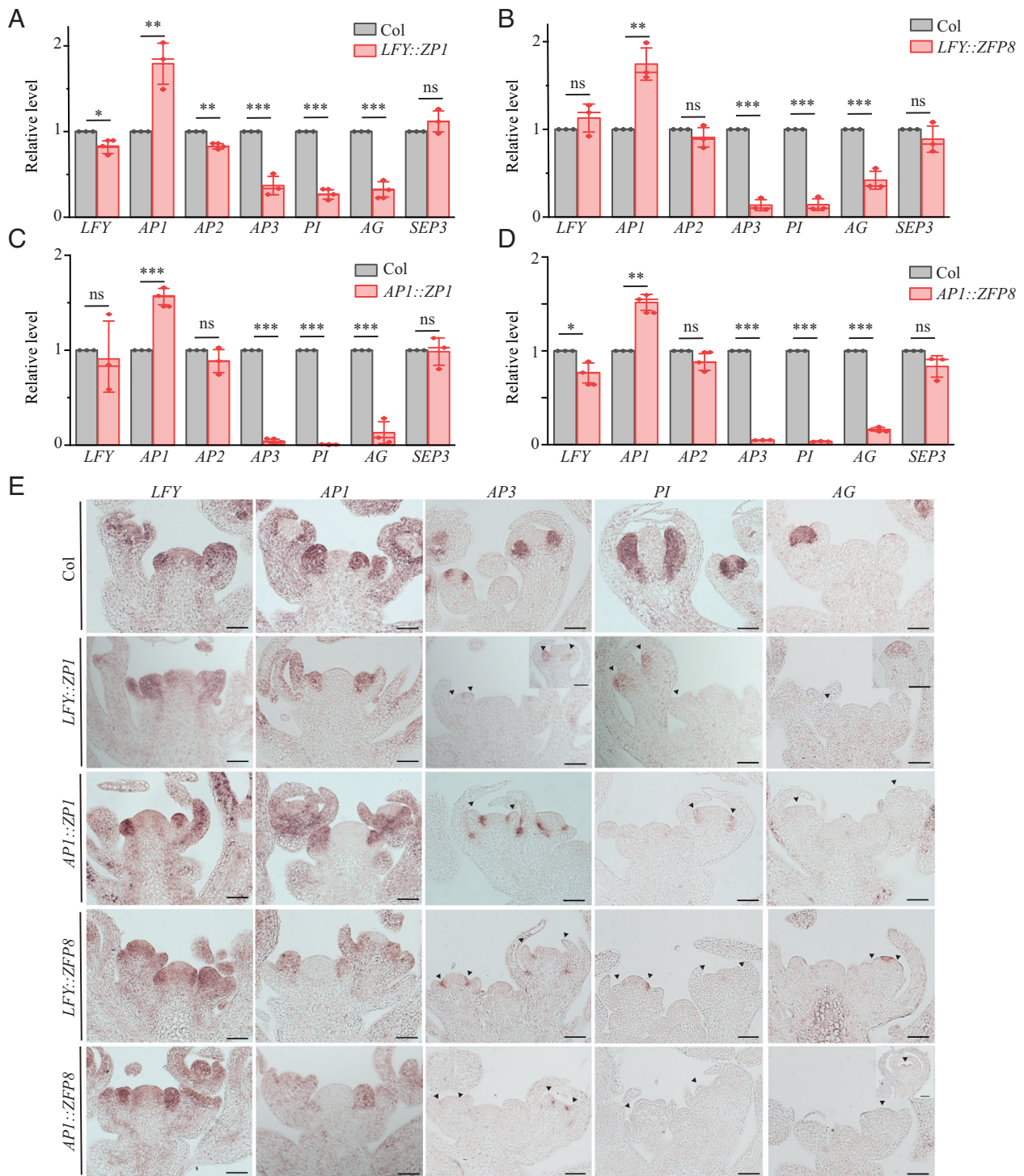


Fig. 4. Expression of ZP1 and ZFP8 driven by LFY promoter and AP1 promoter resulted in downregulation of AP3, PI, and AG. (A–D) qRT-PCR analysis of LFY and ABCE genes in 4-wk-old inflorescence of Col, LFY::ZP1 (A), LFY::ZFP8 (B), AP1::ZP1 (C), and AP1::ZFP8 (D). Transcripts of each gene in Col were set to 1, and values were relative fold to Col. Values represent the mean \pm SE from three biological replicates (diamonds). ns denotes not significant, $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA. (E) In situ hybridization analysis of LFY, AP1, AP3, PI, and AG in 4-wk-old inflorescence apices of Col, LFY::ZP1, AP1::ZP1, LFY::ZFP8, and AP1::ZFP8. (Scale bars: 50 μ m.)

