Letter to the Editor

REF6 promotes lateral root formation through de-repression of PIN1/3/7 genes

Running Title: H3K27 demethylation promotes lateral root formation

Xiaolei Wang†, Jiong Gao†, Shan Gao1,2, Zhongpeng Li1, Benke Kuai†* and Guodong Ren†*

1. State Key Laboratory of Genetic Engineering and Fudan Center for Genetic Diversity and Designing Agriculture, Institute of Plant Biology, School of Life Sciences, Fudan University, Shanghai 200438, China;
2. School of Life Sciences, Qilu Normal University, Jinan 250200, China

†These authors contributed equally to this work.

*Correspondences: Guodong Ren (gdren@fudan.edu.cn, Ren is fully responsible for the distribution of all materials associated with this article); Benke Kuai (bkkuai@fudan.edu.cn)

Edited by: Frans Tax, University of Arizona, USA

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1111/jipb.12726]

This article is protected by copyright. All rights reserved.
Received: July 1, 2018; Accepted: August 31, 2018
Summary
The H3K27 methyltransferase CLF inhibits lateral root (LR) formation through depositing the repressive H3K27me3 mark to the chromatin of PIN1, a key polar auxin transporter gene. Here, we show that the H3K27me3 demethylase REF6 promotes lateral root primordium initiation and LR emergence. REF6 directly binds to the chromatin of PIN1/3/7. Dysfunction in REF6 results in increased levels of H3K27me3 on PIN1/3/7 and suppressed expression of PIN genes. Genetic analysis of the clf ref6 double mutant revealed an antagonistic action between CLF and REF6, in terms of LR formation. Our findings indicate that H3K27 methylation and demethylation activities are likely coordinated to ensure proper LR organogenesis.

The plant root system, which is composed of an embryo-derived primary root and its branches, called lateral roots (LRs), determines the efficiency of water and nutrient acquisition from the soil (Moller et al. 2017). By contrast with the primary root, lateral roots are produced post-embryonically. LRs initiate from individual or pairs of pericycle founder cells located adjacent to the xylem poles (Casimiro et al. 2001; Moller et al. 2017). These cells undergo several rounds of asymmetric cell division to form a LR primordium (LRP) (Malamy and Benfey 1997a). Pre-establishment of an auxin concentration gradient, which is achieved through polar auxin transport, is crucial for LRP initiation and development until they become self-sufficient (Himanen et al. 2002; Benková et al. 2003). Two main classes of transmembrane proteins, i.e. the PIN-FORMED (PIN) auxin efflux carriers and the AUX1/LIKE AUX1 (AUX1/LAXs) auxin influx carriers, function in polar auxin transport across the plasma membrane (PM) (Petrášek et al. 2006; Grones and Friml 2015).

Histone modifications are key regulators of spatio-temporal gene transcription and are dynamically modulated by both “writers” and “erasers”. A recent study showed that an H3K27 methyltransferase, CURLY LEAF (CLF), inhibits lateral root formation through depositing the repressive H3K27me3 mark to the chromatin of
PIN1 (Gu et al. 2014). In this study, we report that RELATIVE OF EARLY FLOWERING 6 (REF6), an H3K27me2/3 demethylase (Lu et al. 2011), is also involved in LR formation through modulating the H3K27me3 levels at the chromatin of the PIN1, 3, and 7 genes.

To investigate whether H3K27me3 demethylation plays a role in root development, we first analyzed the primary root length and numbers of LR in the ref6-1 mutant. Compared with wild-type plants (Wt), the ref6-1 mutant exhibited significantly fewer LR numbers with primary root elongation barely affected (Figure 1A, 1B; Figure S1A). To test how REF6 might affect LR production, we classified LRP into several stages, according to (Malamy and Benfey 1997b). Dysfunction in REF6 caused significantly lower LRP numbers, at all stages, whereas REF6 overexpression resulted in higher LRP numbers at later stages and consequently more LRs (Figures 1A−C, S1B). These data suggested that REF6 may modulate LRP initiation.

We next investigated the expression pattern of REF6 in the root. A GUS reporter driven by a 1028-bp promoter fragment of REF6 was introduced into Col-0 plants. We observed that REF6 was highly expressed in the stele, but was barely detectable in the cortex and epidermis (Figure 1D). We were unable to detect robust pREF6::GUS expression in LRP. Nevertheless, weak pREF6::GUS expression was detected in both young and mature lateral roots (Figure 1D). It is still possible that REF6 is expressed, at low levels, and directly targets PIN genes in LRP. Alternatively, REF6 may regulate PIN gene expression in the stele, which indirectly then affects PIN expression in LRP. Indeed, we detected reduced PIN1-GFP signals in both primary root and LRP in ref6-1 (Figure S5).