two petals, and the average number of petals per flower was significantly higher than that of the double mutants (Fig. 5 A and D). The occurrence of petals was significantly increased in *ap1-10 zpl-3 zfp8* and *lfy-1 zpl-3 zfp8* triple mutants (Fig. 5 A, C, and D), suggesting that class B floral organ identity genes might be up-regulated in these backgrounds. We then examined the transcripts of AP3, PI, and AG by in situ hybridization. Our results showed that AP3, PI, and AG were easily detected in

Col-0 and *zpl-3 zfp8* stage 3 and stage 4 floral meristems, while they could hardly be detected in *lfy-1*. AP3 and PI expression was partially restored, while AG was only slightly restored in *lfy-1 zpl-3 zfp8* stage 3 and stage 4 floral meristems (Fig. 5B). Together, our results suggest that LFY and AP1 promote the activation of AP3, PI, and AG both directly and indirectly, and the indirect pathway involves redundant actions of ZP1, ZFP8, and other factors.

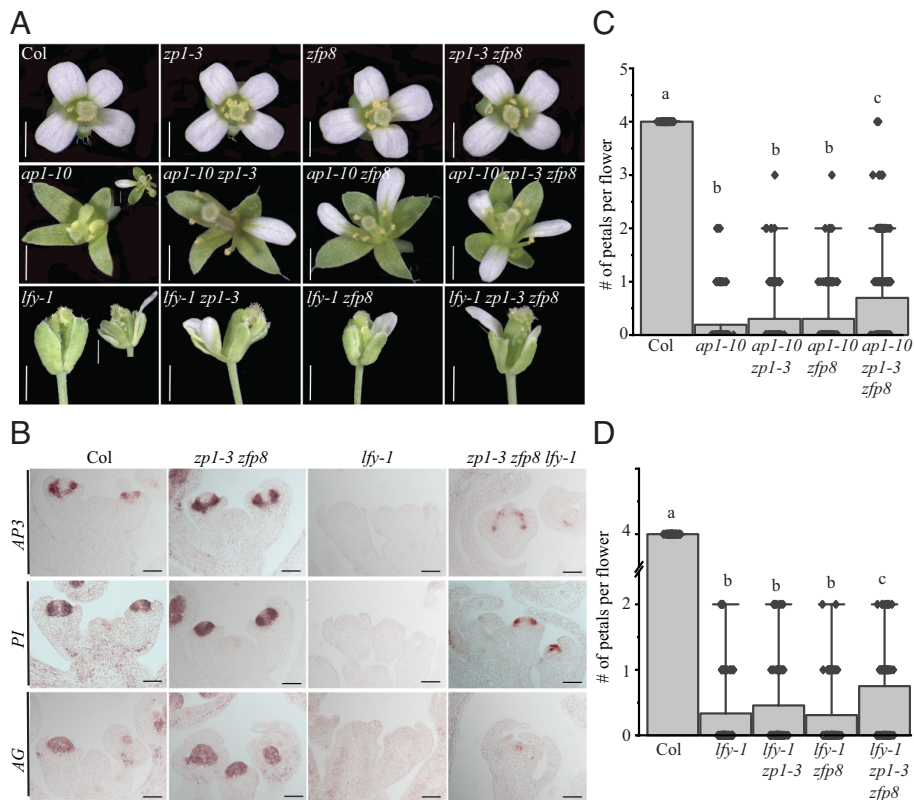


Fig. 5. Loss-of-function *zpl1-3 zfp8* partially rescues petal development in *ap1-10* and *lfy-1*. (A) Flowers from Col and mutants. (Scale bars: 1 mm.) (B) In situ hybridization analysis of *AP3*, *PI*, and *AG* in Col, *zpl1-3 zfp8*, *lfy-1*, and *lfy-1 zpl1-3 zfp8* floral meristems. (Scale bars: 50 μ m.) (C and D) Statistical analysis on the number of petals per flower in *ap1-10 zfp* mutants (C) and *lfy-1 zfp* mutants (D). Shared letters above each group (C and D) indicate not significantly different groups, $p > 0.05$. Different letters above each group indicate significantly different groups. $P < 0.05$, one-way ANOVA.

ZP1 Binds to *AP3*, *PI*, and *AG* Directly to Repress Their Expression in Vegetative Tissues.

ZP1 and *ZFP8* are expressed at relatively high levels in vegetative tissues compared to their levels in flowers at stages 4 to 12, while *AP3*, *PI*, and *AG* are expressed at low levels in 2-wk-old shoot apex when flowers are not visible and are expressed at high levels in 4-wk-old inflorescence when flowers are well developed (Fig. 1). To examine whether *ZP1* and *ZFP8* repress *AP3*, *PI*, and *AG* in vegetative tissues, we fixed 11-d-old LD-grown shoot apices from Col-0, *zpl1-3*, *zfp8*, and *zpl1-3 zfp8* for in situ hybridization analysis. *AP3*, *PI*, and *AG* could not be detected in Col-0 and *zpl1-3*, nor *zfp8*, but could be detected in a few cells in *zpl1-3 zfp8* leaf primordia (Fig. 6A). To examine whether *ZP1* represses *AP3*, *PI*, and *AG* directly, we transformed *ZP1::ZP1-HA* into *zpl1-3* mutant and selected transgenic lines which expressed *ZP1* at a higher level than Col-0 (SI Appendix, Fig. S6H). Our ChIP-qPCR results showed that *ZP1* binds to the region *AP3-2* (close to the TSS, a *LFY* binding site) (8, 22) and *AP3-5* (~400 bp upstream of the TSS). *ZP1* binds to *PI-5* (~1,100 bp upstream of the TSS), but not to *PI-2* (close to TSS, a *LFY* binding site) (Fig. 6B and C) (8). These results showed that *ZP1* could bind to *AP3* and *PI* independent of *LFY*. The ChIP-qPCR results are consistent with the in situ hybridization results that *AP3* and *PI* could be easily detected in *lfy-1 zpl1-3 zfp8*, though in a narrower domain, as well as the phenotypic analysis showing that petal development could be partially rescued in *lfy-1 zpl1-3 zfp8*. Because many regulatory elements for *AG* lie in the second intron of *AG* (23), we examined the binding of *ZP1* to the second intron of *AG*. Our mapping of the *ZP1* binding to *AG* regulatory sequences showed that *ZP1* associates with the 3' end of its second intron, which is also bound by *LFY* and *CURLY LEAF* (*CLF*, a methyltransferase in *PRC2*) (12, 22), but not to the beginning of

the second intron (*AGi-9*) (Fig. 6B and C). This suggests that *ZP1* and *LFY* may compete for binding to *AG*, which is consistent with the in situ hybridization analysis that *AG* could hardly be up-regulated in *lfy-1 zpl1-3 zfp8*, and that carpel development could not be rescued in *lfy-1 zpl1-3 zfp8* (SI Appendix, Fig. S6J). Together, our results showed that *ZP1* binds to *AP3*, *PI*, and *AG* directly in vegetative tissues to repress their expression.

Discussion

Vegetative development precedes reproductive development in most flowering plants. The homeotic genes *AP3* and *AG* are repressed by *PRC2* in vegetative tissues (11, 12). However, transcription factors mediating the repression of *AP3*, *PI*, and *AG* were unknown. Here, we showed that two *ZFP* transcription factors, *ZP1* and *ZFP8*, are highly expressed in vegetative tissues and act redundantly to repress *AP3*, *PI*, and *AG* in these tissues. Activation of *LFY* and *AP1* after floral induction leads directly to the down-regulation of *ZP1* and *ZFP8*. Thus, *LFY* and *AP1* activate *AP3*, *PI*, and *AG* both directly and indirectly (Fig. 6D). Our results suggest a mechanism for the temporal regulation of *AP3*, *PI* and *AG*, which ensures fitness and robust activation of floral program.