Given that auxin plays a central role in LR formation (Benková et al. 2003), we next assessed whether exogenous auxin could rescue the LR defect in ref6-1. Plants were grown for 5 d on vertically oriented agar plates supplemented with 0.5× Murashige and Skoog (MS) medium, and then transferred to new plates containing either 1 μM 1-naphtalene acetic acid (NAA, synthetic auxin) or 10 μM
1-N-naphthylphthalamic acid (NPA, auxin inhibitor).

In both Wt and ref6-1, massive LRs were produced upon NAA treatment, whereas no LR was observed upon NPA treatment (Figure S2). The asymmetric distribution of PINs and AUX1/LAXs in the PM is a premise of polar auxin transport (Grones and Friml 2015). The phenotype of ref6-1 is similar to that of drr1, an alkamide-resistant mutant whose causal gene has not yet been identified. To test whether drr1 is allelic to ref6, we treated ref6-1 with N-decanoyl-l-homoserine lactone (C10-AHL), an alkamide-related chemical that inhibits primary root elongation. The drr1 mutant was shown to be highly insensitive to C10-AHL (Morquecho-Contreras et al. 2010), whereas ref6-1 displayed a similar sensitivity to C10-AHL as the Wt control (Figure S3), suggesting that drr1 is not caused by a mutation in REF6.

As histone H3K27me3 negatively regulates transcription, we surmised that dysfunction in REF6 may lead to suppressed expression of target genes. To this end, we analyzed the relative transcript levels of PIN1/2/3/4/6/7 and AUX1/LAXs in roots of ref6-1 at 11 d after germination (DAG). REF6 dysfunction resulted in decreased expression levels of PIN1/2/3/4/7, but not the other tested genes (Figures 1E, S4). These results implicated the PINs family genes as potential targets of REF6. The pPIN1::PIN1-GFP reporter line was further introduced into ref6-1 by genetic crossing. Indeed, the PIN1-GFP intensities were decreased in all examined root zones (i.e. primary root, LRP and LR) in ref6-1 relative to Wt (Figure S5).

We next examined whether REF6 associates with the PIN genes by ChIP-qPCR analysis. Chromatin isolated from ref6-1+ pREF6::REF6-HA transgenic seedlings was immunoprecipitated with an HA antibody. We observed a strong enrichment of REF6 in the promoter regions of PIN1 (P1 & P2) and coding regions of PIN7 (P2 & P3). REF6 was also enriched in the coding regions of PIN3, albeit to a less extent (Figure 1F, 1G). In contrast, no significant enrichment was detected in PIN2/4/6 genes (Figure S6). Genomic targeting of REF6 is achieved through its recognition and directly
binding of the CTCTGYTY motif (Y=C/T) by its C-terminal zinc finger domain (Cui et al. 2016). We found multiple putative REF6 binding motifs existed near the REF6 enriched sites (i.e. P1 of PIN1, P3 of PIN3, and P2 of PIN7).

To test whether REF6 directly bound to these motifs, we performed an electrophoretic mobility shift assay (EMSA). The C2H2-ZnF domain of REF6 (REF6C, 1239-1360 aa) fused with a GST tag (GST-REF6C) was expressed and purified, as previously described (Cui et al. 2016). With ChIP data as a reference, we first analyzed a CTCTGYTY motif (i.e. CTCTGTYY) located in the PIN1 promoter region (-520~527 bp, Figure 1F). We detected a shifted band when labeled probes were pre-incubated with GST-REF6C. Addition of an excess amount of non-labeled probe, but not motif-mutated ones, abolished the shift, indicating that the binding of REF6 to the probe is CTCTGYTY motif-specific (Figure 1H). REF6 also bound to the CTCTGYTY motifs of PIN3/7, in vitro (Figure S7).

The above results showed that REF6 associates with chromatin of PIN1/3/7 both in vivo and in vitro. We next asked whether loss of REF6 affects the H3K27me3 levels at PIN1/3/7. ChIP-qPCR analysis revealed that the H3K27me3 levels at PIN1 and PIN7 were significantly higher in ref6-1 than those in Wt (Figure 1I), indicating that the decreased expression of PIN1/7 was due to high levels of H3K27me3 in ref6-1. We found constitutive over-expression of PIN1 rescued the LR defects in ref6-1 (Figure S8).