Ectopic expression of *ZP1* or *ZFP8* in floral meristems is sufficient to down-regulate *AP3*, *PI*, and *AG* in floral meristems. However, inactivating *ZP1* and *ZFP8* simultaneously could only slightly derepress *AP3*, *PI*, and *AG* in leaves. This suggests the presence of additional factors that function redundantly with *ZP1* and *ZFP8*. *SUP* encodes a related C2H2 zinc finger protein with an EAR repression domain. *SUP* functions to define the boundary between whorl 3 and whorl 4 to restrict the expression of *AP3* from

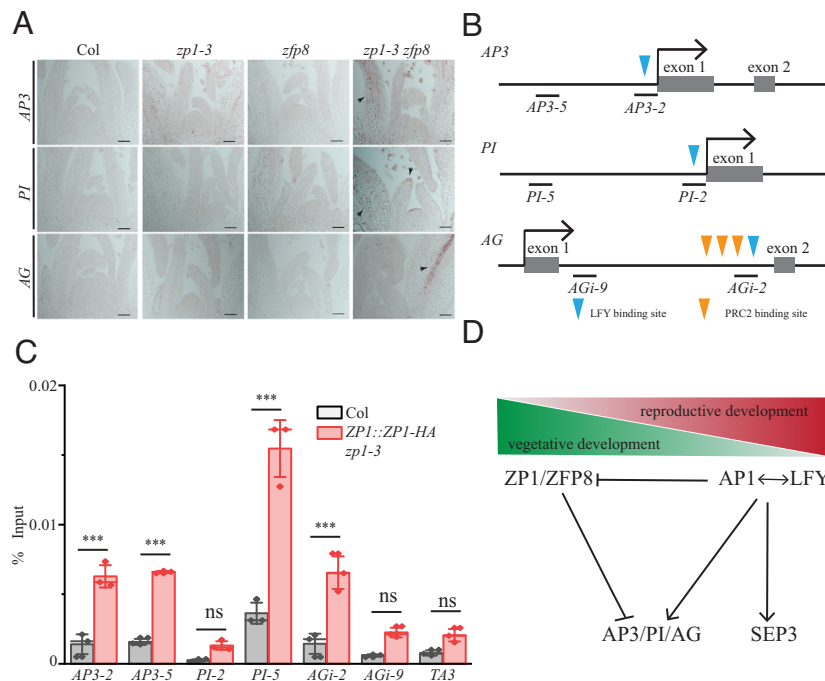


Fig. 6. ZP1 binds to *AG*, *AP3*, and *PI* directly. (A) In situ hybridization analysis of *AP3*, *PI*, and *AG* in LD-grown 11-d old shoot apices from Col, *zp1-3*, *zfp8*, and *zp1-3 zfp8* double mutant. (B) Schematic structure of *AP3*, *PI*, and *AG* and DNA fragments tested for ZP1 binding. Arrow indicates TSS, and gray box represents exons. LFY and PRC2 binding sites are represented as blue and orange triangles, respectively. (C) ChIP-qPCR analysis of *AP3*, *PI*, and *AG* fragments in Col and ZP1::ZP1-HA *zp1-3*. ns denotes not significantly different groups, $P > 0.05$. Stars denote significantly different groups, $***P < 0.001$, two-way ANOVA. (D) Model for activation of *AP3*, *PI*, and *AG*. In vegetative tissues where ZP1 and ZFP8 are highly expressed, they function redundantly (probably together with other zinc finger proteins) to repress *AP3*, *PI*, and *AG*. Once LFY and AP1 are activated in floral meristems, they down-regulate ZP1 and ZFP8 to release the repression of *AP3*, *PI*, and *AG*. LFY and AP1 activate *AP3*, *PI*, and *AG* directly in parallel to the derepression of *AP3*, *PI*, and *AG*. SEP3 is activated by LFY directly and is not repressed by ZP1 and ZFP8.

whorl 4 (24). Mutations in *sup* resulted in an expansion of *AP3* expression into whorl 4 and consequently a change of the cell fate from female to male with additional stamens replacing carpels in the fourth whorl (24–28). RBE, encoding another related C2H2 zinc finger protein, is expressed in cells giving rise to petal primordia, and is required for proper sepal and petal development through repressing *AG* in the outer whorls (29, 30). Though SUP and RBE repress *AP3*, *PI*, and *AG* in floral meristems, it is unlikely that they function redundantly with ZP1 and ZFP8 to maintain the repression of *AP3*, *PI*, and *AG* in vegetative leaves. SUP and RBE are expressed at low levels in vegetative tissues and high in floral meristems, which is opposite to the expression pattern of ZP1 and ZFP8 (SI Appendix, Fig. S3 A and B). Finding zinc finger proteins that function redundantly with ZP1 and ZFP8 in repressing homeotic gene expression in leaves is essential for understanding the temporal regulation of homeotic genes in leaves and flowers.

Another C2H2 zinc finger protein KNUCKLES (KNU), that functions in regulating floral determinacy, shares a conserved C2H2 zinc finger domain and an EAR domain with SUP, ZP1, and ZFP8 (13) (SI Appendix, Fig. S7). Genetic and molecular analyses of KNU, SUP, and ZP1 showed that they are transcriptional repressors (13, 27, 31). The transcriptional repressor activities in them might be carried out through the interaction between the EAR domain and TOPLESS (TPL), which is a transcriptional repressor that recruits histone deacetylase19 (32, 33). Several lines of evidence suggest that their repression activities could also be carried out through interaction with PRC2. SUP represses the auxin biosynthesis genes *YUCCA1/4* (*YUC1/4*) by recruiting PRC2 to *YUC1/4* to fine-tune local auxin signaling to determine floral meristem determinacy (27). KNU recruits PRC2 to initiate and maintain downregulation of *WUSCHEL* (*WUS*) to regulate floral meristem determinacy (31). Our ChIP-qPCR analysis of the ZP1 binding sites at *AG* showed that ZP1 binds to the 3' end of the second intron of *AG*. This region is also bound by PRC2,

suggesting that ZP1 might be involved in recruiting PRC2 to *AG*. In vivo protein–protein interaction analysis showed that both the zinc finger domain and EAR domain are required for KNU and FIE (a protein in the PRC2 complex) interaction and SUP and CLF interaction. However, motif analysis among transcription factors that recruit PRC2 to target loci showed that they share an EAR domain (34), suggesting that the EAR domain may recruit PRC2. The EAR domain in KNU is important for H3ac levels and H3K27me3 levels in vivo, indicating that the EAR domain is important for recruiting histone deacetylases and PRC2 to target loci (34). It remains to be determined whether both the zinc finger domain and EAR domain in ZP1 and ZFP8 are required and how they are required for recruiting PRC2 to *AP3* and *AG* in vegetative leaves.

C2H2 zinc finger proteins contain two cysteine residues and two histidine residues that hold one zinc ion. There are 176 C2H2 zinc finger proteins in *Arabidopsis*, which are divided into three subfamilies (A, B, and C). Among them, 64 are classified in the C1 subclass (35). A lot of the C1 subclass members are involved in stress responses, but relatively little is known about how they function in plant development (13, 35). This study provides insights into how ZP1 and ZFP8 act to repress floral homeotic gene expression in inappropriate developmental times and tissues. Protein sequence alignment analysis of ZP1 and ZFP8 among *Arabidopsis*, wild cabbage, tomato, and soybean showed that the zinc finger domain and the EAR domain are conserved among them, suggesting conserved mechanisms and function in dicots (SI Appendix, Fig. S8).

Materials and Methods

Plants were in the Col-0 background and were grown under 16-h light/8-h dark at 22 °C. Either vegetative apices (1-wk-old or 11-d-old shoot apices) or inflorescences of plants were harvested for gene expression analysis. RNA isolation and quantitative RT-PCR were performed as previously described (36). qRT-PCR and

ChIP qPCR results are mean \pm SEM from three biological replicates. Detailed information on plant material, construction of plasmids for transformation, qRT-PCR analysis, GUS staining analysis, in situ hybridization, and coimmunoprecipitation followed by qPCR analysis is provided in *SI Appendix, Materials and Methods*.

Data, Materials, and Software Availability. The raw RNA-Seq data generated in this study have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) and are publicly accessible under the BioProject ID [PRJNA968041](https://www.ncbi.nlm.nih.gov/bioproject/968041) (<https://www.ncbi.nlm.nih.gov/bioproject/968041>) (37). The associated metadata, including sample information, experimental design, and sequencing details, can be found within the BioProject records. All other study data are included in the article and/or *SI Appendix*.

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