To further explore how H3K27me3 dynamics might regulate LR emergence, we compared the root phenotypes in Wt, ref6-1, early flowering 6-5 (elf6-5), ref6-1 elf6-5, clf-28 and ref6-3 clf-28 (Figure 1J). Loss-of-function mutation of another H3K27me3 demethylase, elf6-5, did not affect root development, and the LR number in ref6-1 elf6-5 was comparable to those in ref6-1, arguing against a role for ELF6 in LR formation. Consistent with previous results, the clf-28 mutant produced more LRs (Gu et al. 2014). The number of LRs in ref6-3 clf-28 was in between those in clf-28 and ref6-1 (ref6-1 and ref6-3 had similar root phenotypes, data not shown), suggesting an
antagonistic action of H3K27 methylation and demethylation on the same target genes (Figure 1K).

The hormone, auxin, plays an important role in plant growth and the PIN-family auxin efflux carrier form a major part of this system (Adamowski and Friml 2015). Our results, along with previous data (Gu et al. 2014), suggest that the spatio-temporal expression of PIN genes, at least for PIN1, are fine-tuned by both H3K27 methylation and demethylation. CLF represses the transcriptional level of PIN1 through writing H3K27me3 on it, whereas our study suggested that REF6 binds to the chromatin of PIN1/3/7, erases H3K27me3, and thereby, increases their expression. Moreover, BRAHMA (BRM), a SWI/SNF chromatin remodeling ATPase recruited by REF6, also targets the chromatin of PIN1/2/3/4/7 (Yang et al. 2015; Li et al. 2016). We propose that this system may provide an excellent model to study how “writers” and “eraser” of the same histone modification coordinate with each other to ensure proper organogenesis and development.

ACKNOWLEDGEMENTS
We thank Xiaofeng Cao for providing the ref6-1, elf6-5, ref6-3 clf-28, ref6-1 elf6-5 mutants and the ref6-1+pREF6::REF6-HA transgenic plants. We also thank Aiwu Dong for providing the clf-28 mutant and Tongda Xu for the pPIN1::PIN1-GFP plants. This work was supported by the Science and Technology Commission of Shanghai Municipality (2015JC1400800 to G.R.) and the National Natural Science Foundation of China (31622009 to G.R., 31700246 to J.G.)

AUTHOR CONTRIBUTIONS
X.W., J.G., G.R., and B.K. conceived the research. X.W., J.G., S.G., and Z.L. performed the experiments. X.W., J.G., G.R., and B.K. analyzed the data and wrote the article. All authors read and approved the manuscript.
REFERENCES


Figures and legends

Figure 1 REF6 promotes lateral root formation through de-repressing of PIN1/3/7

(A) Phenotypes of 11-day-old seedlings of Col-0, ref6-1 and ref6-1+pREF6::REF6-HA (REF6-OE). (B) LR numbers per primary root. (C) LRP numbers at I-II, III-IV, V-VI, and VII stages. (D) GUS staining of a pREF6::GUS transgenic plant root. (E) Relative transcript levels of PIN genes in ref6-1 at 11 DAG. ACT2 was used as an internal control. (F) Distribution of the CTCTGYTY motifs and positions of fragments amplified in ChIP-PCR assays in (G) and (I). (G) Association of REF6-HA to the PIN1/3/7 genes by a ChIP-PCR assay. Eleven-day-old seedlings from Col-0 and ref6-1+pREF6::REF6-HA transgenic plants were used. Relative fold enrichment was calculated as the ratio of ref6-1+pREF6::REF6-HA to Col-0. NAC004 was included as a positive control. P1 to P4 fragments are shown as in (F). (H) Binding of REF6 to the CTCTGYTY motif of the PIN1 promoter fragment in vitro. GST or GST-tagged REF6 was incubated with the biotin-labeled wild-type DNA probe. Excessive amount (40× and 200×) of unlabeled DNA probe was used for competition assays. The m200×, mutated probe added at 200×. Shifted bands indicate the formation of DNA-protein complexes, as marked by arrowheads. “-” represents absence, “+” represents presence. (I) H3K27me3 levels across PIN1/3/7 chromatin regions. Eleven-day-old seedlings were used for ChIP analysis. (J) Phenotypes of 11-day-old seedlings of Col-0,
ref6-1, elf6-5, ref6-1 elf6-5, elf-28, and ref6-3 elf-28. (K) LR numbers per primary root of indicated genotypes. Bar, 3.5 mm. Data are means ± SD (in B, C, n = 12, Unpaired student’s t tests; in E, G, I, n=3, Paired student’s t tests; *, P < 0.05; **, P < 0.01; ***, P < 0.001. In K, n = 12, One-way ANOVA test, P < 0.5.)

SUPPORTING INFORMATION

Figure S1. REF6 promotes lateral root formation
(A) Primary root length. Primary root lengths of 5~11-day-old seedlings were measured. Data are mean ± SD (n=12). Unpaired Student’s t tests, *, P < 0.05. (B) LRP numbers per primary root of 11-day-old seedlings. Data are mean ± SD (n=20). Unpaired Student’s t tests, *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure S2. Root phenotypes upon auxin or auxin transport inhibitor treatment
(A) Col-0 and ref6-1 plants were vertically grown on 0.5× MS medium for 11 days. (B, C) Col-0 and ref6-1 plants were vertically grown on 0.5× MS medium for 5 days and transferred to new 0.5× MS medium containing either 1 μM NAA (B) or 10 μM NPA (C) for another 6 days. Bar = 3.5 mm. Similar results were obtained from three independent experiments.

Figure S3. Effects of C10-AHL on primary root elongation in different genotypes
(A) Phenotypes of Col-0, ref6-1 and REF6-OE plants at 8 DAG, with or without 30 μmol C10-AHL treatment. Bar = 1.0 cm. (B) Primary root length of seedlings at 8 DAG. Data are means ± SD (n=3). One-way ANOVA test, P < 0.05.

Figure S4. Relative transcript levels of AUX1/LAXs
Relative transcript levels of AUX1/LAXs in the root samples of Wt and the ref6-1 mutant at 11 DAG. ACT2 was used as an internal control. Data are mean ± SD (n=3). Paired Student’s t tests, NS, P > 0.05.

Figure S5. Analysis of PIN1-GFP in Col-0 and ref6-1 roots
(A-C) Expression of PIN1-GFP in ref6-1 and Col-0. Different root zones were shown: primary root (A), LRP (B), and LR (C). Bar = 50 μm. (D) Relative GFP intensities
quantified by ImageJ) in roots of Col-0 and ref6-1. Data are mean ± SD (n=8). Unpaired Student’s t tests, ***, P < 0.001.

**Figure S6. Binding of REF6-HA to PIN2/4/6 by ChIP-PCR**

(A) Distribution of the CTCTGYTY motifs across the PIN2/4/6 promoters and coding regions. (B) ChIP analysis of in vivo binding of REF6-HA to the PIN2/4/6 genes in seedlings of 11-day-old Col-0 (wild type) and ref6-1+pREF6::REF6-HA plants. Fold enrichment was calculated as the ratio of ref6-1+pREF6::REF6-HA to Col-0 signal. P1 to P4 primers are listed in Table S1. NAC004 was a positive control. Data are mean ± SD (n=3). Paired Student’s t tests, ***, P < 0.001.

**Figure S7. REF6 binds to the CTCTGYTY motifs of PIN1/3/7 in vitro**

(A, B) REF6 binds to the CTCTGYTY motif of PIN3 (A) and PIN7 (B) in vitro, respectively. GST-tagged REF6 was incubated with the biotin-labeled wild-type DNA probe. Competition experiments were performed by adding 40× and excessive amounts (200×) of unlabeled DNA probe. A mutated probe was used to test the binding specificity. Shifted bands indicate the formation of DNA-protein complexes, are marked by arrowheads. “-” represents absence, “+” represents presence. Sequences of both the wild-type and mutated probes are shown at the bottom of the images, with the CTCTGYTY motif in bold and underlined. (C) REF6 doesn’t bind to the probes with mutated motif. Bio: biotin-labeled DNA probe with the CTCTGYTY motif. Mu-Bio: biotin-labeled DNA probes with motif region mutated.

**Figure S8. Constitutive overexpression of PIN1 in ref6-1 transgenic plants compromises LR formation in 11-day-old seedlings**

(A) Phenotypes of Col-0, ref6-1 and lines of transgenic plants at 11 DAG. (B) Primary root length of the indicated seedlings at 11 DAG. (C) LR numbers per primary root of the indicated seedlings at 11 DAG. (D) The PIN1 transcript levels of the indicated seedlings at 11 DAG. Data are mean ± SD (n=3). Paired Student’s t tests, *, P < 0.05; **, P < 0.01; ***, P < 0.001 compared to Col-0.
Figure 1